Expression of TWIST1 and CD44 as diagnostic and prognostic biomarkers in patients with gastric cancer

Marwa A. El Kholy, Hala A. El Sayed, Eman M. Ahmed

Objective The aim of this study was to investigate the association of TWIST1 and CD44 in gastric cancer (GC) with clinical parameters and their relation to prognosis, which may be beneficial for targeted therapeutic strategies in the future.

Materials and methods The material of this work consisted of 40 primary GC specimens from patients who underwent radical gastrectomy. Patients who received neoadjuvant chemotherapy or chemoradiotherapy, those who presented with other cancers at the same time, or the patients with incomplete clinical data were excluded from the study. Hematoxylin and eosin-stained sections from all cases were re-evaluated and further stained immunohistochemically using antibodies against TWIST1 and CD44.

Results TWIST1 and CD44-positive expressions were significantly increased in GC cases of diffuse type (P=0.019 and 0.002, respectively). Moreover, there was a statistically significant correlation between both markers and tumor grade, stage, and lymphovascular invasion (P=0.027 and 0.010, P=0.002 and 0.012, and P=0.001 and 0.005, respectively). A statistically significant correlation was found between TWIST1 and CD44 expressions in GCs (P=0.000).

Conclusion The presence of TWIST1-positive carcinoma cells and CD44-positive cancer stem-like cells in GC tissue can be used as a diagnostic tool for GC and regarded as a marker of poor prognosis in patients with GC, which may provide potential targets for GC therapy.

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Department of Pathology, Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt

Correspondence to Marwa A. El Kholy, PhD, Department of Pathology, Al-Azhar University, Cairo, 13111, Egypt. Tel: +20 100 636 0819; e-mail: marwapatho@yahoo.com

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Introduction

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer-related death for both sexes in the world [1]. In Egypt, GC is the 12th most common cancer in both sexes, representing 1.6% of the total cancers. It is the 12th leading cause of cancer-related death, representing 2.2% of the total cancer mortality [2].

The survival of patients with GC has improved in the past three decades worldwide [3]. Gastroenterologists have been trying to identify effective biomarkers for evaluating the early detection of GC, which may also be targeted for novel therapies for this cancer [4].

Tumor metastasis is a complex multistep process [5]. In the process of tumor metastasis, cancer cells lose their polarity and intercellular adhesions and acquire the phenotype and invasive characteristics of mesenchymal cells through the epithelial–mesenchymal transition (EMT) to achieve the infiltration of surrounding tissue [6].

First, the EMT was characterized as an important process that is critical for embryonic development and wound healing. There was established evidence that EMT activation in the human stomach is associated with gastric carcinogenesis and tumor progression. Moreover, EMT activation gives gastric epithelial cells characteristics of mesenchymal cells and reduces their epithelial features. Moreover, mesenchymal cells tend to dedifferentiate and acquire stem cell or tumorigenic phenotypes such as invasion, metastasis, and apoptosis resistance as well as drug resistance during EMT progression [7].

TWIST1 is a basic helix-loop-helix transcription factor that plays essential roles in multiple stages of embryonic development and significant shares in tumor metastasis, even tumor initiation, and primary tumor growth. It induces EMT, a key process in the metastases formation of cancer. TWIST1 also promotes the formation of cancer stem cells and facilitates the process of tumorigenesis [8].

Cancer stem-like cells are defined as rare cells in malignant tumors with the ability to self-renew and to differentiate into various heterogeneous cancer cell lineages [9].

The CD44 proteins, which are expressed in many cell types, belong to a polymorphic family of cell surface glycoproteins that were originally described as lymphocyte homing receptors [10]. It is involved in cell–cell adhesion, cell–matrix interactions, and tumor metastasis. CD44 is widely accepted as a cancer stem-like cell marker for GC in many studies [11]. This study was designed to determine the associations of TWIST1 and CD44 with clinical parameters, investigate their relation to prognosis, and provide a better understanding of the context-dependent regulation of TWIST1 and CD44 in GC, which might reveal new therapeutic targets in the treatment of GCs.

