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Phenotypic classification of gastric signet ring cell carcinoma and its relationship with K-ras mutation

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ABSTRACT. We aimed to analyze gastric signet ring cell (SRC) carcinoma subtypes by investigating gastric and intestinal phenotypic marker expression, and explore the relationship between phenotype and K-ras mutation. Immunohistochemistry was performed on 163 SRC carcinoma patient specimens to detect gastric (MUC1, MUC5AC, and MUC6) and intestinal (MUC2 and CDX2) phenotypic markers, and tumors were classified into gastric (G), intestinal (I), and gastrointestinal (GI) phenotypes. DNA was extracted from the formalin-fixed, paraffin-embedded tumor samples, and K-ras mutations in codons 12, 13, and 61 were identified using polymerase chain reaction-based direct DNA sequencing. G, GI, and I phenotypes were observed in 63 (38.6%), 71 (43.5%), and 29 cases (17.8%), respectively. Expression of MUC2 was significantly associated with invasion depth and lymph node

metastasis (P = 0.001 and 0.002, respectively), whereas that of CDX2 significantly corresponded to tumor size and submucosal invasion (P = 0.004 and 0.001, respectively). MUC5AC expression was inversely associated with gastric wall invasion (P = 0.001). Intestinal phenotypic marker expression was positively associated with gastric wall invasion and lymph node metastasis. K-ras mutations, all of which were in codon 12, were detected in 20 (12.27%) tumors, were significantly associated with the I phenotype, and exhibited an inverse relationship with MUC5AC and MUC6 expression. I-phenotype SRC carcinomas should be distinguished from those of the G phenotype because of their increased malignancy regarding invasion and metastasis, and higher K-ras aberration rate. The different K-ras mutation frequencies observed imply distinct genetic mechanisms in the carcinogenesis of I- and G-phenotype gastric SRC carcinomas.

Key words: Gastric carcinoma; Signet ring cell; Phenotype; Mutation; Immunohistochemistry

INTRODUCTION

Gastric adenocarcinoma is the fourth most common malignancy and the second leading cause of cancer-related mortality worldwide (Humar et al., 2007). It has been reported that 3.4-29% of patients with gastric adenocarcinoma demonstrate signet ring cell (SRC) histology (Senapati et al., 2008). Compared with other types of gastric adenocarcinoma, SRC carcinoma tends to be more common among young and female individuals (Yokota et al., 1998). It is characterized by its high frequency of lymph node metastasis, infiltrative growth to the gastric wall, and poor prognosis (Otsuji et al., 1998).

Lauren (1965) classified gastric adenocarcinoma into two major histological types, intestinal and diffuse, based on gland formation tendency. According to this system, gastric SRC carcinoma belongs to the diffuse category. With respect to the histogenesis of these two gastric adenocarcinoma types, intestinal malignancies are thought to result from a multistage process beginning with intestinal metaplasia followed by dysplasia and ultimately, gastric cancer. In contrast, cancers of the diffuse type are believed to arise from the normal gastric mucosa, with no precursor lesion (Tajima et al., 2006). It is believed that these two tumor types involve different genetic pathways during carcinogenesis (Tahara et al., 1996). However, recent reports have shown that both gastric and intestinal phenotypic markers are expressed in gastric adenocarcinoma in a manner that imitates the tissue of origin, irrespective of histological type (Tajima et al., 2001). Tumor phenotypic marker expression has also been associated with tumor aggressiveness in this disease. Tajima et al. (2001, 2004) reported that among patients with gastric carcinomas of the intestinal type, those with gastric (G)-phenotype tumors are at significantly higher risk of peritoneal recurrence and poorer outcome compared to those with intestinal (I)-phenotype malignancies. Conversely, it has been reported that a considerable proportion of gastric SRC carcinomas express intestinal phenotypic markers such as CDX2 and MUC2, and that in this disease, the I phenotype is associated with larger tumors and deeper gastric wall invasion (Bamba et al., 2001; Aihara et al., 2004). Therefore, phenotypic marker expression is closely related to both gastric tumorigenesis and the behavior of gastric adenocarcinoma. It can thus be conjectured that biological differences

exist between SRC carcinomas of the G and I phenotypes.

