Journal of Health Sciences

RESEARCH ARTICLE Open Access

Expression of HER2, CD34, and EBV infection in gastric cancer, do they relate?

Alexandra Pinheiro[1], Diana Martins[1,2,3,4,5], Clara Rocha[6], Fernando Melo[7], Inês Morais[7], Inês Colaço[7], Sara Andrade[6], Rui Caetano[7], Patrícia Carreira[7], Fernando Mendes[1,2,3,4,5,8]*

[1]Polytechnic University of Coimbra, ESTESC, UCPCBL, Coimbra, Portugal, [2]H&TRC - Health and Technology Research Center, Coimbra Health School, Polytechnic University of Coimbra, Coimbra, Portugal, [3]Biophysics Institute of Faculty of Medicine, Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO), University of Coimbra, Coimbra, Portugal, [4]Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal, [5]Clinical Academic Center of Coimbra, Coimbra, Portugal, Polytechnic University of Coimbra, ESTESC, UCPNS, SM Bispo, Coimbra, Portugal, [6]Department of Surgery, Hospital Distrital da Figueira da Foz, E.P.E., Figueira da Foz, Portugal, [7]Centro de Diagnóstico Anátomo-Patológico, Coimbra, Portugal, [8]European Association for Professions in Biomedical Sciences, Brussels, Belgium

ABSTRACT

Introduction: Gastric cancer (GC), as a multifactorial disease, exhibits a complex pathogenesis, having intra and inter-tumor heterogeneity, challenging the efficacy of current treatments. GC is a major global health problem, and better diagnostic and therapeutic strategies are needed, leading to further biomarkers research associated with innovative targeted therapies. Our aim was to study human epidermal growth factor receptor 2 (HER2), Epstein Barr virus (EBV), and CD34 expression in GC samples to understand the relationship between these markers and relate them with the clinicopathological characteristics.

Methods: Thirty samples of primary carcinoma gastrectomy cases performed between February 2017 and December 2021 at the Hospital Distrital da Figueira da Foz, EPE, were studied after approval by the Ethics Committee. Immunohistochemistry assays were performed in sections of the selected tumor representative samples.

Results: All samples were negative for EBV and only two GC samples were positive for HER2. A significant statistical relationship was found between gender and CD34+ stroma cells. Microvascular density and stroma CD34+ cells presented relations with clinicopathological features and a positive tendency between them. Our study was able to identify a relationship between CD34+ stroma cells and females.

Conclusion: With this study, we mainly explored the potential role of CD34 as a biomarker in GC and projected possible associations of prognostic and therapeutic value toward other HER2 and EBV markers.

Keywords: Stomach neoplasms; antigens, CD34; genes, erbB; Epstein-Barr virus infections; tertiary lymphoid structures

INTRODUCTION

Over 1 million cases of gastric cancer (GC) were diagnosed worldwide during the year 2020, being the 5th most common cancer in the world, with a remarkably high mortality rate and male predisposition (1). The global incidence of GC shows a wide variability of geographical distribution, with Asian countries having the greatest number of cases (2,3). More than 95% of GC are adenocarcinomas, which are typically classified based on anatomic location (cardia/proximal or noncardia/distal) and histologic type (diffuse or intestinal) (4). The distinction of the various GC subtypes is relevant in terms of prognosis and therapy approach, as each type differs in clinical, genetic, epidemiology, and invasive features (3,5,6).

As a multifactorial disease, the development of GC is influenced by several risk factors such as diet, alcoholism, smoking, and others, *Helicobacter pylori* infection is the best-described risk factor for non-cardia cancer since it causes chronic inflammation of the mucosa leading to progression to atrophic gastritis and intestinal metaplasia, pre-neoplastic predispositions. Whereas gastroesophageal reflux disease has evidenced a correlation with cardia GC (3,5). Another GC-associated pathogen, the Epstein Barr Virus (EBV), is also noteworthy, since its infection is associated with a better prognosis due to an outstanding response to immune checkpoint inhibitors, as it promotes a tumoral stroma containing an inflammatory infiltrate, mostly of lymphocytes (6–10).

Although GC is mostly sporadic, approximately 10% of diagnosed GC cases have an associated family history, some of which result from inherited germline mutations.

*Corresponding author: Fernando Mendes, Polytechnic of Coimbra, ESTESC, UCPCBL, Coimbra, Portugal.
E-mail: fjmendes@estesc.ipc.pt

Submitted: 19 October 2023/Accepted: 08 December 2023

DOI: https://doi.org/10.17532/jhs.2023.2622

UNIVERSITY OF SARAJEVO
FACULTY OF HEALTH STUDIES

Hereditary diffuse GC is the best-known form of hereditary GC, often found in individuals with mutations in the gene encoding *E-cadherin*. In these cases, prevention is achieved through earlier screening and molecular studies to identify mutations of higher genetic predisposition, compared to sporadic cancers where eradication of *H. pylori* and improved dietary practices are the most adopted measures (3,5).

GC is most effectively diagnosed by endoscopy of the gastrointestinal tract for evaluation of the gastric mucosa and biopsy of suggestive lesions. Staging should be performed by characterizing the lesion observed, and TNM classification. According to the stage defined, more adequate prognosis and therapeutics can be used (2–4). Currently, surgery with complete tumor resection is the only cure, however, remission is quite common and not all patients are eligible as most of them have late diagnoses presenting locally advanced unresectable and metastatic forms of GC. Chemotherapy holds important value as a neoadjuvant and adjuvant therapeutic modality for gastric adenocarcinomas, respectively allowing previous unresectable patients to undergo gastrectomy and minimizing possible recurrences (2–4). Despite substantial progress in medical oncology, the clinical outcome of GC patients remains poor so the development of better therapeutic strategies is needed, leading to further investigation of this disease pathogenesis (5,6,11).

The accentuated intra and inter-tumor heterogeneity creates difficulties in understanding the mechanisms of carcinogenesis involved. Overexpression of growth factors and/or receptors, alterations in DNA damage response, and loss of genomic stability are the mainstays of GC (3,12). One of the most frequently affected genes is *TP53*, which causes the loss of function of a major tumor suppressor gene. Several other mutations also present that characterize the molecular complexity of this pathology. Such as mutations in *Ras homolog gene family, member A* (*RHOA*) that modulates apoptosis and cell motility, and alterations in other genes that reflect features of programme death-Ligand 1 (PD-L1) or PD-L2 overexpression and immune cell infiltration, and among others, epidermal growth factor receptor 2 (HER2) overexpression (2,12).