Materials and methods

The material of this work consisted of 40 primary GC specimens from patients who underwent radical gastrectomy. Patients who received neoadjuvant chemotherapy and chemoradiotherapy, patients who presented with other cancers at the same time, or the patients with incomplete clinical data were excluded from the study. The pathological specimens were obtained from the surgical files of the Histopathology Departments of Al-Azhar University Hospital (Al-Zahraa University Hospitals) and a private laboratory, covering the period from January 2013 to April 2016. In addition, 20 adjacent normal gastric tissues removed for radical gastrectomy were also included as a negative control.

The clinicopathological data such as age, sex, size of the tumor, histologic type, grade, stage, vascular invasion, and lymphatic invasion were obtained from the available histopathological charts.

For histopathological examination, 5-mm-thick sections were prepared from each tissue paraffin block and stained with hematoxylin and eosin for confirmation of the diagnosis. The histologic classification into intestinal and diffuse type adenocarcinomas was performed according to the Laurén classification in 1965 of GC. Although this classification was introduced in 1965, it remains widely accepted and employed, as it constitutes a simple and robust classification approach. The intestinal type corresponds to tubular, papillary, and mucinous adenocarcinoma in The WHO classification issued in 2010, whereas diffuse type includes Signet-ring cell carcinoma and other poorly cohesive carcinomas. The histologic grade of GC was in accordance with the criteria of the WHO (2000), and tumors were grouped as well (I), moderately (II), and poorly (III/IV) differentiated. The TNM stage of the cases was assessed according to the criteria of the sixth edition of the TNM classification of the International Union against Cancer (Union for International Cancer Control, 2002). The depth of tumor invasion, lymph node involvement, and distant metastasis were assessed according to the seventh edition of Union for International Cancer Control/American Joint Committee on Cancer guidelines.

For immunohistochemical staining, two sections were cut from each case on positively charged slides and subjected to immunohistochemical staining using the streptavidin-biotin alkaline phosphate methods. The primary antibodies used for immunohistochemical staining (with clone, manufacture, dilution, incubation period, and positive control) were as follows: monoclonal mouse antibody antihuman TWIST1 (code LS-C191858, diluted 1.200; LSBio, Cambridge, USA) and monoclonal antibody diagnostic kits for CD44 (DF1485, diluted 1.200; Abcam). Negative control samples were processed in parallel to test samples by replacing the primary antibody with phosphate buffer saline. As a positive control, we used sarcoma tissues and normal tonsil for TWIST1 and CD44, respectively (based on the manufacturer's instructions).

The sections were placed in an oven at 50°C for 30 min, and then the sections were deparaffinized in xylene, rehydrated in graded alcohol dilution, washed in PBS, incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, washed in PBS again, and boiled in citrate buffer solution (pH 6.0) using a microwave for 10 min at 60°C for antigen retrieval. After cooling at room temperature, the sections were incubated with primary antibody overnight in a humidified chamber and rinsed with PBS. The sections were then incubated for 30 min at 37°C with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase. After three rinses with PBS, the sections were incubated with diaminobenzidine substrate and then rinsed with distilled water and counterstained with hematoxylin.

All immunohistochemical-stained slides were observed by two expert pathologists (with no knowledge of patient's clinical data) and reported.

Interpretation of immunohistochemical staining

When immunohistochemical staining was evaluated, the whole section was scanned at low power (×100) then by high power of magnification (×400) in each case to calculate the rate of positive staining.

Assessment of TWIST1

The final staining score for TWIST1 either cytoplasmic or nuclear was assisted by the sum of the extent and intensity of staining. The staining intensity was scored as 0 (negative), 1 (very weak), 2 (weak), 3 (medium), and 4 (strong). The extent of staining was scored as 0 (0–10%), 1 (10–30%), 2 (30–50%), 3 (50–75%), and 4 (>75%) according to the percentage of positive-staining cells in relation to the total cancer cells. The expression of TWIST1 in each slide was scored as the sum of intensity and extent of positive-staining cells. The slide with a final staining score of more than 3 was defined as positive expression [5].

Assessment of CD44

For membranous or cytoplasmic expression of the CD44, the percentage of positive cancer cells and the staining intensity were quantified. The mean percentage of positive tumor cells was quantified in at least five fields at ×400 magnification and classified into one of the following five grades: 0 (<5% of cells had positive staining), 1 (5–25% of cells had positive staining), 2 (26–50% of cells had positive staining), 3 (51–75% of cells had positive staining), and 4 (>75% of cells had positive staining). The staining intensity of CD44 was scored as follows: 0 (no staining), 1 (light brown), 2 (brown), and 3 (dark brown). The percentage score and staining intensity score were multiplied to get the final staining score for each tumor specimen. The overall staining scoring system could be categorized into two groups: negative (0–4) and positive (5–12) [4].

