The European Research Journal 2023;9(5):1015-1026 Original Article

DOI: 10.18621/eurj.1296567 *Medical Pathology*

Comparison of human epidermal growth factor receptor 2 and cancer stem cell markers like CD44 and CD133 expressions with clinicopathological parameters in gastric cancer

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ABSTRACT

Objectives: Gastric carcinoma (GC) is the fourth most common cause of cancer-related tumor deaths worldwide. The prognostic significance of CD44, CD133 and human epidermal growth factor receptor 2 (HER2) expression in GC remains controversial. Therefore, we aimed to investigate the relationship of CD44, CD133 and HER2 expression with clinicopathological features in metastatic and non-metastatic GC patients.

Methods: A total of 139 patients with GC (68 with metastasis, 71 without metastasis) diagnosed were retrospectively analyzed. CD44 and CD133 expression were determined by immunohistochemical method in all cases. In addition, HER2 overexpression of the tumor was evaluated in patients with metastatic GC.

Results: The CD133 positivity rate was 90.6% (n = 126) when all cases were considered, and that for CD44 was 84.9% (n = 118). There was no difference in CD133 and CD44 positivity (intensity or density) rates and between the total scores of metastatic and non-metastatic patients with GC ($p > 0.05$). HER2 positivity in metastatic cases was detected in 49 (70.1%) patients by immunohistochemical method. No correlation was found between CD133 total score and age, tumor size or depth, and HER2 scores in metastatic or non-metastatic cases ($p > 0.05$). In the correlation analyzes performed with CD44 scores, only a borderline significant correlation was found between CD44 scores and tumor size (r:0.175; $p = 0.047$) in non-metastatic cases.

Conclusions: We demonstrated associations between CD44/CD133 expression and histological grade in all patients, between CD44 and tumor size in non-metastatic patients, and between HER2 and intestinal type (Lauren) in metastatic patients. The results of this study need to be confirmed by multicenter studies including large case series.

Keywords: Stem cell, CD44, CD133, gastric cancer, HER2

Gastric cancer (GC) (also known as stomach cancer) is the fifth most common cancer in the world, after lung, breast, colorectum, and prostate cancer, and is the fourth most common cause of cancer-

Received: ***May 14, 2023;*** *Accepted:* ***July 13, 2023;*** *Published Online:* ***August 8, 2023***

How to cite this article: *Gecer M, Büyükpınarbaşılı N, Turna S, Beşiroğlu M, Gucin Z. Comparison of human epidermal growth factor receptor 2 and cancer stem cell markers like CD44 and CD133 expressions with clinicopathological parameters in gastric cancer. Eur Res J 2023;1015-1026. DOI: 10.18621/eurj.1296567*

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related death [1]. The incidence of the disease is highest in Eastern Asia (Japan and Mongolia), whereas it is lowest in Northern America, Northern Europe and Africa [1-4].

The incidence and mortality rate of gastric cancer in Turkey has been reported as 14.2 per 100,000 and 12.15 per 100,000, respectively, suggesting that Turkey is one of the countries with the highest incidence of gastric cancer in Europe [2]. In the current WHO classification (2019), gastric carcinomas are classified as adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, and neuroendocrine carcinoma. Gastric adenocarcinoma, the most common type is divided into 5 subtypes: tubular, papillary, poorly cohesive, mucinous and mixed. Signet ring cell carcinoma terminology has been replaced by poorly cohesive carcinoma and is a subtype of adenocarcinoma. However, Lauren's classification of gastric cancers into two major types based on histological features, namely, intestinal (associated with chronic atrophic gastritis and intestinal metaplasia) and diffuse (originates from normal gastric mucosa) types, is more commonly used [5, 6]. The 5-year survival rate in gastric cancer cases is quite low despite aggressive treatments [5, 7].

In recent years, cancer stem cells (CSCs), a subpopulation of cancer cells, have begun to assume increasing importance in cancer studies. It has been shown that CSCs have specific functions such as self-renewal and differentiation as well as differentiation capacities and can acquire tumorigenicity when transferred to an animal host [8]. It has been reported that CSCs affect cancer initiation, progression, metastasis and recurrence, and consequently have a close relationship with the prognosis of the disease [9-17]. Two of the newest and most robust CSC surface markers investigated for GC are CD133 and CD44. CD44 is a principal cell surface glycoprotein for hyaluronic acid and a major component of extracellular matrices. CD44 has been shown to play an important role in adherence to the extracellular matrices, in motility, matrix degradation, proliferation and cell survival [18]. CD133 (also known as prominin-1), a five transmembrane cell-surface glycoprotein, plays a principal role in the maintenance of cell polarity and migration through the interactions of cells with each other [9, 19, 20]. It has been reported that CD133 is associated with a diagnosis of GC [20, 22].

