

Original Article

Clinicopathologic significance of putative stem cell markers, CD44 and nestin, in gastric adenocarcinoma

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Abstract: Cancer stem cells (CSC) are unique subpopulations that have the capacity to drive malignant progression and mediate radio/chemoresistance. The role of nestin as a CSC marker in gastric adenocarcinoma is largely unknown. Our objective was to evaluate immunoeexpression of CSC markers CD44 and nestin in gastric adenocarcinoma versus non-neoplastic gastric mucosae (NNGM) and correlate it with various clinicopathologic factors. Tissue microarray blocks from 174 cases of gastric adenocarcinoma and 41 samples of adjacent NNGM were assembled. Clinical data including patient's age and sex, tumor histologic subtype and grade, and disease stage were obtained. Expression of CD44 and nestin was assessed by immunohistochemistry. Expression of membranous CD44 (51%, 78/152) and cytoplasmic nestin (25%, 43/174) was significantly greater in gastric adenocarcinoma than in NNGM ($P<0.001$). A subset of cases ($n=15$) that co-expressed membranous CD44 and cytoplasmic nestin were significantly more frequent in Lauren intestinal histologic subtype than in diffuse subtype ($P<0.05$). Foci of intestinal metaplasia ($n=6$) showed either CD44 (3/6) or nestin (2/6) expression. This is the first study to report the clinicopathologic significance of nestin expression in gastric cancers, and to correlate the nestin expression with CD44, another stem cell marker. The study shows that nestin and CD44, are significantly expressed in a subset of gastric adenocarcinoma, particularly co-expression of nestin and CD44 is significantly revealed in Lauren intestinal histologic subtype. Their expression is also increased in intestinal metaplasia, a premalignant lesion. These findings suggest that CSCs may have a pathogenic role in the pathway of intestinal metaplasia-intestinal type gastric adenocarcinoma.

Keywords: Stem cell markers, CD44, Nestin, gastric adenocarcinoma

Introduction

Gastric adenocarcinoma is the fourth most common cancer worldwide and the second leading cause of cancer-related death [1]. Although current practice incorporates chemotherapy and/or radiation into treatment protocols, surgical resection is the mainstay of treatment [2]. The overall 5-year survival rate is low (15% to 35%) because of the high recurrence rates, nodal metastasis and the short-lived response to chemotherapy [3].

Gastric carcinogenesis is a multifactorial and multistep process leading to accumulation of multiple genetic and epigenetic alterations. Recently, interest in gastric cancer stem cells

(CSCs) has arisen in the broader context of the CSC hypothesis for solid tumors [4-6]. In early 2006, a working group of the American Association for Cancer Research, operationally defined a CSC as a malignant cell within a tumor that has the ability to self-renew and to give rise to the heterogeneous cancer cell lineages [7]. CSCs fuel tumor growth and confer resistance to current chemotherapy and radiotherapy regimens. Therefore, CSCs are promising new targets for novel therapeutic agents [8].

Studies relying on differential expression of surface markers such as CD133, CD44, CD24 and nestin have identified CSC subpopulations that self-renew and produce phenotypically distinct non-tumorigenic carcinoma cells in various solid

tumors [9-11]. Among these markers CD133, CD44 and nestin are widely used as signatures for CSCs.

CD44 is a transmembrane glycoprotein that participates in many cellular processes, including growth, survival, differentiation, and motility [12]. It is a unique adhesion molecule that plays a role in cancer cell migration and matrix adhesion in response to cellular microenvironment, thus enhancing cellular aggregation and tumor cell growth [13]. The CD44 as a putative stem cell marker has been used to isolate breast CSCs [9], prostate CSCs [14, 15], pancreatic CSCs [16], and colorectal CSCs [17]. A recent study by Du et al reported CD44 to be a more robust marker of CSC, as compared to CD133, in colorectal cancer [18].

Nestin, an intermediate filament protein, is expressed primarily in mammalian nervous tissue during embryonic development [19]. In adults, nestin occurs only in a small subset of tissues under reactive conditions, such as the glial scar that forms after injury to the central nervous system [20], regeneration of injured skeletal muscle tissue [21] and central nervous system tumors [22]. Nestin is also considered a putative marker of CSCs and recent studies have shown that it is also expressed in epithelial tumors, such as pancreatic cancer [23], prostate cancer [24] and breast cancer [25].

