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Original Article

Expression of CD44v6 in gastric cancer and its significance

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Abstract: Aims: This study aimed at investigating the expression and clinicopathological significance of cellular adhesion molecule CD44v6 in gastric cancer (GC), with the hope to facilitate early GC diagnosis and prognosis assessment. Methods: A total of 60 GC resection specimens were collected in our hospital in 2013. The enrolled GC patients were diagnosed pathologically, and did not receive any preoperative anti-tumor therapy. We also collected 14 normal gastric mucosa and 21 precancerous lesions as controls. We investigated CD44v6 expression in all specimens using immunohistochemical staining, studied the positive expression rates in normal gastric mucosa, precancerous lesion, para-cancerous tissue and GC, and analyzed the data using SPSS 20.0, comparing differences between groups using χ^2 test. Results: Positive CD44v6 expression was indicated by brown granules in cytoplasm and/or on cellular membrane. The expression rates were 7.1% in normal gastric mucosa, 23.8% in precancerous lesion, and 76.7% in GC ($P < 0.05$). In para-cancerous tissues, the expression rates were 18.3% and 3.3% for the para-cancerous sites 1 cm and 2 cm from the tumor site, respectively ($P < 0.05$). Based on histological classification, in well, moderately, and poorly differentiated adenocarcinomas the expression rates were 40.0%, 76.0%, and 83.3%, respectively ($P < 0.05$). The expression rates of pT1, pT2, and pT3-4 tumors were 62.5%, 65.0%, and 87.5%, respectively ($P < 0.05$). The expression rates were 82.4% for patients with lymphatic metastasis, and 44.4% for individuals without lymphatic metastasis ($P < 0.05$). For patients with and without distant metastasis, the expression rates were 100.0% and 75.0%, respectively. Stratified by tumor pTNM classification, the expression rates were 55.6% in stage I tumor, 68.8% in stage II tumor, 83.9% in stage III tumor, and 100.0% in stage IV tumor ($P < 0.05$). Conclusions: CD44v6 expression was closely correlated with GC differentiation degree, invasion depth, lymphatic metastasis, and pTNM classification. It might facilitate tumor cell invasion, playing a key role in GC progression. Its detection could assist early GC detection and surgical efficacy assessment.

Keywords: CD44v6, gastric cancer, para-cancerous tissue, precancerous lesion, invasion, metastasis

Introduction

Gastric cancer (GC) is one of the most common and lethal malignancies worldwide, and its incidence is increasing yearly [1]. How to increase early detection and treatment rates of GC thus improving prognosis remains a very intractable issue [2, 3]. Tumor growth, invasion, and metastasis are closely dependent on the function of cellular adhesion molecules (CAMs), among which CD44 is involved in specific intercellular adhesion [4]. CD44v is a transmembrane glycoprotein molecule in the CD44 family, and is a variant protein encoded by the *CD44* gene which contains the v zone exon [5]. Researches have shown that CD44 is significantly associated with malignant genesis, progression, invasion, metastasis, and chemo-resistance [6, 7], and that the variant CD44v, which is rarely expressed in normal tissues, is obviously upregulated in various tumors including colorectal cancer, breast cancer, *etc.* [8, 9]. To the best of our knowledge, CD44v6, a tumor stem cell surface marker, has been rarely studied in GC. Herein this study examined the expression of CD44v6 in normal gastric mucosa, precancerous lesion, and GC tissue using immunohistochemistry (IHC) staining, and investigated its correlation with GC genesis, invasion, and metastasis, with the hope to reveal CD44v6 as

Table 1. General clinicopathological features of enrolled gastric cancer patients

Item	Value
Gender (male/female)	41/19
Age (median, range [years])	61, 31-77
Disease stage (early/advanced)	8/52
Histological differentiation degree (well/moderately/poorly)	5/25/30
pT stage (T1/T2/T3-4)	8/20/32
pN stage (N0/N1-3)	9/51
pM stage (M0/M1)	56/4
pNM classification (I/II/III/IV)	9/16/31/4

a novel early diagnostic maker, anti-GC therapeutic target, and prognosticator.