Apart from these markers, human epidermal growth factor receptor 2 (HER2), also known as CerbB-2 or ERBB2 (erb-b2 receptor tyrosine kinase 2), is a proto-oncogene located on chromosome 17q21 that encodes a transmembrane protein with tyrosine kinase activity and is involved in signal transduction pathways, leading to cell growth and differentiation [23]. It has been shown that HER2 is a negative prognostic factor in GC, and HER2-positive tumors are associated with more aggressive biological behavior, higher recurrence frequencies, and decreased survival [23, 24]. There are very few studies evaluating the prognostic significance of CD44, CD133 and HER2, which together are important CSCs markers in GC patients. In addition, some studies have reported conflicting results regarding the effect of these markers on prognosis [11, 21, 24-29]. In this study, we aimed to investigate the relationship of CD44, CD133 and HER2 expression with clinicopathological features of the disease in patients diagnosed with GC in our center.

METHODS

All participants included in this single-center and cross-sectional study were informed about the scope of the study and their informed written consent was obtained. The study was evaluated and approved by the local ethics committee and adhered to the principles laid down by the Helsinki Declaration. One hundred thirty-nine patients with GC, (71 with metastasis and 68 without metastasis) diagnosed and followed up in our hospital between 2016 and 2019, were retrospectively analyzed. Demographic and clinical characteristics, tumor localization, histological type, tumor size, grade and invasion depth (T), lymph node status (N), and metastasis (M) data of all patients were obtained from file records. Histopathological parameters were re-evaluated from the archive slides. Gastric adenocarcinoma was classified as well differentiated, moderately differentiated, or poorly differentiated. All patients were defined as intestinal, diffuse or mixed type according to the Lauren classification [6]. Tumor stage was determined based on the American Joint Commission for Cancer criteria (AJCC 8th edition) [30]. CD44 and CD133 expression were determined by immunohistochemical (IHC) method in all cases.

In addition, HER2 overexpression of the tumor was evaluated in patients with metastatic GC.

Formalin (10%)-fixed and paraffin wax-embedded gastric adenocarcinoma blocks extracted from the archive of the pathology department were prepared for staining with Hematoxylin-Eosin (HE). For IHC staining, 4 µM thick sections were obtained and left for 1 hour at 60 degrees. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. The slides were buffered in Tris- EDTA (pH = 9) and then placed in a microwave at full power until the buffer reached boiling point. After that, the microwave temperature was reduced to 40°C and all tissues were left in place for 15 min. Then the slides were removed and left at room temperature for 15 min. Then monoclonal antibody diagnostic kits for CD44 (1: 100 dilution; clone MRQ-13, Millipore Sigma, USA), CD133 (1: 100 dilution; clone D4W4N, Cell Signalling Technology), and HER2 (ready to use;c-erbB-2/HER-2/neu Ab-17 (e2-4001+3B5) Thermo Scientific/LabVision) IHC stains were applied to them respectively.After washing, sections were overlaid with a secondary antibody (VECTASTAIN elite ABC kit Universal; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Sections were incubated in 3.0 % hydrogen peroxide in PBS for 30 min to block endogenous peroxidase activity. The reaction was developed using avidin-biotin-peroxidase complex. The peroxidase reaction was developed with 3-amino-9-ethylcarbazole, and sections were counterstained with hematoxylin. Colon cancer sections were used as a positive control. Negative control sections (isotype control) were incubated with normal mouse serum instead of the primary antibody. HE and IHC sections were examined under a light microscope by two expert pathologists.

Scoring of the cytoplasmic or membranous staining of CD133 and membranous staining of the CD44 proteins were evaluated as semi-quantitative according to the expression percentage and intensity of immune positivity. Intensity scoring was as follows: 0: negative expression; 1: poor intensity; 2: moderate intensity; and 3: strong intensity [31]. The scoring of the expression percentage (extent of positivity) was done according to the percentage of cells showing positive staining as follows: a score of 0 if less than 5%, a score of 1 if it was between 5-25%, a score of 2 if it was between 25-50%, and a score of 3 if it was more than 50%. Tumors were categorized based on the following scores: < 1, negative; ≥ 1, positive. Moreover, the total score was determined from 0 to 6 based on an evaluation of the intensity and the percentage of the expression scores when taken together.