Members of the ras family are likely the most important proto-oncogenes in human tumorigenesis. It has been reported that up to 28% of patients with gastric adenocarcinoma carry K-ras mutations (Lee et al., 1995; Arber et al., 1997). Hongyo et al. (1995) reported that carcinoma of the intestinal type often closely resembles colorectal cancer, in that activation of K-ras by point mutation occurs relatively early in tumor progression. Correspondingly, K-ras mutation has been found to be frequent in intestinal-type cases in Japan (Hongyo et al., 1995). However, only limited data are available regarding the frequency of K-ras mutation in diffuse-type cancers such as SRC carcinoma. Moreover, the relationship between expression of phenotypic markers and genetic alterations is unknown. To the best of our knowledge, no studies of phenotypic marker expression and K-ras mutation in gastric SRC carcinoma involving a large number of cases have been carried out. The association between phenotypic marker profile and K-ras mutation in this disease remains unclear.

In the present study, the expression of phenotypic markers and presence of K-ras gene mutations were examined in 163 cases of gastric SRC carcinoma. The purpose of this work was to clarify the expression of various phenotypic markers in this condition and its relationship with K-ras aberration.

MATERIAL AND METHODS

Tissues

In total, 163 patients with gastric SRC carcinoma having undergone gastrectomy between January, 2008 and December, 2013 at the First Affiliated Hospital of Nanchang University (Jiangxi, China) were included. SRC carcinoma was defined as an adenocarcinoma predominantly (>50%) comprising isolated or small groups of malignant cells with eccentric crescent-shaped nuclei pushed by intracytoplasmic mucus against the cell membranes. The study group included 95 women and 68 men, with a median age of 51 years (range: 21-82 years). Gastric carcinoma stage was determined according to the International Union Against Cancer 1997 tumor-node-metastasis (TNM) classification of malignant tumors. Written informed consent was obtained from all participants to perform the present work, and the study was approved by the Nanchang University Ethics Committee.

Phenotypic classification of SRC carcinoma

To classify gastric SRC carcinoma phenotypes, we used MUC2 and CDX2 as intestinal markers. MUC2 is a specific marker of goblet cells, whereas CDX2 is expressed in intestinal epithelial cells and ectopically expressed in intestinal metaplasia and gastric carcinomas of the intestinal type (Jung et al., 2007). We chose MUC1, MUC5AC, and MUC6 as markers of the G phenotype, as these are commonly used for this purpose. MUC1 and MUC5AC are expressed in the superficial foveolar epithelium, whereas MUC6 is expressed in the mucous neck cells of the gastric body and deeper glands of the antrum (Guillem et al., 2000).

According to marker expression, tumors were classified into three different phenotypes, namely, G (positive staining for one or more G-phenotype markers, but no I-phenotype markers), I (positive staining for one or more I-phenotype markers, but no G-phenotype markers), and gastrointestinal (GI; positive staining for both G- and I-phenotype markers).

Immunohistochemistry

Tissue specimens were fixed in 10% formalin and embedded in paraffin. One paraffin-embedded tumorous tissue sample was selected for each case and cut into 4-μm sections. The sections were placed in an oven at 60°C for 4 h, deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 20 min. Antigen retrieval was conducted by heating samples at over 90°C for 20 min in 0.01 M sodium citrate buffer, pH 6.0, using a microwave. The sections were then incubated with primary antibodies at appropriate dilutions (Table 1) at 4°C overnight. Bound primary antibodies were detected using Powervision two-step histostaining reagent (PV-6001; Dako, Glostrup, Denmark) as the secondary antibody. Finally, the slides were stained with 3,3'-diaminobenzidine and counterstained with hematoxylin. Samples of normal gastric and normal small intestinal mucosa were used as positive controls for G- and I-phenotype markers, respectively. Negative controls were created by replacing the primary antibodies with phosphate buffered saline.

Table 1. Antibodies used for immunohistochemistry.

Antibody	Clone	Dilution	Specificity	Company
G-phenotype markers				
MUC1	45M1	1:100	Peptide core of MUC1	DAKO (Carpinteria, CA, USA)
MUC5AC	CLH2	1:100	MUC5AC glycoprotein	DAKO
MUC6	CLH5	1:50	MUC6 glycoprotein	DAKO
I-phenotype markers				
MUC2	Ccp58	1:100	MUC2 glycoprotein	DAKO
CDX2	CDX2-88	1:50	Peptide core of CDX2	DAKO

Two experienced pathologists blind to the clinicopathologic data independently examined the staining results. At least 10 high-power fields were chosen randomly at 400X magnification, and >1000 tumor cells were counted in each section. Expression of MUC1, MUC2, MUC5AC, and MUC6 was observed in the cytoplasm and cell membrane, and that of CDX2 was localized to the nucleus. Cases were defined as positive when the number of positive tumor cells was >10% in each section (6,15,16).

