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EDITORIAL

Pembrolizumab autoimmune related diabetes: Moving forward, keep learning

Jose Angel Garcia, Diego Alcaraz, Esther Holgado, Felipe Couñago

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Abstract

Immune checkpoint inhibitors (and more specifically programmed cell death 1/programmed cell death ligand 1 inhibitors as Pembrolizumab) initiated a revolution in the field of melanoma and have now expanded to several tumor subtypes and in increasingly broader clinical contexts, including the adjuvant and neoadjuvant setting, with potentially curable patients and prolonged survival. The side effects related to these drugs include a wide spectrum of manifestations, with endocrinological adverse events being some of the most frequent. Pembrolizumab-induced type 1 diabetes mellitus is an infrequent but potentially serious and not clearly reversible side effect that possesses characteristic clinical features and has high morbidity and mortality, with a chronic impact on quality of life. The etiopathogenesis of this phenomenom needs to be further investigated and a collaborative effort through the involvement of oncologists and other medical specialists is necessary for the correct identification and management of patients at risk.

Key Words: Immune checkpoints; Pembrolizumab; Immunotherapy; Side effects; Endocrine system; Diabetes mellitus

Core Tip: Pembrolizumab-induced type 1 diabetes mellitus is a rare and potentially serious adverse event of immunotherapy, with a significant number of cases debuting abruptly and in a state of diabetic ketoacidosis without clear predisposing factors. Further research and strict follow-up by oncologists are fundamental tools for prevention and early treatment focusing on reducing the morbidity and mortality associated with this side effect.

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INTRODUCTION

The study published by Bhanderi *et al*[1] in 2023 presents a case report of an oncologic patient on immunotherapy who debuts in a state of rapidly developing diabetic ketoacidosis with low HbA1c levels suggestive of autoimmune diabetes mellitus 1 (DM1) with negative autoantibody studies, which differs from the clinical presentation of classic DM1. This publication suggests that the identification of patients at risk from a genotypic point of view remains unclear based on current literature, highlighting the need for future research to define prognostic biomarkers that may help in the management of these patients.

Immune checkpoint inhibitors (ICI) are currently a standard in daily practice. In the last ten years we have witnessed a revolution that has changed the treatment paradigm for many solid tumors, initiated in 2011 with the Food and Drug Administration (FDA) approval of Ipilimumab (anti-CTLA4) and continued further with the approvals in 2014 of the anti-programmed cell death 1 (PD1)/programmed cell death ligand 1 (PDL1) drugs (Pembrolizumab, Nivolumab). Since the FDA approval in 2014 of Pembrolizumab for advanced melanoma, its use has expanded to more than 20 treatment indications in different solid tumors in 2023, consolidating its position as one of the standard treatments in monotherapy or combined with other drugs, in both disseminated and localized disease (adjuvant and/or neoadjuvant setting)[2,3]. The adverse effect profile of immunotherapy has changed the perspective established by conventional chemotherapy treatments and poses a clinical challenge. In this context endocrinological toxicities are frequent and probably underdiagnosed. Pembrolizumab-induced DM1 is a rare and potentially serious adverse event described in the literature that can have relevant consequences in terms of quality of life.

PD1 inhibitors are associated with approximately 95% of cases of ICI-induced DM1, although incidence is < 1%[4-6]. Pathogenically, it is caused by destruction of insulin-producing pancreatic beta cells, with reports suggesting a deeper and more rapid destruction than in classic DM1. It is unresponsive to corticosteroids and hardly reversible when established[7,8]. The average time to debut varies between 7-17 wk, although there are cases of late development (even months after the end of immunotherapy)[9,10]. It is important to note that 38%-70% of patients who develop this type of DM1 debut in a state of diabetic ketoacidosis with high (but lower than expected) hemoglobin A1C levels, suggesting a rapid development with a sharp drop in insulin secretion[4]. The clinical features of Pembrolizumab-induced DM1 involve acute onset of hyperglycemia, increased frequency of ketosis, rapid decline in C concentrations, and high glycemic variability consistent with the absence of residual beta-cell function[10].

It is because of the high risk of morbidity and mortality that it is important for physicians to be aware of this situation and act accordingly in an early manner[8]. In this context, the European Society for Medical Oncology recommends the monitorization of blood glucose in patients receiving (ICI)[11], while the American Society of Clinical Oncology guidelines recommend measuring it at baseline and with each treatment cycle for 12 wk, and then every 3-6 wk[12].

Since the publication of the first cases of Pembrolizumab-induced DM1 in 2015 we have witnessed a marked increase in reported cases, with > 90 in recent years[13,14]. In our opinion, the growing interest in this field and the efforts of clinicians and researchers should aim towards a stricter follow-up and a better identification of patients with immune-mediated adverse events. However, which group of patients at risk could benefit from a stricter follow-up remains unclear. The work of Magis *et al*[15] in 2018 shows us a prospective study based on the glycemic follow-up of 163 patients under anti-PD1 with a median follow-up of 5.6 months. This study shows the low incidence of this adverse effect (only three patients developed diabetes mellitus with anti-PD1 plus two additional patients in a parallel study with anti-PD1 *vs* Ipilimumab) and also evidences that, in all cases, glycemia prior to treatment was normal, reflecting that glycemia monitoring during treatment may not be sufficient to anticipate this phenomenon. This publication also suggests the potential role of human leukocyte antigen (HLA) determination given that four of the five affected patients were DRB01 03 or 04, (which are known to increase the risk of type 1 diabetes in the general population), noting the role of risk haplotyping to aid in the comprehensive follow-up of these patients. In 2019, Tsang *et al*[16] conducted a retrospective study of 538 melanoma patients treated with anti-PD1 (Pembrolizumab monotherapy and Ipilimumab-Nivolumab combination) over a 3-year period, with ten patients (1.9%) developing potentially immunotherapy-induced diabetes mellitus with Pembrolizumab (six patients) or the combination therapy (four patients). In the ten affected patients a DM1-associated autoantibody test (including anti-glutamic acid decarboxylase antibody, anti-insulin antibody, islet antigen 2 antibody and zinc transporter 8 antibody) was performed, with GADA (20%) being the only positive result in two patients. In addition, HLA typing was performed on the ten affected patients showing that three patients expressed high-risk HLA haplotypes (two patients had DRB104-DQB103:02-DQA103:01, one patient had DRB103:01-DQB102:01-

DQA105:01) , while three patients had an HLA haplotype previously associated with protection against DM1 (one had DRB107:01-DQB103:03-DQA102:01, one had DRB13:01, and one had DRB111-DQB103:01-DQA105:01). This publication concludes that the absence of autoantibodies and lack of clear association with high-risk HLA typing might suggest an entity with its own characteristics. The work of Wu *et al*[17] in 2021 is consistent with the data presented previously and again shows the genetic variability in DM1 cases associated with ICI, including a review of 200 patients of whom 10% had protective haplotypes. This suggests that the association with risk HLA haplotypes appears to be weaker than in DM1 cases and that other factors may be at play, although it appears that the presence of these protective haplotypes was associated with a delayed onset (18 *vs* 9 wk). To note, other authors (Akturk *et al*[18], Clotman *et al*[19]) have reported contradictory results regarding the role of autoimmunity and HLA typing in this under-represented population.

In light of these results and given the low incidence in the published case series, it is difficult to establish with certainty which predisposing factors and the mechanisms are involved. This makes early identification difficult considering that glycemia monitoring as an isolated tool may not be enough. These uncertainties reinforce the idea that joint and collaborative efforts are necessary to understand these mechanisms, correctly identify patients at risk and develop tools that allow for effective follow-up to be established in order to reduce the risk of serious side effects.

CONCLUSION

AntiPD1-PDL1 drugs represent a widely used treatment in oncology, with a different profile of adverse events compared to conventional chemotherapy. DM1 is a rare and potentially serious side effect, with a variable development time and different clinical presentations. The identification of patients at risk remains unclear and more collaborative research is needed due to the small number of subjects affected. According to the main clinical practice guidelines in the world, thorough surveillance by treating oncologists is necessary for early management to help reduce morbidity and mortality, as well as the participation of other medical specialists for an integral management of affected patients.

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EDITORIAL

Core needle biopsy for thyroid nodules assessment-a new horizon?

David D Dolidze, Serghei Covantsev, Grigorii M Chechenin, Natalia V Pichugina, Anastasia V Bedina, Anna Bumbu

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Abstract

Ultrasound-guided fine-needle aspiration is the standard for evaluating thyroid nodules with a high safety profile and a relatively low number of non-diagnostic cytological findings. Nevertheless, this diagnostic method traditionally has its weak points. Several diagnostic categories such as Bethesda I, III and IV are not reliable for thyroid carcinoma risk assessment. Recent advancements in a core needle biopsy made it possible to use this tool as a new method for thyroid nodules evaluation. The main feature of this method is the use of thin needles (18-21G) and guns with an automatic trigger mechanism. The histological material collected with the use of a core needle biopsy is usually superior to cytological. Therefore, the core needle biopsy can be used as a complementary technique to a standard fine needle aspiration in difficult and dubious cases of thyroid neoplasia with uncertain malignant potential.

Key Words: Core-needle biopsy; Thyroid; Follicular tumor; Fine-needle aspiration; Thyroid cancer

Core Tip: Modern oncology is largely based on histopathological examination of preoperative specimens. However, decision to perform thyroid surgery is based on results of fine-needle aspiration cytology. It is considered that core-needle biopsy is an invasive procedure for an abundantly vascularized gland. Advances in technical construction of automatic and semi-automatic guns has led to the possibility of safe and efficient obtainment of thyroid histological specimens before surgery. Ultrasound-guided core-needle biopsy is now a widely implemented procedure in difficult and dubious cases of thyroid neoplasia owing to the advances in thyroid imaging. The latest advances in this field demonstrate that core-needle biopsy is a safe procedure that can decrease the number of unnecessary thyroid surgery and can provide additional information in doubtful cases.

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INTRODUCTION

The incidence of thyroid cancer has increased significantly over the last decades in many countries. The Globocan 2020 incidence rates of thyroid cancer were 10.1 *per* 100000 women and 3.1 *per* 100000 men, while the mortality rates were 0.5 *per* 100000 women and 0.3 *per* 100000 men[1]. The rise in thyroid cancer incidence is mostly attributed to papillary thyroid carcinoma early diagnosis. However, the incidence of stage IV thyroid cancer has increased as well[2]. Of note, papillary thyroid cancer detection has risen by 240% in the last three decades[3]. Of the major histologic types, about 79%-90% are papillary thyroid carcinomas, 4%-5% are follicular thyroid carcinomas, 2%-5% are Hürthle-cell carcinomas, 2%-4% are medullary thyroid carcinomas, and 1%-2% are anaplastic thyroid carcinomas[4,5]. In iodine-deficient countries the incidence of follicular thyroid cancer can be up to 27%-28%, while papillary thyroid cancer is less frequent (47%-53%)[6,7]. In the cases of delayed diagnosis approximately 10%-15% of differentiated thyroid cancer can progress to more aggressive histological types[8].

The diagnosis of thyroid cancer is based on the Bethesda classification system, which plays the central role in interpreting cytology results[9-11]. Fine-needle aspiration has proven to be an efficient, simple and safe method for triage of patients with thyroid nodules[12]. It became the standard diagnostic tool for thyroid nodules in the late 1980s and replaced large-needle biopsy due to its high diagnostic accuracy and low complication rate[13]. However, its use in clinical practice demonstrated several weak points that require strengthening.

The rate of non-informative fine needle aspiration (FNA) is 10%-20% and up to 50% in repeated FNA. However, the FNA diagnostic accuracy depends on the skills of a medical specialist and the pathologist, who interprets the results. As was to be shown, approximately 17%-20% of FNA are classified as insufficient samples[14-16]. Cytological categories Bethesda III and IV do not provide an accurate diagnosis, since they require additional testing or surgery. Moreover, thyroid surgery complication rate is relatively high, while most cases of thyroid follicular neoplasia are benign[17,18]. Additional diagnostic tests are needed to overcome challenges in evaluating thyroid nodules[19].

In the light of these difficulties, large-needle biopsy has re-emerged as a new diagnostic method in the form or core-needle biopsy (CNB). Advanced technical construction of automatic and semi-automatic guns has led to the possibility of safe and efficient obtainment of thyroid histological specimens before surgery. CNB is now a widely implemented procedure used in difficult and suspicious cases of thyroid neoplasia with uncertain malignant potential owing to the progress in thyroid ultrasonography (USG) imaging[20].

CURRENT POSITION PAPERS AND GUIDELINES

Several documents reinforced the use of CNB on a national level. In 2013 and 2017 the Korean Society of Thyroid Radiology published "Core needle biopsy of thyroid nodules: Consensus statement and recommendations"[21,22]. In 2015 the Korean Endocrine Pathology Thyroid CNB Study Group published a positional paper "Pathology Reporting of Thyroid Core Needle Biopsy" [23]. The National Cancer Institute, American Association of Clinical Endocrinologists, American College of Endocrinology, and Associazione Medici Endocrinologi proposed CNB for thyroid nodules with non-diagnostic FNA results and persistently non-diagnostic nodules[22,24,25].

PREPARATION BEFORE THE PROCEDURE

Core needle biopsy is performed under local anesthesia and there is no need for fasting before the procedure. The Korean Society of Thyroid Radiology recommends withdrawal from aspirin and clopidogrel for 7-10 d, warfarin for 3-5 d, and heparin for 4-6 h before the procedure. Initiation of aspirin and clopidogrel bisulfate after CNB is recommended on the next day after procedure followed by heparin administered 2 h later after CNB and warfarin at night[22]. There have been

studies that ultrasound-guided FNA of thyroid nodules can be safely used in patients who receive antiplatelet agents and anticoagulants without increase in adverse events or decrease in diagnostic rate[26]. Similar reports have been published for patients undergoing CNB of other organs[27]. Nevertheless, thyroid gland is a highly vascularized organ and there should be further studies to assess antiplatelet agents and anticoagulants security profile before administering them to the patients undergoing this procedure. Therefore, it seems reasonable that the Korean Society of Thyroid Radiology recommendations should be implemented.

CNB TECHNIQUE

The device for CNB should meet the parameters for the depth of needle penetration, taking into account the size of the mass. The optimal goal is maximum capture of the thyroid mass with minimal damage to the surrounding tissues. As for the thyroid gland, it is optimal to use a needle length of 6-10 cm, with an excursion of 1.5-2.2 cm. An ultrasound probe is insulated with a sterile sleeve, and sterile gel or saline are used to improve conductivity.

The procedure is performed in aseptic conditions. The neck area is disinfected with an antiseptic solution and subcutaneous tissue is infiltrated with local anesthetics. Additionally, it is possible to use non-steroidal anti-inflammatory drugs to reduce pain.

The thyroid gland CNB is performed under ultrasound control with an 18-21G cutting biopsy needle. The size of the needle affects the volume of material obtained; the thinner the needle, the less tissue will be obtained, but it is also related with the lower risk of trauma to the organ and lower complication rate. Therefore, in the majority of cases it is optimal to use 18 G needles. The procedure is performed by trained surgeon and a sonographer using the "free hand" method.

The needle is inserted using ultrasound navigation to the level of the thyroid gland. The needle tip is positioned so it remains on a pre-selected trajectory away from the major vessels (carotid artery and jugular vein) and trachea, and the biopsy needle firing distance is measured. After the procedure, finger pressure is applied to the biopsy area for 20-30 min. There are several ways to collect material: Biopsy of the mass formation itself, marginal biopsy of the mass with the adjacent zone of the capsule and healthy tissue, and a combination of these methods. At least two biopsies of the thyroid nodule are required (Figure 1). The obtained biological material is placed in containers with a 10% formaldehyde solution and they are marked in advance for a subsequent histological and, if necessary, immunohistochemical examination and/or molecular genetic testing.

ULTRASONOGRAPHY-GUIDANCE

The thyroid gland CNB is performed under ultrasonography control with preliminary assessment of the mass blood supply, Thyroid Imaging Reporting and Data System (TI-RADS) score, and identification of potentially malignant areas. Optimal visualization is reached using a linear probe with a frequency of 7-12 MHz. The probe is aimed perpendicular to the skin surface in the projection of the mass. The needle is inserted strictly into the area of interest on the scanning plane. Ultrasound control is carried out parallel to the inserted needle along its length with mandatory visualization of the needle tip. It is necessary that the ultrasound scanning field includes the main vessels (carotid artery and jugular vein) and the trachea in order to avoid their injury. It is possible to assess the penetration distance before firing using the Vernier caliper. After the procedure, a re-evaluation of the mass is performed. The stages of ultrasonography-guidance are presented in Figure 2.

DIAGNOSTIC ACCURACY

A recent meta-analysis that included nine eligible studies, with 2240 patients and 2245 thyroid nodules involved, demonstrated that the pooled proportion for non-diagnostic results, inconclusive results and malignancy was 1.8% (95%CI: 0.4%-3.2%), 25.1% (95%CI: 15.4%–34.9%) and 18.9% (95%CI: 8.4%–29.5%), respectively. The sensitivity of CNB varied, ranging from 44.7% to 85.0%, while the specificity was 100% in all cases[28]. Second CNB in the case of non-diagnostic results provides a definitive diagnosis in almost all cases[29].

Furthermore, as CNB has advantages over FNA in amount of tissue obtained, it is possible to perform immunohistochemistry (IHC) or molecular testing using additional paraffin-embedded tissue sections. A combination of IHC markers consisting of galectin-3, HBME-1, cytokeratin 19 and CD56 is commonly used for the diagnosis of papillary carcinoma [22]. Molecular testing based on the FNA does not always provide adequate results. Rate of false-negative BRAF mutation testing using FNA increases in cases of old age, indeterminate FNA pathology results, and certain thyroid cancer subtypes[30]. The material extracted from CNB is usually sufficient to perform IHC and genetic tests[31,32].

CNB may have its limitation in small thyroid nodules. Some studies demonstrate that superiority of CNB to FNA is found in thyroid nodules larger than 2.0 cm and classified by American College of Radiology as TI-RADS or Korean-TIRADS category 4[33]. CNB is also superior to FNA in cases of possible lymph node metastases[34].

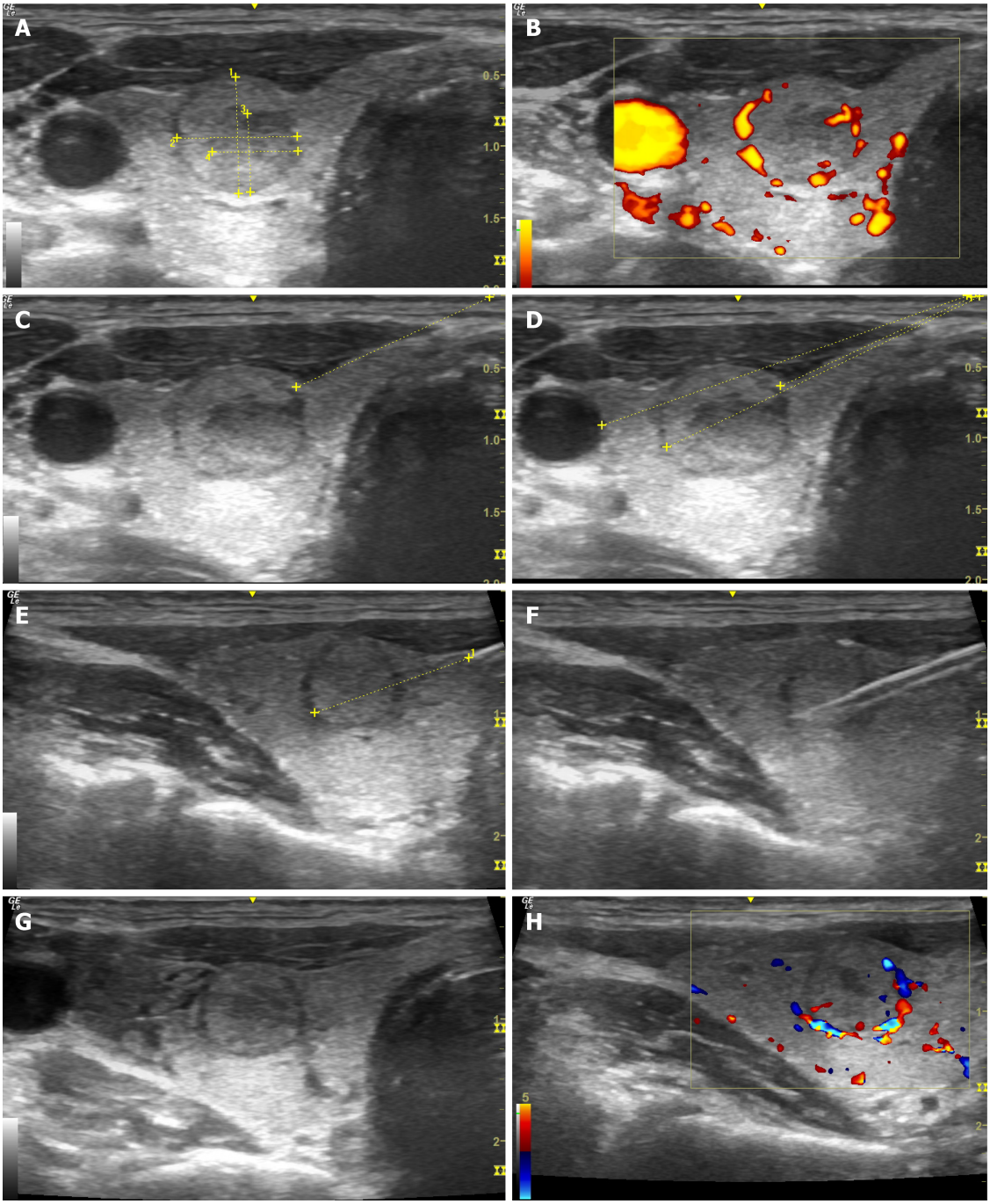

Figure 1 Ultrasonography-guided core-needle biopsy of the thyroid gland. A: Assessment of the size of the mass; B: Assessment in the Doppler mode; C: Marking the optimal trajectory for core-needle biopsy (CNB); D: Calculation of the distance till the mass and major vessels; E: Control of the needle along its length; F: Control during the biopsy; G: Assessment of the mass after CNB; H: Assessment of the node in Doppler mode after CNB.

SAFETY AND COMPLICATIONS

An analysis of 6169 patients who underwent ultrasound-guided CNB demonstrated a complication rate of 0.81% (53 complications, of which only 4 were major). The complications included hematoma, pseudoaneurysm formation, dysphonia, carotid injury, tracheal puncture, dysphagia, oedema, vertebral puncture, and vasovagal reaction[35]. Most of the reported cases of bleeding could be treated with manual pressure[36]. Another study that included 4412 CNB demonstrated that minor complications occurred in 2.2% of CNB, and major in four procedures (0.09%)[29]. As it seems, in high volume centers the complication rate is around 1%-2% and major complications are exceedingly rare.

Treatment tactics for bleeding depends primarily on the blood volume loss and airway patency. In order to assess the degree of surrounding tissues compression, the most optimal method is USG. Decompression can be employed by performing USG-guided puncture or by installing drainage into the bleeding area. In most cases, bleeding stops with conservative therapy. In the case of dyspnea aggravation, uncontrolled hemorrhage or hemodynamic instability, emergency surgical intervention is indicated. Conservative treatment should include antibacterial therapy to prevent abscess formation, and anti-inflammatory therapy to reduce swelling. If a patient condition worsens, emergency

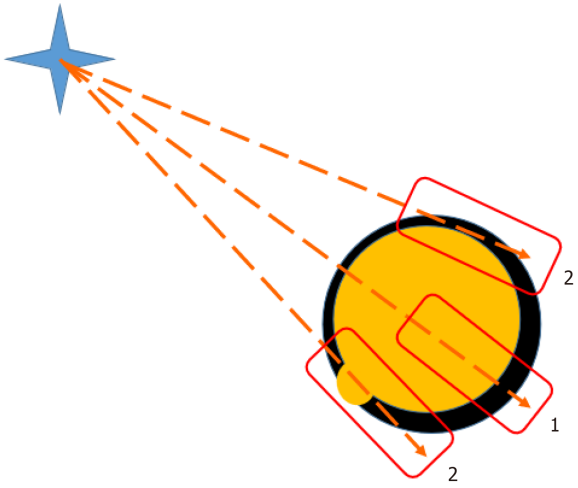

Figure 2 Core-needle biopsy of a thyroid node may be marginal 2 or through the node 1.

intubation to protect airways is indicated and surgical treatment is performed, which most often includes stopping bleeding and hemithyroidectomy[37].

The literature presents isolated cases of transient laryngeal nerve paresis after CNB biopsy of the thyroid gland. In such cases, conservative treatment methods can be used: Drug therapy (neostigmine and other drugs that increase conductivity in the nervous system), exercises with a speech therapist, vocal cord augmentation using botulinum toxin injections and surgery (laryngoplasty, reinnervation)[38].

CONCLUSION

Modern oncology is largely based on histopathological examination of preoperative specimens. Nevertheless, thyroid surgery is carried out on the basis of fine-needle aspiration cytology. It has been presumed that CNB is an invasive procedure for an abundantly vascularized gland. However, latest advances in this filed demonstrate that CNB is a safe procedure that can decrease the number of unnecessary thyroid surgical operations and can provide additional information in undetermined cases.

FOOTNOTES

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EDITORIAL

Bruton's tyrosine kinase inhibitors in primary central nervous system lymphoma: New hopes on the horizon

Leonardo S Lino-Silva, Sabrina B Martínez-Villavicencio, Luisa Fernanda Rivera-Moncada

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Abstract

In this editorial, we comment on the article by Wang *et al*. This manuscript explores the potential synergistic effects of combining zanubrutinib, a novel oral inhibitor of Bruton's tyrosine kinase, with high-dose methotrexate (HD-MTX) as a therapeutic intervention for primary central nervous system lymphoma (PCNSL). The study involves a retrospective analysis of 19 PCNSL patients, highlighting clinicopathological characteristics, treatment outcomes, and genomic biomarkers. The results indicate the combination's good tolerance and strong antitumor activity, with an 84.2% overall response rate. The authors emphasize the potential of zanubrutinib to modulate key genomic features of PCNSL, particularly mutations in myeloid differentiation primary response 88 and cluster of differentiation 79B. Furthermore, the study investigates the role of circulating tumor DNA in cerebrospinal fluid for disease surveillance and treatment response monitoring. In essence, the study provides valuable insights into the potential of combining zanubrutinib with HD-MTX as a frontline therapeutic regimen for PCNSL. The findings underscore the importance of exploring alternative treatment modalities and monitoring genomic and liquid biopsy markers to optimize patient outcomes. While the findings suggest promise, the study's limitations should be considered, and further research is needed to establish the clinical relevance of this therapeutic approach for PCNSL.

Key Words: Primary central nervous system lymphoma; Zanubrutinib; Bruton's tyrosine kinase; Prognosis; Myeloid differentiation primary response 88 gene; Cluster of differentiation 79B gene

Core Tip: The combination of zanubrutinib with high-dose methotrexate shows promise as a therapeutic approach for primary central nervous system lymphoma (PCNSL). In this study involving 19 patients, the treatment demonstrated a notable 84.2% overall response rate with manageable adverse events. The presence of circulating tumor DNA in cerebrospinal fluid emerged as a potential tool for monitoring treatment response. These findings are optimistic, but research in larger patient groups is crucial to validate outcomes and assess long-term effects, especially in different molecular subtypes of PCNSL.

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INTRODUCTION

In this editorial we comment on the article by Wang *et al*[1]. Primary central nervous system lymphoma (PCNSL) is a rare and aggressive subtype of non-Hodgkin lymphoma that primarily affects the central nervous system (CNS), including the brain, spinal cord, and eyes. This malignancy is distinct from systemic lymphomas as it arises and remains confined within the CNS, presenting unique diagnostic and therapeutic challenges. The majority of PCNSL cases are classified as diffuse large B-cell lymphoma (DLBCL), which is often associated with immunocompromised states such as individuals with human immunodeficiency virus/acquired immunodeficiency syndrome or those undergoing immunosuppressive therapy. PCNSL comprises approximately 2%-3% of all primary brain tumors, with an increasing incidence noted in recent years, particularly among the elderly and immunocompromised populations[2]. While the precise etiology remains unclear, immunosuppression, chronic inflammation, and infections such as with the Epstein-Barr virus have been suggested as potential risk factors[3].

PCNSL typically presents with nonspecific neurological symptoms including cognitive decline, focal neurological deficits, and seizure[4]. Ocular involvement, termed primary intraocular lymphoma, may occur concurrently, adding to the complexity of diagnosis[4]. Given the lack of pathognomonic clinical features, the definitive diagnosis often requires brain biopsy, as imaging findings may overlap with other brain lesions[4]. Prognosis in PCNSL is influenced by various factors. Advanced age, immunocompromised status, deep-seated lesions, and elevated lactate dehydrogenase levels at diagnosis are associated with poorer outcomes[5]. Additionally, the blood-brain barrier poses a challenge by limiting the efficacy of systemic treatments, contributing to the overall difficulty in managing PCNSL.

The primary treatment approach for PCNSL involves a combination of high-dose methotrexate (HD-MTX)-based chemotherapy and, in some cases, radiation therapy[6]. HD-MTX, which crosses the blood-brain barrier, forms the backbone of most treatment regimens. The addition of rituximab, an anti-cluster of differentiation 20 (CD20) monoclonal antibody, to chemotherapy protocols has improved outcomes and is now commonly incorporated into treatment strategies. For eligible patients, consolidation therapies such as autologous stem cell transplantation may be considered[6]. Current research in PCNSL focuses on identifying novel therapeutic strategies to enhance treatment efficacy and reduce toxicity. Immunotherapeutic approaches, including chimeric antigen receptor T-cell therapy, are being investigated for their potential in targeting lymphoma cells within the CNS[7]. Additionally, targeted therapies, such as Bruton's tyrosine kinase (BTK) inhibitors, are under exploration, offering promising avenues for future treatment modalities[8]. PCNSL represents a unique and challenging entity within the spectrum of lymphomas. Advances in diagnostic techniques, treatment modalities, and ongoing research are essential for improving outcomes in patients with this rare malignancy.

BTK INHIBITORS AND LYMPHOMAS

Lymphomas, a heterogeneous group of blood cancers, often exhibit dysregulated signaling pathways that contribute to their pathogenesis. One key target for therapeutic intervention in lymphomas is BTK, a crucial enzyme in B-cell receptor signaling. In recent years, the development of BTK inhibitors has shown remarkable promise in the treatment of various lymphoid malignancies.

In the realm of lymphoma therapeutics, inhibitors targeting BTK have emerged as pivotal agents, with notable contenders including ibrutinib, acalabrutinib, and zanubrutinib.

Ibrutinib, which was granted approval for the treatment of chronic lymphocytic leukemia (CLL) and mantle cell lymphoma, has substantiated its efficacy through rigorous clinical trials[9]. Meanwhile, acalabrutinib, indicated for chronic lymphocytic leukemia and mantle cell lymphoma, has garnered attention for its distinctive selectivity and potency, a topic extensively elucidated in the study by Barf *et al*[10]. On a similar note, anubrutinib, specifically designed for adult mantle cell lymphoma, has undergone scrutiny in a phase 1 trial overseen by Tam *et al*[11], shedding light on both its safety profile and clinical effectiveness. Recent investigations have extended the application of BTK inhibitors to PCNSL. Zanubrutinib, in combination with HD-MTX, has emerged as a promising therapeutic avenue for newly diagnosed PCNSL[1]. A notable aspect of these studies involves the exploration of liquid biopsy techniques, particularly the use of circulating tumor DNA (ctDNA) in cerebrospinal fluid. This innovative approach has been proposed as a potential

monitoring tool for treatment response, providing valuable insights into disease surveillance.

BTK is an intracellular tyrosine kinase (non-membrane receptor) composed of 659 amino acids, with its gene located on chromosome Xq21.33-q22. BTK belongs to the tyrosine kinase family known as TEC. The domains that make up the structure of BTK are: a pleckstrin homology domain in the N-terminal region, which facilitates binding to lipid regions of phosphatidylinositol on the plasma membrane; a Src homology 2 (SH2) domain involved in protein-protein interaction binding to phosphorylated tyrosines; an SH3 domain with binding to proline-rich regions, and a C-terminal catalytic domain.

An important point to note is that pharmacological inhibition of BTK activity not only interferes with signaling through the B-cell receptor (BCR) but also with signals from the tumor microenvironment, such as those induced by chemokines and other survival factors for leukemic cells. Thus, BTK inhibitors promote the egress of the leukemic clone from its survival niches in lymphoid tissues, directing it towards cell death.

BTK inhibitors are classified as reversible or irreversible, depending on the site of inhibition on the protein. Irreversible inhibitors covalently bind to the cysteine residue at amino acid position 481 (kinase activity site), blocking adenosine triphosphate (ATP) binding; restoration of activity requires synthesis of new protein. Reversible inhibitors bind tightly to BTK but not covalently, resulting in transient ATP blockade. Irreversible or reversible BTK inhibitors potentially bind to other kinases with lower affinity, particularly those of the TEC family. This binding can lead to side effects and specific toxicity profiles, depending on which and how many kinases are inhibited. Therefore, increasing selectivity for BTK reduces the risk of toxicities.

While the progress in BTK inhibition for lymphomas is encouraging, challenges and questions persist. Further research is warranted to validate these findings in larger cohorts, assess long-term effects, and explore the efficacy of BTK inhibitors across molecular subtypes of lymphomas. The continuous trajectory of research in this domain is characterized by a relentless pursuit of optimizing therapeutic efficacy while minimizing adverse effects, underscoring the dynamic nature of this field.

CLINICAL IMPLICATIONS

The study reports an overall response rate of 84.2%, with a 2-year progression-free survival of 75.6% and an overall survival of 94.1%. The safety profile is described, with a focus on hematological and non-hematological toxicities.

The efficacy results against genotoxic therapy are so successful that the end of chemo-immunotherapy in some neoplasms, including lymphoma, especially for CLL, is now a recognized postulate by leading research groups. Importantly, the manuscript explores the role of ctDNA in cerebrospinal fluid for disease surveillance and treatment response monitoring. Changes in ctDNA levels corresponded to radiographic responses in some patients, highlighting the potential utility of liquid biopsy profiling in PCNSL.