The objective of this study was to evaluate the expression of putative stem cell markers, CD44 and nestin, in gastric adenocarcinomas and non-neoplastic gastric mucosae (NNGM).

Materials and methods

Tissue samples

After Institutional Review Board approval, total and partial gastrectomy specimens from 174 patients with gastric adenocarcinoma were selected from surgical pathology database of resection specimens received during years 1997 to 2006 at the University of Texas M.D. Anderson Cancer Center, Houston, TX. The control group (n=41) comprised of NNGM obtained randomly from these 174 tumor cases. None of the patients had received neoadjuvant therapy. The patient charts were reviewed for clinical parameters including patient age and sex, tumor Lauren classification subtype and histological

grade, disease stage, and survival. Tissues representative of each tumor (case) and the NNGM (controls) were fixed in 10% neutral buffered formalin and embedded in paraffin.

Histologic examination and TMA construction

Hematoxylin and eosin (H&E)-stained slides were reviewed for confirmation of histopathologic diagnoses and selection of adequate specimens for analysis. High-density TMAs were assembled using a Beecher tissue puncher/array system (Beecher Instruments, Silver Spring, MD, USA). Specimens were retrieved from selected regions of the donor paraffin block and are precisely arrayed in a new recipient block. Tissue cores were 1.0 mm in diameter and ranged in length from 1.0 to 3.0 mm depending on the depth of tissue available in the donor block. For each case, two to three core samples of normal and tumor tissue were acquired from two different donor blocks resulting in 336 cores. Cores were assembled in three high-density TMA blocks. One arrayed core contained up to 3200 neoplastic cells.

Immunohistochemistry and scoring

TMA sections were cut at 4µm, then were deparaffinized and rehydrated in a graded series of alcohols. Heat-induced epitope retrieval was performed. The sections were treated with 3% H₂O₂ and rinsed with Tris-buffered saline (TBS/Tween-20). A few drops of diluted normal blocking serum were placed on the tissues, which were incubated at room temperature. The serum was then washed off and the sections were incubated with the following primary antibodies: CD44 (Mouse monoclonal; clone DF1485; 1/100 dilution; Novocastra Ltd, Newcastle upon Tyne, UK) or nestin (Monoclonal mouse; dilution 1/400; Abcam, Cambridge, MA). The incubation was 30 minutes for both CD44 and nestin. The remainder of the immunostaining procedure was carried out on a DAKO autostainer programmed to incubate each slide with diluted biotinylated secondary antibody for 30 minutes. The slides were then rinsed and incubated with DAB (3', 3'-diamino-benzidine chromogen solution, DAKO Envision+ System kit) for 10 minutes. The slides were rinsed again and counterstained with Gill II hematoxylin, treated with xylene and coverslipped. Positive and negative controls run concurrently were noted to react appropriately.

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The slides were reviewed by two pathologists. Cases with membranous immunoexpression of CD44 or cytoplasmic immunoexpression of nestin, were considered positives. The positive cases were further scored semi-quantitatively. The chromogenic signal intensity, for both CD44 and nestin, was graded on a scale of 1+ (weak) to 3+ (strong). The percentage of CD44 and nestin positive cells in each case, was estimated on a scale of 1% to 100% in each of the three cores for that case, and an average percent positivity for each marker was then calculated for that case. A composite score (CS) was then calculated using these data as follows: CS= signal intensity x percent positive cells.

For statistical analysis chi-square test was used to compare CD44 and nestin expression in tumor and NNGM. The z-test was used to compare the difference in frequency of CD44 and nestin co-expression between Lauren histologic subtypes. A P-value <0.05 was considered to be significant for both tests. Survival curves were estimated using the Kaplan-Meier product-limit method and the significance of differences between survival curves was determined using a log-rank test where the P value for the χ^2 statistics was determined.

Results

Clinicopathologic data on the study group are summarized in **Table 1**. The group comprised 174 cases of gastric adenocarcinoma. The age range of patients was 29 to 89 years, with a mean of 63 years. The male to female ratio was 2:1. Data pertaining to Lauren classification were available for 171 cases of which 113 cases were Lauren intestinal subtype and 58 cases were Lauren diffuse subtype. Both Lauren subtypes showed a male predominance with a male to female ratio of 2:1. Among patients with the Lauren intestinal subtype, 53% (60/113) were aged \geq 65 years or older, while a majority (75%) of those with the Lauren diffuse subtype was younger than 65 years. Data on histological grade were available in 170 cases. A large majority (97%) of the cases were of higher histological grade (grade 2 or grade 3). Clinical information about disease stage was available for 172 cases. All stages were well represented in the study group.

