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ORIGINAL ARTICLE

Case Control Study

Does low volume high-intensity interval training elicit superior benefits to continuous low to moderate-intensity training in cancer survivors?

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Abstract

AIM

To determine the impact of low volume high-intensity interval training (LVHIIT) and continuous low to moderate-

intensity exercise training (CLMIT) on cardiovascular disease (CVD) risk and health outcomes in cancer survivors.

METHODS

Sedentary cancer survivors (n = 75, aged 51 ± 12 year) within 24 months of diagnosis, were randomised into three groups for 12 wk of LVHIIT (n = 25), CLMIT (n = 25) or control group (n = 25). The exercise intervention involved 36 sessions (three sessions per week). The LVHIIT group performed 7 x 30 s intervals (⩾ 85% predicted maximal heart rate) with a 60 s rest between intervals, and the CLMIT group performed continuous aerobic training for 20 min (⩽ 55% predicted maximal heart rate) on a stationary bike. Outcome variables were measured at baseline and at 12 weeks and analysed using a 3 x 2 (group x time) repeated measures ANCOVA to evaluate main and interaction effects.

RESULTS

Significant improvements (time) were observed for seven of the 22 variables (ES 0.35-0.97, $P \leqslant 0.05$). There was an interaction effect ($P < 0.01$) after 12 wk in the LVHIIT group for six-minute walk test ($P < 0.01$; d = 0.97; 95%CI: 0.36, 1.56; large), sit to stand test ($P < 0.01$; d = -0.83; 95%CI: -1.40, -0.22; large) and waist circumference reduction (P = 0.01; d = -0.48; 95%CI: -1.10, 0.10; medium). An interaction effect ($P < 0.01$) was also observed for quality of life in both the LVHIIT (d = 1.11; 95%CI: 0.50, 1.72; large) and CLMIT (d = 0.57; 95%CI: -0.00, 1.20; moderate) compared with the control group (d = -0.15; 95%CI: -0.95, 0.65; trivial).

CONCLUSION

Low-volume high-intensity training shows promise as an effective exercise prescription within the cancer population, showing greater improvements in cardio-respiratory fitness, lower body strength and waist circumference compared with traditional CLMIT and control groups. Both LVHIIT and CLMIT improved quality of life. A proposed benefit of LVHIIT is the short duration (3 min) of exercise required, which may entice more cancer survivors to participate in exercise, improving health outcomes and lowing the risk of CVD.

Key words: High-intensity exercise; Health; Oncology; Exercise prescription

Core tip: Low-volume high-intensity training is not commonly used in the rehabilitation of Cancer Survivors. In this study it shows promise as an effective exercise prescription, with greater improvements in cardio-respiratory fitness, lower body strength and waist circumference compared with traditional continuous low to moderate-intensity exercise training (CLMIT) and control groups. Low volume high-intensity interval training (LVHIIT) and CLMIT improved quality of life. A proposed benefit of LVHIIT is the short duration (3 min) of exercise required, which may entice more cancer survivors to participate in exercise, improving health outcomes and lowing the risk of cardiovascular disease.

Toohey K, Pumpa K, McKune A, Cooke J, DuBose KD, Yip D, Craft P, Semple S. Does low volume high-intensity interval training elicit superior benefits to continuous low to moderate-intensity training in cancer survivors? *World J Clin Oncol* 2018; 9(1): 1-12 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i1/1.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i1.1

INTRODUCTION

Worldwide there is an increase in cancer survival rates[1]. This potentially raises the risk of cancer recurrence and other non-communicable diseases (NCDs) such as type Ⅱ diabetes and cardiovascular disease (CVD)[2]. These increased health risks may be due to the effects of cancer treatments and reductions in healthy lifestyle habits, such as physical activity (PA)[3,4]. Physical activity decreases NCD risk in apparently healthy people[5], though it is not conclusive if the same trends are evident in individuals with cancer. Exercise during and after cancer treatment has been shown to be safe, improve fitness levels, and quality of life (QoL)[6,7]. Because of this, there is significant interest in the clinical use of exercise as an adjunctive therapy for improving cancer-related health outcomes.

Evidence is rapidly increasing regarding the benefits of exercise concurrent to treatment in the remediation of adverse clinical outcomes for cancer survivors[8]. It is uncommon for cancer survivors to be advised by clinicians to participate in an exercise program, despite the existence of exercise guidelines[9,10]. This may be due to the generic nature of the guidelines, limited practitioner expertise or the costs associated with some programs[11,12]. The present exercise guidelines lack detail on the type, mode, duration and intensity of exercise necessary to achieve the best outcomes for cancer survivors. Additional research is required to fill the gaps in our current knowledge to further improve exercise recommendations, the evidence-based knowledge and exercise education for cancer survivors and their treating practitioners.

Low volume high-intensity interval training (LVHIIT) is the use of small doses of high-intensity exercise to elicit physiological responses such as improved VO_2 max and positive metabolic changes in skeletal muscle. The physiological changes could potentially be much higher or different to those currently being obtained with participation in other doses of activity, such as continuours, moderate-intensity exercise[13]. LVHIIT has been shown to improve VO_2 peak and insulin sensitivity in apparently healthy individuals in as little as

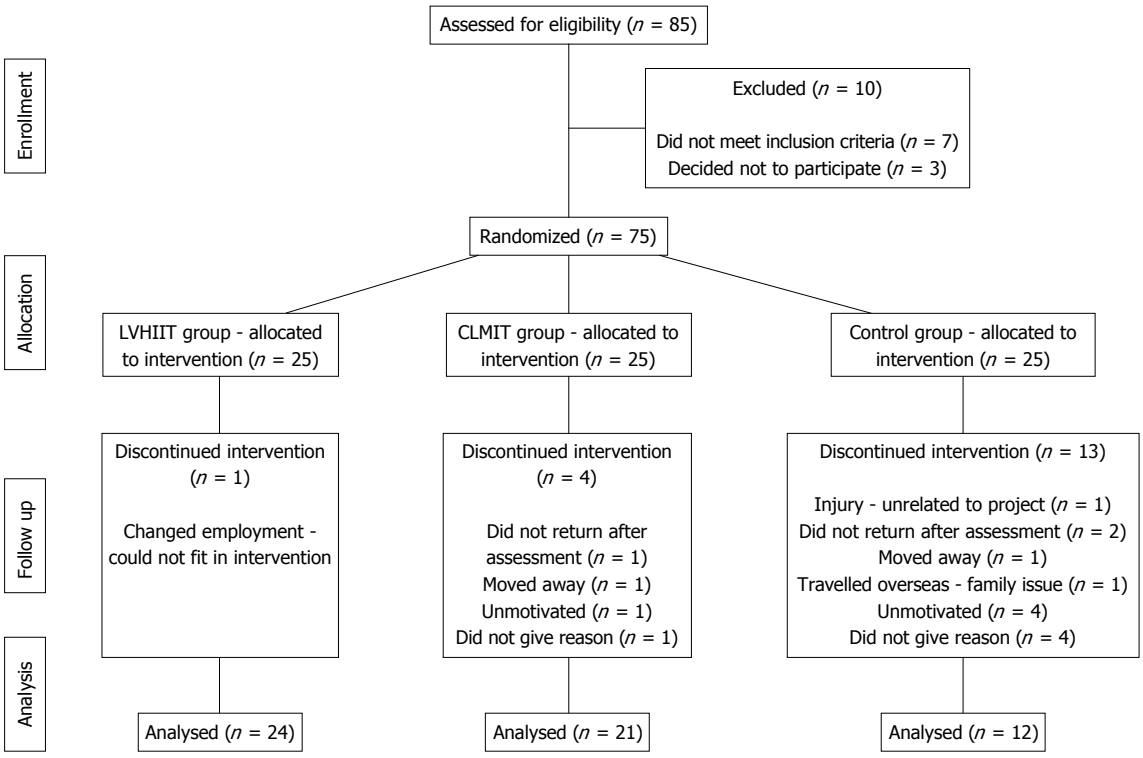

Figure 1 Consort diagram. LVHIIT: Low volume high-intensity interval training; CLMIT: Continuous low to moderate-intensity training.

four weeks[14,15]. There is limited research examining the effects of LVHIIT in improving health outcomes for cancer survivors. Its application as a modality for use with other chronic disease populations is evolving[14,16,17], potentially due to its ability to elicit positive physiological improvements in a short period. The current study aimed to investigate and compare the effects of LVHIIT and the more commonly prescribed continuous low to moderate-intensity training (CLMIT) on improving health outcomes and reducing cardiovascular disease (CVD) risk in cancer survivors.

MATERIALS AND METHODS

Participants

Eighty-five cancer survivors (83 female and 2 male, 51.48 ± 12.45 years) were recruited for the 12-wk study. Recruitment was conducted between September 2014 and June 2016 *via* email, pamphlet distribution, word of mouth and online social media. Referrals were obtained from the medical community and community organisations. The inclusion criteria included: (1) Participants within the first 24 mo of diagnosis; (2) in the post-treatment phase of the "physical activity across the cancer experience" (PEACE) organisational model, once the acute effects of medical treatments had dissipated[18], and (3) sedentary, as described by the American College of Sports Medicine[19]. Participants were excluded if they had: (1) Brain or metastatic bone cancers; (2) bone pain; (3) resting blood pressure > 180/110 mmHg; (4) were pregnant; (5) undergoing psychotherapy treatment; (6) had musculoskeletal injuries or disabilities restricting their ability to participate in exercise.

Randomisation

Of the 85 participants, seven participants did not meet the inclusion criteria, and three decided not to participate due to the timing of exercise sessions. The 75 remaining participants were randomly assigned *via* an online randomisation tool into either LVHIIT (n = 25) or CLMIT (n = 25) or control (n = 25) group (Figure 1). A person independent of the research team used the research randomizer computer software[20] to allocate participant codes into the three groups (LVHIIT, CLMIT, and control).

Of the 75 participants, 57 completed the study (76%). In the LVHIIT group (n = 24), one participant changed employment and could not complete the intervention. In the CLMIT group (n = 21), one participant did not return after baseline assessment, one moved interstate, one was not motivated to continue, and one failed to provide a reason. In the control group (n = 12) one participant sustained an injury (unrelated to the project), two did not return after baseline assessment, one moved interstate, one traveled overseas, four failed to respond to the final evaluation and four did not provide a reason (Figure 1).

Quality of life

The Functional Assessment of Cancer Therapy-General (FACT–G) questionnaire (version 4) was used to measure quality of life (QoL) and functional capacity[21]. The FACT-G is a validated survey containing 27 items. The questions are in four categories: (1) physical well-

being; (2) social/family well-being; (3) emotional well-being; and (4) functional well-being. The questionnaire is regularly used to measure QoL in cancer survivors[22] and was completed at baseline and then after the 12-wk intervention.

Anthropometrics

The Dual X-Ray Absorptiometry (DXA) scan (GE Healthcare, Sydney, NSW, Australia) was used to measure total body composition, including; lean mass, body weight and body fat percentage[23]. The DXA scanner was calibrated each day, using a phantom spine. The manufacturers' guidelines were followed to carry out daily quality control checks. All scans were carried out by trained densitometrists. Participants were asked to fast overnight and wear no jewellery, while being scanned. Hip and waist circumferences were measured using a standard anthropometric tape measure[24,25]. The same individual measured the circumferences at baseline and post-intervention using WHO STEPwise approach measurement protocols[25,26].

Cardiovascular functioning

Pulse wave velocity (PWV) and pulse wave analysis (PWA) were measured using the SphygmoCor XCEL system (SphygmoCoR; At-Cor Medical Pty Ltd., Sydney, Australia). Carotid-femoral PWV is the recognised gold standard measure of aortic stiffness, a strong independent predictor of cardiovascular risk[27,28]. PWA, which included measures of resting heart rate (RHR), augmentation index (AIx), central systolic blood pressure (CSP), central diastolic blood pressure (CDP), central pulse pressure (CPP), augmentation pressure (AP), mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were assessed at baseline and upon completion of the intervention. The pulse pressure (PP) waveform of the left carotid artery was measured with an applanation tonometer. Participants rested supine for 10 min before the measurements were obtained. Twenty continuous waveforms were essential for results to be considered valid and these were used to acquire the PP waveform of the aorta, and PWV was used as a marker of aortic stiffness[29].

Biomarkers

Participants fasted overnight in preparation for blood sample analysis. Samples were analysed by an independent laboratory for high-sensitive C-reactive protein (CRP), insulin, glucose, and full blood count. The analysis was conducted offsite at Capital Pathology, Canberra, Australia.

Functional capacity

Lower body strength was measured using a repeated chair rise test (STS). The participants sat in a chair and were asked to stand and then sit as fast as possible five times, without the use of their arms[30,31]. The six-minute walk test (6MWT) was used to delineate participants' cardiorespiratory fitness levels. Participants were asked to walk as quickly as possible for six minutes and the distance traveled was recorded[32]. The 6MWT is used extensively to assess cardiorespiratory fitness in cancer survivors[33,34].

Intervention

An Accredited Exercise Physiologist (AEP) supervised all exercise sessions performed over the 12-wk intervention period (three sessions per week). Participants were asked to refrain from consuming food and caffeine and participating in any exercise for two hours before baseline and post-intervention assessments. Assessments were carried out within seven days before commencement and within seven days following completion of the program[29]. The exercise sessions were carried out on a stationary bike. The LVHIIT group performed interval training ($\geqslant$ 85% maximal heart rate), which consisted of a five-minute warm up, seven by 30 s intervals, with one-minute rest in between each interval, followed by a five-minute cool down (adapted from Gibala, 2012). A gradual increase in exercise was carried out by the LVHIIT group. Individuals started the first session with three intervals, with one interval added per week over the following four weeks and by the 5th week participants performed all seven intervals[29]. The CLMIT group performed continuous aerobic training ($\leqslant$ 55% predicted maximal heart rate) for 20 min also with a five-minute warm up and cool down[29]. Age-predicted maximal heart rate was used to calculate relative intensity and the two exercise protocols were matched for appropriate energy expenditure using the calculation reported by Rognmo *et al*[36] (2004). For each participant, their heart rate (HR) and rate of perceived exertion (RPE) were logged every minute for the CLMIT sessions. The peak HR and RPE in each interval and the resting HR and RPE between each interval were recorded during the LVHIIT sessions. Blood pressure (BP) was monitored immediately before and after each exercise session.

Statistical analysis

Means and standard deviations were calculated for dependent variables. An ANOVA was performed to determine if pre-intervention differences existed between the three groups. A Bonferroni post-hoc multiple comparison test was conducted to determine where group differences existed. A 3 x 2 (group x time) repeated measures ANCOVA[29] was used to evaluate main and intervention effects and was adjusted by baseline values. Effect sizes (ES) were calculated, and Cohen's *d* values were interpreted as follows: large $\geqslant$ 0.8, medium $\geqslant$ 0.5, small $\geqslant$ 0.2 and trivial < 0.2[37]. The alpha level was set at $P < 0.05$, and SAS 9.3 (Cary, NC, United States) was used for all analyses.

RESULTS

Participant characteristics

Participant cancer diagnosis included 47 (82%) breast

Table 1 Baseline characteristics of participants

Characteristics	LVHIIT *n* = 24	CLMIT *n* = 21	Control *n* = 12
Sociodemographic			
Age, mean (SD), yr	48 (11.9)	52 (12.4)	57 (11.5)
Sex, *n* (%) male	F, 25 (0)	F, 21 (0)	F, 12 (0)
Comorbidity, *n* (%) yes	2 (0.8)	1 (0.4)	0 (0)
Diagnosis, *n* (%)			
Breast	21 (88)	16 (75)	10 (83)
Ovarian		1 (5)	1 (8.5)
Appendix		1 (5)	
Anal			1 (8.5)
Cervical	1 (4)		
Liver	1 (4)		
Esophageal	1 (4)		
Melanoma		1 (5)	
Leiomyosarcoma		1 (5)	
Unknown primary		1 (5)	
Stage of disease, *n* (%)			
Stage Ⅰ-Ⅱ	19 (79)	16 (76)	10 (83)
Stage Ⅱ-Ⅳ	5 (21)	5 (24)	2 (17)
Type of treatment, *n* (%)			
Surgery	21 (88)	20 (95)	12 (100)
Radiation therapy	18 (75)	13 (62)	10 (83)
Hormone therapy	19 (79)	15 (71)	9 (75)
Types of chemotherapy, *n* (%)			
TAC	7 (29)	4 (19)	1 (8)
FEC	5 (21)	3 (14)	3 (25)
TAC/FEC combinations	2 (8)	3 (14)	2 (17)
Capecitabine and oxaliplatin		1 (5)	
Carboplatin and paclitaxel	1 (4)		1 (8)
Cisplatin	1 (4)		
CHOP	1 (4)		
ABVD	1 (4)	1 (5)	1 (8)
Other	2 (8)	3 (14)	
No chemotherapy	4 (17)	6 (29)	4 (33)

LVHIIT: Low volume high-intensity training; CLMIT: Continuous low to moderate-intensity training; TAC: Taxotere, adriamycin, cyclophosphamide; FEC: Fluorouracil, epirubicin, cyclophosphamide; CHOP: Cyclophosphamide, doxorubicin, vincristine, prednisone; ABVD: Doxorubicin, bleomycin, vinblastine, dacarbazine.

cancer, two (3%) ovarian cancer and one diagnosis of appendix, anal, cervical, liver, oesophageal, melanoma, leiomyosarcoma and unknown primary (15%).

The age of the participants was 51.48 ± 12.45 years, with a BMI of 26.43 ± 4.08 kg/m^2 (Table 1). Baseline values for all variables were similar across the three groups except for white blood cell count, which was higher in the LVHIIT group compared with both the CLMIT group and control groups ($P < 0.02$).

For the LVHIIT group, the average HR during the 36 sessions was 147 ± 11 beats per minute (bpm), while RPE was 6 ± 3 using the Borg 1-10 scale[38,39]. The average HR and RPE at the end of each 60s recovery was 117 ± 8 bpm and RPE of 3 ± 2. The average HR for the CLMIT group was 98 ± 6 bpm and RPE was 3 ± 2 during the sessions. Overall mean session compliance was 92% (72%-100%). Ten participants attended all 36 sessions, and one participant missed ten sessions over the intervention period (data were included). There were no adverse events reported in the study.

Fitness, functional and body composition measures

There was a significant interaction effect ($P < 0.01$) for 6MWT. Distance walked was significantly longer for the LVHIIT group ($d = 0.97$; 95%CI: 0.36, 1.56; large) when compared with the CLMIT ($d = 0.17$; CI: -0.23, 0.99; trivial) and control ($d = -0.13$; 95%CI: -0.93, 0.67; trivial) groups (Figure 2A). There was a significant interaction effect ($P < 0.01$) for the STS test with a faster performance in the LVHIIT group ($d = -0.83$; 95%CI: -1.40, -0.22; large) compared with the CLMIT ($d = -0.59$; 95%CI: -1.20, 0.42; unclear) and control ($d = 0.36$; CI: -0.44, 1.17; unclear) groups (Figure 2B). There was a significant interaction ($P = 0.01$) effect for waist circumference; post results were significantly lower for LVHIIT ($d = -0.48$; 95%CI: -1.10, 0.10; medium) compared with CLMIT ($d = -0.05$; 95%CI: -0.66, 0.55; trivial) and control group ($d = 0.11$; 95%CI: -0.69, 0.91; trivial).

Quality of life

Overall: There was an interaction effect for overall QoL ($P < 0.01$). The QoL score was significantly improved in the LVHIIT group ($d = 1.11$; 95%CI: 0.50, 1.72; large) compared with the CLMIT group ($d = 0.57$; 95%CI: -0.00, 1.20; moderate) and the control group ($d =$

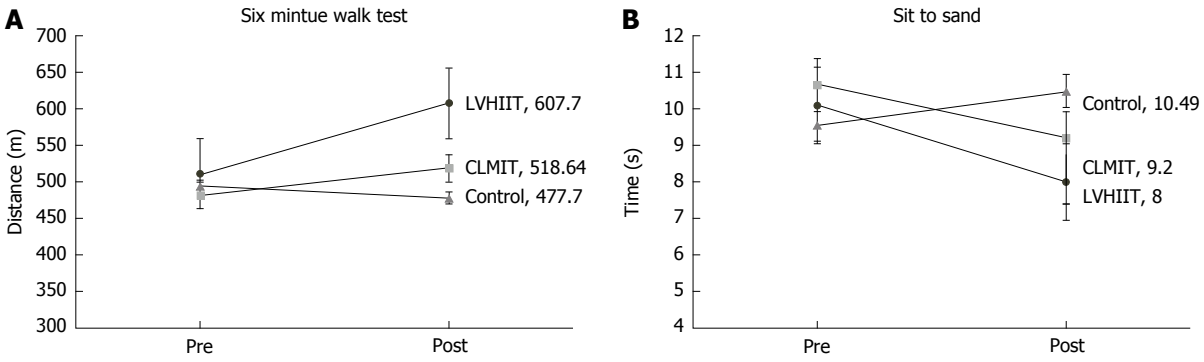

Figure 2 Changes in cardiorespiratory fitness and sit to stand for low volume high-intensity interval training, continuous low to moderate-intensity exercise training and control groups.

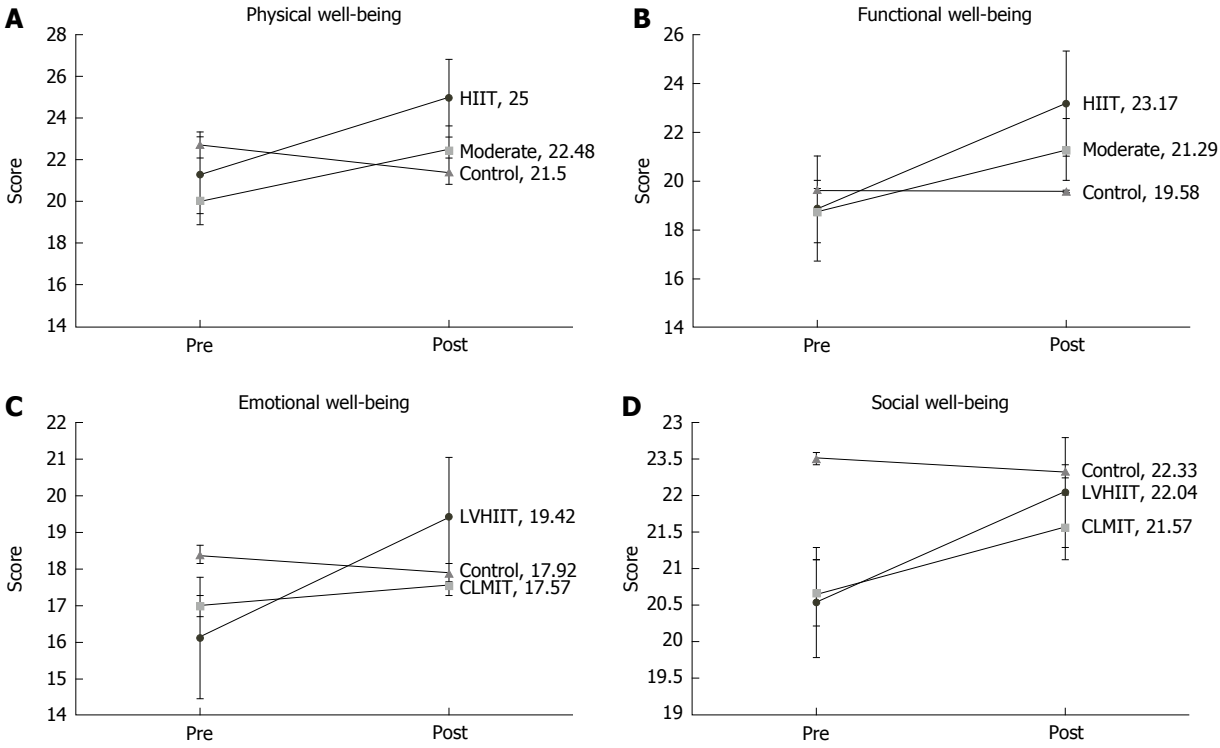

Figure 3 Changes in quality of life subscales for low volume high-intensity interval training, continuous low to moderate-intensity exercise training, and control groups.

-0.15; 95%CI: -0.95, 0.65; trivial) (Figure 3).

Well-being: An interaction effect was observed for physical well-being (P = 0.02). Improvements were seen in the LVHIIT (d = 0.89; 95%CI: 0.30, 1.48; large) and CLMIT (d = 0.61; 95%CI: -0.001, 1.23; medium) compared with the control group (d = -0.30; 95%CI: -1.09, 0.51; unclear) (Figure 3A). There was an interaction effect observed for functional well-being (P = 0.02). The LVHIIT group significantly improved (d = 0.96; 95%CI: 0.37, 1.56; large) compared to the CLMIT group (d = 0.64; 95%CI: 0.02, 1.26; medium) and the control group (d = -0.02; 95%CI: -0.82, 0.78; trivial) (Figure 3B). An interaction effect was also found for emotional well-being (P < 0.01). Improvements were observed for the LVHIIT group (d = 1.04, 95%CI: 0.43, 1.64; large) compared with the CLMIT group (d = 0.15; 95%CI: -0.45, 0.76; trivial) and the control group (d = -0.11; 95%CI: -0.92, 0.69; trivial) (Figure 3C). No interaction effect was observed for social well-being (P = 0.057) (Figure 3D). Changes were observed in the LVHIIT (d = 0.35; 95%CI: -0.22, 0.92; medium) compared to the CLMIT group (d = 0.20; 95%CI: -0.40, 0.80; unclear) and the control group (d = -0.05; 95%CI: -0.85, 0.75; trivial).

Cardiovascular function and biomarkers

There were no interaction effects for vessel stiffness parameters or biomarkers. However, time effects were found for markers of cardiovascular functioning. CSP (P = 0.07) in the LVHIIT group (d = -0.51; 95%CI: -1.07-0.10, moderate) which reduced by 4.94%. PP in

Table 2 Changes in risk factors from pre-to post intervention (Mean ± SD)

Variable	LVHIIT group *n* = 24			CLMIT group *n* = 21			Control group *n* = 12		
	Pre	Post	ES (95%CI)	Pre	Post	ES (95%CI)	Pre	Post	ES (95%CI)
Weight (kg)	72.9 ± 11.7	72.8 ± 11.4	-0.01 (-0.57, 0.56)	69.4 ± 14.3	70.4 ± 14.9	0.06 (-0.54, 0.67)	65.5 ± 13.1	65.9 ± 13.9	0.03 (-0.77, 0.83)
Body fat (%)	42.2 ± 7.9	41.4 ± 7.8	-0.1 (-0.67, 0.46)	43.0 ± 6.7	44.4 ± 9.5	0.17 (-0.44, 0.78)	37.7 ± 9.2	37.0 ± 10.5	-0.07 (-0.87, 0.73)
Fat mass (kg)	30.6 ± 9.1	29.2 ± 8.7	-0.16 (-0.72, 0.41)	30.5 ± 12.4	31.0 ± 12.1	0.05 (-5.6, 0.65)	24.3 ± 9.0	24.4 ± 9.5	0.01 (-0.79, 0.81)
Lean mass (kg)	40.7 ± 6.4	41.0 ± 7.2	0.05 (-0.52, 0.61)	38.8 ± 6.2	39.3 ± 6.8	0.07 (-0.53, 0.68)	38.8 ± 6.7	39.2 ± 6.9	0.06 (-0.74, 0.86)
Waist (cm)[1,2]	91.1 ± 11.7	85.7 ± 10.5[1]	-0.48 (-1.10, 0.10)	93.1 ± 13.1	92.4 ± 13.3	-0.05 (-0.66, 0.55)	89.1 ± 9.5	90.3 ± 11.6	0.11 (-0.69, 0.91)
Hip (cm)[1]	108.3 ± 10.3	104.8 ± 10.5[1]	-0.37 (-0.91, 0.23)	107.9 ± 11.3	107.0 ± 14.8	-0.07 (-0.67, 0.54)	104.1 ± 9.4	104.3 ± 8.9	-0.02 (-0.82, 0.78)
Resting HR (bpm)	76.3 ± 12.0	73.3 ± 12.4	-0.24 (-0.81, 0.32)	73.9 ± 11.9	72.3 ± 9.8	-0.14 (-0.75, 0.46)	74.5 ± 11.9	73.7 ± 12.6	-0.07 (-0.87, 0.74)
SBP (mmHg)	127.3 ± 18.3	121.8 ± 9.7	-0.39 (-0.95, 0.20)	130.9 ± 15.5	130.4 ± 14.7	-0.58 (-0.64, 0.57)	123.9 ± 15.4	128.1 ± 12.7	0.3 (-0.51, 1.10)
DBP (mmHg)	79.7 ± 8.3	77.8 ± 6.9	-0.25 (-0.82, 0.32)	81.5 ± 11.7	81.4 ± 6.5	-0.01 (-0.62, 0.59)	78.2 ± 5.3	79.9 ± 6.1	0.30 (-0.51, 1.10)
MAP	97.2 ± 10.8	93.9 ± 7.9	-0.36 (-0.92, 0.22)	100.7 ± 12.5	98.4 ± 8.9	-0.21 (-0.82, 0.39)	96.6 ± 6.2	95.9 ± 8.6	-0.09 (-0.89, 0.71)
CSP (mmHg)[1]	118.6 ± 17.7	111.3 ± 10.7[1]	-0.51 (-1.07, 0.10)	121.8 ± 15.9	119.9 ± 8.9	-0.14 (-0.75, 0.46)	116.7 ± 12.1	115.3 ± 13.5	-0.11 (-0.91, 0.69)
PP (mmHG)	35.7 ± 12.8	30.8 ± 6.8	-0.5 (-1.05, 0.10)	35.7 ± 10.6	36.0 ± 7.5	0.04 (-0.57, 0.64)	35.0 ± 11.4	34.1 ± 11.4	-0.08 (-0.88, 0.72)
AP (mmHg)[1]	8.1 ± 8.1	5.9 ± 4.4[1]	-0.35 (-0.91, 0.23)	9.3 ± 8.6	7.0 ± 4.8	-0.34 (-0.94, 0.28)	10.3 ± 6.6	8.0 ± 9.2	-0.29 (-1.09, 0.52)
AIx (%)	19.9 ± 14.7	18 ± 11.6	-0.14 (-0.71, 0.42)	22.5 ± 18.4	11.59 ± 18.8	-0.24 (-1.20, 0.03)	26.6 ± 12.5	10.0 ± 18.3	-1.06 (-1.91, -0.20)
CDP (mmHg)	83.25 ± 9.13	80.3 ± 7.9	-0.34 (-0.92, 0.22)	85.9 ± 10.6	82.8 ± 7.1	-0.34 (-0.95, 0.27)	82.7 ± 6.9	81.1 ± 7.1	-0.22 (-1.03, 0.57)
PWV (m/s)	6.6 ± 1.5	6.5 ± 1.2	-0.12 (-0.64, 0.49)	6.8 ± 1.5	6.9 ± 1.0	0.11 (-0.53, 0.68)	6.8 ± 1.5	6.2 ± 1.4	-0.41 (-1.22, 0.40)
STS (s)[1,2]	10.1 ± 2.8	8.1 ± 2.1[1]	-0.83 (-1.40, -0.22)	10.6 ± 2.8	9.2 ± 2.0	-0.59 (-1.20, 0.42)	9.6 ± 2.3	10.49 ± 2.7	0.36 (-0.44, 1.17)
6MWT (m)[1,2]	510.7 ± 114.9	607.7 ± 85.5[1]	0.97 (0.36, 1.56)	483.1 ± 72.3	518.6 ± 94.5	0.17 (-0.23, 0.99)	494.2 ± 128.7	477.7 ± 127.1	-0.13 (-0.93, 0.67)
Glucose (mmol/L)	4.9 ± 0.0	4.8 ± 0.5	-0.28 (-0.85, 0.29)	5.1 ± 0.6	4.9 ± 0.7	-0.31 (-0.92, 0.30)	5.1 ± 0.4	4.9 ± 0.4	-0.5 (-1.31, 0.31)
CRP (mg/L)	2.9 ± 3.5	2.7 ± 3.2	-0.07 (-0.63, 0.50)	4.5 ± 5.1	4.7 ± 4.9	0.04 (-0.56 – 0.64)	2.0 ± 1.1	2.0 ± 1.6	0 (-0.80, 0.80)
Insulin (mU/L)	11.4 ± 6.8	9.0 ± 4.9	-0.41 (-1.36, -0.19)	13.1 ± 10.4	11.3 ± 9.3	-0.18 (-0.79 – 0.42)	9.9 ± 5.6	10.7 ± 6.3	0.13 (-0.67, 0.94)
WBC (x 10^9/L)[1]	6.9 ± 2.5	5.7 ± 2.5[1]	-0.46 (-1.05, 0.10)	5.6 ± 2.0	5.2 ± 1.8	-0.21 (-0.82 – 0.40)	5.4 ± 0.7	4.9 ± 0.9	-0.62 (-1.44, 0.20)

[1]Time effect; [2]Interaction effect. ES: Effect size; HR: Heart rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure; CSP: Central systolic pressure; PP: Central pulse pressure; AP: Arterial pressure; Aix: Augmentation index (stiffness); CDP: Central diastolic pressure; PWV: Pulse wave velocity; STS: Sit-to-stand test; 6MWT: 6-min walk test; CRP: C-reactive protein; WBC: White blood cell.

the LVHIIT group decreased by 7.96% (*d* = 0.50; 95%CI: -1.05 – 0.10, moderate). AP (*P* = 0.02) in the LVHIIT group (*d* = -0.35; 95%CI: -0.91 – 0.23, small) reduced by 27%. A 22% decrease was observed in the CLMIT group (*d* = -0.34; 95%CI: -0.94 – 0.28, small) (Table 2).

DISCUSSION

The aim of this study was to determine the impact of LVHIIT and CLMIT on CVD risk and health outcomes in cancer survivors. There were significant improvements in functional capacity, specifically cardiorespiratory fitness, lower-limb strength, and waist circumference in participants who completed the LVHIIT compared with the CLMIT and control groups. QoL improved in both the LVHIIT group and the CLMIT group when compared with the control group, however a greater effect was observed in the LVHIIT group.

Maintaining or improving functional capacity and lower limb strength in cancer survivors is essential as it enhances survivors' ability to move and carry out physical activities during and after treatment[40]. It also provides the functional strength to start and adhere to an exercise program which increases levels of activity[41,42]. Building lean mass through exercise builds a healthy metabolic profile, which is imperative as it assists in improving risk factors in those who have spent an increased volume of

time sedentary during and following, intensive cancer treatment[43]. Participants in the LVHIIT group gained a larger effect in the sit to stand test compared with the other groups indicating improvements in lower limb strength. Possible mechanisms involved in this change could have been: (1) The increased power-output and the energy system/muscle fibre types recruited during the LVHIIT, similar to those recruited in the STS test; (2) an increased activation of the type Ⅱa and type Ⅱx fibres; and (3) an enhanced level of activation of the ATP/PC and glycolysis systems. An increased level of neuromuscular coordination is required at a higher-intensity of exercise, which also could have contributed to the improved strength and performance in the STS test seen in the LVHIIT group. Improvements were observed in the STS test in the LVHIIT group (20%), and in the CLMIT group (9.5%), highlighting greater increases in lower leg strength and functional capacity for those who completed the LVHIIT program. This finding was identified in our pilot study, however, due to small numbers, it was non- significant[29].

Significant improvements in cardiorespiratory fitness levels were identified through the increased distance covered in the 6MWT, with a larger effect observed in the LVHIIT group. These findings were also detected in the authors pilot study[29] and reported in other randomised controlled trials in cancer survivors[7,29,44]. The greater improvements seen in the LVHIIT group suggests that more comprehensive cardiovascular adaptations may occur with the use of high-intensity exercise. Potential mechanisms involved in increases in cardiorespiratory fitness could be due to the increased level of mitochondrial enzymes recruited in high-intensity exercise[45], which contributes to enhanced aerobic capacity of the skeletal muscles commonly seen after participating in high-intensity training. The positive impacts can contribute to changes in VO_2 difference, thereby increasing maximal aerobic capacity. Improvements in cardiorespiratory fitness using high-intensity interval training has been extensively reported in healthy people, but less in the cancer survivor population[46]. LVHIIT could decrease the risk of developing additional chronic diseases such as CVD and diabetes[47] and increase cancer survival rates[48,49] faster than the commonly prescribed moderate-intensity exercise. It has been shown that cancer survivors have decreased levels of cardiorespiratory fitness levels in comparison with healthy people[50].

A cancer diagnosis has a significant impact on behaviours which in turn can negatively affect QoL[51]. QoL reductions can have a profound effect on the recovery of cancer survivors, reducing the probability of being able to move freely and maintain physical activity levels[52]. A novel and clinically relevant finding of the present study was that QoL improvements were seen in both the LVHIIT and the CLMIT group, with a greater increase observed in the LVHIIT group. To date, very few studies have investigated how different exercise protocols or intensities impact QoL[44]. While current exercise recommendations for cancer survivors prescribe low to moderate intensity exercise, evidence is accumulating that there may be greater improvements in QoL when participating in more vigorous exercise[11]. In most cases QoL changes are monitored from cancer diagnosis, to inform clinical decisions to improve patient outcomes. Linking exercise in with this conversation may assist clinicians in improving healthy behaviours to improve QoL[53].

White blood cell count was significantly reduced from baseline to post-exercise intervention in the exercise groups, with the most pronounced reduction seen in the LVHIIT group, with a small effect found. Lung cancer participants who participated in a program using resistance band exercise over 12 wk in conjunction with chemotherapy treatment showed similar results[54] Normal WBC levels in cancer survivors are important because high levels of WBC's are associated with chronic inflammation, autonomic nervous system imbalances and may contribute to reductions in insulin sensitivity[55]. Similarly, low WBC counts compromises an already compromised patient. Potentially both these factors would contribute to fatigue, obesity and increased risk for CVD[56,57]. To date, little work has been carried out to determine the mechanism involved in the changes in WBC's in cancer survivors after different exercise programs[57]. Insulin levels decreased in the LVHIIT group with a small effect and increased in the CLMIT group by 13.74% with an unclear effect. These results were not significant, yet they are potentially clinically relevant findings[58]. Even a small decrease in insulin is essential in this population because of its reported role in the growth of cells, including the potential growth of tumour cells[58-60].

In the current study, a moderate effect was found in the LVHIIT group for CSP and PP with unclear effects in the CLMIT and control groups. The importance of these changes relates to the negative impact of chemotherapy drugs on the cardiovascular system. Chemotherapy drugs have been found to have a long-lasting anti-angiogenic effect on the cardiovascular system[61]. Specifically, chemotherapy has been shown to negatively impact vessel stiffness and CDP during the treatment period, with increases in BP remaining long after the treatment period[61]. Anthracyclines, cyclophosphamide and tyrosine kinase inhibitors have all been shown to increase oxidative stress[62,63]. Increases in oxidative stress can cause an overproduction of cytokines which damage the vessel wall causing endothelial dysfunction[64-66]. Endothelial dysfunction can also be caused by radiotherapy; with the adverse effects potentially having a lifelong impact on cancer survivors[65,67]. Little is known about the impact of exercise on endothelial dysfunction in cancer survivors. The current study suggests that exercise-induced cardiovascular improvements may be one mechanism whereby exercise diminishes the adverse effects that chemotherapy has on the cardiovascular system.

Limitations of this investigation were that diet; and

daily physical activity levels were not controlled for in the time periods between study sessions. Although 6MWT has been shown to be a valid and reliable measure of fitness in cancer survivors, a more robust cardiorespiratory fitness test such as VO_2 max testing could be used. There were no male participants in this study, and as such the data should be interpreted accordingly. Participants were prescribed different ongoing medications and treatment protocols, all of which could have impacted the results; this does however represent a real world group of cancer survivors.

In conclusion, this study shows promise for the use of LVHIIT in the cancer population. The encouraging results opens up the possibility of introducing LVHIIT in therapy programs, as a shorter and more efficacious exercise to increase the fitness levels in cancer survivors. The LVHIIT protocol improved fitness and functional capacity and decreased waist circumference compared with CLMIT. Both LVHIIT and CLMIT improved QoL. LVHIIT may be an effective alternative to traditional exercise prescription within this population. The benefit of LVHIIT is that for selected variables it produces more pronounced results compared with CMIT and it is short in duration which could entice more cancer survivors to participate in exercise as time is a barrier[68]. This study highlights that the most commonly prescribed CLMIT may not be enough to induce clinically relevant changes in cancer survivors. Additional research is required to fully understand the mechanisms involved in the changes identified in this study in relation to different doses of exercise. This research would be highly beneficial to assist clinicians in the optimisation of clinical exercise recommendations for cancer survivors.

ARTICLE HIGHLIGHTS

Research background

Research into the optimum exercise guidelines for cancer survivors are not conclusive. Little evidence exists for the use of low-volume high-intensity interval training (LVHIIT) within the cancer population, even though it shows promise in other chronic populations. LVHIIT has been used in populations such as stroke, diabetes, cardiovascular disease (CVD), cardiac rehabilitation showing more pronounced health benefits than the more commonly prescribed continuous low-moderate intensity training (CLMIT). Therefore, it should be further investigated for use with cancer survivors as it is a time efficient exercise modality, with greater health benefits.

Research motivation

Using LVHIIT in the cancer population shows promise as a more efficient exercise prescription. The encouraging results of this study has opens the possibility of introducing LVHIIT into rehabilitation programs. LVHIIT is a time efficient exercise modality, which could be used to increase the fitness levels in cancer survivors. The LVHIIT protocol in this study improved fitness and functional capacity and decreased waist circumference compared with CLMIT and the control group. Both LVHIIT and CLMIT improved QoL. LVHIIT may be an effective alternative to traditional exercise prescription within this population. The benefit of LVHIIT is that for selected variables it produces more pronounced results compared with CMIT and it is short, which could entice more cancer survivors to participate in exercise as time is a barrier.

Research objectives

To determine the effectiveness of LVHIIT compared to CLMIT and a control group and to determine if LVHIIT and CLMIT improved CVD risk and health outcomes in cancer survivors. The significance of these objectives is that this form of exercise can be used to achieve more pronounced improvements in health outcomes than the commonly prescribed CLMIT.

Research methods

The experiments and data analysis used in this study were a mix of validated methods used before within this population (6MWT, STS, DXA, hip/waist circumference) and unique protocols which have been used with other populations, but not commonly with cancer survivors (Sphygmocor). The use of the ANCOVA analysis and effect size analysis was chosen due to the robustness that this analysis provides for the data collected.

Research results

There were significant improvements in functional capacity, specifically cardiorespiratory fitness, lower-limb strength, and waist circumference in participants who completed the LVHIIT compared with the CLMIT and control groups. QoL improved in both the LVHIIT group and the CLMIT group when compared with the control group, however a greater effect was observed in the LVHIIT group. Additional research is required to fully understand the mechanisms involved in the changes identified in this study in relation to different doses of exercise. This research would be highly beneficial to assist clinicians in the optimisation of clinical exercise recommendations for cancer survivors.

Research conclusions

Present exercise guidelines for cancer survivors lack detail on the type, mode, duration and intensity of exercise necessary to achieve best outcomes. This current research was required to fill the gaps in current knowledge to further improve exercise recommendations. LVHIIT is the use of small doses of high-intensity exercise to elicit physiological responses such as improved VO_2 max and positive metabolic changes in skeletal muscle which seem greater than the commonly prescribed CLMIT. The LVHIIT physiological changes show potential for use in clinical practice in the rehabilitation of cancer survivors. At present there is limited research examining the effects of LVHIIT in improving health outcomes for cancer survivors. This study shows promise for the use of LVHIIT in the cancer population. The encouraging results opens up the possibility of introducing LVHIIT in therapy programs, as a shorter and more efficacious exercise to increase the fitness levels in cancer survivors. The benefit of LVHIIT is that for selected variables it produces more pronounced results compared with CMIT and it is short in duration which could entice more cancer survivors to participate in exercise. This study highlights that the most commonly prescribed CLMIT may not be enough to induce clinically relevant changes in cancer survivors.

Research perspectives

The use of VO2 max testing would be beneficial if it can be tolerated well by cancer survivors post treatment. Future research should analyse ways to measure the mechanisms involved in the changes seen in the study results and clarify how and why these changes occur.

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ORIGINAL ARTICLE

Observational Study

Palliative surgery for Krukenberg tumors – 12-year experience and review of the literature

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Abstract

AIM

To determine the clinical characteristics of patients undergoing palliative surgery for Krukenberg tumors, including disease presentation, outcomes, and prognostic factors.

METHODS

This was a retrospective clinical study of all patients who underwent palliative surgery for Krukenberg tumors between January 2004 and December 2015. Patient information was obtained from inpatient and outpatient case notes as well as the hospital electronic records. Patients who underwent potentially curative resection, and patients with Krukenberg tumors who did not undergo surgery were also excluded from the study. Palliative surgery was defined as those performed for either alleviation of symptoms or for asymptomatic patients for whom surgical removal of the tumors were deemed necessary following a multidisciplinary consensus. Tumors were diagnosed pre-operatively by computed tomography

scans and all had histologic confirmation of the surgical specimens.

RESULTS

Over the study duration, 38 female patients underwent palliative surgery for Krukenberg tumors at our institution. Mean age was 54.2 ± 11.7 years. The colon was the most frequent primary source of metastases (n = 21) followed by the stomach (n = 4). Prophylactic palliative surgery was performed for eight (21.1%) asymptomatic patients. Median post-operative length of stay was 8 d (IQR 6-12 d). Five patients (13.2%) experienced post-operative complications, although high grade morbidity was only seen in one patient (2.6%). Median overall survival from surgery was 17 mo (95%CI: 12.1-21.9) at a median follow-up duration of 12 mo (IQR 8-17 mo). The median survival was shorter for patients who underwent emergency surgery, younger patients, those with a colorectal primary, larger tumors, or synchronous peritoneal or hepatic metastases.

CONCLUSION

Palliative surgery for Krukenberg tumors can be performed safely with acceptable complication rates. Bilateral oophorectomy should be performed to prevent the risk of symptomatic contralateral tumors.

Key words: Krukenberg tumor; Palliative surgery

Core tip: Krukenberg tumors represent metastases to the ovary and convey a poor prognosis. By reporting our 12-year experience with palliative surgery for Krukenberg tumors and discussing the existing literature, we hope to shed light and establish best practices on the approach to management of the disease. Palliative surgery for patients with Krukenberg tumors can be performed safely in an experienced unit with acceptable complication rates. Where possible, bilateral oophorectomy should be performed to obviate the risk of the contralateral ovary developing symptomatic tumors. Appropriate selection by a multidisciplinary consensus is essential for asymptomatic patients who may benefit from prophylactic surgery.

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INTRODUCTION

A Krukenberg tumor is a rare ovarian tumor which has metastasized from a primary site, accounting for 1%-2% of all tumors of the ovary[1]. The stomach was previously described to be the most common primary site, followed by the colon, appendix and breast[1]. Recent literature reveals an increased incidence of tumors originating from the colon[2]. Compared with primary ovarian tumors, the prognosis of patients with Krukenberg tumors is bleak, even with metastatic disease confined to the ovaries.

Various studies have been published over the past decade with regards to the prognostic factors and outcomes of surgery for Krukenberg tumors. Metastasectomy, or surgical removal of one or both involved ovaries, has been found to improve overall survival[3-5]. Cytoreductive surgery incorporating Krukenberg tumor removal has also been found to have a beneficial effect, with a 7% overall 5-year survival[6]. However, extensive cytoreductive surgery may have significant associated morbidity and mortality. To our knowledge, no previous study focuses exclusively on the group of patients for whom surgery is considered palliative.

Here we discuss the existing literature and report our experience with palliative surgery for Krukenberg tumors, including clinical characteristics of patients, disease presentation, surgical outcomes, safety, and prognostic factors.

MATERIALS AND METHODS

Data was collected for all patients who underwent surgery for Krukenberg tumors between January 2004 and December 2015. Patient information was obtained from inpatient and outpatient case notes as well as the hospital electronic records. Protocols were approved by hospital Institutional Review Board and the data was analyzed retrospectively. Patients with ovarian metastases who underwent potentially curative resection were excluded from the study. Patients with Krukenberg tumors who did not undergo surgery were also excluded from the study.

Over this period, surgery for Krukenberg tumors was performed by experienced surgeons from 3 departments within the same institution; General Surgery, Surgical Oncology, as well as Obstetrics and Gynaecology. The diagnosis of metastatic disease was made pre-operatively at a multidisciplinary tumor board meeting in 37 cases and post-operatively in one patient who underwent emergency surgery. Most Krukenberg tumors were diagnosed pre-operatively by computed tomography (CT) scans and all had histologic confirmation based on the pathological evaluation of the surgical specimens.

Palliative surgery in our series was defined as those performed for either alleviation of symptoms caused by the Krukenberg tumor (*e.g.*, abdominal pain, distension or obstruction), or for asymptomatic patients for whom surgical removal of the tumors were deemed necessary following a multidisciplinary consensus.

Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, United States). Survival was defined as the time from the date of surgery for the Krukenberg tumor to the date of death. Patients in whom the event of death had not occurred

Table 1 Patient characteristics *n* (%)

Variable	Frequency
Age	
⩾ 50	25 (65.8)
< 50	13 (34.2)
Race	
Chinese	33 (86.8)
Malay	3 (7.9)
Others	2 (5.3)
Presenting symptoms	
Pain	15 (39.5)
Distension	9 (23.7)
Intestinal obstruction	3 (7.9)
Obstructive symptoms	3 (7.9)
Asymptomatic	8 (21.1)
ASA score at KT surgery	
2	30 (78.9)
3	8 (21.1)

SD: Standard deviation; ASA: American Society of Anesthesiologists; KT: Krukenberg tumour.

at the time of this study were censored. Survival rates were calculated using the Kaplan-Meier method and the differences in median survival times between groups assessed using the log-rank test. Cox proportional hazards model was used to determine the hazard ratios of variables affecting survival. $P < 0.05$ was taken as significant.

RESULTS

Patient and disease characteristics

Between January 2004 and December 2015, 38 female patients underwent palliative surgery for Krukenberg tumors at our institution. Patient and disease characteristics are summarised in Tables 1 and 2 respectively. Mean patient age was 54.2 ± 11.7 years. Almost 40% of the patients with Krukenberg tumors presented with abdominal pain or had pain as their most significant symptom. Median tumor size was 11.8 cm [Interquartile range (IQR) 7.8–16.0 cm]. Apart from the ovaries, all patients had at least one other site of metastases, mostly commonly the peritoneum or liver. The colon was the most frequent primary source of metastases followed by the stomach. In one patient, the primary tumor was not identified despite extensive investigation. The Krukenberg tumor was diagnosed during the same setting as the primary in just under half of the patients. Amongst the patients who had ovarian metastases detected metachronously, the median duration of diagnosis of the Krukenberg tumor from the primary was 19.5 mo (IQR 9-24 mo).

Twenty-six patients (68.4%) had undergone chemotherapy prior to Krukenberg tumor surgery and of these, 19 continued post-operative palliative chemotherapy. A total of 27 patients (71.1%) received chemotherapy post-operatively, including eight patients who commenced chemotherapy for the first time after surgery. Four patients (10.5%) had never received chemotherapy.

Table 2 Disease characteristics *n* (%)

Variable	Frequency
Site of primary	
Stomach	4 (10.5)
Colon	21 (55.3)
Rectum	1 (2.6)
Pancreas	3 (7.9)
Breast	2 (5.3)
Peritoneum	2 (5.3)
Appendix	1 (2.6)
Endometrium	1 (2.6)
Bladder	1 (2.6)
Lung	1 (2.6)
Unknown	1 (2.6)
Previous surgery for primary	
Yes	17 (44.7)
No	21 (55.3)
Detection of KT and primary	
Synchronous	18 (47.4)
Metachronous	20 (52.6)
KT size	
⩾ 12 cm	19 (50.0)
< 12 cm	19 (50.0)
Ovaries involved	
Unilateral	20 (52.6)
Bilateral	18 (47.4)
Peritoneal metastases	
Yes	21 (55.3)
No	17 (44.7)
Hepatic metastases	
Yes	16 (42.1)
No	22 (57.9)

KT: Krukenberg tumour; SD: Standard deviation.

Surgical characteristics

Surgical procedures performed were; a unilateral or bilateral salphingo-opherectomy alone, or a total hysterectomy in addition to a bilateral salphingo-opherectomy (Table 3). Two other patients underwent additional surgical procedures during the same sitting as the Krukenberg tumor surgery; one patient had a small bowel resection while the other had a right hemicolectomy performed. Prophylactic palliative surgery was performed for eight asymptomatic patients; two were performed for histological confirmation of metastases to guide subsequent treatment, two were for progression of the Krukenberg tumors while on chemotherapy despite adequate extra-ovarian disease control, and four were prophylactic for sizeable tumors which would have likely caused symptoms during the patient's lifetime. Five patients underwent emergent surgery; two for ruptured Krukenberg tumors, the third for a patient with fever and abdominal pain whose pre-operative CT scan was incorrectly reported as tubo-ovarian abscesses, the fourth for ovarian torsion, and in the last patient for acute intestinal obstruction secondary to compression of the bowel from the Krukenberg tumor.

Peri-operative morbidity

Median post-operative length of stay was 8 d (IQR 6-12 d). The majority of patients had an uneventful recovery period (86.8%). Five patients (13.2%) suffered complications; two Clavien-Dindo grade Ⅰ, two grade

Table 3 Surgery characteristics *n* (%)

Variable	Frequency
Urgency	
Elective	33 (86.8)
Emergency	5 (13.2)
Pre-KT surgery chemo	
Yes	26 (68.4)
No	12 (31.6)
Surgery	
USO	10 (26.3)
BSO	12 (31.6)
THBSO	16 (42.1)
Post-op clavien-dindo score	
Grade 0	33 (86.8)
Grade Ⅰ	2 (5.3)
Grade Ⅱ	2 (5.3)
Grade Ⅲa	1 (2.6)

SD: Standard deviation; ASA: American Society of Anesthesiologists; KT: Krukenberg tumour; USO: Unilateral salphingo-opherectomy; BSO: Bilateral salphingo-opherectomy; THBSO: Total hysterectomy and bilateral salphingo-opherectomy.

Table 4 Univariate analyses of variables affecting survival

Variable	HR (95%CI)	*P*-value
Age < 50	2.169 (0.876-5.369)	0.094
ASA score 3	2.830 (0.873-9.171)	0.083
Colorectal primary	1.089 (0.424-2.795)	0.860
Previous surgery for primary	0.894 (0.359-2.226)	0.810
Synchronous lesion	1.240 (0.497-3.096)	0.645
Size ⩾ 12 cm	1.106 (0.424-2.884)	0.837
Bilateral disease	1.473 (0.597-3.636)	0.400
Peritoneal metastases	1.337 (0.509-3.511)	0.555
Hepatic metastases	1.055 (0.419-2.656)	0.909
Emergency surgery	3.382 (1.051-10.89)	0.027
Pre-op chemotherapy	1.575 (0.521-4.758)	0.421
Serum CA 125 > 35 kU/L	0.750 (0.187-3.004)	0.684
Serum CEA ⩾ 30 μg/L	0.785 (0.234-2.626)[1]	0.694[a]

[1]Among patients with a colorectal primary only. HR: Hazard ratio; CI: Confidence interval; ASA: American Society of Anesthesiologists; CEA: Carcinoembryonic antigen.

Ⅱ, and one grade Ⅲa (Table 3). Grade Ⅰ complications included a urinary tract infection requiring antibiotics and supraventricular tachycardia which aborted with medication. Both grade Ⅱ complications were cases of post-operative ileus requiring total parenteral nutrition. In these two patients, peritoneal carcinomatosis was thought to be a likely contributory factor. The grade Ⅲa complication was a post-operative intraabdominal collection requiring radiological guided drainage. This patient had undergone emergency surgery for a large ruptured Krukenberg tumor.

Survival

The median follow-up duration was 12 mo (IQR 8-17 mo). Two patients were lost to follow-up and one patient returned to her country of origin for continued care shortly after discharge. Twenty patients (52.6%) died during the follow-up period. Median overall survival (OS) from Krukenberg tumor surgery was 17 mo (95%CI: 12.1-21.9) (Figure 1). Based on univariate analysis (Table 4), only emergency surgery was associated with a significantly worse survival outcome. The median survival for patients with a colorectal primary compared to all other origins was 15 mo (95%CI: 10.5-19.5) and 38 mo respectively (95%CI: 5.8-70.2), $P = 0.845$. The median OS was also shorter for patients younger than 50 years old (13 mo, 95%CI: 8.0-18.0 *vs* 20 mo, 95%CI: 16.4-23.6), patients with a pre-operative American Society of Anaesthesiologists (ASA) score of 3 *vs* 2 (8 mo, 95%CI: 4.5-12.1 *vs* 29 mo, 95%CI: 18.6-40.1), larger tumors ⩾ 12 cm (14 mo, 95%CI: 9.4-18.6 *vs* 20 mo, 95%CI: 14.8-25.2), those with peritoneal metastases (17 mo, 95%CI: 11.3-22.7 *vs* 20 mo, 95%CI: 9.7-30.3) and those with hepatic metastases (14 mo, 95%CI: 8.4-19.6 *vs* 17 mo, 95%CI: 9.8-24.2).

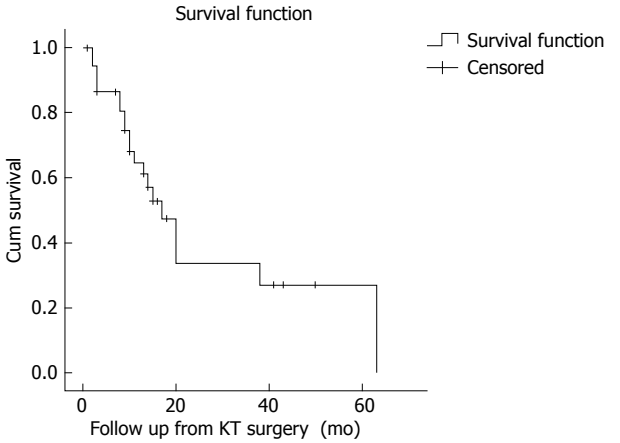

Figure 1 Kaplan-Meier survival analysis of 38 patients who underwent palliative surgery for Krukenberg tumours.

Pre-operative serum tumor marker level did not correlate with OS. Serum CA 125 level was performed in 21 patients (55.3%) before Krukenberg tumor surgery, for which median CA 125 level was 42.4 kU/L (IQR 14.1-206.5). Pre-operative serum carcinoembryonic antigen (CEA) testing was done in 30 patients (78.9%), including 20 out of 21 patients who had a colorectal primary. For these 20 patients, the median CEA level was 30.3 μg/L (IQR 10.8-144.0). Using a cut-off value of 35 and 30 for CA 125 and CEA respectively (Table 4), it appeared that the pre-operative values of these tumor markers did not reflect patient prognosis. These values were chosen based on the normal reference range of CA 125 (0-35 kU/L) and the median CEA level amongst patients with a colorectal primary in our study.

DISCUSSION

The eponymous Krukenberg tumor was first described in 1896 by the German doctor Friedrich Krukenberg (1871-1946). Representing advanced metastatic disease, this entity clearly conveys a poor prognosis. Gastric cancer was previously reported to account for

the majority of primary tumors responsible[7]. However, recent literature suggests an increased frequency of Krukenberg tumors with a colorectal origin[2]. In our study, more than half of the patients had a colorectal primary compared to only 10% with a gastric primary. This is likely related to the increasing frequency of colorectal cancer locally as well as in other developed countries. In Singapore, the age-standardized incidence rate of colorectal cancer is one of the highest among ethnic Chinese populations in the world[8]. With the longer survival generally seen in patients with metastatic colorectal cancer as compared with that of gastric cancer, the option of pursing palliative surgery in these patients may be seemingly more attractive.

Many aspects of Krukenberg tumors still remain controversial, including the mechanism of metastases or even the pathologic tumor characteristics used for diagnosis. The current criteria used by the World Health Organisation, established in 1973 by Serov and Scully[9], may not adequately reflect the complexity of the tumor considering the multiple different primary sites possible. Treatment generally consists of surgery, chemotherapy or radiotherapy but guidelines concerning treatment of choice and appropriate timing of intervention have yet to be established.

Over the past decade, a number of retrospective studies have attempted to determine the prognostic factors for patients with Krukenberg tumors. Current evidence suggests a survival benefit for patients who undergo metastasectomy, compared to those without surgery. A recent multivariate analysis of 128 patients with Krukenberg tumors (58 colorectal, 41 gastric origin) showed that synchronous tumors, pelvic invasion and ascites were independent factors predicting for a poorer overall survival[10]. Other factors found to negatively influence overall prognosis include R1 or suboptimal resection[3], metastatic disease beyond the ovaries[11], tumors of gastric origin compared to colorectal origin[2], and patients with poorer Karnofsky performance status scores[4]. For gastric cancer, metastasectomy in addition to chemotherapy was also found to improve survival compared to palliative chemotherapy alone[12]. For colorectal cancers, ovarian metastases have been shown to be less responsive to chemotherapy compared to extra-ovarian sites[13]. Surgical resection was therefore recommended for these "metastatic sanctuaries" even in the palliative setting, as they would often progress and result in symptoms while on chemotherapy. In a separate study comparing 83 patients with ovarian metastases from a colorectal primary who underwent an oophorectomy *vs* 47 historical controls who did not undergo surgery, metastasectomy conferred a significantly longer OS at 20.8 mo *vs* 10.9 mo[14]. In terms of overall survival, surgery for patients with a colorectal primary may confer a greater advantage over that for a gastric primary in view of less aggressive tumor biology.

In contrast to the usually straightforward oopherectomy, the operative circumstances for patients with Krukenberg tumors may be more challenging. These patients often have undergone previous surgeries with resultant intraabdominal adhesions, or have synchronous pelvic peritoneal disease. Therefore, while the benefit of potentially curative metastasectomy is clear, clinicians may be hesitant to subject patients to palliative surgery, particularly if the patient is asymptomatic or in a debilitated state. In our series, five patients (13.2%) experienced post-operative complications, although high grade morbidity was only seen in one patient (2.6%). Both patients with ileus resumed enteral feeding within two weeks and were discharged well. The 30-d mortality rate was zero and median length of stay was about a week.

Close to half of all patients were found to have bilateral ovarian disease. Of these, the diagnosis of contralateral ovarian involvement was not apparent on pre-operative imaging and only established following surgery and histological examination of the resected specimen in a quarter of the cases (n = 4). Even if not synchronously affected there is a high chance of subsequent contralateral ovary involvement resulting in symptoms. We therefore recommend that bilateral ovarian resection be routine if surgery is to be performed for palliation.

Following our analyses, emergency surgery was the only factor that was found to significantly reduce survival prognosis. Although patients who were less than 50 years old, had a higher ASA score, with tumor size larger than 12 cm, or had hepatic or peritoneal metastases tended to have a shorter median survival, this was not found to be statistically significant. Surprisingly, tumors with a colorectal primary tended to fare worse than tumors of all other origins with a median overall survival of 15 mo *vs* 38 mo. Meaningful comparisons could not be performed for patients with a gastric primary in view of the small sample size (n = 4) with one patient defaulting follow-up and the other continuing further management overseas. While a single study found that a pre-operative CA 125 level of more than 75 kU/L significantly correlated to poorer survival[15], in our series no association could be found with this value nor the reference range cut-off. Pre- and post-operative trending of CA 125 levels therefore remains of questionable utility. CEA levels do not appear to prognosticate survival in patients with a colorectal primary, but may serve as an adjunct to imaging for reflecting disease response to chemotherapy.

Over a fifth of the patients in our series were asymptomatic at the time of surgery, all of whom had uncomplicated post-surgery recovery periods. Appropriate patient selection for prophylactic surgery is essential, with patient fitness and prognosis along with tumor size, location and anticipated development of symptoms being key considerations. With emergency surgery conferring a significantly worse outcome, the role of prophylactic surgery should certainly be explored further. The possibility of Krukenberg tumors being chemo-resistant "metastatic sanctuaries" was also evident in two asymptomatic patients in our study who

had progression of the ovarian tumors despite good systemic control of disease elsewhere.

In conclusion, Palliative surgery for patients with Krukenberg tumors can be performed safely in an experienced unit with acceptable complication rates. The decision to proceed with metastasectomy is influenced by several factors including the presence of symptoms, synchronous disease, and tumor response to chemotherapy, and should be made as part of a multidisciplinary team consensus. Where possible, bilateral oophorectomy should be performed to obviate the significant risk of symptomatic contralateral ovarian involvement. Tumor markers need not be routinely trended peri-operatively. Proper selection is essential for asymptomatic patients who may benefit from prophylactic surgery. Further studies can be done to determine if symptom-free survival can be prolonged or quality of life improved with palliative surgery.

ARTICLE HIGHLIGHTS

Research background

A Krukenberg tumor is a rare ovarian tumor which has metastasized from a primary site, accounting for 1%-2% of all tumors of the ovary, and conveys a poor prognosis even with disease confirmed to the ovaries. A handful of studies have been published over the past decade with regards to the prognostic factors and outcomes of surgery for Krukenberg tumors, but no previous study focuses exclusively on the group of patients for whom surgery is considered palliative.

Research motivation

Many aspects of Krukenberg tumors still remain controversial, and guidelines concerning treatment of choice and appropriate timing of intervention have yet to be established. We report our experience with palliative resection of Krukenberg tumors over the past 12 years and discuss the existing literature, so as to shed light and establish best practices on the approach to management of the disease.

Research objectives

We aimed to determine the clinical characteristics of patients undergoing palliative surgery for Krukenberg tumors, including disease presentation, outcomes, and prognostic factors.

Research methods

This was a retrospective clinical study of all patients who underwent palliative surgery for Krukenberg tumors between January 2004 and December 2015. Patient information was obtained from inpatient and outpatient case notes as well as the hospital electronic records. Patients who underwent potentially curative resection, and patients with Krukenberg tumors who did not undergo surgery were also excluded from the study. Palliative surgery was defined as those performed for either alleviation of symptoms or for asymptomatic patients for whom surgical removal of the tumors were deemed necessary following a multidisciplinary consensus. Tumors were diagnosed pre-operatively by computed tomography scans and all had histologic confirmation of the surgical specimens.

Research results

Over the study duration, 38 female patients underwent palliative surgery for Krukenberg tumors at our institution. Mean age was 54.2 ± 11.7 years. The colon was the most frequent primary source of metastases (n = 21) followed by the stomach (n = 4). Prophylactic palliative surgery was performed for eight (21.1%) asymptomatic patients. Median post-operative length of stay was 8 d (IQR 6-12 d). Five patients (13.2%) experienced post-operative complications, although high grade morbidity was only seen in one patient (2.6%). Median overall survival from surgery was 17 mo (95%CI: 12.1-21.9) at a median follow-up duration of 12 mo (IQR 8-17 mo). The median survival was shorter for patients who underwent emergency surgery, younger patients, those with a colorectal primary, larger tumors, or synchronous peritoneal or hepatic metastases.

Research conclusions

Palliative surgery for patients with Krukenberg tumors can be performed safely in an experienced unit with acceptable complication rates. The decision to proceed with metastasectomy is influenced by several factors including the presence of symptoms, synchronous disease, and tumor response to chemotherapy, and should be made as part of a multidisciplinary team consensus. Where possible, bilateral oophorectomy should be performed to obviate the significant risk of symptomatic contralateral ovarian involvement. Tumor markers need not be routinely trended peri-operatively. Proper selection is essential for asymptomatic patients who may benefit from prophylactic surgery.

Research perspectives

Further studies can be done to determine if symptom-free survival can be prolonged or quality of life improved with palliative surgery.

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CASE REPORT

Yttrium-90 microsphere selective internal radiation therapy for liver metastases following systemic chemotherapy and surgical resection for metastatic adrenocortical carcinoma

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Author contributions: Makary MS, Krishner LS, Wuthrick EJ, Bloomston MP, and Dowell JD performed the research and analyzed the data; Makary MS and Dowell JD wrote the paper.

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Abstract

Adrenocortical carcinoma (ACC) is a rare malignancy with generally poor outcomes and limited treatment options. While surgical resection can be curative for early local disease, most patients present with advanced ACC owing to nonspecific symptoms. For those patients, treatment options include systemic chemotherapy and locoregional therapies including radiofrequency ablation and transarterial chemoembolization. We present the first reported case of utilizing yttrium-90 microsphere selective internal radiation therapy (SIRT) in combination with first line EDP-M (Etoposide, Doxorubicin, Cisplatin, Mitotane) chemotherapy and debulking surgical primary tumor resection for treatment of metastatic ACC. Stable complete radiologic response has been maintained after twelve months with resolution of clinical symptoms. These findings prompt the need for further consideration and studies to elucidate the role of SIRT in combination with systemic and surgical treatment for metastatic ACC.

Key words: Adrenocortical carcinoma; Hepatic metastases; Radioembolization; Yttrium-90

Core tip: Adrenocortical carcinoma (ACC) is a rare malignancy with generally poor outcomes and limited treatment options. Approximately 70% of ACC patients have unresectable stage Ⅲ or Ⅳ disease on initial presentation. Yttrium-90 microsphere selective internal radiation therapy was applied toward hepatic metastases for a patient with metastatic ACC in combination with first line chemotherapy and debulking surgical primary tumor resection. A stable complete radiologic response has been maintained for twelve months with resolution of clinical symptoms.

Makary MS, Krishner LS, Wuthrick EJ, Bloomston MP, Dowell JD. Yttrium-90 microsphere selective internal radiation therapy for liver metastases following systemic chemotherapy and surgical resection for metastatic adrenocortical carcinoma. *World J Clin Oncol* 2018; 9(1): 20-25 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i1/20.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i1.20

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare malignancy that constitutes less than 5% of all adrenal incidentalomas and has an estimated annual incidence of 1-2 cases per million people[1-3]. ACC carries a poor prognosis with an overall 5-year survival of 16%-44%[4-6]. Surgical resection is the treatment of choice with those presenting with only local disease. However, most patients present with advanced disease and are not surgical candidates, and 80% of even those patients with local disease treated surgically develop local or distant recurrence following complete resection[7]. Indeed, given the nonspecific ACC symptoms, approximately 70% of ACC patients have unresectable stage Ⅲ or Ⅳ disease on initial presentation[8]. In those cases, treatment options include mitotane, systemic chemotherapy, external beam radiotherapy, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), or a combination of these therapies. In this case study, we present a complete radiologic and clinical response to yttrium-90 (Y-90) microsphere selective internal radiation therapy (SIRT) treatment of hepatic metastases in conjunction with surgery and chemotherapy for treatment of a patient with advanced disease. A critical review of the current therapies and treatment strategies is also presented.

CASE REPORT

A 60-year-old female patient with a past medical history significant only for poorly controlled hypertension despite medical therapy presented with a large left adrenal mass incidentally discovered on MRA during workup for renal artery stenosis. Abdominal MR imaging revealed a heterogeneous, partially necrotic left adrenal mass measuring 5.6 cm × 8.9 cm as well as multiple bilobar enhancing liver metastases. Histologic (Figure 1A) and immunologic evaluation of a biopsied hepatic lesion showed metastatic carcinoma positive for inhibin (Figure 1B), melan-A (Figure 1C), and CKAE1/3 (Figure 1D) with an immunohistochemical staining profile consistent with metastatic ACC. Serology values revealed normal urine cortisol [14.1 mcg/24 h (normal range: 3-22)], low adrenocorticotropic hormone (ACTH) [5.1 pg/mL (normal range: 9-50)], normal plasma metanephrines [0.20 nmol/L (normal range < 0.50)] and high aldosterone [38.8 ng/dL (normal range: 1-16)].

The Berruti chemotherapy regimen of Etoposide, Doxorubicin and Cisplatin plus Mitotane (EDP-M) was immediately initiated[9] for a total of five 28-d cycles. Subsequent abdominal MRI and PET/CT evaluation demonstrated overall improvement in metastatic disease burden with interval decrease in size of the left adrenal mass. Given the response to chemotherapy and following a multidisciplinary tumor board discussion, decision was made to pursue an open adrenalectomy debulking surgery two months following chemotherapy completion to reduce the hormonal load for clinical symptomatic control. Intraoperatively, the entire adrenal mass was resected *en bloc* without difficulty. Adjacent paraaortic, omental, peripancreatic thickened pearly lymph nodes were resected with a clear periadrenal margin obtained. The adrenal vein and artery were identified with no tumoral involvement visualized. The patient had an uneventful post-operative hospital course and was discharged in 5 d in stable condition.

Additionally, to further control the bilobar liver metastases, decision was made to undergo two lobar Y-90 treatments using Y-90 TheraSphere microspheres (BTG, London, United Kingdom). The patient was evaluated for radioembolization with selective hepatic arteriography (Figure 2). Arterial administration of 4.1 mCi of 99mTc-MAA (Drax Image MAA kit; 99.7% purity) in the proper hepatic artery demonstrated a hepatopulmonary shunt of 0.8% on planar scintigraphy and SPECT, which is within acceptable range (< 10%). Additional pretreatment planning included evaluation with an abdominal CTA which revealed no variant vascular anatomy (Figure 2A). Based on imaging, estimated right hepatic lobe volume of 1339 ml and left hepatic lobe volume of 409 ml were used to calculate the radioactivity required to deliver a desired dose of 120 Gy to each lobe (Figure 2B). Patient underwent treatment of the right hepatic lobe (Figure 2C) followed by the left lobe within a month. Post-treatments, she tolerated the procedures well with low-grade fevers lasting for 24 h and intermittent right upper quadrant pain, both of which are common and expected following this treatment, which resolved within 2-3 wk.

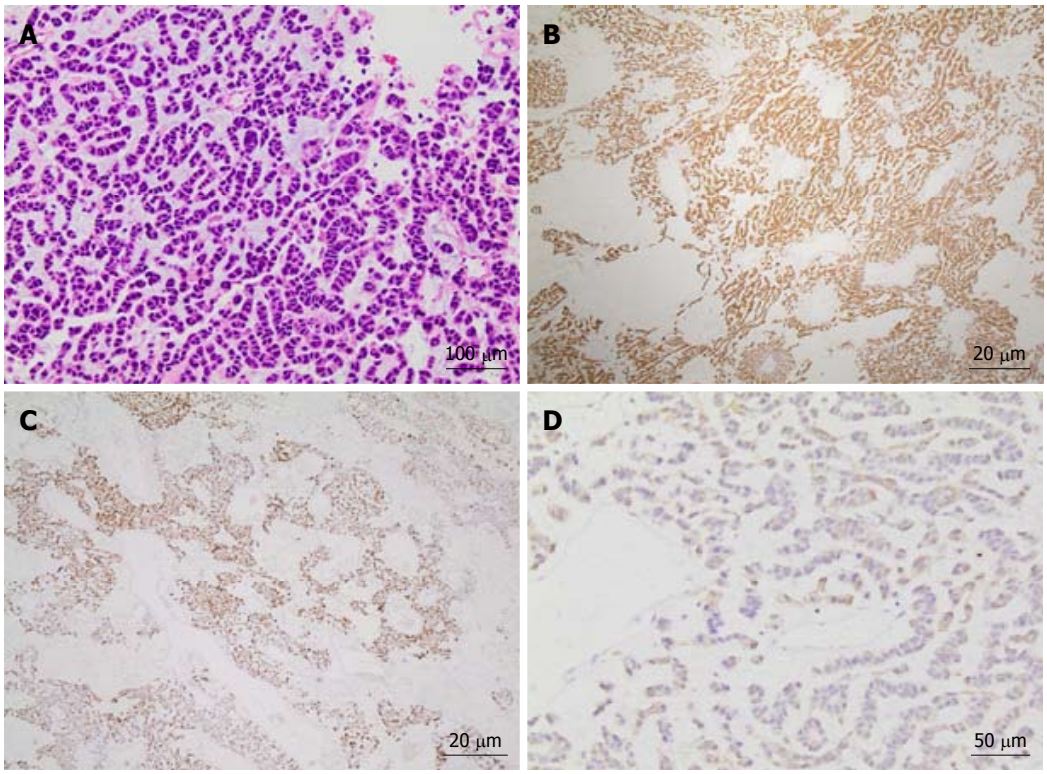

Figure 1 Tissue histopathology of a hepatic lesion confirms metastatic adrenocortical carcinoma. Hematoxylin and eosin (HE) staining (A) at 200 × magnification of tissue from the hepatic mass shows diffuse infiltrating cords of cells with hyperchromasia, eosinophilic cytoplasm, and a mild degree of nuclear pleomorphism in a background of myxoid stroma. Additional positive immunostaining with inhibin (B) and melan-A (C) at 40 × magnification as well as CKAE1/3 (D) at 100 × magnification confirms the diagnosis as adrenocortical carcinoma.

The patient tolerated the treatment well, experienced a drop in aldosterone levels (2.8 ng/dL), and has been normotensive and off of antihypertensive medication following intervention. Post Y-90 therapy imaging at 1 year revealed significantly smaller and less numerous hepatic lesions (Figure 3). Additionally, no mass was identified in the adrenal resection. Patient is currently in observation with periodic surveillance imaging and serology.

DISCUSSION

ACC is a rare malignancy but is highly lethal with limited treatment options. Given its rarity, ACC is difficult to study with most studies being retrospective case series with several key randomized trials[9-11]. In a large landmark Phase Ⅱ trial by Berruti *et al*[9] in 2005, the combination of etoposide, doxorubicin and cisplatin plus mitotane (EDP-M) in the treatment of advanced adrenocortical carcinoma demonstrated an overall response rate of 49%, a median time to progression of 9.1 mo, and an overall survival of 28.5 mo in a cohort of 72 patients[9]. A previous study by Khan *et al*[10] in 2000 reported an overall response rate of 36% and a 2- and 5-year survival of 70% and 33%, respectively, with mitotane plus streptozotocin (M-S). In the First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT) in 2012 of 304 patients, there was no significance difference in the overall survival between EDP-M *vs* M-S (14.8 mo and 12.0 mo, respectively, $P = 0.07$), but the response rates (23.2% *vs* 9.2%, $P < 0.001$) and progression-free survival (5.0 mo *vs* 2.1 mo, $P < 0.001$) were significantly better with EDP-M[11].

In addition to systemic chemotherapy, surgical resection and local treatment modalities such as RFA and TACE are key palliative treatment options. While adrenalectomy for local ACC disease can be curable and is first line therapy, surgical debulking is also often performed, as done here, in the setting of advanced metastatic disease for control of hormone excess[12]. RFA has been also used in addition to surgery or as an alternative to surgery to reduce tumor burden. TACE has been utilized previously to treat liver metastases, although its efficacy has not been assessed specifically for ACC in clinical trials. ACC patients have been rendered disease free following hepatic RFA treatment in retrospective studies, but RFA has limited efficacy in patients with bilobar and multiple lesions as in our patient, highly vascularized metastases, or metastases in proximity to large vessels due to the heat-sink effect[13,14]. In these patients, TACE, or the local intra-arterial delivery of chemotherapy to liver lesions with embolic agents, has been performed. Limited evaluation of TACE in the ACC literature demonstrates a partial response in 21% of patients and stabilization in 62%[15].

