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CD44v6, MMP-7 and nuclear Cdx2 are significant biomarkers for prediction of lymph node metastasis in primary gastric cancer

HIROKAZU OKAYAMA[1], KENSUKE KUMAMOTO[1], KATSUHARU SAITOU[1], SUGURU HAYASE[1], YASUHIDE KOFUNATO[1], YU SATO[1], KOTARO MIYAMOTO[1], IZUMI NAKAMURA[1], SHINJI OHKI[1], KOUJI SEKIKAWA[2] and SEIICHI TAKENOSHITA[1]

[1]Department of Organ Regulatory Surgery, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1295; [2]Department of Surgery, Kawasaki-Saiwai Hospital, 39-1 Kawasaki, Kanagawa 212-0021, Japan

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Abstract. The aim of this study was to examine the expression of CD44v6, CD54, Cdx2, CXCL5, Cyclin B1, MMP-7, nm23, RCAS1 and Survivin in primary gastric cancer and to investigate whether these molecules were useful in predicting the lymph node status. They were selected as candidates for indicators of lymph node metastasis from various kinds of cancer-associated genes reported previously. In 135 cases of radically resected primary gastric adenocarcinoma, we investigated the association between the expression of these molecules and clinocopathologic factors by immunohistochemistry. The results revealed that the expression of CD44v6 and MMP-7 were significantly associated with lymph node status. By contrast, nuclear Cdx2 expression was found to be inversely correlated with lymph node metastasis. Moreover, multivariate analysis demonstrated that CD44v6, MMP-7 and nuclear Cdx2 were independent predictors for lymph node status. In conclusion, our results suggest that positive expression of both CD44v6 and MMP-7, and negative expression of nuclear Cdx2 may serve as powerful predictors of lymph node metastasis in gastric cancer. Combined evaluation of these markers could be further useful to predict lymph node status clinically.

Introduction

Survival of patient with gastric cancer has improved with the development of diagnostic and therapeutic techniques over the past few decades. However, gastric cancer still remains a major cause of cancer death worldwide (1). Lymph node status is one of the most important prognostic factors for patients with radically resected gastric cancer (2-4). The appropriate extension of lymphadectomy on the basis of accurate lymph node status for surgical treatment is still controversial (5,6). In recent years, minimally invasive therapies, including endoscopic resection and laparoscopic surgery, have been performed frequently (7,8). Therefore, predicting lymph node status in patients with gastric cancer is very important in treatment decision. Although numerous molecular markers indicating the lymph node spread in gastric cancer have been investigated, none of them is sufficient enough to be implemented in the clinical setting to predict lymph node status.

In this study, nine biomarkers including CD44v6, CD54, Cdx2, CXCL5, Cyclin B1, MMP-7, nm23, RCAS1 and Survivin, were selected as candidates for predicting lymph node status from many cancer-associated genes, which had shown to be correlated with lymph node metastasis. We performed immunohistochemical analysis in 135 cases of primary gastric cancer, and analyzed the relationship between these biomarkers expression on cancer tissues and clinocopathologic features by multivariate analysis. We also investigated the efficiency of combined analysis of several markers to predict the lymph node status.

Cell adhesion molecules have been examined which involved in cell-cell adhesion, cell-extracellular matrix adhesion and cellular interactions. CD44 glycoproteins, cell surface receptors as adhesion molecules binding to its principal ligand hyaluronan, is known to play a major role in cell-cell adhesion, cell-substrate interaction, lymphocytes recruitement to inflammatory sites, and tumor metastasis (9,10). The standard isoform, CD44s, is expressed in a variety of normal tissues. CD44 isoforms containing variant exon 6 (CD44v6), generated by alternative splicing of at least 12 exons, have been studied most extensively. It has been reported that CD44v6 is correlated with regulating tumor invasion, progression and metastasis in various kinds of malignancies including gastric cancer (11,12). In addition, CD44v6 was significantly correlated with lymph node metastasis in gastric cancer (13-15).

CD54, a member of the immunogloblin superfamily and also called ICAM-1, is a glycoprotein with five immunogloblin-

Correspondence to: Dr Seiichi Takenoshita, Department of Organ Regulatory Surgery, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1295, Japan
E-mail: takenoss@fmu.ac.jp

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like extracellular domains (16). CD54 is expressed by various cells including vascular endothelial cells, fibroblasts, dendritic cells, leukocytes and monocytes. When CD54 binds to its ligands LFA-1 and Mac-1 on the surface of T lymphocytes, it plays an essential role as a signal transmitter of immune response, which mediates tumor cytotoxicity (17). It has been reported that CD54 expression on the surface of tumor cells increases the susceptibility of such tumor cells to lymphocyte-mediated tumor cytotoxity through the CD54/LFA-1 system (18,19). Maruo *et al* suggested that ICAM-1 was overexpressed in cancer cells and released as soluble-ICAM-1, which would promote hematogenous metastasis by suppressing local anticancer immunity (20). By contrast, Yashiro *et al* reported that decreased CD54 expression on cancer cells was significantly correlated with lymphatic spread, which suggested that decreased CD54 might be associated with decreased cytotoxity of immune cells (21).