Statistical analysis

Data were collected, revised, coded, and entered into the statistical package for the social science (IBM SPSS, version 20; IBM). Qualitative data were presented as number and percentages. The comparison between two groups with qualitative data was done by using χ^2 test and/or Fisher's exact test, which was used instead of χ^2 test when the expected count in any cell was found to be less than 5. The confidence interval was set to 95%, and the margin of error accepted was set to 5%. So, the P value was considered significant at less than 0.05.

Results

The clinical and histopathological characteristics of the GC patients enrolled in the study are summarized in Table 1. There were 26 (65%) males and 14 (35%) females, with age in the range of 39–85 years. The tumor sizes were smaller than 5 cm in 47.5% (19 cases), 5–10 cm in 32.5% (13 cases), and larger than 10 cm in 20% (eight cases). Regarding the histopathological types, they were classified as diffuse (23 cases, 57.5%) or intestinal (17 cases, 42.5%). According to the grade of the tumors, well and moderate differentiations were seen in 14 (35%) cases and poorly in 26 (65%) cases. Overall, four (10%) cases had a vascular invasion, eight (20%) cases showed lymphatic invasion, 10 (25%) had both vascular and lymphatic invasion, and 18 (45%) cases were noninvasive. T1 or T2 stage was reported in eight (20%) cases and T3 and T4 in 32 (80%). Distant metastasis was detected in six cases only.

Table 1 Clinicopathologic characteristics of primary gastric cancer

Variables	*n* (%)
Number of cases	40
Age range (years)	39–85
Sex (male and female)	26 (65)/14(35)
Tumor size (≤5, 5 10, and >10 cm)	19(47.5)/13 (32.5)/8 (20)
Pathological type of cancer	
Diffuse	23 (57.5)
Intestinal	17 (42.5)
Grade of tumor	
Well/moderate	14 (35)
Poor	26 (65)
Invasion	
Vascular invasion only	4 (10)
Lymphatic invasion only	8 (20)
Vascular and lymphatic invasion	10 (25)
Without invasion	18 (45)
Gastric wall invasion	
T1–T2	8 (20)
T3–T4	32 (80)
Nodal metastasis	
N0–N1	23 (57.5)
N2–N3	17 (42.5)
Distant metastasis	
MX	34 (85)
M1	6 (15)
TNM stage	
I–II	12 (30)
III–IV	28 (70)

