ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2022 June 24; 13(6): 423-552

Published by Baishideng Publishing Group Inc

World Journal of Clinical Oncology

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Monthly Volume 13 Number 6 June 24, 2022

CASE REPORT

World Journal of Clinical Oncology

Monthly Volume 13 Number 6 June 24, 2022

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INDEXING/ABSTRACTING

The *WJCO* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2021 edition of Journal Citation Reports® cites the 2020 Journal Citation Indicator (JCI) for *WJCO* as 0.48.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Clinical Oncology

ISSN
ISSN 2218-4333 (online)

LAUNCH DATE
November 10, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Hiten RH Patel, Stephen Safe, Jian-Hua Mao, Ken H Young

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2218-4333/editorialboard.htm

PUBLICATION DATE
June 24, 2022

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https://www.wjgnet.com/bpg/gerinfo/204

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https://www.wjgnet.com/bpg/GerInfo/287

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ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 June 24; 13(6): 423-428

DOI: 10.5306/wjco.v13.i6.423

ISSN 2218-4333 (online)

OPINION REVIEW

Implementation of a virtual multicenter gastrointestinal tumor board to reduce cancer disparities in Argentina

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Dhali A, India; El-Nakeep S, Egypt; Tsagkaris C, Switzerland
A-Editor: Liu X, China

Received: January 13, 2022
Peer-review started: January 14, 2022
First decision: March 24, 2022
Revised: April 10, 2022
Accepted: June 4, 2022
Article in press: June 4, 2022
Published online: June 24, 2022

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Abstract

Given the increasing complexity of cancer care, multidisciplinary tumor boards have become essential in daily clinical oncology practice. The Project Extension for Community Healthcare Outcomes (ECHO) initiative developed an innovative telementoring model using a "hub and spoke" design consisting of a team of experts (hub) that offers a full service to multiple participants (the spokes) during regularly scheduled sessions discussing patients' clinical cases. The Alexander Fleming Cancer Institute in Buenos Aires was the first hub in Latin America to implement Project ECHO for gastrointestinal tumors. In our 3-year experience, 80 patients from 37 centers were evaluated within Project ECHO and a range of three

to five cases were discussed in each meeting. From our perspective, the impact of this novel approach was a remarkable strategy to reduce care disparities by equalizing access to high-quality medical knowledge in a multidisciplinary environment for medical discussions. Additionally, it was shown to have a cost-effective impact directly on the patients and the local health system, since relevant costs were saved after unnecessary treatments, studies and travel expenses were avoided.

Key Words: Tumor board; Virtual; Gastrointestinal; Cancer disparities; Oncology; Extension for Community Healthcare Outcomes project

Core Tip: Tumor boards (TBs) have existed for the last 50 years, and at the present time, represent an essential strategy in daily clinical oncology practice. We reported our positive experience and perspective with a novel approach of multidisciplinary virtual TBs using an innovative telementoring model called Project Extension for Community Healthcare Outcomes. This first experience in Latin America for gastrointestinal tumors has shown to reduce care disparities by equalizing access to high-quality medical knowledge in a context of a multidisciplinary environment for medical discussions.

Citation: Esteso F, Tissera NS, O'Connor JM, Luca R, Huertas E, Sánchez Loria F, Pedraza I, Pairola A, Brancato F, Paganini L, Kucharczyk M, Amat M, Pombo MT, Galli M, Bruno L, Caro L, Rodriguez A, Enrico D, Waisberg F, Chacón M. Implementation of a virtual multicenter gastrointestinal tumor board to reduce cancer disparities in Argentina. *World J Clin Oncol* 2022; 13(6): 423-428
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/423.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.423

INTRODUCTION

During recent decades, cancer care has become increasingly complex mainly due to the personalized approach for every single patient. Each patient requires a careful assessment which often involves a multidisciplinary effort and experienced physicians. However, many countries experience the centralization of tertiary referral cancer units in the most populated cities. In many urban centers in Argentina, medical oncologists provide care for patients with multiple types of cancer which challenges practitioners to stay current with the evidence that is necessary to deliver high-quality care. However, given the increasing knowledge of cancer, and the observed trend of subspecialization among oncologists, tumor boards (TBs) have become a widely accepted and successful strategy to promote discussion and evidence-based decisions in a scenario with unequal health access[1,2].

A TB is a multidisciplinary treatment planning approach in which health professionals with different specialties review and discuss the diagnosis and treatment strategies using an integrative approach[3]. In the past few years, TBs have incorporated virtual modalities to make them accessible in remote locations, promoting timely diagnostic and treatment planning for patients in different regions and socioeconomic settings.

In this context, Project Extension for Community Healthcare Outcomes (ECHO) created by Dr. Sanjeev Arora in 2003 in New Mexico, EEUU, uses video conferencing technology to discuss cases and treat rural cases of hepatitis-C[4]. It aims to reduce health disparities by allowing clinicians to share current medical knowledge in underserved and remote areas to cooperate with the care of patients with hepatitis C through innovative telementoring. The ECHO model uses a hub-and-spoke knowledge-sharing approach where expert teams lead virtual meetings amplifying the capacity for providers to deliver best-in-practice care to underserved areas in their communities (Figure 1)[5]. This "hub and spoke" design consists of a model that arranges a network consisting of a team of experts (the hub) that offers a full service to multiple participants (the spokes) during regularly scheduled sessions where patients with clinical cases that need a more accurate treatment are discussed[6,7]. The use of this design also provides the capability to facilitate clinical mentoring and the implementation of regular educational sessions for medical training. Thus, the ECHO approach represents a completely different model than “telemedicine”, wherein a specialist assumes the care of a patient in a typical consultation by using remote technology.

Currently, over 373 academic centers serve as ECHO hubs for multiple severe medical conditions such as infectious diseases, rheumatologic diseases, chronic pain, addiction, human immunodeficiency virus, diabetes, complex multisystem diseases and cancer[8,9].

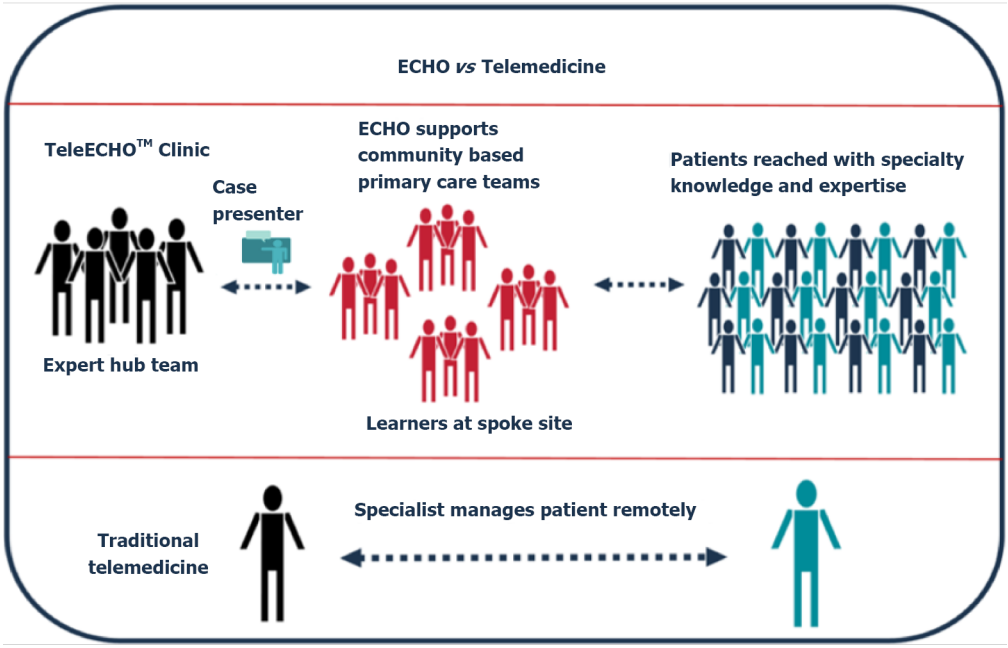

Figure 1 Extension for Community Healthcare Outcomes hub and spoke model. Citation: ECHO. Project ECHO. [cited 11 August 2021]. Available from: https://hsc.unm.edu/echo/. Copyright© Project ECHO 2022. Published by ECHO.

Multidisciplinary virtual TBs represent an opportunity to reduce the existing care disparities by information equality. Under this premise, the Alexander Fleming Cancer Institute in Buenos Aires was the first hub in Latin America to implement Project ECHO for gastrointestinal tumors. Since December 2017, monthly virtual meetings (an hour and a half long) using videoconferencing software have been performed to discuss cases of gastrointestinal cancer. The participating physicians had primary practices in academic and community general hospitals in at least 8 provinces of Argentina. The central hub staff was composed of surgeons, radiotherapists, imaging specialists, pathologists, genetic counselors, gastroenterologists and medical oncologists specifically dedicated to gastrointestinal cancer care. The modality included a case-based discussion followed by a moderated discussion with a final medical recommendation taking into account local available resources. The first 15 min of each meeting were dedicated to an educational lecture.

In our 3-year experience, 80 patients were evaluated within the Project ECHO, and a range of three to five cases was discussed in each meeting (Table 1). During the first 2 years, 15 health centers regularly participated in the virtual meetings. Notably, exponential growth was observed concurrently with the coronavirus disease 2019 (COVID-19) quarantine. Since June 2020, professionals from an average of 37 centers have habitually participated in these monthly meetings. Of note, each participating institution decided and proposed to the expert hub team the most relevant clinical cases that required a multidisciplinary discussion to the expert hub team. The median time from the first oncology visit until the ECHO referral was 16 d (range 12-19).

Most patients included in the program were diagnosed with colorectal cancer (n = 43, 53.75%), followed by neuroendocrine (n = 14, 17.5%), esophagogastric (n = 12, 15%), biliodigestive (n = 7, 8.75%), anal (n = 3, 3.75%) and appendix (n = 1, 1.25%) tumors. Most patients had advanced disease at the time of presentation to the TB (n = 28, 40%). Case discussions included systemic treatment for the advanced scenario, surgical approaches, and adjuvant decisions. Of note, the suggested strategies were mostly managed at local places (n = 60, 75%); other patients were referred to the Alexander Fleming Cancer Institute or tertiary health care centers (n = 8, 10%) for surgery or chemotherapy (n = 10, 12.5%) and a minority of cases were referred for radiotherapy (n = 2, 2.5%). Notably, during the COVID-19 pandemic period, only 6% of the patients were suggested to receive centralized treatment at a tertiary center. In addition, participant satisfaction was evaluated by a centralized digital survey provided to 30 professionals showing the highest level of satisfaction in 25 (83%) participants.

TBs have existed for the last 50 years and have been proven to improve medical training and, in the long run, patient care[10]. Under current conditions, Project ECHO emerges as a collaborative and integrative networking environment for cancer management in remote locations. The impact of novel virtual TB approaches in Argentina is a remarkable strategy to reduce care disparities by equalizing access to a multidisciplinary environment for medical discussions. Furthermore, these models have proven to be consistently cost-effective. Available evidence has highlighted that relevant costs were saved after unnecessary treatments, studies and travel expenses were avoided. The latter is particularly relevant, considering the vast extensions of Argentina[11-14]. As a typical example, a patient with a specific gastrointestinal tumor who would need to travel and have a consultation at a reference cancer

Table 1 Characteristics

Characteristics	*n* = 80
Age (range)	57 yr (48-68)
Sex	
Male	45 (56.25%)
Female	35 (43.75%)
Tumor type	
Colorectal cancer	43 (53.75%)
Neuroendocrine	14 (17.5%)
Esophagogastric	12 (15%)
Biliodigestive	7 (8.75%)
Anal	3 (3.75%)
Appendix	1 (1.25%)
Stage	
Locally/locally advanced	52 (65%)
Metastatic	28 (35%)
Treatment strategy management	
Local institution	60 (75%)
Referred to specialized cancer center	20 (25%)
Reference areas of Argentina (Province)	
North	40 (50%)
Center	24 (30%)
South	16 (20%)

center in Argentina would have to spend approximately 500 USD regardless of the study and treatment. Additionally, in terms of saving time, this strategy could normally take approximately 3 more weeks in delaying the treatment plan decision in Argentina.

Our heal system is heterogeneous, including the private and public sub-systems. Under this circumstance, some patients have to be referred to tertiary or local centers for coverage of treatment and studies to become effective. We believe that the discussion of the clinical cases in a context such as the ECHO initiative represents one of the better chances for high-quality cancer care considering that the referral does not cause a significant delay in the treatment. In our health system context, the virtual ECHO initiative would be more accessible, accurate, affordable and properly developable than the strategy of extending more sub-specialized oncologists in urban and suburban areas.

Our Project ECHO experience has led us to address some important factors that should be improved upon in the future. Internet access, low-quality video-conferencing devices and protected time availability are some of the key areas to expand in the future. The participation of professionals with non-oncology medical specialties should also be promoted to facilitate a comprehensive discussion of the multiple dimensions that are involved in cancer care.

One of the potential limitations of the virtual TB approach could be the lack of complete information on the clinical case for proper and personalized clinical decision-making, given that the medical opinions of the board are based on the case presentation and not on the direct evaluation of the patient. Additionally, monthly meetings may not meet the demand, mainly in situations when a medical decision is urgent.

Although limited, our experience was extremely positive. We are convinced that this strong professional network also creates a unique opportunity to promote national evidence-based recommendations, academic collaborations and clinical cancer research, as well as continuing medical education programs.

CONCLUSION

In our view, multidisciplinary virtual experiences, such as the Project ECHO, should be carefully addressed by health care decision-makers given their popularity and their demonstrated cost-effectiveness. Many of the evaluated barriers require government participation to improve budget and technology access in health care facilities. The COVID-19 pandemic has led to a tremendous need to incorporate modern technology into different work scenarios. Under these circumstances, the implementation of virtual educational and medical activities may be one of the key elements that cannot be excluded in the design and execution of National Cancer Control Programs.

FOOTNOTES

Author contributions: Esteso F and Tissera NS contributed to the conception and design; Enrico D and Waisberg F performed the administrative support; Esteso F and Tissera NS contributed to the collection and assembly of data; Esteso F, Tissera NS, Enrico D, Waisberg F and Andrés R contributed to the data analysis and interpretation; All authors performed the provision of study materials or patients, manuscript writing, final approval of manuscript and are accountable for all aspects of the work.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: Argentina

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 June 24; 13(6): 429-447

DOI: 10.5306/wjco.v13.i6.429

ISSN 2218-4333 (online)

REVIEW

New perspectives in the management of small cell lung cancer

Cristina Pangua, Jacobo Rogado, Gloria Serrano-Montero, José Belda-Sanchís, Beatriz Álvarez Rodríguez, Laura Torrado, Nuria Rodríguez De Dios, Xabier Mielgo-Rubio, Juan Carlos Trujillo, Felipe Couñago

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Chen C, China
A-Editor: Ma L, China

Received: April 18, 2021
Peer-review started: April 18, 2021
First decision: July 16, 2021
Revised: September 5, 2021
Accepted: May 22, 2022
Article in press: May 22, 2022
Published online: June 24, 2022

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Abstract

The treatment of small cell lung cancer (SCLC) is a challenge for all specialists involved. New treatments have been added to the therapeutic armamentarium in recent months, but efforts must continue to improve both survival and quality of life. Advances in surgery and radiotherapy have resulted in prolonged survival times and fewer complications, while more careful patient selection has led to increased staging accuracy. Developments in the field of systemic therapy have resulted in changes to clinical guidelines and the management of patients with advanced disease, mainly with the introduction of immunotherapy. In this article, we describe recent improvements in the management of patients with SCLC,

review current treatments, and discuss future lines of research.

Key Words: Small cell lung cancer; Whole-brain radiotherapy; Prophylactic cranial irradiation; Stereotactic body radiotherapy; Immunotherapy; Atezolizumab; Durvalumab

Core Tip: The treatment of small cell lung cancer (SCLC) continues to be a challenge. Recent studies have described survival benefits achieved by new treatments or combinations of treatments that are both safe and effective. Immunotherapy has a new role in SCLC. Nevertheless, continued research efforts are needed. Here, we review the current management of SCLC and discuss recent improvements and future lines of research.

Citation: Pangua C, Rogado J, Serrano-Montero G, Belda-Sanchís J, Álvarez Rodríguez B, Torrado L, Rodríguez De Dios N, Mielgo-Rubio X, Trujillo JC, Couñago F. New perspectives in the management of small cell lung cancer. *World J Clin Oncol* 2022; 13(6): 429-447
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/429.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.429

INTRODUCTION

Small cell lung cancer (SCLC) accounts for 14% of all lung cancers[1,2], and most cases are associated with tobacco use[3]. Although the global incidence of SCLC is falling, the ratio of male to female cases is currently 1:1[1,2]. SCLC is a fast-growing cancer, and most patients have extensive disease when diagnosed. In approximately one-third of cases, the cancer is limited to the thorax and can be treated with concurrent chemotherapy and radiotherapy. Just a small percentage of patients are amenable to surgery and adjuvant therapy. The goal of treatment in patients with extensive disease is to alleviate symptoms and prolong survival, although long-term survivorship in this setting is rare[4].

LIMITED-STAGE DISEASE

Surgical treatment of SCLC

Early-stage SCLC, stage I and IIA (T1-2N0M0) SCLC in the American Joint Committee on Cancer/International Union Against Cancer classification[5-7], accounts for 7% of all SCLCs and 0.29% of all lung cancers[8]. Numerous studies have shown excellent survival rates in patients with SCLC cT1-2N0M0 treated with surgery as part of a multimodal approach[6,9-28] (Table 1).

Surgical resection followed by adjuvant therapy is currently recommended by most clinical guidelines for operable stage I and IIA SCLC. Choice of adjuvant treatment varies according to pathologic tumor-node-metastasis stage: Chemotherapy for pN0, chemotherapy ± radiotherapy for pN1 and chemoradiotherapy for pN2[29-32] (Figure 1). The indications for the surgical treatment of SCLC can be summarized as follows: (1) Intraoperative diagnosis of a pulmonary SCLC nodule. Between 3% and 5% of SCLCs present as a pulmonary nodule. Multidisciplinary treatment involving surgical resection, systematic nodal dissection, and adjuvant chemotherapy or chemoradiotherapy can achieve survival rates comparable to those seen in non-SCLC[8]; (2) Diagnosis of stage I or IIA SCLC. Local or regional recurrence[33-39] (tumor and/or hilar-mediastinal lymph nodes) is the most common form of disease in patients who relapse after complete remission with chemoradiotherapy[40-45]. Surgery as part of a multimodal approach achieves better local disease control[46-50] than chemoradiotherapy[51-54]; (3) Mixed histology (SCLC with a non-SCLC component). Between 2% and 28% of patients have mixed SCLC/non-SCLC[55-59]. Recurrence or failure to respond to first-line chemotherapy is likely to be due to the non-SCLC component; and (4) Salvage surgery for local chemo-resistant SCLC or exclusively local recurrence after response to chemoradiotherapy. Selected patients in this setting might benefit from surgical resection[60-62].

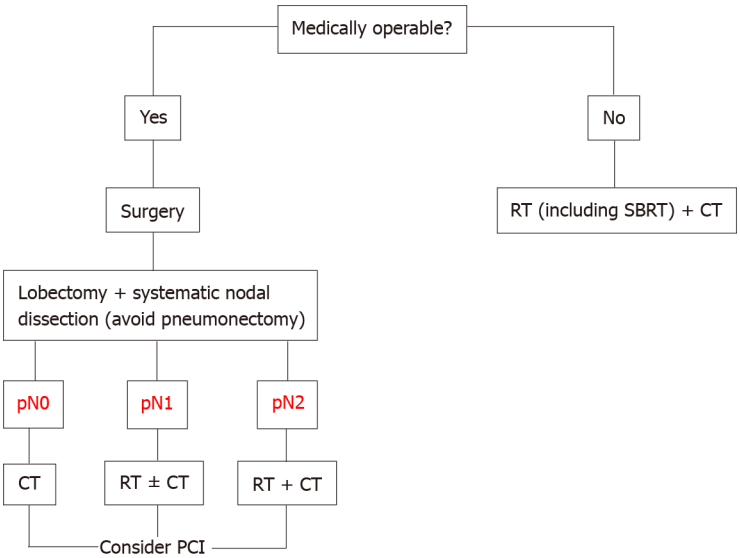
Lobectomy is the preferred procedure for surgical resection, as it is associated with significantly better survival than sublobar resection[40,45,49,54,63]. The significant discrepancies observed between clinical and pathologic stages (mainly due to undetected lymph node metastasis before surgery) highlight the importance of accurate clinical nodal staging and systematic lymph node dissection[47,64]. The recommendations for ruling out hilar and mediastinal lymph node involvement are very similar across the different guidelines. Ideally, clinical staging should be performed using semi-invasive

Table 1 Surgical and survival rates for patients with small cell lung cancer (period time revised 1999-2020) - (dash), lack of information or details

Ref.	Study type & time period. LoE	Inclusion criteria	Number of patients	Neoadjuvant/adjuvant treatments	PCI	Survival data
Jin *et al*[9], 2018	RS; SEER 2004-2013; 3A	cI-II	*n* = 2129; S: 387; RT 1032; S + RT: 154; No S or RT: 556		-	5-yr OS T1N0: 46.0% S *vs* 23.8% RT; 5-yr OS T2N0: 42.6% S *vs* 24.7% RT; T3N0 or T1-2N1 (stage IIB) patients treated with S did not have higher 5-yr OS rates than those treated with RT
Yang *et al*[10], 2018	RS; NCDB 2003-2011; Propensity score match S + AC *vs* CRT; 3A	cT1-2N0M0	S + AC: 501; CRT: 501	S + AC: 501	-	5-yr OS: 47.6% S + AC *vs* 29.8% CRT ($P < 0.01$)
Ahmed *et al*[11], 2017	RS; SEER 2007-2013; 3A	Stage I SCLC	*n* = 1902; S: 427; S + RT: 115	-	-	MST: 50 mo (S); MST: 60 + mo (S + RT)
Wakeam *et al*[12], 2017	RS; NCDB 2004-2013; 3A	cT1-2N0M0	*n* = 5079	-		MST: 25.3 mo
Wakeam *et al*[13], 2017	RS; NCDB 2004-2013; Stage-specific propensity score match S *vs* NST; 3A	cI-III	*n* = 2619	No AD treatment 24% NC or NR 4%; AC 27%; AR 1%; ACR 32%; NC or NR and AC or AR 2%; Other 10%	-	MST cI 38.6 *vs* 22.9 mo S *vs* NST; MST cII 23.4 *vs* 20.7 mo S *vs* NST; MST cIIIA 21.7 *vs* 16.0 mo S *vs* NST
Combs *et al*[14], 2015	RS; NCDB 1998-2011; 3A	cT1-3N0-2 SCLC	*n* = 2476; S 841 cIA, 168 cIB	All; S: 68%	-	5-yr OS: 54% (cIA); 36% (cIB)
Ogawa *et al*[15], 2012	RS; Institutional 1995-2008; 4	cI-III; pI-III SCLC	*n* = 28 (23 SCLC before S); S 21 cI, 5 cII, 7 cIII2	NC 8; AC 19, ACR 2	-	5-yr OS 47%
Ju *et al*[16], 2012	RS; Institutional 1990-2009; 4	pI-III	*n* = 34	NC 3; AC 1, AR 19, 10 CRT	-	5-yr OS 66%
Vallières *et al*[6], 2009	RS; IASLC 1990-2000; 3A	Resected SCLC	*n* = 349 (68 pIA, 91 pIB)	-	-	5-yr OS: 53% (pIA); 44% (pIB)
Lim *et al*[17], 2008	RS; Institutional 1980-2007; 4	cI-cIIIB	*n* = 59	AC 13; AR 2; ACR 1	-	5-yr OS for all patients 52%; No difference in 5-yr survival across; cT and cN categories; No difference in 5-yr survival across; cI to cIII stages
Wang *et al*[18], 2007	RS; Institutional; 4	pI-III	*n* = 122	QT & CRT (not specified)	-	MST 50 mo; 5-yr OS 66%
Veronesi *et al*[19], 2007	RS; Institutional; 4	cI-IIIA	*n* = 23	AC all	-	MST 24 mo
Tsuchiya *et al*[20], 2005	Prospective phase II trial; 1991-1996; 2B	cI-IIIA	*n* = 62	AC 42 (69%)	-	MST not reached in pI; MST 449 d for pII; MST 712 d for pIIIA; 3-yr OS 61%; 3-yr survival rate cI, cII, cIIIA 68%, 56% and 13% respectively
Brock *et al*[21], 2005	RS; Institutional 1976-2002; 4	Resected SCLC	*n* = 82 (24 stage I, S + AC)	AC 55%	23%	5-yr OS: 86% (platinum AC); 42% (non-platinum AC)
Nakamura *et al*[22], 2004	RS; Institutional; 4	cI-III SCLC	*n* = 69	S 37, NC 32, AC 41, ACR 7	-	5-yr survival 48.9 % cI, 33.3 % cII, 20.2 % cIIIA, 0 % cIIIB
Badzio *et al*[23], 2004	Comparative RS; Institutional 1984-1996; 4	cI-III balanced in both, S and NST groups	*n* = 134	S 67 (all AC); NST 67 (all QT)	34% only S group	MST 22 mo (S); MST 11 mo (NST); 5-yr OS S 27%, NST 4%
Lewiński *et al*[24], 2001	R; Institutional 1976-2002; 4	cI-IIIA SCLC	*n* = 75	NC all	If CR to NC	MST N0+1 25 mo; MST N2 14 mo; MST resected 18 mo; 5-yr OS resected 29%
Cataldo *et al*[25], 2000	RS; Institutional 1982-1992; 4	cI-III SCLC	*n* = 60	AC 88%; pII AR (11%); pIII AR (21%)	41%	5-yr survival rate 40% pI, 36% pII and 15% pIII

Inoue *et al*[26], 2000	RS; Institutional 1975-1994; 4	Resected SCLC	*n* = 91 (32 cIA, 30 cIB)	All 78%	5.5%	MST 53 mo, 5-yr OS 49% (cIA); MST 25 mo, 5-yr OS 47% (cIB)
Kobayashi *et al* [27], 2000	RS; Institutional 1982-1992; 4	cI-III SCLC	*n* = 59	NC 71%	-	5-yr survival rate 55% pI, 33% pII, 23% pIII
Eberhardt *et al* [28], 1999	Prospective phase II trial; Institutional 1991-1995; 2B	cIB-cIIIB	*n* = 46	IB/IIA had NC + S; IIB/IIIA had NCR + S	-	MST all patients 36 mo; MST R0 patients 68 mo; 5-yr survival rate all patients 46%; 5-yr survival rate R0 patients 63%

ACR: Adjuvant chemoradiotherapy; AD: Adjuvant; AC: Adjuvant chemotherapy; cIA: Clinical stage IA; cIB: Clinical stage IB; CR: Complete response; CRT: Chemoradiotherapy; IASLC: International Association for the Study of Lung Cancer; ISC-LCSG: The Lung Cancer Study Group of the International Society of Chemotherapy; LoE: Level of evidence; MST: Median survival time; NC: Neoadjuvant chemotherapy; NST: Non-surgical treatment; NCDB: National Cancer Data Base; OS: Overall survival; PCI: Prophylactic cranial irradiation; pIA: Pathologic stage IA; pIB: Pathologic stage IB; pII: Pathologic stage II; pIIIA: Pathologic stage IIIA; pIIIB: Pathologic stage IIIB; QT: Chemotherapy; R0: Complete resection; RS: Retrospective study; RT: Radiotherapy; S: Surgery; SCLC: Small cell lung cancer; SEER: Surveillance, Epidemiology, and End Results database.



DOI: 10.5306/wjco.v13.i6.429

Figure 1 Proposed algorithm for the treatment of early-stage small cell lung cancer focused on surgical treatment. CT: Chemotherapy; PCI: Prophylactic cranial irradiation; RT: Radiotherapy; SBRT: Stereotactic body radiation therapy.

techniques that enable biopsy and the pathologic study of lymph nodes (*e.g.*, transbronchial ultrasound and esophageal echoendoscopy) and invasive techniques such as video mediastinoscopy, anterior mediastinotomy, and videothoracoscopy.

Radiotherapy in limited-stage SCLC

Thoracic radiotherapy and stereotactic body radiotherapy in early-stage SCLC: SCLC is usually classified as limited-stage (LS) or extensive-stage (ES) disease[65]. With adequate treatment, overall survival (OS) is 16-22 mo in patients with LS-SCLC and 8-13 mo in those with ES-SCLC. The corresponding 5-year survival rates are < 20% and < 2%[66]. Radiotherapy is associated with better OS when given in the first few weeks after the start of chemotherapy (ideally during cycle 1 and never later than cycle 3), and the shorter the duration the better[67].

Hypofractionated radiotherapy is well tolerated and produces similar response rates to standard fractionation. Proposed schedules include 40 Gy in 16 fractions with chemotherapy and prophylactic cranial irradiation (PCI)[68] and 55 Gy in 25 once-daily fractions, also with chemotherapy and PCI[69]. Higher complete response rates and longer OS have been observed for hyperfractionated *vs* hypofractionated radiotherapy (45 Gy in 30 fractions twice daily *vs* 42 Gy in 15 fractions twice daily), but the differences were not statistically significant[70].

Treatment must be individualized. Some clinical guidelines recommend surgery and adjuvant chemotherapy for stage I and IIA disease[30,71]. This combination has achieved OS rates of 50%-70%[20,21,72,73]. Nonetheless, stereotactic body radiotherapy (SBRT) should be considered in patients who are unfit for or refuse surgery, as it is not inferior to conventional treatment and has an acceptable safety

profile (toxicity < grade 3)[74-80]. Although the evidence is based on small series, SBRT can achieve local control rates > 85%. No clear benefit, however, has been observed for OS (63%-83% at 1 year, 35%-76% at 2 years, and 21%-26% at 3 years) (Table 2). This could have several explanations. First, SCLC is a fast-spreading tumor (associated with distant metastases in 50% of cases), requiring clinicians to consider neoadjuvant or adjuvant chemotherapy (preferably adjuvant in the case of SBRT due to its short treatment time), particularly in the case of tumors > 2 cm[77-81]. Adjuvant chemotherapy can improve OS by up to 25%[82]. Second, the disease may have been initially understaged. Thus, staging with positron emission tomography-computed tomography (CT) and mediastinoscopy/endobronchial ultrasound is recommended before proposing surgery or SBRT. SBRT should be planned using intensity-modulated techniques (*e.g.*, intensity-modulated radiotherapy, volumetric modulated arc therapy) and delivered with image-guided inter- and/or intrafraction monitoring (*e.g.*, Conebeam, ExacTrac) and respiratory control (*e.g.*, four-dimensional CT, deep inspiration breath hold, active breathing control, gating). The number of fractions can vary, but a biologically effective dose of >100 Gy must be delivered to the isocenter of the tumor. Because SCLC is highly radiosensitive, some groups have suggested using a lower dose, particularly in patients with ultracentral tumors[83].

PCI

Patients with SCLC are at high risk of brain metastases (BM)[84,85]. Research into the potential of PCI began in the late 1970s[86]. Brain magnetic resonance imaging (MRI) should be performed after chemoradiotherapy or systemic therapy[87], as 21.8%-32.5% of patients who achieve complete response subsequently develop BM[88,89]. A meta-analysis published by Aupérin *et al*[90] in 1999 showed that PCI was associated with a reduced incidence of BM at 3 years (59% *vs* 33%) and a 5.4% increase in OS. Subsequent meta-analyses have shown similarly favorable results for PCI in patients who had responded to treatment[91-94]. Most of these studies, however, were published before the introduction of restaging with brain MRI, and therefore the true benefit of PCI in LS-SCLC is not so clear[95,96]. Nonetheless, retrospective studies have described beneficial effects for PCI in patients with a previous negative brain MRI scan[97,98]. Patients who have undergone complete resection should benefit from PCI, except patients with stage I disease, who have a low risk of BM[99-101]. There is a growing interest in the use of brain MRI and stereotactic irradiation rather than PCI in patients with LS-SCLC[102], but prospective randomized trials are needed.

Concomitant treatment in locally advanced disease

Radical treatment with chemotherapy and concomitant radiotherapy are recommended for patients with stage IIB-IIIC disease in good general health[4,103]. Eighty percent of patients with mediastinal involvement treated exclusively with chemotherapy experience local recurrence[104], but the addition of radiotherapy lowers this rate and increases survival[104,105]. The CONVERT trial, which compared fractionated and unfractionated radiotherapy in patients treated with cisplatin-etoposide, reported an overall response rate (ORR) of 70%-90%, an OS of 24-30 mo, and a 5-year OS rate of 25%-30%[106]. Another two trials investigated the combination of bevacizumab, an angiogenic, with conventional chemoradiotherapy, but had to be discontinued because of a relatively high incidence of severe adverse events (tracheoesophageal fistulae)[107].

Perspectives for radiotherapy in LS-SCLC

Radiotherapy with immunotherapy in LS-SCLC: Three trials are currently analyzing the combined use of radiotherapy and immunotherapy in LS-SCLC: The NRG Oncology and Alliance trial (ClinicalTrials.gov Identifier: NCT03811002) investigating chemoradiotherapy with and without atezolizumab; the phase II STIMULI trial (NCT02046733) analyzing nivolumab and ipilimumab after chemoradiotherapy and PCI; and the phase III ADRIATIC trial (NCT03703297) comparing durvalumab, durvalumab plus tremelimumab, and placebo in patients without progression after chemoradiotherapy.

Hippocampal avoidance to reduce the neurotoxicity of PCI: The role of PCI with hippocampal avoidance (HA) in patients with LS- or ES-SCLS without BM is being investigated in three phase III trials: The Dutch NKI/AVL trial (NCT01780675), the NRG Oncology CC003 trial (NCT02635009), and the Spanish PREMER-TRIAL (NCT02397733)[108]. The Dutch group found no significant differences in recall assessed using the revised version of the Hopkins Verbal Learning Test between patients who received PCI and those who received HA-PCI[109]. Using the Free and Cued Selecting Reminding Test, the Spanish group found a significant decline in 3-mo delayed recall [22.22% *vs* 5.08%; odds ratio (OR) = 5.33; 95% confidence interval (CI): 1.44-19.65; P = 0.006) and total recall (20.63% *vs* 6.78%; OR = 3.57; 95%CI: 1.09-11.68; P = 0.02] in the PCI *vs* HA-PCI group[110]. Another potentially interesting line of research is the use of Alzheimer disease drugs to preserve cognition in patients treated with PCI[111].

Proton beam radiation therapy: In non-SCLC, proton therapy has been used to reduce doses to the heart while maintaining high doses to the tumor[112]. Proton beam radiation therapy (PBRT) is potentially beneficial in SCLC, as patients tend to have bulky central disease at diagnosis. In a study of 30 patients at the University of Pennsylvania, PBRT at a median dose of 63.9 cobalt Gy equivalents achieved a promising median OS of 28.2 mo with low toxicity[113]. These results need to be validated in

Table 2 Thoracic radiotherapy and stereotactic body radiotherapy in early-stage small cell lung cancer

Ref.	Sample size	Fractionation	QT	Prophylactic cranial irradiation	Local control	Overall survival	Disease-free survival
Videtic *et al*[76], 2013	*n* = 6	60 Gy (3 fx); 50 Gy (5 fx); 30 Gy (1 fx)	4/6	4/6	100% (1 yr)	63% (1 yr)	75% (1 yr)
Shioyama *et al*[77], 2015	*n* = 64	48 Gy (4 fx)	36/64	10/64	89% (2 yr)	76% (2 yr)	
Stahl *et al*[79], 2017	*n* = 285	48-60 Gy (3-5 fx)	130/285		35% (3 yr). 21.5% (5 yr)		
Verma *et al*[75], 2017	*n* = 74	50 Gy (5 fx)	45/74	17/74	96% (3 yr)		
Shioyama *et al*[78], 2018	*n* = 43	36-60 Gy (3-10 fx)	8/43	8/43	80.2% (2 yr)	72.3% (2 yr)	44.6% (2 yr)
Verma *et al*[74], 2019	*n* = 149	45-60 Gy (3-8 fx)	149/149		83.8% (29.2 mo)		
Newman *et al*[81], 2019	*n* = 239	BED > 100 Gy (max 8 fx)	84/239		27% (5 yr); 36% (5 yr, with QT)		
Singh *et al*[80], 2019	*n* = 21	BED 105.6 Gy (3-5 fx)	4/21		100% (1, 2, 3 yr)	73.1% (1 yr); 36.6% (2 yr)	85.7% (1 yr); 42.9% (2 yr)

BED: Biologically equivalent dose; fx: Fraction; QT: Chemotherapy.

further studies.

ES SCLC

Initial management

Chemotherapy with platinum compounds and etoposide has been the standard treatment for ES-SCLC for many decades. The COCIS meta-analysis showed that cisplatin- and carboplatin-based chemotherapy produced comparable results in terms of OS (9.6 *vs* 9.4 mo), progression free survival (PFS) (5.5 *vs* 5.3 mo), and ORR (67% *vs* 66% mo)[114]. Other strategies attempted, including maintenance treatments and combinations with antiangiogenics, have produced disappointing results[115-117]. The recently published results of the IMpower 133[118] and CASPIAN[119] trials comparing combinations of chemotherapy and immunotherapy followed by immunotherapy with standard platinum and etoposide chemotherapy in ES-SCLC have shown that the combined use of chemotherapy and immunotherapy prolongs OS.

IMpower133 is a phase III trial in which patients received four cycles of carboplatin and etoposide and either atezolizumab or placebo followed by maintenance atezolizumab[118]. The response rates in both arms were similar, but patients in the atezolizumab arm survived for a median of 2.3 mo longer [hazard ratio (HR) = 0.7; 95%CI: 0.54-0.91; P = 0.007]. The updated trial data presented at the 2019 European Society for Medical Oncology congress showed an increase in OS at both 12 mo (39% to 51.9%) and 18 mo (21% to 34%)[120,121].

The phase III CASPIAN trial has three treatment arms. Treatment with durvalumab plus chemotherapy (4-6 cycles of cisplatin or carboplatin plus etoposide) followed by durvalumab maintenance achieved an OS of 12.9 mo (*vs* 10.5 mo for standard chemotherapy) (HR = 0.75; 95%CI: 0.62-0.9; P = 0.0032), a 2-year PFS of 11% (*vs* 2.9%), and a 2-year response rate of 13.5% (*vs* 3.9%)[119,122]. In the third arm, tremelimumab plus durvalumab *vs* chemotherapy showed no benefit in antitumor activity and was associated with increased toxicity[123].

Results from other studies evaluating combinations of anti-programmed death 1 (PD-1) antibodies have been disappointing. While the combined use of pembrolizumab and chemotherapy increased PFS, it did not provide any significant improvements in OS[124]. In the phase II ECOG-ACRIN EA5161 trial, chemotherapy plus nivolumab followed by maintenance treatment achieved a non-significant improvement in PFS (5.5 *vs* 4.7 mo) and OS (11.3 *vs* 8.5 mo)[125] (Table 3). A systematic review and two meta-analyses published in 2020 concluded that a combination of chemotherapy with atezolizumab or durvalumab was the best first-line treatment for ES-SCLC[126,127]. Other options that have been explored include combinations of ipilimumab and chemotherapy (no benefit and greater toxicity)[128,129] and combinations of different chemotherapy agents, such as irinotecan plus etoposide and cisplatin plus irinotecan (also without benefits)[130-132].

Table 3 Combined first-line immunotherapy options for extensive-stage small cell lung cancer

Study	*n*	Design	Treatment	RR	PFS	OS
NCT01450761	1132	Phase III; Randomized, double-blind; Drug: Ipilimumab	Arm A: PE × 4C + ipilimumab × 4C; Control: PE × 4C + placebo × 4C	PR 62% *vs* 62%; SD 26% *vs* 27%; PD 6% *vs* 9%	4.6 *vs* 4.4 mo; HR = 0.85, *P* = 0.0161	11.0 *vs* 10.9 mo; HR = 0.94, *P* = 0.3775
Impower 133	403	Phase III. Randomized, double-blind; Drug: Atezolizumab	Arm A: PE + atezolizumab × 4C/atezolizumab; Control: PE + placebo × 4C/placebo	60% *vs* 64%	5.2 *vs* 4.3 mo; HR = 0.77, *P* = 0.02	12.3 *vs* 10.3 mo; HR = 0.70, *P* = 0.007
CASPIAN	805	Phase III. Randomized, open-label; Drug: Durvalumab	Arm B (*n* = 268): Durvalumab + PE × 4C/durvalumab; Control: PE × 4C	68% *vs* 58%	5.1 *vs* 5.4 mo; HR = 0.78, *P* not tested	13.0 *vs* 10.3 mo; HR = 0.73, *P* = 0.0047
CASPIAN	805	Phase III. Randomized, open-label; Drug: Durvalumab + tremelimumab	Arm A (*n* = 268): Durvalumab + tremelimumab + PE × 4C/durvalumab + tremelimumab. Control: PE × 4C	58% both arms	4.9 *vs* 5.4 mo; HR = 0.84	10.4 *vs* 10.5 mo; HR = 0.82, *P* = 0.045
KEYNOTE 604	453	Phase III; Randomized, double-blind; Drug: Pembrolizumab	Arm A: Pembrolizumab + PE; Control: PE + placebo	71% *vs* 62%	4.5 *vs* 4.3 mo; HR = 0.75, *P* = 0.0023	10.8 *vs* 9.7 mo; HR = 0.80, *P* = 0.0164
ECOG-ACRIN	160	Phase I. Randomized, open-label; Drug: Nivolumab	Arm A: PE + nivolumab × 4C/nivolumab; Control: PE × 4C	52.29% *vs* 47.71%	5.5 *vs* 4.6 mo; HR = 0.65, *P* = 0.012	11.3 *vs* 8.5 mo; HR = 0.67, *P* = 0.038

4C: 4 cycles; OS: Overall survival; PD: Progressive disease; PE: Platinum and etoposide; PFS: Progression free survival; PR: Partial response; RR: Response rate; SD: Stable disease.

PCI in ES-SCLC

The results of the first randomized trial to demonstrate a reduction in the risk of symptomatic BM (14.6% *vs* 40.4% at 1 year) and an improvement in OS (27.1% *vs* 13.3%) in chemotherapy responders who underwent PCI were published in 2007[133]. The results are supported by data from several meta-analyses[134-136], although as a shortcoming of the trial, pre-PCI brain imaging was not performed [133]. The results of a randomized trial conducted in Japan comparing PCI with close MRI follow-up in patients with ES-SCLC who had responded to chemotherapy and had a negative brain MRI were published in 2017. While they did not show an increase in OS (11.6 mo for PCI *vs* 13.7 mo for MRI follow-up; HR = 1.27; 95%CI: 0.96-1.68; *P* = 0.094), they did show a significant decrease in the incidence of BM[137].

A recent meta-analysis showed that PCI was only associated with prolonged OS in studies where brain imaging was not performed between chemotherapy and irradiation (HR = 0.70; 95%CI: 0.57-0.85). In other words, PCI did not offer any significant benefits when preceded by MRI or CT to test for BM (HR = 0.94; 95%CI: 0.74-1.18)[138]. Considering the above results and the neurotoxic effects of PCI[139], it would seem reasonable to consider periodic MRI examination as an alternative to PCI in patients with ES-SCLC. In such cases, a joint evaluation should be made by the medical and radiation oncologists[30]. The recommended dose for PCI is 25 Gy in 10 fractions, as higher doses do not appear to reduce the incidence of BM at 2 years and are associated with higher mortality and chronic neurotoxicity[140].

Treatment of refractory and relapsed SCLC

Relapsed SCLC tends to be resistant to treatment and is associated with an OS of 4-5 mo. Response to second-line treatment varies according to PFS and is 10% in patients with a PFS < 3 mo (refractory SCLC) and 25% in those with a PFS of 3-6 mo (sensitive SCLC)[141-143].

Relapse after PFS > 3 mo: Rechallenge treatment with combinations of platinum-based chemotherapy has been investigated in patients with sensitive SCLC. Patients treated with carboplatin and etoposide had a longer PFS than those treated with topotecan, and the greatest benefits were observed for those who relapsed after 6 mo[144,145].

Relapse after PFS of < 3 mo: Until recently, topotecan was the only drug authorized by the US and Food and Drug Administration (FDA) to treat relapsed SCLC. In the 2006 phase III trial that led to its approval, it significantly improved survival compared with best supportive care only[146]. Another phase III trial comparing topotecan and CAV (cyclophosphamide, doxorubicin, and vincristine) reported similar survival and response rates for the two treatments, but found topotecan to be associated with better symptom control and lower toxicity[147]. An additional study evaluating topotecan plus aflibercept, an antiangiogenic, reported an OS of 5 mo[148].

One recent advance in this setting is the recent approval by the FDA of lurbinectedin as a second-line treatment for SCLC. In a study of patients with SCLC without BM, lurbinectedin achieved an ORR of 35%, and a median response duration of 5.1 mo (> 6 mo in 25% of patients)[149]. The combination of

lurbinectedin and doxorubicin was investigated in two cohorts in a phase I trial and showed disease control rates of 81% and 70% and a median response duration of 4.5 and 5.2 mo[150]. These findings led to the design of the phase III ATLANTIS trial comparing lurbinectedin plus doxorubicin with topotecan and with CAV; a press release, however, announced no improvement in OS[151] (Figure 2).