In our case, we chose to pursue Y-90 SIRT for bilobar liver disease in a patient with metastatic ACC with the

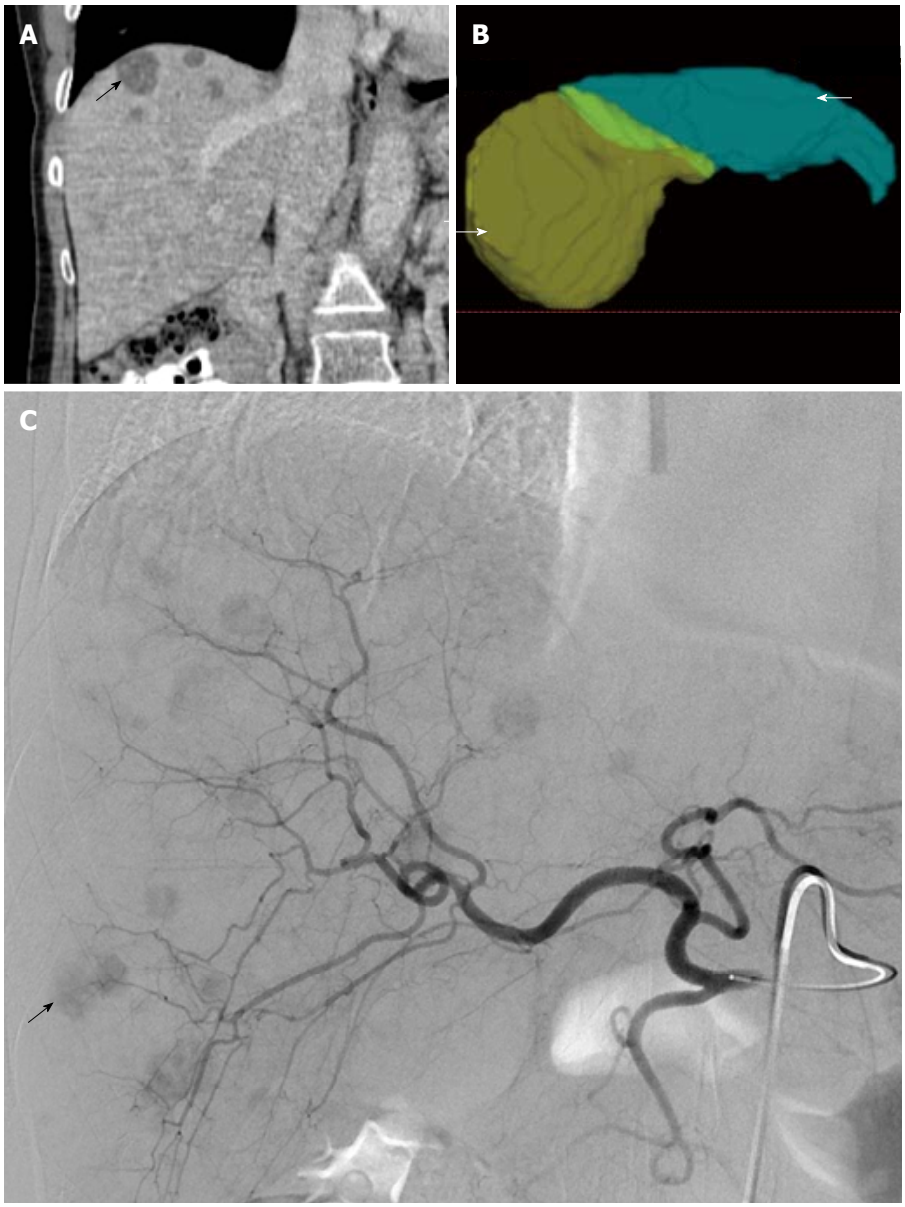

Figure 2 Yttrium-90 planning and radioembolization for treatment of liver metastases from adrenocortical carcinoma. A: Representative coronal CT abdomen image obtained prior to locoregional therapy shows low density lesions within the liver (arrow) consistent with metastatic disease; B: Three-dimensional reconstructions of the liver used for Yttrium-90 dose calculations revealed a proposed treatment volume of 409 mL for the left hepatic lobe (green colored, arrow) and 1339 mL for the right hepatic lobe (yellow colored, arrowhead) for a total hepatic volume of 1749 mL; C: Digitally subtracted contrast angiography reveals numerous hypervascular metastases within the right and left hepatic lobes (representative right hepatic lobe lesion demarked by arrow).

hopes of improving upon the outcomes previously seen with TACE. The benefit of Y-90 over TACE is that it can be delivered in an outpatient setting rather than the inpatient setting as reported previously for ACC, which offers convenience for the patients and is potentially more cost-effective[16]. While Y-90 radioembolization has been primarily studied as a locoregional therapy for hepatic metastases from colorectal carcinoma or for multifocal hepatocellular carcinoma, we present its first successful utility when coupled with surgery and systemic chemotherapy to treat a patient with advanced ACC. Indeed, following a single session to each hepatic lobe, this patient has continued to demonstrate a positive radiologic and clinical response twelve months following treatment. Similar positive outcomes were observed by Hendlisz *et al*[17] in a Phase Ⅲ trial comparing fluorouracil infusion alone or combined with Y-90 radioembolization for metastatic colorectal cancer to the liver, which revealed a longer time to progression in the Y-90 arm (5.5 mo *vs* 2.1 mo, $P = 0.003$). Cosimelli *et al*[18] reported the results of Phase Ⅱ trial showing that radioembolization alone produced meaningful response and disease stabilization in patients with advanced, unresectable and chemorefractory metastatic CRC[18]. Our findings prompt further investigation through similar trials for patients with advanced ACC.

In conclusion, this case study presents the successful treatment of metastatic ACC by combining Y-90 locoregional therapy for bilobar liver disease with first line EDP-M chemotherapy and debulking surgery. This

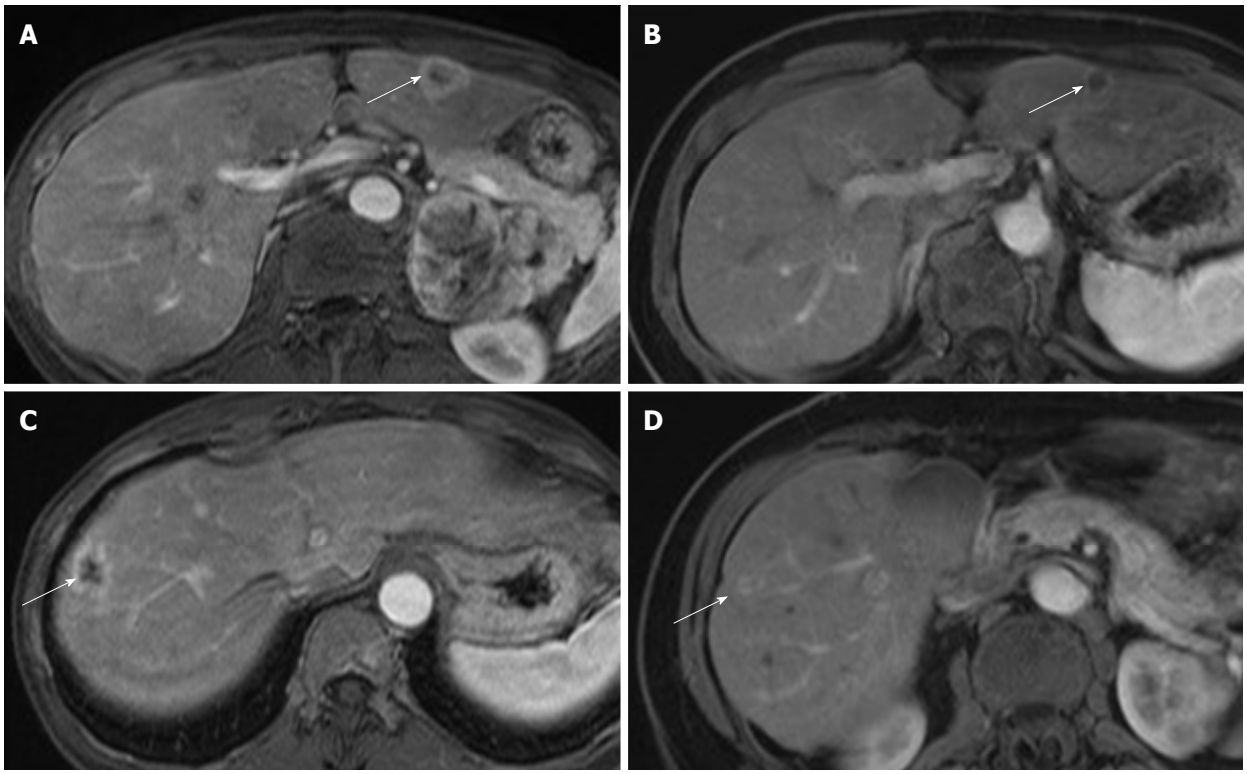

Figure 3 Hepatic metastases from adrenocortical carcinoma respond to Yttrium-90 radioembolization. Index hepatic lesions within the left hepatic lobe (A) and right hepatic lobe (C) demarked by arrows demonstrate significant reduction in size and perfusion one year following single dose treatment (B and D, respectively, arrows) by catheter-directed Yttrium-90 impregnated microspheres.

strategy allowed for disease management for at least 12 mo following a single outpatient Y-90 treatment for each hepatic lobe rather than the repeated inpatient treatments required previously through TACE with possibly superior results. Future prospective trials are needed to further elucidate the role of Y-90 radioembolization for treatment of advanced ACC.

ARTICLE HIGHLIGHTS

Case characteristics

A 60-year-old female patient with a past medical history significant only for poorly controlled hypertension despite medical therapy presented with a large left adrenal mass incidentally discovered on MRA during workup for renal artery stenosis.

Clinical diagnosis

The clinical examination of the patient revealed elevated blood pressure and abdominal pain.

Differential diagnosis

The differential diagnosis includes renal artery stenosis, adrenal pheochromcytoma, adrenocortical carcinoma, hyperaldosteronism, and hyperthyroidism.

Laboratory diagnosis

Serology values revealed normal urine cortisol [14.1 mcg/24 h (normal range: 3-22)], low adrenocorticotropic hormone (ACTH) [5.1 pg/mL (normal range: 9-50)], normal plasma metanephrines [0.20 nmol/L (normal range < 0.50)] and high aldoesterone [38.8 ng/dL (normal range: 1-16)].

Imaging diagnosis

Abdominal MR imaging revealed a heterogeneous, partially necrotic left adrenal mass measuring 5.6 cm × 8.9 cm as well as multiple bilobar enhancing liver metastases.

Pathological diagnosis

Histologic and immunologic evaluation of a biopsied hepatic lesion showed metastatic carcinoma positive for inhibin, melan-A, and CKAE1/3 with an immunohistochemical staining profile consistent with metastatic adrenocortical carcinoma.

Treatment

We present the first reported case of utilizing yttrium-90 microsphere selective internal radiation therapy (SIRT) in combination with first line EDP-M (Etoposide, Doxorubicin, Cisplatin, Mitotane) chemotherapy and debulking surgical primary tumor resection for treatment of metastatic adrenocortical carincoma.

Related reports

While surgical resection can be curative for early local disease, most patients present with advanced ACC owing to nonspecific symptoms. For those patients, previous reports studied treatment options including systemic chemotherapy and locoregional therapies including radiofrequency ablation and transarterial chemoembolization.

Term explanation

Adrenocortical carcinoma (ACC) is a rare malignancy that constitutes less than 5% of all adrenal incidentalomas and has an estimated annual incidence of 1-2 cases per million people. EDP-M (etoposide, doxorubicin and cisplatin plus mitotane) is first line chemotherapy for advanced adrenocortical carcinoma demonstrating an overall response rate of 49%, a median time to progression of 9.1 mo, and an overall survival of 28.5 mo in prior studies.

Experiences and lessons

This case study presents the successful treatment of metastatic ACC by combining Y-90 locoregional therapy for bilobar liver disease with first line EDP-M chemotherapy and debulking surgery. This strategy allowed for disease management for at least 12 mo following a single outpatient Y-90 treatment for each hepatic lobe rather than the repeated inpatient treatments required previously through TACE with possibly superior results.

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MINIREVIEWS

Cell-free DNA integrity for the monitoring of breast cancer: Future perspectives?

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Abstract

Breast cancer (BC) is the most common cancer and the second cause of death in women worldwide. Therapeutic options are increasing, but the response to treatments is not always efficient and the risk of recurrence covers decades. In this perspective, the need to have a proper follow-up for the therapeutic responses and for anticipating recurrence it is urgent in the clinical setting. Liquid biopsy provides the basic principle for a non-invasive method for the routinely monitoring of BC. However, due to the heterogeneity of tumors during onset and progression, the search for tumor DNA mutations of targeted genes in plasma/serum is a limiting factor. A possible approach overtaking this problem comes from the measurement of cell-free DNA integrity, which is an independent factor from the mutational status and theoretically is representative of all tumors. This review summarizes the state-of-the-art of cell-free DNA integrity researches in BC, the controversies and the future perspective.

Key words: cfDNA integrity; Liquid biopsy; Breast cancer; ALU sequences; LINE-1 sequences

Core tip: Despite the potentiality of cell-free DNA integrity as a useful tool for the monitoring of Breast Cancer (BC), evinced in some clinical studies, the scientific community has not reached agreeable conclusions to translate the results from the bench-to-the-bedside yet. The main controversy regards

the targets' choice and the size of circulating cell-free tumor DNA fragments. This work underlines the utility of cell-free DNA Integrity evaluation for BC follow-up and at the same time highlights the common concepts explaining the different results in line of future directions.

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INTRODUCTION

Breast cancer (BC) is still the most common cancer and the second cause of cancer-related death in women worldwide[1]. A timely knowledge of its occurrence, responsiveness to therapies and recurrence is becoming of paramount importance for clinicians to adopt specific and more efficient approaches with regards to any single patient's health assistance. In clinical routine, the evaluation of serum markers as CEA or CA15-3 is still used for BC follow-up, but with a low specificity and sensibility[2-5]. Up to now, one of the most promising frontiers in this field is the liquid biopsy. Recently, the meta-analysis on the clinical utility of circulating tumor cells (CTC) in early BC or in metastatic BC (MBC) provides a solid rationale for their use in oncological settings[6-8]. However, their routinely use is still compromised by the relatively high cost of the technique.

Circulating cell-free DNA and qPCR measurement

From the blood circulation, it is possible to derive CTC, exosomes or cell-free nucleic acids (Figure 1). Cell-free DNA (cfDNA), consists of DNA fragments released after cell death processes from both tumor and normal cells. The circulating tumor DNA (ctDNA) can be differentiated from the rest of the cfDNA by looking at tumor-specific DNA changes, including mutations, gene amplifications, rearrangements and methylations[9] proving it as a valid non-invasive biomarker to monitor tumor growth, spread, clonal evolution and response to therapies[10]. This can be achieved either by a qualitative way (*i.e.*, type of mutations) or quantitative way (*i.e.*, copy number evaluation of mutated genes). However, the known mutations that can be used in liquid biopsy represent a limited percentage of patients. As an example, the most studied *PI3KCA* mutations all together have been found in about 30%-40% of BC patients[11].

Here, both low-cost and easy-to-be-perform methods that are not bound to one or few specific genetic mutations to predict occurrence and monitor disease progression in BC patients will be described in line of what is currently known in literature.

Briefly, real-time polymerase chain reaction-or quantitative PCR (qPCR) is a powerful advancement of PCR technology that enables the measurement of the starting amount of nucleic acids in the reaction without the need for post-PCR gel analysis. This is achieved by the possibility to detect in a real-time manner the amplification process by fluorescence and to measure the amplification products of samples at exponential phases. Through this technology the expression of a target is measured by fluorescent probes or DNA-labelling dyes. Of note, the qPCR dyes do not discriminate between specific or non- specific amplicon products, thus there is a need for an accurate testing of the annealing conditions and buffer reagents to guarantee specificity of the reaction. The quantification of an unknown sample can be absolute by using an internal amplification standard curve obtained with known DNA quantities or it can be relative by comparison of the difference in cycle threshold values (Ct) of a unknown sample with respect to reference (mainly expressed as $^{\Delta\Delta}$Ct values)[12,13]. Finally, to improve the accuracy of measurements, qPCR offers, together with the basic reagents, a passive fluorescein or ROX dyes to remove well-factors. The fluorescein acts as a passive reference dye, providing sufficient background fluorescence before the amplification reaction occurs, removing in this way the well factors-such as pipetting inaccuracies and fluorescence fluctuations-from the plate with the test samples.

Quantification of total circulating cell-free DNA

Some studies have focused on the quantification of total cfDNA levels using *GAPDH*, *Beta-globin*, *Beta2-Microglobulin*, *hTERT* or *LINE-1* as potential target genes, making the higher levels of cfDNA as a way to distinguish benign from malignant BC[14–18]. Also SYBR Green's fluorescence to measure total serum cfDNA has been investigated[19]. However, in our opinion, it is worth to consider how the total cfDNA levels are susceptible to increase also by the presence of other pathological conditions (*e.g.*, infection, inflammation, *etc.*), thus influencing the results.

Quantification of cell-free DNA integrity

The detection of ctDNA levels using cell-free DNA integrity (cfDI) measurement, as ratio between longer and shorter DNA fragments, is more specific than total serum cfDNA and has been explored in BC by qPCR by many authors using SYBRGreen fluorescent dye (Table 1). In principle, normal cells, undergoing apoptosis, release DNA fragments of about 200 bp as the result of enzymatic cleavage of nucleosome units; whereas, tumor cells undergo many different death processes, including necrosis and autophagy, and they can release DNA fragments of different sizes[20,21]. Umetani *et al*[22], using ALU targets proposed cfDI for the first time as a valuable tool to identify primary BC, showing it could be suitable to define lymph node metastasis in a group of 83 patients compared to 51 healthy controls.

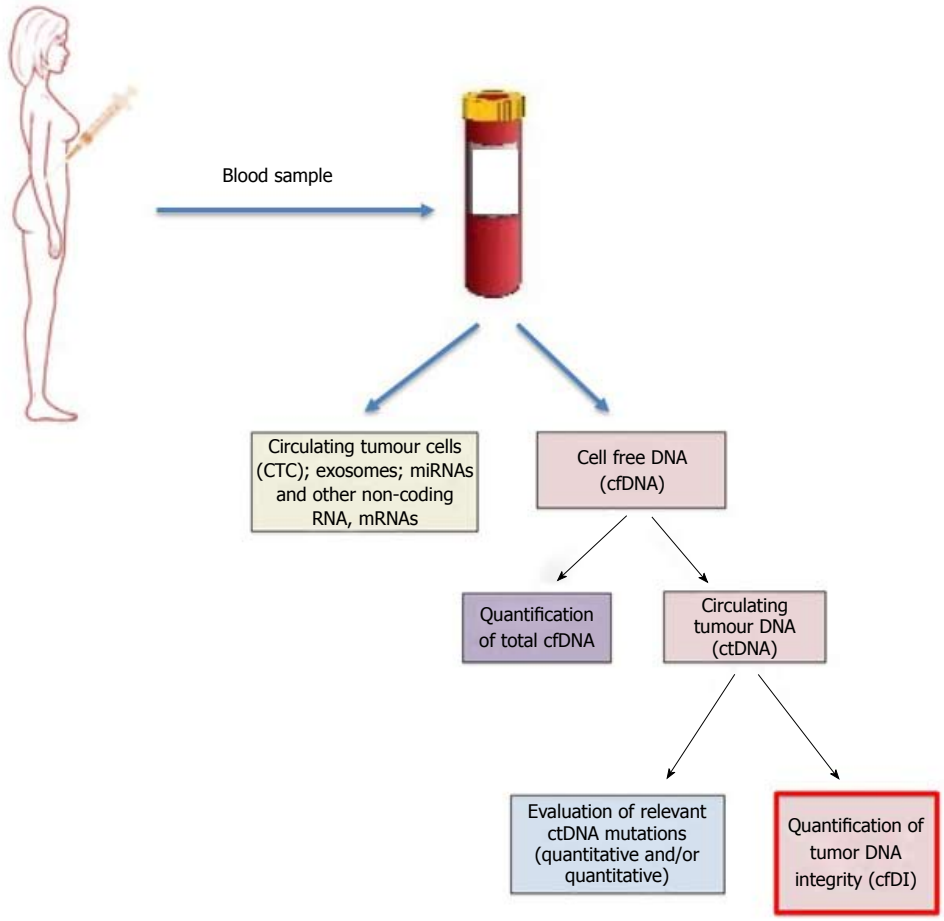

Figure 1 Diagram summarizing the possibility to monitor breast cancer from the blood circulating DNA.

They measured in serum shorter fragments of 115 bp that were considered as derived from apoptotic normal cells and larger ones of 247 bp as ctDNA, derived from necrosis/autophagy of cancer cells. The cfDI value calculated as the ratio quantity of longer over shorter fragments, ALU247/ALU115, was found to be higher in BC patients with high grade cancer compared to healthy controls. Accordingly to Umetani *et al*[22], Agostini *et al*[23] using the same ALU247 bp and ALU115bp targets demonstrated in plasma that cfDI value was twice higher in BC patients (n = 39) *vs* healthy controls (n = 49). Subsequently, Stötzer *et al*[24] proved in plasma that the ratio ALU247/115 were higher in patients with locally confined BC and MBC (n = 47) than benign BC (n = 12) (P < 0.001) but not *vs* healthy controls (n = 28). Moreover, this group evidenced that ALU concentrations alone were very interesting as markers for locally confined BC, while the use of cfDI was limited by the elevated levels found in some healthy controls. However, Iqbal *et al*[25] enrolling a larger number of women (148 patients *vs* 51 healthy controls) confirmed that the cfDI value, represented as ALU247/115 ratio, was significantly higher in serum of patients compared to healthy controls. Moreover, through a multivariate analysis, they showed a correlation between the cfDI value and the tumor size to predict the overall survival (OS) at 5 years and disease-free survival (DFS) at 4 years. Madhavan *et al*[21] also considered cfDI as a useful biomarker for BC in the largest patients' cohort (82 BC and 201 MBC) by using different primer set for ALU sequences and introducing LINE-1 as another DNA repetitive element target. They quantified ALU 260 bp and LINE-1 266 bp amplicons *vs* ALU 111 bp and LINE-197 bp amplicons, respectively. They showed, differently than the other groups, cfDI value was lower in BC patients *vs* healthy control and positively correlated with a decrease in progression-free survival (PFS) (P = 0.0025 for ALU) and OS (P < 0.0001 for both ALU and LINE-1). Similarly, using the same ALU260/111 and LINE-1 266/197 ratios, Cheng *et al*[26] showed that cfDI was significantly lower in recurrent BC (n = 37) *vs* non-recurrent BC (n = 175) (P < 0.001 for both ALU and LINE-1 cfDI values) but they did not provide as an extra measure healthy controls. Interestingly, this latter research group showed that a higher risk of developing recurrence could be predicted by the reduction of cfDI value (P = 0.020 for ALU and P = 0.019 for LINE-1 cfDI values, respectively). Finally, it should be mentioned that Cheng *et al*[27] recently observed that higher cfDI values for both ALU and LINE-1 targets in MBC patients correlated

Table 1 cfDI evaluation for the monitoring of breast cancer

Targets, length of the amplicons and primers' sequences	Patients with primary BC	Results	Ref.
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 51) and BC patients (n = 83) DNA from serum	The ratio ALU247/115 was higher in 51 patients with stage II (P = 0.005), stage III (P < 0.0001), stage IV (0.002) compared to healthy controls but not in 32 patients with stage 0 or I	Umetani *et al*[22], 2006
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 49) and BC patients (n = 39) DNA from plasma	In the group of patients the ratio ALU247/115 was twice higher (P < 0.0001) than in the group of healthy controls	Agostini *et al*[23], 2012
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 28), benign breast disease patients (n = 12), locally confined BC patients (n = 65) and MBC patients (n = 47) DNA from plasma	The ratio ALU247/115 was higher in patients with locally confined BC and metastatic BC than in benign BC (P < 0.001), but not *vs* healthy controls	Stötzer *et al*[24], 2014
ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	Healthy females (n = 100), primary BC patients (n = 82) and MBC patients (n = 201) DNA from plasma	Both the ratios ALU 260/111 and LINE-1 266/97 were lower in primary BC patients (ALU: P = 0.046; LINE-1 P = 0.041) In MBC patients the lower values of cfDI were related to both a decrease in PFS (P = 0.0025 for ALU) and OS (P < 0.0001 for both ALU and LINE-1 fragments)	Madhavan *et al*[21], 2014
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 51) and BC patients (n = 148) DNA from serum	The ratio ALU 247/115 was significantly higher in patients compared to controls (P < 0.001)	Iqbal *et al*[25], 2015
Beta-actin, 100 bp FW: 5′-GCACCACACCTTCTACAATGA-3′ RV: 5′-GTCATCTTCTCGCGGTTGGC-3′ Beta-actin, 400 bp FW: 5-GCACCACACCTTCTACAATGA-3′ (common primer) RV: 5′-TGTCACGCACGATTTCCC-3′	Healthy females (n = 70), benign lesions (n = 95) and BC patients (n = 95) DNA from plasma	cfDI value calculated as difference between 400 bp and 100 bp fragments Higher cfDI values were obtained in BC compared to benign lesions and healthy subjects (P < 0.001)	Kamel *et al*[20], 2016
HER2, 126 bp FW-5-CCAGGGTGTTCCTCAGTTGT-3′ RV-5- -GGAGTTCCTGCAGAGGACAG-3′ HER2, 295 bp FW-5′-CCAGGGTGTTCCTCAGTTGT-3′ RV-5′-TCAGTATGGCCTCACCCTTC-3′ MYC, 128 bp FW-5-GGCATTTAAATTTCGGCTCA-3′ RV-5-AAAAGCCAAATGCCAACTT-3′ MYC, 264 bp FW-5′-TGGAGTAGGGACCGCATATC-3′ RV-5′-ACCCAACACCACGTCCTAAC-3′ BCAS1, 129 bp FW-5-GGGTCAGAGCTTCCTGTGAG-3′ RV-5-TATCATGCCTTGGAGAACCA-3′ BCAS1, 266 bp FW-5′-GGGTCAGAGCTTCCTGTGAG-3′ RV-5′-CGTTGTCCTGAAACAGAGCA-3′ PIK3CA, 129 bp FW-5′CTCCACGACCATCATCATCAGGT-3′ RV-5′-TGGTTATTAATGAGCCTCACGG-3′ PIK3CA, 274 bp FW-5′-CTC CACGAC CAT CATCAGGT-3′ RV-5′-CGAAGGTCACAAAGTCGTCT-3′	Healthy females (n = 10), BC patients (n = 79) DNA from serum	The ratios BCAS1 266/129, MYC 264/128, PIK3CA 274/129 were significantly higher in patients compared to controls (P = 0.002, P = 0.030 and P = 0.004, respectively) No significant values for HER2 targets	Maltoni *et al*[28], 2017

ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′-TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	Non-recurrent BC patients (n = 175) *vs* recurrent-BC patients (n = 37) No healthy females reported DNA from plasma	Both the ratios ALU260/111 and LINE1-266/97 were significantly lower during follow-up in recurrent BC *vs* non recurrent BC (P < 0.001 for both ALU and LINE-1 cfDI), Moreover, BC patients with a lower cfDI had higher risk of developing recurrence compared to patients with higher cfDI (P = 0.020 for ALU cfDI and P = 0.019 for LINE-1 cfDI, respectively)	Cheng *et al*[26], 2017
ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	MBC patients (total n = 268) No healthy females DNA from plasma	Both the ratios ALU260/111 and LINE1-266/97 significantly increased in 268 MBC patients treated with one cycle of chemotherapy (MBCLB) compared to MBC at baseline (MBC1C) (P = 0.00017 for ALU -0.053 *vs* 0.063- and P = 0.0016 for LINE-1-0.45 *vs* 0.49) Moreover, in both MBCBL and MBC1C patients with a higher cfDI (for both ALU and LINE-1) correlated with a higher PFS and OS *vs* lower cfDI MBC patients	Cheng *et al*[27], 2018

BC: Breast cancer; cfDNA: Cell-free DNA; cfDI: Cell-free DNA integrity; ctDNA: Circulating tumour DNA; DFS: Disease free survival; MBC: Metastatic breast cancer; PFS: Progression-free survival; OS: Overall survival; qPCR: Quantitative real-time PCR; ddPCR: Droplet digital PCR.

with longer PFS and OS. However, Kamel *et al*[20] measuring the 400 bp and 100 bp amplicons of the *Beta-actin* from the DNA derived from plasma of 95 BC and 95 benign lesions *vs* 70 healthy controls estimated a cfDI- as difference between longer and shorter fragments- accordingly to Umetani *et al*[22] and the other authors[23-25], while yet differently from Madhavan *et al*[21]. In fact cfDI was found significantly higher in BC samples compared to those of benign and healthy subjects (P < 0.001). Moreover, they related those higher values to TNM stage, suggesting a cut-off to identify the more aggressive BC[20]. In agreement with Kamel *et al*[20], Maltoni *et al*[28] recently showed that tumour cells released longer DNA fragments than normal cells in the bloodstream. They quantified large fragments of 295 bp, 264 bp, 266 bp, 274 bp and short amplicons of 126, 128, 129, 129 bp from *HER2*, *MYC*, *BCAS1* and *PIK3CA,* respectively, from the serum of healthy females (n = 10), non-recurrent BC (n = 58) and recurrent BC (n = 21). They estimated cfDI as the ratio between longer and shorter amplicons of these genes and demonstrated that *BCAS1*, *MYC* and *PIK3CA* long/short amplicons were significantly higher in patients compared to healthy controls (P = 0.002, P = 0.030 and P = 0.004, respectively). On the other hand, there was no significant difference for long/short amplicons of *HER2*[27].

DISCUSSION

The overall literature on cfDI is intriguing as it has an extraordinary potential for the monitoring of BC, but it remains to be clarified what is the expected value of cfDI: some authors claimed that ctDNA is made of longer amplicons than normal cfDNA, explaining why the cfDI increased in BC[20,22-25,27], whereas other research groups, using different primers, claimed the exact opposite[21,26].

Most of the authors, in their measurement of cfDI through the ALU sequences, decided to use a standard DNA curve, as for Umetani *et al*[22], to derive quantifications of their DNA[21-25,27], and used the fluorescein or ROX passive reference dyes to improve the quality of their results[23,25]. Additionally, the specificities of the amplification reactions for the different couple of primers described in the papers have been controlled by means of denaturation curves or gel electrophoresis. This implies that the different results by qPCR hardly can be attributable to the laboratory's methodology, although we cannot completely exclude some variability in sample collection in the studies here described. Of note, differently than the other groups, Stötzer *et al*[24] have adopted a slightly different protocol for ALU amplifications by introducing UDP-DNA glycosidase.

Higher cfDI values in BC *vs* healthy controls were found in larger patients' cohorts derived from independent clinical settings and by using more different targets compared to studies claiming lower cfDI values in the tumor (Figure 2). Of note, higher cfDI in tumor than healthy controls were found in those studies that have analyzed mainly BCs, which did not reach the metastatic setting[22,23,25], whereas lower cfDI than healthy controls were reported in a study using the largest MBC patients' cohort up-to-date[21]. It is interesting to note that Umetani *et al*[22] proposed an increased cfDI value to predict local micrometastasis and recently Cheng *et al*[28] observed that cfDI value particularly decreased in BC patients with visceral metastasis. Thus we

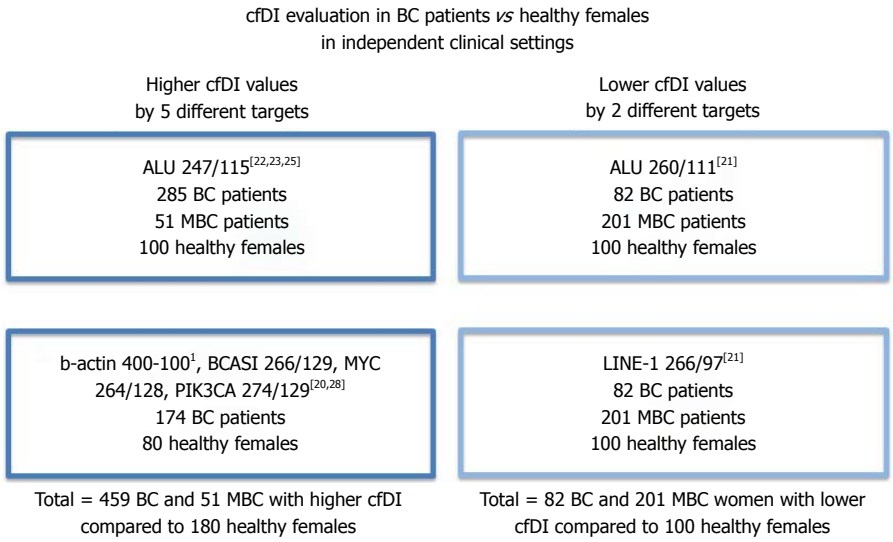

Figure 2 Summary of the literature data on cfDI determination in primary breast cancer *vs* healthy females. [1]Note that cfDI by β-actin was evaluated as difference between large and short amplicons and not as ratio longer to shorter amplicons. BC: Breast cancer; MBC: Metastatic breast cancer.

suggest that cfDI value can increase at initial stages of the BC and decrease in MBC. Surely, the most promising targets for the measurement of cfDI are represented by repetitive elements such as ALU and LINE-1 sequences, accounting for nearly 10% and 17% of the total genome, respectively. It is worth nothing that reproducible results were obtained when independent groups used the same ALU primer pairs, either those demonstrating higher cfDI[22-25] and those demonstrating lower cfDI in BC[21,26]. In our opinion, the methods of DNA extractions merely could have influenced the results. Interestingly, by looking with BLASTN genomic RefSeqGene Human at the target sites of ALU primers' pairs used by the research groups obtaining divergent results, we observed different target sites for ALU247/115 pairs compared to the ALU260/111 ones. We cannot exclude that this could contribute to the opposite cfDI values obtained by the different research groups comparing BC *vs* healthy controls. Moreover, we would like to point out that the qPCR methodology by SYBR Green is not very sensitive in quantifying very small DNA fragments in diluted solutions[29], as it could be in liquid biopsy, and that the variability of amplification efficiency of a sample can be overtaken by many replicates and independent experiments, that are hard to performed with samples derived from liquid biopsy. In this respect, the determination of cfDI in liquid biopsy samples would benefit by more sensitive and accurate technologies such as digital droplet PCR (ddPCR).

In conclusion, monitoring primary and MBC through a non-invasive analysis such as that of circulating DNA remains one of the most interesting goals to achieve. Surely, the mutations in liquid biopsy are of paramount importance for targeted therapies and for monitoring response to treatment. However, the most interesting benefit-to-cost analysis for the follow-up of BC and its recurrence seems to be the evaluation of circulating cfDI. Future investigations for cfDI by ddPCR are warranted for the (1) testing for the choice of best targets; (2) clarification of the clinical significance of larger and shorter DNA fragments origin in serum/plasma; and (3) a better understanding of the potential clinical impact of cfDI in anticipating recurrence and responsiveness to therapies for all patients, independently from the mutational signature of BC.

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ORIGINAL ARTICLE

Observational Study

Clinicopathological predictors of long-term benefit in breast cancer treated with neoadjuvant chemotherapy

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Institutional review board statement: This study was reviewed and approved by the Instituto Nacional de Enfermedades Neoplasicas Institutional Review Board. Personal and filiation data including identity of every patient was protected with an added code in the Excel table. This is a retrospective case series that did not have any activity or contact with the patients.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To investigate the survival impact of clinicopathological factors, including pathological complete response (pCR) and tumor-infiltrating lymphocytes (sTIL) levels according to subtypes, in breast cancer (BC) patients who received neo-adjuvant chemotherapy (NAC).

METHODS

We evaluated 435 BC patients who presented and received NAC at the Instituto Nacional de Enfermedades Neoplasicas from 2003 to 2014. sTIL was analyzed as the proportion of tumor stroma occupied by lymphocytes, and was prospectively evaluated on hematoxylin and eosin-stained sections of the preNAC core biopsy. pCR was considered in the absence of infiltrating cancer cells in primary tumor and axillary lymph nodes. Analysis of statistical association between clinical pathological features, sTIL, pCR and survival were carried out using SPSSvs19.

RESULTS

Median age was 49 years (range 24-84 years) and the most frequent clinical stage was ⅢB (58.3%). Luminal A, Luminal B, HER2-enriched and (triple-negative) TN phenotype was found in 24.6%, 37.9%, 17.7% and 19.8%, respectively. pCR was observed in 11% and median percentage of sTIL was 40% (2%-95%) in the whole population. pCR was associated to Ct1-2 (P = 0.045) and to high sTIL (P = 0.029) in the whole population. There was a slight trend towards significance for sTIL (P = 0.054) in Luminal A. sTIL was associated with grade Ⅲ (P < 0.001), no-Luminal A subtype (P < 0.001), RE-negative (P < 0.001), PgR-negative (P < 0.001), HER2-positive (P = 0.002) and pCR (P = 0.029) in the whole population. Longer disease-free survival was associated with grade Ⅰ-Ⅱ (P = 0.006), cN0 (P < 0.001), clinical stage Ⅱ (P = 0.004), ER-positive (P < 0.001), PgR-positive (P < 0.001), luminal A (P < 0.001) and pCR (P = 0.002). Longer disease-free survival was associated with grade Ⅰ-Ⅱ in Luminal A (P < 0.001), N0-1 in Luminal A (P = 0.045) and TNBC (P = 0.01), clinical stage Ⅱ in Luminal A (P = 0.003) and TNBC (P = 0.038), and pCR in TNBC (P < 0.001). Longer overall survival was associated with grade Ⅰ-Ⅱ (P < 0.001), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001), cN0 (P = 0.002) and pCR (P = 0.002) in the whole population. Overall survival was associated with clinical stage Ⅱ (P = 0.017) in Luminal A, older age (P = 0.042) in Luminal B, and pCR in TNBC (P = 0.005).

CONCLUSION

Predictive and prognostic values of clinicopathological features, like pCR and sTIL, differ depending on the evaluated molecular subtype.

Key words: Breast cancer; Subtype; Tumor-infiltrating lymphocytes; Neoadjuvant therapy; Pathological complete response; Survival

Core tip: The authors evaluated a series of 435 breast cancer (BC) patients who received neoadjuvant chemotherapy. They evaluated the association between stromal tumor-infiltrating lymphocytes levels and pCR in preneoadjuvant chemotherapy samples according to molecular subtypes. The results confirm differences in the predictive and prognostic role of stromal tumor-infiltrating lymphocytes and pathological complete response depending on the tumor subtype. Additionally, the authors evaluate the value of traditional prognostic features in every BC subset. The results increase the understanding of biomarkers in the heterogeneous scenario of BC.

Galvez M, Castaneda CA, Sanchez J, Castillo M, Rebaza LP, Calderon G, De La Cruz M, Cotrina JM, Abugattas J, Dunstan J, Guerra H, Mejia O, Gomez HL. Clinicopathological predictors of long-term benefit in breast cancer treated with neoadjuvant chemotherapy. *World J Clin Oncol* 2018; 9(2): 33-41 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i2/33.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i2.33

INTRODUCTION

Breast cancer (BC) is the second most common cancer in the world and the most frequent cancer among women, with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers), and is the fifth cause of death from cancer overall (522000 deaths)[1]. Neoadjuvant chemotherapy (NAC) is the standard therapy for locally advanced BC and could improve both surgical options and long-term outcome[2]. Response to NAC is considered an *in vivo* test of tumor sensitivity to NAC, and the achievement of a pathological complete response (pCR) is associated with longer disease-free survival (DFS) and greater overall survival (OS)[3-7]. Tumor-infiltrating lymphocytes (TILs) serve to evaluate the host immune system response against a tumor and also constitutes a valuable predictive biomarker of NAC response and survival[8-11].

BC is a heterogeneous disease, and intrinsically different subtypes of BC have been identified in the past years based on gene expression profiles and on the combined immunohistochemical status of hormone and HER2 receptors. Responsiveness to preoperative therapies and outcome after surgery can be predicted by BC subtypes[12-14].

In this study, we investigated the survival impact of different clinicopathological factors, including pCR and TIL levels, according to the subtypes in BC patients who received NAC. The predictive role of different clinicopathological features for having high density TIL and obtaining pCR according to subtypes was also

determined.

MATERIALS AND METHODS

We found 435 patients diagnosed with BC at clinical stage ⅡB to ⅢC at the Medical Department of the Instituto Nacional de Enfermedades Neoplasicas from 2003 to 2014. Eligibility criteria for this retrospective study were a histological diagnosis based on a core needle biopsy, having received NAC regimen and having undergone surgery after NAC. Patient characteristics such as age, clinical stage, histological subtype and grade, presence of estrogen receptors (ERs), progesterone receptors (PgRs) and HER2, and molecular subtype was obtained from the pathology report of preNAC core biopsy. pCR was defined as absence of invasive cancer in the breast and axillary nodes, irrespective of carcinoma *in situ* (ypT0/is ypN0), as previously described[4,15]. Phenotype classification was prospectively concluded through the evaluation of ER, PgR, HER2 and Ki67 as well as histological grade (in cases without Ki67 information): Luminal A (ER ⩾ 10%, PgR ⩾ 20%, HER2-negative and Ki67 < 15% or HG-Ⅰ-Ⅱ), Luminal B (ER ≥ 10% and any PgR < 20%, HER2-positive, Ki67 < 15% or HG-Ⅲ), HER2-enriched (ER < 10%, PgR < 10% and HER2-positive) and triple-negative (TN) (ER < 10%, PgR < 10% and HER2-negative). Stromal (s)TIL was prospectively evaluated in preNAC core biopsy and was defined as percentage of stromal area covered by lymphocytes[16].

Follow-up and recurrence information (date and location) were obtained from patient files. Time-from-last-chemotherapy-to-surgery was considered as the number of months from the date of the last NAC administration to surgery of the primary tumor. OS was calculated from surgery date of the primary breast tumor to death or last follow-up date, and DFS was calculated from surgery date of the primary breast tumor to recurrence or last follow-up date.

Statistical analysis

Categorical comparisons and association analysis between clinical pathological features and pCR were carried out using the chi-square statistic or Fisher's exact test. Survival analysis, regarding OS and DFS, was performed using the Kaplan-Meier method, and differences between curves were estimated by log-rank test. In all cases, the level of alpha was set at 0.05 *a priori*. Statistical analysis was performed using SPSS v19 (IBM Corp., Armonk, NY, United States).

RESULTS

Clinicopathological description

There were 435 patients included in this study, with median age at diagnosis of 49 years (range: 24-84 years), median tumor size of 6.5 cm (range: 1.0-24.0 cm), T3 in 27.8% and T4 in 63.9%. Inflammatory disease was found in 29.2%. The most frequent clinical stages were ⅢB (60.5%) and ⅢA (18.6%). Ductal histology was found in 93.3%, high grade in 52.2%, ER+ status in 62.8%, PgR+ status in 51% and HER2+++ in 32.4%. Luminal A, Luminal B, HER2-enriched and TN phenotype was found in 24.6%, 37.9%, 17.7% and 19.8%, respectively. The most frequent NACs were doxorubicin-cyclophosphamide for 4 cycles followed by 12 weekly paclitaxel (67.18%), doxorubicin-cyclophosphamide for 4 cycles followed by every 3 wk paclitaxel in 4 cycles (18.85%) and doxorubicin-cyclophosphamide for 4 cycles alone (7.32%). The median time from the last chemotherapy to surgery was 63 d (maximum: 982 d). pCR was observed in 48 (11%) patients. Median percentage of sTILs was 40% (2%-95%) in the entire population and 70% (60%-95%) in patients with pCR. Recurrence was found in 35.7%. Median DFS was 7.54 and median OS was 5.16 years (95%CI: 4.16-6.15 years) (Table 1).

Clinicopathological factors associated to pCR according to BC subtypes

Association analysis found that pCR was associated with T1-2 (P = 0.045) and to high sTIL level (P = 0.029) in the entire population (Table 1). Higher sTIL level had a slight trend towards association with pCR (P = 0.054) in Luminal A, and smaller tumor size had a trend towards association with pCR (P = 0.098) in Luminal A. Clinical involvement of axillary lymph nodes was not associated to variation of pCR (Table 2). An additional analysis by level of axillary involvement found that N2-3 had lower rates of pCR than N0-1 only in TNBC (P = 0.018).

Clinicopathological factors associated with sTIL according to BC subtypes

Association analysis found that sTIL level was associated with grade Ⅲ (P < 0.001), no-Luminal A subtype (P < 0.001), ER-negative (P < 0.001), PgR-negative (P < 0.001), HER2-positive (P = 0.002) and pCR (P = 0.029) in the entire population (Table 1). Within each BC subtype, sTIL level remained associated with grade Ⅲ in Luminal B (P = 0.011) and TN (P = 0.006) subtypes, as well as cN+ in Luminal B (P = 0.02) (Table 3).

Prognostic clinicopathological factors according to BC subtypes

Survival analysis found longer DFS was associated with grade Ⅰ-Ⅱ (P = 0.006), cN0 (P < 0.001), clinical stage Ⅱ (P = 0.004), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001) and pCR (P = 0.002). Longer DFS was associated with grade Ⅰ-Ⅱ in Luminal A (P = 0.033), N0-1 in Luminal A (P = 0.045) and TNBC (P = 0.01), clinical stage Ⅱ in Luminal A (P = 0.003) and TNBC (P = 0.038), and pCR in TNBC (P = 0.001) (Table 1).

Table 1 Clinical-pathological features *n* (%)

	Cases	sTIL ⩾ 50%	*P* value	pCR	*P* value	Overall Survival at 5 yr (OS = 50.1%)	*P* value	Progression free survival at 5 yr (DFS = 57.8%)	*P* value
	435	181		48					
Age (yr), median (range)	49 (24-84)	49 (24-84)	0.923	47 (28-80)	0.472		0.512		0.833
< 50	231 (53.1)	96 (35.2)		28 (12.1)		48.8%		59.7%	
⩾ 50	204 (46.9)	85 (36.7)		20 (9.8)		51.7%		55.9%	
Histological subtypes			0.928		0.234		0.512		0.497
Ductal	406 (93.3)	169 (43.6)		43 (10.6)		49.0%		57.5%	
Lobular	21 (4.8)	7 (36.8)		2 (9.5)		61.0%		55.2%	
Others	8 (1.8)	5 (62.5)		3 (37.5)		-		-	
Histological grade			< 0.001		0.170		0.001		0.006
G1-G2	200 (46.0)	59 (32.6)		17 (8.5)		57.1%		64.6%	
G3	227 (52.2)	119 (65.7)		29 (12.8)		42.8%		52.2%	
NR	8 (1.8)	3 (1.7)		2 (25)		83.3%		45.7%	
ER			< 0.001		0.098		< 0.001		0.000
No	162 (37.2)	89 (57.8)		23 (14.2)		36.1%		47.1%	
Yes	273 (62.8)	92 (35.2)		25 (9.2)		58.2%		64.3%	
PgR			0.003		0.246		< 0.001		0.000
No	213 (49)	104 (51.0)		27 (12.7)		41.0%		50.0%	
Yes	222 (51)	77 (36.5)		21 (9.5)		58.4%		64.8%	
HER2			0.002		0.135		0.334		0.135
No	294 (67.6)	106 (38.3)		28 (9.5)		53.7%		60.4%	
Yes	141 (32.4)	75 (54.3)		20 (14.2)		40.8%		52.3%	
Molecular subtypes			< 0.001		0.233		< 0.001		< 0.001
Luminal A	107 (24.6)	30 (29.7)		13 (12)		72.0%		76.1%	
Luminal B	165 (37.9)	61 (38.4)		12 (7)		50.6%		57.7%	
HER2-enriched	77 (17.7)	50 (66.7)		10 (13)		41.5%		54.9%	
Triple-Negative	86 (19.8)	40 (50.0)		13 (15)		32.5%		40.3%	
Tumor size (cm)			0.183		0.019		0.490		0.250
Median (range)	6.5 (1-24)	6.5 (1-16)		6.0 (2-15)					
cT									
cT1-cT2	36 (8.3)	19 (54.3)		8 (22.2)		55.0%		69.2%	
cT3-cT4	399 (91.7)	162 (42.6)		40 (10)		49.6%		56.8%	
cN			0.084		0.743		0.007		0.001
cN0	83 (19.1)	28 (35.0)		10 (12)		65.8%		77.0%	
cN1-cN2-cN3	352 (80.9)	153 (45.7)		38 (10.8)		47.2%		54.2%	
Clinical stage			0.192		0.088		0.155		0.004
Ⅱ	72 (16.6)	26 (36.6)		12 (16.7)		62.1%		74.3%	
Ⅲ	363 (83.4)	155 (45.1)		36 (9.9)		48.1%		55.4%	
sTIL%					0.002		0.598		0.747
Median (range)	40 (2-95)	70 (60-95)		65 (5-95)					
< 50%	266 (61.1)	0 (0)		20 (7.5)		49.6%		55.7%	
⩾ 50%	149 (34.3)	181 (100)		26 (17.4)		53.9%		63.1%	
Missing data	20 (4.6)	20 (0)		2 (10)		-		-	
TLCS (d)			0.411		0.633		0.317		0.156
Median (range)	63 (5-982)	58 (8-982)		65 (8-281)					
Shorter than median	207 (47.6)	91 (45.5)		22 (10.6)		48.5%		55.0%	
Longer than median	211 (48.5)	82 (41.4)		26 (12.3)		56.7%		61.2%	
Missing data	17 (3.9)	8 (47.1)		0 (0)		17.6%		46.3%	
pCR			0.029				0.002		0.002
No	387 (89)	154 (41.7)		0 (0)		47.4%		55.1%	
Yes	48 (11)	27 (58.7)		48 (100)		85.1%		84.9%	
Relapse			0.895		< 0.001		< 0.001		
No	284 (65.3)	118 (43.4)		42 (14.8)		81.6%		-	
Yes	151 (34.7)	63 (44.1)		6 (4)		8.58%		-	

TIL: Tumor-infiltrating lymphocytes; pCR: Pathological complete response; OS: Overall survival; DFS: Disease free survival; PgR: Progesterone; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

Longer OS was associated with grade Ⅰ-Ⅱ (P < 0.001), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001), cN0 (P = 0.007) and pCR (P = 0.002) in the entire population. It was also associated with older age in Luminal B (P = 0.042), to clinical stage Ⅱ in Luminal A (P = 0.017), and to cN0 (P = 0.045) and pCR in TNBC (P = 0.005) (Figure 1). Differences in TILs did not affect survival in the entire nor molecular subtype populations (Table 1 and Figure 2).

DISCUSSION

The biological heterogeneity of BC has been extensively described, and differences between intrinsic subtypes have been confirmed in the recent decade. We explored differences in the survival impact

Table 2 Association between response and Clinical-pathological features regarding molecular subtype *n* (%)

	Lum A			Lum B			HER2			TN		
	Total 107	pCR 13	*P* value	Total 165	pCR 12	*P* value	Total 77	pCR 10	*P* value	Total 86	pCR 13	*P* value
Age (yr)			1.000			0.315			0.507			0.157
median (range)	47 (28-75)	46 (28-62)		51 (25-84)	52 (39-69)		51 (28-80)	46 (29-80)		49 (26-73)	45 (28-68)	
< 50	72 (67)	9 (13)		78 (48)	4 (5)		37 (48)	6 (16.2)		44 (48)	9 (20)	
⩾ 50	35 (33)	4 (11)		87 (52)	8 (9)		40 (52)	4 (10)		42 (52)	4 (10)	
Histological subtypes			0.349			1.000			0.434			0.392
Ductal	97 (91)	11 (11)		153 (93)	11 (7)		73 (95)	9 (12.3)		83 (97)	12 (14)	
Lobular and others	10 (9)	2 (20)		12 (7)	1 (8)		4 (5)	1 (25)		3 (3)	1 (33)	
Histological grade			-			0.213			0.266			1.000
G1-G2	103 (97)	12 (12)		61 (39)	2 (3)		23 (30)	1 (4.3)		13 (15)	2 (15)	
G3	-	-		102 (61)	10 (10)		53 (69)	9 (17)		72 (85)	10 (14)	
NR	4 (3)	1 (25)		2 (1)	0 (0)		1 (1)	0 (0)		1 (0)	1 (100)	
Tumor size (cm)			0.102			0.213			0.511			0.620
Median (range)	6 (2-15)	5 (2-9)		7 (2-20)	6 (2-12)		7 (2.5-14)	6 (4-12)		7 (1-24)	8 (3-15)	
cT1-cT2	10 (7)	3 (30)		12 (7)	2 (17)		5 (6)	1 (20)		9 (10)	2 (22)	
cT3-cT4	97 (93)	10 (10)		153 (93)	10 (7)		72 (94)	9 (12.5)		77 (90)	11 (14)	
cN			0.306			0.222			0.270			0.021
cN0	27 (23)	5 (19)		28 (18)	0 (0)		53 (69)	5 (9.4)		14 (14)	4 (29)	
cN1-cN2–cN3	80 (77)	8 (10)		137 (82)	12 (9)		24 (31)	5 (20.8)		72 (86)	9 (13)	
Clinical stage			0.471			0.652			1.000			0.122
EC II	23 (20)	4 (17)		21 (12)	2 (10)		11 (14)	1 (9.1)		17 (16)	5 (29)	
EC III	84 (80)	9 (11)		144 (88)	10 (7)		66 (86)	9 (13.6)		69 (84)	8 (12)	
sTIL%			0.054			0.750			0.150			1.000
Median (range)	30 (2-90)	50 (10-90)		40 (5-90)	30 (8-90)		60 (5-95)	80 (30-95)		45 (2-90)	50 (5-80)	
< 50	71 (69)	6 (8)		98 (60)	6 (6)		25 (32)	1 (4)		40 (47)	6 (15)	
⩾ 50	30 (24)	7 (23)		61 (37)	5 (8)		50 (66)	9 (18)		40 (47)	6 (15)	
Missing data	6 (6)	0 (0)		6 (3)	1 (17)		2 (3)	0 (0)		6 (7)	1 (17)	
TLCS (d)			0.233			0.238			0.744			0.500
Median (range)	67 (14-458)	80 (16-281)		61 (5-412)	54 (8-140)		60 (11-240)	66 (37-106)		64 (8-982)	66 (14-122)	
Shorter than median	49 (48)	4 (8)		77 (45)	8 (10)		41 (53)	5 (12.2)		40 (48)	5 (13)	
Longer than median	57 (51)	9 (16)		76 (47)	4 (5)		33 (43)	5 (15.2)		45 (51)	8 (18)	
Missing data	1 (1)	0 (0)		12 (8)	0 (0)		3 (4)	0 (0)		1 (1)	0 (0)	
Relapse			0.121			0.753			0.300			< 0.001
No	87 (79)	13 (15)		109 (65)	9 (8)		46 (60)	8 (17.4)		42 (41)	12 (29)	
Yes	20 (21)	0 (0)		56 (35)	3 (5)		31 (40)	2 (6.5)		44 (59)	1 (2)	

TIL: Tumor-infiltrating lymphocytes; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

of tumor features, including pCR and TIL levels in each of the four molecular subtypes. Rates of pCR are lower in Luminal-A (9.2%), HER2-enriched (13%) and TNBC (15.3%) subtypes. pCR is also associated with longer survival in the entire population as well as in TNBC (pCR = 92.3% *vs* not pCR = 26.5% 5-year OS, $P = 0.005$; and trend in Luminal A, Luminal B and HER2-enriched phenotypic subsets of our series). It is widely assumed that patients who achieve pCR have significantly better DFS and OS rates in all molecular subtypes[12-14,17-19]. von Minckwitz *et al*[6] found pCR was not associated with prognosis only in Luminal A tumors in a series of 6377 patients with anthracycline-taxane-based NAC from 7 randomized trials; some authors claim it is related to the observed continuous tumor shrinkage occurring in their ER-positive tumor group during extended NAC, different than early and short-period tumor shrinkage observed in the ER-negative group[6,18-24].

pCR was more frequent in small tumors for both the entire population and the Luminal A subtype in our series. This finding is concordant with the previously mentioned idea that the effect of chemotherapy in Luminal A is slower than in other subtypes. Besides, Baron *et al*[18] found a similar lower rate of pCR in tumor size larger than 5 cm ($P = 0.022$) in their entire series ($n = 608$), but no association in the Luminal setting ($P = 0.411$). Higher grade of axillary involvement (cN2-3) was associated with lower rates of pCR only in the TNBC subset of our series. This lower response in bulky metastases could explain the previously described TNBC paradox phenomena of higher pCR rates but also higher distant relapse[21].

pCR was associated with higher percentage of

Table 3 Association between percentage of tumor-infiltrating lymphocytes and clinical-pathological features regarding molecular subtype *n* (%)

	Lum A			Lum B			HER2			TN		
	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value
	71	30		98	61		25	50		40	40	
Age (yr)			0.181			0.783			0.624			0.074
Median (range)	47 (28-75)	47 (36-74)		52 (28-73)	50 (25-84)		52 (28-66)	49 (29-80)		51 (26-73)	45 (27-73)	
< 50	50 (70)	17 (57)		46 (47)	30 (49)		11 (44)	25 (50)		16 (40)	24 (60)	
⩾ 50	21 (30)	13 (43)		52 (53)	31 (51)		14 (56)	25 (50)		24 (60)	16 (40)	
Histological subtypes			0.445			1.000			0.597			1.000
Ductal	66 (93)	26 (87)		91 (93)	57 (93)		23 (92)	48 (96)		39 (98)	38 (95)	
Lobular and others	5 (7)	4 (13)		7 (7)	4 (7)		2 (8)	2 (4)		1 (3)	2 (5)	
Histological grade			-			0.011			0.514			0.006
G1-G2	69 (97)	28 (93)		43 (44)	15 (25)		9 (36)	14 (28)		11 (28)	2 (5)	
G3	0 (0)	0 (0)		53 (54)	46 (75)		16 (64)	35 (71)		29 (73)	38 (95)	
NR	2 (3)	2 (7)		2 (2)	0 (0)		0 (0)	1 (2)		0 (0)	0 (0)	
Tumor size (cm)												
Median (range)	6 (3-13)	6 (2-15)		6 (3-20)	7 (2-15)		7 (3-14)	7 (3-14)		7 (4-24)	7 (1-16)	
cT			1.000			0.538			0.659			0.263
cT1-cT2	7 (10)	3 (10)		6 (6)	6 (10)		1 (4)	4 (8)		2 (5)	6 (15)	
cT3-cT4	64 (90)	27 (90)		92 (94)	55 (90)		24 (96)	46 (92)		38 (95)	34 (85)	
cN			0.890			0.020			0.631			0.762
cN0	18 (25)	8 (27)		22 (22)	5 (8)		6 (24)	8 (16)		6 (15)	7 (18)	
cN1-cN2-cN3	53 (75)	22 (73)		76 (78)	56 (92)		11 (44)	27 (54)		34 (85)	33 (83)	
Clinical Stage			0.666			0.141			0.742			0.576
EC II	17 (24)	6 (20)		16 (16)	5 (8)		3 (12)	8 (16)		9 (23)	7 (18)	
EC III	54 (76)	24 (80)		82 (84)	56 (92)		22 (88)	42 (84)		31 (78)	33 (83)	
TLCS (d)			0.631			0.882			0.502			0.141
Median (range)	64 (14-449)	70 (19-458)		61 (5-412)	58 (8-285)		68 (16-234)	56 (11-240)		74 (24-230)	51 (14-982)	
Shorter than median	34 (48)	13 (43)		48 (49)	28 (46)		12 (48)	28 (56)		15 (38)	22 (55)	
Longer than median	36 (51)	17 (57)		44 (45)	27 (44)		12 (48)	20 (40)		24 (60)	18 (45)	
Missing data	1 (1)	0 (0)		6 (6)	6 (10)		1 (4)	2 (4)		1 (3)	0 (0)	
pCR			0.054			0.750			0.150			1.000
No	65 (92)	23 (77)		92 (94)	56 (92)		24 (96)	41 (82)		34 (85)	34 (85)	
Yes	6 (8)	7 (23)		6 (6)	5 (8)		1 (4)	9 (18)		6 (15)	6 (15)	
Relapse			0.450			0.201			0.737			0.502
No	59 (83)	23 (77)		61 (62)	44 (72)		16 (64)	30 (60)		18 (45)	21 (53)	
Yes	12 (17)	7 (23)		37 (38)	17 (28)		9 (36)	20 (40)		22 (55)	19 (48)	

%sTIL was performed over 415 cases. There 20 missed values. TIL: Tumor-infiltrating lymphocytes; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

sTILs in the entire population and also within the HER2-enriched subtype (P = 0.02). A trend towards association was found in Luminal A, Luminal B and TNBC. Different studies have found that high TIL levels in preNAC samples are associated to higher pCR rates in the entire BC population[25-27]. Wang *et al*[28] performed a meta-analysis with 23 studies including 13100 BC patients, and similarly found that high TIL level was associated with improved pCR rate in the entire population, and in HER2 and TNBC. A high TIL level significantly predicted longer OS in the entire population (P < 0.001) and in patients with HER2-positive (P = 0.005) BC and in TNBC patients (P < 0.001).

TIL showed association with grade Ⅲ tumors in the entire population and in Luminal B and TNBC subsets in our series. Similarly, Pruneri *et al*[29] describes that higher TIL levels have a trend towards association with HG3 (P = 0.052) and was associated to Ki67 ⩾ 50% (P < 0.0001) in a series of 897 TNBC cases, and could reflect the appearance of a larger amount of neoantigens that elicit an immunomediated response. Involvement of axillary lymph nodes was associated to higher TIL levels only in the Luminal B subset. High density of TILs has previously been described as associated to absence of lymph node involvement in the entire population of BC, and our results indicate that this association could differ by some subtypes[30]. Higher level of sTILs was not associated to longer survival in the entire population nor in any subtype in our series. This finding could be explained by the small size of our series and because the highest impact of TILs is over pCR instead of survival.

Our study has some limitations. First, because of the retrospective design of the study, different chemotherapy schemas were used depending on the oncologist decision and surgical election depending

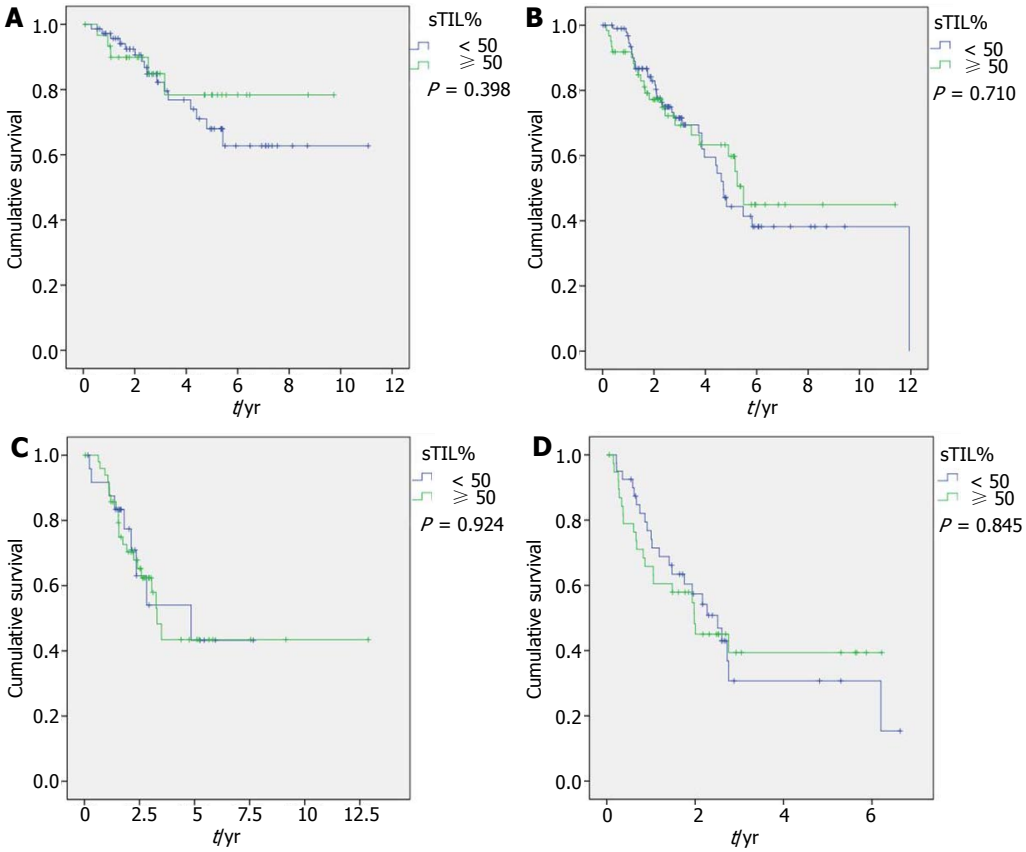

Figure 1 Overall survival regarding tumor-infiltrating lymphocytes (cut-off: 50%) for Luminal A (A), Luminal B (B), HER2-enriched (C) and Triple Negative group (D).

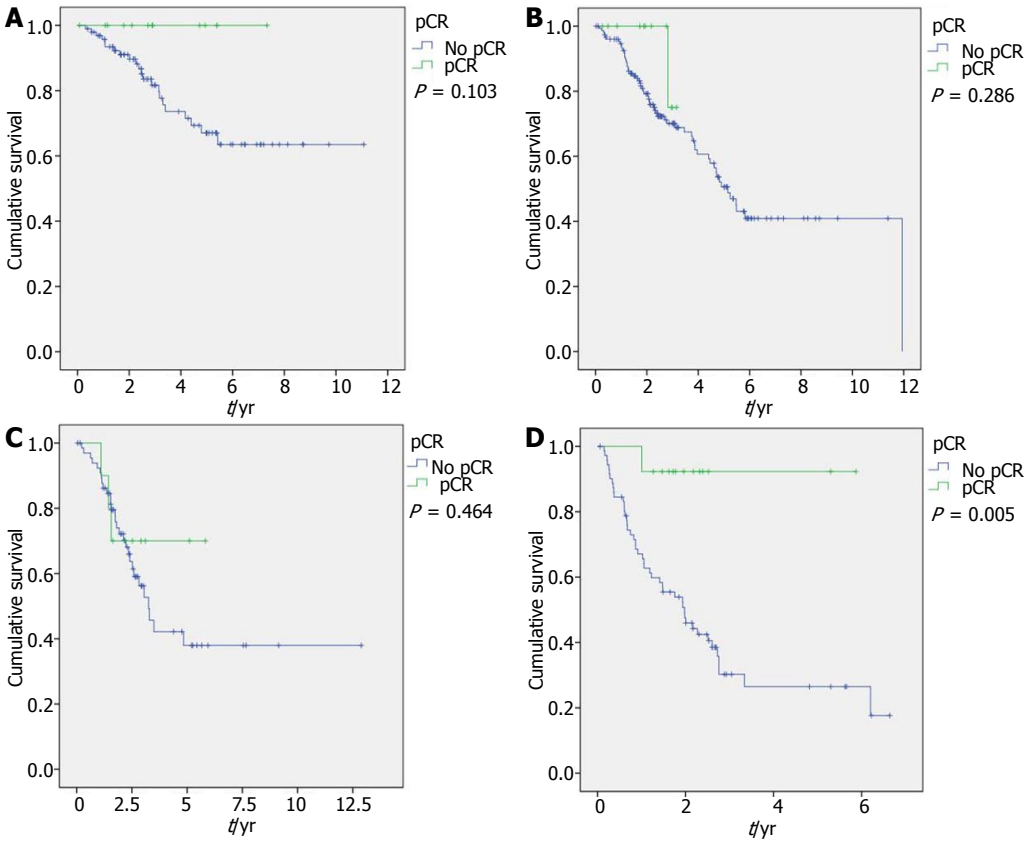

Figure 2 Overall survival regarding pathological complete response for Luminal A (A), Luminal B (B), HER2-enriched (C) and triple negative group (D).

on surgeon. Second, the sample sizes of each BC subgroup are rather small, so the prognostic impact of every clinicopathological feature in each BC subtype should be investigated in a larger population in subsequent studies. Despite these limitations, this is the first comprehensive report of the NAC effect over breast molecular subtypes in a Latin-American population.

ARTICLE HIGHLIGHTS

Research background

Breast cancer can be classified into Luminal A, Luminal B, HER2-enriched and triple-negative. Clinicopathological features can identify breast cancer prognosis and include pathological complete response (tumor sensibility to chemotherapy) and tumor-infiltrating lymphocytes (TILs; host activity against the tumor).

Research motivation

Discussion and new information about molecular breast cancer subtypes have been included in the most relevant cancer-related meeting, and more than 30,000 articles have been published in the last 2 years. Two biomarkers, pathological complete response (pCR) and TILs, have been re-defined and gained pathologist acceptance in the last 3 years.

Research objectives

The main objective is to evaluate the survival impact of different clinicopathological factors, including pCR and TIL levels, according to the subtypes in breast cancer patients who received neoadjuvant chemotherapy.

Research methods

Evaluation of TIL levels was prospectively performed following international guidelines. Breast cancer cases were classified according to 2017 St Gallen Breast Cancer Meeting guidelines.

Research results

pCR was associated with cT1-2 (P = 0.045) and high stromal (s)TILs (P = 0.029) in the entire population. However, this relationship was not found for every molecular subtype, probably because of the small sample size. pCR was associated with longer disease-free survival in the entire population (P = 0.002) and in TNBC (P < 0.001), as well as to longer overall survival in the entire population (P = 0.002) and in TNBC (P = 0.005).

Research conclusions

Predictive and prognostic value of clinicopathological features like pCR and sTIL level differ depending on the molecular subtype being evaluated. Identification of pCR and TIL roles in every molecular subtype will allow for identification of those patients who need more intense chemotherapy and those who will benefit from an immune-modulator treatment.

Research perspectives

No information about the relevance of pCR and TILs in South-American women with breast cancer have been published in. An increase in the knowledge about prognosis impact of pCR and TIL in every molecular breast cancer subtype will allow for obtaining more effective personalized therapies. Furthermore, similar analysis needs to be done with more precise methods to evaluate response to chemotherapy and host immune activity, such as tumor residual burden and CD3/CD8 ratio, respectively.

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REVIEW

Epitranscriptomics of cancer

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Abstract

The functional impact of modifications of cellular RNAs, including mRNAs, miRNAs and lncRNAs, is a field of intense study. The role of such modifications in cancer has started to be elucidated. Diverse and sometimes opposite effects of RNA modifications have been reported. Some RNA modifications promote, while others decrease the growth and invasiveness of cancer. The present manuscript reviews the current knowledge on the potential impacts of N6-Methyladenosine, Pseudouridine, Inosine, 2'O-methylation or methylcytidine in cancer's RNA. It also highlights the remaining questions and provides hints on research avenues and potential therapeutic applications, whereby modulating dynamic RNA modifications may be a new method to treat cancer.

Key words: RNA modifications; N6-methyladenosine; 5-methylcytidine; 2'O-methylation or methylcytidine; Pseudouridine; Inosine

Core tip: The present manuscript reviews the current knowledge on RNA modifications in cancer. The potential impacts of N6-Methyladenosine, Pseudouridine, Inosine, 2'O-methylation or methylcytidine in cancer's RNA is presented and discussed. The review also highlights the remaining questions and provides hints on research avenues and potential therapeutic applications, whereby modulating dynamic RNA modifications may be a new method to treat cancer.

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INTRODUCTION

Diverse and abundant modifications are introduced

posttrancriptionally in cellular RNAs during their maturation. These modifications are made on canonical A, C, G, and U residues, and their formation is catalyzed by numerous specific enzymes or RNA-protein complexes (RNPs). Ribonucleotide residues can bear single or multiple modifications on the purine/pyrimidine ring and/or ribose. To date, over one hundred RNA modifications have been identified and listed in dedicated databases (http://mods.rna.albany.edu/; http://modomics.genesilico.pl)[1,2]. These naturally occurring modified nucleosides play various structural and functional roles in different types of RNAs: Transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), messenger RNAs (mRNAs), small nuclear RNAs (snRNAs), microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). The most widespread RNA modifications are base or ribose methylations, deamination of adenosine to inosine and isomerization of uridine into pseudouridine. Over the past decades these modifications have been studied in the context of malignancies. Frequently, a modification is found to have pro-cancer or anti-cancer effects depending on the type of RNA, the location of the modification and, most importantly, the cell type and context (*e.g.*, hypoxia). This review presents the current knowledge on the potential link between RNA modifications and cancer by systematically addressing the "pro-cancer", "anti-cancer" and mixed effects of RNA modifications. Since such a relationship has been reported for only some abundant modifications and for modifications for which a detection method is available [N6-Methyladenosine (m6A), 5-Methylcytidine (m5C), 2'O-mN, Ψ and I], the present review will focus on these modifications (Figure 1).

M6A IN CANCER RNA

Serendipitously discovered during the characterization of the mRNA 5' cap, methylation of the exocyclic nitrogen of adenosine, named m6A, is by far the most abundant mRNA modification, occurring on an average of three sites per mRNA[3-5]. Recent technological advances have facilitated m6A profiling across eukaryotes, including humans, mice[6], yeasts[7], and plants[8,9], indicating that m6A is a conserved but dynamic modification. m6A has also been identified in rRNA[10], tRNA[11], snRNA[12], miRNA[13] and lncRNA[14].

M6A patterns are attributed to the consensus RRACH sequence (A is methylated; R = A or G; H = A, C, or U; and the first nucleotide next to m6A from the 5' end most frequently is G), with preferential distribution near mRNA stop codons and 3' untranslated regions (UTRs) and within long internal exons. Additionally, the m6A sites are conserved between human and mouse embryonic stem cells (ESCs) and somatic cells. However, distinct m6A patterns can also be detected among different species or cells at different developmental stages[4,7,15,16]. Some m6A signatures are tissue specific[4], and are altered in response to different stimuli[17], pointing to the potential role of m6A in regulating diverse cellular processes. m6A dynamics are assigned to the complex m6A enzymatic machineries, comprising m6A "writers", "readers" and "erasers". Although a plethora of studies suggest crucial and versatile roles of m6A and its machineries, its roles in cancer that have recently emerged are contradictory and require further investigation.

High m6A levels in cancers

"Writers" is a term given to enzymes that are part of the methyltransferase complex that introduces m6A. Components of this complex are methyltransferase-like 3 (METTL3)[18], METTL14[19], Wilms tumor 1-associated protein (WTAP)[20] and KIAA1429[21].

METTL3 protein levels were found to be elevated in lung adenocarcinoma cell lines compared to healthy tissue[22]. Depletion of METTL3 was shown to result in the inhibition of cancer cell growth, decreased invasive ability of cancer cells and increased cell apoptosis in the same study. Additionally, METTL3 was shown to function as an m6A-binding protein ("reader") in a specific subset of m6A-modified mRNAs, where it recruits eIF3 during translation initiation and therefore promotes translation. Expression of several oncogenes, including the mRNA of epidermal growth factor receptor (EGFR) and the Hippo pathway effector transcriptional co-activator with the PDZ-binding motif (TAZ) protein, was found to be promoted upon METTL3 recognition[22].

Similarly, in acute myeloid leukemia (AML), mRNA levels of METTL3 and METTL14 are significantly higher than in most cancers[23]. METTL3 depletion in MOLM13 caused differentiation and increased apoptosis, suggesting that high m6A levels may play a role in sustaining undifferentiated leukemic cells in AML[23] (Table 1).

Low m6A levels in cancers

Two m6A "erasers" have been described: Demethylases fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5)[24,25].

Single nucleotide polymorphisms within FTO known to be involved in the development of obesity in genome-wide association studies have been associated with the risk of developing diverse cancer types: Lung cancer, kidney cancer, high-grade prostate cancer, endometrial cancer, pancreatic cancer, pancreatic cancer in patients with type 2 diabetes, and breast cancer[26-33]. All these cancer types share a single SNP (rsrs9939609): The obesity-associated SNP in intron 1 of the *FTO* gene. This SNP was shown to increase primary transcript levels of the *FTO* gene, suggesting a gain-of-function mutation in cancers associated with this SNP[34].

In human epidermal growth factor receptor type 2 (HER2)-overexpressing subtypes of breast cancer, FTO is highly expressed in comparison to other breast cancer subtypes[35]. Contrary to the studies of high m6A levels in AML discussed in the previous chapter, low m6A levels have also been reported in AML subtypes. FTO expression can be upregulated by certain

Table 1 Relative amount of modification (directly quantified or extrapolated from the expression level of writers/erasers)

RNA modifications	High in cancer	Low in cancer
m6A	Lung adenocarcinoma[22], AML[23]	HER2 overexpressing subtypes breast cancer[35], t(11q23)/MLL-rearranged, t(15;17)/PML-RARA, FLT3-ITD, and/or NPM1-mutated AMLs (ASB2 and RARA)[36], GBM (FOXM1)[39], breast cancer (NANOG)[40]
2'O-methylation	Breast cancer [51,67], primary and metastatic prostate cancers[58], squamous cell cervical carcinoma[57]	
Ψ		Leukemia, lymphoma, multiple myeloma[84-86]
Inosine	BLCA, BRCA, COAD, HNSC, LUAD, THCA[87,88], NSCLC (NEIL1[94], AZIN1[103], miR-381[94]), SCLC (AZIN1)[87], HCC (AZIN1[101], FLNB[90]), GC[91], ESCC (FLNB)[92], cervical cancer[89], CRC (RHOQ)[109], AML (PTPN6)[114]	KIRP, KICH[87,88], Breast cancer (Gabra3)[118], Gastric cancer (PODXL)[91], Glioblastoma (GluR-B)[119], onco miR-21, miR-221, miR-222[128], ESCC (IGFBP7)[120], Glioma (miR-376a*)[129], Melanoma[130] (miR-455-5p)[132]
5mC	Circulating tumor cells in lung cancer[137]	

In brackets are the names of genes that have been analyzed. AZIN1: Antizyme inhibitor 1; RHOQ: Ras homolog family member Q; PODXL: Podocalyxin-like; IGFBP7: Insulin-like growth factor-binding protein 7; PTPN6: Protein tyrosine phosphatase non-receptor type 6; NEIL1: NEI-like protein 1; GluR-B: Glutamate R-B; Gabra3: Alpha-3 subunit of gamma-aminobutyric acid type A; FlnB: Filamin B; ASB2: Ankyrin repeat and SOCS box containing 2; RARA: Retinoic acid receptor alpha; FOXM1: Forkhead box protein M1; GBM: Glioblastoma multiforme; HER2: Human epidermal growth factor receptor type 2; MLL: Mixed lineage leukemia; PML/RARA: Promyelocytic leukemia/retinoic acid receptor alpha; FLT3-ITD: Fms-related tyrosine kinase 3–internal tandem duplication; NPM1: Nucleophosmin 1; NSCLC: Non-small cell lung cancer; HCC: Hepatocellular carcinoma; ESCC: Esophageal cell carcinoma; GC: Gastric cancer; CRC: Colorectal cancer; AML: Acute myeloid leukemia; SCLC: Small cell lung cancer; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; COAD: Colon adenocarcinoma; HNSC: Head and neck squamous cell carcinoma; LUAD: Lung adenocarcinoma; THCA: Thyroid carcinoma; KICH: Kidney chromophobe; KIRP: Kidney renal papillary cell carcinoma.

oncogenic proteins (*e.g.*, mixed lineage leukemia (MLL)-fusion proteins, promyelocytic leukemia/retinoic acid receptor alpha *(*PML-RARA), fms-related tyrosine kinase 3-internal tandem duplication (FLT3-ITD), and nucleophosmin 1 (NPM1) mutant), and dataset analysis of human AML confirmed that FTO was expressed at significantly high levels in t(11q23)/MLL-rearranged, t(15;17)/PML-RARA, FLT3-ITD, and/or NPM1-mutated AMLs[36]. Overexpression of FTO reduces m6A levels in ankyrin repeat and SOCS box containing 2 (ASB2) and retinoic acid receptor alpha (RARA) mRNA transcripts. It has been shown that the loss of m6A markings reduces mRNA stability, resulting in the partial repression of ASB2 and RARA expression in AML cells. In four different AML cohorts, ASB2 and RARA exhibit a significant inverse correlation with FTO expression. ASB2 and RARA are upregulated during normal hematopoiesis and are important regulators of all-*trans*-retinoic acid (ATRA)-induced differentiation of leukemia cells. Through regulating the expression of such targets, FTO inhibits ATRA-induced AML cell differentiation. Both gain- and loss-of-function studies of FTO in leukemic cell models showed an oncogenic role of FTO in these AML subtypes[36]. However, recent studies have suggested that FTO acts as a demethylase of N6-2'O-dimethyladenosine in mRNA 5' caps, having only minor effects on m6A[37]. Thus, the role of FTO in AML might be independent of m6A.