Cdx2 is an intestine-specific caudal-related homeobox transcription factor expressed in the epithelium of the small intestine and colorectum, in which it works for cell proliferation, differentiation, and maintaining intestinal phonotypes (22). Cdx2 expression is not detected in normal gastric mucosa, but ectopically observed in nearly all intestinal metaplasia (23). Recent reports demonstrated that intestinal-type gastric cancer developed from intestinal metaplasia in Cdx2-transgenic mice, suggesting that Cdx2 might be involved in gastric carcinogenesis (24,25). According to the clinical study, Cdx2 expression in gastric cancer was corelated with better prognosis (26,27). Furthermore, a significant negative correlation has been shown between Cdx2 expression and lymph node metastasis (28).

Chemokines which were originally identified in inflammatory pathways stimulating the migration of leucocytes, have been recently reported to induce the migration of tumor cells and their expression was associated with tumor growth, angiogenesis and metastatic potential (29). CXCL5, a member of CXC-type chemokines, functions as a proangiogenic factor and as a powerful chemoattractant for granulocytic immune cells mainly through binding its receptor CXCR2. It has been reported that CXCL5 expression contributes to tumor progression and metastasis (30-32). Park *et al* demonstrated that CXCL5 overexpression was associated with advanced gastric cancer and high N stage, suggesting a role for CXCL5 in the progression of gastric cancer, specifically in lymph node metastasis (33).

Cell division is a critical event in tumor progression and numorous molecules involved with this process have been investigated intensely in tumor biology. Cyclins, which control the cell cycle, have been shown to be overexpressed in various kinds of malignancies (34,35). Cyclin B1, an important molecule in the cell cycle transition from G2-to-M phase, regulates cell division and cell arrest by cooperating with cdc2 kinase. Previous reports revealed that Cyclin B1 overexpression was observed in the cytoplasm of many cancers and related to prognosis (36,37). Korenaga *et al* showed the association between cyclin B1 and lymph node metastasis in human colorectal cancer (38). In gastric cancer, Yasuda *et al* reported that Cyclin B1 overexpression was closely associated with less aggressive tumor behavior, including nodal metastasis and stage of disease (39).

The extracellular matrix (ECM) provides a structural framework to support cells by mediating cell-cell or cell-ECM interactions. Degradation of ECM components is mostly controlled by proteolytic enzymes called matrix metalloproteinases (MMP). The MMP family, zinc-dependent endopeptidases, consists of at least 25 members (40). MMP-7, also known as matrilysin, has proteolytic activities against a wide range of substrates, including not only proteoglycans, fibronectin and laminin, but also type IV collagen (41). MMP-7 is frequently overexpressed in cancer tissues and is associated with cancer progression, which includes multistep process such as cell growth, apoptosis, invasion, metastasis and angiogenesis (40,42,43). Moreover, MMP-7 expression in gastric cancer is associated with aggressive phenotypes including lymph node metastasis (44,45).

Nm23 was originally identified as a metastasis suppressor gene (46). It has two isotypes, nm23-H1 and nm23-H2, have been shown to be identical to nucleoside diphosphate kinase (NDPK) A and B. Many reports exhibited that reduced expression of nm23 (or nm23-H1) was correlated with tumor metastasis or poor prognosis (47-49). In contrast, overexpression of nm23 is related to advanced tumors (50,51), suggesting that the significance of nm23 expression is different depending on cancer types. As for gastric cancer, several researchers reported that reduced nm23 expression was related to lymph node metastasis or poor prognosis (52,53). On the other hand, some studies showed opposite results or no significant findings (54,55).

The tumor-associated antigen, receptor-binding cancer antigen expressed on SiSo cells (RCAS1), is recognized by the mouse monoclonal antibody, 22-1-1, which was isolated from mice immunized with the human uterine cervical adenocarcinoma cell line SiSo (56). RCAS1 is a type II membrane protein and expressed on several kinds of carcinomas (57,58). *In vitro* studies of RCAS1 indicated that it might act as an apoptosis-inducing factor, since RCAS1 induced apoptosis in its receptor expressing cells, such as T, B and NK cells (56). Thus, RCAS1 is considered to play a role in evasion of immune surveillance. RCAS1 expression in gastric cancer cells was classified as polarity pattern or diffuse pattern (59). The diffuse pattern of gastric cancers was implicated in large size, depth of invasion and regional lymph node metastasis, compared with polarity pattern. Moreover, RCAS1 expression in T3 gastric carcinoma was significantly correlated with the histological type and lymph node metastasis (60).

Survivin, a unique member of the inhibitor of apoptosis protein (IAP) family, is a multifunctional protein that inhibits apoptosis, regulates cell division and enhances angiogenesis (61). In many types of cancers, survivin expression is correlated with reduced apoptosis and unfavorable prognosis (62). Miyachi *et al* demonstrated that survivin mRNA expression was significantly higher in patients with lymph node metastasis in gastric cancer (63). Lee *et al* reported that survivin was significantly associated with depth of invasion, tumor stage, poor survival and lymph node metastasis (64). However, there are still conflicting results about survivin expression on gastric cancer (65-67). In the present study, we evaluated the usefulness of these molecules to predict lymph node metastasis in gastric cancer.