Amrubicin is available for the treatment of relapsed SCLC in Japan, but it has not been approved by the FDA. A phase III trial comparing amrubicin with topotecan showed superior symptom control for topotecan but no significant differences in OS[152]. Immune checkpoint inhibitors have also been tested. The CheckMate 032 trial comparing nivolumab alone with nivolumab plus ipilimumab in recurrent SCLC reported improved ORR and OS in both treatment arms regardless of prior treatment or PD-L1 expression[153,154]. With these data, the FDA approved nivolumab for use in previously treated patients.

The phase III CheckMate 331 trial showed similar OS for nivolumab *vs* standard chemotherapy in the second-line treatment of SCLC[155]. Pembrolizumab has also been tested in SCLC. A pooled analysis of the KEYNOTE-028 (phase Ib)[156] and KEYNOTE-158 (II)[157,158] trials found an ORR of 19.3%, leading to FDA approval. Atezolizumab was also tested in a phase II trial, but the primary endpoint was not met[159]. Paclitaxel every 3 wk for 6 cycles plus pembrolizumab after the second cycle until disease progression achieved a disease control rate of 80% and a median OS of 9.2 mo[160]. Other drugs tested in the setting of relapsed SCLC are temozolomide[161,162], irinotecan[163], paclitaxel[164,165], docetaxel[166], gemcitabine[167,168], and vinorelbine[169]. Finally, a recent phase IIb study showed that belotecan was associated with better OS and disease control than topotecan in patients with sensitive SCLC[170].

Recent advances in systemic therapy

New drugs linked to targets with a role in cell proliferation have been developed. These include poly (ADP-ribose) polymerase (PARP) inhibitors, delta-like ligand 3 inhibitors (DLL3), and drugs that selectively inhibit oncogenic transcription. The expression of DNA damage response proteins [especially PARP1/checkpoint kinase 1 (CHK1)] is elevated in SCLC, and *in vitro* studies have shown an antitumor effect for PARP inhibitors[171]. Monotherapy with PARP inhibitors has also been investigated in different clinical trials, but the results have been disappointing. In an early study, talazoparib showed an ORR of 8.7%[172]. No benefit was observed for maintenance treatment with olaparib after first-line chemotherapy with cisplatin and etoposide[173] or for the addition of veliparib *vs* placebo to first-line cisplatin and etoposide, with findings showing no significant differences in PFS (6.1 *vs* 5.5 mo) or OS (10.3 *vs* 8.9 mo)[174,175].

Discordant results have been reported for combinations of chemotherapy and PARP inhibitors in successive treatment lines. No significant differences were found for PFS or OS in a study comparing temozolomide plus veliparib *vs* temozolomide only[176]. Temozolomide combined with olaparib, however, was associated with a response rate of 41.7%, a PFS of 4.2 mo, and an OS of 8.5 mo in a phase I/II clinical trial[177]. No benefits have been observed for the combined use of PARP inhibitors and immunotherapy (durvalumab with olaparib, among others)[178]. Future actions targeting this actionable molecular pathway in SCLC will probably involve combinations of PARP inhibitors and chemotherapy agents and immunotherapy, or new molecules. Promising results have been reported for CHK1 (SRA737) combined with low-dose gemcitabine and anti-PD-1/programmed death ligand 1 (PD-L1) immune checkpoint inhibitors[179] and for PARP inhibitors combined with WEE1 inhibitors, which act at the cell-cycle level[180].

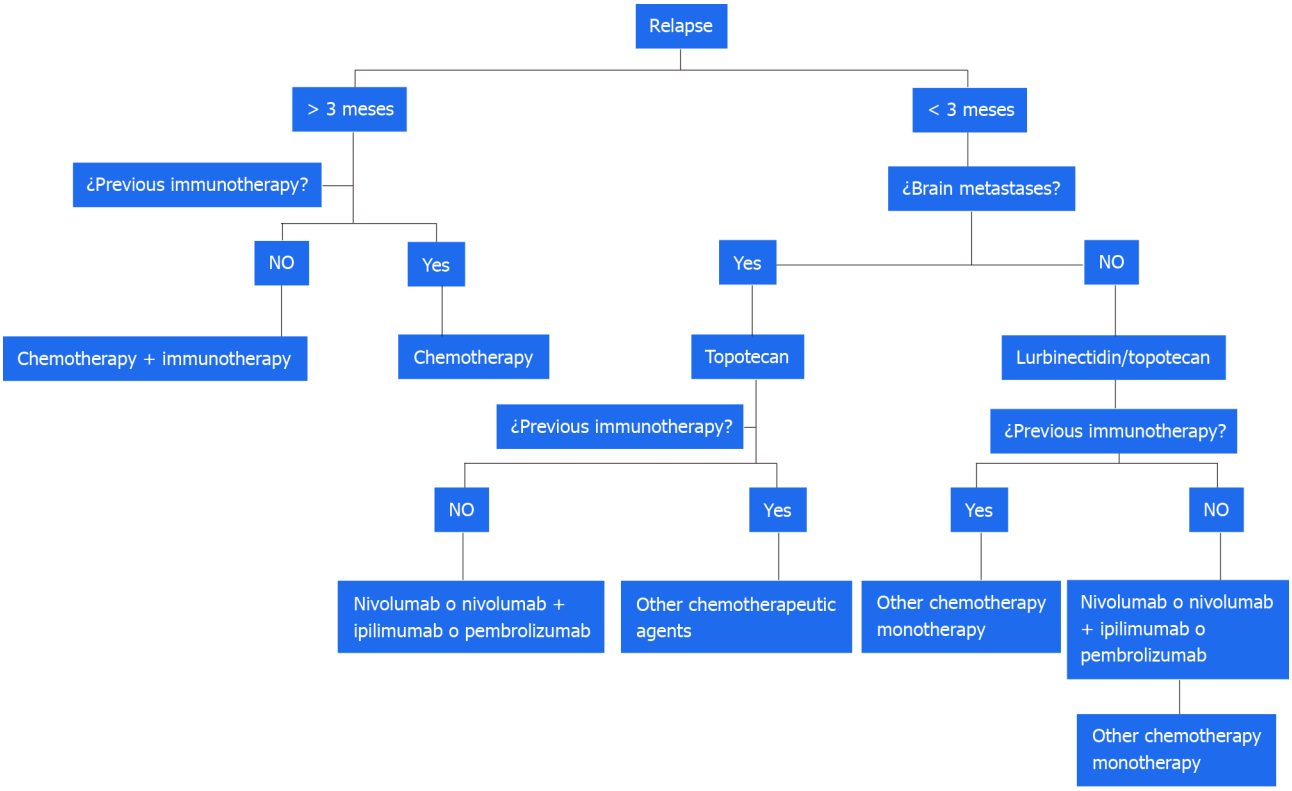
Other treatments have also yielded positive results. Lurbinectedin, a selective oncogenic transcription inhibitor, was recently evaluated in combination with irinotecan in pretreated patients in a phase Ib/II basket trial. The results for the SCLC cohort showed an ORR of 62%, a clinical benefit rate of 81%, a disease control rate of 90%, and a PFS of 6.1 mo[181]. Other new molecules with different ligands under investigation include DLL3 inhibitors, such as rovalpituzumab-tesirine. This is a promising drug in pretreated patients expressing DLL3, although recent reports have described greater toxicity and little benefit compared with topotecan[182-184]. AMG 757, a half-life extended DLL3 bispecific T-cell engager, has also shown promising results in pretreated patients in an ongoing phase I trial, with an ORR of 14%, a disease control rate of 37%, and a very promising median duration of 6.2 mo[185].

Perspectives for radiotherapy in ES-SCLC

Numerous questions remain to be answered regarding the role of radiotherapy in ES-SCLC.

Consolidation radiotherapy in extensive SCLC: What is the optimal radiation dose or indication for patients with complete thoracic response or partial distant response? The Chinese phase III trial (NCT02675088) is comparing 45 Gy at 3 Gy/d in 15 fractions *vs* 10 fractions (CREST trial schedule) with a primary endpoint of OS at 2 years[186]. How can radiotherapy be best combined with immunotherapy? The RAPTOR phase II/III trial (NCT04402788) is evaluating the use of radiotherapy to the chest and distant lesions after 4-6 cycles of carboplatin and etoposide plus atezolizumab.

Stereotactic radiosurgery to treat BM: Stereotactic radiosurgery has not traditionally been investigated in SCLC due to the high incidence of BM and poor prognosis. Nonetheless, there is growing evidence



DOI: 10.5306/wjco.v13.i6.429

Figure 2 Proposed algorithm for the treatment of relapsed small cell lung cancer.

that it may be appropriate[187]. ENCEPHALON, a phase II trial (NCT03297788) is currently comparing stereotactic radiosurgery with whole-brain radiotherapy in patients with SCLC and 1-10 BM.

CONCLUSION

The treatment of SCLC will continue to be a challenge. Immunotherapy has a new role lung cancer and will be the future treatment standard alone or in combination, as well as the new radiotherapy techniques. As has been occurred in non-SCLC, the future of treatments in both early and advanced stages is through immunotherapy and targeted treatments. Furthermore, the use of different combinations of chemoimmunotherapy in recent months has improved the prognosis of patients with advanced SCLC. Nevertheless, continued research efforts are needed. Different lines of investigation are open and we hope that their findings will continue to improve prognosis and quality of life in this setting.

FOOTNOTES

Author contributions: Pangua C, Rogado J, Serrano-Montero G, Belda-Sanchís J, Álvarez Rodríguez B, Torrado L, and Rodríguez De Dios N performed the research and wrote the paper; Mielgo-Rubio X, Trujillo JC, and Couñago F contributed to the critical review of the manuscript for important intellectual content.

Conflict-of-interest statement: Mielgo-Rubio X declares the following conflicts of interest: Advisory role; Boehringer-Ingelheim, Astra Zeneca, Brystol Myers Squibb. Speakers' bureau; Roche, Astra Zeneca, Brystol Myers Squibb, MSD, Abbott. Research funding; Brystol Myers Squibb. The other authors have no conflicts of interest to declare.

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S-Editor: Wang JJ
L-Editor: Filipodia
P-Editor: Wang JJ

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World J Clin Oncol 2022 June 24; 13(6): 448-472

DOI: 10.5306/wjco.v13.i6.448

ISSN 2218-4333 (online)

REVIEW

Immunotherapy for advanced hepatocellular carcinoma: From clinical trials to real-world data and future advances

Kathrine S Rallis, Dimitrios Makrakis, Ioannis A Ziogas, Georgios Tsoulfas

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Li Y, China; Ling Q, China; Zeng YY, China
A-Editor: Liu X, China

Received: January 31, 2022
Peer-review started: January 31, 2022
First decision: April 13, 2022
Revised: April 27, 2022
Accepted: May 28, 2022
Article in press: May 28, 2022
Published online: June 24, 2022

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-associated mortality worldwide. HCC is an inflammation-associated immunogenic cancer that frequently arises in chronically inflamed livers. Advanced HCC is managed with systemic therapies; the tyrosine kinase inhibitor (TKI) sorafenib has been used in 1st-line setting since 2007. Immunotherapies have emerged as promising treatments across solid tumors including HCC for which immune checkpoint inhibitors (ICIs) are licensed in 1st- and 2nd-line treatment setting. The treatment field of advanced HCC is continuously evolving. Several clinical trials are investigating novel ICI candidates as well as new ICI regimens in combination with other therapeutic modalities including systemic agents, such as other ICIs, TKIs, and anti-angiogenics. Novel immunotherapies including adoptive cell transfer, vaccine-based approaches, and virotherapy are also being brought to the fore. Yet, despite advances, several challenges persist. Lack of real-world data on the use of immunotherapy for advanced HCC in patients outside of clinical trials constitutes a main limitation hindering the breadth of application and generalizability of data to this larger and more diverse patient cohort. Consequently, issues encountered in real-world practice include patient ineligibly for immunotherapy because of contraindications, comorbidities, or poor performance status; lack of response,

efficacy, and safety data; and cost-effectiveness. Further real-world data from high-quality large prospective cohort studies of immunotherapy in patients with advanced HCC is mandated to aid evidence-based clinical decision-making. This review provides a critical and comprehensive overview of clinical trials and real-world data of immunotherapy for HCC, with a focus on ICIs, as well as novel immunotherapy strategies underway.

Key Words: Hepatocellular carcinoma; Liver cancer; Immunotherapy; Immune checkpoint inhibitors; Clinical trials; Real-world data

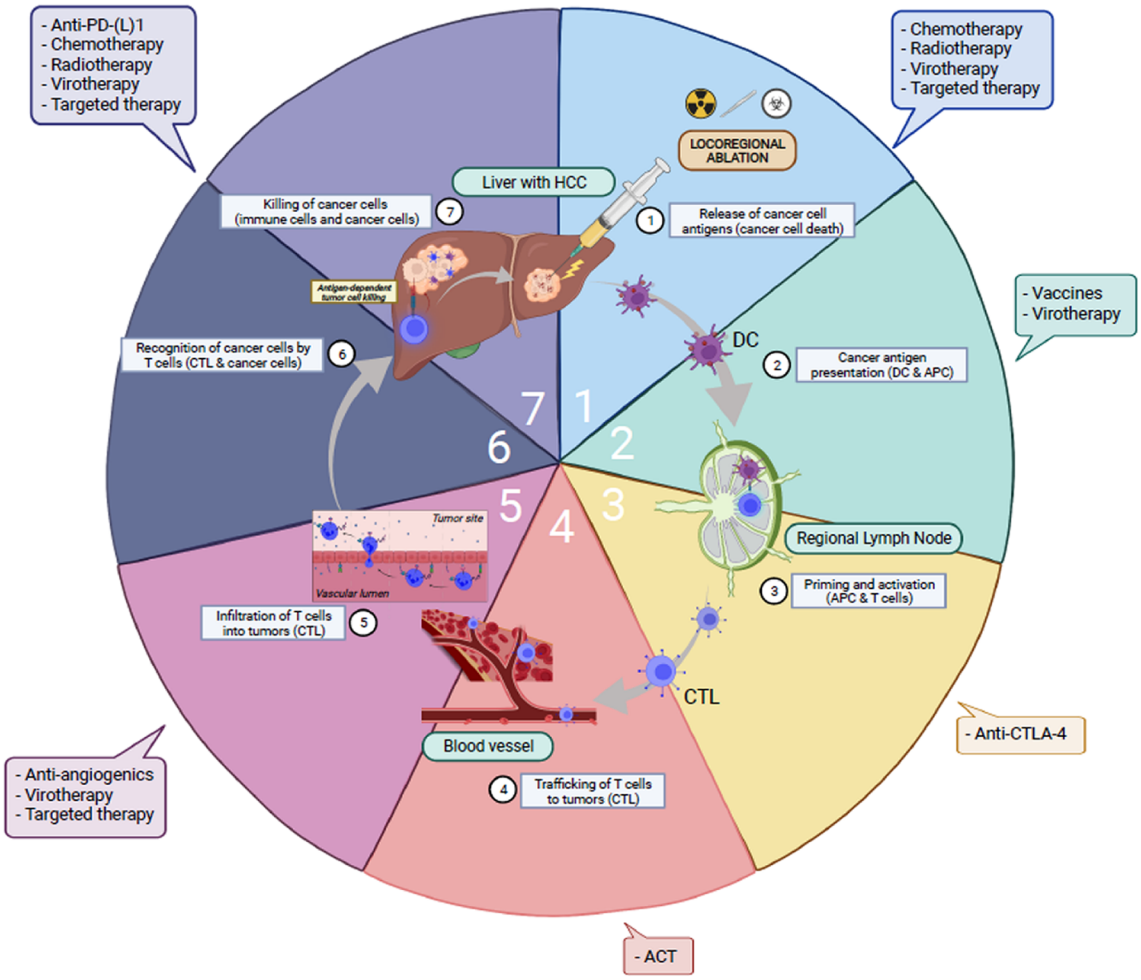
Core Tip: In the last five years, immune checkpoint inhibitors (ICIs) have entered the treatment landscape of hepatocellular carcinoma (HCC) in the 1st and 2nd line setting. However, due to restrictions in clinical trial inclusion and exclusion criteria, there remains a need for further real-world data on the efficacy, toxicity, and cost-effectiveness of ICIs in a broader cohort of HCC patients. New trials are underway investigating further ICI regimens, including combination therapy strategies, while novel immunotherapies are also being brought to the fore. This review discusses key clinical trials, real-world data, and future advances of immunotherapy for HCC, with a focus on ICIs.

Citation: Rallis KS, Makrakis D, Ziogas IA, Tsoulfas G. Immunotherapy for advanced hepatocellular carcinoma: From clinical trials to real-world data and future advances. *World J Clin Oncol* 2022; 13(6): 448-472
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/448.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.448

INTRODUCTION

Liver cancer is the third leading cause of cancer-related mortality worldwide and sixth in terms of incidence accounting for 830180 deaths and 905677 cases in 2020[1]. Hepatocellular carcinoma (HCC) is the leading type of primary liver cancer representing 85%-90% of cases[2]. The incidence of HCC is expected to continue to increase in countries, including the United States, until 2030. Asia and Africa feature the highest incidence of disease due to the endemic prevalence of hepatitis B or C virus (HBV or HCV) which, when untreated, lead to chronic liver disease and subsequent development of HCC. Global vaccination efforts against HBV and HCV are expected to lower the incidence of HCC, with effects becoming apparent after a latency period of 20-30 years correlating to the time required from liver damage to cancer development[3]. Second to viral hepatitis, alcohol abuse is another main cause of HCC development[4]. Diabetes, aflatoxin-B1 exposure, obesity, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease, and metabolic syndrome represent other leading contributors to HCC development[5]. NASH typically develops in patients with obesity, type 2 diabetes, dyslipidemia, and hypertension, therefore being a leading risk factor for HCC in rich developed countries such as the United States[3]. Both incidence and mortality of liver cancer are expected to double in the next two decades[6]. With a 5-year survival rate of less than 20%, liver cancer carries one of the worst cancer prognoses after pancreatic cancer[7]. Although this figure represents a significant improvement compared to the 3% 5-year survival observed in the 1970s[7], further research is warranted to improve treatments, especially for individuals with distant and regional metastatic disease which feature a 3% and 12% 5-year survival, respectively[8].

The current treatment landscape of HCC depends on disease stage (Figure 1). Surgical resection, liver transplantation (LT), and locoregional ablation therapies (2nd-line) are used with curative intent in early and intermediate disease. Yet, recurrence rates are high, while only 30%-40% of patients qualify for the above treatments[9,10]. Advanced HCC (aHCC) is managed with systemic therapies. Historically, systemic chemotherapies have largely been ineffective in HCC due to high rates of chemoresistance and liver impairment with associated susceptibility to toxicities[9]. Starting with the Food and Drug Administration (FDA) approval of sorafenib, a multiple tyrosine kinase inhibitor (TKI) with antiangiogenic and antiproliferative action, as a frontline systemic therapy for HCC in 2007, systemic therapies for HCC have evolved remarkably[11]. In 2018, following several randomized controlled trials exploring systemic therapies, which failed to surpass sorafenib, the multikinase inhibitor, lenvatinib, gained FDA approval as another 1st-line therapy in HCC following results of a phase III non-inferiority trial[12]. Subsequently, the TKIs regorafenib[13], cabozantinib[14], and ramucirumab[15] received approval in refractory HCC.



DOI: 10.5306/wjco.v13.i6.448

Figure 1 Schematic of the cancer-immunity cycle and strategies to overcome mechanisms of resistance in each step by enhancing necessary immune stages *via* different anti-cancer therapeutic modalities in advanced hepatocellular carcinoma. ACT: Adoptive cell transfer; APC: Antigen presenting cell; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell.

In the last decade, the field of cancer immunotherapy has evolved tremendously, largely owing to the success of monoclonal antibodies (mAbs) directed against negative regulator molecules of T-cell activation, namely cytotoxic T-lymphocyte protein-4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand, PD-L1. Immune checkpoint inhibitors (ICIs) reverse the immunosuppressive cancer phenotype by binding to and blocking co-inhibitory immune signalling molecules that are upregulated in cancer providing a means of systemic immune recognition and targeting of malignant cells. Following the approval of ipilimumab (anti-CTLA-4) for metastatic melanoma in 2011, ICIs have gradually been trialled and expanded across solid tumors. To date, four ICI regimens have been approved for HCC: nivolumab (anti-PD-1), approved as 2nd-line in 2017[16]; pembrolizumab (anti-PD-1), approved as 2nd-line in 2018[17]; nivolumab plus ipilimumab, approved as 2nd-line in 2020[18]; and atezolizumab (anti-PD-L1) plus bevacizumab (anti-vascular endothelial growth factor [VEGF] mAb), approved as 1st-line in 2020[19].

The complex interdependent relationship between chronic inflammation and anticancer immunity in HCC represents a possible opportunity and challenge for immunotherapy. Intelligent therapeutic strategy design that balances enhancing anti-tumor immunity whilst minimizing pro-tumorigenic inflammation and immunosuppressive adaptations lies at the center of successful immunotherapeutic regimens for HCC. Furthermore, effective anti-cancer immunity to overcome cancer immune escape involves multiple steps. Hence, new immunotherapies continue to be investigated for HCC, with novel adoptive cell transfer (ACT), therapeutic cancer vaccines, and virotherapy being developed as monotherapies or in combination strategies. This review summarizes updates and future directions for immunotherapies and their combinations in HCC.

IMMUNOGENICITY IN HCC

Immunotherapies are potentially promising therapeutic strategies in HCC. A complex interdependent relationship exists between chronic inflammation and anticancer immunity in the normal liver and in HCC, representing an opportunity and challenge for immunotherapy in HCC.

The liver itself is an immune organ with rich and unique immune cell populations (*e.g.*, Kupffer cells), functional anatomy, and immune functions. Under normal conditions, the liver finetunes immune tolerance, systemic inflammation and immunity, and anti-tumor immunity (*reviewed in*[20]). The tolerogenic potential of the liver – required for the modulation of host response to gut flora – underlies its capacity to generate potent immune tolerance to tumors when liver metastases occur from other primary cancers[21]. This same tolerogenic potential of the liver also underlies its ability to fully accept allograft LT and safely discontinue immunosuppressants in some LT patients[22]. Immune tolerance within the liver develops through complex interactions between liver-resident cells and peripheral leukocytes involving poor or incomplete activation of CD4+ and CD8+ T cells, elevated expression of immune checkpoints, and an immunosuppressive environment mediated by IL-10 and TGFβ[23,24]. Indeed, through new technologies and machine learning algorithms tumor immune microenvironment features have been correlated with patient prognostication to classify patients into separate groups based on response to immunotherapy and other treatments[25-28].

HCC represents a typical inflammation-associated immunogenic cancer as it often arises in chronically inflamed livers (necroinflammation)[29]. It is well known that chronic inflammation causes local and systemic immunosuppression of innate and adaptive immunity due to chronically elevated pro-inflammatory stimuli[20], while scar tissue itself impedes immunosurveillance[30]. Chronic antigen stimulation results in T-cell exhaustion, immune inhibitory receptor upregulation (*e.g.*, PD-1), and progressive loss of polyfunctional cytokine production[20]. Moreover, cirrhotic patients are systemically immunocompromised, due to loss of synthetic liver functions, and are susceptible to life-threatening infections[31]. Locally, both tumor cells and surrounding stroma orchestrate tissue remodeling with concurrent functional and phenotypical immunobiology adaptations resulting in a dysfunctional and immunosuppressive tumor milieu[32]. Simultaneously, successive chronic inflammatory stresses cause hepatocellular DNA damage, whereby genetic and epigenetic mutations give rise to immunogenic pathogen-associated proteins (abnormal amino acid sequences) through transcription and translation of mutated genetic sequences. In turn, tumor associated antigens (TAA) and neo-antigens may result that act as recognizable epitope targets to facilitate effector T-cell recognition of a non-self antigen against which to mount an immune response, so long as strong human leukocyte antigen binding and immunological synapse is possible against the new abnormal peptide sequence[32].

ICIs and other emerging forms of immunotherapy display high efficacy in cancers expressing targetable TAAs and neo-antigens. Indeed, some forms of immunotherapy incorporate molecular recognition of specific TAAs and neo-antigens in their mechanistic design[33]. Tumor mutational burden (TMB) is regarded as a surrogate marker for the expression of TAA and neo-antigens, and hence immunotherapy efficacy, as seen in the case of melanoma[34]. HCC has been shown to feature a low-to-moderate TMB compared to other tumors[35]. Although this theoretically corresponds to lower probability of immunotherapy efficacy, the antigenicity and immunogenicity of any resultant TAAs and neo-antigens in HCC is not well characterized and these may still be sufficiently potent targets for immunotherapies[36].

Intelligent therapeutic strategies that achieve an acceptable balance between enhancing anti-tumor immune surveillance and destruction whilst minimizing pro-tumorigenic inflammation and immunosuppression lie at the center of successful immunotherapy regimen design for HCC.

IMMUNE CHECKPOINT INHIBITORS IN HCC

ICI monotherapy in aHCC

The first study to investigate the efficacy of ICIs in HCC was CheckMate 040, a phase I/II clinical trial of nivolumab with or without ipilimumab in aHCC. Patients treated in the nivolumab monotherapy arm demonstrated an objective response rate (ORR) of 20% [95% confidence interval (CI): 15-26%] and a manageable toxicity profile; 25% of patients experienced grade 3-4 treatment-related adverse events (AEs). Of interest, 68% (n = 145 of 216) of patients in the expansion phase had previously received sorafenib in the 1^{s}-line setting. Analysis was stratified by PD-L1 expression but not by receipt of previous treatment[16]. Nivolumab received accelerated approval in the 2nd-line setting for the treatment of aHCC following results from this trial.

Another phase I/II study of ICI therapy for aHCC was NCT01693562, a trial assessing the efficacy of durvalumab in advanced solid tumors, including aHCC. In this trial, 93% of patients had been previously treated with sorafenib. ORR for the whole cohort was 10% (95%CI: 2.9%-24.2%) with a median overall survival (mOS) of 13.2 mo (95%CI: 6.3-21.1). Grade 3-4 AEs were noted in 20% of patients, with elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) among the most common (7.5% and 5.0%, respectively). Overall, durvalumab was shown to exert promising

activity over aHCC with an acceptable toxicity profile. The above studies established ICIs as tolerable and effective alternative (2nd-line) options to sorafenib in patients with aHCC. ICIs were subsequently trialed in 1s-line setting against sorafenib.

CheckMate 459 was a phase III study of nivolumab *vs* sorafenib as a 1st-line therapy in patients with aHCC. No statistically significant difference in mOS was found between treatment arms (nivolumab: 15.2 mo *vs* sorafenib: 14.7 mo); however, treatment-related AEs were more favorable with nivolumab [37]. Although results from CheckMate 495 have not yet been strong enough to justify approval of nivolumab as a 1st-line therapy for aHCC over sorafenib – due to the prespecified significance boundary for superior OS compared to sorafenib not being met – conclusions from this trial are significant as they indicate nivolumab as an alternative treatment option that should be offered to patients who cannot receive anti-angiogenics and TKIs because of contraindications or AE severity. This is reflected in the National Comprehensive Cancer Network Clinical Practice Guidelines in the USA. However, it has not been adopted in the European or Asian guidelines to date. Following this trial, the accelerated FDA approval of nivolumab monotherapy was withdrawn as it did not meet the post-marketing requirements. Importantly, to date this is the only phase III clinical trial completed to investigate single-agent anti-PD-1 or anti-PD-L1 monotherapy against single agent TKI monotherapy in the 1st-line setting for aHCC.

In the 2nd-line setting for the treatment of aHCC, the use of ICIs has been investigated with more success leading to clinical approvals. Keynote 224 was a phase II study of 2nd-line pembrolizumab in patients with aHCC that had previously been treated with, or were intolerant to, sorafenib. ORR for pembrolizumab was 17% (95%CI: 11%-26%) while treatment-related AEs were noted in 73% of patients, with 25% experiencing grade 3 AEs. Of note, most common AEs were increased AST and AL in 7% and 4% of patients, respectively. Based on these results, pembrolizumab has been granted FDA approval in the 2nd-line setting for patients with aHCC who previously received treatment with sorafenib.

In another study investigating the anti-PD1 agent camrelizumab as 2nd-line therapy in patients with aHCC previously treated with sorafenib ORR was 14.7% (95%CI: 10.3%-20.2%). Grade 3-4 AEs were encountered in 22% of patients, with increased AST being the most common AE (5%). This study demonstrated that camrelizumab also had a manageable toxicity profile[38].

Following the successful results of Keynote 224, Keynote 240, a randomized phase III study of 2nd-line pembrolizumab *vs* placebo in patients treated with 1st-line sorafenib, was initiated. The study showed a benefit in both mOS [13.9 *vs* 10.6 mo, hazard ratio (HR): 0.78, 95%CI: 0.61-0.99] and median progression-free survival (mPFS) (3.0 *vs* 2.8 mo, HR: 0.71, 95%CI: 0.57-0.90) in favor of pembrolizumab. The trial did not meet the prespecified criteria for mOS and mPFS despite the superior results observed with pembrolizumab. However, pembrolizumab had a favorable risk-to-benefit ratio and received accelerated FDA approval as a 2nd-line treatment for aHCC[39]. These results were similar to those of Keynote 224, suggesting that ICIs could become the preferred treatment of choice for patients who are at high risk for AEs.

More recently, the anti-PD-L1 agent avelumab showed moderate efficacy in a phase II trial in 30 patients previously treated with sorafenib (NCT03389126)[40]. The mOS and mPFS were 14.2 mo (95%CI: 9.5-18.9) and 3.5 mo (95%CI, 2.0–5.1), respectively. Treatment was well-tolerated, with 23% of patients exhibiting grade 3 AEs – commonest being increased AST/ALT (13%)—and none experiencing grade 4 AEs.

Clinical trials of ICI monotherapy in HCC attest to their efficacy and tolerability; however, these failed to demonstrate clear superiority over sorafenib, prompting investigators towards combination strategies to increase efficacy.

ICI combination therapies

ICI duplet therapy: The use of ICI combinations has gained attention in the last few years across a wide spectrum of solid tumors. Combinations of ICIs usually include an anti-PD-L1/PD-1 and an anti-CTLA-4 agent and demonstrate better responses compared to single-agent therapy, but also higher rates of AEs, especially serious and life-threatening ones[41]. Several clinical trials have assessed the efficacy and safety of anti-CTLA-4 and anti-PD-L1 combination regimens for aHCC.

A phase II trial (NCT02519348) assessed the efficacy of 2nd-line tremelimumab, an anti-CTLA-4 antibody, combined with durvalumab in patients with aHCC that were intolerant to, progressed to, or refused sorafenib. Patients were randomized into 4 different arms (tremelimumab 300 mg with durvalumab for the 1st cycle followed by durvalumab; durvalumab monotherapy; tremelimumab monotherapy; and tremelimumab 75mg with durvalumab for 4 cycles followed by durvalumab alone). Grade 3-4 AEs were observed in 35.1%, 17.8%, 42.0% and 24.4% of patients, respectively. ORRs were 22.7%, 9.6%, 7.2% and 9.5%, respectively, while mOS was 18.7 [95%CI: 10.8-not reached (NR)], 11.7 (95%CI: 8.5-16.9), 17.1 (95%CI: 10.9-NR) and 11.3 (95%CI: 8.4-14.6) months, respectively. Between treatment arms, tremelimumab 300 mg with durvalumab demonstrated the best benefit-to-risk profile [42]. After these results, the combination of tremelimumab-durvalumab was subsequently investigated in 1st-line setting in HIMALAYA (NCT03298451), a phase III trial that showed better outcomes with tremelimumab-durvalumab compared to sorafenib; mOS was 16.4 *vs* 3.8 mo, respectively (HR: 0.78%, 95%CI: 0.65-0.92), and ORR was 20.1% *vs* 17.0%, respectively[43].

In the nivolumab-ipilimumab combination part of the Checkmate 040 trial of patients with aHCC pre-treated with sorafenib, participants were randomized 1:1:1 into 3 different groups: Group A patients received nivolumab 1 mg/Kg every 2 wk & ipilimumab 3 mg/Kg every 3 wk for the first 3 mo, followed by nivolumab 1 mg/Kg every 2 wk; those in group B received nivolumab 3 mg/Kg every 2 wk and ipilimumab 1 mg/Kg every 3 wk for the first 3 mo, followed by nivolumab 3 mg/Kg every 2 wk; and those in group C were treated with nivolumab 1 mg/kg every 2 wk and ipilimumab 3 mg/Kg every 6 wk. ORR was 32% (95%CI, 20%-47%) for group A, 27% (95%CI: 15%-41%) for group B, and 29% (95%CI: 17%-43%) for group C. Median duration of response was not reached for patients in group A and was 15.2 and 21.7 mo for groups B and C, respectively. Although the total number of patients that experienced AEs of any grade was high (94% for group A, 71% for B, and 76% for C), serious AEs were not very common; in group A, 10% of patients reported grade 3-4 AEs, compared to 4% for group B, and 2% for group C. Overall, the study showed promising results on the efficacy and tolerability of ICI combinations for advanced HCC[18]. Following results from this trial, the combination of nivolumab-ipilimumab received FDA approval for aHCC becoming the new standard of care in the 2nd-line setting for patients who progress on prior TKI therapy and who do not have a contraindication to ICIs and are fit and able tolerate the higher toxicity observed in double ICI combination. Consequently ICI monotherapy with pembrolizumab (or nivolumab off-license) in the 2nd-line setting was reserved for less fit patients. The success of Checkmate 040 in establishing the efficacy of nivolumab-ipilimumab combination therapy for aHCC lead to Checkmate 9DW, another ongoing phase III trial comparing nivolumab-ipilimumab combination *vs* sorafenib or lenvatinib monotherapy.

ICI+TKI: The combination of ICIs with other factors with proven efficacy for aHCC has been investigated extensively through several trials subsequent to the negative results reported from Checkmate 459. TKIs have been among the most commonly tested agents. A recent phase Ib study investigated the use of pembrolizumab-lenvatinib combination therapy for patients with unresectable HCC. ORR was 36% (95%CI: 26.6%-46.2%), with a 12.6-mo duration of response, a mOS of 22 mo, and a mPFS of 8.6 mo. Grade ≥ 3 AEs were observed in 67% of patients[44].

The RESCUE trial was a phase II study of patients with aHCC treated with camrelizumab, a PD-1 inhibitor, combined with apatinib – another VEGFR-2 TKI that has demonstrated activity as 1st- and 2nd-line therapy for aHCC[45,46]. Patients were enrolled into the study irrespective of previous treatment status and analyses were stratified by line of therapy. For patients treated with 1st-line camrelizumab-apatinib combination, ORR was 34% (95%CI: 23.3%-46.6%) and mPFS was 5.7 mo. For 2nd-line therapy patients, ORR was 23% (95%CI: 15.4%-31.0%) and mPFS was 5.5 mo. Interestingly, grade ≥ 3 AEs were experienced by 77% of patients, while serious AEs were witnessed in 28.9%[47].

Another trial, COSMIC-132, is a randomized phase III trial comparing atezolizumab-cabozantinib combination *vs* cabozantinib *vs* sorafenib as a 1st-line therapy for patients with aHCC. mPFS for the combination therapy group was significantly improved over sorafenib monotherapy (6.8 *vs* 4.2 mo, HR: 0.63, 95%CI: 0.44-0.91). As expected, reported grade ≥ 3 AEs were much higher in the combination group (54%) as opposed to the sorafenib monotherapy group (32%)[48].

Results of ICI and TKI combination therapies have been promising; however, combination regimens have also been associated with much higher rates of AEs – especially grade ≥ 3 AEs – compared to ICI monotherapy or double ICI combinations. Several ongoing clinical trials are investigating combinations of ICIs, such as nivolumab, with TKIs; such studies will provide more information on the efficacy and tolerability of ICI-TKI combinations (Table 1). In the 2nd-line treatment setting of aHCC, clinicians may reserve TKI-ICI combinations for patients who are fitter, and offer double ICI combinations and ICI monotherapy options for patients who are less fit and least fit, respectively.

ICI+VEGF: Anti-VEGF agents are another popular category of therapeutic factors used in combination with ICIs. Existing evidence points towards a synergistic effect of anti-VEGF factors and ICIs through reversal of VEGF-mediated immunosuppression, and promotion of T-cell tumor infiltration[49,50]. IMbrave150, a phase III clinical trial of atezolizumab-bevacizumab combination *vs* sorafenib in treatment-naïve patients with aHCC, was the first study to demonstrate a benefit with such a combination. Six- and 12-mo OS was significantly better for the combination arm (85% and 67% respectively) compared to sorafenib (72% and 55% respectively), while mOS was not reached in the combination arm after 17 mo compared to a mOS of 13.2 mo for sorafenib. mPFS was also longer with atezolizumab-bevacizumab combination (6.8 *vs* 4.3 mo for sorafenib, HR: 0.59, 95%CI: 0.47-076), while ORR was also better in the combination arm (27%, 95%CI: 22.5-32.5 *vs* 12%, 95%CI: 7.4-18.0 for sorafenib). The toxicity profile of the combination therapy was manageable. As expected, rates of serious AEs were slightly higher[19]. Following results from this trial, atezolizumab-bevacizumab combination received FDA approval for aHCC becoming the new standard of care in the 1st-line setting for patients without contraindication to ICIs or anti-angiogenics; TKIs sorafenib or lenvatinib may be reserved as 1st-line treatment for patients who: (1) are less fit, and thus unlikely to tolerate atezolizumab-bevacizumab combination; and (2) those with contraindications to ICIs or anti-angiogenics[51].

NCT04393220 is another phase II clinical trial comparing nivolumab-bevacizumab combination as 1st-line therapy in aHCC. This trial was recently completed with results pending. Several other trials at various stages of completion are currently investigating combinations of ICIs with anti-VEGF agents

Table 1 Ongoing clinical trials investigating immune checkpoint inhibitor - oral tyrosine kinase inhibitor combinations

NCT	Phase	Study drugs	Treatment line	Endpoint	Estimated End of Trial
NCT04194775	3	CS1003 +LENVATINIB *vs* LENVATINIB	1	OS, PFS	June 2023
NCT04344158	3	PENPULIMAB + ANLOTINIB *vs* SORAFENIB	1	OS	December 2024
NCT03713593	3	PEMBROLIZUMAB + LENVATINIB *vs* LENVATINIB	1	OS, PFS	May 2022
NCT04411706	2	SINTILIMAB + APATINIB + CAPECITABINE	1	ORR	June 2022
NCT04042805	2	SINTILIMAB + LENVATINIB	1	ORR	August 2024
NCT04444167	2	BISPECIFIC AK104 + LENVATINIB	1	ORR	March 2022
NCT04183088	2	TISLELIZUMAB + REGORAFENIB	1	ORR, PFS, Safety	March 2025
NCT04310709	2	NIVOLUMAB + REGORAFENIB	1	ORR	May 2023
NCT04442581	2	PEMBROLIZUMAB + CABOZANTINIB	1	ORR	September 2024
NCT03439891	2	NIVOLUMAB + SORAFENIB	1	MTD, ORR	May 2022
NCT04170556	2	NIVOLUMAB + REGORAFENIB	2	Safety	December 2022
NCT04401800	1b/2	TISLELIZUMAB + LENVATINIB	1	ORR	December 2022
NCT04443309	1b/2	CAMRELIZUMAB + LENVATINIB	1	ORR	August 2024
NCT03347292	1	PEMBROLIZUMAB + REGORAFENIB	1	DLT, Safety	October 2022

NCT: Number of the clinical trial (Clinicaltrials.gov); OS: Overall survival; PFS: Progression-free survival; ORR: Overall response rate; MTD: Maximum tolerated dose; DLT: Dose-limiting toxicities.

(Table 2).

Immunotherapy with locoregional ablation: Another means of enhancing the immune response is through stress-induced tissue damage which stimulates inflammation and immunogenicity. Chemotherapy and radiotherapy cause immunogenic cell death, enhance T-cell activation and priming, induce tumor T-cell trafficking and infiltration, and enhance effector T-cell function whilst depleting tolerogenic T-cells[52-54]. Early and intermediate HCC is routinely treated with percutaneous and intraarterial locoregional therapies, including radiofrequency, thermal, and non-thermal ablation, and TACE[55,56]. These approaches may be ideal candidates in sequential or simultaneous combination therapy with immune-based treatments to enhance efficacy through immune modulation[57]. Aside from local immune effects, locoregional ablation methods produce systemic immune effects in innate and adaptive immune cells stimulating immunological tumor regression in tumor sites distant to the primary site of ablation through the abscopal effect. Upregulation of local and systemic immune checkpoint expression and cytokine production are also observed (*reviewed in*[58]).

Trials have investigated the combination of ablation with immunotherapy. In a proof-of-concept study (NCT03939975), radiofrequency or microwave ablation successfully increased response rates from 10% to 24% in patients undergoing therapy with nivolumab or pembrolizumab who exhibited stable disease or atypical progressive disease; toxicity was tolerated and there was a relative improvement in median survival[59]. Vice versa, immunotherapy may also be used as an adjunct to radiofrequency ablation, with one study demonstrating superior survival from anti-PD-1 (camrelizumab) immunotherapy and radiofrequency ablation compared to radiofrequency ablation monotherapy[60]. Evidence from a phase II trial (NCT01853618) in patients receiving anti-CTLA-4 immunotherapy with tremelimumab supports the added benefit from combination with radiofrequency ablation or TACE [61]. Partial response rate was 26% (95%CI: 9.1%-51.2%) and mOS was 12.3 mo (95%CI: 9.3-15.4 mo). Tumor biopsies taken at 6 wk exhibited a clear increase in CD8+ T cells in the patients who observed a clinical benefit, and 86% of patients with active HCV infection experienced a marked reduction in viral load demonstrating positive clinical activity. Additionally, phase I and II studies (NCT02837029, NCT03380130) demonstrated the safety and tolerability of nivolumab in combination or sequential therapy with selective internal radiation therapy containing yttrium-90 resin in patients who were ineligible for TACE, offering good disease control without increasing the adverse event rate in patients with advanced Child-Pugh scores[62]. Promising results from these trials have encouraged further clinical trials to evaluate ICI combinations with ablation methods (Table 3).

ICIs in the adjuvant/neoadjuvant setting

The use of ICIs may not be exclusive only for advanced stage disease as per BLBC criteria; they have also been investigated in adjuvant and neoadjuvant setting. In the neoadjuvant setting, preliminary

Table 2 Ongoing clinical trials investigating combinations of immune checkpoint inhibitors and anti-vascular endothelial growth factors factors

NCT	Phase	Study drugs	Treatment line	Endpoint	Estimated End of Trial
NCT03794440	2/3	SINTILIMAB + BEVACIZUMABBIOSIMILAR	1	OS, ORR	December 2022
NCT03970616	1b/2	DURVALUMAB + TIVOZANIB	1	Safety	August 2022
NCT03973112	2	HLX-10+BEVACIZUMAB BIOSIMILAR	1	ORR	June 2022

NCT: Number of the clinical trial (Clinicaltrials.gov); OS: Overall survival; ORR: Overall response rate.

results from a phase II study of camrelizumab-apatinib combination for systemic treatment-naïve, resectable HCC, showed a major pathologic response rate of 29% and a pathologic complete response rate of 6%, while demonstrating a manageable toxicity profile with 30% of patients experiencing grade 3 treatment-related AEs. No grade 4-5 AEs were observed[63]. Plenty of ongoing trials are investigating the use of ICIs with or without other agents in the perioperative setting for HCC (Table 4).

Safety of ICIs and HCC

In recent years, ICIs have demonstrated efficacy across a broad spectrum of tumors including HCC, prompting significant interest into their therapeutic value. Due to their involvement in the immune response, ICIs have been linked to immune-related AEs (IRAEs) of varying significance, from mild to life-threatening conditions such as myocarditis, colitis, pneumonitis and hepatitis[64]. Although the precise mechanisms by which ICIs exert these AEs is not known, evidence suggests that ICI administration leads to changes in T-cell population with emergence of autoreactive T-cells, along with increased B-cell clonality and germinal center activation, and display of autoantibodies against thyroid antigens[65,66] and pancreatic islet cells[67,68]. Expression or upregulation of target molecules such as CTLA-4 or PD-1/PD-L1 in normal tissues has also been associated with risk of IRAEs targeting the respective cells[69,70]. The composition of gut microbiota is implicated in the risk for IRAEs development[71]; the former is known to be associated with response to ICIs[72] and active modification through probiotic supplementation has been shown to enhance ICI activity and responses[73]. Systemic administration of antibiotics is known to affect gut microbiota composition[74] and has been associated with worse responses to ICIs[75,76]. The risk for IRAEs also includes reactivation of pre-existing autoimmune conditions, and other complications in patient populations where IRAEs have not been extensively studied, such as transplant patients and those with chronic viral infections[77].