Recently, both FTO and ALKBH5 have been found to play similar roles in glioblastoma stem cells (GSCs) and their tumorigenesis[38]. These studies shed light on their crucial roles in the regulation of mRNA m6A levels for maintaining GSC growth, self-renewal, and tumor development. Enhanced growth and self-renewal of GSCs *in vitro* were detected upon the depletion of METTL3 or METTL14, resulting in reduced mRNA m6A levels, and promoted the ability of GSCs to form brain tumors *in vivo*. Accordingly, treatment with the FTO inhibitor MA2, the ethyl ester form of meclofenamic acid, increased mRNA m6A levels and suppressed GSC growth *in vitro* and GSC-initiated tumorigenesis, ultimately prolonging the survival of GSC-engrafted mice.

In a similar study, the authors checked the expression levels of m6A regulators in available datasets for glioblastoma multiforme (GBM) and discovered elevated expression of m6A demethylase ALKBH5 that correlated with poor clinical outcomes for GBM patients[39]. Stable knockdowns in cultured human GSCs showed that the loss of ALKBH5 decreases GSC proliferation and reduces the expression of the stemness markers Nestin, Sox2, Nanog, and Oct4, which are normally expressed in GSCs. In rescue experiments, wild-type, but not catalytically inactive, ALKBH5 recover the phenotype, suggesting that it plays a role in stemness maintenance and that the proliferation of GSCs is solely based on demethylation activity. Moreover, these authors examined the expression of transcription factor FOXM1 (forkhead box m1), which is known to play a pivotal role in regulating GSC proliferation, self-renewal, and tumorigenicity, and found that it depends on ALKBH5 demethylating activity. All these findings were based on m6A hyper erasing, which opens new possibilities for promising targeted treatments in glioblastoma (Table 1).

It has been reported that the hypoxia-inducible factors (HIFs) HIF-1α and HIF-2α activate *ALKBH5* gene transcription under hypoxic conditions in breast cancer cells, thus inducing m6A demethylation. This demethylation was shown to stabilize NANOG mRNA and promote the breast cancer stem cell (BCSC)

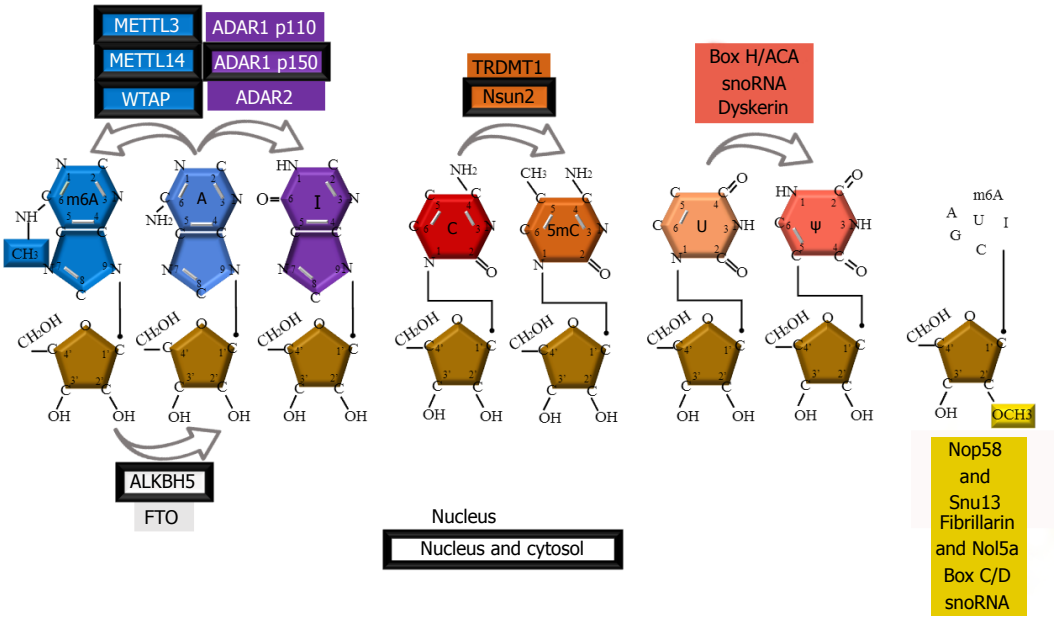

Figure 1 Ribonucleotide RNA modifications known to be of relevance in cancer and their enzymatic machineries. m6A: N6-methyladenosine; METTL3: Methyltransferase like 3; METTL14: Methyltransferase like 14; WTAP: Wilms' tumor 1-associating protein; m6A erasers (ALKBH5: Alkylation repair homologue protein 5; FTO: Fat mass and obesity-associated protein); Inosine (I) writers [ADAR1 (p110 and p150) and ADAR2: Adenosine deaminase acting on RNA 1 and 2]; 5-methylcytosine (5mC) (NSUN2: NOP2/Sun domain protein 2; TRDMT1: tRNA aspartic acid MTase 1); 2'O-methylation writers (Nol5a: Nucleolar Protein 5A; Nop58: Nucleolar protein 58; Snu13: Small Nuclear Ribonucleoprotein 13; SnoRNA: Small nucleolar RNA).

phenotype. Depletion of ALKBH5 in hypoxic breast cancer cells was identified as an effective strategy to decrease NANOG expression and limit the presence of BCSCs *in vivo*[40] (Table 1).

Mixed role of m6A in cancer

The primary microRNA (pri-miRNA) junction region between the hairpin stem and the flanking single-stranded RNA was found to be abundant in m6A consensus motifs. The recognition of the junction regions is mediated by Dicer, followed by the recruitment of the ribonuclease Drosha (the microprocessor complex), which cleaves the RNA duplex to yield the premiRNA product. Depletion of HNRNPA2B1 (a nuclear "reader") or METTL3 knockdown in HEK293 and MDA-MB-231 cells resulted in a significant reduction in the expression levels of the mature forms of a number of m^6A-marked miRNAs. The tumor-suppressor miRNA let-7 was significantly reduced upon the depletion of METTL3 possibly due to diminished Dicer binding to pri-miRNAs, thus preventing the formation of mature miRNAs. However, these METTL3-depletion experiments also showed a decrease in the expression of onco-miRNAs, such as miR-221 and miR-222[13,41]. Taken together, the presence of m6A affects diverse pri-miRNA and mature miRNA subpopulations, but its relevance in the context of cancer still needs to be investigated.

2'O-METHYLATION IN CANCER RNA

Methylation of the 2'-hydroxyl group of ribose is one of the predominant internal modifications of rRNA and snRNA[10,42]. This modification is also found in tRNA and mRNA, mostly at the first and second nucleotides in Cap1 and Cap2 structures, respectively.

Introducing 2'O-methylation on ribose is mediated by complexes of guide RNA and proteins named small nucleolar ribonucleoprotein (snoRNP) complexes or by methyltransferases: Human cap1 and 2, 2'-*O*-ribose methyltransferase, hMTr1 and hMTr2[43-45]. snoRNP complexes consist of Fibrillarin (the catalytic component in humans, also known as Nop1p in yeast), Nol5a (Nop56p), Nop58 and Snu13 subunits[46-48], which are guided by C/D Box snoRNAs to the appropriate base[49,50].

High 2'O-methylation levels in cancer

Tumor suppressor p53 and Fibrillarin seem to be linked[51]. Knockdown of p53 in cellular models of breast and colon cancer resulted in the overexpression of Fibrillarin at both the mRNA and protein levels. It is suggested that tumorigenesis associated to mutated p53 promotes an increase in the methylation status of rRNAs, which alters their ribozyme activity, thus affecting their translation fidelity and rate. Through the methylation of rRNA, Fibrillarin stimulates the translation of cancer-promoting proteins: (1) Insulin-like growth factor 1 receptor (IGF1R), which plays a role in tumor progression, cell survival, and the response to chemotherapy (reviewed by Pollak *et al*[52]); (2) c-Myc, a pleiotropic pro-oncogene (reviewed by Dang *et al* [53]); (3) Fibroblast growth factor 1/2 (FGF1/2), which is involved in epithelial-mesenchymal transition[54]; and (4) Vascular endothelial growth factor

A (VEGFA), which acts in tumoral angiogenesis[51,55].

Translation of these proteins relies on internal ribosome entry site (IRES) in the mRNA, which is a 5′ cap-independent translation mechanism that may be used in specific conditions. The inhibition of rRNA methylation was shown to impair IRES translation initiation by perturbing the association of the 40S and 60S subunits[56]. Therefore, it is conceivable that enhanced ribosomal methylation increases the translation of IRES-containing mRNAs. Nevertheless, clinical analysis shows that a high level of Fibrillarin in primary breast tumors is associated with poor survival, independent of other biological markers[51]. Elevated expression levels of Fibrillarin were previously reported in primary and metastatic prostate cancers and in squamous cell cervical carcinoma (Table 1)[57,58].

NOL5A gene was found to be overexpressed in Burkitt's lymphoma-associated c-Myc mutants[59], and human NOP58 mRNA levels were found to be elevated in metastatic melanoma lesions[60].

Low 2'O-methylation levels in cancer

Contrary to Fibrillarin's indirect promotion of IRES-driven translation, in MCF-7, a breast cancer cell line, Fibrillarin knockdown resulted in the accumulation of p53, possibly affecting the UTR of the p53 mRNA and increasing IRES-driven *de novo* synthesis[61]. These studies suggest a complex interplay between p53 and Fibrillarin, while IRES-dependent translation is not exclusively stimulated by increased rRNA methylation.

Mixed 2'O-methylation in cancer

SnoRNA expression profiles were investigated in endometrial, lung and prostate cancers, as well as in glioma and chronic lymphocytic leukemia. High-throughput screening of snoRNAs in cancerous versus normal tissues underlined their overexpression or underexpression as common molecular events in tumorigenesis, with the former being more pronounced than the latter[62-66]. Analysis of blood serum has shown the possibility of detecting snoRNAs in breast cancer patient samples and the associated upregulation of a specific snoRNA, U6, in active disease[67]. Therefore, profiling snoRNAs with their respective RNA 2'O methylation modification signatures might be used as a noninvasive biomarker in the diagnosis and prognosis of cancer.

PSEUDOURIDINE IN CANCER RNA

The fifth base, known as pseudouridine (Ψ)[68], is one of the most abundant nucleotide modifications present in all three life domains[2]. After its initial detection in rRNA and tRNA, pseudouridine was detected in mRNA, lncRNA, and snRNAs, such as U2 snRNA and snoRNA[69,70]. Introducing Ψ in eukaryotic RNA can be mediated through guide RNA-dependent H/ACA BOX snoRNA pseudouridine synthases (PUSs) or guide RNA-independent PUSs. A recent review by Penzo *et al*[71] reports on the functional roles of pseudouridines and related human pathologies.

Only low Ψ levels have been reported in cancer tissues/cells; thus, this chapter will contain only a section titled "low Ψ levels in cancer". Surprisingly, elevated levels of circulating Ψ have been measured in the body fluids of cancer patients, but its role and origin are not well defined, so this finding will not be further discussed here.

Low Ψ levels in cancer

The highly conserved protein dyskerin is the human PUS that catalyzes the pseudouridylation of snoRNPs that assemble during the transcription of guide H/ACA RNA. Mutations in the *Dkc1* gene coding for dyskerin can be found in the X-linked form of dyskeratosis congenita (DC). DC is a rare, inherited disorder that is characterized by mucocutaneous abnormalities and bone marrow failure. DC can be inherited as an X-linked recessive, autosomal dominant or autosomal recessive disease[72]. Although the absence of dyskerin, which results in the loss of pseudouridine in rRNA, was suggested as a primary cause of DC, a recent study assigned telomerase dysfunction as the primary cause of DC[73]. Namely, mutations in H/ACA-resembling domains in the RNA component of telomerase RNP, which are required for telomerase accumulation, stability, 3′ end processing and function, are associated with an autosomal form of DC[74-76].

In patients with DC, a higher predisposition to cancer has been reported, although low mutational frequency in the *DKC1* gene was shown in primary tumors[77]. This predisposition might be a synergistic outcome of impaired pseudouridylation. Most likely, the dysregulation of rRNA pseudouridylation precedes disease onset, as studies in hypomorphic *Dkc1*-mutant mice suggest. A specific defect of the internal ribosome entry site also occurs upon DKC1 loss, causing a specific defect in the translation of some IRES-containing mRNAs. Ribosomes that lack pseudouridine modifications show a direct impairment in binding to IRES elements[78]. Consequently, in hypomorphic DKC-1 mice, cap-dependent translation of mRNA is not compromised, but translation of IRES-containing mRNAs, including the tumor suppressors p27 and p53, is perturbed[79-82], resulting in a higher incidence of cancer development in these mice. Thus, this impaired translation of tumor suppressor mRNA might also be a driver of cancer in DC patients. Moreover, recent identification of Ψ in mRNA[83] brings an additional level of complexity and regulation of the expression of target RNAs.

In hematological cancers, such as leukemias, lymphoma and multiple myeloma, downregulation of specific subsets of dyskerin-associated H/ACA snoRNAs has been demonstrated[84-86] (Table 1). Thus, lower pseudouridylation levels are a widespread feature of cancer.

INOSINE IN CANCER RNA

Inosine is an RNA modification resulting from the hydrolytic deamination of adenosine catalyzed by adenosine

deaminase enzymes acting on double-stranded RNA (ADAR) or adenosine deaminase acting on transfer RNA (ADAT), which are families known to function in A-to-I RNA editing. Enzymes of the ADAR family are catalytically active ADAR1, ADAR2 and ADAR3, which still has an unknown function.

ADARs introduce inosine in coding and non-coding RNAs and have drastic impacts on the cellular transcriptome and translatome. The hypo- or hyper-editome has been associated with diverse types of cancer. The role of ADAT in cancer has not been reported.

High editing levels in cancer

Most frequently editing locations are long, partially complementary RNAs formed from inverted non-coding repeats, such as *Arthrobacter luteus* (Alu) and long interspersed element (LINE) located in mRNA UTRs and introns. Two major studies have investigated RNA-editing patterns in tumors versus normal tissues. Each of the studies employed RNA-Seq datasets from The Cancer Genome Atlas (TCGA) project (https://cancergenome.nih.gov/) and compared them to reference datasets of editing sites. High-confidence RNA editing sites are annotated in the Rigorously Annotated Database of A-to-I RNA Editing (RADAR, http://rnaedit.com/), where one study focused on detecting Alu and non-Alu RNA editing events in 17 cancers, whereas the other study focused on Alu RNA editing events in 9 different cancers[87,88].

In general, elevated Alu editing activity in tumors compared to matched normal tissues was found in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD) and thyroid carcinoma (THCA). This hyperediting of Alu was attributed to ADAR1, whose expression levels matched in all these types of cancer, except COAD. Similarly, a study by Han *et al*[87] where more patient samples and non-Alu edited sequences were included, confirmed hyperediting in BLCA, BRCA, HNSC, LUAD, THCA compared to normal tissues. Again, increased editing levels correlated with the mRNA levels of ADAR1.

Increased ADAR-1 levels were reported in non-small cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), esophageal cell carcinoma (ESCC), gastric (GC) and cervical cancer, suggesting that tight regulation of editing levels might have implications in cancer development and that ADAR1 might act as an oncogene[89-92] (Table 1 and Figure 2).

Recoding editing: In non-small cell lung cancer samples, *ADAR1* gene amplification was shown to increase the editing of the DNA base excision repair glycosylase enzyme NEI-like protein 1 (NEIL1). Pre-mRNA editing of NEIL-1 causes a lysine to arginine (K242R) change in the lesion recognition loop of the protein. The edited NEIL1 protein removes thymine glycol from duplex DNA at a lower rate compared to the unedited form, while repair of the guanidinohydantoin lesion is enhanced by edited NEIL1[93]. In overexpression experiments, transfection of edited NEIL1 enhanced the growth of A459 cells in comparison with the transfection of unedited transcripts. Thus, increased recoding editing of NEIL1 as a proposed target of ADAR1 could contribute to the phenotype of lung cancer cells[94].

AZIN1 (encoding antizyme inhibitor 1) is edited by ADAR1, which has increased expression levels in HCC and was found to positively correlate with AZIN1 editing frequency. AZIN1 is an antizyme inhibitor whose activity is crucial in limiting cellular proliferation. Antizyme binds and induces the degradation of the growth-promoting proteins ornithine decarboxylase (ODC) and cyclin D1 (CCND1)[95,96]. AZIN1 is homologous to ODC and has a greater binding affinity to antizyme compared to ODC. Binding of AZIN1 to antizyme prevents the degradation of ODC[97]. Thereby, AZIN1 acts as an oncogene by inhibiting the tumor-suppressor activities of antizyme[96]. AZIN1 expression was found to be substantially elevated in cancers of the prostate, brain, breast and liver, and gene expression data have identified alterations in the AZIN1-to-antizyme ratio in many human cancers, confirming its role in promoting growth[98-100]. In HCC, increased A-I editing of the AZIN1 transcript introduces serine-to-glycine substitution at residue 367 in the protein. This recoding editing is associated with conformational changes and translocation from the nucleus to the cytoplasm and results in a higher-binding affinity to antizyme and greater protein stability, thus promoting cell proliferation. AZIN1 editing increases during the progression from primary liver cancer and cirrhosis to advanced HCC with recurrence and metastasis, suggesting its use as a prognostic marker[101]. It is plausible that similar editing events occur in other types of cancer, as has been confirmed in esophageal squamous cell carcinoma (ESCC) and breast cancer[87,92,102]. Recently, tumorigenesis of NSCLC was also attributed to high levels of AZIN1 editing[103]. It has been reported that AZIN1 editing levels correlate with sensitivity to drug treatment in cancer cell lines. Cancer cells lines with increased levels of AZIN1 editing showed more sensitivity to some of the chemotherapies used in small cell lung cancer (SCLC): Paclitaxel, irinotecan, and topotecan[87].

Filamin B (FLNB) is an actin cross-linking protein and, together with filamin A, it forms homo- and heterodimers mediating orthogonal branching of actin filaments[104]. Filamin B is known to be a target for editing[105], and interestingly, one recoding editing event was shown to be increased in two types of cancer. In HCC, ADAR1 and ADAR2 were both reported to mediate FLNB transcript editing in codon 2269, resulting in the amino acid change Met→Val. Increased editing of FLNB compared to matched non-tumor liver tissues has been closely associated with HCC pathogenesis from normal to clinically verified HCC. In this study, ADAR1 levels were shown to be increased, while ADAR2 levels were decreased in HCC samples compared to non-tumor

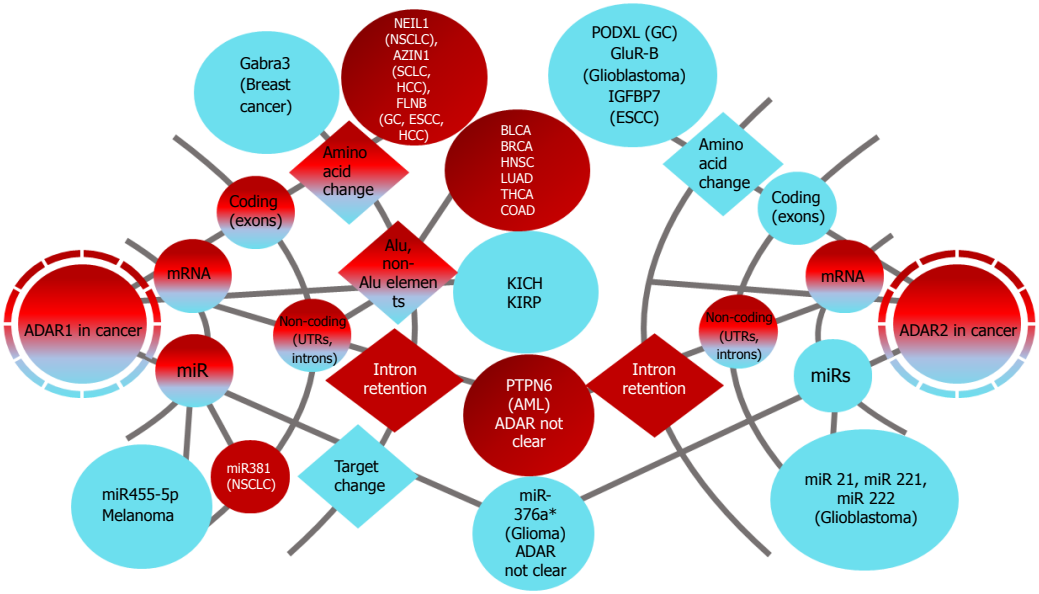

Figure 2 Editing of double-stranded RNA on mRNA and miRNA. Below, high ADAR1 levels are associated with breast cancer, NSCLC, colon cancer and cervical cancer, while low ADAR1 levels are shown in melanoma. Low levels of ADAR2 are present in glioblastoma. HCC, ESCC and gastric cancer are indicated as ADAR2-low and ADAR1-high cancers. The potential roles of ADAR1 and ADAR2 in cancer are depicted by mind map. Red means high expression/inosine content and blue means low expression/inosine content. In circles are the names of molecules/cancers and in diamonds are the mechanisms. NSCLC: Non-small cell lung cancer; HCC: Hepatocellular carcinoma; ESCC: Esophageal cell carcinoma; GC: Gastric cancer; ESCC: Esophageal squamous cell carcinoma; AML: Acute myeloid leukemia; SCLC: Small cell lung cancer; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; COAD: Colon adenocarcinoma; HNSC: Head and neck squamous cell carcinoma; LUAD: Lung adenocarcinoma; THCA: Carcinoma; KICH: Kidney chromophobe; KIRP: Kidney renal papillary cell carcinoma; NEIL1: NEI-like protein 1; Gabra3: Alpha-3 subunit of gamma-aminobutyric acid type A; FlnB: Filamin B; PTPN6: Protein tyrosine phosphatase non-receptor type 6; PODXL: Podocalyxin-like; GluR-B: Glutamate R-B; IGFBP7: Insulin-like growth factor-binding protein 7.

liver tissues[90]. The same recoding editing was found in ESCC, where unlike HCC, only ADAR1 was responsible for this hyperediting. FLNB hyperediting correlated with ADAR1 levels in ESCC samples[92]. The functional role of FLNB editing is not known.

Ras homolog family member Q (RHOQ) belongs to a family of Rho GTPases that are known to be intracellular signaling molecules regulating the actin cytoskeleton and thereby cellular functions, such as cell polarity, migration, and vesicular trafficking. Rho GTPases are present as either an active GTP-bound form or an inactive GDP-bound form[106]. Activation of Rho GTPases is implicated in the development and progression of many types of human malignancies, including CRC[107]. RhoQ has been most extensively studied for its central role in insulin-stimulated GLUT4 transport in adipocytes[108]. Amino acid substitution of asparagine with serine (N136S) in the edited RhoQ was identified in colorectal cancer (CRC). The ADAR responsible has not been identified. This editing was suggested to change RhoQ protein–protein interactions and induce increased levels of RhoQ binding of GTP, causing actin cytoskeletal reorganization and increased invasion potential without affecting proliferation in CRC cell lines. Moreover, edited RHOQ was associated with recurrence of CRC when present in the tumor[109].

Protein tyrosine phosphatase non-receptor type 6 (PTPN6) is a cytoplasmic protein expressed in hematopoietic cell development, proliferation and the receptor-mediated mitogenic signaling pathway[110,111]. In bone marrow mononuclear cells (BMMCs) of patients with acute myeloid leukemia, a novel PTPN6 transcript retaining intron 3 has been identified. This transcript arises from an alternative splicing reaction where editing-mediated deamination of A7866 in intron 3 erases this branch formation site, making it invisible to the splicing machinery. The ADAR responsible has not been identified. It is suggested that this retention results in the translation of a nonfunctional protein where the intron 3-encoded sequence is located in the N-terminal Src homology 2 (SH2) domain. PTPN6 binding with partner proteins, such as proto-oncogene receptor tyrosine kinase-c-Kit[112], and its self-inhibition of phosphatase activity occurs *via* its N terminal domain[113]. All this information suggests that its deregulation ultimately leads to uncontrolled hematopoietic growth and function. The tumor-specific editing seen in AML might correlate with the clinical course of the disease since low levels of intron-retaining transcripts in patient BMMCs at remission compared to those at diagnosis suggest that editing promotes tumorigenesis[114].

High miRNA editing levels in cancer: *ADAR1* gene amplification in NSCLC demonstrated ADAR1 overexpression in patients with early-stage lung cancer, underlining its potential oncogenic role in this cancer. Increased levels of ADAR1 corresponded with edited miR-381 levels in NSCLC. Overexpression of edited miR-381 in NSCLC possibly contributes to stemness and chemoresistance[94].

Low editing levels in cancer

Low levels of mRNA open reading frame editing in cancer: The same correlation but in the opposite direction was shown in hypoedited cancers, such as kidney chromophobe (KICH) and kidney renal papillary cell carcinoma (KIRP), with ADAR1 mRNA paired editing levels. The ADAR2 levels checked in both studies showed a complex expression pattern but no matching editing levels (Table 1 and Figure 2)[87,88]. The role of ADAR1 in breast cancer is not fully understood. A recent study reported high ADAR1 expression in half of the examined triple-negative-cancer patients[115]. Conversely, it has been proposed that ADAR1 prevents tumor progression by editing the transcript coding for the alpha-3 subunit of gamma-aminobutyric acid type A (Gabra3).

The chloride-permeable gamma-aminobutyric acid type A ($GABA_A$) receptors are crucial mediators of fast inhibitory neurotransmission in the central nervous system[116]. The Gabra3 transcript undergoes recoding editing of isoleucine to methionine (I/M) in the third transmembrane region. This substitution was found to affect $GABA_A$ surface presentation and its cellular trafficking[117]. In addition to being normally expressed in normal neuronal tissues, Gabra3 has been identified in breast cancer, where its high expression inversely correlates with breast cancer survival. ADAR1-edited Gabra3 was found in non-invasive breast cancer cell lines and was linked with the protein kinase B (Akt) pathway. A proposed mechanism for the non-invasive phenotype is that Gabra3 editing reduces its surface expression and indirectly prevents Akt activation, thereby preventing cell proliferation and invasiveness. Thus, the unedited form of Gabra3 in breast cancer is suggested to promote tumor progression, invasion and metastatic potential[118].

Lower ADAR2 levels are recognized in gastric cancer, glioblastoma, HCC and ESCC[91,119,120] (Figure 2). ADAR2 levels were found to correlate with changes in podocalyxin-like (PODXL) and GluR-B functions. The PODXL RNA editing event is an amino acid substitution from histidine (His) to arginine (Arg) at codon 241. This editing in the gastric cancer cell line MKN28 was shown to prevent increased growth rates and invasive capability compared to cells with the unedited form. Moreover, recoding editing of a single position located in the channel-pore-loop domain in GluR subunit B (GluR-B) (the Q/R-site) from Gln to Arg results in a channel that is impermeable to Ca^{2+}[121]. Tight regulation of editing is essential for the adequate function of this channel. Hypoedited PODXL and GluR-B with altered functions are associated with gastric cancer and malignant glioblastoma, respectively. Consequently, a tumor-suppressor role has been attributed to ADAR2[91,119].

In ESCC, it has been reported that ADAR2 promotes apoptosis by editing and stabilizing insulin-like growth factor-binding protein 7 (IGFBP7) RNA. IGFBP7 is a secreted factor binding to and interfering with the activation of IGF1R. Through receptor occupation, IGFBP7 blocks downstream phosphatidylinositol 3-kinase (PI3K)-AKT signaling, resulting in the inhibition of protein synthesis and cell apoptosis[122]. IGFBP7 was previously reported to be an apoptotic promoter in prostate cancer[123], colorectal cancer[124] and breast cancer[125]. The editing site in IGFBP7 is at position 284 of the coding sequence, and codon 95 is changed from AAG (lysine) to AIG, which is read as AGG (arginine) (K95R). This editing was shown to protect IGFBP7 against matriptase proteolysis in ESCC culture and xenografts, thus enabling the proapoptotic function of IGFBP7. ADAR2 is known to be downregulated in ESCC, and its upregulation induces apoptosis in ESCC cell lines *in vitro*, suggesting that IGFBP7 under editing may promote tumorigenesis in esophageal squamous cell carcinoma[120].

Low miRNA editing levels in cancer: ADAR2 rescue in glioblastoma cells was shown to inhibit cell proliferation and migration, confirming its possible tumor-suppressor role[126]. This anti-tumor effect might be explained through the regulation of onco-miRNAs in glioblastoma. Three particularly investigated onco-miRNAs, miR-221, miR-222 and miR-21, are overexpressed in glioblastoma[127]. ADAR2 can edit miR-222/221 and miR-21 precursors and decrease the expression of the corresponding mature onco-miRNAs in the normal mouse brain and in different lines. Decreased levels of ADAR2 identified in glioblastoma probably push the balance of onco-miRNA/tumor-suppressor miRNA towards increased expression of onco-miRNAs, such as miR-221, miR-222 and miR-21, thereby supporting tumor progression[128].

In the human brain, the miR-376 cluster encodes 4 pri-miRs that give rise to 5 distinct mature miRNAs, which are subjected to specific A-to-I RNA editing on 9 adenosines. In noninvasive U87 glioma cells, the expression of the unedited miR-376a* was shown to promote aggressive tumor migration and invasion of these cells both *in vitro* and *in vivo*. The editing reaction missing in the GBM cell lines generally occurs in the seed region of pri-miR-376a1 at the +9 site, ultimately giving rise to mature edited miR-376a*. The absence of this editing changes the specific targets of the miRNA. It has been identified that non edited miR-376a*, through its binding to 3′ UTR, has a novel target, RAP2A, which is a member of the RAS oncogene family with an unknown function. However, the non edited miR-376a* targeting of RAP2A is unable to target the autocrine motility factor receptor (AMFR), resulting in its upregulation and possibly contributing to increased migration and invasiveness of glioma cells[129].

Melanoma is the most aggressive type of skin cancer. It has been reported that there is a significant decrease in ADAR1 expression in approximately 65% of metastatic melanoma specimens compared to melanocytes[130] (Table 1 and Figure 2). ADAR-1 transcripts were found to be targeted by miR-17 and miR-432, thus decreasing

ADAR1 expression. Both miR-17 and miR-432 were identified to be overexpressed in melanoma possibly due to the amplification of encoding genes[130]. However, studies suggest that ADAR1 insufficiencies contribute to the enhancement of proliferation of melanoma cells through editing the independent regulation of miRNA biogenesis. miRNA-455-5p was identified as a target of ADAR1 in low-metastatic melanoma cells but not in highly metastatic cell lines. ADAR1 was shown to edit pri-miR-455-5p at +2 and +17 positions. This editing probably results in the reduction of the processing of pri-miRNA by Dicer or Drosha by lowering the binding affinity. However, it is also possible that ADAR1 binds to Dicer since the amount of miR-455-5p bound to Dicer and Drosha was inversely correlated with ADAR1 expression. ADAR1 was shown to form a complex with Dicer through protein-protein interactions[131]. In this study, the authors gave a model of RNA editing in the context of melanoma progression and metastasis, where cAMP responses element binding (CREB) downregulates ADAR1 and gives rise to non-edited miR-455-5p. Expression of miR-455-5p suppresses the tumor suppressor gene cytoplasmic polyadenylation element-binding protein 1 (CPEB1), resulting in growth promotion and metastasis in melanoma cells[132].

M5C IN CANCER RNA

Cytosine base methylation - m5C has been identified in rRNA, tRNA and recently in mRNAs and is particularly enriched in untranslated regions and near Argonaute-binding regions[133]. The enzymes responsible for the introduction of m5C are members of the DNA methyltransferase homolog (Dnmt2) and the NOP2/Sun (NSun 2 and 4) RNA methyltransferase family[134-136]. The role of these enzymes in the methylating activities of tumorigenesis is currently unknown. However, in circulating tumor cells from lung cancer patients, increased RNA m5C levels were shown compared to those in whole blood cells[137]. Further investigation of the role of m5C in cancer is required.

CONCLUSION

Tight regulation of the writing, reading and eventual erasing of RNA modifications is essential for RNA metabolism. Misbalanced expression of the enzymes responsible for introducing, and in some cases removing, these modifications are considered a possible signature for specific types of cancer (Table 1). Considering the broad effect of RNA modifications on tumor cell biology, future methylome, pseudome and editome studies will shed light on those relatively unexplored epitranscriptomic mechanisms in tumors. Those studies will pave the way for the development of anti-cancer drugs that could act by steering RNA modifications.

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EDITORIAL

Quality of life and oral potentially malignant disorders: Critical appraisal and prospects

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Abstract

Quality of life (QoL) is a vital and often required health outcome measure that is relevant to patient care. A healthy oral cavity enables person to perform daily activities without any limitations. However, any disturbance may result in impaired QoL. The oral health-remains an essential element of people's health and well-being. In recent years, the tradition of clinical practice and research has been changed by incorporating QoL assessment, as it helps in assessment of patients' needs and monitoring treatment responses. Oral potentially malignant disorders (OPMDs) are a group of chronic disorders including oral leukoplakia (OL), oral lichen planus and oral submucous fibrosis (OSF). It is evident that patients with OPMDs experience significant health-related symptoms, functional limitations and psycho-social impairment, compromising their QoL. Moreover, the worsening of QoL has been associated with advanced stages of OPMDs. Despite of increasing number of OPMD cases in recent decades, limited literature is available regarding QoL in this population. Although, there is higher prevalence of habit-related OPMDs, particularly OSF and OL in Southern Asian countries, only a few studies have been performed in these populations. Moreover, these studies administered generic QoL instruments, which offer less sensitivity to clinical changes. However, condition-

specific instruments are more sensitive and allows better measurement of QoL. As the impacts of different conditions on OHRQoL may vary, the development and validation of a QoL instrument specific to each clinical entity of OPMDs is currently needed.

Key words: Quality of life; Oral potentially malignant disorders; Oral submucous fibrosis; Oral lichen planus; Oral leukoplakia

Core tip: The quality of life (QoL) assessment has become an essential tool in clinical practice to better understand patient reported outcomes in recent years. It definitely helps to better understand the impact of oral health on the lives of patients with oral potentially malignant disorders (OPMDs) and their families and to monitor the outcomes of treatments. It is a foremost pre-requisite to employ the best available QoL instrument when treating OPMDs. In view of the scarcity of research on QoL assessments in OPMDs, the development and application of condition-specific QoL instruments can allow them to become tools to better understand and shape the state of clinical practice, dental research and dental education.

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INTRODUCTION

The World Health Organization (WHO) has defined quality of life (QoL) as "an individual's perception of his position in life in the context of the culture and value system in which he lives and in relation to his goals, expectations and standards and concerns"[1]. In recent years, OHRQoL has become increasingly important in patient care and extensively applied as a part of daily practice[2]. A healthy oral cavity empowers an individual to perform routine daily activities without any physical and psycho-social limitations. However, any disturbance related with the oral cavity may disturb normal oral functions. Persistent discomfort and a functionally impaired oral cavity may subsequently result in decreased self-confidence and social communication of the individual, compromising his or her QoL. It is well-known that OHRQoL remains an essential element of people's health and well-being and helps in assessment of patients' needs and to monitor treatment responses[3,4]. Even though the impacts of oral diseases can be assessed by traditional methods, there is growing trend of availing patients' perspectives. Therefore, the new era demands QoL assessment using patient reported outcomes (PROs) and experiences (PREs) as a part of day-to-day practice[5]. Moreover, deciding proper treatment protocols and measuring treatment outcomes based on PROs and PREs is definitely helpful and has changed the tradition of clinical practice, surveys and research in recent years.

Oral potentially malignant disorders (OPMDs) are a group of chronic disorders with increased morbidity and mortality due to cancerous changes[6]. Per recent literature, the values of the malignant potential of oral leukoplakia (OL), oral lichen planus (OLP) and oral submucous fibrosis (OSF) are 3.5% (range, 0.13-34.0%)[7], 1.1%[8] and 7%-13%[9], respectively. Careful monitoring of these lesions by an experienced specialist is highly recommended to identify any malignant changes in the early stages to reduce the cancer burden. It has been documented that patients with OPMDs experience significant health-related symptoms affecting their QoL[10]. Moreover, OPMD patients shown psychological impairment due to their fear of developing cancer[11]. These patients also reported to have social and emotional imbalance. Although oral cancer (OC) and OPMDs presents relatively similar health comorbidities; compromising the QoL[12], the available OHRQoL instruments are OC/head and neck cancer specific, and thus, the OHRQoL of patients suffering from OPMDs is seldom assessed. Moreover, the literature on QoL assessment in this population is scanty in contrast to the plentiful literature on QoL in OC/head and neck cancer patients[13,14].

OSF is an OPMD that is highly prevalent in Indian subcontinents and South-East Asia, affecting 5 million people in India alone[9]. Its etiology is multifactorial but arecoline in the areca nut is the main causative agent in initiating the disease process. OSF is clinically characterized by a early sign and a symptoms of burning sensation, vesiculation and ulceration in the oral cavity and lately followed by blanching of the oral mucosa. This results in to increasing stiffeness and marked rigidity of the tissues leading to reduced mouth opening, significantly compromising the patient's QoL. It is evident that OSF have detrimental effects on OHRQoL and the worsening of QoL has been associated with advanced stages of OSF[15].

OLP is a chronic inflammatory disorder with etiopathogenesis that is still poorly understood. OLP affects approximately 1%-2% of the population worldwide[16] and is more prevalent in middle-aged females. It is characterized by outbreaks or flares of different types of clinical presentations, which has been categorized by Eisen[17] into three subtypes: (1) reticular form; (2) erosive/atrophic form; and (3) ulcerative form. Even though the reticular form is asymptomatic, erosive and ulcerative forms are often painful and disabling and are variants with burning sensations of the oral mucosa. The persistent painful symptoms can have a significant negative impact on daily life activities including eating, swallowing or speaking. Moreover, OLP has been linked

with impaired psychosocial morbidity and QoL[4,18].

The prevalence of OL is approximately 1%, with a greater number of cases seen in adults. The etiology of OL includes chewing or smoking of tobacco and related products. Clinically, OL can be classified into homogenous and non-homogenous subtypes, with the highest malignant potential reported in proliferative verrucous leukoplakia and speckled leukoplakia. OHRQoL of patients with OL was evaluated in a few past studies[19,20].

Our recent systematic review demonstrated that the QoL of patients affected by different OPMDs has been studied and successfully assessed by various authors using different QoL instruments in European countries. However, most of these studies have focused on QoL in patients with OLP, which is not at all applicable to all OPMDs[21]. Despite the fact that habit-related OPMDs, such as OSF and OL are highly prevalent in Southern Asian countries[22], surprisingly, only a few studies have assessed QoL in patients with OSF and OL in this population to our knowledge. Moreover, all these studies administered QoL instruments, namely the Oral Health Impact Profile (OHIP), University of Washington Quality of Life Questionnaire (UW-QOL), Chronic Oral Mucosal Disease Questionnaire (COMDQ) and Oral Health Related Quality of Life-UK (OHQoL-UK). However, these instruments are generic to a range of chronic oral mucosal diseases and are not condition-specific. The generic questionnaires offer less sensitivity to clinical changes than disease-specific tools[23], as they are applicable to a wide variety of population and disease states. In contrast, it is well-known that condition-specific instruments allow for better measurement of QoL than generic questionnaires, as they evaluates the effects of a concerned disease on life quality of an individual. A condition specific QoL tool for OPMD, *i.e.,* the OPMDQoL questionnaire study, observed a significant impact of OLP and OSF compared to OL on the QoL of affected patients especially in the subscales of "physical impairment and functional limitations"[24]. Recently, we developed and validated a condition-specific instrument for OSF patients. This was found reliable in QoL evaluation tool in an Indian population[25].

We believe that QoL assessment has become a necessity to determine the feelings and perceptions of patients as well as to increase effective communication between health care professionals and patients. This definitely provides clues not only to better understand the influence of oral diseases on the patients and their families but also to monitor the outcomes of the treatments provided. Currently, increased incidence of OPMDs specifically OSF and OL in South Asian countries, is an alarming situation as far as oral cancer is concerned. This might be due to the increased popularity of commercially available areca nut and tobacco preparations, especially in India. In addition, an increasing number of young people are becoming addicted to this ancient, socially acceptable habit due to easy access, effective price changes and marketing strategies. In view of the scarcity of research on QoL assessment in OPMDs, there is a dire need for more studies to better understand this situation. It is evident that researchers have been continuously focusing on improving the QoL of affected individuals. Therefore, it is a foremost pre-requisite to employ the best available QoL instrument in OPMDs. Furthermore, due to differences in their pathogenesis and clinical presentations and thus, differing impacts on OHRQoL, the development and validation of a QoL instrument specific to each clinical entity of OPMD separately is needed. Such condition-specific instruments can become tools of choice in future researches and help to improve QoL of affected individuals.

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MINIREVIEWS

Polyubiquitination inhibition of estrogen receptor alpha and its implications in breast cancer

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Abstract

Estrogen receptor alpha (ERα) is detected in more than 70% of the cases of breast cancer. Nuclear activity of ERα, a transcriptional regulator, is linked to the development of mammary tumors, whereas the extranuclear activity of ERα is related to endocrine therapy resistance. ERα polyubiquitination is induced by the estradiol hormone, and also by selective estrogen receptor degraders, resulting in ERα degradation *via* the ubiquitin proteasome system. Moreover, polyubiquitination is related to the ERα transcription cycle, and some E3-ubiquitin ligases also function as coactivators for ERα. Several studies have demonstrated that ERα polyubiquitination is inhibited by multiple mechanisms that include posttranslational modifications, interactions with coregulators, and formation of specific protein complexes with ERα. These events are responsible for an increase in ERα protein levels and deregulation of its signaling in breast cancers. Thus, ERα polyubiquitination inhibition may be a key factor in the progression of breast cancer and resistance to endocrine therapy.

Key words: Estrogen receptor alpha polyubiquitination; Breast cancer; Estrogen receptor alpha

Core tip: The inhibition of the estrogen receptor alpha polyubiquitination and degradation by several molecular mechanisms is related to the progression of breast cancer and resistance to endocrine therapy.

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INTRODUCTION

Estrogen receptor alpha (ERα) protein, also known as nuclear receptor subfamily3 group A member 1 (NR3A1), comprises of 595 amino acids, organized in two activation function domains (AF-1 and AF-2), a DNA-binding domain (DBD), a ligand-binding domain (LBD) that recognize the 17beta-estradiol hormone (E2), and a hinge region that connects the DBD and the LBD[1-3] (Figure 1). Many nuclear functions of ERα are triggered by the binding of E2 to the receptor[4,5], inducing ERα homodimers to bind to estrogen responsive elements (ERE) within the enhancer and promoter regions of E2-target genes[6,7]. In these events, pioneer factors expose chromatin sections, facilitating the association of ERα with EREs[8]. Moreover, transcriptional coregulators are recruited by the AF-1 and AF-2 domains of the receptor for the remodeling of the chromatin structure[9,10] and promotion of chromatin loops that modulate E2-responsive gene expression[11,12]. In addition, there is crosstalk between ERα and other signaling pathways: ERα acts as a coregulator by interacting with other transcription factors, such as activator protein 1 (AP-1), specificity protein 1 (Sp1), and nuclear factor-κB (NF-κB)[3,5,13-17]. Additionally, ERα is phosphorylated and transcriptionally activated in response to growth factors such as the epidermal growth factor (EGF) and insulin-like growth factor (IGF)[13,14,18-20]. Recently, progesterone receptor (PR) was shown as an ERα interacting protein that modulates and re–directs the binding of ERα to the chromatin and the expression of specific genes in breast cancer cells [21] (Figure 2).

ERα also exhibits extranuclear activity by associating with the cell membrane *via* palmitoylation, and with the help of protein complexes, linked to the cell membrane or cytoplasm[22] (Figure 2). Thereafter, ERα transduces rapid extranuclear signaling that can trigger second messengers such as calcium and cAMP, and activate kinases such as ERK/MAPK, PI3K/AKT, PKC and Src kinase[13,23,24]. Both nuclear and extranuclear signaling of ERα are connected and are critical in about 70% of breast cancer cases (ERα+ breast cancer)[13,24,25]. Consequently, ERα is a target for endocrine therapy *via* the use of selective estrogen receptor modulators (SERMs), such as tamoxifen (Tam), which competes with E2 by binding to ERα to inhibit its transcriptional activity, as well as, *via* the use of selective estrogen receptor degraders (SERDs) such as fulvestrant that decreases the ERα stability[8,14,26,27]. The acquisition of resistance to these treatments commonly occurs in ERα+ breast cancer, and although the mechanisms are unclear, the extranuclear signaling of ERα is strongly activated under this condition[19,20,26,28-31].

The activation or inhibition of ERα activity is modulated by its transcriptional coregulators, by phosphorylation induced by E2 hormones and growth factors, and by other posttranslational modifications such as ubiquitination. Remarkably, several studies have emerged to demonstrate that multiple mechanisms are activated in ERα+ breast cancers to inhibit ERα polyubiquitination, increasing its signaling pathways (Figure 2), which have crucial implications in the progression of this cancer type, as we will describe in the following sections.

GENERALITIES OF THE POSTTRANSLATIONAL MODIFICATION "UBIQUITINATION" FOR ERα IN BREAST CANCER CELLS

ERα is a monoubiquitination and polyubiquitination-target. However, fewer reports are available to demonstrate monoubiquitination of ERα, in comparison to those that exhibit polyubiquitination of this receptor. Nevertheless, these studies clearly show that ERα monoubiquitination is decreased by E2, and that, this modification is important, both for stability and for the transcriptional activity of this receptor in breast cancer. In contrast, polyubiquitination is induced by E2, resulting in a signal to direct ERα degradation *via* the UPS[14,32,33], facilitated by the concerted action of the enzymes E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase)[32,33]. The specific covalent binding of ubiquitin to ERα lysine residues is mediated by several E3 ubiquitin ligases for ERα, that include CHIP[34], E6AP[35], BRCA1[36], BARD1[37], SKP2[38], MDM2[39], and Hbo1[40]. Importantly, E2 treatment induces ERα polyubiquitination, followed by its degradation by the UPS[14,17,33,41-43].

Although polyubiquitination leads to ERα downregulation through its degradation by the 26S proteasome, it is important to note that, this modification and the proteasome activity, have also been reported as elements required for the transcriptional cycle of ERα. Likewise, it has been evidenced that ERα bound to ERE can recruit coactivators, some of which possess E3-ubiquitin ligase activity, such as SKP2[17], E6AP, and RNF8. As coactivators enhance the activity of ERα, and the activity of E3-ubiquitin ligases mediate the downregulation of this receptor, the recruitment of these proteins with dual function may maintain a balance in the level and activity of ERα[17,44,45].

ERα residues, K302 and K303, have been suggested as the lysine targets for ubiquitination and degradation, in response to E2 and fulvestrant, but the same residues are also important for ERα stability in untreated breast cancer cells[46]. Against this background, it maybe envisaged that, several factors delicately modulate the stability and degradation of ERα, which may be altered

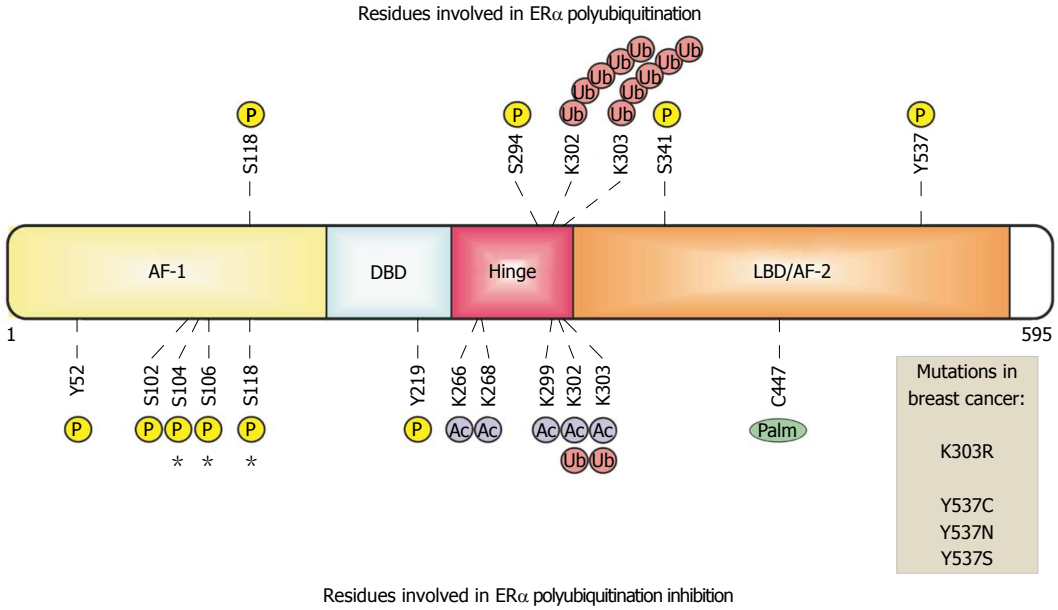

Figure 1 Estrogen receptor α in breast cancer cells. ERα is organized in functional domains. The transactivation domains AF-1 and AF-2 recruit both coactivators and corepressors. The DNA-binding domain (DBD) recognizes and binds to estrogen response elements in enhancers or promoters. The ligand-binding domain (LBD) is recognized and activated by the 17 beta estradiol hormone. The hinge domain links LBD and DBD allowing the conformational changes of this receptor. Some residues are modified by phosphorylation, acetylation, ubiquitination and palmitoylation , which are related with ERα polyubiquitination. Sites of phosphorylation or mutations in ERα that have been identified in breast–cancer biopsy samples are indicated.

in breast cancer.

Additionally, the ubiquitination of ERα is also related to its phosphorylation state. Several kinases, such as CDK11p58[47], cyclin E-CDK2[17], Src[35], PKC[42], p38MAPK[38], and ERK7[48] have been reported as modifiers of ERα in breast cancer. The main residues of ERα that are phosphorylated in E2-response, and have been associated with its polyubiquitination and degradation, are S118[49], S294[38], S341[17], and Y537[35]. A key example is the sequential modification of ERα, where, first, the ERα Y537 residue is phosphorylated by Src kinase in E2-treated cells, followed by E6AP, an E3-ubiquitin ligase, which induces ERα polyubiquitination and its degradation[35]. Thus, phosphorylation and ubiquitination of ERα are interconnected in order to control both, the abundance and the functions of this receptor.

IS ERα IN BREAST CANCER CELLS POLYUBIQUITINATED AND DEGRADED?

In recent years, several studies have emerged to demonstrate the inhibition of polyubiquitination of ERα and consequently, a decrease in its degradation *via* the UPS, increasing its protein stability in breast cancer cells, through several mechanisms and ERα-associated proteins. Here, we describe these evidences.

ERα polyubiquitination inhibitor proteins in breast cancer cells

ERα polyubiquitination inhibitor proteins (EPIP). There has been a progressive increase in the number of ERα polyubiquitination inhibitor proteins that have been discovered in breast cancer cells, which we have grouped and identified as EPIP. So far, it has been reported that proteins such as Mucin 1 (MUC1), PIN1, GSK3, LMTK3, RNF8, RNF31, RB, ABL, SHARPIN, and SMURF1 have the ability to interact with ERα, conferring it protection against polyubiquitination and degradation. Interestingly, not all of these proteins have related sequences and structures, but some of them are functionally similar.

MUC1 and Protein interacting with Never in mitosis A (PIN1), for example, induce the formation of stable transcription complexes on the DNA[49,50]. MUC1 interacts with ERα to inhibit its polyubiquitination and degradation, and recruits coactivators such as SRC1 and GRIP on E2-regulated promoters to enhance gene transcription linked to cellular proliferation, migration, tumorigenicity, and endocrine resistance[50-54]. Likewise, PIN1 interacts with ERα phosphorylated at S118, inducing its cis/trans isomerization. Moreover, PIN1 blocks the polyubiquitination and degradation of ERα by preventing its interaction with the E6AP E3 ligase, hence enhancing its stability, binding to EREs, and the subsequent transcriptional activity of ERα[10,49,55-57]. High levels of PIN1 and ERα, and low levels of E6AP are observed in endocrine resistance[49].

Other examples are GSK3, LMTK3, and ABL1 kinases that phosphorylate ERα to inhibit its polyubiquitination[58,59]. First, the glycogen synthase kinase-3 (GSK3) isoforms interact with and phosphorylate ERα at S102, S104, S106, and S118. GSK3 depletion decreases phosphorylation and E2-induced transcriptional activity by increasing polyubiquitination and degradation of this receptor[59-61]. Thereafter, LMTK3 (lemur tyrosine kinase 3) interacts with and phosphorylates ERα to protect it from

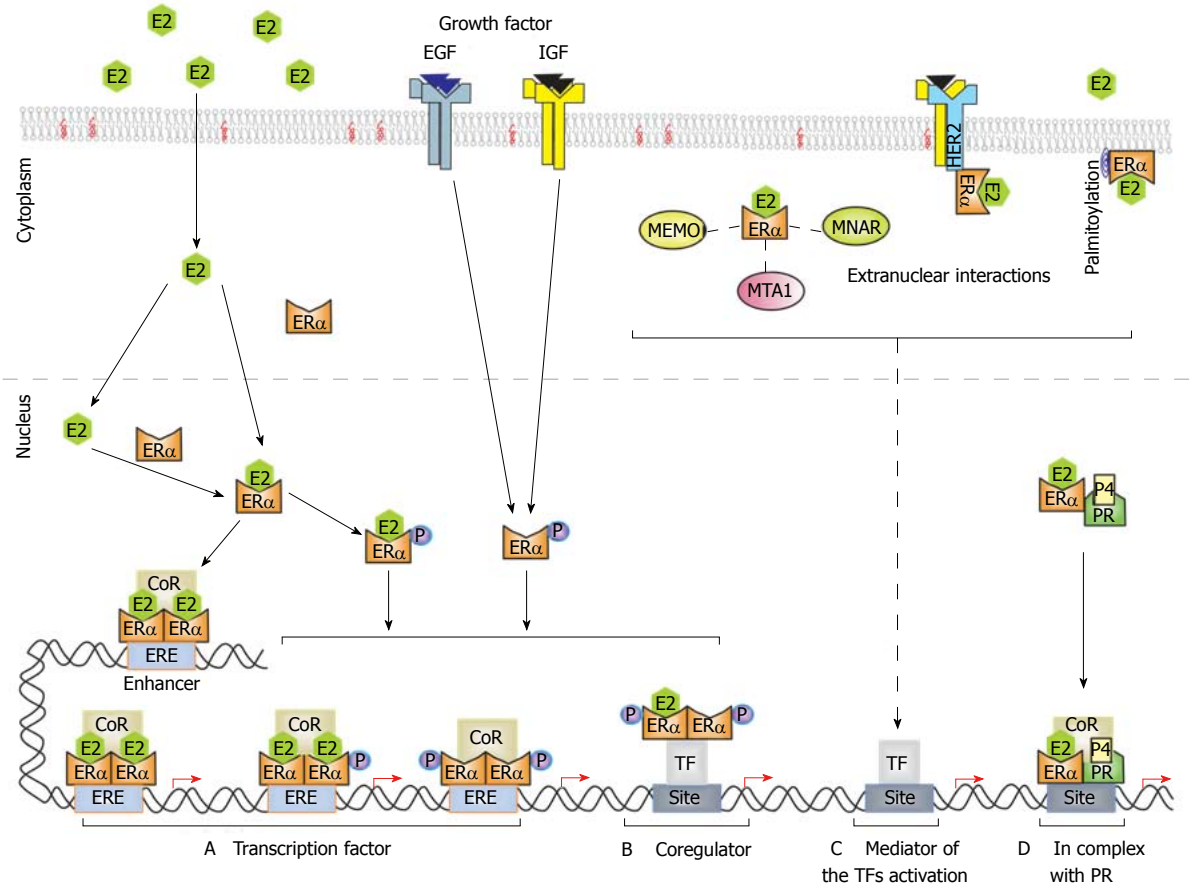

Figure 2 Nuclear and extranuclear signaling of estrogen receptor α. E2 binds to ERα in the cytoplasm and/or nucleus. Then ERα forms homodimers that recognize the ERE sequence (AGGTCAnnnTGACCT) in target enhancers and promoters, recruiting coregulator (CoR) complexes such as coactivators to induce gene expression. ERα phosphorylation can be induced by E2 to modulate its activity as a transcription regulator. A and B: Growth factors (epidermal growth factor and insulin-like growth factor) also induce ERα phosphorylation in an E2-independent manner to promote ERα activity as a transcription factor or CoR for some transcription factors (*i.e.*, AP-1, Sp1, and NF-κB); C: Cell membrane-associated ERα (*via* palmitoylation) associated with transmembranal receptors (*i.e.*, HER2) or with cytoplasmatic proteins as (*i.e.*, MEMO, MTA1 and MNAR). These extranuclear interactions can induce kinase–dependent signaling that could finalize in the activation of some transcription factors; D: PR can associate with ERα to coordinate the binding of ERα to the chromatin modulating the expression of specific genes.

polyubiquitination and degradation *via* the UPS in breast cancer cells[58]. Similarly, ABL (ABL proto-oncogene 1, non-receptor tyrosine kinase) interacts with and phosphorylates ERα at Y52 and Y219, increasing the ERα stability and resistance to Tam; both proteins are increased in breast tumor tissue samples[62,63].

On the other hand, RB induces the assembly of ERα with chaperone proteins[64]. Hence, retinoblastoma (RB) interacts with ERα, HSP90, and p23 in the cytoplasm to protect ERα from polyubiquitination and degradation by the UPS. ERα is highly ubiquitinated and degraded in RB-knockdown cells; however, its levels are restored with MG132 (a proteasome inhibitor) treatment in breast cancer[64].

Interestingly, E3 ubiquitin ligases such as RNF8, RNF31, SHARPIN, and SMURF1 interact with ERα to block its polyubiquitination and to promote the proliferation of breast cancer cells. RNF8, RNF31, and SHARPIN inhibit ERα polyubiquitination by catalyzing monoubiquitination of this receptor, and as a result, ERα protein levels and E2-dependent transcriptional activity are enhanced in breast cancer cells[65]. SHARPIN could monoubiquitinate the ERα K302/303, but whether these residues are also modified by RNF8 and/or RNF31 is unclear. Moreover, RNF8 also acts as a coactivator for ERα in breast cancer cells. Instead, SMURF1 apparently inhibits polyubiquitination of ERα, but the implicated mechanisms need to be studied[65-68].

Other proteins and modifications that inhibit ERα polyubiquitination

ERα polyubiquitination indirect inhibitors (EPII), intriguingly, the inhibition of ERα polyubiquitination also occurs with the help of other proteins that lack the ability to directly interact with ERα. For instance, it has been suggested that Src-dependent phosphorylation of ERα allows E6AP to polyubiquitinate and induce the degradation of this receptor. However, PEBP4 (phosphatidyl ethanolamine-binding protein 4) protein[69,70] interacts with Src, blocking the phosphorylation and degradation of ERα induced by Src[69].

Furthermore, although the mechanisms are unclear,

it has been reported that ERα protein levels decrease in cells with low levels of REGγ (PA28γ, a nuclear proteasome coactivator), but when the proteasome is inhibited by MG132 treatment, ERα protein levels are recovered, suggesting that downregulation of REGγ promotes ERα polyubiquitination and degradation. High levels of REGγ and ERα in breast tumors correlated with poor prognosis in patients with breast cancer[69].

Additionally, some posttranslational modifications are also associated with ERα polyubiquitination inhibition. Hence, ERα acetylation induced by trichostatin (a deacetylase inhibitor) increases the p300 levels and the stability of the receptor in breast cancer cells, but the mechanisms implicated need to be investigated (Figure 1)[71]. Palmitoylation has also been linked to ERα polyubiquitination since it has been shown that the ERα mutants that cannot be palmitoylated are polyubiquitinated and degraded *via* UPS[72].

Mutations and modifications that affect ERα polyubiquitination detected in mammary tumors from patients

ERα polyubiquitination has a clinical relevance, since mutations and/or posttranslational modifications such as phosphorylation in residues of ERα have been identified in tumor tissues from samples of patients with breast cancer, and these residues have been linked to the polyubiquitination and downregulation by degradation of this receptor. Thus, the Y537 residue is required for the ERα phosphorylation, and this modification subsequently promotes polyubiquitination and degradation of the receptor[35]. However, mutations in the residues Y537N, Y537C, and Y537S are detected in mammary tumors of patients with metastasis and endocrine resistance. Accordingly, ERα polyubiquitination and degradation is prevented by experimentally induced mutations at the Y537 residue, and similarly, these mutations have been associated with the development of endocrine therapy resistance in breast cancer[15,73,74]. In the same way, the K303 residue is needed for ERα polyubiquitination and degradation, but this residue has been identified to be mutated as K303R in tumors of patients who have poor survival outcome and prognosis[46,74]. Other residues, such as S104, S106, S118, and S294, that seem to be related with ERα stability, have been found to be phosphorylated in breast tumor samples[15,73].

ERα POLYUBIQUITINATION INHIBITION IN BREAST CANCER AS A KEY FACTOR FOR THERAPEUTIC STRATEGY

ERα polyubiquitination for its downregulation *via* the UPS, is a central mechanism of some endocrine therapies with SERDs, such as fulvestrant[46,75]. Clearly, the induction of ERα polyubiquitination for its degradation decreases the abundance and pro-tumor activity of ERα, consequently novel drugs including AZD9496[76], GDC-0810[77], bazedoxifene[78], and RAD1901[79] have been synthetized as SERDs, but more studies are required. Despite the importance of SERDs in the therapy of breast cancer, EPIP are promising targets for the management of this disease. Remarkably, the proteins that inhibit the ERα polyubiquitination are enhanced in ERα+ breast cancers, contributing to disease progression. For this reason, EPIP may be useful as a biomarker for breast cancer and as a therapeutic target.

PIN1 is overexpressed in breast cancer and is related to mammary tumor growth, and epithelial-mesenchymal transition, and natural and synthetic inhibitors are being probed to control its activity[55,57,80-87]. Similarly, LMTK3 overexpression stimulates cellular proliferation and tumor formation, and correlates with shorter survival times in ERα+ breast cancer, and resistance to Tam treatment, but these events are reduced when LMTK3 expression is decreased[58,88-90]. Moreover, CG0009, is a GSK3 inhibitor that decreases proliferation of breast cancer cells[61,73,91-94].

Another molecule is RNF31, whose overexpression increases ERα protein levels, expression of ERα target genes and the growth of breast cancer cells, and these events are decreased when RNF31 is abated[65]. Lastly, the loss of RB expression seems to be related to the loss of ERα stability in ERα negative (ERα−) breast cancers and with poor responses to hormonal therapies in patients[64,95-98]. Thus, these proteins can be potential biomarkers and target for the treatment of ERα+ breast cancer.

Among EPIIs, PEBP4 inhibits ERα polyubiquitination and enhances its transcriptional activity in breast cancer cells. Because PEBP4 is overexpressed in breast cancer and competes with ERα for components of the UPS, this protein may be an important target for breast cancer. Additionally, specific posttranslational modifications, such as palmitoylation, acetylation and phosphorylation, as well as, mutations of sites linked to ERα polyubiquitination and degradation, demands more research to find new strategies for detection and treatment of breast cancer.

Muc1 is an EPIP in breast cancer

Mucin 1 (MUC1) is a heterodimeric glycoprotein conformed by MUC1 N-terminal (MUC1-N) and MUC1 C-terminal (MUC1-C) subunits[52]. MUC1-N is an extracellular glycosylated subunit and MUC1-C is a transmembrane subunit with a cytoplasmic domain that interacts with diverse proteins[54]. MUC1 is localized on the apical borders in normal mammary epithelium, but under breast cancer conditions, it also localizes to the nucleus. An aberrant expression of MUC1-C is detected in breast cancer cells through a regulation loop that implicates Rab31 protein inhibits the lysosomal degradation of MUC1-C, and *Rab31* gene expression is induced by MUC1-C[52-54,99]. Furthermore, *MUC1* is upregulated in 90% of breast cancers, where the expression of *Rab31* gene and other genes associated with endocrine resistance are modu-

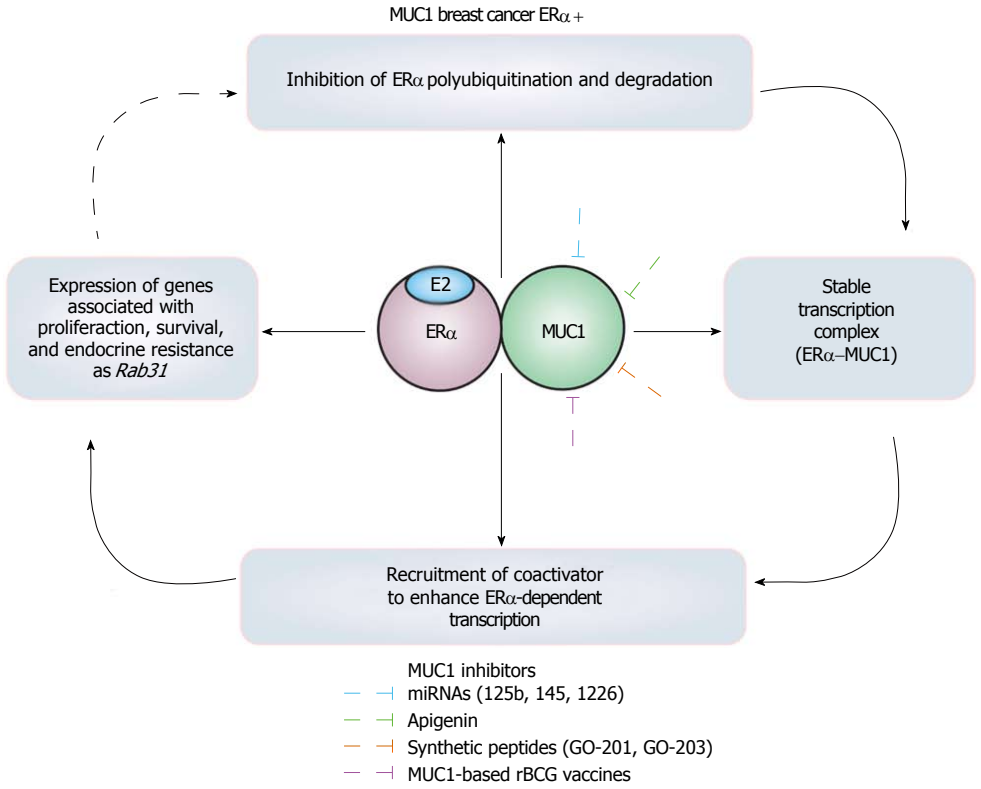

Figure 3 Mucin 1 is an estrogen receptor α polyubiquitination inhibitor protein in breast cancer cells.

lated by the MUC1-C/ERα complex. For these reasons, MUC1 has been suggested as a potential biomarker of breast cancer and predictor of resistance to Tam treatment[51,100,101] (Figure 3).

Interestingly, MUC1-C subunit interacts with DBD of ERα promoting (1) Inhibition of ERα polyubiquitination maintaining high levels of this receptor; (2) a stable complex between MUC1-C and ERα; and (3) an enhancement in the pro-tumor transcriptional activity of ERα since SRC1 and GRIP coactivators with histone acetyltransferase activity are recruited by MUC1[50]. Thus, MUC1-C increases the growth and survival induced by E2 in breast cancer cells, but also transformation, loss of cellular polarity, cellular proliferation and migration, anchorage-independent growth , and tumorigenicity in transgenic mouse models[51,99,102-104].

Remarkably, MUC1 is an EPIP involved in proliferation and endocrine resistance[50,53,54,100,105], inhibited by miR-125b[106], miR-145[104], miR-1226[103], and by specific siRNAs, inducing apoptosis, reducing cell proliferation, and increasing sensitivity to Tam[100]. Similarly, apigenin[107], and the synthetic peptides GO-201[54] and GO-203[100], affect localization and dimerization of MUC1, and as a result, tumor development is decreased, and sensitivity to Tam is increased[54,100,107]. Moreover, MUC1-based rBCG (Bacillus Calmette-Guerin) vaccines induce anti-MUC immune responses inhibiting the growth of tumors in mice[108,109]. Interestingly, high levels of Rab31 antigen have been associated with a proliferative status, a high tumor grade, and with poor 5-year disease-free survival in patients with ERα+ breast cancer. Consequently, the Rab31 antigen levels in mammary tumors have been suggested as a biomarker for ERα+ breast cancers that may also to be useful in the selection of patients for MUC1-targeted therapeutic strategies[110].

CONCLUSION

Several mechanisms seem to cooperate to inhibit ERα polyubiquitination, decreasing its degradation in ERα+ breast cancer cells. These cells become resistant to ERα polyubiquitination due to the evident upregulation of proteins, modifications, and mutations that protect it from ubiquitination. There is no pattern of the characteristics of the inhibitor or protector proteins for ERα polyubiquitination. Some of the reported EPIPs are MUC1, GSK3, LMTK3, RNF8, RNF31, SHARPIN, SMURF1, RB, and PIN1. All of them inhibit ERα polyubiquitination and its degradation in a dissimilar manner, *via* subcellular compartments or mechanisms. Some of them can be grouped as coactivators for ERα (MUC1, PIN1, and RNF8), kinases for ERα (GSK3, LMTK3, and ABL1), E3 ubiquitin ligase (RNF8, RNF31, SHARPIN, and SMURF1), and scaffold protein (RB). Amongst these different mechanisms, the participation of E3-ubiquitin ligases, such as RNF8, RNF31, and SHARPIN, are interesting, since they catalyze ERα monoubiquitination, suggesting a possible competition between monoubiquitination and polyubiquitination of this receptor.

Considering the findings described above, inhibition

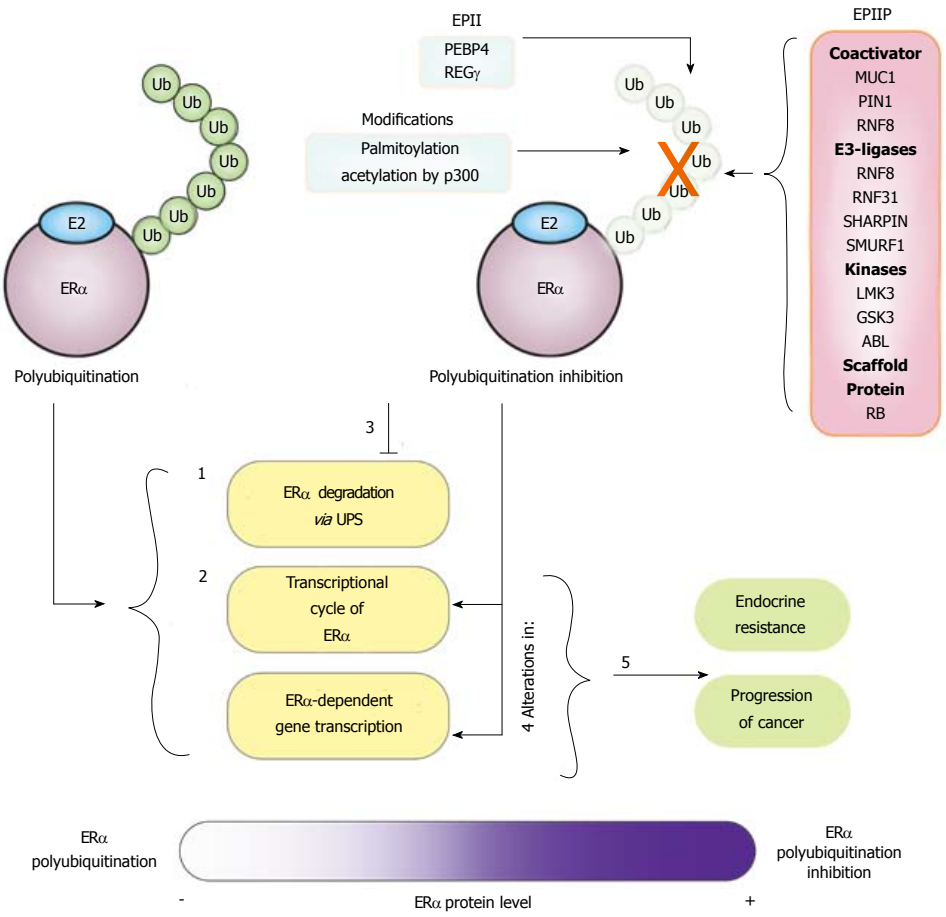

Figure 4 Mechanisms implicated in the estrogen receptor α polyubiquitination inhibition. Half-life of estrogen receptor α protein oscillates between 3-5 h under basal condition. E2 treatment induces ERα polyubiquitination, and as result: (1) Degradation of this receptor is promoted, decreasing its protein levels starting from 1h after treatment; (2) the ERα transcriptional cycle is activated. ERα polyubiquitination inhibitor proteins (EPIP) and ERα polyubiquitination indirect inhibitors (EPII) and other modifications increased in breast cancer cells can inhibit the basal and E2-induced polyubiquitination of ERα; resulting in (3) the inhibition of its degradation and an enhancement in the ERα protein levels; (4) alterations in the transcription cycle of this receptor and the expression of its targets genes; and (5) these events seem to be associated with endocrine resistance and progression of breast cancer.

of ERα polyubiquitination, increases its abundance, and the expression of E2-dependent genes linked to proliferation and tumor development. In addition, inhibition of ERα polyubiquitination may have other serious implications, since it has been reported that this modification and proteasome activity are coupled to the transcriptional cycle of this receptor[45]. Moreover, it has been proposed that high ERα protein levels are related to ERα binding to other DNA regulatory regions of genes that are atypically activated under this condition[111]. Thus, inhibition of ERα polyubiquitination and its degradation increases the stability of this receptor, but also affects ERα/E2 signaling and its transcriptional activity, involved with the development of tumor and endocrine resistance[111,112] (Figure 4).

Importantly, there is an interplay between inhibition of ERα polyubiquitination and endocrine therapy resistance in ERα+ breast cancer, promoted by EPIP and EPII [49,50,58,65]. In contrast, in luminal B breast cancers or ERα− breast cancers, RB is commonly lost or dysfunctional, leading to high levels of polyubiquitination and degradation of ERα, with a poor prognosis for patients. Therefore, EPIP, EPII, and mutations and modifications that inhibit ERα polyubiquitination and degradation may act in a cooperative manner to enhance the stability of the receptor in the progression of breast cancer. Consequently, the mechanisms involved in the inhibition of ERα polyubiquitination represent useful biomarkers, therapeutic targets, and prognostic indicators of endocrine therapy in breast cancer.

In conclusion, EPIP, EPII, and mutations and modifications associated to ERα polyubiquitination inhibition, enhance the signaling pathways of this receptor. These findings represent a new field in breast cancer, for the establishment of potential biomarkers, as well as, in the design of effective therapeutic targets to control the progression of this disease. Integration between the molecular basis of ERα inhibition and its correlation with the progression of breast tumors remains to be elicited.

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EDITORIAL

Cancer prevention in patients with human immunodeficiency virus infection

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Abstract

Cancer is a leading cause of death in patients with human immunodeficiency virus (HIV) infection. With the advent of antiretroviral treatment, the risk of AIDS-defining cancers declined but the ageing of this population resulted in the emergence of other common cancers, particularly lung and hepatocellular cancer. Accordingly, screening programs similar to the general population should be implemented in patients with HIV infection. Vaccination against common oncogenic viruses is also essential. However, rates of cancer screening and vaccination against HPV and HBV are considerably low in this population, highlighting a pressing need to educate patients and healthcare professionals about the importance of cancer preventive measures in these vulnerable patients.

Key words: Antiretroviral treatment; Prevention; Human immunodeficiency virus infection; Cancer; Vaccination

Core tip: Cancer is a leading cause of death in patients with human immunodeficiency virus (HIV) infection. With the advent of antiretroviral treatment, the risk of AIDS-defining cancers declined but the ageing of this population resulted in the emergence of other common cancers, particularly lung and hepatocellular cancer. Accordingly, screening programs similar to the general population should be implemented in patients with HIV infection. Vaccination against common oncogenic viruses is also essential.

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Cancer is a leading cause of death in patients with human immunodeficiency virus (HIV) infection[1,2]. In recent decades, overall mortality declined in this population in industrialized countries but the percentage of deaths due to non-AIDS related cancer increased and currently represent almost one fourth of all deaths[1,2]. Moreover, HIV infection is associated with increased incidence of several cancers, including Kaposi's sarcoma, certain types of aggressive B-cell lymphomas and invasive cervical cancer, which are classified as AIDS-defining cancers[3-5]. However, several non-AIDS related cancers, including lung and hepatocellular cancer, are also observed more frequently in patients with HIV infection[3,6]. Therefore, the prevention of cancer is of paramount importance in this population.

The introduction of antiretroviral treatment (ART) resulted in substantial reductions in the incidence of AIDS-defining cancers[3-5]. Moreover, immunosuppresion also increases the risk of non-AIDS-defining cancers in this population[7,8]. Therefore, timely implementation of ART is essential for cancer prevention in patients with HIV infection. However, the cost/health benefit ratio of early implementation of ART and persistent suppression of HIV replication should also be considered, particularly in resource-poor settings.

It has been reported that almost 40% of cancers that affect patients with HIV infection are due to oncogenic virus, specifically hepatitis B and C virus infection-related hepatocellular carcinoma (HCC) and human papillomavirus (HPV) infection-related cervical, vulvar, penile, anal, oral and pharyngeal cancer[8,9]. Accordingly, vaccination against hepatitis B is recommended in all seronegative patients with HIV infection and repeat doses should be administered until anti-HBs titers $\geqslant$ 10-100 IU/mL are achieved[10]. Double doses might be indicated in patients with low CD4 count and high HIV viral load[10]. Vaccination against HPV is also recommended in patients with HIV infection < 26-year-old or < 40-year-old in men who have sex with men (MSM)[10]. Three doses of the 9-valent HPV vaccine should be used where available[10].