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s and methods

Clinical materials. Primary gastric adenocarcinoma specimens were obtained from 135 patients who underwent radical resection at Fukushima Medical University between January 1991 and December 2004. No patients received chemotherapy or radiotherapy before surgery. Among the 135 patients, 91 were male and 44 female. Ages ranged between 31 and 87 years with the mean age of 63.4 years. All patients were treated by lymph node dissection of D1 or D2 according to the Japanese Classification of Gastric Cancer (JSGC), which were defined by location of lymph node relative to the primary site. The resected and examined lymph nodes were at least 15 for each case. The mean number of examined lymph nodes was 27.56 (15-60) and the mean number of metastatic nodes was 3.62 (0-32). No lymph node metastasis was found in 61 (45.2%) cases, whereas, lymph node involvement were present in 74 (54.8%). Consequently, patients with distant metastases, peritoneal dissemination or extension to other organs (SI) were excluded from the analysis. Patients with only mucosal infiltration (M) were also excluded. Histological type was devided into differentiated and undifferentiated type. Well- and moderately differentiated tubular or papillary adenocarcinomas were classified as differentiated type, and poorly differentiated adenocarcinomas and signet-ring cell carcinomas were classified undifferentiated type. Mucinous adenocarcinomas were classified as either type depending upon the other predominant elements. Of the 135 patients, tubular adenocarcinoma, papillary adenocarcinoma, poorly differentiated adenocarcinoma, signet-ring cell carcinoma and mucinous carcinoma were present in 60, 6, 55, 10 and 4 patients, respectively. Sixty-five (48.1%) were classified as differentiated, 70 (51.9%) were undifferentiated. Depth of tumor invasion was recorded using T classification, tumor invasion of mucosa (M) or submucosa (SM) was defined as T1, that of muscularis propria (MP) or subserosa (SS) was T2, tumor penetration of serosa (SE) was T3, tumor invasion of adjacent structures (SI) was T4. As for the depth of invasion, 43 cases were T1, 54 were T2 and 38 were T3. Sixty-two cases were allocated to stage I, 28 to stage II, 45 to stage III. The extent of lymphatic (ly) and venous (v) invasion was divided into present or absent. Those clinicopathological findings were determined according to the Japanese Classification of Gastric Cancer (JCGC) (68). Table I shows the clinicopathological features of patients with or without lymph node metastasis. There was no significant difference between the lymph node metastasis and gender, age and histological type. In contrast, the depth of invasion ($p<0.001$), lymphatic invasion ($p=0.003$) and venous invasion ($p<0.001$) showed significant association with the presence of lymph node involvement.

Table I. Clinicopathological features.

		LN metastasis		
	Total n=135	Positive n=74	Negative n=61	P-value
Age				0.295
>65	73	37	36	
≤65	62	37	25	
Gender				0.965
Male	91	50	41	
Female	44	24	20	
Histological type				0.051
Differentiated	65	30	35	
Undifferentiated	70	44	26	
Depth of invasion				<0.001
T1	43	11	32	
T2	54	31	23	
T3	38	32	6	
Lymphatic invasion				0.003
Present	25	7	18	
Absent	110	67	43	
Venous invasion				<0.001
Present	39	11	28	
Absent	96	63	33	

Immunohistochemistry. All specimens were fixed in formalin and embedded in paraffin. Ten serial sections (4-μm) were prepared and one of them was used for hematoxylin and eosin (H&E) staining, while the others were used for immunohistochemistry. Sections were deparaffinized in xylene and hydrated through a graded series of ethanol. After the sections were rinsed three times in phosphate-buffered saline (PBS), endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. Antigens were retrieved by autoclaving sections on slides in 0.01 M pH 6.0 citrate buffer for 10 min (except for nm23 and Cdx2). After being rinsed in PBS, the sections were incubated with each primary antibody overnight at 4˚C. The primary antibodies were anti-CD44v6 (clone 2F10; R&D Systems, Minneapolis, MN, USA, 1:100), anti-CD54 (clone 23G12; Novocastra, Newcastle, UK, 1:100), anti-Cdx2 (clone CDX2-88; Biogenex, San Ramon, CA, USA, ready for use), anti-CXCL5 (clone 33160; R&D Systems, 1:50), anti-Cyclin B1 (clone 7A9; Novocastra, 1:50), anti-MMP7 (clone 141-7B2; Daiichi Fine Chemical, Toyama, Japan, 1:200), anti-nm23 (clone 37.6; Novocastra, 1:500), anti-RCAS1 (clone 22-1-1; Medical & Biological Laboratories, Nagoya, Japan, ready for use), and anti-Survivin (Rabbit polyclonal; Novus Biologicals, Littleton, CO, USA, 1:1000). A further wash in PBS was followed by treatment with peroxidase-labeled polymer conjugated to gout anti-mouse or anti-rabbit immunogloblins (Envison+kit; Dako, Glostrup, Denmark) as the secondary antibody for 30 min at room temprature. The staining was visualized with diaminobenzidine (DAB), followed by counterstaining with hematoxylin.