In patients with HCC, the most common IRAEs observed with single-agent therapy include rash (up to 23% of patients), pruritus (up to 19% of patients), and diarrhea (up to 17%). For anti-CTLA-4 and anti-PD-L1 combination treatment, incidence rates are up to 29%, 45%, and 24%, respectively. These results align with the evidence of higher risk of IRAEs with double ICI therapy[78]. Higher rates of AEs observed with combination therapy regimens are a limiting factor that should be considered in clinicians' therapeutic decision-making. Patient eligibility for combination regimens much be considered on a case-by-case basis. Hepatic-related IRAEs such as AST/ALT elevation, defined as an increase of either AST or ALT 1-2.5 times the Upper Normal Limit (UNL)[79] are more common in patients with HCC compared to other tumors. Transaminitis of any grade has been observed in up to 14% of patients with HCC compared to 3% among patients with other tumor types[78], while there are reports that estimate incidence as high as 30%[80]. The association of IRAEs and viral infections is particularly important in HCC as 50%-60% of patients with HCC in the United States are infected with HCV, while 10%-15% are infected with HBV[81]. Recent evidence suggests that ICI therapy for advanced cancer in HBV/HCV positive patients is associated with an increased risk for reactivation of hepatitis. Interestingly, the risk for hepatitis was not significantly different for patients with HCC compared to other malignancies[82]. Although IRAEs can complicate treatment with ICIs, evidence suggests that IRAE incidence positively correlates with better response to ICIs. In a study of patients with HCC treated with ICIs, patients with history of IRAEs had longer PFS, OS and higher Disease Control Rate compared to those who did not experience IRAEs[83].

Treatment of immune-related hepatitis/transaminitis is dependent upon AST/ALT levels. Temporary hold of treatment is indicated for enzyme level elevations between 2 to 5 times the UNL, while permanent discontinuation of the associated checkpoint inhibitor is indicated for elevations greater than 5 times the UNL[84-86]. For patients with an elevation 5- to 10-times the UNL, a course of 1-2 mg/kg/d prednisone is indicated with possible escalation to IV methylprednisolone if no improvement is seen in 3-5 d. Further treatment escalation to mycophenolate mofetil (1000 mg twice daily) should be considered in patients that do not improve after maximum steroid treatment[87].

Table 3 Ongoing clinical trials investigating combinations of locoregional therapy and immune checkpoint inhibitors

NCT	Phase	Locoregional Therapy	Systemic Therapy	Endpoint	Estimated End of Trial
NCT03817736	2	TACE + SBRT	ICI	Sequential	February 2024
NCT03638141	2	DEB-TACE	DURVALUMAB + TREMELIMUMAB	Sequential	November 2023
NCT03143270	1	TACE	NIVOLUMAB	Combination	April 2022
NCT03572582	2	TACE	NIVOLUMAB	Combination	June 2023
NCT03397654	1/2	TACE	PEMBROLIZUMAB	Sequential	December 2021 (*results awaited*)
NCT03383458	3	Curative resection or ablation	NIVOLUMAB	Adjuvant	June 2025
NCT02821754	2	TACE, RFA, Cryo	DURVALUMAB, TREMELIMUMAB	Combination	December 2022
NCT03033446	2	Y90-Radioembolization	NIVOLUMAB	Combination	December 2021 (*results awaited*)
NCT03099564	1	Y90-Radioembolization	PEMBROLIZUMAB	Combination	July 2022
NCT03259867	2	TATE	NIVOLUMAB OR PEMBROLIZUMAB	Combination	December 2022
NCT03937830	2	TACE	DURVALUMAB + TREMELIMUMAB + BEVACIZUMAB	Combination	December 2023
NCT03778957	3	TACE	DURVALUMAB or DURVALUMAB + BEVACIZUMAB	Combination	August 2024
NCT04340193	3	TACE	NIVOLUMAB + IPILIMUMAB or NIVOLUMAB MONOTHERAPY or DOUBLE PLACEBO	Combination	January 2024
NCT04246177	3	TACE	PEMBROLIZUMAB + LENVATINIB	Combination	December 2029
NCT04268888	2/3	TACE/TAE	NIVOLUMAB	Combination	June 2026
NCT05162898	N/A	RFA	TORIPALIMAB + LENVATINIB	Combination	December 2025
NCT05057845	2	Cryo	TISLELIZUMAB + LENVATINIB	Combination	September 2024
NCT04988945	2	TACE + SBRT	DURVALUMAB + TREMELIMUMAB	Sequential (for downstaging)	December 2026
NCT04727307	2	RFA	ATEZOLIZUMAB (neoadjuvant) + ATEZOLIZUMAB-BEVACIZUMAB (adjuvant)	Combination	July 2027
NCT04663035	2	Ablation	TISLELIZUMAB	Combination	December 2025
NCT04652440	1/2	RFA	TISLELIZUMAB	Combination	November 2023
NCT04639180	3	Curative resection or ablation	CAMRELIZUMAB + APATINIB	Adjuvant	July 2024
NCT04220944	1	MWA+TACE	SINTILIMAB	Combination	September 2022
NCT04102098	3	Surgical resection or ablation	ATEZOLIZUMAB+ BEVACIZUMAB	Adjuvant	July 2027
NCT03867084	3	Surgical resection or local ablation	PEMBROLIZUMAB	Adjuvant	June 2025
NCT03864211	1/2	Thermal ablation (MWA or RFA)	TORIPALIMAB	Combination	June 2023
NCT03753659	2	MWA or RFA or Brachy-therapy or TACE	PEMBROLIZUMAB	Combination	June 2024
NCT03630640	2	Electroporation	NIVOLUMAB (neoadjuvant & adjuvant)	Combination	November 2023

NCT: Number of the clinical trial (Clinicaltrials.gov); TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiotherapy; DEB: Drug eluting bead; RFA: Radiofrequency ablation; Cryo: Cryoablation; TATE: Transarterial tirapazamine embolization; TAE: Transarterial embolization; MWA: Microwave ablation.

REAL-WORLD DATA FOR IMMUNOTHERAPY IN HCC

Despite the advances in ICI therapies for HCC in recent years, phase II/III studies are generally limited by strict inclusion and exclusion criteria, thus lacking ecological validity and generalizability to real-life clinical practice outside of clinical trial setting[88]. Real-world data describe health-related information gathered outside of clinical trials. Gathering and reporting real-world data through cohort and observational studies is important for clinicians who aim to apply approved clinical trial regimens to a broader patient group.

Immunotherapy ineligibility

In clinical trials of systemic therapies in HCC, patients who have aHCC or intermediate disease and are not suitable for locoregional therapies are enrolled; however, in real-world clinical practice, a large proportion of such patients are ineligible to receive immunotherapy due to contraindications.

In unselected HCC patients in the general population, no more than a third are amenable to ICIs as a 1st-line approach and this figure decreases considerably in combination therapy with anti-VEGF or TKI agents according to an analysis of the Italian Liver Cancer (ITA.LI.CA) database involving 2483 patients across liver dysfunction stages[55,89]. When considering only aHCC and intermediate HCC that was unresponsive to locoregional ablation (n = 1514), eligibility increased from 21% with nivolumab and 11% with pembrolizumab to 35% and 18%, respectively. Overall, the main contraindications to frontline ICI were Child-Pugh class > A (24%, n = 601), uncontrolled ascites (15%, n = 380), performance status > 1 (13%, n = 343), active alcohol intake (13%, n = 323), thrombocytopenia (12%, n = 299), hepatic encephalopathy (6%, n = 155), aminotransferase levels > 5 times the UNL (5%, n = 123), and concurrent autoimmune diseases (2%, n = 57)[89]. In the 2nd-line, ICI eligibility was substantially lower with 5% and 8% of patients amenable to nivolumab and pembrolizumab, respectively[89]. When repeating this analysis to take into account anti-VEGF and TKI combination therapy with ICIs, atezolizumab-bevacizumab eligibility drops to 18% in the whole HCC population and 29% in aHCC or intermediate HCC patients who are not eligible for surgery or locoregional procedures[55]. Reasons for the exclusion of these additional patients were clinically significant heart disease (n = 52), chronic non-healing skin ulcerations (n = 15), uncontrolled hypertension (n = 10), and non-liver-related coagulative abnormalities increasing the risk of bleeding (n = 1).

The expert opinion panel of ASCO has acknowledged the role of ICIs in the treatment of patients with aHCC and especially patients with contraindications, or intolerance to, TKIs who may derive immense benefit from immune therapies[51]. However, they also highlight that patients and clinicians should be aware of life-threatening toxicities that may occur with ICIs. Future research may provide additional information on specific patient subpopulations within this subgroup that may have a favorable risk to benefit ratio.

Immunotherapy in patients with liver dysfunction (Child-Pugh class B and above, hepatitis, NASH)

Due to the lack of Child-Pugh class B and above patients in HCC trials, which often specify Child-Pugh class A in the inclusion criteria, there is a large unmet need for data to support treatment efficacy and toxicity profiles in this cohort[51]. As a result, published recommendations and guidelines for systemic therapy in HCC are often limited to patients with Child-Pugh class A[51]. Experts recommend cautious consideration of systemic therapies for Child-Pugh class B HCC patients with good performance status, taking into account their liver function, bleeding risk, presence of portal hypertension, extent of extrahepatic spread, tumor burden, and major vascular invasion[51]. A handful of studies have compared explorative primary outcomes in Child-Pugh class B patients treated with immunotherapy with different outcomes. Use of the Barcelona Clinic Liver Cancer (BCLC) staging criteria helps avoid the unselect exclusion of all Child-Pugh class B and above patients by allowing for holistic patient scoring and selection based on other performance status criteria.

One retrospective case series of 18 Child-Pugh class B HCC patients treated with nivolumab monotherapy reported a higher rate of AEs compared to those observed in Child-Pugh A patients in CheckMate 040; however, the majority of serious AEs and other AEs were associated with complications of comorbid liver dysfunction and advanced tumor burden, including 11% *vs* 4% of serious treatment-related AEs, and 28% *vs* 19% treatment-related AEs grade ≥ 3. The ORR was comparable in both studies (17% *vs* 20%)[90]. Notably, the mOS in this case series was 5.9 mo, which is lower compared to the 7.6-mo mOS reported in the analogous CheckMate 040 cohort, though higher compared to the limited mOS data reported for analogous patients treated with sorafenib (3-5 mo). Comparable safety and efficacy of nivolumab and pembrolizumab across Child-Pugh class and line of therapy has been confirmed in other real-world data studies with no significant difference observed in ORR and toxicity in terms of AEs; however, mOS and OS tends to be shorter in Child□Pugh B and above patients[91-93].

In a study of 34 HCC patients (5/29 BLBC B/C; 19/14/1 Child–Pugh A/B/C) including sorafenib pre-treated individuals, nivolumab was safe and efficacious with reported 6% (n = 2) grade 3 toxicity, 12% (n = 4) partial response, and 24% (n = 8) stable disease[94]. However, mOS was only 7.5 wk as 59% (n = 20) of patients had died on assessment due to tumor progression (80%, n = 16), acute liver failure (15%, n = 3), and variceal bleeding (5%, n = 1)[94]. On analysis, 24% of patients (n = 8) were still on

Table 4 Ongoing clinical trials investigating immune checkpoint inhibitor - based clinical trials in the adjuvant and neoadjuvant setting

NCT	Phase	Study drugs	Treatment setting	Endpoint	Estimated End of Trial
NCT03383458	3	NIVOLUMAB *vs* PLACEBO	Adjuvant	RFS	June 2025
NCT03867084	3	PEMBROLIZUMAB *vs* PLACEBO	Adjuvant	RFS, OS	June 2025
NCT03847428	3	DURVALUMAB + BEVACIZUMAB *vs* PLACEBO	Adjuvant	RFS	September 2023
NCT04102098	3	ATEZOLIZUMAB + BEVACIZUMAB *vs* PLACEBO	Adjuvant	RFS	July 2027
NCT03859128	2/3	TORIPALIMAB *vs* PLACEBO	Adjuvant	RFS	April 2024
NCT03839550	2	CAMRELIZUMAB + APATINIB	Adjuvant	RFS	February 2023
NCT04418401	2	ANTI-PD1 + DONAFINIB	Adjuvant	RFS	June 2023
NCT03510871	2	NIVOLUMAB + IPILIMUMAB	Neoadjuvant	ORR, downstaging rate	December 2022
NCT04123379	2	NIVOLUMAB + CCR2/5-inhibitor *vs* NIVOLUMAB +ANTI-IL8	Neoadjuvant	Safety	October 2024
NCT03222076	2	NIVOLUMAB	Neoadjuvant	Safety	September 2022
NCT03682276	1/2	NIVOLUMAB + IPILIMUMAB	Neoadjuvant	Safety, Delay to surgery	September 2022
NCT03383458	1	NIVOLUMAB *vs* PLACEBO	Adjuvant	RFS	June 2025
NCT04425226	N/A	PEMBROLIZUMAB + LENVATINIB	Neoadjuvant	RFS, ORR	December 2025

NCT: Number of the clinical trial (Clinicaltrials.gov); RFS: Recurrence-free survival; OS: Overall survival; RFS: Recurrence-free survival; ORR: Overall response rate.

nivolumab treatment and 18% (n = 6) had stopped treatment for other reasons [patients wish (n = 5), toxicity (n = 1)]. On multivariate analysis, Child–Pugh stage was the only significant independent risk factor for survival (HR 7.72, 95%CI: 2.62-22.78, P < 0.001). Although safe and efficacious, the study concluded that patients with advanced liver disease require further prospective evaluation due to probable limited efficacy of nivolumab. Overall, efficacy was approximately half of that observed in Checkmate 040. Results from this study are in agreement with latest evidence indicating that the survival of patients with aHCC treated with nivolumab is correlated to the Child-Pugh liver function score at baseline[95], as reported in aforementioned studies as well[92-94]. Equally, the finding that unselected Child-Pugh B and above patients exhibit an unsatisfactory response to and survival with nivolumab has been echoed in another retrospective study of 203 HCC patients; (ORR 3% *vs* 16% in Child–Pugh class B/A, P = 0.01; mOS 11.3 *vs* 42.9 wk in Child–Pugh class B/A, adjusted HR, 2.10, P < 0.001)[96].

Aside from Child-Pugh class, other liver dysfunction causes have also been examined in real-world studies. Safety and antitumor activity had been demonstrated in hepatitis-induced cirrhosis and in patients with active hepatitis viral load, even those on anti-viral treatment, while viral hepatitis status has been suggested as a possible predictive biomarker for response since it is clearly not a contradiction against the use of ICIs, yet further prospective studies are mandated[93,97,98]. Conversely, the same cannot be said for patients with underlying NASH and those with HCCs with activated Wnt/β-catenin signaling which observe reduced efficacy from ICI therapy according to pre-clinical and clinical data (*reviewed in*[99]).

Given that the level of liver dysfunction in this cohort may have significant implications on guidelines regarding the optimal selection of drugs in each line of therapy, further data is needed to guide the evidence-based use of systemic immune therapies in Child-Pugh class B HCC, as supported by the above limited data.

Macrovascular invasion

Another indicator of poor performance status is macrovascular invasion (MVI). Approximately 10%-40% of HCC patients present with MVI at diagnosis[100], and as such are not amenable to curative treatment and exhibit very poor prognosis[101]. Despite MVI being common, patients are often excluded from clinical trials. Thus, real-world data are needed to demonstrate the relative efficacy of ICIs in this cohort.

Tsai *et al*[102] retrospectively compared the efficacy of PD-1 inhibitors in 34 HCC patients with vascular metastases in the portal vein and inferior vena cava *vs* 34 patients without tumor thrombi; ORR and survival were comparable between both cohorts. The response rate of vascular tumor thrombosis

was 52.9%, and responders exhibited a superior survival benefit than non-responders. MVI responsiveness closely correlated with the maintenance of optimal liver function and a lower occurrence of distal metastases. These findings are in agreement with those from an earlier study showing that the magnitude of treatment response is significantly more intense in vascular invasion compared to hepatic tumors, and vascular response is also an independent prognostic factor that is significantly associated with PFS, while ECOG (Eastern Cooperative Oncology Group) performance status was a significant independent predictor of OS[103]. Moreover, similar findings have been observed in renal cell carcinoma displaying inferior vena cava thrombus treated with ICIs that proposed the response of vascular thrombi to ICIs is stronger in a high T-cell inflamed tumor microenvironment[104]. ICIs markedly decrease or stabilize tumor thrombus volume, and this response may be affected by the diversity of tumor microenvironments[102,103,105,106]. Vascular metastasis regression helps preserve organ function while the use of ICIs in these patients also delays distant metastases[102]. Therefore, ICIs should be prioritized in patients with MVI in an attempt to prevent further progression as well as mediate vascular tumor response, to hopefully improve outcomes.

Autoimmune disease

The incidence of autoimmune diseases in cancer patients has been reported at 13%-30% with hypothyroidism, rheumatoid arthritis, type 1 diabetes and psoriasis representing the most common conditions[107,108]. Hepatobiliary autoimmune diseases, such as autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are known risk factors for HCC[109,110]. The risk of HCC is lower with PSC compared to AIH[110], especially when the latter co-exists with cirrhosis[111]. Yet, patients with underlying autoimmune disease are typically excluded from immunotherapy trials because of the risk of immune-mediated flares of their underlying autoimmune disease [99].

In an observational, retrospective study including 15 patients with pre-existing autoimmune diseases, including 4 HCC patients treated with nivolumab, only 4 (27%) patients experienced an autoimmune disease exacerbation with ICIs, including 1 of the HCC patients[112]. Moreover, the most frequent cause of treatment discontinuation was disease progression rather than toxicity. Studies in other cancers (mostly melanoma and non-small cell lung cancer) report a wide range of incidence of flare-ups and IRAEs in patients with underlying autoimmune disease that receive ICI treatment[113-117]. One systematic review of 123 cancer patients from 49 publications reported incidences of 41% exacerbation of previous autoimmune disease, 25% de novo IRAEs, and 11% of both; no difference was observed between those with active *vs* inactive disease[118]. Patients receiving immunosuppressive therapy at initiation of ICI therapy appeared to experience fewer AEs than those not receiving treatment. AEs improved in over half of patients without ICI discontinuation while 3 patients died. The incidence of IRAEs is higher in patients with autoimmune disease treated with ICIs compared to incidences quoted in studies and trials of patients without autoimmune conditions. In terms of efficacy, studies show no difference in ORR, PFS, and OS in patients with underlying autoimmunity compared to those without [113,116]. However, the evidence is conflicting regarding the response rate depending on concomitant immunosuppressive therapy at the time of ICI initiation with some studies quoting lower response rates [114] and others quoting no association[117]. Recently, the use of selective immunosuppressive drugs over non-selective immunosuppressants in patients with underlying autoimmune disease for ICI therapy has been recommended, as the former may be less likely to adversely affect ICI efficacy[119].

Overall, although limited, these data support the administration of immunotherapy in cancer patients with a pre-existing (controlled) autoimmune disease with adequate follow-up and early management if flare ups or IRAEs occur; immune exacerbations can usually be management with steroids or other immunosuppressants without treatment discontinuation. Therefore, every cancer patient with underlying autoimmune disease should be considered for ICI therapy with a decision on management achieved through multidisciplinary team discussion that weighs up the risks and benefits[120]. Recommendations state that ICIs should be avoided: (1) whenever autoimmune disease reactivation may be life threatening, (2) in patients with neurological or neuromuscular disorders, and (3) in patients with poorly controlled autoimmune disease or on high doses of immunosuppression[120]; TKIs should be considered 1st-line in these cases[101].

In the case of HCC particularly, there is a lack of data. Further large prospective studies are needed to establish the incidence of IRAEs and autoimmune disease exacerbations in patients with pre-existing autoimmune conditions treated with immunotherapy to evaluate the overall risk-to-benefit ratio and generate practical evidence-based management guidelines for this subpopulation.

Therapeutic decisions: Radiological progression

The concurrent availability of several systemic therapy regimens in the 1st- and 2nd-line settings offers clinicians and patients a wider selection of drugs to choose from. Decisions regarding the selection of a specific agent over another is not only determined by the availability and accessibility to a specific drug, but also, more importantly, by the efficacy and tolerability that is expected or indeed observed in a patient. Thus, the decision of which agent to choose, or switch to, is largely dependent on individual patient characteristics. As previously mentioned, there are inherent difficulties regarding the lack of wide representation of patients of poor performance status in immunotherapy clinical trials that affect a

clinician's ability to triage toxicity risk in these patients. Additionally, assessing tumor progression and response to immunotherapy, which also governs treatment selection and switching, is challenging for several reasons both inside and outside of clinical trials.

Immunotherapies are known to produce an atypical response patten featuring pseudo-progression (PP) and hyper-progressive disease (HPD). Therefore, multiple variations of Response Evaluation Criteria in Solid Tumors (RECIST) have been proposed, with RECIST 1.1 recommended for primary endpoints and immune RECIST (iRECIST) for exploratory analyses. Importantly, patients in clinical trials undergo thorough radiological assessment from specialized radiologists to a more robust standard than what is available outside of trial setting. Decisions about switching or continuing past progression (assumed PP) are usually made based on the trial radiologist report in conjunction with the opinion of experienced oncologists involved in the trial; usually, treatment is stopped in trials when patients progress on immunotherapy whereas in real life a decision may be made to continue treatment if the patient reports benefits and if the drug is well-tolerated. A retrospective multicenter analysis of 31 HCC patients treated with nivolumab in real-life practice assessed radiological response to treatment using both RECIST 1.1 and iRECIST and found that response rates were similar to those reported in prospective clinical trials[122]. However, authors highlight the heterogeneity in response and progression patters, and emphasize the risk of misinterpretation of results in terms of endpoints as well as the difficulty of deciding when to stop treatment past progression. Additional real-life studies such as the above are warranted to support these findings.

In terms of HPD – which remains a controversial concept that is doubted by some clinicians due to lack of robust data to differentiate it from natural cancer progression in non-responders – the above study reported an occurrence of this phenomenon in four cases (13%). All of these patients presented at baseline with massive tumor burden involving different anatomical regions (burden 76-159 mm; 6-15 measurable lesions per patient) before nivolumab initiation[121]. These findings are consistent with those reported in a separate case series of 47 patients in which 3 exhibited HPD (including 1 aHCC patient) when treated with nivolumab; the main characteristics in the hyper-progressors were age < 75 years, ≥2 metastatic sites, PD-L1 < 50%, neutrophil-to-lymphocyte ratio > 3, and elevated lactate dehydrogenase[122]. This and other studies support the notion that high metastatic burden at baseline may be a clinical predictor of HPD during ICI therapy. Other predictors of HPD have been proposed with contradictory data (*reviewed in*[122]).

Cost-effectiveness

In 1st-line setting, the combination of atezolizumab-bevacizumab *vs* sorafenib for aHCC has been shown to lack cost-effectiveness from a US payer perspective despite offering a significant clinical survival benefit, according to data from the IMBRAVE150 clinical trial; an incremental cost-effectiveness ratio of $322500 per quality-adjusted life-year (QALY) gained was observed[123]. In a threshold analysis, prices for atezolizumab and bevacizumab would have to be reduced by 37% and 47%, respectively, to be considered a cost-effective alternative at common willingness-to-pay thresholds of $150000 or $100000 compared to sorafenib[123]. However, it should be noted that patients in the IMBRAVE150 disproportionately represented patients with well-preserved liver function (Child-Pugh class A) and good performance status (ECOG score 0-1), meaning that it is unclear how generalizable the benefits and/or risks, and therefore the cost-effectiveness, of these drugs are in clinical practice, where patients present with more severe disease[124]. IMBRAVE150 also disproportionately included fewer patients of Black and Hispanic ethnicity. Higher age specified mortality and incidence of aHCC are observed in Asian, Black, and Hispanics compared to non-Hispanic White individuals, while Black and Hispanic patients also tend to present with more advanced tumor burden and have worse survival compared with non-Hispanic White patients[125]. Lack of such ethnic representation in IMBRAVE150 means that the above cost-effectiveness analysis model does not accurately represent disease demographics across ethnicity and is thus likely to lack in generalizability[124]. Moreover, racial and ethnic disparities are known to occur in immunotherapy receipt; atezolizumab-bevacizumab regimes are likely to widen existing disparities in HCC mortality, especially given lack of ethnic representation in the aforementioned cost-effectiveness analysis model[124]. Aforementioned results are echoed in a separate study which indicated that 1st-line TKI followed by 2nd-line immunotherapy was the most cost-effective strategy for aHCC[125].

Lack of ICI cost-effectiveness for aHCC also stands true in the 2nd-line setting in the US, where results from a separate cost-effectiveness analysis of pembrolizumab for aHCC based on data from the KEYNOTE-240 trial reported an incremental cost-effectiveness ratio of $340409 per QALY gained[126]. The price of pembrolizumab would need to be reduced by 58% to achieve cost-effectiveness, with a willingness-to-pay threshold of $150000 per QALY. These results are likely to be very similar for nivolumab monotherapy as an alternative anti-PD-1 agent for aHCC in 2nd-line setting; however, further cost-effectiveness analyses are warrant especially for the combination of nivolumab-ipilimumab in the 2nd-line setting for aHCC[126]. It should be noted that other 2nd-line non-immunotherapy alternatives for aHCC including regorafenib, cabozantinib, and ramucirumab have also been shown to have an incremental cost-effectiveness ratio with $224362 and over $1 million per QALY for regorafenib and cabozantinib, respectively, while no cost-effectiveness data has been published for ramucirumab which is also unlikely to be cost-effective[126]. The lack of robust head-to-head trials comparing different 2nd-

line therapies means that it is difficult to undertake a robust cost-effectiveness comparison of agents in this setting[126].

From a patients' perspective, sadly, differences in drug costs are often an important factor that impact patient decision as some cancer therapies pose a more significant financial burden than others. Additionally, potential burdens—financial or otherwise—associated with regular travel to a treatment center for IV infusion therapy may render a patient more likely to opt for a treatment regimen composed of oral medications which they can take at home. In the case of the latter, the importance of medication compliance, even in the presence of adverse events, as well as patient safety-netting must be stressed. Occasionally, providers themselves may have financial biases for supporting some regimens over others when there is no significant difference in treatment effectiveness.

Immunotherapy in LT

One of the questions yet to be answered is the role of immunotherapy in LT. Immunotherapy post-LT may prevent or be useful in the management of recurrent HCC as well as other post-transplant secondary malignancies. Current guidelines state that immunotherapy approaches should be avoided in patients who recur following LT because of the high rates (40%) of allograft rejection and mortality, owing to stimulation of the host immune response by these agents; however, aside from rejection, anti-tumor efficacy and tolerability are promising[127,128]. A review of 25 patients receiving immunotherapy post-transplant identified immunotherapy initiation after short duration from transplant and graft PD-L1 positivity as potential risk factors for rejection[129]. Despite the limited amount of data, in the current era it is not safe to advise for the use of immunotherapy to prevent or treat disease recurrence after transplant. Elucidating better predictors of patients that are at higher risk of experiencing transplant rejection may help identify a subset of patients who are more likely to observe a favorable benefit-to-risk ratio from immunotherapy after transplant, and may thus be eligible for this approach.

In the pre-LT setting, the role of neoadjuvant immunotherapy is even less clear. Immunotherapy pre-LT may facilitate downstaging of unresectable HCC bridging to subsequent surgical eligibility, thus offering these patients their only chance of disease cure; however, this potentially comes at a higher risk of donor graft rejection[127]. The latest study published on this topic identified seven patients from their center and three from the literature who received anti-PD-1 ICIs pre-LT[130]. Eight patients (80%) observed partial response, and the disease control rate was 100%. Acute rejection occurred in 30% of patients with two patients dying as a result, despite treatment with immunosuppressive medications. Despite this growing body of evidence, further research is warranted. Currently, a phase II multicenter clinical trial is underway to investigate the role of durvalumab and tremelimumab for patients with HCC listed for LT (NCT05027425). Such trials are needed to determine the safety and efficacy of immunotherapy as a potential bridging strategy to LT.

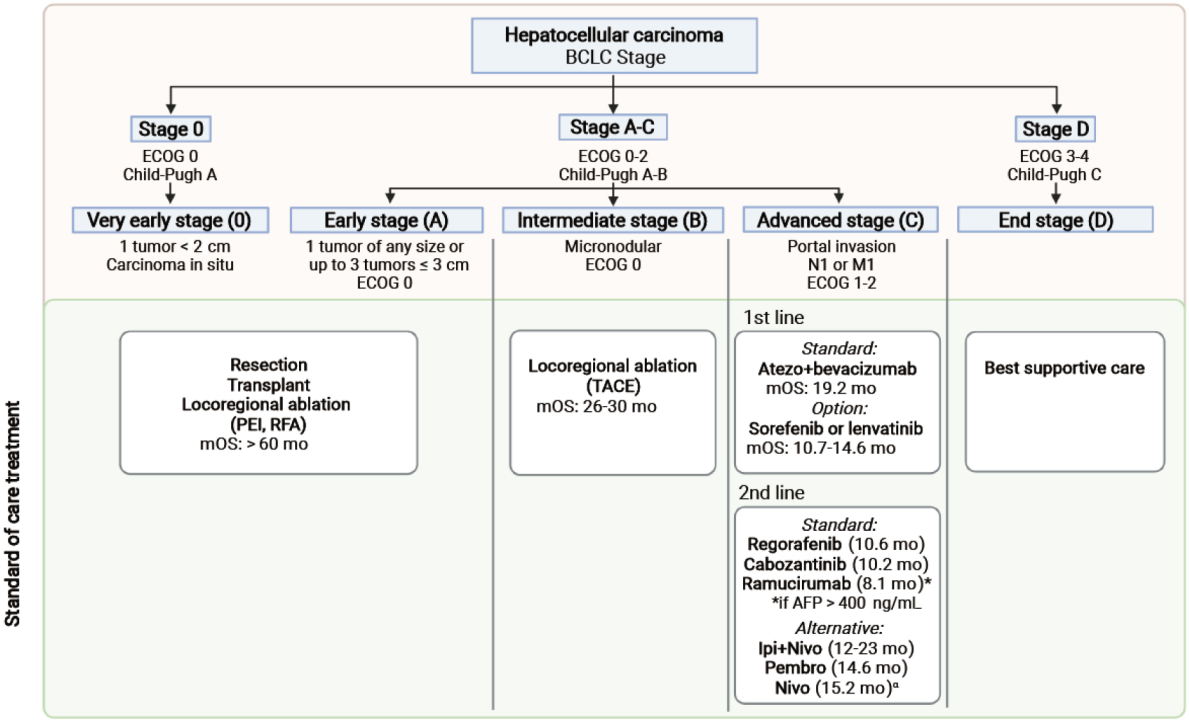
FUTURE DIRECTIONS

Immunotherapy beyond ICIs: ACT, vaccination, and virotherapy

Other promising forms of immunotherapy for HCC aside from ICIs include ACT strategies, anti-tumor cancer vaccines, and transgenic therapy applied through viral vectors. These strategies have begun to be investigated clinically as monotherapy options in HCC and as combination therapies, to enhance the efficacy of other treatments. The potential of such immunotherapies as combination treatments is highly promising, particularly in the context of combination with other immunotherapies, such as ICIs, as they improve immunogenicity. Combining immunotherapeutics with different mechanisms of action and primary immune effects is a well-recognized approach to counteract the multiplicity of tumor immune evasion mechanisms and ensure all necessary steps for the successful mounting of an anti-tumor immune response are met, as described in the *cancer immunity cycle* theory[31,131,132]. The same principle stands true as the underlying biological rationale to justify combination therapy of ICIs with different mechanisms of action – an example being combination with anti-PD-1 and anti-CTLA-4 agents which has been shown to significantly improve ORR, at the expense of increased but tolerable toxicity (Figure 2).

ACT: Following the success and approval of a plethora of ACT strategies in hematologic cancers[133], researchers have explored various forms of ACT in solid tumors. ACT involves the autologous or allogeneic transplant of tumor-infiltrating lymphocytes (TILs), or genetically modified T-cells engineered to express novel T-cell receptors (TCR) or chimeric antigen receptors (CAR)[134]. Cytokine-induced killer (CIK) cells represent another form of ACT wherein T-cells are co-cultured *in vitro* under cytokine manipulation to express natural killer (NK) cell-surface markers in addition to TCR. Cytotoxic cells with this double T/NK phenotype are capable of lysing a broad array of tumor cell targets in a non-MHC-restricted manner[135].

The primary immune effects of ACT result in supplementation of immune effector cells[31]. Compared to other immunotherapies, one of the advantages of ACT is that it is considered a "living"



DOI: 10.5306/wjco.v13.i6.448

Figure 2 Treatment algorithm for immunotherapy in hepatocellular carcinoma according to American Society of Clinical Oncology guidelines. Atezo: Atezolizumab; Ipi: Ipilimumab; Nivo: Nivolumab; Pembro: Pembrolizumab; mOS: Median overall survival; mo: Months; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; ECOG: Eastern Cooperative Oncology Group; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha fetoprotein; cm: centimeter; N1: Regional nodal spread; M1: Metastatic spread.

treatment method as it exhibits the capability to become active and replicate *in vivo* for long lasting anti-tumor effect[136]. In theory, this grants the possibility of disease cure due to the accrual of long-term immunological memory; however, in practice this is often not achieved due to multifactorial lack of cell persistence[137]. Currently, most novel ACT strategies, such as CARs, are bespoke to each patient and are manufactured specifically for them. Off-the-shelf ACT strategies are being investigated as a means to improve costs, time and ease of manufacturing, and allow for universal applicability across patients [133]. Due to the presence of TAAs with an acceptable specificity, HCC in one of the most promising organs for ACT in solid tumors, as TAA specificity decreases chances of on-target off-tumor recognition and subsequent toxicity due to target antigen expression on normal cells which are then destroyed[138].

Combinations of ACT with ICIs have not yet been trialed in HCC, while in other solid malignancies such combinations have been shown to be feasible and safe[139]. Conversely, ACT in combination with ablative therapies in HCC is undergoing investigation in phase I/II setting[58]. Regarding ACT monotherapy for HCC, early phase I/II studies have shown feasibility and tolerability with TILs, TCR, and CAR cell variants while several other phase I/II trials are ongoing (*reviewed in*[35,138,140]). CIK is the only ACT that has been investigated for HCC in a phase III setting (NCT00699816). This multicenter trial involved 230 HCC patients who had undergone curative surgical resection, radiofrequency ablation, or percutaneous ethanol injection that were randomized to receive adjuvant CIK immunotherapy (injection of 6.4 × 10^9 autologous CIK cells, 16 times over 60 wk) or no adjuvant therapy (controls)[141]. mPFS was significantly prolonged in the CIK arm *vs* control (44 mo *vs* 30 mo; HR: 0.63, 95%CI: 0.06-0.75). All-cause death and cancer-related death were also significantly reduced in the experimental arm. AE occurrence was higher in the experimental arm but SAEs did not differ significantly. In combination with transarterial chemoembolization (TACE), an international registry analysis of 106 clinical trials including 10225 patients of which 4889 patients in over 30 distinct tumor entities were treated with CIK cells alone or in combination with conventional or novel therapies, CIK has been shown to significantly improve mPFS and mOS (27 trials), and 5-year survival rate (9 trials) with mild AEs and graft-versus-host diseases[142]. Additionally, a systematic review and meta-analysis of 6 randomized controlled trials including 844 HCC patients concluded that adjuvant autologous CIK after curative resection significantly improved 1-year, 2-year, and 3-year disease-free survival and OS but did not significantly extend these at 4 and 5 years; AEs were comparable in CIK and control patients [143]. Furthermore, combination of CIK with ICIs has been trialed in solid and hematologic cancer with promising results and potential to be trialed in HCC in the future[142]. Despite these positive results, ACT including CIK is still not used in most centers as an adjuvant therapy, probably due to the limitations of in-house cell therapy facilities[35].

Vaccination and virotherapy: Following results of several early phase trials involving relatively small numbers of HCC patients in the past decade, vaccines and virotherapy are currently being investigated as enhancer strategies in combination with other forms of therapy as opposed to a viable monotherapy option[31,35].

Vaccines: The underlying primary immune effect of vaccines lies in their ability to enhance T-cell priming and expansion[31]. Cancer vaccines are being constructed to enhance presentation of tumor-associated epitopes to host immunity to overcome tumor-specific tolerance in the context of immune stimulation by activating and selectively expanding tumor-specific lymphocytes within the native effector cell repertoire while maintaining immune-regulatory protection against autoimmunity[144]. Vaccine-mediated stimulation of tumor-specific immunity provides a physiologic stimulus for T-cell activation, fostering a potentially more-sustained native immune response with greater durability for long-term antitumor surveillance. Alike engineered ACT, cancer vaccines may be designed to target TAAs or neoantigens, of which HCC exhibits many with high specificity[10]. Classical cancer vaccines rely on exogenous administration of antigens or antigen-pulsed dendritic cells (DCs). The only cancer therapy vaccine to be approved by the FDA to date is Sipuleucel-T, a DC-like anticancer vaccine for prostate cancer[145]. In HCC, vaccine constructs are mainly based on RNA, peptides, proteins, or DCs [31,140].

Early investigations in cancer patients and pre-clinical models have demonstrated the synergistic capacity of cancer vaccines in combination with anti-PD-1 and anti-CTLA-4 checkpoint blockade, with evidence also reported for HCC[146-148]. Vaccines can reverse immune tolerance and exhaustion seen in patients treated with ICIs by providing a stimulus to prime and expand tumor-specific T-cells that preserve their effector functions through the effect of ICIs[31]. Moreover, phase I/II studies have shown that vaccines are tolerable and can reduce recurrence rate[149] and prolong recurrence-free survival[150, 151] in patients treated with ablative therapy. However, trials with tumor lysate vaccines have failed to show promising results in terms of efficacy[31,151]. Today, combination of vaccines with ICIs are being investigated clinically in phase I/II trials in HCC (NCT04912765, NCT04248569, NCT04251117), as are combinations with ablative therapies (NCT03674073, NCT03942328), and other treatments.

Virotherapy: Cancer virotherapy represents the most common type of cancer gene therapy and involves the transfer of genetic material (transgenes) into cells to modify their gene-expression profiles *via* viral vectors[31]. Oncolytic viruses (OVs) are a type of cancer virotherapy incorporating modified viral agents that selectively replicate in cancerous cells resulting in tumor cell lysis[152]. By contrast to classical vaccines, OVs represent an in situ cancer vaccine[35]. The primary immune effect aimed with virotherapy is to reduce tumor burden and broaden TCR repertoire[31]. Yet, scientists have increasingly realized that most anti-cancer efficacy observed with OVs is attributable to enhanced immune response activation triggered by immunogenic cell death caused by the destruction of cancer cells and uptake of tumor antigens by antigen presenting cells – often enhanced by the arming of OVs with cytokine encoding genes, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) – rather than their oncolytic properties[153]. Additionally, OVs display short-lived efficacy and are not effective on repeated doses, due to brief vector replication and transgene expression as well as neutralizing antibody development after first vector administration, respectively. However, long-term vectors may broaden applications[31]. Hence, OVs are better candidates as an adjunct therapy to trigger adaptive antitumor responses which require maintenance and expansion with additional immunotherapies[154]. To date, three OVs have been approved for cancer therapy: RIGVIR for melanoma[155], Oncorine for head and neck cancer[156], and T-Vec for melanoma[157], though only the latter has been granted FDA approval.

In HCC, several OVs have been investigated clinically with some featuring promising results. JX-594 (Pexa-Vec), an oncolytic poxvirus carrying human GM-CSF genes, is the only OV to have successfully reached phase III investigation. Unfortunately, phase IIb/III trials showed that Pexa-Vec failed to improve treatment efficacy in patients previously treated with sorafenib[158], in combination with sorafenib[159], and in sorafenib-naïve patients[160]. Investigation of Pexa-Vec combination with nivolumab was also terminated early due to futility in other pivotal trials[161]. Still, phase I/II trials are ongoing to investigate different virotherapy agents as monotherapy (NCT00028496, NCT04246671), and in combination with pembrolizumab (NCT02509507, NCT02432963) for HCC.

CONCLUSION

The progress achieved within the landscape of immunotherapy for HCC is remarkable. In the last five years, ICIs have become a cornerstone systemic treatment approach in the routine clinical management of aHCC. The year 2020 saw ICIs become frontline treatments for aHCC in combination with bavacizumab, rendering sorafenib frontline only for patients who are ineligible for or contraindicated to receive immunotherapy or anti-angiogenics. In 2nd-line setting, several ICIs continue to be standard of care, with more agents emerging in the horizon. Still, much progress is yet to be made, especially concerning the lack of real-world data to support the generalizability and applicability of clinical trial

findings to a broader cohort of aHCC patients who are not subjectable to stringent clinical trial inclusion and exclusion criteria. The main obstacles to immunotherapy frequently encountered in real-world practice surround patient ineligibility for immunotherapy because of contraindications, comorbidities, or poor performance status; lack of response, efficacy, and safety data; and cost-effectiveness. Hence, the reality of immunotherapy treatment for HCC outside of trial setting is far from ideal. Further real-world data from high-quality large prospective cohort studies as well as evidence from institutional experiences of immunotherapy in patients with aHCC outside of clinical trials is mandated to aid evidence-based clinical decision-making for this cohort of individuals who indeed represent the vast majority of patients encountered. At the same time, ongoing trials investigating novel approaches to optimize systemic regimens and enhance ICI efficacy through combination with locoregional ablation, other systemic agents, and novel immune-based approaches are necessary to break new grounds. With multiple ICI agents undergoing investigation, more ICIs are likely to enter the treatment landscape for aHCC. The development of new models such the *cancer immune cycle* theory to better understand and reverse limiting steps in cancer immune evasion; the characterization and subgrouping of different tumor immune microenvironment phenotypes in aHCC; and novel means of employing machine learning algorithms to predict patient response to select targeted therapies further advance the field of precision medicine in HCC into a new era. In the years to come, ACT including CAR T cells and CIK cells are likely to become part of the treatment armamentarium against aHCC. We eagerly await to monitor the field as it advances.

FOOTNOTES

Author contributions: Rallis KS and Makrakis D contributed equally to the writing, reviewing, and designing of the manuscript and are equal joint first authors; Ziogas IA and Tsoulfas G lead the initial conceptualization, designing, reviewing, and supervision of the work in this manuscript; all authors have read and approved the final manuscript.

Conflict-of-interest statement: All the authors declare that they have no conflict of interest.

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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 June 24; 13(6): 473-484

DOI: 10.5306/wjco.v13.i6.473

ISSN 2218-4333 (online)

MINIREVIEWS

Circulating tumor DNA for diagnosis, prognosis and treatment of gastrointestinal malignancies

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gu J, China

Received: March 1, 2021
Peer-review started: March 1, 2021
First decision: April 27, 2021
Revised: May 6, 2021
Accepted: May 26, 2022
Article in press: May 26, 2022
Published online: June 24, 2022

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Abstract

Minimally invasive detection of circulating tumor DNA (ctDNA) in peripheral blood or other body fluids of patients with gastrointestinal malignancies *via* liquid biopsy has emerged as a promising biomarker. This is urgently needed, as conventional imaging and plasma protein-derived biomarkers lack sensitivity and specificity in prognosis, early detection of relapse or treatment monitoring. This review summarizes the potential role of liquid biopsy in diagnosis, prognosis and treatment monitoring of gastrointestinal malignancies, including upper gastrointestinal, liver, bile duct, pancreatic and colorectal cancer. CtDNA can now be part of the clinical routine as a promising, highly sensitive and specific biomarker with a broad range of applicability. Liquid-biopsy based postoperative relapse prediction could lead to improved survival by intensification of adjuvant treatment in patients identified to be at risk of early recurrence. Moreover, ctDNA allows monitoring of antineoplastic treatment success, with identification of potentially developed resistance or therapeutic targets during the course of treatment. It may also assist in early change of chemotherapy in metastatic gastrointestinal malignancies prior to imaging findings of relapse. Nevertheless, clinical utility is dependent on the tumor's entity and burden.

Key Words: Cell-free tumor DNA; Circulating tumor DNA; Gastrointestinal cancer; Liquid biopsy; Esophageal cancer; Gastric cancer; Liver cancer; Bile duct cancer; Pancreatic cancer; Colorectal cancer

Core Tip: This review provides an update on the state-of-the-art circulating tumor DNA detection *via* liquid biopsy for diagnosis, prognosis and treatment in gastrointestinal malignancies and presents the strengths and limitations of this innovative method.

Citation: Kirchweger P, Wundsam HV, Rumpold H. Circulating tumor DNA for diagnosis, prognosis and treatment of gastrointestinal malignancies. *World J Clin Oncol* 2022; 13(6): 473-484
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/473.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.473

INTRODUCTION

Historically, tissue biopsy or plasma protein-derived tumor markers have been the fundamental pillars of cancer diagnosis, selection of treatment, monitoring of treatment effect and estimation of prognosis [1]. As cancer is a dynamic and likely progressive disease, histological analysis of a single lesion (*i.e.*, primary tumor or metastasis) at a single time point is now being replaced by minimally invasive detection of cell-free deoxyribonucleic acid (cfDNA) *via* liquid biopsy to monitor the continual change of the disease process[1-3]. Furthermore, disconcordance and genetic differences within the primary tumor tissue over time (temporal heterogeneity) or between the primary tumor and its metastases (spatial heterogeneity) can be observed *via* next-generation sequencing (NGS)[2,4]. Thus, single conventional biopsies do not accurately reflect the cellular and genetic composition of malignancies[1]. In contrast, liquid biopsies include nucleic acids or cancer cells from the entire tumor burden of the patient and can easily be conducted serially[4].