Immunoexpression of CD44 and nestin in human gastric adenocarcinoma with respect to

Table 1. Clinicopathologic data on 174 patients with gastric adenocarcinoma

Characteristic	N (No. of cases)
Age in years, Mean (range)	63(29-89)
Gender	
Male	116
Female	58
Lauren classification subtype	171
Intestinal	113
Diffuse	58
Histological grade	170
Well differentiated (G1)	5
Moderately differentiated (G2)	73
Poorly differentiated (G3)	92
Stage grouping	172
I	47
II	43
III	66
IV	16
Median Survival (since diagnosis)	
CD44 positive cases	44 months
CD44 negative cases	20 months
Nestin positive cases	21 months
Nestin negative cases	29 months
	128

non-neoplastic gastric mucosa, Lauren's classification subtype, histological grade and tumor stage is presented in **Table 2**. Tissue sections for optimal assessment of CD44 expression were available for 152 of the 174 cases. The suboptimal sections did not have sufficient tumor tissue due to missing or partial tissue cores with too few tumor cells. However, nestin expression was analyzed optimally in all 174 cases. The cellular immunolocalization of CD44 was predominantly membranous and that of nestin was cytoplasmic (**Figures 1** and **2**). Nestin expression was also observed in endothelial cells in normal gastric mucosa and in tumors, which served as positive internal controls (**Figure 2**).

The positive expression rates of CD44 (78/152, 51%) and nestin (43/174, 25%) in gastric adenocarcinoma tissues were significantly higher than those in NNGM (P<0.001). A majority of the cases positive for these markers (CD44 65%; nestin 72%) showed high expression intensity and large expression area, as indicated by CS \geq 50 (**Figures 1B, 1D, 2B, 2D**). Of the 41 NNGM, six (15%) showed CD44 expression and two (5%) showed mild (1+) focal nestin expression. Six of the NNGM specimen had foci of intestinal metaplasia. These foci showed membranous CD44 expression in three cases and cytoplasmic nestin expression in two cases

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Table 2. Expression of CD44 and nestin in human gastric adenocarcinoma with respect to non-neoplastic gastric mucosa, Lauren's classification subtype, histological grade and tumor stage

Pathologic variables		CD44			Nestin		
		Total cases	Positive cases (%)	<i>p</i> -value	Total cases	Positive cases (%)	<i>p</i> -value
GAC		152	78(51)	<0.001	174	43(25)	<0.001
NNGM		41	6(15)		41	2(5)	
Histologic subtype	Intestinal	92	51(55)	0.13(NS)*	107	28(26)	NS*
	Diffuse	45	18(40)		51	13(25)	
Histologic Grade	Well differentiated (G1)	5	3(60)	NS*	5	0(0)	NS*
	Moderately differentiated (G2)	62	32(52)		69	18(26)	
	Poorly differentiated (G3)	69	34(49)		83	23(28)	
Tumor Stage	Stage I	40	18(45)	NS*	41	10(24)	NS*
	Stage II	32	15(47)		38	10(24)	
	Stage III	51	26(51)		64	16(25)	
	Stage IV	15	10(67)		16	5(31)	