Methods and materials

Patients and specimens

A total of 60 GC specimens (45 cases of type II/III adenocarcinomas of esophagogastric junction (AEG) [10, 11], 8 adenocarcinomas of gastric body, and 7 adenocarcinomas of gastric antrum) from patients enrolled in our department in 2013 were collected. The patients with surgical gastric resection did not receive any preoperational chemoradiotherapy, and were pathologically diagnosed as GC. For each specimen, we obtained the malignant lesion and para-cancerous tissues (1 cm and 2 cm from the tumor site), respectively. The detailed clinicopathological information and surgical report for each patient were also retrieved. Tumors were categorized according to the 7th TNM classification system proposed by the American Joint Committee on Cancer (AJCC) [12, 13]. The general clinicopathological characteristics of the selected GC patients are shown in **Table 1**. Besides, we collected 14 cases of normal gastric mucosa and 21 precancerous tissues from the gastroscopy room as comparisons. Written informed consent was obtained from each investigated individual, and our study was permitted by the Institutional Review Board of the First Affiliated Hospital of Anhui Medical University and carried out according to the Declaration of Helsinki [14] and Good Clinical Practice guidelines [15].

Reagents

The mouse-anti-human CD44v6 monoclonal antibody working solution, streptavidin-perosidase (SP) ultra-sensitive ready-to-use reagent box, and diaminobenzidine (DAB) enzyme substrate coloration kit were all purchased from the Maixin Biotechnology Development Company (Fuzhou, China).

IHC

We investigated CD44v6 expression in all specimens using IHC staining as previously described [11], studying the positive expression rates in normal gastric mucosae, precancerous lesion, para-cancerous tissue, and GC. In short, all specimens were fixed by 10% neutral formalin solution, embedded in paraffin, serially sectioned into 5 μm slices, gradient-dehydrated, and stained using the routine hematoxylin-eosin (HE) and IHCSP methods, respectively. The para-cancerous tissues 1 cm and 2 cm from the tumors were also collected, and underwent the same procedures as described above. The staining procedures were carried out strictly according to the kit instructions. The known positive tissue section was selected as the positive control, and the negative control was obtained by replacing the primary antibody by phosphate buffer saline (PBS).

Assessment criteria

Positive CD44v6 staining was illustrated by brown granules in cytoplasm and/or on cellular membrane. The results were stratified based on the proportion of positive-staining cells in contrast to tumor cells: negative (-), ≤ 5% positive cells; weakly positive (+), 6%-25% positive cells; moderately positive (++), 26%-50% positive cells; and strongly positive (+++), > 50% positive cells. Staining results were determined by 2 independent pathologists using the double blind procedure. In case of discrepancy, a third investigator was consulted and agreement was then reached by consensus.

Statistical analysis

We processed the experimental data with the IBM SPSS Statistics 20.0 software, comparing enumeration results using χ^2 test. A difference was statistically significant with $P < 0.05$, and very significant with $P < 0.01$.

Table 2. Expression of CD44v6 according to gastric lesion type, malignant degree, differentiation grade and pTNM stage

Category	Item	n	CD44v6 expression			Positive rate (%)	χ^2	*P*
			-	+	++			
Benign gastric lesion	Gastric polyp	10	9	1	0	10.0	1.975	0.373
	Chronic gastritis	6	4	2	0	33.3		
	Gastric ulcer	5	3	2	0	40.0		
Gastric disease	Precancerous lesion	21	16	5	0	23.8	23.263	< 0.0001
	Early gastric cancer	8	3	3	2	62.5		
	Advanced gastric cancer	52	11	14	27	78.8		
Para-cancerous tissue	2 cm to tumor	60	58	2	0	3.3	7.007	0.008
	1 cm to tumor	60	49	9	2	18.3		
Differentiation grade	Well	5	3	1	1	40.0	4.682	0.031
	Moderately	25	6	9	10	76.0		
	Poorly	30	5	7	18	83.3		
pT	T1	8	3	3	2	62.5	5.983	0.014
	T2	20	7	6	7	65.0		
	T3-4	32	4	8	20	87.5		
pN	N0	9	5	2	2	44.4	5.416	0.020
	N1-3	51	9	15	27	82.4		
pM	M0	56	14	16	26	75.0	1.611	0.204
	M1	4	0	1	3	100.0		
pTNM	I	9	4	2	3	55.6	7.708	0.006
	II	16	5	8	3	68.8		
	III	31	5	6	20	83.9		
	IV	4	0	1	3	100.0		

Results

Expression of CD44v6 in gastric tissues

In CD44v6 positive cells, the brown particles were mostly distributed on the cellular membrane and/or in the cytoplasm, and for the typical strongly-positive cells, the stained cell membranes were connected with each other, forming a net-like structure. In normal gastric mucosa, CD44v6 expression rate was 7.1% (1/14). There was no significance difference in CD44v6 expression among different types of benign gastric lesions ($P > 0.05$). The expression rates were 23.8% (5/21) in precancerous tissues, and 76.7% (46/60) in GC (early stage disease, 46.7%; advanced stage disease, 77.8%), indicating that CD44v6 was more highly expressed in cancerous tissues, especially in advanced GC ($P < 0.05$) (**Table 2** and **Figure 1**).