HER2 positive GC is a unique subtype of the disease, which demonstrates increased expression of the human HER affecting its crucial role in the cell signalling processes that control normal growth and development (11,13). As this transmembrane glycoprotein does not have a specific activating ligand and the heterodimerization with other family members (HER1 and/or HER3) seems to be able to constitutively activate this receptor, easily a continuous activation of HER2 receptors and cellular pathways involved occurs, such as mitogen-activated protein kinase, protein kinase C, and phosphatidylinositol-4,5-bisphosphate 3-kinases promoting cell survival, angiogenesis, and metastasis. When overexpression is detected, HER2 is suggestive of poor prognosis and enhanced tumor invasiveness (11,14–16).

Interestingly, some studies suggest that HER2 overexpression is not usually coexistent with GC positive for EBV infection. Emphasizing the value of such biomarkers in screening programs, as they may serve as a key to identifying patients with better therapeutic outcomes (7,8,10). The potential of targeted therapies has already been explored in other types of cancer, such as successful anti-HER2 therapy in breast cancer (BC). Trastuzumab is the best-known and widely used monoclonal antibody against HER-2 receptors, however, in GC, it does not offer desirable performance. Thus, the investigation of possible combination therapies appears to be an innovative approach to maximizing their efficacy (11,13,17).

Considering the importance of angiogenesis for cancer progression by sustaining tumor growth and metastasis, the optimization of anti-angiogenic therapies is relevant for tumor containment. As these therapies have limited efficacy, it is necessary to select patients who can benefit from them (13,17). In malignant tumors, angiogenesis can be studied by microvascular density present (MVD), microvascular area size, or by quantification of angiogenic molecules and receptors in tumor tissue. Since CD34 is an endothelium marker with high sensitivity and strong expression in small vessels, it represents a useful indicator of MVD and, consequently, the extent of angiogenesis (13,17,18). CD34 is also widely known as a marker of immaturity, prominent in other progenitor cells and hematopoietic cells, with expression associated with high proliferation and regulation of cell differentiation. Although it cannot characterize a phenotype of an encountered stem cell population in isolation, it is a useful identifier of progenitor cells, that have an important involvement in cancer progression (19,20).

Of relevance is also the association of CD34 with tertiary lymphoid organs (TLS), strongly organized structures that allow interactions between recirculating T and B cells and antigen-presenting cells (APC), since their functions depend fundamentally on the activity of specialized stromal cell populations, including CD34-positive stromal cells. The formation of these structures usually indicates an adaptation of the organism to an increased requirement for a localized immune response, which makes their study interesting, since most solid tumors have a reactive stroma (19,21,22).

The HER2 overexpression is associated with more intense angiogenesis, so it would make the application of effective therapies targeting HER2 and angiogenesis possible in GC patients who express concomitantly these conditions (13). The correlation between HER2 overexpression and the intensity of angiogenesis at the molecular level had been demonstrated in BC, but in GC, it is still under investigation (13,17).

Our aim was to study HER2, EBV and CD34 expression in GC samples to better understand the relationship between these markers as well as to relate them with the clinicopathological characteristics, as they might have a potential role for combined targeted therapy in GC patients.

METHODS

In this retrospective study, we analyzed 30 samples of primary carcinoma gastrectomy cases performed between February 2017 and December 2021 at the Hospital Distrital da Figueira da Foz, EPE (HDFF, EPE), after approval by the Ethics Committee (OBS.SF.054-2022) while respecting the principles of the Declaration of Helsinki, ensuring maximum protection and confidentiality of the data obtained by the participants.

The clinicopathological features of the cases, regarding age, gender, diagnosis, anatomic localization of the lesion, differentiation grade, stage, TMN classification (specifically lymph node infiltration and metastasis), type of treatment, disease-free survival (DFS), and overall survival (OS) were included in the analysis, considered relevant for further tumor characterization.

Samples were prepared according to laboratory routine, fixed in formalin with 10% neutral buffer, and embedded in paraffin. After the appropriate histological process, routinely stained Hematoxylin and Eosin (HE) specimens were analyzed by pathologists to select those with a representative area of GC lesion.

Conventional 4μm histological sections of the selected samples were made ("Leica RM2125 Rotary Microtome") to perform the immunohistochemistry (IHC) on appropriate adhesive slides (Matsunami TOMO, Japan) conducted in automated equipment "Ventana BenchMark Ultra" (Roche, USA), according to manufacturer's instructions.

The biomarkers of interest, HER2, CD34, and EBV are studied by commercial antibodies anti-HER2, ready-to-use (anti-HER2/neu [4B5] Rabbit Monoclonal Primary Antibody from Roche), anti-CD34, diluted 1:100 (anti-CD34 anti-human, PE – clone AC136), and anti-EBV (anti-LMP1, latent membrane protein 1), diluted 1:50 (anti-EBV antibody cocktail including CS1, CS2, CS3, and CS4 from Invitrogen), respectively, used according manufacturer's instructions. Without associated symptomatology, most of the population carries the EBV virus through past infections, often not previously detected. To identify an active EBV infection from latently infected cells, the LMP1 protein is quantified. This transmembrane protein is one of the main products of viral gene expression. Therefore, the target of the immunohistochemical technique in our study of EBV is this oncoprotein, generically referred to as anti-EBV by some authors.

Each IHC slide studied for GC has positive and negative controls of the respective markers, crucial to making a reliable interpretation of the stains.

After IHQ assays, the results were observed blindly by two independent observers microscopically and interpreted based on defined immunoreactivity criteria. For the interpretation of HER2 expression on cell membranes of GC tissues, there is a defined score from 0 to 3, depending on the intensity of the staining detected. A score of 0 or 1 represents negativity, while a score of 3 indicates a strong positivity for HER2 in the tissues. A score of 2 is borderline, requiring a fluorescent *in situ* hybridization (FISH) study to be able to define overexpression as negative or positive (11,13,17). Within these samples, the HER2 staining obtained only 0 and 3+ scores, as for a simpler statistical analysis were categorized respectively as negative and positive for overexpression of this protein.

The CD34 expression was interpreted according to 2 different methodologies that provided information about the MVD and expression pattern in $CD34^+$ stromal cells. For the MVD evaluation, microvessels were counted (field x200; by light microscopy) in each of the five most vascularized, separately located areas (hot spots), which were previously identified during mosaic scanning of the whole section at low magnification (xlOO) as areas with the highest density of $CD34^+$ cells. The interpretation was based on the Weiner method's criteria, with only considering the blood vessels in the stroma of the tumor, surrounded by malignant glands, with a clearly defined lumen or a linear vessel shape, but not single endothelial cells. The MVD value is represented by the mean count of vessels immunolabeled with CD34 in each case (23,24). Regarding CD34 expression in stromal cells, the interpretation of immunoreactivity used vascular endothelial cells as positive internal control, to define low or high expression of the marker compared to the basal expression verified (22).

In contrast, cells are simply interpreted as positive or negative for EBV infection, depending on the presence of staining.

The relationship of the expression pattern between the studied markers was determined using statistical methods and measures for which the analyses were performed using IBM Statistical Package for the Social Sciences version 28.0 for Windows.