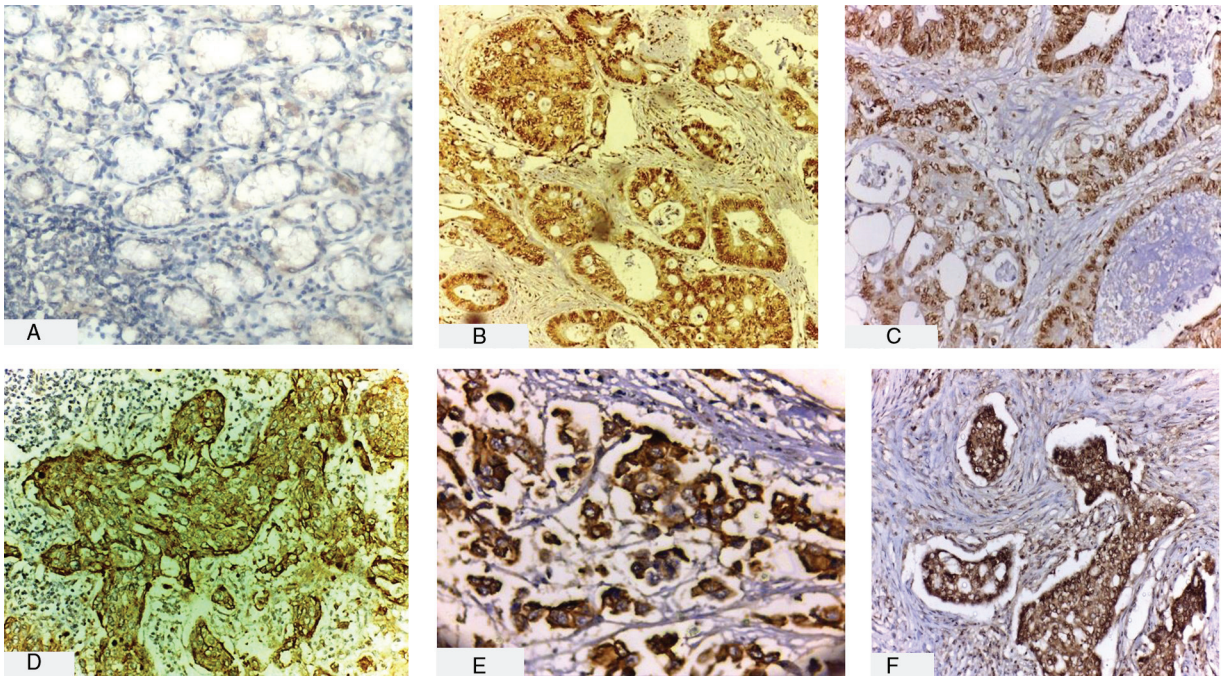
Immunohistochemical results

TWIST1 immunoexpression

TWIST1 was localized in the cytoplasm or nucleus of primary cancer cells. It was detected in 18 of 40 (45%) cases of human GC. However, it showed negative expression in the normal gastric epithelial cells (Fig. 1).

TWIST1 expression was statistically insignificant with age, sex, and tumor size. Regarding tumors, histological type statistical analysis showed a significant increase in expression of TWIST1 in

Figure 1



Immunohistochemical staining for TWIST in (a) noncancerous tissues (negative control) and (b, c) gastric cancer lesions intestinal type. TWIST positive expression was prominent in tumor tissue and seemed mostly to localize in the cell cytoplasm as a diffuse staining or nuclear, respectively (×100). (d) TWIST was highly expressed in poorly differentiated adenocarcinoma (magnifications were ×100). (e) TWIST was highly expressed in Signet-ring cell carcinoma (×400). (f) TWIST was highly expressed in gastric carcinoma with lymph vascular invasion (×100).

diffuse type compared with intestinal type, with 14 of 23 cases of diffuse type were positive for TWIST1, whereas four of 17 cases of intestinal type were positive. A highly significant statistical correlation was detected (P=0.001) between cases of GCs with vascular, lymphatic, or lymphovascular invasion and TWIST1 expression. When the grade of the tumor increased, there was a statistically significant increase in TWIST1 expression of tumor cells, with three of 14 cases with well/moderate differentiated tumor and 15 of 26 cases with poorly differentiated tumor were positive for TWIST1. When considering tumor stage there was a significant correlation between TWIST1 expression and higher tumor stage, although nodal and distant metastasis give insignificant statistical correlation with TWIST1 expression (Table 2).

CD44 immunoexpression

CD44 expression was detected in the membrane and cytoplasm of cancer cells in GC samples. Among 40 cases of GC, there was a positive expression of CD44 in 25 (62.5%) of 40 cases. However, the normal gastric epithelial cells showed negative expression of CD44 (Fig. 2).

Regarding age, sex, and size, there were statistically insignificant correlations with CD44. Statistical analysis showed a significant increase in expression of CD44 in diffuse type compared with intestinal type, with 19 of 23 cases with diffuse-type tumors were positive for CD44, whereas six of 17 cases of intestinal-type tumors were positive for CD44. When talking about lymphovascular invasion, there was a significant statistical correlation (P=0.005) between CD44 expression and vascular, lymphatic, or lymphovascular invasion compared with cases without invasion. Moreover, with a higher grade of the tumor, there was a higher CD44 expression in tumor cells, with a statistically significant difference between well/moderate and poorly differentiated groups. This study recorded higher CD44 expression in advanced GC stages, with statistically significant correlation, and there was a highly significant correlation between primary tumor (T) and CD44 expression (Table 2).

The relation between CD44 and TWIST1 expression in gastric carcinomas

Statistical analysis showed a significant association between CD44 and TWIST1 expression in GCs (P=0.000) (Table 3).