Analysis of K-ras mutation

DNA extraction

Microdissection and DNA extraction were performed on 8-10 paraffin-embedded tissue sections, each 8 μm in thickness. Using the hematoxylin-eosin(HE)-stained section as a guide, precisely identified tumor tissue was obtained with care using a needle, to ensure that more than 75% of the recovered material constituted tumor cells, rather than unremarkable connective tissue components, necrotic debris, or cell populations associated with inflammation or hemorrhage. The procured tissue was suspended in 1000 μL lysis buffer [50 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate, and 20 μg/mL proteinase K] and incubated at 55°C overnight. Following its extraction using the phenol-chloroform method, genomic DNA was washed with 75% ethanol and dissolved in 40 μL Tris-EDTA buffer for use in polymerase chain reaction (PCR).

PCR assay

PCR was carried out in a PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). A 227-bp sequence across codons 12 and 13, and a 213-bp sequence in codon 61 of the K-ras gene were amplified using the following primer pairs, respectively: 5'-AGG-CCT-GCT-GAA-AAT-GAC-TG-3' (sense) and 5'-TCA-AAG-AAT-GGT-CCT-GCA-CC-3' (antisense); and 5'-TGT-AAT-AAT-CCA-GAC-TGT-GTT-TCT-CC-3' (sense) and 5'-AGC-TTA-TTA-TAT-TCA-ATT-TAA-ACC-CAC-C-3' (antisense). The PCR mixture (50 μL) contained 2 μL genomic DNA, 25 μL SYBR Green Realtime PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan), 19 μL double-distilled H_2O, and 2 μL each primer. Template DNA was initially denatured for 7 min at 94°C, then 35 cycles of amplification were carried out, each comprising 45 s at 94°C, 45 s at 62°C, and 45 s at 72°C. The reaction was incubated at 72°C for 7 min after the final cycle, and cooled to 4°C before DNA sequencing. Negative controls lacking a DNA template were also processed to exclude the possibility of reagent contamination. Samples from colorectal carcinomas known to be homozygous for K-ras mutations in codons 12, 13, and 61 were used as positive controls.

DNA sequencing

PCR products from each sample were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced on an ABI 3730*xl* sequencer (Genomics Company, Shanghai, China) using a BigDye Terminator v2.0 kit following the manufacturer protocol. Both strands were sequenced for each product, and genomic DNA from control samples was sequenced in parallel to confirm mutations.

Statistical analysis

All data were analyzed using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Associations between protein expression, K-ras mutation, and various clinicopathologic parameters were analyzed using the chi-square test, two-sided Fisher's test, and Spearman's rank correlation analysis. P values ≤ 0.05 were considered to represent statistical significance.

RESULTS

Expression of phenotypic markers and classification of gastric SRC carcinoma phenotype

Both gastric and intestinal markers showed a heterogeneous staining pattern (Figure 1). Expression of MUC1, MUC5AC, MUC6, MUC2, and CDX2 was observed in 20.9 (34), 73.6 (120), 28.8 (47), 46.6 (76), and 39.3% (64) of the 163 gastric SRC carcinomas, respectively.

According to the expression of phenotypic markers, the 163 cases were classified into three phenotypes: 63 (38.6%) exhibited the G phenotype, 71 (43.5%) the GI phenotype, and 29 (17.9%) the I phenotype.