*Not Significant

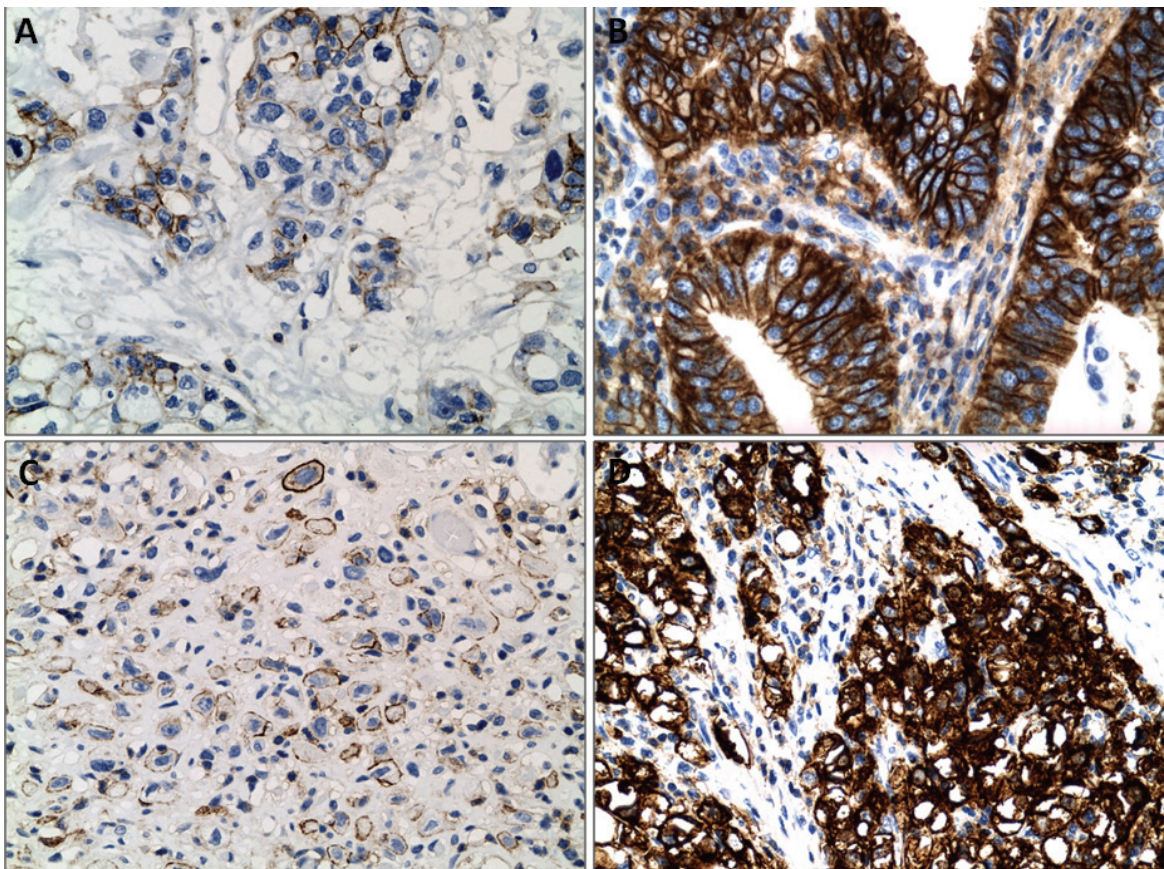


Figure 1. Membranous CD44 expression (A) Gastric adenocarcinoma, intestinal subtype, CS of immunopositivity <50 (B) Gastric adenocarcinoma, intestinal subtype, CS >50 (C) Gastric adenocarcinoma, diffuse subtype, CS <50 (D) Gastric adenocarcinoma, diffuse subtype, CS >50. Immunoperoxidase, $\times 400$ (A,B,C,D).

(data not shown). Two of these CD44- positive foci showed high expression, with CS ≥ 50 .

Pathologic information concerning Lauren classification of gastric adenocarcinoma was avail-