HER2 IHC scoring was evaluated according to the scoring system proposed by Hofmann *et al*. [32] as follows: a score of 0: 0 or < 10% staining in tumor cells; a score of 1: weak or incomplete membranous staining in > 10% of tumor cells; a score of 2: weak-moderate staining in > 10% of tumor cells; and a score of 3: moderate-strong, complete or basolateral staining in > 10% of tumor cells. Scores of 0 and 1 were classified as no or low HER2 expression, while scores of 2 and 3 were evaluated as being HER2 positive (+). Silver enhanced in situ hybridization (SISH) was applied to the samples with +2 and +3 IHC scores. The SISH method was performed according to the manufacturer's protocols for VENTANA HER2 Dual ISH DNA Probe Cocktail (https://www.diagnostics.roche.com). The SISH evaluation was performed by analyzing the HER2 gene and the chromosome 17 centromere signals of at least twenty consecutive cells under a light microscope (with 40× magnification). As a result of this evaluation, samples with a HER2 centromeric probe for chromosome 17 (CEP17) and a ratio ≥ 2 were considered as HER2 positive.

Statistical Analysis

SPSS 26.0 (IBM Corporation, Armonk, New York, United States) program was used in the analysis of the variables. The normal distribution of the data was evaluated with the Shapiro-Wilk Francia test, with the Levene test used to evaluate the homogeneity of variance. In the comparison of the quantitative data of two independent groups, the Independent-samples t-test with the Bootstrap results or the Mann-Whitney U test (with Monte Carlo Simulation technique) was used. In the comparison of more than two groups according to quantitative variables, the Jonckheere-Terpstra test and the Kruskal-Wallis H tests with the Monte Carlo Simulation technique were used and the Dunn's Test was used for Post hoc analyses. In the comparison of categorical variables, the Pearson Chi-square, Fisher exact and Fisher-Freeman-Halton tests with the Monte Carlo Simulation technique were used, and the comparison of column ratios with each other was expressed with the Benjamini-Hochberg corrected *p* -

Table 1. Comparison of anthropometric and clinicopathological features of gastric cancer patients according to metastasis status

	Total (n = 139)	Metastasis		*p value*
		Yes (n = 71)	No (n = 68)	
Age (years)	63 (21-89)	63 (30-86)	61.5 (21-89)	0.820[u]
Gender, n (%)				0.999[c]
Female	38 (27.3)	19 (26.8)	19 (27.9)	
Male	101 (72.7)	52 (73.2)	49 (72.1)	
Tumor type (Lauren), n (%)				**< 0.001[c]**
Diffuse	12 (8.6)	0 (0)	12 (17.6)[A]	
Intestinal	127 (91.4)	71 (100)[B]	56 (82.4)	
Tumor location, n (%)				0.948[ff]
Antrum	58 (41.7)	30 (42.3)	28 (41.2)	
Cardia	33 (23.7)	18 (25.4)	15 (22.1)	
Corpus	30 (21.6)	14 (19.7)	16 (23.5)	
Entire Stomach	15 (10.8)	7 (9.9)	8 (11.8)	
Fundus	3 (2.2)	2 (2.8)	1 (1.5)	
Tumor grade, n (%)				**< 0.001[ff]**
Poor	80 (63.0)	35 (49.3)	45 (80.4)[A]	
Moderate	38 (29.9)	29 (40.8)[B]	9 (16.1)	
Well	9 (7.1)	7 (9.9)	2 (3.6)	
Tumor depth (T), n (%)				**< 0.001[ff]**
T0	2 (1.4)	2 (2.8)	0 (0)	
T1	5 (3.6)	5 (7)	0 (0)	
T2	26 (18.7)	26 (36.6)[B]	0 (0)	
T3	38 (27.3)	38 (53.5)[B]	0 (0)	
T4	68 (48.9)	0 (0)	68 (100)[A]	
CD133 score, n (%)				0.364[c]
0	13 (9.4)	8 (11.3)	5 (7.4)	
1	35 (25.2)	21 (29.6)	14 (20.6)	
2	50 (36.0)	25 (35.2)	25 (36.8)	
3	41 (29.5)	17 (23.9)	24 (35.3)	
CD44 score, n (%)				0.698[c]
0	21 (15.1)	13 (18.3)	8 (11.8)	
1	35 (25.2)	17 (23.9)	18 (26.5)	
2	40 (28.8)	21 (29.6)	19 (27.9)	
3	43 (30.9)	20 (28.2)	23 (33.8)	
CD133 total score	3 (0-6)	3 (0-6)	4 (0-6)	0.117[u]
CD44total score	3 (0-6)	3 (0-6)	3 (0-6)	0.446[u]
Tumor depth (T)	3 (0-4)	3 (0-3)	4 (4-4)	**< 0.001[u]**
Tumor size (cm)	4.5 (0.7-17.5)	4 (0.7-17.5)	5 (2.5-15)	0.229[u]

Data are shown as median (minimum-maximum or n (%).