Discovered in 1989 in patients with gastrointestinal (GI) malignancies, circulating tumor DNA (ctDNA) derives from apoptotic, necrotic, or circulating cancer cells, and constitutes a small subset (< 0.01%) of cfDNA in the plasma of peripheral blood or other body fluids[5]. cfDNA has become a promising tool for diagnosis, monitoring of antineoplastic treatment effect, and early detection of relapse, in addition to evaluating potential new drug targets[6-8]. ctDNA is thought to be actively released *via* microvesicles (exons) of double-stranded DNA. Passive release of DNA fragments into the circulation from apoptotic and necrotic cells has been demonstrated[3,9]. The amount of cfDNA is significantly higher in cancer patients than in healthy individuals, but serum levels can easily be biased by various factors; ctDNA is considered tumor-specific and more robust[1]. Nevertheless, both values are strongly influenced by preanalytical and analytical variables. The influence of differences in type of sample collection tubes, sample storage time, performing the assay with plasma or serum, use of short or long amplification assays, or the time of blood collection have been evaluated[10]. Plasma is considered superior to serum because of its robust DNA data with higher KRAS allele frequency. A greater absolute amount of DNA is yielded by serum samples, but is also more affected by contamination or lysis)[10]. Although the American Society of Clinical Oncology and the College of American Pathologists highly recommend plasma analysis for DNA detection, many investigators in the past used serum samples. Two reviews[10,11] published in 2018 claimed that 100% of gastrointestinal stromal tumor (commonly known as GIST) studies, 62% of gastric cancer studies, 29% of esophageal cancer studies, and 20% of colorectal cancer (CRC) studies used serum samples for ctDNA analysis.

Over the years, several amplification techniques, such as real-time quantitative polymerase chain reaction (referred to as qPCR)[12], digital droplet PCR (ddPCR)[13,14], beads, emulsion amplification, and magnetics (BEAMing)[15] or NGS[16] have been adopted for clinical use[4]. The most commonly employed are digital PCR (dPCR) or ddPCR techniques using water-in-oil emulsion droplets with dispersed individual DNA strands. These fluorescently labeled samples allow a binary identification system of target mutations (*i.e.* mutant *vs* wild-type alleles), leading to a very low limit of detection (LOD) ranging from 0.1%-0.001%[4]. One of the most used dPCR systems for ctDNA detection is the Bio-Rad QX-200 platform[14]. BEAMing provides a high analytical sensitivity of < 0.01% minor allele frequency (MAF) by combining emulsion PCR and flow cytometry with a focus on rare mutations in *a priori* known target mutations[4]. NGS, on the other hand, can cover a broad range of mutations in multiple cancer-associated genes but is less sensitive than dPCR (~ 1%)[4]. Safe-SeqS is one of the first and most commonly used NGS platforms (LOD 1%)[17], whereas CAPP-Seq/iDES is a newer NGS technique with LODs of 0.002%-0.00025%[18]. Depending on the entity under investigation, approaches have emerged for detection within samples with a known mutation target and those without a known mutation. In the following section we describe currently promising prospects of this new and easily harvested biomarker for diagnosis, early relapse detection, and treatment efficacy.

UPPER GI CANCER

Diagnosis

Upper GI (UGI) cancer subsumes esophageal cancer, cancer of the gastroesophageal junction, and the gastric cancer. Unfortunately, detection rates of UGI cancers are low in the early stages (approximately 20%) and reporting studies, thus, have low case numbers[19]. However, potential targets for molecular tracking are: HOXD10 (higher methylation rates in more advanced disease); ZIC1, RUNX3, and TP53 (53%); or receptor tyrosine kinases, including KRAS (15%), FGFR2, EGFR (17%), ERBB2, PIK3CA (13%), or HER2 (17%)[7,20,21]. NGS of metastatic UGI cancer in small case studies revealed detection rates of up to 87.5%[21]. Detection rates greatly depend on the MAF in the site of associated metastases, with only 23.3% in the lung, 19.2% in the liver, and only 2.5% in peritoneal metastases; the primary tumor burden is represented by tumor volume[20].

Prognosis

Relapse prediction following neoadjuvant treatment is a substantial issue in UGI cancer that affects almost all patients undergoing surgery. A study including more than 1600 patients reported postsurgical detection rates of up to about 32% and that MAF cutoff levels of > 0.25% (100% sensitivity) were associated with worse progression-free survival (PFS) (12.5 mo *vs* not reached, $P = 0.03$, $n = 22$)[20]. A significant survival disadvantage was observed in patients undergoing treatment with checkpoint inhibitors when detecting a MAF of > 3.5% prior to treatment initiation (8.8 mo *vs* 2.5 mo, $P = 0.04$, $n = 27$)[20]. If detectable, some mutations like PIK3CA (3.8 mo *vs* 13.6 mo, $P = 0.006$) or BRAF (5.6 mo *vs* 13.7 mo) indicate especially poor survival among stage IV patients[20]. On the other hand, targeted therapy, when detecting HER2 or EGFR mutation, can lead to significant survival benefits (21.1 mo *vs* 14.4 mo, $P = 0.001$)[20]. These findings need to be evaluated in larger prospective studies.

Treatment monitoring

Serial measurement of ctDNA in stage IV UGI cancer has found a significant survival benefit for patients with a > 50% decrease of the maximum MAF (13.7 mo *vs*. 8.6 mo, $P = 0.02$, $n = 35$) during the course of first-line therapy[21]. The Personalized Antibodies for Gastroesophageal Adenocarcinoma ("PANGEA") study revealed promising results in 68 patients undergoing ctDNA-guided individualized monoclonal antibody treatment compared with historical chemotherapy controls (1-year survival of 66%, median overall survival (OS) of 15.7 mo, $P = 0.0024$, median PFS of 8.2 mo, and first-line response rate of 74% *vs* about 50%)[22].

LIVER AND BILE DUCT CANCER

Diagnosis

CtDNA has been investigated in liver cancer patients for several years, and although it is still not in routine clinical use, liquid biopsy was shown to be superior to conventional plasma-derived biomarkers. For example, alpha-fetoprotein has a diagnostic sensitivity of 50% for hepatocellular carcinoma (HCC)[23]. Unfortunately, HCC has a broad range of potentially mutated genes. The most common are TP53 (c.747G>T), TERT (c.1-124C>T), and CTNNB1 (c.121A>G and c.133T>C)[24]. Generally, detection rates using liquid biopsy are expected to reach 56% in resectable HCC patients (ddPCR of 48 samples)[24]. A study published in 2006 reported sensitivity and specificity values of 69% and 93%, respectively, for discrimination of HCC and controls using cfDNA cutoff levels[25]. Subsequently, the presence of a combination of different methylated tumor suppressor genes, which rarely occur in the DNA of healthy tissue, had a reported sensitivity of 83.3% and specificity of 90.5% for detection of HCC[26]. Apart from detection of malignancies, liquid biopsy and stratification following detection of methylated peroxisome proliferator-activated receptor gamma (commonly known as PPARg) gene promoter has also shown promise for prediction of fibrosis grade in nonalcoholic fatty liver disease[27].

On the contrary, data on mutation detection *via* ctDNA in bile duct cancer is sparse, as cholangiocarcinoma is a rare disease. It has an estimated incidence 0.5-3.5/100.000), is often diagnosed at a metastasized stage, and the reported data is frequently pooled with liver or pancreatic cancer[28]. Overall, about 28% of patients with bile duct cancer show TP53 mutations, followed by 17% with ARID1A mutations and 16% with KRAS mutations[28]. However, bile duct cancer has very heterogenous mutation patterns. Using liquid biopsy in cases with a histologically verified mutation, Ettrich *et al*[28] reported a detection rate of 92% in intrahepatic cholangiocarcinoma (IHCC) and only a 55% detection rate in extrahepatic cholangiocarcinoma (referred to as EHCC).

Prognosis

Both the untargeted (cfDNA) and targeted detection of mutation, primarily of TP53 (32%), CTNNB1 (17%), and TERT (51%), has shown prognostic potential indicating poorer disease-free survival and OS in patients with HCC, regardless of tumor stage[23,24,29-31]. Moreover, vascular invasion, tumor mass,

and level of postoperative cfDNA have emerged as independent risk factors for recurrence in patients with resectable HCC[32].

Regarding cholangiocarcinoma, some studies reported poorer PFS when detecting mutations *via* liquid biopsy, especially in cases with ctDNA assay of TP53, KRAS, BAP1, or PBRM1 in settings of both curative and palliative intent, as compared with patients with nondetectable mutation[33-35]. Again, the data was obtained in IHCC patients; most studies could not detect a significant correlation regarding PFS in EHCC patients[28].

Treatment monitoring

Serial ctDNA measurement in advanced HCC has revealed progression of the disease before imaging or alpha-fetoprotein dynamics could indicate recurrence, but the studies included small case numbers[36]. As ctDNA MAF of both IHCC and EHCC correlate with tumor load, some authors estimate a potential for treatment efficacy detection in bile duct cancer, but that needs further evaluation, as serial measurement for treatment monitoring has not yet been performed[28,37]. In a January 2021 publication, Felden *et al*[31] reported prospective findings of ultra-deep sequencing and ddPCR in 121 patients that supported the treatment-monitoring potential of ctDNA as a biomarker response to antineoplastic treatment.

PANCREATIC CANCER

Diagnosis

While surgical resection can improve 5-year survival by 15%-25%, fewer than 20% of patients qualify for a primarily surgical approach[38]. In 2018, more than 50% of patients were diagnosed with distant metastases and had a 5-year survival rate of only about 3%[39]. The mean 5-year survival of all stages of pancreatic ductal adenocarcinoma (PDAC) stages is reported to be about 6%-8%, which is also due to the early systemic spread of the disease[39]. Thus, highly sensitive and reliable biomarkers are urgently needed for earlier diagnosis. Theoretically, PDAC, which accounts for 90% of all pancreatic cancers, could be an ideal entity for agnostically driven ctDNA determination as a screening biomarker because of the high rate of histologically detectable early KRAS mutations (> 90%)[40,41]. However, detection rates in histologically verified PDAC *via* liquid biopsy are significantly lower, controversially reported in literature, and very much depend on the stage of the disease (43%-54% in stages I–II; 67% in stage IV, and up to 95% if a mutation had already been detected in the tissue)[42-44]. Another study suggests much lower detection rates for early-stage PDAC[45]. Using ddPCR, Berger *et al*[46] reported in 2016 that mean cfDNA values (KRAS, GNAS) discriminated potentially premalignant cysts (*e.g.*, intraductal papillary mucinous neoplasms) and harmless pancreatic cysts. Nevertheless, the sensitivity and specificity were too low for applicability as a potential screening method. Thus, ctDNA offers little clinical application in diagnosis of localized pancreatic cancer, as it is inferior to the plasma-protein derived tumor marker CA 19-9, which has high sensitivity (70%-95%) and specificity (70%-90%). It also has a high vulnerability to coincident Lewis-negative blood group, acute cholangitis, obstructive jaundice, or chronic pancreatitis[47-51]. Therefore, the gold standard for diagnosis remains imaging combined with histological verification with endoscopic ultrasound and fine needle aspiration (commonly known as EUS-FNA)[38].

Prognosis

Although lacking usability in the initial diagnosis of PDAC, several studies demonstrated a significant correlation between both pre- and postoperative ctDNA positivity and OS [hazard ratio (HR): 2.093, P = 0.028] and PFS (HR: 4.543, P = 0.006) for both localized and metastatic PDAC (referred to as lPDAC and mPDAC, respectively)[52-55]. In 2010, Chen *et al*[56] reported a median OS of 3.9 mo *vs* 10.2 mo (P < 0.001) positivity in 91 patients with mPDAC, associated with mutKRAS ctDNA. In 2019, Lee *et al*[57] reported an OS of 5.8 mo *vs* 16.3 mo in lPDAC. The same group showed 100% of patients remaining ctDNA-positive after systemic neoadjuvant treatment following an early relapse, with a median PFS of 5 mo.

Treatment monitoring

Liquid biopsy allows earlier detection of relapse compared with plasma protein-derived tumor markers (CA 19-9), with lead times of 1 mo to 2 mo, and is more sensitive (83%) to changes in ctDNA levels[43,58]. This could indicate a potential opportunity for monitoring treatment during palliative chemotherapy using serial liquid biopsies, and ultimately making a change of the antineoplastic agent. Data on serial measurement in advanced PDAC mostly lacks large patient numbers, although promising results have raised the hope of early response to therapy and, ultimately, relapse identification[43]. Kruger and colleagues[43] were the first to report the potential of ctDNAs to indicate response as early as 14 d after treatment initiation, demonstrating major superiority to plasma-protein derived tumor markers, with a specificity of 100%. Nevertheless, the clinical survival benefit by eventual change of treatment in

patients with detected relapse using serial ctDNA measurements still needs to be explored. Targeted therapy could be another promising field of future research. Liquid biopsy has already found usage in PDAC patients suffering from BRCA1/2 mutations by providing PARP-inhibitors[59].

CRC

Diagnosis

CRC is the third leading newly diagnosed malignancy worldwide[60]. CtDNA is detectable in about 73% of stage II–III cases and 90% of patients with localized and metastatic CRC, positioning this entity as the ideal target for liquid biopsy[61,62]. Until now, liquid biopsy has not been included in the routine screening for CRC, but samples are easily assessable. Tests are becoming more cost effective and the presence of ctDNA in early-stage CRC (46% detection rate in stage I) was reported in 2015[63]. Acceptance of liquid biopsy in the general population appears to be high, based on a 2014 German study finding that patients not willing to undergo colonoscopy preferred blood tests over other noninvasive screening tools, like fecal occult blood tests[64].

Prognosis

Multivariate analyses conducted in several studies have confirmed that postoperative detection of ctDNA is an independent marker of recurrence, regardless of stage and location of the primary tumor. The 3-year PFS was 33% *vs* 87%[65,66]. In 2019, Tie *et al*[65] serially measured plasma ctDNA in 159 patients with locally advanced rectal carcinoma (T3/4 and/or N+) and treatment-naïve stage, post-chemoradiotherapy, of 4–10 wk after primary curative resection with adjuvant treatment. In an analysis that was blinded to ctDNA status, HRs and 3-year PFS significantly differed with positive liquid biopsy results. The 3-year PFS was 33% *vs* 87% after chemoradiotherapy (HR: 6.6, $P < 0.001$) and was 13.0 ($P < 0.001$) after resection, which allowed for stratification of patients with very high and very low risk of relapse[65]. Based on those findings, several ongoing prospective international studies are evaluating the potential additional clinical benefit from postoperative ctDNA positivity in CRC to identify patients at high risk of recurrence[7].

CtDNA-positive patients with stage II disease (*i.e.* with no clear recommendation for adjuvant treatment) might benefit from additional adjuvant chemotherapy. That is being tested in the DYNAMIC (ACTRN-12615000381583), COBRA (NCT04068103), and CIRCULATE AIO-KRK/PRODIGE 70 (NCT04089631/NCT04120701) trials. Whether stratifying stage III CRC patients by ctDNA results can guide decision making for intensification or de-escalation of adjuvant treatment or surveillance is being tested in the DYNAMIC-III (ACTRN-12617001566325) study[7,67-69]. Postoperative ctDNA detection has proven to be a strong indicator of distant recurrence, with a median lead time of 10 mo compared with conventional modalities such as computed tomography (commonly known as CT) and plasma-derived biomarkers like carcinoembryonic antigen (CEA)[70]. Regarding metastatic CRC, studies have demonstrated a significant survival benefit of wild-type carrier patients compared with patients with detectable ctDNA, dependent on the particular mutation[71].

Treatment monitoring

At diagnosis, approximately 80% of patients with CRC present without distant metastases and undergo primary curative resection. Over 50% of patients with stage II or III cancer also show rather unspecific abnormalities, such as elevated CEA (20%), CT abnormalities (40%), or both (13%) during the 5-year surveillance recommended by the American Society of Clinical Oncology (known as the ASCO)[72-74]. Nevertheless, until now there is no evidence of OS improvement resulting from 5-year surveillance, including clinical and endoscopic examinations, CEA measurement, and imaging[75,76]. Liquid biopsy could help to clarify uncertain findings. For example, ctDNA is positive in 85% of persons who experience imaging-verified relapse, whereas increased CEA levels are observed in only about 41% of radiologically verified recurrences[77]. Moreover, lead time of liquid biopsy compared with CEA is reported to be about 8 mo[76]. Thus, serial ctDNA measurement as a postoperative treatment monitoring method during surveillance could provide earlier detection of relapse.

The clinical benefit for OS has to be evaluated in future studies. Among other ongoing ctDNA studies investigating the benefit of adjuvant chemotherapy based on liquid biopsy findings, Danish investigators (IMPROVE-IT2; NCT04084249) have evaluated the surveillance improvement when implementing supplementary fludeoxyglucose-positron emission tomography/CT follow-up evaluation every 3 mo, based on ctDNA positivity (*i.e.* ddPCR every 4 wk) for 2 years after surgery for stage II–III CRC and early detection of relapse[78]. Most centers use NGS prior to the start of antineoplastic treatment for identification of potential therapeutic targets, providing in advance a mutational target for liquid biopsy. Interim analysis of our own ongoing study revealed detection rates of more than 92% in metastatic CRC with ddPCR of 28.5 mL plasma samples and known mutations found in tissue samples prior to analysis. That is in line with the 8% disconcordance rate in a BEAMing analysis of 236 patients reported in 2018[79].

Serial liquid biopsies allow response prediction prior to that obtained by conventional methods in metastatic-stage patients undergoing palliative chemotherapy[80]. Furthermore, studies have demonstrated the PFS and OS benefits of repeated mutational status determination for eventual rechallenge with cetuximab/irinotecan-based regimes in initially RAS/BRAF wild-type patients or patients with acquired resistance during the course of treatment[81,82].

CONCLUSION

CtDNA is ready to be integrated into routine clinical use in order to improve survival and relapse prediction in nonmetastatic GI cancers. It also allows for monitoring of antineoplastic treatment success for early detection of nonresponders, with potential early change of chemotherapy in metastatic GI malignancies prior to imaging findings of relapse. For some entities, especially CRC, rapid progress in liquid biopsy research could lead to fundamental changes in therapeutical strategies, accompanied by the desired survival improvement. The test is simple, cost effective, and easily assessable, although there are large differences in suitability, detection rate, progress of research (Table 1), tumor volume, and site of metastasis. Overall, lymph node metastases or peritoneal carcinosis lead to significantly lower amounts of detectable ctDNA compared with liver or lung metastases. Various techniques of target mutation detection have been established in clinical trials, and several potential preanalytical variables have to be taken into account when implementing these into routine clinical practice. Depending on the technologies in clinical use, the limits of detection range from about 1% (qPCR, Safe-SeqS) to 0.01% (ddPCR, BEAMing, CAPP-Seq/iDES)[4]. Nevertheless, mutation detection *via* liquid biopsy has several potential pitfalls and limitations. Firstly, standardization of sample drawing and the processing methods could help avoid common mistakes leading to very heterogenous sensitivity and specificity. Secondly, measured ctDNA levels are strongly affected by the period of time between blood draw and surgery or the initiation of chemotherapy. The ideal post-interventional interval for sample assessment needs to be further explored and eventually standardized, as ctDNA levels initially increase but continuously decline over the following weeks. The same applies for systemic antineoplastic treatment, as Maron *et al*[20] reported considerable differences in ctDNA MAF in untreated stage IV UGI patients (mean MAF: 11.6%) compared with patients receiving treatment up to 14 d prior to sample collection (mean MAF: 5%).

UGI cancer

Liquid biopsy could provide essential benefits for adjuvant and palliative treatment decision making, but low detection rates in nonmetastatic UGI cancers hinders this. Positive ctDNA after neoadjuvant treatment can identify patients with significantly increased risk of relapse (HR: 18.7), distant metastases (HR: 32.1), and cancer-associated death (HR: 23.1), but identifying how this issue should be addressed for significant survival benefit is a key question for further studies[7]. Moreover, liquid biopsy may become integrated into treatment response prediction, especially of immunotherapy, in advanced UGI cancers[83].

Liver cancer

Liquid biopsy offers significant prognostic potential in resectable HCC and was recently established as a promising biomarker for early response prediction of systemic therapy in advanced HCC; although, improvement regarding the LOD is necessary to implement these findings into clinical practice[31]. Ongoing studies are attempting to lower the LOD using multifocal screening panels, which could establish ctDNA as a valuable diagnostic and predictive biomarker for HCC patients, regardless of the disease stage[23].

Bile duct cancer

Until now, no prospective studies have investigated the benefits of liquid biopsy in bile duct cancer. Detection rates of mutations *via* liquid biopsy in histologically verified patients distinguishes between extra- and intrahepatic cholangiocarcinoma in favor of IHCC[28]. Nevertheless, screening for certain mutations, like IDH1 or FGFR, could help to establish personalized first-line palliative antineoplastic treatment, for example with ivosidenib (IDH1) or FGFR-kinase-inhibitors in the future[84,85].

Pancreatic cancer

For localized or locally advanced pancreatic cancer, ctDNA positivity prior to treatment is predictive of survival and relapse. This finding could assist decision making for additional perioperative or adjuvant antineoplastic treatment of high-risk patients. Negative ctDNA, on the other hand, holds no additional informative value in those with pancreatic cancer. Since 2014, pancreatic cancer has been known to release significantly lower amounts of detectable circulating tumor cells into the bloodstream compared with most other tumors, including colorectal, gastric, lung, breast, ovarian, prostate, bladder, or renal cancer[86]. However, some recent, small pilot studies have shown promising screening rates using specially designed detection methods. For example, hTERT promoter-regulated oncolytic herpes

Table 1 Detection rates and impact on outcome of circulating tumor DNA in gastrointestinal cancer

Entity	Detection rate	Common target	OS ctDNA −/ +	PFS ctDNA −/ +
mPDAC	67%-75%[43]	> 90% KRAS, but also TP53, SMAD4	8.4 *vs* 3.2[89]	5 *vs* 3.9[43]
lPDAC	21%-69%[42]		16.3 *vs* 5.8[57]	19 *vs* 8[57]
mCRC	> 90%[79]	KRAS, NRAS, BRAF, PIK3CA, NRAS, APC, TP53, EGFR, ERBB3/4	36.5 *vs* 17.1[90]	RAS 8.3 *vs* BRAF 4.5 *vs* wild-type 22.9[72]
lCRC	73%(43%-80%)[62,63]		-	87% *vs* 33%[65]
				3-yr PFS
mUGIC	87.5%[21]	TP53, HER2, MET, EGFR, KRAS	13.7 *vs* 8.6[20]	7.4 *vs* 4.9[83]
lUGIC	20%[19]		66.9 *vs* 37.7[10]	12.5 *vs* not reached[20]
HCC	56.3%[24]	TP53, CTNNB1, TERT	61% *vs* 24%[29]	47% *vs* 22%[29]
			3-yr OS	3-yr PFS
mIHCC	92%[28]	TP53, KRAS, ARID1A	16.4 *vs* 7.4[91]	8.2 *vs* 4.6[91]
mEHCC	55%[28]		NS[28]	NS[28]

−/+: ctDNA negative/positive; ctDNA: Circulating tumor DNA; HCC: Hepatocellular carcinoma; lCRC: Localized colorectal carcinoma; lPDAC: Localized pancreatic ductal adenocarcinoma; lUGIC: Localized upper gastrointestinal carcinoma; mCRC: Metastatic colorectal carcinoma; mEHCC: Metastatic extrahepatic cholangiocarcinoma; mIHCC: Metastatic intrahepatic cholangiocarcinoma; mPDAC: Metastatic pancreatic ductal adenocarcinoma; mUGIC: Metastatic upper gastrointestinal carcinoma; NS: Not significant; OS: Overall survival in months; PFS: Progressive-free survival in months.

simplex virus-1 targeting telomerase reverse transcriptase was positive in 88.2% of 17 patients with PDAC in all stages of disease. A parallel-flow microfluidic chip detected 91.7% of 12 mPDAC patients[4,87,88]. Methods like these need further study before they can be integrated into the clinical routine.

CRC

Significant progress has been made in ongoing trials of liquid biopsy in nonmetastatic CRC, especially on ctDNA-guided change in adjuvant therapeutic regimes, which may have a fundamental impact in future care. CRC is the ideal entity for liquid biopsy because of high rates of mutation detection and the total amount of cf/ctDNA in the plasma. This is in addition to the fact that tissue samples for Safe-SeqS/NGS are available for a sufficient proportion of patients to allow for guided mutation detection, thus resulting in very high specificity and sensitivity rates. Metastatic CRC offers even higher detection rates and could optimally benefit from the use of liquid biopsy in prognosis estimation and treatment evaluation in the future.

FOOTNOTES

Author contributions: Kirchweger P drafted the manuscript; Wundsam HV did extensive revisions; Rumpold H had the idea and co-drafted the manuscript and did revisions.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

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World J Clin Oncol 2022 June 24; 13(6): 485-495

DOI: 10.5306/wjco.v13.i6.485

ISSN 2218-4333 (online)

MINIREVIEWS

Current treatment landscape for oligometastatic non-small cell lung cancer

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kermenli T, Turkey; Ko R, Japan

Received: April 25, 2021
Peer-review started: April 25, 2021
First decision: June 7, 2021
Revised: June 24, 2021
Accepted: May 12, 2022
Article in press: May 12, 2022
Published online: June 24, 2022

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Abstract

The management of patients with advanced non-small cell lung carcinoma (NSCLC) has undergone major changes in recent years. On the one hand, improved sensitivity of diagnostic tests, both radiological and endoscopic, has altered the way patients are staged. On the other hand, the arrival of new drugs with antitumoral activity, such as targeted therapies or immunotherapy, has changed the prognosis of patients, improving disease control and prolonging survival. Finally, the development of radiotherapy and surgical and interventional radiology techniques means that radical ablative treatments can be performed on metastases in any location in the body. All of these advances have impacted the treatment of patients with advanced lung cancer, especially in a subgroup of these patients in which all of these treatment modalities converge. This poses a challenge for physicians who must decide upon the best treatment strategy for each patient, without solid evidence for one optimal mode of treatment in this patient population. The aim of this article is to review, from a practical and multidisciplinary perspective, published evidence on the management of oligometastatic NSCLC patients. We evaluate the different alternatives for radical ablative treatments, the role of primary tumor resection or radiation, the impact of systemic treatments, and the therapeutic sequence. In short, the present document aims to provide clinicians with a practical guide for the treatment of oligometastatic patients in routine clinical practice.

Key Words: Oligometastatic; Non-small cell lung carcinoma; Non-small cell lung cancer; Oligometastasis

Core Tip: The treatment of oligometastatic non-small cell lung cancer patients remains controversial. The lack of solid evidence for the best therapeutic strategy and the multiple options currently available for both systemic and local treatments make this particular population of patients a challenge for clinicians. Improvement of surgical and radiotherapy techniques and the appearance of different ablative methods, such as radiofrequency or cryoablation, have made it possible to radically treat metastases in any location. In addition, recent prospective studies suggest that combining these ablative therapies with systemic treatments improve patient outcomes. We discuss the current status of the management of oligometastatic patients.

Citation: Garde-Noguera J, Martín-Martín M, Obeso A, López-Mata M, Crespo IR, Pelari-Mici L, Juan Vidal O, Mielgo-Rubio X, Trujillo-Reyes JC, Couñago F. Current treatment landscape for oligometastatic non-small cell lung cancer. *World J Clin Oncol* 2022; 13(6): 485-495
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/485.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.485

INTRODUCTION

Up to two-thirds of patients diagnosed with non-small cell lung cancer (NSCLC) present with advanced disease on diagnosis, or develop incurable metastases during the course of the disease[1]. Despite the heterogeneity of this group of patients, their treatment is largely systemic. In recent years, new systemic treatments have appeared, such as tyrosine kinase inhibitors (TKIs) with molecular targets or immunotherapy (ICI), which have significantly improved the efficacy of these systemic treatments, leading to prolonged survival in candidates for targeted therapies or immunotherapy. Moreover, the prognosis is very different for patients with a low metastatic volume. This is reflected in the 8th tumor-node-metastasis (TNM) classification, which distinguishes between patients with a single extra-thoracic metastasis, M1b stage IVA, and patients with multiple lesions in one or multiple organs, M1c stage IVB [2]. Patients with a low metastatic burden, also referred to as oligometastatic, can benefit from local treatment of the primary tumor and metastatic sites. The term oligometastatic, coined by Hellman and Wichselabum in 1995[3,4], refers to an intermediate situation between potentially curable local neoplastic disease and incurable widespread metastatic cancer. In the case of NSCLC, oligometastatic patients constitute 26% to 50% of patients with advanced disease, depending on whether the cutoff is taken at ≤ 3 or ≤ 5 metastatic locations[5,6]. Precisely, the main challenge for an optimal approach to oligometastatic disease has been the lack of consensus in its definition. Recently, the European Consensus defined oligometastatic state as a maximum of five metastases from up to three different sites[7], although this definition is not unanimously accepted by the scientific community, and some

prospective studies developed in this context define oligometastatic stage as a maximum of three metastases.

Initially, surgery was the only radical treatment that could be offered to these patients. Now, however, thanks to technological advancements, they can receive ablative irradiation doses by stereotactic body radiotherapy (SBRT) at cranial and extracranial levels, which is both safe and well-tolerated. When local treatment of metastases is combined with systemic treatment, 5-year survival rates between 8.3% and 86% can be achieved[8]. Three randomized studies in oligometastatic patients have shown that this radical local treatment of metastatic locations increases progression-free survival (PFS) and even benefits overall survival (OS)[9-11]. However, it is still unclear which patients can benefit from this strategy. Although there is a lack of consensus about the definition of oligometastases, for some patients with oligoprogression, local treatment of these sites can increase PFS without exhausting new lines of systemic chemotherapy (CT).

In this review, we propose to explore the most controversial aspects of patients with oligometastatic NSCLC, examining in greater depth aspects such as: the definition of this condition, the selection of patients, and the combination of systemic and local treatments.

THE DEFINITION OF OLIGOMETASTATIC DISEASE

Oligometastatic lung cancer refers to a group of patients with stage IV NSCLC, who present with limited metastatic disease in terms of the number of lesions and organs affected. The incidence of oligometastatic NSCLC has been estimated at between 27% and 55%, depending on the series published [12]. The most frequent oligometastatic location is the brain (36%), followed by the contralateral lung (34%), suprarenal gland (13%), bones (9%), and liver (2%)[13]. Oligometastatic disease, more accurately referred to as an oligometastatic state, can have a more indolent biology than widespread metastases, or at the least, microscopic disease that can be eradicated with systemic therapy. This limited metastatic phenotype could benefit from local aggressive therapy known as consolidation therapy. In fact, an ongoing study is examining different epigenetic markers such as microRNAs[14], to determine their ability to distinguish between the oligometastatic state and widespread metastases. This distinction together with the determination of different prognostic factors are crucial to select patients in whom radical treatment of the primary tumor and of the oligometastases could improve PFS and OS[9].

Currently, the concept of limited metastatic disease is not clearly defined and there is some discrepancy among authors. A European multidisciplinary group recently agreed to accept the definition of oligometastatic disease as the presence of up to five metastases in three different organs[7]. However, this is not universally accepted and additional studies are required to standardize the concept of oligometastases. Within the oligometastatic state, different patterns of presentation of the disease and its response to treatment can be clearly distinguished. The term synchronic or "*de novo*" oligometastatic disease refers to the initial simultaneous diagnosis of both the primary lung tumor and a limited number of metastases. This presentation pattern appears to have a worse prognosis than metachronic oligometastatic disease or oligorecurrence, in which the patient develops distance metastases after having received radical treatment with curative intent of the primary lung tumor, with an apparent local control of the disease[12,15]. In both patterns, the oligometastatic phenotype seems to reflect the biology of the underlying tumor rather than being related to any specific previous therapy. Another two patterns correspond to patients with initially widespread metastases who receive systemic treatment and achieve a partial response, consisting of the stable persistence of a small number of oligometastases (oligopersistent disease or "induced oligometastasis") with possible later progression (oligoprogression). These scenarios are more common among patients treated with targeted therapies who present acquired resistance to treatment.

ALTERNATIVES TO ABLATIVE TREATMENT OF METASTASES

The main local treatments in oligometastatic disease correspond to surgical resection, radiotherapy treatment, and ablative radiofrequency techniques[16]. Although there are no prospective studies that compare the efficacy of these treatments, the main characteristics and published evidence for each of these therapeutic alternatives are described below.

SURGERY

Traditionally, surgery has always been the elective approach in oligometastatic patients[17].Surgical indication depends on several factors relating to the metastases (size, number, and locations), and also on patient-related factors (age, performance status, comorbidities, and prognosis). Over the past decade, the rate of metastectomies among NSCLC patients has increased and these mainly correspond to

interventions on lung, brain, and adrenal gland metastases. Moreover, mortality has declined with the development of less invasive advanced surgical techniques[18]. Most evidence for the benefits of surgery can be found in studies on patients with brain metastases. Patchell *et al*[19] randomized 48 patients with a single brain metastasis (77% of whom were diagnosed with NSCLC) to whole brain radiotherapy (WBRT) or surgical resection of the metastasis followed by WBRT. The results demonstrated an increased local control and OS in the group treated surgically. Few studies on the surgical resection of extracranial metastases have been published and most of these are retrospective and highly heterogeneous regarding time of onset of the metastases and their location[20-22].

RADIOTHERAPY

Thanks to technological advances in recent years, large doses of radiation can be delivered with high precision to several sites. Brain metastases are treated with stereotactic radiosurgery and extracerebral lesions with SBRT, or stereotactic ablative radiotherapy (SABR). One of the advantages of these treatments is that they require fewer sessions, each of a short duration. They are also safe, produce minimum toxicity, and do not require long interruptions in systemic chemotherapy.

Most studies are retrospective, but some prospective randomized phase II studies focusing on the efficacy and safety of these techniques have produced promising results[8-10,23-28] (Table 1). Results are pending, over the next few years, for several ongoing phase 3 studies[29,30] (Table 2).

RADIOFREQUENCY ABLATION

The radiofrequency ablation (RFA) technique consists of applying high frequency microwaves by means of a catheter inserted inside the tumor to destroy the tissue with heat. RFA has been used for both primary lung tumors and pulmonary metastases. Simon *et al*[31] treated 153 patients with primary or medically-inoperable metastatic NSCLC with RFA. For stage I NSCLC, they reported OS at 1, 2, and 5 years of 78%, 57%, and 27%, respectively. Tumoral control was 83%, 64%, and 47% at 1, 2, and 5 years for tumors of 3 cm or less, and for tumors larger than 3 cm, was 45%, 25%, and 25%, respectively. The incidence of pneumothorax was 28.4% (52 of 183 sessions) and 9.8% (18 cases) required placement of a drain. More recently, Picchi *et al*[32] reported a retrospective series of 174 patients with lung cancer treated with 264 CT-guided ablation sessions. In patients with primary lung lesions, the OS rates were 66.73% at 1 year, 23.13% at 3 years, and 16.19% at 5 years. In patients affected by metastatic lung lesions, the OS rates were 85.11%, 48.86%, and 43.33%, respectively, at 1, 3, and 5 years[32]. Although evidence is scarce, these experiences support CT-guided RFA in patients with primary and metastatic lung cancer as an alternative therapy in non-surgical candidates.

CRYOABLATION

This technique destroys the tissues by extreme cold and freezing. Cryoablation is currently used routinely to treat lung cancers with specific clinical indications. Bronchoscopic cryoablation is an accepted, standard-of-care for the safe and effective treatment of obstructing endobronchial tumors in the central airways[33-36]. Cryoablation has also been used as a treatment option for unresectable primary and secondary peripheral lung tumors[37,38].

Recently, high rates of tumoral control and promising survival outcomes have been reported in a series of patients with metastatic lung cancer lesions treated with this technique[39], although more research is required to verify these findings.

THE ROLE OF SURGERY AND RADIOTHERAPY IN PRIMARY TUMORS

Local therapies in primary tumors should conform with the principles governing a good control of the pulmonary neoplastic disease and, in this context, the concept of oligometastatic disease has become a different entity. The most important prognostic factor is the stage of spread according to the TNM classification, but in recent years histological subtype, lymphovascular spread, and genetic and molecular alterations have gained in importance[40].

In the treatment of primary tumors per se, the type of patients is an important prognostic factor that can affect survival[41]. The lymph nodes should be examined thoroughly to rule out pathological mediastinal or hilar involvement. This is an important prognostic factor as it could indicate lymphatic and hematogenic spread, thus limiting the options of intrathoracic control and would also increase the risk of spread of the metastatic disease[12].

Table 1 Main studies on stereotactic body radiotherapy for the treatment of oligometastatic non-small cell lung carcinoma

Ref.	Year	Patients (*n*)	Site of oligo-metastasis	N	Dose (Gy/fraction)	Systemic therapy	Median follow-up (mo)	Median PFS (mo)	Median OS (mo)
Retrospective studies									
Inoue *et al*[27]	2010	41[1]	Brain, lung, adrenal	< 5	48/8 (adrenal)35-60/4-8 (lung)	NA	20	3-yr PFS 20%	24
Hasselle *et al*[28]	2012	25	Multiple	< 5	24-70/3-20	Various	21	4.2 (all); 12 (1 met)	23 (1 met)
De Rose *et al*[26]	2016	60	Lung	< 5	48-60/3-8	Chemo	28	32.2 (actuarial)	32.1 (actuarial)
Single arm prospective trials									
Salama *et al*[23]	2012	61[1]	Multiple	< 5	24-48/3	Chemo	20.9	2-yr PFS 22%	2-yr OS 56.7%
De Ruysscher *et al* [20]	2012	40	Multiple	< 5	54/3[2]	Chemo	27.7	12.1	13.5
Collen *et al*[29]	2014	26	Multiple	< 5	50/10	Chemo	16.4	11.2	23
Randomized phase II trials									
Gomez *et al*[25]	2016	49	Multiple	< 3	NR	Chemo	12.4	14.2 *vs* 4.4	41.2 *vs* 17
Iyengar *et al*[10]	2018	29	Multiple	< 5	21-37.5/1-5	Chemo	9.6	9.7 *vs* 3.5	Not reached *vs* 17
Palma *et al*[11]	2019	99	Multiple	< 5	35-60/3-8	Chemo	25	12 *vs* 6	41 *vs* 28

[1]Diverse primary histology including non-small cell lung carcinoma.
[2]Only 1patient received stereotactic body radiotherapy.
Chemo: Chemotherapy; N: Number of oligometastatic lesions per patient; NA: Not applicable; NR: Not reported; OS: Overall survival; PFS: Progression-free survival.

On the other hand, the type of local therapy chosen should guarantee complete local control. Surgery is the most frequent local treatment in published studies[41]. Moreover, for a therapeutic approach to oligometastatic disease, complete resection (R0) must be performed[42]. In the case of surgery, the patient's clinical condition must be good enough to ensure not only that the tumor can be resected, but that the patient can withstand an operation. In other words, that the patient's overall cardiologic and respiratory functional status are sufficient to permit surgical intervention.

The role of radiotherapy and its modalities depend upon the stage of the primary tumor. In the case of external curative radiotherapy (EBRT), this is defined by delivery of a biologically effective dose (BED) higher than or equal to $60Gy_{10}$. In the case of SBRT with intention-to-treat, a BED higher than or equal to 100 Gy_{10} is required[43]. In the initial stages, SBRT is indicated when surgical intervention is not possible, or when the patient refuses surgery[43]. EBRT is only used in non-operable patients, who do not fulfil criteria for SBRT. In stage III, if the lesion is potentially resectable, the combination of radiotherapy and chemotherapy plays a dominant role within multimodal treatments, either pre or post-operatively[44].

In this stage, if the tumor is unresectable, the elective treatment is radiotherapy delivered concurrently with chemotherapy. Sequential administration is possible if the size of the tumor makes it difficult to deliver sufficient radiation. Radiation therapy can also be delivered alone if chemotherapy is contraindicated[45].

SYSTEMIC TREATMENT IN THE OLIGOMETASTATIC PATIENT

In the management of oligometastatic NSCLC patients, local treatments of surgery or radiotherapy have been used to reduce tumoral burden and prolong OS and PFS. For years, the evidence supporting this strategy was mainly provided by retrospective studies in which encouraging results were observed in patients treated with local ablative therapies compared to those receiving systemic therapy alone[7]. The recent publication of some randomized prospective studies has provided valuable information to help

Table 2 Ongoing studies on stereotactic body radiotherapy in oligometastatic non-small cell lung carcinoma

Title	Patients	Study design	Estimated completion
Stereotactic Ablative Radiotherapy for Oligometastatic Non-Small Cell Lung Cancer. A Randomised Phase III Trial Institution: University College London Clinical Trials.gov identifier: NCT02417662	340	Phase 3 multicenter: chemotherapy alone or chemotherapy + radical radiotherapy (conventional RT and SABR) Primary histology: all NSCLC 1-3 oligometastatic lesions Primary outcome measure: OS	August 2022
Maintenance Systemic Therapy Versus Local Consolidative Therapy (LCT) Plus Maintenance Systemic Therapy for Limited Metastatic Non-Small Cell Lung Cancer (NSCLC): A Randomized Phase II/III Trial (NRG LU-002) Institution: NRG Oncology Clinical Trials.gov identifier: NCT03137771	400	Phase 2/3 multicenter: maintenance chemotherapy or SBRT + maintenance chemotherapy Primary histology: all NSCLC 1-3 oligometastatic lesions Primary outcome measure: PFS	August 2022
Randomized Phase III Trial of Local Consolidation Therapy after Nivolumab and Ipilimumab for Immunotherapy-naive Patients with Metastatic NSCLC (LONESTAR)-Strategic Alliance: BMS Institution: M.D. Anderson Cancer Center Clinical Trials.gov identifier: NCT03391869	360	Phase 3 multicenter; systemic treatment only with nivolumab and ipilimumab, or induction nivolumab and ipilimumab followed by local consolidative therapy with surgery and/or radiotherapy Primary histology: all NSCLC 1 oligometastatic lesions Primary outcome: OS	December 2022
A Randomised Trial of Conventional Care Versus Radioablation (Stereotactic Body Radiotherapy) for Extracranial Oligometastases Institution: Royal Marsden NHS Foundation Trust Clinical Trials.gov identifier: NCT02759783	245	Phase 2/3 multicenter: standard of care + SBRTPrimary histology: breast, prostate or NSCLC 1-3 oligometastatic lesions Primary outcome measure: PFS	October 2024
A Randomized Phase III Trial of Stereotactic Ablative Radiotherapy for the Comprehensive Treatment of 4-10 Oligometastatic Tumors (SABR-COMET 10) Institution: Lawson Health Research Institute Clinical Trials.gov identifier: NCT03721341	159	Phase 3 multicenter: stereotactic ablative radiotherapy, plus standard of care treatment; chemotherapy, immunotherapy, hormones, or observation given at the discretion of the treating oncologist Various histology including NSCLC 4-10 oligometastatic lesions Primary outcome: OS	January 2029
Randomized Phase II Trial of Osimertinib With or Without Local Consolidation Therapy (LCT) for Patients With EGFR-Mutant Metastatic NSCLC (NORTHSTAR) Institution: M.D. Anderson Cancer Center Clinical Trials.gov identifier: NCT03410043	143	Phase 2 multicenter: osimertinib followed by local consolidative therapy with surgery and/or radiotherapy or maintenance osimertinib alonePrimary histology: NSCLC > 1oligometastatic lesion Primary outcome: PFS	January 2023
A Multicenter Single Arm Phase II Trial Assessing the Efficacy of Immunotherapy, Chemotherapy and Stereotactic Radiotherapy to Metastases Followed by Definitive Surgery or Radiotherapy to the Primary Tumor, in Patients With Synchronous Oligometastatic Non-small Cell Lung Cancer Institution: European Thoracic Oncology Platform Clinical Trials.gov identifier: NCT03965468	47	Phase 2 multicenter: durvalumab, carboplatin/paclitaxel chemotherapy, followed by SBRT to all oligometastases. Restaging at 3 mo definitive local treatment with surgical resection of primary tumor or RT 60-66 Gy to the primary tumor if not disease progression 1-3 oligometastatic lesions Primary outcome: PFS	December 2023

OS: Overall survival; PFS: Progression-free survival; RT: Radiotherapy; SABR: Stereotactic ablation radiotherapy; SBRT: Stereotactic body radiotherapy.

treatment decisions in this setting.

The SABR-COMET study is a phase II prospective clinical trial in which 99 patients with different types of oligometastatic tumor (a maximum of 5 metastatic sites) were randomized to receive SBRT and standard systemic treatment, or systemic treatment alone[11]. Ablative treatment with SBRT significantly increased OS [41 mo *vs* 28 mo, hazard ratio (HR): 0.57, 95% confidence interval (CI): 0.30-1.10]. Only 18 patients in this cohort had a primary lung tumor, thus making it difficult to extrapolate

the results for application in this patient group. In the SBRT-treated group, a higher proportion of patients had breast and prostate cancer. The less aggressive history of these entities could also affect outcomes. However, a post-hoc analysis which excluded patients with breast and prostate cancer still found a significant benefit for patients receiving ablative radical treatment, with a survival rate at 5 years of 33% compared with 16% in patients receiving the standard treatment.