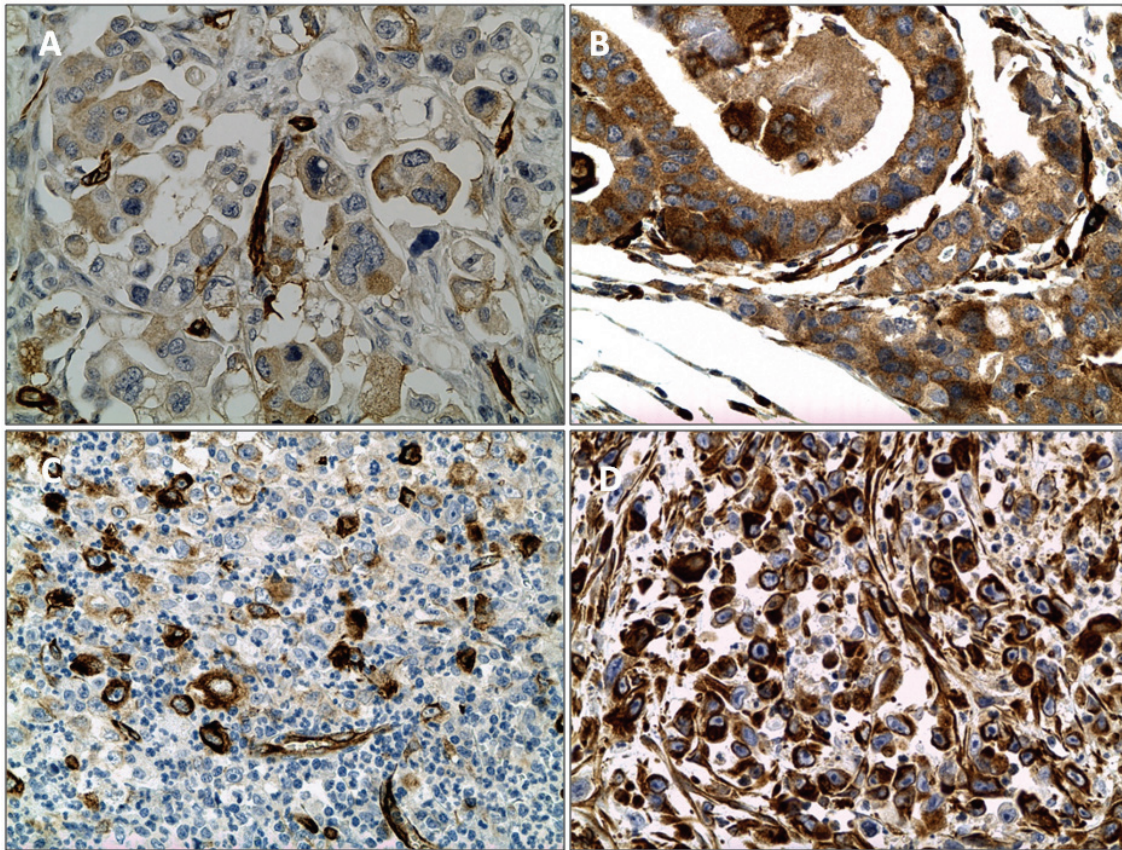


Figure 2. Cytoplasmic nestin expression (A).Gastric adenocarcinoma, intestinal subtype, CS of immunoexpression<50 (B) Gastric adenocarcinoma, intestinal subtype, CS>50 (C) Gastric adenocarcinoma, diffuse subtype, CS<50 (D) Gastric adenocarcinoma, diffuse subtype, CS>50 (E). Immunoperoxidase, × 400 (A,B,C,D).

able in 137 cases evaluated for CD44 expression and in 158 cases analyzed for nestin expression. There was a trend towards increased CD44 positivity in the intestinal subtype as compared to the diffuse subtype ($P=0.13$) but this trend did not reach statistical significance. No difference in nestin expression between Lauren subtypes was observed. **Figures 1** and **2** show CD44 and nestin expression in Lauren histologic subtypes. **Figures 1A** and **1C** show CD44 expression with CS<50, **Figures 1B** and **1D** show CD44 expression with CS>50, and in intestinal and diffuse subtype. **Figures 2A** and **2C** show nestin expression with CS<50 in the Lauren histologic subtypes and **Figures 2B** and **2D** show nestin with CS>50.

Pathologic data on histological grade were available in 136 cases evaluated for CD44 and 157 cases evaluated for nestin. The study group included very few ($n=5$) well-differentiated (G1)

tumors as compared to moderate (G2, $n=62$) and poorly differentiated (G3, $n=69$) tumors. No significant difference in expression of either CD44 or nestin was observed between histological grades G2 and G3.

Clinico-pathologic information on stage was available for 138 cases analyzed for CD44 and in 159 cases analyzed for nestin. All stages showed proportionately similar rates of expression of CD44 and nestin. No significant difference in expression of either protein was observed amongst different stage groups.

Table 3 highlights clinico-pathologic data in the subset of cases with CD44 and nestin co-expression. Of the 17 cases that co-expressed these two markers, data on Lauren classification, histological grade and stage were available for 15. Co-expression occurred significantly more frequently in the intestinal subtype than in

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Table 3. Clinicopathologic distribution of cases with co-expression of CD44 and Nestin

Groups	N*** (%)	P-value
Lauren classification subtype	15	
Intestinal	11(73)	*0.031
Diffuse	4(27)	
Histological grade	15	
Well differentiated (G1)	0	**NS
Moderately differentiated (G2)	7(47)	
Poorly differentiated (G3)	8(53)	
Disease Stage	15	
Stage I	3(20)	**NS
Stage II	2(13)	
Stage III	7(47)	
Stage IV	3(20)	
Median survival (since diagnosis)		
57 months	15	

*P-value significant <0.05, **NS, Not significant, ***N= number of cases

diffuse subtype (P-value=0.031). No correlation was observed with histological grade or stage.