[u]Mann-Whitney U test(Monte Carlo), [c]Pearson Chi-Square test(Monte Carlo), Post-hoc Test = Benjamini-Hochberg correction

[A]Expresses significance according to the groups without metastasis, [B]Expresses significance according to groups with metastases

value. Kendall's tau-b test was used to analyze the correlations of quantitative variables. While quantitative variables were expressed as mean (standard deviation) and median (Minimum-Maximum) in the tables, categorical variables were shown as n (%). Variables were analyzed at a 95% confidence level, and a p - value of less than 0.05 was considered significant.

RESULTS

A total of 139 GC patients, 68 with metastases (median age 63 years; 52 males and 19 females) and 71 without metastases (median age 61.5 years, 59 males, and 19 females) were included in the study. The groups with and without metastases were similar in terms of age, gender, tumor location, and tumor size ($p > 0.05$) (Table 1). In patients with metastatic GC, the frequency of diffuse type, poorly differentiated and T4 stage were significantly higher than those in the non-metastatic group ($p < 0.05$). According to IHC evaluation, CD133/CD44 positivity was 88.7% (n = 63) and 81.7% (n = 58) (respectively). In metastatic cases, CD133/CD44 staining negativity was 7.4% (n = 5) and 11.8% (n = 8), CD133/CD44 positivity was 92.6% (n = 63) and 88.2% (n = 60), respectively ($p > 0.05$). Considering all cases, CD133 positivity (mild/moderate/intense) was 90.6% (n = 126) and CD44 positivity (mild/moderate/intense) rate was 84.9% (n = 118) (Fig. 1a-d). There was no difference in CD133 and CD44 positivity (intensity or density) rates and the total scores of metastatic and non-metastatic patients with GC ($p > 0.05$) (Table 1).

When metastatic cases (n = 68) were evaluated in terms of HER2 IHC positivity, 19 (27.9%) cases had a HER2 IHC score of 0 or 1, 35 (51.5%) cases had a score of 2 and 14 (20.6%) cases a score of 3. The SISH method was applied to cases with a HER2 score of 2 or 3 according to IHC (n = 49), and 26 (53.1%) cases were found to be negative, with 23 (46.9%) cases positive according to the SISH method. HER2 scores determined by the IHC method were divided into two

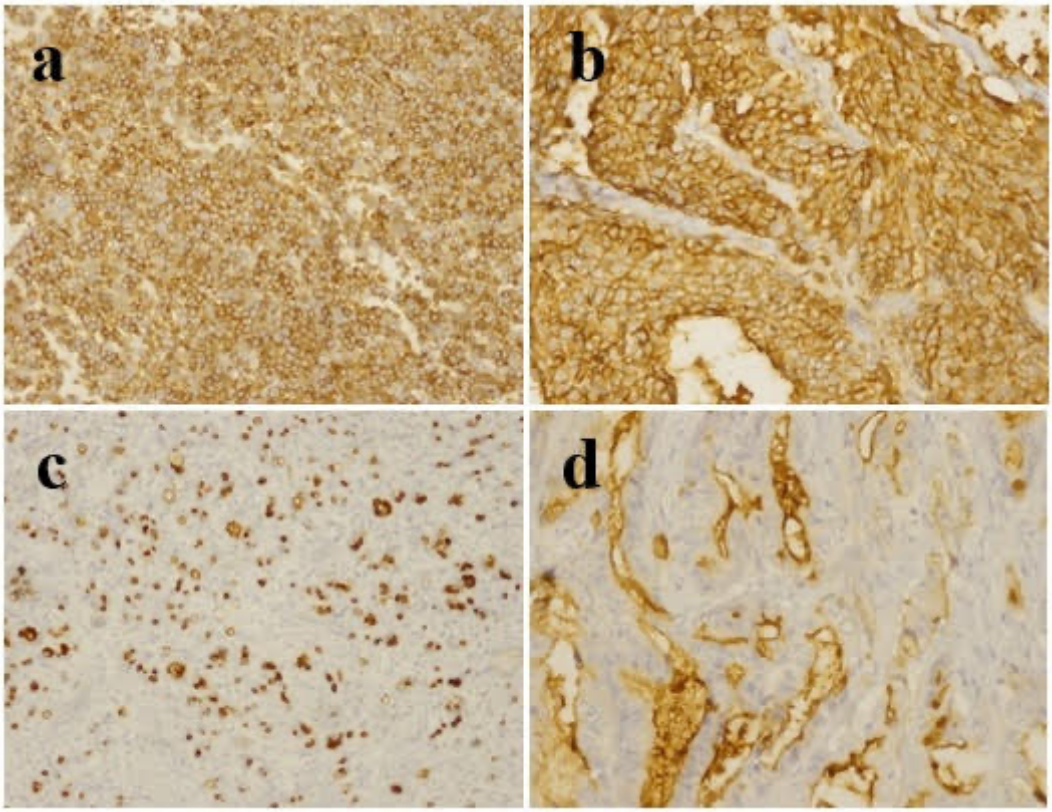

Fig. 1. (a) Membranous strong positivity of CD44 in poorly differentiated gastric carcinoma with ×100, (b) Membranous strong positivity of CD44 in moderately differentiated gastric carcinoma with ×200, (c) Membranous (luminal) staining of CD133 in poorly differentiated gastric carcinoma with ×100, and (d) Membranous (luminal) staining of CD133 in moderately differentiated gastric carcinoma with ×200.