More recently, the findings of several phase II clinical trials in patients with lung cancer have been published. Iyengar *et al*[10] published the results of a phase II clinical trial in 29 patients with oligometastatic advanced NSCLC who had completed induction chemotherapy with disease response or stabilization. Patients were randomized to receive maintenance chemotherapy *vs* SBRT on all tumoral sites followed by maintenance chemotherapy[10]. A significant increase in PFS was observed in the patient group receiving the radical treatment (9.7 mo *vs* 3.5 mo; P = 0.01), with excellent local control of irradiated sites and no rise in toxicity. Similarly, Gomez *et al*[9] published the results of a phase II trial in which 49 patients with advanced lung cancer, with three or fewer metastases at diagnosis, had been treated with induction therapy and were randomized to receive local radical treatment and maintenance with standard systemic therapy *vs* systemic therapy exclusively. They found significant differences in both PFS (14.2 mo *vs* 4.4 mo; P = 0.022), and in OS (41.2 mo *vs* 17 mo; P = 0.017) in favor of the combined treatment[9].

More recently, the annual conference of the American Society of Clinical Oncology reported the results of the SINDAS study. This phase III randomized clinical trial explored the role of stereotactic radiotherapy combined with first or second generation tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR) (tyrosine kinase inhibitor [TKI]-EGFR) *vs* TKI-EGFR alone in first-line treatment of patients with EGFR-mutant advanced oligometastatic lung adenocarcinoma, with five or fewer metastatic lesions[46]. A total of 133 patients were included (65 in the TKI arm) and 68 in the TKI-SBRT arm, finding a significant difference in favor of the experimental arm for both PFS [20.2 mo *vs* 12.5 mo, HR: 0.6188 (95%CI: 0.3949-0.9697); P < 0.001) and OS [25.5 mo *vs* 17.4 mo, HR: 0.6824 (95%CI: 0. 4654-1.001); P < 0.001), with no increase in toxicity[46].

CONCLUSION

Oligometastatic disease (OMD) is a unique condition characterized by a limited number of metastases and an indolent evolution. Because of the different prognosis of this condition, the TNM classification considers stage IV with a single metastatic site as a different category, called stage IV1b[2]. However, the World Health Organization classification is much more heterogeneous and includes patients with a greater number of metastases (up to 5 for some authors). Because of this difference, in an attempt to reach a consensus, the European Organization for Research and Treatment of Cancer established the most accepted definition to be the presence of five metastases and three affected organs after staging with computed tomography/positron emission tomography (CT/PET) and brain magnetic resonance imaging[14]. Noteworthy, the main points to consider in OMD are that all lesions (primary lesions and metastases) can be managed with an intention-to-treat approach and that the goal of treatment must be curative.

Growing interest in OMD has arisen from three main developments. First, an improvement in diagnostic techniques, mainly with the use of CT/PET in lung cancer staging, has resulted in an increasing number of patients being diagnosed with fewer metastases. The prognosis of this group is also better[21]. Moreover, technological advances in the field of radiotherapeutic oncology mean that high doses of radiation can be applied to specific sites. This non-surgical approach is preferred by patients with OMD as they can avoid the morbidity and mortality derived from surgical intervention. Over the past few years, the number of studies into the use of SBRT in OMD has been increasing. These have not only focused on brain metastases, but also on metastases of liver, lung, bone, and multiple organs, reporting local control rates of 70%-90% and a toxicity ≥ grade 3 lower than 10%[47]. The final aspect to consider, but not the least important, concerns the employment of immunotherapy in lung cancer treatment. Ionizing radiations can alter the tumor (beyond merely reducing the number of viable cells) and also its microenvironment, producing a specific immune response (antigenic tumoral death) that can trigger an immune response in non-irradiated sites (abscopal effect)[48]. This immunogenic effect is more pronounced with SBRT, in which high doses are delivered in few fractions[49], making this even more attractive as a treatment of OMD.

A question frequently posed in OMD is whether the better prognosis is due to the ablative treatment or the more indolent course of the disease. In a retrospective study of 90 patients with ≤ 3 metastases, after adjusting for factors that could potentially affect OS and PFS, it was found that patients who received local intensive treatment with CT + radiotherapy or surgery, or both, had better OS and PFS than those who received less intensive treatments, such as palliative CT alone[50]. However, randomized studies in both the general population[9,11] and in the population with EGFR mutations [46] have shown that the addition of local ablative treatment to systemic treatment is associated with increased PFS and OS.

One of the greatest remaining challenges is to distinguish between patients with OMD, characterized by a reduced number of metastases, and those with pre-widely metastatic disease, in other words, those diagnosed as having a small number of metastases but who develop multiple metastases in the following weeks or months. A search is currently underway for genetic profiles[51,52], either epigenetic modifications by overexpression or inhibition of microRNA[53,54] or methylations of genetic loci, that regulate the expression of the microRNA they encode[55]. These could possibly explain the limited rather than extensive spread of the disease in these patients. However, a greater knowledge of the immune system has revealed the importance of its interaction with the tumor for tumoral control or spread. Pitroda *et al*[56] *via* integrated transcriptional analysis, describe three molecular subtypes of liver metastases of colon cancer, all biologically different and each with a clinical course that is independent of known clinical risk factors. Canonical and stromal subtypes are characterized by a lack of, or a reduction in, T cell infiltration and the expression of non-immune inflammatory pathways, and are linked to a higher recurrence rate and a greater number of metastases. By contrast, the immune subtype, characterized by upregulation of the immune genes and a greater infiltration of T cells in the tumor, is associated with better survival, with relapse limited to between one and three metastases. These findings are in line with studies that show that the adaptive immune response plays a key role in controlling metastatic spread[57]. From these findings, it could be hypothesized that OMD would represent a point of equilibrium between tumoral growth and its inhibition by the immune system.

Over the next few years, further research into the immune and molecular profiles of OMD patients, combined with the application of radiotherapy with its immunogenic role, and treatment with new immunomodulator agents could be beneficial for these patients.

FOOTNOTES

Author contributions: All authors contributed equally to the writing and critical revision of the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 June 24; 13(6): 496-504

DOI: 10.5306/wjco.v13.i6.496

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Basic Study

Molecular docking of DS-3032B, a mouse double minute 2 enzyme antagonist with potential for oncology treatment development

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Odhar HA, Iraq
A-Editor: Kołat D, Poland

Received: August 8, 2021
Peer-review started: August 8, 2021
First decision: September 2, 2021
Revised: September 16, 2021
Accepted: May 28, 2022
Article in press: May 28, 2022
Published online: June 24, 2022

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Abstract

BACKGROUND

It is known that p53 suppression is an important marker of poor prognosis of cancers, especially in solid tumors of the breast, lung, stomach, and esophagus; liposarcomas, glioblastomas, and leukemias. Because p53 has mouse double minute 2 (MDM2) as its primary negative regulator, this molecular docking study seeks to answer the following hypotheses: Is the interaction between DS-3032B and MDM2 stable enough for this drug to be considered as a promising neoplastic inhibitor?

AIM

To analyze, *in silico*, the chemical bonds between the antagonist DS-3032B and its binding site in MDM2.

METHODS

For molecular docking simulations, the file containing structures of MDM2 (receptor) and the drug DS-3032B (ligand) were selected. The three-dimensional structure of MDM2 was obtained from Protein Data Bank, and the one for DS-3032B was obtained from PubChem database. The location and dimensions of the Grid box was determined using AutoDock Tools software. In this case, the dimensions of the Grid encompassed the entire receptor. The ligand DS-3032B interacts with the MDM2 receptor in a physiological environment with pH 7.4; thus, to simulate more reliably, its interaction was made with the calculation for the prediction of its protonation state using the MarvinSketch® software. Both ligands, with and without the protonation, were prepared for molecular docking

using the AutoDock Tools software. This software detects the torsion points of the drug and calculates the angle of the torsions. Molecular docking simulations were performed using the tools of the AutoDock platform connected to the Vina software. The analyses of the amino acid residues involved in the interactions between the receptor and the ligand as well as the twists of the ligand, atoms involved in the interactions, and type, strength, and length of the interactions were performed using the PyMol software (pymol.org/2) and Discovery Studio from BIOVIA®.

RESULTS

The global alignment indicated crystal structure 5SWK was more suitable for docking simulations by presenting the p53 binding site. The three-dimensional structure 5SWK for MDM2 was selected from Protein Data Bank and the three-dimensional structure of DS-3032B was selected from PubChem (Compound CID: 73297272; Milademetan). After molecular docking simulations, the most stable conformer was selected for both protonated and non-protonated DS-3032B. The interaction between MDM2 and DS-3032B occurs with high affinity; no significant difference was observed in the affinity energies between the MDM2/pronated DS-3032B (-9.9 kcal/mol) and MDM2/non-protonated DS-3032B conformers (-10.0 kcal/mol). Sixteen amino acid residues of MDM2 are involved in chemical bonds with the protonated DS-3032B; these 16 residues of MDM2 belong to the p53 biding site region and provide high affinity to interaction and stability to drug-protein complex.

CONCLUSION

Molecular docking indicated that DS-3032B antagonist binds to the same region of the p53 binding site in the MDM2 with high affinity and stability, and this suggests therapeutic efficiency.

Key Words: DS-3032B; Mouse double minute 2 antagonist; Molecular docking; Tumor suppressor p53

Core Tip: The knowledge, at the molecular level, of the complexes formed by therapeutic drugs and their target in the body are relevant to understand the efficiency of the drug. These data can be provided, with high reliability, by bioinformatics tools, which saves time in relation to *in vitro* and *in vivo* analyses. The drug DS-3032B has been a potential candidate for oncogenic treatment in preclinical trials, but clinical studies are scarce. This work shows data on chemical interactions between this drug and its target, mouse double minute 2, that corroborate the preclinical data and demonstrate the stability of the therapeutic complex.

Citation: da Mota VHS, Freire de Melo F, de Brito BB, Silva FAFD, Teixeira KN. Molecular docking of DS-3032B, a mouse double minute 2 enzyme antagonist with potential for oncology treatment development. *World J Clin Oncol* 2022; 13(6): 496-504
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/496.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.496

INTRODUCTION

Cancer is a genetic disease whose evolution leads to numerous changes in DNA. According to a survey conducted by the International Agency for Research on Cancer in 2018, cancer was considered the second leading cause of death in the world, affecting 18.1 million people and causing the death of 9.6 million people around the world[1]. For Brazil, the estimate for the triennium 2020-2022 predicts that 625000 new cases of cancer will occur[2].

While proto-oncogenes are genes responsible for the positive regulation of cell proliferation, tumor suppressor genes are genes responsible for negative regulation; in other words, it inhibits cell multiplication. An example of this class is the p53 gene, which is found mutated in about half of human cancers[3]. p53 is a transcription factor activated by signs of stress, such as DNA damage, oncogenes activation, and nutritional deprivation[4]; it also has an essential function in DNA damage repair and antioxidant response regulation[5].

Therefore, overactivation of p53 is considered as an option for selective therapies against cancer, providing a targeting of neoplastic cells and sparing unaffected normal tissue[6]. One proposed treatment aims to inhibit tumor growth by activating the tumor suppressor protein p53 through inhibition of the mouse double minute 2 (MDM2)[7].

MDM2 is an E3 ubiquitin ligase enzyme that has a negative regulatory role of the tumor suppressor p53. This enzyme controls transcriptional activity and stability of p53. MDM2 expression is regulated in several tumors, resulting in loss of p53-dependent activities, such as apoptosis and cell cycle arrest[8].

p53 is targeted for degradation by the proteasome by MDM2. Through E3 ubiquitin ligase activity, MDM2 promotes ubiquitination of p53, leading to increased p53 degradation. In some human tumors, MDM2 has been shown to be abnormally upregulated leading to enhanced degradation and reduction of p53 activity[9].

The molecule DS-3032B (Milademetan or RAIN-32) is an MDM2 antagonist that prevents its interaction with p53. Clinical trials with DS-3032B have been conducted by the National Institute of Health (phase I) in patients with leukemia and lymphoma and showed clinical efficacy[10]. A phase I trial evaluated the safety, tolerability, efficacy, and pharmacokinetics of DS-3032B in Japanese patients with solid tumors who relapsed after or refractory to standard therapy, and dose-limiting toxicities, safety, tolerability, maximum tolerated dose, pharmacokinetics, and recommended dose for phase II clinical trial were determined[11].

However, the trials are empirical, as the mechanism and biochemistry of interaction of DS-3032B with MDM2 are not known. Although there is a lot of preclinical evidence of the action of MDM2 inhibitors as monotherapy or in combination, clinical experience with these agents is limited. Thus, information, at the molecular level, about the complex formed between the inhibitor and its target will help to clarify the nature of the interaction and its stability[9].

Such information is important to understand the functioning of the compound and even increase its efficiency through structural alterations. Since obtaining these data by crystallization and X-ray diffraction is laborious and time consuming, a plausible alternative has been facilitated by computational methods, such as molecular docking that has proven useful and reliable for predicting the possible interactions and affinity of ligands with macromolecules. *In silico* methods have been gaining increasing prominence since the experimental determination of complex three-dimensional structures is quite complex and costly[12]. Thus, the aim of this study was to analyze the interaction of the DS-3032B to its binding site in MDM2, the chemical bonds between drug and protein, and the affinity of the formed complex in order to clarify the stability of the interaction and thus help in elucidating the molecular mechanism of therapeutic action of the antagonist.

MATERIALS AND METHODS

Receptor preparation

For molecular docking simulations, the structures of MDM2 (receptor) and the drug DS-3032B (ligand) were selected. The three-dimensional structure of MDM2 was selected from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) after a previous global alignment of all available primary sequences using CLUSTAL X 2.0 software. The 3D structure file was obtained in the extension ".pdb" (input file). The selected receptor's three-dimensional structure was prepared for molecular docking simulations using AutoDock Tools software; the water molecules were deleted, since they do not belong to the molecule and can interfere in the docking process and hydrogen atoms were also added. Then it was determined through the AutoDock Tools software the location and dimensions of the Grid (virtual box that delimits the region where the ligand will perform possible interactions with the receptor). In this case, the dimensions of the Grid encompassed the entire receptor. The Grid data and coordinates were used in molecular docking.

Ligand preparation

The three-dimensional structure of the drug DS-3032B was solved experimentally and deposited in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/); the file was obtained in the extension ".sdf" and converted to ".pdb" (input file). The ligand DS-3032B interacts with the MDM2 receptor in a physiological environment with pH 7.4; thus, to simulate more reliably, its interaction was made with the calculation for the prediction of its protonation state using the MarvinSketch 5.7® software from ChemAxon®. Both ligands, with and without the protonation, were prepared for molecular docking using the AutoDock Tools 1.5.6 software. This software detects the torsion points of the drug and calculates the angle of the torsions.

Molecular docking

Molecular docking procedures for a rigid protein and a flexible ligand were used. A grid of points in x, y, and z directions was built with a grid spacing of 1.0 Å using the AutoGrid component of the software. Molecular docking simulations were performed using the tools of the AutoDock platform (http://autodock.scripps.edu/) connected to the Vina 1.1.2 software (http://vina.scripps.edu/). The software used associates two components: A search algorithm and a score function. First, the algorithm was responsible for the search of possible combinations in the bonds, exploring the rotational, translational, and conformational degrees of freedom of the ligand as well as of the proteins. Then, the score function was used to choose the best binding modes. These functions were obtained according to the force fields

of molecular mechanics and empirical parameters from free energy calculations.

In silico analysis of drug-protein interactions

The analyses of the amino acid residues involved in the interactions between the receptor and the ligand, as well as the twists of the ligand, atoms involved in the interactions, and type, strength, and length of the interactions were performed using the PyMol 2.5 software (pymol.org/2) and Discovery Studio from BIOVIA®.

RESULTS

Receptor and ligand selected

During the search for the three-dimensional structure of the receptor, the alignment of primary sequences of three-dimensional structures available in the PDB corresponding to MDM2 was performed. The 5SWK, from *Homo sapiens*, was indicated as the best structure by having structure resolved by X-ray crystallography with high resolution (1.92 Å) and greater coverage of the site responsible for the antagonism of the protein. The antagonist binding site is located in chain A of MDM2, so chain A was isolated to perform molecular docking simulations. Figure 1 shows the primary sequence alignment between 5SWK chain A (153 residues) from PDB and whole MDM2 (466 residues). The consensus region presents the binding site for the MDM2 receptor antagonist. The three-dimensional structure of the antagonist DS-3032B was obtained from PubChem database with the CID (compound identification number) 73297272. MarvinSketch® software showed that at pH 7.4, 97.37% of DS-3021B was distributed in its protonated form.

Analysis of the drug-protein complex

A ranking of nine conformations presenting different affinity energies was obtained in molecular docking for each ligand (protonated and non-protonated), and the conformation with the lowest energy (Figure 2) was selected for subsequent analysis of drug-protein interactions. In addition, two root mean square deviation (RMSD) metric variants are also available: RMSD/L.b. (lower limit of RMSD) and RMSD/u.b. (upper limit RMSD), which differ by the way the atoms are matched in the distance calculation. While in RMSD/u.b. each atom is matched in one conformation to itself in the other conformation, ignoring any symmetry, in RMSD/L.b., each atom is matched in one conformation to the nearest atom of the same element type in the other conformation. No significant difference was observed between the affinity energies between both MDM2/protonated DS-3032B and MDM2/non-protonated DS-3032B conformers (Table 1); therefore, the protonated form, which prevails under physiological conditions, was better analyzed in this study. Sixteen amino acid residues of MDM2 are involved in chemical bonds with the protonated DS-3032B. Polar bond, hydrophobic interactions (pi-sigma and alkyl), and Van der Waals were observed (Table 2). These 16 residues of MDM2 chain A belong to the p53 biding site region. Four out of 16 interactions are more relevant, and they range from 2.18 to 3.96 Å; the shortest bond is a hydrogen bond between an oxygen atom of leucine residue 54 (LEU 54) of MDM2 and a nitrogen atom of one of the rings of the DS-3032B antagonist (Figure 3).

DISCUSSION

The MDM2, also called E3 ubiquitin ligase enzyme, is commonly overexpressed in various cancers[13], inactivating directly p53 by interacting with its transcriptional activation domain and inducing its degradation through ubiquitination[4]. DS-3032B is a compound derived from dispiropyrrolidine, also called milademetan. It impairs the binding of MDM2 to the transcriptional activation domain of p53[14].

Pharmacological inhibition of the p53-MDM2 interaction has been evaluated as a therapeutic approach to exert p53-mediated antitumor effects. Because MDM2 antagonists can produce nongenotoxic activation of wild-type p53, leading to anticancer activity, these agents are candidates to improve the therapeutic index of current chemotherapy regimens while minimizing the risk of resistance to single-agent MDM2 inhibition[15]. MDM2 inhibitors have demonstrated in preclinical and clinical studies their antineoplastic effects arising from p53 activation caused by negative regulation of MDM2 in solid and hematological tumors.

Identifying the interactions between drugs and their targets is critical in the discovery of new drugs. This helps to understand better the mechanism of the disease and to identify unexpected therapeutic activity or adverse side effects of the drugs. Therefore, the prediction of interaction between drugs and targets becomes important in the context of pharmacology and drug redefinition[16]. The precise and efficient identification of interactions between drugs and their targets in the body can reveal hidden functions of these drugs and target proteins as well as speed up the drug development process[17]. Drug development is a time-consuming process, the experimental identification of interactions between drugs and their targets is very costly, and modern technologies have mitigated this problem. The

Table 1 Affinity energy and RMSD values of both conformers mouse double minute 2/protonated DS-3032B and mouse double minute 2/non-protonated DS-3032B

Conformer	Affinity energy (kcal/mol)	Dist. From	
		RMSD l.b.	RMSD u.b.
MDM2/protonated DS-3032B	-10.0	0.000	0.000
MDM2/non-protonated DS-3032B	-9.9	0.000	0.000

MDM2: Mouse double minute 2; RMSD: root mean square deviation; RMSD l.b.: RMSD lower limit; RMSB u.b.: RMSD upper limit.

Table 2 Chemical interactions between mouse double minute 2 receptor and protonated DS-3032B antagonist

Residues of MDM2	Interaction	Bond size (Å)
Leu 54	Hydrogen bond	2.18
Leu 57	Alkyl	3.92
Ile 61	Alkyl	3.91
Tyr 67	Pi-Sigma	3.96
Ile 103	Van der Waals	-
Phe 86	Van der Waals	-
Phe 91	Van der Waals	-
Ile 99	Van der Waals	-
Tyr 100	Van der Waals	-
Val 75	Van der Waals	-
Val 93	Van der Waals	-
Gln 71	Van der Waals	-
Gln 72	Van der Waals	-
Gly 58	Van der Waals	-
His 73	Van der Waals	-
Met 62	Van der Waals	-

MDM2: Mouse double minute 2.

computational prediction of drug target interactions has been shown to be fundamental for the study of drugs, since it reduces the time and costs of the process[18].

In fact, through *in silico* approach, it was possible to observe that DS-3032B is able to connect to the p53 binding site of MDM2 chain A with a significant affinity. The interaction between the antagonist and MDM2 involves 16 amino acid residues by polar and nonpolar bonds throughout the entire structure of the drug. The arrangement of the connections contributes to the low bind energy value and consequently to the stability of the complex. Complex stability and high affinity indicate promising therapeutic potential for DS-3032B.

There are three clinical studies of MDM2 inhibitors in various cancers, including the MDM2 antagonist, DS-3032B, still in early phase (solid tumors and lymphoma: NCT01877382; myeloma: NCT02579824; leukemia: NCT02319369; acute myeloid leukemia: NCT03671564; acute myeloid leukemia, being associated with Quizartinib: NCT03552029; refractory leukemia, being associated with Cicarabine: NCT03634228)[19]. Clinical responses in these trials have been limited overall, but some patients have clearly achieved clinical benefit through monotherapy with MDM2 inhibitors[20]. Although monotherapy with MDM2 inhibitors has benefits, clinical responses are usually modest; association with other inhibitors has shown synergism and more efficient clinical responses[9]. However, the association of more medications can increase the presence of unwanted effects and even decrease patients' adherence to therapy. The action of drugs is related to their interaction with their therapeutic target; drug-protein affinity predicts complex stability, longer interaction time, and greater pharmacological effectiveness.

```
MDM2          ---MCNTNMSVPTDGAVTTSQIPASEQETL-----------------------VLFYL  32
SSWK_A|Chains GPHMCNTNMSVPTDGAVTTSQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKEVLFYL  60
                 ***************************                       *****

MDM2          GQYIMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMIYRNLVVVNQQESSD  92
SSWK_A|Chains GQYIMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMIYRNLVVVNQQESSD 120
              ************************************************************

MDM2          SGTSVSENRCHLEGGSDQKDLVQELQEEKPSSSHLVSRPSTSSRRRAISETEENSDELSG 152
SSWK_A|Chains SGTSVSENRCHLEGGSDQKDLVQELQEEKPSSS---------------------------- 153
              ********************************

MDM2          ERQRKRHKSDSISLSFDESLALCVIREICCERSSSSESTGTPSNPDLDAGVSEHSGDWLD 212
SSWK_A|Chains ------------------------------------------------------------ 153

MDM2          QDSVSDQFSVEFEVESLDSEDYSLSEEGQELSDEDDEVYQVTVYQAGESDTDSFEEDPEI 272
SSWK_A|Chains ------------------------------------------------------------ 153

MDM2          SLADYWKCTSCNEMNPPLPSHCNRCWALRENWLPEDKGKDKGEISEKAKLENSTQAEEGF 332
SSWK_A|Chains ------------------------------------------------------------ 153

MDM2          DVPDCKKTIVNDSRESCVEENDDKITQASQSQESEDYFQPSTSSSIIYSCQEDVKEFERE 392
SSWK_A|Chains ------------------------------------------------------------ 153

MDM2          ETQDKEESVESSLPLNAIEPCVICQGRPKNGCIVHGKTGHLMACFTCAKKLKKRNKPCPV 452
SSWK_A|Chains ------------------------------------------------------------ 153

MDM2          CRQPIQMIVLTYFP 466
SSWK_A|Chains -------------- 153
```

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Figure 1 Global alignment between 5SWK chain A (153 residues) and whole mouse double minute 2 (466 residues). Consensus region is located at position 56 - 153 of 5SWK chain A. The color of the residues represents the chemical characteristic of their side chains. MDM2: Mouse double minute 2.

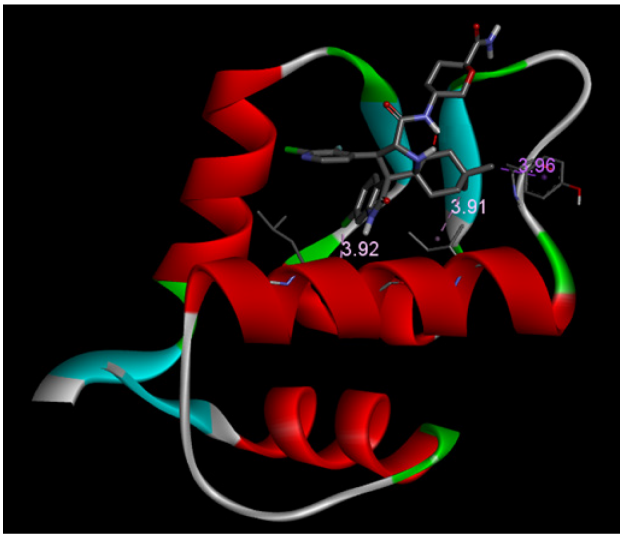

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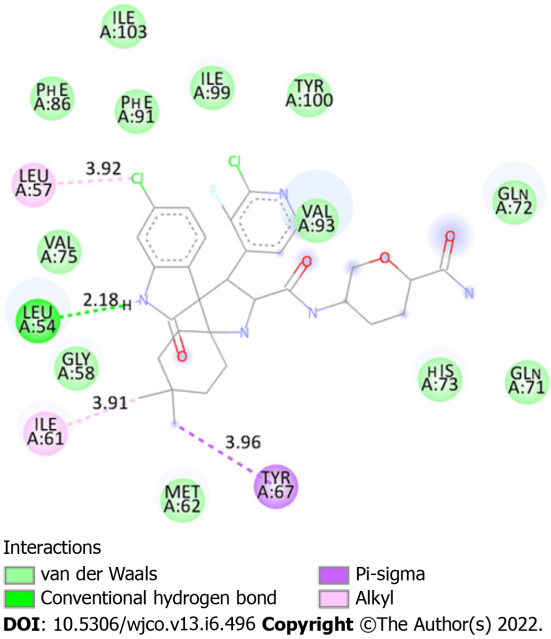
Figure 2 Mouse double minute 2/protonated DS-3032B conformer. Mouse double minute 2 (MDM2) receptor is shown in ribbon and DS-3032B is shown in sticks. Only LEU 57, ILE 61, and TYR 67 bonds are shown. Bond size in Å.

Thus, knowledge about the molecular interactions between the test drug and its therapeutic target becomes interesting to predict the behavior of the drug in the body, predict its efficiency and stability, and can help predict how this drug can affect the physiology of the individual. Since *in silico* studies can provide this data satisfactorily and reliably and much faster compared to *in vitro* and *in vivo* studies, this methodology has been used to support scientific studies. The analyses performed in this study, with the DS-3032B, indicate that the complex formed between this drug and its target is quite stable, indicating high therapeutic efficiency. This efficiency, measured indirectly, through affinity energy may be responsible for the good preclinical results of ds-3032B, and it may be more effective as monotherapy



DOI: 10.5306/wjco.v13.i6.496

Figure 3 Two-dimensional diagram demonstrating interactions between mouse double minute 2 chain A (5SWK) and protonated DS-3032B. Light green: Residues involved in Van der Waals interactions. Green: Residue involved in hydrogen bond. Lilac: Residue involved Pi-Sigma interaction. Pink: Residues involved in alkyl interactions. Numbers in spheres indicate the residue position. Bond size are shown (Å).

than current inhibitors.

Regarding the *in silico* analyses performed, the ligand position is selected based on calculations that are ranked according to docking score that represents the binding affinity between the ligand and the receptor and is expressed in kcal/mol[21]. Molecular docking is an established *in silico* structure-based method widely used in drug discovery. Docking enables the identification of novel compounds of therapeutic interest, predicting ligand-target interactions at a molecular level[22]. In this study it was obtained as a result of molecular docking, the ranking of the nine conformations with the highest affinities for the receptor, in parallel with two metric variants of RMSD: RMSD/L.b. and RMSD/u.b., which differ by the way the atoms are matched in the distance calculation. While in RMSD/u.b. each atom is matched in one conformation to itself in the other conformation, ignoring any symmetry, in RMSD/L.b. each atom is matched in one conformation to the nearest atom of the same element type in the other conformation. Whereas that for RMSD, the tolerance value respected is at most 2.0Å[23]. In addition, the affinity of the conformation presents the binding energy between the receptor and the ligand, being considered significant values less than -6.0 kcal/mol[13]. The compound that requires lower energy for the interaction to occur forms a more stable complex, in other words, has greater biological activity[24].

It is important to highlight that the DS-3032B, in addition to presenting satisfactory preclinical data regarding its anti-tumor potential, which are supported by computational findings, this drug is administered orally[11]. Thus, the DS-3032B becomes an attractive therapy compared to invasive and uncomfortable administrations for the patient.

CONCLUSION

In this study, higher biological activity means greater antagonism of MDM2 and consequent restoration of the tumor suppressor, p53. The confirmation was provided by the results obtained during molecular docking calculations, given that the conformer with the highest affinity showed -9.9 kcal/mol and therefore can be considered a promising candidate to inhibit the MDM2 protein. These dyspyrolidine-derived compounds may represent a starting point for the development of new drugs to treat cancers with overexpression of the MDM2 protein. The identified results reinforce that bioinformatics offers great direction in the search and validation of treatment targets, because it presents itself as a starting point for improving the knowledge involving drug- protein interactions. In addition, it also promotes cost and time reduction when compared to traditional research methods and directs the treatment so that the new drugs have their side effects minimized. However, *in silico* processes are complementary and do not rule out the need for *in vitro* and *in vivo* tests.

ARTICLE HIGHLIGHTS

Research background

Mouse double minute 2 (MDM2) is the main negative regulator of tumor suppressor p53; in this context, the effective inhibition of MDM2 is an alternative for cancer treatments.

Research motivation

DS-3032B is an MDM2 antagonist, and its activity is known only empirically, so bioinformatics analyses can point to molecular characteristics of complex interaction.

Research objectives

To analyze, *in silico*, the interactions between the antagonist DS-3032B and MDM2 and infer the antineoplastic potential of the drug.

Research methods

The analysis of chemical bonds, interaction of the drug-protein complex, and its stability were done by molecular docking.

Research results

Molecular docking simulations between MDM2 chain A (PDB: 5SWK) and DS-3032B (CID: 73297272) in its protonabed form indicated a complex with significant affinity energy, -10.0 kcal/mol. The results indicate a stable complex, maintained by hydrophilic and hydrophobic bonds involving 16 amino acid residues of MDM2.

Research conclusions

DS-3032B is able to bind to MDM2 with high affinity and stability, suggesting therapeutic efficiency.

Research perspectives

Analyze the DS-3032B/MDM2 complex using molecular dynamics and verify the possibility of structural changes of the drug to increase its efficiency.

FOOTNOTES

Author contributions: da Mota VHS, Freire de Melo F, and Teixeira KN contributed to study conceptualization, methodology, validation, and investigation and writing of the original draft; Freire de Melo F contributed to study conceptualization, methodology, and investigation, and manuscript review; de Brito BB and Silva FAFD contributed to study validation, visualization, and formal analysis and manuscript writing, review, and editing; Silva FAFD contributed to study validation, visualization, and formal analysis, manuscript writing, review, and editing, and supervision; Teixeira KN contributed to study conceptualization, methodology, and investigation, original draft writing, and supervision.

Institutional review board statement: This research was carried out in In silico models, not requiring an Institutional Review Board Statement.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data available.

Country/Territory of origin: Brazil

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 June 24; 13(6): 505-519

DOI: 10.5306/wjco.v13.i6.505

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Basic Study

Nicotinic receptors modulate antitumor therapy response in triple negative breast cancer cells

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Khaled I, Egypt; Rossi T, Italy; Xie S, China

Received: December 27, 2021
Peer-review started: December 27, 2021
First decision: February 15, 2022
Revised: February 24, 2022
Accepted: April 26, 2022
Article in press: April 26, 2022
Published online: June 24, 2022

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Abstract

BACKGROUND

Triple negative breast cancer is more aggressive than other breast cancer subtypes and constitutes a public health problem worldwide since it has high morbidity and mortality due to the lack of defined therapeutic targets. Resistance to chemotherapy complicates the course of patients' treatment. Several authors have highlighted the participation of nicotinic acetylcholine receptors (nAChR) in the modulation of conventional chemotherapy treatment in cancers of the airways. However, in breast cancer, less is known about the effect of nAChR activation by nicotine on chemotherapy treatment in smoking patients.

AIM

To investigate the effect of nicotine on paclitaxel treatment and the signaling pathways involved in human breast MDA-MB-231 tumor cells.

METHODS

Cells were treated with paclitaxel alone or in combination with nicotine, administered for one or three 48-h cycles. The effect of the addition of nicotine (at a concentration similar to that found in passive smokers' blood) on the treatment with paclitaxel (at a therapeutic concentration) was determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The signaling mediators involved in this effect were determined using selective inhibitors. We also investigated nAChR expression, and ATP "binding cassette" G2 drug transporter (ABCG2) expression and its modulation by the different treatments with Western blot. The effect of the treatments on apoptosis induction was determined by flow cytometry using annexin-V and 7AAD markers.

RESULTS

Our results confirmed that treatment with paclitaxel reduced MDA-MB-231 cell viability in a concentration-dependent manner and that the presence of nicotine

reversed the cytotoxic effect induced by paclitaxel by involving the expression of functional α7 and α9 nAChRs in these cells. The action of nicotine on paclitaxel treatment was linked to modulation of the protein kinase C, mitogen-activated protein kinase, extracellular signal-regulated kinase, and NF-κB signaling pathways, and to an up-regulation of ABCG2 protein expression. We also detected that nicotine significantly reduced the increase in cell apoptosis induced by paclitaxel treatment. Moreover, the presence of nicotine reduced the efficacy of paclitaxel treatment administered in three cycles to MDA-MB-231 tumor cells.

CONCLUSION

Our findings point to nAChRs as responsible for the decrease in the chemotherapeutic effect of paclitaxel in triple negative tumors. Thus, nAChRs should be considered as targets in smoking patients.

Key Words: Breast cancer; Paclitaxel; Nicotinic acetylcholine receptors; Drug therapy; Signal transduction; Drug transporter

Core Tip: Smokers with lung tumors are more likely to generate resistance to chemotherapy than non-smokers. However, little is known about the effect of nicotinic activation during the treatment of breast cancer, a cancer which arises close to the lung. In triple negative human breast cells, nicotine reduces the chemotherapeutic effect of paclitaxel through the participation of several kinases, as well as by modulating ATP "binding cassette" G2 drug transporter expression and inducing resistance to treatment. These results indicate that nicotinic acetylcholine receptors are a new possible target in antitumor therapy for this subtype of breast cancer.

Citation: Español A, Sanchez Y, Salem A, Obregon J, Sales ME. Nicotinic receptors modulate antitumor therapy response in triple negative breast cancer cells. *World J Clin Oncol* 2022; 13(6): 505-519
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/505.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.505

INTRODUCTION

Cigarette smoke contains many harmful components for human health[1]. Among them, nicotine (NIC), which has addictive properties[2], exerts its effects by activating nicotinic acetylcholine receptors (nAChRs)[3]. These receptors belong to the Cys-loop family of pentameric ionic channels activated by ligands[4]. nAChRs can be made up of 17 different subunits, whose assembly creates several homopentameric and heteropentameric channel subtypes in the cell membrane[5].

Originally, nAChRs were found in the nervous system. However, their expression has been described in several organs such as the lungs[6,7], kidney[8,9], intestine[10,11] and breast, where mainly the α7 and α9 subunits are expressed[12,13]. The activation of nAChRs can induce an increase in the levels of intracellular calcium[14], which has been related to tumorigenesis in the lungs[15], liver[16], pancreas [17] and brain[18]. This increase in intracellular calcium can in turn activate kinase signaling pathways [19,20], which regulate different parameters of tumor biology such as proliferation, migration and invasion[21-23].

Previous evidence has demonstrated that the activation of these receptors can decrease the effectiveness of different antitumor agents in various tumor types such as those from the oral and nasal cavity [14,24], pancreas[25], head and neck[26] and lungs[27-29]. In the lungs, many authors have described that NIC may exert its modulatory effect on the actions of chemotherapeutic agents through the activation of signaling pathways involving protein kinases[30-33]. However, little is known about the effect of nicotinic activation during the treatment of breast cancer, a cancer that arises close to the lungs.

Breast cancer is characterized by a high incidence that causes a high number of deaths in women worldwide[34]. According to their genetic profile, breast tumors are classified in different subtypes, a fact that allows doctors to choose the most effective antitumor therapy. Tumors can be categorized according to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 protein by immunohistochemistry. This allows defining four subtypes of breast tumors: luminal A, luminal B, HER2+ and normal-like or triple negative (TN)[35,36]. TN tumors lack the expression of ER, PR and HER2, but present high expression of Ki-67 protein and are usually very invasive and aggressive[37]. Since these tumors do not exhibit a defined therapeutic

target, there is no specific treatment, and they respond poorly to taxanes like paclitaxel (PX), usually administered as conventional chemotherapy to other breast cancer patients.

PX is an effective drug used not only in the treatment of breast cancer, but also in non-small cell lung cancer, prostate cancer, and head and neck cancer[38]. It is a cytostatic compound that causes hyper-stabilization of polymerized microtubules, inhibiting the mitotic spindle and arresting cells in G_2/M phases[39]. The persistence of cell arrest eventually produces cell death by apoptosis[40]. Thus, a low level of apoptosis could be an important factor in the development of resistance to treatment.

In this study, we evaluated the ability of NIC to interfere in the treatment of human TN breast cancer MDA-MB-231 cells with PX and the signaling pathway involved in this action.

MATERIALS AND METHODS

Cell culture

The human breast adenocarcinoma cell lines MDA-MB-231 (TN, CRM-HTB-26), MDA-MB-468 (TN, HTB-132) and MCF-7 (luminal A, HTB-22) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Inc., Carlsbad, CA, United States) supplemented with 100 mL/L heat inactivated fetal bovine serum (FBS) (Internegocios SA, Mercedes, Buenos Aires, Argentina), L-glutamine (0.3 mg/L) and gentamicin (80 mg/L). The cultures were maintained at 37 °C in humidified air with 50 mL/L CO_2 and the medium was replaced three times a week. Cells were detached with 250 mg/L trypsin in Ca^{2+}- and Mg^{2+}-free PBS containing 20 mg/L EDTA. Cell viability was determined by the Trypan blue exclusion test and the absence of mycoplasma was observed by Hoechst staining[41].

Cell viability assay

Cell viability after treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method (Life Technologies, Eugene, OR, United States). A suspension containing 4×10^3 cells/well was added to each well of a 96-well plate in culture medium supplemented with 50 mL/L FBS and cells were then left to attach overnight. When cells reached 60%-70% of confluence, they were deprived of FBS for 24 h to induce the synchronization of cultures. Then, cells were treated with PX (Bristol-Myers Squibb, Buenos Aires, Argentina) and/or NIC (which is a non-selective nAChR agonist) in medium supplemented with 20 mL/L FBS, for 48 h in triplicate. To inhibit the action of the nicotinic agonist, cells were previously treated with the antagonists mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) at 10^{-6} mol/L; these three are respectively nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists. To determine the participation of kinases in the effects of PX, cells were previously treated with inhibitors of: protein kinase C (PKC) [staurosporine (Stau), 10^{-8} mol/L], mitogen-activated protein kinase kinase (MEK) [PD098059 (PD), 10^{-5} mol/L], Ras [S-trans, trans-farnesylthiosalicylic acid (FTS), 10^{-6} mol/L], extracellular signal-regulated kinases (ERK1/2) (U126, 10^{-5} mol/L), p38 mitogen-activated protein kinases (p38MAPK) [SB203580 (SB), 10^{-5} mol/L] or the mediator of the activation of the NF-κB pathway IκB kinase (IKKβ) [IMD354 (IMD), 5×10^{-8} mol/L].

To determine the modulation of the sensitivity of MDA-MB-231 cells to chemotherapy, cells were treated with PX in the absence or presence of NIC for three 48-h cycles with 24 h intercycles without treatment. Then, surviving cells were treated with a new cycle of PX for 48 h. After treatment, the medium was removed and 100 μL of MTT solution (500 mg/L medium free of phenol red and FBS) was added. Plates were incubated for 4 h at 37°C and the production of formazan was measured by analyzing the absorbance at 540 nm with an ELISA reader (BioTek, Winooski, VT, United States). Values are indicated as mean ± SD and expressed as the percentage of cell viability in comparison to cells without treatment considered 100%.

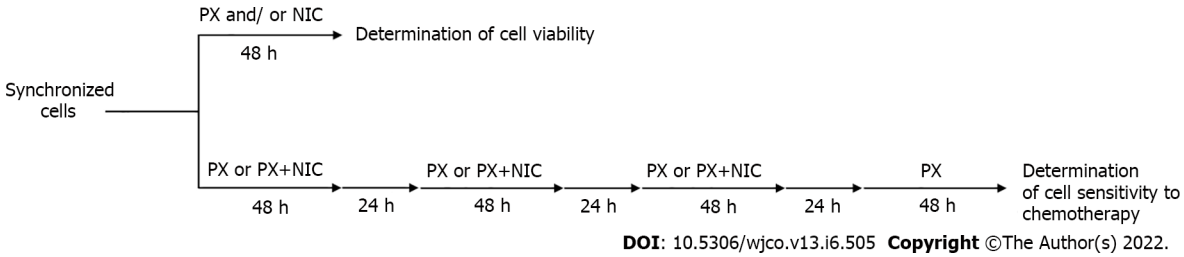
A diagram of the administration schedule for the determination of cell viability or cell sensitivity to chemotherapy is shown in Figure 1.

Calculation of the effective concentration 50

Dose–response data were transformed, changed to percentage and fitted to a sigmoidal curve, following a maximal effective concentration (Emax) model with at least six data points, using the GraphPad Prism 6 software. This allowed calculating the effective concentration 50 (EC50) and Emax values. Only data with a coefficient of variation lower than 20% were considered for the EC50 values.

Detection of nicotinic receptors by Western blot

MDA-MB-231 cell proteins were extracted by washing them with a buffer containing 6 g/L Tris-HCl, 3 g/L NaCl, 210 mg/L NaF, 480 mg/L $MgCl_2$, 300 mg/L EDTA, 380 mg/L EGTA, 870 mg/L phenylmethanesulfonyl fluoride, 10 mL/L Triton X-100 and 10 mg/L trypsin inhibitor, aprotinin and leupeptin, at pH 7.4. Samples were incubated on ice for 1 h and centrifuged at 800 G for 20 min at 4°C, afterwards the supernatants were collected and saved at -80°C. Protein concentrations were determined by the



DOI: 10.5306/wjco.v13.i6.505

Figure 1 Diagram of the administration schedule for the determination of cell viability or cell sensitivity to chemotherapy. PX: Paclitaxel; NIC: Nicotine.

Bradford assay[42].

Samples (80 μg protein per lane) were separated by 10% SDS-PAGE minigel electrophoresis and then transferred to nitrocellulose membranes. They were then blocked with 50 g/L skim milk and incubated overnight with rat anti-human-α7 nAChRs monoclonal antibody or mouse anti-human-α9 nAChRs monoclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX, United States), both diluted 1:200. Then, strips were incubated with anti-rat or anti-mouse IgG coupled to horseradish peroxidase diluted 1:10000 in buffer containing 2.4 g/L Tris-HCl buffer, 9 g/L NaCl and 500 mg/L Tween 20 (TBS-T) at 37°C for 1 h. α7 nAChR and α9 nAChR bands were detected by chemiluminescence and quantified by densitometric analysis using Image J software (NIH). The results are expressed as optical density (O.D.) units relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology Inc., Dallas, TX, United States), which was used as the loading control[43].

Trypan blue exclusion assay

Cell viability was also determined by the trypan blue dye exclusion test. Briefly, cells were treated with the corresponding drugs for 48 h in 48-well plates at a density of 10^4 cells/well. Cells were collected and centrifuged at 900 r/min for 10 min. Then, pellets were resuspended in DMEM and the trypan blue solution was added in a 1:1 ratio. The number of viable cells, identified as non-stained cells, was counted using a hemocytometer under an inverted microscope at 10X magnification and the percentage of these cells with respect to the total cell number was calculated.

Apoptosis determination by flow cytometry

For apoptosis determination, MDA-MB-231 cells were grown in six-well plates and treated for 48 h with PX, NIC or their combination, in the presence or absence of selective and non-selective nicotinic antagonists and kinase inhibitors. Then, cells were harvested and resuspended in binding buffer, and 2 μL of AnnexinV-FITC was added to each sample. Cells were then incubated for 15 min at room temperature in the dark. After that, 2 μL of 7AAD was added and the samples were immediately analyzed by the BD Accuri C6 Plus Flow Cytometer. Data were analyzed by the BD Accuri C6 Plus software.