Lung cancer is more frequent in patients with HIV infection and is a leading cause of death in this population[3,6]. The higher prevalence of smoking in patients with HIV infection might partly contribute to this association[11,12]. Therefore, current guidelines state that these patients should be made aware of the detrimental effects of smoking on health and smokers should be informed about the benefits of smoking cessation[10]. For those willing to quit smoking, pharmacotherapy (including nicotine replacement therapy, varenicline and bupropion), cognitive behavioral counseling and/or motivational strategies can be employed to help quitting[10]. On the other hand, computed tomography screening appears to have low yield in patients with HIV infection, probably due to the young age of most of these patients[13].

Cancer screening recommendations in patients with HIV infection are similar to the general population, since there are no studies that specifically evaluated the benefits and harms of these strategies in this population[10]. Mammography is recommended every 1-3 years in women 50-70-year-old and measurement of prostate specific antigen is recommended every 2-4 years in men > 50 years with life expectancy > 10 years[10]. Annual faecal occult blood test, sigmoidoscopy every 5 years or colonoscopy every 10 years are recommended in subjects > 50 years with life expectancy > 10 years[10]. In patients with cirrhosis and in those with HBV co-infection and either a history of elevated transaminases or risk factors for HCC (family history of HCC, Asians, Africans), abdominal ultrasound and measurement of alpha-fetoprotein levels are recommended every 6 mo to enable the early diagnosis of HCC[10]. Regarding AIDS-defining cancers, liquid-based cervical cytology test every 1-3 years is recommended in women > 21 years or within 1 year after sexual debut[10]. Digital rectal examination with or without anal cytology is also recommended in MSM and patients with HPV-associated dysplasia[10]. Finally, careful inspection of the skin should be performed regularly to detect cancers such as Kaposi's sarcoma, basal cell carcinoma and malignant melanoma[10].

In conclusion, cancer is a frequent cause of death in patients with HIV infection. With the advent of ART, the risk of AIDS-defining cancers declined but the ageing of this population resulted in the emergence of other common cancers, particularly lung cancer and HCC. Accordingly, screening programs similar to the general population should be implemented in patients with HIV infection. Vaccination against common oncogenic viruses is also essential. In addition, primary prevention of cancer by implementing educational programs stressing the importance of healthy lifestyle are equally important in patients with HIV infection. However, rates of cancer screening and vaccination against HPV and HBV are considerably low in this population[14,15], highlighting a pressing need to educate patients and healthcare professionals about the importance of cancer preventive measures in these vulnerable patients.

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REVIEW

Oxytocin and cancer: An emerging link

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Abstract

The neuropeptide hormone oxytocin, which is released from the posterior pituitary gland, is involved in a number of physiological processes. Understanding of its effects is gradually increasing due to new research in this area. While mostly recognized as a reproductive system hormone, oxytocin also regulates other organ systems such as the brain and cardiovascular system. Recently, research has focused on unraveling its involvement in cancer, and emerging evidence suggests a potential role for oxytocin as a cancer biomarker. This review summarizes observations linking oxytocin and cancer, with a special emphasis on prostate cancer, where it may promote cell proliferation. Research suggests that oxytocin effects may depend on cell type, concentration of the hormone, its interactions with other hormones in the microenvironment, and the precise localization of its receptor on the cell membrane. Future research is needed to further elucidate the involvement of oxytocin in cancer, and whether it could be a clinical cancer biomarker or therapeutic target.

Key words: Oxytocin; Cancer; Prostate; Pancreas; Exercise

Core tip: Oxytocin's role outside of the reproductive system and social bonding has yet to be fully elucidated. Apparently, its role in cancer may vary depending on location and cell type. This review summarizes the current state of our understanding of the potential role of oxytocin in cancer.

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INTRODUCTION

Oxytocin is a central nervous system (CNS) neuropeptide hormone, which is composed of nine amino acids. The synthesis of oxytocin begins in the hypothalamus, where the paraventricular nucleus and supra-optic neurons express high levels of oxytocin, which is released from the posterior pituitary gland[1]. Oxytocin is biologically similar to vasopressin (also known as antidiuretic hormone), and they are often studied in parallel, as both hormones also share some functions. Originally thought of as a hormone with a role limited to the uterus and milk ejection - oxytocin means "quick birth" in Greek[2] - further research has expanded understanding of its function across sexes and organ systems. Furthermore, it has become clear that in addition to physical function, oxytocin also wields important impact on social behaviors, which include stress and trust, anxiety, social interaction and bonding, and parental care[3], and thereby on neuropsychiatric disorders linked to these social behaviors. Interestingly, emerging evidence has linked oxytocin to somewhat conflicting roles in carcinogenesis, as oxytocin is implicated in either fostering development or, conversely, inhibition of cancer-related cellular functional phenomena.

PHYSIOLOGICAL FUNCTIONS OF OXYTOCIN

Oxytocin in the reproductive system

Oxytocin exerts its effects primarily through a single receptor, which has been well characterized. The oxytocin receptor is a class- Ⅰ G-protein-coupled receptor with seven transmembrane domains, and can be bound by several ligands, including oxytocin, oxytocin agonists and antagonists, as well as vasopressin[4]. These receptors are found in the endometrium, myometrium, trophoblast, osteoblasts, reproductive organs, and throughout the CNS (Table 1). Notably, oxytocin receptor has also been implicated in various cancers related to tissues in which it is expressed, including endometrial cancer, glioblastomas, neuroblastomas[5], and others. Several oxytocin receptor antagonists have been identified, with the most common being Atosiban[6,7]. While studies of oxytocin antagonist in cancer has been limited, atosiban has been implicated in inhibiting cell growth of DU145 prostate cancer[8] and various breast cancer cell lines[9]. However, little evidence indicates whether this strategy is efficacious *in vivo*.

The effectors of the oxytocin receptor may vary. While the primary signaling mechanisms of oxytocin have not been fully elucidated, recent studies show that the main mitogenic signaling mechanism of the oxytocin receptor involves the Gq alpha subunit protein (Gαq)/phospholipase C (PLC)/inositol 1, 4, 5 triphosphate (InsP3) pathway. Through this activation, the G protein couples to PLC-B, resulting in the release of calcium from intracellular stores[10], triggering smooth muscle contractions[11], for example in the uterus or in the myoepithelial cells of the mammary gland. The main oxytocin pathways described in this paper are depicted in Figure 1.

Indeed, the first functional role attributed to oxytocin centered on the female reproductive system, specifically in uterine contraction and in lactation. Uterine sensitivity to oxytocin increases around the onset of labor, and upon labor oxytocin stimulation becomes more efficient. Therefore, exogenous administration of oxytocin is also clinically used to induce labor. Following parturition, the density of oxytocin receptors are declines. In lactation, the oxytocin pathway is activated when the infant begins sucking on the nipple[12]. A sensory impulse is sent from the nipple to the spinal cord, and from there transmitted to the oxytocinergic neurons in the hypothalamus. These neurons generate action potentials that lead to a substantial release of oxytocin into the blood stream, which subsequently elicits milk ejection via a contraction of myoepithelial cells[4]. Interestingly, this cascade can be triggered even before suckling occurs, by an event such as a baby crying[13], suggesting that it may involve reflex neural pathways. Increased plasma oxytocin has also been linked to an increased risk of pregnancy-induced hypertension[14], and exogenous oxytocin administration has been associated with angiotensin II-induced hypertension[15]. Interestingly, though, there is also a potential link between increased oxytocin and reduced risk of hypertension (which might be acquired *in utero* in association with intrauterine growth retardation)[16], so that oxytocin has been attributed with eliciting a reduction in blood pressure[17,18].

The reproductive function of oxytocin is not limited to females, as it stimulates contractility of the seminiferous tubules, epididymis, and the prostate gland[19]. Due to its production locale in the testes, oxytocin has been studied as a paracrine regulator of the prostate gland, specifically of growth and muscle contractility[20]. In males, oxytocin has been thought to induce erection and play a role in ejaculation[19,21]. Specifically, in the prostate, oxytocin has been suggested to induce prostatic smooth muscle cell contraction[22]. Its postulated involvement in ejaculation includes stimulation of the reproductive tract to promote sperm release[19]. Oxytocin's role in aggravating and potentially facilitating the development of benign prostatic hyperplasia, and the oxytocin-induced proliferative effect, are likely mediated through the extracellular signal regulated kinase (ERK) pathway[23].

Oxytocin in the CNS

Given its ubiquitous distribution in the CNS, the role of oxytocin in cognition and social behavior has also been studied extensively, especially over the past decade.

Table 1 Human tissue with known expression of the oxytocin receptor

Tissue	Expression type
Myometrium	mRNA and protein[7,11]
Lung	mRNA[109]
Breast	mRNA and protein[16]
Prostate	mRNA[22,79]
Uterus	mRNA and Protein[11]
Heart	mRNA[17]
Vascular endothelium	mRNA[110]
Brain	mRNA[31,42,43]
Thymus	mRNA[111]
Pancreas	mRNA[112]
Blood	mRNA and protein[113]
Bone	mRNA[114]

It has been shown that oxytocin can enhance positive social interactions, and importantly can enhance trust[24]. Oxytocin activity was found to be decreased in women who suffered abuse in their youth[25]. Conversely, and perhaps related to this, a study reported increased oxytocin levels in individuals enjoying heightened levels of partner support[26]. Oxytocin also has been reported to improve social cognition[27,28]. However, it must be noted that studies on the social effects of oxytocin are somewhat inconsistent[29], suggesting that at least socially, additional factors are likely to be at play.

One of the aims of oxytocin research stems from an attempt to establish it as a tool to predict, diagnose, and potentially treat neuropsychiatric disorders[30], and has mostly focused on anxiety and depression. Oxytocin intake has been shown to reduce anxiety symptoms[31,32]. In concordance with its pivotal role during birth, the majority of the research regarding depression in humans has revolved around pregnancies and the mother's ability to recover from postnatal depression (PND). Studies in this field have discovered lowered oxytocin in mothers with PND, and that increased oxytocin levels bestow positive effects on mothers with PND and on their interactions with infants[33,34]. On the other hand, abnormal post-prandial oxytocin secretion has also been demonstrated in women with anorexia nervosa, possibly as an adaptive response to food-related symptoms of anxiety and depression[35]. One of the emerging "hot topics" in neuropsychiatric research links oxytocin with autism[36-38], with recent studies beginning to identify oxytocin as a potential medical therapy to alleviate social anxiety caused by autism[39].

Oxytocin in the cardiovascular system

The potential regulatory role of oxytocin in other organ systems has also raised considerable interest. Oxytocin contributes to several forms of cardiovascular regulation, as it has been shown that preconditioning rats with oxytocin reduces cardiac arrhythmias[40], and that oxytocin can lower blood pressure[41], increase anti-inflammatory and antioxidant activity, and exert beneficial metabolic effects. Therefore, its cardiovascular activity seems to aim largely at restoring homeostasis.

Notably, oxytocin seems to also exert cardiovascular regulation during elevated levels of physical activity. Oxytocin levels have been shown to rise in response to exercise[42], which activates oxytocinergic projections[43] and oxytocinergic modulatory loops that adjust cardiac output, assisting in keeping cardiovascular control over the blood supply[44]. The rise in oxytocin has been traced to the lumbar spinal cord[45]. Oxytocin also reduces the rise of exercise-induced adrenocorticotropic hormone (ACTH) and cortisol[46,47], furthering support on its effects on cortisol levels. Furthermore, exogenously administered oxytocin along with exercise have been shown to protect ovariectomized rats from myocardial infarction[48]. On the other hand, its contribution to cardiovascular regulation may depend on the type or intensity of exercise, because plasma oxytocin in cyclists remains unchanged during intense exercise[49].

OXYTOCIN IN CANCER

Less is understood about the connection between oxytocin and cancer, partly due to lack of adequate research in this area, and partly due to some inconsistency in the current data (Figure 2). The first link of oxytocin to cancer was reported in 1984, when oxytocin was described to be structurally and genomically related to vasopressin, an endogenous hormone that is also secreted by the pituitary, and that in addition to its physiological functions has been found to constitute a biomarker of small-cell lung cancer[50]. Furthermore, oxytocin and vasopressin are co-expressed in these cells, where they have been proposed to induce mitogenic effects[51]. Oxytocin's link to vasopressin and its potential role as a biomarker was subsequently proposed in 1990[52]. Shortly thereafter, it was suggested that oxytocin may modulate growth in breast cancer[53], which was subsequently demonstrated[54]. These observations have instigated additional research into oxytocin's potential involvement in various forms of cancer.

Oxytocin in breast cancer

Interestingly, subsequent studies have shown that oxytocin in fact inhibits proliferation of breast cancer cell lines, such as MDA-MB231, MCF7, and T47D[55,56], as well as the canine mammary cell line CMT-U27[57], mouse mammary carcinoma cell line TS/A, and rat mammary carcinoma cell line D-R3230AC[9]. This effect was shown to be mediated via the cyclic adenosine monophosphate protein kinase A in human cell lines[58]. Importantly, anti-proliferative and tumor inhibitory properties were also observed *in vivo* in both rat and mouse experimental models, and attributed to both oxytocin and its analogue F314[9]. Recently, it was suggested that exercise training, by inducing oxytocin secretion, may reduce the expression of specific signaling proteins involved in breast cancer[59].

Lactation has long been linked to a reduced risk

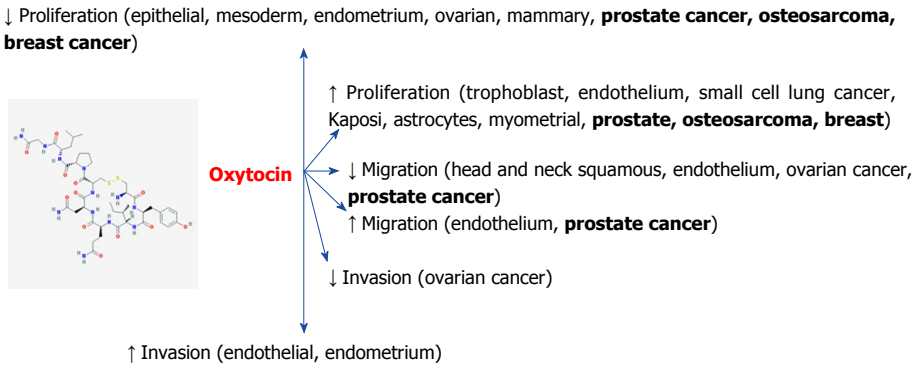

Figure 1 The potential role of oxytocin in various cancers and cell types. Bold font indicates conflicting observations.

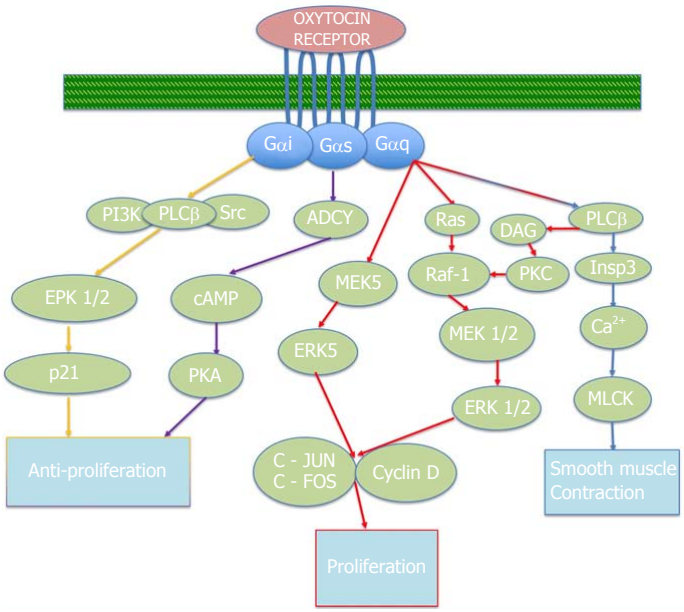

Figure 2 Mechanisms of action of oxytocin. Yellow and purple are anti-proliferation (*via* different subunits), red is proliferation, and blue is smooth muscle contraction. Gαi: Guanine nucleotide binding protein subunit alpha I; Gαs: Guanine nucleotide binding protein subunit alpha s; Gαq: Guanine nucleotide binding protein subunit alpha q; PI3K: Phosphoinositide 3-kinase; PLCβ: Phospholipase beta; Src: Tyrosine protein kinase; ERK: Extracellular signal-regulated kinase; p21: Cyclin-dependent kinase inhibitor 1; ADCY: Adenylate cyclase; cAMP: Cyclic AMP; PKA: Protein kinase A; DAG: Diacylglycerol; PKC: Protein kinase C; Insp3: Inositol triphosphate 3; MLCK: Myosin light chain kinase.

of cancer, with research dating back to as early as the 1950's[60-63]. Worldwide, it has been shown that breastfeeding reduces the risk of both breast and uterine cancer, with prolonged durations of breastfeeding (usually involving multiple children breastfed) correlating with a progressive fall in the risks of both breast and uterine cancer[64-68]. The relationship with uterine cancer might be related to the action of oxytocin as a paracrine and endocrine hormone in lactation. Nevertheless, while the relationship between oxytocin, lactation, and breastfeeding with reduced risk of breast and uterine cancer are all well documented individually, more research needs to be conducted to determine if the relationship between oxytocin, lactation, and breastfeeding with reduced breast and uterine cancer is causal. Elucidating such a connection may establish new therapeutic targets in cancer.

Oxytocin in ovarian cancer

In addition to breast and uterine cancer, the potential participation of oxytocin in the pathogenesis of other cancers in the reproductive system has been investigated. Oxytocin was found to inhibit the progression of ovarian carcinoma (Figure 2) both *in vitro* and *in vivo*. Using cell viability, invasion, and migration assays, it was demonstrated that oxytocin inhibited proliferation, migration and invasion of ovarian cancer cells *in vitro*, and its administration also attenuated the dissemination of ovarian cancer using mean tumor burden as a measure[69]. The same investigators had demonstrated in a previous study expression of the oxytocin receptor in various human ovarian carcinoma tissues and cell lines, and identified placental leucine aminopeptidase (P-LAP) as an oxytocin-degrading oxytocinase in certain adenocarcinoma tissues[70]. This team of investigators,

therefore, proposed that a system involving P-LAP and oxytocin plays a role in the regulation of human endometrial adenocarcinoma, in which P-LAP exerts a functionally positive impact on carcinoma cell growth by degrading suppressive peptides such as oxytocin. More recently, these effects have also been linked with a cross-talk network between oxytocin and the stress hormone cortisol, whereby oxytocin reversed the carcinogenic effects of cortisol via autophagy (cellular self-degradation)[71]. Interestingly, pertinent to the postulated connection between oxytocin and symptoms of autism[39], oxytocin and cancer have also demonstrated an inverse relationship in autistic children[72].

Oxytocin in the gastrointestinal tract

Oxytocin receptors are expressed throughout the gastrointestinal (GI) tract[73], but little is known about their function in the GI tract, especially in relation to cancer. Some studies have suggested a link between oxytocin and its receptor in GI-related cancers, such as esophageal, gastric, and pancreatic cancers. For example, some studies showed an inverse relationship between the duration of breastfeeding and risk of esophageal cancer[74,75], gastric cancer[76], and pancreatic[77] cancer. In fact, Yu *et al*[78] showed a 54% decreased risk of developing esophageal cancer in women who breastfed for over 12 mo.

Unpublished data from our laboratory shows that the messenger ribonucleic acid (mRNA) expression of oxytocin is twofold higher in PANC-1 (a human pancreatic cancer cell line highly unresponsive to the chemotherapeutic agents, gemcitabine and 5-FU) compared to L3.6pl (a highly responsive human pancreatic cancer cell line). We also found that oxytocin receptor protein expression is also higher in PANC-1 than in L3.6pl. Further, inhibition of the oxytocin receptor decreased cell proliferation of PANC-1 and L3.6pl cells. Our analysis of data from the cBioPortal database revealed that up to 5% of pancreatic cancer patients included in The Cancer Genome Atlas showed genetic alterations (primarily upregulation of mRNA expression) in oxytocin and its receptor. Patients with these alterations had poorer survival outcomes as compared to those without these alterations. These interesting data warrant further investigation on the molecular mechanisms implicating oxytocin and its receptor in pancreatic cancer and other GI cancers.

Oxytocin in prostate cancer

As a role for oxytocin in the regulation of prostate function is established, its potential involvement in the development of prostate cancer has been proposed. Data from over two decades ago implicated oxytocin in the pathophysiology of benign prostatic hyperplasia, where the peptide might contribute to both the physical enlargement and dynamic tone of the gland[19]. More recently, immunohistochemical staining has detected oxytocin expression in stromal and epithelial cell lines and in tissue from patients with benign prostatic hyperplasia, which was significantly reduced in tissues of invasive prostate cancer in comparison to both benign prostatic hyperplasia tissues and normal human prostate epithelial cells[79]. This inverse relationship might implicate a fall in oxytocin levels in progression of prostate cancer. Within the prostate, oxytocin has been shown to affect gland growth both directly and *via* its interaction with androgen metabolism, and oxytocin concentrations are positively correlated with androgens[20,80]. Indeed, while in the absence of androgens oxytocin had no effect on prostate cancer cell lines (LNCaP and PC-3), in the presence of testosterone low oxytocin doses stimulated proliferation of PC-3 cells[81], supporting the notion that changes in levels of oxytocin in the prostate in aging and cancer may promote prostate epithelial cell proliferation. It is possible that increased levels of oxytocin might be involved in the mechanisms by which high ejaculation frequency is related to decreased risk of prostate cancer[82]. This hypothesis needs to be further investigated.

Conversely, a different study recently revealed that oxytocin increased the expression of APPL1, a protein with the ability to interact with tumor suppressor proteins. *In vitro* studies showed that oxytocin increased prostate cancer cell proliferation, and expression of APPL1. Analysis of serum and tissue samples identified increased oxytocin levels in the serum of prostate cancer patients, and high expression of oxytocin and its receptor in prostate tissues collected from prostate cancer patients in comparison to those collected from patients without prostate cancer. The oxytocin receptor has also been implicated in the migration of prostate cancer cells, and possibly modulation of prostate cancer metastasis[83]. Taken together, these observations of oxytocin in prostate cancer cells both *in vivo* and *in vitro,* suggest that oxytocin could serve as a prostate cancer biomarker[84].

Several explanations have been offered for the apparent differences in the data from different studies regarding the role of oxytocin in prostate cancer. One explanation is the notable difference in the numbers of participants involved in each study. Secondly, some of the studies included prostate cancer patients that had undergone neo-adjuvant therapy, which can affect oxytocin levels[85]. Thirdly, oxytocin is likely to activate a wide range of signaling mechanisms to elicit variable cellular responses, possibly depending on the density or precise localization of the oxytocin receptor on the plasma membrane[86]. This may also account for the dichotomy in the observations reported regarding the role of oxytocin in cancer. Clearly, additional studies are needed to elucidate the involvement of oxytocin and oxytocin receptor in progression or regression of human prostate cancer.

Perspectives and future directions

There is clearly some evidence implicating oxytocin in carcinogenesis, although its precise effect and underlying mechanisms are still unclear. It is possible that in some

individuals, cell types, or types of cancers, oxytocin may not act as a sole regulator of carcinogenesis, but may mediate or modulate other coexisting factors in the microenvironment.

For example, research generally supports the hypothesis that exercise can inhibit the progression of cancer[87-89]. The positive impact of exercise in blunting development of cancer and facilitating recovery may potentially be partly mediated through oxytocin. For example, oxytocin has been proposed to mediate an exercise-induced reduction in the expression of specific signaling molecules involved in breast cancer[59]. It has also recently been shown *in vivo* that the combination of exogenous oxytocin with exercise improves cardiac function, which might be associated with improved cancer survival. Cardiac dysfunction and cancer have such a strong link that it has even spawned its own subspecialty in cardio-oncology[90-93], although much of the research has centered around long-term survival and cardiac complications in cancer patients. While this potential mediating effect of oxytocin has been hypothesized[94], it appears that little primary research has been conducted to address this postulation. The ability to knockout or silence oxytocin is available, and its social effects are already documented[95,96]. Thus, a thorough study is certainly possible. Similarly, oxytocin may mediate the inhibitory effects of lactation on development of breast cancer, and of ejaculation on development of prostate cancer, and additional studies could prove to be invaluable in revealing its involvement.

It must be noted that most systems and bodily processes in living organisms are tightly inter-connected. Untangling this complexity in the presence of confounding elements is often difficult, especially in relation to psychosocial factors. Therefore, interpretation of oxytocin's expression and levels alone might be over-simplistic and under-informative. For example, instead of being increased as a direct response to exercise, oxytocin may be induced to assist its "companion" hormone, vasopressin, a well-known hormonal regulator of body fluid homeostasis[97]. Cortisol, the "stress hormone" and the rhythm surrounding its release has been linked to the progression and survival from various cancers[98-101], and has also been linked to oxytocin[71,72]. The ability to completely elucidate various pathways and mechanisms of actions would go a long way to showing if the established connection between the different hormones extends to cancer. Furthermore, while outside the scope of this review, there are several other diseases and pathologic states possibly linked to oxytocin. Pain, depression, and anxiety have all been linked to oxytocin[102,103]. Oxytocin's role in depression management was mentioned previously in this article, but oxytocin also seems to be a promising target in pain management[104-107], and in immunotherapy, especially through its interactions in the gut[108].

CONCLUSION

Most knowledge of oxytocin centers on its role as a reproductive hormone. Since its discovery, its other roles have progressively become clearer, including involvement in social behavior, cardiovascular regulation, and carcinogenesis. While it is currently difficult to pinpoint and precisely define oxytocin's oncogenic roles, it is hoped that this review will encourage greater intensity in researching the details of the role of oxytocin in cancer. Future research has a number of plausible and exciting directions to follow and will hopefully clarify some of the ambiguities concerning the role of oxytocin in cancer.

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REVIEW

Role of polymorphisms in genes that encode cytokines and *Helicobacter pylori* virulence factors in gastric carcinogenesis

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Abstract

The *Helicobacter pylori* (*H. pylori*) infection is a determinant factor in gastric cancer (GC) development. However, the infection outcomes are variable and depend on both host and bacterial characteristics. Some host cytokines such as interleukin (IL)-1β, IL-1Ra, IL-8, IL-10 and tumor necrosis factor-α play important roles in the host immune system response to the pathogen, in the development of gastric mucosal lesions and in cell malignant transformation. Therefore, these host factors are crucial in neoplastic processes. Certain polymorphisms in genes that encode these cytokines have been associated with an increased risk of GC. On the other hand, various virulence factors found in distinct *H. pylori* bacterial strains, including cytotoxin-associated antigen A, vacuolating cytotoxin, duodenal ulcer promoting gene A protein, outer inflammatory protein and blood group antigen binding adhesin, have been associated with the pathogenesis of different gastric diseases. The virulent factors mentioned above allow the successful infection by the bacterium and play crucial roles in gastric mucosa lesions, including malignant transformation. Moreover, the role of host polymorphisms and bacterial virulence factors in gastric carcinogenesis seems to vary among different countries and populations. The identification of host and bacterium factors that are associated with an increased risk of GC development may be useful in determining the prognosis of infection in patients, what could help in clinical decision-making and in providing of an optimized clinical approach.

Key words: *Helicobacter pylori*; Virulence factors; Cytokines; Gene polymorphisms; Gastric cancer

Core tip: Various polymorphisms in host genes that encode cytokines and *Helicobacter pylori* virulence factors have been associated with different tendencies of gastric diseases development. Several reviews have been written on the role of host and bacterial isolated factors in gastric carcinogenesis. However, only a small amount of reviews unites the important characteristics of both bacterium and host in carcinogenesis. General overviews about polymorphisms in genes that encode cytokines are also scarce. We aimed to join the main polymorphisms in genes that encode cytokines and bacterial virulent factors related to gastric carcinogenesis and to provide a broad overview about these themes.

de Brito BB, da Silva FAF, de Melo FF. Role of polymorphisms in genes that encode cytokines and *Helicobacter pylori* virulence factors in gastric carcinogenesis. *World J Clin Oncol* 2018; 9(5): 83-89 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i5/83.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i5.83

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram negative bacterium, which inhabits the gastric epithelial tissue of most people in the world[1], and it is considered a determinant factor in the initiation of gastric carcinogenesis[2]. Gastric cancer (GC) is one of the four most prevalent neoplasms and the second biggest cause of deaths in consequence of cancer worldwide[3]. Despite the importance of *H. pylori* in gastric carcinogenesis, the development of GC only occurs in a minority of infected people, demonstrating that the infection outcomes are variable. It is believed that multifactorial precancerous processes associated with both host mucosal inflammatory response and pathogen characteristics are determinant in the severity of the disease[4].

The host immune system response plays a crucial role in the outcomes of *H. pylori* infection. Polymorphisms in genes that encode cytokines have been reported and associated with the severity of gastric mucosa inflammation and GC development. Some of these determinant variations are present in genes that encode cytokines such as interleukin (IL)-1β, IL-1Ra, IL-8, IL-10 and tumor necrosis factor (TNF)-α[5-13]. These polymorphisms are important aspects in understanding gastric carcinogenesis, since chronic inflammation induced by the bacterium is critical in the emergence and evolution of GC precursor lesions (Figure 1)[14].

On the other hand, the virulence factors of *H. pylori* are determinant in the interaction with host cells. Cytotoxin associated antigen A (CagA), vacuolating cytotoxin (VacA), duodenal ulcer promoting gene A protein (DupA), outer inflammatory protein (OipA) and blood group antigen binding adhesin (BabA) are some virulent factors that seem to be associated with different risks of GC development[15]. Furthermore, *H. pylori* with EPIYA-D or more than one EPIYA-C segment in its *CagA* gene have been associated with a higher risk of gastric carcinogenesis[16-20].

POLYMORPHISMS IN GENES THAT ENCODE CYTOKINES AND GASTRIC CARCINOGENESIS

Gastric carcinogenesis is a process in which chronic inflammatory status plays a crucial role. The increase of inflammatory cytokine levels, due to *H. pylori* infection, seems to be determinant in the initiation and progression of GC[12]. The intensity of the expression of cytokines can be modified by functional polymorphisms in the promoter regions of the genes, which has the potential to alter the affinity of transcription factors, interfering in the expression levels of the messenger ribonucleic acid (mRNA) of specific inflammatory mediators related to the susceptibility of GC initiation[21].

IL-1

IL-1 is a family of cytokines that possesses 11 described members, among which IL-1β and IL-1 receptor antagonist (IL-1Ra), combined with *H. pylori* infection, seem to be key factors in GC development[22-24]. Signaling through the IL-1 receptor is a necessary event for the beginning and sustenance of various responses of the immune system[25].

The promoter regions of *IL1B* and *IL1RN* genes, which encode IL-1β and IL-1Ra respectively, have SNPs that modify the expression of the genes and affect the inflammatory response[26]. These SNPs increase the IL-1β/IL-1Ra ratio, which unleashes processes that result in gastric hypochlorhydria, favoring GC development[15,27].

IL-1β is an important cytokine for host-response to pathogens; however, this mediator can exacerbate damage during chronic diseases[28]. High levels of IL-1β in *H. pylori* infections lead to gastrin overexpression, increased gastric inflammation, hypochlorhydria, and gastric atrophy[29]. Moreover, IL-1β might promote neoplastic growth[30]. The *IL1B* gene can be composed by three different SNPs: C-T base transition at IL-1B-511 (rs16944), T-C base transition at IL-1B-31 (rs1143627) and IL-1B-3954 (rs1143634), and all of them are strongly associated with increased production of proinflammatory cytokines, hypochlorhydria and increased GC risk, mainly intestinal type, among Caucasians, but not among Asians or Hispanics[31-34].

IL-1Ra inhibits IL-1α and IL-1β by means of binding to IL-1 receptors. IL1RN possesses a changeable number of tandem repeats in intron 2, forming long alleles (IL1RN1) with 3-6 repeats or a short allele (IL1RN2) with 2 repeats[35]. The IL1RN2 allele is associated with severe gastric lesions and higher risk for GC, besides raised IL-1β expression in Caucasians[33-36].

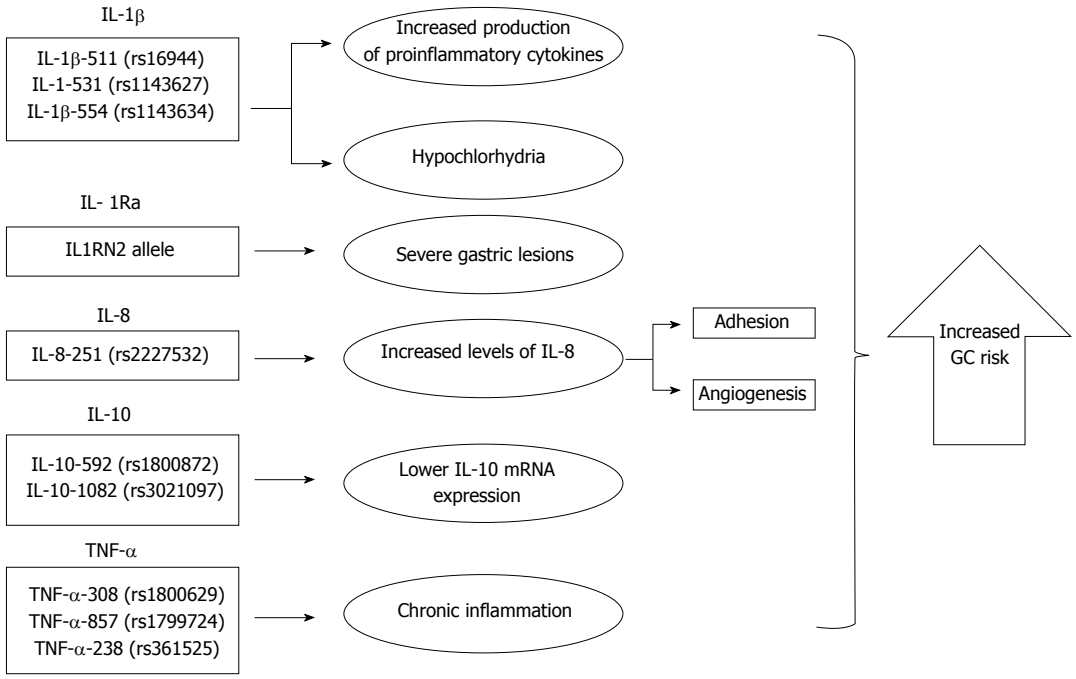

Figure 1 Potential functions of the host genetic polymorphisms in gastric carcinogenesis. IL: Interleukin; GC: Gastric cancer; TNF: Tumor necrosis factor.

IL-8

IL-8 is a potent cytokine that induces the directed migration of cells to inflammatory sites, acting as a chemoattractant[37]. IL-8 secretion can be increased by different stimuli, such as live bacteria (including *H. pylori*) and lipopolysaccharides (LPS), besides others inflammatory cytokines, including IL-1 and TNF[38]. The association of IL-8 with angiogenesis, adhesion and tumorigenesis have been related[39,40].

The gene *CXCL8*, which encodes IL-8, is located on 4q12-21 chromosome and possesses four exons and three introns[41]. An A/T SNP in the -251 position of this gene (*rs2227532*) has been associated with the development of various inflammatory diseases and cancer, including GC in Asians, but not in Europeans[42,43]. Furthermore, the IL-8-251 A allele was related to increased levels of IL-8[41].

IL-10

In opposition to the cytokines mentioned above, IL-10 is an anti-inflammatory cytokine, and it is involved in the cytotoxic response of inflammation and in cell downregulation. Moreover, this mediator prevents the production of pro-inflammatory cytokines, including TNF-α and IL-8[44]. Some studies have demonstrated that SNPs, particularly IL-10-592 (rs1800872) and IL-10-1082 (rs3021097) alleles, might modulate transcriptional activation and affect IL-10 production in vitro. These IL-10 polymorphisms are related to lower mRNA expression of this cytokine and it have been associated with GC development in Asians[45-48].

TNF-α

TNF-α composes the TNF/TNFR cytokine superfamily and it is involved in maintenance and homeostasis of the immune system and host defense[49]. However, this cytokine is related to various pathologic processes, including autoimmunity, chronic inflammatory processes and malignant disease[50]. According to studies, TNF-α signaling through TNFR1 (TNF-α receptor) is important for gastric tumor development[51,52].

Some SNPs in the TNF-α gene are related to increased expression of this cytokine. Among these polymorphisms, TNF-α-857 C/T (rs1799724), TNF-α-308 G/A (rs1800629) and TNF-α-238 G/A (rs361525) are the most studied ones. TNF-α-308 G/A was significantly associated with GC only in Caucasians, while TNF-α-857 and TNF-α-238 were related to an increased risk of gastric tumorigenesis in Asians, but not in Caucasians[53-55].

H. PYLORI VIRULENCE FACTORS AND CARCINOGENESIS

The capacity of *H. pylori* bacteria to trigger a carcinogenic process is not limited to the intense immune response that they unleash, but it also depends on various bacterial factors that can start and modulate neoplastic processes[56]. Different virulent factors found in distinct bacterial strains have been closely associated with the emergence of gastric carcinogenesis. However, genetic variations in genes that encode these virulence factors as well as geographic differences can influence the role of these proteins in GC emergence[15].

CagA

CagA is encoded by the *cagA* gene, present in a DNA

segment containing 30 genes called cag pathogenicity island (*cag PAI*). Infections by strains containing CagA are more capable to induce carcinogenic processes, mainly those with EPIYA-D or more than one EPIYA-C segment[57]. Various *cag PAI* genes are involved in the codification of elements of a pilus structure named type Ⅳ secretion system (TFSS), which has the function of transporting CagA from bacterium to the cytoplasm of the cells from gastric epithelium[58].

After being injected into host cells by TFSS, CagA suffers tyrosine phosphorylation at a carboxi-terminal segment compound by distinct number of EPIYA (Glu-Pro-Ile-Tyr-Ala) regions. There are different EPIYA segments -A, B, C and D-, which contain distinct amino acids in their structure[20]. EPIYA A and B segments are present in most CagA proteins and are followed either by 0-3 EPIYA-C segments in *H. pylori* strains from Occidental countries or by EPIYA-D segments in Eastern countries[59].

Following EPIYA-C or EPIYA-D phosphorylation, an interaction between these segments and SHP-2 possessing SH2 domain occurs, unleashing SHP-2/mitogen-activated protein kinases (MAPK), ERK1, 2-JAK and STAT3 pathways[20]. Cytotoxin associated antigen containing EPIYA-D or more than one EPIYA-C segment ties to SHP-2 more strongly, being more effective in the activation of the pathways mentioned above[60]. This process, activated by CagA, leads to dysfunction of cell growth and of cell-to-cell contact inhibition, cell migration, epithelial cell elongation, and increase of epithelial cell turnover, increasing the propensity of acquirement of precancerous genetic changes by damaged cells[61]. Furthermore, it was demonstrated that relatives of GC patients are more often infected by *H. pylori* strains with more than one EPIYA-C segment in CagA structure[62]. Another study carried out by this same group, performed in a Brazilian population, showed that the host signal transducer and activator of transcription protein 3 (STAT3) rs7744166 polymorphism, as well as being infected by *H. pylori* with CagA containing more than one EPIYA-C segment, are independent predisposing factors for GC[20].

VacA

VacA is another determinant virulence factor in *H. pylori* infection and in gastric carcinogenesis. Patients infected with VacA-positive *H. pylori* strains have a higher propensity for GC development when compared with patients colonized by VacA-negative strains, either in American or in Asian people[63]. Particularly, individuals infected with *H. pylori* strains VacA s1, m1 and s1m1 had an increased risk for gastric carcinogenic unleash in Middle East, Africa and Latin America populations[64]. The peptide mentioned above has only two functional domains in its structure. One of them, the p55-58 domain, has the function of binding to receptors of gastric epithelial cells. The other functional domain, p33-37, produces the cytotoxic effect[65].

VacA is a 90 kDa exotoxin that is activated in a low pH environment[66]. This toxin promotes the generation of numerous acidic vacuoles in gastric epithelial cell cytoplasm[67]. In this process, VacA affects the structure and function of the membrane, the endoplasmic reticulum, the Golgi apparatus and the mitochondria, which can lead to cell death. Furthermore, vacuolating cytotoxin also plays an important role in the activation and suppression of the immune response[68]. This peptide induces a powerful inhibition over T lymphocyte proliferation by means of an interaction with dendritic cells, which are reprogrammed to a tolerogenic genotype[69]. The damage and the immunomodulation performed by this toxin contributes to the increase of gastric mucosa inflammation, ulceration and carcinogenesis in mammals[68].

DupA

Unlike the other virulence factors mentioned in this article, DupA seems to be a protective condition for GC. The *dupA* gene is constituted by two homologue genes of *virB4*, *jhp0917* and *jhp091*, which constitute a continuous gene. The real function of the protein encoded by dupA is still obscure, however, its mechanisms seems to be related to the increase of the production of IL-8 in the gastric antrum, contributing to the development of gastritis that predominates in that gastric region, a process that leads to duodenal ulcer formation[70]. DupA has been significantly associated with duodenal ulcer formation in Asian countries, but this relation was not observed in the Western population[71]. Furthermore, DupA-positive *H. pylori* has been associated with eradication failure[72].

OipA

OipA constitutes a group of peptides described as outer membrane proteins (OMPs), a *H. pylori* protein family composed of 32 components[73]. OipA has been described as a better marker for severe clinical outcomes than CagA, since the infection by strains possessing OipA is an independent determinant risk factor of GC *vs* gastritis in Americans[74,75]. OipA enhances IL-8 production and leads to an increased inflammation status of gastric epithelium. Moreover, it was observed that OipA could inhibit the maturation of dendritic cells in vitro, which might contribute to the immunomodulatory processes performed by *H. pylori*[76].

BabA

babA is a gene that encodes an adhesin whom allows the specific binding to the b and H-1 Lewis antigens, which are expressed in the surface of the gastric mucosa cells[77]. The adhesion of the *H. pylori* to the gastric epithelium mediated by blood group antigen binding adhesin (BabA) appears to play a critical function in the transference of bacterial virulence factors to the host cells. This process contributes to the development of tissue lesions, and a high correlation

of *babA*-positive strains of *H. pylori* with GC has been described[78,79].

CONCLUSION

Despite the wide knowledge about host and *H. pylori* interaction developed since the discovery of its colonization in human stomach, many characteristics that contribute to the infection outcomes are still obscure. The understandings about host polymorphisms in genes that encode cytokines and bacterium virulence factors in GC development are important not only for the determination of patients' prognosis, but it is also a potential way for the development of new preventive and therapeutic strategies.

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MINIREVIEWS

Resistance to FLT3 inhibitors in acute myeloid leukemia: Molecular mechanisms and resensitizing strategies

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Abstract

FMS-like tyrosine kinase 3 (FLT3) is classified as a type Ⅲ receptor tyrosine kinase, which exerts a key role in regulation of normal hematopoiesis. *FLT3* mutation is the most common genetic mutation in acute myeloid leukemia (AML) and represents an attractive therapeutic target. Targeted therapy with FLT3 inhibitors in AML shows modest promising results in current ongoing clinical trials suggesting the complexity of FLT3 targeting in therapeutics. Importantly, resistance to FLT3 inhibitors may explain the lack of overwhelming response and could obstruct the successful treatment for AML. Here, we summarize the molecular mechanisms of primary resistance and acquired resistance to FLT3 inhibitors and discuss the strategies to circumvent the emergency of drug resistance and to develop novel treatment intervention.

Key words: FMS-like tyrosine kinase 3; Tyrosine kinase domain; Internal tandem duplication; FLT3 inhibitor; Drug resistance; Acute myeloid leukemia; Combination therapy

Core tip: FMS-like tyrosine kinase 3 (FLT3) mutations including internal tandem duplication (ITD) or point mutation in tyrosine kinase domain are common genetic

abnormalities in acute myeloid leukemia (AML), predicting dismal outcome. The Federal Drug Administration granted the use of Midostaurin (Novartis) in newly diagnosed FLT3-ITD positive AML in April 2017. A number of other FLT3 inhibitors are in different phases of clinical trials. However, emerging drug resistance poses a major challenge for clinicians to use FLT3 inhibitors. In this manuscript, we systematically reviewed mechanism of primary resistance and acquired resistance to FLT3 inhibitors. We then propose different strategies to overcome drug resistance and novel treatment options for FLT3-ITD positive AML.

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INTRODUCTION

Acute myeloid leukemia (AML) consists of a group of different disease characterized with diverse cellular morphologies and various genetic abnormalities[1-5]. Many of these genetic lesions are of clinical importance because they not only implicate in the pathology of AML, but also have prognostic values[6-8]. Mutation in in FMS-like tyrosine kinase 3 (FLT3) confers inferior response to chemotherapy and poor overall survival in AML patients[9-11]. Since the discovery of FLT3 mutations in 1996[12], intensive research effort has provided a better understanding of the molecular mechanism of normal and aberrant FLT3 signaling transduction pathways. Internal tandem duplications (ITDs) in the juxtamembrane domain and activating point mutations in the second tyrosine kinase domain (TKD) occur in near 30% and 10% of patients with AML respectively[13-15].

FLT3 mutations constitutively activate PI3K-AKT, RAS-MEK-MAPK, and STAT5 pathways and result in uncontrolled cell proliferation and cell survival[16-19]. On the other hand, FLT3 mutations suppress myeloid transcription factors PU.1, CCAAT/enhancer-binding protein α (C/EBPα), which result in blocking of myeloid differentiation[20,21]. Thus, FLT3 mutations exert a key role in the pathology of AML, and have been validated as promising intervening targets[22-25]. Currently, Midostaurin (PKC412, Novartis) has been granted by the Federal Drug Administration (FDA) in the use in newly diagnosed FLT3-ITD positive AML in combination with chemotherapy[26]. Moreover, there are about a dozen of other FLT3 inhibitors in different phases of clinical development[27]. Despite most FLT3 inhibitors display strong effectiveness in cell culture system, most of AML patients in trials haven't achieved durable response[28-30]. Notably, AML patients inevitably don't respond to these drugs when they are administrated as single agent for a period. Scientists first observed this resistance phenomenon in patients with chronic myeloid leukemia (CML) who received imatinib mesylate (Gleevec), the first small molecule kinase inhibitor targeting BCR-ABL fusion protein[31].

Here we review published literature on preclinical and clinical findings and molecular mechanisms of primary resistance and acquired resistance to FLT3 inhibitors. We further discuss the strategies to circumvent the emergency of drug resistance and development of novel treatment intervention.

PRIMARY RESISTANCE TO FLT3 INHIBITORS

The identification of a number of de novo and secondary point mutations in the BCR-ABL kinase domain from imatinib-resistant patients promotes researchers to investigate variable sensitivity of FLT3 inhibitors between different activating point mutations in the kinase domain of FLT3.

Based on the mutations identified in AML cases, Grundler *et al*[32] employed site-directed mutagenesis method to create Asp835Tyr, Ile836del and Ile836Met + Arg (numbering is based on the human FLT3) into the cDNA of murine wild-type FLT3. These vectors were then transfected into murine Ba/F3 and 32Dcl3 cells, rendering them independent from growth factors. Tyrphostin AG1296 does not inhibit FLT3 Asp835Tyr (D835Y)-induced proliferation, inhibition of apoptosis, as well as the downstream signaling, and phosphorylation of STAT5. AG1296 is effective on the inhibition of signaling from FLT3 Ile836del (I836del), -ITD and to the less extent, from Ile836Met + Arg (I836M + R). Staurosporin derivative PKC412 is sensitive to all the 3 catalytic domain mutations, but less sensitivity to - *ITD* mutant. Indolinone compound SU5416 shows similar inhibition profile as PKC412. This study suggests that different inhibitors exert a divergent sensitivity toward different mutations in the FLT3 receptors.

A similar approach was used to introduce each FLT3 activation loop mutant, including D835Y, Asp835Ala (D835A), Asp835Glu (D835E), Asp835Gly (D835G), Asp835His (D835H), Asp835Asn (D835N), Asp835Val (D835V), and D835del into human FLT3 cDNA[33]. Ba/F3 cells were transformed with each vector. These 8 activation loop mutations display variable sensitivity toward quinazoline-based inhibitor MLN518 with more than a 10-fold range. I836del is as sensitive as ITD with IC_{50} 0.55 μmol/L. The IC_{50} of D835E, D835A, D835N, D835H ranges from 0.99 to 2.65 μmol/L. D835del, D835V and D835Y confer relative resistance to MLN518 with much higher IC_{50} up to greater than 10 μmol/L.

This phenomenon could be explained by the assumption that the mutations in the amino acid sequence change the conformation of the catalytic domain of FLT3, resulting in a weaken affinity with FLT3 inhibitors[32,33]. However, the structural analysis of these inhibitors in the context of various mutants is not available in these

papers. These findings are of great clinical interesting. Patients enrolled in the FLT3 inhibitor trials potentially can be screened for all known activation loop mutations. In addition, sensitivity of specific inhibitor can potentially be evaluated *ex vivo* prior to clinical administration to avoid known primary resistant cases.

About 1% to 2% of newly diagnosed AML patients carry both ITD and TKD (FLT3-ITD-TKD) with worse outcome when compared with patients with either ITD or TKD mutation alone[34-36]. Similarly, an in vitro study using Ba/F3 cells transfected with FLT3-ITD-TKD dual mutants, for example ITD-D835N and ITD-D835Y, can induce resistance toward not only FLT3 inhibitor SU5614, but also cytotoxic drug Daunorubicin[37]. Molecular study reveals these dual mutants promote overactivation of STAT5 pathway, and result in upregulation of downstream target Bcl-xL and RAD51 and arrest in the G_2/M phase of the cell cycle[37]. Overexpression of Bcl-2 is also detected in primary AML patient samples with FLT3-ITD-Y591 duplication, correlated to high levels of phosphorylated p53. However, whether this mutant induces resistance to FLT3 inhibitors has not been tested[38].

Other possible mechanisms of primary resistance to TKIs have been investigated. P-glycoprotein (p-gp, also named multi-drug resistance 1, MDR1), a major membrane efflux pump, Primary AML blasts co-expressing p-gp and FLT3-ITD, are resistant to herbimycin A, a tyrosine kinase inhibitor, and AG1296, but not to PKC412[39]. The difference could be due to the fact that PKC421 has dually inhibitory roles in FLT3 and protein kinase C (PKC), which can induce phosphorylation of p-gp, resulting in subversion of p-gp mediated MDR. However, other study shows no association between FLT3 mutations and high levels of MDR1 gene expression in AML patients[40].

In contrast to earlier studies, Siendones *et al*[41] demonstrate that inhibition of FLT3-ITD activity does not necessary block the phosphorylation of AKT, ERK and STAT5, which are the 3 major pathways activated by FLT3 mutations, in some primary AML cells. This could be one reason for the limited anti-tumor effect of FLT3 inhibitors used as monotherapy in clinical trials. In addition, a new "niche and leukemia stem cell" model was proposed to explain the limitation of single agent[42]. If FLT3 - ITD is presented in CD34 + CD38 - CD123 + leukemia stem and progenitor cells (LSPC) from primary AML samples, they are more resistant to FLT3 inhibitor in culture under defined nice conditions (fibronectin, IL - 3, SCF, IL - 6 and Ang-). This result is consistent with an earlier finding that patients whose CD34+CD33- precursors harbor FLT3 - ITD have worse outcome than patients whose CD34 + CD33 + progenitors have FLT3 - ITD[43]. These data indicate that FLT3 - ITD AML derived from the less mature progenitors may be associated with drug resistance.

ACQUIRED (SECONDARY) RESISTANCE TO FLT3 INHIBITORS

Pioneer researches in imatinib-resistant CML patients revealed two different resistant mechanisms including increased copy number of BCR-ABL fusion and point mutations in its adenosine triphosphate (ATP) binding motif[44]. These initial discoveries facilitate our understanding of acquired resistance to FLT3 inhibitors. As demonstrated by imatinib-resistant CML studies, over expression of a mutated FLT3 could also be a common mechanism for drug desensitization and leading to resistance. Weisberg and Boulton *et al*[45] first address this issue using a Ba / F3 - FLT3 - ITD resistant polyclonal subline developed by coculture with increasing concentration of PKC412 (up to 40 nmol/L) with the parental cell line over 2 mo. The protein level of FLT3-ITD is significantly increased in this resistant subline compared to the parental Ba / F3 - FLT3 - ITD, leading to desensitize PKC412. It is not clear that FLT3 - ITD protein over expression was regulated on transcriptional (gene amplification) or post-translational levels (increased protein stability). Also, there is no further study on whether this resistant subline harbors point mutation(s) in the TKD domain.

Other resistant lines, designated as Ba / F3 - ITD - R1 to R4 derived from the same parental Ba / F3 - FLT3 - ITD have been developed in the presence of escalatory dose of SU5614[46]. The average IC_{50} of these lines is 17 - fold higher than the parent line. Consistent with their resistant phenotypes, on the molecular level, the phosphorylation of MAPK and STAT5 in these sublines is not inhibited by higher dose up to 10 μmol/L SU5614, while 1 μmol/L of SU5614 effectively decreases activity of MAPK and STAT5 in the parental line. They are also completely resistant to AG1295, which is structurally similar to SU5614. But, Ba / F3 - ITD - R1 to R4 display a similar sensitivity to a structural unrelated FLT3 inhibitor PKC412, a general TKI, Genistein and a chemotherapeutic agent cytosine arabinoside (Ara-C) as the parent ITD cells. Both flow cytometric analysis and western blot demonstrate elevated amount of FLT3 receptor in the resistant Ba / F3 - ITD - R1 to R4 compared with the parent line. Sequence analysis of TKD domain identifies Y842H mutation in ITD-R1 and –R2 cells, and D835 mutation in ITD-R3 and -R4 cells. These data indicate that both FLT3 target desensitization and acquired mutations in the activation kinase domain can contribute to secondary resistance *in vitro*.

Using random PCR mutagenesis to introduce point mutations in ATP-binding pocket of the KD of MSCV-FLT3-ITD, Cools *et al*[47] identified 4 different point mutations (Ala627, Asn676, Phe691, or Gly697) in Ba/F3 cells that render resistance to PKC412, SU5614 or K-252a. The G697R mutation is the most resistant clone to all the three inhibitors tested. Accordingly, PCK412 fails to reduce phosphorylation of FLT3 up to 400 nmol/L in cells with G697R and they are cross-resistant to other structurally different FLT3 inhibitors (GTP-14546, AGL2043, D-64406, D-64476, TMPPP and DQPPC). Modeling crystal structure of FLT3 receptor in complex with PCK412 indicates that the amino acid Gly697 and Phe691 directly contact with PKC412 and substitution

Table 1 Summary of main studies on primary and acquired resistance to FMS-like tyrosine kinase 3 inhibitors

	Ref.	Disease model (method and material)	Mechanisms of resistance
Primary resistance	Grundler *et al*[32], 2003	Site-directed mutagenesis, murine Ba/F3	TKD mutation, deletion or insertion
	Clark *et al*[33], 2004	Site-directed mutagenesis, murine Ba/F3	TKD mutation
	Bagrintseva *et al*[37], 2005	Site-directed mutagenesis, murine Ba/F3	ITD-TKD mutation, Bcl-xL overexpression
Acquired resistance	Weisberg *et al*[45], 2002	Coculture with PKC412, murine Ba/F3-FLT3-ITD	FLT3 protein overexpression
	Bagrintseva *et al*[46], 2004	Coculture with SU5614, murine Ba/F3-FLT3-ITD	ITD-TKD mutation, FLT3 protein overexpression
	Cools *et al*[47], 2004	Random PCR mutagenesis, murine Ba/F3-FLT3-ITD	ITD-TKD mutation
	Heidel *et al*[48], 2006	PKC412 clinical trial, relapsed AML with ITD	ITD-TKD (N676K) mutation
	Piloto *et al*[49], 2007	Coculture with CEP-5214 and CEP-701, human MOLM-14, Hb1119 and SEM-K2	RTK amplification, N-Ras mutation
	Zhou *et al*[50], 2009	Coculture with ABT-869, human MV4-11	Overactivation of STAT, overexpression of Survivin

FLT3: FMS-like tyrosine kinase 3; ITD: Internal tandem duplication; TKD: Tyrosine kinase domain; RTK: Receptor tyrosine kinase; STAT: Signal transducer and activator of transcription.

Gly697 with a larger amino acid will decrease its binding affinity due to possible steric clash with the FLT3 inhibitors. Importantly, mutation in Asn676 (N676K) has been reported in 1 of 6 patients with FLT3-ITD AML who relapsed after PKC412 treatment in a phase 2 clinical trial[48]. The authors were able to rule out other common mechanisms of drug resistance including gene amplification, overexpression of FLT3 protein, drug metabolism, drug efflux, inhibition by serum proteins and major deficiency in apoptosis pathway. Although the identification of N676K is clinically significant to elucidate the mechanism of resistance and relapse, so far acquired point mutation of TKD has been reported only in a FLT3 inhibitor treated, and relapsed AML patient. In addition, transfection of FLT3-ITD-N676K in 32D cells confers resistance to PKC412[48]. This finding is in consistent with the clinical observation that this mutant could be the sole reason of secondary resistance.

Most of the initial pre-clinical studies on mutations were conducted in murine cell lines transfected with FLT3 cDNA[32,33,37,45-47]. We and others to have further investigated the molecular mechanisms of acquired resistance to FLT3 inhibitors. Human leukemia cell lines with FLT3 mutations are valuable and relevant models for molecular biology and drug sensitivity studies. MV4-11 and MOLM-14 cell lines were derived from primary AML cells, while MV4-11 has two FLT3-ITD alleles; MOLM-14 harbors one mutant FLT3-ITD allele, while the other allele is wild-type (WT). Leukemia cell line Hb1119 and SEM-K2 were derived from primary ALL (acute lymphoblastic leukemia) cells. Hb1119 harbors FLT3-D836H, whereas SEM-K2 over expresses wild-type FLT3. Piloto *et al*[49] reported that prolonged coculture of MOLM-14, Hb1119 and SEM-K2 cells with CEP-5214 and CEP-701 respectively leaded to the development of resistant lines including M14(R)5214, M14(R)701, Hb(R)5214, Hb(R)701, SEM(R)5214 and SEM(R)701. They are cross resistant to PKC412 and AG1295, a structurally related FLT3 inhibitor[49]. Although TKIs can inhibit phosphorylation of FLT3 receptor in most of the resistant clones as demonstrated in this study, the downstream Akt and/or MAPK signaling remain activated, thus providing cells sustained survival and proliferative signaling. Acquired N-Ras mutations have been identified in 2 [M14 (R) 5214 and M14 (R) 701] out of the 6 resistant lines. Transducing N-Ras-G12V mutation into MOLM-14 cells results in resistance to CEP-701[49]. So, activation of parallel signaling pathway independent to FLT3 signaling may contribute to secondary resistance in some cases.

Through long-term culture of MV4-11 cell line with the FLT3 inhibitor, ABT-860, a FLT3 inhibitor-resistant line, MV4-11-R was generated[50]. The IC_{50} of ABT-869 for MV4-11-R line is 52 nmol/L *vs* 6 nmol/L for the parental MV4-11 cell line. Importantly, other structurally unrelated inhibitors including SU5416, AG1296 and a FLT3 inhibitor Ⅲ from MERCK, were not effective to MV4-11-R line anymore, suggesting a cross resistant circumstance. Sequencing analysis showed normal sequence of FLT3-TKD in MV4-11-R cells. Western blot and FACS analysis excluded the overexpression of p-FLT3, FLT3 and three multidrug resistance related proteins (MDR, MRP1 and LRP) in this resistant line. But, overexpression of FLT3LG and Survivin was demonstrated at the both transcript and protein level. Down-regulation of suppressor of cytokine signaling (SOCS) proteins (negative regulators of STAT pathways) was also observed in the presence of overactivation of the STAT1, STAT3 and STAT5 pathways in this resistant line. In conclusion, our findings show that overactivation of STAT pathways and subsequently increased expressions of surviving genes are the main mechanism of resistance to FLT3 inhibitors. A total of 9 main studies regarding to primary and acquired resistance is summarized in Table 1.

STRATEGIES TO CONQUER RESISTANCE

The understanding of molecular mechanisms of primary and secondary resistance to FLT3 inhibitors (Figure 1) provides the foundation for establishing strategies to conquer or reduce resistance. Combination of FLT3 inhibitors with cytotoxic drugs or other small molecule inhibitors targeting different pathways has been extensively searched and tested *in vitro*, in murine

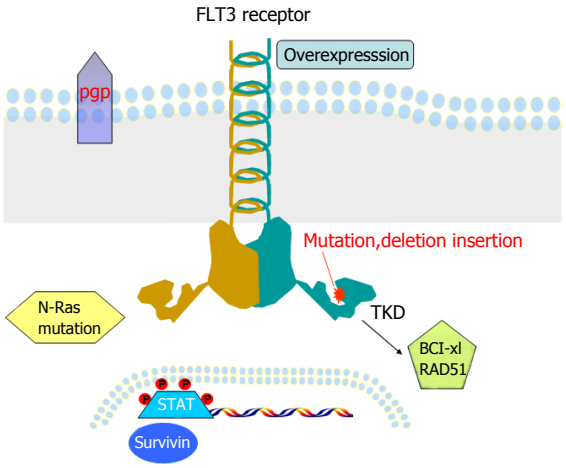

Figure 1 Schematic representation of various published mechanisms of resistance to FMS-like tyrosine kinase 3 inhibitors. This figure shows a number of models of resistance including desensitization of drug targets by *FLT3* gene amplification or protein overexpression, decreased drug binding affinity by mutation, deletion or insertion in TKD, increased drug efflux by p-gp, activation of survival and proliferative pathways (molecules) such as RAS pathway, STAT pathway, anti-apoptotic Bcl-xL, Survivin and DNA repair molecule RAD51. FLT3: FMS-like tyrosine kinase 3; TKD: Tyrosine kinase domain; STAT: Signal transducer and activator of transcription.

xenograft models and some in clinical trials.

We and other investigators have demonstrated that combination of FLT3 inhibitors with conventional chemotherapy drugs, such as cytarabine and doxorubicin, can achieve synergistic effect[30,51-54]. The optimal combination sequence should start with chemotherapy, followed by FLT3 inhibitors to maximize synergism and potential to reduce and/or overcome resistance[30,53].

Combination of FLT3 inhibitors with a spectrum of small molecules inhibitors targeting downstream or independent signaling pathways have been evaluated in pre-clinical studies and showed early promises. Rapamycin, an mTOR inhibitor, sensitizes not only Imatinib-resistant BCR-ABL positive cells[55] but also TKI-resistant Ba/FLT3 dual mutant (ITD and TKD) cell[46,55]. Approaches targeting cellular apoptosis machinery also have been explored. BH3 mimetic ABT-737, a potent inhibitor of anti-apoptotic Bcl2, effectively neutralizes resistance to FLT3 inhibitors in primary AML blasts[56]. The proapoptotic inhibitor LBW242, a Smac (one member of the inhibitor of apoptosis, IAP) mimetic, can overcome resistance to PKC412 when used in combination with PKC412[57]. Combination of FLT3 inhibitor GTP14564 with a HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), produces synergism via STAT5 pathway[58]. Concurrent treatment with histone deacetylase inhibitor (HDACi) LAQ824 and PCK412 can synergistically induced apoptosis in human cell line and primary AML samples with FLT3 mutations[59]. We demonstrate that either treatment with IDR E804, an inhibitor of CDKs and the SRC-STAT pathway, or targeting Survivin by shRNA or a dominant-negative vector (survivin-T34A) sensitize MV4-11-R to ABT-869 induce apoptosis[50].

Other compounds such as bis(1*H*-indol-2-yl) methanone Cpd.98, Cpd.102 and Sorafenib (B-Raf inhibitor) also overcome resistance to FLT3 inhibitors[60,61]. Downregulation of FLT3 expression by RNAi increases sensitivity to FLT3 inhibitor MLN518 in human AML cell lines, a potential approach to override resistance[62]. The PIM family of serine/threonine kinases (PIM-1, -2 and -3) has been shown to be cytoprotective[63]. Constitutively activated FLT3 signaling up-regulates the PIM-1 expression via STAT5 pathway, which results in phosphorylation of BAD protein (pSer112 and pSer136), exerting anti-apoptotic effect[63,64]. PIM-2 also phosphorylates BAD at Ser-112, blocking BAD-inducing cell death[65]. Silencing PIM-2 or PIM-1 sensitizes resistant cells to FLT3 inhibitors[66]. IMC-EB10, an anti-FLT3 monoclonal antibody, is still effective in FLT3-TKI resistant clones, because it mediates antibody-dependent, cell-mediated cytotoxicity (ADCC) which is independent of the FLT3-ITD signaling pathway[49].

CONCLUSION

Primary and secondary resistance to TKI therapy is challenging issue in modern anti-cancer warfare for various cancers including AML. At present time, monotherapy using FLT3 inhibitors showed limited benefit in relapsed AML clinical trials. We now began to better understand the molecular mechanisms of resistance in FLT3 targeting. Ongoing early phase clinical trials are important to further shed light on various potential mechanisms of resistance, and will eventually facilitate better strategies to prevent and overcome resistance. Sequel combination of FLT3 inhibitors with chemotherapy or other small molecule inhibitors targeting mTOR, HDAC, HSP90, STAT3, Bcl2, PIM family, IAPs (Survivin and Smac) and others are ongoing strategies. The correlative studies with these ongoing trials for identifying resistance mechanisms among trial patients, will help investigators in refining the design for next generation trial protocols. In addition, by determining "oncogenic signature" of each patient prior to treatment should guide the proper choice of most efficient combinations targeting the specific "oncogenic signature" individually.

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ORIGINAL ARTICLE

Basic Study

Tunable structure priors for Bayesian rule learning for knowledge integrated biomarker discovery

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Abstract

AIM

To develop a framework to incorporate background domain knowledge into classification rule learning for knowledge discovery in biomedicine.

METHODS

Bayesian rule learning (BRL) is a rule-based classifier that uses a greedy best-first search over a space of Bayesian belief-networks (BN) to find the optimal BN to explain the input dataset, and then infers classification rules from this BN. BRL uses a Bayesian score to evaluate the quality of BNs. In this paper, we extended the Bayesian score to include informative structure priors, which encodes our prior domain knowledge about the dataset. We call this extension of BRL as BRL$_p$. The structure prior has a λ hyperparameter that allows the user to tune the degree of incorporation of the prior knowledge in the model learning process. We studied the effect of λ on model learning using a simulated dataset and a real-world lung cancer prognostic biomarker dataset, by measuring the degree of incorporation of our specified prior knowledge. We also monitored its effect on the model predictive performance. Finally, we compared BRL$_p$ to other state-of-the-art classifiers commonly used in biomedicine.

RESULTS

We evaluated the degree of incorporation of prior knowledge into BRL$_p$, with simulated data by measuring the Graph Edit Distance between the true data-generating model and the model learned by BRL$_p$. We specified the true model using informative structure

priors. We observed that by increasing the value of λ we were able to increase the influence of the specified structure priors on model learning. A large value of λ of BRL_p caused it to return the true model. This also led to a gain in predictive performance measured by area under the receiver operator characteristic curve (AUC). We then obtained a publicly available real-world lung cancer prognostic biomarker dataset and specified a known biomarker from literature [the epidermal growth factor receptor (*EGFR*) gene]. We again observed that larger values of λ led to an increased incorporation of EGFR into the final BRL_p model. This relevant background knowledge also led to a gain in AUC.

CONCLUSION

BRL_p enables tunable structure priors to be incorporated during Bayesian classification rule learning that integrates data and knowledge as demonstrated using lung cancer biomarker data.

Key words: Supervised machine learning; Rule-based models; Bayesian methods; Background knowledge; Informative priors; Biomarker discovery

Core tip: Bayesian rule learning is a unique rule learning algorithm that infers rule models from searched Bayesian networks. We extended it to allow the incorporation of prior domain knowledge using a mathematically robust Bayesian framework with structure priors. The hyperparameter of the structure priors enables the user to control the influence of their specified prior knowledge. This opens up many possibilities including incorporating uncertain knowledge that can interact with data accordingly during inference.

Balasubramanian JB, Gopalakrishnan V. Tunable structure priors for Bayesian rule learning for knowledge integrated biomarker discovery. *World J Clin Oncol* 2018; 9(5): 98-109 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i5/98.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i5.98

INTRODUCTION

Knowledge discovery from databases (KDD) is the non-trivial extraction of valid novel, potentially useful, and understandable patterns from the dataset[1]. Data mining is the computational process of the extraction of these patterns. In biomedicine, data mining is extensively applied for knowledge discovery[2]. The recent advances in biomedical research, triggering an explosion of data, have encouraged these applications. Particularly, the development of high-throughput "omic" technologies has generated a large number of datasets, which provide a holistic view of a biological process. These datasets present opportunities to discover new knowledge in the domain. They also present some challenges, especially from their high-dimensionality. High-dimensional datasets are challenging to data mining algorithms because several thousands of candidate variables (*e.g.,* gene expressions or SNPs) can potentially explain an outcome variable of interest (*e.g.,* phenotypes or disease states) but have only a few instances as evidence to support an explanation. These large numbers of candidate variables generate a model search space that is very large for data mining algorithms to explore efficiently, and having only a few instances generates uncertainty for the algorithm to determine the correctness of any candidate model. In such model search spaces, data mining algorithms can easily get stuck in local optima or they may infer associations between spurious variables and the outcome variable, by chance.

Fayyad *et al*[3], emphasized the importance of domain prior knowledge in all steps of the KDD process. In biomedicine, often in addition to the dataset, we have some prior domain knowledge about the dataset. This domain knowledge can help guide the data mining algorithm to focus on regions in the model search space that are either objectively more promising for a given problem or subjectively more interesting to a user. The prior knowledge can come from domain literature (*e.g.,* searching through PubMed), a domain expert (*e.g.,* a physician), domain knowledge-bases (*e.g.,* Gene Ontology) or from other related datasets [*e.g.,* from public data repositories like Gene Expression Omnibus (GEO)]. It is now imperative to develop data mining methods that can leverage domain knowledge to assist with the data mining process.

Rule learning methods are among the oldest, well-developed, and widely applied methods in machine learning. They are particularly attractive for KDD tasks because they generate interpretable models with understandable patterns and have good predictive performance. Interpretable models are succinct, human-readable models that explain the reasoning behind their predictions. Bayesian rule learning (BRL) is a rule learning method that has been shown to perform better than state-of-the-art interpretable classifiers on high-dimensional biomedical datasets[4,5]. BRL takes a dataset as input and searches over a space of Bayesian belief-networks (BN) to identify the BN that best explains the input dataset. BRL then infers a rule model from this BN. BRL uses the Bayesian score[6] as a heuristic to evaluate a BN during search. The score allows the user to specify a prior belief distribution over the space of BNs that encodes our prior beliefs about which models are more likely to be correct than others with respect to our domain knowledge. Typically in literature uninformative priors are used, which means that we claim that a priori all models are equally likely to be correct. As we saw earlier, often along with the dataset, additional domain knowledge is available that can assist with the data mining process. These sources lead us to believe that some models are more likely to

be correct than others even before we see the dataset. We can specify this belief using informative priors. Two approaches to using informative priors in literature have shown promise[7,8]. In the Methods and Materials section of this paper, we discuss each of the two approaches and describe ways to extend BRL to specify such informative priors that can incorporate domain knowledge.

In this paper, we implemented an approach to incorporate prior domain knowledge into the BRL learning process using informative priors. We evaluated the effect of this prior knowledge on model learning using experiments with simulated and a real-world lung cancer prognostic dataset.

MATERIALS AND METHODS

In this section, we describe our implementation in BRL to incorporate prior domain knowledge, and then describe two experiments we conducted to evaluate this implementation. Specifically, we describe a BRL greedy best-first search algorithm, the heuristic score used by the search to evaluate candidate models, and our approach to extend this heuristic score to incorporate prior background domain knowledge using informative priors. We call this extension to BRL as BRL$_P$ (BRL with informative priors). After describing our implementation of BRL$_P$, we describe two experiments we conducted to study the effects of informative priors in model learning: (1) using simulated data; and (2) on a real-world lung cancer prognostic dataset.

BRL

BRL is a rule-based classifier that takes as input, a dataset D, and returns a rule set model. Let the dataset D be an observed instantiation of a system with a probability distribution over a set of n random variables and a target random variable of interest,$D = \{X_i,T_i; i \in 1...n\}$. Here, T is the target variable of interest, which is the dependent variable for the prediction task. Every other variable, X_i in D is an independent random variable that may help predict T. There are a total of m instances in D. In the classification problem, our task is to accurately predict the value of the target variable. For example, consider a diagnostic problem of predicting a disease outcome for a patient, say lung cancer outcome (either Case or Normal), using gene expression biomarker data, measured for each patient. Here, the dataset D would be composed of a set of m patients, each with n gene expression measurements $\{X_i; i \in 1...n\}$. The target variable T is the binary-valued lung cancer outcome variable, $T = \{Case, Normal\}$, for each patient in the dataset.

The BRL search algorithm explores a space of BNs, learned from the observed dataset D, and returns the most optimal BN found during the search. A BN is a graphical representation of the probabilistic dependencies of the different variables in the system under study. They are represented as a directed acyclic graph (DAG). In our lung cancer diagnostic problem example, an example of probabilistic dependence could be some hypothetical gene expression, say the binary-valued $X_A = \{Up; Down\}$ with a value for up-regulated and a value for down-regulated gene A, is known to be predictive of the outcome T. Then an optimal BN should contain a directed edge from $X_A \rightarrow T$. In other words, the lung cancer outcome depends upon whether or not gene X_A is expressed. In such a BN, the probability distribution, $P(T \mid X_A)$ is the parameter of the BN.

The parameters of the BN can be represented in form of a conditional probability table (CPT). The CPT is often stored in form of decision trees[9,10]. The BRL generates a mutually exclusive and exhaustive set of inference rules from this decision tree for prediction of class of any new test instances. Here, each path from root to leaf of the decision tree is interpreted as a rule. The BRL rules are represented in the form of explicit propositional logic: IF antecedent THEN consequent. The rule antecedent is the condition made up of conjunctions (ANDing) of the independent random variable-value pairs, which when matched to a test instance, implies the rule consequent composed of the dependent target variable-value. Continuing with our example, a learned rule can be IF ($X_A = Up$) THEN ($T = Case$). In other words, if the gene X_A is up-regulated then the patient is classified to have a lung cancer outcome as a *Case*. There are several types of BRL search algorithms[4,5,11] to help find the optimal BN. In this paper, we will only discuss a simple greedy best-first search algorithm from our previous work[4] and is summarized in the next sub-section.

BRL greedy best-first search algorithm: The BRL greedy best-first search algorithm is described in detail in the paper by Gopalakrishnan *et al*[4], where it is referred to as BRL$_1$. In this paper, we will refer to this algorithm simply as BRL. We will summarize the algorithm in this subsection. The BRL algorithm initializes the search with a network structure with just the variable T and no parent nodes. In each iteration of the algorithm, one new parent is added to T among the n random variables that is not already a parent of T. This BN implies the hypothesis that T is dependent upon the set of variables added as parents to T. This process is called model specialization. The resulting models from that iteration is added to a priority queue. The priority queue sorts these specialized models by evaluating them using a heuristic score called the Bayesian score, which evaluates the likelihood that the observed dataset was generated by a given hypothesized BN model. This score is described in detail in the next subsection. The greedy search picks the model in the head of the priority queue at the end of the iteration. This model is evaluated to be the best scoring model among the specializations in that iteration. In the next iteration, this model is selected for further specialization by adding more parents. The search terminates when a subsequent specialization step fails to improve the

heuristic score. The search also terminates if the model has reached a limit on the maximum number of parents allowed for *T*. This search parameter is called maximum conjuncts. Finally, BRL generates a rule model inferred from the model returned by the search.

BRL heuristic score (Bayesian score): BRL search evaluates the quality of a candidate BN model using a heuristic score called the Bayesian score[9]. In this sub-section, we describe this score. We represent a BN model as the tuple $B = (B_S, B_P)$, where B_S is the network structure with a subset of π discrete-valued nodes, and B_P is the numerical parameters of the network. The posterior probability of the candidate structure given the observed dataset, *D*, is calculated as in Equation 1.

$$P(B_S \mid D) = P(B_S, D)/P(D) \quad (1)$$

Since we are comparing Bayesian networks learned from the same dataset *D*, the denominator does not affect our decision. Only the numerator helps with model selection as shown in Equation 2.

$$P(B_S \mid D) \propto P(B_S, D) \quad (2)$$

The joint probability of the network structure and the observed dataset, $P(B_S, D)$, is equal to the prior probability of the network structure, $P(B_S)$ and the likelihood that the observed data was generated by that network structure,$P(D \mid B_S)$. This is shown in Equation 3.

$$P(B_S, D) = P(B_S) \cdot P(D \mid B_S) \quad (3)$$

To compute the joint probability of the network structure and the observed dataset, $P(B_S, D)$, we use the BDeu score[6]. We get Equation 4.

$$P(B_S, D; \alpha) = P(B_S) \cdot \prod_{i=1}^{n} \prod_{j=1}^{q_i} \frac{\Gamma\left(\frac{\alpha}{q_i}\right)}{\Gamma\left(N_{ij} + \frac{\alpha}{q_i}\right)} \prod_{k=1}^{r_i} \frac{\Gamma\left(N_{ijk} + \frac{\alpha}{r_i q_i}\right)}{\Gamma\left(\frac{\alpha}{r_i q_i}\right)} \quad (4)$$

Here, *i* iterates through each node in the BN with *n* nodes. Index *j* iterates though all, q_i, possible variable-value instantiations of the parents of the i^{th} node. Index *k* iterates through all r_i values of the i^{th} node. N_{ijk} is the number of instances in *D*, where the variable *i* takes the k^{th} value and its parent variables take the j^{th} variable-value instantiation, and $N_{ij} = \sum_k N_{ijk}$. The Gamma function is defined as $\Gamma(x) = (x\text{-}1)!$. The α is a user-defined parameter called prior equivalent sample size (*pess*). We set $\alpha = 1$, which allows the data to easily dominate the score[9]. The $P(B_S)$ term is called the structure prior (see[9] section 18.3.6.1 for details) that represents the prior belief distribution over all network structures before we look at the data. The remaining terms in Equation 4 compose the likelihood term that infers the likelihood of the network from the observed data.

In the classification task using BRL, we do not learn a fully generalized BN but only care about the relationship of the variables with a specific target variable of interest, *T*. Variable *T* is discrete with different values. The set of parents of the i^{th} variable is represented as π_i. In BRL, we learn a constrained BN with node *T* and its set of parents, π_T. The set π_i can have q_T possible attribute-value instantiations. So, for BN search in BRL, we optimize the heuristic score in Equation 5.

$$P(B_S, D) = P(B_S) \cdot \prod_{j=1}^{q_T} \frac{\Gamma\left(\frac{\alpha}{q_T}\right)}{\Gamma\left(N_j + \frac{\alpha}{q_T}\right)} \prod_{k=1}^{r_i} \frac{\Gamma\left(N_{jk} + \frac{\alpha}{r_T q_T}\right)}{\Gamma\left(\frac{\alpha}{r_T q_T}\right)} \quad (5)$$

The expectation of each parameter value of the BN is computed with Equation 6.

$$\mathbb{E}[\theta_{jk} \mid D, B_S] = \frac{N_{jk} + \frac{\alpha}{r_T q_T}}{N_j + \frac{\alpha}{q_T}} \quad (6)$$

We use this value as the posterior probability of the rule. The number of rules inferred by BRL is equal to the number of θ_{jk} values in the BN. The expectation of this value shows the degree of support a rule has in the observed dataset.