Table 2. Evaluation of HER2 expression evaluated by two different methods in terms of anthropometric and clinicopathological features

	HER2 status (IHC)			HER2 status (SISH)		
	Negative (IHC0/1) (n = 19)	**Positive (IHC2/3) (n = 49)**	***p value***	**Negative (n = 26)**	**Positive (n = 23)**	***p value***
Age(years)	63 (31-80)	59 (21-89)	0.591[u]	58 (21-89)	63 (38-75)	0.194[u]
Tumor Depth (T)	4 (4-4)	4 (4-4)	0.999[u]	4 (4-4)	4 (4-4)	0.999[u]
Tumor size (cm)	5.5 (2.5-11.7)	4.5 (2.5-15)	0.082[u]	4.55 (2.5-15)	4.3 (2.5-10)	0.807[u]
Gender			0.766[c]			0.532[c]
Female	6 (31.6)	13 (26.5)		8 (30.8)	5 (21.7)	
Male	13 (68.4)	36 (73.5)		18 (69.2)	18 (78.3)	
Tumor type (Lauren)			0.294[f]			**0.011[f]**
Diffuse	5 (26.3)	7 (14.3)		7 (26.9)[B]	0 (0.0)	
Intestinal	14 (73.7)	42 (85.7)		19 (73.1)	23 (100.0)[A]	
Tumor location			0.119[ff]			0.155[ff]
Antrum	10 (52.6)	18 (36.7)		12 (46.2)	6 (26.1)	
Cardia	1 (5.3)	14 (28.6)		4 (15.4)	10 (43.5)	
Corpus	4 (21.1)	12 (24.5)		6 (23.1)	6 (26.1)	
Entire stomach	4 (21.1)	4 (8.2)		3 (11.5)	1 (4.3)	
Fundus	0 (0.0)	1 (2.0)		1 (3.8)	0 (0.0)	
Tumor grade			0.552[ff]			0.710[ff]
Poor	13 (92.9)	32 (76.2)		15 (78.9)	17 (73.9)	
Moderate	1 (7.1)	8 (19.0)		4 (21.1)	4 (17.4)	
Well	0 (0.0)	2 (4.8)		0 (0.0)	2 (8.7)	

IHC = immunohistochemical method, SISH = Silver enhanced in situ hybridization

[u]Mann Whitney U test (Monte Carlo), [c]Pearson Chi-Square test (Monte Carlo), [ff]Fisher Freeman Halton Test (Monte Carlo), [f]Fisher exact test (Monte Carlo)

[A]Expresses significance when compared with SISH-negative groups, [B]Expresses significance when compared with SISH positive groups

groups as negative (IHC 0/1) and positive (IHC 2/3). The gender, age, tumor depth, tumor size, tumor type (Lauren), tumor location, and tumor grade characteristics of these two groups were similar ($p > 0.05$). In addition, all of the HER2 positive cases (n = 23, 100%) in the SISH method were of the intestinal type, which was statistically significant according to the distribution of the HER2 negative patients ($p = 0.011$). Age, gender, tumor depth and size, tumor location and tumor grade characteristics of HER2 positive and negative cases regarding the SISH method were similar ($p > 0.05$) (Table 2).

The comparison of CD44 and CD133 scores of both metastatic and non-metastatic GC patients with various parameters are shown in Table 3. CD44 and CD133 scores were similar in terms of gender, HER2 positivity (IHC or SISH methods), tumor type (Lauren) and tumor location ($p > 0.05$). On the other hand, when CD133 and CD44 scores were compared according to disease grade in all patients, they were significantly higher in poorly differentiated cases ($p < 0.05$). CD133 and CD44 scores according to tumor grade in non-metastatic cases and CD133 scores according to disease grade in metastatic cases were similar ($p > 0.05$). CD44 scores in metastatic cases were significantly higher in poorly differentiated cases ($p <$

Table 3. Evaluation of CD44, CD133 and HER2 (evaluated by two different methods) expressions according to the metastasis status of gastric cancer patients