Assessment of staining. Sections were considered positive for CD44v6, CD54, CXCL5, Cyclin B1, MMP-7, RCAS1, when more than 5% of tumor cells were stained in the cytoplasm or

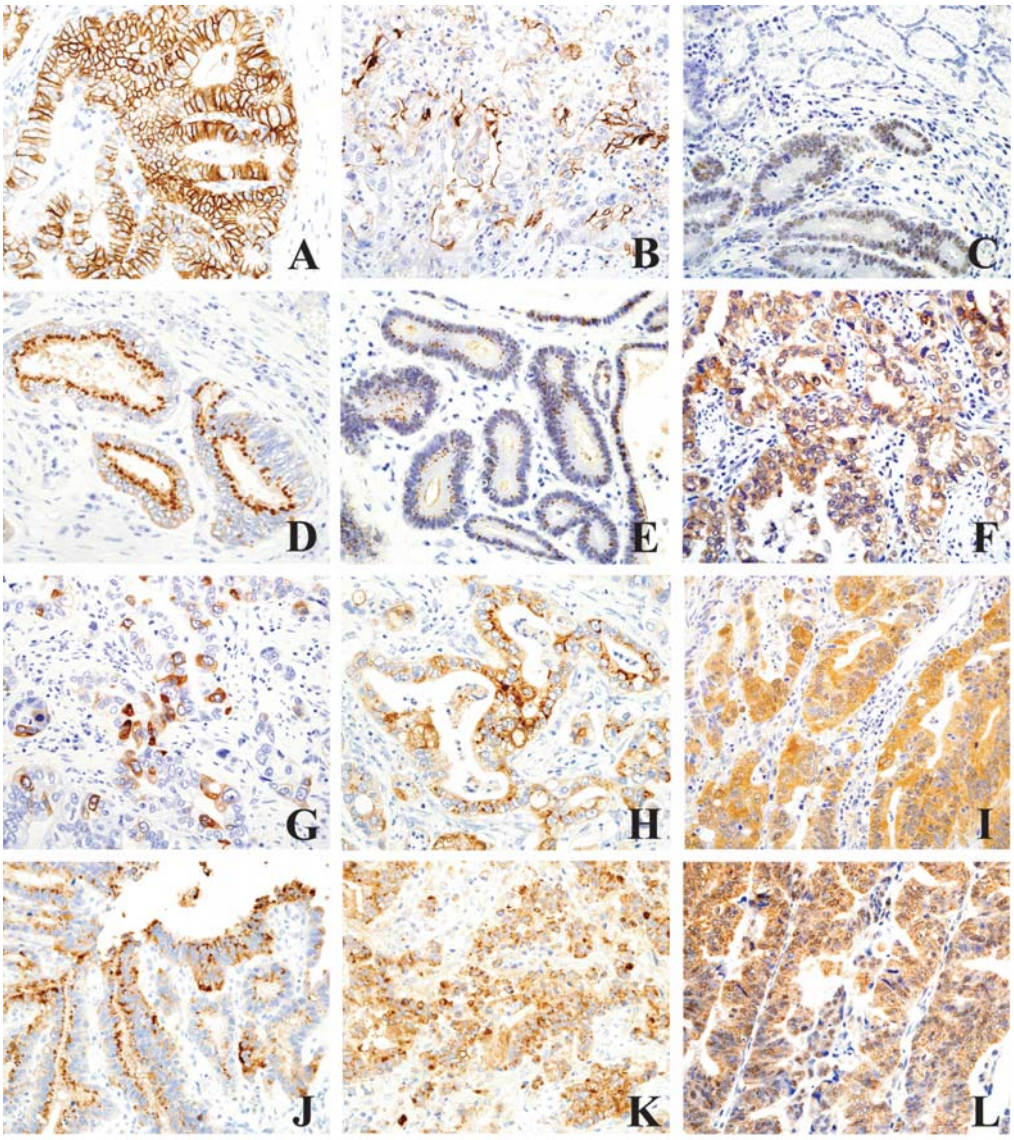

Figure 1. Representative images of immunohistochemical staining for CD44v6, CD54, Cdx2, CXCL5, Cyclin B1, MMP-7, nm23, RCAS1 and Survivin in gastric cancer (original magnification, x40). (A) CD44v6 expressed prominently in membrane of cancer cells. (B) Strong membranous staining of CD54. (C) expression of Cdx2 in nuclei of tumor cells but absent in normal gastric mucosa adjacent to cancer tissue. (D) Cdx2 granular staining detected in cytoplasm. (E) Both nuclear and cytoplasmic staining of Cdx2. (F) CXCL5 positive expression in cytoplasm. (G) Cyclin B1 expressed heterogeneously in the cytoplasm. (H) MMP-7 stained in the cytoplasm of cancer cells at the invasive part of the tumor. (I) nm23 observed diffusely in cytoplasm and nuclei of tumor cells. (J) RCAS1 defined as P pattern. (K) RCAS1 defined as D pattern. (L) Survivin expression, strong staining in nuclei and diffuse granular staining in cytoplasm of cancer cells.

cell membrane. The expression of Cdx2 was evaluated cytoplasmic and nuclear staining respectively, and sections with >5% stained tumor cells were considered positive. For the expression of nm23, the cases with >30% tumor cells in a section were regarded as positive. To quantitate survivin expression, a scoring method was used as described previously (64,66). Staining intensity and percentage of staining area were classified and calculated, sections were then categorized as high expression or low expression. In addition to the evaluation mentioned above, RCAS1 expression was classified into two patterns as described previously (59). Granular staining enriched in the grandular side of cytoplasm with polarity was defined as a P pattern, and granular staining scattered diffusely in the cytoplasm and on the cell membrane was defined D pattern. Assessment of the staining for these molecules was evaluated by two independent investigators (H.O. and K.K.) without knowledge of the clinicopathological data and clinical outcomes of the patients.