Discussion

GC is the fourth most common type of cancer and the second leading cause of cancer-associated mortality

Table 2 Correlation of CD44 and TWIST1 expression of gastric cancer with clinicopathological variables

	CD44 [*n* (%)]		*P* value	TWIST1 [*n* (%)]		*P* value
	Negative (*N*=15)	Positive (*N*=25)		Negative (*N*=22)	Positive (*N*=18)	
Sex						
Female	4 (26.7)	10 (40.0)	0.392	6 (27.3)	8 (44.4)	0.257
Male	11 (73.3)	15 (60.0)		16 (72.7)	10 (55.6)	
Age (years)						
<50	7 (46.7)	10 (40.0)	0.679	11 (50.0)	6 (33.3)	0.288
>50	8 (53.3)	15 (60.0)		11 (50.0)	12 (66.7)	
Size						
≤5	7 (46.7)	12 (48.0)	0.621	10 (45.5)	9 (50.0)	0.351
5–10	6 (40.0)	7 (28.0)		9 (40.9)	4 (22.2)	
> 10	2 (13.3)	6 (24.0)		3 (13.6)	5 (27.8)	
Pathological type of cancer						
Diffuse	4 (26.7)	19 (76.0)	**0.002**	9 (40.9)	14 (77.8)	**0.019**
Intestinal	11 (73.3)	6 (24.0)		13 (59.1)	4 (22.2)	
Invasion						
No invasion	12 (80.0)	6 (24.0)	**0.005**	16 (72.7)	2 (11.1)	**0.001**
Vascular invasion	1 (6.7)	3 (12.0)		1 (4.5)	3 (16.7)	
Lymphatic invasion	0 (0.0)	8 (32.0)		2 (9.1)	6 (33.3)	
Vascular and lymphatic invasion	2 (13.3)	8 (32.0)		3 (13.6)	7 (38.9)	
Grade						
Well/moderate	9 (60.0)	5 (20.0)	**0.010**	11 (50.0)	3 (16.7)	**0.027**
Poor	6 (40.0)	20 (80.0)		11 (50.0)	15 (83.3)	
Gastric wall invasion (T)						
T1–T2	7 (46.7)	1 (4.0)	**0.001**	7 (31.8)	1 (5.6)	**0.039**
T3–T4	8 (53.3)	24 (96.0)		15 (68.2)	17 (94.4)	
Nodal metastasis (N)						
N0–N1	12 (80.0)	11 (44.0)	**0.026**	14 (63.6)	9 (50.0)	0.385
N2–N3	3 (20.0)	14 (56.0)		8 (36.4)	9 (50.0)	
Distant metastasis (M)						
Mx	15 (100.0)	19 (76.0)	**0.039**	20 (90.9)	14 (77.8)	0.247
M1	0 (0.0)	6 (24.0)		2 (9.1)	4 (22.2)	
TNM stage						
I–II	8 (53.3)	4 (16.0)	**0.012**	11 (50.0)	1 (5.6)	**0.002**
III–VI	7 (46.7)	21 (84.0)		11 (50.0)	17 (94.4)	