The treatments for PCNSL and DLBCL differ significantly. HD-MTX stands as the primary therapy for PCNSL. Zanubrutinib, a novel oral BTK inhibitor, holds promise as a therapeutic intervention targeting BCR and toll-like receptor signaling pathways. BTKs traverse the blood-brain barrier. This study investigates the oncological outcomes of a combined regimen of methotrexate with zanubrutinib. An advantage of this study is the molecular profiling of tumors, either from paraffin-embedded tissue, peripheral blood, or cerebrospinal fluid. Complete response was achieved in 11 patients, partial response in 5 patients, and disease progression in 3 patients. The overall response rate was 84.2%, with a 5-year overall survival of 94.1%, notably better in cases with a germinal center genotype. Perhaps the most significant point of this article is the observed survival rate, which surpasses the estimated rate in the literature for this lymphoma subtype, typically below 30%. The only potential confounder in this series and its results is the median age of patients (57 years), as age over 60 is an adverse prognostic factor in this lymphoma; however, this median age aligns with literature estimates. One should approach these findings with caution due to the retrospective nature of the study, where the selection criteria for the 19 patients treated with zanubrutinib are not precisely defined. Although inclusion and exclusion criteria were established, it remains unclear whether cases were consecutively recruited and the criteria for initiating treatment with zanubrutinib.

The study's findings have future implications, including the necessity for larger-scale prospective cohort studies and longer follow-up periods to validate the results, and the study underscores the potential of zanubrutinib as a frontline therapeutic regimen for PCNSL, paving the way for further research into optimizing treatment strategies.

CONCLUSION

In summary, BTK inhibitors represent a transformative approach within the landscape of treatments for lymphomas. The evolving understanding of their mechanisms, coupled with developments of innovative applications in PCNSL and liquid biopsy monitoring, signifies a promising era in lymphoma therapeutics. Continued research endeavors are crucial to fully exploit the potential of BTK inhibitors for enhanced patient outcomes.

FOOTNOTES

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EDITORIAL

Feasibility and limitations of combined treatment for lateral pelvic lymph node metastases in rectal cancer

Ying-Zi Zheng, Fang-Fang Yan, Lian-Xiang Luo

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Abstract

Colorectal cancer ranks among the most commonly diagnosed cancers globally, and is associated with a high rate of pelvic recurrence after surgery. In efforts to mitigate recurrence, pelvic lymph node dissection (PLND) is commonly advocated as an adjunct to radical surgery. Neoadjuvant chemoradiotherapy (NACRT) is a therapeutic approach employed in managing locally advanced rectal cancer, and has been found to increase the survival rates. Chua *et al* have proposed a combination of NACRT with selective PLND for addressing lateral pelvic lymph node metastases in rectal cancer patients, with the aim of reducing recurrence and improving survival outcomes. Nevertheless, certain studies have indicated that the addition of PLND to NACRT and total mesorectal excision did not yield a significant reduction in local recurrence rates or improvement in survival. Consequently, meticulous patient selection and perioperative chemotherapy may prove indispensable in ensuring the efficacy of PLND.

Key Words: Rectal cancer; Lateral pelvic lymph nodes metastases; Pelvic lymph node dissection; Neoadjuvant chemoradiotherapy; Total mesorectal excision

Core Tip: Management of lateral pelvic lymph node metastases in rectal cancer patients who receive neoadjuvant chemoradiotherapy and selective pelvic lymph node dissection (PLND), can be effective in reducing recurrence rates and extending survival. However, meticulous patient selection and aggressive perioperative chemotherapy are crucial factors contributing to the success of PLND in this context.

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INTRODUCTION

As one of the most frequently identified types of cancer globally, colorectal cancer is the second greatest source of cancer mortality[1]. Surgery is still the main treatment for colorectal cancer, while there is a high incidence of pelvic recurrence after the procedure, which may be closely related to the perirectal lymph nodes in the mesentery. Research has shown that if total mesorectal is done, the rate of recurrence decreases significantly to 6.5%[2,3]. The recurrence rate of colorectal cancer in a local area is connected to lateral pelvic lymph node metastases (mLLN). According to Gerota and Villemin, a noteworthy lateral lymph flows from the lower rectum to the iliac lymph nodes. It has been reported that 15%-20% of people with locally advanced middle and low rectal cancer experience mLLN, and the treatment of lateral pelvic lymph node disease is quite complex from both an oncological and technical standpoint[4]. Chua *et al*[5] suggested that the best approach to treating mLLN is a multi-modal one, which includes neoadjuvant chemoradiotherapy (NACRT) and selective pelvic lymph node dissection (PLND). Based on some reports, PLND is more advantageous in terms of prognostic survival than conservative or conventional resection in colorectal cancer and mLLN cases. The primary method for predicting mLLN is based on the size diagnosis of the maximum short axis diameter (SAD) of the lymph nodes on computed tomography or magnetic resonance imaging (MRI) after NACRT. However, it has recently been suggested that it is more appropriate to use the initial lateral pelvic lymph nodes (LPN) size rather than the post-chemoradiotherapy (CRT) LPN size as a predictor of mLLN, because in clinical practice, there are many cases where CRT is not performed before surgery for various reasons, such as patient rejection, old age, or complication[6]. Therefore, PLND is advocated as an adjunct to radical surgery for colorectal cancer. There exist different views from East to West regarding the current indications for PLND. Malakorn *et al*[7] recommend using a 5mm threshold for lymph node SAD after neoadjuvant therapy, aiming to diagnose PLND. Chua *et al*[5] proposed that based on neoadjuvant pre-MRI, it would be prudent to select a SAD of 7 mm or the presence of suspicious features as criteria for selective PLND until more reliable data are available. The results from Chinese studies suggested that PLND should be considered for patients with MRI SAD ≥ 7 mm after CRT and poor/signet/mucinous adenocarcinoma. However, for the patients who suffered from LPN enlarged beyond the obturator region or the intrailiac region or involving 3 or more LPN, even PLND was performed after preoperative CRT, the prognosis is still unsatisfactory and poor. Therefore, PLND should be carefully considered for such patients[8]. Studies from Japan proved that the optimal indications for PLND in rectal cancer are the initial lymph node ≥ 8 mm and the distance between the anus and the tumor edge > 5 cm or the initial lymph node ≥ 6 mm and the distance between the anus and the tumor edge ≤ 5 cm[6,9]. Malakorn *et al*[7] showed no evidence of local or distant recurrence of disease after 2 years of follow-up by using minimally invasive robot-assisted PLND to treat patients with persistent lateral pelvic lymph node enlargement after NACRT, which confirmed that Chua *et al*[5] The proposed robotic assistance may be a useful aid for PLND. In addition, studies retrospectively compared the clinical results of elderly and non-elderly patients and concluded that selective lateral PLND after NACRT in elderly patients with locally advanced rectal cancer was safe[10]. Lateral PLND may improve the tumor prognosis in some rectal cancer patients but may adversely affect the functional prognosis. Cribb *et al*[11] compared the functional outcomes of lateral PLND patients with non-lateral PLND patients and concluded that lateral PLND is associated with male sexual dysfunction compared to standard surgical resection. Another study indicated that additional lateral PLND use not only increased postoperative complications, urinary dysfunction, and sexual dysfunction, but also did not improve recurrence rates or enhance long-term survival [12]. Law *et al*'s research revealed that lateral PLND patients did not have a statistically significant decrease in 3-year and 5-year local recurrence rates in comparison to non-lateral PLND patients (P = 0.10 and P = 0.12, respectively)[13]. Additionally, there was no meaningful change in overall survival rate for 3-year and 5-year periods (P = 0.81 and P = 0.57, respectively). Therefore, the addition of lateral PLND to NACRT and total mesorectal excision (TME) did not have a substantial effect on local recurrence rates or survival. The study by Zhou *et al*[14] also illustrated that patients who experienced pathological lateral lymph node metastasis and received TME + lateral PLND after NACRT still showed a higher overall recurrence rate after surgery. To understand the role of PLND in low rectal cancer, further studies are required. Both NACRT and lateral PLND have the potential for residual tumors, and adjuvant chemotherapy (ACT) after radical surgery can help eliminate micrometastases, thus preventing distant metastasis and improving prognosis. Jiang *et al*[15] explored the effectiveness of ACT in the new era of intensive local therapy (*i.e.*, post-NACRT TME and lateral PLND in patients with clinically suspected mLLN, and the results showed that the efficacy of adding ACT to TME combined with lateral PLND after NACRT was not confirmed in patients. Besides, patients with age ≥ 64 years and those with ypStage 0 may not receive benefit from ACT after NACRT followed by TME plus PLND. Therefore, it is necessary to further explore the effectiveness of ACT in clinically suspected mLLN patients.

CONCLUSION

The ideal management of mLLN in rectal cancer patients requires a comprehensive approach involving NACRT comb-

ined with selective PLND. Criteria for the latter include SAD in lymph nodes after neoadjuvant use of 5 mm, SAD in lymph nodes as displayed on pre-neoadjuvant MRI of 7mm, or any suspicious features. To guarantee the efficacy of lateral PLND, careful patient selection and thorough perioperative chemotherapy must be used.

FOOTNOTES

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EDITORIAL

Navigating breast cancer brain metastasis: Risk factors, prognostic indicators, and treatment perspectives

Jayalingappa Karthik, Amit Sehrawat, Mayank Kapoor, Deepak Sundriyal

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Abstract

In this editorial, we comment on the article by Chen *et al*. We specifically focus on the risk factors, prognostic factors, and management of brain metastasis (BM) in breast cancer (BC). BC is the second most common cancer to have BM after lung cancer. Independent risk factors for BM in BC are: HER-2 positive BC, triple-negative BC, and germline *BRCA* mutation. Other factors associated with BM are lung metastasis, age less than 40 years, and African and American ancestry. Even though risk factors associated with BM in BC are elucidated, there is a lack of data on predictive models for BM in BC. Few studies have been made to formulate predictive models or nomograms to address this issue, where age, grade of tumor, HER-2 receptor status, and number of metastatic sites (1 *vs* > 1) were predictive of BM in metastatic BC. However, none have been used in clinical practice. National Comprehensive Cancer Network recommends screening of BM in advanced BC only when the patient is symptomatic or suspicious of central nervous system symptoms; routine screening for BM in BC is not recommended in the guidelines. BM decreases the quality of life and will have a significant psychological impact. Further studies are required for designing validated nomograms or predictive models for BM in BC; these models can be used in the future to develop treatment approaches to prevent BM, which improves the quality of life and overall survival.

Key Words: Breast cancer; Brain metastasis; HER2 positive; Metastatic breast cancer; Risk factors, Predictive models

Core Tip: Breast cancer brain metastasis management faces many challenges. Key risk factors include HER-2 positivity, triple-negative subtype, and germline *BRCA* mutation. Limited predictive models emphasize the need for validated nomograms. Current guidelines recommend screening when symptomatic. Chen *et al* highlight HER-2 and triple-negative associations, impacting treatment strategies. Proactive research is crucial for preventive strategies, blood-brain barrier-penetrating therapies, and validated predictive models.

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INTRODUCTION

Breast cancer (BC) is one of the three most common cancers in women, apart from lung cancer and colorectal cancer. BC accounts for 30% of all cancer in women and is the leading cause of death in the age group between 20 to 59 years. At the same time, survival of BC is about 90%[1]. BC is the second most common cancer to metastasize to the brain after lung cancer; about 30%-50% of metastatic BC (MBC) develop brain metastasis (BM)[2]. The incidence of breast cancer brain metastasis (BCBM) has increased in recent times; reasons could be due to the increased survival rate of MBCs, the inability of some drugs to cross the brain barrier, the use of targeted agents to control systemic disease[3] and the use of newer imaging techniques to diagnose the central nervous system (CNS) metastasis.

BM significantly reduces the quality of life and overall survival (OS) and poses significant challenges in the treatment. Median OS of BCBM ranges around 4.5 to 13.8 months. Factors influencing the survival include patient age, Karnofsky performance status > 70, single BM, size of BM < 5 cm, and control of extracranial disease.

Considering the challenges in the management, poor quality of life, and poor OS with BCBM, there is a need for an hour to formulate a nomogram or identify the risk factors associated with BM and prevention of it. Future research should focus on systemic therapy, which can penetrate the blood-brain barrier and target metastatic lesions. In our discussion, we focus on elucidating risk factors associated with BM and prospects for managing BM in BC.

In the recent issue of *World Journal of Clinical Oncology*, Chen *et al*[4] published a retrospective analysis of clinicopathological features and prognostic factors of BCBM. The study involved 68 patients with BCBM, treated between 2000 and 2022; these BCBM patients were matched with BC patients without BM in the ratio of 1:2. Cohort consisted of 19.1% of luminal A subtype, 32.2% of luminal B subtype, 20.6% of HER-2 overexpressing subtype, 20.6% of triple-negative BC (TNBC) subtype. The median age of diagnosis of BC was 47 years. The median age from diagnosis of BM in BCBM was 50.5 years. The incidence of BM in the study population was 4.42%, and the prevalence of BM at the time of diagnosis was 0.089%. The cumulative incidence of BM in stage IV was 10.3%. The median time from diagnosis of BC to development of BM was 33.5 months (0-181). In multivariate analysis, stage III/IV tumor at initial diagnosis [hazard ratio (HR): 5.58, 95% confidence interval (CI): 1.99-15.68], lung metastasis (HR: 24.18, 95%CI: 6.40-91.43), and HER2-overexpressing and TNBC were significantly associated with BM and were more prone for BM in BCBM. The presence or absence of bone metastasis, molecular type, and presence or absence of neurological symptoms were significantly associated with the prognosis of patients with BCBM. These results were consistent with previous population-based studies regarding the incidence of BM in BC patients (5.1%) and molecular subtype association with BM in BC patients. In contrast, the cumulative incidence of BM after diagnosis of MBC was higher in previous studies A retrospective study by Darlix *et al* [5]. involving 16703 MBC from French epidemiological strategy and medical economics reported that 7.2% of the study population had BM at the time of diagnosis of MBC, and 24.6% of the study population developed BM during follow-up, median time 17 months for BM. Differences in these studies can be attributed to smaller sample size and inclusion of earlier stages of BC in Chen *et al*[4]. When considered among the early-stage BC, the incidence of BC was similar to previous studies. A study by Pestalozzi *et al*[6], aimed at identifying BC patients at risk of BM in the trial of an international BC study group, showed 10-year cumulative BM incidence was 5.2% and significantly associated with lymph node (> 4, 2.2%; $P < 0.01$), HER-2 expression (2.7%, $P < 0.01$), large tumor size (1.7%, $P < 0.01$) and ER-negative tumors (2.3%, $P < 0.01$) in univariate analysis. Similar observations were made in Chen *et al*[4] study.

The study highlighted the HER-2 overexpression in BCBM. In the study population, HER-2 overexpression was seen in 20.6%, which was similar to other previous studies, and it was statistically significant with BCBM in both univariate and multivariate analysis. HER-2 is an oncogene that encodes 185-kDa transmembrane glycoprotein receptor with intracellular tyrosine kinase activity and belongs to the epidermal growth factor receptors family. These receptors are involved in intracellular signal transduction pathways such as PI3K/Akt, Ras/MEK/ERK, and JAK/STAT, which control epithelial cell growth, angiogenesis, migration differentiation, and survival. In humans, HER-2 overexpression is seen in 25% of BCs; overexpression is mainly attributed to HER-2 gene amplification and HER-2 signaling pathways. HER-2 overexpression association with CNS metastasis in BC has been shown in both pre- and post-trastuzumab era, indicating biological predisposition of HER-2 positive BC for BM and increased survival of HER-2 positive BC with the use of trastuzumab. The study by Chen *et al*[4] was unique in that it included cohorts from the pre-trastuzumab and post-trastuzumab eras in China. Among 68 patients, 52.9% received HER-2 targeted therapy, and the median OS of the trastuzumab arm was 17 months, which was similar to the reported median OS in previous studies in the range of 13.1-

17.5 months[7-9] and the highest for capecitabine + tyrosine kinase inhibitor (TKI) arm of 54 months. However, the study did not highlight the secondary prevention of BM in patients receiving HER-2 targeted therapy without BM. Western studies have shown a longer median time to BCBM with trastuzumab (15 months *vs* 10 months, $P = 0.035$) and capecitabine + TKI[10,11]. These findings could have helped us understand the disease biology and design clinical trials to prevent BM.

TNBC accounts for 15% to 20 % of all BC diagnosed worldwide, more commonly seen in young age and older African American women[12-14]. TNBC is a highly aggressive variant lacking ER/PR and HER-2 expression. Due to the aggressive nature of the disease, distant metastasis is seen in the early stage of TNBC. Almost one-third of patients with TNBC will develop BM[15]. BM occurs early in the course of the disease compared to HR-positive and HER-2-positive BC [5,16]. Even early-stage BC treated with curative intent has distant recurrence within five years and is more prone to metastasis to the brain, liver, lungs, and other organs. In a retrospective study of 2448 patients involving stage I to stage III BC, the cumulative incidence rate of developing BM as the first site of recurrence at five years was 2.8%, 4.6%, and 9.6% among patients with stage I, II, and III disease, respectively ($P < 0.0001$)[17]. Similarly, the incidence of BM in TNBC at the time of diagnosis of MBC is around 14%. Recent studies have shown that the cumulative incidence rate of BM in TNBC after diagnosis of MBC continued to increase over the period[5,18,19]. Similar observations were made by Chen *et al*[4], where TNBC accounted for 20.6% with an incidence rate of 20%-30% and was significantly associated with BM in multivariate analysis[HR: 4.34 (1.55-12.11), $P = 0.005$] and had a shorter survival time of 8 months. Management of TNBC with BM poses a unique challenge as targeted therapy and hormonal therapy, which are used in HER2 and HR-positive subtypes, are ineffective, and only a few systemic chemotherapy agents can penetrate the intact blood-brain barrier. Considering the aggressive nature of the disease and challenges in the management of BM, OS, and prognosis are poor for TNBC when compared to HR-positive and HER-2-positive tumors[18,19].

Data are lacking in the secondary prevention of BM in MBC and routine screening for BM in MBC. As BM is associated with poor survival and poor quality of life, identifying the early metastatic brain lesion before the appearance of symptoms and managing it with its local therapy would improve the quality of life and survival; this approach showed significant differences in small cell lung cancer (SCLC) of the lung.

Data still need to be included, and only a few studies have been done in this regard to address occult BM. A study involving 155 screening imaging studies from four clinical trials conducted between 1998 and 2001 showed occult BM in 14.8% of patients. These clinical trials excluded established BM. Survival among patients with occult BM and symptomatic BM were similar. Clinical trials are ongoing to evaluate the role of periodic magnetic resonance imaging brain screening and its benefit in terms of quality of life and survival outcomes (NCT03881605, NCT04030507, NCT03617341).

The success of prophylactic cranial radiation (PCI) in SCLC, which prevented BM, was partly due to the high incidence of BM with no PCI intervention in SCLC (59%-67%).

However, in metastatic breast lesions, overall incidence is about 30%-50%, lowest for ER/PR subtype and highest for HER-2 positive and TNBC. Data are lacking on the benefits of PCI in MBC. Murine models and extrapolating these to computational models have shown promising results of the PCI role[20,21]. With recent advances in hippocampal sparing whole brain radiation therapy, which has shown a lesser incidence of neuropsychological adverse events, a newer approach is needed to prevent and treat HER-2/TNBC.

One such approach is identifying the high-risk individuals who will benefit from PCI in MBC. Predictive models are needed to determine the patients with MBC who will develop BM and benefit from PCI. Such an approach to formulating predictive models for identifying BM was used by Graesslin *et al*[22] in 2011, who made a nomogram to predict the risk of BM in MBC. The study involved 2136 patients of MBC, out of which 362 patients developed subsequent BM. In multivariate analysis, age, grade of the tumor, ER/PR negative and HER-2 positive status, a number of metastatic sites (1 *vs* > 1), and short disease-free survival were all independent factors associated with BM in MBC. Race, primary tumor size, and nodal stage were not significantly associated with BM in MBC. A nomogram was made using this data, and to test the accuracy and performance of the model, discrimination and calibration metrics were quantified. In the external validation set, discrimination was good, with an area under the curve (AUC) of 0.74 (95%CI: 0.70-0.79), and the calibration of the set showed no significant differences between the probabilities of observed and predicted probabilities of BM ($P = 1$). Genre *et al*[23] validated this predictive model by retrospective analysis and the study included 70 MBC patients. Multivariate analysis showed that risk factors associated with BM in MBC were HER-2+ status, TNBC subtype, and number of extracranial metastases. Validation cohort characteristics were similar in age, histological type, immunohistochemical subtypes, and number of brain metastases. Quantified discrimination and calibration were comparable with Graesslin's nomogram model [AUC of 0.695 (95%CI: 0.61-0.77) *vs* AUC of 0.74 (95%CI: 0.70-0.79)][22]. Such predictive models are required in the future to identify the high-risk individuals who are likely to develop BM and design the trail to evaluate the role of PCI or chemotherapy/targeted therapy/immunotherapy. Temozolomide has shown promising results in the secondary prevention of BM in the metastatic breast in HER-2 positive subtype cancer in the phase I trial, and further evaluation is ongoing in phase II[24-26].

CONCLUSION

Management of BCBM is a growing challenge; future research is needed to formulate predictive models for BM in BC, overcome the difficulties of drug transport through the blood-brain barrier, and early intervention of asymptomatic BM. Research focusing on these topics would significantly reduce BM's burden and improve survival and quality of life in BCBM.

FOOTNOTES

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EDITORIAL

Colorectal cancer: Getting the perspective and context right

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Abstract

Colorectal cancer (CRC) is a significant global health burden, being the third leading cancer globally. Its incidence has been observed to be higher in developed regions such as North America and Europe with geographical variations in mortality rates. Efforts to address this disease burden include promoting early detection through screening and implementing treatment strategies to improve patient outcomes. With the growing and aging population, the incidence of CRC will undoubtedly increase. These epidemiological trends will mean that healthcare professionals will increasingly encounter CRC in more complex patients. Hence, it becomes imperative to have a deeper appreciation of the pathophysiology of CRC and understand the intricate interplay between a patient's physiology and their goals of care before offering treatment. This review article will aim to encapsulate the important nuances and perspectives of managing this disease in the context of an elderly patient.

Key Words: Colorectal cancer; Cancer epidemiology; Management; Holistic care; Screening

Core tip: Colorectal cancer (CRC) is a global health concern, especially among older individuals. A holistic, patient-centered approach, incorporating a multidisciplinary approach and shared decision-making is crucial for optimal management. Continued research and collaboration are vital for advancing knowledge and improving patient care in CRC treatment and prevention.

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INTRODUCTION

Colorectal cancer (CRC) represents itself as a significant global health burden, being the third leading cancer globally with approximately 1.14 million new cases and 538136 deaths in 2020[1]. Its incidence has been observed to be higher in developed regions such as North America and Europe, while lower rates have been observed in Africa and Asia[2]. There also appears to be geographical variations in mortality rates, most notably higher in low and middle-income countries due to limited access to public healthcare services[3-5]. Efforts to address this burden include promoting early detection through screening and implementing treatment strategies to improve patient outcomes.

CRC is strongly associated with aging, with incidence rates increasing substantially in older populations. Around 87% of cases are diagnosed in individuals aged 50 or older[6] with Zaki *et al*[7] finding that the incidence of CRC increases sharply after the age of 50. These findings underscore the significance of age as a major risk factor and emphasize the importance of early detection efforts through age-appropriate screening to mitigate the disease burden.

As the population of a country increases, so will the incidence of CRC. These epidemiological trends will mean that healthcare professionals will increasingly encounter CRC in more complex patients. Hence, it becomes imperative to have a deeper appreciation of the pathophysiology of CRC and understand the intricate interplay between a patient's physiology and their goals of care before offering treatment. As the population continues to grow and age, the burden and complexity of CRC patients will undoubtedly increase, further emphasizing the importance of a comprehensive and multidisciplinary approach to its management.

While certain risk factors for CRC such as the male gender, smoking and processed meat consumption are well-established in available literature, other risk factors lack robust data mostly being from observational studies. Pinheiro *et al*[8] has rightly addressed controversies surrounding the efficacy of certain interventions in reducing CRC risk such as vitamin supplements and dietary fiber. In particular, the popular notion of dietary fiber being a protective factor for CRC is not supported by robust scientific evidence with several studies yielding conflicting results. In the meta-analysis by Park *et al*[9] it was found that dietary fiber intake was not significantly associated with CRC risk. Similarly, Aune *et al*[10] found no significant association between dietary fiber intake and CRC risk in both men and women.

However, health promotion campaigns in certain countries still focus on dietary fiber as a protective factor against CRC. The Dietary Guidelines for Americans has reinforced the role of diet and increasing dietary fiber intake as important determinants of CRC prevention[11]. While dietary fiber is important for digestive health, the evidence to CRC prevention is lacking. It becomes important to take a step back to reconcile new perspectives and promote scientifically backed recommendations in healthcare policies and guidelines.

The implementation of policies to promote a healthy lifestyle and diet can prove itself to be a colossal task. Initiatives that focus on diet and exercise may face resistance due to entrenched societal norms and economic disparities. Furthermore, changing behaviors often rely on individual choices which are easily influenced by complex interactions between psychological, social, economic, and environmental factors. This makes it difficult to enforce policy changes solely through government mandates. Encouraging individuals to adopt and maintain healthy behaviors will require a multifaceted approach that can adequately address the aforementioned factors. Despite these challenges, some countries have implemented successful programs to promote healthy lifestyles. For example, Finland's North Karelia Project was first implemented in 1972, focusing on reducing cardiovascular disease risk factors through community-based interventions. This included diet modification, smoking cessation, and exercise. It achieved significant reductions in cardiovascular disease mortality rates over the years, demonstrating the feasibility and effectiveness of population-level interventions[12]. This highlights the potential impact of nation-directed policies although caution must be taken to interpret their success as it often hinges on large-scale collaborative efforts across multiple stakeholders.

CRC screening is of paramount importance due to its ability to detect precancerous lesions and early-stage cancer. This can significantly reduce mortality rates if treated early[13]. Studies have consistently demonstrated the efficacy of screening modalities such as colonoscopy, fecal occult blood tests, and sigmoidoscopy in reducing both incidence and mortality rates of CRC. The European Randomized Study of Screening for Colorectal Cancer and the National Health Service Bowel Cancer Screening Program in the United Kingdom have demonstrated significant reductions in CRC mortality rates through screening programs[14,15]. Most recently, the Nordic-European Initiative on Colorectal Cancer trial revealed an 18% relative risk reduction of CRC when comparing individuals who received a screening colonoscopy to individuals who had no screening[16]. However, achieving a perfect screening program for CRC remains elusive due to various challenges. These include issues related to accessibility, adherence, and cost-effectiveness. Moreover, disparities in healthcare access and resources contribute to the level of screening uptake and effectiveness across different populations and regions.

Hence, a more disruptive way of CRC prevention needs to be devised and implemented. Perhaps inspiration can be drawn from the strategy of human papilloma virus (HPV) vaccination for cervical cancer. Just as HPV vaccination targets the viral infection central to the pathogenesis of cervical cancer, chemo-preventive agents for CRC could focus on inhibiting inflammatory pathways[17], modulating gut microbiota dysbiosis[18], or targeting key signaling pathways in gene mutations that causes malignant transformation[19]. By leveraging on these strategies and incorporating innovative approaches targeting CRC risk factors, there is potential to significantly reduce the burden of CRC and improve public

health outcomes.

Efforts in medical care have primarily been focused on the pathology of CRC. However, a holistic assessment of a patient diagnosed with CRC is essential before deciding on the subsequent medical management of their disease. This allows for the optimization of medical outcomes and provides patient-centered care. This approach considers not only the pathology of the disease but also the patient's physiological status, psychosocial needs, and personal preferences. This can translate to lower rates of unnecessary imaging tests and invasive procedures in CRC patients, leading to cost savings and better patient experiences[20]. This approach emphasizes shared decision-making, respect for patient autonomy, and tailoring treatment plans to align with patients' goals and preferences. It has been shown that comprehensive assessment and tailored management plans can result in better patient outcomes and satisfaction[21]. Multidisciplinary team meetings, where patient cases are discussed comprehensively can lead to improved decision-making and reduced mortality rates in CRC patients[22]. Furthermore, in an increasingly aging population with longer life expectancies, care will become increasingly complex due to the higher prevalence of comorbidities and the need for individualized care plans. Global life expectancy has increased steadily, and this highlights the importance of addressing the unique needs of patients in CRC management.

CONCLUSION

In conclusion, the future of CRC surgery has only just begun, and it is imperative for healthcare professionals to be equipped with the latest knowledge so that they can work in a transdisciplinary team that aspires to deliver the best possible care. This review article has summarized the key issues and principles in appreciating the context of treating CRC in the elderly. We hope that this article can serve as a platform to stimulate more research and collaboration in the near future.

FOOTNOTES

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MINIREVIEWS

Receptor tyrosine kinase-like orphan receptor 1: A novel antitumor target in gastrointestinal cancers

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Abstract

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a member of the type I receptor tyrosine kinase family. ROR1 is pivotal in embryonic development and cancer, and serves as a biomarker and therapeutic target. It has soluble and membrane-bound subtypes, with the latter highly expressed in tumors. ROR1 is conserved throughout evolution and may play a role in the development of gastrointestinal cancer through multiple signaling pathways and molecular mechanisms. Studies suggest that overexpression of ROR1 may increase tumor invasiveness and metastasis. Additionally, ROR1 may regulate the cell cycle, stem cell characteristics, and interact with other signaling pathways to affect cancer progression. This review explores the structure, expression and role of ROR1 in the development of gastrointestinal cancers. It discusses current antitumor strategies, outlining challenges and prospects for treatment.

Key Words: Receptor tyrosine kinase-like orphan receptor 1; Gastrointestinal cancers; Therapeutic target; Molecular mechanisms; Antitumor strategies

Core Tip: Delve into a comprehensive review spotlighting receptor tyrosine kinase-like orphan receptor 1 (ROR1) pivotal involvement in gastrointestinal cancer advancement and its promising prospects as an anti-tumor remedy. Explore pivotal cellular pathways governing ROR1 regulation and pertinent insights into existing commercial therapeutic offerings. Emphasizing ROR1's crucial role in gastrointestinal cancer treatment, this review offers a nuanced blend of historical context and scientific insight, illuminating the dynamic landscape of this evolving field.

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INTRODUCTION

Receptor tyrosine kinase-like orphan receptor (ROR) 1 and ROR2 belong to the ROR subfamily of type I receptor tyrosine kinase (RTK) family. They were first identified in human neuroblastoma cells in 1992 and are termed orphan receptors as their ligands are not known[1-3]. The two proteins share 58% sequence identity. ROR1 has recently been shown to be involved in embryonic development and cancer. It is recognized as a diagnostic biomarker and is a research hotspot for the targeted treatment of various malignancies. ROR1 has two subtypes; an intact membrane receptor and a truncated variant. The truncated versions include a membrane-bound form that lacks an extracellular domain and a soluble form that contains only the extracellular structure[4]. Expression of the soluble isoform does not increase in cancer patients, showing minimal or undetectable levels in the serum that are not dependent on disease progression or severity, whereas expression of the intact membrane-bound isoform increases significantly[5].

ROR1 proteins are found in a variety of species. Drosophila melanogaster has a protein called Dror, which resembles an RTK. The kinase and structural extracellular domains of Dror display 61% and 36% sequence identity, respectively, with the corresponding domains of human ROR1[6], while human and mouse ROR1 are 97% identical. This evolutionary conservation of ROR family proteins indicates the importance of their physiological functions during embryonic and organ development. Many recent studies on ROR1 have demonstrated its aberrant expression in various forms of cancer. Abnormal expression of ROR1 was first observed in chronic lymphocytic leukemia (CLL)[2,7]. Although specific disease-related mutations have yet to be identified in ROR1, it has become a widely studied therapeutic target for localized ischemia, diabetes, and malignancies[8-11].

Gastrointestinal cancers, including liver, gastric, colorectal, and pancreatic cancer, are some of the most frequently diagnosed tumors and contribute significantly to cancer-associated mortality in China and worldwide. Overexpression of ROR1 is seen in many gastrointestinal cancers, where it is linked with poor prognosis, indicating the essential roles of ROR1 in these tumors. This paper reviews the structure and expression patterns of ROR1 in gastrointestinal cancers, as well as its involvement in cancer development. Current antitumor strategies for gastrointestinal cancers involving ROR1 are discussed and the challenges and prospects in treating these diseases are summarized.

STRUCTURE AND EXPRESSION PATTERNS OF ROR1

ROR1 is a type I transmembrane protein, 937 residues in length, with a molecular weight of approximately 105 kDa. The protein includes extracellular, transmembrane, and intracellular regions (Figure 1). The extracellular region includes several extracellular domains, specifically, immunoglobulin-like (Ig-like), frizzled (FZD), and Kringle (KD) domains. FZD is a cell membrane receptor for the secreted glycoprotein Wnt. It contains a cysteine-rich domain located at the N terminus outside the cell that can bind to Wnt. The FZD domain regulates nonclassical Wnt signaling through interaction with its ligand, Wnt5a, while KD mediates interactions between ROR1 and other receptors, including ROR2. The intracellular domains include three domain types; one tyrosine kinase (TK)-like and two serine/threonine-rich domains, together with a proline-rich domain. The serine/threonine-rich domains bind to bridging proteins such as 14-3-3ζ to prevent apoptosis[12], while the proline-rich domain binds to Src homology 3 (SH3) domains in various proteins, such as hematopoietic lineage cell-specific protein 1 (HS1), dedicator of cytokinesis protein 2 (DOCK2), and cortactin, to promote cell migration and proliferation[13-15]. However, although ROR1 is an RTK, the function of its TK domain remains essentially unknown.

ROR1 expression is primarily limited to embryonic development with low levels seen in postnatal tissue. Nevertheless, some normal tissues, including adipose tissue, endocrine glands, and the gastrointestinal tract, display high levels of ROR1 expression[16,17]. ROR1 knockout is fatal in the embryo, providing evidence of its crucial role in embryonic development[18]. In contrast to the relatively low or minimal expression observed in normal tissues, ROR1 expression increased markedly in a variety of hematological cancers and solid tumors, including CLL, breast cancer, melanoma, and gastrointestinal cancers, such as colorectal cancers and pancreatic ductal adenocarcinomas[19-22]. This feature suggests that ROR1 may be a promising candidate for cancer therapy.