To establish the $CD34^+$ stroma expression with the clinicopathological characteristic data, such as gender, diagnosis, and TMN classification: Metastasis, a Chi-square test was applied. The remaining features, such as anatomic localization of the lesion, differentiation grade, stage, TMN: Lymph node status, age, type of treatment, and DFS were studied by the Mann–Whitney U test. Only the OS relation was determined by a different test, t-student, as the criteria for applying parametric measures were fulfilled.

For the relation between MVD measurement and clinicopathological characteristics data, such as gender, diagnosis, anatomical location of the lesion, cell differentiation, TMN classification: Metastasis, and treatment type, the Chi-square test was applied. While the rho-spearman correlation test was applied to the rest features of the stage, TMN: Lymph node status, age, DFS, and OS. The relationship among the markers, $CD34^+$ stroma cell expression, and MVD was measured through a t-student test since the requirements for parametric measures were met.

To obtain a more concise analysis, the TMN classification was considered only for the presence or absence of nodular infiltration and metastases.

Other statistical data relevant, such as the mean, median, standard deviation, frequency tables for qualitative variables, or quartiles for quantitative variables, were calculated using descriptive statistical measures. The interpretation of these statistical data is significant, at $p < 0.05$.

RESULTS

A total of 30 patients with primary CG from HDFF and EPE were included in this retrospective study to access HER2 status, CD34, and EBV infection. All clinicopathological data used in this investigation were collected from medical records, with tumor staging based on the American Joint Committee on Cancer Staging Manual. The clinicopathological characteristics of all patients studied are summarized in Table 1. In the 30 GC patients, the median age observed was 72.37 years (from min.=34 to max.=89 years), with a higher incidence in males (n = 17, 56.7%).

TABLE 1. Association between low and high expression of HER2, MVD values and low or high expression CD34+ stroma cells with the patients clinicopathological characteristics as gender, diagnosis, anatomic localization of the lesion, differentiation grade, stage, TMN classification, type of treatment, disease-free survival and overall survival

	n (%)	Mean ± SD	HER2				CD34								
							MVD			CD34+ Stroma Cells Density					
			Negative		Positive		Md	Rho	*p*	Low			High		*p*
			n (%)	Mean ± SD	n (%)	Mean ± SD	(Q1-Q3)			n (%)	Mean ± SD	Md (Q1-Q3)	n (%)	Mean ± SD	Md (Q1-Q3)	
Female	13(43,3%)		13 (100%)		0		13 (6,50-27)		0.457	7 (53,8%)			6(46,2%)			0.049
Male	17(56,7%)		15 (88,2%)		2 (11,8%)		16 (11-23,50)			15 (88,2%)			2(11,8%)			
Total (years)		72,37 ±11,892		72,21±2,271		74,50±9,500		0.58	0.76		72,05±2,663	75,50 (64,75-80,75)		73,25±3,816	74 (66,75-82)	1
Female (years)		74,31±10,475		74,31±2,905		0					77,14±3,562			71,00±4,698		
Male (years)		70,88±12,985		70,40±3,442		74,50±9,500					69,67±3,435			80,00±4,000		
Carcinoma	11(37,7%)		11 (100%)		0		13 (9-28)		0.735	10 (90,9%)			1 (9,1%)			0.199
Adenocarcinoma	19(63,3%)		17 (89,5%)		2 (10,5%)		15 (13-24)			12 (63,2%)			7 (36,8%)			
Cardia	3 (10%)		3 (100%)		0		24 (6-?)		0.579	2 (66,7%)			1 (33,3%)			*
Body	8(26,7%)		8 (100%)		0		12 (5,50-26,50)			6 (75%)			2 (25%)			
Antrum	19(63,3%)		17 (89,5%)		2 (10,5%)		15 (13-22)			14 (73,7%)			5 (26,3%)			
Not Assessable	7(23,3%)		6 (100%)		0			0.24	0.898	6 (100%)		1 (0-2)	0		2 (1-2)	0.136
Poorly differentiated	12 (40%)		10 (100%)		0					7 (70%)			3 (30%)			
Moderately differentiated	14(48,7%)		10 (83,3%)		2 (16,7%)					8 (66,7%)			4 (33,3%)			
Well differentiated	2(6,7%)		2 (100%)		0					1 (50%)			1 (50%)			
I	9(30%)		9 (100%)		0			-0.251	0.18	8 (88,9%)		3 (1-3,25)	1 (11,1%)		3 (2,25-3)	0.823
II	2(6,7%)		2 (100%)		0					1 (50%)			1 (50%)			
III	14(46,7%)		13 (92,9%)		1 (7,1%)					8 (57,1%)			6 (42,9%)			
IV	5(16,7%)		4 (80%)		1 (20%)					5 (100%)			0			
With Lymph Node Infiltration	18(63,3%)		16 (88,9%)		2 (11,1%)			-0.17	0.93	11 (61,1%)		0,50 (0-2,25)	7 (38,9%)		2(1-3,75)	0.129
Without Lymph Node Infiltration	12(37,7%)		12 (100%)		0					11 (91,7%)			1 (8,3%)			
With Metastis	5(17,7%)		4 (80%)		1 (20%)		13 (5,50-25)		0.503	5 (100%)			0			0.281
Without Metastis	25(83,3%)		24 (96%)		1 (4%)		16 (10-24,50)			17 (68%)			8 (32%)			
Surgery	17(56,7%)		15 (100%)		0		13 (5-30)		0.589	12 (80%)		1 (1-6)	3 (20%)		6 (1-6)	0.417
Surgery + CT	13(43,3%)		13 (86,7%)		2 (13,3%)		16(13-22)			10 (66,7%)			5 (33,3%)			
		13,03±15,248		13,68±15,559		4,00± 5,657		-0.58	0.762		13,82±3,478	8 (0-23,50)		10,88±4,438	8 (0,75-16,50)	0.945
		16,80±15,232		17,07±15,729		13,00±4,243		-0.3	0.107		18,77±15,895			11,38±12,546		0.246

n: absolute frequency; %: relative frequency; SD: Standard Deviation; Md: Median; Q1- Q3: interquartile range; p: probability of significance; Rho: Rho-spearman correlation coefficient; QT: quimiotherapy

In general, adenocarcinoma stands out as the most frequent type of tumor, present in 19 (63.3%) patients of the remaining 11 (37.7%) diagnosed as carcinoma, with the antrum region showing the highest prevalence of lesions (63.3%), as shown in Table 1.

Although not all cases provided this information, according to staging, only 11 cases were in lower stages, I (n = 9, 30%) and II (n = 2, 6%), whereas most tumors were in advanced stages, III (n = 14, 46.7%) and IV (n = 5, 16.7%). The TMN classification also shows that 18 (63.3%) tumors have lymph node infiltration (N1, N2, N3a, and N3b), and 5 (17.7%) are metastatic (M1). Furthermore, the accessible database revealed that only 2 (6.7%) patients had well-differentiated tumors, of the others, 12 (40%) had poorly and 14 (48.7%) moderately differentiated tumors (Table 1).