Statistical analysis. Associations between categorical variables were evaluated with χ^2 test, Fisher's exact test or Mann-Whitney U test. The factors found to be significant in univariate analysis were included in subsequent stepwise multivariate logistic regeression analysis to identify the independent predictors for the lymph node metastasis. Differences at $p<0.05$ were considered significant. All statistical analyses were performed using SPSS 11.0 software (SPSS Inc., Chicago, IL).

Features of expression and relation to clinicopathological factors. In the normal gastric tissue surrounding the cancerous lesions, few normal epithelial cells stained weakly for CD44v6. Among the 135 of primary gastric cancer examined, 84 (62.2%) cases showed positive expression for CD44v6. The staining pattern was intense mainly on the cell membrane and cytoplasm of cancer cells (Fig. 1A). The distribution of CD44v6-expressed cells were found particularly at the invasive region of tumor. As shown in Table II, positive expression of CD44v6 was significantly more frequent when lymph node metastasis (p=0.001) or lymphatic invasion (p=0.037) were present. CD44v6 expression was observed in 55 (74.3%) of 74 cases with lymph node metastasis and 73 (66.4%) of 110 cases with lymphatic invasion. Also as the depth of invasion or stage increased, positive expression rate of CD44v6 became significantly higher (p=0.036, 0.010, respectively). No significant correlations could be determined between CD44v6 and other variables such as age, gender, histological type and venous invasion.

CD54 was commonly observed in the vascular endothelial cells and monocytes, but not in the normal gastric epithelial cells. The expression of CD54 was found predominantly in membrane of gastric cancer cells, and also detected in the cytoplasm (Fig. 1B). Fifty one cases (37.8%) were determined as positive for CD54. CD54 positive expression was correlated with increased depth of invasion (p=0.007), advanced stage (p=0.033) or presence of venous invasion (p=0.008). No relationship was found between CD54 expression and age, gender, histological type, lymphatic invasion and lymph node status.

Cdx2 was expressed in the nuclei or cytoplasm of tumor cells (Fig. 1C-E). No staining was observed in normal epithelial cells. Nuclear Cdx2 expression was found in 55 cases (40.7%), whereas cytoplasmic expression was found in 69 cases (51.1%). Twenty-five cases (18.5%) showed positive expression of both nucleus and cytoplasm. The positive expression of nuclear Cdx2 was significantly higher in males than in females (p=0.001) and in differentiated types than in undifferentiated types (p=0.003). There was a significant inverse correlation between nuclear Cdx2 and depth of invasion (p=0.001) or stage (p<0.001), respectively. Furthermore, significantly less expression was observed in cases with lymph node involvement than in those without lymph node involvement (p<0.001) and in presence of venous invasion than absence of venous invasion (p=0.018). Nuclear Cdx2 was positive in 20 (27.0%) of 74 cases with lymph node metastasis, and in 35 (57.4%) of 61 cases without lymph node metastasis. By contrast, cytoplasmic expression of Cdx2 was only related to histological type (p=0.032) and gender (p=0.006).

CXCL5 expression existed focally in the cytoplasm of normal gastric epithelial cells and gastric cancer cells (Fig. 1F). Seventy-three cases (54.1%) were defined positive for CXCL5. The expression of CXCL5 positive rate was related to advanced depth of invasion (p=0.029) or advanced stage (p=0.008), respectively. However, correlations with any of these clinicopathological variables were not observed.

Weak and focal expression of Cyclin B1 was detected in the cytoplasm of normal gastric epithelial cells. In gastric cancer, Cyclin B1 was observed more intense predominantly in the cytoplasm of tumor cells (Fig. 1G). The positive expression of Cyclin B1 was found in 56 cases (41.5%). No significant association was observed between Cyclin B1 expression and clinicopathologic features, except for histological type (p=0.002).

The expression of MMP-7 was hardly detected in normal gastric mucosa. In gastric cancer, MMP-7 was expressed in cytoplasm and membrane of cancer cells (Fig. 1H). Stained cells were found heterogeneously in cancerous tissue, especially in invasive region of the tumor. MMP-7 positive cases were detected in 96 (71.1%) of 135 cases. MMP-7 positive expression was significantly correlated with presence of lymph node metastasis (p<0.001), depth of invasion (p<0.001) and stage (p<0.001). Sixty-two (83.8%) of 74 cases with lymph node metastasis showed positive for MMP-7. On the other hand, MMP-7 expression was not significantly associated with other variables, including gender, histological type, lymphatic or venous invasion.