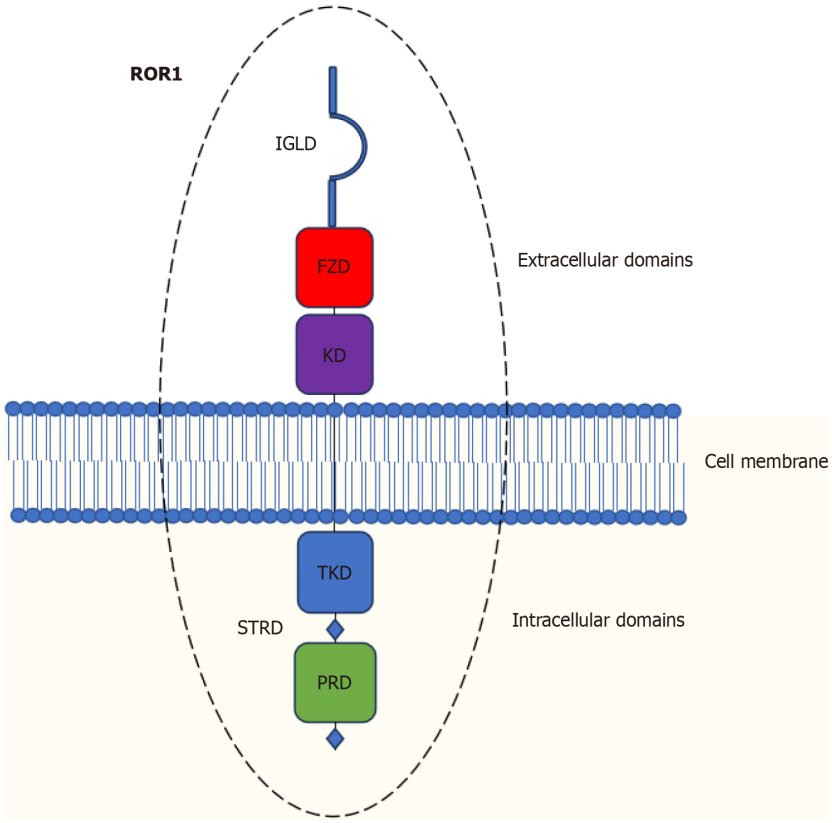

Figure 1 Diagram of the receptor tyrosine kinase-like orphan receptor 1 domain structure. IG: Ig-like domain; FZD: Frizzled domain; KD: Kringle domain; TKD: Tyrosine kinase domain; S/T: Serine/Threonine-rich domain; PRD: Proline-rich domain.

REGULATORY MECHANISMS OF SIGNALING PATHWAYS INVOLVED IN ROR1

Aberrant expression of ROR1 protein in CLL cells was identified using monoclonal antibodies[19]. These authors also found that Wnt5a bound specifically to ROR1 to promote cancer growth and survival, thus ending the orphan status of ROR1. Wnt5a has been shown to activate the non-classical Wnt pathway through interaction with the FZD domain of ROR1[18,23], indicating the potential importance of ROR1 in various biological processes through non-canonical Wnt signaling. Wnt5a is also involved in phosphorylation of the Nuclear Factor-kappa B (NF-κB) subunit p65, activating NF-κB signaling in cancer cells, and thus promoting tumorigenesis, the epithelial-mesenchymal transition (EMT), and metastasis. NF-κB signaling is also closely linked with both inflammation and immune regulation, and shows significant activation in various tumor types.

The Wnt5a pathway is a vital component of the Wnt signaling pathway family and plays a significant role in cancer progression. It affects cell behaviors such as proliferation, differentiation, and migration, which can impact cancer development. Dysregulation of this pathway has been linked to various cancers. By regulating cell cycle and migration, it restricts cancer cell growth and invasion. Additionally, it may impact cancer stem cell characteristics and immune responses within the tumor microenvironment. Furthermore, it interacts with neighboring tissues, promotes angiogenesis, and induces inflammation. Its functions differ among various types of cancer, leading to investigations into its regulatory mechanisms as potential therapeutic targets.

The FZD gene family includes seven transmembrane proteins that are receptors for Wnt ligands. FZD5 appears to be the receptor for Wnt5a. As shown in Figure 2, Wnt5a can activate both FZD5 and ROR1, leading to activation of dishevelled 2/3 (Dvl2/3) and protein kinase B (Akt) phosphorylation. This leads to Akt-mediated phosphorylation of IκB kinase α (IKKα) and activation of IκB kinase (IKK), inducing Inhibitor of kappa B α (IκBα) degradation and NF-κB subunit p65 phosphorylation. After phosphorylation, p65 translocates to the nucleus where it promotes the transcription of various genes including Cyclin D1, cellular myelocytomatosis (c-Myc), matrix metalloproteinase-9, and Vascular Endothelial Growth Factor, as well as Wnt5a to induce an autonomous feedback loop. Continued stimulation of the ROR1/Akt/p65 pathway within this loop further promotes the production of proinflammatory factors such as interleukin-6 (IL-6) and chemokines such as CCL2[24]. Further research found a positive correlation between ROR1 expression and increased transcription of Yes-associated protein/Transcriptional co-Activator with PDZ-binding motif (YAP/TAZ), promoting both tumorigenesis and chemotherapy resistance[25,26]. Complexation of Wnt5a with ROR1/FZD promotes binding between G protein subunit alpha 12/13 (Gα12/13) and the transforming protein Ras homolog gene family, member A (RhoA), inhibiting the activity of large tumor suppressor kinase 1/2 (Lats1/2) and stimulating YAP/TAZ dephosphorylation and nuclear translocation. Gα12/13 belongs to the gα protein family, where it differs from other members of the family, G protein subunit alpha s (Gαs), G protein subunit alpha i (Gαi), and G protein subunit alpha q (Gαq), and modulates cytoskeletal reorganization and morphological changes in the cell by activating the small

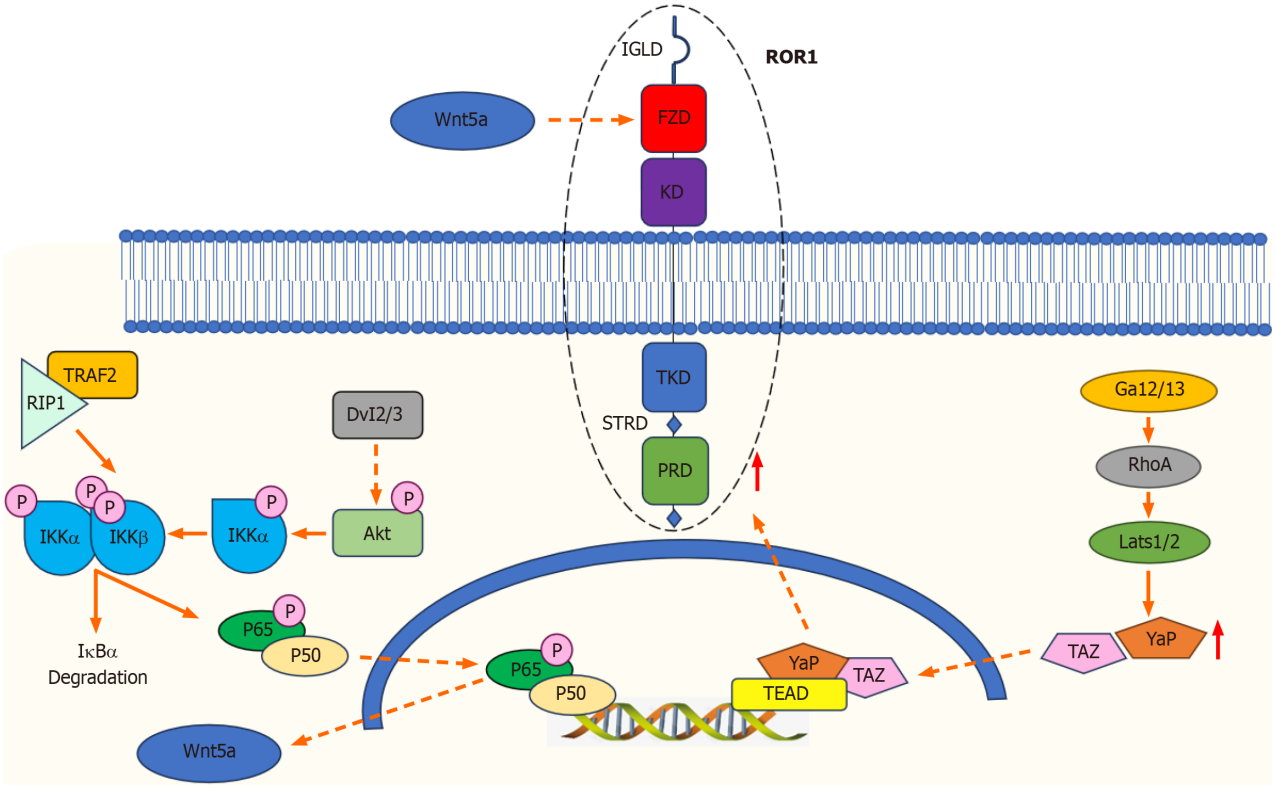

Figure 2 Signaling pathways regulated by receptor tyrosine kinase-like orphan receptor 1. Dvl2/3: Dishevelled 2/3; Akt: Protein kinase B; Ikkα: IκB kinase α; IκBα: Inhibitor of kappa B α; p65: Nuclear factor NF-kappa-B p65 subunit; RhoA: Ras homolog gene family, member A; Gα12/13: G protein subunit alpha 12/13; Lats1/2: Large tumor suppressor kinase 1/2; YAP/TAZ: Yes-associated protein/Transcriptional co-Activator with PDZ-binding motif. Binding of Wnt5a to receptor tyrosine kinase-like orphan receptor 1 (ROR1) induces Dvl2/3 activation and Akt phosphorylation, leading to phosphorylation of Ikkα, activation of the IκB kinase complex, degradation of IκBα, and p65 phosphorylation. Subsequently, p65 is transferred to the nucleus where it promotes the transcription of specific genes, including Wnt5a, producing an autonomous feedback loop. Binding between Wnt5a and the ROR1/frizzled complex leads to activation of RhoA *via* Gα12/13. This activation inhibits Lats1/2 activity, leading to the dephosphorylation and nuclear translocation of YAP/TAZ. The binding of YAP/TAZ to transcription enhanced association domain increases the transcription of genes linked to tumorigenesis and stemness while increased levels of YAP/TAZ enhance ROR1 expression.

Guanosine Triphosphatase (GTPase) Rho. Gα12/13 is also involved in processes such as cell proliferation, migration, and apoptosis. Nuclear translocation of YAP/TAZ, together with transcription enhanced association domain (TEAD), increases the transcription of genes associated with stem cell renewal, proliferation, and tumorigenesis. The upregulation of ROR1 expression is, in turn, stimulated by increased YAP/TAZ transcription.

ROLE OF ROR1 IN GASTROINTESTINAL CANCERS

The mechanisms of ROR1 action in diverse malignancies, encompassing gastrointestinal tumors such as liver, gastric, colorectal, and pancreatic cancer, exhibit heterogeneity. Distinct signaling pathways or processes influenced by ROR1 may be implicated in each specific cancer type. Therefore, it is necessary to discuss the function of ROR1 in these cancers individually.

ROR1 and liver cancer

Liver cancer is one of the six most common cancers worldwide and falls into three main types, namely, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, and mixed cell carcinoma[27]. HCC is the most prevalent and lethal variety, representing approximately 90% of all liver cancers. Liver tumors may also be derived from metastasis of other malignancies, such as lung tumors. Due to its high mortality rate and ease of metastasis, HCC is a subject of significant interest in cancer research[28].

A study by Cetin *et al*[29] assessed ROR1 in HCC, demonstrating its presence in both human and mouse cells, without limitation to metastatic or mesenchymal cell lines. Induction of EMT by transforming growth factor-β (TGF-β) reduced ROR1 levels, while ROR1 knockdown inhibited both the proliferation and migration of HCC cells and increased resistance to apoptosis. Chemotherapeutic agent uptake was modified in ROR1 knockdown epithelial-type HCC cells, leading to resistance to chemotherapy-induced apoptosis. ROR1 has been shown to promote HCC progression through the modulation of various pathways, and changes in ROR1 expression may be useful in diagnosis and prognostic prediction. Meng *et al*[30] reported that ROR1 expression is substantially increased in acid-treated cancer cells and significantly promotes invasion and migration of HCC. ROR1 knockdown through siRNA successfully inhibited acid-

induced tumor cell migration, invasion, and EMT. Nevertheless, the underlying regulatory actions of ROR1 in HCC are not yet clear.

ROR1 and gastric cancer

Gastric cancer (GC) is a heterogeneous disease with a high mortality rate. It is known that infection with *Helicobacter pylori* is both necessary and insufficient for the development of GC[31] which appears to involve a multifactorial and multistep pathogenesis[32].

There is limited information on ROR1 in GC. Kotoh *et al*[33] found an association between Wnt5a and GC invasion and metastasis, showing that Wnt5a bound to FZD/ROR1 and was linked to various signaling pathways including β-catenin-TCF/LEF, Dishevelled–RhoA–Rho-associated protein kinase (DVL–RhoA–ROCK), and mitogen-activated protein kinase kinase kinase 7–nuclear factor-B (MAP3K7–NF-κB) in specific contexts. ROR1 also associates with c-Src, promoting its phosphorylation. C-Src is a non-RTK and known oncogene that has been linked to the development of numerous human cancers, including colon, gastric, lung, breast, and prostate cancers. Once activated, c-Src can regulate a variety of processes, both normal and cancer-associated, including cell survival, proliferation, motility, differentiation, and angiogenesis[34,35].

ROR1 and colorectal cancer

Colorectal cancer (CRC) is among the most prevalent cancer types globally, ranking fifth in incidence in China. Advanced CRC is associated with both recurrence and drug resistance, reducing patient survival. Thus, investigation of the mechanisms underlying CRC is important to improve survival and develop new drugs. Zhou and colleagues investigated expression of ROR1 in CRC[36], observing markedly increased levels in CRC tissues and in approximately 94% of patients with CRC. However, the molecular mechanisms underlying these increases remain unknown.

Wnt signaling is closely associated with regulation of morphogenesis during embryonic development and repair processes. The Wnt ligands are key to these pathways, and are known to bind different receptors, including proteins of the FZD family and ROR1. The interactions trigger downstream cascades involving multiple pathways to influence the arrangement of the cytoskeleton, transcription of genes associated with proliferation and cell growth, and the behavior of intracellular organelles. Dysregulated Wnt signaling has been linked to various malignancies, including CRC, through the modulation of cells associated with the tumor microenvironment (TME)[37].

ROR1 and pancreatic cancer

Pancreatic cancer is an especially lethal form of cancer. Despite significant progress in elucidating its molecular pathology, the prognosis remains extremely poor, mostly due to treatment resistance and the formation of distant metastases in the early stages of the disease[28]. Therefore, uncovering the underlying molecular mechanisms of pancreatic cancer to create new targeted therapeutic strategies remains a pressing research priority.

Yamazaki *et al*[38] demonstrated that ROR1 promotes the proliferation of pancreatic ductal adenocarcinoma (PDAC) by activating E2F *via* c-Myc and inducing expression of aurora kinase B. It was also found that transcription of ROR1 was dependent on the binding of YAP/BRD4 to its promoter region. Prevention of this binding reduced ROR1 expression and inhibited PDAC growth. These findings suggest that increased levels of ROR1 are closely involved in tumorigenesis, highlighting its importance in PDAC progression and its potential as a target for treating the cancer.

ANTITUMOR STRATEGIES TARGETING ROR1 IN GASTROINTESTINAL CANCERS

There has been an increased focus on ROR1 as a potential antitumor target for gastrointestinal cancers, involving various therapeutic approaches and ongoing clinical trials and preclinical studies. These include the use of monoclonal antibodies (mAbs), antibody–drug conjugates (ADCs), bispecific antibodies, small molecule inhibitors, and chimeric antigen receptor (CAR)-T cell therapies (Table 1).

Anti-ROR1 monoclonal antibodies

The application of anti-ROR1 mAbs to block binding between the receptor and the Wnt5a ligand prevents activation of downstream signaling and activates the immune system to promote removal of tumor cells[39]. Zilovertamab, also known as cirmtuzumab or UC-961, is an mAb targeting ROR1. Zilovertamab binding to ROR1 blocks Wnt5a signaling, thus inhibiting tumorigenic behavior, such as cell growth and survival, and inducing differentiation. This mAb has shown significant antitumor efficacy against both hematological and solid malignancies, such as GC and pancreatic cancer, in Phase I clinical trials, and is currently the only antibody on the market that has undergone clinical evaluations [40]. A phase I/II trial of a combination of zilovertamab and paclitaxel for treating breast cancer is currently underway.

Chen *et al*[24] reported that the Wnt5a/ROR1 axis can activate the NF-κB pathway in tumor cells, resulting in the production of interleukin-6 and signal transducer and activator of transcription 3 phosphorylation. Zilovertamab has the potential to inhibit these processes. According to Hasan *et al*[15], Wnt5a promotes complexation between ROR1 and DOCK2, leading to the activation of Rac1/2. Silencing of DOCK2 specifically reduces Wnt5a-mediated activation of Rac1/2 and tumor cell proliferation. The proline-rich domain (PRD) of ROR1 has been implicated in the Wnt5a interaction with DOCK2 and activation of Rac1/2 in MEC1 CLL cells. This effect is blocked by zilovertamab, which also has the advantages of having a long half-life in the plasma and no dose-limiting toxicity. The use of this antibody showed reductions in the activities of RhoA and HS1, while transcriptome analysis indicates reductions in the expression of stemness genes in CLL *in vivo*. It is apparent that zilovertamab is both safe and effective for the inhibition of ROR1 in

Table 1 Receptor tyrosine kinase-like orphan receptor 1-targetd therapies in clinical trials or preclinical studies

Therapy types	Therapy drugs	Indications	ClinicalTrials.gov Identifier/Ref.
mAb	Zilovertamab	Gastric cancer Pancreatic cancer	[42]
ADC	VLS-101	Solid tumor	NCT04504916
	LCB71		NCT05279300
	NBE-002		NCT04441099
	miR-29b		[47]
	OSU-2S		[48]
Bispecific antibody	R11×v9-BiAb	Solid tumor	[50]
	NVG-111		NCT04763083
	SFG.ROR1-BiTE	Pancreatic cancer	[51]
Small-molecule inhibitors	KAN0439834	Pancreatic cancer	[54]
	KAN04415711C		[55]
	miR-27b-3p	Gastric cancer	[57]

mAb: Monoclonal antibodies; ADC: Antibody drug conjugate.

various tumor cells[40].

Antibody-drug conjugates

ADCs involve linkage between mAbs and small cytotoxic molecules *via* specific chemical linkers. ADCs targeting ROR1 are able to transfer cytotoxic agents and regulatory RNAs to ROR1-expressing tumor cells.

VLS-101 (MK-2140) is an ADC consisting of a lead antibody to effectively target ROR1, for treating hematological malignancies and solid tumors. VLS-101 represents a combination of zilovertamab, a lysyl iminohexene-valine-citrulline p-aminobenzoate linker, and a cytotoxic agent monomethyl auristatin E (MMAE) that targets microtubules. Within cells, MMAE is released from the linker to block microtubule polymerization, thus adversely affecting mitosis and cell division [41]. The drug also safely promotes tumor regression in patient-derived xenograft models previously found to be resistant to CAR-T cell, ibrutinib, and/or venetoclax treatment[42]. These findings provide valuable insights into the increasing clinical demand for VLS-101 in gastrointestinal cancers.

LCB71 (CS5001) is an ADC based on an mAb that targets ROR1. LCB71 has a distinctive design involving a unique β-glucosidic acid linker and a pyrrolobenzodiazepine (PBD) pre-toxin dimer. Both the linker and the pretoxin are cleaved by lysosomal β-glucuronidase, an enzyme significantly overexpressed in many types of cancer cells. Thus, LCB71 can eliminate cancerous cells after arrival at the tumor site and cleavage of the linker to release the PBD pre-toxin, which is subsequently activated within the tumor cell. The dual control mechanism of the linker plus precursor toxin resolves typical toxicity issues associated with traditional PBD loads, resulting in an improved safety profile. LCB71 has shown notable dose-dependent antitumor activity in several xenograft tumor models of condylomatous lymphoma and breast cancer and is thus a promising drug candidate with potential for precision therapy in both hematological and solid malignant tumors expressing high levels of ROR1. The ADC NBE-002 is composed of a humanized antibody, huXBR1-402, conjugated to the anthracycline derivative, PNU-159682. HuXBR1-402 is a chimeric rabbit/human mAb identified from a rabbit antibody library using phage display. NBE-002 has been shown to be effective in xenograft models of various human tumors[43]. The drug is currently in a clinical Phase I study. Chiang *et al*[44] connected an anti-ROR1 mAb to a regulatory miRNA (miR-29b). This ADC treatment reduced the expression of the miR-29 targets, DNA methyltransferase (DNMT) 1 and DNMT3A, in cells expressing ROR1. It also led to alterations in global DNA methylation patterns, downregulation of SP1, and upregulation of p21. Another ADC used an mAb against ROR1 linked to nanoparticles containing OSU-2S, a sphingosine analog with anti-tumor capabilities, that activated protein phosphatase 2A and induced SHP1 phosphorylation and nuclear translocation[45], resulting in apoptosis.

Bispecific antibody

Bispecific antibodies (BiAbs) are synthetic antibodies created through cell fusion or recombinant DNA technology. They are formed by two single-chain antibody fragments, or single-chain fragment variable (scFvs), together with different variable regions composed of both heavy and light chains and a common constant region, or Fc. Bispecific T-cell conjugates (BiTes) are fusion proteins composed of two separate scFvs without an Fc. One arm can bind to the Kringle domain on ROR1 while the other interacts with the CD3 subunit of the T cell receptor, thus recruiting polyclonal T cells to the site of the tumor. BiTEs do not rely on antigen presentation and can activate T-cell binding, resulting in the killing of neighboring target cells. Both BiAbs and BiTes can bind simultaneously to two different antigens or epitopes on the same antigen. Due to their specificity and bifunctionality, the use of BiAbs and BiTes is a trending research topic in antibody

engineering and has a broad range of applications in tumor therapy and autoimmune diseases.

Qi *et al*[46] co-conjugated multiple anti-ROR1 single-chain antibodies to a single-chain antibody against CD3 with glycosylated IgG1-Fc, generating various BiAbs. They found that targeting scFv using ROR1 with the proximal epitope R11 of the membrane was the most effective one for recruiting T cells to ROR1-positive solid cancer cell lines. This BiAb exhibited promising antitumor activity both in vitro and *in vivo*. In a similar investigation, Wang *et al*[47] also used a BiAb composed of an scFv and Fc that targeted both ROR1 and CD3; this was termed R11×v9-biAb. When tested on multiple ROR1-positive solid cancer cell lines, the observed cytotoxicity was found to be dependent on the R11×v9-biAb dosage. The BiAb targeted ROR1-positive cancerous cells, induced tumor cell death, and recruited significant numbers of CD4+ and CD8+ T cells to the tumor through the secretion of T-cell-derived proinflammatory factors and promoting perforin production by granzyme B and CD8+ T cells.

NVG-111 is a unique humanized BiTE recognizing both ROR1 and CD3, and is composed of tandem scFvs against ROR1 and CD3. NVG-111 is the first BiAb that utilized the inherent cytotoxicity of T cells. Ongoing clinical trials of NVG-111 have shown promising results in both CLL and solid tumors, with favorable cytotoxicity[48]. Another bispecific T-cell splicer, SFG. ROR1-BITE, includes scFvs targeting the cysteine-rich coiled-coil structural domain of ROR1 and CD3, and showed strong cytotoxicity against pancreatic cancer cells expressing ROR1 at low concentrations, as well as significant tumor reduction in a mouse model of pancreatic tumors[49].

Small-molecule inhibitors of ROR1

The lack of clearly defined kinase activity in ROR1 has limited the identification of small-molecule inhibitors that could target this activity. Although some catalytic activity was observed in immunoprecipitated ROR1 suggesting the possibility of autophosphorylation *in vivo*, this was found to be negligible[50,51].

Nevertheless, Hojjat-Farsangi *et al*[52] identified a promising inhibitor, KAN0439834, against ROR1 by screening a library of 110000 small molecules. This compound targeted the TK domain, preventing phosphorylation of ROR1 by Wnt5a and thus blocking the activation of downstream proteins such as Src, Akt, protein kinase C, and MAPK. Further research demonstrated that KAN0439834 was more effective in inducing apoptosis in cancer cells than mAbs with a potency equivalent to that of venetoclax, an inhibitor of Bcl-2 used for the treatment of patients with CLL. Clinical trials have also shown that combinations of KAN0439834 with erlotinib and ibrutinib, small-molecule inhibitors of epidermal growth factor receptor and Bruton's tyrosine kinase, act synergistically in the treatment of patients with pancreatic cancer [53]. The same group screened KAN04415711C, a second-generation compound targeting ROR1, which was found to be effective against tumors in a zebrafish model[54]. KAN04415711C was also effective in vitro when combined with venetoclax. Its mechanism of action, however, requires further investigation.

ROR1-targeted CAR-T cell therapy

CAR-T therapy involves the transfer of structural domains that specifically recognize antigens and genetic material promoting T cell activation signals into T-cells. This enables the T cells to target the specific antigens on the surfaces of cancer cells, leading to the release of cytotoxic compounds such as perforin and granzyme B. Cytokines may also be released to recruit endogenous immune cells for cotreatment. Additionally, the formation of memory T cells can establish a long-lasting antitumor mechanism *in vivo*[55-57].

ROR1 is currently considered a promising target in the design of CAR-T cell therapy. Hudecek *et al*[58] and Hudecek *et al*[59] designed a range of CAR-T cells targeting ROR1, featuring a concise “hinge-only” extracellular spacer, that was found to be highly effective in destroying ROR1-positive tumor cells and inducing T cell effector functions. The safety of this therapy is currently being evaluated due to concerns about cross-reaction with normal tissues by the scFv[60,61]. Lee *et al*[62] developed two CAR-T cell constructs targeting ROR1 by utilization of the antigen-recognition domains of zilovertamab and different hinge regions on the antibody. It was found that the construct with a truncated IgG4 hinge fragment was the most successful in terms of expression and *in vitro* functionality. Additionally, it effectively controlled tumor growth in xenograft mouse models with no significant toxicity. This study demonstrates the potential of Zilovertamab-derived ROR1 CAR-T for the clinical treatment of solid tumors.

The vast majority of candidate CAR targets in solid tumors, such as HER2, GD2, and mesothelin, are co-expressed in a variety of normal tissues, making off-tumor toxicity a significant risk for immunotherapies targeting these molecules. This makes it crucial to study the toxicity of ROR1 targeted therapy for gastrointestinal tumors, as ROR1 is no exception. SNIP CARs provide a solution to CAR-T therapy toxicities by utilizing a regulated protease-based system controlled by an FDA-approved small molecule. They offer improved potency and safety in various models, enabling remote control over CAR activity for treating solid tumors[63].

A study by Srivastava and colleagues[64] found that ROR1 CAR-T therapy led to fatal bone marrow failure in mice due to the recognition of ROR1-positive stromal cells. To enhance selectivity, they integrated synthetic Notch (synNotch) receptors specifically recognizing epithelial cell adhesion molecule or B7-H3 into T cells. These receptors were expressed on ROR1-positive cancer cells but not on stromal cells expressing ROR1. This induced a selective ROR1 CAR-T effect, leading to successful tumor ablation without toxicity in normal ROR1-positive cells. The study demonstrated that the use of synNotch receptors for combined antigen sensing can prevent CAR-T-cell-mediated lethal toxicity to normal tissue, provided that the tumor and normal tissue were spatially separated. However, if they are highly colocalized at the same site, this approach may not be effective. Moreover, the same group further discovered that ROR1 CAR-T cells showed poor tumor infiltration and malfunctioned. The inclusion of oxaliplatin, however, in the regimen allowed the recruitment of T cells by activated tumor macrophages[65], improving CAR-T-cell infiltration to modify the TME and increasing the sensitivity of the tumor to immunotherapy. Thus, the ROR1 CAR-T combination therapy provides an alternative strategy for the enhancement of CAR-T cell efficacy in clinical settings.

Some metabolites and small-molecule drugs can boost the anticancer actions of CAR-T cells. It was found that some short-chain fatty acids, such as butyrate and valerate, could enhance the actions of cytotoxic T lymphocytes and CAR-T cells through both epigenetic and metabolic means[66]. Furthermore, SD-208, a kinase inhibitor, could enhance the cytolytic activity, cytokine production, viability, and proliferation of ROR1-CAR T cells by blocking TGF-β receptor signaling[67]. These findings underscore the significance of novel combination therapies in enhancing the efficacy of ROR1 CAR T cells for treating gastrointestinal cancers.

CONCLUSION

ROR1 is a potentially effective therapeutic target for various gastrointestinal cancers. However, high expression of ROR1 on the surface of normal tissues poses potential risks for off-target tumor toxicity. Therefore, the use of treatments targeting ROR1 requires monitoring of potential toxicity to healthy tissues to ensure safety. The effectiveness of mAbs, ADCs, or BiTEs against ROR1 on solid tumors, including gastrointestinal cancers, may be restricted due to the characteristics of the TME and tumor heterogeneity. Therefore, if long-term localized expression of anti-ROR1 BiTEs or mAbs can be induced within tumors, this could recruit and activate tumor-infiltrating lymphocytes for effective antitumor effects. Oncolytic viruses can serve as vectors for delivering ROR1 BiTEs or mAbs, potentially offering a solution to this problem. Our group has constructed a novel oncolytic adenovirus that delivers anti-ROR1-BiTE, which has demonstrated efficient inhibition of the growth of tumor cells in a mouse xenograft model. This highlights the importance of the combination of oncolytic virotherapy with ROR1 CAR T cell therapy. Overall, the in-depth research into the use of treatments targeting ROR1 suggests that better strategies for the clinical treatment of gastrointestinal cancer will be developed in the future.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Different types of tumor microvessels in stage I-IIIA squamous cell lung cancer and their clinical significance

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Abstract

BACKGROUND

Lung cancer (LC) is the leading cause of morbidity and mortality among malignant neoplasms. Improving the diagnosis and treatment of LC remains an urgent task of modern oncology. Previously, we established that in gastric, breast and cervical cancer, tumor microvessels (MVs) differ in morphology and have different prognostic significance. The connection between different types of tumor MVs and the progression of LC is not well understood.

AIM

To evaluate the morphological features and clinical significance of tumor MVs in lung squamous cell carcinoma (LUSC).

METHODS

A single-center retrospective cohort study examined medical records and archival paraffin blocks of 62 and 180 patients with stage I-IIIA LUSC in the training and main cohorts, respectively. All patients underwent radical surgery (R0) at the Orenburg Regional Cancer Clinic from May/20/2009 to December/14/2021. Tumor sections were routinely processed, and routine Mayer's hematoxylin and eosin staining and immunohistochemical staining for cluster of differentiation 34 (CD34), podoplanin, Snail and hypoxia-inducible factor-1 alpha were performed. The morphological features of different types of tumor MVs, tumor parenchyma and stroma were studied according to clinicopathological characteristics and LUSC prognosis. Statistical analysis was performed using Statistica 10.0 software. Univariate and multivariate logistic regression analyses were performed to

identify potential risk factors for LUSC metastasis to regional lymph nodes (RLNs) and disease recurrence. Receiver operating characteristic curves were constructed to discriminate between patients with and without metastases in RLNs and those with and without disease recurrence. The effectiveness of the predictive models was assessed by the area under the curve. Survival was analyzed using the Kaplan-Meier method. The log-rank test was used to compare survival curves between patient subgroups. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Depending on the morphology, we classified tumor vessels into the following types: normal MVs, dilated capillaries (DCs), atypical DCs, DCs with weak expression of CD34, "contact-type" DCs, structures with partial endothelial linings, capillaries in the tumor solid component and lymphatic vessels in lymphoid and polymorphocellular infiltrates. We also evaluated the presence of loose, fine fibrous connective tissue (LFFCT) and retraction clefts in the tumor stroma, tumor spread into the alveolar air spaces (AASs) and fragmentation of the tumor solid component. According to multivariate analysis, the independent predictors of LUSC metastasis in RLNs were central tumor location ($P < 0.00001$), the presence of retraction clefts ($P = 0.003$), capillaries in the tumor solid component ($P = 0.023$) and fragmentation in the tumor solid component ($P = 0.009$), whereas the independent predictors of LUSC recurrence were tumor grade 3 (G3) ($P = 0.001$), stage N2 ($P = 0.016$), the presence of LFFCT in the tumor stroma ($P < 0.00001$), fragmentation of the tumor solid component ($P = 0.0001$), and the absence of tumor spread through the AASs ($P = 0.0083$).

CONCLUSION

The results obtained confirm the correctness of our previously proposed classification of different types of tumor vessels and may contribute to improving the diagnosis and treatment of LUSC.

Key Words: Lung cancer; Lung squamous cell carcinoma; Tumor microvessels; Tumor stroma; Regional lymph node metastases; Disease recurrence; Disease prognosis

Core Tip: In this retrospective study, we examined the morphology of different types of tumor microvessels, tumor parenchyma, and tumor stroma and their associations with the risk of regional metastasis and disease recurrence in lung squamous cell carcinoma (LUSC) patients. Independent predictors of LUSC metastases in regional lymph nodes were the central location of the tumor ($P < 0.00001$), the presence of retraction clefts ($P = 0.003$), capillaries in the solid component of the tumor ($P = 0.023$) and fragmentation of the solid component of the tumor ($P = 0.009$), while independent predictors of LUSC recurrence were tumor grade 3 ($P = 0.001$), N2 stage ($P = 0.016$), the presence of loose fine fibrous connective tissue in the tumor stroma ($P < 0.00001$), fragmentation of the tumor solid component ($P = 0.0001$) and the absence of tumor spread through the alveolar air spaces ($P = 0.0083$). These findings may help improve the diagnosis and treatment of LUSC.

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INTRODUCTION

Lung cancer (LC) is the leading cause of morbidity and mortality among malignant neoplasms[1,2]. There are two main histological forms of LC: Non-small cell LC (NSCLC) and small cell LC (SCLC). NSCLC accounts for up to 80% of all LC cases[3], 20% to 67.5% of which are lung squamous cell carcinomas (LUSC)[4-6]. Despite a decrease in the incidence of NSCLC, mortality rates remain very high and are associated with late diagnosis, an aggressive course of the disease even in the early stages, and the presence of severe comorbidities in patients older than 60 years; these conditions prevail among patients with NSCLC, and radical treatment is often impossible for these patients.

Although both LUSC and lung adenocarcinoma are classified as NSCLC, increasing evidence suggests that these cancers have different origins, different mutational profiles, different biological behaviors, different sensitivities to drugs and radiation therapy (RT), and different disease prognoses[4,7,8]. The development of LUSC is preceded by squamous metaplasia of the bronchial epithelium, which is closely associated with smoking. In addition, alcohol consumption; infections such as human papillomavirus and Epstein–Barr virus[4,7]; and environmental pollution also increase the risk of LUSC[9]. LUSC can have both central and peripheral localization and is characterized by a high rate of genetic mutations, chromosomal instability, and a high degree of cellular heterogeneity[7,10]. Late diagnosis and the lack of treatment methods specific for this disease determine the high mortality rate from this pathology, which is almost 30%

greater than the mortality rate from lung adenocarcinoma[4,8].