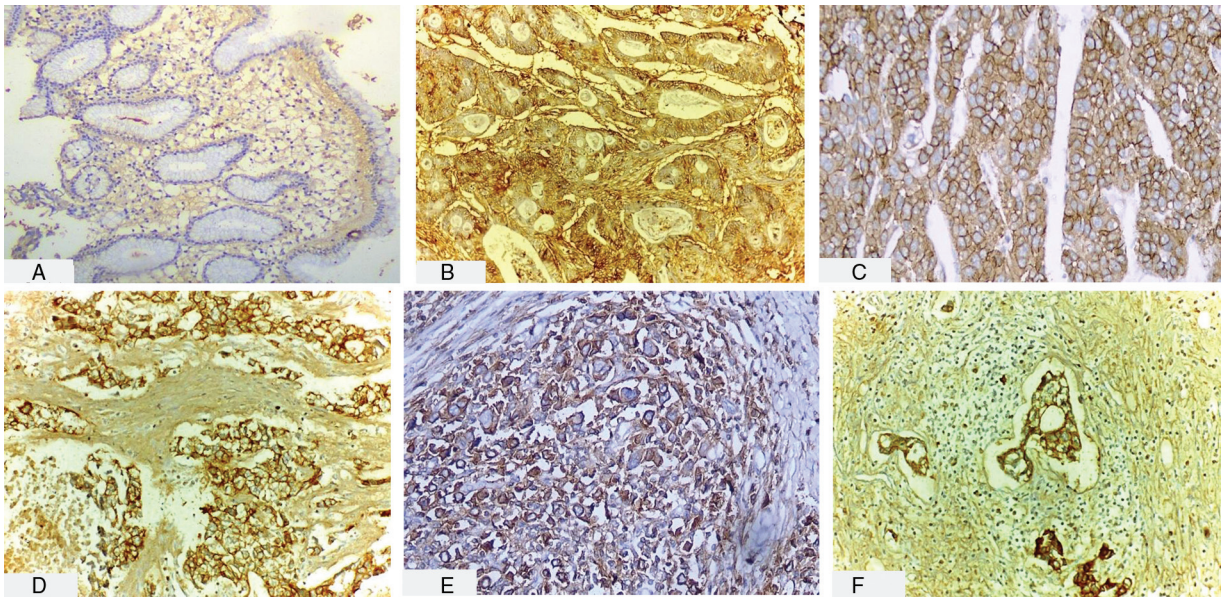
worldwide [12]. Worldwide mortality rates for GC have decreased in the past 10 years; however, the survival rate still remains low [13]. So, investigations into its initiation and development are of great importance [7]. GC is a highly heterogeneous disease, which presents a variety of biological behaviors [14]. Certain genes or proteins exhibit different levels of expression in GC [15].

These genes may constitute biomarkers. However, the specific role of such biomarkers remains to be elucidated to predict the prognosis of GC [16].

There are a number of molecules that indicate the stage of EMT and stem cell markers. In this study, associations of TWIST1 and CD44 with age, sex of the patients, and size of the tumor have been examined. The age of the patients ranged from 39 to 85 years, and there were 26 male patients and 14 female patients. The tumor size was more than or equal to 5 cm in 19 cases, 5–10 cm in 13 cases, and more than 10 cm in eight cases. There were no statistically significant correlations detected between TWIST1 or CD44 and these clinical parameters. However, these results were not in line with Guo-Qing *et al*. [17] who demonstrated significant correlation between TWIST1 and increasing age of the patient or tumor size. Moreover, Nosrati *et al*. [18] found that there was a significant correlation of CD44 only with increased tumor size but no statistically significant association between CD44 expression and patient age.

Among 40 cases of GC, a positive expression of TWIST1 was observed in 18 (45%) of 40 cases. However, the normal gastric epithelial cells showed negative expression of TWIST1 in contrast to CD44, which was detected in 25 (62.5%) of 40 cases of human

Figure 2



Immunohistochemical staining for CD44 in (a) noncancerous tissues (negative control) and (b) gastric cancer lesions intestinal type. CD44-positive expression was prominent in tumor tissue and seemed mostly to localize in the membrane and cytoplasm of cancer cells (×100). (c) Gastric cancer lesions intestinal type CD44-positive expression was prominent in the membrane and cytoplasm of cancer cells (×400). (d) CD44 was highly expressed in poorly differentiated adenocarcinoma (×100). (e) CD44 was highly expressed in Signet-ring cell carcinoma (×400). (f) CD44 was highly expressed in gastric carcinoma with lymphovascular invasion (×100).

Table 3 Relation between CD44 and TWIST1 expression in gastric carcinomas

	TWIST1 (*n* (%)]		χ^2 test	
CD44	Negative	Positive	χ^2	*P* value
Negative	15 (68.2)	0 (0.0)	19.636	0.000
Positive	7 (31.8)	18 (100.0)		

GC. However, it showed negative expression in the normal gastric epithelial cells.