BRL with structure priors: In Equation 5, the $P(B_S)$ term is the structure prior that represents the prior distribution over all network structures. Here, we can specify our prior bias of certain network structure over others to skew the BRL search to focus on certain network structures more than others. Typically, in literature uninformative priors are used, which means that a priori we claim that we do not have any preference of network structures over the others. BRL in this case lets the data alone decide the final learned model. The challenge of specifying these priors is that the total number of network structures grows super-exponentially with the number of variables *n*[12]. It often becomes infeasible to specify structure priors for each of these network structures for even moderately sized datasets. So far in BRL, we had been using an uninformative prior by setting $P(B_S) = 1$, in Equation 5.

Castelo and Siebes[7] describe a promising approach to elicit structure priors by specifying the probability of the presence or absence of each edge in the network structure. The user only needs to specify the probability of a subset of edges in the network structure. The probabilities for all the remaining edges are assigned a discrete uniform distribution value. A challenge using this approach is to specify the values of these probabilities. In our experiments with BRL using these priors, we observed that the likelihood term in Equation 5 always dominates the structure prior term. It would help us if we could control the influence of structure priors over the likelihood term using a scaling factor. As we described earlier in the introduction section, the background knowledge, we specify, itself has uncertainty associated with it. A scaling factor would help us control the influence of data and our prior knowledge.

Mukherjee and Speed[8] propose an informative prior that uses a log-linear combination of weighted real-valued function of the network structure, $f_i(B_S)$. This function is called the concordance function. It can be any function that monotonically increases with the increase in agreement between the learned network structure and the prior beliefs of the user. This is shown in Equation 7.

$$P(B_S) \propto exp\left[\lambda \cdot \sum w_i f_i(B_S)\right] \quad (7)$$

The hyperparameter w_i are the positive weights that represent the relative importance of each function. The hyperparameter λ is a scaling factor that helps to

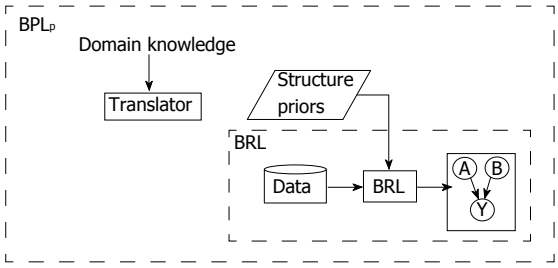

Figure 1 The Bayesian rule learning framework that can incorporate domain knowledge. BRL: Bayesian rule learning.

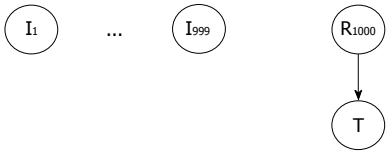

Figure 2 The data-generating graph for the simulated data.

control the overall influence of the structure prior. This will help us quantify the uncertainty in the validity of our prior knowledge.

The structure prior we used for BRLp comes from an instantiation of the general form of this prior, shown in Equation 7, as described by Mukherjee and Speed[8]. It allows the user to specify their prior beliefs about the presence and absence of the edges in the network structure. This instantiation is shown in Equation 8.

$$P(Bs) \propto exp\,[\lambda \cdot (|E(Bs) \cap E_{+}| - |E(Bs) \cap E_{-}|)] \quad (8)$$

Here, set E_{+} (positive edge-set) represents the set of edges the user believes should be present in the model, and set E_{-} (negative edge set) represents the set of edges the user believes should be absent from the model. So, the concordance function in this instantiation simply gives a positive count for if the candidate graph contains an edge from the positive edge-set, and a negative count (penalty) when it contains an edge from the negative edge-set. In this instantiation, the weights hyperparameter is set to 1, since our counts are all valued 1. We need to learn the value of the hyperparameter λ. The range of values it can take depends upon the well-known Jeffrey's scale[13]. When $\lambda = 0$, the whole exponent becomes 0, and $P(Bs) = exp(0) = 1$, which is the uninformative prior. In other words, when $\lambda = 0$, BRLp should have no effect of structure prior and so would behave the same as the baseline model, BRL. As we increase the value of λ, the effect of the structure prior would have an increased influence over the likelihood term in Equation 5.

To summarize, BRLp uses a heuristic score called the BDeu score, shown in Equation 5, and encodes the structure prior in that score using Equation 8. The BRLp framework is shown in Figure 1. The inner dotted box, labeled "BRL", is the classic BRL without prior knowledge, which takes in an input dataset, uses BRL algorithm to learn and output a model. The outer dotted box is our extension, BRLp that can incorporate domain knowledge. The translator process, currently done manually, converts knowledge from various sources to input into Equation 8.

Experiment design

In this section, we describe our experiment design that we used to demonstrate the functionality of BRLp. We examined its behavior on both, simulated dataset, and on a real-world dataset. We were mainly interested in the ability of BRLp to incorporate the supplied prior domain knowledge with respect to the structure prior hyperparameter λ. Additionally, we also monitored the changes in the predictive power of the learned model resulting from the influence of the supplied prior domain knowledge. We studied the functionality of BRLp on a simulated dataset, and then on a real-world dataset. Each is described, in detail, in the following sub-sections.

Simulated data analysis: We first generated simulated data to study the behavior of BRLp. We can control the properties of the simulated dataset, which gave us a controlled environment to check if BRLp was behaving as we expected on a dataset with the specified properties.

Data generation: We generated a simulated dataset with 1000 variables in addition to the target variable, *T*. We show the data-generating graph in Figure 2. Out of the 1000 candidate variables that can predict *T*, only one variable, R_{1000}, is relevant. A relevant variable is a variable that helps to predict *T*. All the remaining 999 variables, $\{I_1...I_{1000}\}$, are irrelevant. Irrelevant variables are random values that do not help predict *T*. All the random variables in the graph are binary {0, 1}. The conditional distributions in the graph are Bernoulli with the success parameter *p* depending upon the value instantiation of their parent variables. The irrelevant and relevant variable values were randomly sampled with $p = 0.5$. The *T* variable value was sampled with $p = 0.9$ if its parent, R_{1000}, took the value 1, and $p = 0.1$ otherwise.

Data background knowledge: In a simulation problem, we already knew the true data-generating graph as shown in Figure 2. We knew that in the learned network structure from BRLp, there should be an edge present between R_{1000} and *T*. So, in Equation 8, the positive edge-set only contained this edge, $E_{+} = \{(R_{1000}, T)\}$. All the edges between irrelevant variables and *T* should be absent in the BRLp model, so they went to the negative edge-set, $E_{+} = \{(I_k, T); k = 1...999\}$. We evaluated the impact of the λ hyperparameter value of the structure prior on the final model learned by BRLp.

Methods evaluated: We evaluated the method BRLp here. We set the user-defined, search algorithm

parameter of BRL$_p$ of maximum conjuncts (constraint on maximum number of parents of *T*) to 8. We evaluated the effect of the hyperparameter λ by assigning =its values λ = {0,1,2,3,4,5,6,7,8,9,10}. The value of λ = 0 represents the baseline model of BRL with no structure priors.

Evaluation metrics: We evaluated BRL$_p$ with two metrics: (1) graph edit distance (GED); and (2) area under the receiver operator characteristics curve (AUC). We evaluated them over 5 runs of 10-fold cross-validation. In each run, the dataset was randomly shuffled to produce a different set of 10 stratified folds. GED measures how much of the prior domain knowledge gets incorporated into the model learning process. Specifically, how much does the model learned by BRL$_p$ agree with the supplied prior knowledge? This metric is described in detail in the next paragraph. We monitored the BRL$_p$ model predictive power by measuring the average AUC across the 5 runs of 10-fold cross-validation. The AUC helped us monitor the influence of structure priors in model predictive performance.

GED[14] is a metric of similarity between two graphs. In this experiment, we compared two constrained BNs. Specifically, we were interested in measuring how closely our BRL$_p$ predicted BN, $\widehat{B}_S$ (learned by BRL$_p$) resembled the true BN, B_S, which generated the simulated dataset (Figure 2 in this experiment). This was used to estimate the value of adding structure prior knowledge for model learning when the true model is available for comparison. We computed this metric using Equation 9.

$$d_{v_{min}}(B_S, \widehat{B}_S) = \min_{v \in \Upsilon(B_S, \widehat{B}_S)} \sum_{e_i \in v} c(e_i) \qquad (9)$$

Here, $d_{v\,min} = [B_S, \widehat{B}_S]$ is a function that returns the GED between the two BNs. A specific e_i is an edit operation to transform one graph into another. For the constrained BN we have two available edit operations - delete edge, and insert edge. There is a cost c (e_i) associated with each edit operation. We set c (e_i) = -1, for both the edit operations. A *v* is an edit path containing a sequence of edit operations to transform graph B_S into $\widehat{B}_S$. The set γ [B_S, $\widehat{B}_S$] is a set of all possible edit paths. To compute the graph edit distance, we find the edit path, *v*, that minimizes the overall cost and then return this minimum cost value indicating the minimum number of operations needed to transform one graph to another. Therefore, an edit distance of 0 indicates that the predicted graph is identical to the true graph. Since the maximum parents resulted from BRL is constrained to 8 from the user parameter, the worst possible model contains all 8 irrelevant variables. So, we get $d_{v\,min} = 9$ (8 edge deletion operations from irrelevant variables, 1 insert edge operation to the relevant variables).

Real-world lung cancer prognostic biomarker data analysis: We obtained a real-world dataset for our analysis from Gene Expression Omnibus[15] (GEO), a public gene-expression data repository. We extracted the dataset from a study[16] that collected both tumor and normal tissue samples from 60 female non-small cell lung cancer (NSCLC) patients in Taiwan. As a result, there were 120 samples in this dataset (60 patients, each with paired tumor and normal tissue). RNA was extracted from these paired tumor and normal tissues for gene expression analysis on the Affymetrix Human Genome U133 Plus 2.0 Array platform. The platform has 54675 probes. The accession ID for this study on GEO database is GSE19804.

Data pre-processing: The raw dataset extracted from GEO contained 54675 probes and 120 instances. We needed to pre-process the data to prepare it for data analysis. The dataset pre-processing was done using Bioconductor (version 3.6) packages in R (version 3.4.3). We extracted the raw dataset using the *affy* package[17]. We used Robust Multichip Analysis (RMA) for background correction, quantile normalization, and probe summarization. We mapped probes to the genes they represented. Multiple probes can map to a single gene. In the final dataset, we would like to have just one random variable representing a unique gene. Among the multiple probes that map to a single gene, we chose the probe with the largest inter-quantile range to represent the gene. This process is called inter-quantile range (IQR) filtering. Finally, we also extracted the tissue phenotype (tumor or normal) for each sample and add to this dataset. The outcome variable of interest was this tissue phenotype. After this pre-processing step, we were left with 16382 genes. So, the final dataset for our analysis had 16382 variables and 120 instances. The R script we used for data pre-processing is available in the GitHub repository linked in the Conclusion section.

Many classification algorithms, including BRL, cannot handle continuous-valued variables, and require the input data to be discretized. Moreover, supervised discretization can help improve the performance of several classifiers including Support Vector Machines and Random Forests[18]. This is because supervised discretization acts as a feature selector that only retains variables with meaningful discretization bins. Biomedical datasets are high dimensional, there can be many noisy and redundant variables. Supervised discretization can help remove some of these variables from the model learning process. We discretized the dataset using efficient Bayesian discretization (EBD), a supervised discretization method, which has been shown to obtain better classification performance and stability but less robust when compared to the popular Fayyad-Irani supervised discretization method on several biomedical datasets[19]. We set the user-defined lambda parameter of EBD, to 0.5, as the recommended default value in the paper. During model learning, we split the data into 10 folds for cross-validation. For each train-test fold pair, supervised discretization bins were learned on the train dataset alone. The learned bins were applied to the test

Table 1 Clinical features of the 60 non-small cell lung cancer patients in the real-world lung cancer prognostic dataset

Attribute	Value	*n* (%)
Gender	Women	60 (100)
	Men	0 (0)
Tumor type	Adenocarcinoma	56 (93)
	Bronchioloalveolar carcinoma	3 (5)
	Squamous	1 (2)
	Others	0 (0)
Smoking history	Yes	0 (0)
	No	60 (100)

Statistics extracted from the paper by Lu *et al*[16].

dataset. So, during supervised discretization, we did not look at the test dataset.

Data background knowledge: We explored the medical literature for known prognostic markers that may assist in model learning with BRL$_p$. Before exploring, we first sought to understand more about the dataset, which turned out to have some interesting characteristics making it highly worthy of study. Of note, only tissue samples taken from non-smokers who were all women, who had contracted lung cancer were analyzed in this study. Table 1 summarizes some clinical features known about the 60 Taiwanese NSCLC patients studied in the dataset as described in the paper of the study[16].

We noted from the Table 1 that the subjects in the dataset were all women (60 out of 60 patients), contain mainly adenocarcinoma patients (56 out of 60 patients), and none of them had any smoking history (60 out of 60 patients). Additionally, we also knew that all the patients were from Taiwan. So, we explored the medical literature to find known prognostic markers for this sub-population. Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase is prognostic marker known to be frequently over-expressed in NSCLC[20]. EGFR encodes a transmembrane glycoprotein, a receptor for members of the epidermal growth factor family. A ligand binding to this receptor induces dimerization and tyrosine autophosphorylation, and leads to cell proliferation (referred from RefSeq, June 2016). In NSCLC patients, Shigematsu *et al*[21] observed that EGFR domain mutations are statistically significantly more frequent in women than men (42% *vs* 14%), in adenocarcinomas than other histologies (40% *vs* 3%), in non-smokers than smokers (51% *vs* 10%), and in East Asians than other ethnicities (30% *vs* 8%); all with a *P*-value of < 0.001. This description is very similar to the subjects in the dataset we are studying. Therefore, EGFR gene expression was potentially a good candidate to be incorporated as prior domain knowledge into model learning with BRL$_p$ on this dataset.

Methods compared: We again evaluated BRL$_p$ here. We set its of maximum conjuncts to 8. We evaluated the effect of the hyperparameter λ by assigning it values of $\lambda = \{0,1,2,4,6,8,10,20\}$. The value $\lambda = 0$ represents the baseline model of BRL with no structure priors. We included $\lambda = 20$, to study the scenario where the structure priors overwhelmingly dominates the likelihood score. Additionally, we compared these models with some state-of-the-art classifiers including three interpretable class of classifiers namely - C4.5[22], RIPPER[23], and PART[24]; and three complex and non-interpretable classifiers namely- Random Forests[25], naïve Bayes[26], and Support Vector Machines[27]. C4.5[22] is a popular decision tree learning algorithm, where each path of the decision tree can be interpreted as rules. RIPPER[23] (Repeated Incremental Pruning to Produce Error Reduction) is a propositional rule learning algorithm that uses a divide-and-conquer strategy during model training. PART[24] is a rule learning method that combines the approaches of both C4.5 and RIPPER by building partial decision trees, inferring rules from the trees, and using a divide-and-conquer strategy to build the rule model. Random Forest[25] is an ensemble learning method that learns a number of decision trees during training, and combines predictions from them during inference. The naive Bayes[26] classifier is a simple probabilistic classifier that learns a network with strong independence assumption between the variables, and uses the Bayes theorem for inference from the learned network. Support Vector Machines[27] is an algorithm that learns a hyperplane function to differentiate the classes in the problem space. We ran these classifiers from the Weka[28] workbench (version 3.8.1) using the default parameters for each classifier.

Evaluation metrics: We evaluated BRL$_p$ with two metrics: (1) Prior Frequency (PF); and (2) AUC. We evaluated the dataset over 5 runs of 10-fold cross-validation. For this real-world scenario, we used PF to measure the gain of the background knowledge into BRL$_p$. With the simulated dataset, we had evaluated using GED because we knew the true data-generating graph. In most real-world problems, we do not know the true model that generated the data and so, we cannot use GED. PF measures the fraction of models learned on each of the 50 folds (5 runs of 10-fold cross-validation) that incorporates the specified prior domain knowledge. In this experiment, we measured the fraction of the models that contained an edge between EGFR and *T* in the learned BRL$_p$ model.

RESULTS

In this section, we present the results from our experiments examining the effects of the λ hyperparameter of the structure prior, and consequentially the influence of the specified prior knowledge on model learning. We show our results using the simulated data first, and then from the real-world lung cancer prognostic dataset.

Simulation data analysis results

The results from the 5 runs of 10-fold cross-validation

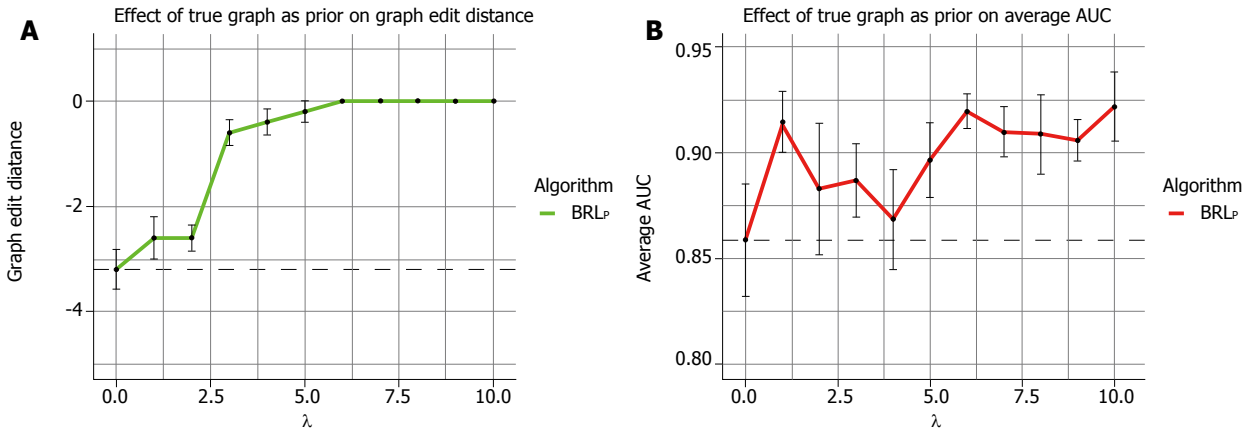

Figure 3 Evaluation metrics on Bayesian rule learning model learning with simulated data. A: Graph edit distance between BRLp and true data-generating model; B: Area under the receiver operator characteristic curve of the BRLp model. BRLp: Bayesian rule learning with informative priors; AUC: Area under the receiver operator characteristic curve.

1. IF (RV1000 = 0) THEN (T = 0)
Posterior Probability = 0.9944, TP = 44, FP = 0, Pos = 48, Neg = 52

2. IF (RV1000 = 1) THEN (T = 1)
Posterior Probability = 0.9248, TP = 52, FP = 4, Pos = 52, Neg = 48

Figure 4 Bayesian rule learning generated rule model with λ=10 (highest average area under the receiver operator characteristic curve) on the simulated dataset. Each rule has its posterior probability, the number of true positives (TP), false positives (FP), total number of examples that match the rules consequent target value (Pos), and total number that do not match the right hand side of the rule (Neg). The TP measures examples that correctly match the rules left and right hand sides, while FP measures examples that correctly match the rules condition or left-hand-side, but have a different consequent or right-hand-side.

are summarized in Figure 3. In Figure 3A, the various values of the hyperparameter λ is shown in the x-axis, while the y-axis shows the average GED. This average is obtained across the 10-folds of each run, and then averaged across the 5 runs. Each data-point in the graph is this average deviation from the true model as measured by the GED, and the error bars represent the standard error of mean. The dotted line shows the value of BRL$_P$ with λ = 0, which as we mentioned earlier is the same as BRL, where we use uninformative priors. We saw that even with λ = 1, the structure priors helped improve the GED thereby bringing the learned model closer to the data-generating model. We saw a sharp gain of GED from λ = 2 to 3. For λ ⩾ 6, BRL$_P$ returned the true data-generating model specified by the structure priors. This showed that BRL$_P$ effectively and correctly incorporates the specified domain knowledge. The degree of incorporation is controlled by λ.

Figure 3B displays the average AUC. The overall trend is a gain in AUC but the trend is noisy, especially with low λ values when the GED > 0. This region indicated models that picked up irrelevant variables, which were spurious and were associated with *T*, by chance. Their AUC fluctuated a lot because random associations were found. When λ ⩾ 6, the GED reached the perfect 0, we saw a rise in AUC. The noise reduced in this region of the graph. Random samplings from our simulation generated slightly different values of the parameters, which were reflected in the fluctuations here. So, from the AUC graph we saw a gradual gain in predictive performance with the incorporation of prior knowledge of the truth.

Figure 4 shows a BRL$_P$ rule model obtained when λ = 10, which achieved the largest average AUC from our experiments (AUC = 0.92). The particular run achieved an AUC of 0.96 on the 10-fold cross-validation and a GED of a perfect 0. The posterior probability was computed using Equation 6. TP and FP refers to the total true positives and false positives. Pos and Neg are the total positives and negative examples. Our simulation design only had one relevant variable, R_{1000}, and 999 irrelevant variables, $\{I_1 \ldots I_{1000}\}$. The rule model in Figure 4 correctly picked up only the relevant variable. We had designed the simulation such that if the relevant variable took the value 1, then *T* would be sampled with a Bernouli distribution with $p = 0.9$, this was reflected in Rule 2. So, BRL$_P$ accurately retrieved the true data-generating model assisted by informed structure priors.

Real-world lung cancer prognostic data analysis results

The results from the 5 runs of 10-fold cross-validation on the real-world lung cancer prognostic dataset are summarized in Figure 5. We specified the structure prior of an edge between EGFR and the outcome *Class* variable to be present. We altered the values of λ and observed its effect on the learned model. Figure 5A, shows the effect of the different values of λ on PF, the fraction of models that contained EGFR. From λ = 2 to 6,

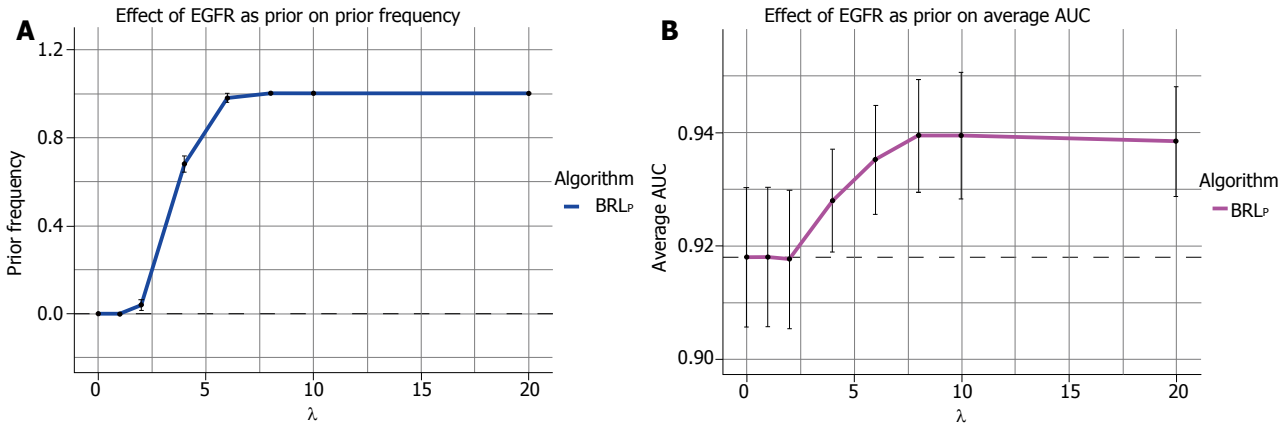

Figure 5 Evaluation metrics on Bayesian rule learning model learning with real-world lung cancer prognostic dataset. A: Prior frequency of the edge between epidermal growth factor receptor and T in BRLp model; B: Area under the receiver operator characteristic curve of the BRLp model. BRLp: Bayesian rule learning with informative priors; EGFR: Epidermal growth factor receptor; AUC: Area under the receiver operator characteristic curve.

we saw a steep gain in PF. For $\lambda \geqslant 8$, EGFR was present in every learned model. This again showed that BRL$_p$ effectively incorporated the specified prior knowledge and the λ hyperparameter allowed the user to determine the degree of incorporation of this knowledge by BRL$_p$.

Figure 5B, shows the gain of average AUC across 5 runs of 10-fold cross-validation. We observe a steady gain of AUC for $\lambda > 2$. For $\lambda \geqslant 8$, the AUC gain tapers off. The results show that the EGFR prior knowledge helped improve the AUC of BRL$_p$.

BRL$_p$ with $\lambda = 8$ generated the highest average AUC of 0.935. Figure 6 shows the rule model from one of the runs, which had achieved a cross-validation AUC of 0.967 and PF of 1. Rule 1 had the highest amount of evidence (38 true positives and no false positives) for the outcome Control (normal tissue). This rule had the EGFR value range from negative infinity to 10.8. In other words, EGFR was under-expressed in these 38 normal tissue instances. Rule 15 had the highest amount of evidence (15 true positives) for the outcome Case. This rule had EGFR value range from 10.8 to positive infinity. In other words, EGFR was over-expressed in these 15 tumor tissue instances. These rules also lent support to what we had found in the literature about EGFR being over-expressed in tumor cells. In addition to EGFR, which was incorporated from the structure prior, the model picked up 3 other variables during model learning from the dataset. They were ephrin A4 (EFNA4), killer cell lectin like receptor G2 (KLRG2), and C2 calcium dependent domain containing 6 (C2CD6).

Finally, we compared two BRL$_p$ models with state-of-the-art classifiers using average AUC achieved across 5 runs of 10-fold cross-validation. The two BRL$_p$ models were (1) with $\lambda = 0$, which represented the baseline BRL model with uninformative priors, and (2) with $\lambda = 8$ that incorporated EGFR into the structure prior, which achieved the highest average AUC of 0.935. The state-of-the-art classifiers compared were C4.5, RIPPER, PART, Random Forests, naïve Bayes, and Support Vector Machines. This comparison is shown in Figure 7.

The first two bars in Figure 7 are BRL$_p$ algorithms, BRL$_p$ with $\lambda = 0$ is indicated as BRL, and then BRL$_p$ with $\lambda = 8$. We saw a gain in performance crom incorporating EGFR as structure priors. The next three bars - C4.5, RIPPER, and PART are interpretable class of models, which are human readable. C4.5 is a decision tree learning algorithm. RIPPER and PART are rule learning algorithms. We noticed that these three algorithms performed worse than both BRL$_p$ algorithms in this dataset. The last three bars in Figure 7 are - Random Forest, naïve Bayes, and Support Vector Machines. These are examples of complex models that use all variables in the dataset to generate a classifier. It is not easy to explain the reasoning behind their predictions. But all three algorithms here outperformed BRL$_p$ on this dataset. This comparison shows the trade-off of predictive performance and interpretability. On this dataset, BRL$_p$ offered an interpretable model that outperformed other popular interpretable models but did not perform as well as the complex models.

DISCUSSION

An important practical consideration to note while specifying structure priors is to avoid specifying priors that introduce bias into the model search. Informative priors can be biased if they are inferred based on the predictions, of some predictive model, on the test dataset. For example, if we notice that our learned model predicts poorly on a subset of test instances, and we notice some independent variable(s) strongly associated with the target variable in that subset of test instances. Specifying, our newly found association from the predictions on the test dataset, into the structure priors to re-learn the model will return a biased model and must be avoided.

Mukherjee and Speed[8] show how the general form of the score in Equation 7 can be extended to

1.IF ((EFNA4 = -inf to 6.9) (KLRG2 = 6.4 to inf) (EGFR = -inf to 10.8) (C2CD6 = -inf to 3.9)) THEN (Class = Control)
Posterior Probability=0.9995, TP=38, FP=0, Pos=60, Neg=60

2.IF ((EFNA4 = 6.9 to 7.5) (KLRG2 = 6.4 to inf) (EGFR = -inf to 10.8) (C2CD6 = -inf to 3.9)) THEN (Class = Control)
Posterior Probability=0.9977, TP=9, FP=0, Pos=60, Neg=60

3.IF ((EFNA4 = -inf to 6.9) (KLRG2 = 6.4 to inf) (EGFR = 10.8 to inf) (C2CD6 = -inf to 3.9)) THEN (Class = Control)
Posterior Probability=0.9959, TP=5, FP=0, Pos=60, Neg=60

4.IF ((EFNA4 = -inf to 6.9) (KLRG2 = -inf to 6.4) (EGFR = -inf to 10.8) (C2CD6 = -inf to 3.9)) THEN (Class = Control)
Posterior Probability=0.9959, TP=5, FP=0, Pos=60, Neg=60

5.IF ((EFNA4 = 7.5 to inf) (KLRG2 = 6.4 to inf) (EGFR = 10.8 to inf) (C2CD6 = 3.9 to inf)) THEN (Class = Control)
Posterior Probability=0.9898, TP=2, FP=0, Pos=60, Neg=60

6.IF ((EFNA4 = 6.9 to 7.5) (KLRG2 = 6.4 to inf) (EGFR = 10.8 to inf) (C2CD6 = -inf to 3.9)) THEN (Class = Control)
Posterior Probability=0.98, TP=1, FP=0, Pos=60, Neg=60

Rules 7 through 14 match 0 instances and so are removed from display.

15.IF ((EFNA4 = 7.5 to inf) (KLRG2 = -inf to 6.4) (EGFR = 10.8 to inf) (C2CD6 = -inf to 3.9)) THEN (Class = Case)
Posterior Probability=0.9986, TP=15, FP=0, Pos=60, Neg=60

16.IF ((EFNA4 = 7.5 to inf) (KLRG2 = -inf to 6.4) (EGFR = -inf to 10.8) (C2CD6 = -inf to 3.9)) THEN (Class = Case)
Posterior Probability=0.9985, TP=14, FP=0, Pos=60, Neg=60

17.IF ((EFNA4 = 7.5 to inf) (KLRG2 = 6.4 to inf) (EGFR = 10.8 to inf) (C2CD6 = -inf to 3.9)) THEN (Class = Case)
Posterior Probability=0.9974, TP=8, FP=0, Pos=60, Neg=60

18.IF ((EFNA4 = 7.5 to inf) (KLRG2 = -inf to 6.4) (EGFR = 10.8 to inf) (C2CD6 = 3.9 to inf)) THEN (Class = Case)
Posterior Probability=0.997, TP=7, FP=0, Pos=60, Neg=60

19.IF ((EFNA4 = 7.5 to inf) (KLRG2 = -inf to 6.4) (EGFR = -inf to 10.8) (C2CD6 = 3.9 to inf)) THEN (Class = Case)
Posterior Probability=0.9959, TP=5, FP=0, Pos=60, Neg=60

20.IF ((EFNA4 = 7.5 to inf) (KLRG2 = 6.4 to inf) (EGFR = -inf to 10.8) (C2CD6 = -inf to 3.9)) THEN (Class = Case)
Posterior Probability=0.9948, TP=4, FP=0, Pos=60, Neg=60

21.IF ((EFNA4 = 6.9 to 7.5) (KLRG2 = -inf to 6.4) (EGFR = 10.8 to inf) (C2CD6 = -inf to 3.9)) THEN (Class = Case)
Posterior Probability=0.9932, TP=3, FP=0, Pos=60, Neg=60

22.IF ((EFNA4 = 7.5 to inf) (KLRG2 = 6.4 to inf) (EGFR = -inf to 10.8) (C2CD6 = 3.9 to inf)) THEN (Class = Case)
Posterior Probability=0.9898, TP=2, FP=0, Pos=60, Neg=60

23.IF ((EFNA4 = 6.9 to 7.5) (KLRG2 = -inf to 6.4) (EGFR = -inf to 10.8) (C2CD6 = 3.9 to inf)) THEN (Class = Case)
Posterior Probability=0.98, TP=1, FP=0, Pos=60, Neg=60

24.IF ((EFNA4 = 6.9 to 7.5) (KLRG2 = -inf to 6.4) (EGFR = 10.8 to inf) (C2CD6 = 3.9 to inf)) THEN (Class = Case)
Posterior Probability=0.98, TP=1, FP=0, Pos=60, Neg=60

Figure 6 Bayesian rule learning generated rule model with λ=8 (highest average area under the receiver operator characteristics curve) on the real-world lung cancer prognostic dataset. TP: True positives; FP: False positives; Pos: Total number of examples that match the rules consequent target value; Neg: Total number that do not match the right hand side of the rule; EGFR: Epidermal growth factor receptor.

incorporate other kinds of prior knowledge including rewarding network sparsity, where structure priors can be used as a regularization term. In the introduction section, we had discussed other sources of prior knowledge than literature, including - input from a domain expert (*e.g.*, A physician), domain ontology (*e.g.*, Gene Ontology), and models learned from other related datasets. In the future, we will explore the incorporation of knowledge from these other sources. In novel biomarker discovery, we could place all of our known knowledge into the negative edge-set in Equation 8. Models learned from such a structure prior would be penalized for learning already known biomarkers and would encourage discovery of novel biomarkers. We used an instantiation of the general form of the score, in Equation 7, where the relative weights, w_i, of each of i^{th} network are set to 1. It would be interesting to explore different relative weights for different network features and see its impact on model learning. In this paper, we performed a grid search over the hyperparameter λ. We would like to explore if we can come up with better ways to optimize the value of this hyperparameter.

In this paper, we implemented BRL_P, a method

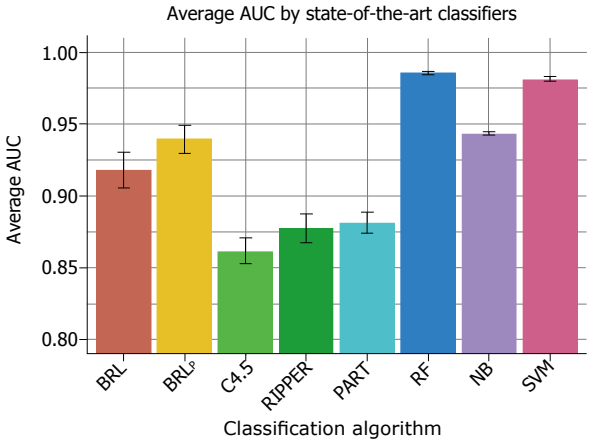

Figure 7 Comparison of area under the receiver operator characteristics curve achieved by Bayesian rule learning with state-of-the-art classifiers. AUC: Area under the receiver operator characteristic curve.

that extended BRL to allow it to integrate prior domain knowledge using structure priors into the model learning process. We demonstrated the ability of BRLp to incorporate this knowledge on simulated data and a real-world lung cancer prognostic dataset. We observed that the λ hyperparameter allowed us to control the degree of incorporation of prior knowledge. This parameter can be helpful if we were uncertain about our specified prior knowledge. We also observed that relevant prior knowledge could sometimes help improve the predictive performance of BRLp. Methods developed in this paper, the simulation data experiment code, and the R script for data extraction and processing of the prognostic dataset, are all made publicly available in an online repository (https://github.com/jeya-pitt/brl-structure-priors). We envision that BRLp will be very beneficial in data mining tasks across domains where some prior domain knowledge is available.

ARTICLE HIGHLIGHTS

Research background

Biomedicine is increasingly a data-driven science, owing largely to the explosion in data, especially from the development of high-throughput technologies. Such datasets often suffer from the problem of high-dimensionality, where a very large number of candidate variables can explain the outcome variable of interest but have few instances to support any model hypothesis. In many applications, in addition to the data itself, some domain knowledge is available that may assist in the data mining process to help learn more meaningful models. It is important to develop data mining tools to leverage this available domain knowledge. However, currently, there is a dearth of data mining methods that can incorporate this available domain knowledge.

Research motivation

Developing data mining methods that can incorporate domain knowledge will help learn more meaningful models and will benefit many domains, especially the ones that suffer from data scarcity but have some domain knowledge that can assist with the data mining process (for example - biomedicine).

Research objectives

In this work, our objective was to extend a rule learning algorithm, called Bayesian rule learning (BRL), to make it capable of incorporating prior domain knowledge. BRL is a good candidate because it has been shown to be successful in application to high-dimensional biomedical data analysis tasks. We implemented such a tool, called BRLp that has tunable priors, which means the user can control the degree of incorporation of their specified knowledge. BRLp is a novel data mining tool that allows the user to specify their domain knowledge (including uncertain domain knowledge) and incorporates it into the model search process.

Research methods

BRL searches over a space of Bayesian belief network models (BNs) to find the optimal network and infers a rule set from that model. We implemented a way for the BN to incorporate informative priors, a distribution encoding the relative importance of each model prior to seeing the training data. This allowed BRL to incorporate user-specified domain knowledge into the data mining process called BRLp. BRLp has a hyperparameter λ that allows the user to adjust the degree of incorporation of their specified prior knowledge.

We evaluated BRLp by comparing it to BRL (without informative priors) and other state-of-the-art classifiers on a simple simulated dataset, and a real-world lung cancer prognostic dataset. We measured the degree of acceptance of the specified prior knowledge with respect to the hyperparameter λ in BRLp. We also observed the changes in predictive power using AUC.

Research results

We observed, in both the experiments with simulated data and the real-world lung cancer prognostic data that with increasing values of λ the degree of incorporation of the specified prior knowledge also increased. We also observed that specifying prior knowledge relevant to the problem dataset could sometimes help find models with better predictive performance. When BRLp is compared to the state-of-the-art classifiers, we observed that it performed better than other interpretable models but the more complex and non-interpretable models achieved better predictive performance than BRLp.

Research conclusions

BRLp allows the user to incorporate their specified domain knowledge into the data mining task and allows them to control the degree of incorporation with a hyperparameter. This is a novel rule learning algorithm that we have made available to the general public via GitHub. We anticipate its use in many applications especially the ones suffering from data scarcity but have additional domain knowledge available that may assist in the data mining task.

Research perspectives

In this paper, we explored specifications of simple domain knowledge. We need to further explore the incorporation of more complex forms of knowledge. In this paper, we incorporate domain knowledge from literature. We also want to explore domain knowledge available in other sources. These future directions may motivate further developments to BRLp.

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ORIGINAL ARTICLE

Retrospective Study

FOLFIRI3-aflibercept in previously treated patients with metastatic colorectal cancer

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Abstract

AIM

To evaluate the efficacy and safety of the modified FOLFIRI3-aflibercept as second-line therapy in patients with metastatic colorectal cancer.

METHODS

This is a retrospective multicenter cohort, evaluating the efficacy and safety of the association of aflibercept with FOLFIRI3 (day 1: aflibercept 4 mg/kg, folinic acid 400 mg/m^2, irinotecan 90 mg/m^2, 5-fluorouracil infusion 2400 mg/m^2 per 46 h; day 3: irinotecan 90 mg/m^2) in patients with previously treated metastatic colorectal cancer. The primary endpoint was overall response rate (ORR). Secondary endpoints were disease control rate (DCR), progression-free survival (PFS), overall survival (OS), and safety.

RESULTS

Among 74 patients treated in four French centers, nine were excluded due to prior use of aflibercept (n = 3), more than one prior treatment line in irinotecan-naïve patients (n = 3), and inadequate liver function (n = 3). In the "irinotecan-naïve" patients (n = 30), ORR was 43.3% and DCR was 76.7%. Median PFS and OS were 11.3 mo (95%CI: 6.1-29.0) and 17.0 mo (95%CI: 13.0-17.3), respectively. The most common (> 5%) grade 3-4 adverse events were diarrhea (37.9%), neutropenia (14.3%), stomatitis and anemia (10.4%), and hypertension (6.7%). In the "pre-exposed irinotecan" patients (n = 35), 20 (57.1%) received $\geqslant$ 2 prior lines of treatment. ORR was 34.3% and DCR was 60.0%. Median PFS and OS were 5.7 mo (95%CI: 3.9-10.4) and 14.3 mo (95%CI: 12.8-19.5), respectively.

CONCLUSION

Minimally modified FOLFIRI has improvement dramatically the FOLFIRI3-aflibercept efficacy, whatever prior use of irinotecan. A prospective randomized trial is warranted to compare FOLFIRI-aflibercept to FOLFIRI3-aflibercept.

Key words: Chemotherapy; Irinotecan; Aflibercept; Second-line; Colorectal cancer

Core tip: Results obtained in this retrospective study show that minimally modified FOLFIRI has improvement dramatically the efficacy of the FOLFIRI3-aflibercept combination with high response rates and survivals in patients with previously treated metastatic colorectal cancer, whatever prior use of irinotecan. A prospective randomized trial is planned to compare FOLFIRI-aflibercept to FOLFIRI3-aflibercept.

Carola C, Ghiringhelli F, Kim S, André T, Barlet J, Bengrine-Lefevre L, Marijon H, Garcia-Larnicol ML, Borg C, Dainese L, Steuer N, Richa H, Benetkiewicz M, Larsen AK, de Gramont A, Chibaudel B. FOLFIRI3-aflibercept in previously treated patients with metastatic colorectal cancer. *World J Clin Oncol* 2018; 9(5): 110-118 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i5/110.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i5.110

INTRODUCTION

Standard second-line therapy in patients with previously treated metastatic colorectal cancer (mCRC) is doublet fluoropyrimidine-based chemotherapy with either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), depending on the regimen used in first-line, in association with antiangiogenic agents (*e.g.*, bevacizumab, aflibercept, ramucirumab) or anti-EGFR agents in absence of *RAS* tumor gene mutation (*e.g.*, cetuximab, panitumumab)[1-8].

The standard FOLFIRI regimen was optimized by splitting the dose of irinotecan on day 1 [half dose before 5-fluorouracil (5-FU)] and day 3 (half dose after 5-FU) in the so-called FOLFIRI3 regimen[9]. Drugs and doses are similar to FOLFIRI, except for suppression of the 5-FU bolus. The response rate was higher than that reported for FOLFIRI[9,10]. Based on these results, FOLFIRI3 became the second-line regimen of choice in some centers. Adding bevacizumab to FOLFIRI3 has shown promising efficacy results in two prior retrospective trials [response rate 22% and 35%, median progression-free survival (PFS) 7.0 and 6.2 mo, median overall survival (OS) 13.0 and 10.8 mo, respectively][11,12]. The addition of aflibercept to FOLFIRI in patients with pretreated mCRC increased response rate from 11% to 20% and improved median PFS from 4.7 to 6.9 mo [hazard ratio (HR) = 0.76] and median OS from 12.1 to 13.5 mo (HR = 0.82)[4]. Aflibercept was approved by the Food and Drug Administration on August 3, 2012 and by the European Medicines Agency on February 1, 2013 in combination with FOLFIRI for the treatment of patients with mCRC resistant to an oxaliplatin-containing regimen[13]. Based on the VELOUR study results and non-randomized FOLFIRI3 studies, we retrospectively analyzed the safety and efficacy of the FOLFIRI3-aflibercept combination as second or later-line therapy in patients with mCRC.

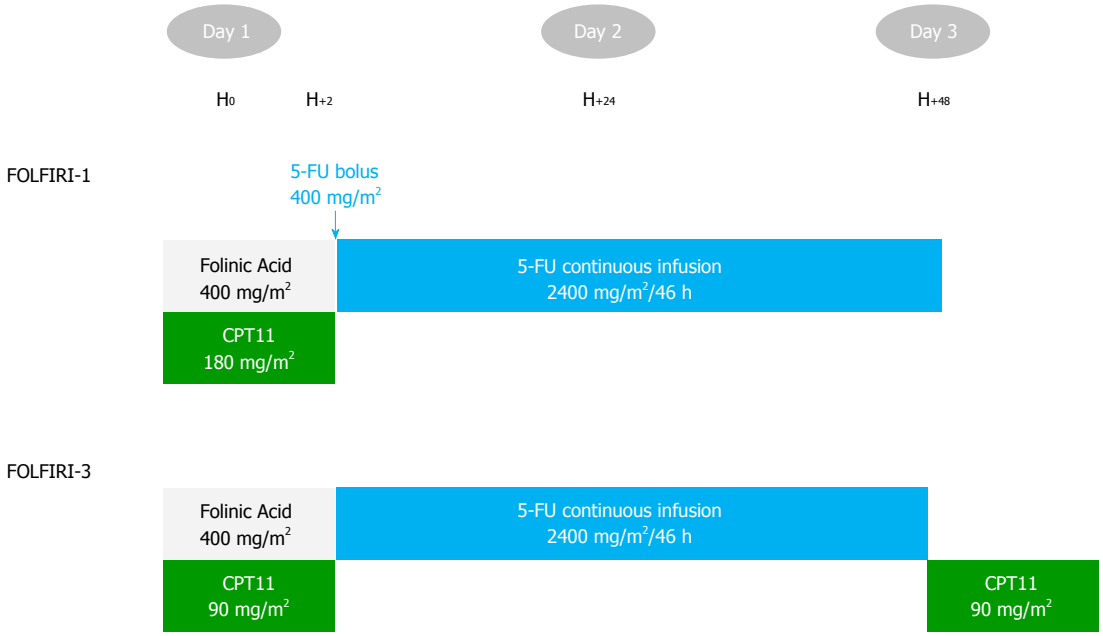

Figure 1 Comparison of the FOLFIRI-1and FOLFIRI-3 schedules.

MATERIALS AND METHODS

This study was a retrospective, multicenter cohort, conducted in four French institutions (Centre Georges François Leclerc, Franco-British Hospital, University Hospital Besançon, and Saint-Antoine University Hospital) from September 2014 to December 2016.The main objective of this study was to evaluate the efficacy and safety profile of the aflibercept-FOLFIRI3 combination.

Population

All patients with previously treated mCRC and with FOLFIRI3-aflibercept administered from September 2014 to December 2016 were included. During the inclusion period, the decision to give FOLFIRI3-aflibercept to each patient or another treatment regimen was at physician's discretion. Prior use of bevacizumab was allowed, but prior exposure to aflibercept was not permitted. Patients were divided into two subgroups depending on the prior use of irinotecan and the number of previous treatment lines for metastatic disease: (1) "irinotecan-naive" population including patients with no more than one prior line of treatment for metastatic disease; and (2) the "irinotecan pre-exposed" population including patients for whom the number of prior treatment lines for metastatic disease was not restricted.

Treatment administration

Treatment cycles were given intravenously every 14 d, as follows: Aflibercept 4 mg/kg over 1-h infusion (day 1), folinic acid 400 mg/m² over 2-h infusion (day 1), irinotecan 90 mg/m² over 60-90 min infusion (day 1), followed by continuous 5-FU 2400 mg/m² as a 46-h infusion (days 1 to 3), then irinotecan 90 mg/m² over 60-90 min infusion (day 3; Figure 1). Treatment information (date of treatment, doses) was collected using CHIMIO® 5.4 (Computer Engineering, Paris, France) or BPC (GCS Emosist, Région Franche-Comté, France) softwares.

Endpoints

Treatment efficacy was evaluated with tumor response, PFS, and OS. The objective response rate (ORR) was defined as the proportion of patients having either complete response (CR) or partial response (PR) according to RECIST version 1.1. The best ORR was defined as the best response recorded from the start of treatment until progressive disease (PD). Disease control rate (DCR) was the sum of ORR and stable disease (SD). PFS was defined as the time from the date of starting treatment to the date of progression or death (from any cause). OS was defined as the time from the date of starting treatment to the date of patient death (from any cause) or to the last date the patient was known to be alive. Toxicity was evaluated according to the United States National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03.

Statistical analysis

Follow-up and survival were estimated using the reverse Kaplan-Meier method and the Kaplan-Meier method, respectively, and were described using median with 95% confidence interval (CI). Qualitative variables were described using percent and means and continuous variables using medians (minimum-maximum). The cut-off date for statistical analysis was June 15, 2017. The final analysis was performed on the irinotecan-naïve and irinotecan pre-exposed

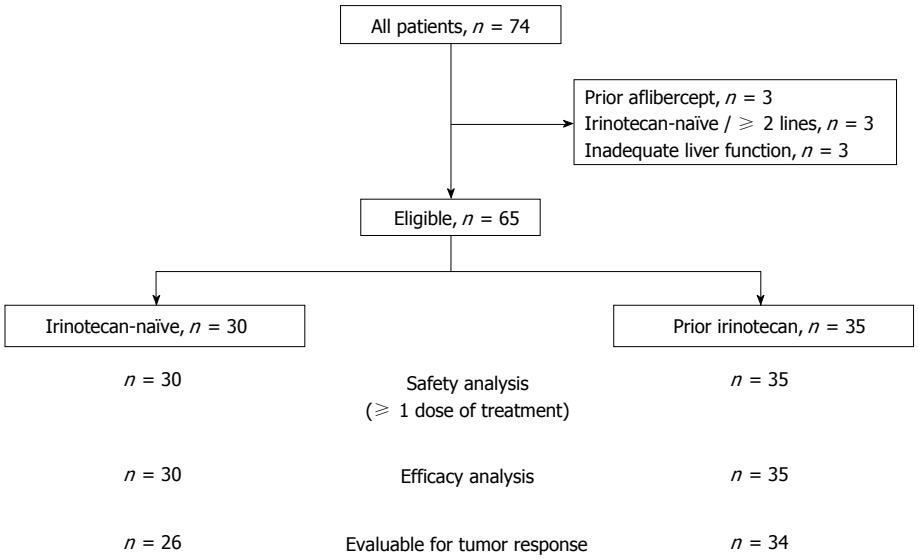

Figure 2 Flow diagram.

populations.

RESULTS

A total of 74 patients were treated (Figure 2). Nine patients were excluded from the analysis due to: Prior use of aflibercept (n = 3), more than one prior line of treatment in irinotecan-naïve patients (n = 3), or inadequate liver function (pretreatment alkaline phosphatase level > 7 × upper normal limit, n = 3). Thirty patients did not receive prior irinotecan (the irinotecan-naïve population) and 35 were previously exposed to irinotecan (the pre-exposed population).

Overall, 25 (38.5%) patients had an Eastern Cooperative Oncology Group performance status 0. The mean age was 63.1 years (range: 31.9-82.1); 23 (35.4%) had a single metastatic site, 49 (75.4%) had *RAS* mutated tumors, and two (3.1%) had *BRAF* mutated tumors. Prior use of bevacizumab and anti-EGFR were reported in 47 (72.3%) and 6 (9.2%) patients, respectively.

In the irinotecan-naïve population, five patients did not receive first-line therapy for metastatic disease (n = 4, early relapse after FOLFOX adjuvant therapy or n = 1, radiochemotherapy). In irinotecan pre-exposed population, 20 (57.1%) patients received more than two prior lines of treatment. Various irinotecan regimens (FOLFIRINOX, n = 21; FOLFIRI, n = 10; FOLFIRI3, n = 4) were previously given. The portion of patients with increased level of lactate dehydrogenase in the irinotecan pre-exposed population was higher than in that with the irinotecan-naïve patients (56.0% *vs* 17.6%; P = 0.027; Table 1).

Treatment exposure

In the irinotecan-naïve population, chemotherapy drugs (irinotecan and 5-FU) were given at standard dose in 12 (40.0%) patients. A lower dose of irinotecan and 5-FU were given in 15 (50.0%) and 4 (13.3%) patients, respectively. The median number of cycles was 8 (range: 1-19), and the median treatment duration was 3.7 mo (95%CI: 2.4-5.7). Dose reductions during treatment were performed in 7 (23.3%), 13 (43.3%), and 6 (20.0%) patients for aflibercept, irinotecan, and 5-FU, respectively. Granulocyte colony-stimulating factor (G-CSF) was given as primary prophylaxis in 14 (46.7%) patients and as secondary prevention in 3 (10.0%) patients. Erythropoietin was used in 5 (17.9%) patients. At the time of analysis, the treatment was still ongoing in 2 patients. The main reasons for stopping therapy were the occurrence of a limiting adverse event in 14 (46.7%) patients (diarrhea, n = 8; bleeding, n = 1; bowel perforation, n = 1; asthenia, n = 1; other, n = 3) or progression in 11 (36.7%) patients.

In the irinotecan pre-exposed population, chemotherapy drugs (irinotecan and 5-FU) were given at standard dose in 22 (62.9%) patients. A lower dose of irinotecan and 5-FU were given in 10 (28.6%), and 6 (17.1%) patients, respectively. The median number of cycles was 6 (range: 1-20), and the median treatment duration was 3.5 mo (95%CI: 2.1-5.6). Dose reductions during treatment were performed in 5 (14.3%), 9 (25.7%), and 7 (20.0%) patients for aflibercept, irinotecan, and 5-FU, respectively. G-CSF was given as primary prophylaxis in 13 (37.1%) patients and as secondary prevention in 2 (5.7%) patients. Erythropoietin was used in 3 (8.6%) patients. At the time of analysis, the treatment was still ongoing in 5 patients. The main reasons for stopping therapy were disease progression in 22 (62.9%) patients and the occurrence of limiting adverse events in 3 (8.6%) patients (diarrhea, n = 2; skin reactions, n = 1).

Response rate

In the irinotecan-naïve population, 4 (13.3%) patients

Table 1 Patient characteristics *n* (%)

Characteristics	Irinotecan-naive cohort	Irinotecan-pre-exposed cohort	*P*-value
Age (yr)			0.793
< 70	21 (70.0)	23 (65.7)	
≥ 70	9 (30.0)	12 (34.3)	
Sex			0.623
Male	16 (53.3)	21 (60.0)	
Female	14 (46.7)	14 (40.0)	
ECOG PS			0.227
0	14 (46.7)	11 (31.4)	
1	13 (43.3)	15 (42.9)	
2	3 (10.0)	9 (25.7)	
Time to metastasis			0.314
Metachronous	9 (30.0)	15 (42.9)	
Synchronous	21 (70.0)	20 (57.1)	
No. of metastatic sites			0.118
1	14 (46.7)	9 (25.7)	
> 1	16 (53.3)	26 (74.3)	
No. of prior lines for metastatic disease			-
0	5 (16.7)	0 (0.0)	
1	25 (83.3)	15 (42.9)	
> 1	-	20 (57.1)	
Prior drug exposure			0.699
Oxaliplatin	29 (96.7)	35 (100.0)	
Bevacizumab	18 (60.0)	29 (82.9)	
Anti-EGFR	2 (6.7)	4 (11.4)	

ECOG PS: Eastern Cooperative Oncology Group performance status.

Table 2 Summary of the efficacy results

	Irinotecan-naïve, *n* = 30			Prior irinotecan, *n* = 35		
Response rate						
	n	% (ITT)	% (evaluable)	*n*	% (ITT)	% (evaluable)
CR	0	0	0	1	2.8	2.9
PR	13	43.3	50	11	31.4	32.4
SD	10	33.3	38.5	9	25.7	26.5
PD	3	10	11.5	13	37.1	38.2
NE	4	13.3	-	1	2.8	-
ORR	13	43.3	50	12	34.3	35.3
DCR	23	76.7	88.5	21	60	61.8
Survivals						
	median, mo	95%CI		median, mo	95%CI	
PFS	11.3	6.1-29.0		5.7	3.9-10.4	
OS	17.0	13.0-17.3		14.3	12.8-19.5	

RR: Response rate; ITT: Intention-to-treat; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NE: Not evaluable; ORR: Objective response rate; DCR: Disease control rate; PFS: Progression-free survival; OS: Overall survival.

were not evaluated for tumor response due to an early stop for limiting toxicity. ORR was reported in 13 patients [43.3%, intention-to-treat (ITT); 50.0%, evaluable patients] without CR, and DCR was reported in 23 patients (76.7%, ITT; 88.5%, evaluable patients). Three (10.0%) patients had PD at the first tumor evaluation (Table 2).

In the irinotecan pre-exposed population, one patient was not evaluable for tumor response (switch to intra-arterial chemotherapy after 2 treatment cycles). ORR was reported in 12 patients (34.3%, ITT; 35.3%, evaluable patients) including one CR, and DCR was reported in 21 patients (60.0%, ITT; 61.8%, evaluable population). Thirteen (37.1%) patients had PD at the first tumor evaluation (Table 2). Among seven patients refractory to irinotecan, 1 (2.9%) had PR with FOLFIRI3-aflibercept, 2 (5.7%) had stable disease, 3 (8.6%) had PD, and 1 (2.6%) was not evaluable (Table 3).

Survival

The median follow-up was 13.6 mo (95%CI: 9.6-17.7) in the irinotecan-naïve population and 14.2 mo (95%CI: 11.0-21.5) in the pre-exposed population (*P*

Table 3 Contingency table of tumor response with FOLFIRI3-aflibercept according to prior tumor response with irinotecan [*n* = 35, *n* (%)]

		FOLFIRI3-aflibercept				
		CR/PR	SD	PD	NE	All
Prior irinotecan-based regimen	**CR/PR**	5	3	7	0	15 (42.8)
	SD	4	4	2	0	10 (28.6)
	PD	1	2	3	1	7 (20.0)
	NE	2	0	1	0	3 (8.6)
	All	12 (34.3)	9 (25.7)	13 (37.1)	1 (2.8)	**35**

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NE: Not evaluable.

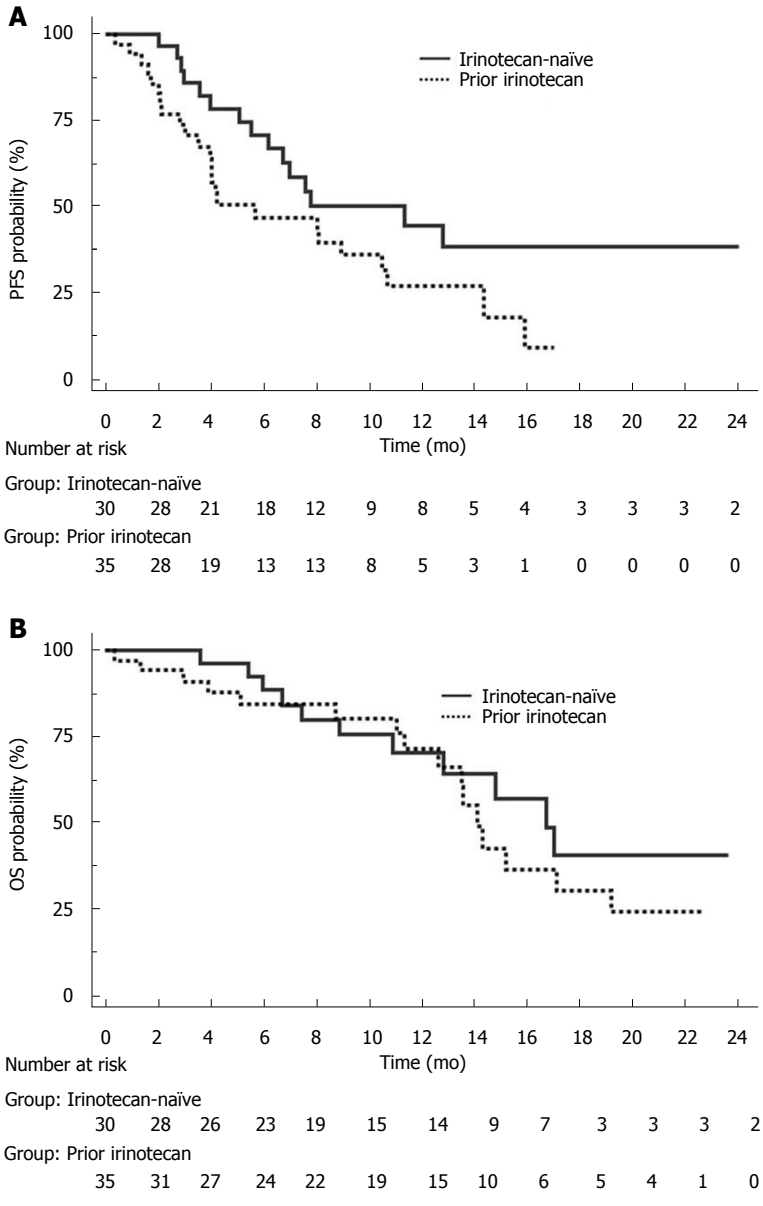

Figure 3 Progression-free survival and overall survival according to prior exposure to irinotecan (*n* = 65). A: Progression-free survival; B: Overall survival.

= 0.692). In the irinotecan-naïve population, median PFS was 11.3 mo (95%CI: 6.1-29.0) and median OS was 17.0 mo (95%CI: 13.0-17.3; Figure 3A). A lower starting dose of irinotecan (< 90 mg/m²) did not impact PFS (P = 0.518) and OS (P = 0.311), but decreased the incidence of severe neutropenia (0.0% *vs* 30.8%, respectively, P = 0.041). In the irinotecan pre-exposed population, median PFS was 5.7 mo (95%CI: 3.9-10.4) and median OS was 14.3 mo (95%CI: 12.8 -19.5; Figure 3B).

Table 4 Selected (≥ 5%) grade 3-4 adverse events (NCI CTCAE version 4.0) *n* (%)

SOC	PT	Irinotecan-naïve	Prior irinotecan	All
Any		17 (56.7)	15 (42.9)	32 (49.2)
Blood	Neutropenia	4 (13.3)	1 (2.9)	5 (7.7)
	Anemia	3 (10.0)	0 (0.0)	3 (4.6)
	Thrombocytopenia	0 (0.0)	0 (0.0)	0 (0.0)
Gastrointestinal	Nausea	2 (6.7)	0 (0.0)	2 (3.1)
	Vomiting	1 (3.3)	0 (0.0)	1 (1.5)
	Mucositis	3 (10.0)	3 (8.6)	6 (9.2)
	Diarrhea	11 (36.7)	9 (25.7)	20 (30.8)
Vascular	Hypertension	2 (6.7)	4 (11.4)	6 (9.2)

SOC: System Organ Class; PT: Preferred term.

Safety

In the irinotecan-naïve cohort, 17 (56.7%) patients experienced grade ≥ 3 toxicity (Table 4). The most common (≥ 5%) grade 3-4 adverse events were diarrhea (*n* = 11, 36.7%), neutropenia (*n* = 4, 13.3%), anemia and mucositis (*n* = 3, 10.0%), and nausea and hypertension (*n* = 2, 6.7%). Any grade hemorrhage was reported in 4 (13.8%) patients, gastrointestinal perforation in 1 (3.3%) patient, and arterial thromboembolic event in 1 (3.3%) patient.

In the irinotecan pre-exposed cohort, 15 (42.9%) patients experienced grade ≥ 3 toxicity (Table 4). The most common (≥ 5%) grade 3-4 adverse events were diarrhea (*n* = 9, 25.7%), hypertension (*n* = 4, 11.4%), and mucositis (*n* = 3, 8.6%).

Salvage surgery

Salvage surgery for metastatic disease was performed in 7 (10.0%) patients (*n* = 4, liver; *n* = 1, lung; *n* = 1, liver and lung; *n* = 1, peritoneum). A complete (R0) resection and liver pathological complete response were observed in all and one patient, respectively.

DISCUSSION

To our knowledge, this is the first report evaluating the FOLFIRI3-aflibercept combination in patients with previously treated mCRC. The response rate, which is a strong indicator of treatment efficacy, was unusually high not only in irinotecan-naïve patients (43%, ITT; 50%, evaluable), but also in irinotecan pre-exposed patients (34%, ITT, 35%, evaluable).

The median 11.3 mo PFS and median 17.0 mo OS in the irinotecan-naïve population receiving FOLFIRI3-aflibercept as second-line therapy compared favorably to the FOLFIRI-aflibercept combination in the pivotal phase Ⅲ VELOUR study (response rate 19.8%, median 7.2 mo PFS, median 13.2 mo OS) and the FOLFIRI3 regimen without targeted agent (response rate 17%-23%, median 4-7 mo PFS, median 9-12 mo OS)[4,9,10,14].

In the irinotecan pre-exposed population, patients received FOLFIRI3-aflibercept as salvage therapy. Yet, median PFS and OS were 5.7 mo and 14.3 mo, respectively, and were comparable to figures observed in second-line trials[8,15].

A high portion (27%) of patients had to stop the FOLFIRI3-aflibercept combination because of limiting toxicity, mainly diarrhea. Its frequency (38%) was twice as common as in the VELOUR study (19%), but in the same range as in previous studies using FOLFIRI3. It has been demonstrated that severe diarrhea induced by aflibercept is due to microscopic colitis, which can be managed successfully using oral budesonide and/or mesalamine treatment[16,17]. Placental growth factor (PlGF) could play a role in the occurrence of diarrhea. The absence of PlGF blocks dextransodium sulfate-induced colonic mucosal angiogenesis and increases mucosal hypoxia[18,19]. Knockout of PlGF aggravates disease course in acute colitis[20]. Neutropenia and stomatitis were at a lower incidence than in the FOLFIRI-aflibercept arm of the pivotal VELOUR study (13.3% *vs* 36.7%, neutropenia; 10.0% *vs* 13.8%, stomatitis), which can be explained by deletion of the 5-FU bolus in the FOLFIRI3 regimen and use of G-CSF in 49% of patients. In the irinotecan-naïve population, a lower starting dose of irinotecan (< 90 mg/m²) did not impact treatment efficacy, but decreased the incidence of severe neutropenia (0.0% *vs* 30.8%, *P* = 0.041).

The conversion to surgery of metastasis in second-line is another key finding that could modify the strategy in patients suitable for salvage surgery in case of response (sequential doublets versus triplets). The main limitation of this study is the retrospective design with a low number of patients. In conclusion, the combination of aflibercept and FOLFIRI3 in our study shows the encouraging efficacy results with high response rates and longer survivals in patients with previously treated mCRC, whatever the prior exposition to irinotecan. A randomized trial is warranted to compare FOLFIRI-aflibercept to FOLFIRI3-aflibercept.

ARTICLE HIGHLIGHTS

Research background

FOLFIRI3 is the second-line regimen of choice in patients with previously treated mCRC in some centers. Adding bevacizumab to FOLFIRI3 has shown

promising efficacy results in two prior retrospective trials. The addition of aflibercept to FOLFIRI in patients with pretreated mCRC increased response rate from 11% to 20% and improved median PFS from 4.7 to 6.9 mo.

Research motivation

The phase III VELOUR and non-randomized FOLFIRI3 studies results provide a backbone for our study.

Research objectives

The main objective of the study is to evaluate the safety and efficacy of the FOLFIRI3-aflibercept combination as second or later-line therapy in patients with mCRC.

Research methods

Patients with previously treated mCRC were given the aflibercept-FOLFIRI3 combination and were divided into "irinotecan-naive" population including patients with no more than one prior line of treatment for metastatic disease, and the "irinotecan pre-exposed" population including patients for whom the number of prior treatment lines for metastatic disease was not restricted. The primary endpoint was overall response rate (ORR). Secondary endpoints were disease control rate, progression-free survival (PFS), overall survival (OS), and safety. Toxicity was evaluated according to the United States National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03.

Research results

Minimally modified FOLFIRI has improvement dramatically the efficacy of the FOLFIRI3-aflibercept combination with high response rates (43% in irinotecan-naïve patients and 34% in irinotecan pre-exposed patients) and survivals (median PFS: 11.3 mo, OS: 17.0 mo and PFS: 5.7 mo and OS: 14.3 mo, respectively) in patients with previously treated mCRC, whatever prior use of irinotecan.

Research conclusions

The combination of aflibercept and FOLFIRI3 shows encouraging efficacy results in patients with previously treated mCRC.

Research perspectives

A prospective randomized trial is planned to compare FOLFIRI-aflibercept to FOLFIRI3-aflibercept.

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EDITORIAL

Risk of colorectal cancer in patients with diverticular disease

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Abstract

Colorectal cancer constitutes an important burden on the healthcare system. Screening at-risk populations to reduce colorectal cancer-related morbidity and mortality has become part of good clinical practice. However, recommendations regarding subgroups of patients with diverticular disease are subject to controversy.

Herein, we review the most recent literature regarding the prevalence of colorectal cancer in patients with diverticular disease, diverticulitis and uncomplicated diverticulitis.

The recent literature does not identify diverticular disease as a long-term risk factor for colorectal cancer. However, the risk of colorectal cancer is increased in the short-term period after hospitalization related to diverticular disease. According to a recent systematic review and meta-analysis, the prevalence of colorectal cancer is 1.6% in patients with acute diverticulitis who underwent colonoscopy. The risk of having colorectal cancer after an episode of acute diverticulitis is 44-fold higher than that of an age- and gender-adjusted reference population. Despite lower among patients with uncomplicated episode, the risk of colorectal cancer remains 40-fold higher in that subpopulation than that in the reference population.

To conclude, the recent literature describes an increased risk of colorectal cancer among patients with acute diverticulitis compared to the reference population. Colonoscopy is therefore recommended in patients with diverticulitis to exclude colorectal cancer.

Key words: Diverticulosis; Diverticulitis; Colonoscopy; Screening; Tumor; Risk factor

Core tip: The present manuscript reviews the current literature reporting on the prevalence of colorectal cancer

in patients with diverticular disease. The prevalence of colorectal cancer among subgroups of patients with diverticulitis and uncomplicated diverticulitis is discussed, with the objective of providing recommendations for colorectal cancer screening.

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INTRODUCTION

In 2018, colorectal cancer (CRC) will represent 8.1% of estimated cancer cases and will account for 15.6% of estimated cancer deaths in the United States[1]. At-risk populations are screened to reduce CRC-related morbidity and mortality and to reduce healthcare costs.

CRC IN DIVERTICULAR DISEASE

Some authors consider diverticular disease a risk factor for CRC. For instance, Stefansson *et al*[2] reported a standardized incidence ratio (SIR) of 5.8 (95%CI: 4.6-7.3) over a 2-year period for CRC in patients with diverticular disease compared to controls without diverticular disease. That risk persisted for 10 years for patients with left-sided disease. Later, Granlund *et al*[3] performed a case-control population-based study including 41037 CRC patients matched with 82074 controls. They identified that hospitalization for diverticular disease in the previous 6 mo was a risk factor for CRC with an odd ratio (OR) ranging between 22.75 (95%CI: 14.06-36.82) and 31.49 (95%CI: 19.00-52.21). Thereafter, that risk was not significantly different from that of the population without diverticular disease[3]. More recently, Huang *et al*[4] followed 41359 Asian patients with diverticular disease and 165436 age- and gender-matched patients without diverticular disease. They found annual incidences of CRC of 0.62% and 0.15%, respectively, in these populations. Among patients with diverticular disease, the hazard ratio (HR) for CRC was 5.08 (95%CI: 4.19-4.91). After excluding the first year of follow-up, the adjusted HR dropped to 0.98 (95%CI: 0.85-1.13)[4]. Therefore, both of these large-scale population-based studies, which included 2 geographically distinct populations, were distinct from the findings of Stefansson *et al*[2] by revealing an increased risk of CRC only during the short period of follow-up after hospitalization/diagnosis for diverticular disease. Although the identification of uncomplicated diverticular disease as a risk factor for CRC remains a subject of controversy[5-8], we note that the usual reasons for diverticular disease-related hospitalization are diverticular bleeding and diverticulitis.

CRC IN DIVERTICULITIS

Diverticulitis accounts for more than 200000 hospital admissions per year in the United States[9]. The prevalence of CRC in patients suffering from diverticulitis has remained poorly documented for decades, but has recently come under the spotlight. In 2013, Sharma *et al*[10] performed a systematic review and meta-analysis of colonoscopy results from 1970 patients with diverticulitis treated with nonsurgical management. They described a prevalence of CRC of 1.6% in that population[10]. This prevalence was, however, not compared to the prevalence of a reference population. Moreover, selection bias for colonoscopy could not be excluded. To this end, our group compared the incidence of CRC in 506 patients with computed tomography-proven diverticulitis to that of an age- and gender-matched reference population. We found a one-year incidence of CRC of 1.9% in patients with diverticulitis, an incidence that was 44-fold higher (95%CI: 18.58-75.96) than that in the reference population[11]. One year later, Grahnat *et al*[12] confirmed these results by describing a SIR of 20 (95%CI: 10.2-35.7) for sigmoid cancer in 890 patients with CRC compared to their reference population. We also note that Sharma *et al*[10] did not include recent population-based large-scale studies in their systematic review and meta-analysis. The population-based study of Huang *et al*[4] reported an annual incidence of CRC of 0.49% in the subgroup of 31216 Asian patients with diverticulitis, whereas Mortensen *et al*[13] reported that 977 of the 40496 patients hospitalized for diverticulitis were diagnosed with CRC within a year, for an annual incidence of 2.41%. In the latest study, diverticulitis constituted a risk factor for CRC, with an OR of 2.20 (95%CI: 2.08-2.32)[13]. That increased risk was not identified in the Asian study [HR of 1.08 (95%CI: 0.91-1.28) for CRC among the subgroup of patients with diverticulitis][4].

Nevertheless, it seems reasonable to consider that the risk of CRC is increased in the first year after diagnosis of diverticulitis, especially in Western societies. Fifty-seven percent of CRC cases in the study by Mortensen *et al*[13] were diagnosed during that period. The risk of CRC might be increased due to difficulties in distinguishing CRC from diverticulitis based on computed tomography, resulting in an initial misdiagnosis, despite the contribution of information bias that cannot be excluded. Indeed, Mortensen *et al*[13] reported an increased rate of colonoscopy in the diverticulitis population (57%) when compared to the reference population (10%). Taken together, this evidence led professional societies to recommend performing colonoscopy after an episode of diverticulitis[14,15].

Furthermore, we note that some authors reported a low prevalence of CRC among patients with uncomplicated diverticulitis and suggested exempting them from colonoscopy[16-19]. According to the systematic review and meta-analysis by Sharma *et al*[10], the pre-

valence of CRC in these patients was 0.7%. However, in our study, we described the prevalence of CRC in patients with uncomplicated diverticulitis to be 40-fold higher than that in the reference population[11]. Unfortunately, large-scale population-based studies have not distinguished uncomplicated diverticulitis from complicated diverticulitis. Therefore, evidence is lacking to provide recommendations for this subgroup of patients.

Some studies did not identify cases of CRC among young Asian or Hispanic patients with diverticulitis[20,21]. Further, in their large-scale study, Huang *et al*[4] described a higher HR for CRC among old patients than among young patients. Moreover, in our study[11] and the study by Grahnat *et al*[12], all CRC patients were older than 70 years. Additionally, the increased risk documented by our study was age-adjusted, confirming that the increased risk observed among diverticulitis patients was not only due to a global increase in CRC prevalence among older patients. Therefore, the prevalence of CRC seems to be lower among young patients than among old patients, but this remains to be confirmed by large-scale studies.

CONCLUSION

To conclude, the recent literature describes an increased risk of CRC among patients with acute diverticulitis compared to the reference age-matched population. Colonoscopy is therefore recommended in patients with diverticulitis to exclude CRC. However, the evidence is not convincing enough to determine whether subgroups of patients - those with an uncomplicated episode of diverticulitis and/or young patients - might be exempted from colonoscopy. Future studies are needed to determine the prevalence of CRC in patients with uncomplicated diverticulitis, looking more specifically at age groups and providing large-scale population-based comparisons with reference populations. A new systematic review and meta-analysis aggregating the most recent studies, including the latest population-based studies, is necessary. In addition, to our knowledge, no study provides recommendations for patients with a recent colonoscopy to exclude interval colorectal cancer. These questions are of critical importance to better identify at-risk patients in order to provide the best benefit/risk ratio for CRC screening in patients with diverticulitis.

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ORIGINAL ARTICLE

Basic Study

Lymphocyte subsets predictive value and possible involvement of human papilloma virus infection on breast cancer molecular subtypes

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Abstract

AIM

To detect human papilloma virus (HPV) presence and to characterize cellular immune response in breast cancer patients.

METHODS

A total of 74 women were included, of which 48 samples were from patients diagnosed with breast cancer and 26 patients with benign pathology of the breast. Molecular subtype classification was performed based on the immunohistochemical reports of the tumor piece. HPV genome detection and genotyping from fresh breast biopsies was performed using the INNO-LIPA HPV Genotyping Extra test (Innogenetics, Ghent, Belgium). CD3+, CD4+, CD8+ and natural killer (NK)+ cells levels from peripheral blood samples from patients with breast cancer and benign pathology were measured by flow cytometry.

RESULTS

Luminal A was the most frequent breast cancer molecular subtype (33.33%). HPV was detected in 25%

of the breast cancer patients, and genotype 18 was the most frequent in the studied population. The mean of CD3+, CD4+ and CD8+ subpopulations were decreased in patients with breast cancer, in relation to those with benign pathology, with a statistically significant difference in CD8+ values (P = 0.048). The mean of NK+ cells was increased in the benign pathology group. The average level of CD3+, CD4+, CD8+ and NK+ cells decreased as the disease progressed. HER2+ and Luminal B HER2+ tumors had the lowest counts of cell subsets. HPV breast cancer patients had elevated counts of cellular subsets.

CONCLUSION

Determining level changes in cellular subsets in breast cancer patients is a useful tool to evaluate treatment response.

Key words: Breast cancer; Human papilloma virus; Molecular subtypes; Immune response; T lymphocytes; NK cells

Core tip: This work detected the presence of the human papilloma virus (HPV) genome in patients with breast cancer and measured the levels of cellular subsets as predictor factors. The viral genome was found in 25% of breast cancer cases, with the high-risk 18 genotype the most frequent. Luminal A tumors represented 33.33% of the sample. The average level of CD3+, CD4+, CD8+ and NK+ cells was decreased in cancer patients compared to the benign pathology group, while the reverse effect was observed in HPV positive patients.

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INTRODUCTION

With more than 408200 new cases and over 92000 deaths, breast cancer is the first cancer in the Americas, in terms of new cases, and the second in terms of women cancer deaths[1]. In Venezuela, according to the Ministry of Popular Power for Health in 2012, breast cancer ranked first in cancer incidence with 5063 new cases and was the first cause of cancer death in the female population. This was followed by cervix cancer, with 2067 deaths, representing 22.88% of new diagnoses due to cancer and 18.25% of deaths caused by this pathology[2].

The 13th Conference of St Gallen in 2013 stablished that it was unnecessary to perform genetic tests on each patient to classify molecular subtypes, which was proposed by Perou *et al*[3] in 2000, since histopathological results were comparable. The use of molecular diagnosis was necessary only in atypical behavior cases[4]. Luminal A breast cancer cases are the most frequent and have good prognosis and response to hormonal treatment. Luminal B HER2- and Luminal B HER2+ breast cancers represent between 15% and 20% of breast tumors, and their response to hormonal therapy is less. HER2+ cancer accounts for 15%-20% of breast cancer subtypes, showing more aggressive biological and clinical behavior, with increased sensitivity to chemotherapy. The triple negative (TN) subtype represents from 8% to 37% of all breast cancers. These tumors are infiltrating ductal types characterized by a solid growth pattern, aggressive clinical behavior and high rate of metastasis to brain and lungs[4,5]. Molecular subtypes are currently used as predictive factors among breast cancer patients[6].

Nearly 50% of newly diagnosed breast cancer cases are related to hormonal factors; only 5% to 10% are related to genetic factors, although it is known that these greatly increase the risk of developing the disease. Several studies have determined the physiological, environmental and lifestyle factors related to the incidence of breast cancer, some of which are modifiable through preventive interventions[1].