The choice of optimal treatment for LUSC, determining the indications for neoadjuvant and adjuvant therapy, is based on an assessment of the risk of disease relapse[11,12]. Currently, this assessment is based on disease stage, histopathological features and genetic alterations. However, for LUSC patients with the same clinicopathological and molecular genetic characteristics, the response to treatment and survival rates can differ significantly[11,13,14]. Thus, the search for new prognostic and predictive markers of LUSC has not lost relevance.

Angiogenesis is one of the key factors in tumor progression[15,16]. Its activation is associated with hypoxia, inflammatory processes, epithelial–mesenchymal transition (EMT), the formation of the phenotype of stem cells, and other factors[16-18]. Assessment of angiogenesis activity is currently considered the most important factor associated with disease prognosis and therapeutic effectiveness[16,19,20].

Previously, in gastric cancer, breast cancer, and squamous cell carcinoma of the cervix, we proposed a classification of tumor microvessels (MVs) based on their morphology and correlations with the clinical and pathological characteristics and prognosis of these diseases[21-23]. The aim of this study was to evaluate the morphological features and clinical significance of various types of tumor MVs, as well as tumor parenchymal and stromal components, in LUSC.

MATERIALS AND METHODS

Patient characteristics

This single-center retrospective cohort study of the "case–control" type was conducted in accordance with the Helsinki Declaration and internationally recognized guidelines after receiving study approval from the Ethics Committee of Orenburg State Medical University (No. 281, dated 30 September 2021). One hundred eighty archived paraffin blocks of patients with LUSC stages I-IIIA were retrieved from the tumor bank of the Orenburg Regional Cancer Clinic. All patients underwent radical surgery (R0) at this clinic from May/20/2009 to December/14/2021. None of the patients included in the study underwent preoperative chemotherapy (ChT) or RT. The patients did not receive steroids, nonsteroidal anti-inflammatory drugs, or antihistamines and had no significant comorbid pathologies in the decompensation stage.

The age of the patients was 61.6 ± 6.7 years (median 62 years). There were 174 men and 6 women. The right lung was affected in 93 (51.7%) patients, and the left lung was affected in 87 patients (48.3%). The tumor was localized in the upper lobe in 102 (56.7%) patients, in the middle lobe in 5 (2.8%), in the lower lobe in 49 (27.2%), and in the main bronchus in 24 (13.3%).

To study the features of the tumor MV morphology and their correlations with clinical and morphological characteristics and disease prognosis, 20 archived paraffin blocks of patients with stage I LUSC, 22 with stage II LUSC and 20 with stage IIIA LUSC were randomly selected. The patients composed the training cohort. There were no differences in age, type, comorbidity status, histology or tumor grade between the main and training groups of patients, while differences in the volume of surgery and adjuvant therapy were because, in the main cohort, there was a greater percentage of patients with locally advanced LUSC. In addition, it should be noted that in the training cohort, a greater percentage of patients received postoperative radiotherapy as an adjuvant treatment. This is because patients in this group received treatment between 2009 and 2013. During this period, radiotherapy was most commonly used as an adjuvant treatment for LUSC. However, over the past 10 years, ChT or chemoradiotherapy has been used most often as an adjuvant treatment for LC. The baseline patient clinicopathological and treatment information is shown in Table 1.

During the observation period, 27 (15.0%) patients were diagnosed with cancer in other locations: Prostate cancer in 12 (6.7%), nasal mucosa cancer in 4 (2.2%), skin cancer in 7 (3.9%), bladder cancer in 1 (0.6%), laryngeal cancer in 1 (0.6%), breast cancer in 1 (0.6%), and colon cancer in 1 (0.6%).

As of January/31/2023, 84 (46.7%) patients were alive. LUSC recurrence was diagnosed in 95 (52.8%) patients, 83 of whom died from disease progression; 13 (7.2%) patients with LC died from nononcological pathology. LUSC recurrence was local in 44 (24.4%) patients, systemic in 41 (22.7%), and local and systemic in 7 (3.9%). In particular, metastases to the lungs and pleura were detected in 30 (16.7%) patients; to the mediastinum, 15 (8.3%); to the liver, 10 (5.6%); to the brain, 5 (2.8%); to the cervical lymph nodes, 3 (1.7%); and to the bones, 6 (3.3%). Multiple metastases were detected in 24 (13.3%) patients. Forty-four patients received 1 to 4 courses of mono- or polychemotherapy due to LC recurrence. Eight patients with LC relapse continued to receive monochemotherapy.

In 2020-2022, six patients were treated for COVID-19, and one patient died from a new coronavirus infection. The median follow-up period was 66 months for patients in the main cohort and 72 months for patients in the training cohort.

Pathology

Sections (4 μm) were cut on a microtome and transferred to glass slides (SuperFrost® Plus, Menzel, Thermo Scientific, United States). Sections were stained with Mayer's hematoxylin and eosin (H&E) and studied *via* light microscopy (Levenhuk D740T digital microscope connected to a 5.1 MP camera, Russia). At 200 × magnification, the presence or absence of loose, fine fibrous connective tissue (LFFCT) in the tumor stroma, fragmentation in the tumor solid component, retraction clefts and tumor spread through the alveolar air spaces (AASs) were determined.

Immunohistochemistry

The morphology of the tumor MVs was studied *via* immunohistochemical staining of histological sections with antibodies against cluster of differentiation 34 (CD34) and podoplanin (PDPN). For immunohistochemistry (IHC), 4-μm sections were stained with the following antibodies: Monoclonal antibody against CD34, 1:100 dilution; polyclonal antibody

Table 1 Baseline patient clinicopathological and treatment information

Clinical and pathological data	Patient groups				Significance level (*P* value)
	Main cohort (*n* = 180)		Training cohort (*n* = 62)		
	n	%	*n*	%	
Age (yr)					
< 60	60	33.3	20	32.3	0.897
60-69	98	54.5	33	53.2	
≥ 70	22	12.2	9	14.5	
Tumor location					
Central	119	66.1	38	61.3	0.493
Peripheral	61	33.9	24	38.7	
Comorbidity					
Absence	22	12.2	8	12.3	0.888
Presence	158	87.8	54	87.7	
Histology					
KSCC	56	31.1	21	33.9	0.687
NKSCC	124	68.9	41	66.1	
Tumor grade					
G1	57	31.7	23	37.1	0.325
G2	88	48.9	32	51.6	
G3	35	19.4	7	11.3	
T stage					
T1a	9	5.0	8	12.9	0.039[a]
T1b	15	8.3	6	9.7	
T2a	82	45.6	34	54.8	
T2b	17	9.4	5	8.1	
T3	57	31.7	9	14.5	
N stage					
N0	48	26.7	22	35.5	0.411
N1	88	48.9	26	41.9	
N2	44	24.4	14	22.6	
Stage					
I	30	16.7	20	32.3	0.03[a]
II	73	40.6	22	32.4	
III	77	42.8	20	32.3	
Type of surgery					
Lobectomy	84	46.7	40	64.4	0.001[a]
Bilobectomy	6	3.3	5	8.1	
Pneumonectomy	86	47.8	13	21.0	
Segmentectomy	4	2.2	4	6.5	
Adjuvant therapy					
Absent	51	28.3	27	43.5	0.002[a]
Chemotherapy	68	37.8	10	16.1	

Radiation therapy	38	21.1	21	33.9
Chemo- and radiotherapy	23	12.8	4	6.5

[a]$P < 0.05$, the differences between groups are statistically significant.
KSCC: Keratinizing squamous cell carcinoma; NKSCC: Nonkeratinizing squamous cell carcinoma.

against PDPN, 1:50 dilution; polyclonal antibody against Snail homolog 1 (SNAI1), 1:100 dilution; and polyclonal antibody against hypoxia-inducible factor-1 alpha (HIF-1a), 1:200 dilution (Cloud-Clone Corp®, Texas, United States). Staining with antibodies against SNAI1 and HIF-1a was performed when fragmentation in the solid component of the tumor was detected in the test samples. The staining procedure was performed according to the manufacturers' protocols as described previously[23]. For the negative control sections, the primary antibodies were replaced with phosphate-buffered saline, and the sections were processed in the same manner. All sections were carefully and completely scanned by two of the authors (Evgeniy A Kalinin and Marina A Senchukova) without knowledge of the clinical and pathological data. The MV density (MVD) was assessed by counting the number of CD34-positive and PDPN-positive normal MVs in accordance with the international consensus on the methodology and criteria for the quantitative evaluation of angiogenesis in human solid tumors[24]. The MVD was determined in five fields of view at high magnification (× 800) at the selected "hot spots", that is, areas where positively stained vessels were highly concentrated.

The density of dilated capillaries (DCs), atypical DCs (ADCs) and structures with a partial endothelial lining (SPELs) was estimated at low magnification (× 200) in the three fields of view in the selected "hot spots". The presence or absence of DCs with weak expression of CD34, contact-type DCs, lymphatic vessels (LVs) in lymphoid and polymorphic cell infiltrates and tumor emboli in CD34-positive and PDPN-positive vessels was also assessed in the tumor samples.

Statistical analysis

Statistical analysis was performed using Statistica 10.0 software. Quantitative data are presented as the mean ± SD, while categorical variables are presented as numbers and percentages (*n*, %). Correlations between different data were evaluated using the nonparametric Spearman's rank correlation or gamma correlation. Chi-square tests were carried out to analyze the differences in distribution among the categorized data. The Mann–Whitney *U* nonparametric test was used to compare the values of the quantitative data. Both univariate and multivariate logistic regression analyses were performed to identify potential risk factors for LUSC metastasis to regional lymph nodes (RLNs) and disease recurrence. Receiver operating characteristic (ROC) curves were constructed to discriminate between patients with and without LUSC metastases in the RLNs and those with and without LUSC recurrence. The best threshold (cutoff) values were determined by the largest Youden's index (J = sensitivity + specificity - 1). The effectiveness of the predictive models was assessed by the area under the curve (AUC). Survival was analyzed using the Kaplan–Meier method. The log-rank test was used to compare survival curves between patient subgroups. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Previously, in squamous cell carcinoma of the cervix, we proposed classifying tumor MVs into eight types according to their size, shape, clarity of contours, localization, characteristics of the endothelial lining, intensity of CD34 and PDPN staining, and content of the MV lumen[23]. The types of MVs described included normal MVs, DCs, ADCs, DCs with weak CD34 expression, contact-type DCs, SPELs, capillaries in the tumor solid component, and LVs in lymphoid and polymorphocellular infiltrates. These tumor MVs not only differed in morphology but were also associated with different clinical and morphological characteristics of squamous cell carcinoma of the cervix and long-term treatment results.

Identification of different types of tumor microvessels in LUSC specimens from the training patient cohort

In the first phase of this study, we identified eight previously described tumor MVs and endothelial-lined structures in 62 LUSC specimens (from the training patient cohort) *via* immunohistochemical staining with antibodies against CD34 and PDPN (Figure 1). Here, is a brief description of them:

Normal MVs: The normal MVs were capillaries with a diameter of 5-40 microns (Figure 1A). The MVD was 14.0 ± 4.7 per conventional unit area (range 2.4–28.8, median 13.3). The cytoplasm of some vessels of this type was positive when stained with antibodies against PDPN.

DCs: DCs were defined as MVs larger than 40 microns in size that were regular in shape with clear, even contours (Figure 1B). The DC density was 4.4 ± 3.3 per conventional unit area (range 0–12.4, median 4.1), and it was significantly greater along the invasive edge of the tumor than in the central region ($P = 0.002$). The cytoplasm of the endothelial cells in these vessels was negative when stained with antibodies against PDPN.

ADCs: The ADCs were MVs larger than 40 microns in size and irregular in shape with a chaotic arrangement of endothelial lining cells (Figure 1C). Tumor emboli and CD34-positive cells were often observed in the lumen of these vessels. The endothelial lining of dilated lymphatic capillaries had similar characteristics, so we believe that some ADCs

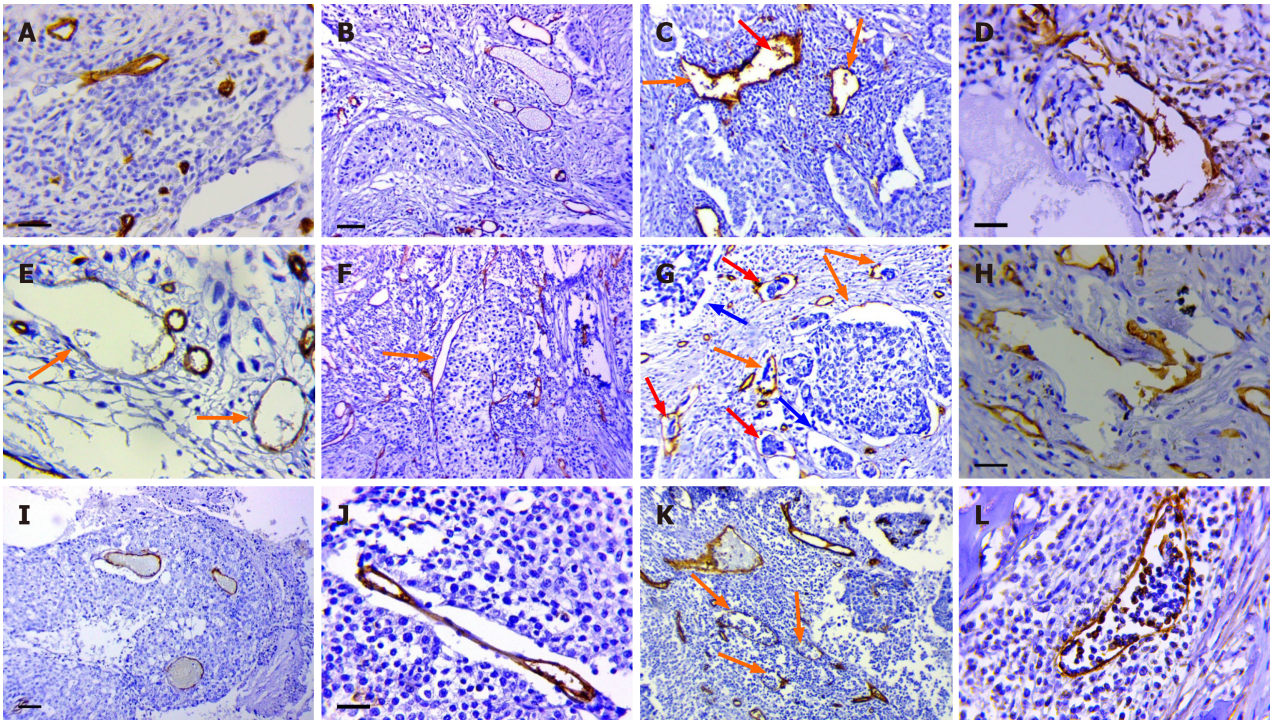


Figure 1 Different types of tumor microvessels. A: Normal microvessels. Scale bar = 10 μm; B: Dilated capillaries (DCs). Scale bar = 100 μm; C: Atypical DCs with tumor emboli (orange arrows) and cluster of differentiation 34 (CD34)-positive cells (red arrow) in their lumen, Scale bar = 100 μm; D: Lymphatic capillary with the chaotic arrangement of the endothelial cells. Scale bar = 10 μm; E: DCs with weak expression of CD34 (orange arrows). Scale bar = 10 μm; F: Contact-type DCs (orange arrow). Scale bar = 100 μm; G: Structures with partial endothelial lining (type 1, orange arrows), structures without endothelial lining (blue arrows) and vessels with complexes of tumor cells in their lumen (red arrows). Scale bar = 100 μm; H: Structures with partial endothelial lining (type 2). Scale bar = 10 μm; I: Capillaries in the tumor solid component (type 1). Scale bar = 100 μm; J: Capillaries in the tumor solid component (type 2). Scale bar = 10 μm; K: Lymphatic capillary in lymphoid and polymorphic cell infiltrates. Scale bar = 100 μm; L: Lymphatic capillary in lymphoid and polymorphic cell infiltrates. Scale bar = 10 μm scale; A-C, E-K: Staining with antibodies against CD34; D and L: Staining with antibodies against PDPN.

were LVs (Figure 1D). The density of ADCs (CD34) was 7.7 ± 3.9 per conventional unit area (range 1.0–19.4, median 7.5), and the density of dilated LVs was 2.1 ± 1.2 per conventional unit area (range 0.3–5.0, median 1.9).

DCs with weak expression of CD34: These vessels were defined as capillaries with a regular shape and smooth contours and very weak, sometimes barely detectable, expression of CD34 in the endothelial cell cytoplasm. These cells had large, pale nuclei with weakly condensed chromatin (Figure 1E). The average diameter of vessels of this type was 68.7 ± 29.5 μm. The described vessels were observed only in LFFCT and were not stained with antibodies against PDPN; that is, they were blood vessels. These vessels were identified in 26 (41.9%) of the LUSC samples.

The contact-type DCs: The described vessels were MVs, the walls of which were in direct contact with tumor cells. The described MVs had a regular shape and clear, even contours (Figure 1F). The average diameter was 68.7 ± 29.5 μm. Some vessels of this type were stained with PDPN; that is, they were LVs. Contact-type DCs were found in 36 (58.1%) of the studied samples.

Structures with partial endothelial linings: Structures with partial endothelial linings were first described in gastric cancer[21]. We observed two types of SPELs in both LUSC and squamous cell carcinoma of the cervix. The first type was associated with retraction clefts. In tumor samples with type 1 SPELs, structures without an endothelial lining (retraction clefts) and vessels with complexes of tumor cells in their lumen were often observed (Figure 1G). The described SPELs were identified in 28 (45.2%) of the samples. In most cases, these structures were positive when stained with antibodies against PDPN.

Type 2 SPELs were observed predominantly in the peritumoral stroma; they were often linear or irregular in shape and were positive when stained with antibodies against CD34 and PDPN (Figure 1H). The density of CD34-positive SPELs was 3.2 ± 1.8 per arbitrary unit area (range 0.3-7.6, median 2.8), and the density of PDPN-positive SPELs was 1.2 ± 1.0 (range 0–4.4, median 1.1).

Capillaries in the solid component of the tumor: We observed 2 types of such vessels. The first type of vessel consisted of capillaries, the walls of which were in direct contact with tumor cells (Figure 1I). The average diameter of these vessels was 34 ± 32.1 μm. There was no content in the lumen of the vessels, or it was represented by erythrocytes. Type 1 capillaries were found in 29.1% of the LUSC samples. The second type of vessel was linear capillaries whose walls were retracted from tumor cells (Figure 1J). Vessels of the second type were found in 32.3% of the samples. In 35% of the samples, capillaries of both types were observed in the solid component of the tumor. Both types of vessels were negative

when stained with antibodies against PDPN.

LVs in lymphoid and polymorphic cell infiltrates: LVs in lymphoid and polymorphic cell infiltrates had very thin, sometimes slightly noticeable endothelial linings when stained with antibodies against CD34 and were not visible when stained with Mayer's H&E (Figure 1K). Lymphocytes or other leukocytes were found in large numbers in the lumen. All vessels of this type were positive when stained with antibodies against PDPN (Figure 1L). The described vessels were found in 32.3% of the LUSC samples.

Features of parenchymal and stromal tumor components in LUSC specimens from the training patient cohort

Considering the published data and our earlier results, we included the following features of the parenchymal and stromal components of the tumor in the analysis:

The presence of LFFCT in the tumor stroma: LFFCT was rich in cells with large pale nuclei and weakly condensed chromatin (Figure 2A). LFFCT was most often detected peritumorally and was noted in 57.8% of the LUSC samples.

The tumor spread through the AASs: In our study, tumor spread through the AASs was observed in 41 (66.1%) samples. Only in 6 samples did tumor cells spread through relatively unchanged AASs (Figure 2B). In other samples, the walls of the alveoli were thickened and contained DCs, sometimes merging with each other (Figure 2C). In the AASs, both unchanged clusters of tumor cells and completely necrotic tumor masses were observed. In a number of samples, fragmentation of tumor masses that spread through the AASs was noted (Figure 2D).

The fragmentation of the tumor solid component: This phenomenon was defined as the appearance of individual fibroblast-like tumor cells with nuclear expression of HIF-1a and Snail in the tumor solid component or in the tumor masses spreading through the AASs (Figure 2E and F). This phenomenon was noted in 46 (68%) of the LUSC samples.

The presence of retraction clefts: This phenomenon has been described in some tumor types and involves the presence of empty spaces around tumor nests[25,26]. Several studies have demonstrated that the presence of retraction clefts may be associated with a poor prognosis in patients with various malignant tumors[27-29] or more aggressive tumors[30]. In LUSC, retraction clefts were detected in 77% of the samples (Figure 2G). In 28 (45.2%) samples, the described structures had partial endothelial linings (Figure 2H).

It should be noted that in some samples, the described phenomenon had to be differentiated from tumor spread through AASs. The retraction of tumor nests from a pronounced stroma, presented by fibrous connective tissue, was regarded as a retraction cleft.

Associations of different types of tumor microvessels and features of parenchymal and stromal tumor components with clinical characteristics and prognosis in LUSC

The density of normal MVs (MVD), DCs, ADCs and SPELs according to the clinical and pathological characteristics and prognosis of patients with LUSC are presented in Supplementary Table 1.

According to the obtained results, neither the density of the described vessels nor the SPELs were associated with the presence of metastases in RLNs or the prognosis of LC. MVD was greater in patients younger than 60 years, in stage T2a and T2b, in stage N0 and N1, in nonkeratinizing squamous cell carcinoma (NKSCC), in the presence of LVI in the tumor samples, and in patients with a high or moderate degree of tumor malignancy (G3 and G2); however, the differences in the groups were not statistically significant ($P > 0.05$). The DC density was significantly lower in G3 than in G1 and G2 ($P = 0.04$) and somewhat lower in the presence of LVI than in the absence of LVI; however, these differences were not statistically significant ($P > 0.05$). Correlations between the density of ADCs or SPELs (CD34) and clinical and pathological characteristics and LUSC prognosis have not been established; however, the LV density and density of SPELs (PDPN) were significantly greater in peripheral LCs than in central LCs ($P = 0.003$ and $P = 0.007$, respectively). In addition, the density of SPELs (PDPN) was greater in the presence of LVI than in the absence of LVI ($P = 0.04$).

Similarly, according to the ROC analysis data, neither the density of the described vessels nor the SPELs were associated with the presence of RLN metastases (RLNM) or the prognosis of LC. The ROC analysis data are presented in Supplementary Figure 1.

The data on the frequency of DCs with weak expression of CD34, DCs of contact type, capillaries in the solid component of the tumor and LVs in lymphoid and polymorphic cell infiltrates, depending on the clinical and pathological characteristics and prognosis of LUSC, are presented in Supplementary Table 2.

As shown in the data, DCs with weak expression of CD34 were detected significantly more often in patients with LUSC recurrence than in patients without LUSC recurrence and somewhat more often in patients with keratinizing squamous cell carcinoma (KSCC) than in patients with NKSCC ($P > 0.05$). In patients with and without disease relapse, these vessels were detected in 61.5% and 27.8%, respectively ($P = 0.008$).

The analysis showed that contact-type DCs were detected significantly more often in stage I and IIIA LUSC than in stage II ($P = 0.04$) and somewhat more often in peripheral LUSC than in central LUSC ($P > 0.05$) and in stage N0 and N2 than in stage N1 ($P > 0.05$). However, in patients with stage II and IIIA LUSC, contact-type DCs were significantly more often detected at stage IIIA than at stage II (70.0% *vs* 36.4%, respectively, $P = 0.03$); moreover, contact-type DCs were more common in the presence of disease recurrence than in its absence (61.9% *vs* 33.3%, respectively, $P = 0.06$). It can be cautiously assumed that for small tumors, vessels of this type can adequately provide tumor cells with oxygen, while for large tumors, they can contribute to tumor progression, for example, by activating epithelial–mesenchymal transformation.

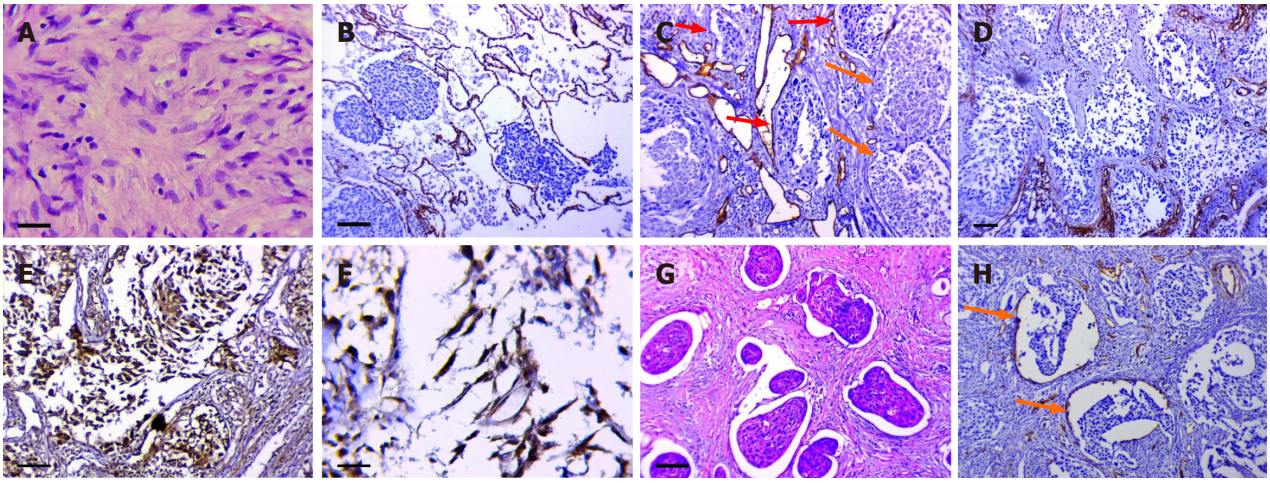


Figure 2 Features of parenchymal and stromal tumor components. A: Loose, fine fibrous connective tissue in the tumor stroma. Scale bar = 10 μm; B: Tumor spread through the unchanged alveolar air spaces (AASs). Scale bar = 100 μm; C: Spread of the relatively unchanged clusters of tumor cells (red arrows) and the completely necrotic tumor masses (orange arrows) through the AASs having thickened walls with dilated capillaries. Scale bar = 100 μm; D: Fragmentation of the tumor masses spreading through the AASs. Scale bar = 100 μm; E: Positive nuclear expression of hypoxia-inducible factor-1 alpha (HIF-1a) in fibroblast-like tumor cells. Scale bar = 10 μm; F: Positive nuclear expression of Snail in fibroblast-like tumor cells. Scale bar = 10 μm; G: Retraction clefts. Scale bar = 100 μm; H: Structures with partial endothelial lining (orange arrows). Scale bar = 100 μm. A and G: Mayer's hematoxylin and eosin staining; B, C and H: Staining with antibodies against cluster of differentiation 34; E: Staining with antibodies against HIF-1a; F: Staining with antibodies against Snail.

There were no significant differences in the frequency of LVs in lymphoid and polymorphic cell infiltrates according to clinical and morphological characteristics or the LUSC prognosis. These vessels were somewhat more common in stage T1a and T3 than in stage T1b-T2b (P = 0.054), in stage I and IIIA than in stage II, in KSCC than in NKSCC, and in the presence of LVI in the tumor samples than in the absence of LVI; however, the differences among the groups were not statistically significant (P > 0.05).

We also investigated the correlations between tumor parenchymal and stromal component features and LUSC clinical and pathological characteristics and disease prognosis. The data are presented in Supplementary Table 3.

The analysis revealed that LFFCT in the tumor stroma was significantly more common in the tumor samples from patients with LUSC recurrence than in those from patients without LUSC recurrence (76.9% *vs* 44.4%, P = 0.01) and was somewhat more common in the tumor samples from patients older than 70 years; in patients younger than 60 years, aged 60 to 70 years and older than 70 years, LFFCT was detected in 45.0%, 57.6% and 88.9% of the tumor samples, respectively (P = 0.16).

In turn, fragmentation in the tumor solid component was observed in 79.5%, 81.3% and 42.9% of the tumor samples from patients with G1, G2 and G3, respectively (P = 0.09); in 70.0%, 72.7% and 100.0% of the tumor samples from patients younger than 60 years, aged 60 to 70 years and older than 70 years, respectively (P = 0.18); in 50%, 50%, 79.4%, 100% and 88.9% of the tumor samples from patients in stages T1a, T1b, T2a, T2b and T3, respectively (P = 0.13); in 63.6%, 81.8% and 85.0% of the tumor samples from patients in stages N0, N1 and N2, respectively (P = 0.24); in 60.0%, 81.8% and 85.0% of the tumor samples from patients in stages I, II and IIIA, respectively (P = 0.13); and in 84.0% and 69.4% of the tumor samples from patients with and without disease recurrence, respectively (P = 0.17).

Retraction clefts were detected in 54.5%, 61.5% and 78.6% of the tumor samples from patients with stage N0, N1 and N2 LUSC, respectively (P = 0.049).

Tumor spread through the AASs was significantly more often detected in patients with peripheral LUSC than in those with central LUSC (78.9% *vs* 41.7%, P = 0.003); in the N0 stage than in the N1 and N2 stages (90.9% *vs* 50.0% and 50.0%, P = 0.006); and in patients with stage I LUSC than in those with stage II or IIIA LUSC (90.0% *vs* 54.5% and 50.0%, respectively, P = 0.03).

Correlations between the different types of tumor microvessels and characteristics of the parenchymal and stromal components of LUSC

Considering that tumor MVs form in the altered stroma, we analyzed the correlations of different types of tumor MVs with the features of the stromal and solid components of the tumor. The results are shown in Table 2.

According to the data obtained, the most significant correlations (P < 0.01) were: (1) A negative correlation of MVD density with tumor spread in the AASs; (2) positive correlations of ADC density with the presence of tumor destruction and fragmentation in the tumor solid component and a negative correlation with the presence of LFFCT in the tumor stroma; (3) positive correlations of SPEL density with retraction clefts and tumor spread in the AASs; (4) positive correlations of DCs with weak expression of CD34 with LFFC in the tumor stroma; and (5) positive correlations of contact-type DCs with the presence of LFFC in the tumor stroma and the presence of retraction clefts.

Univariate and multivariate logistic regression results

Considering the obtained results, to evaluate the independent predictors associated with the risk of LUSC metastasis in

Table 2 Correlations between different types of tumor microvessels and characteristics of the parenchymal and stromal components of lung squamous cell carcinoma

	Fragmentation in the tumor solid component		The presence of LFFCT in the tumor stroma		Retraction clefts		Tumor spread in the AASs	
	Gamma	*P* value	Gamma	*P* value	Gamma	*P* value	Gamma	*P* value
MVD	0.240	0.24	0.034	0.84	0.042	0.79	-0.537	0.002[a]
DCs	0.064	0.66	0.014	0.90	0.211	0.06	-0.251	0.04[a]
ADCs	0.555	0.0006[a]	-0.537	0.0005[a]	0.115	0.45	0.248	0.15
SPELs	0.071	0.65	0.124	0.43	0.517	0.0006[a]	0.435	0.007[a]
DCs with weak expression of CD34	0.297	0.14	0.943	0.0000[a]	0.193	0.21	-0.210	0.22
Contact-type DCs	0.344	0.03[a]	0.502	0.001[a]	0.666	0.0000[a]	-0.077	0.66
The capillaries in the tumor solid component	0.040	0.82	0.009	0.96	0.231	0.16	-0.069	0.72
LVs in lymphoid and polymorphic cell infiltrates	-0.538	0.001[a]	0.054	0.75	0.284	0.07	0.302	0.08

[a]$P < 0.05$, the differences between groups are statistically significant.

AASs: Alveolar air spaces; ADCs: Atypical dilated capillaries; DCs: Dilated capillaries; LFFCT: Loose, fine fibrous connective tissue in the tumor stroma; LVs: Lymphatic vessels; MVD: Microvessel density; SPELs: Structures with partial endothelial lining; CD34: Cluster of differentiation 34.

RLNs and the risk of disease recurrence in the main group of patients, the following predictors were included in the univariate and multivariate analyses: Patient age, tumor location, histology, tumor grade, T stage, N stage, TNM stage, DCs with weak expression of CD34, contact-type DCs, capillaries in the tumor solid component, LFFCT in the tumor stroma, fragmentation in the tumor solid component, retraction clefts and tumor spread through the AASs.

Risk factors for regional lymph node metastases in patients with LUSC

The results of univariate and multifactorial logistic regression analyses of risk factors for RLNM in patients with LUSC are presented in Table 3.

According to the univariate logistic regression analysis, we identified 5 prognostic factors associated with the risk of RLNM, namely, tumor location, T stage, capillaries in the tumor solid component, fragmentation in the solid component of the tumor and retraction clefts. All of these factors, with the exception of T stage, were found to be independent predictors of RLNM. The risk of RLNM was significantly greater in patients with capillaries in the tumor solid component, fragmentation in the tumor solid component and retraction clefts in the tumor tissue and in patients with central LUSC.

We summarized the ORs of the independent predictors for each patient in the main and training groups. For example, for a patient with peripheral LUSC [odds ratio (OR) = 1], capillaries in the solid component of the tumor (OR = 2.72) and retraction clefts (OR = 3.53), without fragmentation in the solid component of the tumor (OR = 1), this number was 8.25 (1 + 2.72 + 3.53 + 1). On the basis of these results, ROC curves were constructed to discriminate between patients with and without RLNM (Figure 3A and B).

For patients in the main cohort, the AUC was 0.784 (DI = 0.71 - 0.915, $P < 0.0001$), and for the training cohort, the AUC was 0.853 (DI = 0.766 - 0.94, $P < 0.0001$). When the OR sum (ORS) was greater than or equal to 12.52 (cutoff), 89 (87.25%) of the 102 patients in the main groups and 23 (100%) of the 23 patients in the training group had metastases in the RLNs. The sensitivity, accuracy and specificity of the method for the main cohort were 66.9%, 68.9% and 72.9%, respectively; for the training cohort, they were 60%, 74.2% and 100%, respectively. Interestingly, in 8 of the 13 patients in the main group with an ORS of 12.52 or more without metastases in RLNs, local (4 patients) or systemic (4 patients) recurrence of the disease was noted within 3 years.

Risk factors associated with LUSC recurrence

The results of univariate and multivariate analyses evaluating the independent factors associated with the risk of LUSC recurrence are presented in Table 4.