ATP binding cassette transporter G2 detection by Western blot

For the detection of ATP "binding cassette" G2 drug transporter (ABCG2), cells (2×10^6) were treated for 48 h with the different drugs and samples were prepared as indicated to detect nicotinic receptors. Then, samples (80 μg protein per lane) were separated by 12% SDS-PAGE minigel electrophoresis, transferred to nitrocellulose membranes, blocked with 50 g/L skim milk and incubated overnight with a rabbit anti-human ABCG2 polyclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX, United States) diluted 1:200. Then, strips were incubated with horseradish peroxidase-linked anti-rabbit IgG, diluted 1:10000 in TBS-T at 37°C for 1 h. Bands were detected by chemiluminescence and then quantified by densitometric analysis using the Image J program (NIH) and expressed as O.D. units in comparison to the expression of GAPDH, which was used as the loading control[44].

Statistical analysis

Results are expressed as mean ± SD and statistical analysis was performed using the GraphPad Prism6 software. To determine differences between mean values, one-way ANOVA was performed with Tukey´s post-hoc analysis. $P < 0.05$ was accepted as statistically significant. The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology [45].

RESULTS

Effect of NIC on MDA-MB-231 cell viability

First, we analyzed the effect of NIC administered for 48 h to MDA-MB-231 cells in culture. The addition of different concentrations of NIC increased cell viability from 10^{-10} mol/L, with an Emax of 189.3% ± 5.2% (Figure 2A). This effect was reduced by the pre-treatment of cells with different nicotinic antagonists: MM (non-selective) (Emax: 118.4% ± 3.36%), MLA (α7 nAChR selective) (Emax: 143.6% ± 1.38%), or Lut (α9 nAChR selective) (Emax: 135.6% ± 5.69%), all of them added at 10^{-6} mol/L (all P < 0.001 *vs* NIC treatment) (Figure 2A). The nicotinic antagonists alone did not modify cell viability (data not shown). In addition, expression of α7 and α9 nAChRs in MDA-MB-231 cells was detected by Western blot assay (Figure 2B).

Paclitaxel treatment of MDA-MB-231 cells in the presence of NIC

In the next set of experiments, we analyzed the action of PX on tumor cells in the presence of NIC. First, we confirmed that PX reduced MDA-MB-231 cell viability in a concentration-dependent manner and that the PX effect was significant at concentrations equal to or higher than 10^{-8} mol/L (EC50: 1.0 × 10^{-7} mol/L) (Figure 3). On the other hand, the presence of the first effective concentration of NIC (10^{-10} mol/L) shifted the concentration-response curve to the right, increasing the EC50 value by more than one order (EC50: 1.2 × 10^{-6} mol/L) (Figure 3).

After three cycles of PX (48 h each) in the presence of NIC, followed by 24 h without drugs, surviving cells were less sensitive to a subsequent PX cycle in comparison to the same treatment in the absence of NIC. The latter was evidenced by an increase in the EC50 value obtained from the concentration-response-curve (PX+NIC EC50: 3.6 × 10^{-6} mol/L; PX EC50: 4.8 × 10^{-7} mol/L) (Figure 4).

It has been documented that 10^{-10} mol/L can be considered a concentration of NIC similar to that present in the bloodstream of passive smoking patients[46]. To analyze the ability of NIC to interfere with the action of therapeutic concentrations of PX, we treated MDA-MB-231 cells with a combination of 10^{-10} mol/L NIC and 10^{-7} mol/L PX for 48 h. We determined that NIC reduced PX effectiveness by increasing tumor cell viability to 110.8% ± 1.9% in comparison to PX treatment in the absence of NIC (51.1% ± 3.9%, P < 0.01). This effect of NIC on PX action was partially reduced by the pre-treatment of cells with nicotinic antagonists (MM, MLA or Lut) added at 10^{-6} mol/L (P < 0.001 *vs* PX+NIC) (Figure 5A) in a manner similar to that of NIC treatment alone (NIC: 138.16 ± 6.08; NIC+MM: 116.52 ± 0.12; NIC+Lut: 126.16 ± 5.96; NIC+MLA: 119.69 ± 4.26; P < 0.001; P < 0.05 and P < 0.001 *vs* NIC, respectively). We confirmed that the PX effect was independent of nAChR activation since pre-treatment with nicotinic antagonists did not modify the effect of PX treatment alone (PX+MM: 52.12 ± 6.65; PX+Lut: 49.97 ± 5.88; PX+MLA: 54.6 ± 4.01). To confirm the action of drugs on cell viability, we next analyzed the ratio of living cells by the Trypan blue exclusion test after the different treatments. The results plotted in Figure 5B (control: 100 ± 3.3; NIC: 129.3 ± 1.1, P < 0.001; PX: 64.1 ± 4.3, P < 0.001; PX+NIC: 92.3 ± 2.5, P=ns; PX+NIC+MM: 56.3 ± 3.4, P < 0.001; PX+NIC+MLA: 66.7 ± 5.4, P < 0.001; PX+NIC+Lut: 52.3 ± 6.0, P < 0.001; all being % or significance with respect to the control) show values similar to those obtained by the MTT assay (Figure 5A).

To confirm that nicotinic agonists can modulate the action of PX in reducing viability in other breast cancer cells, we tested the effect of PX (10^{-7} mol/L) in the absence or presence of NIC (10^{-10} mol/L) on MDA-MB-468 and MCF-7 cell viability. We determined that the presence of NIC reduced the effect of PX in MDA-MB-468 and MCF-7 cells, and that these effects were prevented by pre-incubating cells with 10^{-6} mol/L of MM (Table 1).

Signal transduction pathways and mechanism involved in the effect of paclitaxel on NIC-treated tumor cells

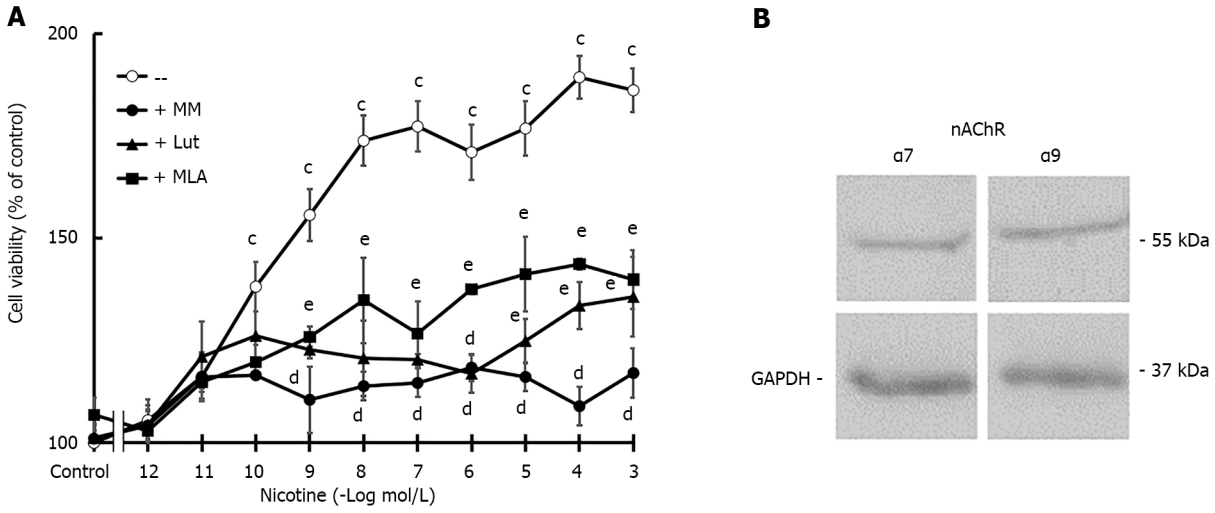
Previous reports have indicated that the activation of protein kinases is essential to modulate cell viability by triggering pro/anti-apoptotic gene transcription[47,48]. Additionally, the induction of the expression of these proteins could implicate activation of the NF-κB pathway. In the present study, we observed that the effect of PX on MDA-MB-231 cells is mediated, at least in part, by the Ras, p38MAPK (P < 0.01 *vs* PX) and NF-κB pathways (P < 0.05 *vs* PX) since the addition of their specific inhibitors FTS, SB or IMD respectively modified PX action (Figure 6A). On the other hand, the presence of NIC during PX treatment not only involved the previously mentioned molecules, but also the PKC (P < 0.05 *vs* PX), MEK (P < 0.01 *vs* PX) and ERK1/2 (P < 0.01 *vs* PX) pathways, as revealed by the action of Stau, PD and U126, respectively, which were added to the cultures (Figure 6B). We confirmed that the inhibitors alone did not modify cell viability (data not shown).

Apoptosis is a cell death mechanism that could improve antitumor actions of chemotherapy, and the activation of protein kinases is frequently associated with the activation of pro/anti-apoptotic signaling pathways. As expected, we found a reduction in cell viability due to the apoptosis induced by PX (PX: 16.7% ± 1.4%; control: 6.1% ± 0.7%, P < 0.001). The presence of NIC reduced the effect of PX. The percentage of apoptotic cells was 9.3 ± 0.6 (P < 0.05 *vs* control or P < 0.001 *vs* PX) (Figure 7). We also observed that the effect of NIC on PX-treated cells was totally reversed in the presence of nicotinic antagonists (P > 0.05 *vs* PX) (Figure 7).

Table 1 Effect of the combination of paclitaxel with nicotine on MDA-MB-468 and MCF-7 cell viability

Treatment	MDA-MB-468, cell viability (% of control)	MCF-7, cell viability (% of control)
PX	61.01 ± 3.79	65.36 ± 4.86
NIC	137.79 ± 3.69[c]	141.94 ± 4.07[c]
PX+NIC	79.15 ± 6.94[a]	117.99 ± 10.06[c]
PX+NIC+MM	62.37 ± 4.71	69.13 ± 7.22

[a]$P < 0.05$ *vs* control.
[c]$P < 0.001$ *vs* control.



DOI: 10.5306/wjco.v13.i6.505

Figure 2 MDA-MB-231 cell viability. A: Concentration-response curves of nicotine on cell viability in the absence or presence of nicotinic antagonists: mecamylamine [non-selective for nicotinic acetylcholine receptors (nAChRs)], methyllycaconitine (selective for α7 nAChRs), or luteolin (selective for α9 nAChRs) at a concentration of 10^{-6} mol/L. Values are the mean ± SD of five experiments performed in duplicate. [c]$P < 0.001$ *vs* control; [d]$P < 0.001$ *vs* nicotine; [e]$P < 0.001$ *vs* control or nicotine; B: Western blot assay to detect α7 and α9 nAChR expression. Molecular weights are indicated on the right. The expression of glyceraldehyde 3-phosphate dehydrogenase was used as the loading control. One representative experiment of three is shown. MM: Mecamylamine; MLA: Methyllycaconitine; Lut: Luteolin; nAChRs: Nicotinic acetylcholine receptors; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

An important aspect in cancer chemotherapy is the development of resistance. This phenomenon has been linked to several proteins, including ABCG2, which is a drug extrusion pump that decreases the effectiveness of antitumor drugs. Thus, we next analyzed ABCG2 expression by Western blot. As shown in Figure 8, MDA-MB-231 cells expressed this protein and the addition of PX caused a significant increase in its expression ($P < 0.05$). The presence of NIC during PX treatment potently increased ABCG2 levels in tumor cells ($P < 0.001$ *vs* PX). The pretreatment of cells with nicotinic antagonists reduced NIC action on ABCG2 expression ($P > 0.05$ *vs* PX+NIC) (Figure 8).

DISCUSSION

Our results reveal the mechanisms by which NIC decreases the cytotoxic effects of PX on human TN breast cancer MDA-MB-231 cells. Several authors have described the effect of nAChR activation on cell proliferation. Regarding the latter, NIC from tobacco smoke stimulates nAChRs expressed in the oral cavity[49], esophagus[50], stomach[51], intestine[52] and lungs[53-55]. NIC can also trigger malignant transformation in smoking patients and increases the risk of developing lung cancer[56,57]. Less is known about the effects of NIC on developing tumors or promoting malignant growth in other organs near the lung that also express nAChRs, such as is the case of the breast. Previous studies have shown that human breast tumors express the α7 and α9 nAChR subtypes[58,59]. In the present study, we confirmed that these receptors are functional in MDA-MB-231 tumor cells as treatment with NIC increased cell viability in a concentration-dependent manner. This effect was reversed by the pre-treatment of cells with selective nicotinic antagonists.

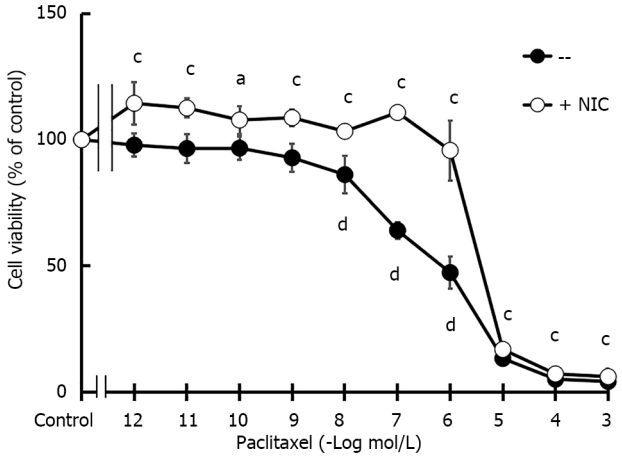


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Figure 3 MDA-MB-231 cell viability. Concentration-response curves of paclitaxel on cell viability in the absence or presence of nicotine (NIC) (10^{-10} mol/L). Values are the mean ± SD of six experiments performed in duplicate. $^{a}P < 0.05$; $^{c}P < 0.001$ *vs* Control; $^{d}P < 0.001$ *vs* +NIC. NIC: Nicotine.

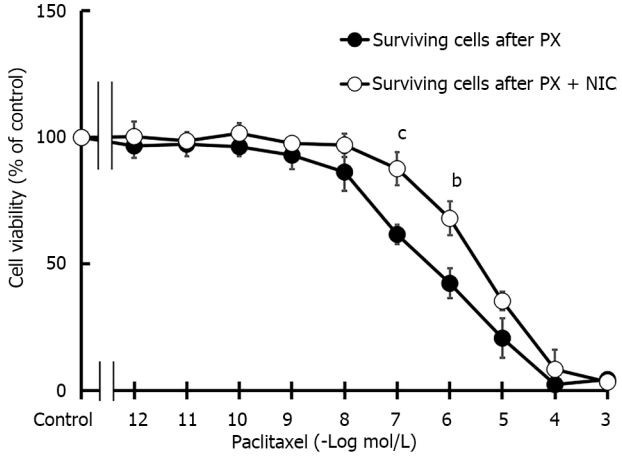

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Figure 4 Sensitivity of MDA-MB-231 cells to chemotherapy. Concentration-response curves of paclitaxel (PX) on the viability of surviving cells after three cycles of PX treatment (10^{-7} mol/L) in the absence or presence of nicotine (10^{-10} mol/L). Values are the mean ± SD of three experiments performed in duplicate. $^{b}P < 0.01$; $^{c}P < 0.001$ *vs* surviving cells after three cycles of PX treatment. PX: Paclitaxel; NIC: Nicotine.

Previous reports have indicated that the presence of NIC (due to smoking) reduces the effectiveness of chemotherapy in lung cancer patients[60]. Less evidence is available about the effect of NIC on breast cancer patients during chemotherapy administration. PX is a first-choice drug in breast cancer treatment due to its antimitotic ability and its effect on inhibiting different tumor progression pathways[61]. Our results confirm that PX acts on MDA-MB-231 tumor cells by reducing their viability in a dose-dependent manner. The presence of NIC at a concentration similar to that present in the blood of passive smokers[62,63] reduced the potency of PX by more than one order of magnitude. It is also important to highlight that the administration of NIC is effective in reducing the PX effect in other TN tumor cells such as MDA-MB-468, and in luminal A MCF-7 tumor cells. In a human gastric cancer model, Tu *et al*[64] observed a reduction in the effect of PX on cell viability due to NIC through the activation of α7 nAChRs. Similarly, here we demonstrated that both the α7 and α9 nAChR subtypes are involved in this effect, a fact also evident when PX chemotherapy was administered in cycles to TN breast tumor cells.

Several authors have demonstrated that chemotherapeutic drugs control tumor tissue growth by reducing cell viability[28,65,66] and activating distinct signaling transduction pathways that involve PKC[67], MEK[68], ERK1/2[69], Ras[70], p38MAPK[71] and NF-κB[72]. In particular, PX exerts its effects *via* the activation of different kinase signaling pathways depending on the cell type analyzed[73-75]. In our model, PX reduced cell viability through activation of the Ras, p38MAPK and NF-κB pathways. These results are in line with those obtained by Lu *et al*[76], who described that treatment with PX decreases ovarian carcinoma cell viability by activating the p38MAPK pathway, as well as with those of Okano and Rustgi[77], who observed that the treatment of human esophageal squamous cancer

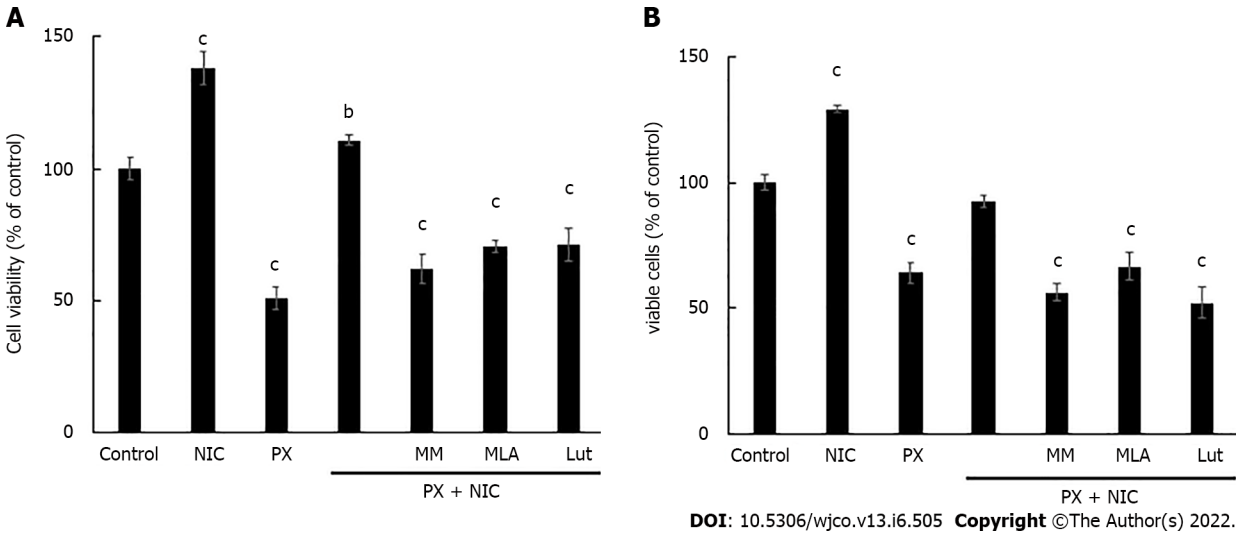

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Figure 5 Effect of nicotine on paclitaxel treatment. A: Viability determination of MDA-MB-231 cells treated with nicotine (10^{-10} mol/L) and paclitaxel (10^{-7} mol/L) alone or in combination, in the absence or presence of nicotinic antagonists: mecamylamine [non-selective for nicotinic acetylcholine receptors (nAChRs)], methyllycaconitine (selective for α7 nAChRs), or luteolin (selective for α9 nAChRs) at a concentration of 10^{-6} mol/L; B: Determination of percentage of living MDA-MB-231 cells treated with the same drug combinations as those shown in Figure 5A. Values are the mean ± SD of four experiments performed in duplicate. $^{b}P < 0.01$; $^{c}P < 0.001$ *vs* control, considered as 100%. MM: Mecamylamine; MLA: Methyllycaconitine; Lut: Luteolin; PX: Paclitaxel; NIC: Nicotine.

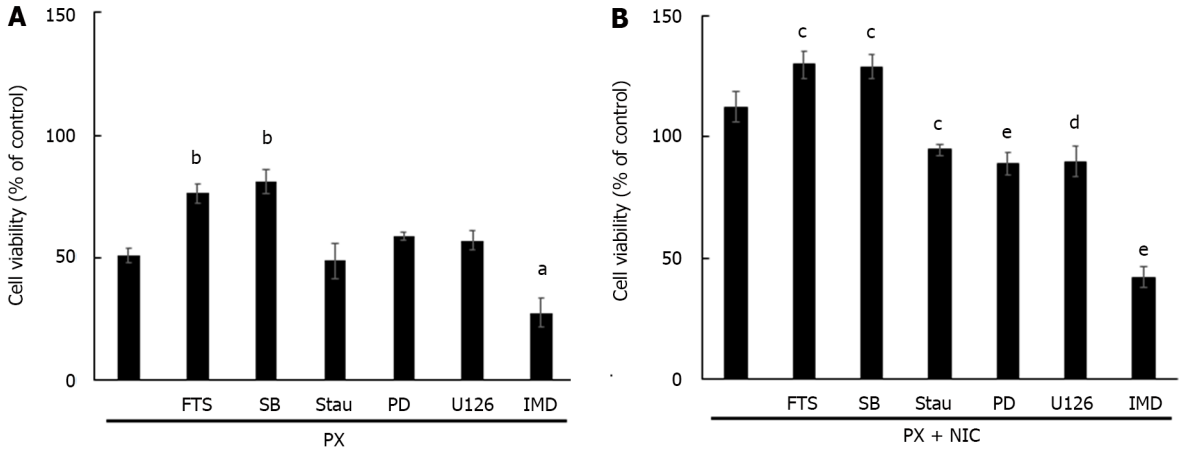

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Figure 6 Effect of paclitaxel and nicotine on MDA-MB-231 cell viability. A: Cells were treated with paclitaxel (PX) (10^{-7} mol/L) and the mediators were evaluated in the absence or presence of the kinase inhibitors for: PKC (staurosporine, 10^{-8} mol/L), MEK (PD098059 PD, 10^{-5} mol/L), Ras (S-trans, trans-farnesylthiosalicylic acid, 10^{-6} mol/L), ERK1/2 (U126, 10^{-5} mol/L), p38MAPK (SB203580, 10^{-5} mol/L) or IKKβ (IMD354, 5×10^{-8} mol/L); B: Cells were treated with the combination of PX and nicotine (NIC) (10^{-10} mol/L) as well as with the same inhibitors as those shown in Figure 6A. Values are the mean ± SD of four experiments performed in duplicate. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* PX. $^{c}P < 0.05$; $^{d}P < 0.01$; $^{e}P < 0.001$ *vs* PX+NIC. FTS: S-trans, trans-farnesylthiosalicylic acid; SB: SB203580; Stau: Staurosporine; PD: PD098059; PX: Paclitaxel; NIC: Nicotine.

cells with PX increases cell death through the activation of Ras.

In the present study, when we analyzed signaling pathways involved in PX treatment in the presence of NIC, we determined that PKC, MEK and ERK1/2 also participate in the reduction of breast tumor cell viability. These results are similar to those of Chernyavsky *et al*[78], who described that, in human corneal epithelial cells, the stimulation of α7 nAChRs can activate the PKC-MEK-ERK1/2 signaling pathway through an increase in intracellular calcium, which induces an up-regulation of E cadherin expression related with corneal re-epithelization. In the same line of evidence, Wang *et al*[79] described that the activation of α7 nAChRs can mediate the proliferation of hepatocellular carcinoma through a TRAF6/NF-κB-dependent mechanism similar to that observed in the present study. Our results and those of others thus indicate the presence of antagonistic effects between PX and NIC on cell viability.

PX reduces breast cell viability, at least in part, through the induction of apoptosis[71]. We confirmed that this effect also occurs in our model as an increase in MDA-MB-231 cell apoptosis was observed after 48 h of treatment. The effect of PX on cellular apoptosis was attenuated by the activation of α7 and α9 nAChRs with NIC.

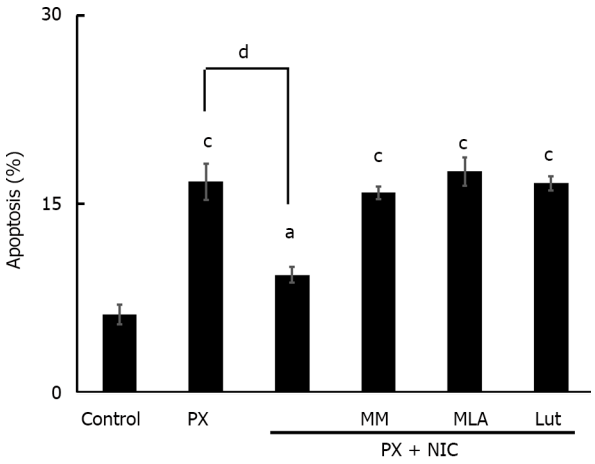

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Figure 7 Effect of nicotine on paclitaxel-induced apoptosis in MDA-MB-231 cells. Tumor cells were treated with paclitaxel (10^{-7} mol/L) in the absence or presence of the following nicotinic antagonists: mecamylamine [non-selective for nicotinic acetylcholine receptors (nAChRs)], methyllycaconitine (selective for α7 nAChRs), or luteolin (selective for α9 nAChRs) at a concentration of 10^{-6} mol/L. The percentage of apoptotic cells was determined by flow cytometry. Values are the mean ± SD of four experiments performed in duplicate. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ *vs* control; $^{d}P < 0.001$. MM: Mecamylamine; MLA: Methyllycaconitine; Lut: Luteolin; PX: Paclitaxel; NIC: Nicotine.

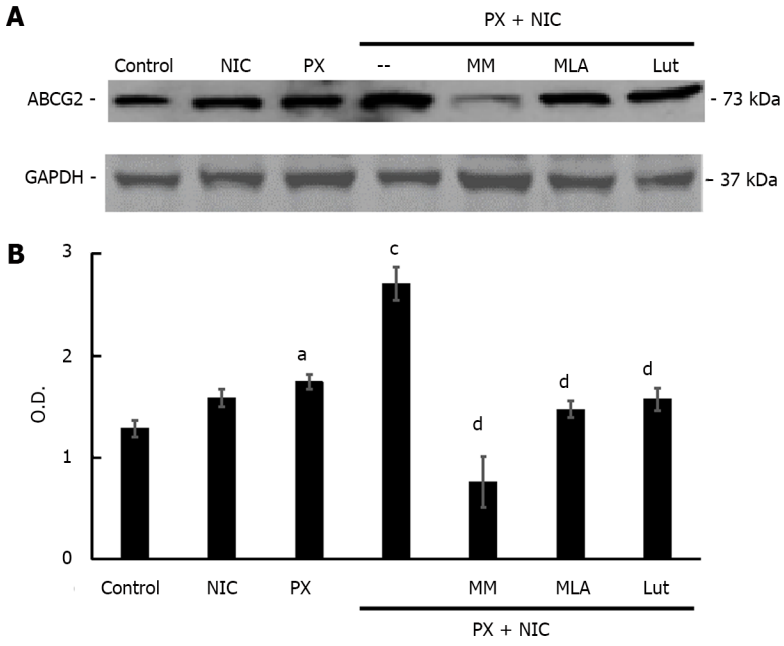

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Figure 8 Effect of nicotine on paclitaxel-induced expression of ATP binding cassette transporter G2 protein in MDA-MB-231 cells. A: ATP binding cassette transporter G2 expression was determined by Western blot assays in cells treated with paclitaxel (10^{-7} mol/L), nicotine (10^{-10} mol/L) or both, in the absence or presence of the nicotinic antagonists mecamylamine [non-selective for nicotinic acetylcholine receptors (nAChRs)], methyllycaconitine (selective for α7 nAChRs) or luteolin (selective for α9 nAChRs) at a concentration of 10^{-6} mol/L. Molecular weights are indicated on the right; B: The densitometric analysis of the bands is expressed as optical density units relative to the expression of glyceraldehyde 3-phosphate dehydrogenase protein used as the loading control. One representative experiment of three is shown. Values are the mean ± SD of three experiments. $^{a}P < 0.05$; $^{c}P < 0.001$ *vs* Control; $^{d}P < 0.001$ *vs* PX+NIC. ABCG2: ATP "binding cassette" G2 drug transporter; O.D.: Optical density; MM: Mecamylamine; MLA: Methyllycaconitine; Lut: Luteolin; nAChRs: Nicotinic acetylcholine receptors; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PX: Paclitaxel; NIC: Nicotine.

A frequent undesirable effect of antitumor therapy is innate or acquired resistance. In particular, PX can induce acquired resistance[80,81] by the transactivation of signaling pathways[82] or by triggering the overexpression of several proteins[83,84]. Considering the latter, the ABCG2 transporter plays an important role in the generation of resistance to PX treatment in breast adenocarcinomas[85]. In the present study, PX induced an increase in ABCG2 expression and this effect was potentiated by α7 and α 9 nAChR activation by NIC. The increase in ABCG2 expression should lead to a higher rate of PX extrusion, partly explaining the reduction in the cytostatic effect of PX in the presence of NIC, as well as

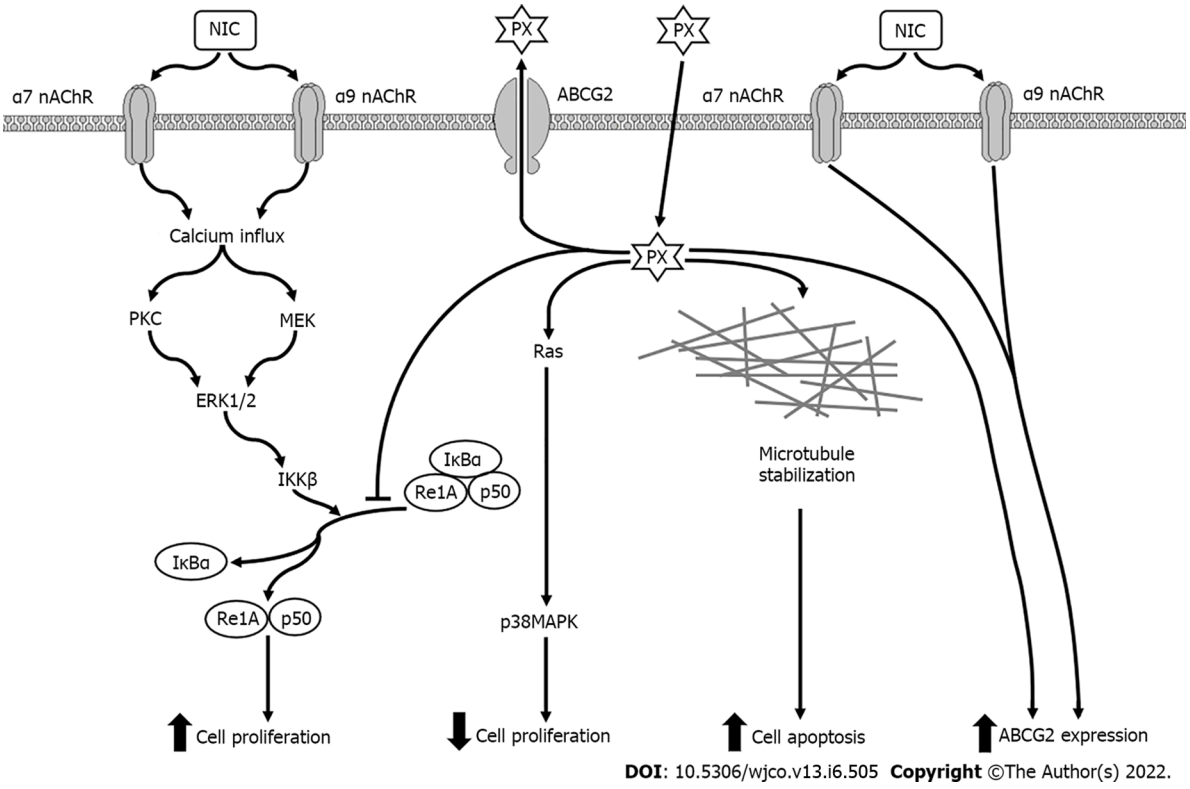

Figure 9 Possible signal transduction pathways in MDA-MB-231 cells activated by paclitaxel in the absence or presence of nicotine. NIC: Nicotine; PX: Paclitaxel; nAChR: Nicotinic acetylcholine receptors; ABCG2: ATP "binding cassette" G2 drug transporter; PKC: Protein kinase C; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated kinases; p38MAPK: p38 Mitogen-activated protein kinases; IKKβ: IκB kinase; IκBα: κB inhibitors.

the decrease in the sensitivity to PX when administered in cycles in the presence of NIC. Similar results have been obtained by Mukherjee *et al*[86] and Amawi *et al*[87], who described that PX treatment increases the activation and expression of ABCG2 in different breast cancer cell lines and that this factor could mediate the resistance to treatment[88]. Moreover, Nimmakayala *et al*[89] and An *et al*[90] observed that the exposure of pancreatic and lung tumor cells to cigarette smoke caused an increase in the expression of this pump.

A possible mechanism of action of PX treatment in the presence of NIC in a TN tumor-bearing smoking patient is proposed in Figure 9.

CONCLUSION

Our results demonstrate that NIC at a concentration similar to that present in passive smokers' plasma can negatively modulate the cytotoxic/apoptotic effect of PX in TN breast tumors. The reduction in the sensitivity to PX could be due to an increase in the expression of the ABCG2 transporter in malignant cells. Additionally, our findings demonstrated the participation of different kinases and the NF-κB pathway, which would modulate cell viability in this effect. This information could allow the development of better strategies to improve TN breast cancer therapy, such as blocking nAChRs together with PX during chemotherapy administration to passive smoking patients.

ARTICLE HIGHLIGHTS

Research background

Triple negative is the subtype of breast cancer with the worst prognosis, showing an increase in resistance to chemotherapy in smoking patients, who have high levels of nicotine in their blood. In lung cancer, it has been proposed that the activation of nicotinic acetylcholine receptors could be responsible for the modulation of several parameters of tumor biology and the loss of effectiveness of chemotherapeutic treatment, but it is not known what occurs in a nearby organ such as the breast.

Research motivation

Given that breast tumor-bearing patients have a low efficiency to antitumor therapy, knowledge of the signaling pathways involved in this phenomenon is important to generate new therapeutic targets that improve sensitivity to treatment.

Research objectives

This research aimed to determine the signaling pathways involved in the nicotinic modulation of the cytostatic effect of paclitaxel in human triple negative breast cancer cells.

Research methods

The modulatory effect of nicotine on paclitaxel treatment was assessed by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The trypan blue exclusion assay was used to evaluate viable cells in response to different treatments. Protein expression levels were evaluated by Western blot assays and apoptosis was determined using immunofluorescence assays with annexin V and 7AAD.

Research results

Nicotine decreased paclitaxel's inhibition of viability and apoptosis in MDA-MB-231 breast cancer cells. This modulation of viability is mediated by the activation of α7 and α9 nicotinic acetylcholine receptors and protein kinases PKC, Ras, MEK, ERK, p38MAPK and the NF-κB pathway. Cells surviving paclitaxel treatment in the presence of nicotine are less sensitive to another cycle with the chemotherapeutic agent probably due an increase in the protein expression of ATP binding cassette transporter G2.

Research conclusions

Nicotine modulates the cytotoxic/apoptotic effects of paclitaxel and knowledge of its signaling pathway mediators could allow the development of better strategies to improve triple negative breast cancer therapy, such as nicotinic acetylcholine receptors blockage together with paclitaxel during chemotherapy administration to smoking patients.

Research perspectives

Knowledge of the mediators that participate in the nicotinic modulation of paclitaxel's effect will allow the development of new antitumor strategies that could be applied not only to other subtypes of mammary tumors, but also to tumors in other organs of smoking patients.

FOOTNOTES

Author contributions: Español A performed cell assays, supervised the work, analyzed and interpreted the data, and wrote the manuscript; Sanchez Y and Salem A performed cell assays and contributed to the writing of the manuscript; Obregon J carried out lab work as part of her grade thesis, and helped to analyze and interpret the data; Sales ME supervised the work and edited the manuscript draft; all authors read and approved the final manuscript.

Supported by University of Buenos Aires (UBA) UBACYT 2018-2022, No. 20020170100227; National Research Council (CONICET) PIP 2015-2017, No. 2015-0239; and National Agency for Scientific and Technological Promotion (ANPCyT) PICT 2015-2017, No. 2015-2396.

Institutional review board statement: In the realization of the manuscript, no human samples or patients were used, so the authorization of the Institutional Review Board was not required.

Institutional animal care and use committee statement: In the realization of the manuscript, no animals were used, so the authorization of the Institutional Animal Care and Use Committee was not required.

Conflict-of-interest statement: The authors certify that they have no conflicts of interest (including but not limited to commercial, personal, political, intellectual or religious interests) for this article.

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at aespanol@fmed.uba.ar. Participants gave informed consent for data sharing.

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S-Editor: Gao CC
L-Editor: Webster JR
P-Editor: Cai YX

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 June 24; 13(6): 520-528

DOI: 10.5306/wjco.v13.i6.520

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Retrospective Study

Risk factors for lymph node metastasis in patients with pancreatic neuroendocrine neoplasms

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Sun Y, China

Received: April 10, 2021
Peer-review started: April 10, 2021
First decision: July 6, 2021
Revised: October 24, 2021
Accepted: May 21, 2022
Article in press: May 21, 2022
Published online: June 24, 2022

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Abstract

BACKGROUND

Although PNENs generally have a better prognosis than pancreatic cancers, some PNENs display malignant behavior including lymph node (LN) metastasis. Complete tumor resection can be the only potentially curative treatment for patients with resectable PNENs. However, the indications for LN dissection are still controversial. Over the last decade, minimally invasive surgery such as laparoscopic pancreatic surgery (LPS) has been increasingly performed for pancreatic tumors including PNENs.

AIM

To investigate the risk factors for LN metastasis in PNENs and to select appropriate patients for limited surgery by LPS.

METHODS

From April 2001 to December 2019, 92 patients underwent pancreatic resection for PNENs at Kumamoto University Hospital. Finally, 82 patients were enrolled in this study. Using perioperative factors, we examined the predictive factors for LN metastasis in PNENs.

RESULTS

Among the 82 patients, the percentage of LN metastasis according to the pathological findings was 12% (10/82 cases). The median tumor size was 12 mm (range: 5-90 mm). The median tumor size in the LN-positive group (37 mm) was significantly larger than that in the LN-negative group (12 mm) (P = 0.0001). Multivariate analyses revealed that larger tumor size (≥ 20 mm) was an inde-

pendent risk factor for LN metastasis (odds ratio 16.8, $P = 0.0062$). In patients with small tumors (≤ 10 mm), LN metastasis was not found.

CONCLUSION
Larger tumor size (≥ 20 mm) is an independent risk factor for LN metastasis in PNENs. In smaller PNENs (≤ 10 mm), we may be able to choose limited surgery without LN dissection.

Key Words: Lymph node metastasis; Pancreatic neuroendocrine neoplasms; Risk factor; Tumor size

Core Tip: Among the 82 patients, the percentage with lymph node (LN) metastasis according to the pathological findings was 12% (10/82 cases). The median tumor size was 12 mm (range: 5-90 mm). The median tumor size in the LN-positive group (37 mm) was significantly larger than that in the LN-negative group (12 mm) ($P = 0.0001$). Multivariate analyses revealed that large tumor size (≥ 20 mm) was an independent risk factor for LN metastasis. In patients with small tumors (≤ 10 mm), LN metastasis was not found. In conclusion, large tumor size (≥ 20 mm) is an independent risk factor for LN metastasis in PNENs. In smaller PNENs (≤ 10 mm), we may be able to choose limited surgery without LN dissection.

Citation: Nakao Y, Hayashi H, Yamashita YI, Takashi O, Matsumura K, Uemura N, Kitamura F, Itoyama R, Yusa T, Taki K, Miyata T, Higashi T, Nakagawa S, Okabe H, Imai K, Baba H. Risk factors for lymph node metastasis in patients with pancreatic neuroendocrine neoplasms. *World J Clin Oncol* 2022; 13(6): 520-528
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/520.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.520

INTRODUCTION

Pancreatic neuroendocrine neoplasms (PNENs) are relatively rare and represent 1%-2% of all pancreatic neoplasms[1]. Although patients with PNENs generally have better prognosis than those with pancreatic cancers, some PNENs display malignant behavior including local invasion, lymph node (LN) metastasis, and distant metastasis[2]. The natural history of PNENs is not fully understood because of their relative rarity, and therefore, it is difficult to predict the malignant potential of PNENs precisely.

Complete tumor resection can be the only potentially curative treatment for patients with resectable PNENs. However, optimal surgical management procedures have not yet been established[3,4]. Especially, the indications for LN dissection are still controversial, especially in early PNENs. This is partly caused by the difficulty of predicting LN metastasis. Therefore, it is important to establish appropriate indications for LN dissection to treat PNENs.

Over the last decade, minimally invasive surgery such as laparoscopic pancreatic surgery (LPS) has been increasingly performed for pancreatic tumors including PNENs[5-7]. Non-comparative studies have shown that LPS for pancreatic tumors is safe and equivalently effective to open pancreatic surgery (OPS)[8-10]. In well-selected groups of patients with pancreatic lesions, LPS provides good peri and post operative outcomes, such as reduced intraoperative blood loss, and postoperative pain and length of postoperative day[8,10-13]. As a limited type of LPS, laparoscopic spleen-preserving distal pancreatectomy and excisional resection for PNENs has also been performed in selected cases[7,14,15]. However, the indications for limited surgery by LPS for patients with PNENs remain unclear.

The aims of this study are to investigate the risk factors for LN metastasis in PNENs and to select appropriate patients for limited LPS.

MATERIALS AND METHODS

Study cohort

From April 2001 to December 2019, 92 patients underwent pancreatic resection for PNENs at Kumamoto University Hospital. Of them, 10 patients (11%) were excluded from this analysis because of distant metastases and coexisting tumors other than PNENs. Finally, 82 patients were enrolled in this study. The patients were identified retrospectively from a prospectively maintained database, and additional data were obtained by reviewing each patient's medical records. Written informed consent was obtained from all patients before treatment, and this study was approved by the Institutional Review Board of Kumamoto University (number 1291).

Treatment strategy

Before treatment, all patients underwent routine diagnostic laboratory tests and imaging modalities including enhanced computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS). The final diagnoses were confirmed pathologically using resected specimens. Tumors were classified as functional PNENs according to the clinical signs and symptoms of hormonal excess and increased levels of corresponding serum peptides and hormones. Tumors were classified as non-functional if they were not associated with distinct clinical manifestations or hormonal alterations[16]. Surgical procedures were selected based on each tumor's location and extent and the patient's general condition. Pancreatic resection was considered the first-choice treatment for patients with PNENs.

Postoperative workup

After treatment, all of the patients underwent regular follow-up examinations including routine laboratory tests and imaging studies including EUS, CT, or MRI to detect any pancreatic recurrence or distant metastasis, as described previously[17]. When tumor recurrence was confirmed, various treatment modalities were selected, including repeat surgery, chemotherapy, or a combination of these methods, according to tumor location and patient condition.

Statistical analysis

Continuous variables were expressed as median (range). Continuous and categorical variables were compared using Mann-Whitney U and χ^2 tests, respectively. Survival analyses were performed using the Kaplan-Meier method, with comparisons using the log rank test. Overall survival (OS) was calculated from the date of surgery until death or last follow-up. Variables in which the *P* value for LN metastasis was < 0.05 in univariate analysis were subjected to subsequent multivariate analysis by stepwise backward elimination procedures. All statistical analyses were performed using JMP® version 13.1 (SAS institute, Cary, NC, United States). All *P* values were two-sided, and $P < 0.05$ was considered as statistically significant.

RESULTS

The 82 patients' demographic and clinical characteristics are summarized in Table 1. There were 41 male and 41 female patients, with a median age of 59 years (range, 18-81 years). Thirty five patients (43%) had symptoms at the first consultation. Preoperative contrast-enhanced CT showed that the majority of patients had tumors with hyper enhanced pattern (72 patients, 88%). Of the 31 patients (38%) who had functional PNENs, the most frequent type of functional PNEN was insulinoma (26 patients, 32%), followed by glucagonoma (2, 2.5%), gastrinoma (2, 2.5%), and VIPoma (1, 1%). There were 51 patients (62%) who had non-functional PNENs. Their 2017 WHO classifications were: G1, 70 (85%); G2, 9 (11%); and G3 or NEC, 3 (4%). Fourteen patients (17%) had multiple tumors, and the median tumor size was 12 mm (range, 5-90 mm). Among the 82 patients, 23 (28%) received pancreatoduodenectomy (PD), 38 (46%) received distal pancreatectomy, 2 (2.5%) received PD + DP, and 19 (23%) received enucleation or partial pancreatectomy.