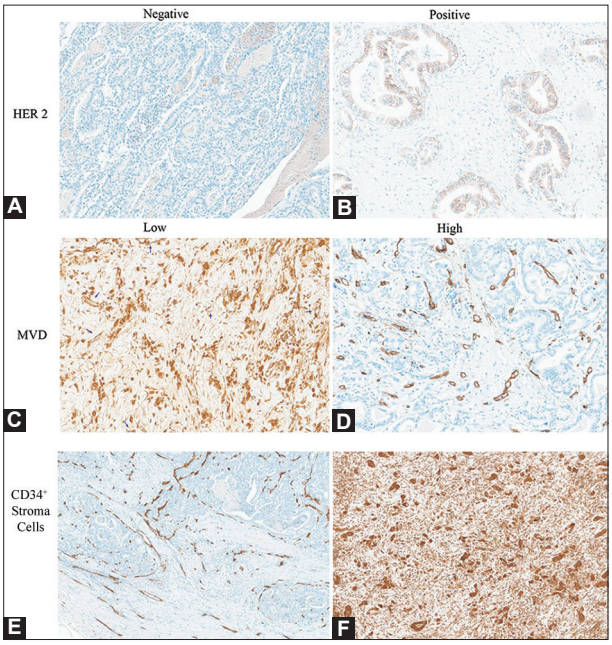

FIGURE 1. Immunohistochemical staining of HER2 and CD34, as MVD and CD34+ stroma cells in primary gastric cancer patients. (A) A representative image of negative HER2 expression, score 0; (B) A representative image of a HER2 positive expression, score 3+; (C) A representative image of a low MVD; (D) A representative image of a high MVD; (E) A representative image of a low CD34⁺ stroma cells expression; (F) A representative image of a high CD34⁺ stroma cells expression.

Regarding the type of treatment, 13 (43.3%) patients underwent surgery and CT, whereas the remaining 17 (56.7%) were submitted only to surgery, as shown in Table 1.

All the gastric samples in this study were negative for EBV infection.

Only two samples were positive for HER2 overexpression. Both HER2-positive cases were male (n = 2, 11.8%), with an adenocarcinoma-type tumor (n = 2, 10.5%) whose lesion was anatomically located in the antrum (n = 2, 10.5%). The tumors of these cases are of moderate cell differentiation (n = 2, 16.7%) of stages III (n = 1, 7.1%) and IV (n = 1, 20%). Lymph node infiltration is seen in both cases (n = 2, 11.1%), but metastasis only in one case (n = 1, 20%). Treatment of the HER2-positive patients was surgery and CT (n = 2, 13.3%), representative images of HER2, MVD and CD34, as observed in Figure 1.

No significant relationship was found between MVD and any clinicopathological characteristic in the samples studied, as shown in Table 1.

Regarding gender (p = 0.457), it was observed that the median MVD value was higher in males than in females, with 50% of the female patients involved in this study having up to 13 density (Md = 13, Q_1 = 6.50; Q_3 = 27), compared to 16 in the 50% of the male patients (Md = 16, Q_1 = 11; Q_3 = 23.50).

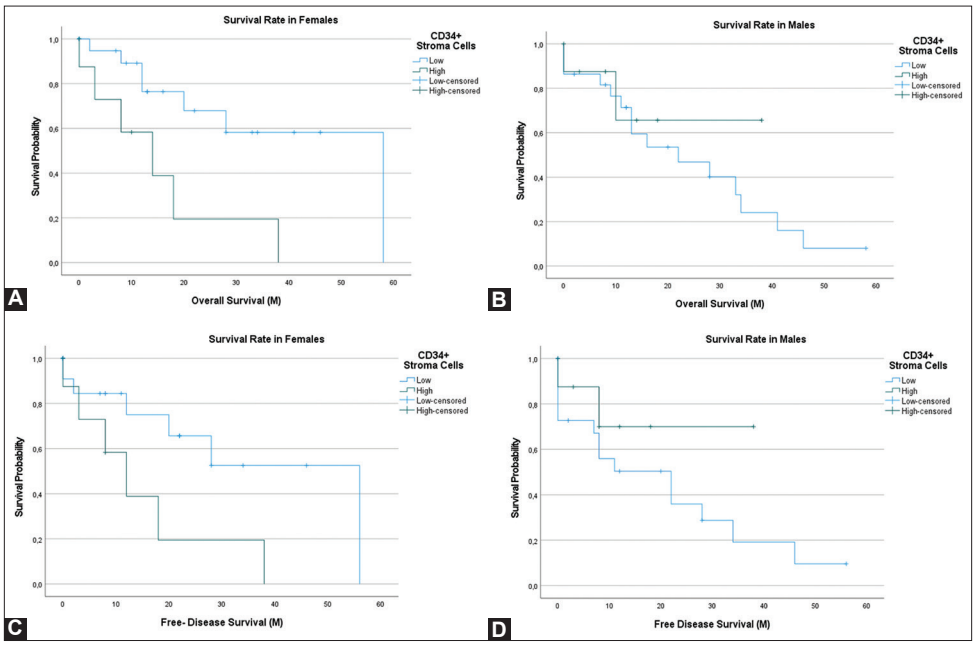

FIGURE 2. Kaplan–Meier survival analysis according to gender in CD34+ tumor stroma cells expression. (A) The free-disease survival of female patients with high CD34 stroma cells were considerably worse than on patients with lower CD34⁺ stroma cells expression. (B) The overall survival of female patients with high CD34⁺ stroma cells were considerably worse than on patients with lower CD34⁺ stroma cells expression. (C) The free-disease survival of male patients with high CD34⁺ stroma cells were substantially worse than on patients with lower CD34⁺ stroma cells expression. (D) The overall survival of male patients with high CD34⁺ stroma cells were substantially worse than on patients with lower CD34⁺ stroma cells expression.