The present study revealed the statistically significant correlation between TWIST1 and CD44 expression and histopathological types of GCs, with six of 17 cases of intestinal type tumors expressing CD44 and 19 of 23 cases of diffuse-type tumors revealing high expression. In contrast to TWIST1 expression, which was positive in four of 17 intestinal-type cases and in 14 of 23 diffuse cases. Regarding tumor grade, of 14 well/moderate differentiated grades, there were nine cases and 11 cases negative for CD44 and TWIST1, respectively, whereas the 26 poorly differentiated grades revealed negative expression in six and 11 cases and positive expression in 20 and 15 cases for CD44 and TWIST1, respectively. These results were in line with those of Rosivatz *et al.* [19] who demonstrated up-regulation of TWIST1 in a diffuse-type GC in contrast to a well-differentiated intestinal carcinoma and another result of Guo-Qing *et al.* [17] which approved positive correlation of TWIST1 with tumor differentiation. Studies by Chen *et al.* [4] found an association between high CD44 expression and clinicopathological characteristics, indicative of increased malignant potential, such as gross type and tumor differentiation. However Nosrati *et al.* [18] and Dhingra *et al.* [20] found that the expression of CD44 in intestinal-type GC was significantly higher than the diffuse type and was not correlated with other pathologic and clinical parameters.

In the current work, the expression pattern of TWIST1 in GC and its association with lymphovascular invasion and TNM staging of the tumor have been carefully investigated. The results demonstrated that positive TWIST1 expression was significantly correlated with depth of invasion, lymph node and distant metastases, and TNM stage. This is in concordance with previous studies by Xing-Hui *et al.* [5] and Guo-Qing *et al.* [17], who demonstrated a significant correlation between TWIST1 expression and lymph node, distant metastases, and TNM stage.

The result suggested that TWIST1 overexpression is likely to play a role in the EMT process, progression, and metastasis of GC. In the process of tumor metastasis, cancer cells lose their polarity and intercellular adhesions and acquire the phenotype and invasive characteristics of mesenchymal cells

through EMT to achieve the infiltration of surrounding tissue [6].

The mechanism by which TWIST1 regulated is still in debate [21]. The findings of Yang *et al*. [22] have shown that TWIST1 could play as the inducer in the EMT by binding DNA with similar E-box sequence motifs. Other reports by Watanabe *et al*. [23] indicate that TWIST1 is regulated by Wnt/β-catenin signaling in mouse mammary cell differentiation; this effect could contribute to mammary carcinogenesis.Hsu *et al*. [24] found that the activation of the NOTCH1/STAT3/TWIST1 axis promotes colony formation, migration, and invasion of GC cells *in vitro* and tumor growth metastasis *in vivo*. Interestingly, Fas-ligand, an important regulator of cell apoptosis, exhibits mesenchymal-inducing properties in gastrointestinal (GI) cancer cells. Fas signaling down-regulates epithelial marker, up-regulates mesenchymal marker, and promotes GI cancer cell motility, which is blocked by knockdown of TWIST1 expression. In-vivo studies demonstrate that Fas-ligand expression increases whereas E-cadherin decreases during GI cancer progression, further confirming the role of Fas signaling in EMT and metastasis in GI cancers [25]

On the basis of our results, the CD44 expression was positively correlated with lymphovascular invasion, depth of invasion, nodal metastasis, and TNM staging. However, these findings were not in line with those of Nosrati *et al*. [18] who demonstrated an inverse relationship with the extent of invasion in a way that the highest expression was in tumors without invasion. Another study by Kim *et al*. [26] found that the expression of CD44 was shown to be related to tumor size and stage, vascular invasion, lymph node metastases, and perineural invasion as in the current study. Moreover, the study by Min *et al*. [27] on CD44 found a significant association with certain clinicopathological features, such as the T category, the N category, distant metastasis, lymphatic invasion, and TNM stage. These discrepancies may be explained by the existence of numerous CD44 isoforms, which may have considerable homology in their antigenic instructions, thus increasing the possibility of cross-reactivity between the antibodies. Another reason is probably using different techniques.

To the best of our knowledge, significant direct relationships between TWIST1 and CD44 expression in GC clinicopathologic parameters have been elucidated, which may be explained by the fact that the EMT not only gives epithelial cells the mesenchymal cells characteristics but also these cells dedifferentiate and acquire stem cell phenotypes such as invasion, metastasis, and apoptosis.

Conclusion

In conclusion, the application of TWIST1 and CD44 as a routine immunohistochemistry method in GC tissue can be used as diagnostic markers for GC, and their upregulation can be regarded as a marker of poor prognosis in patients with GC. The EMT regulation can provide potential targets for GC therapy. Furthermore, assessment of CD44 expression in the future may guide the clinician in delineating targeted strategies for therapy in patients with GC. However, a future larger scale study may be needed to verify our results.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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