No statistically significant differences in survival were seen between subgroups of GAC cases categorized based on CD44 and/or nestin stem cell marker expression (data not shown). The median survival, assessed from date of diagnosis, was longer (44 months) for CD44 positive cases as compared to CD44 negative (20 months) cases. For the nestin positive group, the median survival was less (21 months) as compared to nestin negative (29 months) group (p-value=0.1655). The median survival for nestin+/CD44+ group was 57 months.

Discussion

Gastric adenocarcinoma represents roughly 2% of all new cancer cases yearly in the United States. It is widely prevalent in many parts of the world outside of the United States such as East Asia and South America. Noncardia gastric cancer is classified according to the Lauren system as the intestinal or the diffuse subtype. The intestinal subtype usually occurs at a later age and shows a male predominance, whereas the diffuse subtype tends to occur at a younger age and affects men and women equally [5]. We observed similar trends in this study cohort except that both subtypes showed a male predominance. Male predominance in diffuse subtype in our study group could be due to small patient numbers.

Our results show significant overexpression of CD44 (51%) and nestin (25%) in tumors as compared to NNGM (CD44 15%, nestin 5%). Furthermore, we showed that some foci of intestinal metaplasia expressed nestin and CD44. There is a statistically significant higher frequency of intestinal subtype of tumor cases that co-expressed CD44 and nestin compared to diffuse subtype of tumor cases that co-expressed CD44 and nestin. We also observed a higher frequency of CD44 expression in the intestinal subtype than in the diffuse subtype, but this trend is statistically insignificant (P-value=0.13). We observed no correlations in this cohort between CD44 or nestin expression and other clinicopathologic parameters such as histological grade, disease stage or prognosis in terms of survival. To reduce the chances of sampling error due to smaller tissue used in

TMA, 3 core samples per case were acquired in current study, and a needle core diameter of 1 mm rather than the conventional 0.6 mm was used to improve tissue representation. Nevertheless, relatively consistent profiling of nestin and CD44 immunoreactivity patterns in gastric cancers has been shown regardless of tumor types. In addition, it has been demonstrated in our earlier work that even focally expressed proteins, such as Cables, may be assessed using TMAs if a large number of samples are evaluated [26]. Thus we are confident that the sample cores used in the TMAs in our study are a reasonable representation of the tumors.

Several experimental studies on mouse models have shown that CSCs play a major role in initiating and sustaining the gastric cancers, as they do in other gastrointestinal epithelial tumors [1, 5, 6, 27, 28]. In a recent study, Takaishi et al identified gastric cancer-initiating cells from a panel of human gastric cancer cell lines using cell surface marker CD44 [1]. Similarly Ishimoto et al discovered a population of CD44-expressing stem cell-like slow-cycling cells in gastric glands at the squamo-columnar junction in normal mice. They concluded that these stem cell-like slow-cycling cells may contribute to the development of lethal gastric tumors [28]. Few reported studies have used immunohistochemical techniques to evaluate the clinicopathologic significance of CSCs in human gastric adenocarcinoma [29-34]. These reports include use of CD133 and CD44 (including standard and vari-

ant isoforms) as CSC markers. To the best of our knowledge, expression of nestin as a stem cell marker in gastric adenocarcinoma and its clinicopathologic significance has never been reported in the literature.

CD44 is a member of widely expressed family of adhesion receptors. The CD44 gene is located on chromosome 11p12-13, occupies 20 exons and has many isoforms. The standard isoform (CD44s) has at least 10 of the 20 exons and predominates in normal tissues. Post-translational modification such as alternative splicing gives rise to many variant isoforms, CD44v, identified by specific exons such as CD44v6 or CD44v8-10 [35]. Some studies on gastric cancer have evaluated CD44s [29] and some have used the CD44v6 isoform [32, 33] whereas others have used both CD44s and CD44v6 [30, 34]. The antibodies used in this study detect the CD44s form which lacks all variant exons and is widely expressed in lymphoid and non-lymphoid tissues.