Expression of CD44v6 in para-cancerous tissues

Overall, the expression rates were 18.3% (11/60) and 3.3% (2/60) in para-cancerous tissues 1 cm and 2 cm from the malignant lesions, respectively ($P < 0.05$). Interestingly, in pT1 tumors, CD44v6 was negatively expressed in the para-cancerous tissues. The CD44v6 expressions in para-cancerous tissues were significantly lower than the corresponding tumor lesions ($P < 0.05$) (**Table 2** and **Figure 2**).

Correlation between CD44v6 expression and histological differentiation grade

CD44v6 expression rates were 40.0% (2/5), 76.0% (19/25), and 83.3% (25/30) in well, moderately, and poorly differentiated gastric adenocarcinomas, respectively, suggesting that the worse the tumor histopathological differentiation was, the higher positive expression rate the tumor had. The difference between groups was statistically significant ($P < 0.05$) (**Table 2**).

Correlation between CD44v6 expression and pathological pT, pN and pM stages

CD44v6 positive expression rates were 62.5% (5/8), 65.0% (13/20), and 87.5% (28/32) in

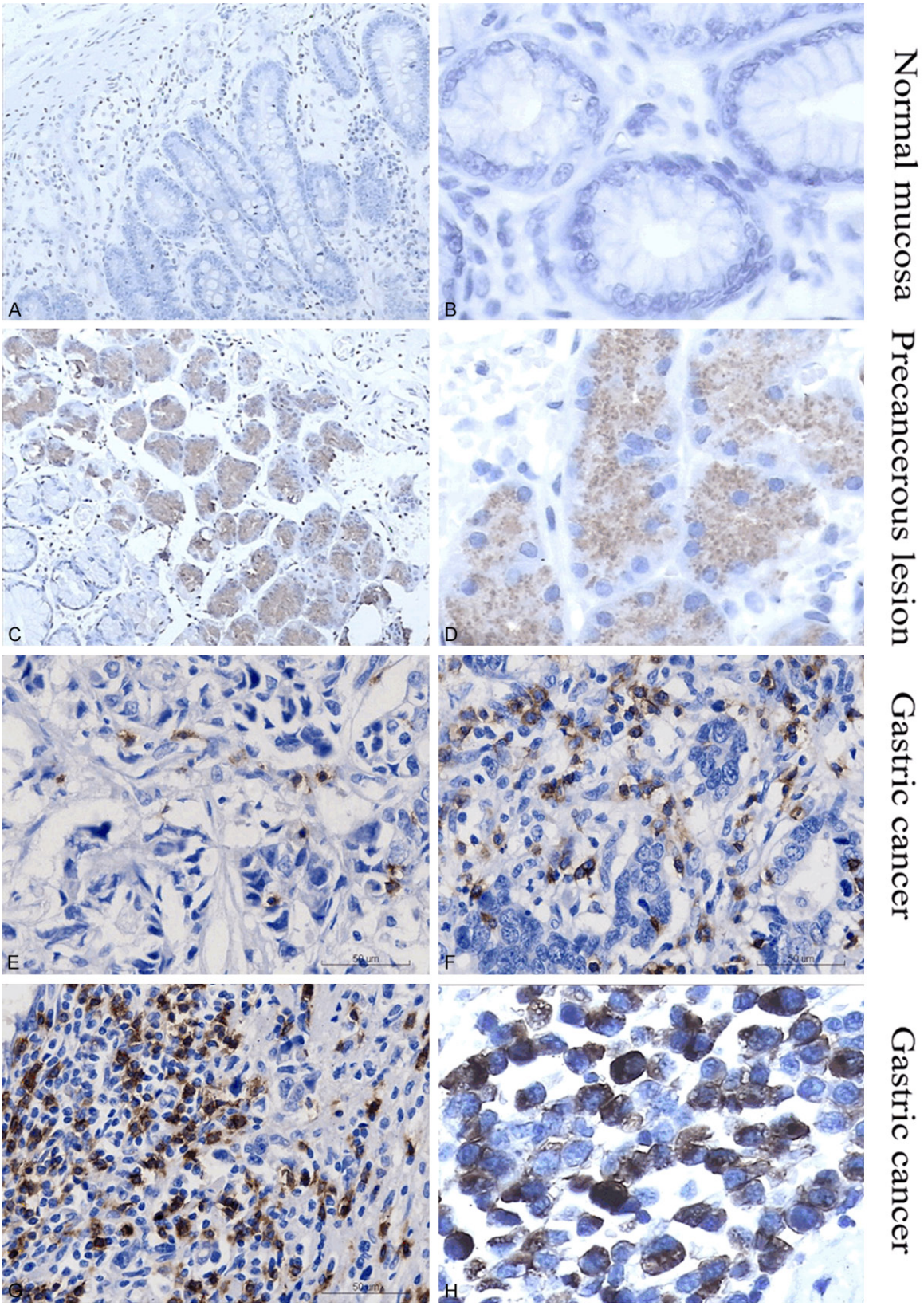

Figure 1. Expression of CD44v6 in normal gastric mucosa (negative; A: × 100; B: × 400), precancerous lesion (moderately positive; C: × 100; D: × 400), and gastric cancer (weakly positive: E: × 100; moderately positive: F: × 100; strongly positive: G: × 100 and H: × 400).

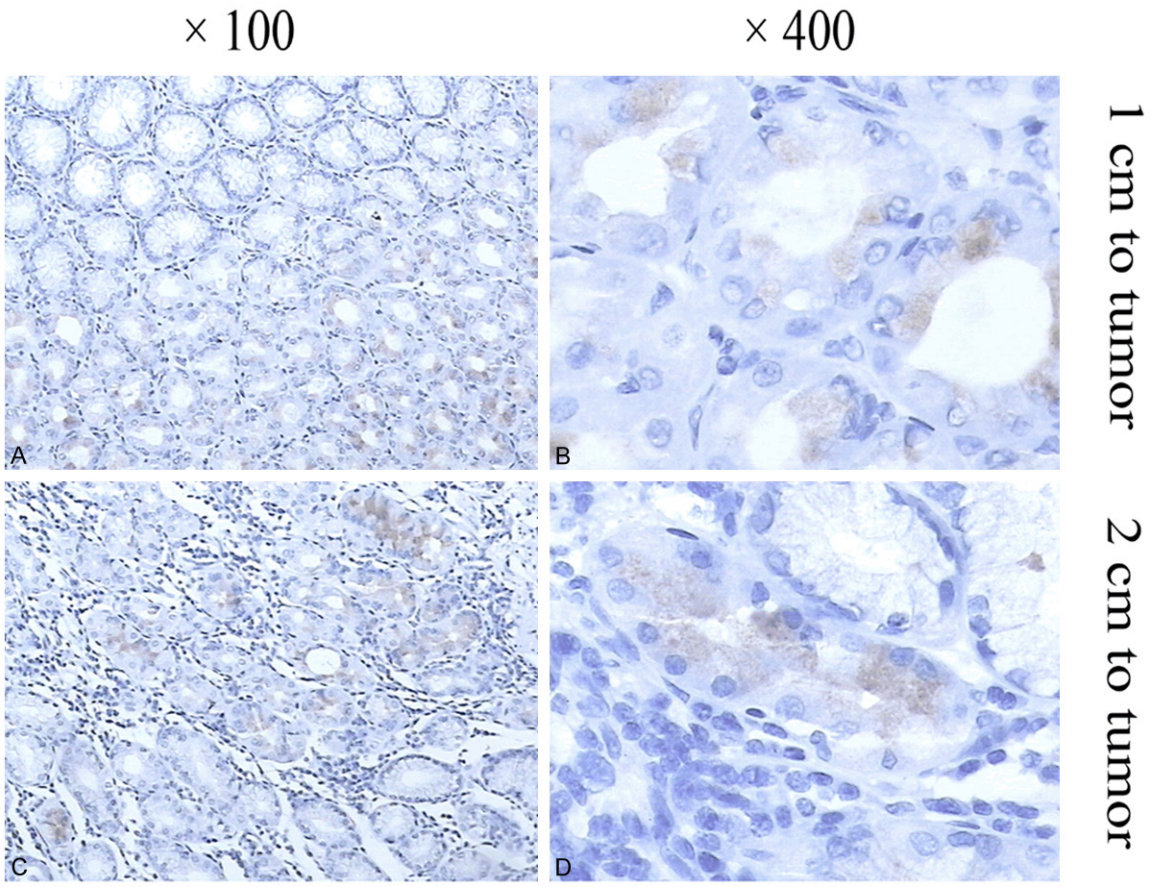

Figure 2. Expression of CD44v6 in para-cancerous tissues 1 cm (A: × 100; B: × 400) and 2 cm (C: × 100; D: × 400) from tumor.

pT1, pT2, and pT3-4 tumors respectively, with significant differences between groups ($P < 0.05$), indicating that the expression rate rose with the deepening of invasion. CD44v6 expression rate was significantly higher in patients with lymphatic metastasis (82.4% [42/51]) than in those without lymphatic metastasis (44.4% [4/9]) ($P < 0.05$). The expression rate was higher in patients with distant metastasis (100.0% [4/4]) than in those without distant metastasis (75.0% [42/56]), however, the difference was not statistically significant, possibly due to the small number of metastatic cases (**Table 2**).