Almost all normal gastric epithelial cells were stained homogeneously for nm23. In gastric cancer, nm23 was found in cytoplasm and nuclei of cancer cells that were more intense than normal tissue (Fig. 1I). The majority of cancer specimens were stained diffusely, 113 cases (83.7%) were in fact determined as positive for nm23. Among 22 cases of nm23 negative group, 20 cases (90.9%) were undifferentiated type. In addition, in ten signet-ring cell carcinoma cases, eight (80.0%) were markedly negative for nm23. The negative expression of nm23 was significantly correlated with younger age (p=0.022), undifferentiated histological type (p<0.001) and increased stage (p=0.046), but not with other parameters including lymph node metastasis.

RCAS1 expression was present in both cytoplasm and membrane of normal epithelial cells and cancer cells. All 135 gastric cancer specimens showed positive expression of RCAS1. Therefore, tumors were further categorized to P pattern or D pattern as previously described (59) (Fig. 1J and K). Of 135 cases, 62 (45.9%) and 73 (54.1%) were defined as P pattern and D pattern, respectively. There was a significant difference between RCAS1 expression pattern and several clinicopathological parameters such as histological type (p=0.005), depth of invasion (p<0.001), venous invasion (p=0.002), lymph node metastasis (p=0.006) and stage (p<0.001). D pattern of RCAS1 expression were associated with undifferentiated histological type, incidence of venous invasion, presence of lymph node metastasis, increased depth of invasion and advanced clinical stage. Forty-eight (65.8%) of 73 cases defined as D pattern had lymph node involvement.

The expression of survivin was observed primarily in the nuclei but was also weakly present in the cytoplasm. Survivin was detected strongly in gastric cancer cells, while weak staining was diffusely detected in normal epithelial cells (Fig. 1L). High expression of survivin was more frequently observed in males than in females (p=0.009), and in differentiated type (p=0.001) than in unddifferentiated type. No significant association was detected between survivin expression and any other variables.

Table II. Correlation between markers and clinicopathological features.

	CD44v6		CD54		Cdx2 cytoplasmic		Cdx2 nuclear		CXCL5	
	Positive n=84	Negative n=51	Positive n=51	Negative n=84	Positive n=69	Negative n=66	Positive n=55	Negative n=80	Positive n=73	Negative n=62
Age										
>65	45	28	31	42	34	39	30	43	34	39
≤65	39	23	20	42	35	27	25	37	39	23
		p=0.880		p=0.223		p=0.253		p=0.927		p=0.058
Gender										
Male	46	35	33	58	39	52	46	45	44	47
Female	28	16	18	26	30	14	9	35	29	15
		p=0.814		p=0.602		p=0.006		p=0.001		p=0.055
Histological type										
Differentiated	38	27	23	42	27	38	35	30	32	33
Undifferentiated	46	24	28	42	42	28	20	50	41	29
		p=0.385		p=0.580		p=0.032		p=0.003		p=0.277
Depth of invasion										
T1	21	22	10	33	17	26	22	21	16	27
T2	36	18	21	33	32	22	28	26	34	20
T3	27	11	20	18	20	18	5	33	23	15
		p=0.036		p=0.007		p=0.209		p=0.001		p=0.029
Lymphatic invasion										
Present	73	37	44	66	58	52	44	66	62	48
Absent	11	14	7	18	11	44	11	14	11	14
		p=0.037		p=0.264		p=0.431		p=0.713		p=0.263
Venous invasion										
Present	62	34	43	53	48	48	33	63	53	43
Absent	22	17	8	31	21	18	22	17	20	19
		p=0.375		p=0.008		p=0.685		p=0.018		p=0.678
LN metastasis										
Positive	55	19	32	42	38	36	20	54	45	29
Negative	29	32	19	42	31	30	35	26	28	33
		p=0.001		p=0.149		p=0.951		p<0.001		p=0.084
Stage										
I	31	31	17	45	27	35	38	24	25	37
II	20	8	13	15	17	11	9	19	19	9
III	33	12	21	24	25	20	8	37	29	16
		p=0.010		p=0.033		p=0.177		p<0.001		p=0.008

Continued.

	Cyclin B1		MMP7		nm23		RCASI		Survivin	
	Positive n=56	Negative n=79	Positive n=96	Negative n=39	Positive n=113	Negative n=22	P n=62	D n=73	High n=99	Low n=36
Age										
>65	32	41	46	27	66	7	29	44	55	18
≤65	74	38	50	12	47	15	33	29	44	18
		p=0.547		p=0.024		p=0.022		p=0.117		p=0.567
Gender										
Male	41	50	62	29	78	13	45	46	73	18
Female	15	29	34	10	35	9	17	27	26	18
		p=0.226		p=0.272		p=0.363		p=0.237		p=0.009
Histological type										
Differentiated	36	29	42	23	63	2	38	27	56	9
Undifferentiated	20	50	54	16	50	20	24	46	43	27
		p=0.002		p=0.109		p=0.001		p=0.005		p=0.001
Depth of invasion										
T1	15	28	23	20	37	6	29	14	35	8
T2	30	24	39	15	45	9	27	27	38	16
T3	11	27	34	4	31	7	6	32	26	12
		p=0.698		p=0.001		p=0.585		p=0.001		p=0.178
Lymphatic invasion										
Present	49	61	82	28	93	17	49	61	78	32
Absent	7	18	14	11	20	5	13	12	21	4
		p=0.130		p=0.065		p=0.579		p=0.500		p=0.182
Venous invasion										
Present	42	54	72	24	79	17	36	60	69	27
Absent	14	25	24	15	34	5	26	13	30	9
		p=0.401		p=0.118		p=0.486		p=0.002		p=0.548
LN metastasis										
Positive	35	39	62	12	59	15	26	48	53	21
Negative	21	40	34	27	54	7	36	25	46	15
		p=0.131		p=0.001		p=0.169		p<0.006		p=0.620
Stage										
I	23	39	32	28	55	7	38	24	48	14
II	16	12	21	7	25	3	15	13	23	5
III	17	28	41	4	33	12	9	36	28	17
		p=0.767		p=0.001		p=0.046		p<0.001		p=0.111