M: Months

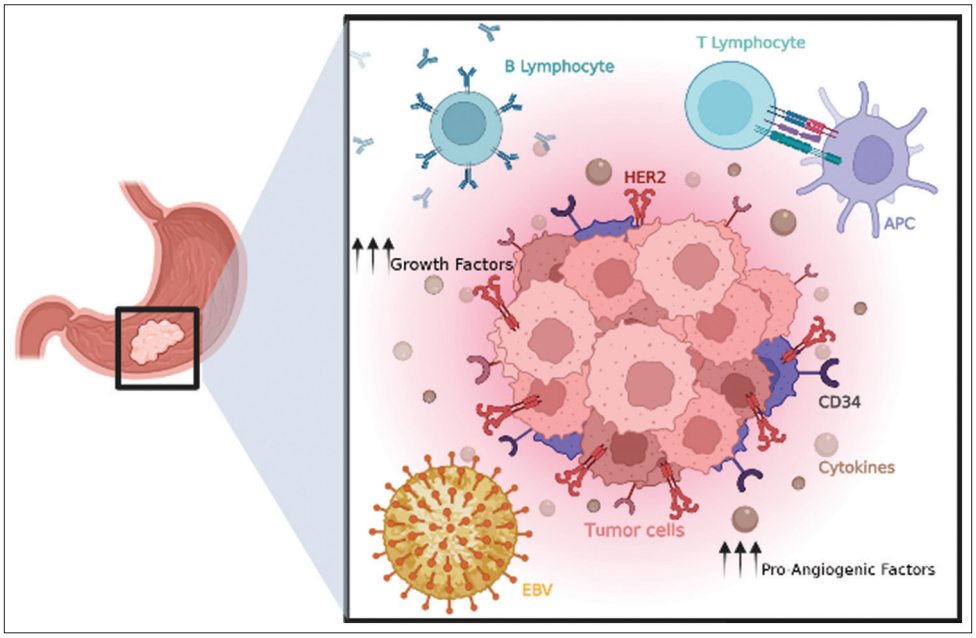

FIGURE 3. Visual representation of the HER2, EBV and CD34 markers studied in primary gastric cancer patients as well as the involvement of the tumor stroma, and its components, in the support and stimulation of the tumor, namely portrayed at the level of growth and angiogenic promotion.

HER2: Human epidermal growth factor receptor 2; EBV: epstein-barr virus; APC: antigen-presenting cells

For diagnosis (p = 0.735), the median MVD value was higher in adenocarcinomas (Md = 15, Q1 = 13; Q3 = 24) compared to carcinomas (Md = 13, Q_1 = 9; Q_3 = 28), meaning that 50% of adenocarcinomas involved in this study had up to 15 density, whereas 50% of carcinomas had up to 13. In relation with the anatomical location of the lesion (p = 0.579), the median MVD value was 24 for the cardia (Md = 24, Q_1 = 6; Q_3 =-), 12 for body (Md = 12, Q_1 = 5.50; Q_3 = 26. 50) and 15 for the antrum (Md = 15, Q_1 = 13; Q_3 = 22), which represents that 50% of the cardiac lesions had a density of up to 24, 50% of the body lesions had a density of up to 12, and 50% of the antrum lesions had a density of up to 15. Regarding the degree of tumor differentiation with MVD (p = 0.898) a weak positive correlation was observed (rho = 0.24). On the other hand, MVD shows a weak negative relationship with staging (rho = –0.251; p = 0.18) and with lymph node infiltration (rho = –0.17; p = 0.93). No relation could be established between MVD with TMN: metastasis as intended (p = 0.503), the median MVD of the cases with metastases was 13 (Md = 13, Q_1 = 5.50; Q_3 = 25), and 16 in the cases without metastases (Md = 16, Q_1 = 10; Q_3 = 24.50). In the type of treatment (p = 0.589), it was found that the median MVD value for patients undergoing surgery only is 13 (Md = 13, Q_1= = 5; Q_3 = 30), whereas for those undergoing surgery and QC, the value is 16 (Md = 16, Q_1 = 13; Q_3 = 22). For age, a moderate positive correlation was observed with MVD (rho = 0.58; p = 0.76).

The expression of CD34⁺ stromal cells showed an association with statistical significance with gender (r = 0.367; p = 0.046), revealing a higher density of markers of these cells in females than in males (n = 6, 46.2% vs. n = 2, 11.8%), as showed in Table 1.

Regarding the type of tumor diagnosed (p = 0.199), the high expression of CD34⁺ cells in the stroma was more incident in adenocarcinomas (n = 7, 36.8%) compared to carcinomas (n = 1, 9.1%).

From the anatomical location of the lesions, it is possible to observe that between tumors with low and high CD34⁺ stromal expression, there are differences in incidence, respectively, in the cardia (n = 2, 66.7% vs. n = 1, 33.3%), in the body (n = 6, 75% vs. n = 2, 25%), and in the antrum (n = 14, 73.7% vs. n = 5, 26.3%).

Regarding the degree of cell differentiation (p = 0.136), high expression of CD34⁺ stromal cells has a median of 2 (Md=, Q1 = 13; Q3 = 22) whereas low expression of CD34 stromal cells has a median of 1 (Md =, Q1 = 0; Q3 = 2) meaning that 50% of tumors with high expression are classified as moderately differentiated grade whereas 50% of low expression tumors are in a poorly differentiated grade.

For staging (p = 0.823) the medians of CD34⁺ stroma cells expression, high (Md = 3; Q1 = 1; Q3 = 3.25) and low expression (Md = 3; Q1 = 2.25; Q3 = 3) show that both 50% of high expression and 50% of low expression tumors are up to stage 3. Regarding lymph node infiltration and CD34⁺ stroma cells expression (p = 0.129), it is possible to verify that high-expression tumors have a lower incidence of infiltration (n = 7, 38.9%) compared to low-expression tumors (n = 11, 61.1%). A similar pattern was found for metastases (p = 0.281), with low-expression CD34⁺ stroma cells having more metastatic cases than high-expression tumors (n = 12, 80% vs. n = 3, 20%). Regarding the type of treatment (p = 0.417), we observed different medians for CD34⁺ stroma cells expression depending on whether they are high or low-expression tumors, respectively (Md = 6; Q1 = 1; Q3=6 vs. Md = 1; Q1 = 1; Q3 = 6) representing 50% of high expression tumors include up to surgery with CT whereas 50% of those with low expression include surgery only. For age (p = 1), the median of high expression CD34⁺ stroma cells, Md = 74; Q1 = 66.75; Q3 = 82, indicates that 50% of patients are up to 74 years old compared to 50% of those with high expression of the marker are up to 75.50 years old, Md = 75.50; Q1 = 64.75; Q3 = 80.75.

There was no significant correlation between $CD34^+$ stroma cell expression and MVD, as the $p = 0.266$ (t = 1.134; $p = 0.266$).

In general, the OS and DFS rates of the patients in the study were not statistically significant, only concluded that patients had a mean of 13.03 ± 15.248 and 16.80 ± 15.232 months, respectively. The only two HER2-positive GC cases in this study presented a mean DFS survival rate of 4.00 ± 5.657 and 13.00 ± 4.243 OS rate, as shown in Figure 2.

Regarding MVD, a moderate and weak negative correlation (rho = −0.58; $p = 0.762$ and rho = −0.3; $p = 0.107$) was observed with the DFS and OS rates, respectively.