Correlation between CD44v6 expression and pTNM classification

The expression rates were 55.6% (5/9) in stage I tumor, 68.8% (11/16) in stage II tumor, 83.9% (26/31) in stage III tumor, and 100.0% (4/4) in stage IV tumor. The expression rate increased with the elevating of the tumor pTNM stage ($P < 0.05$) (**Table 2**).

Discussion

During tumor genesis and progression, metastasis occurs when parts of malignant cells penetrate basement membrane, entering the surrounding tissue or invading vascular and lymphatic vessels, after their gaining the invasive and metastatic abilities. The intra-cell and cell-extracellular matrix (ECM) interactions, which are significantly mediated by surface CAMs of tumor cells, play vital roles in this procedure [16]. As a CAM, CD44 is widely distributed, and its major functions are acting as an oriented receptor, mediating the lymphocyte-endotheliocyte adhesion which is involved in the activation signal transduction of lymphocytes, and participating theimmune cell homing. The *CD44* gene is located on Chromosome 11p, and can be translated into two kinds of pro-

teins, standard CD44s and variant CD44v, via alternative splicing of the exon. CD44v could endow malignant cells with great invasive and metastatic abilities through adhesion to hyaluronic acid and endothelial cells in the ECM [17]. CD44v6 is a translational product after variant alternative splicing of the v zone exons in the gene. It plays an important role in tumorigenesis and metastasis. It is scarcely expressed in normal tissues, and is significantly correlated with invasion depth, lymphatic metastasis, TNM classification, and differentiation grade in malignancies [18, 19]. So far as we know, the clinicopathological significance of CD44v6 in GC has scarcely been previously reported.

CD44v6 expression is closely associated with GC genesis and progression. It was shown in our study that CD44v6 was rarely expressed in normal gastric mucosa, and was significantly more highly expressed in GC tissues than in precancerous lesions. The phenomenon that CD44v6 widely existed in GC and partially in precancerous tissues indicates that it might be involved in the carcinogenesis process of gastric mucosa, and might play a certain role in GC progression. With the increase of the invasion depth and lymphatic metastasis, CD44v6 was more highly expressed in GC cells, suggesting that high CD44v6 expression might facilitate tumor cell invasion, which is in accordance with Liang's research [20]. This research also revealed that histological differentiation degree of GC was significantly correlated with the expression of CD44v6, and that the more poorly the cancer was differentiated, the higher the expression rate was, which is also consistent with Liang's study [20]. Our results indicated that the positive expression rate of CD44v6 was significantly higher in GC patients with lymphatic metastasis than in those without lymphatic metastasis, which is consistent with Okayama's study [21]. This could be possibly explained by the fact that endothelial cells are vulnerable to invasion by tumor cells mediated by the CAM CD44v6, contributing to penetration into the lymphatic vessel. Some scholars also hypothesize that CD44v6-positive cancer cells could gain the camouflage as lymphocytes, which facilitates their entering the lymph node and metastasis [22]. The distribution of CD44v6 might suggest its function mainly through intra-cell and cell-ECM interactions. This study also uncovered that the positive CD44v6 expression rate was significantly lower in the para-cancerous tissues (1 cm and 2 cm from the tumor site) than in the corresponding tumor lesions, based on which we propose that we might have a good understanding of whether there is any remnant cancer cell via investigating the CD44v6 expression at the cutting edge of the resected specimen to assess the surgery efficacy, especially for AEG with a relatively high location of the tumor mass and with usually unsatisfactory resection length. The negative correlation between CD44v6 expression in para-cancerous tissue and the distance from tumor site suggest that it might be helpful to determine the safety and efficacy of surgery by investigating its expression at the cutting edge.

In conclusion, CD44v6 expression was closely correlated with GC differentiation degree, invasion depth, lymphatic metastasis, and TNM classification. It might facilitate tumor cell invasion and metastasis, playing a key role in GC progression. Its detection could assist early GC detection and surgical efficacy assessment. The development of strategies against this protein could lead to new therapeutic approaches. We are now further gathering data concerning the post-surgical recurrence and survival rate, with the hope to develop CD44v6 as a novel GC prognosticator.

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Disclosureof conflict of interest

None.

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