Ghaffarzadehgan et al evaluated 100 cases of gastric adenocarcinoma and found CD44s expression in 65% cases [29]. Kim et al studied CD44 expression, both CD44s and CD44v6 in 211 cases of gastric adenocarcinoma; they found 11.4% positivity for CD44s but did not detect CD44v6 protein expression in any of their cases [30]. Yamaguchi et al reported a 47.3% positivity for the CD44v6 variant in a cohort of 95 patients [33]. In our study of 174 cases, the positivity rate was 51% positivity for CD44 expression and 25% for nestin expression. This wide range of reported frequencies of CD44 expression (11.4% to 65%) could be a reflection of geographic variation and different sensitivities of utilized antibodies, which targeted various isoforms of CD44 in identifying CSCs.

We observed CD44 and nestin expression in a small percentage of NNGM specimens. This contrasts with other studies that have reported no CD44 expression in NNGM [29, 32]. Of the six cases that showed foci of intestinal metaplasia (IM) three foci showed CD44 expression and two showed nestin expression. Two of the three cases with CD44 positivity and both cases with nestin positivity also showed CD44 and nestin co-expression in tumor tissue. Since intestinal metaplasia is thought to be a precursor of gastric adenocarcinoma, the expression of stem

cell markers indicates an epigenetic change at this level and supports the role of stem cells in progression of the Correa pathway in gastric tumors of intestinal subtype. Dammrich et al reported CD44v6 expression in intestinal metaplasia in cases of chronic atrophic gastritis and gastric adenoma with dysplastic changes [32].

The non-significant trend in our data (P-value=0.13) towards a higher frequency of CD44 expression in Lauren intestinal subtype than in the diffuse subtype and the increase of statistical strength (P<0.05) of this observation when we evaluated cases with co-expression of CD44 and nestin, is consistent with previous reports in the literature of significantly higher expression of CD44 (CD44s or CD44v6) in the intestinal subtype. This finding emphasizes the need to subclassify tumors by Lauren classification when evaluating the clinical significance of CD44 expression in gastric cancers [29, 32-34].

This study found no correlation of CD44 immunorexpression with tumor histological grade, tumor stage or prognosis in terms of survival. Kim et al evaluated CD44 immunorexpression (along with NFκB and osteopontin expression) in 211 cases of gastric carcinoma and correlated the expression with tumor size, pathologic stage, patient survival, angioinvasion, perineural invasion and lymph node metastasis [30]. Similar to our study they reported no significant clinicopathologic correlation with CD44 expression. However, Ghaffarzadehgan et al reported significant correlation between CD44 expression and histological grade and patient survival [29]. Other studies have reported a correlation between CD44 expression and metastatic spread as well as survival [29, 32, 33].

We also looked at clinicopathologic correlation of nestin expression in gastric adenocarcinomas and found no significant correlation with Lauren classification subtype, histological grade, tumor stage or survival.

CD44, besides being a surrogate marker for CSCs is also recognized as a cell adhesion molecule. To better assess the specificity of CD44 as a CSC marker, we further evaluated the 17 cases that co-expressed nestin and CD44. In the 15 cases for which clinicopathologic information was available, co-expression of both CD44 and nestin was significantly more frequent in the Lauren intestinal

subtype cases than in the diffuse subtype, as discussed earlier, while no correlation with any other clinico-pathologic parameter was found. This finding underscores the importance of using Lauren classification when evaluating expression of stem cell markers in gastric cancers.

To summarize, this is the first study to report the clinicopathologic significance of nestin expression in gastric cancers, and to correlate nestin expression with CD44, another putative stem cell marker. The study shows that nestin and CD44, are significantly expressed in a subset of gastric adenocarcinoma, particularly co-expression of nestin and CD44 is significantly revealed in Lauren intestinal histological subtype. Their expression is also increased in intestinal metaplasia, a premalignant lesion. These findings suggest that CSCs may have a pathogenetic role in the pathway of intestinal metaplasia-intestinal type gastric adenocarcinoma. Development of therapies targeted towards CSCs may help to improve the management of gastric cancers. In this era of personalized medicine, readily convenient detection of CSCs by immunohistochemistry in formalin-fixed paraffin-embedded tissues may potentially help to select the subset of patients to enroll clinical trials which may eventually lead to specific targeted therapies for the subgroup of patients.

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