The mean DFS ($p = 0.945$) and OS ($p = 0.246$) rates for $CD34^+$ stroma cell expression in patients with high expression are 10.88 ± 4.438 and 11.38 ± 12.546, respectively, whereas for patients with low expression are 13.82 ± 3.478 and 18.77 ± 15.895.

DISCUSSION

The relationship between HER2 overexpression, angiogenesis, $CD34^+$ cell activity in the stroma and EBV infection in GC has not been studied to date. Several studies have aimed to investigate some of these markers in this type of cancer, but few have conclusive data (13,17,25). Thus, our project intended to deepen the knowledge of such a prevalent pathology nowadays, with specific focus on EBV, HER2 and CD34 markers, given their strong potential for prognostic, diagnostic and therapeutic applications.

The literature approaches GC as a very heterogeneous pathology, with several types and subtypes, among which tumors of glandular differentiation predominate (4,5,12,23). Additionally, it is widely described as being more related to the male gender, and to advanced stages of disease as the diagnoses are typically late (3,12). According to our study, of the 30 cases studied, 19 were gastric adenocarcinomas (63.3%) and 17 were males (56.7%), with most tumors belonging to stages III and IV, consistent with previous findings.

HER2, a transmembrane receptor that acts as a proto-oncogene in several tumors, is the target of many trials in gastric tumors because of its structural diversity, which conditions targeted therapeutic applications that in other organs show results (Figure 3). This heterogeneity also influences staining patterns during immunohistochemical studies, where incomplete staining may occur, affecting tissue interpretation and scoring, and further contributing to the difficulty in obtaining conclusive data on this marker (11,13,17,26,27).

Several authors associate HER2 overexpression to GC with poor prognosis, due to a more aggressive form of the disease (11,27–29). Lei et al. showed results indicating that patients with more advanced stages of the disease, distant metastasis, and lymph node infiltration, namely clinicopathological features affiliated with a poor prognosis, tended to have higher HER2 expression levels (28). However, the prognostic value of this marker in GC is still controversial due to some contradictory results, which is why many do not consider it an independent prognostic factor for GC patients (3,27,28,30,31). As evidenced in research by Ciesielski et al. where a correlation was found between HER2-positive status and well-known features that suggest a better prognosis (less advanced TNM stage and better histological differentiation) (13).

Although our study could not establish statistical relationships with this marker due to insufficient sampling of HER-2 positive GC, a possible trend was observed in the 2 cases studied towards more advanced staging, a moderate degree of differentiation, the existence of lymph node infiltration and, in one of the patients, even with metastization when compared to the rest of the sample population, all HER2 negative. These results agree with the previous studies by Lei et al. and perhaps might hold statistical significance in a larger and more representative sample (28).

We investigated the expression of CD34 in two distinct ways, considering the different potential outcomes this marker can provide. Given its typical expression in vascular endothelial cells, CD34 was primarily useful to detect new blood vessels, ultimately defining a MVD, beneficial to understand the extent of angiogenesis and overall tumoral progression of each case (13,17,31,32) (Figure 3). Malignant tumors have the highest MVD values and are therefore associated with a higher risk of metastasis and recurrence and a correspondingly poor prognosis. Accordingly, MVD values are predicted to be positively related to invasiveness characteristics, such as more advanced disease stages, bigger tumors, and metastasis (17,24,31–33). Our results suggest two opposite-to-expected correlations between MVD and staging and with differentiation grade, which contrasts with other authors. Bădescu et al. concluded with their study that MVD values had a positive relation with staging, finding a higher number of new vessels in patients with a more advanced stage of disease. They also reported a significant increase in the number of intratumoral neovessels as tumor differentiation is lost (31).

This disagreement of results is not novel, as inconsistencies have been reported at the level of the MVD study mainly due to different evaluation criteria for the measurement of vascular density. Variability in the number of fields evaluated, the vessel counting conditions, and even the microscopic magnification used affects the results. Weidner's method, a well-known technique in this area of defined evaluation criteria, may also involve missed "hot spots". As a consequence of this lack of unanimity among some studies, the prognostic value of MDV is still a debatable question (13,17,23,31,32).

With our study we would like to help clarify the currently questionable prognostic values of HER2 and MVD by demonstrating an association between these and a worse evolution of GC.

While our study did not have the sampling conditions to do so, we found that sample size was a common limitation shared by other authors who have attempted to analyze this relationship, allowing only a trend between markers to be established (13,31). So far, only Li et al. have succeeded to established a statistically significant relationship between HER and MVD in GC samples (17).

The other way in which CD34 was evaluated in our study consisted of defining expression patterns of $CD34^+$ cells in the tumor stroma. $CD34^+$ cells are constitutive components

of the stroma of most tissues, carrying a variety of designations according to their characteristics, location, and organization in the tissues. Typically, their functions are to support maturation and proliferation of mesenchymal stem cells and adjacent epithelial cells and to mediate immune responses. At tumor stroma level, $CD34^+$ fibroblastic stromal cells (SFC) are of particular interest, given their ability to synthesize molecules, such as growth factors and cytokines, constituting prominent modifiers of tumor progression upon eventual stromal reaction (19,20,22,33–36). Their activity is further responsible to sustain a highly organized matrix that allows facilitated interactions between T, B lymphocytes and APC at sites of infection or chronic immune stimulation, known as tertiary lymphoid organs (21,35,37,38).

As TLS are referenced for their ability to amplify the immune response, when present in tumors, are often associated with more severe disease profiles (8,21,37,39). In this way, observation of higher CD34 expression patterns reveals more active tumor stromas and possible detection of TLS. Throughout our study, our only statistically significant relationship established is between the $CD34^+$ stromal cells and gender, with women having a higher incidence of GC with larger expression of the marker, and being, therefore, associated with more reactive stromas and possible presence of TLS. To our knowledge, this relationship has not been described before. One explanation behind this finding may be the ratio of tumor-stroma, whereby Ahn et al. described a tendency for women with gastric tumors to have a high percentage of tumor stroma compared to men (40).

Any other relationships were not conclusive, including with MVD, in which we attempted to explore the role of angiogenesis in tumor progression through involvement with tumor stromal activity. However a positive tendency was observed with MVD, since the stimulus of new vessel formation is a result of the interaction and shifts in the balance of pro- and anti-angiogenic substances produced by the tumor stroma (24,38). Although the prognostic role of TLS in oncology remains debatable, as it differs according to cancer type and stage, recent studies have concluded that TLS formation in solid tumors led to better therapeutic outcomes with immune checkpoint inhibitors (39).

GC is one of today's most deadly cancers, so DFS and OS rates are, in the samples studied, approximately just over a year. A striking contrast was observed regarding the average survival rates of the 2 HER2-positive cases, whose DFS was only 4 months, whereas after the initiation of therapy, the OS was approximately 1 year. Although there was no statistical relationship, our study observed a clear association between HER2 overexpression and worse prognosis with shorter life span, consistent with the study by Li et al (17).