	Total		Non-metastatic		Metastatic	
	CD133 total scores	**CD44 total scores**	**CD133 total scores**	**CD44 total scores**	**CD133 total scores**	**CD44 total scores**
	n / Median (Min-Max)	**n / Median (Min-Max)**	**n / Median (Min-Max)**	**n / Median (Min-Max)**	**n / Median (Min-Max)**	**n / Median (Min-Max)**
Gender						
Female	38 / 3 (0-6)	38 / 3 (0-6)	19 / 3 (2-6)	19 / 3 (0-6)	19 / 3 (0-6)	19 / 3 (0-6)
Male	101 / 3 (0-6)	101 / 3 (0-6)	52 / 3 (0-6)	52 / 3 (0-6)	49 / 4 (0-6)	49 / 3 (0-6)
p value	0.626[u]	0.398[u]	0.197[u]	0.569[u]	0.644[u]	0.517[u]
HER2 (SISH)						
Negative	32 / 3.5 (0-6)	32 / 3 (0-6)	-	-	32 / 3.5 (0-6)	32 / 3 (0-6)
Positive	23 / 3 (0-6)	23 / 4 (1-6)	-	-	23 / 3 (0-6)	23 / 4 (1-6)
p value	0.774 [u]	0.071[u]	-	-	0.774[u]	0.071[u]
HER2 (IHC)						
IHC-0/1	19 / 4 (0-6)	19 / 3 (0-6)	-	-	19 / 4 (0-6)	19 / 3 (0-6)
IHC-2	35 / 3 (0-6)	35 / 3 (0-6)	-	-	35 / 3 (0-6)	35 / 3 (0-6)
IHC-3	14 / 5 (0-6)	14 / 4.5 (2-6)	-	-	14 / 5 (0-6)	14 / 4.5 (2-6)
p value	0.623[j]	0.121[j]	-	-	0.623[j]	0.121[j]
Tumor type (Lauren)						
Diffuse	12 / 3.5 (0-6)	12 / 3.5 (0-6)	-	-	12 / 3.5 (0-6)	12 / 3.5 (0-6)
Intestinal	127 / 3 (0-6)	127 / 3 (0-6)	71 / 3 (0-6)	71 / 3 (0-6)	56 / 4 (0-6)	56 / 3 (0-6)
p value	0.677[u]	0.810 [u]	-	-	0.677[u]	0.810[u]
Tumor location						
Antrum	58 / 3 (0-6)	58 / 3 (0-6)	30 / 3 (0-6)	30 / 3 (0-6)	28 / 3.5 (0-6)	28 / 2.5 (0-6)
Cardia	33 / 4 (0-6)	33 / 3 (0-6)	18 / 3 (2-5)	18 / 3.5 (0-6)	15 / 5 (0-6)	15 / 3 (2-6)
Corpus	30 / 3 (0-6)	30 / 4 (0-6)	14 / 2.5 (0-6)	14 / 4 (0-6)	16 / 3 (0-5)	16 / 3.5 (0-6)
Entire stomach	15 / 3 (0-6)	15 / 4 (0-6)	7 / 1 (0-5)	7 / 4 (0-6)	8 / 3.5 (2-6)	8 / 4.5 (1-6)
Fundus [excluted]	3 / 2 (2-5)[excluded]	3 / 6 (6-6)[excluded]	2 / 3.5 (2-5)[excluded]	2 / 6 (6-6)[excluded]	1 / 2 (2-2)[excluded]	1 / 6 (6-6)[excluded]
p value	0.144[k]	0.359[k]	0.078[k]	0.795[k]	0.294 [k]	0.433[k]
Tumor grade						
Poor	80 / 4 (0-6)	80 / 4 (0-6)	35 / 4 (0-6)	35 / 4 (0-6)	45 / 4 (0-6)	45 / 4 (0-6)
Moderate	38 / 3 (0-6)	38 / 2 (0-6)	29 / 3 (0-6)	29 / 2 (0-6)	9 / 3 (0-5)	9 / 2 (0-6)
Well	9 / 2 (1-6)	9 / 2 (2-6)	7 / 2 (1-4)	7 / 2 (2-6)	2 / 4 (2-6) [excluded]	2 / 3 (2-4)[excluted]
p value	**0.015**[j]	**0.004**[j]	0.054[j]	0.109[j]	0.201[u]	**0.006**[u]
	***P* (Poor-Moderate) = 0.043**	***P* (Poor-Moderate) = 0.004**	-	-	-	-

IHC = immunohistochemical method, SISH = Silver enhanced in situ hybridization

[u]Mann Whitney U Test (Monte Carlo), [k]Kruskal-Wallis Test (Monte Carlo), [j]Jonckheere-Terpstra Test (Monte Carlo); Post Hoc Test: Dun's Test,

0.05) (Table 3).