According to the univariate logistic regression analysis, we identified 8 prognostic factors associated with the risk of LUSC recurrence, namely, G3, N2 stage, stage IIIA, the presence of adjuvant ChT (A-ChT), the presence of DCs with weak expression of CD34, capillaries in the tumor solid component, LFFCT in the tumor stroma and fragmentation in the tumor solid component, as well as the absence of tumor spread through the AASs. However, only tumor grade 3, N2 stage, the presence of LFFCT in the tumor stroma, fragmentation of the tumor solid component, and the absence of tumor spread through the AASs were found to be independent predictors of a high risk of LUSC recurrence.

Table 3 Univariate and multifactorial logistic regression analysis of risk factors for regional lymph node metastases in patients with lung squamous cell carcinoma

Characteristic	Univariate analysis, OR (95%CI)	*P* value	Multivariate analysis, OR (95%CI)	*P* value
Age				
< 60	1	-		
60-69	1.48 (0.71-3.07)	0.291		
≥ 70	0.75 (0.27-2.10)	0.584		
Tumor location				
Peripheral	1	-	1	-
Central	4.77 (2.36-9.63)	0.0000[a]	7.80 (3.33-18.26)	0.0000[a]
Histology				
KSCC	1	-		
NKSCC	0.77 (0.37-1.60)	0.482		
Tumor grade				
G1	1	-		
G2	1.07 (0.50-2.29)	0.858		
G3	0.78 (0.31-1.97)	0.597		
T stage				
T1a	1	-	1	-
T1b	5.50 (0.91-33.18)	0.063	4.93 (0.76-31.78)	0.093
T2a	6.20 (1.42-27.09)	0.015[a]	2.82 (0.59-13.53)	0.195
T2b	9.33 (1.45-60.21)	0.019[a]	5.27 (0.75-37.28)	0.096
T3	5.60 (1.24-25.25)	0.026[a]	1.66 (0.31-8.80)	0.551
DCs with weak expression of CD34				
Absence	1	-		
Presence	1.10 (0.56-2.15)	0.783		
DCs of "contact type"				
Presence	1	-		
Absence	1.67 (0.86-3.25)	0.13		
The capillaries in the tumor solid component				
Absence	1	-	1	-
Presence	2.89 (1.33-6.28)	0.008[a]	2.72 (1.14-6.48)	0.023[a]
LFFCT in the tumor stroma				
Absence	1	-		
Presence	0.70 (0.32-1.55)	0.383		
Fragmentation in the tumor solid component				
Absence	1	-	1	-
Presence	2.55 (1.28-5.06)	0.008[a]	3.03 (1.33-6.94)	0.009[a]
Retraction clefts				
Absence	1	-	1	-
Presence	2.62 (1.33-5.16)	0.005[a]	3.52 (1.56-7.96)	0.003[a]
Tumor spread through the AAS				
Absence	1	-		

Presence	0.72 (0.37-1.42)	0.342

[a]$P < 0.05$, the differences between groups are statistically significant.

AASs: Alveolar air spaces; DCs: Dilated capillaries; KSCC: Keratinizing squamous cell carcinoma; LFFCT: Loose, fine fibrous connective tissue in the tumor stroma; NKSCC: Nonkeratinizing squamous cell carcinoma; OR: Odds ratio.

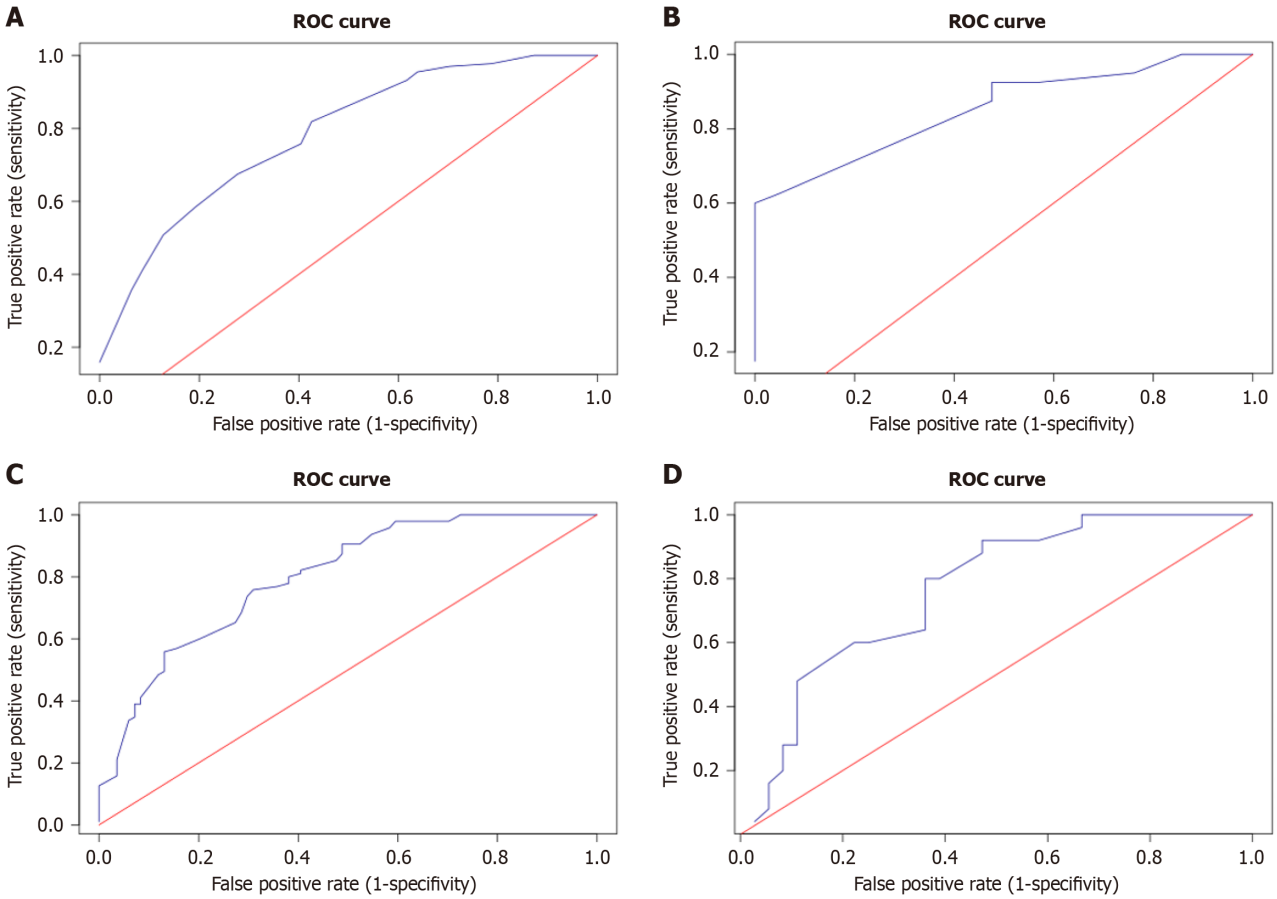


Figure 3 Receiver operating characteristic curves discriminating between patients. A and B: Receiver operating characteristic (ROC) curves discriminating between patients with and without regional lymph node metastases. ROC curve for the main cohort (A); ROC curve for the training cohort (B); C and D: ROC curves discriminating between patients with and without disease recurrence. ROC curve for the main cohort (C); ROC curve for the training cohort (D). ROC: Receiver operating characteristic.

Similarly, we summarized the ORs of independent predictors of the risk of disease recurrence in patients in the main and training groups, and based on the results obtained, we constructed ROC curves that distinguish between patients with and without disease recurrence (Figure 3C and D).

For patients in the main cohort, the AUC was 0.799 (DI = 0.735 - 0.863, $P < 0.0001$), and for the training cohort, the AUC was 0.767 (DI = 0.648 - 0.885, $P < 0.0001$). When the ORS was less than or equal to 12.11 (cutoff), 35 (94.59%) of the 37 patients in the main cohort and 20 (90.91%) of the 22 patients in the training cohort had no LUSC recurrence. The sensitivity, accuracy and specificity of the method for the main cohort were 41.18%, 71.10% and 97.89%, respectively, and those for the training cohort were 54.05%, 69.35% and 92.00%, respectively.

Survival analysis

We analyzed the survival of LUSC patients according to the established predictors of disease recurrence risk. The recurrence free survival (RFS) curves are shown in Figure 4.

The overall survival (OS) curves according to the predictors of a high risk of LC recurrence are shown in Figure 5.

Survival analysis indicated that the OS and disease-free survival (DFS) of patients with LUSC were significantly lower in G3 than in G1 and G2, in N2 than in N0 and N1, and in the presence of LFFCT in the tumor stroma and fragmentation in the tumor solid component than in the absence of LFFCT. The best survival rates were observed for patients with an ORS ≤ 12.11. Moreover, the distribution of patients according to LUSC stage did not significantly differ between the groups of patients in whom the ORS was ≤ 12.11 and those in whom the ORS was > 12.11 ($P = 0.483$). In particular, 29.7%, 37.8% and 32.4% of patients in the first group had stage I, II and IIIA LUSC, respectively, and 13.3%, 41.3% and 45.5%, respectively, of patients in the second group. Tumor spread through the AASs had the least effect on DFS and was not

Table 4 Univariate and multivariate analyses for the predictors of disease recurrence in patients with lung squamous cell carcinoma

Characteristic	Univariate analysis, OR (95%CI)	*P* value	Multivariate analysis, OR (95%CI)	*P* value
Age				
< 60	1	-		
60-69	1.87 (0.97-3.58)	0.06		
≥ 70	2.45 (0.89-6.72)	0.082		
Tumor location				
Peripheral	1	-	1	-
Central	1.86 (0.99-3.47)	0.052[a]	1.04	0.92
Histology				
KSCC	1	-		
NKSCC	1.79 (0.94-3.38)	0.075		
Tumor grade				
G1	1	-	1	-
G2	1.51 (0.76-2.95)	0.232	1.36 (0.59-3.01)	0.466
G3	3.44 (1.39-8.48)	0.007[a]	4.24 (1.37-13.14)	0.001[a]
T stage				
T1a	1	-		
T1b	7.00 (1.04-46.95)	0.045[a]		
T2a	4.26 (0.83-21.74)	0.082		
T2b	5.00 (0.79-31.62)	0.087		
T3	3.38 (0.65-17.69)	0.149		
N stage				
N0	1	-	1	-
N1	2.00 (0.97-4.11)	0.059	1.89 (0.81-4.39)	0.137
N2	3.22 (1.37-7.57)	0.007[a]	3.43 (1.26-9.34)	0.016[a]
Stage				
I	1	-	1	-
II	2.17 (0.89-5.27)	0.088	1.87 (0.45-7.69)	0.385
III	3.13 (1.29-7.60)	0.012[a]	3.44 (0.50-23.49)	0.207
Adjuvant therapy				
Radiation therapy	1	-	1	-
Chemo- and radiotherapy	1.41 (0.50-3.97)	0.513	0.57 (0.16-1.98)	0.376
Chemotherapy	3.02	0.008	2.07 (0.78-5.53)	0.146
DCs with weak expression of CD34				
Absence	1	-	1	-
Presence	4.26 (2.25-8.05)	0.0000[a]	1.18 (0.43-3.23)	0.749
Contact-type DCs				
Absence	1	-		
Presence	0.97 (0.54-1.75)	0.934		
The capillaries in the tumor solid component				
Absence	1	-	1	-

Presence	2.32 (1.24-4.35)	0.008[a]	1.20 (0.56-2.59)	0.638
LFFCT in the tumor stroma				
Absence	1	-	1	-
Presence	6.24 (2.85-13.67)	0.0000[a]	7.34 (3.03-17.76)	0.0000[a]
Fragmentation of the tumor solid component				
Absence	1	-	1	-
Presence	3.03 (1.58-5.82)	0.0009[a]	5.23 (2.27-12.07)	0.0001[a]
Retraction clefts				
Absence	1	-		
Presence	0.71 (0.38-1.29)	0.262		
Tumor spread through the AASs				
Presence	1	-	1	-
Absence	2.06 (1.13-3.76)	0.019[a]	3.37 (1.22-5.66)	0.0083[a]

[a]$P < 0.05$, the differences between groups are statistically significant.

AASs: Alveolar air spaces; DCs: Dilated capillaries; KSCC: Keratinizing squamous cell carcinoma; LFFCT: Loose, fine fibrous connective tissue in the tumor stroma; NKSCC: Nonkeratinizing squamous cell carcinoma; OR: Odds ratio; CD34: Cluster of differentiation 34.

associated with OS; moreover, the nonsignificant difference in survival in the training cohort, depending on tumor grade and fragmentation in the tumor solid component, seems to be due to the small number of patients in individual subgroups.

According to multivariate analysis, although the type of adjuvant therapy was not an independent prognostic factor, the 5-year RFS in the absence of adjuvant therapy, with A-ChT, with adjuvant RT (A-RT) and with A-chemoradiotherapy were 64.6%, 30.4%, 57.9% and 47.8%, respectively ($P = 0.0027$). In these groups, 60.4%, 14.6%, 0% and 0% of patients had stage I LUSC; 25.0%, 47.8%, 60.5% and 21.7% had stage II LUSC; and 14.6%, 50.7%, 39.5% and 78.3% had stage IIIA LUSC ($P < 0.0001$).

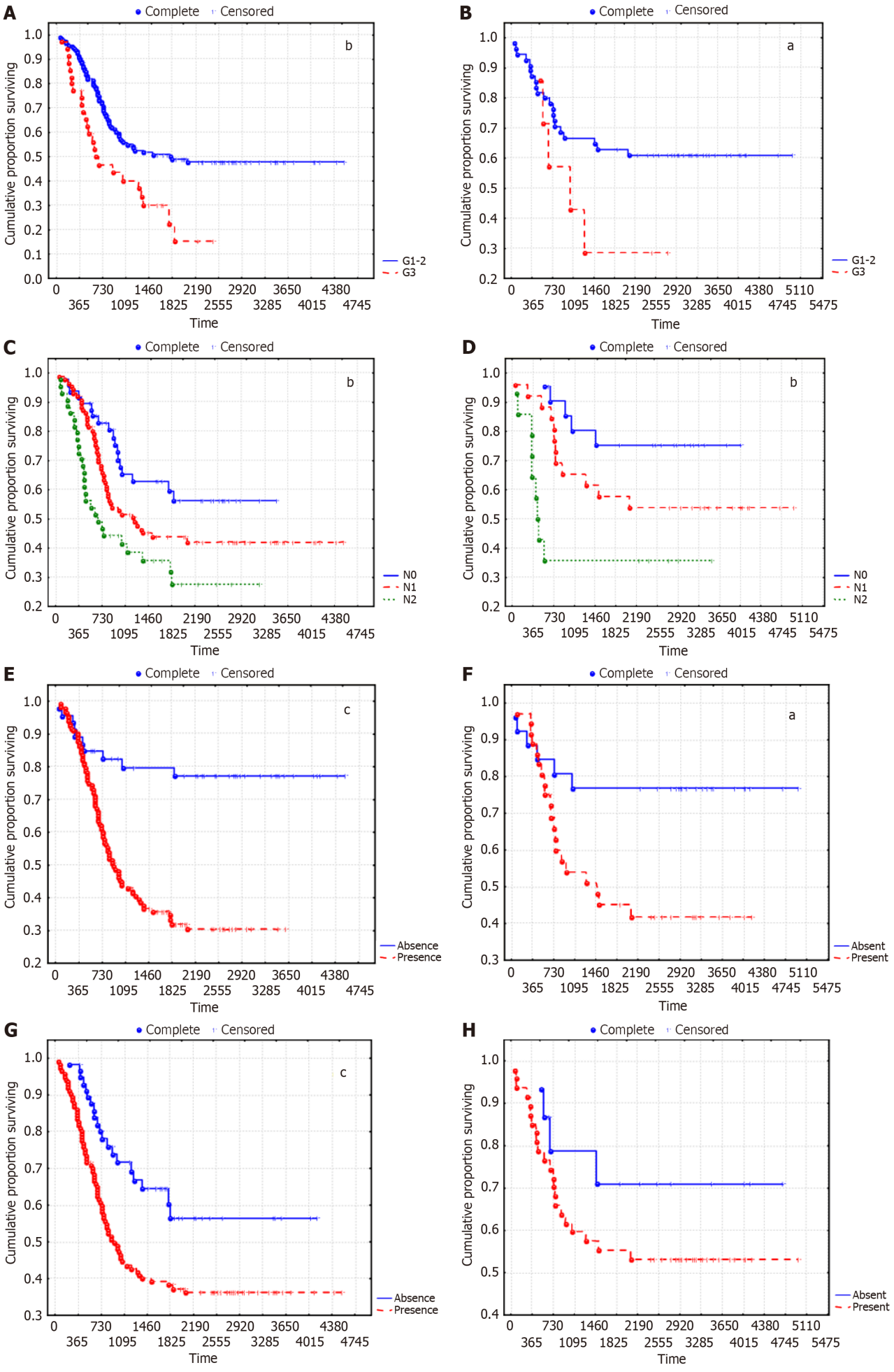
DISCUSSION

LC is an important medical, social and economic problem in most countries worldwide[1,2,31]. Unsatisfactory long-term treatment results for this pathology are associated with late diagnosis, either due to a lack of symptoms in early-stage disease or due to presentation with nonspecific symptoms common with a broad range of alternative diagnoses. According to a large meta-analysis of 78979 R0 resections for NSCLC, the 5-year survival rates ranged from 40% to 74% for stage IA, 38% to 68% for IB, 28% to 53% for stage II, and 18% to 39% for stage IIIA[12]. Thus, early detection of this pathology is critical for reducing mortality in patients with NSCLC.

NSCLC is a very heterogeneous disease. The most common subtypes of NSCLC are adenocarcinoma and LUSC, the incidence of which is 19%–60% and 20%–67.5%, respectively, of the total number of NSCLC cases[4-6]. Despite major breakthroughs in the treatment of lung adenocarcinoma, there are no treatment methods specific for LUSC; as a result, the prognosis of this disease has been poor, especially in the late stages[4,7,8]. The treatment efficacy of LUSC will be largely determined by the choice of optimal treatment regimens and will depend on the sensitivity of the tumor to radiation, ChT and targeted therapy. Thus, determining the risk of disease relapse, including assessing the status of RLNs, is of key importance for choosing the optimal treatment for LUSC. In addition, research in this direction can contribute to understanding the mechanisms of LUSC progression and, consequently, to new approaches for the treatment of this disease.

Currently, angiogenesis is regarded as both an important factor in disease prognosis[15,16] and in the effectiveness of angiogenesis blockers, ChT and targeted therapy[16,20,32-34].

Methodological approaches for assessing angiogenesis in NSCLC vary widely. In most studies, the authors limit themselves to a quantitative assessment of MVD or the level of vascular endothelial growth factor (VEGF) expression. In addition, when calculating the MVD, researchers often consider only normal MVs, *i.e.*, vessels whose lumen does not exceed the diameter of erythrocytes; other types of tumor MVs are excluded from the assessment. As a result of these studies, correlations between MVD and various factors associated with tumor progression have been established; for example, correlations between MVD and the expression of markers such as TFIIB-related factor 2[3], ASK1-interacting protein-1[13] and chitinase 3-like 1[35] in tumor tissue, as well as between MVD and the levels of CXC chemokine ligand 4 in plasma[36]. Several studies have noted a correlation between the prognosis of NSCLC and MVD[37] and between the prognosis of NSCLC and the expression of VEGF-A and angiopoietin-2 (Ang-2)[38]. However, this dependence was observed predominantly for lung adenocarcinoma and not for LUSC. The serum levels of VEGF, Ang-2, and Interleukin-8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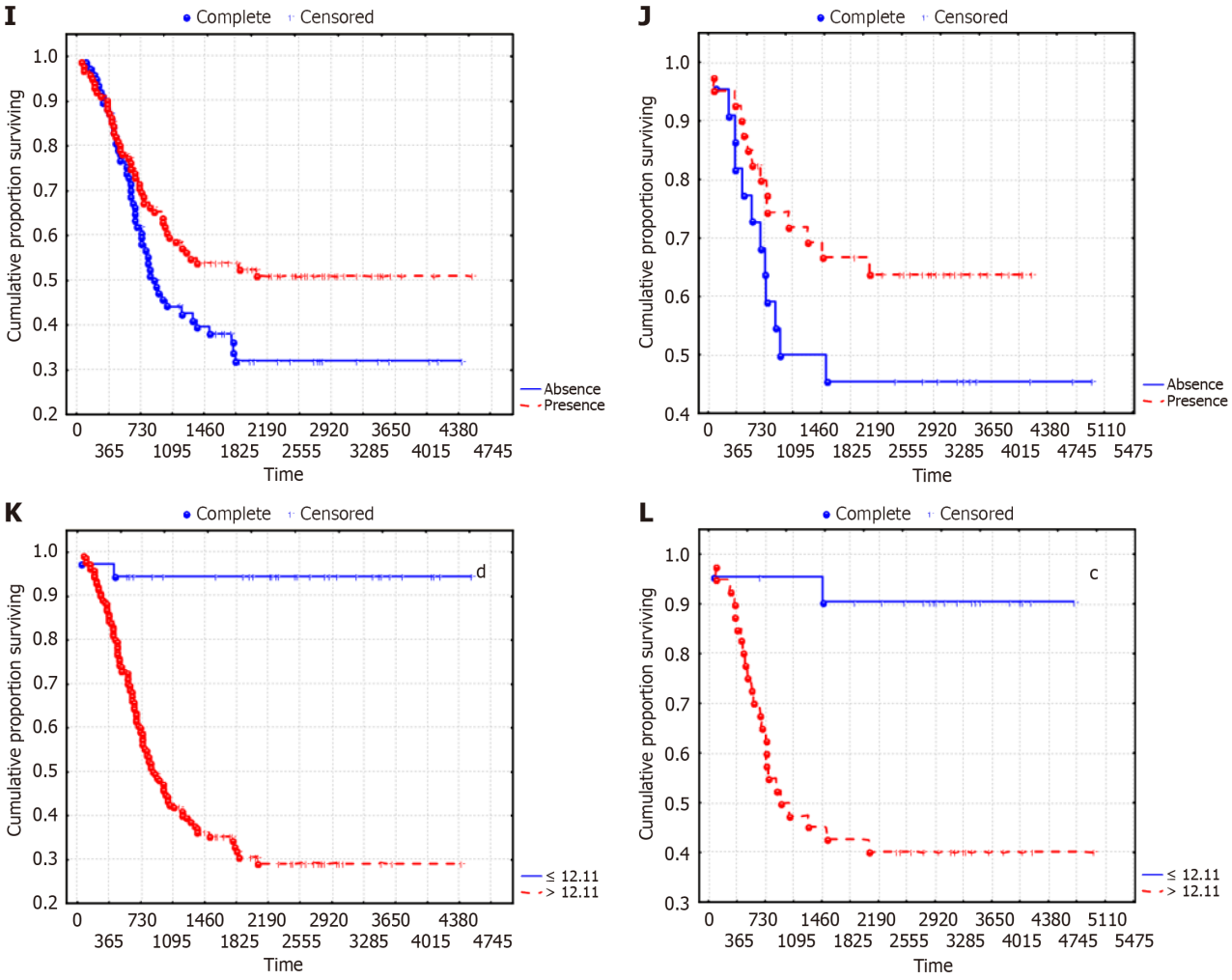


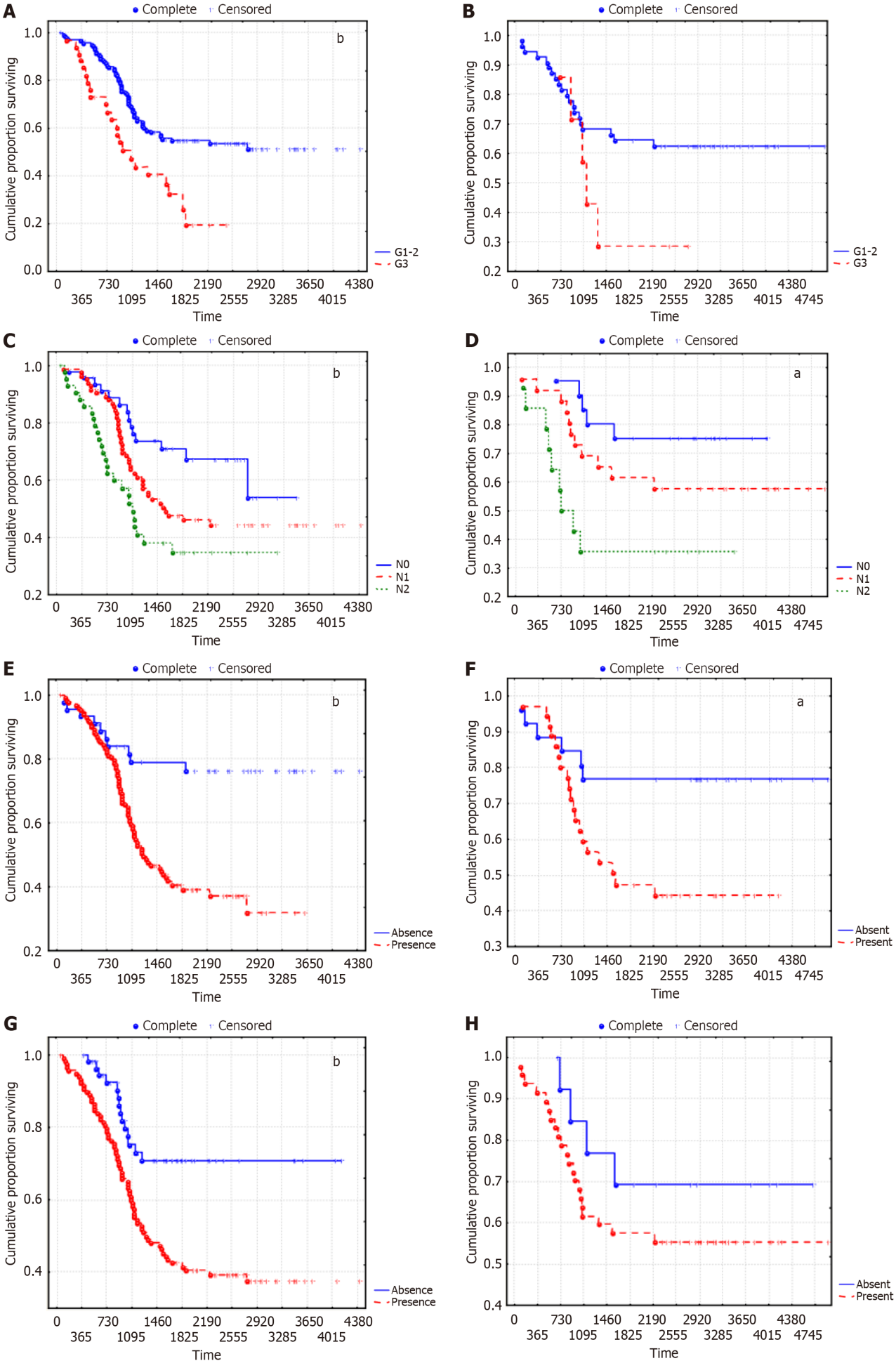
Figure 4 Disease-free survival curves according to the independent predictors of lung squamous cell carcinoma recurrence risk (log-rank test). A and B: Tumor grade; C and D: N stage; E and F: Loose, fine fibrous connective tissue; G and H: Fragmentation of the tumor solid component; I and J: Tumor spread through the alveolar air spaces; K and L: Odds ratio sum (ORS) ≤ 12.11 or ORS > 12.11. A, C, E, G, I and K: Recurrence free survival (RFS) curves for the main cohort of patients; B, D, F, H, J and L: RFS curves for the training cohort of patients. [a]P < 0.05, [b]P < 0.01, [c]P < 0.001, [d]P < 0.0001.

were also not associated with survival in patients with NSCLC[39]. Notably, the assessment of MVD in patients with advanced NSCLC (stage IIIA) using an antibody against CD31 showed that a high MVD is associated with a decrease in the 2-year survival of patients but is not correlated with VEGF-A expression[40].

We believe that the ambiguity of the results obtained may be associated with the heterogeneity of tumor MVs, which differ not only in origin and morphology but also in clinical significance[41,42]. Paulsen *et al*[43], investigating tumor MVs in NSCLC in accordance with the classification proposed by Pezzella *et al*[44], identified three angiogenic subtypes of tumor blood supply, namely, the basal, diffuse and papillary subtypes and the nonangiogenic alveolar subtype[43]. The authors revealed correlations of different vascular patterns with the immune microenvironment of the tumor, the severity of hypoxia and EMF markers. Moreover, the relationships of angiogenic subtypes with clinical and pathological characteristics and the prognosis of NSCLC have not been established. In lung adenocarcinomas, when a nonangiogenic alveolar pattern was detected, there was a significant decrease in patient survival, but in LUSC, such a dependence was not noted[43].

Over the past few years, we have been actively studying the different types of tumor MVs in gastric cancer, breast cancer and cervical cancer[21-23]. In this study, we investigated the features of different types of tumor MVs in LUSC and confirmed the acceptability of the previously proposed classification of tumor MVs. In this study, we also characterized the features of the tumor stromal and parenchymal components as factors that, on the one hand, can influence the formation of certain types of vessels, while on the other hand, different types of tumor vessels can provide the tumor tissue with oxygen and nutrients to different degrees; consequently, they can influence the behavior of the tumor, as well as its tendency to invade and metastasize[45].

The present study established a number of independent predictors associated with the risk of LUSC metastasis in RLNs and the risk of disease recurrence. The first group of predictors included tumor location, the presence of capillaries in the tumor solid component, the presence of fragmentation in the tumor solid component, and the presence of retraction clefts. We summarized the ORs of the independent predictors for each patient and, on the basis of these results, constructed ROC curves that discriminate between patients with and without RLNM. When the ORS was greater than or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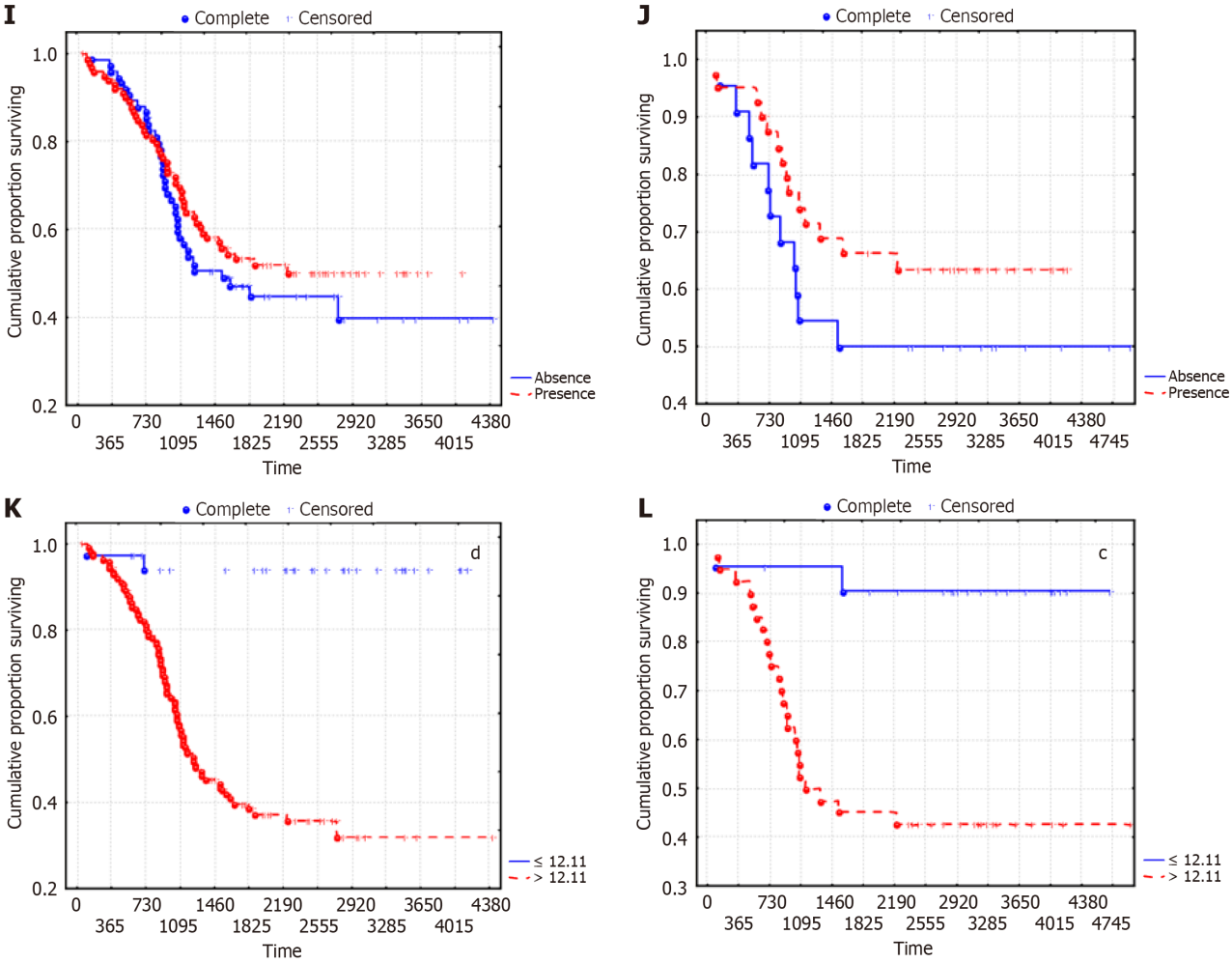

Figure 5 Overall survival curves according to the independent predictors of lung squamous cell carcinoma recurrence risk (log-rank test). A and B: Tumor grade; C and D: N stage; E and F: Loose, fine fibrous connective tissue; G and H: Fragmentation of the tumor solid component; I and J: Tumor spread through the alveolar air spaces; K and L: Odds ratio sum (ORS) ≤ 12.11 or ORS > 12.11. A, C, E, G, I and L: Overall survival (OS) curves for the main cohort of patients; B, D, F, H, J and L: OS curves for the training cohort of patients. [a]P < 0.05, [b]P < 0.01, [c]P < 0.001, [d]P < 0.0001.

equal to 12.52 (cutoff), 89 (87.25%) of the 102 patients in the main cohort and 23 (100%) of the 23 patients in the training cohort had metastases in the RLNs. Furthermore, in 8 out of the 13 patients in the main group with an ORS of 12.52 or more without metastases in RLNs, local (4 patients) or systemic (4 patients) disease recurrence was noted within 3 years.