The non-genetic factors include: Age of diagnosis after 65 years, which is the most important risk factor, early menarche, menopause after 55-years-old, first live birth after 30-years-old, nulliparity, breast biopsies history, diagnosis of atypical hyperplasia by breast biopsy, obesity, alcohol consumption, hormone replacement therapy and excessive exposure to radiation. Other possible risk factors include high-in-fats and low-in-fiber diet, and little exercise[7].

Approximately 18% of human cancers are caused by infectious factors, and it is recognized that breast cancer is strongly related to environmental factors, such as viruses, diet, radiation, among others[8]. Human papilloma virus (HPV) infection distribution reports in breast cancer are controversial. In 2015, an overall prevalence between 0% and 86% was reported, with an average of 30.30%, with the highest frequency reported in Oceania at 44.30%. South America presented 14.60% of HPV infection, exceeding 10.70% of North America[9].

HPV types are classified as low-risk and high-risk types based on the hability to induce carcinogenesis. HPV 6 and HPV 11 are low-risk subtypes and cause more than 90% of genital warts. High-risk HPV subtypes such as HPV 16, 18, 31, 33, 45 and 52, cause squamous intraepithelial lesions that can progress to invasive squamous cell carcinomas[10]. This double strand DNA virus expresses *E6* and *E7* oncogenes, which interact with p53 and pRB proteins, respectively, promoting the development of neoplasias due to uncon-

trolled cell cycle activation and inhibition of apoptosis[11]. The high-risk HPV genome could be integrated into the host genome during the carcinogenesis process, causing the loss of *E6* and *E7* transcription regulation by the interruption of *E2* gene open reading frame[12].

Besides clinical and treatment parameters, the host immune response might influence the prognosis of cancer patients after standard treatment[13]. Breast tumor progression is due to a systematic action, affecting the host's physiological processes and triggering responses in peripheral blood cells[14]. It is known that CD4+ and CD8+ T cells are required for an effective anti-tumor immune response. CD4+ T cells are critical for priming tumor-specific CD8+ T cells and for the secondary expansion and memory of CD8+ T cells as well[15]. CD8+ T cells have been shown to be mediators of antitumor immunity and act directly over tumor cells. Recent studies have suggested its clinical importance, reporting that an increase of CD8+ T cells correlates with increased survival in large cohorts of various human cancer patients[13].

The natural killer (NK)+ cells have the ability to produce lysis in tumor cells and cells infected with intracellular viruses or parasites, through cytotoxic mechanisms mediated by preformed molecules, such as perforins and granzymes. They also have the ability to secrete cytokines such as interferon types Ⅰ and Ⅱ[16]. NK cells appears to protect against tumor development and progression[17].

Evaluation of circulating T lymphocytes, B lymphocytes and NK+ cells may be one of the beneficial ways to understand immune response, assist in clinical diagnosis, provide evidence of pathogenesis, course, and prognosis of disease and determine clinical treatment[18]. This work aimed to evaluate the possible role of cellular subsets as predictive factors and the association of HPV in patients with breast cancer, according to the molecular subtypes, being the first study of its kind reported in Venezuela.

MATERIALS AND METHODS

Study population

We evaluated prospectively from February 2011 to October 2013 patients attending at the Breast Pathology Unit, in the Gynecology Department, from the University Hospital of Caracas. A total of 74 women were included, of which 48 samples were from patients diagnosed with breast cancer and 26 patients diagnosed with benign pathology of the breast. Patients were invited to participate in the study, with prior information on the design and protocol. Each one signed an informed consent, approved by the hospital Bioethics Committee.

Patients with other tumors or immune system-related disease were excluded. None of the patients had received any form of medical or surgical therapy such as radiotherapy, chemotherapy or treatment with steroids or immunosuppressants prior to the investigation.

Breast cancer molecular subtypes classification

Determination of breast cancer molecular subtype was performed based on the immunohistochemical reports of the tumor piece, which were obtained from the clinical histories of each patient. According to markers expression, tumors were classified as: (1) Luminal A: RE+; RP+ (≥ 20%); Ki67 (< 14%), HER2-; (2) Luminal B HER2-: RE+; RP (< 20%); Ki67 (≥ 14%), HER2-; (3) Luminal B HER2+: RE+; RP indifferent; Ki67 (≥ 14%); HER2+; (4) HER2+: RE-, RP-, Ki67 (≥ 14%); HER2+; and (5) TN: RE-, RP-, Ki67 (≥ 14%); HER2-[5].

Tissue samples

Fresh biopsies were obtained from tumors of patients who underwent surgery. Biopsies were frozen at -70 ℃ for molecular analysis.

DNA extraction

To perform DNA extraction from fresh breast biopsies, QIAmp DNA mini kit (250) was used (Qiagen, Hilden, Germany), following the manufacturer's instructions.

HPV detection and genotyping

HPV genome detection and genotyping from fresh breast biopsies was performed using the INNO-LIPA HPV Genotyping Extra test (Innogenetics, Ghent, Belgium), following the manufacturer's instructions. Based on the reverse hybridization principle, 28 different HPV genotypes were identified by detecting specific sequences in the L1 region. The assay uses the proven SPF10 primer set for highly sensitive amplification of the most clinically relevant HPV genotypes.

Blood samples

Before surgery, 5 mL of venous blood was obtained from each patient. The samples were drawn into heparinized tubes and transported to Oncology and Hematology Institute for processing.

Cellular subsets

Cellular subsets quantification was performed by flow cytometry. Fifty microliters of whole blood was taken, and 5 μL of monoclonal antibody was added. The sample was briefly vortexed and incubated for 15 min at room temperature in the dark. Eight hundred microliters of BD 1X lysis solution was added and incubated for 10 min at room temperature in the dark. The sample was centrifuged for 5 min at high speed, and the supernatant was discarded. The pellet was resuspended with 300 μL of Facs Flow and vortexed. Finally, the tube was acquired in the BD Facs Canto Ⅱ cytometer, of six colors (configuration 4-2), with the BD FACS Diva 6.2.2 application.

Cell surface marker analysis was performed using CD4-FTIC/CD8-PE/CD3-PC5 (Beckman Coulter) for CD3+, CD4+ and CD8+ T cells and CD3-APC, CD16-FITC, CD56-PE for NK+ cells (Beckman Coulter, Pasadena, CA, United States). Absolute cell counts were calculated by multiplying the cell subset percentage by the total lymphocyte concentration present in peripheral blood.

Table 1 Demographic characteristics for the breast cancer and benign pathology groups

	Breast cancer (n = 48)	Benign pathology (n = 26)	P value[1]
Mean age (yr)	57.79 ± 14.13 (range: 31-85)	32.54 ± 11.63 (range: 14-60)	0.000
Menarche (yr)	12.42 ± 1.67 (range: 9-16)	12.62 ± 1.69 (range: 9-17)	0.763
Term pregnancy	2.63 ± 2.27 (range: 0-11)	1.50 ± 1.55 (range: 0-5)	0.066
Sexual partners	1.98 ± 1.15 (range: 0-5)	1.76 ± 1.09 (range: 1-3)	0.750
Oral contraceptives	47.92%	65.38%	0.352
Tobacco	50.00%	11.53%	0.002
Alcohol	47.92%	46.15%	0.678
Breast cancer family history	37.50%	19.23%	0.199

[1]Breast cancer group *vs* benign pathology group.

Table 2 Mean absolute values for the cellular subsets in the breast cancer and benign pathology groups

	Breast cancer (cel/mm^3)	Benign pathology (cel/mm^3)	P value[1]
TCD3+	1517.95 ± 666.23	1861.68 ± 760.52	0.102
TCD4+	888.73 ± 445.18	974.01 ± 390.17	0.504
TCD8+	551.34 ± 284.35	764.54 ± 431.10	0.048
CD4+/CD8+	1.94 (range: 0.59-6.33)	1.57 (range: 0.64-4.36)	0.271

[1]Breast cancer group *vs* benign pathology group.

Statistical analysis

Measures of central tendency and dispersion were used for continuous variables; frequency analysis and contingency tables were used for discrete variables. Analysis of variance between groups, *t*-Student Parametric Test for two independent samples and non-parametric Mann-Whitney *U* test were used to perform hypothesis contrast. Significance level was fixed at $P < 0.05$ (Statistical Software: SPSS in its Version 20 in Spanish; IBM Corp., Armonk, NY, United States).

RESULTS

The study included 74 patients, with menarche age between 9 and 17 years, with 60.81% of menarche age between 12 and 14 years. The average sexual partners number was between 0 and 5, and 74.32% of the patients had 0 to 2 sexual partners. Regarding pregnancy at term, the cases registered were between 0 to 11 per patient, with the highest proportion between 0 and 2 deliveries (67.57%). Overall, 47.29% had an alcoholic habit, 36.49% had a tobacco habit, 54.05% of the patients used oral contraceptives, and 59.46% had a family history of cancer.

Table 1 shows demographic characteristics for the study groups, where 50% of breast cancer patients reported smoking and alcohol habit, and about 40% reported a family history of breast cancer. Mean age and tobacco habit showed a statistically significant difference between breast cancer patients and benign pathology patients.

Of the breast cancer samples, 50.00% corresponded to stage Ⅱ, followed by stage Ⅰ (20.83%), stage Ⅲ (14.58%), stage 0 (8.33%) and stage Ⅳ (6.25%). Regarding the histopathological diagnosis, the breast cancer tumors were infiltrating ductal carcinoma (79.16%), ductal carcinoma *in situ* (8.33%), lobullar carcinoma (8.33%) and mucinous carcinoma (4.17%). Luminal A was the most frequent breast cancer molecular subtype (33.33%), followed by Luminal B HER2- (29.17%), Luminal B HER2+ (14.58%), TN (12.50%) and HER2+ (10.42%).

HPV was detected in 25.00% of the breast cancer patients, and genotype 18 was the most frequent in the studied group, followed by types 16, 6 and 31. 41.67% of the patients presented mixed infections, and 75.00% showed infection with high oncogenic risk genotypes in breast fresh tissue biopsies. In the benign pathology group, HPV genome was detected in 7.69%, finding genotypes 18 and 33 of high oncogenic risk, in single infections. Fifty percent of the HPV positive breast tumors were Luminal A, followed by the HER2+ type, with 25%. Luminal B HER2- and TN types represent 16.67% and 8.33%, respectively, among HPV positive breast tumors ($P = 0.027$).

Table 2 shows the mean absolute values for each cellular subset of the breast cancer patients and benign pathology patients. Breast cancer patients showed a decrease in mean values compared to those of benign pathology, with statistically significant difference in CD8+ count between both study groups. The mean of each cellular subset decreased as the stage of the disease increased ($P > 0.005$). Regarding breast cancer molecular subtypes, the HER2+ tumors had the lowest CD4+ and CD8+ values (Table 3).

The NK+ cells counts were elevated in the benign pathology group, with 1217.04 ± 778.69 cel/mm^3 (range: 48.30-3193.18), in comparison with breast cancer patients (1053.79 ± 690.56 cel/mm^3 (range: 187.55-3675.00)) ($P = 0.651$). A decrease in the mean

Table 3 Correlations between the breast cancer molecular subtypes and the cellular subtypes

	CD4+ (cel/mm^3)	CD8+ (cel/mm^3)	NK+ (cel/mm^3)
Luminal A	916 ± 358.81	645 ± 299.91	1064 ± 455.61
Luminal B HER2-	877 ± 494.16	544 ± 273.04	1120 ± 818.37
Luminal B HER2+	914 ± 318.73	491 ± 208.07	926 ± 500.35
HER2+	680 ± 444.86	426 ± 319.13	834 ± 836.75
TN	992 ± 568.02	483 ± 214.90	1183 ± 837.52
P value[1]	0.842	0.527	0.912

[1]Between breast cancer molecular subtypes. TN: Triple negative.

Table 4 Correlations between the breast cancer HPV status and the cellular subtypes

	HPV+ (*n* = 14)	HPV- (*n* = 34)	*P* value[1]
CD3+ (cel/mm^3)	1832.12 ± 537.08	1399.56 ± 665.69	0.563
CD4+ (cel/mm^3)	1139.92 ± 416.84	796.56 ± 416.84	0.091
CD8+ (cel/mm^3)	630.66 ± 271.00	518.58 ± 281.53	0.748
CD4+/CD8+	2.11 (range: 0.71-4.17)	1.88 (range: 0.59-6.33)	0.029
NK+ (cel/mm^3)	1350.21 ± 736.79	950.47 ± 634.35	0.082

[1]HPV+ *vs* HPV-. HPV: Human papilloma virus; NK: Natural killer.

of NK+ cells was observed as the stage of the disease increased (P = 0.0827). Regarding breast cancer molecular subtypes, HER2+ tumors and Luminal B HER2+ had the lowest values of NK+, while TN tumors had the highest values (Table 3).

As shown in Table 4, HPV+ breast cancer tumors had elevated cellular subtype counts compared to HPV- tumors. A decrease in the mean of NK+ cells was observed as the disease stage increased in the HPV+ tumors.

DISCUSSION

Breast cancer is the most frequently diagnosed neoplastic disease during menopause, leading to a significant reduction of women's quality of life[19]. In developing countries, the disease emerges as a serious public health problem due to the high economic and social costs associated with its care[20].

Breast tumors have a very wide phenotypic diversity, which is accompanied by a large variability in gene expression patterns[3]. Gene signatures are used as predictors of therapy response, and protein gene products have direct roles in the biology and clinical behavior of cancer cells and are potential targets to develop novel therapies[5].

According to different reports, Luminal A represents 50% to 60% of breast cancer cases, while Luminal B, both HER2- and HER2+, represent 15% to 20% of cases. HER2+ tumors are detected between 15% and 20%, and TN tumors are found between 8% and 37% of breast tumors[4,5]. In this study, Luminal A was the most frequent group, followed by Luminal B HER2-, Luminal B HER2+, TN and HER2+.

In Venezuela, few studies have characterized breast cancer molecular subtypes. Uribe *et al*[21] reported Luminal A as the more frequent subtype (60.94%), followed by TN (28.75%). López *et al*[22] found that of 110 patients evaluated, 40.0% presented with Luminal B HER2- tumors, followed by Luminal A (20.91%).

Identification of genetic and risk factors, such as environmental and hormonal factors, have increasing value and play an important role in breast cancer prevention[23]. These risk factors increase neoplastic process development probability and depend on the exposure time or individual genetic predisposition. Therefore, they can influence cancer development, even if they do not directly cause the disease[24].

Regarding non-genetic factors, in our study, 35.42% of patients had a diagnosis age greater than 65 years, 58.33% had menarche before 12 years, 14.58% were nulliparous at the time of the study, and 47.92% indicated alcohol consumption. However, many of the patients who developed breast cancer did not have any identifiable risk factor.

Epidemiological studies gave a first indication of an association between viral agents and specific human cancers. Infectious factors are responsible for approximately 18% of human cancers, and it is well accepted that human breast cancer is highly associated with environmental factors. Among many microorganisms studied, viral infections have been suggested to play a role in cancer development, especially those cancers caused by HPV[8].

In this study, HPV was detected in 25.00% of the samples. High oncogenic risk genotypes were the most common, with a higher prevalence of type 18 over 16, unlike cases of benign pathology, where the virus detection reached 4.76%. Similar reports indicate frequencies between 15.0% and 29.4% for HPV positivity[25-29], and Aguayo *et al*[30] reported the lowest of this group of studies, with 8.7% in South America. As

for genotypes found, all studies found HPV type 16 was the most frequent in single infections or mixed infections with genotype 18.

However, other studies performed viral detection by polymerase chain reaction and genotyping by sequencing and reported a greater presence of type 18. In 2005, Kan *et al*[31] showed 48% positivity for HPV in breast cancer biopsies, with 100% for genotype 18. In addition, Heng *et al*[32], Antonsson *et al*[33], Glenn *et al*[34] and Lawson *et al*[35] found genotype 18 in greater proportion in breast cancer tissue. Since adenocarcinoma constitutes the majority of histological breast carcinoma types, it is understandable that HPV 18 was similar or even a little higher than HPV 16 in these studies. Hence, the distribution of high-risk HPV type for breast carcinoma is probably different than that for cervical cancer[36].

To our knowledge, there are no reports about the correlation between HPV presence and breast cancer molecular subtypes, so our research is the first study to demonstrate this association. Piana *et al*[29] correlated HPV presence with TN tumors and showed a viral detection of 15%. However, the authors used paraffin embedded biopsies and did not discriminate between all the molecular subtypes.

More studies are necessary to evaluate the correlations between HPV presence and breast cancer molecular subtypes. With this knowledge, we could determine if the presence of HPV is associated with better prognosis tumors that are more responsive to chemotherapy treatments, as occurs in HPV positive oropharyngeal squamous cell carcinoma (OPSCC). Multiple studies have confirmed that HPV positive OPSCC shows a better response to chemotherapy and radiation, independently of treatment scheme[37].

Recently, several studies focused on DNA deaminase APOBEC3B (A3B), a source of uracil dependent genomic mutations that is associated with mutagenesis in multiple human cancers, including cancers in breast, head and neck, cervix, bladder, lung, ovary and other tissues. This enzyme belongs to a protein family that has broad and overlapping functions in innate immunity *via* the restriction of viruses, transposons and other foreign DNA elements[38]. Therefore, some authors have suggested a possible role for viral infections, such as HPV and EBV, in the regulation A3B gene expression in some cases of breast cancer[38,39].

A3B expression levels are low in most of normal tissues. The E6 HPV oncoprotein offers the first contact in viral infection and A3B-mediated mutagenesis. In one model, high-risk HPV E6 protein inactivates p53, causing the elimination of A3B gene transcription[38] for cervix cancer, head and neck cancer[40], and HPV positive breast cancer[39]. Given the roles of the proteins p53, A3B and E6, when levels of DNA damage and mutations are raised, answers to these damages and apoptosis are prevented.

Regarding the immune response, previous studies have shown a decrease in T lymphocytes proliferation, low CD3+ and CD4+ count, an increase in CD8+ count and a decrease in CD4+/CD8+ ratio. Other studies have reported a gradual decrease in the CD4+/CD8+ ratio, proportional to the progression of breast cancer[18]. Currently, data about the cytotoxicity of NK cells and blood levels are contradictory, and there is a lack of information regarding the tumor microenvironment in patients with breast cancer[41].

An important strength of this work was the inclusion of breast cancer patients samples, which were taken prior to surgical, systemic and radiant treatment. This allowed us to evaluate the differences in the immunological status of the patients without the influence of treatment.

In the breast cancer patients group, there was a decrease in the concentration of TCD4+ and TCD8+ lymphocytes compared to the benign pathology group, while the CD4+/CD8+ ratio was higher in the breast cancer. TCD8+ concentration variation showed a statistically significant difference (P = 0.048) in respect to the benign pathology group, representing a possible predictive marker. Regarding the behavior of lymphocytes in relation to breast cancer staging, the CD4+/CD8+ ratio decreased as the stage of the disease increased, which is consistent with Jia *et al*[18], who reported a rate decrease in advanced breast cancer.

The average absolute values for NK+ cells in the breast cancer patients group was less than those of the benign pathology group. Verma *et al*[42] also reported a lower NK+ cell count in breast cancer patients. They evaluated the variation of these values during and after treatment and found that neoadjuvant therapy increased NK+ cell values above that reported in the healthy group. It has been reported that chemotherapy, by damaging or stressing cells, promotes the release of various signals that activate dendritic and NK+ cells and induces the release of pro-inflammatory cytokines[43].

It is known that functional capacity of immune cells decline with aging. Diminished phagocytic capacity of dendritic cells leads to impaired antigen presentation and activation of the adaptive immune system. In addition, thymus involution decreases the production of naïve T cells, and memory T cells accumulate, diminishing the T-cell repertoire[44,45]. Postmenopausal women exhibit a reduced number of total lymphocytes, mainly B and CD4+ cells. Similarly, after surgical menopause, the CD4+/CD8+ ratio and the circulating B cells are decreased, while NK cells are increased[45]. The breast cancer patients evaluated did not show statistically significant differences with respect to cell subsets and age groups (data not shown).

Regarding breast cancer molecular subtypes, we found that the CD4+ count and the CD4+/CD8+ ratio were decreased in the HER2+ tumor group, and that TN tumors showed an increase in the NK+ cell count. Particularly, the HER2+ group and the Luminal B HER2+ group showed a considerable decrease of NK+ with respect to the rest of the molecular subtypes.

Jia *et al*[18] reported a decrease in the CD4+ count

and the CD4+/CD8+ ratio and an increase in CD8+ and NK+ cells in RE-, HER2+, TN tumors, with Ki67 ⩾ 14%, indicating a greater failure of the immunological response in those tumors with aggressive phenotype. Previous studies have shown that estrogen plays an important role in regulating the activation of T lymphocytes, particularly CD4+ and CD8+; suggesting that the absence of estrogen receptors in HER2+ tumors is related to what was observed in this study population and was affecting the concentration of both CD4+ and NK+ cells.

ER- and TN breast tumors have a worse prognosis than ER positive tumors. These findings indicate a greater degree of immune function suppression and anti-tumor activation, which is reflected in the aggressiveness of HER2+ and TN tumors[18]. Kim *et al*[13] revealed that the decreased number of CD8+ T cells was significantly associated with aggressiveness and malignant features of tumors, including lymph node metastasis, higher stage and higher Ki67. Therefore, immunotherapy is one of the most promising approaches for breast cancer therapy. If new research establishes a role for lymphocyte subsets in the etiology of aggressive phenotypes, such as those characterized by being ER-, HER2+, presenting a Ki67 ⩾ 14% and TN subtype, new treatment strategies for these breast cancer types should include immunotherapy.

The cellular subset values were increased in the HPV+ tumors compared to those of HPV- patients, and there was a statistically significant difference with respect to the CD4+/CD8+ ratio (P = 0.029), due to a considerable increase in the CD4+ count of HPV+ patients. Despite the HPV evasion mechanisms of the immune response, about 90% of genital and skin lesions resolve on average in 2 years. Immunohistochemical studies show that regression of cutaneous, oral and genital warts in animals and humans is characterized by a massive local infiltration with CD4+, CD8+ and macrophages into the lesion, and the expression of Th1 cytokines profile. Despite the intense local response, the systemic antigen-specific T cell responses are weak, transient and difficult to measure[46].

Compromised adaptive immunity is the basis for high-risk HPV infection to cervical cancer progression. Different immune cell profiles characterize the different stages of the disease progression in cervical intraepithelial neoplasm and invasive cancer. The high-risk HPV infection changes and modifications induced include the adaptation of the immune system to create a suitable microenvironment for persistent infection and lesion progression[47].

During HPV persistent infection, pro-inflammatory cytokines are not released, and the Langerhans cell and dendritic cell activation and recruitment signals are absent. In fact, cells with viral late gene expression, which may contain high levels of viral proteins, are shed from the surface of the epithelium away from immune surveillance. In general, a failure in developing an effective host immune response correlates with persistent infection and an increased probability of progression toward invasive cancer[11].

Although no previous studies have evaluated the profile of cellular subsets in patients with HPV positive breast tumors, we observed that the virus infected group showed higher values of CD4+, CD8+ and NK+ cells in comparison with negative patients. It is possible that these increases are due to an activation of the immune response to viral infection in the mammary tissue.

It has been described in HPV positive cases of OPSCC that patients show a greater proportion of circulating TCD8+ lymphocytes compared with HPV negative cases and that these lymphocyte levels are better predictors of treatment response than HPV status. Other prognostic markers could include CD4+/CD8+ ratio, the circulating levels of the T cell group, the presence of infiltrating lymphocytes in the tumor microenvironment, the expression of MHC class Ⅰ and the characterization of the immune response by microarray[48].

It is known that CD8+ T cells play a major role in elimination of viral infection, secreting interferon and displaying cytolytic effects mediated by granzyme and perforin. CD4+ T cells also secrete interferon but instead mediate killing primarily by engagement with ligands for death receptors, such as Fas or TNF-related apoptosis-inducing ligand, resulting in caspase-mediated apoptosis[49]. The presence of CD8 T cells in cervical lesions is associated with a favorable prognosis, with their numbers inversely correlating with tumor progression.

On the other hand, infection with high-risk HPV genotypes compromises the activation of NK+ cells. In the case of the cervix, NK+ cells predominate in early stages of infection and in low-grade squamous intraepithelial lesions, whereas in cases of cervical cancer, NK+ activation receptors are considerably diminished. These findings imply a low cytotoxic activity by the NK+, thereby facilitating the progression of the lesion and carcinogenesis[47].

In patients with breast cancer and HPV positive breast tissue, a decrease in the NK+ cell count was observed as the severity of the disease increased, implying a failure in the cytotoxic activity performed by the cells studied. It has been observed that the presence of HPV in mammary tissue modifies the activity of the evaluated cells, at the level of the cellular immune response.

It is well known that the incidence of cervical cancer due to HPV infection is much higher compared to non-genital cancers or oropharyngeal cancers associated with the presence of the virus, so the distribution and application of HPV vaccines for preventing cervical cancer remains as a public health priority. However, by 2008, non-genital and oropharyngeal cancers represented about 80000 new cases of cancer associated with HPV infection worldwide, implying a major health problem[50].

Evidence obtained from clinical trials indicates that

current HPV vaccines can prevent HPV infections in vulva, vagina, anus and mouth as well as pre-cancerous anogenital lesions in women and oral and anogenital infections, and pre-cancerous lesions in men. However, data comparing the efficacy and effectiveness data of cervical infections and high-grade lesions with those types of injuries are limited[50,51].

As yet, the implications of HPV vaccination for prevention of non-cervical cancers have not been fully explored. Some countries have recommended HPV vaccination for young males, based on the hypothesis that vaccination will prevent HPV-associated cancers in men as well as theoretical benefits in preventing HPV transmission to women[50].

Therefore, if HPV vaccines contribute to decrease the rate of non-genital cancers, we could have a group of patients that develop breast cancer due to HPV infection, which could benefit from the use of vaccines available in the international market, which include the bivalent that protects against types 16 and 18, the tetravalent, for types 6, 11, 16 and 18, and the nonavalent, approved in 2014 for types 6, 11, 16, 18, 31, 33, 45, 52 and 58[52].

ARTICLE HIGHLIGHTS

Research background

Breast cancer is the leading cause of death among women, classified in molecular subtypes according to a genetic profile. Approximately 18% of human cancers are caused by infectious factors, and it is recognized that breast cancer is strongly related to environmental factors, such as viruses, diet, radiation, among others. Human papilloma virus (HPV) has been detected in 0% to 86% of breast cancer tumors, and it represents a possible risk factor. The host immune response might influence the prognosis of cancer patients after standard treatment.

Research motivation

It was recently suggested that HPV presence may act as a risk factor in breast cancer development, but it has not been correlated with molecular subtypes. In addition, it is important to evaluate the immune response of breast cancer patients and to be able to suggest some prognostic values that make it possible to offer better patients treatment.

Research objectives

The main objective is to detect HPV presence and to characterize the cellular immune response in breast cancer patients based on the molecular subtypes.

Research methods

The patients inclusion was done prospectively, and the breast cancer molecular classification was made according to the St. Gallen International Breast Cancer Conference. HPV detection and genotyping were performed using HPV INNO-LIPA Genotyping Extra test, and lymphocyte subsets were measured by flow cytometry.

Research results

Luminal A was the most frequent breast cancer molecular subtype (33.33%), and HPV was detected in 25% of the breast cancer patients, with genotype 18 as the most frequent in the studied population. The means of CD3+, CD4+ and CD8+ subsets were decreased in patients with breast cancer, respective to benign pathology, with a statistically significant difference between CD8+ values (P = 0.048). The mean of NK+ cells was increased in the benign pathology group. HER2+ and Luminal B HER2+ tumors had the lowest counts of cell subsets. The HPV breast cancer patients had elevated counts of cellular subsets.

Research conclusions

It can be observed that HPV positive breast cancer tumors have a better prognosis, correlated with Luminal A subtypes, and show a better cellular immune response, specifically in relation with TCD8+ cells counts, suggesting a better response to chemotherapy and radiant treatment, as in the case of HPV positive oropharynx tumors.

Research perspectives

Future effforts should focus on evaluating patients disease-free survival, based on HPV positivity in the tumor tissue. In addition, viral load and HPV genome integration, the identification of HPV variants by sequencing, and the infiltrating lymphocytes in the tumor bed should also be studied. As a final consideration, the experience of working with fresh samples is complex and involves a process of sample collection in the operating room, in addition to the management of bioethical parameters, and our experience will allow for the possibility of obtaining more accurate results.

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ORIGINAL ARTICLE

Case Control Study

Mismatch repair protein expression and intratumoral budding in rectal cancer are associated with an increased pathological complete response to preoperative chemoradiotherapy: A case-control study

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Institutional review board statement: This work was authorized by the institutional review board of the National Cancer Institute of Mexico with the number REV/16/87.

Informed consent statement: A waiver form informed consent was provided due the retrospective nature of the study and data were collected from clinical files and pathology database.

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Abstract

AIM

To determine whether the association of rectal adenocarcinoma with a defective-mismatch repair system (dMMR) was associated with a pathological complete response (pCR) to preoperative chemoradiotherapy.

METHODS
A case-control study was designed with the aim of determining if patients with rectal adenocarcinoma with dMMR had an associated high pCR rate in response to neoadjuvant chemoradiotherapy (nCRT).

RESULTS
Seventy-two cases with pCR were compared against 144 controls without pCR. Across 216 cases, the mean age was 56.8 years, 140 (64.8%) were men, and 63 (29.2%) demonstrated the dMMR system. The pCR was associated with G1 tumors, dMMR, the absence of vascular invasion, and low tumor budding in the pretreatment biopsy. In a multivariant analysis, the factors associated with pCR were dMMR (OR: 2.61; 95%CI: 1.355-5.040, P = 0.004) and a low degree of tumor budding (OR: 2.52; 95%CI: 1.366-4.894, P = 0.025).

CONCLUSION
We found an independent association between dMMR and a low rate of tumor budding, with a higher rate of pCR, in the basal biopsies of patients with rectal carcinoma subjected to nCRT.

Key words: Rectal cancer; Chemoradiotherapy; Tumor budding; Mismatch repair; Pathological complete response

Core tip: Defective Mismatch repair (dMMR) and a low number of buds in the pretreatment biopsy were independently associated with a high rate of the pathological complete response. Tumor budding and MMR status should be considered as tools to be implemented in studies that predict the pathological response to preoperative chemo-radiotherapy in rectal cancer.

Lino-Silva LS, Gamboa-Domínguez A, Zúñiga-Tamayo D, Salcedo-Hernández RA, Cetina L, Cantú-de-León D. Mismatch repair protein expression and intratumoral budding in rectal cancer are associated with an increased pathological complete response to preoperative chemoradiotherapy: A case-control study. *World J Clin Oncol* 2018; 9(7): 133-139 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i7/133.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i7.133

INTRODUCTION

Rectal carcinoma is the third most common cancer, only after lung, breast, and prostate cancers, but it is the second leading cause of cancer-related mortality[1]. The treatment in locally advanced stages is neoadjuvant chemoradiotherapy (nCRT), with the aim of decreasing the tumor size, inducing favorable changes in staging (downstaging), increasing the probability of anal sphincter preservation, and decreasing the local recurrence rate and the possibility of a pathological complete response (pCR)[2], which is defined as the absence of neoplastic cells in both the rectal wall and lymph nodes. A pCR is reported in between 10% and 30% of these patients, and it is suggested that it is associated with a better oncologic outcome, a low local recurrence and an improved survival[3-4].

Unfortunately, the pCR is only indicated by a histopathological study of the surgical specimen, and in theory, elements that anticipate a pCR in the pretreatment biopsy would be very useful because elements, such as preoperative biopsy findings (post-nCRT), endoscopic examination, or imaging studies after completion of nCRT, are not sensitive or specific enough to predict pCR.

Identifying characteristics of the tumors that will respond completely to nCRT could help avoid the radical resection of the rectum, a procedure that reduces the quality of life of the patient. Several histopathological, immunohistochemical, and molecular markers have been analyzed, but with contradictory results[5].

The fundamental damage caused by nCRT is direct nuclear damage, and then, some studies have associated the mismatch repair system (MMR) status with pCR with inconclusive results[6]. During DNA damage, the presence of proteins, such as MSH2, MSH6, MLH1, and PMS2 (associated with the MMR), increases[7]. We hypothesized that deficient MMR (dMMR) neoplastic cells may be especially prone to death from agents that induce genetic damage, such as nCRT. Other factors that might predict this response are the immune response to the tumor and the tumor budding (individual neoplastic cells at the tumor stroma).

Our aim was to determine if rectal adenocarcinomas with a dMMR system were associated with a higher pCR rate to nCRT compared to cases that were MMR-efficient (eMMR). Second, we evaluated the association between the inflammatory tumoral response and intratumoral budding with the pCR rate.

MATERIALS AND METHODS

A case-control study was performed

Ethical statement: This work was authorized by the ethics and research committee of our institution, with a waiver of informed consent, because of its retrospective nature (Instituto Nacional de Cancerología, approval number: Rev/16/21).

Inclusion criteria: From a prospective maintained rectal cancer database from 2010 to 2016 at one large cancer center (INCan), we searched for patients with stage Ⅲ adenocarcinoma of the lower two-thirds of the rectum that received and completed nCRT and were subjected to radical rectal surgery. For each case, the

initial biopsy (pre-nCRT) was retrieved, and the MMR protein status was determined by immunohistochemistry in this tissue. For all the cases, the pathological material from the surgical specimens were available, and all the resections were systematically evaluated according to the procedure recommended by Quirke and were standardized in our institution with the entire rectal wall examination in the case of a macroscopic absence of a tumor (in other words, the totality of the rectal wall was pathologically examined)[8,9]. We excluded patients who had previously undergone chemotherapy or radiotherapy for another neoplasm.

Case definition: Individuals with rectal cancer after nCRT who do not have histological evidence of cancer in both the rectum and lymph nodes of the surgical specimen (pCR, ypT0N0M0).

Controls definition: Individuals with rectal cancer who had histological evidence of residual neoplasm after nCRT. We took two controls per case that were matched by sex and age (± 5 years).

The outcome of interest was the presence or absence of pCR. An odds ratio (OR) of 2.0, for the primary objective, was the base for the sample size calculation, considering an alpha-value of 95%, a beta-value of 80%, a known proportion of MMR of 14%[10], and a 15% loss; the calculated number of cases per group was 72.

The clinical, demographic, and histopathological data were retrieved from the selected cases and controls. The cancer staging was determined preoperatively based on the Tumor Node Metastasis (TNM) classification of the American Joint Committee on Cancer (seventh edition)[11]. The expression of the MMR proteins was evaluated separately and independently by three pathologists with experience in gastrointestinal pathology who were blinded to the clinical data. If discordance was found, a consensus was achieved in a multi-observer microscope. The expression was evaluated according to the following criteria[12]: (1) The nuclear expression of all markers in each sample, allowing for the classification of adenocarcinoma, as with an efficient MMR system (eMMR); and (2) The absence of the nuclear expression of any of the markers in each sample, allowing for the classification of adenocarcinoma, as with a dMMR.

Additionally, for the MMR evaluation, each case the inflammatory infiltrate was categorized as present (either peritumoral in the form of lymphoid aggregates, or intratumoral) or absent. The evaluation of intratumoral budding was carried out by examining 0.785 mm^2 of the peritumoral stroma of the biopsy specimen, following the guidelines described by Lugli *et al*[13]. We defined low intratumoral budding as the presence of 0 to 4 buds/0.785 mm^2, and high intratumoral budding occurred when there were ⩾ 5 buds/0.785 mm^2.

Statistical analysis

Data analysis was performed using the statistical package SPSS 22.0 (IBM, California, United States). The ORs were calculated and contrasted with the Mantel-Haenszen method, with a significance level of 0.05. For the comparison of the numerical variables, we used a *t* test to compare the means, the Mann-Whitney test for medians and the chi-square test or Fischer's exact test for categorical variables. A multivariate analysis was performed with a conditional logistic regression to determine the independent predictors of pCR, with the variables at a significance level of $P < 0.05$ in the univariate analysis as components of the regression model.

RESULTS

Population and clinical characteristics

Seventy-two cases with pCR were evaluated, and two controls were selected per case, totaling 144 controls, for a total sample of 216 cases. The mean age was 56.8 ± 12.5 years, with 192 (88.9%) cases younger than 40 years. Of the 216 cases, 140 (64.8%) were men.

All the cases were in stage Ⅲ and at the time of the presentation, 123 cases (56.9%) had a cT3 stage rectal tumor, whereas 44 (20.4%) had cT2, 31 (14.4%) had cT4a, and 18 (8.3%) were cT4b. Seventy-seven (35.6%) patients were classified as having nodal disease by radiological studies and endoscopic ultrasound.

Pathological features

Of the total tumors, 109 were histologically grade 3, corresponding to 50.5%, whereas grade 1 was presented in 57 (26.4%) cases. Regarding budding, there was a mean of 6.3 ± 5.7 buds per 0.785 mm^2 (range, 0-45). In 23 (10.6%) cases, lymphovascular invasion was identified.

For tumor-associated inflammatory infiltrate, mainly lymphocytes and plasma cells were observed; in 41 (19%) cases, nodular lymphoid aggregates were immediately adjacent to the neoplastic cells. The mean number of intratumoral lymphocytes was 1.69 ± 1.74 / mm^2 (range, 0 to 10).

Of the 216 cases, a dMMR system was demonstrated in 63 (29.2%) patients, with the main alterations in the co-absence of PMS2 and MLH1 expression (74.6%) followed by an isolated loss of MSH6 expression (25.4%) and an isolated loss of MSH2 expression (17.4%).

Univariant analysis

The basal characteristics of the patients are summarized in Table 1, which shows that the tumors with a higher pCR rate were associated with grade 1 tumors, dMMR, an increased presence of intratumoral lymphocytes, and the absence of vascular invasion in the pretreatment biopsy. Factors that were statistically associated with the presentation of pCR were dMMR, the absence of lymphovascular invasion, and low intratumoral budding

Table 1 Clinical-pathological characteristic of 216 patients with rectal carcinoma with preoperative chemo-radiotherapy, divided by type of pathological response *n* (%)

	No pathological complete response group (controls) (*n* = 144)	Pathological complete response group (cases) (*n* = 72)	*P*
Median age (yr ± SD)	56.67 ± 11.94	57.22 ± 13.65	0.759[3]
Sex			
Female	48 (33.3)	24 (33.3)	1.0[1]
Male	96 (66.7)	48 (66.7)	
Histologic grade			
G1	30 (20.8)	27 (37.5)	
G2	44 (30.6)	6 (8.3)	< 0.001[1]
G3	70 (48.6)	39 (54.2)	0.147[1]
Budding			
Low (0-4 buds)	63 (43.8)	44 (61.6)	0.016[1]
High (5 or more buds)	81 (56.2)	28 (38.4)	
Missmatch repair status			
MMR proficient	112 (77.8)	41 (56.9)	0.001[1]
MMR defective	32 (22.2)	31 (43.1)	
CDX2 expression			
Absent	8 (5.6)	7 (9.7)	0.195[1]
Present	136 (94.4)	65 (90.3)	
MLH1			
Non expressed	23 (16)	26 (36.1)	0.001[1]
Normal	121 (84)	46 (63.9)	
PMS2			
Non expressed	23 (16)	26 (36.1)	0.001[1]
Normal	121 (84)	46 (63.6)	
MSH2			
Non expressed	11 (7.6)	0	0.010[2]
Normal	133 (92.4)	72 (100)	
MSH6			
Non expressed	12 (8.3)	4 (5.6)	0.587[2]
Normal	132 (91.7)	68 (94.4)	
Lymphovascular invasion			
Absent	130 (90.3)	63 (87.5)	0.105[1]
Present	14 (9.7)	9 (12.5)	

[1]Value calculated form *Chi square* test with the first group as the reference; [2]Values calculated from *Fischer's exact* test; [3]Value calculated from a Student *t*-test. MMR: Mismatch repair system; SD: Standard deviation.

(Table 2).

Multivariate analysis

The factors associated with pCR were a dMMR status and a low degree of budding (Table 2).

DISCUSSION

We found an independent prognostic value of dMMR and intratumoral budding in pretreatment biopsies of patients with rectal adenocarcinoma treated with nCRT. The rest of the factors examined did not correlate with pCR. Our study is the first to demonstrate the independent predictive value of intratumoral budding for increased pCR in these patients.

To date, no clinical, endoscopic, pathological, radiological or molecular features have been sensitive and specific enough to predict pCR. Several studies report promising results and a few have investigated the pretreatment histopathological features. Some of the characteristics associated with pCR are early T and N stage, small tumor size, histologic low-grade and low carcinoembryonic antigen serum levels[14,15]. However, these results are controversial[16].

We found that a dMMR status was associated with pCR. This is explained by the fact that the ability of a cell to survive is affected by defects in DNA repair against damage. These mechanisms in the rectal cancer response have been poorly studied because of the consensus that in colon cancer a dMMR is associated with poor response to fluoropyrimidine[17]. In the setting of concomitant radiation, the impact of fluoropyrimidine given to dMMR tumors is controversial, and the predictive and prognostic roles of MMR genes in colorectal cancer are still unclear[18]. Huh *et al*[19] evaluated MMR protein expression in 209 patients with locally advanced rectal cancers treated with nCRT and subsequent surgery, where a pCR of 14.4% was observed. They did not observe any differences in the MMR protein expressions between patients with pCR and patients without pCR. In another study, de Rosa *et al*[6], studied 62 dMMR colorectal cancers and identified 29 with stage Ⅱ-Ⅲ cancers who received nCRT and surgical resection and found that 8 (27.6%) had a pCR; this pCR was elevated compared to a rate of 18% reported by Brouquet *et al*[20]. As is clear, there is some evidence pointing out the possible value

Table 2 Analysis of 216 patients with rectal carcinoma with preoperative chemo-radiotherapy, regarding their association with presence of complete pathological response

	Univariate analysis			Multivariate analysis[1]		
	OR	*P*	95%CI	OR	*P*	95%CI
MMR status (defective *vs* proficient)	2.64	0.002	1.438-4.870	2.61	0.004	1.355-5.040
Lymphovascular invasion (present *vs* absent)	0.7	0.094	0.066-2.860			
Histologic Grade (G1-G2 *vs* G3)	1.24	0.442	0.709-2.203			
Tumor budding [Low budding (0-4 buds) *vs* High budding (5 or more buds)]	2.495	0.017	1.278-4.881	2.52	0.025	1.366-4.894
Inflammatory infiltrate (present *vs* absent)	1.194	0.624	0.587-2.429			

[1]Adjusted by MMR status and high tumor budding. OR: Odds ratio; CI: Confidence interval; MMR: Mismatch repair system.

of MMR proteins in the prediction of pCR. We found an increase in the pCR in patients with the dMMR system.

We found that 29% of our cases showed dMMR, which was a higher number than most reported studies. However, this high dMMR prevalence was according to the dMMR prevalence in Latin American studies[10,21].

Tumor budding is observed in approximately 40% of colorectal cancer cases, and several publications have informed the prognostic value of tumor budding in stage Ⅱ[22,23]. However, it has not been studied in patients prone to receive nCRT, and its predictive value for pCR has not been studied. Tumor budding has been studied in both biopsies and surgical specimens, and there is a lot of controversy about where and how to measure tumor buds[13]. In our study, we reported the number of buds in the intratumoral stroma of the biopsies and dichotomized the tumor budding into two grades. The consensus recommends the evaluation in the invasive front of the surgical specimens, which clearly demonstrates its prognostic significance, even when evaluated in biopsy specimens[24].

It is believed that tumor budding represents the first step in cancer metastasis and is considered a histological representation of the epithelial-mesenchymal transition (EMT). This hypothesis has not been validated thus far, and the mechanisms by which the bud cells separate from the main tumor are unclear. Individual cells at the stroma are thought to migrate through the extracellular matrix, invade lymphovascular structures, and form colonies of metastatic tumors in the lymph nodes and distant sites[24]. Additionally, tumor cells that undergo EMT are characterized by a gene expression switch from genes associated with epithelial differentiation to genes associated with mesenchymal properties[25]. We think that these events give neoplastic cells an intrinsic capacity to resist nCRT. Although the underlying mechanisms are not clear, this is a future line of investigation.

The last outcome evaluated by our study was the inflammatory infiltrate, which did not demonstrate an association with pCR. This parameter was very difficult to evaluate because of the complexity of the cellular response at tumor invasion. More complex studies specifically aimed to explore this response are needed.

The main limitation of our study was the unexplored reproducibility of the intratumoral budding in the literature in general, especially in the biopsy specimens. MMR protein evaluation is also a potential source of bias because, sometimes, the heterogeneous immunohistochemical reaction of the proteins is difficult to interpret. However, the standardized technique in our center (with external validation at UK-NEQAS), the consensus evaluation of the reaction, and the evaluation in whole tissue slides decreased this bias.

In conclusion, the response of rectal cancer to nCRT is a variable and very complex phenomenon. There is no individual molecular or genetic mechanism clearly associated with pCR. The key action that may help to predict pCR is a model that involves the combination of data from imaging, endoscopy, immunohistochemistry, gene expression, and histopathological data (such as intratumoral budding and dMMR).

ARTICLE HIGHLIGHTS

Research background

Several histopathological, immunohistochemical, and molecular markers have been analyzed in rectal carcinoma in an attempt to predict the pathological complete response (pCR) to neoadjuvant chemoradiotherapy (nCRT) with contradictory results.

Research motivation

Identifying characteristics of tumors that will respond to nCRT could help avoid surgery.

Research objectives

To determine whether the association of rectal adenocarcinoma with a defective-Mismatch repair system (dMMR) are associated with the rate of pCR. To identify histologic features in the diagnostic biopsy associated with the rate of pCR.

Research methods

A case-control study design paired 2:1 was performed.

Research results

The pCR was associated with well-differentiated tumors, dMMR, the absence of vascular invasion, and low tumor budding in the diagnostic biopsy. In the multivariant analysis, the factors independently associated with the pCR were dMMR and a low degree of tumor budding.

Research conclusions

Tumors with pCR were associated with the dMMR status and low tumor

budding in the diagnostic biopsy.

Research perspectives

Adding the status of both MMR and tumor budding to the current and future prognostic indexes could help predict the pCR of a rectal adenocarcinoma and evaluate alternative strategies for the care of these patients.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Interconversion of two commonly used performance tools: An analysis of 5844 paired assessments in 1501 lung cancer patients

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Abstract

AIM

To establish the Karnofsky performance status (KPS) categories which would facilitate the interconversion of the KPS scale to the Eastern Cooperative Oncology Group (ECOG) performance status (PS) scale.

METHODS

This was a retrospective analysis of all patients attending the lung cancer clinic at a tertiary care center over a 5-year period (September 2009 to August 2014). All patients were assessed with both KPS and ECOG PS scales at each visit. Correlation between KPS and ECOG PS was assessed using Spearman's correlation coefficient. KPS categories equivalent to ECOG PS scores were compared using hit rate and weighted kappa (κ_w).

RESULTS

A total of 1501 patients were assessed over the study period, providing 5844 paired KPS and ECOG PS assess-

ments. The study cohort had a mean (standard deviation; SD) age of 58.4 (10.8) years, with the majority being current or ex-smokers (76.9%) and males (82.3%). Non-small cell lung cancer was the most common histological type (n = 1196, 79.7%) with the majority having advanced (stage ⅢB/Ⅳ) disease (83.4%). Mean baseline KPS and ECOG PS scores were 77.6 (SD = 14.4) and 1.5 (SD = 1) respectively. The most frequent KPS score was 80 (29%), and the most frequent ECOG PS score was 1 (43%). The overall correlation between KPS and ECOG PS was good (Spearman r = -0.84, P < 0.0001) but ranged from -0.727 to -0.972 between visits. KPS categories derived from our cohort [10-40 (ECOG 4), 50-60 (ECOG 3), 70 (ECOG 2), 80-90 (ECOG 1), 100 (ECOG 0)] performed better [hit rate 78.1%, κ_W = 0.749 (0.736-0.762) P < 0.0001] than those suggested in the past literature.

CONCLUSION

The current study provides the largest set of paired KPS-ECOG assessments to date. We suggest that the KPS categories 10-40, 50-60, 70, 80-90, and 100 are equivalent to ECOG PS categories of 4, 3, 2, 1, and 0 respectively.

Key words: Karnofsky performance status; Eastern Cooperative Oncology Group; Performance status; Lung cancer; Chemotherapy

Core tip: Karnofsky performance status (KPS) scale and Eastern Cooperative Oncology Group (ECOG) scale are the most commonly used performance status (PS) tools worldwide for patients with cancer. Since the number of scoring points in each scale is different, these scales are not readily interconvertible. However, most clinical studies use only one of these two scales (either KPS or ECOG PS) to assess PS, rendering interpopulation comparisons difficult. In this study, we analyze the largest set of paired KPS-ECOG assessments to date in a cohort of lung cancer patients for a solution.

Prasad KT, Kaur H, Muthu V, Aggarwal AN, Behera D, Singh N. Interconversion of two commonly used performance tools: An analysis of 5844 paired assessments in 1501 lung cancer patients. *World J Clin Oncol* 2018; 9(7): 140-147 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i7/140.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i7.140

INTRODUCTION

Performance status (PS) is a measure of a subject's ability to perform the activities of daily living. PS has been shown to predict survival in patients with cancer[1-4]. Patients with poor PS often do not tolerate more aggressive treatment strategies like surgery or chemotherapy, and, hence, PS is often taken into account while deciding the therapeutic strategy for cancer. It is a well-known fact that patients with poor PS are excluded from most clinical trials in cancer[5,6].

Several tools are available for the assessment of PS, including the Karnofsky performance status (KPS) scale, the Eastern Cooperative Oncology Group (ECOG) PS scale, the palliative performance scale, and the Australia-modified KPS. Among these tools, the KPS scale and the ECOG PS scale are the most commonly used worldwide[7]. The KPS scale is an 11-point numerical scale, with scores ranging from 100 (normal functional status) to 0 (death), in decremental steps of 10[8]. The ECOG PS scale is a 6-point numerical scale, with scores ranging from 0 (normal functional status) to 5 (death), in incremental steps of 1[9]. No conclusive evidence exists in the literature to suggest that one scale is better than the other. However, the ECOG PS scale is often preferred, as it is simpler to apply with a smaller number of choices. Both the scores have been shown to be good predictors of mortality[1,4,10,11]. The ECOG PS may have a slightly better prognostic value compared to KPS[10]. Although both the scales have been used to predict treatment response in lung cancer, results have been variable[12].

Several studies have shown PS assessment made using the KPS and ECOG PS by different healthcare professionals (doctors, medical students, nurses) to have moderate to high interobserver correlation, albeit with considerable variation[13-16]. Assessments made by technical staff or patients have been shown to have a relatively larger variability compared to assessments by healthcare workers[11,13,16]. These differences could be attributed to variation in the level of overall training and exposure of the individual, which might affect the interpretation of the existing disability. Moreover, interobserver variability can get aggravated when assessments are made on patients with lower KPS scores[17]. Closer attention to certain behavioral issues might help to improve the PS assessments in such situations[17]. However, neither of these two scales have been shown to be consistently superior to the other with respect to interobserver variability[13-15].

Since PS is an important factor which can influence clinical outcomes, researchers making interpopulation comparisons across studies should ensure that the PSs of the populations analyzed are equivalent. However, most clinical trials usually utilize only one of the two scales in their study population (either KPS or ECOG PS)[7]. Hence, the comparison of PS between different patient populations is difficult as the two scales are not readily interconvertible, with different number of scoring points (11 *vs* 6). To overcome this hindrance, several attempts have been made to examine the possibility of interconversion between KPS and ECOG PS scales (Table 1)[7,10,18-20]. Despite these suggestions, confusion still exists regarding the optimal KPS categories for interconversion to ECOG. Herein, we attempt to determine the KPS categories equivalent to ECOG PS

Table 1 Karnofsky performance status categories suggested for interconversion to Eastern Cooperative Oncology Group performance status in the literature

Author, year	*n* and patients, assessments	Setting	KPS categories	ECOG
AJCC[34], 1977	-	-	90-100	0
			70-80	1
			50-60	2
			30-40	3
			10-20	4
Minna *et al*[20], 1985	-	-	100	0
			80-90	1
			60-70	2
			40-50	3
			20-30	4
Buccheri *et al*[10], 1996	536 (1656)	Subjects with lung cancer visiting a cancer clinic of a tertiary care centre	80-100	0-1
			60-70	2
			10-50	3-4
Ma *et al*[19], 2010	1385 (1385)	Subjects visiting an oncology palliative care clinic, or admitted to an acute cancer palliative care unit	100	0
			80-90	1
			60-70	2
			40-50	3
			10-30	4
de Kock *et al*[18], 2013	955 (674)	Subjects with advanced life-limiting illnesses (cancer and non-cancer) in acute care and community settings	60-100	1
			50	2
			30-40	3
			10-20	4
Current study	1501 (5844)	Subjects with lung cancer visiting a cancer clinic of a tertiary care center	100	0
			80-90	1
			70	2
			50-60	3
			10-40	4

AJCC: American Joint Committee on Cancer; ECOG: Eastern Cooperative Oncology Group; KPS: Karnofsky performance status; PS: Performance status.

scores by analyzing data from a large cohort of lung cancer patients.

MATERIALS AND METHODS

Study design

We performed a retrospective analysis of data collected in the lung cancer clinic of our center over a 5-year period (September 2009 to August 2014). Informed consent was obtained from all the subjects.

Patients

All subjects with lung cancer who visited the lung cancer clinic for chemotherapy and had at least one paired assessment of KPS and ECOG PS were included in the study. Subjects with a diagnosis of intrathoracic malignancy other than lung cancer were not included in this study. Subjects who did not receive chemotherapy and received only alternative forms of therapy, like surgery, radiotherapy, or targeted therapy, were also excluded from this study. The PS assessments made during the first 10 visits of each patient, starting from the date of the first cycle of chemotherapy, were included in this study.

Standard of care

The subjects were treated appropriately with chemotherapy, tyrosine kinase inhibitors, radiotherapy, or surgery as guided by the tumor histopathology, mutation status, and clinical status, as described previously[21-24]. Briefly, subjects with adenocarcinoma without any driver mutation were treated with pemetrexed-based platinum doublet followed by maintenance pemetrexed therapy until disease progression. Subjects with squamous histology were treated with docetaxel or gemcitabine-based platinum doublet. Subjects with small cell lung cancer received irinotecan-based platinum doublet. All patients receiving chemotherapy were administered at least four cycles of chemotherapy before response assessment. Subjects who showed a partial response received an additional two cycles, for a total of six cycles. Subjects with sensitizing *EGFR* gene mutation or *ALK* gene rearrangements were treated with appropriate EGFR tyrosine kinase or ALK inhibitors, respectively.

Data collection and assessments

All subjects underwent a systematic assessment at baseline and follow-up as described previously[25-29]. At baseline, the following parameters were collected: age, gender, smoking status, body mass index, tumor histopathology, TNM stage (7th edition), KPS, and ECOG-PS[30]. The frequency of scheduled visits to the clinic by the patient varied depending upon the ongoing treatment modality. At every visit to the lung cancer clinic, the subjects were assessed with both the KPS and the ECOG PS scales by the treating physician.

Table 2 Baseline characteristics of the study population *n* (%)

Characteristic	Total, *n* = 1501
Age in yr	58.4 (10.8)
Males	1236 (82.3)
Smokers	1155 (76.9)
Body mass index in kg/m^2	20.2 (4.2)
Histopathology	
NSCLC: Squamous	553 (36.8)
NSCLC: Adeno	514 (34.2)
NSCLC: Undifferentiated	87 (5.8)
NSCLC: Other	42 (2.8)
SCLC	305 (20.3)
NSCLC/SCLC stage[1]	
I and II	44 (2.9)
IIIA	193 (12.9)
IIIB	426 (28.4)
IV	767 (51.1)
SCLC stage	
Limited disease	145 (9.7)
Extensive disease	160 (10.7)
Baseline performance status	
KPS	77.6 (14.4)
ECOG PS	1.5 (1)

[1]Total does not add up to 1501 as TNM staging was available for only some of the patients with SCLC. All values are mean (standard deviation) unless otherwise specified. ECOG PS: Eastern Cooperative Oncology Group performance status; KPS: Karnofsky performance status; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer.

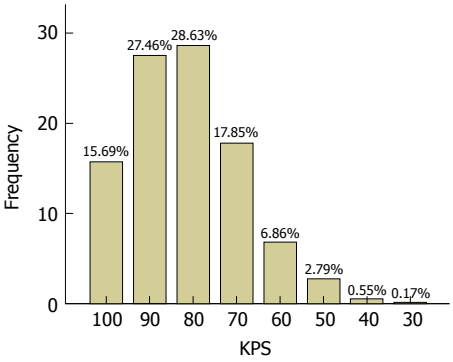

Figure 1 Frequency distribution of karnofsky performance status scores (*n* = 5844 assessments). KPS: Karnofsky performance status.

Statistical analysis

Descriptive data are presented as numbers and percentages or mean ± SD unless otherwise stated. Correlation between KPS and ECOG PS was assessed using Spearman's correlation coefficient (*r*), with values ranging from -1.00 to +1.00. An *r*-value of -1.00 was considered to represent a perfect negative correlation and a value of +1.00 was considered to represent a perfect positive correlation[31]. The original 11-point KPS scale was converted into a 5-point scale for comparison with the ECOG PS. The agreement between these KPS categories and the actual ECOG PS measurements was assessed using hit rate and the weighted kappa coefficient (κ_w). Hit rate was calculated as the proportion of assessments with a perfect agreement between the KPS categories and the measured ECOG PS scale. The κ_w was calculated to assess the level of agreement beyond chance. Agreement between the scales was classified based on the kappa values as poor (< 0.00), slight (0.00 to 0.20), fair (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80), or almost perfect (0.81 to 1.00)[32]. Statistical analyses were performed with the help of Statistical Package for Social Sciences software (IBM SPSS Statistics, version 22; IBM Corporation, Armonk, NY, United States). All statistical tests were performed as two-sided. $P < 0.05$ was considered statistically significant.

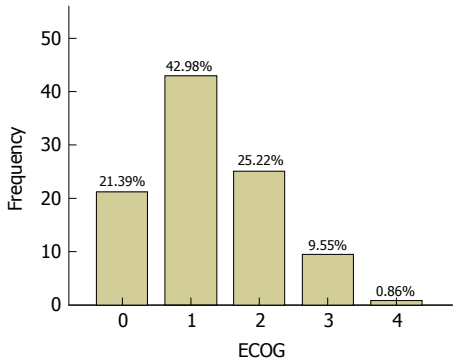

Figure 2 Frequency distribution of Eastern Cooperative Oncology Group status scores (*n* = 5844 assessments). ECOG: Eastern Cooperative Oncology Group.

RESULTS

During the study period, 1664 patients visited the lung cancer clinic. Among them, 150 patients did not receive any chemotherapy and were excluded. An additional 13 patients who had intrathoracic malignancies other than lung cancer were also excluded. A total of 1501 patients were assessed during the study period, providing 5844 paired PS assessments (KPS and ECOG PS). The mean (standard deviation; SD) age was 58.4 (10.8) years, with the majority being current or ex-smokers (76.9%) and males (82.3%) (Table 2). Non-small cell lung cancer was the most common histological type (*n* = 1196, 79.7%), with the majority having advanced (stage ⅢB or Ⅳ) disease (83.4%).

Mean baseline KPS and ECOG PS scores were 77.6 (SD = 14.4) and 1.5 (SD = 1) respectively. The most frequent KPS score was 80 (29%) (Figure 1) and the most frequent ECOG PS score was 1 (43%) (Figure 2). Among the total of 5844 paired PS assessments, a vast majority of the KPS scores were between 70 and 100 (89.6%) and similarly most ECOG PS scores were between 0 and 2 (89.6%). The overall correlation between KPS and ECOG PS was good [Spearman ρ = (-) 0.84, $P < 0.0001$] but ranged from -0.727 to -0.972 between visits (Table 3 and Figure 3). As expected, the number of assessments available at each progressive follow-up visit showed a reduction

Table 3 Correlation between Karnofsky performance status and Eastern Cooperative Oncology Group performance status

Visit number	Number of assessments	Spearman's correlation coefficient[1]
1	1426	-0.727
2	1099	-0.890
3	957	-0.876
4	823	-0.863
5	602	-0.858
6	464	-0.867
7	270	-0.901
8	129	-0.854
9	54	-0.773
10	20	-0.972
Overall	5844	-0.840

[1]$P < 0.0001$ for all individual visits and overall.

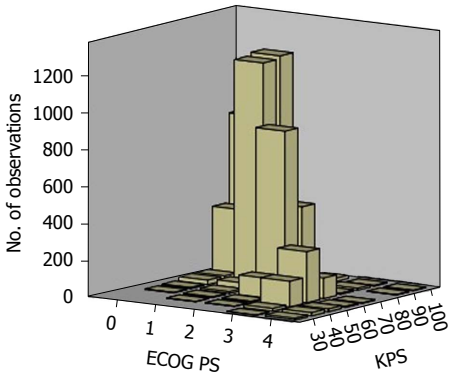

Figure 3 Distribution of karnofsky performance status and Eastern Cooperative Oncology Group performance status scores (n = 5844 paired assessments). ECOG PS: Eastern Cooperative Oncology Group performance status; KPS: Karnofsky performance status.

due to reduction in number of patients due to death or loss to follow-up.

Visual inspection of cross-tabulated KPS and ECOG PS data was performed to arrive at the best possible KPS categories to allow conversion of KPS to ECOG PS scale, namely: 100 (ECOG 0), 80-90 (ECOG 1), 70 (ECOG 2), 50-60 (ECOG 3), and 10-40 (ECOG 4)[10]. We also analyzed the performance of KPS categories suggested in the past literature, on our cohort (Table 4). We found a substantial agreement with all the suggested conversion categories, except those suggested by de Kock *et al*[18] and the American Joint Committee on Cancer (AJCC), which had slight and fair agreements respectively. KPS categories derived from our cohort performed better (hit rate 78.4%, κ_w = 0.674) than those suggested in the past literature[19,33]. The highest hit rate (83.2%) was observed with the interconversion categories suggested by Buccheri *et al*[10]. The highest κ_w was observed with the categories derived from our cohort (κ_w = 0.749). The interconversion categories suggested by de Kock *et al*[18] had the worst hit rate (43.2%) and κ_w (0.079).

DISCUSSION

In this study, we propose KPS categories which can aid interconversion of KPS scores to ECOG PS scores by retrospectively analyzing paired PS assessments (KPS and ECOG PS) made in a large cohort of lung cancer patients. We also compare the performance of KPS categories in previous literature for interconversion of KPS to ECOG PS on our cohort.

It is well-known that the PS is a predictor of mortality[1-4]. Hence, when comparing clinical characteristics or the outcomes of a therapeutic modality in different patient populations with lung cancer, it is essential that their PS is also matched. Failure to do so may result in erroneous conclusions. However, such comparisons are difficult when the tool used for assessment of PS in the study population is different (either KPS or ECOG PS). To overcome this difficulty, several investigators have suggested KPS categories for interconversion to ECOG PS scale (Table 1)[7,10,18-20].

The AJCC and Minna *et al*[20] were among the first to have suggested KPS categories for interconversion to ECOG PS[34]. However, these suggestions were not evidence-based. Verger *et al*[7] compared these two suggestions in a cohort of 150 patients with cancer attending a radiotherapy clinic and found that the categories suggested by Minna *et al*[20] performed better. They also stressed that at lower PS levels, interconversion between the two scores would be difficult as they observed a wide spread of values. Subsequently, Buccheri *et al*[10] studied 536 patients with lung cancer and found that a 3-point conversion scale for KPS resulted in the best agreement (hit rate 84%) between the two PS scales. Ma and colleagues had assessed KPS and ECOG PS in 1385 subjects with various cancers attending or admitted to an oncology palliative care clinic[19]. They analyzed several KPS categories and suggested a KPS category which closely resembled that suggested by Minna *et al*[20] and had the best combination of hit rate (75%) and κ_w (0.84, $P < 0.0001$). de Kock *et al*[18] studied 955 subjects with advanced life-limiting illnesses and suggested a 4-point scale, which had the best combination of hit rate (57%) and κ (0.7, 95%CI: 0.66-0.73).

Among all the interconversion categories for KPS suggested in the literature, the KPS categories which we suggest in this study had the best agreement with the actual PS measurements. Although, the categories suggested by Minna *et al*[20] and Ma *et al*[19] had the next best agreement, the categories suggested in our study appear more appropriate clinically. This can be illustrated by the following two examples. A patient who requires occasional assistance for daily activities (KPS 60), would be classified as ECOG 2 (capable of all self-care) using the categories suggested by Minna *et al*[20] or Ma *et al*[19], while they will be classified more appropriately as ECOG 3 (capable of limited self-care) using the categories suggested in the current study. Similarly, a patient who is disabled and requires special care and assistance (KPS 40) would be classified as ECOG 3 (capable of limited self-care) using the categories suggested by Minna *et al*[20] or Ma *et al*[19], while they will be classified more appropriately as ECOG

Table 4 Comparison of the performance of various suggested Karnofsky performance status categories (for interconversion to the Eastern Cooperative Oncology Group performance status scale) in the current cohort

Author, year	Hit rate[1]	κ_w[1]
AJCC *et al*[34], 1977	43.60%	0.376 (0.363-0.389) $P < 0.0001$
Minna *et al*[20], 1985	75.20%	0.701 (0.687-0.714) $P < 0.0001$
Buccheri *et al*[10], 1996	83.20%	0.695 (0.679-0.711) $P < 0.0001$
Ma *et al*[19], 2010	75.20%	0.701 (0.687-0.714) $P < 0.0001$
de Kock *et al*[18], 2013	43.20%	0.079 (0.068-0.090) $P < 0.0001$
Current study	78.10%	0.749 (0.736-0.762) $P < 0.0001$

[1]Performance in the current cohort shown as hit rate (%) or weighted kappa (95% confidence limits) along with the *P*-value.

4 (cannot carry out any self-care) using the categories suggested in the current study. It should also be borne in mind that a change in the ECOG scale by a single score in the above situations entails a change in 1-year survival by at least 10%[4].

The highest hit rate (83.2%) was observed with the KPS categories suggested by Buccheri *et al*[10]. However, this could have been because of the consolidation of the KPS categories into a smaller number of groups (3 groups instead of 5): KPS 10-50 (ECOG PS 3-4), KPS 60-70 (ECOG PS 2), KPS 80-100 (ECOG PS 0-1). Despite this, the level of agreement was lower when compared to the current study (κ_w 0.645 *vs* 0.749).

We observed the lowest hit rate (43.2%) and the least agreement (κ_w = 0.079) with the KPS categories suggested by de Kock *et al*[18]. This observation could have been because a majority of the population in that study had a poor PS, which is known to affect the interobserver rating[17]. In fact, 42.7% of their subjects had an ECOG PS of 3, and 66.2% had a KPS score between 30 and 50. Additionally, the authors did not assign KPS categories for ECOG PS 0.

Our study is not without limitations. The inherent limitations of retrospective studies apply to our study as well. Since the PS assessments were made by various treating physicians, there could have been some variability in the assessments. However, it has been demonstrated that both the KPS and ECOG PS scored by physicians have substantial interobserver reliability[13-16]. Moreover, each paired assessment was made by a single observer. On the other hand, this presumed limitation could be considered as an advantage as our study is more likely to approximate a real-world scenario. A majority of our subjects had good PS (KPS score $\geqslant$ 70 and ECOG PS $\geqslant$ 2) as they visited the clinic for treatment purposes. Hence, the suggested KPS categories may not apply to patients with poorer PS (*e.g.*, palliative care clinics). Finally, we did not have survival data for our cohort, as this was not the intended purpose of the study. However, despite these limitations, the current study provides the largest set of paired KPS-ECOG assessments to date in a real-world setting.

In conclusion, we suggest that the KPS categories 10-40, 50-60, 70, 80-90, and 100 are equivalent to ECOG PS categories of 4, 3, 2, 1, and 0 respectively. These categories may be useful for interconversion of KPS to ECOG PS scale when attempting to compare patient populations across different studies in whom investigators have used one of the two different scales (KPS or ECOG PS) for assessment of PS.

ARTICLE HIGHLIGHTS

Research background

Performance status (PS) is an estimate of a subject's ability to perform activities of daily living. Several tools are available to estimate the PS. Among them, the Karnofsky performance status (KPS) scale and the Eastern Cooperative Oncology Group (ECOG) scale are the most commonly used PS scales worldwide for patients with cancer. The KPS scale is an 11-point numerical scale, with scores ranging from 100 (normal functional status) to 0 (death), in decremental steps of 10. The ECOG PS scale is a 6-point numerical scale, with scores ranging from 0 (normal functional status) to 5 (death), in incremental steps of 1. Since the number of scoring points in each scale is different, these scales are not readily interconvertible.

Research motivation

PS is an important clinical factor which affects prognosis and influences treatment decisions in subjects with lung cancer. Hence, researchers who attempt to compare clinical characteristics or outcomes across different patient populations should ensure that their PS levels are matched. Failure to do so may result in erroneous conclusions. Most clinical studies employ only one of these two scales (either KPS or ECOG PS) in their study population for assessment of PS. When the PS scale used in the studies are different (either KPS or ECOG PS) this may lead to difficulty. Several investigators have tried to overcome this hindrance by suggesting KPS categories for interconversion to the ECOG PS scale. However, the performance of these suggested KPS categories has been variable.

Research objectives

We attempted to establish the KPS categories which would facilitate the interconversion of the KPS scale to the ECOG PS scale.

Research methods

We retrospectively analyzed the data of 1501 patients from a lung cancer clinic. In these patients, at every visit, paired assessments of PS had been made using both the KPS and ECOG PS scales by physicians. We also studied the performance of other KPS categories suggested in the literature, on our patient cohort. We used statistical methods called hit rate and weighted kappa to test the agreement between the KPS categories and the actual observations.

Research results

We found that the KPS categories 10-40, 50-60, 70, 80-90, and 100 were equivalent to ECOG PS categories of 4, 3, 2, 1, and 0 respectively. We also found that the agreement between the KPS categories suggested in the past literature (for interconversion to ECOG PS) and the paired KPS-ECOG PS assessments made in our cohort was variable.

Research conclusions

The current study is the largest set of paired KPS-ECOG assessments published in the literature in patients with lung cancer to date. The suggested KPS categories will facilitate interconversion of the KPS to the ECOG PS scale and will enhance communication between researchers utilizing either of the two scales.

Research perspectives

The KPS categories suggested in our study may be prospectively evaluated to test their validity. The applicability of the suggested categories may be evaluated in other populations to study the effect of cultural and regional variations.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of the eighth version of the American Joint Committee on Cancer manual to the seventh version for colorectal cancer: A retrospective review of our data

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Abstract

AIM

To analyze the survival trends in colorectal cancer (CRC) based on the different classifications recommended by the seventh and eighth editions of the American Joint Committee on Cancer staging system (AJCC-7th and AJCC-8th).

METHODS

The database from our institution was queried to identify patients with pathologically confirmed stage 0-Ⅳ CRC diagnosed between 2006 and 2012. Data from 2080 cases were collected and 1090 cases were evaluated through standardized inclusion and exclusion criteria. CRC was staged by AJCC-7th and then restaged by AJCC-8th. Five-year disease-free survival (DFS) and overall survival (OS) were compared. SPSS 21.0 software was used for all data. DFS and OS were compared and analyzed by Kaplan-Meier and Log-rank test.

RESULTS

Linear regression and automatic linear regression showed lymph node positive functional equations by tumor-node-metastasis staging from AJCC-7th and tumor-node-metastasis staging from AJCC-8th. Neurological

invasion, venous infiltration, lymphatic infiltration, and tumor deposition put forward stricter requirements for pathological examination in AJCC-8th compared to AJCC-7th. After re-analyzing our cohort with AJCC-8th, the percentage of stage ⅣB cases decreased from 2.8% to 0.8%. As a result 2% of the cases were classified under the new ⅣC staging. DFS and OS was significantly shorter (P = 0.012) in stage ⅣC patients compared to stage ⅣB patients.

CONCLUSION

The addition of stage ⅣC in AJCC-8th has shown that peritoneal metastasis has a worse prognosis than distant organ metastasis in our institution's CRC cohort. Additional datasets should be analyzed to confirm these findings.

Key words: Colorectal cancer; Tumor-node-metastasis staging; Prognosis; Peritoneal metastasis; Disease-free survival

Core tip: Since the promulgation of the eighth edition of the American Joint Committee on Cancer staging system manual (AJCC-8th), it has attracted the attention of many clinicians around the world and guided clinical work. Using our institution data we explored the prognostic differences between AJCC-8th and the seventh edition of the AJCC manual (AJCC-7th) for colorectal cancer. We found that patients with stage ⅣC colorectal cancer have a worse prognosis. This shows that peritoneal metastasis has a worse prognosis than organ metastasis. Considering many prognostic factors, individualized treatment is particularly important to improve the survival time of stage Ⅳ patients, especially stage ⅣC patients.

Tong GJ, Zhang GY, Liu J, Zheng ZZ, Chen Y, Niu PP, Xu XT. Comparison of the eighth version of the American Joint Committee on Cancer manual to the seventh version for colorectal cancer: A retrospective review of our data. *World J Clin Oncol* 2018; 9(7): 148-161 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i7/148.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i7.148

INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor[1-2]. In 2016, the incidence and mortality in the United States were respectively ranked fourth and second[3-7]. In 2015, 376000 patients were newly diagnosed with CRC in China and 191000 patients died from the disease[8]. Surgical resection remains the mainstay of treatment for local and regional disease[9-14]. Adjuvant chemotherapy is frequently used in advanced colon cancer and CRC, but remains controversial for stage Ⅱ disease[15-21]. Understanding the pathologic staging in conjunction with prognostic values is essential to making therapeutic decisions. The American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging model has provided this universal modality since its first edition in 1977[22]. Since then, the AJCC has repeatedly revised this guideline (Figure 1) to continuously guide clinical treatment.

The eighth edition of the AJCC staging system (AJCC-8th) was released on October 6, 2016 in Chicago, IL, United States, and was implemented globally on January 1, 2018, which included significant changes for CRC patients with stage Ⅳ disease[23]. The Cancer Council under the American College of Surgeons required the use of the AJCC-8th staging system as the "primary language" for cancer reporting. In 2013, AJCC established the "Evidence-Based Medicine and Statistics Core Group" of the 8th edition of the staging system. The organization is composed of clinical physicians, statisticians, and methodologists. It is responsible for determining the level of evidence for any updated content of the AJCC staging system.

The level of evidence is divided into four levels, and the quality of evidence represented by it gradually decreases from level Ⅰ to level Ⅳ. Level Ⅰ requires that the evidence is from multiple large national or international studies, has consistent results, has good research requirement design and implementation, was conducted in appropriate patient populations with appropriate study endpoints and appropriate treatment options, either as prospective studies or review-based studies based on patient populations, but all studies must be methodologically assessed. Level Ⅱ requires that the evidence comes from at least one large study and had good design and implementation, was conducted in a suitable patient population with a suitable study endpoint, and has external reliability (generally the representative and extrapolated capabilities of the study are better). Level Ⅲ includes evidence from a study with certain flaws, defects in the number of possible subjects, size, or quality of the study, or the consistency of multiple findings, the appropriateness of the patient population, and the appropriateness of the results. Level Ⅳ includes evidence wherein no reasonable research had been done. Only evidence from levels Ⅰ-Ⅲ could be included in the 8th version of the staging system.

A major difference between AJCC-7th, and AJCC-8th is that the CRC staging system was revised to include a new stage involving peritoneal metastasis (named stage ⅣC) (see Tables 1 and 2 for details). Based on a variety of evidence-based medical evidence[24,25], the AJCC-8th CRC staging system continues to recommend vascular lymphatic vessel infiltration and tumor deposition as prognostic level information, while microsatellite instability status and *BRAF* gene status are used as prognostic factors, and *BRAF*, *KRAS*, and degeneration of the *NRAS* gene were used as a predictor of efficacy (Table 3)[26].

The increased complexity of the AJCC-8th staging

Table 1 Comparison of the tumor-node-metastasis stages between the 7th edition and the 8th edition

7th edition	8th edition
Tx: Primary tumor cannot be assessed	Tx: Primary tumor cannot be assessed
T0: No evidence of primary tumor	T0: No evidence of primary tumor
Tis: Carcinoma *in situ*, limited to intraepithelial or invasive lamina propria	Tis: Carcinoma *in situ*, limited to intraepithelial or invasive lamina propria
T1: Tumor invading submucosa	T1: Tumor invading submucosa
T2: Tumor invading the muscularis propria	T2: Tumor invading the muscularis propria
T3: Tumor penetrating the muscularis propria and arriving at colorectal fat tissue	T3: Tumor penetrating the muscularis propria and arriving at colorectal fat tissue
T4: Tumor directly invading other organs or structures	T4: Tumor directly invading other organs or structures
T4a: Tumor penetrating visceral peritoneum	T4a: Tumor penetrating visceral peritoneum
T4b: Tumor directly invading or adhering to other organs or structures	T4b: Tumor directly invading or adhering to other organs or structures
Nx: Regional lymph nodes cannot be assessed	Nx: Regional lymph nodes cannot be assessed
N0: No lymph node metastasis and no tumor deposits (TD)	N0: No lymph node metastasis and no TD
N1: 1-3 lymph nodes metastases	N1: 1-3 lymph nodes metastases
N1a: 1 lymph node metastases	N1a: 1 lymph node metastases
N1b: 2-3 lymph nodes metastases	N1b: 2-3 lymph nodes metastases
N1c: Although there was no regional lymph node metastasis, TDs were submucosal, mesangial, or peritoneum-covered para-colorectal tissue.	N1c: Although there was no regional lymph node metastasis, TDs were submucosal, mesangial or peritoneum-covered para-colorectal tissue.
N2: More than or equal to 4 lymph node metastases	N2: More than or equal to 4 lymph node metastases
N2a: 4-6 regional lymph node metastases	N2a: 4-6 regional lymph node metastases
N2b: More than or equal to 7 lymph node metastases	N2b: More than or equal to 7 lymph node metastases
M1: There is distant lymph node metastasis	M1: There is distant lymph node metastasis
M1a: Metastasis is limited to one organ or site (*e.g.*, liver, lung, ovary, and extra-regional lymph node metastases)	M1a: Metastasis is limited to one organ or site (*e.g.*, liver, lung, ovary, and extra-regional lymph node metastases)
M1b: Transfer more than one organ or site, or to the peritoneum[1]	M1b: Transfer more than one organ or site[1]
	M1c: Peritoneal metastases with or without metastasis of other organs[1]

[1]Differences between the two versions.