Table III. Univariate and multivariate logistic regression analysis with respect to lymph node metastasis.

		Univariate			Multivariate		
		Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Age	≤65 vs. >65	0.694	0.350-1.376	0.296			
Gender	Male vs. Female	1.016	0.493-2.064	0.965			
Histological type	Differentiated vs. Undifferentiated	1.974	0.993-3.927	0.053			NS
Depth of invasion	T1 vs. T2/T3	6.320	2.800-14.264	<0.001	4.524	1.876-10.911	0.001
Lymphatic invasion	Absent vs. Present	4.007	1.544-10.395	0.004			NS
Venous invasion	Absent vs. Present	4.860	2.152-10.976	<0.001			NS
CD44v6	Negative vs. Positive	3.194	1.548-6.590	0.002	2.390	1.036-5.512	0.041
CD54	Negative vs. Positive	1.684	0.827-3.428	0.151			
cytoplasmic Cdx2	Negative vs. Positive	1.022	0.519-2.012	0.951			
nuclear Cdx2	Negative vs. Positive	0.275	0.134-0.566	<0.001	0.284	0.125-0.641	0.002
CXCL5	Negative vs. Positive	1.829	0.920-3.634	0.085			
Cyclin B1	Negative vs. Positive	1.709	0.851-3.435	0.132			
MMP-7	Negative vs. Positive	4.103	1.847-9.116	0.001	2.667	1.075-6.612	0.034
nm23	Negative vs. Positive	0.510	0.193-1.345	0.174			
RCAS1	P pattern vs. D pattern	2.658	1.322-5.346	0.006			NS
Survivin	Low vs. High	1.215	0.562-2.628	0.621			

CI, confidence interval; NS, not significant.

Table IV. Multivariate analysis with respect to lymph node metastasis in submucosal and advanced cancer group.

		Submucosal cancer group (n=43)			Advanced cancer group (n=92)		
		Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Histological type	Differentiated vs. Undifferentiated			NS			NS
Depth of invasion	T1 vs. T2/T3			NS			NS
Lymphatic invasion	Absent vs. Present			NS			NS
Venous invasion	Absent vs. Present			NS	9.090	2.065-40.018	0.004
CD44v6	Negative vs. Positive	7.500	1.382-40.689	0.020			NS
nuclear Cdx2	Negative vs. Positive			NS	0.323	0.115-0.908	0.032
MMP-7	Negative vs. Positive			NS	3.785	1.164-12.308	0.027
RCAS1	P pattern vs. D pattern			NS			NS

CI, confidence interval; NS, not significant.

Multivariate analysis of factors related to lymph node metastasis. As shown in Table III, among factors shown to have a correlation with the presence of lymph node metastasis by univariate analyses, subsequent multivariate analysis using the logistic regression model demonstrated that several conventional clinicopathological factors, including depth of invasion and venous invasion were significant independent predictors for lymph node metastasis. CD44v6 and MMP-7 expression were also found to be independently related to lymph node metastasis. Moreover, expression Cdx2 in nuclei was a significant negative predictor of lymph node metastasis. In the submucosal cancer group, only CD44v6 expression was found to be significantly and independently related to lymph node metastasis. In advanced cancer group, positive MMP-7 and negative nuclear Cdx2 expression, respectively, were significant predictors for lymph node metastasis (Table IV). To investigate the implications of combined analysis, cases could be categorized into two groups: CD44v6 (+), MMP7 (+), nuclear Cdx2 (-) or others. The former group also proved to be independent indicator of lymph node metastasis (Table V).

Multivariate logistic regression analysis with respect to lymph node metastasis.

		Odds ratio	95% CI	P-value
Histological type	Differentiated vs. Undifferentiated			NS
Depth of invasion	T1 vs. T2/T3	3.169	1.265-7.940	0.014
Lymphatic invasion	Absent vs. Present			NS
Venous invasion	Absent vs. Present	2.678	1.054-6.808	0.038
CD44v6, MMP-7, nuclear Cdx2	+ / + / - vs. others	3.280	1.320-8.149	0.011

CI, confidence interval; NS, not significant.