Among the 82 patients, 10 (12%) were identified as having LN metastasis. The demographic and clinical characteristics of the 10 patients with LN metastasis were compared with those of the 72 patients without LN metastasis (Table 1). LN metastases of PNENs were positively associated with pathological grade: 6%, 44%, and 67% of cases with LN metastases were classified as G1, G2, and G3/NEC, respectively ($P = 0.0009$). In the LN metastasis-positive group, tumor size was significantly larger than that in the negative group (median, 12 *vs* 37, $P = 0.0001$). Univariate analysis showed that the following three factors were related to LN metastasis: tumor size ≥ 20 mm [Odds Ratio (OR) 31.5, $P < 0.0001$], WHO 2017 classification ≥ G2 (OR 20.1, $P = 0.0001$), and non-functional type of PNEN (OR 6.43, $P = 0.035$). Multivariate logistic regression analyses revealed that tumor size ≥ 20 mm was an independent risk factor for LN metastasis (OR 16.8, $P = 0.0062$) (Table 2).

Figure 1 shows the rate of LN metastasis according to tumor size. The rates of LN metastasis according to tumor size were as follows: 0% (0/29 cases, ≤ 10 mm group), 3% (1/31 cases, 11 mm-20 mm group), 25% (2/8 cases, 21-30 mm group), 50% (3/6 cases, 31-40 mm group), and 50% (4/8 cases, > 40 mm group) (Figure 1). The median length of follow-up after surgery was 51.8 months (range, 0.4-224.2). The cumulative OS rate after surgery for patients with no LN metastasis was significantly higher than that for those with LN metastasis ($P = 0.009$) (Figure 2).

DISCUSSION

PNENs are rare tumors[1]. The oncological history is not yet fully understood due to their often-lazy course, because it is not easy to find correct diagnosis and treatment. Furthermore, PNENs have wide variety biological behaviors, such as benign tumors and malignant status[18]. Because of the hetero-

Table 1 Comparisons of patients' characteristics according to the presence of lymph node metastasis of pancreatic neuroendocrine neoplasm

Variables	Total (*n* = 82)	N^- (*n* = 72)	N^+ (*n* = 10)	*P* value
Age, median (range)	59 (18-81)	58 (18-80)	63 (18-81)	0.65
Gender (male/female)	41/41	35/37	6/4	0.50
Tumor size, median, mm (range)	12 (5-90)	12 (5-90)	37 (12-75)	0.0001
Tumor number (single/multiple)	68/14	59/13	9/1	0.50
Tumor location (Ph/Pb/Pt/Ph and Pt)	32/23/25/2	23/22/25/2	9/1/0/0	0.15
Symptoms (yes/no)	35/47	31/41	4/6	0.85
CT Enhancement (hyper/hypo)	72/10	64/8	8/2	0.17
Type of PNEN, *n* (%)				NS
Insulinoma	26 (32)	26	0	
Gastrinoma	2 (2.5)	1	1	
Glucagonoma	2 (2.5)	2	0	
VIPoma	1 (1)	1	0	
Non functional	51 (62)	42	9	
WHO classification 2017, *n* (%)				0.0009
NET G1	70 (85)	66	4 (6%)	
NET G2	9 (11)	5	4 (44%)	
NET G3/NEC	3 (4)	1	2 (67%)	
Surgical procedure, *n* (%)				NS
Pancreatoduodenectomy (PD)	23 (28)	15	8	
Distal pancreatectomy (DP)	38 (46)	37	1	
PD + DP	2 (2.5)	2	0	
Enucleation/partial pancreatectomy	19 (23)	18	1	

N^-: Negative for lymph node metastasis; N^+: Positive for lymph node metastasis; NEN: Neuroendocrine neoplasms; WHO: World Health Organization; NET: Neuroendocrine tumor; PD: Pancreatoduodenectomy; DP: Distal pancreatectomy; CT: Computed tomography; PNEN: Pancreatic neuroendocrine neoplasm. NS: Not significant.

geneity of PNENs, it is very difficult both to construct the effective clinical treatment policy systems and to confirm the surgical method for cure.

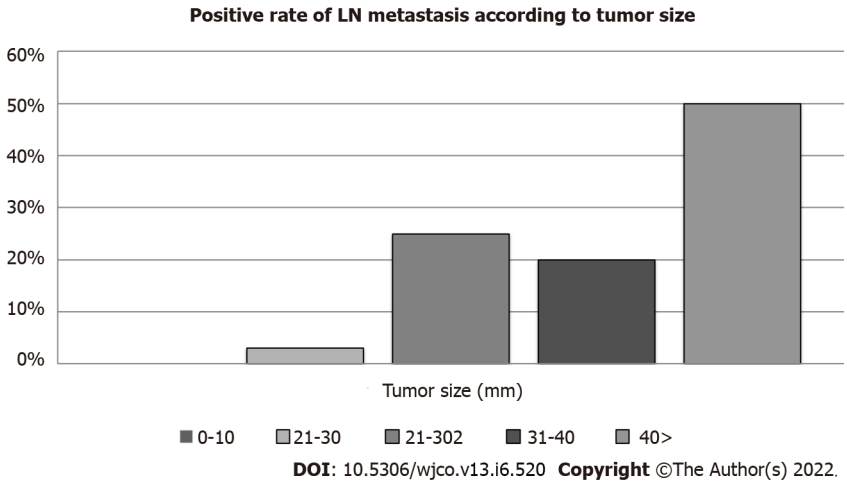
Some reports have associated LN metastasis with shorter OS[3,19-25], while others have found that LN status did not affect survival[26-29]. LN metastasis is positively correlated with pathological grade, with 15%-20%, 30%-40%, and > 50% of patients with LN metastasis classified as G1, G2, and G3, respectively[30]. In our study, we also reported that the LN metastasis-positive group of PNENs had poor OS after surgery. Further, we reported that LN metastases of PNENs are positively associated with pathological grade, with 6%, 44%, and 67% of patients with LN metastases classified as G1, G2, and G3/NEC, respectively (P = 0.0009; Table 1). Therefore, patients with PNENs and LN metastasis have poor prognosis and high malignant potential. However, previous reports have not clearly shown that to omit LN dissection may increase the possibility of recurrence. Some previous studies shows that local LN metastases of PNENs have oncologic effects[30,31]. A past study related with non-functional G1 PNENs who underwent surgery of pancreas reported that LN metastases of PNENs do not adversely affect oncological outcomes and do not require routine local lymphadenectomy[32]. Partelli *et al*[33] reported that a lot of insulinomas (well-differentiated) and non-functional PNENs located in the distal pancreas are very small, rarely associated with LN metastases, and there is no radiographic evidence of positive of LN metastases. Thus, the significance of LN metastasis in patients with PNENs is very complicated, and the indications for regional LN dissection are still controversial.

Previous studies have focused on the associations of LN metastasis or and/or prognosis with tumor size[17,21,34-39]. Although LN metastasis has been seen even in patients with tumors < 10 mm, LN metastasis occurs more often in patients with large tumors than in those with smaller ones. In our study, there were no cases of LN metastasis in patients with tumors ≤ 10 mm. LN metastases of PNENs were

Table 2 Factors related to lymph node metastasis of pancreatic neuroendocrine neoplasm

Factors	Univariate analysis		Multivariate analysis		
	Odds ratio	*P* value	Odds ratio	95%CI	*P* value
Age ≥ 60	0.54	0.40			
Gender (male)	0.63	0.50			
Symptoms (yes)	1.13	0.85			
CT Enhancement (hyper)	4	0.17			
Tumor number (multiple)	1.98	0.50			
Tumor size (≥ 20 mm)	31.5	< 0.0001	16.8	2.15-35.4	0.0062
WHO classification 2017 (≥ G2)	20.1	0.0001		NS	
Type of PNEN (non functional)	6.43	0.035		NS	

NEN: Neuroendocrine neoplasms; WHO: World Health Organization; CT: Computed tomography; PNEN: Pancreatic neuroendocrine neoplasm; NS: Not significant.

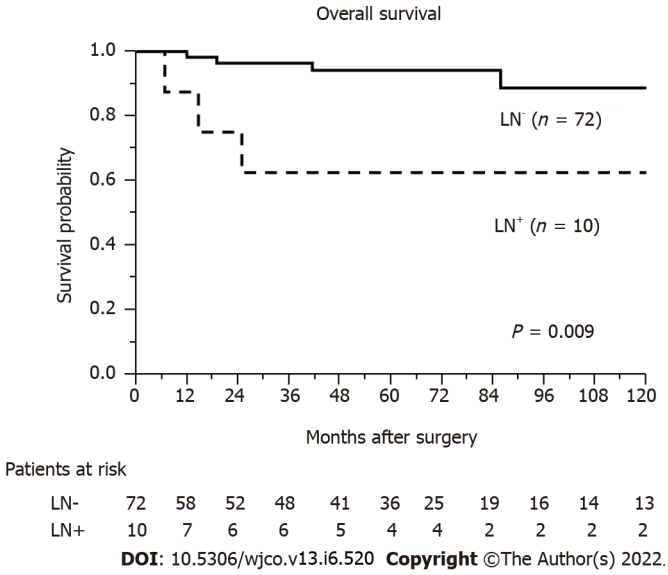


DOI: 10.5306/wjco.v13.i6.520

Figure 1 Rate of lymph node metastasis according to tumor size. The rates of lymph node metastasis according to tumor size were as follows: 0% (0/29 cases, ≤ 10 mm group), 3% (1/31 cases, 11 mm-20 mm group), 25% (2/8 cases, 21-30 mm group), 50% (3/6 cases, 31-40 mm group), and 50% (4/8 cases, > 40 mm group). LN: Lymph node.

positively associated with tumor size, being present in 0%, 3%, 25%, and 50% of patients with primary tumors ≤ 10 mm, 11-20 mm, 21-30 mm, and > 30 mm, respectively. If we can predict the presence of LN metastasis according to tumor size, we can select appropriate patients for limited LPS.

Over the last decade, the use of laparoscopy in pancreatic surgery has increased significantly, and previously almost all open surgery can now be performed in a minimally invasive method. In general, these minimally invasive surgery should be limited to high-volume centers with extensive experience in pancreatic surgery with open surgery. Patients with small-sized PNENs in the body and tail of the pancreas are particularly well suited for minimally invasive surgery, and the laparoscopic procedures gives better result than open surgical method[40,41]. Laparoscopic distal pancreatectomy have the potential to be superior to the open surgical method in patients with benign tumors, resulting in less operative bleeding, shorter postoperative days, and equivalent rates of complications[13]. According to the review by 11 studies, which involve 906 PNENs patients, of whom 22% and 78% underwent LPS and OPS, respectively, it reported that overall complication rate of laparoscopic method was significantly lower (38% *vs* 46%, $P < 0.001$) and the postoperative days in hospital is shorter ($P < 0.001$) [40]. LPS is now considered to be a safe approach for PNENs and should be included in the patient's surgical equipment. Many surgeons have reported that the rates of overall complication in small or benign tumors were lower with LPS than OPS. Although in the cases of patients with malignant PNENs, we need advanced surgical skills, LPS was not associated with compromised oncologic resection and provided benefits including reduced postoperative pain, shorter hospital stay, and shorter postoperative recovery period. Thus, it is important to investigate the risk factors of LN metastases in PNENs and to select appropriate patients for limited LPS. Our results offer certain recommendations in this regard.



DOI: 10.5306/wjco.v13.i6.520

Figure 2 Overall survival after surgery of 82 patients according to the presence of lymph node metastasis. The cumulative overall survival rate after surgery among patients who had no lymph node (LN) metastasis was significantly higher than that for those who had LN metastasis. LN: Lymph node.

However, this study had several limitations, including its retrospective design, the small number of subjects, and the lack of data on certain pathologic variables (especially the Ki-67 indices and mitotic rates) for all patients. The number of examined LNs was not sufficient, and data on the number of positive LNs were not available for all PNENs. Therefore, further research regarding advanced PNENs is required.

CONCLUSION

In conclusion, larger tumor size (≥ 20 mm) is an independent risk factor for LN metastasis in PNENs. In smaller PNENs (≤ 10 mm), we may be able to choose limited surgery without LN dissection.

ARTICLE HIGHLIGHTS

Research background

The indications for lymph node (LN) dissection are still controversial.

Research motivation

Over the last decade, minimally invasive surgery such as laparoscopic pancreatic surgery (LPS) has been increasingly performed for pancreatic tumors including pancreatic neuroendocrine neoplasms (PNENs).

Research objectives

The aim of this study was to investigate the risk factors for LN metastasis in PNENs and to select appropriate patients for limited surgery by LPS.

Research methods

From April 2001 to December 2019, 92 patients underwent pancreatic resection for PNENs at Kumamoto University Hospital. Finally, 82 patients were enrolled in this study. Using perioperative factors, we examined the predictive factors for LN metastasis in PNENs.

Research results

Among the 82 patients, the percentage of LN metastasis according to the pathological findings was 12% (10/82 cases). The median tumor size was 12 mm (range: 5-90 mm). The median tumor size in the LN-positive group (37 mm) was significantly larger than that in the LN-negative group (12 mm) (P = 0.0001). Multivariate analyses revealed that large tumor size (≥ 20 mm) was an independent risk factor for LN metastasis (odds ratio 16.8, P = 0.0062). In patients with small tumors (≤ 10 mm), LN metastasis

was not found.

Research conclusions

Large tumor size (≥ 20 mm) is an independent risk factor for LN metastasis in PNENs. In smaller PNENs (≤ 10 mm), we may be able to choose limited surgery without LN dissection.

Research perspectives

In smaller PNENs (≤ 10 mm), we may be able to choose limited surgery without LN dissection.

FOOTNOTES

Author contributions: Nakao Y, Hayashi H and Yamashita Y designed the research study; Nakao Y, Takashi O, Matsumura K, Uemura N, Kitamura F, Itoyama R, Yusa T, Taki K, Miyata T, Higashi T, Nakagawa S, Okabe H and Imai K performed the research; Yamashita Y and Baba H contributed new reagents and analytic tools; Nakao Y, Hayashi H and Takashi O analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

Institutional review board statement: Institutional Review Board of Kumamoto University (number 1291).

Informed consent statement: Consent was obtained from the patient and family according to Institutional Review Board protocols.

Conflict-of-interest statement: All the authors have no conflicts of interest in association with this study. No financial support was received for the work described in this manuscript.

Data sharing statement: No additional data are available.

Country/Territory of origin: Japan

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S-Editor: Liu JH
L-Editor: Kerr C
P-Editor: Liu JH

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 June 24; 13(6): 529-539

DOI: 10.5306/wjco.v13.i6.529

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Prospective Study

Significance of serum carcinoembryonic antigen in metastatic breast cancer patients: A prospective study

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Liang Z, China; Wang J, China
A-Editor: Liu X, China

Received: January 7, 2022
Peer-review started: January 7, 2022
First decision: April 13, 2022
Revised: April 16, 2022
Accepted: May 28, 2022
Article in press: May 28, 2022
Published online: June 24, 2022

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Abstract

BACKGROUND

Carcinoembryonic antigen (CEA) is an important serum tumour marker with a substantial role in diagnosis and monitoring of various solid tumours. About 36%-70% of breast cancers have elevated serum CEA. And the available studies show discrepancy in addressing the prognostic significance of CEA in advanced breast cancer.

AIM

To estimate the serum CEA level in our metastatic breast cancer patients and correlate it with response to treatment and clinical outcome.

METHODS

This was a prospective clinical study conducted on 50 metastatic breast cancer patients treated at breast clinic, with newly diagnosed metastatic breast cancer planned for palliative chemotherapy, targeted therapy, and hormonal treatment. We estimated the proportion of patients with elevated serum CEA level at baseline and after palliative treatment and also studied the association of serum CEA levels with known prognostic factors. The response to treatment was correlated with the serum CEA levels in the context of responders and non-responders.

RESULTS

The median pre-treatment and post-treatment CEA levels were 7.9 (1.8-40.7) ng/mL and 4.39 (1.4-12.15) ng/mL, respectively, in the whole study population (P = 0.032). No statistically significant difference was seen in baseline serum CEA between responders and non-responders. Even in the luminal group, pre-treatment serum CEA was not a predictor of response, but post-treatment CEA was a significant predictor of tumour progression. In patients with liver and lung metastases, post-treatment CEA level difference was not statistically significant in both responders and non-responders though the values were higher in non-

responders. Among those with bone metastases, 69.5% had elevated post-treatment serum CEA, and only 37.5% had elevated serum CEA in those with no bone metastases.

CONCLUSION
Elevated post-treatment serum CEA levels are associated with disease progression and poor response to therapy. Persistently elevated post-treatment serum CEA levels are significantly associated with bone metastases. Elevated serum CEA and hormonal status are significant predictors of treatment response.

Key Words: Carcinoembryonic antigen; Metastatic breast cancer; Serum tumour marker; Luminal and non-luminal metastatic breast cancer; Palliative chemotherapy

Core Tip: In breast cancer patients, elevated serum carcinoembryonic antigen (CEA) levels are particularly noted in advanced disease. Our study suggested that serum CEA has potential clinical value in monitoring the treatment response of metastatic breast cancer patients, especially in those with bone metastasis.

Citation: Anoop TM, Joseph P R, Soman S, Chacko S, Mathew M. Significance of serum carcinoembryonic antigen in metastatic breast cancer patients: A prospective study. *World J Clin Oncol* 2022; 13(6): 529-539
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/529.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.529

INTRODUCTION

Breast cancer, one of the leading causes of malignancy related morbidity and mortality among women, comprises of a spectrum of clinically and histologically heterogeneous group of diseases with distinct molecular portraits[1]. In spite of increasing awareness, advanced screening, and diagnostic methodologies, we still witness a significant proportion of patients who present with advanced stage disease. Deciding optimal treatment and monitoring strategies for patients with metastatic and recurrent disease remains a diagnostic challenge for physicians.

Carcinoembryonic antigen (CEA) is an important serum tumour marker with a substantial role in diagnosis and monitoring of colorectal cancer. Globally, cancer antigen 15-3 (CA15-3) and CEA are used serum tumor markers in breast cancer[2-4]. In breast cancer patients, elevated serum CEA levels are particularly noted in metastatic and recurrent disease. Studies have reported a varying incidence of serum CEA positivity ranging from 36%-70%[5]. Elevated levels are known to positively correlate with tumour burden, grade of tumour, and site of metastasis, and they also translate into poor overall survival (OS) and progression-free survival[6]. The clinical utility of serial tumour marker measurements is not indicated in asymptomatic women for surveillance after treatment of breast cancer [7-9]. The main applications are used in metastatic disease monitoring during treatment, especially CA15-3. Among serum tumour markers in breast cancer, CA15–3 and CEA have been the commonly used ones[10-13]. Hence, serum CEA estimation can be proposed as an auxiliary tool for response assessment, monitoring, and gaining prognostic information. In spite of these, due to discordant results, their clinical utility remains unclear[14-16]. There are very few studies addressing the prognostic significance of CEA and the available studies show discrepancy. Hence, we conducted this study to estimate the serum CEA level in our metastatic breast cancer (MBC) patients and correlate it with response to treatment and clinical outcome.

MATERIALS AND METHODS

This was a prospective experimental study conducted on 50 MBC patients treated at Breast Clinic, Department of Medical Oncology during the period December 2019 to November 2020. Patients with newly diagnosed MBC planned for palliative chemotherapy, targeted therapy, and hormonal treatment were included. Routine protocol for MBC work-up included biopsy from breast lump or metastatic lesion, histopathology and immunohistochemistry for oestrogen, progesterone, and Her2 receptors, computed tomography of the chest, abdomen, and pelvis, bone scan, and serum biochemistry. Patients with inflammatory breast cancer and active inflammatory conditions were excluded in this study due to the fact that they could cause elevation of serum CEA levels. Five milliliters of venous blood was drawn

from MBC patients who consented for study participation and serum was isolated after centrifugation at 3000 rpm for 10 min, transported into new disposable tubes, and stored at -20 ℃. In patients with hormone positive MBC with visceral crisis and triple negative breast cancer (TNBC) patients, sample for serum CEA levels was collected before initiation of first cycle of palliative chemotherapy and after completion of six cycles of chemotherapy. In patients with hormone positive MBC without visceral crisis, serum CEA sample was collected before initiation of endocrine agents and at 6 mo after initiation. In patients with HER2 positive MBC, blood sample was collected before initiation of first cycle of palliative chemotherapy plus trastuzumab and after completion of six cycles of chemotherapy plus trastuzumab.

Concentrations of the serum tumour marker CEA were measured with an automated sandwich ELISA test system using the manufacturer's recommended kits (ELISA 2010, Roche Company). CEA concentrations were recorded in nanogram *per* millilitre. CEA value more than 3.8 ng/mL was considered positive. Patient treatment and response evaluation were as *per* the institutional protocol. Treatment and follow-up details of the patients were noted from the medical case records. We estimated the proportion of patients with elevated serum CEA level in MBC and also studied the association of serum CEA levels with known prognostic factors. The radiological response was assessed using Response Evaluation Criteria in Solid tumours (RECIST1.1). The response to treatment were correlated with the serum CEA levels in the context of responders and non-responders.

Statistical analysis

Statistical calculations were performed using the SPSS for Windows, version 15.0 (SPSS, Inc., Chicago, United States). Categorical variables are expressed using frequencies and percentages. Continuous variables are presented in terms of the mean and standard deviation. Association between two categorical variables was analyzed using Chi square or fisher's exact test. Non-parametric tests were used for finding the statistical significance. Wilcoxon signed rank test was used for comparing pre- and post-treatment serum CEA in different categories. Comparison of serum CEA in different clinical categories was carried out using Mann-Whitney test and Kruskal-Wallis test.

The optimal cut-off values of the CEA were determined using receiver operator characteristic (ROC) curve. A P value < 0.05 was considered significant.

RESULTS

The median age of diagnosis was 57.5 (48.7-63.2) years. Median duration of symptoms was 4 (1.75-6.0) mo. About 24% (12/50) of the patients were premenopausal and 76% (38/50) were post-menopausal. The main comorbidities were diabetes mellitus (24%; 12/50), hypertension (28%; 14/50), and coronary heart disease (4%; 2/50). About 64% (32/50) of the patients had distant nodal metastases, 50% (25/50) had bone metastases, 72% (36/50) had lung metastases, 36% (18/50) had liver metastases, and 6% (3/50) had oligometastatic diseases. About 96% (48/50) had invasive ductal carcinoma (IDC) and 4% (2/50) had other histology. Approximately 72% (36/50) were hormone positive and 38% (19/50) were HER2 positive. Grade 2 IDC accounted for 24% (12/50) and grade 3 IDC accounted for 76% (38/50). Among the study population, luminal type was seen in 70% (35/50), HER2 positive type in 8% (4/50), and TNBC in 22% (11). The pre-chemotherapy CEA levels were more than 3.8 in 72% (36/50) of the patients. About 82% (41/50) were treated with chemotherapy and 18% (9/50) treated with hormonal agents. Anti-Her2 treatment was received by 16% (8/50) of the patients. The median number of cycles of chemotherapy was 6 (4-6). The main palliative chemotherapy agents were docetaxel (68%; 34/50), paclitaxel (4%; 2/50), capecitabine (2%; 1/50), doxorubicin plus cyclophosphamide (2%; 1/50), carboplatin (2%; 1/50), and paclitaxel plus carboplatin (4%; 2/50). About 6% (3/50) of the patients received palliative radiation to their painful bone metastases.

About 36% (18/50) of the patients progressed on treatment while 64% (32/50) had responded to palliative systemic treatment. Among responders (64%), 2% (1/50) had complete remission, 32% (16/50) had partial response, and 30% (15/50) had stable disease. About 36% (18/50) had progressive disease.

Serum CEA and its correlation with other variables

Serum CEA value more than 3.8 ng/mL was considered positive. Baseline serum CEA and its correlation with other variables in MBC are given in Table 1. None of the factors like menstrual status, grade of the tumour, number and sites of metastases, presence or absence of metastases, HER2 status, and TNBC status showed any statistical significance except luminal type (P = 0.016).

Serum CEA as a predictor of response to treatment

The median pre-treatment and post-treatment CEA levels were 7.9 (1.8-40.7) ng/mL and 4.39 (1.4-12.15) ng/mL, respectively, in the whole study population (P = 0.032). Serum CEA and response to treatment in responders and non-responders are given in Table 2. Among responders, median pre-treatment CEA was 8.87 (2-49.6) ng/mL and post-treatment CEA was 2.07 (1-8.7) ng/mL (P = 0.001). Among non-responders, median pre-treatment CEA was 5.4 (1.7-36.01) ng/mL and post-treatment CEA was 11

Table 1 Association of baseline serum carcinoembryonic antigen with other variables in study population

CEA level	Less than or equal to 3.8	More than 3.8	*P* value
Pre-menopausal	3	9	0.79
Post-menopausal	11	27	
Grade 2	3	9	0.79
Grade 3	11	27	
Luminal	6	30	0.016
Her2 Neu	1	2	
TNBC	7	4	
Luminal	6	30	0.012
Non luminal	8	6	
Bone metastases	6	19	0.682
No bone metastases	7	17	
Lung metastases	11	25	0.487
No lung metastases	2	11	
Liver metastases	3	15	0.392
No liver metastases	10	21	
Less than 5 metastases	1	2	0.78
More than 5	12	34	
PR/SD/CR	9	23	0.79
Progression	5	13	

CEA: Carcinoembryonic antigen; TNBC: Triple negative breast cancer; PR/SD/CR: Partial response/stable disease/complete response.

Table 2 Serum carcinoembryonic antigen and response to treatment in responders and non-responders

Serum CEA	Responders	Non-responders	*P* value
Median pre-treatment serum CEA	8.87 (2-49.6)	5.4 (1.7-36.01)	0.527
Median post-treatment serum CEA	2.07 (1-8.7)	11 (4.65-22.5)	0.002
P value	0.001	0.06	

CEA: Carcinoembryonic antigen.

(4.65-22.5) ng/mL (P = 0.06). Since there was no statistically significant difference between responders and non-responders in baseline serum CEA, it cannot be taken as a predictor of response but post-treatment increase in CEA was associated with non-response or progression.

Pre-treatment and post-treatment ROC curves for the whole study population and luminal type breast cancer are given in Figure 1. We tried to find optimal pre-treatment cut-off for serum CEA in luminal breast cancer using ROC curve. The cut-off can be taken as 29.7 ng/mL as a predictor of tumour progression, with a sensitivity of 50% and specificity of 64%, but that cut-off was not statistically significant. ROC curve analysis for finding the cut-off for post-treatment CEA was also done. Post-treatment CEA for predicting the progression was taken as 2.16 ng/mL, with a sensitivity of 94.1% and specificity of 54.8%. For hormone positive tumours, post-treatment cut-off can be taken as 9.46 ng/mL with a sensitivity of 88.9% and specificity of 75.9% (P = 0.02). With a cut-off of 9.41, we found statistical significance in the whole group of patients (P = 0.006).

Serum CEA and luminal and non-luminal MBC

Table 3 shows serum CEA and response to treatment in responders and non-responders according to breast cancer type. Among responders, median pre-treatment CEA for luminal type was 14.7 (5.4-50.6) ng/mL and post-treatment CEA was 3.0 (1-10) ng/mL (P = 0. 001). Even in the luminal group, pre-

Table 3 Serum carcinoembryonic antigen and response to treatment in responders and non-responders according to breast cancer type

Classification		Responders			Non-responders		
		Median pre-CEA	Median post-CEA	*P* value	Median pre-CEA	Median post-CEA	*P* value
Hormonal classification	Luminal	14.7 (5.4-50.6)	3 (1-10)	0.001	22.39 (3.9-84.4)	21.00 (10.6-164.15)	0.26
	Non-luminal	1.85 (1-3.65)	1.25 (0.5-3)	0.046	4.15 (0.85-10.17)	5.65 (2.65-12.05)	0.161
Genomic classification	Luminal	14.7 (5.4-50.6)	3 (1-10)	0.001	22.39 (3.9-84.47)	20.67 (10.6-164.17)	0.260
	HER2	4 (1.2-4)	3.25 (0.5-3.25)	0.18	11.7	13	_
	TNBC	1.85 (0.74-2.4)	1.25 (0.67-1.88)	0.144	4 (0.5-5.6)	5.3 (2.2-9.2)	0.237

TNBC: Triple negative breast cancer; CEA: Carcinoembryonic antigen.

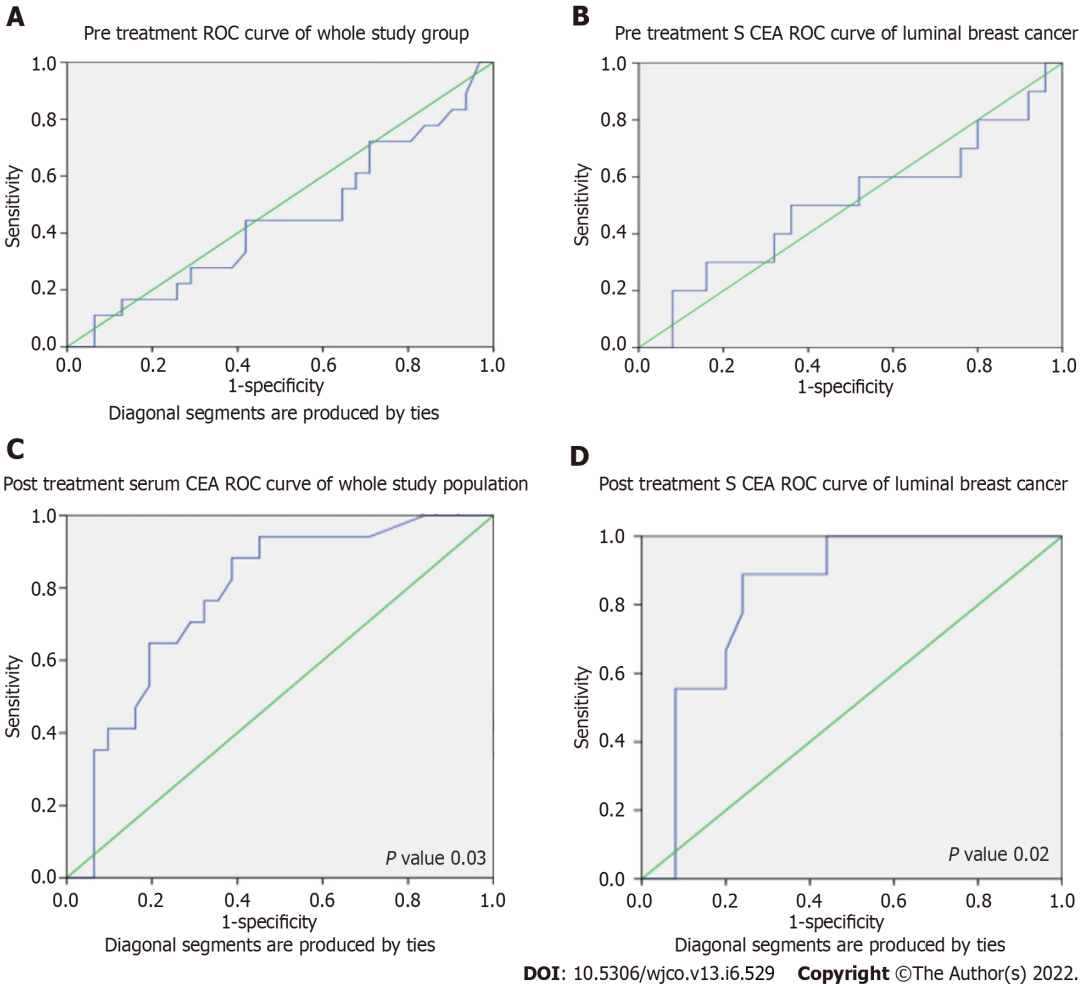

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Figure 1 Receiver operator characteristic curves. A: Pre-treatment receiver operator characteristic (ROC) curve for whole study population; B: Pre-treatment ROC curve for luminal type; C: Post-treatment ROC curve for whole study population; D: Post-treatment ROC curve for luminal type. ROC: Receiver operator characteristic; CEA: Carcinoembryonic antigen.

treatment serum CEA was not a predictor of response, but post-treatment CEA was a significant predictor of tumour progression (Figure 2).

Association of serum CEA with various sites of MBC

Figure 3 shows median pre-treatment and post-treatment serum CEA levels in responders and non-responders according to site of metastasis. Among responders, median pre-treatment serum CEA levels of patients with bone metastases, lung metastases, and liver metastases were 27.2 ng/mL, 8.4 ng/mL, and 24.5 ng/mL respectively. Among non-responders, median post-treatment serum CEA levels of

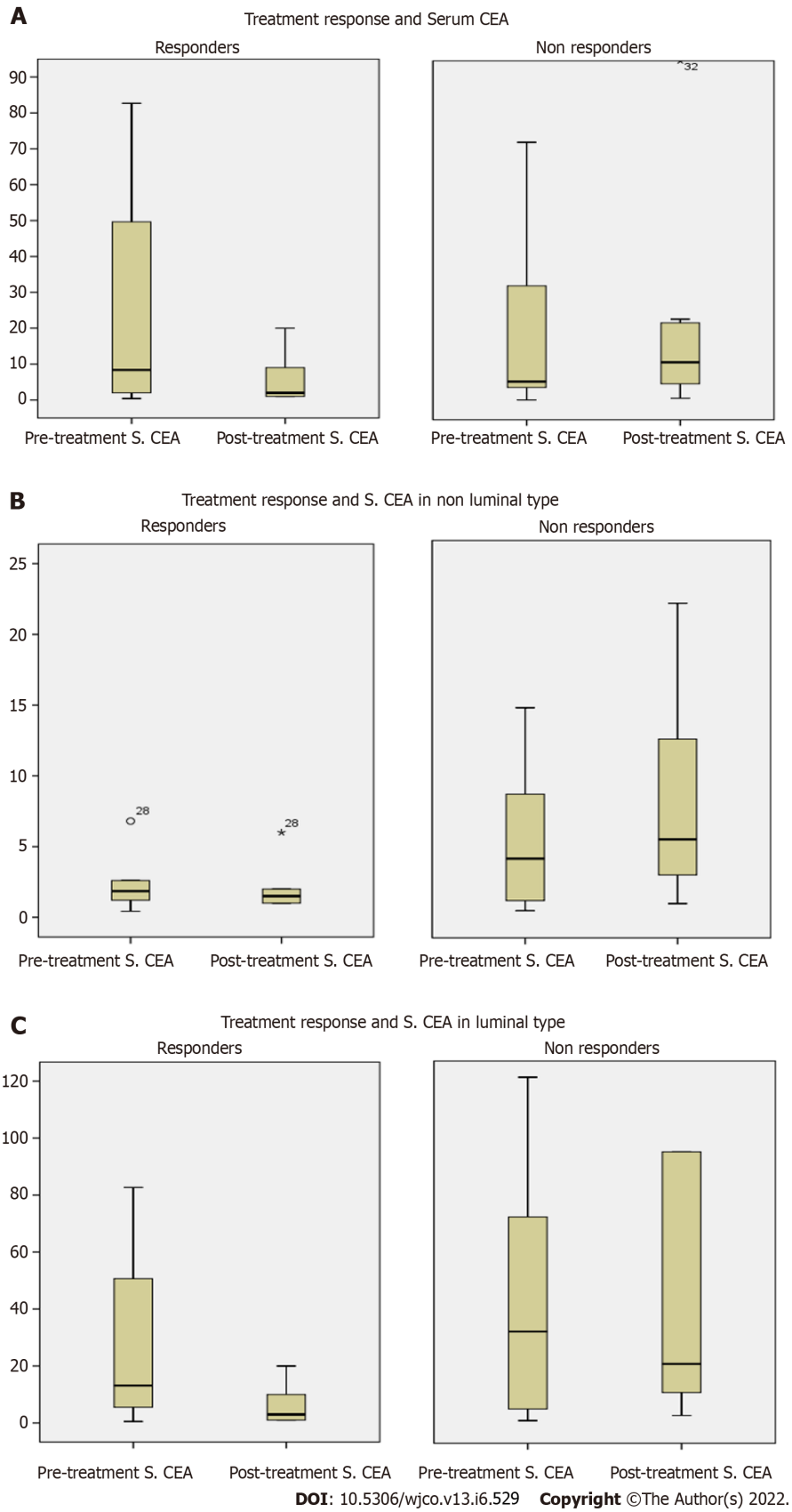

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Figure 2 Treatment response. A: Association of treatment response with serum carcinoembryonic antigen (CEA) (pre-treatment and post-treatment) in whole study population; B: Association of treatment response with serum CEA in non-luminal type; C: Association of treatment response with serum CEA in luminal type. CEA: Carcinoembryonic antigen.

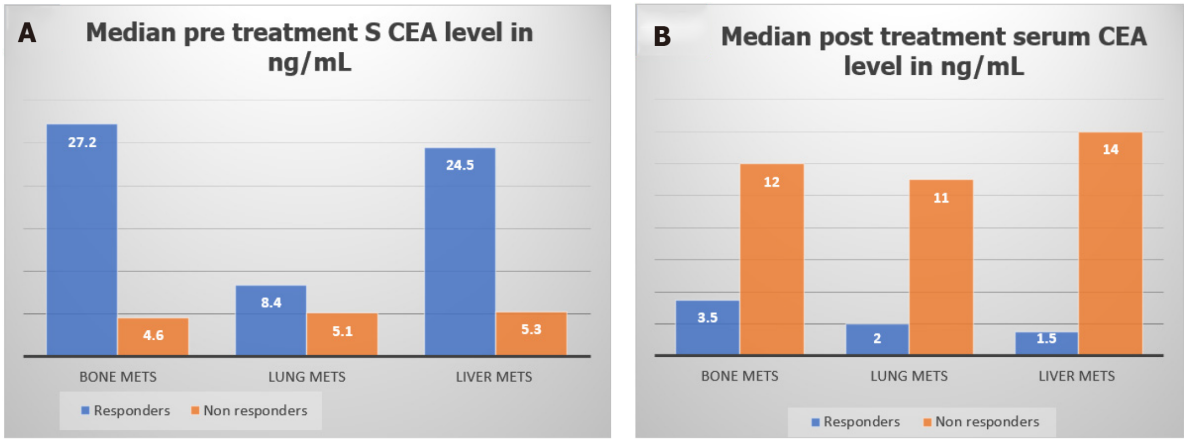

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Figure 3 Median pre-treatment and post-treatment serum carcinoembryonic antigen levels in responders and non-responders according to various sites of metastasis. A: Median pre-treatment serum carcinoembryonic antigen (CEA) level; B: Median post-treatment serum CEA level. CEA: Carcinoembryonic antigen.

patients with bone metastases, lung metastases, and liver metastases were 12 ng/mL, 11 ng/mL, and 14 ng/mL, respectively.

Table 4 shows serum CEA and response to treatment in bone, liver, and lung metastases**.** In patients with liver and lung metastases, post-treatment CEA level difference was not statistically significant in both responders and non-responders though the values were higher in non-responders.

In non-responders, comparing patients with or without bone metastases, the median post-treatment serum CEA of patients with bone metastases was 12 ng/dL whereas median post-treatment serum CEA in those without bone metastases was 10 ng/mL; post-treatment CEA level difference was statistically significant (P = 0.063). Among those with bone metastases, 69.5% had elevated post-treatment serum CEA, and only 37.5% had elevated serum CEA in those with no bone metastases (Figure 4).

DISCUSSION

The measurement of serum tumour marker levels could provide useful information for earlier detection of recurrence or accurate prediction of outcomes after recurrence in various cancers. They are more useful when patients have elevated level at baseline. The commonly studied tumour markers in breast cancer are CA15-3 and CEA. The significance of these markers remains unclear[17,18]. Even though the prognostic value of CA15-3 in breast cancer had been documented in some studies, serum CEA is less widely investigated as a prognostic factor than CA15-3 because of its poor sensitivity and specificity[18,19]. Elevated serum levels of CA15-3 and CEA preoperatively were significantly associated with tumour size, axillary node metastasis, and advanced stage[20-23]. A recent meta-analysis investigated the prognostic value of these two markers (serum CA15-3 and CEA) in 12993 breast cancer patients and indicated that elevated CA15-3 level significantly corresponded with poor disease-free survival and OS of breast cancer[23].

In our study, no clinically meaningful significance was seen in factors like menstrual status, grade of the tumour, number and sites of metastases, presence or absence of metastases, HER2 status, and TNBC status except luminal type. This finding was consistent with a study by Geng *et al*[23]. Elevated CEA levels were significantly associated with breast cancer molecular subtypes and luminal subtypes exhibited a higher percentage of elevated CEA levels compared to non-luminal subtypes. The reason for this differential expression of CEA is that the expression patterns of luminal, HER2 positive, and basal-like tumours are closely associated with their maturation and differentiation. Luminal subtypes have high expression of hormone receptor related genes, whereas HER2 positive or basal-like tumours have low expression of hormone receptor related genes, which explains the association between CEA elevation and luminal subtype. Our study showed that pre-treatment serum CEA cannot be taken as a predictor of response even in luminal subtype but post-treatment CEA was a significant predictor of tumour progression. Hence, we can conclude that monitoring CEA levels in luminal MBC at the end of treatment is a significant predictor of treatment response.

The correlation between tumour marker levels and various metastatic sites in MBC is poorly defined [24,25]. A study by Yerushalmi *et al*[26] identified that tumour marker elevation was documented in the majority of patients with MBC and luminal subtypes expressed more frequently compared with the non-luminal groups[26]. CEA elevation was not different between different sites of metastasis. Whereas in our study, in patients with liver and lung metastases, post-treatment CEA level difference was not

Table 4 Serum carcinoembryonic antigen and response to treatment in bone, liver, and lung metastases

Serum CEA (ng/mL)	Bone metastases	No bone metastases	*P* value
Median pre-treatment serum CEA	11.7 (2.9-48.4)	6.8 (2-32.3)	0.788
Median post-treatment serum CEA	9 (2-20)	2 (1-9)	0.063
	Liver metastases	**No liver metastases**	
Median pre-treatment serum CEA	11.7 (4.4-62.7)	6.8 (1.9-22.7)	0.244
Median post-treatment serum CEA	8 (1.2-19.75)	3 (1.25-11.5)	0.352
	Lung metastases	**No lung metastases**	
Median pre-treatment serum CEA	7.8 (1.9-31.3)	9.78 (5.15-66.64)	0.353
Median post-treatment serum CEA	3.5 (1-13.75)	5 (1.5-10)	0.93

CEA: Carcinoembryonic antigen.

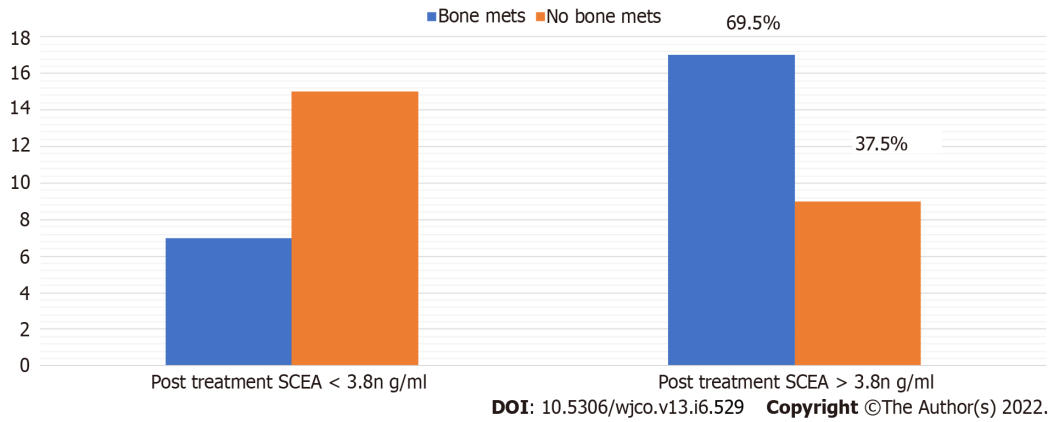

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Figure 4 Association of post-treatment serum carcinoembryonic antigen with bone metastases. CEA: Carcinoembryonic antigen.

statistically significant in both responders and non-responders even though the values were higher in non-responders.

A study by Yazdani *et al*[27] showed that age, menopausal status, number of axillary lymph node metastases, tumor size, and ALP were identified as prognostic factors for bone metastasis in patients with breast cancer, whereas significantly persistent elevated post-treatment serum CEA levels were seen with bone metastases in our study[27]. Kosaka *et al*[28] proposed that in hormone receptor positive breast cancer, nodal metastasis and elevated serum CEA were associated with a poor prognosis and there was a significant rate of recurrence in those with high serum CEA levels compared with those with low levels of CEA[28]. Elevated serum levels of HER2, BCL2, CA15-3, and CEA in breast cancer patients are useful markers for predicting aggressive behaviour and relapse[29,30].

One major limitation of our study is the small sample size (50 patients) and it limits the predictive power of these markers and needs larger studies to confirm the findings.

CONCLUSION

Pretreatment serum CEA is elevated in luminal subtype. With treatment, responders have a significant fall in serum CEA level but it is clinically significant in luminal breast cancer type. Elevated post-treatment serum CEA levels are associated with disease progression and poor response to therapy. Persistently elevated post treatment serum CEA levels are associated with bone metastases. Elevated serum CEA and hormonal status are significant predictors of treatment response.

ARTICLE HIGHLIGHTS

Research background

In breast cancer patients, elevated serum carcinoembryonic antigen (CEA) levels are particularly noted in metastatic and recurrent disease and its significance in clinical practice is doubtful.