Regarding MVD we also only observed in our study a weak correlation with survival rates, which further supports the study of Li et al. by showing a tendency for cases with higher microvascular densities to have lower survival rates. Interestingly, in our study, the GC cases with the best survival rates were the cases with less reactive tumor stromas, by expressing a lower amount of $CD34^+$ stroma cells, so once again we find more reactive stromal tumors, and the possible presence of TLS to be associated with poor prognosis.

CONCLUSION

Over the years, major advances have been made in the oncology area. Despite the many adversities faced in GC, mainly due to its intra and intertumor diversity, our study offers new perspectives. Consolidating the predictive power and expanding the benefits of studying markers such as CD34 in GC, where a more complete tumor characterization occurs through MVD measurements, CD34 expression patterns in the stroma, and detection of TLS.

Our results suggest a significant role of these factors in more aggressive cancer profiles, thereby constituting potential therapeutic targets. Ideally, links between CD34, HER2, and EBV would also have been established for innovative combinations in targeted therapy, maximizing their efficacy.

However, our study presented some limitations, the main one being the small sample size, not representative of all markers we intended to study. In addition, not all clinicopathological data were available, as the differentiation grade was inaccessible in seven cases. The EBV detection method used in this study was IHC, whereas the gold standard is *in situ* hybridization for its greater sensitivity, possible false negatives cannot be excluded.

Many questions remain unclear, so hypotheses such as a mutually exclusive relationship between HER2 and EBV, the association of HER2 with angiogenesis, and EBV causing more reactive stromas and TLS formation, among other pertinent points should be further investigated in upcoming studies.

DECLARATION OF INTERESTS

Authors declare no conflict of interest.

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021 Aug 15;149(4):778–89.
https://doi.org/10.1002/ijc.33588
2. Johnston FM, Beckman M. Updates on Management of Gastric Cancer. Curr Oncol Rep. 2019 Aug 24;21(8):67.
https://doi.org/10.1007/s11912-019-0820-4
3. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. The Lancet. 2020 Aug;396(10251):635–48.
https://doi.org/10.1016/S0140-6736(20)31288-5
4. Ajani JA, D'Amico TA, Bentrem DJ, Chao J, Cooke D, Corvera C, et al. Gastric Cancer, Version 2.2022. Vol. 20, JNCCN Journal of the National Comprehensive Cancer Network. Harborside Press; 2022. p. 167–92.
https://doi.org/10.6004/jnccn.2022.0008
5. Machlowska J, Baj J, Sitarz M, Maciejewski R, Sitarz R. Gastric cancer: Epidemiology, risk factors, classification, genomic characteristics and treatment strategies. Int J Mol Sci. 2020;21(11).
https://doi.org/10.3390/ijms21114012
6. Sexton RE, Al Hallak MN, Diab M, Azmi AS. Gastric cancer: a comprehensive review of current and future treatment strategies. Vol. 39, Cancer and Metastasis Reviews. 2020. p. 1179–203.
https://doi.org/10.1007/s10555-020-09925-3
7. Zhang Y wang, He D, Tan C, Dong M, Zhou L, Shao C kui. Differential expression of HER2 and downstream proteins in prediction of advanced tumor phenotypes and overall survival of patients with Epstein-Barr virus-positive vs. negative gastric cancers. Pathol Res Pract. 2019;215(11):152675.
https://doi.org/10.1016/j.prp.2019.152675
8. Gullo I, Oliveira P, Athelogou M, Gonçalves G, Pinto ML, Carvalho J, et al. New insights into the inflamed tumor immune microenvironment of gastric cancer with lymphoid stroma: from morphology and digital analysis to gene expression. Gastric Cancer. 2019 Jan 22;22(1):77–90.
https://doi.org/10.1007/s10120-018-0836-8
9. Moore A, Hikri E, Goshen-Lago T, Barkan T, Morgenstern S, Brook E, et al. Young-onset gastric cancer and Epstein-Barr Virus (EBV) - a major player in the pathogenesis? BMC Cancer. 2020 Jan 14;20(1).

https://doi.org/10.1186/s12885-020-6517-0

10. Stanek L, Gurlich R, Musil Z, Havluj L, Whitley A. Monitoring EBV infection, MSI, PDL-1 expression, Her-2/neu amplification as a biomarker for PD-1 inhibition in gastric cancer. Bratisl Lek Listy. 2022;123(2):83–6.

    https://doi.org/10.4149/BLL_2022_013

11. Roviello G, Catalano M, Iannone LF, Marano L, Brugia M, Rossi G, et al. Current status and future perspectives in HER2 positive advanced gastric cancer. Vol. 24, Clinical and Translational Oncology. Clin Transl Oncol; 2022. p. 981–96.

    https://doi.org/10.1007/s12094-021-02760-0

12. Seeneevassen L, Bessède E, Mégraud F, Lehours P, Dubus P, Varon C. Gastric Cancer: Advances in Carcinogenesis Research and New Therapeutic Strategies. Int J Mol Sci. 2021 Mar 26;22(7):3418.

    https://doi.org/10.3390/ijms22073418

13. Ciesielski M, Szajewski M, Pęksa R, Lewandowska MA, Zieliński J, Walczak J, et al. The relationship between HER2 overexpression and angiogenesis in gastric cancer. Medicine. 2018 Oct;97(42):e12854.

    https://doi.org/10.1097/MD.0000000000012854

14. Kocsmár É, Kocsmár I, Szalai L, Lendvai G, Szijártó A, Schaff Z, et al. Cross-testing of major molecular markers indicates distinct pathways of tumorigenesis in gastric adenocarcinomas and synchronous gastrointestinal stromal tumors. Sci Rep. 2020 Dec 1;10(1).

    https://doi.org/10.1038/s41598-020-78232-2

15. Roviello G, Aprile G, D'Angelo A, Iannone LF, Roviello F, Polom K, et al. Human epidermal growth factor receptor 2 (HER2) in advanced gastric cancer: where do we stand? Vol. 24, Gastric Cancer. Springer Japan; 2021. p. 765–79.

    https://doi.org/10.1007/s10120-021-01182-9

16. Meric-Bernstam F, Johnson AM, Ileana Dumbrava EE, Raghav K, Balaji K, Bhatt M, et al. Advances in HER2-targeted therapy: Novel agents and opportunities beyond breast and gastric cancer. Vol. 25, Clinical Cancer Research. Clin Cancer Res; 2019. p. 2033–41.

    https://doi.org/10.1158/1078-0432.CCR-18-2275

17. Li F, Meng G, Tan B, Chen Z, Ji Q, Wang X, et al. Relationship between HER2 expression and tumor interstitial angiogenesis in primary gastric cancer and its effect on prognosis. Pathol Res Pract. 2021 Jan 1;217:153280.