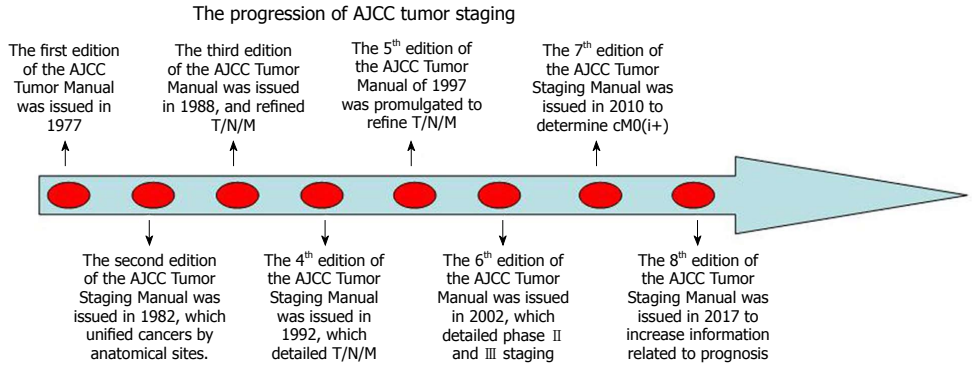


Figure 1 The progression of American Joint Committee on Cancer tumor staging. AJCC: American Joint Committee on Cancer.

model was intended to improve the prognostic staging of CRC, but the impact of these changes remains unclear. In this study, we used data from our institutional registries to compare the prognostic accuracy of criteria from AJCC-7th and AJCC-8th in patients with stage 0-Ⅳ through survival models. We also explored the relationship between positive node and tumor size, differentiation, tumor invasion, chemotherapy, tumor-node-metastasis (TNM) staging from AJCC-7th, and TNM staging from AJCC-8th. In addition, we also discussed the pathological importance of lymph invasion, vein invasion, and nerve invasion according to AJCC-8th.

MATERIALS AND METHODS

Patients

A total of 2080 patients with pathologically confirmed stage 0-Ⅳ CRC between 2006 and 2012 were collected from our institutional database. Then the following inclusion and exclusion criteria were applied to this cohort: (1) on the basis of a colonoscopy, computed tomography, pathological diagnosis of CRC, in or outside the hospital diagnosis in our hospital; (2) patients undergoing colorectal surgery in our hospital (including radical surgery and non-radical surgery); (3) diagnosis as a recurrence of the primary tumor or as a result of the death of the primary tumor; (4) cases with complete and detailed clinical and pathological data; and (5) cases with complete follow-up data and accurate data. Exclusion criteria were: (1) a serious heart, brain, liver, or lung disease led to intolerant surgery; (2) the non-CRC factors that led to the death of the pathological interstitial tumor, neuronal tumor, lymphoma, melanoma and other non-adenocarcinoma in addition to other malignant tumors;

Table 2 Colorectal cancer tumor-node-metastasis staging American Joint Committee on Cancer 7th and 8th editions

7th edition				8th edition			
Stage	T	N	M	Stage	T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
Ⅰ	T1-2	N0	M0	Ⅰ	T1-2	N0	M0
ⅡA	T3	N0	M0	ⅡA	T3	N0	M0
ⅡB	T4a	N0	M0	ⅡB	T4a	N0	M0
ⅡC	T4b	N0	M0	ⅡC	T4b	N0	M0
ⅢA	T1-2	N1/N1c	M0	ⅢA	T1-2	N1/N1c	M0
	T1	N2a	M0		T1	N2a	M0
ⅢB	T3-4a	N1/N1c	M0	ⅢB	T3-4a	N1/N1c	M0
	T2-3	N2a	M0		T2-3	N2a	M0
	T1-2	N2b	M0		T1-2	N2b	M0
ⅢC	T4a	N2a	M0	ⅢC	T4a	N2a	M0
	T3-4a	N2b	M0		T3-4a	N2b	M0
	T4b	N1-2	M0		T4b	N1-2	M0
ⅣA	Any T	Any N	M1a	ⅣA	Any T	Any N	M1a
ⅣB	Any T	Any N	M1b	ⅣB	Any T	Any N	M1b
				ⅣC	Any T	Any N	M1c

Table 3 American Joint Committee on Cancer 8th edition updates for the colorectal cancer staging system

Update points	Update details	Level of evidence
Definition of distant transfer (M)	Introduction of M1c, specifically peritoneal metastasis, is an indicator of poor prognosis	Ⅰ
Definition of regional lymph nodes (N)	Further introduce the definition of tumor deposit	Ⅱ
Recommended additional indicators for guiding clinical practice	Lymphatic vessel infiltration: Reintroducing the meaning of L and V[1] positive to correctly understand lymphatic and vascular invasion	Ⅰ
Recommended additional indicators for guiding clinical practice	Microsatellite instability: Further explaining its importance as a prognostic risk and efficacy predictor	Ⅰ
Recommended additional indicators for guiding clinical practice	Determine the *KRAS*, *NRAS*, and *BRAF* mutations as very important prognostic risk and efficacy predictors	Ⅰ, Ⅱ

[1]L-positive infiltrates for medics and V-positive for venous infiltration.

and (3) cases with incomplete clinical-pathologic data and cases with incomplete follow-up data. As a result, 990 cases were excluded. Therefore our analysis focused on the remaining 1090 cases.

Follow-up

Patients were routinely followed in the outpatient clinic 2 wk after surgery for 3 mo and every 3 mo for the first year, then every 6 mo for the second year and every year for the next 3 year. Follow-up data was complemented by phone contact as well as contact with written mail.

Ethics statement

This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Huzhou Central Hospital.

Preliminary processing of data

Using the extent of disease codes, tumor invasion (T staging), lymph node positivity (N staging), tumor metastasis (M staging) status, CRC was staged based on the AJCC-7th and AJCC-8th (Table 4). The patients were divided into three groups (N0, N1, N2) by the number of positive lymph nodes. Clinicopathological data were analyzed between the three groups. Patient status was designated into three outcome categories for disease-free survival (DFS): (1) death from CRC; (2) recurrence from CRC; or (3) alive at the last follow-up. Patient status was designated into two outcome categories for overall survival (OS): (1) death from CRC; or (2) alive at the last follow-up.

Statistical analysis

SPSS 21 (Chicago, IL, United States) was used for data analysis. Intergroup measurement data were analyzed using ANOVA analysis of variance and count data were analyzed using Cross-Tab χ^2 analysis.

The relationship between positive lymph node and tumor size, differentiation, tumor invasion, chemotherapy, and TNM staging from AJCC-7th, and TNM staging from AJCC-8th were analyzed by linear and automatic linear regression and the functional equations were established.

Survival curves were generated using Kaplan-Meier estimates, and 5-year DFS and OS were compared using the Log-rank test. Kaplan-Meier was also used to calculate the survival rate of DFS and OS in each group. Afterwards, Cross-table was used to compare the DFS

Table 4 Two-way classification table of staging based on tumor-node-metastasis staging from AJCC-7th *vs* tumor-node-metastasis staging from AJCC-8th for patients with stages 0-Ⅳ colorectal cancer from 2006-2012 (*n* = 1090)

		TNM staging from AJCC-7th										Total
		0	Ⅰ	ⅡA	ⅡB	ⅡC	ⅢA	ⅢB	ⅢC	ⅣA	ⅣB	
TNM staging from AJCC-8th	0	16	0	0	0	0	0	0	0	0	0	16
	Ⅰ	0	131	0	0	0	0	0	0	0	0	131
	ⅡA	0	0	138	0	0	0	0	0	0	0	138
	ⅡB	0	0	0	56	0	0	0	0	0	0	56
	ⅡC	0	0	0	0	31	0	0	0	0	0	31
	ⅢA	0	0	0	0	0	136	0	0	0	0	136
	ⅢB	0	0	0	0	0	0	400	0	0	0	400
	ⅢC	0	0	0	0	0	0	0	127	0	0	127
	ⅣA	0	0	0	0	0	0	0	0	24	0	24
	ⅣB	0	0	0	0	0	0	0	0	0	9	9
	ⅣC	0	0	0	0	0	0	0	0	0	22	22
		16	131	138	56	31	136	400	127	24	31	1090

TNM: Tumor-node-metastasis.

and OS survival rates of sub-periods between AJCC-7th and AJCC-8th groups, and a histogram was generated. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Lymph staging (N) and clinicopathologic characteristics

During the 6-year study period, 2080 patients with stage 0-Ⅳ CRC were identified but only 1090 met our inclusion criteria. The median age at diagnosis was 66 years [interquartile range (IQR): 55-73] and median follow-up was 60 mo (IQR: 54-60). The N staging did not change between AJCC-7th and AJCC-8th, therefore we used N staging to analyze clinical pathology data. Patient demographics and pathological features were summarized in Table 5. This table also compared staging of CRC with AJCC-7th *vs* AJCC-8th criteria. Although there was no difference in the total number of patients with stage Ⅳ CRC, the distribution of patients in this period was different. The χ^2 test was performed for all sub-stages of CRC, and significance exited between ⅣA and ⅣB according to AJCC-7th ($P = 0.001$), and between ⅣA, ⅣB, and ⅣC according to AJCC-8th ($P = 0.05$).

Linear model between the number of positive lymph nodes and tumor size, differentiation, tumor invasion, chemotherapy, TNM staging from AJCC-7th, and TNM staging from AJCC-8th

The number of positive lymph nodes was related to the N anatomical stages in AJCC-7th and AJCC-8th. An automated linear model found that the number of positive lymph nodes and tumor size, tumor differentiation, depth of tumor invasion, chemotherapy, TNM staging from AJCC-7th, and TNM staging from AJCC-8th were indicators of good fit and showed significance ($P < 0.05$). The fitting degree for TNM staging from AJCC-7th was 61.3% (Figure 2A), and the index that had a significant influence on positive lymph nodes was shown in Figure 2B. However, chemotherapy was not included in the predictive importance index (Figure 2C). The importance of TNM staging from AJCC-7th was 77%, and the importance of tumor invasion was 19%, the importance of tumor size was 3%, the degree of tumor differentiation was 1%. Figure 2D showed significant parameters of each coding amount and constant coefficient. The fitness for TNM staging from AJCC-8th was 63.3% (Figure 3A), and the indexes that had a significant influence on positive lymph nodes were shown in Figure 3B. Chemotherapy was also included in the predictive importance index (Figure 3C). The importance of TNM staging from AJCC-8th was 72%, the importance of tumor invasion was 20%, the importance of chemotherapy was 4%, the importance of tumor size was 3%, the degree of tumor differentiation was 1%. Figure 3D showed significant parameters of each coding amount and constant coefficient.

Then the linear model calculated the functional equation for these variables and positive lymph node relationships. Outcome showed that $Y_A = -0.918 + 0.409X_B + 0.18X_C - 0.583X_D - 0.460X_E + 0.669X_F$ and $Y_A = -0.821 + 0.404X_B + 0.183X_C - 0.587X_D - 0.491X_E + 0.658X_G$ (A: Positive lymph node; B: Tumor size; C: Differentiation; D: Tumor invasion; E: Chemotherapy; F: TNM staging from AJCC-7th; G: TNM staging from AJCC-8th).

DFS and OS between AJCC-7th and AJCC-8th criteria

Using Kaplan-Meier univariate analysis and Log-rank test, the 5-year survival rate of DFS and OS in 1090 patients was calculated and compared by stage and sub-stage according AJCC-7th and AJCC-8th criteria. DFS and OS survival rate between the two editions did not change from stage 0-Ⅳ and from substage 0-ⅣB. However, when the 5-year DFS and OS survival rate were compared from stage ⅣB from AJCC-7th and from stage ⅣB and ⅣC from AJCC-8th the survival curve of DFS and OS showed a significant right shift for stage ⅣB and a significant left shift for stage ⅣC ($P = 0.001$ and $P < 0.001$, respectively). Details were shown in Table 6

Table 5 Demographic and clinical characteristics of patients with stage 0-Ⅳ colorectal cancer from 2006-2012 (*n*, mean ± SD)

	N0	N1	N2	F or χ^2	*P*
Gender				2.895	0.235
Male	242	182	126		
Female	234	201	105		
Age (yr)	62.46 ± 14.43	62.17 ± 14.43	61.98 ± 14.70	0.095	0.909
ASA				6.011	0.198
1	362	277	158		
2	102	94	68		
3	12	12	5		
Primary site				4.94	0.895
Ileocecum	36	26	11		
Right colon	43	30	22		
Transverse colon	70	64	40		
Left colon	88	72	46		
Sigmoid colon	53	34	21		
Rectum	186	157	91		
Tumor size (cm)	3.31 ± 1.17	3.76 ± 0.82	4.11 ± 0.74	56.008	< 0.001
Operation method				8.233	0.411
RHC	97	67	43		
LHC	186	154	91		
HO	9	6	9		
AR	145	112	70		
APR	39	44	18		
Operation time (m)	151.59 ± 36.31	156.40 ± 34.94	153.17 ± 31.30	2.044	0.130
Resection length (cm)	27.96 ± 9.92	27.26 ± 9.83	27.65 ± 9.92	0.533	0.587
Blood loss (mL)	184.39 ± 94.25	185.23 ± 95.26	194.30 ± 107.32	0.879	0.416
Tumor invasion				131.640	< 0.001
Tis	16	0	0		
T1	85	17	9		
T2	92	75	43		
T3	162	127	132		
T4a	82	108	22		
T4b	39	56	25		
Differentiation				188.64	< 0.001
Well	150	31	13		
Moderate	276	296	124		
Poor or undifferentiated	50	56	94		
Number of LNs examined	14.70 ± 1.88	14.13 ± 1.78	14.26 ± 1.85	0.408	0.665
Number of positive LNs	0	1.85 ± 0.73	5.46 ± 1.64	3050.47	< 0.001
Complication				4.088	0.130
No	436	349	201		
Yes	40	34	30		
Chemotherapy				295.36	< 0.001
Yes	283	383	229		
No	193	0	2		
TNM staging AJCC-7th				887.08	< 0.001
0	16	0	0		
Ⅰ	131	0	0		
ⅡA	138	0	0		
ⅡB	56	0	0		
ⅡC	31	0	0		
ⅢA	45	82	9		
ⅢB	49	234	117		
ⅢC	9	47	71		
ⅣA	1	15	8		
ⅣB	0	5	26		
TNM staging AJCC-8th				887.32	< 0.001
0	16	0	0		
Ⅰ	131	0	0		
ⅡA	138	0	0		
ⅡB	56	0	0		
ⅡC	31	0	0		
ⅢA	45	82	9		
ⅢB	49	234	117		
ⅢC	9	47	71		
ⅣA	1	15	8		
ⅣB	0	1	8		
ⅣC	0	4	18		

TNM: Tumor-node-metastasis.

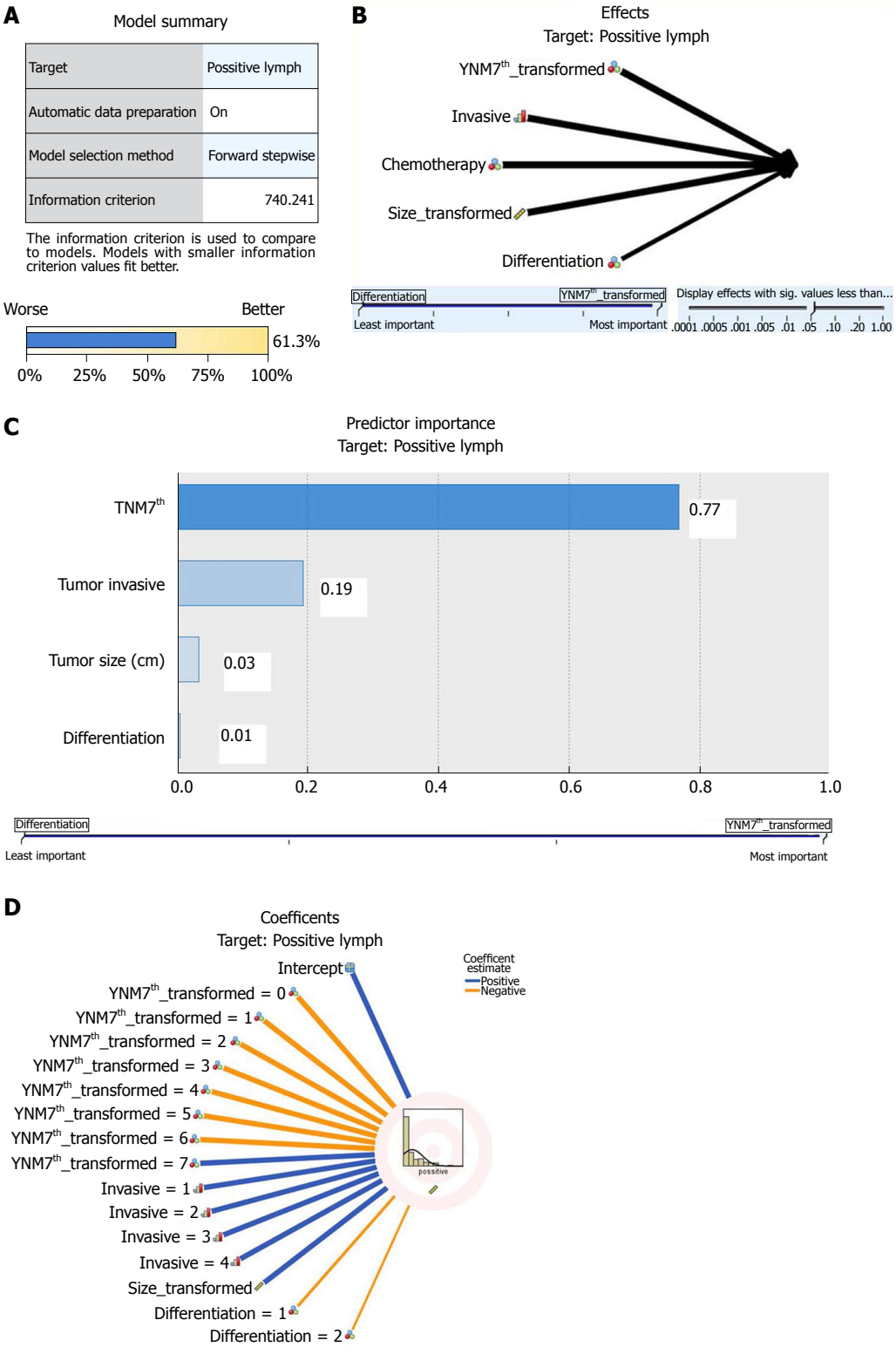


Figure 2 Automatic linear regression about positive lymph nodes and clinicopathologic parameters with tumor-node-metastasis staging from AJCC-7th. A: Clinical pathological parameters fitting degree. Fitting value is 61.3%; B: Significant effect parameters ($P < 0.05$); C: Predictor importance of positive lymph nodes and clinicopathological parameters. The values of tumor-node-metastasis staging from AJCC-7th, tumor invasion, tumor size, and differentiation are 0.77, 0.19, 0.03, and 0.01, respectively; D: Coefficients about positive nodes and clinicopathological parameters.

and Figure 4.

Nerve invasion, vein invasion, Lymphatic invasion and tumor deposit between AJCC-7th and AJCC-8th

AJCC-8th further emphasized the clinical value of tumor lymphatic invasion, vein invasion, nerve invasion, and tumor deposit (TD) and were included in "evidence-based medicine" evidence level (Table 3). Since the release of AJCC-7th, our institution's pathologist has attached great importance to this aspect of the test and has described

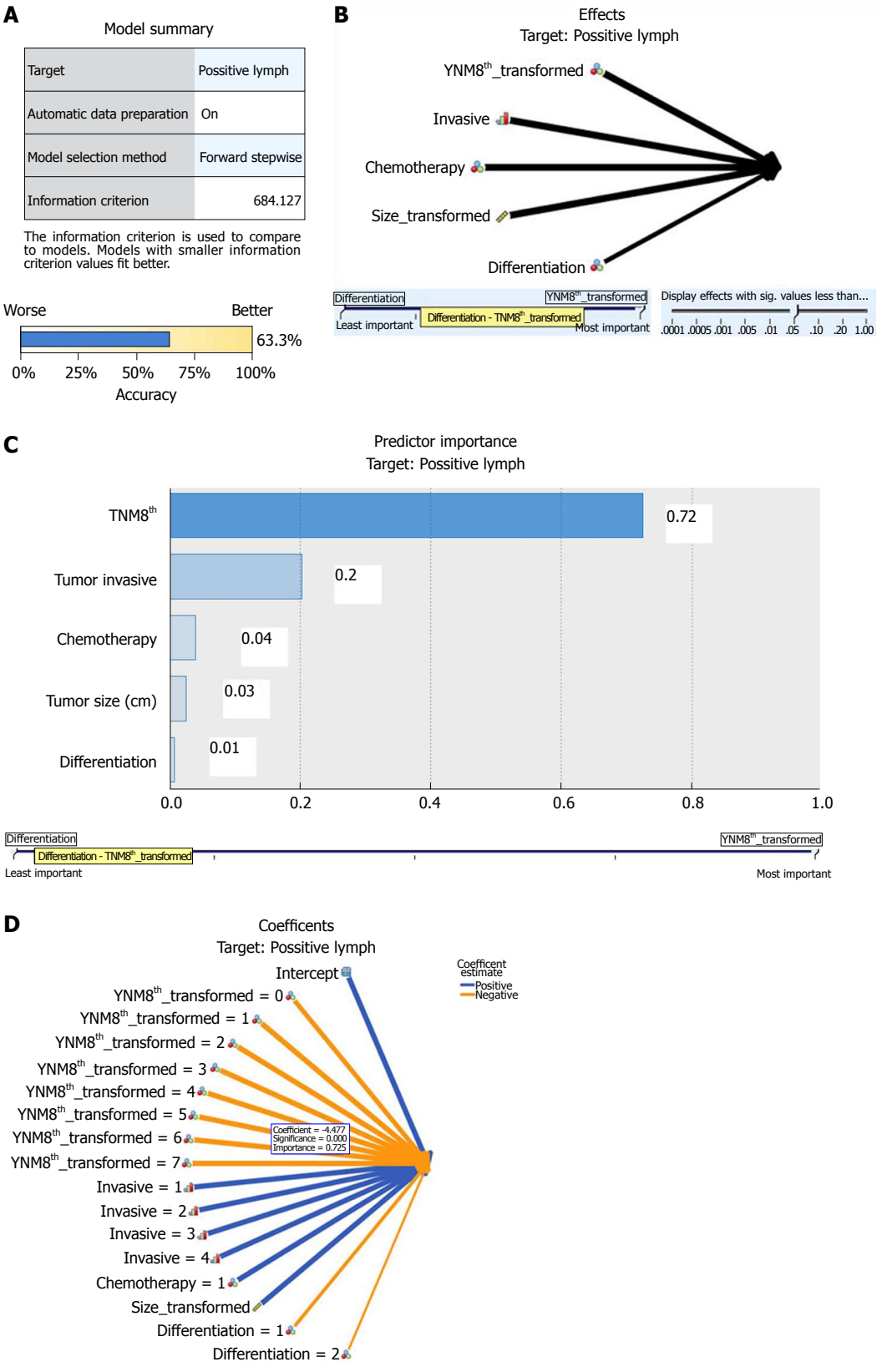


Figure 3 Automatic linear regression about positive lymph nodes and clinicopathologic parameters with tumor-node-metastasis staging from AJCC-8th. A: Clinical pathological parameters fitting degree. The fitting value is 63.3%; B: Significant effect parameters ($P < 0.05$); C: Predictor importance of positive lymph nodes and clinicopathological parameters. The values of tumor-node-metastasis staging from AJCC-8th, tumor invasion, chemotherapy, tumor size, and differentiation are 0.72, 0.2, 0.04, 0.03 and 0.01, respectively; D: Coefficients about positive nodes and clinicopathological parameters.

Table 6 Comparison of 5-year disease-free survival and overall survival rate for stage and sub-stage using American Joint Committee on Cancer-7th edition and American Joint Committee on Cancer-8th edition (%)

			0	Ⅰ	ⅡA	ⅡB	ⅡC	ⅢA	ⅢB	ⅢC	ⅣA	ⅣB	ⅣC	Log-rank χ^2	*P*
OS	Sub-stage	AJCC-7	100	98.5	82.6	76.8	67.7	65.4	60	44.9	8.3	0	-	1423.53	< 0.01
		AJCC-8	100	98.5	82.6	76.8 Ⅱ	67.7	65.4	60 Ⅲ	44.9	8.3	0 Ⅳ	0	1608.11	< 0.01
	Stage	AJCC-7	100	98.5		79.1			58.2			3.6		913.56	< 0.01
		AJCC-8	100	98.5		79.1			58.2			3.6		875.46	
DFS	Sub-stage	AJCC-7	100	93.1	78.3	73.2	61.3	65.4	56.3	37	8.3	0	-	1418.9	< 0.01
		AJCC-8		93.1	78.3	73.2 Ⅱ	61.3	65.4	56.3 Ⅲ	37	8.3	0 Ⅳ	0	1603.4	< 0.01
	Stage	AJCC-7	100	93.1		74.7			54.4			3.6		875.46	< 0.01
		AJCC-8		93.1		74.7			54.4			3.6		875.46	< 0.01

DFS: Disease-free survival; OS: Overall survival; AJCC: American Joint Committee on Cancer.

them in detail (Figure 5).

DISCUSSION

In 1977, AJCC established the first edition of the cancer staging system. Revision to the system have been made every 6-8 years and until recently it has been regarded as the most comprehensive tool for prognostic and predictive grouping of patients with colon cancer[24]. However, when AJCC-6th was released in 2002[27], it elicited criticism because survival of patients with stage ⅢA colon cancer was superior to that of patients with stage ⅡB colon cancer[28]. In 2010, the AJCC cancer staging system was updated to the 7th edition[22,29]. This edition included both the refinement of the classic TNM "anatomic blood" diagnostic system, the increase in tumor regression scores, and the risk of prognoses and curative effects for circumferential resection margins.

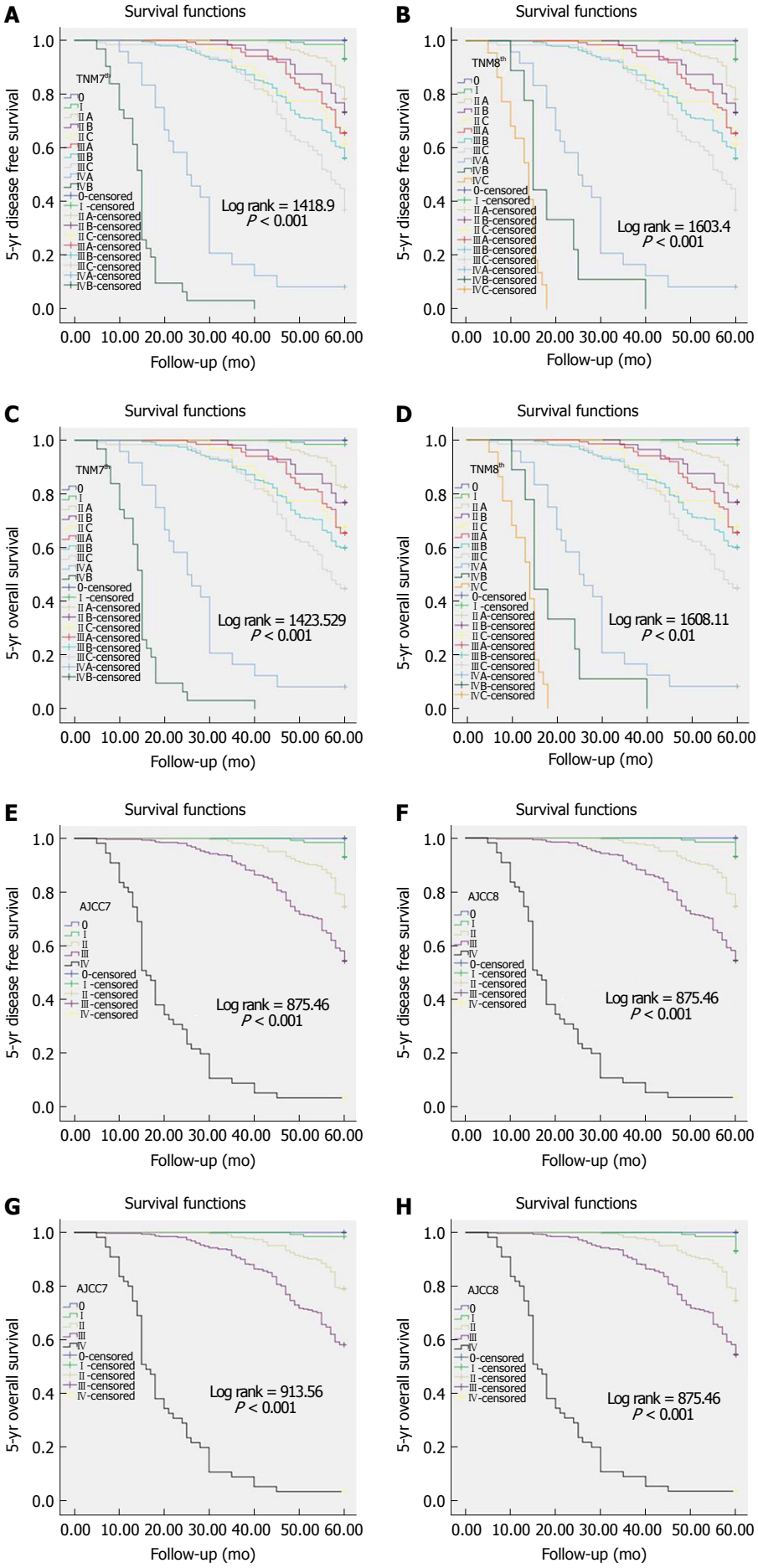
Evaluation index

The problem with AJCC staging of CRC was initially attributed to inadequate lymph node (LN) assessment. Previous studies demonstrated that the number of examined LNs impacted survival[30-34]. Subsequent studies showed a strong correlation between outcomes and compliance with 12-LN minimum[35-39]. In our study, in addition to analyzing the distribution of LN numbers in different N stages, we also focused on the effect of positive LN numbers on lymphatic pathology, and established a linear function.

In recent years, researchers have recognized the importance of tumorigenesis and the role of non-anatomic markers in establishing the prognosis and anticipated response to therapy[40-45]. Of these factors, the circumferential margin of the resected non-peritonealized surface of the specimen (CRM) is relevant for prognostic assessment of patients with tumors in the ascending and descending colon[46,47]. Microsatellite instability, KRAS mutation and the 18q LOH have been shown to have clinical prognostic significance[48,49]. These factors have not been incorporated into the staging system because it is not clear how they should be used to determine prognosis or the need for adjuvant chemotherapy. In 2013, AJCC established the "Evidence-Based Medicine and Statistics Core Group" of the eighth edition system, which was responsible for determining the level of evidence for any updated content of the AJCC staging system. New evidence had to reach an evidence quality level of Ⅰ-Ⅲ to be factored into the staging system for the eighth edition.

AJCC-8th did not include any updates for tumor staging. The definition of TD and N1c in the N-stage was further interpreted as the presence of encouraging tumor nodules in the lymphatic drainage area of the primary tumor, and no lymph node, vessel, or nerve structure identified during the period. The presence of TD did not alter the T stage of the primary tumor, but if it was not accompanied by lymph node metastasis, the TDs would change N stage (from N0 to N1c). If there was combined lymph node metastasis, the number of TDs did not need to be counted in the number of positive lymph nodes. The latest version reaffirmed the definition of lymphatic infiltrating vessels. Any vessel lesions with or without residual vascular walls could be identified as lymphocytic infiltrates in storage vessels and become a routine item in the pathology report of the American College of Pathology. Our institutional pathologist recognized this and described them in the report (Figure 5). Vascular lymphatic infiltration could be subdivided into small vessel infiltration (lymphatic or venular infiltration, defined as "L" positive) and venous infiltration (a structure surrounded by tumor immersion and endothelial cells, which contain red blood cells coated with smooth muscle machinery was defined as "V" positive). At the same time, it was found that tumor immersion and nerve tissue were defined as infiltration around the nerve. Lymphatic infiltration and perineural invasion were both important prognostic factors[50-56].

AJCC-7th classified the metastasis stage M1 as M1a (metastasis in one organ or site) and M1b (metastasis in


A
Survival functions
5-yr disease free survival
TNM7th
0
I
II A
II B
II C
III A
III B
III C
IV A
IV B
0-censored
I -censored
II A-censored
II B-censored
II C-censored
III A-censored
III B-censored
III C-censored
IV A-censored
IV B-censored
Log rank = 1418.9
P < 0.001
Follow-up (mo)
B
Survival functions
5-yr disease free survival
TNM8th
0
I
II A
II B
II C
III A
III B
III C
IV A
IV B
IV C
0-censored
I -censored
II A-censored
II B-censored
II C-censored
III A-censored
III B-censored
III C-censored
IV A-censored
IV B-censored
IV C-censored
Log rank = 1603.4
P < 0.001
Follow-up (mo)
C
Survival functions
5-yr overall survival
TNM7th
Log rank = 1423.529
P < 0.001
Follow-up (mo)
D
Survival functions
5-yr overall survival
TNM8th
Log rank = 1608.11
P < 0.01
Follow-up (mo)
E
Survival functions
5-yr disease free survival
AJCC7
0
I
II
III
IV
0-censored
I -censored
II -censored
III -censored
IV -censored
Log rank = 875.46
P < 0.001
Follow-up (mo)
F
Survival functions
5-yr disease free survival
AJCC8
Log rank = 875.46
P < 0.001
Follow-up (mo)
G
Survival functions
5-yr overall survival
AJCC7
Log rank = 913.56
P < 0.001
Follow-up (mo)
H
Survival functions
5-yr overall survival
AJCC8
Log rank = 875.46
P < 0.001
Follow-up (mo)

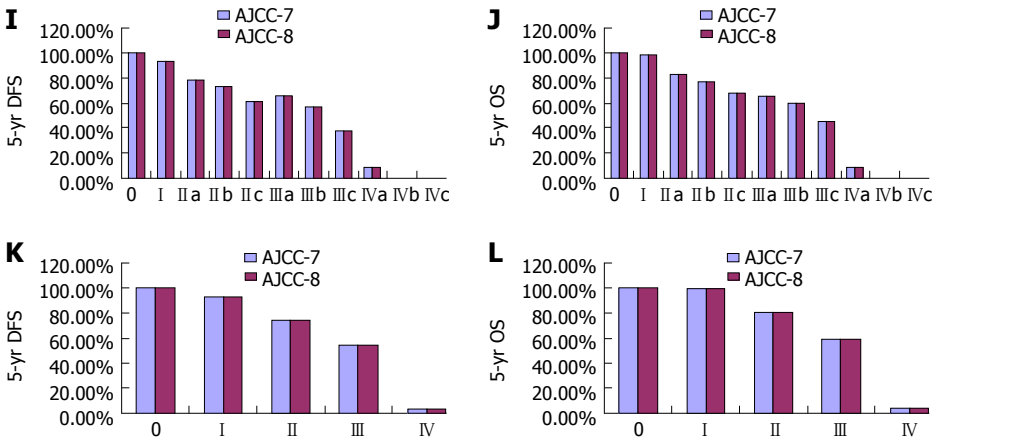


Figure 4 Disease-free survival and overall survival curves and histograms between tumor-node-metastasis staging from AJCC-7th and tumor-node-metastasis staging from AJCC-8th. A: Comparison of 5-year disease-free survival (DFS) by sub-stage from AJCC-7th ($P < 0.001$); B: Comparison of 5-year DFS by sub-stage from AJCC-8th ($P < 0.001$); C: Comparison of 5-year overall survival (OS) by sub-stage from AJCC-7th ($P < 0.001$); D: Comparison of 5-year OS by sub-stage for from AJCC-8th ($P < 0.001$); E: Comparison of 5-year DFS by stage from AJCC-7th ($P < 0.001$); F: Comparison of 5-year DFS by stage from AJCC-8th ($P<0.001$); G: Comparison of 5-year OS by stage from AJCC-7th ($P < 0.001$); H: Comparison of 5-year OS by stage for from AJCC-8th ($P < 0.001$); A *vs* B and C *vs* D: Survival curves of DFS and OS in stage ⅣB shift right and those in stage ⅣC shift left; I-L: Comparison of DFS and OS by sub-stage and stage between staging from AJCC-7th and staging from AJCC-8th, all $P < 0.01$. DFS: Disease-free survival; OS: Overall survival; TNM: Tumor-node-metastasis.

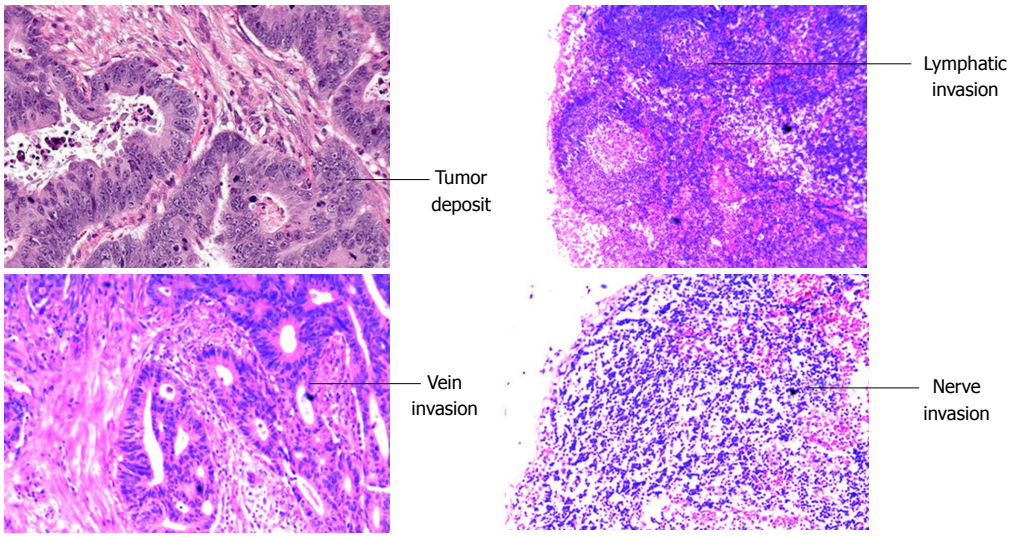


Figure 5 The focus of the eighth edition of the American Joint Committee on Cancer pathology test description. A: Tumor deposit; B: Lymphatic invasion; C: Vein invasion; D: Nerve invasion.

more than one organ or site, or in the peritoneum). In AJCC-8th, another stage was added to describe colorectal peritoneal metastases (whether or not with metastasis of other organ sites). This is called M1c, and M1a and M1b were redefined as metastasis limited to one organ or site (such as liver, lung, ovary, extra-nodal lymph nodes, *etc*.) and transition beyond one organ or site, but without peritoneal metastasis, respectively. The reason for the change is that although peritoneal metastasis occur in 1% to 4% of patients with CRC, the prognosis is far worse than that of M1a and M1b patients who have metastasis of substantial organs[57-61].

We reclassified our cohort according to the AJCC-8th criteria. The results showed that the DFS and OS of the M1a stage remained unchanged, while that of the M1b stage improved, and that of the M1c stage decreased significantly. This demonstrated that the M stage refinement was necessary. This additional classification in the eighth edition will have a positive and far-reaching effect on cancer treatment that will promote the individualized diagnosis and treatment of CRC patients. However, further analysis with additional institutional databases is needed to confirm our findings.

In conclusion, the addition of a sub-stage to classify peritoneal metastasis separately from distant organ metastasis in the AJCC-8th manual has shown that peritoneal metastasis has a worse prognosis than organ metastasis in our cohort.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a common malignant tumors. Clinicians have been using the American Joint Committee on Cancer (AJCC) system to guide clinical

diagnosis and treatment for CRC. The eighth edition of the AJCC (AJCC-8th) has received extensive attention since its promulgation in 2016. Compared to the previous version, AJCC-8th refined the stage Ⅳ classification to separate peritoneal metastasis and organ metastasis.

Research motivation

In China, there are still many hospital surgeons and physicians who still use the old version to guide clinical practice and are uneducated about the new AJCC-8th classifications.

Research objectives

We analyzed our institution's CRC cohort to determine differences in the survival trends based on the diagnostic classifications between AJCC-8th and the previous version.

Research methods

A total 1090 patients of 2080 CRC patients were included in the study. The data were classified by AJCC-7th and AJCC-8th standards. Five-year disease-free survival (DFS) and overall survival (OS) were compared.

Research results

Linear regression and automatic linear regression showed lymph node positive functional equations by TNM staging from AJCC-7 and TNM staging from AJCC-8th. Neurological invasion, venous infiltration, lymphatic infiltration, and tumor deposition put forward stricter requirements for pathological examination. AJCC-8th staging yielded a proportional decrease of ⅣB from 2.8% to 0.8% and a new staging of ⅣC to 2%. Log-rank test showed that DFS and OS survival time of patients with ⅣC *vs* ⅣB was significantly shorter (P = 0.012).

Research conclusions

The addition of a sub-stage to classify peritoneal metastasis separately from distant organ metastasis in the AJCC-8th manual has shown that peritoneal metastasis has a worse prognosis than organ metastasis in our cohort. Considering many prognostic factors, individualized treatment is particularly important to improve the survival time of stage Ⅳ patients, especially ⅣC patients.

Research perspective

Further studies can be done to improve outcomes for peritoneal metastasis CRC patients. Further analysis of additional institutional databases is needed to confirm our findings.

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CASE REPORT

Giant exophytic renal angiomyolipoma masquerading as a retroperitoneal liposarcoma: A case report and review of literature

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Author contributions: Sharma G, Jain A, and Garg PK designed the report; all the authors actively managed the patient; Sharma G, Jain A, and Garg PK collected the patient's clinical data; Sharma S provided the histopathological images; Rathi V provided the radiological images. All the authors analyzed the case, drafted the manuscript and finally approved it.

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Abstract

A 42-years-old lady, presented with a large retroperitoneal mass which was preoperatively diagnosed as a retroperitoneal liposarcoma following an image guided core biopsy. She underwent a margin-negative resection of the retroperitoneal mass (multi visceral resection - enbloc excision of the retroperitoneal mass with a left nephrectomy and a segmental descending colectomy). The final histopathological examination of the resected specimen confirmed an exophytic renal angiomyolipoma (AML) which was extending into the retroperitoneum. AML is a rare benign tumor arising most commonly from the kidney. It can sometimes present as a diagnostic challenge as it mimics a retroperitoneal liposarcoma or a fat-containing renal cell carcinomas closely. We present this case to share our experience of managing a case of giant exophytic AML which resembled retroperitoneal liposarcoma closely and resulted into an aggressive

surgery.

Key words: Angiomyolipoma; Retroperitoneum; Liposarcoma; Diagnosis; Biopsy

Core tip: A giant exophytic renal angiomyolipoma (AML) can pose a serious diagnostic challenge and may be confused with a retroperitoneal sarcoma. A discordance of the radiological and core biopsy findings in a suspected case of an exophytic renal AML must lead to re-evaluation of the case and repeat biopsies may further clarify the diagnosis.

Sharma G, Jain A, Sharma P, Sharma S, Rathi V, Garg PK. Giant exophytic renal angiomyolipoma masquerading as a retroperitoneal liposarcoma: A case report and review of literature. *World J Clin Oncol* 2018; 9(7): 162-166 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i7/162.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i7.162

INTRODUCTION

Angiomyolipoma (AML) is a benign tumor primarily containing fat, atypical blood vessels and smooth muscles in varying proportions[1]. The common site of AML is kidney and it presents primarily as an intra-renal mass. Occasionally, a renal AML may outgrow exophytically in the retroperitoneum and closely mimics a liposarcoma due to the high fat content on a radiological imaging. The diagnostic dilemma is further compounded as the core-cut biopsy may also be erroneously reported as liposarcoma[2].This may result in an aggressive surgery, and at times multi-visceral resections, to achieve a margin-negative resection. A post-resection histopathological diagnosis of AML may come as a surprise for both the surgeons and the patients, and may even lead to litigations. We are reporting a case of a giant exophytic AML which was misdiagnosed preoperatively as a retroperitoneal liposarcoma following an image guided core biopsy examination.

CASE REPORT

A 42-years-old lady presented to us with complaints of abdominal pain and fullness over the left side of the abdomen for two months duration. The pain was mild, dull aching, localized and continuous with a gradual increase in intensity over a period of time. She denied having hematuria, flank pain, dysuria, anorexia, rectal bleeding, malena or vaginal bleeding. She also denied any previous history of fever or blood transfusion. She reported to had undergone bilateral salpingo-oophorectomy around 3 years back for a left adnexal mass; no medical documents were available for this surgery. She developed amenorrhea following bilateral salpingo-oopherectomy, and she was not taking hormone replacement therapy. She also did not report any other significant past medical or family history. She had three children; all were borne as normal vaginal delivery and were hale and hearty. Her general physical examination was unremarkable. Her body mass index was 27.5. Her abdominal examination revealed a firm, non-tender, immobile, bimanually palpable intra-abdominal lump of size 25 × 20 cm involving left hypochondrium, left lumbar, umbilical, and left iliac fossa. Rest of her physical examination was unremarkable. Her blood investigations showed a hemoglobin of 6.6 g/dL and a total leucocyte count of 12000/μL. Her platelet count, liver function tests and renal function tests were within normal limits. Her contrast-enhanced computed tomography (CT) of the abdomen suggested two fairly well defined, predominantly fat density rounded lesions in the left kidney. The peripheral capsule of the smaller lesion was ill defined and was continuous with a large perirenal mass showing prominent vessels (Figure 1). There was also a peripheral hematoma in the perirenal lipomatous mass. An ultrasonography guided core biopsy of the retroperitoneal mass suggested liposarcoma in view of the variable sized adipocytes with atypia (Figure 2). Surgical exploration revealed a large retroperitoneal mass involving the left kidney and a segment of descending colon. The patient underwent a margin-negative resection of the retroperitoneal mass (multi visceral resection - enbloc excision of the retroperitoneal mass with a left nephrectomy and a segmental descending colectomy). Figure 3 displays the resected specimen. Her postoperative period was uneventful. Final histopathological examination of the resected specimen confirmed an exophytic renal AML which was extending into the retroperitoneum (Figure 4). A retrospective evaluation of the patient did not reveal any clinical stigmata of neurofibromatosis.

DISCUSSION

AML is a rare benign tumor which has a tri-phasic morphology - blood vessels, smooth muscles, and fat cells. Based on the fat content, AML is typically divided into classical or fat-rich type, and fat-poor AML. Both of these types are benign tumors with no metastatic potential. A third rare type is an epithelioid AML which is rare but has a malignant potential. Though fat-poor AML poses a diagnostic challenge due to the presence of scattered fat cells in the smooth muscles, classical renal AML can be easily diagnosed based on the contrast enhancement CT features: (A) the demonstration of the fat cells as the presence of regions of interest containing attenuations less than < 10-20 HU; and (B) the location of the fat cells within the lesion and not in another structure near it[3]. However, one must not forget that there are other kinds of lesions which may contain fat and mimic AML.

When this fat-rich renal AML grows exophytically and

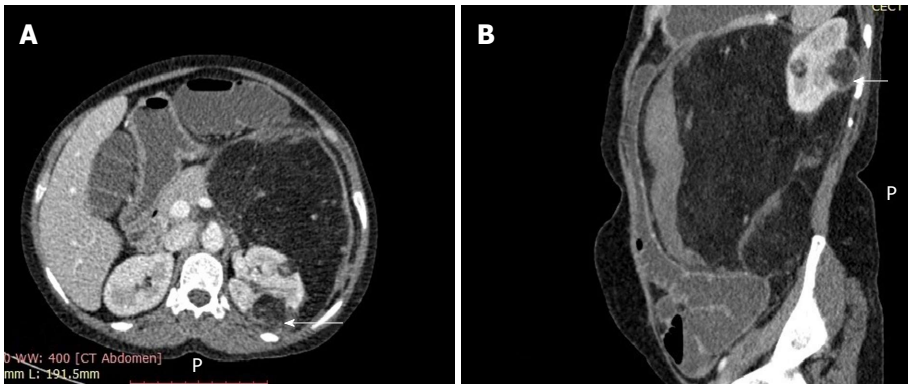

Figure 1 Axial section of contrast enhancement computed tomography abdomen. A: It shows two fairly well defined, predominantly fat density rounded lesions, in the left kidney. The peripheral capsule (arrow) of the smaller lesion is ill defined and continuous with a very large perirenal angiomyolipoma showing prominent vessels (+); B: Sagittal reconstruction shows a peripheral hematoma (arrow) in the perirenal lipomatous mass.

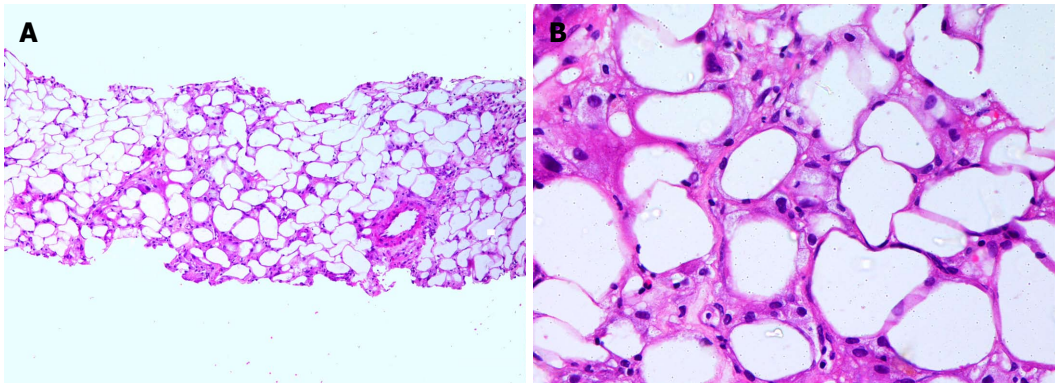

Figure 2 Preoperative core biopsy of tumor mass. A: Variable sized adipocytes (10 ×); B: Variable sized adipocytes with atypia (40 ×).

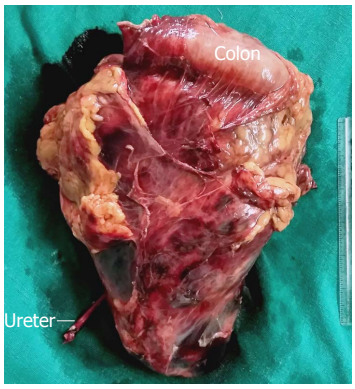

Figure 3 Resected enbloc specimens of retroperitoneal mass, left kidney, and colon.

extends into the retroperitoneum, it becomes difficult to differentiate it from a retroperitoneal sarcoma, especially a liposarcoma. As the management strategies are entirely different from these two entities - wide margin-negative resection for liposarcoma *vs* conservative approach for AML, it is of utmost importance to differentiate them radiologically. In a retrospective study of CT images of 15 large exophytic renal AMLs and 12 well-differentiated perirenal liposarcomas, Israel *et al*[4] suggested that a defect in the renal parenchyma combined with the presence of the enlarged vessels may help differentiate an exophytic AML from a retroperitoneal sarcoma. In a study of 11 patients with the perirenal liposarcoma and 9 patients with the giant exophytic AMLs, Ellingson *et al*[5] also concluded that the presence of tumoral vessel extending into the renal cortex or a renal parenchyma defect at the site of the tumor contact points strongly towards the diagnosis of AML while intra-tumoral calcification favors the presence of liposarcoma. However, Wang *et al*[6] suggested that the intra-tumoral calcification can be seen in both AML and liposarcoma. They highlighted that the characteristics of a perinephric AML are intratumoral linear vascularity, aneurysmal dilatation, bridging vessel sign, hematoma, beak sign, and discrete intra-renal fatty tumors while the CT characteristics of a perinephric liposarcoma are a non-fatty soft tissue mass. Other close differentials of an exophytic AML include lipoma, leiomyoma with fatty change or a fat containing renal cell carcinoma.

A small biopsy of the lesion to confirm the diagnosis may also yield erroneous results due to the non-representative sample in view of the heterogenous nature of the AML[5]. A positive immune-reactivity for human melanoma black 45, is characteristic of AMLs and may differentiate AMLs from other similar appearing

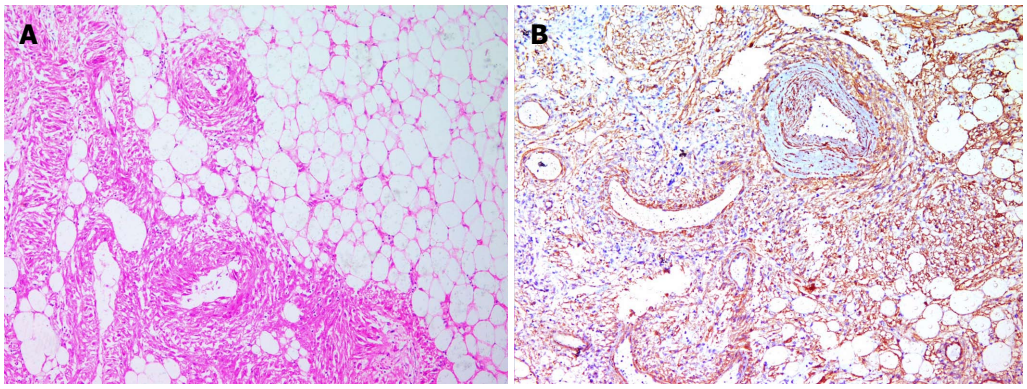

Figure 4 Final histopathology of surgical specimen. A: Components of all the three tissues, *i.e.*, mature adipocytes, spindle shaped smooth muscle cells and blood vessel (10 ×); B: Immunohistochemical staining for smooth muscle actin shows strong positivity in final histology specimen.

lesions such as liposarcomas, lipomas, leiomyosarcomas or, leiomyomas[7].

Our patient had a giant symptomatic exophytic AML. Though radiological findings were suggestive of AML, our patient was operated upon with a preoperative diagnosis of retroperitoneal liposarcoma due to the small image-guided biopsy which was reported as liposarcoma. This is likely to be due to a non-representative sample of the lesion. Though various conservative approaches including radiofrequency ablation, cryoablation, microwave ablation, selective angio-embolization are described to manage AML[8], our patient would have otherwise also required surgical excision in view of the large size, the symptomatic nature, and the risk of potential life-threatening hemorrhage. However, a correct preoperative diagnosis would have helped us to take a more conservative resection.

We conclude that a giant exophytic renal AML can pose a serious diagnostic challenge and may be confused with a retroperitoneal liposarcoma. A discordance of the radiological and core biopsy findings in a suspected case of exophytic renal AML must lead to re-evaluation of the case and repeat biopsies may further clarify the diagnosis.

ARTICLE HIGHLIGHTS

Case characteristics

A 42-years-old lady came to our hospital with complaints of abdominal pain and distension of two months duration. The physical examination of the patient revealed large retroperitoneal lump present on the left side of the abdomen.

Clinical diagnosis

The clinical diagnosis was a retroperitoneal mass, likely to be malignant in nature.

Differential diagnosis

The differential diagnosis included retroperitoneal sarcoma, renal neoplasm, multicystic kidney, cold abscess, or hydatid cyst.

Laboratory diagnosis

Routine blood investigations did not reveal any abnormality.

Imaging diagnosis

Abdominal computed tomography revealed a large perirenal mass lesion displaying prominent vessels. There were also two fairly well defined, predominantly fat density rounded lesions in the left kidney; the smaller lesion was continuous with the large perirenal mass.

Pathological diagnosis

Preoperative image guided biopsy of the retroperitoneal mass suggested liposarcoma.

Treatment

In view of preoperative pathological diagnosis of liposarcoma, the patient underwent a margin-negative resection of the retroperitoneal mass (multi visceral resection - enbloc excision of retroperitoneal mass with left nephrectomy and segmental descending colectomy).

Related reports

The final histopathological report of the resected specimen confirmed angiomyolipoma.

Term explanation

AML is a tumor of tri-phasic morphology - blood vessels, smooth muscles, and fat cells. Majority of the AML tumors are benign in nature with almost no malignant potential.

Experiences and lessons

A large exophytic renal AML may be confused with a retroperitoneal sarcoma on a small biopsy specimen. A discordance of the radiological and core biopsy findings in a suspected case of exophytic renal AML must alert the surgeon and a re-evaluation of the case with repeat biopsies may clarify the diagnosis.

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EDITORIAL

Malignant peritoneal effusion acting as a tumor environment in ovarian cancer progression: Impact and significance

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Abstract

Until recently, ovarian cancer research has mainly focused on the tumor cells themselves ignoring for the most part the surrounding tumor environment which includes malignant peritoneal effusions. However, one of the major conceptual advances in oncology over the last few years has been the appreciation that cancer progression cannot be explained by aberrations in cancer cells themselves and is strongly influenced by the surrounding tumor environment. The mechanisms of ovarian cancer progression differ from that of other solid tumors because ovarian cancer cells primarily disseminate within the peritoneal cavity. Malignant peritoneal effusion accumulates in the peritoneal cavity during ovarian cancer progression. These exudative fluids act as a unique tumor environment providing a framework that orchestrates cellular and molecular changes contributing to aggressiveness and disease progression. The composition of ascites, which includes cellular and acellular components, constantly adapts during the course of the disease in response to various cellular cues originating from both tumor and stromal cells. The tumor environment that represents peritoneal effusions closely constitute an ecosystem, with specific cell types and signaling molecules increasing and decreasing during the course of the disease progression creating a single complex network. Although recent advances aiming to understand the ovarian tumor environment have focused one at a time on components, the net impact of the whole environment cannot be understood simply from its parts or outside is environmental context.

Key words: Ovarian cancer; Tumor environment; Peritoneal effusions; Ascites; Dissemination; Multicellular spheroids

Core tip: The malignant peritoneal effusion that accumulates during ovarian cancer dissemination and progression constitutes a unique tumor environment. Bidirectional communications between tumor cells and their surrounding environment influence ovarian cancer dissemination, progression and patient prognosis. To solve the complexity of this tumor environment and understand how it affects cancer progression, a paradigm shift is necessary. Peritoneal effusions should be studied as integrated systems with innovative modeling approaches.

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MAIN TEXT

Malignant peritoneal effusions (ascites) commonly occur during the progression of certain types of cancers such as ovarian and pancreatic adenocarcinomas[1]. These exudative fluids contain a large number of tumor cells and are by definition metastatic. For example, patients with ovarian cancer will often present at diagnostic with multiple omental and peritoneal tumor implants. These patients will usually have accumulation of large amount of peritoneal fluids (often several liters). The presence of malignant effusion is associated with a poor prognosis. Because of their nature, malignant effusions, in which tumor cells proliferate and metastasize, constitute a distinct and unique tumor environment compared to solid cancers that develop in a tissue microenvironment. The cell-free fraction of malignant effusions is rich in cytokines, chemokines, growth factors and extracellular matrix components[2-4]. In addition to tumor cells, effusions contain of a large number of non-malignant cells such as fibroblasts and mesothelial cells, and various immune cells including macrophages and lymphocytes[5]. Although the importance of the bidirectional communication between tumor cells and their adjacent tissue microenvironment for supporting cancer progression is well recognized now, much less is known regarding the role peritoneal effusions in cancer progression. Nonetheless, it has been shown that extracellular cues provided by soluble factors contain in effusions can drive certain aspects of cancer progression such as cell proliferation, migration, and invasion. For example, effusion-associated hepatocyte growth factor[6-8], lysophosphatidic acid[9] and CCL18[10] have been shown to promote ovarian cancer cell growth and survival. Furthermore, cell-free ascites can promote drug resistance in ovarian cancer cells[11,12]. In support of the critical role of cell-free ascites, a number of factors contained in effusions are predictor of clinical prognosis[13,14]. The overall effect of cell-free ascites results from the complex integration of various extracellular cues, which leading to receptor-induced signaling in both tumor cells and stromal cells. For example, in contact with ascites, mesothelial cells undergo a pro-tumoral shift[15] whereas ovarian cancer cells display a more invasive phenotype with a shift along an epithelial-to-mesenchymal transition (EMT)[16]. As a result of this extensive cellular crosstalk, peritoneal effusions are constantly adapting during the course of the disease in response to the different cues[1]. The functional role of these changes in effusion composition during cancer dissemination and progression is mostly unknown.

Beyond the potential contribution of specific soluble factors to cancer progression, non-malignant cells present in peritoneal effusions probably play an essential supporting role, particularly in the early stages of dissemination, by preventing anoikis and stimulating proliferation of tumor cells. Paracrine signaling through mesenchymal stem cells can confer resistance to chemotherapy in ovarian cancer cells via IL-6/IL-6 R pathway and CXCL12[17,18]. Similarly, paracrine secretion of anti-apoptotic from ascites-primed mesothelial cells promote ovarian cancer cell drug resistance[15]. Cancer-associated fibroblasts isolated from omentum can be activated by tumor cells to promote ovarian cancer growth, adhesion and invasiveness through TGFβ1 pathway[19]. Expression of HOXA9 by ovarian cancer cells induce TGFβ2 secretion, which in turn, promote by secretion of IL-6 and CXCL12 by fibroblasts leading to tumor growth[20]. The presence of tumor-infiltrating $CD8^+$ T cells in primary tumors is associated with prolonged progression-free and overall survival in ovarian cancer patients[21,22]. These data suggest that several cell types within peritoneal effusions may have a robust influence on ovarian cancer cells. Bidirectional communications between tumor cells and their surrounding environment are at play, however identifying these crosstalks and validating their functional roles in a systemic approach remains challenging because of complex interactions among multiple cell types in effusions.

Getting a better understanding of the complex ecosystem that constitute malignant peritoneal effusions also have the potential to enhance our insight to limit the accumulation of these effusions and to overcome platinum resistance in ovarian cancer, which represents a yet unmet need. Both soluble factors and cancer-associated stromal and immune cells constitute potential targets for novel therapies. Examples of

soluble factors in the tumor environment of ovarian cancer targeted by novel therapies (mostly humanized monoclonal antibodies) include VEGF, VEGFR, PDFR, PDGFR, c-kit, PD-1 and PD-L1[23]. However, drug-associated toxicity and resistance often limit the clinical benefits associated with these novel therapies. These studies highlight the fact that targeting a single component of the tumor environment is likely to have limited clinical benefits and the secretome of malignant peritoneal effusions should be considered as a whole that cannot be understood simply from its parts. Given the progression-enhancing role of cancer-associated stromal cells in malignant peritoneal effusions, cell therapy may represent of novel approach to limit ovarian cancer dissemination. For example, it has been shown that genetically-engineered human mesothelial cells with the Herpes Simplex virus thymidime kinase (HSV-tk) efficiently deliver anticancer modalities to ovarian cancer cells within the peritoneal cavity when mice were treated with ganciclovir[24]. Conversion of ganciclovir pro-drug in a toxic apoptosis-inducing drug by HSV-TK results in the transfer of apoptotic bodies to adjacent tumor cells. Alternatively, the cells could be used to locally deliver in the peritoneal cavity other anti-cancer molecules potentially circumventing the toxicity associated with systemic delivery.

Tumor such as ovarian cancer does not usually use the classic patterns of metastasis via the hematogenous route[25]. The current view is that ovarian cancer spreads mainly via the peritoneal circulation with a high affinity for the omentum. Cells detach from the primary tumor to form free-floating multicellular spheroids, which then travel through the peritoneal fluid at distant sites onto the mesothelial lining where metastatic outgrowth occurs[26]. It is believed that the formation of multicellular spheroids is an essential step in the initiation of peritoneal implantation metastasis for ovarian cancer. In this context, spheroids can be considered the primary vehicles of ovarian cancer dissemination. However, the mechanisms of spheroid formation are for the most part unknown. Before tumor cells detach, they will usually undergo an EMT characterized by decreased expression of E-cadherin acquiring therefore a proliferative and invasive phenotype. However, it is not clear if tumor cells detached as single cell entity and then aggregate together to form homotypic multicellular spheroids or if clumps of the primary tumor exfoliate and stay together to form heterotypic spheroids. Alternatively, exfoliated single tumor cells could aggregate with floating mesothelial cells forming heterotypic unvascularized multicellular spheroids. Recent studies aiming to characterize ascites-derived spheroids suggested that spheroids are heterotypic containing mesothelial-derived myofibroblatic cells[27] and macrophages[28]. The binding of ovarian cancer cells to mesothelial cells to form spheroids could be mediated by integrins, CD44 and MUC16[27,29,30]. Further work however is needed to gain a comprehensive understanding of the cellular composition of the multicellular spheroids found in ascites and how this influences cancer progression. Another interesting hypothesis is that multicellular spheroids potentially constitute a chemoresistant niche that continuously repopulates the peritoneal cavity despite chemotherapy treatments as they are potentially endowed with a high tumor-initiating potential and increased drug resistance linked to the expression of stemness-associated genes[31]. However, the presence of mesenchymal stem cells in ovarian cancer spheroids remains to be demonstrated. The generation of unvascularized 3D spheroids would generate a structure with a metabolite density gradient that can inhibit the access of chemotherapy agents to internal cells[32]. Ovarian cancer cell grown as spheroids display enhanced resistance to common chemotherapeutic drugs such as taxol and cisplatin compared monolayers[31,33]. Because of their role in ovarian cancer dissemination and their potential role in disease recurrence after chemotherapy, a much better understanding of multicellular spheroid biology is necessary.

PERSPECTIVE

As highlighted here, most research on the ovarian cancer environment has focused one at a time on components, such as particular signaling cascades, specific ascites factors or specific cell types, *etc*. This knowledge is essential, but obviously not sufficient. The tumor environment that represents peritoneal effusions closely resembles an ecosystem, with specific cell types and signaling molecules increasing and decreasing during the course of the disease progression creating a single complex network. This environment is an archetypical complex system in which the functioning of the whole cannot be understood simply from its parts or outside is environmental context. Perhaps to understand the role of peritoneal effusions on cancer progression, a paradigm shift is necessary and effusions should be studied as integrated systems using bioinformatic modeling to quantify system-level

biodiversity changes[34].

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EDITORIAL

Current status of PI3K-mTOR inhibition in hormone-receptor positive, HER2-negative breast cancer

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Abstract

Breast cancer (BC) is the most common cancer in women and second only to lung cancer in terms of mortality. Among the three different BC subtypes, the oestrogen receptor positive represents nearly 70% of all cases and it is usually treated with anti-oestrogen drugs. However, the majority of hormone receptor positive metastatic BC patients develop resistance to anti-oestrogen treatments. The need for more down-stream therapies brought to the development of therapeutic strategies inhibiting the phosphatidylinositol 3-kinase-mammalian target of rapamycin (mTOR) pathway. Inhibitors of the mTOR have been tested in different clinical trials; everolimus has been Food and Drug Administration approved for the treatment of oestrogen receptor positive/human epidermal growth factor receptor 2 negative BC patients in combination with exemestane in patients who have progressed to anastrozole or letrozole after the encouraging results coming from BOLERO-2 trial. Similar results were obtained by the TAMRAD investigatory study testing tamoxifen in combination with everolimus in advanced BC. This editorial focuses on the results from BOLERO-2, BOLERO 4 and BOLERO-6, which tested the clinical importance of mTOR inhibition. We comment also on the role of phosphatidylinositol 3-kinase-mTOR inhibition as reported in the BELLE-2 and BELLE-3 trials and the future directions for the inhibition of this tumour metabolic axis.

Key words: Hormone receptor positive/Her2-negative breast cancer; PI3K; mTOR; TORC1/2; Akt; Everolimus

Core tip: The phosphatidylinositol 3-kinase-mammalian target of rapamycin (PI3K-mTOR) pathway sustains cancer progression and drug resistance. The first Food and Drug Administration approved molecule against this pathway was everolimus, an mTOR inhibitor, to be used in combination with exemestane in hormone receptor positive/human epidermal growth factor receptor 2 negative breast cancers progressing to non-steroidal aromatase inhibitors. Drugs targeting other effectors such as PI3K, PI3Kα, Akt and mTORC1/2 have gained clinical interest. Nevertheless, everolimus

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remains the best option due to the relevant toxicity of the other drugs targeting the PI3K-mTOR pathway. Future directions point towards the development of biomarkers that would identify those patients who would benefit from the PI3K-mTOR inhibitors for improving overall survival.

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INTRODUCTION

With the introduction of molecular technologies, breast cancer (BC) has been divided into four main subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2), or triple negative. The luminal variants, due to the hormone receptor positive (HR+) expression, have a generally favourable prognosis in the non-metastatic setting. Some of the most commonly used drugs for the treatment of HR+ disease have been anti-oestrogen therapies, *e.g*. tamoxifen and aromatase inhibitors (AI) - such as anastrozole or letrozole or exemestane. In BC survivors, metastatic breast cancer (MBC) is a treatable but still incurable disease. The majority of HR+ MBCs develop resistance to anti-oestrogen treatments[1,2]. Cancer driver mutations and/or constitutive activation in the key signalling pathway genes of phosphatidylinositol 3-kinase-mammalian target of rapamycin (PI3K-mTOR) cascade are capable of overriding anti-oestrogen treatments in BC cells[3]. In particular, *PIK3CA* is mutated in 20%-40% of BC[4,5], representing therefore an interesting target for therapies. For these reasons, inhibitors of the PI3K-mTOR pathway was pursued in clinical development until one of them, an mTOR inhibitor-a derivative of rapamycin - named everolimus, reached Food and Drug Administration (FDA) approval for the treatment of the disease in 2012, in combination with exemestane, for second line treatment of HR+/HER2- BCs that have previously regressed to anastrozole or letrozole treatment.

This commentary is focused on the outcomes coming from the most important clinical trials, testing everolimus in combination with other drugs: TAMRAD (Tamoxifen Plus Everolimus), BOLERO-2 (OraL EveROlimus-2), BOLERO-4, BOLERO-6, BELLE-2 and BELLE-3. Future directions and perspectives testing therapeutical combinations targeting different effectors are also discussed.

PI3K-mTOR PATHWAY

PI3K-mTOR signaling events play an important role in the modulation of cell survival, proliferation, motility and apoptosis: activation of PI3K phosphorylates Akt, shifting thereby the protein in the activated form that localizes in the plasma membrane. Subsequently a cascade of downstream events are activated, including the inhibition of p27 and the increase of the transcription factor CREB levels and, consequently, of the cell cycle promoter cyclin A, leading to mTOR activation that promotes the expression of genes related to cell proliferation. The aberrant activation of the PI3K-mTOR pathway sustains cancer cell survival and proliferation and also contributes to anti-oestrogen treatment resistance.

PIK3CA mutations constitutively activate the PI3K signal transduction pathway, which ultimately leads to the phosphorylation of PIP_2 into PIP_3, thus activating the phosphatidylinositol signalling bringing to higher cell survival and to inhibition of apoptosis. Predominantly mutations of *PI3KCA* occur around hotspots E542/5 (exon 9) and H1047 (exon 20) at the catalytic subunit[6-9]. Additionally, *AKT* or *PTEN* mutations overrides anti-oestrogen therapies[4,10]. Eventually, PIP_3 phosphorylates Akt (pAkt is the active form), which then activates the mTOR.

Of note, PI3K activation has been shown to cause a decrease in ER levels and therefore a lesser degree of response to anti-oestrogen therapies[11]. Targeting PI3K could be capable of restoring ER sensitivity[12]. Therefore, PI3K-mTOR pathway inhibition plus endocrine therapies represent an interesting strategy to pursue. There is a link between ER and mTORC1: mTORC1 can phosphorylate ER at S167 through

p70S6K or it can also phosphorylate ER at S104/S106, thus resulting in ER activation[13]. Of note, recently, Sonnenblick *et al*[14] discovered a gene signature related to the phosphorylation of Akt (pAkt) and mTOR (p-mTOR) in early ER+ BC. Analyzing gene expression and proteomic data collected from over 7000 early BC, they found pAkt correlated to luminal A and a good prognosis, whereas p-mTOR was found in worse prognosis luminal B cases. Interestingly, the signatures of pAkt and p-mTOR correlated to *PI3KCA* mutations positively and negatively, respectively. The authors underlined the different role at the molecular level of pAkt and p-mTOR effectors in early luminal BC, a matter that further contributes to the heterogeneity of the disease[14].

CLINICAL TRIALS

The discovery of everolimus led to the hypothesis of synergism in co-targeting two different pathways in HR+/HER2- advanced BC patients: the mTOR and the HRs pathways. Such hypothesis has been tested in several clinical trials.

The TAMRAD trial was a phase 2, randomized clinical trial that tested the combination of everolimus with tamoxifen in postmenopausal women with MBC and resistance to AI. Besides proving to be a safe approach, it demonstrated that the combination of these treatments was capable of increasing the clinical benefit rate (CBR) from 42% with tamoxifen to 61% with the combination. The time to progression increased from 4.5 mo in patients receiving only tamoxifen to 8.6 mo in patients receiving both tamoxifen and everolimus. The death risk was reduced by 55% after treating patients with tamoxifen plus everolimus in comparison to tamoxifen alone[15]. Interestingly, the combination was efficient in patients who responded to AIs but subsequently became resistant to AIs. Contrarily, the combination was not effective in *de novo* treated MBC HR+[16].

The BOLERO-2 trial was a phase 3, randomized, double-blinded, multicentre clinical trial that recruited 724 HR+ MBC patients. This trial evaluated everolimus with the steroideal AI as exemestane compared to exemestane and placebo. Patients who were enrolled in this clinical trial had previously received AI anastrozole or letrozole. This clinical trial showed an improved progression free survival (PFS) of 11 mo in the exemestane + everolimus arm compared to the 4.1 mo placebo + exemestane ones [$P < 0.0001$; hazard ratio = 0.38; 95% confidence interval (CI): 0.31-0.48][17]. This shows that the combination of everolimus with exemestane doubled up the PFS time versus placebo plus exemestane alone; these results led to the FDA approval of everolimus with exemestane in the treatment of HR+ MBC patients who did not respond to letrozole nor anastrozole treatments.

Recent studies further tested everolimus in combination with other therapies as first line in HR+ BC patients. The BOLERO-4 (NCT01698918) and BOLERO-6 (NCT01783444) tested everolimus with different combinations of drugs. BOLERO-4 was a single arm, open labeled phase 2 study evaluating in 202 ER+, HER2- advanced BC patients the PFS of first line everolimus + letrozole and in a quarter of them whose disease progressed, everolimus + exemestane as second line. This study demonstrated the efficacy of everolimus + letrozole with a median PFS of 22 mo, whereas the second line everolimus + exemestane, which was used in some of the progressed patients, gave limited results with a median PFS of only 3.7 mo[18]. The BOLERO-6 was a randomized open-labeled study, comparing second line therapies everolimus + exemestane against everolimus or capecitabine alone in 309 AI-resistant ER+, HER2- MBC patients. Consistent with the BOLERO-2 study, robust evidence of superiority in median PFS was registered for everolimus + exemestane versus everolimus alone but surprisingly not versus capecitabine alone. However, the authors pointed out that there were some limits to the study, such as censored data and baseline imbalance in Kaplan Meyer curves, that could have inferred on the evaluation of the capecitabine arm[19]. Thus, the combination of everolimus + exemestane was confirmed to benefit these patients, whereas the superiority/equivalence/inferiority of capecitabine needs further clinical evaluation.

Inhibition of PI3K has been also investigated as an alternative strategy in targeted therapies of the mTOR pathway. As an example, buparlisib (BKM120), which is a PI3K inhibitor, has been tested in combination with fulvestrant in patients progressing AIs (BELLE-2, NCT01610284) and in patients progressing mTOR inhibition (BELLE-3, NCT01633060).

Buparlisib has been tested in 1147 patients as second line in the randomized, double-blinded BELLE-2 clinical trial, alone or in combination with fulvestrant. It was clear that the combination with the PI3K inhibitor had slightly increased PFS compared to fulvestrant alone, with the exception of *PI3KCA* mutated patients who

improved the PFS of about 4 mo. Of note, the serious toxicity of buparsilib limited the efficacy of the targeted therapy[20]. BELLE-3 was a randomized double-blinded phase 2 trial enrolling 432 patients who progressed AIs to evaluate PFS in buparlisib + fulvestrant arm compared to fulvestrant + placebo. The combination with buparlisib improved the PFS of only 2 mo while showing a high toxicity profile that hampered the clinical benefit[21]. Finally, an exploratory strategy is aiming for the simultaneous inhibition of PI3K and mTOR by alpelisib (BYL719) and everolimus, respectively. The safety of the combination of alpelisib and everolimus alone or everolimus + exemestane has been tested in a Phase 1 trial in various solid tumours, including BC (NCT02077933).

Worth of note is the combination of letrozole and alpelisib, a selective oral inhibitor of the class I PI3K catalytic subunit p110α, which has shown synergistic antitumour activity with endocrine therapy against ER+/*PIK3CA*-mutated BC cells. The combination was safe with reversible toxicities. Clinical activity was observed independently of *PIK3CA* mutation status, although clinical benefit was seen in a higher proportion of patients with *PIK3CA*-mutated tumours. Phase 2 and 3 trials of alpelisib and endocrine therapy in patients with ER^+ BC are ongoing[22].

PERSPECTIVE

In addition to the previous therapies targeting mTOR, other research groups sought to investigate alternative strategies targeting molecules belonging to the mTOR complex or other effectors of the PI3K-mTOR pathway. An investigatory strategy consists in the use of sapanisertib (TAK228) for TORC1/2 inhibition by targeting mTOR kinase. TORC1/2 are complexes of proteins that regulate many vital cellular processes and proliferation. This drug has been tested in the neoadjuvant setting with tamoxifen (NCT02988986) or letrozole (NCT02619669) in the treatment of HR+ BC patients. AZD5363 is a specific Akt inhibitor, which has been tested either as a monotherapy (NCT01226316) or as a combination with fulvestrant in HR+ MBC patients who progressed AIs (NCT01992952). Table 1 summarizes all the clinical trials testing PI3K-mTOR pathway inhibitors. In our opinion, there is a need for predictors of the efficacy of such inhibitors: a pooled analysis for overall survival, disease free survival and PFS meta-analysis using clinical studies investigating the prognostic role of *PI3KCA* mutations in BC, proved that mutations of this gene are predictive of a worse clinical survival outcome for the patients (HR = 1.67, 95%CI: 1.15-2.43; P = 0.007)[23]. Unfortunately, there was not enough clinical evidence to prove that *PI3KCA* mutations could be predictive for therapy-efficacy and further clinical trials investigating the role of such mutations could be precious for guiding clinicians to choose those patients who would benefit from PI3K-mTOR inhibition treatment.

In conclusion, the inhibition of the PI3K-mTOR pathway remains one of the major goals for the treatment of HR+ advanced BC patients. It is known that targeting more upstream effectors, such as PI3K, which proved to be very promising based on *in vitro* evidence, lead to major side effects in clinical studies, limiting the clinical development of this type of drugs. This class of inhibitors, however, seems to be beneficial for patients harboring *PI3KCA* mutations, thus producing less toxicity.

Currently, everolimus remains the best option in the clinical setting in combinational therapies for HR+ BC patients. Moreover, several studies are directed to explore the clinical benefits of everolimus in combination with other drugs, as illustrated in Table 1. Of note, 10%-15% of patients are intrinsically resistant to everolimus treatment[24]. For this reason, dual inhibition of the pathway by targeting mTORC1/2 proteins have been tested (Table 1), and some phase 1 studies are exploring new PI3K inhibitors, such as gedatolisib (NCT02626507) in neoadjuvant setting for ER+, HER2- BC patients. CUDC-907, an histone deacetylase and PI3K inhibitor (NCT02307240), and AZD8186, a PI3K-β inhibitor (NCT03218826), have been tested in advanced solid tumours, including BC. Having biomarkers from the metastatic setting to predict the responsiveness/resistance to anti-PI3K/mTOR/Akt drugs are thus an urgent task, and many efforts in this direction are expected. Finally, with the advancement of immune-therapy, trials testing the combination of immune-therapy with everolimus are warranted.