The results of the correlation analysis of CD133 and CD44 scores and various parameters are shown in Table 4. No correlation was found between CD133 total score and age, tumor size or depth, and HER2 scores in metastatic or non-metastatic cases ($p > 0.05$). In the correlation analyzes performed with CD44 scores, only a borderline significant correlation was

Table 4. Correlation analysis of CD44, CD133 total scores with age, HER2 expression, tumor size and tumor depth

	CD133 total scores		CD44 total scores	
	r	*p* value	r	*p* value
All patients				
Tumor depth (T)	0.082	0.240	0.069	0.325
Tumor size (cm)	0.008	0.893	0.121	0.052
HER2 (IHC)	-0.035	0.726	0.165	0.099
Age (years)	0.003	0.960	0.005	0.937
Non-metastatic patients				
Tumor depth (T)	-0.088	0.384	0.072	0.480
Tumor size (cm)	-0.023	0.793	0.175	**0.047**
Age (years)	-0.030	0.728	-0.042	0.635
Metastatic patients				
Tumor size (cm)	0.025	0.779	0.041	0647
HER2 (IHC)	-0.035	0.726	0.165	0.099
Age (years)	0.052	0.561	0.046	0.609

IHC = immunohistochemical method, Kendall's tau-b Test, r = Correlation Coefficient,

found between CD44 scores and tumor size (r:0.175; $p = 0.047$) in non-metastatic cases (Table 4).

DISCUSSION

Despite medical advances, significant improvements in the prognosis of GC have not been achieved and it still remains a serious public health problem. In recent years, various molecular and histochemical studies have been carried out to investigate the presence of various cell markers in CSCs [the most common markers: CD44, CD133] thought to initiate tumor development, and to be associated with metastasis and disease recurrence- and to consequently identify novel prognostic indicators and targeted biological approaches in the treatment of GC [18-29, 33, 34]. In most of these studies, these markers were considered separately, but in the current study, we evaluated the expression status of these three markers together. In addition, we examined whether there was a difference in the expression of these markers in metastatic and non-metastatic patients and in their relationship with clinicopathological features.

The expression positivity rate of CD44, one of the major components of the extracellular matrix, in GC cells has been shown to vary between 17.7% and 65.0% in various studies [11-13, 27, 35, 36]. In the current study, when all cases were evaluated, the rate of CD44 expression positivity was 84.9% (88.2% in metastatic patientsand 81.7% in non-metastatic patients). The difference in the frequency of CD44 positivity in GC in various reported studies may be related to geographic/racial characteristics, use of different cut-off values, or differences in the CD44 antibodies used. Numerous studies have been conducted on CD44 expression related to clinicopathological features, disease progression and the prognosis of patients with GC. Wakamatsu *et al*. [11] reported that CD44 may be one of the good markers associated with tumor invasion, distant metastasis and survival in patients with GC. In a study by Chen *et al*. [12], it is shown that high expression of CD44 is associated with poor differentiation, the presence of distant metastases, advanced TNM stage and tumor recurrence. In another study conducted by Düzcü *et al*. [15], it was shown that while CD44 was associated with histological grade, intestinal type, lymphovascular and perineural invasion, T-stage, and N-stage, it was not associated with distant metastasis, as in our study. Numerous

studies, including recent systematic reviews and meta-analysis studies, confirm that CD44 overexpression is associated with lymph node invasion, distant metastasis, poor prognosis, tumor size, and poor 5-year survival and as a result, it is suggested that CD44 is one of the most important guiding biomarkers in predicting the poor prognostic outcomes of GC [13, 36-43]. In our study, we found that CD44 expression was associated with poor differentiation and tumor size in our GC patients; however, unlike previous studies, no relationship was found between CD44 expression and tumor type (Lauren), tumor location and T-stage in our patients. On the other hand, we could not perform disease recurrence, prognosis or survival analyzes due to the lack of long-term follow-up of our patients.

CD133 is one of the best known CSC makers and has been shown to be expressed in various cancers including hepatocellular carcinoma, GC, colorectal cancer, pancreatic cancer, and ovarian cancer. Experimental studies suggest that CD133 expression in cancer patients is associated with resistance to various chemotherapeutic agents such as 5-fluorouracil and cisplatin. In addition, it has been shown in some studies that anti-CD133 antibody treatment inhibits the growth of cancer cells and induces apoptosis [44]. Therefore, determining CD133 expression status in patients may be a guide for the use of different treatment modalities. CD133 expression positivity rates in GC cells have been reported as 49.5% [13], 57.4% [46], and 58.4% [19] in various studies. In the current study, when all cases were taken into account, CD133 positivity (mild/moderate/intense) was seen to be 90.6%, and there was no difference in CD133 expression frequency between metastatic and non-metastatic patient groups. The difference in the expression frequency of CD133 may be related to the use of different antibodies in the histopathological evaluation or the use of different cut-off values. Moreover, in many studies, including meta-analysis studies, it has been shown that CD133 overexpression is associated with tumor size, histological grade, intestinal subtype, lymphatic infiltration, vascular invasion, TNM stage, depth of invasion, distant metastasis, tumor recurrence and reduced survival [9, 11-13, 15, 19, 22, 42, 46]. On the other hand, in a study by Sarıcanbaz *et al*. [21], it was shown that CD133 expression was not associated with nodal involvement, tumor size, T-stage, N-stage, and histological grade. In the current study, we demonstrated a relationship between CD133 expression and histological grade, but similar to the study of Sarıcanbaz *et al*. [21]. CD133 expression was not associated with age, tumor depth, tumor size, tumor type (Lauren), and tumor location. These conflicting results indicate the need for further studies with large case series.