In turn, tumor grade 3, N2 stage, the presence of LFFCT in the tumor stroma, fragmentation in the tumor solid component and tumor spread through the AASs were found to be independent predictors of a high risk of LUSC recurrence. Similarly, the constructed ROC curves made it possible to identify a group of patients with a low risk of LUSC recurrence (less than 90%). When the ORS was less than or equal to 12.11 (cutoff), 35 (94.59%) of the 37 patients in the main cohort and 20 (90.91%) of the 22 patients in the training cohort had no LUSC recurrence.

The main cause of death in cancer patients is metastasis. A number of studies have shown that circulating clusters of tumor cells are associated with a greater risk of metastasis and relapse in NSCLC patients than single circulating tumor cells[46-48]. Previously, we proposed a possible mechanism for the formation of tumor cell clusters in tumor MVs[21]. This mechanism is associated with the retraction of tumor cells from the underlying stroma and the formation of hollow structures with tumor cells in the lumen, which corresponds to the previously described phenomenon of retraction clefts [27,28,29]. Subsequently, the inner surface of the described hollow structures may be partially or completely lined with endothelium. Characteristic features of this stage include the presence of SPELs and vascular invasion with clusters of tumor cells in the lumen. When the formed cavity structures merge with blood or LVs, clusters of tumor cells may enter the blood or lymphatic bed. In gastric cancer, breast cancer and cervical cancer, the presence of retraction clefts and SPELs was correlated with the presence of metastases in RLNs and was associated with a high risk of disease recurrence[21-23]. In a study by Hisakane *et al*[49], a vascular invasion size greater than 425 μm was the most significant factor associated with poor prognosis in patients with stage I LUSC. Tumor cells within larger areas of vascular invasion expressed higher levels of PDPN and cancer stem cell markers; a greater MVD; and greater numbers of CD204 (+) macrophages and α-SMA (+) myofibroblasts than did those within smaller vascular invasion areas[49].

Importantly, increasing evidence suggests that intravasation of single, apoptotic or clusters of tumor cells can occur *via* leaky neoangiogenetic vessels in the tumor core and not by crossing the adjacent tumor stroma after EMT[48]. Hamilton *et al*[48] emphasized that some tumor emboli may be located in blind nonfunctioning tumor vessels, which may limit

their entry into the bloodstream. We believe that these data indirectly confirm the possibility of the formation of circulating clusters of tumor cells by the mechanism of cavity-type angiogenesis described above. Indeed, until the cavity structures merge with the blood and LVs, tumor cell clusters cannot reach the bloodstream. In addition, the large size of tumor emboli may also limit their entry into the bloodstream. For example, in LUSC and squamous cell carcinoma of the cervix, SPELs and vascular invasion with very large tumor emboli (more than 100 μm) were observed in the tumor stroma, the entry of which into the bloodstream is highly questionable.

In our study, we also observed fragmentation of the solid component of the tumor, which was manifested by the appearance of fibroblast-like cells expressing HIF-1α and Snail, indicating its connection with the mechanisms of EMT. According to multivariate analysis, the described phenomenon was an independent predictor of a high risk of LUSC metastasis and disease relapse. Similar results were obtained for squamous cell carcinoma of the cervix[23]. Thus, fragmentation of the solid component of the tumor through EMT may be involved in the intravasation of tumor cells into tumor MVs, thereby promoting the formation of metastases.

Of particular interest is also the association between LFFCT and a high risk of disease relapse. A number of studies have shown that tumor tissue contains several types of noncancerous regenerative cells, including embryonic stem cells, mesenchymal stem cells and adult stem cells. These cells are the source of cancer-associated fibroblasts (CAFs), tumor endothelial cells, and tumor-associated macrophages. These cells play key roles not only in cancer progression but also in the development of drug resistance[50]. It can be assumed that the cells with large pale nuclei and weakly condensed chromatin observed in LFFCT may belong to one of these cell types. In our study, LFFCT was detected predominantly at the invasive margin of the tumor. Several studies have shown that tumor-promoting stromal cells, including PDPN-positive CAFs, CD204-positive tumor-associated macrophages, and CD34+ microvascular cells, are also more frequently recruited to invasive tumor margins[51].

Notably, the majority of the LUSC specimens studied (68%) exhibited tumor spread through AASs. Currently, tumor spread through AASs is regarded as a factor associated with an unfavorable prognosis in patients with NSCLC[52-54]. A decrease in patient survival is likely associated with co-option of the interalveolar septum vessels by the tumor, which leads to a decrease in the effectiveness of systemic therapy due to the development of resistance to antiangiogenic agents and chemotherapy[55,56]. However, in our study, tumor spread through AASs had no significant effect on the survival of patients with LUSC. Moreover, according to multivariate analysis, the absence of tumor spread through the AASs was an independent predictor associated with the risk of LUSC recurrence. It can be assumed that tumor spread through AASs leads to co-option of the vessels of the interalveolar septa by tumors, which, on the one hand, reduces hypoxia in the tumor and the need for the formation of new vessels and, on the other hand, can increase the sensitivity of the LUSC to radiation and chemotherapy. This assumption is supported by the negative correlations of the MVD and density of DCs with tumor spread through AASs. However, this assumption requires verification.

We would also like to note that according to the clinical guidelines for the treatment of NSCLC, A-RT is considered to be a factor associated with worse survival in patients[57]. However, in our study, the five-year survival rate of patients who received A-RT was even greater than that of patients who received A-ChT. We believe that this may be because most studies of NSCLC have not taken tumor histology into account when analyzing the effect of treatment modalities on long-term outcomes. For example, in a meta-analysis by Smeltzer *et al*[12], more than 50% of patients with NSCLC were diagnosed with adenocarcinoma, which is known to be less sensitive to RT than is squamous cell carcinoma. Moreover, it can be assumed that in patients with LUSC, the spread of tumors along AASs can even increase the effectiveness of RT by reducing hypoxia in tumor tissue, which was reflected in the treatment results of patients with LUSC. However, these assumptions require confirmation in clinical studies.

CONCLUSION

Thus, according to the results of this study, the independent predictors associated with a high risk of LUSC metastasis to RLNs are the central location of the tumor, the presence of capillaries in the solid component of the tumor, fragmentation of the tumor solid component, and the presence of peritumoral retraction clefts. In turn, tumor grade 3, N2 stage, the presence of LFFCT in the tumor stroma, fragmentation of the tumor solid component, and absence of tumor spread through the AASs are independent predictors of a high risk of LUSC recurrence. Notably, these results are similar to those obtained when studying different types of tumor vessels in gastric, breast and squamous cell carcinomas of the cervix. This study has several limitations. First, this was a single-center retrospective study; therefore, the results obtained need to be confirmed in other prospective clinical studies. Second, there is a need to standardize the quantitative indicators of the studied markers for their correct use in clinical practice. Third, the small sample size (180 patients) may have led to selection bias despite the use of a fairly homogeneous group of LUSC patients. Thus, future prospective multicenter studies with larger cohorts are needed to further explore the prognostic and predictive significance of different types of tumor MVs in patients with LUSC. Considering the data on the relationship of a certain type of vessel with the characteristics of the stromal and parenchymal components of the tumor, further research in this direction may also be of great scientific and practical interest.

FOOTNOTES

Author contributions: Senchukova MA designed and performed the research, and wrote the paper; Kalinin EA acquired and analyzed the data and contributed substantially to the conception and design of the study; Volchenko NN participated in the discussion of related

data and revised and approved the final version; All the authors wrote and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Orenburg State Medical University (Russia, Orenburg), No. 281, dated 30 September 2021.

Informed consent statement: Patients were not required to give informed consent to the study because the study was retrospective and analyses were performed with anonymous clinical data obtained after each patient agreed to treatment *via* written consent.

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

Data sharing statement: Data from patients included in the study in Statistica10 table or Excel table format can be provided upon request to the corresponding author at masenchukova@yandex.com.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Human epidermal growth factor receptor 2 expression level and combined positive score can evaluate efficacy of advanced gastric cancer

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Abstract

BACKGROUND

Although treatment options for gastric cancer (GC) continue to advance, the overall prognosis for patients with GC remains poor. At present, the predictors of treatment efficacy remain controversial except for high microsatellite instability.

AIM

To develop methods to identify groups of patients with GC who would benefit the most from receiving the combination of a programmed cell death protein 1 (PD-1) inhibitor and chemotherapy.

METHODS

We acquired data from 63 patients with human epidermal growth factor receptor 2 (HER2)-negative GC with a histological diagnosis of GC at the Cancer Hospital, Chinese Academy of Medical Sciences between November 2020 and October 2022. All of the patients screened received a PD-1 inhibitor combined with chemotherapy as the first-line treatment.

RESULTS

As of July 1, 2023, the objective response rate was 61.9%, and the disease control rate was 96.8%. The median progression-free survival (mPFS) for all patients was 6.3 months. The median overall survival was not achieved. Survival analysis showed that patients with a combined positive score (CPS) ≥ 1 exhibited an extended trend in progression-free survival (PFS) when compared to patients with a CPS of 0 after receiving a PD-1 inhibitor combined with oxaliplatin and tegafur as the first-line treatment. PFS exhibited a trend for prolongation as the expression level of HER2 increased. Based on PFS, we divided patients into two

groups: A treatment group with excellent efficacy and a treatment group with poor efficacy. The mPFS of the excellent efficacy group was 8 months, with a mPFS of 9.1 months after excluding a cohort of patients who received interrupted therapy due to surgery. The mPFS was 4.5 months in patients in the group with poor efficacy who did not receive surgery. Using good/poor efficacy as the endpoint of our study, univariate analysis revealed that both CPS score (P = 0.004) and HER2 expression level (P = 0.015) were both factors that exerted significant influence on the efficacy of treatment the combination of a PD-1 inhibitor and chemotherapy in patients with advanced GC (AGC). Finally, multivariate analysis confirmed that CPS score was a significant influencing factor.

CONCLUSION

CPS score and HER2 expression both impacted the efficacy of immunotherapy combined with chemotherapy in AGC patients who were non-positive for HER2.

Key Words: First line; Gastric cancer; Human epidermal growth factor receptor 2; Programmed cell death protein 1; Progression-free survival

Core Tip: For when considering non-positive human epidermal growth factor receptor 2 (HER2) advanced gastric cancer (AGC), combined positive score and HER2 expression are represent influencing factors for the treatment efficacy of AGC patients receiving immunotherapy combined with chemotherapy. Survival analysis with When using progression-free survival (PFS) as the end point, survival analysis suggested that the HER2 expression level was levels are suggestive of the efficacy of treatment featuring a programmed cell death protein 1 inhibitor combined with and chemotherapy. With the increase of HER2 expression, PFS also showed a tendency to prolong increase with an increase in HER2 expression.

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INTRODUCTION

Gastric cancer (GC) is one of the most common forms of malignant tumor with the fifth highest incidence of cancer in the world; in addition, GC ranks third in terms of global fatalities from cancer, thus representing a serious threat to population health[1]. Surgical resection remains as the main radical treatment for GC at present. However, most patients with GC are in advanced stages of the disease at diagnosis; consequently, < 50% of patients can achieve R0 resection. A previous study reported that > 80% of GC patients are diagnosed at an advanced stage, and most are accompanied by extensive invasion and distant metastasis, thus missing the opportunity for radical surgery[2]. Despite the continuous progression of treatment options for GC, the overall prognosis for GC remains poor, traditional chemotherapy drugs have entered a bottleneck period, and the selection of targeted drugs is limited.

Over recent years, immune checkpoint inhibitors (ICIs), such as programmed cell death protein 1 (PD-1)/programmed cell death 1 Ligand 1 (PD-L1) inhibitors or cytotoxic T lymphocyte-associated antigen-4 inhibitors, have become the main treatment options for many types of cancer. For the first-line treatment of advanced GC (AGC), the combination of a PD-1 inhibitor and chemotherapy has gradually become the new standard mode of treatment. Multiple clinical studies have reported that approximately 85% of human epidermal growth factor receptor 2 (HER2)-negative (HER2 IHC 0 or 1+ or 2+/FISH-) patients achieved efficacious results following first-line treatment involving the combination of a PD-1 antibody and chemotherapy in the first-line treatment of AGC *via* the administration of ICIs and different chemotherapy regimens, when considering the comprehensive positive score (CPS) of PD-L1 expression on immunohistochemistry and different research endpoint indicators[3-6]. However, not all patients can benefit from treatment based on PD-1 inhibitors. In addition to the clear status of high microsatellite instability, the predictive value of the PD-L1 CPS score remains controversial, and other predictive factors, such as high tumor mutational burden, remain uncertain. Therefore, there is a clear need to develop a method to identify groups of patients with AGC who might benefit the most from the combination of a PD-1 inhibitor and chemotherapy.

In this study, we analyzed the efficacy of a first-line PD-1 inhibitor combined with chemotherapy in patients with AGC. We aimed to identify relevant risk factors that can predict treatment efficacy in order to provide a method to identify potential beneficiaries as early as possible and provide a basis for clinical practice.

MATERIALS AND METHODS

Patient population

We reviewed and collected data from 63 patients with HER2-negative patients with AGC who were diagnosed histologically at the Cancer Hospital, Chinese Academy of Medical Sciences from November 2020 to October 2022 (Table 1). All patients had proficient mismatch repair (pMMR). These patients were treated with oxaliplatin and tegafur (SOX) chemotherapy combined with tislelizumab (a PD-1 monoclonal antibody). All patients were at least 18 years of age and had measurable lesions according to the RECIST 1.1 criteria. The initial TNM staging was stage IV or recurrence and metastasis after radical surgery. Common distant metastatic sites included retroperitoneal lymph nodes, supraclavicular lymph nodes, peritoneum, liver, and ovaries. Patients with initial stage IV had not previously received anti-tumor therapy, and patients with postoperative recurrence had an interval of more than 6 months between adjuvant treatment after radical resection. The main exclusion criteria included previous chemotherapy or targeted therapy, ICI treatment history (excluding patients with recurrence and metastasis more than 6 months after adjuvant or neoadjuvant chemotherapy), and other malignant tumors over the previous 5 years (excluding radical cervical cancer, skin squamous cancer, or basal-cell carcinoma).

This study was approved by the Ethics Committee of the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College.

Treatment plan

All of the selected patients received tislelizumab combined with chemotherapy every 3 wk. The specific dose of SOX regimen was 130 mg/m^2 oxaliplatin (2 h intravenous infusion) on day 1, and tegafur was taken orally at doses of 80 mg, 100 mg, or 120 mg/d (< 1.25 m^2, 1.25 to 1.5 m^2, or > 1.5 m^2) depending on body surface area, on days 1 to 14. Tislelizumab was given every 3 wk at a standard dose of 200 mg. If the combination treatment reached six cycles, we continued to maintain tegafur combined with tislelizumab after stopping oxaliplatin. The clinicians followed institutional guidelines for the use of premedication anti-emetics and growth factors. The dose of the chemotherapy regimen was reduced at the discretion of the clinicians. Some patients who were evaluated as operable by multi-disciplinary treatment after chemotherapy underwent radical gastrectomy.

Evaluation indicators

We collected the following baseline demographic and clinical data: age, sex, performance status (PS) score, primary cancer site, metastatic organs, HER2 expression level, PD-L1 CPS score[7], Epstein-Barr virus-encoded small RNA (EBER) status, treatment plan and drug dosage adjustment, all of which were recorded in our medical record system.

All patients underwent tumor imaging examinations (computed tomography and/or magnetic resonance imaging) every two cycles, and were evaluated according to the response evaluation criteria in solid tumors 1.1 criteria. Objective response rate (ORR) was defined as the sum of the proportions of complete remission (CR) and partial remission (PR). Disease control rate (DCR) was defined as the proportion (%) of patients who achieved remission (PR + CR) and stable disease following treatment. Progression-free survival (PFS) was defined as the time from the beginning of chemotherapy to the date of imaging confirmation of disease progression or the date of radical surgical resection, or the time of death from any cause before disease progression (whichever came first). Overall survival (OS) was defined as the time from the beginning of chemotherapy to death due to any cause.

Statistical analysis

Data were statistically analyzed with R-language. PFS was estimated by Kaplan-Meir survival curve analysis. The Cox proportional risk model was used to estimate the risk ratio. All statistical tests were bidirectional, and P value of < 0.05 was considered significant. Logistic regression model analysis was performed to determine whether gender, age, PS score, PD-L1 CPS score, HER2 expression, EBER expression, and the number of distant metastasized organs were risk factors for efficacy. The Kruskal-Wallis test was used to analyze the variance of all patients after two cycles of treatment.

RESULTS

Patient characteristics

The clinical and pathological features of the patients included in this study are shown in Table 1. As of July 1, 2023, all patients had been followed-up for more than 6 months from enrollment to date. A total of 63 patients were included in this study, including 34 males and 29 females. The initial diagnosis was stage IV in 56 patients and postoperative recurrence occurred in seven patients. The included patients ranged in age from 27 to 76 years, with a mean and median age of 54 and 56 years, respectively. PS score ranged from 0 to 2. Distant sites of metastasis mainly included the liver, peritoneum, ovary and distant lymph nodes. pMMR status was determined by immunohistochemistry. HER2 was either not expressed or expressed at low levels (1+/2+ and FISH was not amplified). EBER status and PD-L1 CPS score were also determined by immunohistochemistry.

Efficacy

As of the July 1, 2023, the numbers of patients experiencing disease progression and death were 40 and 17, respectively. The ORR was 61.9% (range: 48.8%-73.4%) and the DCR was 96.8% (range: 89.0%-99.6%). The median PFS (mPFS) for the

Table 1 Baseline characteristics, *n* (%)

Variable	All (63)	Excellent efficacy group (42)	Poor efficacy group (21)
Age	56 (51, 58)	56 (50, 58)	59 (50, 62)
Sex			
Male	34 (54.7)	20 (47.6)	14 (66.7)
Female	29 (45.3)	22 (52.4)	7 (33.3)
ECOG-PS			
0	17 (27.0)	12 (28.6)	5 (23.8))
1	39 (61.9)	26 (61.9)	13 (61.9)
2	7 (11.1)	4 (9.5)	3 (14.3)
Disease status			
Unresectable	55 (87.3)	38 (90.5)	17 (81.0)
Recurrent	8 (12.7)	4 (9.5)	4 (19.0)
Number of organs involved			
1	40 (63.5)	27 (64.3)	13 (61.9)
≥ 2	23 (36.5)	15 (35.7)	8 (38.1)
HER2 status			
0	31 (49.2)	17 (40.5)	14 (66.7)
1	15 (23.8)	10 (23.8)	5 (23.8)
2	11 (17.5)	11 (26.2)	0 (0)
Unmeasured	6 (9.5)	4 (9.5)	2 (9.5)
CPS			
0	8 (12.7)	2 (4.8)	6 (28.6)
1 ≤ CPS < 5	11 (17.5)	7 (16.7)	4 (19.0)
5 ≤ CPS < 10	12 (19.0)	9 (21.4)	3 (14.3)
CPS ≥ 10	21 (33.3)	18 (42.9)	3 (14.3)
Unmeasured	11 (17.5)	6 (14.3)	5 (23.8)
EBER			
Positive	7 (11.1)	6 (14.3)	1 (4.8)
Negative	42 (66.7)	26 (61.9)	16 (76.2)
Unmeasured	14 (22.2)	10 (23.8)	4 (19.0)

ECOG-PS: Eastern cooperative oncology group-performance status; CPS: Combined positive score; HER2: Human epidermal growth factor receptor 2; EBER: Epstein-Barr virus-encoded small RNA.

entire cohort was 6.3 months (range: 6.0-7.8 months). The median OS was not attained. Survival analysis was performed for patient age, sex, PS score, PD-L1 CPS score, HER2 expression, EBER status, and number of metastatic organs (Figure 1). Analysis showed that patients with a CPS ≥ 1 showed an extended trend in PFS when compared to the population of patients with a CPS score of 0 after receiving a PD-1 inhibitor combined with chemotherapy as the first-line treatment (P = 0.06). PFS exhibited a trend for prolongation as the expression level of HER2 increased (P = 0.074).

Predictive effect of clinicopathological factors on immunotherapy efficacy in patients with GC

We divided patients into two groups based on their mPFS: A treatment group with excellent efficacy and a treatment group with poor efficacy. The excellent efficacy group included patients with a PFS > 6.3 months, or those who met surgical standards (the primary lesion was resectable and the metastases were indistinct) within 6.3 months and achieved radical resection. The poor efficacy group included patients with a PFS not exceeding 6.3 months or those who died from any cause within 6.3 months. The two sets of baseline characteristics are shown in Table 1.

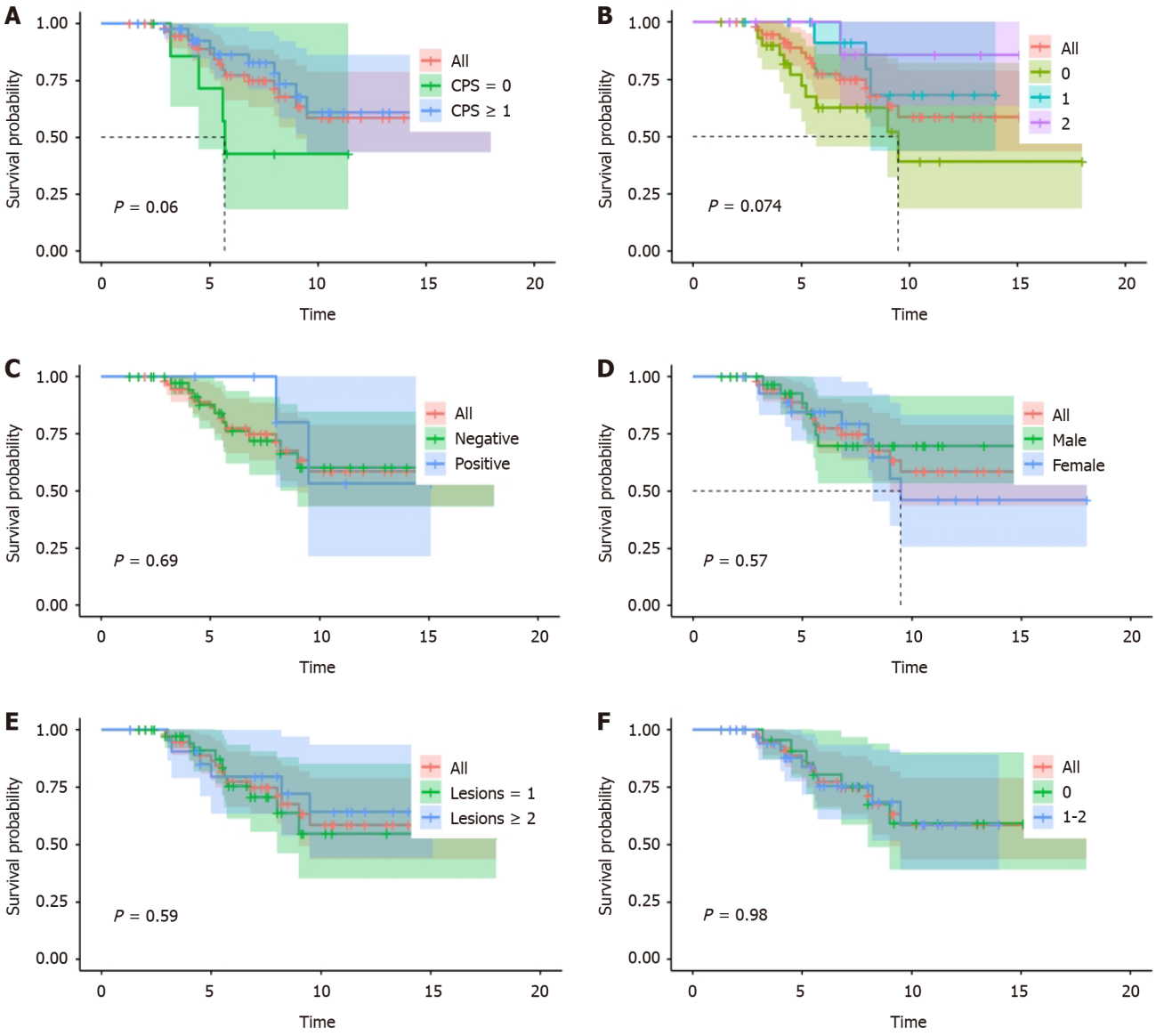

Figure 1 Progression-free survival of clinicopathological factors. A: The progression-free survival (PFS) of different combined positive scores of programmed cell death 1 ligand 1; B: PFS according to different human epidermal growth factor receptor 2 expression levels; C: PFS according to Epstein-Barr virus-encoded small RNA status; D: PFS according to patient gender; E: PFS according to different numbers of metastatic organs; F: PFS according to different performance status scores.

The mPFS of the excellent efficacy group was 8 months (range: 6.9-9.2 months and 16 patients underwent surgery in this group. Excluding those who experienced an interruption of immunotherapy due to surgery, the remaining 26 patients had a mPFS of 9.1 months (range: 8.8-11.1 months). Patients in the group with poor efficacy who did not receive surgery had a mPFS of 4.5 months (range: 3.7-4.9 months).

Next, we attempted to identify risk factors by analyzing patient age, sex, PS score, PD-L1 CPS score, HER2 expression, EBER status, and the number of metastatic organs. Univariate analysis revealed that age, sex, PS score, EBER status, and number of metastatic organs were not significant factors influencing the efficacy of AGC patients receiving a combination of a PD-1 inhibitor and chemotherapy. However, CPS score (P = 0.004) and HER2 expression (P = 0.015) were identified as factors influencing the efficacy of AGC patients receiving a combination of a PD-1 inhibitor and chemotherapy (Figure 2). The therapeutic effect of a CPS ≥ 1 was significantly better than that of a CPS = 0, and the therapeutic effect of a CPS ≥ 5 was significantly better than that of a CPS < 5. However, no significant difference in efficacy was detected between the two groups with a CPS ≥ 10 when compared with patients with a CPS < 10. The higher the expression of HER2, the better the therapeutic effect. Multivariate analysis further suggested that CPS score was a factor influencing the efficacy of AGC patients receiving a combination of a PD-1 inhibitor and chemotherapy, further confirming the role of CPS score in predicting the efficacy of immunotherapy in GC patients (Supplementary material).

The predictive effect of short-term objective remission on immunotherapy efficacy in patients with GC

Finally, we analyzed the objective efficacy of all patients after two cycles of treatment, with excellent/poor efficacy as the research endpoint. Of the 42 patients in the excellent efficacy group, 30 patients achieved PR after two cycles of treatment,

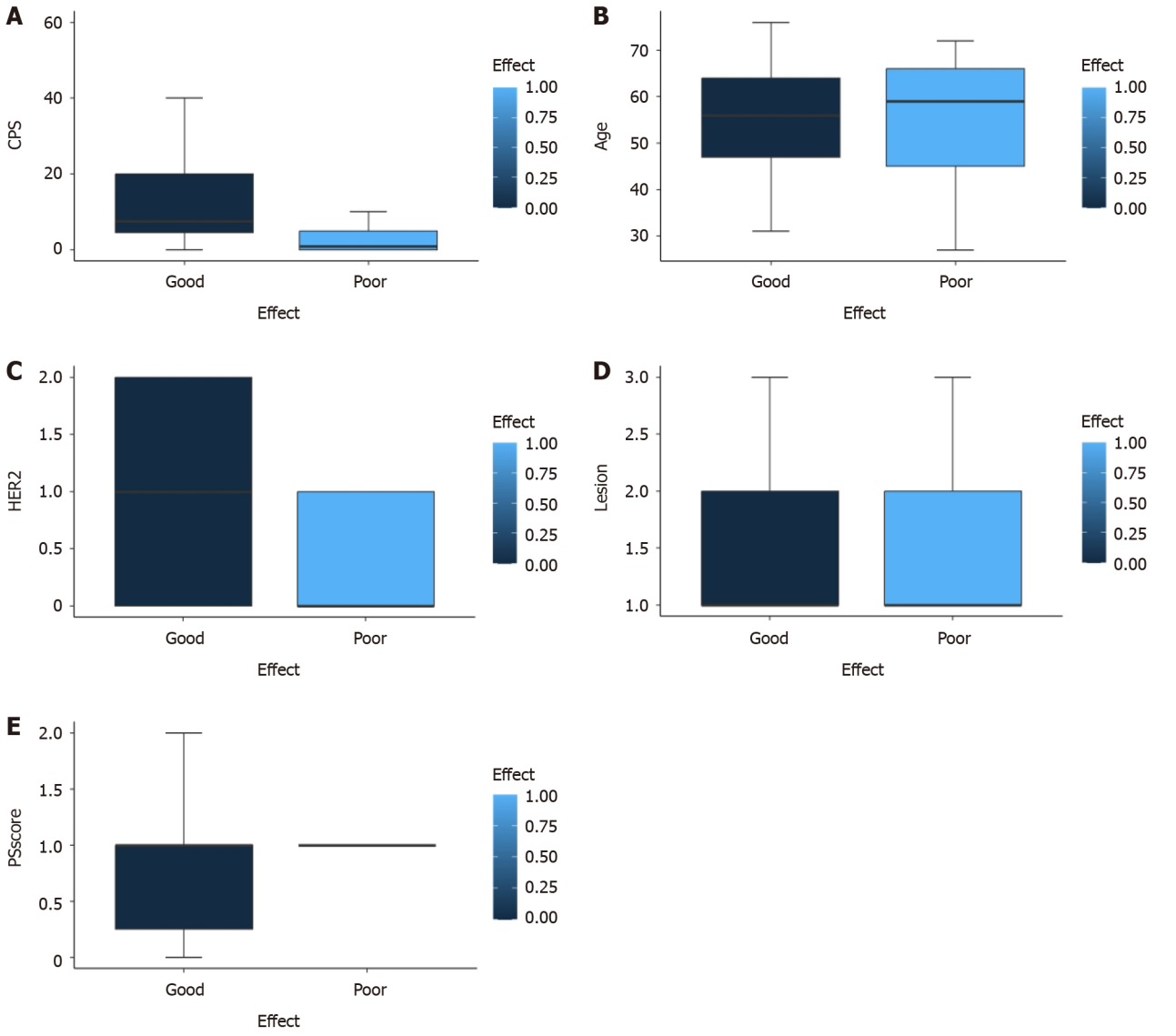

Figure 2 Risk factor analysis of clinicopathological factors. A: Risk factor analysis of programmed cell death 1 Ligand 1 combined positive score scores; B: Risk factor analysis of patient age; C: Risk factor analysis of different numbers of metastatic organs; D: Risk factor analysis of human epidermal growth factor receptor 2 expression; E: Risk factor analysis of performance status scores. PS: Performance status; CPS: Combined positive score; HER2: Human epidermal growth factor receptor 2.

with an ORR of 71.4% (range: 55.4%-84.3%). Of the 21 individuals in the poor efficacy group, nine achieved PR after two cycles of treatment, with an ORR of 42.9% (range: 21.8%-66.0%). There was a significant difference between the two groups in terms of ORR (P = 0.029) (Table 2). These findings suggested that patients who achieved PR in the first efficacy evaluation after two cycles of treatment were more likely to experience PFS for more than 6 months or have the opportunity for radical surgery.

DISCUSSION

In this study, we found that HER2-negative, pMMR AGC, patients with a CPS ≥ 1 experienced significantly better treatment efficacy and a longer PFS when compared to those with a CPS of 0. Furthermore, PFS also exhibited a trend for prolongation as the expression level of HER2 increased.

HER2 is an important member of the ERBB family of receptors encoded by the *ERBB2* gene, and can bind to other family members to form heterodimers and transmit proliferation and survival signals to cells by activating the downstream RAS-RAF-MEK-ERK or P13K-AKT-mTOR pathways. When cells overexpress HER2 or exert increased functionality, excessive cell proliferation signals can be transmitted downstream; this can lead to tumor formation[8]. At present, the prognostic value of HER2 status in GC remains controversial. Some studies have reported that HER2 positivity is a poor prognostic factor, while other studies have linked HER2 to better survival; other studies have reported that there is no association between HER2 and patient survival[9-11]. Continuous advancement in the field of precision therapy and the advent of ADC drugs has redefined HER2 expression into different classifications: HER2-negative (IHC

Table 2 Best overall response (patients with measurable lesions)

	Group A (*N* = 42)		Group B (*N* = 21)		*P* value
	n (%)	95%CI	*n* (%)	95%CI	
Best overall response					
Complete response (CR)	0	-	0	-	-
Partial response (PR)	30 (71.4)	-	9 (42.9)	-	-
Stable disease (SD)	12 (28.6)	-	10 (47.6)	-	-
Progressive disease	0 (0)	-	2 (9.5)	-	-
Overall response rate (CR + PR)	30 (71.4)	55.4-84.3	9 (42.9)	21.8-66.0	0.03
Disease control rate (CR + PR + SD)	42 (100)	91.6-1.00	19 (90.5)	69.6-98.8	2.527e-13

N: The total number of subjects in the treatment group. It is the denominator for percentage (%) calculation. *n*: Number of subjects who are at the corresponding category. The exact 95%CI for the frequency distribution of each variable were computed using Clopper and Pearson method.

0), HER2-low expression (IHC 1+/2+ and FISH negative) and HER2-positive (IHC 3+ or 2+ and FISH positive). Zhang *et al*[12] analyzed the clinical characteristics and prognosis of these HER2 expression subtypes in breast cancer and found that when compared with patients who were HER2-negative, those with low levels of HER2 expression were more inclined to express low levels of Ki67 and have a better trend for long-term survival. Previous data analysis of 2310 non-HER2-amplified patients showed that breast cancer patients with low levels of HER2 expression were significantly different from those who were HER2-negative in terms of biological behavior, clinicopathological features, treatment response, and clinical outcomes[13]. According to previous studies, low levels of HER2 expression account for 40% to 60% of all GC patients[14]. In a study involving patients with stage III GC, Gao *et al*[15] reported that the prognosis of patients with an HER2 score of 1 or 2+ was significantly better than that of patients with an HER2 score of 0 or 3+. In addition, most previous studies focused on the relationship between HER2 and tumor cell growth and apoptosis; relatively few studies have been conducted on the role of HER2 with respect to the immune cycle of tumors and the role of immunotherapy.

The tumor immune microenvironment is composed of various immune cells and immune-active substances. Tumor infiltrating lymphocytes (TILs), macrophages, and neutrophils are the main immune cells that are found in the tumor microenvironment. PD-1/PD-L1 is considered an important pathway for immune escape in tumor cells. Changes in the immune microenvironment play an important role in the recurrence and metastasis of GC patients, and PD-L1 positivity has also been proven to be sensitive to immunotherapy. The immune response of the body to tumors mainly involves CD8+T cells, which infiltrate into tumor tissue and participate in the tumor immune cycle[16,17]. In the early stages of disease, the body can clear tumors by immune monitoring functionality; tumor cells can also suppress the immune system by immune editing functionality, and even evade immune system attacks *via* a range of different mechanisms[18]. No studies have directly analyzed the tumor-associated immune environment associated with low levels of HER2 expression and HER2-negative expression. However, a previous study involving breast ductal carcinoma *in situ* (DCIS), researchers found that the densities of intraepithelial lymphocytes, CD3+T cells, CD3+CD8 T cells, CD3+FOXP3+T cells and CD8+Ki67+T cells in HER2 positive DCIS were significantly higher than those of immune cell subsets in HER2-negative DCIS ($P < 0.05$)[19]. In another study, Mutka *et al*[20] reported that HER2-positive tumors had more TILs.