    https://doi.org/10.1016/j.prp.2020.153280

18. Rai B, Shukla J, Henry TD, Quesada O. Angiogenic CD34 stem cell therapy in coronary microvascular repair—A systematic review. Vol. 10, Cells. Multidisciplinary Digital Publishing Institute; 2021. p. 1137.

    https://doi.org/10.3390/cells10051137

19. Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: Evidence for CD34 as a common marker for diverse progenitors. Vol. 32, Stem Cells. Wiley-Blackwell; 2014. p. 1380–9.

    https://doi.org/10.1002/stem.1661

20. Shin SJ, Jeung HC, Ahn JB, Rha SY, Yoo NC, Roh JK, et al. Mobilized CD34+ cells as a biomarker candidate for the efficacy of combined maximal tolerance dose and continuous infusional chemotherapy and G-CSF surge in gastric cancer. Cancer Lett. 2008;270(2):269–76.

    https://doi.org/10.1016/j.canlet.2008.05.011

21. Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, Lambrecht BN. Tertiary lymphoid organs in infection and autoimmunity. Trends Immunol. 2012;33(6):297–305.

    https://doi.org/10.1016/j.it.2012.04.006

22. Nakayama H, Enzan H, Miyazaki E, Kuroda N, Naruse K, Kiyoku H, et al. CD34 positive stromal cells in gastric adenocarcinomas. J Clin Pathol. 2001;54(11):846–8.

    https://doi.org/10.1136/jcp.54.11.846

23. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med. 1991 Jan;324(1):1–8.

    https://doi.org/10.1056/NEJM199101033240101

24. Tenderenda M, Rutkowski P, Jesionek-Kupnicka D, Kubiak R. Expression of CD34 in gastric cancer and its correlation with histology, stage, proliferation activity, p53 expression and apoptotic index. Pathology and Oncology Research. 2001;7(2):129–34.

    https://doi.org/10.1007/BF03032579

25. Li H, Li L, Zhang N, Wang Z, Xu N, Linghu E, et al. Relationship between HER2 overexpression and long-term outcomes of early gastric cancer: a prospective observational study with a 6-year follow-up. BMC Gastroenterol. 2022;22(1):1–7.

    https://doi.org/10.1186/s12876-022-02309-7

26. Zhang H, Wang Y, Wang Y, Wu D, Lin E, Xia Q. Intratumoral and intertumoral heterogeneity of HER2 immunohistochemical expression in gastric cancer. Pathol Res Pract. 2020;216(11):153229.

    https://doi.org/10.1016/j.prp.2020.153229

27. Motoshima S, Yonemoto K, Kamei H, Morita M, Yamaguchi R. Prognostic implications of HER2 heterogeneity in gastric cancer. Oncotarget. 2018;9(10):9262–72.

    https://doi.org/10.18632/oncotarget.24265

28. Lei Y ying, Huang J yu, Zhao Q rui, Jiang N, Xu H mian, Wang Z ning, et al. The clinicopathological parameters and prognostic significance of HER2 expression in gastric cancer patients: A meta-analysis of literature. World J Surg Oncol. 2017;15(1):1–7.

    https://doi.org/10.1186/s12957-017-1132-5

29. Chen L, Wang L, Li X, Zhang G, Li Z, Wang Y. Clinic-pathological characteristics and prognostic value of pd-l1 and her2 in gastric cancer. DNA Cell Biol. 2021;40(2):405–13.

    https://doi.org/10.1089/dna.2020.6232

30. Kataoka Y, Okabe H, Yoshizawa A, Minamiguchi S, Yoshimura K, Haga H, et al. HER2 expression and its clinicopathological features in resectable gastric cancer. Gastric Cancer. 2013;16(1):84–93.

    https://doi.org/10.1007/s10120-012-0150-9

31. Bădescu A, Georgescu CV, Vere CC, Crăiţoiu Ş, Grigore D. Correlations between Her2 oncoprotein, VEGF expression, MVD and clinicopathological parameters in gastric cancer. Romanian Journal of Morphology and Embryology. 2012;53(4):997–1005.

32. Hong WG, Ko YS, Pyo JS. Clinicopathological significance and prognostic role of microvessel density in gastric cancer: A meta-analysis. Pathol Res Pract. 2017;213(12):1459–63.

    https://doi.org/10.1016/j.prp.2017.11.001

33. Li B, Nie Z, Zhang D, Wu J, Peng B, Guo X, et al. Roles of circulating endothelial progenitor cells and endothelial cells in gastric carcinoma. Oncol Lett. 2018;15(1):324–30.

    https://doi.org/10.3892/ol.2017.7272

34. Bure I, Braun A, Kayser C, Geddert H, Schaefer IM, Cameron S, et al. The expression of hematopoietic progenitor cell antigen CD34 is regulated by DNA methylation in a site-dependent manner in gastrointestinal stromal tumours. Int J Cancer. 2017;141(11):2296–304.

    https://doi.org/10.1002/ijc.30905

35. Díaz-Flores L, Gutiérrez R, García MP, Sáez FJ, Díaz-Flores L, Valladares F, et al. CD34+ stromal cells/fibroblasts/fibrocytes/telocytes as a tissue reserve and a principal source of mesenchymal cells. Location, morphology, function and role in pathology. Histol Histopathol. 2014;29(7):831–70.

    https://doi.org/10.14670/HH-29.831

36. Kim HS, Won YJ, Shim JH, Kim HJ, Kim J, Hong HN, et al. Morphological characteristics of vasculogenic mimicry and its correlation with EphA2 expression in gastric adenocarcinoma. Sci Rep. 2019;9(1):1–13.

    https://doi.org/10.1038/s41598-019-40265-7

37. Sitnik KM, Wendland K, Weishaupt H, Uronen-Hansson H, White AJ, Anderson G, et al. Context-Dependent Development of Lymphoid Stroma from Adult CD34+ Adventitial Progenitors. Cell Rep. 2016;14(10):2375–88.

    https://doi.org/10.1016/j.celrep.2016.02.033

38. San Martin R, Barron DA, Tuxhorn JA, Ressler SJ, Hayward SW, Shen X, et al. Recruitment of CD34+ fibroblasts in tumor-associated reactive stroma: The reactive microvasculature hypothesis. American Journal of Pathology. 2014;184(6):1860–70.

    https://doi.org/10.1016/j.ajpath.2014.02.021

39. Asam S, Nayar S, Gardner D, Barone F. Stromal cells in tertiary lymphoid structures: Architects of autoimmunity. Immunol Rev. 2021;302(1):184–95.

    https://doi.org/10.1111/imr.12987

40. Ahn B, Chae YS, Kim CH, Lee Y, Lee JH, Kim JY. Tumor microenvironmental factors have prognostic significances in advanced gastric cancer. Apmis. 2018;126(10):814–21.

    https://doi.org/10.1111/apm.12889