Table 1 Clinical trials inhibiting PI3K-mTOR pathway in HR+, HER2- breast cancer patients

Clinical trial identifier	Phase	Year	Administered drug(s) (target)	Primary endpoint	Target/s of the PI3K-mTOR pathway
NCT00699491	1/2	18.06.2008	Cixutumumab + temsirolimus	ORR	mTOR
Bolero 2; NCT00863655 [16]	3	18.03.2009	Everolimus + exemestane or placebo + exemestane	PFS	mTOR
NCT00876395	3	06.04.2009	Everolimus + placebo	PFS	mTOR
NCT01219699	1	13.10.2010	BYL719 + fulvestrant	MTD	PI3Kα
NCT01283789	2	26.01.2011	Everolimus + lapatinib	ORR	mTOR
TAMRAD; NCT01298713 [14]	2	18.02.2011	Everolimus + tamoxifen	CBR	mTOR
BELLE-2; NCT01610284	3	04.06.2012	Fulvestrant ± BKM120 (AIs refractory pts)	PFS	PI3K
BELLE-3; NCT01633060	3	04.06.2012	Fulvestrant ± BKM120 (Pts previously on mTOR inhibitors)	PFS	PI3K
NCT01627067	2	25.06.2012	Everolimus + exemestane + metformin	PFS	mTOR
NCT01674140	3	28.08.2012	Everolimus + standard adjuvant endocrine therapy (tamoxifen citrate, goserelin acetate, leuprolide acetate, anastrozole, letrozole, or exemestane)	Invasive DFS	mTOR
Bolero 4; NCT01698918	2	03.10.2012	Everolimus + letrozole	PFS	mTOR
NCT01776008	2	25.01.2013	everolimus + exemestane; MK2206 + anastrozole ± goserelin acetate	CRR	Akt
NCT01783444; Bolero-6	2	05.02.2013	Everolimus or capecitabine or everolimus + exemestane	PFS	mTOR
NCT01791478	1	15.02.2013	BYL719 + letrozole	DLT	PI3Kα
NCT01805271	3	06.03.2013	Everolimus + placebo	DFS	mTOR
CLEE011X2107; NCT01872260	1b/2	07.06.2013	Letrozole + ribociclib + alpesilib	DLT	PI3K
INPRES; NCT01948960	4	24.09.2013	Everolimus	AUC correlation with age and/or obesity	mTOR
NCT01992952	1/2	25.11.2013	Fulvestrant ± AZD5363 or placebo	MTD	Akt
PEARL; NCT02028364	2	07.01.2014	Everolimus + exemestane	PFS and response by PET	mTOR
NCT02035813	2	14.01.2014	Everolimus + eribulin	PFS	mTOR
NCT02049957	1b/2	30.01.2014	MLN0128 + exemestane *vs* MLN0128 + fulvestrant	Participant with AE and Clinical Benefit Rate	mTORC1/2
NCT02057133	1b	06.02.2014	LY2835219 + exemestane + everolimus *vs* exemestane + everolimus *vs* LY2835219 + trastuzumab *vs* LY2835219 + anastrazole *vs* LY2835219 + tamoxifen *vs* LY2835219 + letrozole *vs* LY3023414 + LY2835219 + fulvestrant	Safety	mTOR
NCT02058381	1b	10.02.2014	BYL719 + BKM120	MTD	PI3Kα + PI3K
NCT02077933	1	04.03.2014	Alpelisib + everolimus + exemestane	DLT and MTD	PI3K + mTOR

NCT02123823	1b	28.04.2014	BI 836845 + everolimus + exemestane	PFS, MTD and DLT	mTOR
NCT02216786	2	15.08.2014	Everolimus + fulvestrant *vs* AZD2014 + fulvestrant	PFS	mTOR + mTORC1/2
NCT02236572	2	10.09.2014	Everolimus + aromatase inhibitor	Preoperative Endocrine Prognostic Index	mTOR
NCT02269670	2	21.10.2014	Everolimus + endocrine therapy (anastrozole, letrozole, tamoxifen citrate, fulvestrant or megestrol acetate)	PFS and ORR	mTOR
NCT02285179	1/2	16.11.2014	Taselisib + tamoxifen	MTD	PI3K
NCT02291913	2	17.11.2014	Everolimus + anti-estrogen therapy (exemestane, tamoxifen, fulvestrant, anastrozole, letrozole, toremifine)	PFS	mTOR
Sandpiper NCT02340221	3	16.01.2015	Taselisib + fulvestrant *vs* placebo + fulvestrant	PFS	PI3K
FEVEX; NCT02404051	3	31.03.2015	Everolimus + exemestane *vs* everolimus + exemestane (at progression fulvestrant)	PFS	mTOR
NCT02404844	2	01.04.2015	BKM120 + tamoxifen	PFS	PI3K
NCT02379247		04.05.2015	BYL719 + Nab-paclitaxel	DLT , ORR for Phase II	PI3Kα
SOLAR-1; NCT02437318	3	07.05.2015	Fulvestrant ± alpelisib (AIs refractory pts)	PFS	PI3K
NCT02506556	2	23.07.2015	BYl719	ORR	PI3Kα
NCT02511639	3	30.07.2015	Everolimus + aromatase inhibitors	PFS	mTOR
NCT02520063	1/2	11.08.2015	Letrozole + everolimus + TRC105	MTD	mTOR
NCT02619669	1	02.12.2015	Sapanisertib + letrozole	TAE	mTORC1/2
TRINITI-1; NCT02732119	1/2	08.04.2016	Everolimus + ribociclib + exemestane	MTD	mTOR
NCT02742051	2	18.04.2016	Everolimus + letrozole *vs* neoadjuvant+ fluorouracil, epirubicin + cyclophosphamide (FEC)	ORR	mTOR
NCT02871791	2	18.08.2016	Everolimus + exemestane	DLT	mTOR
NCT02988986	2	12.12.2016	Sapanisertib + tamoxifen	Change in Ki67 expression	mTORC1/2
CLEVER; NCT03032406	2	26.01.2017	Everolimus ± hydroxychloroquine	AE	mTOR
NCT03056755	2	17.02.2017	Alpelisib + fulvestrant + letrozole (AIs and CDK4/6 refractory pts)	DFS	PI3K
NCT03128619	1/2	25.04.2017	Copanlisib + letrozole + palbociclib	MTD	PI3K
EVEREXES; NCT03176238	3	05.06.2017	Everolimus + exemestane	AE	mTOR
NCT03207529	1	02.07.2017	BYL719 + enzalutamide	MTD	PI3Kα
NCT03377101	2	19.12.2017	Fulvestrant + palbociclib ± copanlisib	DLT	PI3K

DLT: Dose limiting toxicity; PFS: Progression free survival; DFS: Disease free survival; MTD: Maximum tolerated dose; AE: Adverse events; CBR: Clinical benefit rate; ORR: Overall response rate; CRR: Clinical response rate; PI3K-mTOR: Phosphatidylinositol 3-kinase-mammalian target of rapamycin.

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MINIREVIEWS

Role of senescence induction in cancer treatment

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Abstract

Cellular senescence is a form of permanent cell cycle arrest that can be triggered by a variety of cell-intrinsic and extrinsic stimuli, including telomere shortening, DNA damage, oxidative stress, and exposure to chemotherapeutic agents and ionizing radiation. Although the induction of apoptotic cell death is a desirable outcome in cancer therapy, mutations and/or deficiencies in the apoptotic signaling pathways have been frequently identified in many human cancer types, suggesting the importance of alternative apoptosis-independent therapeutic approaches for cancer treatment. A growing body of evidence has documented that senescence induction in tumor cells is a frequent response to many anticancer modalities including cyclin-dependent kinases 4/6 small molecule inhibitor-based targeted therapeutics and T helper-1 cytokine-mediated immunotherapy. This review discusses the recent advances and clinical relevance of therapy-induced senescence in cancer treatment.

Key words: Cellular senescence; Cancer treatment; Chemotherapy; Ionizing radiation; Cyclin-dependent kinases 4/6 inhibitor; Aurora kinase inhibitor; Immunotherapy; T helper-1 cells; T helper-1 cytokines

Core tip: Both *in vitro* and *in vivo* studies have revealed that senescence induction in human cancer cells is a prominent response to chemotherapy and irradiation. A senescent phenotype has been detected in clinical tumor samples of breast cancer patients following preoperative neoadjuvant chemotherapy. Immunotherapy-induced senescence of cancer cells contributes to tumor regression *in vivo*. The induction of cancer cell senescence appears to be a major mechanism of action of cyclin-dependent kinases 4/6 small molecule inhibitor-based targeted therapy. Collectively, these preclinical and clinical observations have demonstrated an important role for senescence induction in cancer treatment.

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INTRODUCTION

Cellular senescence is an anti-proliferative program that restrains tumorigenesis *via* limiting the propagation and transformation of aging and damaged cells[1,2]. Senescence can be induced by a variety of cell-intrinsic and extrinsic stimuli, including telomere shortening, DNA damage, oxidative stress, chemotherapeutic agent treatment, radiation exposure, and the activation of oncogenes such as *Ras*[3-6]. Senescent cells are in a state of irreversible cell cycle arrest and are characterized by a flat and enlarged morphology, elevated senescence-associated β-galactosidase activity, and the activation of the p53-p21 and p16-Rb signaling pathways[2,7]. Additional characteristics of senescence cells include the presence of senescence-associated heterochromatic foci and the senescence-associated secretory phenotype[8,9]. Traditionally, senescence induction is considered to be an important mechanism of cancer prevention and cellular aging[10]. However, numerous recent studies have revealed that senescence is a prominent solid tumor response to therapy in which cancer cells evade apoptosis and instead enter into a stable and prolonged cell cycle arrest[3,5]. Furthermore, targeted therapeutics and cancer immunotherapies also have been shown to cause cancer regression *via* the induction of senescence in tumor cells[11,12]. These findings underscore the significant implications of senescence induction in cancer treatment.

INDUCTION OF TUMOR CELL SENESCENCE IS AN IMPORTANT OUTCOME OF CHEMOTHERAPY

Cellular senescence is a state of permanent cell growth arrest that often has been included with apoptosis as one of the terminal outcomes of cancer treatment. It is well-established that different classes of chemotherapeutic agents and ionizing radiation (IR) induce senescent-like phenotypes in tumor cells both *in vitro* and *in vivo*[2,5,11,13]. Our recent studies indicate that resveratrol induces premature senescence in lung cancer cells *via* promoting reactive oxygen species (ROS)-mediated DNA damage[14]. Inactivation of Myc results in tumor regression through induction of senescence but not apoptosis in hepatocellular carcinoma and lymphoma cells[15]. In addition, it has been reported that the restoration of p53 promotes tumor regression *in vivo via* induction of senescence in tumor cells[16,17]. More importantly, Schmitt *et al*[13] showed that senescence induction contributes directly to the outcome of chemotherapy *in vivo*. In agreement with this finding, there is evidence that the senescent phenotype is present in clinical tumor samples of breast cancer patients after undergoing preoperative neoadjuvant chemotherapy[18]. Moreover, our recent studies found that the number of senescent cells is markedly increased in tumor samples of lung cancer patients as compared to normal lung tissues (Figure 1). However, the clinical relevance of the presence of senescent cells in tumor samples has yet to be determined.

Notably, human tumors may harbor various types of defects in the apoptotic signaling pathways (*e.g.*, loss of p53 and overexpression of BCL2) and, as a result, are resistant to apoptosis-based anticancer therapies[19,20]. In these conditions, a senescence-targeted strategy is likely to be a more effective and practical than traditional apoptosis-inducing approaches. A welcome benefit to this approach is that therapy-induced senescence can be achieved using much lower doses of therapy than those required to induce apoptosis[2,14]. Compared to the traditional apoptosis-inducing strategies, this low dose approach would significantly reduce the side effects of anticancer therapy and thus improve the quality of life for cancer patients.

ROLE OF SENESCENCE INDUCTION IN CANCER RADIOTHERAPY

Radiotherapy is used in over 50% of patients during the course of cancer treatment and is effective both as a curative modality and for palliation[21]. However, many epithelial-derived tumors including lung cancer have been shown to be resistant to

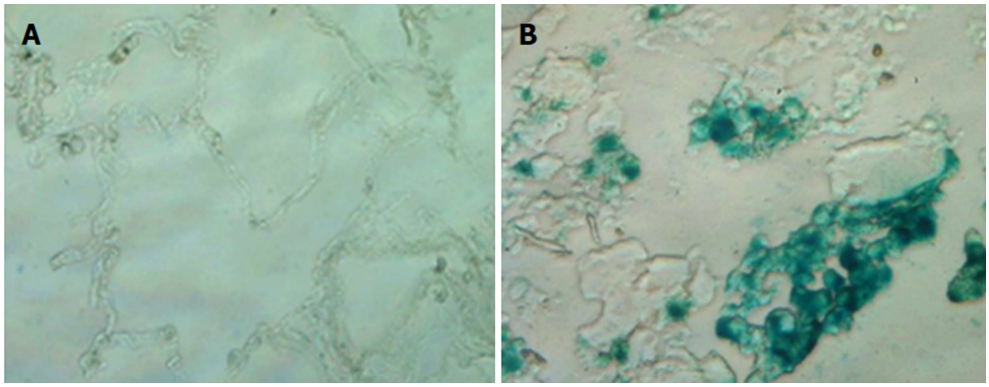

Figure 1 Number of senescent cells is significantly higher in tumor tissues of lung cancer patients than in normal lung tissues. Senescence-associated β-galactosidase staining was performed to analyze senescent cells in normal human lung tissues (A) and tumor tissues of lung cancer patients (B), respectively.

radiation-induced apoptosis[6,20]. Consistent with these observations, our recent studies have demonstrated that IR primarily induces premature senescence rather than apoptosis in human non-small cell lung cancer (NSCLC) cells[6]. Subsequent mechanistic studies revealed that the p53-p21 signaling pathway plays a critical role in modulating IR-induced senescence in NSCLC cells (Figure 2). More importantly, we showed that pharmacologic activation of p53 by Nutilin-3 sensitizes NSCLC cells to radiation by enhancing IR-induced senescence[6]. These results suggest that pharmacological promotion of senescence induction can be exploited as a novel and effective therapeutic approach to improve the efficacy of lung cancer radiotherapy.

MicroRNA-34a (miR-34a) has been shown to be a p53 responsive miRNA that is involved in regulating senescence induction[22-24]. Our recent studies showed that the expression of miRNA-34a was increased substantially in human NSCLC cells after exposure to IR[25]. Moreover, we found that treatment with synthetic miR-34a mimics enhances the anti-cancer effects of irradiation by promoting senescence induction *via* targeting the Myc oncoprotein in NSCLC cells (Figure 2). These findings not only provide new insights into the mechanisms by which IR induces senescence in lung cancer cells but also support the hypothesis that pharmacological manipulation of senescence induction should be explored as a new therapeutic strategy for improving the outcome of cancer radiotherapy.

INDUCTION OF SENESCENCE IS A MAJOR MECHANISM OF ACTION OF CYCLIN-DEPENDENT KINASES 4/6 INHIBITION-BASED TARGETED THERAPEUTICS

The cyclin-dependent kinases (CDKs) are a large family of serine-threonine kinases that play a pivotal role in regulating cell cycle progression. Commitment to cell cycle entry occurs during the G1 phase, when CDK4 and CDK6 form active complexes with one of the three D-type cyclins (D1, D2, or D3). Cyclin D-CDK4/6 complexes promote G1/S transition by phosphorylating the Retinoblastoma tumor suppressor (Rb), which in turn releases its suppression of the E2F transcription factor, resulting in initiation of E2F-dependent gene transcription and DNA synthesis. CDK4 and CDK6 are overexpressed in the majority of human cancers and they presumably promote tumorigenesis by suppressing senescence in cancer cells[11,26,27]. Both preclinical studies and clinic trials have demonstrated the therapeutic potential of CDK4/6 small molecule inhibitors against several solid tumors[28-31]. Recently, a CDK4/6-specific inhibitor, palbociclib (also known as PD033299[1], was approved by the FDA for the treatment of advanced estrogen receptor-positive breast cancer[32].

It is well established that CDK4/6 inhibitors suppress tumor growth by the induction of senescence in various type of cancer cells[11,26,27,32]. Mechanistic studies have revealed that CDK4/6 suppress tumor cell senescence *via* phosphorylating and activating the Forkhead Box M1 (FOXM1) transcription factor, and that activation of FOXM1 may inhibit the induction of senescence in tumor cells by repressing ROS-mediated oxidative stress[33]. However, the precise mechanisms whereby FOXO1 controls ROS and oxidative stress remain unclear. Promyelocytic leukemia (PML) acts as a tumor suppressor by inducing senescence in response to oncogenic stress[34,35]. Interestingly, Acevedo *et al*[36] showed that CDK4/6 suppress PML-induced senescence in tumor cells, suggesting that CDK4/6 inhibitors such as PD0332991 may

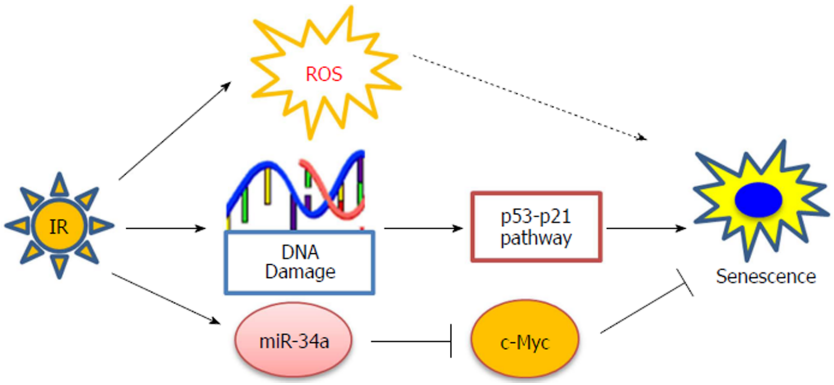

Figure 2 This model summarizes the mechanisms underlying ionizing radiation-induced senescence in non-small cell lung cancer cells. Ionizing radiation (IR) exposure increases the levels of reactive oxygen species in tumor cells and oxidative stress is known to trigger the induction of senescence. IR exposure leads to DNA damage which in turn can activate the p53-p21 senescence pathway. Irradiation up-regulates the expression of miR-34a that inhibits c-Myc, resulting in the induction of senescence. ROS: Reactive oxygen species; IR: Ionizing radiation.

induce senescence in cancer cells by blocking CDK4/6-mediated suppression of PML's senescence-inducing function. Moreover, CDK4/6 inhibition has been shown to be efficacious against therapy-resistant HER2 positive breast cancers[37]. These results imply that although some tumors may be resistant to apoptosis-inducing treatments, it is likely that they are still responsive to senescence-targeted therapies.

AURORA KINASE INHIBITION INDUCES SENESCENCE IN TUMOR CELLS BOTH *IN VITRO* AND *IN VIVO*

Aurora kinases are a family of serine/threonine mitotic kinases that regulate a diverse set of mitotic processes including spindle formation, centrosome segregation, checkpoint activation and kinetochore-microtubule connections[38,39]. Aurora A kinase (AurA) is located on 20q13.2, a chromosomal region that is frequently amplified in many human cancers. Overexpression of AurA correlates with poor clinical outcomes in patients with hormone-related cancers[40]. Aurora kinase inhibitors (AKIs) are a promising class of drugs for cancer treatment. Several small molecule AKIs including MK-0457, PHA-739358 and MLN8237 have been investigated in clinical trials for the treatment of human cancers[41-43].

Alisertib (MLN8237) is an orally bioavailable, second-generation selective inhibitor of Aurora kinases which binds to AurA and prevents its phosphorylation and activation[44]. It has been shown that senescence is likely a terminal outcome of AurA inhibition and that pharmacological inhibition of AurA induces senescence in colon cancer cells both *in vitro* and *in vivo*[45]. AurA is overexpressed in gliomas and treatment with Alisertib induces senescence and differentiation in glioblastoma cells[46]. Moreover, recent studies have revealed that p53 and p73 tumor suppressors play a critical role in modulating tumor cell responses to AKIs. Tentler *et al*[47] reported that Alisertib treatment resulted in apoptosis in human triple-negative breast cancer (TNBC) cells with functional p53 and p73, whereas it induced senescence in cells lacking p53 and p73. These findings suggest that AKI may induce growth arrest in TNBC through a p53- and p73-independent mechanism. Nevertheless, further studies are warranted to better understand the in-depth mechanisms whereby AKIs induce senescence in human tumor cells.

INDUCTION OF CANCER CELL SENESCENCE CONTRIBUTES TO IMMUNOTHERAPY-INDUCED TUMOR REGRESSION

Immunotherapy has shown promising efficacy against human cancers both in preclinical studies and in clinical trials[48-50]. T helper-1 (Th1) cells play a critical role in mediating the antitumor adoptive immune response[51,52]. Th1 T-cell infiltration is associated with better outcomes and Th1 cytokines are more effective at promoting the antitumor functions of CD40L-activated macrophages as compared to Th2 cytokines[53]. Moreover, Müller-Hermelink *et al*[54] showed that treatment with T

antigen (Tag)-specific Th1 cells induced tumor dormancy and doubled the survival of tumor-bearing mice by arresting tumor growth, without detectable evidence of tumor cell cytotoxicity, necrosis or apoptosis. These findings suggest that other alternative non-cytotoxic mechanisms are likely involved in Th1- and Th1 cytokine-mediated immunotherapy. In agreement with this idea, it has been shown that treatment with Th1 cytokines, IFN-γ and tumor necrosis factor (TNF) induces senescence in cancer cells[12].

It has been reported that cancer immunotherapy causes tumor growth arrest and regression without any clear evidence of tumor cell death or cellular toxicity[55,56]. Moreover, it has been shown that immunotherapy-induced tumor growth arrest was associated with an increase in interferon (IFN)-γ-producing $CD4^+$ Th1 cells, but not $CD8^+$ cytotoxic T lymphocytes[55-57]. These observations suggest that senescence induction may contribute to the outcome of immunotherapy. In agreement with this, Braumüller *et al*[12] showed that Th1 immunotherapy arrests tumor progression through IFN-γ- and TNF-induced cancer cell senescence *in vivo*. Their subsequent mechanistic studies revealed that TNFR1 signaling is required for Th1 immunotherapy-induced tumor cell senescence and that activation of the p16-Rb pathway is involved in senescence induction[12]. In line with these findings, it has been shown recently that CDK4/6 inhibition-induced tumor cell senescence promotes antitumor immunity in preclinical models[58,59]. Furthermore, there is evidence that IL-12 inhibits the growth of human sarcoma cells by senescence induction[60]. Taken together, these results highlight a novel link between cancer immunotherapy and the induction of senescence in tumor cells.

CONCLUSION

Given the fact that many human cancers may harbor different defects in the apoptotic signaling pathways, and thus are inherently resistant to chemoradiotherapy-induced apoptosis, there is a critical need for the development of innovative apoptosis-independent approaches for cancer therapeutics. The induction of tumor cell senescence has been well-established as a prominent therapeutic response of cancer cells to chemotherapy, radiation, small-molecule inhibitor-based targeted therapeutics and immunotherapy. These results underscore the important implications of senescence induction in cancer treatment. Although senescent cells were detected in clinical tumor samples of cancer patients, the clinical significance and applications of therapy-induced senescence remain incompletely understood. It is still unclear if senescence markers in patient tumor samples will be useful for prognosis prediction and/or therapeutic efficacy evaluation in the clinic. In addition, further studies, particularly clinical investigations, are necessary to better elucidate the clinical relevance and significance of therapy-induced senescence in the treatment of cancer.

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ORIGINAL ARTICLE

Basic Study

Combination immunotherapy with Survivin and luteinizing hormone-releasing hormone fusion protein in murine breast cancer model

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Abstract

AIM

To investigate the therapeutic potential of two recombinant proteins, Survivin and luteinizing hormone-releasing hormone (LHRH) fusion protein [LHRH(6leu)-LTB] for immunotherapy of breast cancer.

METHODS

Murine 4T-1 breast cancer model was used to evaluate the efficacy of recombinant proteins *in vivo*. Twenty four Balb/c mice were divided into 4 groups of 6 mice each. Recombinant Survivin and LHRH fusion protein, alone or in combination, were administered along with immunomodulator *Mycobacterium indicus pranii* (MIP) in Balb/c mice. Unimmunized or control group mice were administered with phosphate buffer saline. Each group was then challenged with syngeneic 4T-1 cells to induce the growth of breast tumor. Tumor growth was monitored to evaluate the efficacy of immune-response in preventing the growth of cancer cells.

RESULTS

Preventive immunization with 20 μg recombinant Survivin and MIP was effective in suppressing growth of 4T-1 mouse model of breast cancer (P = 0.04) but 50 μg dose was ineffective in suppressing tumor growth. However, combination of Survivin and LHRH fusion protein was more effective in suppressing tumor growth (P = 0.02) as well as metastasis *in vivo* in comparison to LHRH fusion protein as vaccine antigen alone.

CONCLUSION

Recombinant Survivin and MIP suppress tumor growth significantly. Combining LHRH fusion protein with Survivin and MIP enhances tumor suppressive effects marginally which provides evidence for recombinant Survivin and LHRH fusion protein as candidates for translating the combination cancer immunotherapy approaches.

Key words: Immunotherapy; Survivin; Luteinizing hormone-releasing hormone fusion protein; Combination immunotherapy; Breast cancer

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Core tip: Targeting Survivin for treatment of cancer is an emerging trend in cancer immunotherapy. In this study we optimized the dose of recombinant full-length Survivin for tumor growth inhibition. Since luteinizing hormone-releasing hormone (LHRH) is also known to support breast cancer in case of premenopausal women, we combined recombinant LHRH fusion protein with Survivin and *Mycobacterium indicus pranii* and obtained a positive anti-tumor response. We report our results of using recombinant Survivin protein and LHRH fusion protein as potential vaccine antigens for immunotherapy of murine model of breast cancer which was developed by injecting 4T-1 mammary cell line in syngeneic Balb/c mice. Immunized mice challenged with syngeneic 4T-1 cells exhibited suppression of tumor growth and metastasis in lungs in comparison to control mice.

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INTRODUCTION

Combination methods that combine two or more therapeutic agents have become a principal treatment modality for breast cancer, especially for metastatic breast cancer[1]. The rationale behind combination approach is to enhance therapeutic response by targeting multiple pathways involved in the complex process of oncogenesis. However, none of the empirically defined combination of drugs such as taxanes/anthracyclins has been able to overcome the problems of drug resistance and metastasis in breast cancer and impart a satisfactory toxicity profile when administered to patients[2,3]. In order to overcome the drawbacks associated with prevalent drug combinations, immunotherapy is proposed as a viable approach for developing targeted and safe treatment options for cancer[4,5]. Advancements in molecular mechanisms of cancer cell survival have led to identification of new targets which can be employed for immunotherapy[6].

Cancer immunotherapy approach using immunomodulators, monoclonal antibodies or vaccines against tumor growth promoters, have shown benefit in preclinical models of many cancers and clinical trials[7,8]. However since tumor cells often develop immune evasion mechanisms[9], combination of tumor antigens may be used to counteract immune resistance by tumor cells. We hypothesize that combination immunotherapy based on potent tumor antigen and hormone, may present a viable approach to enhance the therapeutic landscape and effectively counteract the heterogeneous process of tumor development in breast cancer.

Survivin is a tumor antigen exclusively expressed on tumor cells, essential for cancer cell survival, and its overexpression is associated with aggressiveness of the disease[10,11]. It has been widely explored as a candidate antigen for cancer immunotherapy[12]. Similarly, many tumor cells from breast, prostate, ovary or endometrial origin are hormone dependent for their survival[13]. Therefore, immune system mediated neutralization of hormones is also a key therapeutic strategy for hormone dependent cancers[14]. Immunization against hormones such as luteinizing hormone-releasing hormone (LHRH) using LHRH based peptide vaccines have shown efficacy as vaccine antigens in preclinical models of hormone dependent cancers such as breast and prostate cancer[15].

We have previously shown that preventive immunization with a combination of recombinant Survivin protein and an immunomodulator *Mycobacterium indicus pranii* (MIP) provides protection to Balb/c mice when challenged with syngeneic mammary tumor 4T-1 cells[16]. This communication describes the studies undertaken to determine the optimum dose of Survivin antigen for maximum tumor suppressive effect. Also investigated is the synergy between Survivin and LHRH fusion protein as vaccine antigens for immunotherapy of murine model of breast cancer. 4T-1 murine breast cancer cells not only display Survivin as tumor antigen but also have receptors for LHRH[17,18]. Thus active immunization against both the Survivin and LHRH, is likely to neutralize Survivin and also make LHRH unavailable for uptake by the

cancer cells, hence generate enhanced protective immune response directed against the cancer cells.

MATERIALS AND METHODS

Cloning and expression of recombinant mouse Survivin and LHRH fusion protein

Survivin and LHRH fusion protein, were used as vaccine antigens. These antigens were expressed as recombinant proteins using *E. coli* based host-vector systems as described previously[16,19]. Survivin and LHRH fusion protein were purified as approximately 17 kDa and 14 kDa size proteins, respectively using affinity based Ni-NTA chromatography. Purified proteins were adsorbed on alum before administration as immunogens in mice. MIP was used as immunomodulator along with the recombinantly made vaccine antigens.

Immunogenicity and efficacy of Survivin vaccine in vivo

Eight-10 week old inbred Balb/c mice were used for conducting the immunogenicity and efficacy studies of the Survivin vaccine alone *in vivo*. For determining the optimal Survivin dose, study was carried out in 3 groups of mice. Mice were divided into 3 groups randomly with 6 animals in each group. Group 1 was administered phosphate buffer saline (PBS) buffer. While group 2 mice was administered alum adsorbed 20 μg Survivin vaccine (+ MIP), group 3 mice received alum adsorbed 50 μg Survivin dose along with MIP. Five × 10^6 cells of heat-killed MIP were used in each dose wherever required. Each group of mice was given 2 boosters of its respective dose at 15 d interval after the primary immunization. Sixty days after the last immunization, mice in all the groups were challenged with 3.0 × 10^5 4T-1 murine breast cancer cells subcutaneously. Third booster of the vaccine was administered on day 18^{th} after the challenge, when tumors were palpable. Tumor size was measured every alternate day till the end of the study. Tumor volume was measured bi-dimensionally with digital vernier calipers and tumor volume calculated using the formula: $(a \times b^2)/2$, where "a" is the largest diameter and "b" is the perpendicular diameter. Tumor volumes were measured till day 35 post challenge with 4T-1 cells. Results are expressed as mean ± SD of tumor volume for each group.

Immunogenicity and efficacy of combination of Survivin and LHRH fusion protein in comparison to Survivin and LHRH fusion protein alone

Studies were designed in 4 groups of mice each consisting of 6 of 8-10 week old inbred Balb/c mice. Recombinant antigen(s) were administered intramuscularly along with heat-killed 5.0 × 10^6 cells of MIP. Group 1 of mice received 20 μg of Survivin protein alone, group 2 received 20 μg of LHRH(6leu)-LTB (LTB- heat-labile enterotoxin of *E. coli*) protein alone and group 3 received combination of 20 μg of Survivin and 20 μg of LHRH(6leu)-LTB proteins. Each group of mice was given 2 boosters of its respective vaccine dose at 15 d interval after the primary immunization. A control group of mice was administered PBS buffer. Fifty-two days after first dose, mice were inoculated with 4T-1 breast cancer cells (3.0 × 10^5 cells in 100 μL of PBS buffer) subcutaneously on right flank. It was recorded as day 0 of tumor challenge. Tumors were palpable in mice in 18 d after tumor cell inoculation. Third booster of vaccine antigen(s) along with immunomodulator MIP was given intramuscularly at the same day when tumors were palpable. Tumor volume was measured till the end of the study. Mice were sacrificed at the end of the study and lungs were examined for the presence of pulmonary metastases.

Estimation of Interferon-γ levels in sera

Sera were collected from mice and assayed for Interferon gamma levels using commercially available enzyme linked immunosorbent assay (ELISA) kit (E Biosciences, United States) as per the manufacturer's protocol. Briefly, 96-well microtitre plate was coated with 100 μL/well of IFN-γ capture antibody at a dilution of 1:1000 and incubated overnight at 4 °C. Plates were then washed thrice with PBST buffer (PBS buffer supplemented with 0.05% Tween-20) and then blocked with ELISA diluent (250 μL/well) at 37 °C for 2 h. Standard was serially diluted in ELISA diluent to obtain concentration in the range of 15-2000 pg/mL and 100 μL of standard of each concentration was added to the corresponding wells. Serum samples were pooled for each group of mice, diluted in ELISA diluent and added to the respective wells at 100 μL/well. Plate was then incubated for 2 h at 37 °C. Biotinylated anti-IFN-γ detection antibody was added to each well (100 μL/well) at a dilution of 1:1000 followed by incubation of 100 μL/well of Avidin-HRP diluted at 1:250 for 30 min. After washing, TMB was added in each well and incubated in dark for 15 min. Reaction was stopped with 2N H_2SO_4 and absorbance was read at 450 nm. Standard curve was prepared by

plotting the average absorbance of each standard on the vertical axis versus the corresponding IFN-γ standard concentration on the horizontal axis and fitted using 4 parameter logistic (4PL) regression analysis. Actual concentration of IFN-γ in mouse serum samples was calculated by extrapolating OD values using the 4PL curve.

ELISA

ELISA technique was used for the analysis of immunogenicity of the recombinant proteins in mice. ELISA plate (Nunc, Thermo Fisher Scientific, United States) was coated with 100 μL (5 μg/mL concentration) of recombinant Survivin or LHRH(6leu)-LTB protein, and incubated overnight at 4 °C. Plate was washed thrice with 10 mmol/L PBS, pH 7.4 supplemented with 0.05% Tween 20 followed by blocking with 2% Bovine serum albumin (BSA) in PBS buffer. Plate was washed with PBST buffer thrice. One hundred micro lift of the serum diluted at 1:5000 in PBS buffer, was added to the wells of the plate, and incubated for 1 h at 37°C. HRP-labeled goat anti-mouse antibody was added at a dilution of 1:10000 to each well and color was developed by adding 100 μL of O-phenylenediamine (OPD) solution containing Hydrogen Peroxide. The reaction was stopped by addition of 50 μL of 2N H_2SO_4. Plate was read at 492 nm in an ELISA plate reader (ELX 800MS, Biotek, United States) and results were expressed as mean ± SD of absorbance values.

Ethics statement

Pathogen free female Balb/c mice used in the study were procured from National Institute of Nutrition, Hyderabad. Animals were housed at animal facility of Amity University Uttar Pradesh and were given ad libitum access to water and food. All experimental procedures were in strict agreement with committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines for the care and use of laboratory animals and approved by institutional animal ethics committee (IAEC) of Amity University Uttar Pradesh.

Statistical analysis

Data was analyzed using two tailed student's *t* test. *P* value < 0.05 was reported as significant. Data was analyzed using graph pad prism software.

RESULTS

Optimum dose of Survivin antigen dose for the effective inhibition of tumor growth in 4T-1 model

We reported previously that the recombinant Survivin vaccine is efficacious in preventing the growth of 4T-1 based murine model of breast cancer[16]. Increase in vaccine dose from 5 μg to 10 μg, led to better immunogenicity which resulted in significant reduction in tumor suppression[16]. Therefore, it was decided to test if it was possible to employ doses higher than previously used 10 μg dose to inhibit the tumor maximally. Thus the studies were designed to evaluate the efficacy of the vaccine at 20 μg and 50 μg doses. Pathogen-free mice of similar weight (6 mice/group) were first immunized with alum adsorbed recombinant Survivin vaccine along with immune-modulator MIP. Mice were then challenged with syngeneic 4T-1 cells and observed for the tumor growth in presence of pre-formed anti-Survivin antibodies. Three immunizations of alum adsorbed recombinant Survivin and MIP were administered at 15 d interval following which 4T-1 cancer cells were injected at day 90. A booster of the Survivin vaccine was given at day 108 when tumors were palpable. Tumor volume was measured and continued till 35 d post injection of cancer cells. Figure 1 shows the volume of the tumor developed in mice immunized with 20 μg and 50 μg of Survivin protein administered along with MIP. It is evident from the Figure that significant decrease in tumor volume was observed in mice immunized with 20 μg dose of the vaccine ($P = 0.04$) in comparison to control mice. However increase in vaccine dose to 50 μg did not lead to any significant inhibition in tumor volume thus reflecting dose-inhibition related effects. Figure 2 shows the IFN-γ levels determined in the sera collected from immunized mice. Survivin at 20 μg dose induced higher levels of IFN-γ in comparison to 50 μg dose, which is in accordance with the observed efficacy of the anti-Survivin antibodies in preventing the growth of tumor at 20 μg dose. The mice in each group did not show any visible adverse effects of the treatment.

Active immunization against a combination of Survivin and LHRH fusion protein

After finding the optimal dose of Survivin vaccine, studies were advanced to investigate the additive effect of LHRH vaccine, if any, in terms preventing the 4T-1

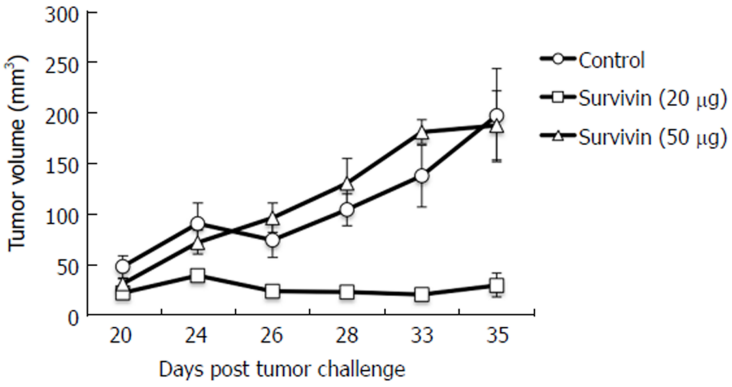

Figure 1 4T-1 breast tumor volume in mice injected with phosphate buffer saline buffer (circle), 20 μg Survivin + *Mycobacterium indicus pranii* (square) and 50 μg Survivin + *Mycobacterium indicus pranii* (triangle) at different time points after tumor was palpable.

breast cancer cells. Studies were carried out in 4 groups of pathogen free mice (6 mice/group). A group of mice was immunized by combination of alum adsorbed Survivin and LHRH(6leu)-LTB proteins along with immunomodulator MIP. Two groups of mice were injected with Survivin alone (+ MIP) and LHRH(6leu)-LTB alone (+ MIP), respectively. Mice receiving the PBS buffer formed the control group. Mice were immunized thrice at 15 d interval with combination or individual antigens following which 4T-1 tumor cells were injected at 50th day. Boosters of the vaccine antigens were administered when the tumors became palpable. The tumor size was measured post 35 d of tumor cell challenge. Though LHRH(6leu)-LTB alone prevented the growth of tumor size in comparison to control group mice but did not reach statistical significance, inhibition was more significant with Survivin alone (P = 0.04). Combining it with LHRH fusion protein led to marginally better tumor suppression, P = 0.02 (Figure 3). There were no adverse effects of the treatment observed in the mice of each group.

Evaluation of metastasis of primary tumor to lungs

Mice challenged with the tumor cells were followed to evaluate the efficacy of the active immunization by Survivin and LHRH fusion protein alone or in combination in terms of preventing cancer metastasis. The secondary growth of the cancer was studied by counting the number of nodules in the lungs of the mice challenged with the tumor cells. Table 1 shows the mean of number of nodules counted in various groups at the end of the study. Mice immunized with the combination of Survivin and LHRH(6leu)-LTB along with MIP did not show any visible nodules in the lungs 30 d after challenge with 4T-1 cells whereas mice immunized with either Survivin + MIP or LHRH(6leu)-LTB + MIP showed presence of tumor nodules in lungs. Thus combination of Survivin and LHRH(6leu)-LTB along with immunomodulator MIP is capable of controlling the pulmonary metastasis besides suppressing the primary tumor growth.

Characterization of total IgG response

Sera collected from each group of mice were checked for the total IgG titre against the respective antigen(s) pre and post challenge with tumour cells. Both Survivin and LHRH(6leu)-LTB antigens were able to induce antibody response when used to immunize the mice alone. Combined immunization with both the antigens, also led to induction of both anti-Survivin and anti-LHRH antibodies thus confirming their immunogenicity (Figure 4).

DISCUSSION

Tumor antigens have been proposed as important therapeutic targets for cancer immunotherapy[20]. Immunotherapy approaches incorporating Survivin as tumor antigen have shown efficacy for elimination of cancer cells. Various approaches such as peptide vaccines[21-23], multi epitope vaccines[24,25] or DNA vaccines incorporating Survivin as vaccine antigen[26,27], have shown efficacy in preclinical studies. It has also been suggested that combination of Survivin derived peptides with immunomodulators or immunomodulatory cytokines such as IFN alpha or induces a significant overall survival in patients with advanced or recurrent urothelial cancer[28].

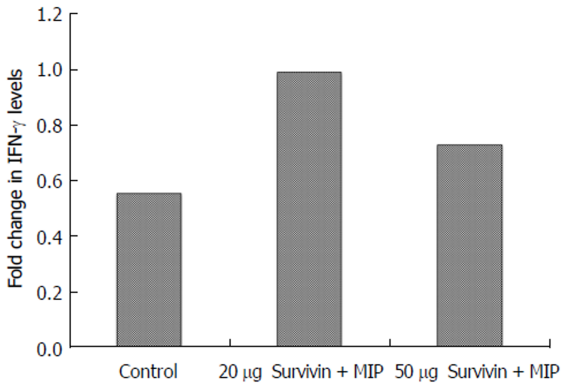

Figure 2 Interferon-gamma levels measured in mice immunized with 20 μg Survivin (+ *Mycobacterium indicus pranii*) and 50 μg Survivin (+ *Mycobacterium indicus pranii*) in comparison to phosphate buffer saline administered control mice and *Mycobacterium indicus pranii* administered mice. Interferon-gamma is expressed as fold change relative to the levels obtained in phosphate buffer saline administered mice before the tumor challenge. MIP: *Mycobacterium indicus pranii*.

We have also previously reported that combination of Survivin along with MIP has potent dose dependent anti-tumor effects in a mouse model of breast carcinoma[16]. Our data suggests that MIP alone is not effective in preventing tumor growth (data not shown) however combining MIP with tumor antigen such as Survivin gives significant tumor suppression. MIP may work as immunomodulator and serves to enhance the immune response against Survivin antigen.

We have demonstrated in the present study that increasing the dose of recombinant Survivin to 20 μg induces most effective tumor suppression in murine 4T-1 model of breast cancer. Interestingly, further increasing the dose of antigen does not induce a tumor suppressive effect. Suppression of immune response at supraoptimal doses of antigen has also been previously reported[29-31]. We hypothesize that high dose Survivin antigen may induce a suboptimal or anergic immune response which may not be able to inhibit the tumor growth.

Interferon-γ secreted by Th1 CD4+ T, NK cells, CD8+ T cells, APCs and B-cells cells is known for its anti-tumor effects[32]. It has role in rejection of transplanted tumors and inhibition of formation of spontaneous tumors[33,34]. Data presented in the present studies also show higher levels of IFN-γ at 20 μg dose of Survivin than the 50 μg dose which is in agreement with observed better suppression of tumor with 20 μg dose. Previous studies have confirmed the important role of humoral immune response in providing in anti-tumor immunity[35]. Our studies also indicate that antigen specific antibodies were induced after immunization with tumor antigens which possibly have contributed to tumor suppression. A recent study by Fenstermaker *et al*[36] has also demonstrated therapeutic effect of monoclonal antibody against Survivin in vivo in murine glioma model.

Many of the premenopausal breast cancers are also hormone-dependent which rationalizes the use of LHRH agonists as an effective treatment option in breast cancers[37]. Various combinations of LHRH agonists such as cisplatin loaded LHRH nanoparticles which have shown good anti-tumor response in 4T1 breast tumor model by accumulating higher cisplatin in tumors[18]. Another conjugate of LHRH, Pt-Mal-LHRH (activated cisplatin linked to LHRH peptide with a malonate linker) showed targeted delivery to cells expressing LHRH receptors. Treatment of 4T1 breast cancer with Pt-Mal-LHRH reduced tumor volume and metastasis to lungs[38].

We evaluated the possibility of using an immunotherapy based on a combination of Survivin and LHRH fusion protein in 4T-1 murine model of breast cancer. LHRH(6leu)-LTB, a vaccine developed for the immunotherapy of androgen dependent cancers, was used as an immunogen in combination with Survivin along with immunomodulator, MIP. The idea was to generate anti-LHRH antibodies to neutralize LHRH making it unavailable for intake by the breast cancer cells through the receptors. It was observed that efficacy of the combination was marginally better than the Survivin alone but significantly better when only LHRH(6leu)-LTB was used along with MIP. More importantly, combination was best at preventing metastasis of primary tumors to lungs.

Our results show promising outcome in a prophylactic scenario. We undertook the prophylactic approach for two reasons: (1) to investigate if the immunization induces a tumor protective response *in vivo*; and (2) this prophylactic approach also closely mimics the clinical situation where primary tumors have been removed or adjuvant

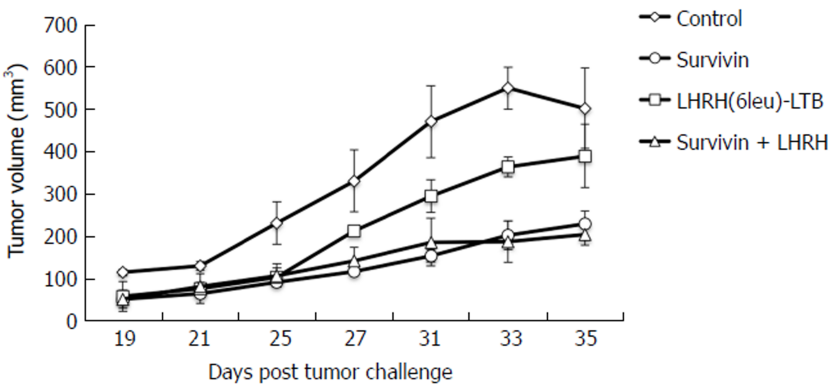

Figure 3 4T-1 Breast tumor volume in mice immunized with PBS buffer, 20 μg Survivin + *Mycobacterium indicus pranii*, 20 μg luteinizing hormone-releasing hormone + *Mycobacterium indicus pranii* and combination of 20 μg Survivin and 20 μg luteinizing hormone-releasing hormone along with *Mycobacterium indicus pranii*. Tumor volume is measured at different time points from day 19th when tumor was palpable. LHRH: Luteinizing hormone-releasing hormone.

treatment for cancer patients has been initiated. In both these settings, the patients undergo surgery. Though patients are rendered free of tumor, chances of recurrence are very high. If translation of LHRH fusion protein and Survivin based combination approach is successful, it may find applications in such clinical scenario. We have also investigated the therapeutic potential of this approach by immunizing the mice with implanted and palpable tumor, which has showed similar suppression of tumor growth (data not shown).

In conclusion, we have shown the Survivin (+ MIP) at a dose of 20 μg is most effective in preventing the growth of 4T-1 breast tumor cells in mice. Incorporation of anti-LHRH vaccine exercises a synergistic effect. A schematic diagram depicting the strategy used in the present study has been shown as Figure 5. Although our results show the protective role of combination immunotherapy with Survivin and LHRH antigens in murine tumor model, yet the study is limited by the fact the results need to be validated in other tumor models and further translational development needs to be undertaken for appropriate human application. Though it did not lead to much benefit in preventing the growth of primary tumor, it was highly effective in blocking the pulmonary metastasis. Our data suggests that combination of Survivin and LHRH fusion protein may hold immense promise for further development of immunotherapeutic approaches in management of breast cancers. The combination enhances immune response that is involved in inhibiting tumor growth, thus it will be effective in immune competent organisms and has to be supplemented with other therapies for use in immune compromised individuals. Furthermore, investigating the molecular mechanism of action of the combination leading to tumor inhibition may also lead to development of novel targeted therapies for cancer.

Table 1 Number of nodules in the lungs of mice challenged with 4T-1 breast cancer cells post immunization with PBS buffer, Survivin, LHRH(6leu)-LTB and combination of Survivin and LHRH(6leu)-LTB

Immunization group	Average No. of lung nodules
PBS (untreated)	> 20
Survivin	9
LHRH(6leu)-LTB	9
Survivin + LHRH(6leu)-LTB	0

LHRH: Luteinizing hormone-releasing hormone.

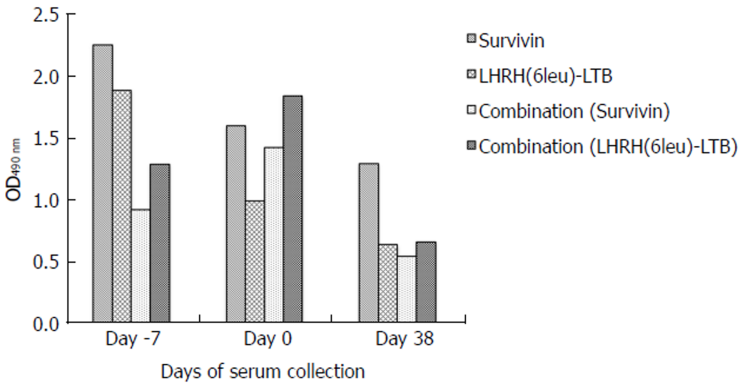

Figure 4 Graph representing the comparative level of total IgG antibody measured in mice immunized with Survivin (+ *Mycobacterium indicus pranii*), LHRH(6leu)LTB (+ *Mycobacterium indicus pranii*) and combination of Survivin and LHRH(6leu)-LTB (+ *Mycobacterium indicus pranii*). The serum was collected pre tumor challenge (Day -7), on tumor challenge (Day 0) and at the end of the study (Day 38). The total IgG titer was determined against respective antigens in each group. Groups of mice receiving Survivin alone and combination were checked for anti-Survivin antibody titers. Similarly, anti-LHRH titres were checked in mice in groups corresponding to LHRH(6leu)-LTB alone and combination. LHRH: Luteinizing hormone-releasing hormone.

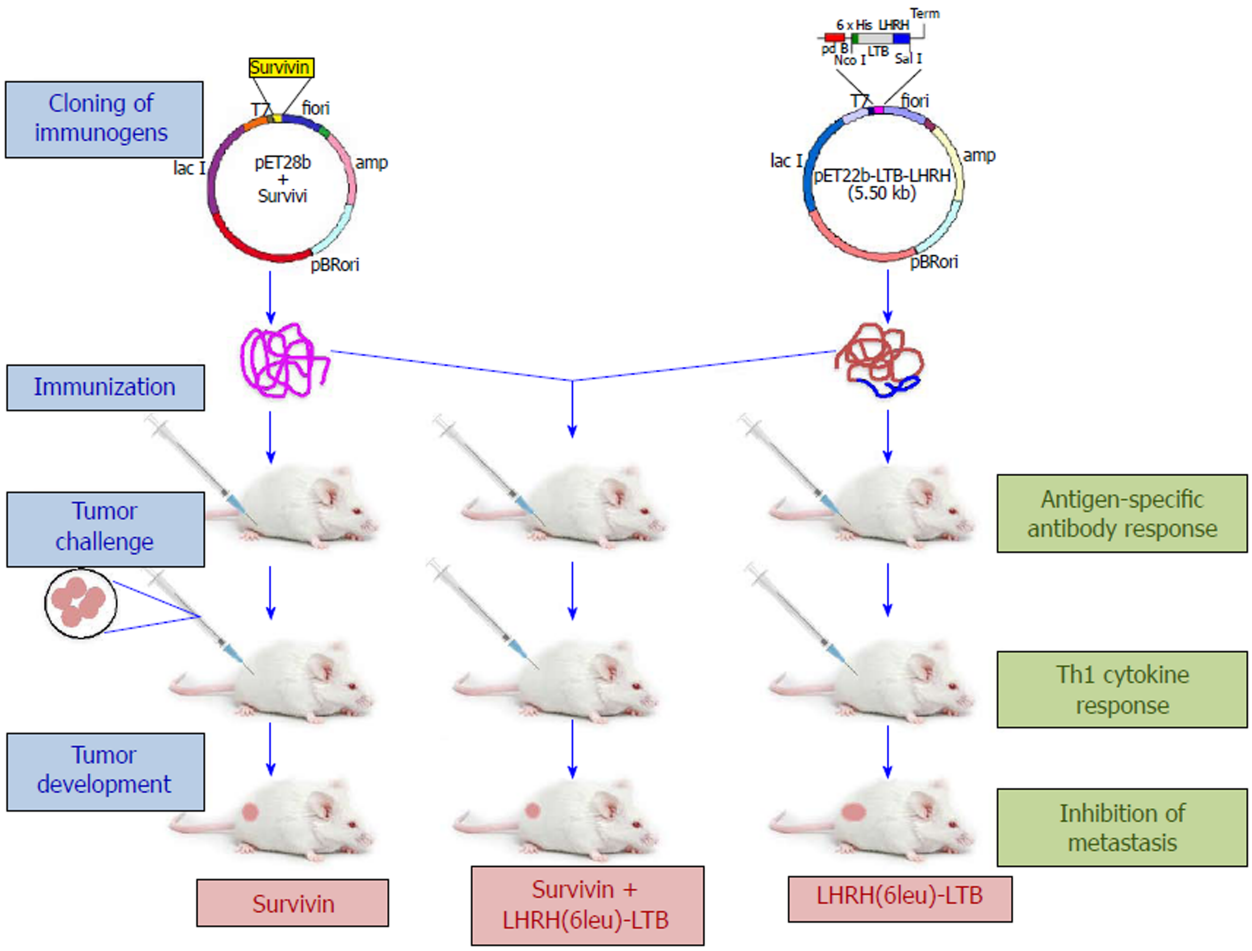

Figure 5 Schematic representation of the study of anti-tumor effect of Survivin, luteinizing hormone-releasing hormone fusion protein individually and in combination. The study was conducted on four groups of mice: Untreated or control, Survivin alone, LHRH(6leu)LTB alone and Survivin + LHRH(6leu)LTB. Individual recombinant proteins were purified and used as immunogen along with *Mycobacterium indicus pranii* as an immunomodulator in murine model. The mice from all the groups were further challenged with tumor cells, 4T1 and tumor development was observed in each group. LHRH fusion protein was not effective in suppressing tumor alone. However, the combination of both Survivin and LHRH(6leu)LTB inhibited tumor growth substantially followed by Survivin alone. LHRH: Luteinizing hormone-releasing hormone.

ARTICLE HIGHLIGHTS

Research background

Tumor cells often develop immune evasion mechanisms, thus combination of tumor antigens may be used to counteract immune resistance. Survivin is a tumor antigen exclusively expressed on tumor cells, essential for cancer cell survival, and its overexpression is associated with aggressiveness of the disease. Similarly, many tumor cells from breast, prostate, ovary or endometrial origin are hormone dependent for their survival. Immunization against hormones such as luteinizing hormone-releasing hormone (LHRH) using LHRH based peptide vaccines have shown efficacy as vaccine antigens in preclinical models of hormone dependent cancers such as breast and prostate cancer. This communication describes the studies undertaken to determine the optimum dose of Survivin antigen for maximum tumor suppressive effect and the synergy between Survivin and LHRH as vaccine antigens for immunotherapy of murine model of breast cancer, 4T-1.

Research motivation

We undertook the prophylactic approach for two reasons: (1) to investigate if the immunization induces a tumor protective response *in vivo* and (2) this prophylactic approach also closely mimics the clinical situation where primary tumors have been removed or adjuvant treatment for cancer patients has been initiated. Major issues in cancer treatment are the recurrence of tumor after surgery and chemoresistance. Our study was aimed at targeting these issues and developing a strategy, which may prevent tumor spread and recurrence and can overcome resistance. Generating immune response in the body against the tumor antigens will be a safer way of treatment. Anti-Survivin approach may help in overcoming the problem of resistance to therapy.

Research objectives

The aim of this study was to evaluate the possibility of using an immunotherapy based on a combination of Survivin and LHRH fusion protein in 4T-1 murine model of breast cancer. LHRH(6leu)-LTB was used as an immunogen in combination with Survivin along with an immunomodulator, *Mycobacterium indicus pranii* (MIP). It was observed that efficacy of the combination was marginally better than the Survivin alone but significantly better when only LHRH(6leu)-LTB was used along with MIP. More importantly, combination was best at preventing metastasis of primary tumors to lungs. In most cancer cases, the patients undergo surgery. Though patients are rendered free of tumor, chances of recurrence are very high. If translation of LHRH fusion protein and Survivin based combination approach is successful, it may find applications in such clinical scenario.

Research methods

Survivin and LHRH fusion protein were used as vaccine antigens. These antigens were expressed as recombinant proteins using *E. coli* based host-vector systems. Purified proteins were adsorbed on alum before administration as immunogens in mice. MIP was used as immunomodulator along with the recombinantly made vaccine antigens. Inbred Balb/c mice were used for conducting the immunogenicity and efficacy studies of the Survivin vaccine *in vivo*. Tumor volume was measured bi-dimensionally with digital vernier calipers and results are expressed as mean ± SD of tumor volume for each group. Anti-tumor efficacy of combination of Survivin and LHRH fusion protein in comparison to Survivin and LHRH fusion protein alone was also determined. Sera were collected from mice and assayed for Interferon gamma levels using ELISA kit. ELISA was also performed for the analysis of immunogenicity of the recombinant proteins in mice.

Research results

We have shown that Survivin (+ MIP) at a dose of 20 μg is most effective in preventing the growth of 4T-1 breast tumor cells in mice and incorporation of anti-LHRH vaccine exercises a synergistic effect. The study is limited by the fact that the results need to be validated in other tumor models and further translational development needs to be undertaken for appropriate human application. The combination will be effective in immune competent organisms and has to be supplemented with other therapies for use in immune compromised individuals. Furthermore, investigating the molecular mechanism of action of the combination leading to tumor inhibition may also lead to development of novel targeted therapies for cancer.

Research conclusions

Our results show the protective role of combination immunotherapy with Survivin and LHRH antigens in murine tumor model. Though it did not lead to much benefit in preventing the growth of primary tumor, it was highly effective in blocking the pulmonary metastasis. Combination of Survivin and LHRH fusion protein may hold immense promise for further development of immunotherapeutic approaches in management of breast cancers.

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CASE REPORT

Radiation-associated epithelial-myoepithelial carcinoma among five secondary malignancies: A case report and review of literature

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Abstract

BACKGROUND

Epithelial-myoepithelial carcinoma (EMC) is a rare, low-grade, malignant tumor that constitutes less than one percent of all salivary gland tumors. To date, only one other case report has described radiation-associated EMC in the English language medical literature.

CASE SUMMARY

In this report, we describe the case of a 56-year-old male patient who presented with a neck mass diagnosed as EMC of the left submandibular gland approximately 30 years after mantle field radiation and chemotherapy for Hodgkin lymphoma. Treatment included resection, re-resection with nodal dissection, and adjuvant chemoradiotherapy. This patient was also diagnosed with 4 other secondary malignancies, including stage IV diffuse large B cell lymphoma in the abdomen with subsequent brain metastases, low-grade neuroendocrine carcinoma of the lung, Hurthle cell adenoma, and small B cell lymphoma before the patient expired. This case provides important information regarding the pathology, clinical sequelae, and management of a patient diagnosed with radiation-associated EMC amidst four concurrent malignancies.

CONCLUSION

Further investigation is needed on the efficacy of adjuvant radiotherapy in EMC, especially atypical EMC.

Key words: Secondary malignancy; Radiation-associated malignancy; Epithelial-myoepithelial carcinoma; Cancer survivorship; Case report

Core tip: We describe the second known case of radiation-associated epithelial-myoepithelial carcinoma (EMC). This patient received chemotherapy and radiation therapy thirty years prior for Hodgkin lymphoma, and in addition to EMC was also diagnosed with four other malignancies in a span of five years. Because of worrisome

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histopathologic features atypical for EMC and pathologic stage of this patient, this patient was treated more aggressively with adjuvant chemoradiotherapy. This case provides important information regarding the pathology, clinical sequelae, and management of radiation-associated EMC. Further investigation is needed on the efficacy of adjuvant radiotherapy in EMC, especially atypical EMC.

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INTRODUCTION

Epithelial-myoepithelial carcinoma (EMC) is a rare, low-grade malignant tumor of the salivary gland. Originally described by Donath *et al*[1] in 1972, EMC first appeared in the World Health Organization classification of salivary gland tumors in 1991[2]. Representing less than one percent of all salivary gland tumors, EMC typically manifests as a bulky, slowly growing mass primarily within the parotid gland or less commonly the submandibular glands, minor salivary glands, or palate[3]. EMC is more prevalent in females and most often presents in the seventh decade of life[3]. EMC is recognizable histologically as a biphasic tumor, characterized by inner ductal epithelial cells and abundant outer clear myoepithelial cells[2,3]. Pathologic subcategorizations of EMC include double clear EMC, oncocytic EMC, sebaceous EMC, apocrine EMC, EMC ex pleomorphic adenoma, and EMC with high-grade transformation (HGT)[4]. HGT is a transition from low-grade to high-grade morphology with loss of the original histological characteristics. EMC with HGT is exceedingly rare[4]. The minimum recommended treatment for EMC is surgical resection, although local recurrence after resection has been reported in 23%-50% of cases[3]. Chemotherapy and radiotherapy (RT) are also appropriate therapeutic options especially with nodal involvement or metastatic disease, although very few data exist regarding their efficacy[5].

To the best of our knowledge, only one case report has thus far described RT-associated EMC in the English language medical literature[6]. In this previous report, a 48-year-old male was diagnosed with right submandibular high-grade EMC 25 years after mantle field RT. Multiple resections as well as adjuvant RT and chemotherapy were required due to multiple recurrences over a 4-year period. Although EMC is typically a low-grade tumor, this report conjectured an etiologic association between the extensive necrosis profile and high mitotic activity evident on pathological exam, as well as the unusually young age of the patient, and the course of previous RT.

While seldom utilized today, mantle field RT was the standard of care for Hodgkin lymphoma in the 1960's because of enhanced curative potential compared to more focal treatment. Historically, RT treatment paradigms were built on the concept of extended field radiation therapy (EF-RT), wherein both the primary site and regional lymphatic groups were treated. EF-RT was categorized into mantle field RT and inverted-Y field RT. Mantle field RT covered the cervical, mid-chest, and axillary lymph nodes, while inverted-Y field RT covered the subdiaphragmatic field including all para-aortic, iliac, upper femoral, and inguinal lymph nodes[7]. Notably, mantle field RT has been associated with an increased risk of breast cancer[8]. According to De Bruin *et al*[8], mantle field RT (involving the axillary, mediastinal, and neck nodes) was associated with a 2.7-fold increased risk of breast cancer compared with similarly dosed (36 to 44 Gy) mediastinal nodes alone. Mantle field RT has also been associated with increased cardiac morbidity and mortality[9].

CASE PRESENTATION

Chief complaints

After subtotal resection of EMC, the patient presented to our institution for evaluation. The patient reported that, from discovery until dissection, the neck mass was without symptomatic changes and was neither painful nor enlarging.

History of present illness

At the age of 56, the patient presented with a chief complaint of a neck mass detected while shaving. Computerized tomography (CT) scan of his neck showed a heterogeneous ovoid mass in the left submandibular region measuring 2.9 cm × 1.8 cm (Figure 1). The CT scan also demonstrated an enlarged left paramedial submental lymph node measuring 1.6 cm × 0.9 cm. Biopsy of the neck mass revealed EMC staining positive for CK7, CKAE1/AE3, CK903, S100, and smooth muscle actin and negative for CK20, PAX8, GATA, CDX2, GFAP. Infiltrates into soft tissue were noted along with fragments of salivary gland with fibrosis and atrophy. The left submandibular mass was then resected in piecemeal fashion, with final pathology report confirming EMC.

History of past illness

At the age of 19, this male patient presented with Hodgkin lymphoma and underwent mantle field RT, chemotherapy, and splenectomy. He did not exhibit evidence of recurrence. At the age of 52, stomach biopsy revealed diffuse large B cell lymphoma (DLBCL) with positron emission tomography (PET) scan displaying diffuse disease in the right axilla, mediastinum, multiple abdominal lymph nodes, appendicular skeleton, and liver. For DLBCL treatment, the patient received denosumab and six cycles of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone. Shortly after completing therapy, he presented with dizziness, ataxia, and memory loss. Brain MRI showed two ring-enhancing lesions consistent with metastasis, one in the right temporal lobe and the other in the left posterior fossa adjacent to the fourth ventricle. He underwent subsequent whole brain radiation therapy with 3960 cGy delivered in 22 fractions as well as intrathecal cytarabine.

In addition to his oncologic history, his past medical history was notable for coronary artery disease, hypertension, hypothyroidism, gastroesophageal reflux disease, and mini-stroke. His surgical history was remarkable coronary artery bypass grafting x 4 vessels, right carotid endarterectomy, appendectomy, and aortic valve replacement. Family history of cancer included a maternal grandfather with lung cancer, a maternal uncle with colon cancer, and a paternal grandfather with lung cancer. There were no first degree relatives with a history of cancer, heart disease, alcoholism, asthma, bleeding disorder, diabetes, or thyroid disease. Social history was remarkable for a 32-pack year smoking history, asbestos exposure, and no alcohol or illicit drug use.

Physical examination

Physical exam was notable for bilateral dense fibrosis.

Laboratory testing

Laboratory diagnosis was deferred, as definitive pathological diagnosis was available.

Imaging examination

MRI and PET did not show evidence of visible residual local EMC.

Genetic testing

Genetic testing was deferred.

FINAL DIAGNOSIS

Given the piecemeal manner in which the cancer was removed, a multidisciplinary tumor board concluded that the patient required revision surgery with additional neck dissection for concerning nodes unaddressed by the original surgery. The patient proceeded with resection of the left submandibular gland with dissection of level IA, IB, and suprahyoid neck nodes. Histologic examination revealed EMC in 1/2 left level IB lymph nodes and 0/3 left level IA lymph nodes staged as pT2pN1M0. The pathology showed extensive lymphovascular invasion and positive margins on the primary mass (Figure 2). Based on the high probability of residual microscopic disease, the patient elected to proceed with adjuvant chemoradiation.

TREATMENT

For adjuvant treatment of EMC, the patient's left hemineck received intensity-modulated radiation therapy consisting of 5040 cGy in 28 fractions, followed by a 3D-boost of 1620 cGy in 9 fractions to the left submandibular tumor bed and adjacent

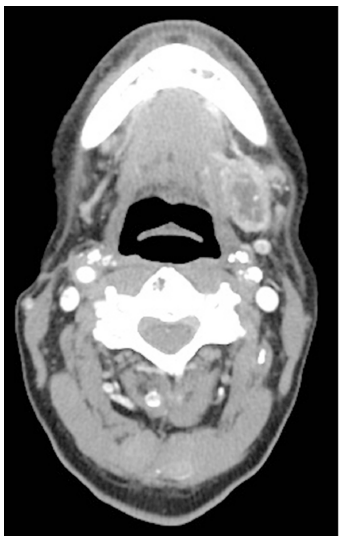

Figure 1 Primary epithelial-myoepithelial carcinoma presenting computerized tomography scan. Computerized tomography scan showing heterogeneous mass determined to be primary epithelial-myoepithelial carcinoma in the left submandibular gland.

level IB lymph node region. The cumulative dose totaled 6660 cGy in 37 fractions. Concurrent chemotherapy consisted of weekly of carboplatin and paclitaxel. After completing chemoradiotherapy, the patient showed no evidence of EMC disease on history, physical exam, and CT imaging. At this time, he reported some sequelae of treatment including xerostomia and fibrosis of the neck tissues.

OUTCOME AND FOLLOW-UP

While awaiting chemoradiation for EMC, at the age of 56, a restaging PET scan demonstrated mild to moderate 2-^{18}F-fluoro-deoxy-D-glucose (FDG) uptake in an irregular nodular density measuring 1.7 cm × 1.3 cm in the right upper lobe of the lung (Figure 3). Comparison to a previous CT scan showed interval growth of the nodule from 1.4 cm × 1.0 cm. Bronchoscopy with biopsy revealed a neuroendocrine tumor (NET). Immunopanel showed the tumor to be positive for CAM 5.2, TTF1, synaptophysin, and chromogranin. A Ki67 immunoperoxidase stain exhibited a low proliferation rate of less than 3%, indicative of a low-grade neoplasm. Notably, the patient denied any cough, shortness of breath, diarrhea, flushing, or weight loss, and consequently elected for observation with close monitoring.

The PET scan also demonstrated an intensely FDG focus measuring 0.6 cm × 0.7 cm in the anterior medial aspect of the left thyroid lobe (Figure 4). Thyroid ultrasound with Doppler displayed an oblong mildly hypoechoic nodule with peripheral and internal vascularity. Fine needle aspiration revealed a follicular lesion of undetermined significance composed predominantly of Hurthle cells. Given the benign nodular hyperplasia of the thyroid nodule compared to the micrometastatic EMC, the patient elected to first proceed with chemoradiation for EMC followed by hemithyroidectomy for the Hurthle-cell adenomatoid nodule.

Ten months post-chemoradiotherapy, a follow-up CT scan displayed interval growth of the right upper lobe NET along with several new bilateral pulmonary nodules. PET scan noted a new intensely FDG avid focus in the left pterygoid muscle along with intensely avid hilar lymphadenopathy and moderately avid mediastinal lymph nodes. Biopsy of the pulmonary nodules and mediastinal lymph nodes was negative for malignancy. Based on the interval growth of the NET, the patient elected for stereotactic body radiation therapy (SBRT) to the right lung. This lesion received a total dose of 5250 cGy over 5 fractions with 1 isocenter and 2 VMAT beams. In each fraction, 1050 cGy covered 95% of the planned treatment volume (PTV) while 90% of the prescription dose covered 99% of the PTV.

Two months after completing SBRT, the patient presented to the emergency department with epistaxis and a supratherapeutic international normalized ratio secondary to warfarin. A fall while hospitalized lead to altered mental status and hypoxic respiratory failure, prompting transfer to the intensive care unit and intubation. He subsequently developed pneumonia and sepsis with a vasopressor requirement as well as bilateral pleural effusions requiring repeated therapeutic

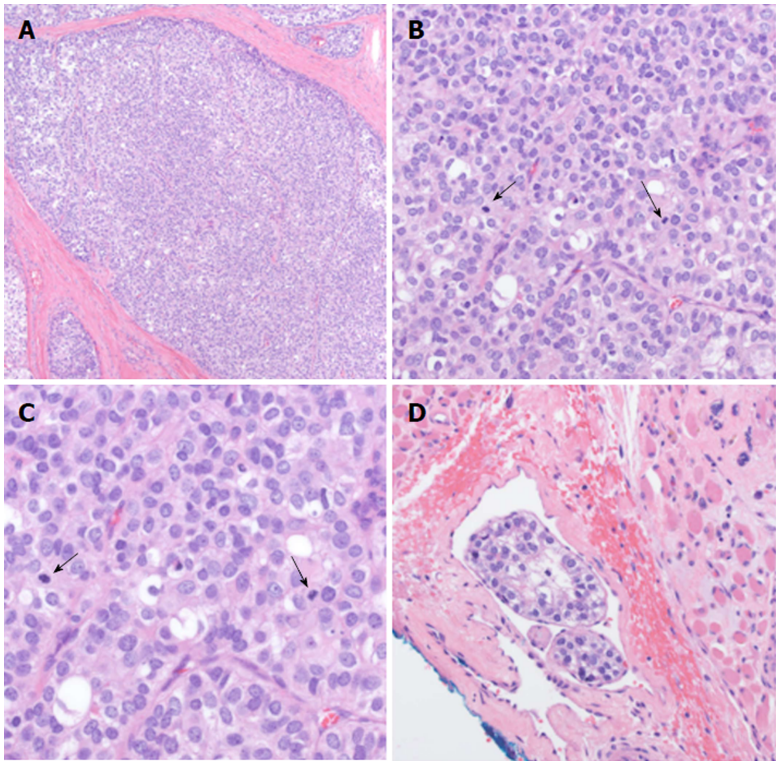

Figure 2 Hematoxylin and eosin stain of epithelial-myoepithelial carcinoma specimen. A: Epithelial-myoepithelial carcinoma (EMC), 10 × magnification; B: EMC, 40 × magnification; C: EMC, 60 × magnification; D: Lymphovasuclar invasion, 40 × magnification. Arrows indicate mitotic figures.

thoracentesis. Flow cytometry of the pleural fluid was notable for a CD10-positive lambda-skewed B cell population suspicious for a small B cell lymphoma *vs* follicular lymphoma, with a negative peripheral blood flow cytometry. After discussion with his family regarding goals of care, he transferred to the palliative care unit and subsequently inpatient hospice, where he expired shortly thereafter.

DISCUSSION

Secondary malignancy after Hodgkin lymphoma is not an infrequent occurrence. According to Schaapvel and co-authors, even 40 years after treatment, survivors of Hodgkin lymphoma are at an increased risk for secondary cancers, with the cumulative incidence approaching 48.5%[10]. Additionally, a recent retrospective study by Patel *et al*. investigating 1541 stage I and stage II Hodgkin lymphoma patients treated from 1968 to 2007 revealed that, after median follow-up of 15.2 years (35% of patients with > 20 years of follow up), 395 patients had died of all causes, 85 patients had died from Hodgkin lymphoma, 168 had died from secondary malignancies (25 hematologic and 143 nonhematologic), 70 had died from cardiovascular causes, and 21 had died from pulmonary causes[11]. Remarkably, as these data show, more patients died from secondary malignancies than from the primary Hodgkin lymphoma.

The patient presented in this case report developed multiple malignancies, including DLBCL, EMC, Hurthle cell adenoma, low grade NET, and small B cell lymphoma after chemotherapy and RT of Hodgkin lymphoma three decades prior. Of these 4 malignancies, EMC is the most likely to be secondary to RT in this patient. Its location in left submandibular gland is within the previous mantle field RT treatment area, fulfilling Cahan's criteria for RT-associated secondary malignancy[12]. Additionally, the pathology of this patient's EMC was atypical. While characteristically a low-grade, indolent tumor, this EMC was an aggressive, invasive malignancy, in keeping with the only previously reported case of RT-associatec EMC[6]. Other RT-induced secondary malignancies, including glioma, sarcoma, and meningioma, are more often higher grade and more aggressive than their sporadic counterparts[13-15]. Regarding the other malignant entities in this patient, including DLBCL, Hurthle cell adenoma, and low-grade NET, these may be secondary to RT for Hodgkin lymphoma, as they do fulfill Cahan's criteria. Thyroid malignancies secondary to RT including Hurthle cell adenoma are well-described, and there are

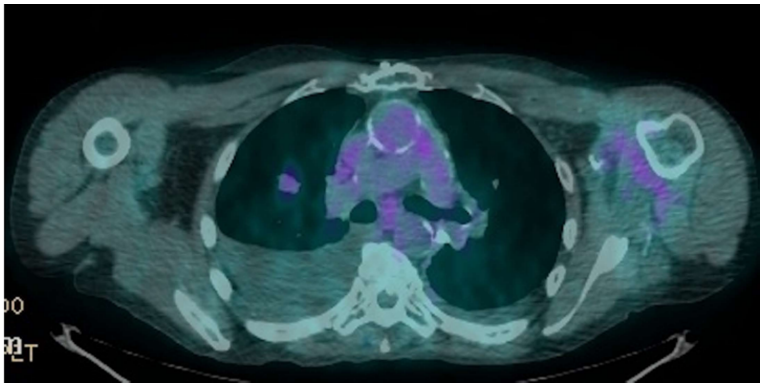

Figure 3 Neuroendocrine tumor. Positron emission tomography/computerized tomography chest scan showing moderately 2-^{18}F-fluoro-deoxy-D-glucose avid nodule of the right upper lobe.

reports of DLBCL secondary to radiation[16-18]. A case of quadruple neoplasms following RT therapy has also been published[19]. While family history was negative for first degree relatives with cancer, this patient may have had an unstable genomic background more susceptible to mutagenic insult that, in combination with a strong tobacco history, resulted in a lower threshold for carcinogenesis secondary to mantle field RT.

Importantly, clinical management guidelines of DLBCL, Hurthle cell adenoma, NET, and small B cell lymphoma are well-described; however, EMC, a rare entity, is less well-understood, especially even rarer high grade variants. EMC is most typically treated with surgery, although local recurrence occurs in one-fourth to one-half of all cases[3]. To risk stratify EMC based on pathological features, a retrospective study evaluated 61 tumors and reported positive margin status, angiolymphatic invasion, tumor necrosis, and myoepithelial anaplasia as significantly associated with shortened disease free survival[20]. This patient with positive margins, lymphovascular invasion, and nodal involvement was certainly at an elevated risk for recurrence. However, very few data have been reported on treatment paradigms for high risk patients, and the decision to pursue adjuvant chemoradiotherapy in this patient was in part based on evidence extrapolated from other salivary gland tumors. The largest study of EMC retrospectively analyzed the Surveillance, Epidemiology, and End Result (SEER) database and did not find a disease specific survival benefit for patients receiving adjuvant RT[21]. However, such data are more applicable to classic quiescent EMC than higher grade or RT-associated EMC. RT has been included in treatment plans since WHO inclusion in 1991, and some authors advocate for a refined incorporation of adjuvant RT in the setting of recurrent or high risk EMC[22,23]. Metastatic cases, while uncommon, have a poor prognosis when observed without chemoradiotherapy[24]. Finally, a biological basis may exist for an enhanced response to RT for more aggressive cancers compared to more indolent tumors[25].

In summary, given the scarcity of information about EMC after Hodgkin lymphoma, this case report provides vital information regarding the pathology, clinical sequelae, and treatment of EMC after Hodgkin lymphoma. Furthermore, this case provides a unique illustration of a quintet of malignancies following RT and chemotherapy for Hodgkin lymphoma. Patients with a history of treated Hodgkin lymphoma merit long-term follow-up due to increased risk of secondary malignancy. Further investigation is needed on the efficacy of adjuvant RT in EMC, especially atypical EMC.

EXPERIENCES AND LESSONS

Patients with a history Hodgkin lymphoma treated with mantle field RT are at risk for secondary malignancies. This includes on rare occasions EMC. We illustrate that radiation-associated EMC can be treated with resection and adjuvant chemoradiation.

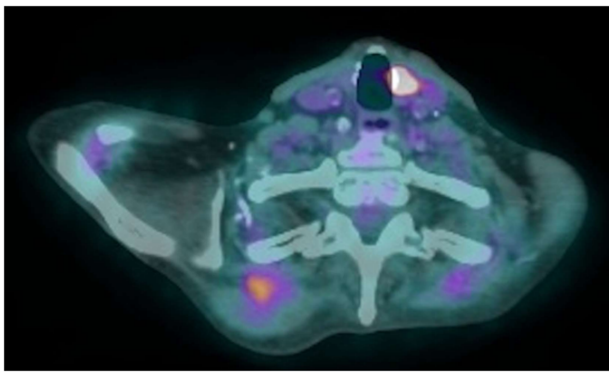

Figure 4 Hurthle cell adenoma. Positron emission tomography/computerized tomography scan neck scan showing intensely 2-^{18}F-fluoro-deoxy-D-glucose avid focus in the left medial thyroid.

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