In the present study, the included population were all HER2-negative or HER2-low expression patients. We conducted subgroup analysis of our patient population. Multivariate analysis demonstrated that the expression levels of HER2 did not present an independent risk factor. However, survival analysis, using PFS as the endpoint, still indicated the predictive effect of HER2 expression on the efficacy of treatments involving a PD-1 inhibitor combined with chemotherapy. However, some previous reports contradicted our current findings. Some studies have found that HER2 bound to the carboxyl end tail of STING, and inhibited TBKL activity by phosphorylating specific positions of AKTl, thus preventing STING from binding to TBKL and inhibiting anti-tumor immune activity. These findings suggest that the level and activity of HER2 in tumor cells may also be a decisive factor in controlling immune perception ability[21-23]. Therefore, the relationship between HER2 expression level and the tumor immune environment needs to be further investigated by clinical research and basic scientific experiments with larger data sets.

Multiple large phase III randomized controlled studies have confirmed the efficacy of immunotherapy combined with chemotherapy for non-positive HER2 AGC; the most recent published data state that the mPFS is around 7 months[3-6]. Our study differed from other studies in that we directly used the PFS of the patient population as the cut-off point and the outcome endpoint as the binary variable to analyze efficacy. In addition, we also analyzed efficacy for all patients receiving two cycles of treatment to determine the predictive value of the first efficacy evaluation after treatment for subsequent efficacy.

There were some limitations to this study that need to be considered. First, the small sample size of patients and the lack of sufficient universality in a study based in a single center made it difficult to generalize our findings. Second, the small sample size caused by sub-grouping limited further subgroup analysis. In the future, we will conduct a multi-center study with a larger cohort to further validate our findings.

CONCLUSION

Analysis showed that CPS score and HER2 expression were both influencing factors for the efficacy of AGC patients who were non-positive for HER2 and receiving immunotherapy combined with chemotherapy. Multivariate analysis further confirmed the role of CPS score in predicting the efficacy of immunotherapy in patients with GC. Survival analysis, using PFS as the end point, suggested that the level of HER2 expression was suggestive of the efficacy of treatment involving the combination of a PD-1 inhibitor and chemotherapy. Finally, PFS showed a tendency to increase with an increased expression level of HER2.

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Impact of the economic crisis and drug shortage on Lebanese cancer patients' care

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Abstract

BACKGROUND

As a consequence of the economic crisis, the sociopolitical instability and the advent of the coronavirus disease-19 pandemic, nested challenges faced the Lebanese healthcare system. These have resulted in critical shortages of essential resources, including medications vital for oncologic patients.

AIM

To assess the ramifications of the ongoing economic crisis on oncology patient care focusing on our outpatient oncology department.

METHODS

A questionnaire was distributed during the month of February 2022 to oncology patients in Hôtel Dieu de France University Hospital in Beirut during their outpatient therapy. The primary objective was to assess the far-reaching impact of the economic crisis on patient care and the resulting psychological implications.

RESULTS

Among 182 interviewed patients, 31.87% experienced treatment interruption mainly due to acute drug shortages. Despite 87.91% of the patients benefiting from third-party coverage, 69.60% had to self-pay for their medications leading to 69.78% of patients perceiving that healthcare was more difficult to access after

2020. Psychologically, one-third of the patients exhibited symptoms of anxiety and/or depression, with 7 patients reporting suicidal ideations. Notably, 37.93% of patients who interrupted cancer treatment reported a history of comorbidities, and 89.66% who altered their treatment cited financial difficulties.

CONCLUSION

Lebanese cancer patients face complex challenges spanning economic, healthcare, and psychological realms. Income inequalities exacerbated by the economic crisis hindered healthcare access.

Key Words: Cancer care; Drug shortage; Economic crisis; Cancer psychology; Healthcare access

Core Tip: The Lebanese healthcare system, strained by economic crisis, sociopolitical unrest, and the coronavirus disease 2019 pandemic, faces critical shortages, impacting vital oncology medications. In February 2022, a questionnaire was administered to 182 oncologic patients at Hôtel Dieu de France University Hospital, Beirut during outpatient therapy. Results revealed that 31.87% experienced treatment interruptions due to acute drug scarcities. Despite 87.91% having third-party coverage, 69.60% self-funded medications, leading to 69.78% perceiving limited healthcare access post-2020. Psychologically, one-third exhibited anxiety/depression symptoms, and 7 patients reported suicidal thoughts. Most patients altered their treatment plan, citing financial strains.

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INTRODUCTION

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic in March 2020, the global landscape has grappled with a profound economic downturn[1]. Lebanon, amidst this worldwide crisis, has faced a multitude of deficiencies across various sectors. As per the World Bank, the economic upheaval in Lebanon stands among the foremost three significant global economic crises since the mid-19th century[2]. The Lebanese Pound (LBP) experienced unprecedented fluctuations, plummeting from 1500 LBP to 93000 LBP for 1 United States Dollar[3], notably exacerbated after the catastrophic Beirut blast on August 4, 2020. Despite 82% of the country's healthcare sector being reliant on private hospitals, the Lebanese government is indebted to them for a staggering 1.3 trillion dollars, a sum escalating perpetually [4].

The dearth of funds, resources, and foreign currency has placed the country in a precarious position in confronting the challenges posed by the COVID-19 pandemic. Furthermore, the government's inability to allocate a stimulus package has left both public and private hospitals ill-equipped with necessary resources[5]. Consequently, hospitals are grappling with financial constraints in procuring supplies and equipment and meeting the salaries of healthcare workers[4]. This strain on the socioeconomic health system has impacted patient care in various fields (infectious disease, cardiovascular disease, and others)[6,7].

Amidst this economic instability within the healthcare sector, the Lebanese oncology patient also faces formidable barriers in accessing adequate healthcare. Not only is treatment inherently expensive, but it has also become susceptible to smuggling across neighboring countries, hoarding by individuals and local warehouses, illicit trafficking of counterfeit drugs, and the collapse of third-party payer coverage[8].

In this backdrop, we assessed the ramifications of the ongoing economic crisis on oncology patient care at the outpatient oncology department at Hôtel Dieu de France University Hospital, a tertiary multidisciplinary university hospital located at the heart of Beirut, Lebanon. This hospital serves 15% of the Lebanese population and hosts almost 20% of cancer patients on a national scale.

MATERIALS AND METHODS

This study was based on a questionnaire that we established and distributed during the month of February 2022 to Lebanese oncology patients treated in the 1-d oncology ward at Hôtel Dieu de France University Hospital. The aim of the questionnaire was to assess their access to medical care as well as the physical and psychological impacts of the crisis and the lack of medication. Patients were questioned during their outpatient course of therapy, for around 10 min. They received an explanation of the study objectives, consented to fill out the questionnaire, and were assisted by one of the interns (who are also authors of this manuscript) during its completion on-site.

The study and the questionnaire were approved by the department's Institutional Review Board (No. CEHDF 1903).

Data collection

The following data were obtained for each patient: Age; sex; nationality; work status; marital status; educational level; and monthly income. The presence of other chronic illnesses in the patient and their family members and the presence or absence of healthcare coverage were also reported. Patients were questioned about social and financial difficulties that they have faced during the period of economic crisis and about difficulties in accessing healthcare or getting financial coverage for their healthcare expenses. The questionnaire also contained the Patient Health Questionnaire-4 (PHQ-4) items[9] that addressed the impact of the crisis on the patients' mental health (Supplementary Table 1) and one question about any suicidal thoughts the patient had during the prior 3 month. The Arabic and English versions of the questionnaire (both distributed to our patients who could choose either of the two versions) are represented in Supplementary material.

Statistical analyses

We estimated descriptive statistics to study the sociodemographic characteristics of patients. Patient characteristics were presented as mean and standard deviation in the cases of continuous data and absolute and relative frequencies in the cases of categorical data. Pearson's χ^2 and Student's t test were performed to determine statistical significance. Results were considered significant with a P value < 0.05.

The data collection was conducted through a Google Form. Statistical analyses were performed using R statistical software version 3.5.3 (packages: PrettyR, tableone).

RESULTS

Demographic and financial profiles among the population

A total of 182 patients were interviewed, consisting of 79 males (43.41%) and 103 females (56.59%) with an average age of 61.18 ± 13.32 years. Among them, 173 were Lebanese nationals (95.05%), while the remaining individuals hailed from diverse backgrounds such as France (n = 4), Iraq (n = 1), the Philippines (n = 1), Morocco (n = 1), Palestine (n = 1), and South Africa (n = 1).

Regarding residency, 122 participants (67.03%) lived within urban areas. Notably, the majority of the participants were non-working individuals during the survey period (63.38%) and were married (78.02%). Approximately half of the participants held university degrees, while 8.79% were characterized as being illiterate (Table 1).

Concerning monthly household income, 78 patients (42.86%) reported earnings below $300, while 56 patients (30.77%) fell within the $300-$1000 bracket. Forty-eight patients (26.37%) reported incomes exceeding $1000 per month. On average, this income supported 3.00 ± 1.54 individuals within the same household.

Around 34.07% of patients identified a family member with a severe chronic illness managed within the same income used for their own treatment (Table 2).

Medical condition and coverage of patients and their relatives during the economic crisis

Approximately 31.87% of patients recently halted or interrupted at least one oncological treatment, predominantly chemotherapy. Primary reasons cited were drug scarcity (70.69%) and unaffordable prices compounded by insurance coverage rejections (13.79%). These factors significantly influenced 44.51% of the population to change at least one medication (Table 3).

The vast majority (87.91%) of patients benefited from third-party payer coverage through either public social coverage or private insurance, the latter being the predominant means of insurance (51.25%). The remaining coverage included the National Social Security Fund or Caisse Nationale de Sécurité Sociale (32.5%), the Ministry of Public Health (8.13%), the Lebanese army (3.12%), the syndicate (2.5%), and the Caisse Française de l'Etranger (2.5%; Table 3).

Third-party payer limitations forced 69.60% of patients to self-finance their medication, with 11.20% relying on donations. Therefore, 79.12% of the population reported facing financial challenges due to healthcare expenses, and 23.08% resorted to self-medication instead of seeking professional care or emergency room assistance due to financial constraints. Also, 25 individuals (13.74%) had to forego treatments, surgeries, or procedures due to cost considerations, with 12 patients canceling chemotherapy sessions. Moreover, nearly half of the patients (51.65%) grappled with additional chronic illnesses, and 76% of these encountered barriers accessing specific treatments (Table 4).

The perceived access to healthcare post-2020 was considered more difficult by 69.78% of respondents. Yet, a quarter of patients (26.92%) noticed no changes before and after 2020, with only 3.30% perceiving healthcare as more accessible after 2020 (Figure 1).

Mental health of cancer patients during the economic crisis

We assessed the mental health status of patients during the crisis period through their responses to the 4-item PHQ-4 questionnaire. One-third of the patients expressed at least one symptom of anxiety and/or depression (Figure 2). Seven patients (4%) acknowledged suicidal ideations (Figure 3), of whom two patients had a PHQ-4 within the normal range.

Correlations among different characteristics

Analyzing these findings using the χ^2 test revealed significant relationships. Notably, a link between other personal

Table 1 Sociodemographic and clinical characteristics of patients, *n* (%)

Sociodemographic variables	Total, *n* = 182
Age, mean ± SD	61.18 ± 13.32
Sex	
Male	79 (43.41)
Female	103 (56.59)
Nationality	
Lebanese	173 (95.05)
Other	9 (4.95)
Place of residency	
City	122 (67.03)
Outside of the city	60 (32.97)
Work status	
Worker	63 (34.62)
Non-worker	119 (63.38)
Educational level	
Illiterate	16 (8.79)
Less than high school	11 (6.04)
High school	25 (13.74)
College	35 (19.23)
University	95 (52.20)
Status	
Married	142 (78.02)
Single	29 (15.93)
Divorced	1 (0.55)
Widowed	10 (5.50)

Table 2 Characteristics of the patients' households, *n* (%)

Characteristics	Total, *n* = 182
Total household income/month in USD	
< 300	78 (42.86)
Between 300 and 1000	56 (30.77)
> 1000	48 (26.37)
Number of people in household	3.00 ± 1.54
Household members-excluding the patient-suffering from serious chronic disease	
Yes	62 (34.07)
No	120 (65.93)

USD: United States Dollar.

Table 3 Reasons for treatment disruption, *n* (%)

Characteristics	Value, *n* = 182
Stopped or interrupted any drugs including chemotherapy	
Yes	58 (31.87)
Reason:	
Shortage	41 (70.69)
Price/coverage	8 (13.79)
COVID-19	1 (1.72)
Other	8 (13.79)
No	124 (68.13)
Changed any of their medications	
Yes	81 (44.51)
Reason:	
Shortage	74 (91.36)
Price	3 (3.70)
Coverage	4 (4.94)
No	101 (55.49)
Had a third-party payer	
Yes	160 (87.91)
Syndicate	4 (2.50)
Insurance	82 (51.25)
CNSS	52 (32.50)
CFE	4 (2.50)
Ministry of Public Health	13 (8.13)
Army	5 (3.12)
No	22 (12.09)
Source of medication payment	
Out-of-pocket	87 (69.60)
Donations	14 (11.20)
Family	9 (7.20)
Primary healthcare centers	14 (11.20)
Ministry of Public Health	1 (0.80)

CFE: Caisse Française de l'Etranger; CNSS: Caisse Nationale de Sécurité Sociale or National Social Security Fund; COVID-19: Coronavirus disease-19.

comorbidities and interrupting cancer treatment (P = 0.01) emerged, with 37.93% of patients who interrupted cancer treatment reporting a history of comorbidities.

Interruptions in cancer treatment correlated significantly with patient financial status (P = 0.017): 89.66% who altered their treatment cited financial issues compared to 15.79% who maintained their original medication.

Our data analysis also showed that individuals earning less than $300 were more prone to healthcare access-related financial difficulties compared to those earning more ($300 or above) with a statistically significant P value of 0.0022.

For those encountering financial distress, a notable association existed with patients avoiding consultations. Specifically, 95.24% of self-medicating individuals due to financial constraints reported difficulties stemming from healthcare expenses.

Additionally, among patients identified as severely prone to depression and/or anxiety *via* the PHQ-4 questionnaire, a significant link emerged with the presence of comorbidities. Nevertheless, no substantial correlation was observed between mental health status and financial situations or judgments regarding healthcare access.

Table 4 Financial coverage of the patient population, *n* (%)

Characteristics	Total, *n* = 182
Changing third-party payer due to coverage problems	
Yes	11 (6.04)
No	171 (93.96)
Reporting financial difficulties due to health care spending	
Yes	144 (79.12)
No	38 (20.88)
Self-medication instead of seeking medical treatment due to financial difficulties	
Yes	42 (23.08)
No	140 (76.92)
Cancelled a treatment, surgery, or any procedure because of its cost during the last year	
Yes	25 (13.74)[1]
No	157 (86.26)
Suffering from other chronic diseases necessitating chronic treatments	
Yes	94 (51.65)
No	88 (48.35)
Any problems accessing specific treatments for other chronic diseases	
Yes	76 (80.85)
No	18 (19.15)

[1]Twelve out of 25 canceled at least one course of chemotherapy.

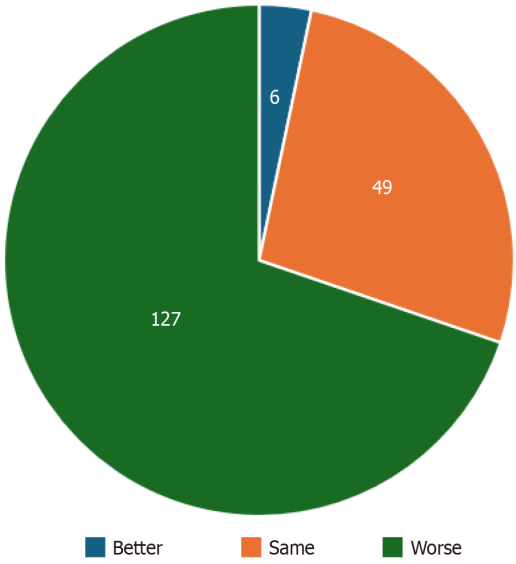

Figure 1 Patients' opinions on their access to healthcare before and after 2020.

DISCUSSION

The enduring economic crisis in Lebanon continues to exert profound strain, particularly on chronically ill patients. Within our cohort, these individuals represent a minority but are among the most susceptible to the ramifications of this economic upheaval. Despite this, it is evident that the COVID-19 pandemic has significantly exacerbated the challenges associated with accessing healthcare in Lebanon.

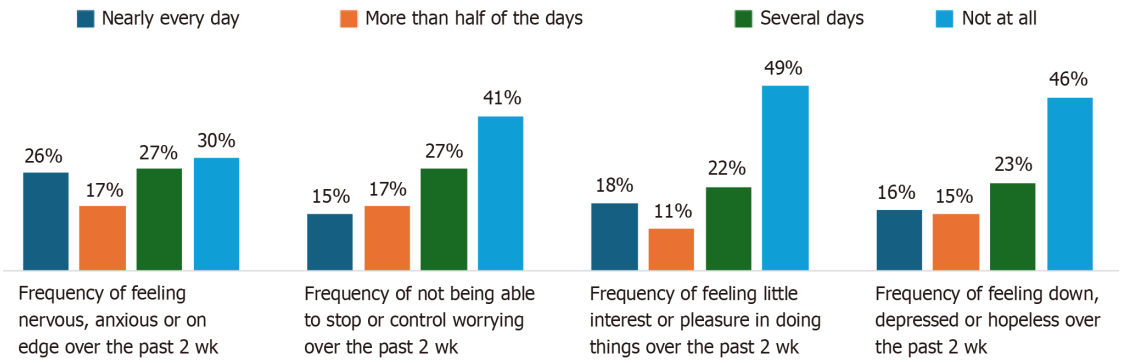

Figure 2 Responses to the patient health questionaire-4 assessment.

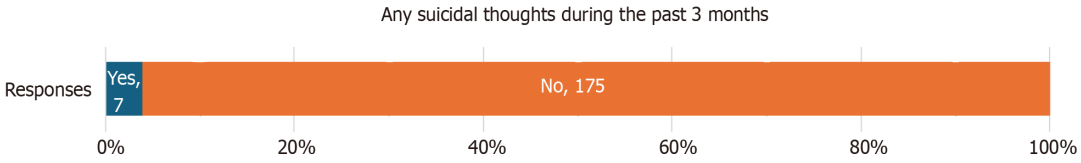

Figure 3 Responses of the population to an extra question about suicidal ideation.

Our investigation was tailored to measure the impact of this economic crisis specifically on cancer patients' healthcare access. Our objective was to analyze different material-related and mental health-related aspects of this impact and seek any discernable links between them.

While certain relationships yielded statistically significant findings, others proved inconsequential. For instance, we showed that individuals earning less than $300 were more susceptible to healthcare access-related financial difficulties compared to those earning more. However, when juxtaposing their healthcare system judgment (worse or not) with their household income, the study unveiled no statistically proven correlation, suggesting a nuanced relationship between income and access to treatment.

Moreover, Lebanon grapples with medication shortages and treatment unavailability, independent of financial means, thereby impeding access even for affluent individuals. Notably, the burden of cancer treatment poses a significant strain on the country's economic fabric[10].

A study published by Elias *et al*[11] in 2016 demonstrated the impact of government-financed cancer treatment between 2008 and 2013. The Ministry of Public Health financed cancer medication for all patients who did not have private insurance, which amounted to an average of 6475 United States Dollars over the 5 years. Interestingly, the cost exponentially increased during the study period due to excellent compliance of the patients to the treatment program. Our investigation revealed that since 2019 cancer drugs are no longer covered by the Ministry of Public Health due to the collapse of the government's financial structure. This forced patients to either pay out of pocket or interrupt their treatment.

Another study by Kattan *et al*[12] showcased how Lebanese oncologists struggle with drug shortages and coverage issues by resorting to switching brands, using on/off prescription policies and suboptimal drug doses, and deviating from recommended drug intake modes. However, these coping strategies adversely affect cancer patients.

Our findings revealed a prevalent trend of avoiding doctor consultations among the studied population, which was significantly associated with financial struggles. Surprisingly, no significant relationship emerged between skipping appointments due to financial issues and total household income. This could imply that affording medical expenses is difficult, even for people with a better income, due to paying out of pocket in the absence of third-party coverage.

Interestingly, perceptions of poor healthcare access did not align with the presence of a third-party payer, suggesting complexities within the policies of private insurance in Lebanon, particularly in covering cancer-related medications. Affording medication out of pocket did not directly impact perceptions of healthcare access.

Of note, the Caisse Nationale de Sécurité Sociale covered almost 90% of health expenses before the crisis, whereas the Ministry of Public Health used to cover all the expenses of patients who did not have any other coverage means. However, these policies were subject to perpetual changes since the beginning of inflation and with the daily change in currency rates. Some measures included tightening the area of coverage.

Moreover, our analyses underscored a noteworthy connection between personal comorbidities and discontinuation of cancer treatment. This could be attributed to patients diverting resources from cancer treatment to address other medical conditions. However, external factors, such as the country's ability to provide adequate treatment, could also influence treatment discontinuation.

Additionally, a worse mental health status was significantly correlated to the presence of comorbidities but was not correlated with the perception of worse healthcare access. This highlights the multifaceted nature of patients' mental health influenced by Lebanon's ongoing challenges. Not all 7 patients who expressed suicidal thoughts were within the severe risk range for anxiety and/or depression per the PHQ-4 test. This discrepancy raises queries about the sources of

suicidal thoughts beyond mental health concerns, emphasizing the need for comprehensive evaluations beyond the PHQ-4 test.

According to the World Health Organization, suicide is tightly linked to mental health conditions such as depression. However, many suicide attempts are reported in situations of crisis (financial difficulties, interpersonal problems, chronic illness) without previous mental health disorder. This is related to an impaired ability to handle life stress[13]. The suicidal rate among Lebanese people was 2.76 per 100000 in 2019[14] and according to the 2022 annual report of EMBRACE Lebanon one person in Lebanon attempts suicide every 6 h[15].

In our establishment, cancer patients who report suicidal thoughts are systematically referred to the psychiatry department for assessment. Unfortunately, psychiatry consultations and medications are not covered by any of the third-party payers in Lebanon. Therefore, most of these patients did not show up for their appointment.

Ultimately, our study underscored the intricate interplay between economic, healthcare, and mental health challenges in Lebanon. Addressing these multifaceted issues demands a comprehensive approach, integrating economic reforms, healthcare policy revisions, and tailored mental health interventions for better patient outcomes in this challenging landscape[16]. This could be achieved by promoting local potential and local manufacturers, standardizing medical consultation tariffs and private insurance packages, and implementing the national mental health strategy vision for 2023-2030 without delay[17]. Better supervision of healthcare structures and parties by the government is needed to identify corruption, and a wider cooperation with the private sector to shift medical aid to the most deprived patients would be beneficial.

The robustness of our study lies in the substantial number of patients interviewed, allowing for the collection of comprehensive data that potentially reflects the experiences of Lebanese cancer patients across the broader population. The diversity within our patient cohort offers insights into the varying economic circumstances prevalent within our country. Patients' financial situations might be different and easier to manage in secondary hospitals where costs are usually lower. However, we think that our results could still be extrapolated to the global population since cancer care in Lebanon is primarily concentrated in tertiary university hospitals and less often at secondary hospitals[18]. Moreover, cancer management would be less than optimal in secondary centers with the financial deterioration we are witnessing.

However, certain limitations are inherent in the study, primarily attributable to the ongoing instability and fluidity of circumstances during data collection. Factors such as fluctuating medication pricing, accessibility issues, and volatile exchange rates pose challenges in capturing a complete and static snapshot. A crucial aspect to acknowledge is that our study encompassed patients who could access hospitals and maintain their treatment regimen. Yet, it is imperative to note that a subset of patients discontinued hospital visits, which remains unaccounted for in our research. This group's absence from our study potentially alters the findings. Obtaining data from these patients would allow a more comprehensive view of the state of cancer patients in Lebanon.

Given this perspective and the continuously evolving economic landscape, the elaboration and implementation of a national cancer plan is imperative to enhance access to healthcare systems in Lebanon. These guidelines should address the multifaceted challenges faced by cancer patients, ensuring equitable access to essential treatments and support systems amidst these turbulent times. In the meantime, patients will continue to rely on individual donations, fundraising organized on social media, telethons that support cancer patients/cancer organizations, and remittances from the Lebanese diaspora[19].

CONCLUSION

Undoubtedly, the emergence of COVID-19 as a global pandemic has significantly impacted various sectors worldwide and notably the medical field. In Lebanon, the concurrent onset of the COVID-19 outbreak and the accompanying economic crisis has compounded the challenges faced by patients, particularly those battling cancer. These individuals, already navigating a challenging journey due to their medical condition, are now confronted with additional hurdles stemming from the pandemic and economic turmoil. Our study provided a glimpse into the collective experiences of cancer patients, highlighting how the economic crisis, exacerbated by the pandemic, has profoundly affected them both financially and psychologically.

FOOTNOTES

Author contributions: Eid D and Jabbour J contributed equally to the data collection and analysis and writing of the manuscript; Moujaes E supervised the research study and contributed to the final version of the manuscript; Kourie HR, Eid D, and Jabbour J conceived the idea of the research study; Safieddine M analyzed the data and revised the statistics; Kattan J designed and supervised the research study.

Institutional review board statement: The Institutional Review Board of Hôtel Dieu de France University Hospital provided approval for this study (IRB No. CEHDF 1903).

Informed consent statement: Patients received an explanation of the study objectives, consented to fill out the questionnaire, and were assisted by one of the interns during its completion on-site.

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

Data sharing statement: The data that support the findings of this study were collected anonymously within a Google Sheet Form that can only be shared between the team members working on this study. They are available from the corresponding author (Dollen Eid, dolleneid@gmail.com) upon reasonable request.

STROBE statement: The authors have read the STROBE Statement–checklist of items, and the manuscript was prepared and revised according to the STROBE Statement–checklist of items.

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ORIGINAL ARTICLE

Basic Study

In silico prospective analysis of the medicinal plants activity on the CagA oncoprotein from *Helicobacter pylori*

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Abstract

BACKGROUND

Colonization with *Helicobacter pylori* (*H. pylori*) has a strong correlation with gastric cancer, and the virulence factor CagA is implicated in carcinogenesis. Studies have been conducted using medicinal plants with the aim of eliminating the pathogen; however, the possibility of blocking *H. pylori*-induced cell differentiation to prevent the onset and/or progression of tumors has not been addressed. This type of study is expensive and time-consuming, requiring *in vitro* and/or *in vivo* tests, which can be solved using bioinformatics. Therefore, prospective computational analyses were conducted to assess the feasibility of interaction between phenolic compounds from medicinal plants and the CagA oncoprotein.

AIM

To perform a computational prospecting of the interactions between phenolic compounds from medicinal plants and the CagA oncoprotein of *H. pylori*.

METHODS

In this *in silico* study, the structures of the phenolic compounds (ligands) kaempferol, myricetin, quercetin, ponciretin (flavonoids), and chlorogenic acid (phenolic acid) were selected from the PubChem database. These phenolic compounds were chosen based on previous studies that suggested medicinal plants as non-drug treatments to eliminate *H. pylori* infection. The three-dime-

nsional structure model of the CagA oncoprotein of *H. pylori* (receptor) was obtained through molecular modeling using computational tools from the I-Tasser platform, employing the threading methodology. The primary sequence of CagA was sourced from GenBank (BAK52797.1). A screening was conducted to identify binding sites in the structure of the CagA oncoprotein that could potentially interact with the ligands, utilizing the GRaSP online platform. Both the ligands and receptor were prepared for molecular docking using AutoDock Tools 4 (ADT) software, and the simulations were carried out using a combination of ADT and AutoDock Vina v.1.2.0 software. Two sets of simulations were performed: One involving the central region of CagA with phenolic compounds, and another involving the carboxy-terminus region of CagA with phenolic compounds. The receptor-ligand complexes were then analyzed using PyMol and BIOVIA Discovery Studio software.

RESULTS

The structure model obtained for the CagA oncoprotein exhibited high quality (C-score = 0.09) and was validated using parameters from the MolProbity platform. The GRaSP online platform identified 24 residues (phenylalanine and leucine) as potential binding sites on the CagA oncoprotein. Molecular docking simulations were conducted with the three-dimensional model of the CagA oncoprotein. No complexes were observed in the simulations between the carboxy-terminus region of CagA and the phenolic compounds; however, all phenolic compounds interacted with the central region of the oncoprotein. Phenolic compounds and CagA exhibited significant affinity energy (-7.9 to -9.1 kcal/mol): CagA/kaempferol formed 28 chemical bonds, CagA/myricetin formed 18 chemical bonds, CagA/quercetin formed 16 chemical bonds, CagA/ponciretin formed 13 chemical bonds, and CagA/chlorogenic acid formed 17 chemical bonds. Although none of the phenolic compounds directly bound to the amino acid residues of the K-Xn-R-X-R membrane binding motif, all of them bound to residues, mostly positively or negatively charged, located near this region.

CONCLUSION

In silico, the tested phenolic compounds formed stable complexes with CagA. Therefore, they could be tested *in vitro* and/or *in vivo* to validate the findings, and to assess interference in CagA/cellular target interactions and in the oncogenic differentiation of gastric cells.

Key Words: CagA oncoprotein; Phenolic compounds; *Helicobacter pylori*; *In silico* analyses; Medicinal plants; Prospective analysis

Core Tip: Commonly, studies on the effects of medicinal plants on *Helicobacter pylori* (*H. pylori*) infection assess the antimicrobial activity of these plants. However, in this study, the authors conducted a prospective *in silico* analysis of the activity of certain phenolic compounds from plants used to treat *H. pylori* infection on stomach cells affected by CagA, aiming to prevent or block the oncogenic differentiation of these cells.

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INTRODUCTION

Colonization of gastric epithelial cells by *Helicobacter pylori* (*H. pylori*) has a strong positive correlation with gastric diseases such as peptic ulcers and stomach cancer, owing to the virulence factors and evasion capabilities of the bacteria [1]. The standard treatment for eradicating *H. pylori* is complex and relies on antibiotics[2,3], proton pump inhibitors[4], bismuth salts[5], and H2 blockers[6], typically used in combination for an extended duration[7].

Multiple factors contribute to hindering the eradication of *H. pylori*, among which drug inefficiency and the bacteria's antibiotic resistance are prominent[7]. In 2017, the World Health Organization classified *H. pylori* as resistant to clarithromycin, metronidazole, and levofloxacin, emphasizing the urgent need for research into new antibiotics targeting the bacteria[8].

In this context, medicinal plants and their secondary metabolites have emerged as an alternative for managing *H. pylori* infection. The literature reports several plant species with antimicrobial activity against *H. pylori*, which act by inhibiting, reducing, and delaying gastric colonization. Plants such as Pistacia lentiscus, Brassica oleracea, Curcuma longa, Coptis chinensis, and Glycyrrhiza glabra were tested in rodents (mice and rats); Vaccinium macrocarpon, Glycyrrhiza glabra, and Nigella sativa were tested in humans, with observed results indicating biological activity against the bacteria and the progression of infection[9]. Dinat *et al*[10] discuss the use of medicinal plants in treating *H. pylori* infection and identified

antimicrobial activity in several of them, such as Hibiscus sabdariffa and Piper longum.

Commonly, the use of medicinal plants to treat *H. pylori* infection aims at antimicrobial action to eliminate the pathogen and consequently prevent the development of associated pathologies such as gastritis, ulcers, and especially gastric cancer. Although studies have demonstrated antimicrobial activity in many plants, most are classified as having weak or weak to moderate activity based on the minimum inhibitory concentration test. Many secondary metabolites of medicinal plants with anti- *H. pylori* activity have been reported, including phenolic compounds, coumarins, quinones, terpenoids, and alkaloids[11]. Several studies attribute the biological activity of medicinal plants to phenolic compounds, which include anti-cancer[12-16], anti-proliferative, anti-angiogenic, and antimicrobial activities[17,18].

Although studies have primarily focused on antimicrobial activity, medicinal plants and their metabolites may also interfere with pathogenic cellular processes induced by *H. pylori*. In cases of infection with CagA-positive *H. pylori* strains, where the colonization process is advanced, it would be crucial not only to eliminate the pathogen but also to block the cell differentiation induced by CagA to prevent the development of cancer. Therefore, addressing the treatment of *H. pylori* infection through targeting the CagA would be of interest.

The CagA is an oncoprotein, a virulence factor of *H. pylori* responsible for inducing genetic mutations and alterations in gastric cells[19]. This oncoprotein is encoded by the pathogenicity island (cag-PAI) and is transported into cells *via* a type 4 secretion system (T4SS)[20]. Inside cells, CagA attaches to the plasma membrane in two distinct ways: Through the interaction of basic amino acids, including those in the K-Xn-R-X-R binding motif located in the central region of CagA, or through the carboxy-terminus region. Subsequently, CagA undergoes tyrosine phosphorylation on the EPIYA (Glu-Pro-Ile-Tyr-Ala) motif, which is present in multiple copies in the carboxy-terminus polymorphic region, by Src kinase members such as c-Src, Yes, Fyn, and Abl kinase[21]. Phosphorylated CagA interferes with cell signaling pathways, including the MAP kinase pathway, leading to mitogenic imbalance, induction of a pro-inflammatory state, cytoskeleton damage, and disruption of cell-cell junctions[20,22,23].

The carboxy-terminus end of the CagA oncoprotein also contains the CagA-multimerization (CM) motif, which facilitates its dimerization or multimerization. CM motif consists of 16 amino acid residues and is located immediately distal to the last EPIYA segment. Apart from facilitating multimerization, the CM motif enables CagA to interact with regulatory molecules that affect proper cell signaling[21]. The cumulative effect of dysregulated cell signaling, disordered cell growth, endothelial injury, and loss of mucosal integrity caused by the CagA oncoprotein predisposes individuals to precancerous lesions in the stomach, explaining the higher incidence of cancer in individuals colonized with CagA-positive *H. pylori*[22].