It is known that HER2 expression plays a prominent role in the clinicopathological features and the poor prognosis of breast cancer [47]. However, conflicting data have been reported regarding the relationship between HER2-positivity and the clinicopathological features and prognosis of GC [24, 27, 28, 48-50]. In many studies, the rate of HER2 positivity with the IHC method varies between 4.4% and 53.4%, which may be due to geographic differences, tumor heterogeneity, the application of different scoring systems, and dependence on the examining pathologist [24]. In the current study, we found HER2 positivity to be 72.1% in patients with metastasis using the IHC method. In addition, in cases that were found to be HER2 positive by the IHC method, 46.9% of the cases were found to be HER2 positive by the SISH method. In one study, the sensitivity and specificity of the SISH method were reported as 56% and 100%, respectively, and the false-negative rate was 44%, according to the IHC method. These findings indicate that false negativity is very high in the SISH method, which is consistent with the findings of our study [51]. Studies investigating the relationship between clinicopathological features and HER2 expression have reported a close relationship between HER2 overexpression and gender, tumor differentiation, tumor location, tumor invasion, TNM stage, lymph node metastasis, and Lauren classification [48, 50, 52, 53]. In our study, when the SISH and IHC methods were taken into account separately, no relationship between HER2 expression according to the IHC method and demographic and clinicopathological features was found, only between HER2 expression and intestinal type GC in the SISH method, which is in line with the findings of Tanner *et al*. [54], was a relationship found. In addition, similar to our study, some studies reported that HER2 expression is not associated with gender, age, TNM stage tumor differentiation, tumor location, and tumor invasion [27, 28]. In addition, there are studies reporting that disease prognosis is associated with HER2 expression in patients with GC [48, 52, 54]; other studies dif-

fer [27, 28, 50, 55]. It has been reported in a study that the evaluation of HER2 is important because it guides the use of anti-HER2 agents in patients with GC [56]. Since our study did not include long-term follow-up of the patients, its effect on the prognosis could not be evaluated.

Limitations

The strength of this study lies in its evaluation of CD44, CD133 and HER2 expressions together in patients with GC, unlike previous studies, and its investigation of the relationship with clinical features. On the other hand, this study has some limitations. First of all, analysis related to prognosis could not be presented due to the lack of long-term follow-up of the patients. In addition, the number of patients included in the study was relatively small, and the results of subgroup analyzes may have been negatively affected by this. The negative effects of the difference of antibodies used in CD44, CD133 and HER2 measurements in the studies and the use of different cut-off values in determining the positivity of the results were not taken into account.

CONCLUSION

In conclusion, in this study, we investigated the presence of stem cells with CD133 and CD44 in metastatic, non-metastatic cases and in metastatic and HER2 positive cases. We reported associations between CD44 / CD133 expressions and histological grade in all patients, between CD44 and tumor size in non-metastatic patients, and between HER2 (detected by the SISH method) and intestinal type (Lauren) in metastatic patients. In addition, the association of all three markers with age, gender, metastasis, tumor invasion, or tumor location could not be demonstrated. We did not find any relationship between expressions of HER2 and CD44 and CD133. The results of this study need to be confirmed by multicenter studies including large case series.

Authors' Contribution

Study Conception: MG, NB, ST, MB, ZG; Study Design: MG, NB, ST, MB, ZG; Supervision: MG, NB, ST, MB, ZG; Funding: MG, ZG; Materials MG, NB, ST; Data Collection and/or Processing: MG, MB, ZG; Statistical Analysis and/or Data Interpretation: MG, NB, ZG; Literature Review: MG, NB, ST, ZG; Manuscript Preparation: MG, ZG and Critical Review: MG, NB, ZG.

Ethical Statement

The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated.

Informed Consent

Written informed consent was obtained from all participants before the study commenced.

Ethics Committee Approval:

Bezmialem Vakif University, Non-Interventional Research Ethics Committee, Decision number: 02/30, Date: 05/05/2020.

Conflict of Interest

The author disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The author disclosed that they did not receive any grant during conduction or writing of this study.

Acknowledgement

This article has been presented as oral presentations at 29th National Congress of Pathology, October 23-26, 2019, Trabzon, Turkey

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