Research motivation

We aimed to estimate the serum CEA level in our metastatic breast cancer patients and correlate it with response to treatment and clinical outcome.

Research objectives

To evaluate the efficacy of serum CEA levels as a prognostic marker in metastatic breast cancer patients.

Research methods

This is a prospective clinical study of 50 patients with metastatic breast cancer treated at a breast clinic with newly diagnosed metastatic breast cancer planned for palliative chemotherapy, targeted therapy, and hormone therapy. We estimated the proportion of patients with elevated serum CEA levels at baseline and after palliative care, and investigated the association of serum CEA levels with known prognostic factors. Response to treatment was correlated with serum CEA levels in both responders and non-responders.

Research results

Pretreatment serum CEA was elevated in luminal subtype. With treatment, responders had a significant fall in serum CEA level but it was clinically significant in luminal breast cancer type. Metastatic breast cancer patients with bone metastases had significantly elevated post-treatment serum CEA levels after treatment.

Research conclusions

Based on our results, we suggest that serum CEA has potential clinical value in monitoring the treatment response of metastatic breast cancer patients, especially in patients with bone metastasis.

Research perspectives

Serum CEA as a tumour marker warrants further studies in metastatic breast cancer especially with bone metastases.

FOOTNOTES

Author contributions: Anoop TM designed the study, drafted the manuscript, and supervised the study and treatment; Joseph P R participated in the design and supervision of the study and treatment; Chacko S participated in the design of the study; Soman S was involved in data collection, analysis, and statistics; Mathew M participated in data collection; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board/EC according to the ethical norms and regulations.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Data are available from the corresponding author upon reasonable request.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

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S-Editor: Fan JR

L-Editor: Wang TQ
P-Editor: Qi WW

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World J Clin Oncol 2022 June 24; 13(6): 540-552

DOI: 10.5306/wjco.v13.i6.540

ISSN 2218-4333 (online)

CASE REPORT

Epstein-Barr virus-associated smooth muscle tumors in immunocompromised patients: Six case reports

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ishikawa E, Japan; Liu D, China

Received: January 12, 2022
Peer-review started: January 12, 2022
First decision: March 24, 2022
Revised: April 6, 2022
Accepted: May 12, 2022
Article in press: May 12, 2022
Published online: June 24, 2022

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Abstract

BACKGROUND

Epstein-Barr virus associated smooth muscle tumor (EBV-SMT) is a rare oncological entity. However, there is an increasing incidence of EBV-SMTs, as the frequency of organ transplantation and immunosuppression grows. EBV-SMT diagnosis relies on histopathology and immunochemical staining to distinguish it from post-transplant lymphoproliferative disorder (PTLD). There is no clear consensus on the treatment of EBV-SMTs. However, surgical resection, chemotherapy, radiation therapy, and immunosuppression reduction have been explored with varying degrees of success.

CASE SUMMARY

Our case series includes six cases of EBV-SMTs across different age groups, with different treatment modalities, adding to the limited existing literature on this rare tumor. The median latency time between immunosuppression and disease diagnosis is four years. EBV-SMTs present with variable degrees of aggressiveness and seem to have worse clinical outcomes in patients with tumor multiplicity and worse immunocompetency.

CONCLUSION
It is imperative to continue building on this knowledge and keeping EBV-SMTs on the differential in immunocompromised individuals.

Key Words: Epstein-Barr virus; Smooth muscle tumors; Human immunodeficiency virus; Epstein-Barr virus-associated smooth muscle tumors; Immunocompromised; Solid Organ Transplant; Orthotopic heart transplant; Orthotopic liver transplant; Living related kidney transplant; Post-transplant lymphoproliferative disorders; Case report

Core Tip: Epstein-Barr virus associated smooth muscle tumor (EBV-SMT) is a rare oncological entity. Only a handful of case series have shed light on the presence of EBV-SMT in individuals, most of whom are immunocompromised. EBV-SMT should not be confused with post-transplant lymphoproliferative disorder. Histopathology should help guide the diagnosis.

Citation: Khan AA, Estfan BN, Yalamanchali A, Niang D, Savage EC, Fulmer CG, Gosnell HL, Modaresi Esfeh J. Epstein-Barr virus-associated smooth muscle tumors in immunocompromised patients: Six case reports. *World J Clin Oncol* 2022; 13(6): 540-552
URL: https://www.wjgnet.com/2218-4333/full/v13/i6/540.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i6.540

INTRODUCTION

Epstein-Barr virus associated smooth muscle tumor (EBV-SMT), first reported in 1970[1], is a rare oncological entity. Though EBV is present in 50%-89% of children and > 90% of adults worldwide, the virus often remains dormant until an individual becomes immunocompromised[2,3]. EBV is better known for other malignancies including nasopharyngeal carcinomas and lymphomas but in a minority of cases, it can trigger the proliferation of smooth muscle cells, resulting in mesenchymal tumors termed EBV-SMT.

There are three different types of EBV-SMTs identified to date: (1) Post-transplant associated smooth muscle tumors (PT-SMT); (2) Human immunodeficiency virus (HIV) associated smooth muscle tumors (HIV-SMT); and (3) Congenital immunodeficiency associated smooth muscle tumors (CI-SMT) such as in GATA2 and CARMIL2 deficiency[4]. EBV-SMTs can be encountered at any age, though it is more common in children[5,6]. EBV-SMTs can arise in any organ system, however, they are most common in the liver, followed by the lungs, central nervous system (CNS), adrenal glands, and gastrointestinal tract [7,8]. Unlike primary leiomyosarcomas where the histological grade is associated with disease severity, EBV-SMTs behave with variable severity, independent of their histological grade[9]. Clinical presentation is thus non-specific and depends on the location, size, and degree of organ involvement. EBV-SMTs can manifest either synchronously or metachronously in multiple organ systems and have a low propensity to metastasize[7,8].

PT-SMT can be confused with EBV associated post-transplant lymphoproliferative disorder (EBV-PTLD) as they are both caused by the same virus and occur in immunocompromised patients. Clinical presentation and radiographic findings cannot be used to tell the two entities apart. Instead, histopathology, immunohistochemistry, and EBV-encoded small RNA *in situ* hybridization (EBER ISH) are used to aid the diagnosis. Given its rarity, it is difficult to quantify the incidence of EBV-SMTs, however, it is estimated each subtype above may impact < 1%-5% of individuals. EBV-PTLD, on the other hand, is more prevalent than EBV-SMT and has an incidence ranging from 1%-20%[10] with mortality rates around 50%-90%[11,12].

Given how rare these tumors are, there is no standard treatment for EBV-SMTs which are instead treated on a case-by-case basis. Individuals with HIV-SMT are kept on appropriate antiretroviral treatment, and those with PT-SMT are treated with a reduction of immunosuppression. Surgical removal of tumors is considered when tumors impinge on the involved organ. Chemotherapy and radiation have also been utilized but without any obvious benefits on the disease course[6]. Allogeneic hematopoietic stem cell transplantation has also been used to treat CI-SMT with good outcomes[13,14].

Given their rarity, much of what we know about EBV-SMTs is through case reports and case series. However, as the frequency of organ transplantation grows with reliance on immunosuppression to prevent graft loss, it is pertinent to continue gathering information on EBV-SMTs. Many questions remain regarding incidence, prevalence, latency period, survival rates, and appropriate treatment

strategies, amongst other crucial facts. Here, we describe six cases of EBV-SMT at a quaternary academic referral center.

CASE PRESENTATION

Chief complaints

Case 1: Fever, thrush, diarrhea.

Case 2: Weight loss, headaches, and myalgias.

Case 3: Severe headaches and altered mental status.

Case 4: New-onset hematuria.

Case 5: Shortness of breath.

Case 6: Neck, back, and shoulder pain.

History of present illness

Case 1: A five-year-old female with a history of idiopathic dilated cardiomyopathy status post orthotopic heart transplant (OHT) at eight months old, on tacrolimus, and previous EBV viremia at age 22 months presented with her third infection-related hospitalization in the previous six months.

Case 2: A 20-year-old male with a history of Idiopathic dilated cardiomyopathy status post OHT at age 17 on tacrolimus and mycophenolate mofetil (MMF) presented to the hospital with weight loss, headaches, and myalgias.

Case 3: A 16-year-old female with a history of dilated cardiomyopathy status post OHT at 12-year-old, on MMF and tacrolimus presented to the hospital with severe headaches and altered mental status.

Case 4: A 61-year-old male with a pre-transplant negative cytomegalovirus (CMV) and EBV serology underwent orthotopic heart and liver transplant from a CMV and EBV positive donor (CMV Donor+/Recipient-, EBV Donor+/Recipient-) at the age of 58 years for cirrhosis secondary to Hereditary Familial Amyloidosis (Thr60A1a mutation) was admitted for new-onset hematuria and acute kidney injury.

Case 5: A 63-year-old male with a history of a living-related donor kidney transplant at age 55 for end-stage renal disease of unknown etiology was hospitalized for community-acquired pneumonia.

Case 6: A 45-year-old female with a long-standing history of HIV (CD 4 count unknown) on anti-retroviral therapy presented to the primary care clinic with neck, back, and shoulder pain with associated proximal and distal left upper extremity numbness and paresthesias.

History of past illness

Case 1, 2: Idiopathic dilated cardiomyopathy status post OHT.

Case 3: History of dilated cardiomyopathy status post OHT at 12-year-old.

Case 4: Status post Orthotopic heart and liver transplant from a CMV and EBV positive donor (CMV Donor+/Recipient-, EBV Donor+/Recipient-) for cirrhosis secondary to Hereditary Familial Amyloidosis (Thr60A1a mutation).

Case 5: History of a living-related donor kidney transplant for end-stage renal disease of unknown etiology.

Case 6: Long-standing history of HIV (CD 4 count unknown) on anti-retroviral therapy with dolutegravir, abacavir, and lamivudine

Personal and family history

Cases 1-6: No relevant family history.

Physical examination

Physical examination findings are not applicable.

Laboratory examinations

Laboratory findings are summarized in Table 1 for cases 1-6.

Table 1 Demographic characteristics and clinical summary of six Epstein-Barr virus associated smooth muscle tumor at a major quarternary care hospital

Cases	Sex	Type of transplant/IS	IS	Age at transplant (yr)	Age at diagnosis (yr)	EBV titer at diagnosis (copies per mL)	Tumor location	Histopathology findings	Treatment	Outcome (years post EBV-SMT)	Follow up
1	F	OHT	Tacrolimus	8 mo	6	286000	Colon	Smooth muscle spindle cell proliferation with strongly positive *in-situ* EBER hybridization and focal desmin+	Tacrolimus reduced	Alive (age 16)	None
2	M	OHT	Tacrolimus, MMF	17	20	161476	Thymus, lung, liver, mesenteric, and retroperitoneal lymph nodes, ascending colon, and proximal left femoral bone marrow	The spindled cells were positive with antibodies to smooth muscle antigen and desmin. Tumor cells were focally positive with antibodies to caldesmon. The spindle cells were negative with antibodies to S100 protein and cytokeratin AE1/3. *In situ* hybridization revealed that up to 80% of tumor cell nuclei were positive for the presence of EBER	Supportive	Death (age 20)	NA
3	F	OHT	Tacrolimus; Sirolimus	12	16	11848	Brain, lung, kidney	Hepatic parenchyma focally replaced by spindle cell proliferation. The spindle cells are arranged in a haphazard pattern and contain nuclei with minimal nuclear pleomorphism and abundant eosinophilic cytoplasm. (1) The tumor cells are strongly positive for h-caldesmon and smooth muscle actin; (2) Desmin+; (3) EBER positive by CISH; (4) CD20+, MUM-1+. CD3-, CD10-. EBER ISH+ (CNS); and (5) H-caldesmon+, smooth muscle actin+, desmin+ (focal). EBER ISH+ (liver)	Intrathecal rituximab, methotrexate, systemic chemotherapy, whole brain radiation, and T-cell therapy	Alive (age 22); course c/b seizures	Yearly brain and liver MRI
4	M	OHT/OLT	Tacrolimus	58	61	25458	Kidney, liver, lung	(1) Monotonous proliferative of relatively uniform spindle cells arranged in intersecting fascicles with pale eosinophilic cytoplasm and elongated, blunt-ended nuclei with dark vesicular chromatin.; (2) Mitotic figures are not readily identified and there is no significant cytologic atypia, pleomorphism, or necrosis; (3) The tumor cells are strongly immunoreactive for SMA and negative for CD117, DOG-1, desmin, S100, SOX10, CD34, and STAT6; and (4) EBER positive by CISH	L lateral segmentectomy w superficial wedge resection;Tacrolimus reduced, stopped, and eventually started given concern for ACR	Death (age 61)	NA
5	M	LRD kidney	Prednisone, MMF, Sirolimus	55	63	4765	Liver	(1) Fascicles of malignant appearing spindle cells diffusely positive for desmin and H-caldesmon; and (2) EBER positive by CISH	MMF. Sirolimus dosing decreased	Alive (age 73)	Repeat MRI with stable/decreased size hepatic lesions; no new lesions noticed
6	F	HIV	Abacavir-Lamivudine-	NA	45	NA	Thoracic spine	(1) Spindle cell neoplasm staining positive for smooth muscle actin; (2) Negative for desmin;	s/p T8-T10 Laminectomy; no change in HAART	Alive (age 55); total hip	None

	Dolutegravir for HIV	and (3) EBV positive by CISH	arthroplasty for bl avascular necrosis

IS: Immunosuppressant; EBV: Epstein-Barr virus; EBER: EBV-encoded small RNA; MRI: Magnetic resonance imaging; F: Female; M: Male; OHT: Orthotopic heart transplant; MMF: Mycophenolate mofetil; NA: Not available; OHT/OLT: Combined orthotopic heart and liver transplant; LRD: Living related donor; HIV: Human immunodeficiency virus.

Imaging examinations

Case 1: Unrevealing total body computed tomography (CT) scan. EGD and colonoscopy with 6-10 mm polypoid lesions with central ulceration in the sigmoid colon and rectum. Biopsy of lesions confirmed EBV-SMT (Figures 1 and 2).

Case 2: Neck magnetic resonance imaging (MRI) revealed a mass in the right Meckel's cave. Liver biopsy confirmed EBV-SMT (Figure 3). PET scan showed multiple fluorodeoxyglucose avid foci (Figure 4).

Case 3: MRI brain showed a left temporal ring-enhancing lesion (Figure 5). Whole body PET scan showed right hepatic mass (Figure 6).

Case 4: CT scan of the liver revealed multiple indeterminate liver masses (Figure 7).

Case 5: MRI imaging of the abdomen showed over 20 cystic hepatic masses, with the largest measuring 12.5 cm x 9.2 cm, resolved on subsequent imaging (Figure 8).

Case 6: Cervical and thoracic spine MRI which showed a 1.9 cm x 1.2 cm x 2.4 cm intradural heterogeneous mass centered within the left T9-T10 neuroforamina, with abutment of the left lateral spinal cord resulting in rightward cord displacement as well as moderate canal stenosis (Figure 9).

Case series details

Case 1: A five-year-old female with a history of idiopathic dilated cardiomyopathy status post orthotopic heart transplant (OHT) at eight months old, on tacrolimus, and previous EBV viremia at age 22 mo presented with her third infection-related hospitalization in the previous six months. Presenting symptoms included fever, thrush, diarrhea, and neutropenia with an absolute neutrophil count of 260 cells per μL. She was placed on broad-spectrum antibiotics and antifungals but continued to have diarrhea, failure to thrive, as well as new oral ulcers. Due to concern for PTLD, she underwent total body CT which was unrevealing. She also underwent esophagogastroduodenoscopy and colonoscopy. In addition to the presence of colonic candidiasis, colonoscopy revealed 6-10 mm polypoid lesions with central ulceration in the sigmoid colon and rectum with biopsies consistent with an EBV-driven smooth muscle tumor (Figures 1 and 2). The patient was EBV negative before transplant with repeat EBV titers high on admission (Table 1). She was treated for colonic candidiasis as well as a pseudallescheria boydii complex infection of her lungs with symptomatic improvement. She did not undergo any EBV-SMT-directed treatment or surveillance and remains well at age 16 years.

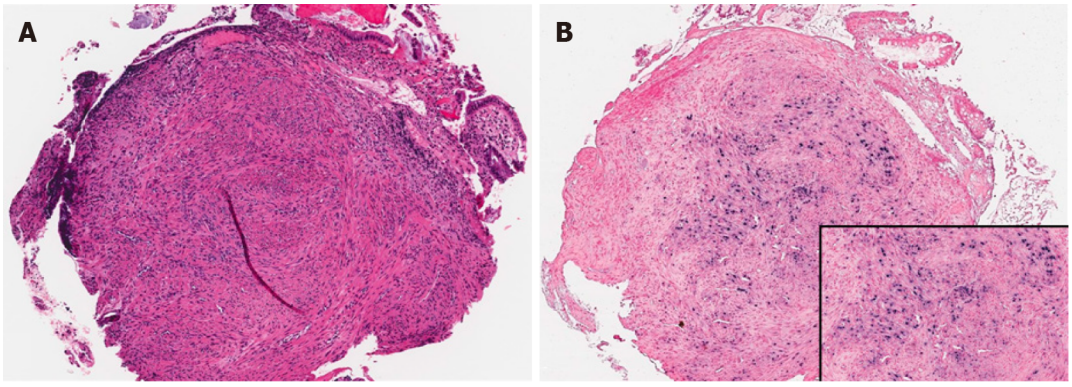

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Figure 1 Epstein-Barr virus associated smooth muscle tumor in the sigmoid colon in case 1. A: Proliferation of smooth muscle cells undermining and distorting the colonic mucosa (HE stain, 70 x); B: Epstein-Barr virus-encoded small RNA *in situ* hybridization is positive within the smooth muscle cell population (70 x, inset box 200 x).

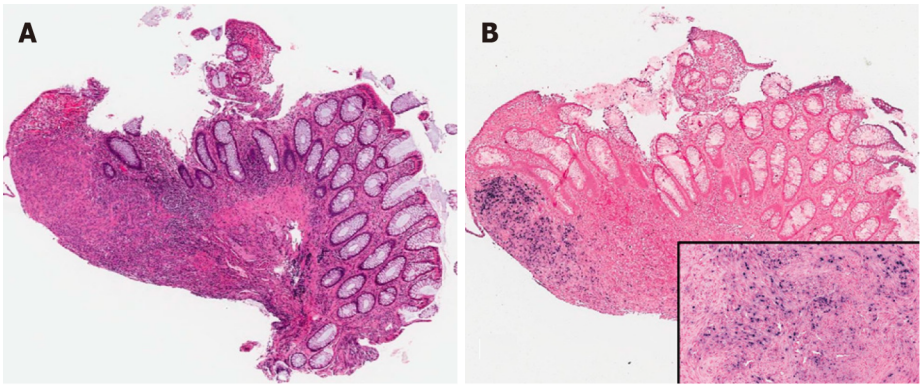

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Figure 2 Epstein-Barr virus associated smooth muscle tumor in the rectum in case 1. A: Proliferation of smooth muscle cells undermining the rectal mucosa (HE stain, 40 x); B: Epstein-Barr virus-encoded small RNA *in situ* hybridization is positive within the smooth muscle cell population (40 x, inset box 200 x).

Case 2: A 20-year-old male with a history of idiopathic dilated cardiomyopathy status post OHT at age 17 on tacrolimus and mycophenolate mofetil (MMF), Crohn's disease, alopecia, juvenile idiopathic arthritis, and common variable immune deficiency (CVID) presented to the hospital with weight loss, headaches, and myalgias. The patient had abruptly stopped his immunosuppression agents a month before admission. He was treated with antibiotics for Group A streptococcus infection. The patient was previously EBV seronegative before transplant but had elevated titers on admission (Table 1). A whole-body positron emission tomography (PET) scan showed multiple fluorodeoxyglucose (FDG) avid foci suspicious for widespread PTLD in the thymus, lung, liver, mesenteric lymph nodes, retroperitoneal lymph nodes, ascending colon, and proximal left femoral bone marrow (Figure 4). A neck magnetic resonance image (MRI) revealed a mass in the right Meckel's cave. The patient's tacrolimus was deceased due to suspicion for PTLD. Biopsies taken during colonoscopy and bone marrow biopsies were negative for PTLD. A liver biopsy was performed and showed a proliferation of bland spindle cells with pale eosinophilic cytoplasm and ovoid nuclei with smooth contours and pale chromatin (Figure 3A). Immunohistochemical stain for smooth muscle actin showed strong, diffuse reactivity in the neoplastic cells (Figure 3B). EBER highlighted the presence of viral genetic material, confirming the diagnosis of PT-SMT (Figure 3C). During this admission, he became febrile prompting a chest X-ray which revealed left upper lobe haziness, concerning for pneumonia. He rapidly deteriorated, developing acute renal failure requiring dialysis and respiratory distress with encephalopathy requiring intubation. He then sustained a fatal cardiac arrest. An autopsy revealed severe acute cellular rejection, negative for antibody-mediated rejection, and confirmed multisite PT-SMT.

Case 3: A 16-year-old female with a history of dilated cardiomyopathy status post OHT at 12-year-old, on MMF and tacrolimus presented to the hospital with severe headaches and altered mental status. MRI brain showed a left temporal ring-enhancing lesion (Figure 5). The patient was previously EBV seronegative before transplant but had elevated titers on admission (Table 1). She underwent a brain

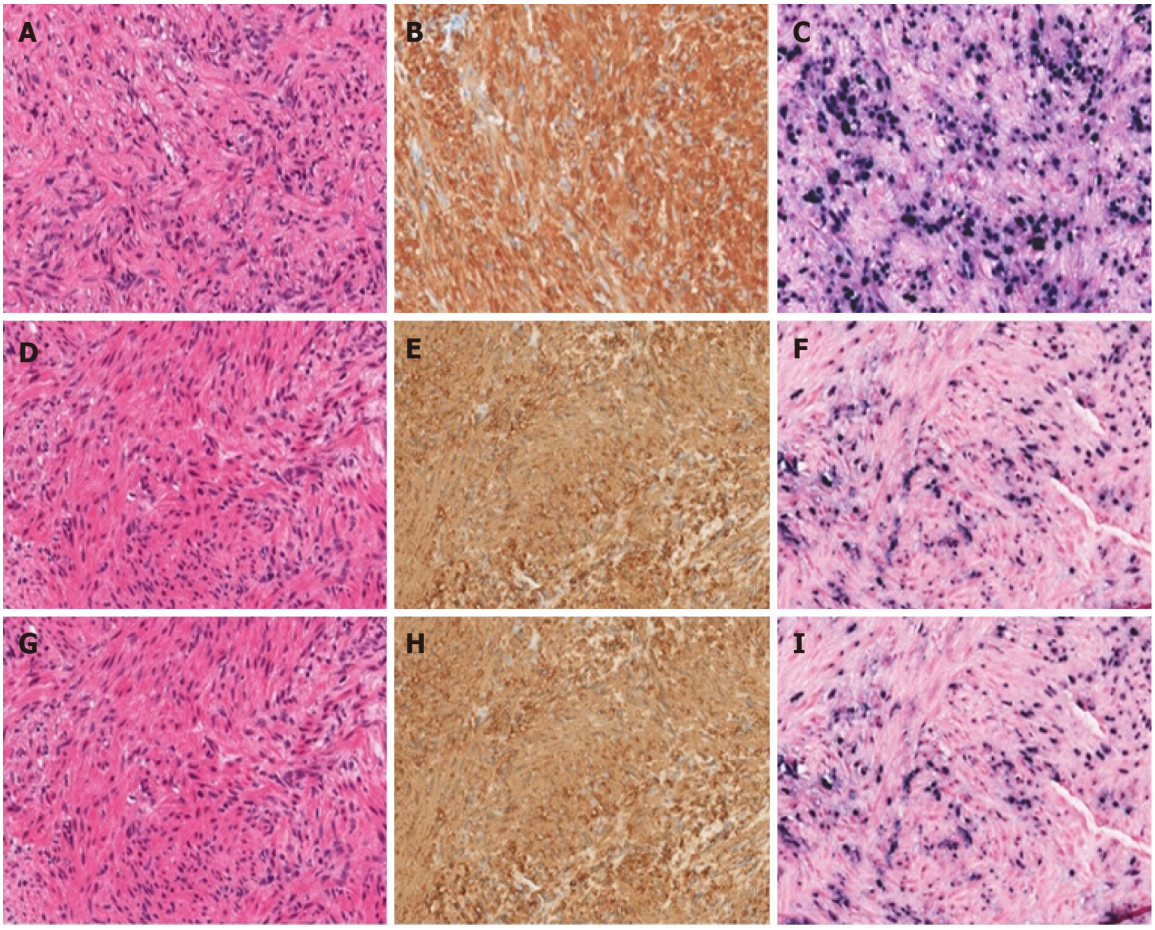

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Figure 3 Epstein-Barr virus associated smooth muscle tumor liver biopsies for cases 2-4 (HE stain, 200 x). Hematoxylin and eosin-stained tissue sections showed fascicles of well-differentiated spindle cells with pale eosinophilic cytoplasm and blunt-ended, ovoid nuclei with smooth nuclear contours. A, D, G: No significant cytologic atypia or nuclear pleomorphism is appreciated; B, E, H: All three cases show strong, diffuse reactivity for smooth muscle actin, confirming smooth muscle differentiation, confirming smooth muscle tumor lineage; C, F, I: Chromogenic *in situ* hybridization studies for the Epstein-Barr virus-encoded small RNA confirm the presence of viral genetic material in all three cases.

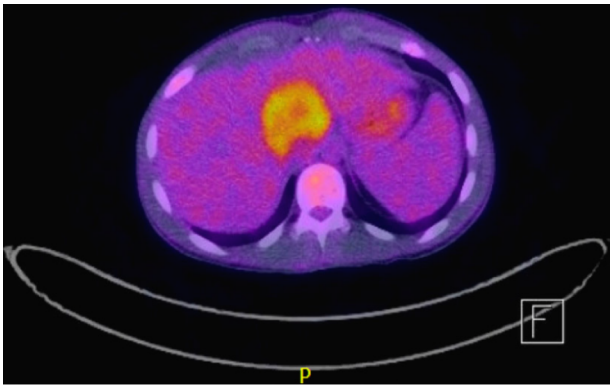

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Figure 4 Positron emission tomography scan showing a hypermetabolic mass arising from the medial segment of the left liver lobe, measuring about 5.1 cm x 4.7 cm in the axial and anteroposterior dimension and 6.9 cm in the craniocaudal dimension in case 2.

biopsy which showed EBV-PTLD, with morphology consistent with diffuse large B-cell lymphoma. Her immunosuppression therapy was reduced. She underwent a CT abdomen which showed a 2 cm low-attenuating hypervascular lesion in the right hepatic dome and another 0.6 cm lesion in the right anterior inferior hepatic lobe, redemonstrated on a PET scan (Figure 6). Liver biopsy showed a proliferation of spindle cells with eosinophilic cytoplasm, mild to moderate nuclear pleomorphism, and focal

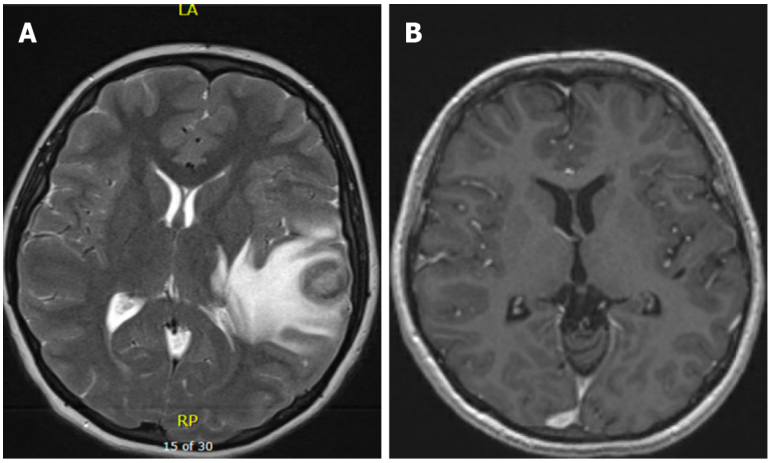

Figure 5 Magnetic resonance imaging brain. A: T2 weighted turbo spin echo magnetic resonance images of the brain showing ring-enhancing lesion in the left temporal region in case 3; B: T1 FI3d magnetic resonance images of the brain showing resolution of ring-enhancing lesion six years post-treatment in case 3.

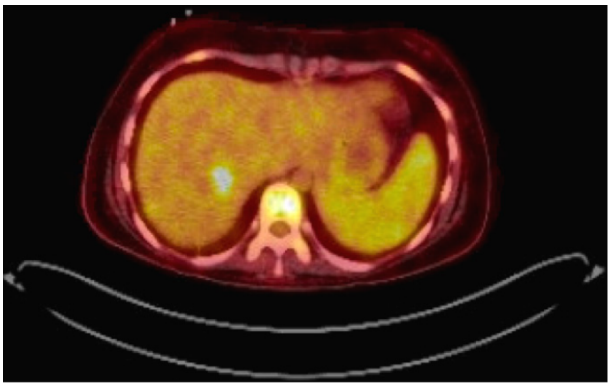

Figure 6 Positron emission tomography scan showing a hypermetabolic right hepatic mass in case 3.

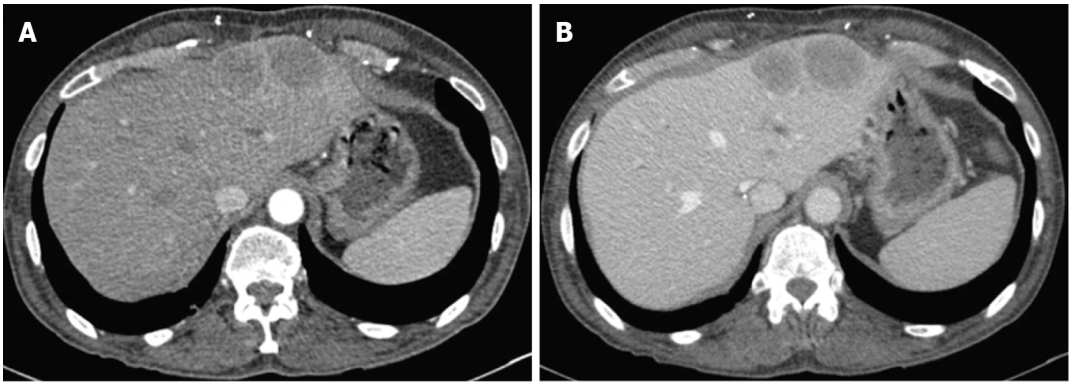

Figure 7 Computed tomography scan. A: The liver with intravenous contrast in arterial phase showing multiple hepatic lesions in case 4; B: The liver in venous phase showing multiple hepatic lesions in case 4.

tumor necrosis (Figure 3D). No severe nuclear pleomorphism or mitotic activity was identified. The neoplastic cells were strongly immunoreactive for smooth muscle actin, and showed diffuse nuclear reactivity for EBER, confirming the diagnosis of PT-SMT (Figure 3E and F). A subsequent CT scan of the abdomen and pelvis showed two nonspecific small low density lesions in the left kidney, three months after the initial diagnosis of PTLD. For her CNS-PTLD, the patient had a ventriculoperitoneal shunt

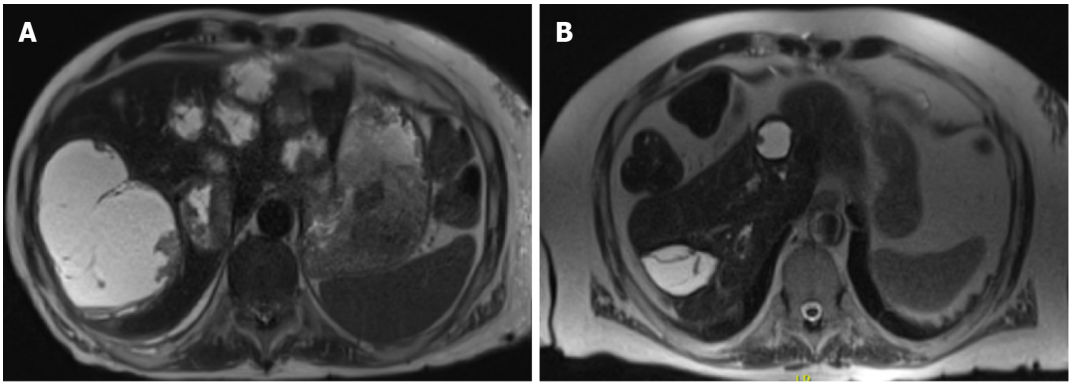

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Figure 8 Axial HASTE sequence abdominal magnetic resonance imaging. A: demonstrating numerous cystic appearing hepatic Epstein-Barr virus associated smooth muscle tumors, including the largest lesion in segment VII in case 5; B: Demonstrating stable/deceased hepatic lesions with no new lesions in case 5.

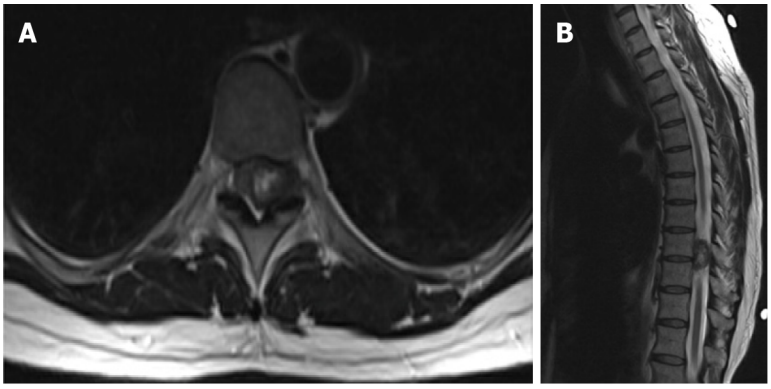

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Figure 9 Axial (A) and sagittal (B) cuts of a T2 weighted magnetic resonance imaging of the thoracic spine demonstrating an Epstein-Barr virus associated smooth muscle tumor centered in the left T9/T10 neuroforamina with a rightward displacement of the spinal cord in case 6.

placed and underwent six cycles of intrathecal rituximab with methotrexate, systemic chemotherapy, whole-brain radiation, and T-cell therapy with subsequent decrease and ultimate resolution in the CNS lesion on subsequent imaging (Figure 5). Her cerebrospinal fluid studies were negative for infection. She started having generalized tonic-clonic seizures in the post-treatment setting and was treated with anti-epileptics. She remains well at the age of 22 years, with yearly liver MRI showing stable hepatic lesions (Figure 6).

Case 4: A 61-year-old male with a pre-transplant negative CMV and EBV serology underwent orthotopic heart and liver transplant from a CMV and EBV positive donor (CMV Donor+/Recipient-, EBV Donor+/Recipient-) at the age of 58 years for cirrhosis secondary to Hereditary Familial Amyloidosis (Thr60A1a mutation). He was on tacrolimus for immunosuppression and post-transplant developed asymptomatic EBV and CMV viremia as well as stage 3 chronic kidney disease secondary to calcineurin inhibitor nephrotoxicity. He was admitted for new-onset hematuria and acute kidney injury. A kidney ultrasound noted a 2 cm hypoechoic lesion in the right inferior liver lobe. A CT scan of the liver revealed multiple indeterminate liver masses (Figure 7A and B). Biopsy of one lesion showed a monotonous proliferation of relatively uniform spindle cells arranged in intersecting fascicles with pale eosinophilic cytoplasm and elongated, blunt-ended nuclei with darkly staining vesicular chromatin (Figure 3G). There was again, no significant cytologic atypia, nuclear pleomorphism, or tumor necrosis. The tumor cells were strongly immunoreactive for smooth muscle actin and showed diffuse nuclear reactivity for EBER (Figure 3H and I). These morphologic and immunohistochemical findings were consistent with a diagnosis of PT-SMT. He subsequently underwent a left lateral liver segmentectomy with additional superficial hepatic wedge resections in segments 4A, 4B, and 8. Pathology showed positive margins. Immunosuppression was initially lowered and eventually discontinued. He was later found to have elevated liver enzymes on follow-up testing and a liver biopsy confirmed severe acute

cellular rejection (ACR). He was treated with steroids and restarted on sirolimus and tacrolimus with resolution of ACR as proven on subsequent biopsy and improvement of liver enzymes on laboratory testing. He also completed a course of valganciclovir for CMV viremia. However, he developed worsening neutropenia with bone marrow suppression, low-grade fevers, altered mental status requiring intubation, and anuria requiring dialysis. He was empirically started on broad-spectrum antibiotics. Infectious workup demonstrated streptococcus Gordonii bacteremia. Despite optimal treatment and supportive measures, his condition deteriorated rapidly and ended up having multi-organ failure, followed by a fatal cardiac arrest.

Case 5: A 63-year-old male with a history of a living-related donor kidney transplant at age 55 for end-stage renal disease of unknown etiology, was maintained on prednisone, MMF, and sirolimus. The patient was hospitalized for community-acquired pneumonia complicated by a parapneumonic effusion necessitating antibiotics and decortication. On CT and subsequent MRI imaging of the abdomen, he was incidentally found to have over 20 cystic hepatic masses, with the largest measuring 12.5 cm x 9.2 cm (Figure 8A). Fine needle aspiration confirmed PT-SMT. EBV DNA quantification was at 4,765 copies per milliliter with no prior pre-transplant levels. He had no evidence of distant or intra-cranial disease on imaging. MMF was stopped and sirolimus was decreased from 1.5 mg to 1 mg daily initially, and down to 0.5 mg one year later. He was also treated with a course of valganciclovir at the time of diagnosis. Annual hepatic MRIs demonstrated initial size reduction followed by stable disease without any new lesions, with the most recent imaging performed at age 72 (Figure 8B). He continues to do well with intact kidney function, despite decreasing his immunosuppression.

Case 6: A 45-year-old female with a long-standing history of HIV (CD 4 count unknown) on anti-retroviral therapy with dolutegravir, abacavir, and lamivudine presented to the primary care clinic with neck, back, and shoulder pain with associated proximal and distal left upper extremity numbness and paresthesia. With worsening symptoms after conservative therapy, she underwent cervical and thoracic spine MRI which showed a 1.9 cm x 1.2 cm x 2.4 cm intradural heterogeneous mass centered within the left T9-T10 neuroforamina, with abutment of the left lateral spinal cord resulting in rightward cord displacement as well as moderate canal stenosis (Figure 9). She underwent T8-T10 Laminectomy with mass excision, with pathology consistent with EBV-SMT. Subsequent PET revealed no FDG avid lesions. Serum EBV viral levels were not obtained and prior seronegative status was also unknown. She did not undergo any further treatment or surveillance and has not had any complications of her disease, now at age 55.

FINAL DIAGNOSIS

EBV-SMT in all six cases.

TREATMENT

Case 1

Immunosuppression reduction.

Case 2

Supportive treatment.

Case 3

Intrathecal rituximab, methotrexate, systemic chemotherapy, whole brain radiation, and T-cell therapy.

Case 4

Left lateral segmentectomy with superficial wedge resection; reduction in immunosuppression, and eventually started given concern for ACR.

Case 5

Reduction in immunosuppression.

Case 6

T8-T10 Laminectomy; no change in HAART regimen.

OUTCOME AND FOLLOW-UP

Cases 2 and 4 died. Cases 1, 3, 5, 6 alive and well with no recurrence in EBV-SMT.

DISCUSSION

Here we report six cases of EBV-SMT, two of which were in pediatric patients with ages ranging from 6-61. There was an equal number of males and females in the cohort. Five out of six cases were PT-SMT while the sixth patient had HIV-SMT. One patient had CVID in addition to being a transplant recipient. Two patients died at the time of diagnosis, though neither death was attributed directly to the SMTs. Three patients in the group had single organ involvement while the rest had multiple organs involved. Two patients with CNS involvement (one with EBV-PTLD and another with HIV-SMT) underwent surgical removal of the tumor without recurrence. Interestingly, one patient in this cohort had both biopsy-proven PTLD and EBV-SMT.

A systemic review by Chen *et al*[15] on EBV-SMT with CNS invasion found that HIV-SMTs have a predilection for the central nervous system. This was seen in our patient with HIV-SMT whose EBV-SMT was found in the thoracic spine. In contrast, patients with PT-SMT have a propensity for extra-CNS involvement, primarily lung and liver as confirmed in a study by Jonigk *et al*[7]. The pathophysiology is unclear but it has been proposed these organ systems are hypervascular and may attract the proliferation of smooth muscle tumors. This is also seen in other lymphoproliferative disorders such as PTLD and non-Hodgkin lymphomas in patients with Acquired Immunodeficiency Syndrome (AIDS). Interestingly, Chen *et al*[15] notice a concomitant lung and liver involvement in patients with PT-SMT. This was true for two of our patients who had both lung and liver SMTs; one patient with solitary lung involvement. This raises the question of whether a liver lesion increases the chance of getting a lung lesion and not vice versa. Regardless, concomitant lung and liver lesions should be kept in mind during workup.

The latency period between either HIV infection or immunosuppression initiation and the occurrence of EBV-SMT is variable. In PT-SMT patients, previous studies have found an average latency period of three years in children compared to four years in adults[7,15]. The latency period in our patients ranged from three to eight years. For HIV-SMT patients, it was more difficult to determine the timeline between HIV infection and diagnosis of AIDS. However, latency time could be as high as 8.5 years[16,17]. For our patient with HIV, we were unable to determine this latency period. In contrast, PTLD can develop at any point after transplant, up to 10 years later, whereby a majority of cases occur within the first year post transplantation[13,14].

Additionally, multiple cases of synchronous or metachronous EBV-SMTs were seen in our patients, consistent with prior publications[15,18,19]. Jonigk *et al*[7] showed that patients with multiple organs involvement had worse overall survival than those with single organ involvement, while individuals with intracranial disease had the worst outcomes. However, in the study by Chen *et al*[15], presence of CNS SMTs, tumor multiplicity, or pre-existing medical conditions did not impact the survival rate. In our case series, one-third of our patients died and had tumor multiplicity in addition to a multitude of chronic medical conditions. These patients did not die directly due to EBV-SMTs but died of the disease while battling other complications. Age differences did not impact survival, raising the question whether age has any role in prognosis. However, it does supplement the theory that the degree of immunocompetency may determine the degree of disease aggressiveness and subsequent survival rates.

EBV-SMTs have been generally thought of as slow-growing tumors with a 1-year overall survival rate of 50%-76% for patients with HIV-SMT and PT-SMT patients and 0% for CI-SMT[8,15]. 5-year survival rate is estimated at 60% for patients with CNS involvement[15]. Patients with HIV-SMTs have been known to have higher survival but with a shorter follow-up period. A separate analysis by Jonigk *et al*[7] suggested that PT-SMT and CI-SMT have better outcomes than HIV-SMT. Four out of six patients in our group continue to live with stable disease or no evidence of disease. It will be prudent to continue follow-up for all EBV-SMTs to help us study the disease course and prognosis over time.

Previous studies have found that pre-transplant EBV seronegativity is a risk factor for EBV-PTLD[20]. However, the role of this factor is not known for EBV-SMT, though it is postulated that pre-transplant seronegativity and post-transplant primary EBV infection could be considered a risk factor for PT-SMT[21]. In our study, EBV status was not known for HIV patient, however, the other transplant patients were seronegative at the time of transplant and became highly seropositive at the time of diagnosis (table 1). It would be interesting to see if any absolute levels of EBV titers have any bearing on the severity of disease course and outcomes.

There is no standardized treatment for EBV-SMT, given its rarity. In the study by Jonigk *et al*[7], patients who underwent surgical resection had similar outcomes to those who underwent reduced immunosuppression alone without any surgery, suggesting that either may be an appropriate strategy. Most patients with CNS involvement undergo surgical resection to alleviate parenchymal tumor compression[15]. No statistically significant difference was seen in outcomes between PT-SMT and HIV-SMT in these patients. Surgical resection to alleviate parenchymal tumor compression in individuals

with CNS involvement is a reasonable strategy.

CONCLUSION

EBV-SMTs are a rare oncological entity found in immunocompromised patients with either primary immunodeficiency as in Congenital Immunodeficiency or secondary immunodeficiency as seen in patients with HIV or post-transplant patients on long term immunosuppression. As the number of patients who undergo organ transplantation increases with time, the incidence of EBV-SMT may also increase. It is imperative to keep EBV-SMT on the differential in immunosuppressed individuals who develop tumors. Questions regarding the best treatment modality remain as patients are treated on a case-by-case basis.

ACKNOWLEDGEMENTS

We owe this publication to the brave patients who harbor Epstein-Barr virus associated smooth muscle tumors and have graciously allowed us to help inform the world about their rare disease.

FOOTNOTES

Author contributions: Khan AA wrote the manuscript; Bassam BN and Esfeh JM conceptualized and proofread the paper; Yalamanchali A and Niang D contributed to the paper; Savage E, Fulmer CG, and Gosnell HL provided input on pathology including preparing slides; and All authors have read and approved the final manuscript.

Informed consent statement: Informed written consent was obtained from all six patients or healthcare power of attorney, where applicable, for the publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare no conflicts of interest.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016). The manuscript was prepared and revised according to the CARE Checklist (2016).

Country/Territory of origin: United States

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S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

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