Discussion

Lymph node metastasis is known to be one of the most important prognostic factors for gastric cancer. There are several distinct steps in the process of lymphatic metastasis of cancer cells, including tumor lymphangiogenesis, infiltration into lymphatic vessels, shedding and floating in lymphatic vessels, migration, adhesion, and proliferation into lymph nodes. Various kinds of molecular markers have been investigated to find as predictive factors for lymph node status in gastric cancer. Those include oncogenes, tumor suppressor genes, growth factors, adhesion molecules, cytokines, chemokines, proteolytic molecules and angiogenic factors. However, the significance of these markers was not consistent with the results of other studies which showed conflicting results or different outcome. It might be caused by research design, technical procedure, different antibodies and the method for the assessment of staining. In the present study, we selected nine molecules from many previous reports as candidate predictors for lymph node metastasis and attempted to clarify which could be the most useful clinically.

The expression of CD44 splice variants (CD44v) on tumor cells, coupled with evidence that upregulation of CD44v confers metastatic potential *in vivo* and results in poor prognosis (69-71), has focused attention on CD44v in the biology of various human malignancies. However, the direct role of CD44v in the metastatic process has remained obscure. It has been reported that CD44v6 in gastric cancer was related to tumor growth and metastasis, especially in lymph node metastasis (13,14). In our study, we demonstrated a significant relationship between CD44v6 expression and lymph node metastasis, lymphatic invasion, depth of invasion and stage by univariate analyses. Multivariate analysis revealed that CD44v6 represents an independent predictor of lymph node metastasis. Additionally, in submucosal carcinoma group, CD44v6 might be the only indicator of lymph node metastasis.

MMP-7 overexpression has been identified generally in a variety of malignant tumors. There is substantial evidence that overexpression of MMP-7 correlates with a more aggressive phenotype. It was initially believed that MMP-7 expressing tumors could proteolytically break down the physical barriers, through degradation of ECM components, thereby initiating tumor invasion, intravasation into vessels, extravasation from the circulation, and local migration at metastatic sites (72,73). Furthermore, recent studies revealed MMP-7 to have multiple biological functions associated with tumor behavior, such as growth, invasion, apoptosis and angiogenesis, by modifying other MMP members and non-ECM molecules (41,73). Our results revealed that MMP-7 stained cells were detected in invasive front of the tumor, and MMP-7 positive tumors were found to be significantly correlated with aggressive clinicopathological factors, such as depth of invasion, stage and lymph node metastasis, whereas, no significant relationship was detected in histological type and lymphatic or venous invasion. We further demonstrated that MMP-7 was useful as an independent predictor of lymph node metastasis by multivariate logistic analysis.

Previous reports have shown that intestinal metaplasia increases the risk for the development of gastric cancer, therefore intestinal metaplasia is an important precancerous lesion in the multistep gastric carcinogenesis (74). Cdx2 is an intestine-specific transcription factor expressed in the epithelium from the duodenum to the distal colon, but not in normal gastric mucosa. As shown in the present study, we investigated the expression of Cdx2 in both nuclei and cytoplasm of tumor cells, 55 cases (40.7%) and 69 cases (51.1%) in 135 gastric cancers, respectively, were found to be positive expression. We showed that negative expression of nuclear Cdx2 was significantly associated with variables suggesting aggressive phenotypes such as undifferentiated type, advanced T stage, increased stage and incidence of lymph node metastasis, by univariate analyses. In contrast, cytoplasmic Cdx2 might not be useful as an indicator of clinicopathological parameters. Multivariate analysis showed that nuclear Cdx2 expression was a significant independent indicator of lymph node status. Our results are consistent with previous studies which suggest that Cdx2 may play a suppressive role in progression and carcinogenesis of gastric carcinoma (28,75).

Given that three indicators, including CD44v6, MMP-7 and nuclear Cdx2, were independent predictive factors for lymph node involvement, we further analyzed the significance of combined expression of these markers. Multivariate analysis revealed that combined CD44v6 (+)/MMP-7 (+)/ nuclear Cdx2 (-) cases were significantly correlated with lymph node metastasis.

Unlike the three molecules mentioned above, regarding other markers, the expression of CD54 and CXCL5 were significantly correlated with deeper depth of invasion and advanced stage of tumor, but not with lymph node status. Likewise, Cyclin B1, nm23 and survivin were not related to lymph node metastasis, although they were significantly higher in differentiated type than in undifferentiated type. Therefore, these findings suggest that CD54, CXCL5, Cyclin

B1, nm23 and survivin might be involved in tumor proliferation or differentiation, whereas not be useful in predicting lymphatic metastasis. On the other hand, RCAS1 was detected in all tumor specimens, and the expression pattern of RCAS1 was found to be related to several clinicopathological variables, including histological type, depth of invasion, venous invasion, stage of tumor and lymph node status. However, multivariate analysis showed that RCAS1 could not independently predict lymph node involvement.

In conclusion, our results provide evidence that positive expression of both CD44v6 and MMP-7, and negative expression of nuclear Cdx2 may serve as powerful indicators for predicting lymph node metastasis in gastric cancer. In addition, combined evaluation of these markers can be clinically useful to predict lymph node metastasis in patients with gastric cancer. This may be helpful to determine the appropriate treatment of gastric cancer.

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