Therefore, approaches could be explored to interfere with the CagA-cellular targets interaction, aiming to reduce or inhibit the activity of this protein and, consequently, its oncogenic potential. Phenolic compounds from plants are capable of crossing the plasma membrane of human cells and interacting with proteins and enzymes of cellular signaling cascades, altering the course of signal transduction[24]. Thus, this study aimed a prospective computational analysis of the action of certain phenolic compounds from medicinal plants, which are reported in the treatment of *H. pylori* infection, on the CagA oncoprotein, and consequently, on the cellular signaling pathways that are important for CagA-dependent gastric carcinogenesis. This prospecting may guide *in vitro* and/or *in vivo* studies to reduce financial and labor burdens.

MATERIALS AND METHODS

Molecular modeling

Molecular modeling of the CagA oncoprotein was carried out using the primary sequence BAK52797.1 of the CagA oncoprotein from *H. pylori*, which contains 1194 residues including the EPIYA-A, B, C, and CM motifs, in FASTA format, selected from GenBank (ncbi.nlm.nih.gov/genbank). The FASTA sequence was utilized to search for X-ray diffraction solved three-dimensional structures deposited in online public databases. However, the structures found in the databases did not include either the amino-terminus or the carboxy-terminus ends of the CagA oncoprotein. Global and local alignment analyses of CagA were performed using the BioEdit software (Informer Technologies, Inc.) and the Basic Local Alignment Search Tool algorithm (National Institutes of Health/United States), respectively. Since no satisfactory homologous templates were found, the CagA oncoprotein was modeled using the structure prediction methodology-Threading, employing tools from the I-Tasser platform[25], and the model was validated using the MolProbity platform [26].

Prediction of binding sites in the CagA oncoprotein

The modeled CagA oncoprotein was analyzed using the GRaSP online platform[27] to predict residues that could serve as potential binding sites. This data will be compared with the results obtained from molecular docking.

Preparation of ligands and receptor

Chemical compounds selected as ligands were phenolic compounds, and their two-dimensional structures were obtained from the PubChem Database (pubchem.ncbi.nlm.nih.gov)-kaempferol (PubChem: 6325460), myricetin (PubChem: 5281672), quercetin (PubChem: 5280343), ponciretin (PubChem: 25201019), and chlorogenic acid (PubChem: 1794427). The two-dimensional structures were converted to three-dimensional form using PyMol software (Schrödinger, Inc.). The protonation state of each phenolic compound at physiological pH 7.4 was predicted using MarvinSketch software (ChemAxon) before proceeding with docking simulations. All phenolic compounds were selected from studies involving medicinal plants used for the treatment of *H. pylori* infection[28,29]. AutoDock Tools 4 (ADT) software[30] was used to

detect and calculate the points and angles of torsion, respectively. The CagA oncoprotein was prepared using the same software to add missing hydrogen atoms.

Molecular docking

Molecular docking was performed using the flexible ligand-rigid receptor methodology[31]. The simulations were conducted by associating ADT with AutoDock Vina v.1.2.0 software[32], enabling the establishment of an algorithm that searches for potential bond combinations, including rotational, translational, and conformational degrees of freedom. This algorithm also establishes scoring criteria to select the best ligand-receptor interactions. Points are assigned according to the molecular force field and the free energy of the bond, with interactions considered stable if the affinity energy is lower than -6.0 kcal/mol[33]. Two sets of molecular docking simulations were carried out: (1) Central region of CagA + phenolic compound; and (2) carboxy-terminus region of CagA + phenolic compound. The membrane binding motif (K-Xn-R-X-R) is located in the central region, while the EPIYA and CM motifs are in the carboxy-terminus region of the CagA oncoprotein. Gridbox dimensions and coordinates were determined separately to allocate the central region and the carboxy-terminus end of the CagA (receptor). Evaluation of the receptor/ligand complexes was performed using PyMol and BIOVIA Discovery Studio (Dassault Systemes) software. Physicochemical analysis of the residues involved in the chemical bonds was carried out using the ProtParam tool[34]. The data was analyzed using descriptive statistics (mean and relative values).

RESULTS

Predicted three-dimensional model of *H. pylori* CagA oncoprotein

Five structural models were constructed based on 10 three-dimensional structures, and the most satisfactory model, with a C-score value of 0.09, was selected (Figure 1A). The C-score assesses the quality of models predicted by I-Tasser; it typically ranges from -5 to 2, with higher values indicating models with higher reliability. MolProbity indicated that approximately 98% of the amino acid residues in the model were located within the allowed regions of the Ramachandran plot.

In silico predicted binding sites of the CagA oncoprotein

A total of 24 amino acid residues were predicted as possible binding sites for ligands. The algorithm indicated 19 phenylalanine (F) and 5 leucine (L) residues with a probability above 50% of interacting with other molecules. This value represents the algorithm's cut-off point. Among the 24 residues, only one coincided with the molecular docking results (F426) (Table 1). The residues are distributed across the surface of CagA, and no clusters were observed (Figure 1B).

CagA/phenolic compound complexes

The phenolic compounds analyzed formed complexes with the CagA oncoprotein *in silico* (Figure 2); all five compounds bound to the central region of the protein close to the spatial region of the membrane-binding motif (residues 621 to 626) (Figure 3). No *in silico* interactions were observed between the phenolic compounds and the carboxy-terminal region of CagA where the EPIYA motifs are located. The affinity energies were considered satisfactory and ranged from -7.9 kcal/mol (CagA/chlorogenic acid) to -9.1 kcal/mol (CagA/kaempferol). The other complexes showed similar affinity energy values-CagA/quercetin = -8.1 kcal/mol, CagA/myricetin = -8.2 kcal/mol, CagA/ponciretin = -8.4 kcal/mol. In all the complexes, covalent bonds and reversible bonds (hydrogen bond and van der Waals) were observed. The CagA/kaempferol complex showed 28 bonds (2.1Å - 2.3Å) involving 23 amino acid residues; this was the complex with the highest number of chemical bonds. The CagA/myricetin complex showed 18 bonds (2.0Å - 2.9Å) involving 15 residues; CagA/quercetin complex-16 bonds (2.2Å) involving 13 residues; CagA/ponciretin complex-13 bonds (2.5Å - 3.4Å) involving 11 residues; CagA/chlorogenic acid complex-17 bonds (1.9Å - 3.3Å) involving 15 residues. All the chemical bonds present in each complex are shown in Figure 4 in a two-dimensional diagram, and the residues are listed in Table 1. Fifty-three amino acid residues participated in chemical bonds with at least one of the phenolic compounds analyzed *in silico*. Of these, eight made bonds with two compounds, and eight made bonds with three compounds, most of which were positively (L) or negatively (D/E) charged residues.

DISCUSSION

H. pylori strains harboring the gene CagA-CagA-positive strains-significantly increase the risk of developing gastric cancer when compared to CagA-negative strains[35]. The cellular modifications triggered by the CagA oncoprotein, after being injected into stomach epithelial cells, involve the EPIYA motifs becoming targets for phosphorylation and recruitment to enzymes and adaptor proteins. These events disrupt the standard cellular metabolism, triggering pre-lesion processes[21].

The results of this *in silico* study suggest that, after being injected into the cytoplasm of gastric epithelial cells *via* T4SS, the CagA oncoprotein can interact with xenobiotics that cross the plasma membrane of these cells, such as phenolic compounds from medicinal plants. The computational search for binding sites in CagA indicated several phenylalanine and leucine residues with this potential. Since these residues did not form clusters, it is possible that they represent the starting point for ligand binding, and the site itself is composed of more residues that become closer together after ligand

Table 1 Amino acid residues bound to phenolic compounds by molecular docking and binding sites predicted by GRaSP

Molecular docking						Binding sites by GRaSP
Phenolic compounds						
	Kaempferol	Myricetin	Quercetin	Ponciretin	Chlorogenic acid	Residue[1]/position
Residue[1]/position	A601	A439	D432	D432	D403	F23
	D403	D432	N400	E383	E396	F31
	E406	E383	E383	E397	F451	F41
	E422	E429	E396	E429	G607	F60
	F407	F378	E397	K382	I463	F88
	F426[2]	K382	E429	K401	K425	F122
	G496	K401	K382	L393	K604	F161
	H500	L393	K401	N400	L454	F188
	K425	N375	L393	N428	L462	F269
	K499	N428	N428	Q385	N360	F291
	K604	Q385	Q385	Y381	R399	F426[2]
	L418	Q390	S394		S453	F537
	L471	S377	Y440		T464	F543
	L494	Y381			V402	F582
	M504	Y440			Y609	F596
	N417					F637
	N597					F702
	Q410					F805
	Q495					F818
	S419					L226
	S497					L260
	V600					L688
	Y473					L835
						L964

[1]The residues are identified by the one-letter code.
[2]Coincident amino acid residue in molecular docking and GRaSP.

binding due to a conformational change; in other words, the ligand would actually be an allosteric modulator[36].

Due to the chemical nature of the side chains of these residues, they are expected to form hydrophobic bonds with compounds of a similar chemical nature. This increases the likelihood of secondary metabolites that are more hydrophobic than those evaluated interacting with the CagA oncoprotein, such as terpenes and terpenoids[37].

The phenolic compounds evaluated in this *in silico* study (ponciretin, kaempferol, quercetin, myricetin, and chlorogenic acid) bound to the central region of the CagA oncoprotein, near the membrane phospholipid-binding motif-K-Xn-R-X-R motif. Due to the proximity of the bonds formed by the phenolic compounds to this motif and the high interaction affinity considered, these compounds could induce conformational changes in the protein, thus destabilizing its previous interactions with cellular targets. Even if the carboxy-terminus region, where the EPIYA and CM motifs are found, does not bind to the analyzed phenolic compounds, the interaction between CagA and the plasma membrane is still necessary for the phosphorylation process. Therefore, interference through the membrane-binding domain could be effective in blocking this process.

Given the consideration of phenolic compounds as an alternative for interfering with CagA oncoprotein activity within epithelial cells, it is important to note that these compounds are absorbed in the human intestine through the action of bile salts, passive diffusion, or transporters[38]. After absorption, phenolic compounds can undergo conjugation with glucuronic acid in enterocytes or hepatocytes, or they can circulate in the bloodstream bound to albumin[39]. Conjugated phenolic compounds can cross cell membranes through carrier proteins and can reach different tissues[38], including the stomach.

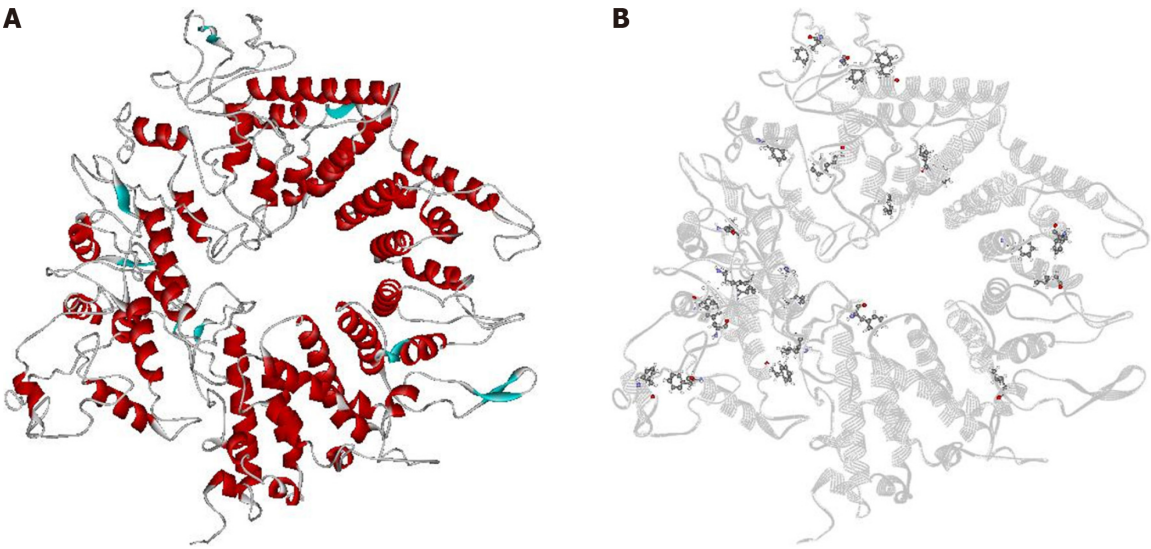

Figure 1 Three-dimensional structure model of *Helicobacter pylori*'s CagA and predicted binding sites *in silico*. A: CagA structure model. Blue: β-sheets; red: α-helices; gray: loops; B: CagA structure model in gray flat ribbon with predicted binding sites (residues) highlighted in ball and stick format.

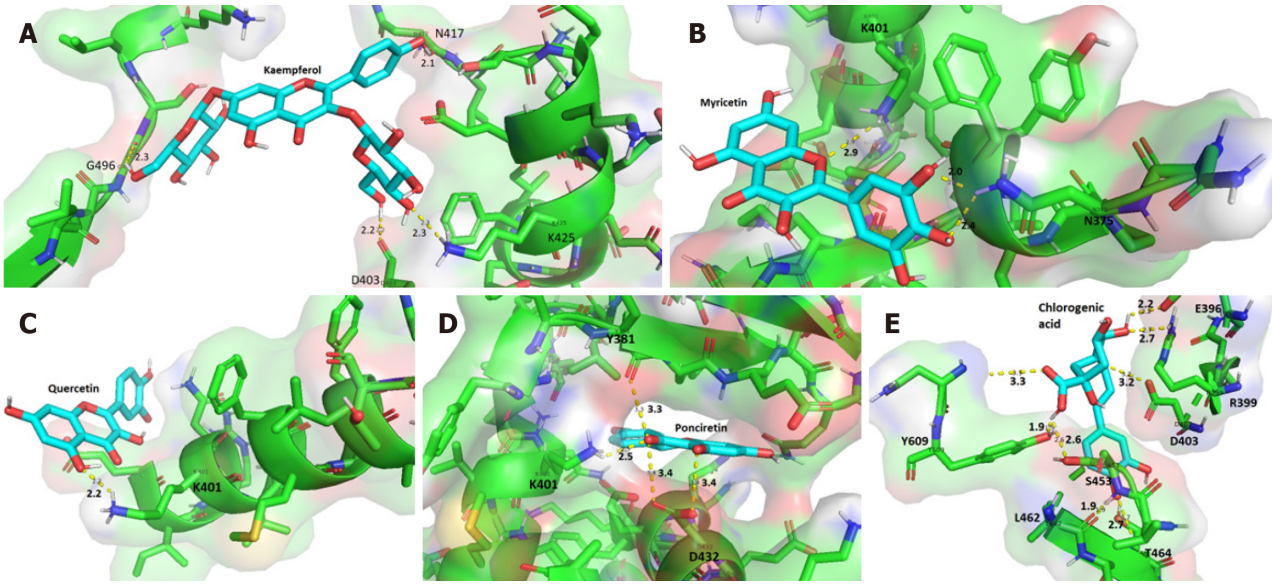

Figure 2 Interaction of phenolic compounds with amino acid residues in the central region of the CagA oncoprotein. A: Cag/kaempferol complex; B: Cag/myricetin complex; C: CagA/quercetin complex; D: CagA/poncíretin complex; E: CagA/chlorogenic acid complex. Residues are represented by their one-letter code and position in the primary sequence. Chemical bonds are depicted by dashed lines, and the bond length is measured in Angstroms (Å). Only a subset of the chemical bonds are showed in the figure.

Therefore, based on the *in silico* results and the capacity of phenolic compounds to penetrate cells, these polyphenols have potential for subsequent *in vitro* and *in vivo* studies for the treatment of gastric *H. pylori* infections. They not only exhibit antimicrobial activity, as described for plants containing these compounds, but also have the ability to bind to and destabilize the interaction between CagA and epithelial cells. This interference could potentially prevent the initiation of changes leading to malignant transformation of gastric cells, such as the activation of PAR1/MARK kinases causing loss of cell polarization, and the inactivation of p53 resulting in uncontrolled and disordered cell proliferation[20,29].

A study by Castillo-Juárez *et al*[40] demonstrated the anti-*H. pylori* activity of some plants commonly used in traditional Mexican medicine, including Moussonia deppeana. This plant, popularly known as tlanchichinol, contains one of the five phenolic compounds analyzed in this study-chlorogenic acid. It was observed that this phenolic acid exhibited better results than the antibiotic metronidazole in inhibiting bacterial growth *in vitro*[29,40]. Furthermore, the *in silico* binding of chlorogenic acid with the central region of CagA suggests its potential for *in vitro* and/or *in vivo* testing to assess its effectiveness in interfering with the interaction between CagA and the plasma membrane, thereby potentially affecting the cell signaling pathway related to gastric cell differentiation.

Szewczyk *et al*[41], in a separate study, investigated the antimicrobial properties of plants from the Balsaminaceae family, including the species Impatiens glandulifera, (*Himalayan balsam*), through an *in vitro* study. The study revealed

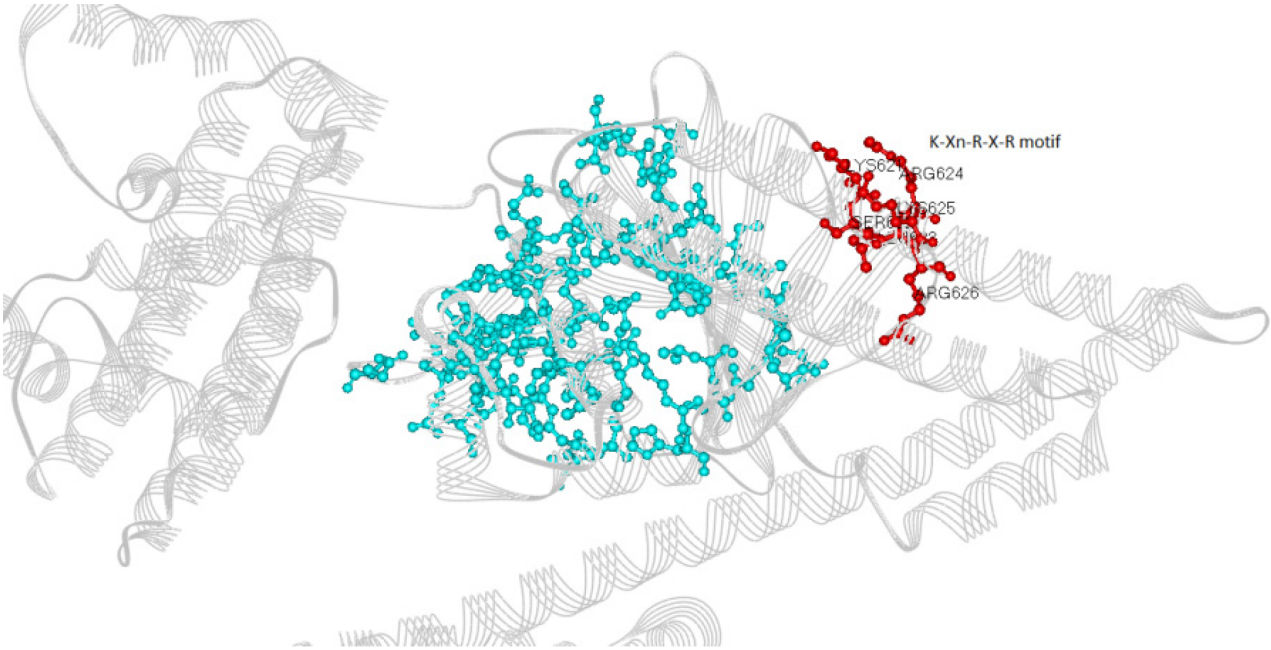

Figure 3 Localization of the K-Xn-R-X-R membrane-binding motif (621-626) and all the amino acid residues that have bound to phenolic compounds *in silico*. Red: Residues of the K-Xn-R-X-R motif. Blue: Residues that have bound to phenolic compounds.

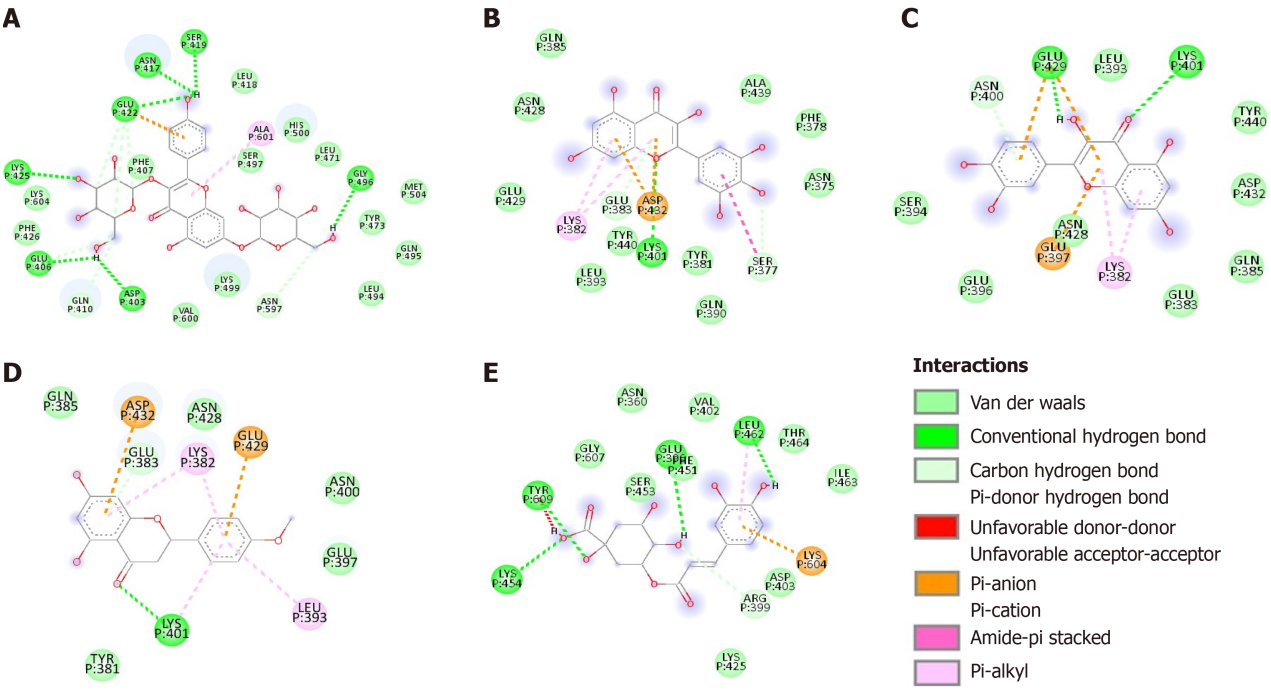

Figure 4 Two-dimensional diagram of the chemical bonds between phenolic compounds and the CagA oncoprotein. A: Cag/kaempferol complex; B: Cag/myricetin complex; C: CagA/quercetin complex; D: CagA/ponciretin complex; E: CagA/chlorogenic acid complex. Residues are represented by three-letter codes and their positions in the primary sequence. Chemical bonds are depicted with dashed lines, except for van der Waals interactions. The internal legend indicates the type of chemical bond.

that *I. glandulifera* exhibits high concentrations of phenolic acids, particularly in its aerial parts, and significant antioxidant and antimicrobial activity against Staphylococcus aureus, *S. epidermidis*, Micrococcus luteus, Bacillus subtilis, *B. cereus*, Streptococcus pneumoniae, and *S. pyogenes*. However, direct studies confirming the antimicrobial activity of *I. glandulifera* against *H. pylori* are lacking.

Nevertheless, Vieira *et al*[28] isolated some flavonoids from this plant through chromatography-kaempferol, quercetin, and myricetin-which, according to our findings, demonstrate *in silico* potential as interferents of the CagA oncoprotein. Among these, due to its binding site, kaempferol appears to be the most effective in destabilizing the interaction between CagA and the epithelial cell membrane. Therefore, *I. glandulifera* warrants further exploration to evaluate its efficacy in

halting precancerous changes induced by CagA-positive *H. pylori* strains.

The plant Buddleja indica, known for its richness in kaempferol, caffeic acid (a metabolite of chlorogenic acid), and quercetin, is reported to possess anti-diabetic, hepatoprotective, antioxidant, and antimicrobial properties[42,43]. Youssef *et al*[42] highlighted the bacteriostatic activity of *B. indica* against *H. pylori* in an *in vitro* study. Given that this plant contains two of the compounds discussed in this study, it could be explored in further research on anti-carcinogenic therapy.

Other plants that could be investigated for the presence of the phenolic compounds discussed in this study include Polygonum tinctorium, or indigo (kaempferol, quercetin, and caffeic acid), known for their bactericidal and bacteriostatic activity[44,45]; Rubus ulmifolius, or blackberry (kaempferol, quercetin, and caffeic acid), with bactericidal action[46,47]; Poncirus trifoliata (ponciretin), exhibiting bacteriostatic action[48-50]; Oliveira decumbens (kaempferol), and Hibiscus rosa-sinensis (myricetin, quercetin, kaempferol), with anti-urease and bacteriostatic activity[51-53]. In our study, among the phenolic compounds tested, kaempferol and chlorogenic acid appear to be the most promising candidates for interfering with the interaction between CagA and the phospholipids of the plasma membrane, as they exhibit lower affinity energies, indicating greater stability of the complexes.

The studies correlating flavonoids/phenolic acids and *H. pylori* primarily focus on the action of these compounds as bactericides, bacteriostats, and anti-urease agents, rather than addressing virulence factors that directly damage host cells, such as CagA. Most research aims to identify alternatives to antibiotics due to the process of bacterial resistance. Indeed, the eradication of *H. pylori* infection is crucial due to its pathogenic factors, as well as the physiological and biochemical mechanisms that can lead to the malignancy of gastric cells[54].

The utilization of bioinformatics in scientific research is increasingly contributing to the study of molecular interactions [55]. Among these studies, González *et al*[56] investigated several flavonoids, including kaempferol, quercetin, and myricetin, for their ability to bind to and inactivate the homeostatic stress regulatory protein, yielding promising results. As this protein is crucial for fundamental *H. pylori* activities such as energy metabolism and genetic material replication, its inactivation results in either bacterial death or reduced multiplication. This study aligns with the same rationale as the present work, wherein the binding of compounds to bacterial components leads to detrimental implications for pathogenic progression.

Inhibiting the action of CagA has the potential to halt the progression of gastric *H. pylori* lesions to malignancy, as this protein disrupts multiple pathways regulating cellular homeostasis. Upon entry into cells through T4SS, phosphorylation of EPIYA motifs occurs, leading to the recruitment of various molecules to the plasma membrane of gastric cells, thereby modulating and altering multiple cell signaling pathways[57]. EPIYA-C motifs are phosphorylated by Src family kinases; *H. pylori* strains containing higher numbers of EPIYA-C motifs are associated with an increased likelihood of gastric cancer emergence. CagA interacts with the tyrosine phosphatase S *H. pylori*-2 and potentiates the action of the Erk-MAP kinase, with or without utilizing the Ras protein, while also inactivating the focal adhesion kinase FAK. This pathway results in the hummingbird phenotype, characterized by a rearrangement of the cytoskeleton in gastric cells, leading to enhanced cell motility and elongation[58,59], and is implicated in the malignancy process[60]. Therefore, since the transition from a precancerous state to cancer is characterized by accelerated and disordered tissue growth, inhibiting the CagA oncoprotein could prevent or slow down this process.

Since the molecular events leading to the hummingbird phenotype are dependent on the action of the Cag oncoprotein, blocking this protein could prevent the cellular oncogenic process. One way to block it would be to prevent CagA from interacting with the plasma membrane of gastric cells, which could be achieved by using a phenolic compound, as suggested by the data from this *in silico* study (Figure 5).

Indeed, a study on the post-translational processing of the CagA oncoprotein revealed that this process may be involved in the pathogenesis of *H. pylori* infection, as CagA fragmentation alters its functionality, thereby reducing the induction of the hummingbird phenotype[61]. This suggests that interventions targeting CagA may reduce carcinogenic predisposition, similar to the observations made in this *in silico* study. It is a fact that the tertiary conformation of a protein is determined by the interaction between its amino acid residues, and the binding, whether transient or otherwise, of external molecules to this assembly can lead to a state of inactivity[62]. Thus, by binding to the central region of CagA, the phenolic compounds in this study, especially kaempferol and chlorogenic acid, may induce conformational and functional changes in this virulence factor.

Finally, the present study has some limitations regarding its application in clinical treatments, as the functioning of the human organism is complex, involving a myriad of biochemical and physiological cascades that interact to achieve homeostasis. Therefore, since computational tools simulate only some physiological parameters, the environment in which the interaction between CagA and phenolic compounds was tested does not fully reflect the physiological environment, and all potential metabolic influences were not considered. Hence, further *in vitro* and *in vivo* studies should be conducted to complement the data obtained.

CONCLUSION

The *in silico* data suggest that phenolic compounds (flavonoids and phenolic acids) present in medicinal plants used to treat *H. pylori* infection bind to the CagA oncoprotein in its central region, close to the membrane anchoring site. Furthermore, none of the amino acid residues of CagA predicted as binding sites are involved in the interaction with the phenolic compounds analyzed in this study. It is possible that these residues interact with other secondary metabolites in medicinal plants, which would increase the chance of interfering with CagA's action. Therefore, medicinal plants have the potential to eliminate *H. pylori* infection due to their antimicrobial activities already proven in the literature and could

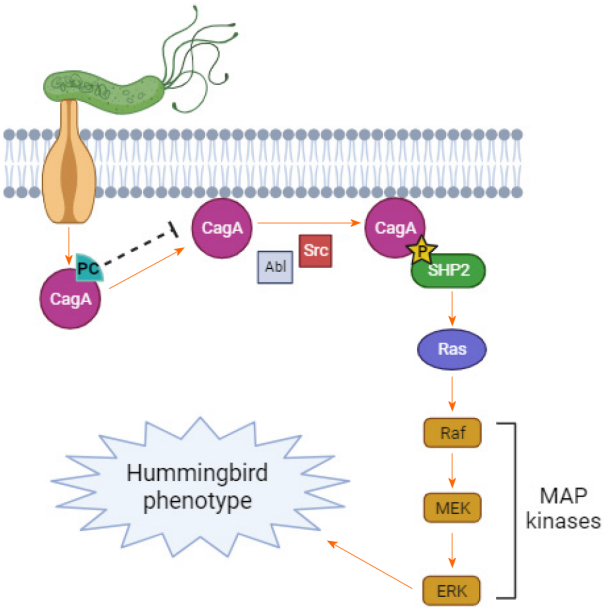

Figure 5 Proposed action of phenolic compounds on the CagA oncoprotein and the gastric epithelial cell signaling pathway. PC: Phenolic compounds.

also interfere with the action of CagA after being injected into the gastric epithelial cell. This interference could affect the cell differentiation process that culminates in the hummingbird phenotype and, consequently, prevent and/or block the onset of gastric cancer. It is important to emphasize that this is a computational study and, therefore, has limitations; thus, the data obtained must be analyzed *in vitro* and *in vivo* to validate the findings.

FOOTNOTES

Author contributions: Peiter GC performed the *in silico* experiments and analysis; Vieira RV and Teixeira KN interpreted the data and wrote the manuscript; Zarpelon-Schutz AC, de Melo FF and Teixeira KN performed the critical analysis of the results and coordinated the study; all authors approved the final version of the manuscript.

Institutional review board statement: Institutional review board declares awareness of the study and states that neither humans nor animals were involved in the research.

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LETTER TO THE EDITOR

Integrating disulfidptosis-related long noncoding RNAs in colorectal cancer prognosis: A path to precision medicine

Shi-Yan Zhang

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Abstract

This commentary explores the burgeoning field of disulfidptosis-related long noncoding RNAs (lncRNAs) in the prognosis and therapeutic targeting of colorectal cancer (CRC). By evaluating recent research, including the pivotal study "Predicting colorectal cancer prognosis based on long noncoding RNAs of disulfidptosis genes" by Wang *et al*, this analysis underscores the critical role of lncRNAs in deciphering the molecular complexities of CRC. Highlighting the innovative methodologies and significant findings, I discuss the implications for patient survival, therapeutic response, and the potential of lncRNAs as biomarkers for precision medicine. The integration of bioinformatics, clinical databases, and molecular biology in these studies offers a promising avenue for advancing CRC treatment strategies and improving patient outcomes.

Key Words: Colorectal cancer; Disulfidptosis; Long noncoding RNAs; Prognosis; Precision medicine

Core Tip: This commentary emphasizes the novel role of disulfidptosis-related long noncoding RNAs (lncRNAs) in colorectal cancer prognosis. Focusing on the intersection of genetic research and clinical practice, it highlights how the study of lncRNAs can facilitate the development of targeted therapies and prognostic models. The critical evaluation of recent research underscores the potential of lncRNAs as biomarkers and therapeutic targets, marking a significant step towards personalized medicine in colorectal cancer treatment.

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TO THE EDITOR

I recently read the insightful study titled "Predicting colorectal cancer prognosis based on long noncoding RNAs of disulfidptosis genes" by Wang *et al*[1] published in *World Journal of Clinical Oncology*. The study pioneers the exploration into the prognostic value of disulfidptosis-related lncRNAs (DRLs) in colorectal cancer (CRC). Through meticulous analysis and innovative approaches, the research uncovers eight significant lncRNAs associated with disulfidptosis, shedding light on their potential as prognostic markers and therapeutic targets.

Key methodologies like leveraging The Cancer Genome Atlas for data collection and the development of a prognostic model through bioinformatics techniques, showcasing the integration of clinical and genetic data for precision medicine. The findings suggest a profound connection between lncRNAs, immune response, and CRC prognosis, offering a novel perspective on cancer treatment strategies. Furthermore, drug sensitivity analysis indicated that Epirubicin, bortezomib, teniposide, and BMS-754807 exhibit the least sensitivity among the assessed immunotherapy drugs, underscoring the necessity for customized therapeutic approaches to augment cancer treatment efficacy.

The relevance of this study is further underscored by related literature, such as the validation of lncRNA prognostic models in CRC, the prognostic significance of disulfidptosis-associated lncRNA signatures, and their implications for the tumor microenvironment and therapeutic options[2-4]. These complementary studies highlight the potential of lncRNAs in understanding CRC's molecular mechanisms and in developing targeted therapies.

The study's findings are pivotal, offering a novel perspective on CRC treatment strategies through DRL utilization. This advancement could revolutionize precision medicine by enabling more personalized and effective treatments. Future research is essential to understand DRLs' impact on CRC progression and treatment response, and their viability as therapeutic targets, potentially leading to innovative treatments that significantly improve patient outcomes[5]. Validating the functions of disulfidptosis-related lncRNAs and immune checkpoints' anti-cancer mechanisms through comprehensive animal and cell studies is imperative.

FOOTNOTES

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