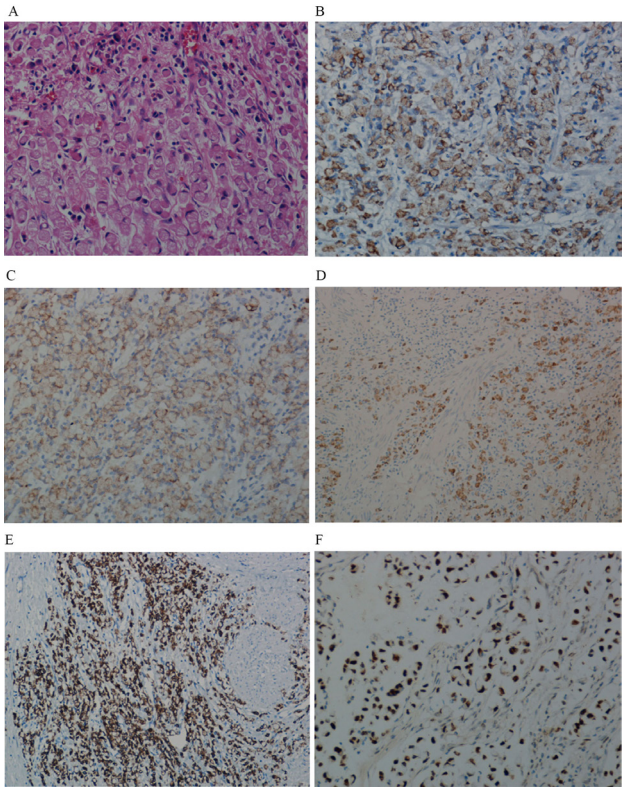

Figure 1. Signet ring cell adenocarcinoma of the gastrointestinal phenotype showing positive MUC1, MUC5AC, MUC6, MUC2, and CDX2 immunohistochemical staining. **A.** Hematoxylin and eosin (400X magnification); **B.** MUC1 [3,3'-diaminobenzidine (DAB), 400X]; **C.** MUC5AC (DAB, 400X); **D.** MUC6 (DAB, 400X); **E.** MUC2 (DAB, 200X); **F.** CDX2 (DAB, 200X). Positive staining for MUC1, MUC5AC, MUC6, and MUC2 can be seen in the signet ring cell cytoplasm and/or membrane, and that for CDX2 in the nucleus.

Relationship between phenotypic markers and clinicopathologic parameters

The relationship between phenotypic marker expression and clinicopathologic parameters is shown in Table 2. Compared with MUC2-negative tumors, MUC2-positive malignancies demonstrated a significantly higher lymph node metastasis rate (31.0 *vs* 55.3%, respectively, P = 0.002) and deeper wall invasion (51.7 *vs* 80.3%, respectively, P = 0.001). No significant correlation was found between MUC2 expression and sex, age, vascular invasion, neural invasion, or TNM stage. In comparison to those negative for CDX2, CDX2-positive tumors were significantly larger (diameter > 5.0 cm; 48.5 *vs* 53.2%, respectively, P = 0.004) and were associated with higher TNM stage (III+IV; 39.4 *vs* 46.9%, respectively, P = 0.002) and deeper wall invasion (47.5 *vs* 92.2%, respectively, P = 0.001). CDX2 expression showed no relationship with other clinicopathologic parameters. Positive MUC5AC staining was inversely associated with submucosal invasion [56.7 (positive) *vs* 88.4% (negative), P = 0.001]. However, no significant correlation was established between MUC5AC expression and other clinicopathologic characteristics. No significant associations were observed between expression of MUC1 or MUC6 and any of the parameters examined.

Table 2. Associations between phenotypic marker expression [N (%)] and clinicopathologic features.

Factor	Cases	MUC2 expression		CDX2 expression		MUC1 expression		MUC5AC expression		MUC6 expression	
		Positive (N = 76)	Negative (N = 87)	Positive (N = 64)	Negative (N = 99)	Positive (N = 34)	Negative (N = 129)	Positive (N = 120)	Negative (N = 43)	Positive (N = 47)	Negative (N = 116)
Gender											
Female	95	46 (60.5)	49 (56.3)	40 (62.5)	55 (55.6)	18 (52.9)	77 (59.7)	73 (60.8)	22 (51.2)	31 (66.0)	64 (55.2)
Male	68	30 (39.5)	38 (43.7)	24 (37.5)	44 (44.4)	16 (47.1)	52 (40.3)	47 (39.2)	21 (48.8)	16 (34.0)	52 (44.8)
Age (years)											
≤50	62	32 (42.1)	30 (34.5)	27 (42.2)	35 (35.4)	17 (50.0)	45 (34.9)	46 (38.3)	16 (37.2)	15 (31.9)	47 (40.5)
>50	101	44 (57.9)	57 (65.5)	37 (57.8)	64 (64.6)	17 (50.0)	84 (65.1)	74 (61.7)	27 (62.8)	32 (68.1)	69 (59.5)
Tumor diameter											
≤5.0 cm	81	35 (46.1)	46 (52.9)	30 (46.8)	51 (51.5)	13 (38.2)	68 (52.7)	61 (50.8)	20 (46.5)	23 (48.9)	58 (50.0)
>5.0 cm	82	41 (53.9)	41 (47.1)	34 (53.2)[a]	48 (48.5)[a]	21 (61.8)	61 (47.3)	59 (49.2)	23 (53.5)	24 (51.1)	58 (50.0)
Depth of wall invasion											
T1	57	15 (19.7)[b]	42 (48.3)[b]	5 (7.8)[c]	52 (52.5)[c]	8 (23.5)	49 (38.0)	52 (43.3)[d]	5 (11.6)[d]	11 (23.4)	46 (39.7)
T2	34	22 (28.9)	12 (13.8)	25 (39.1)	9 (9.1)	5 (14.7)	29 (22.5)	18 (15.0)	16 (37.2)	14 (29.8)	20 (17.2)
T3	54	29 (38.2)	25 (28.7)	28 (43.8)	26 (26.3)	17 (50.0)	37 (28.7)	37 (30.8)	17 (39.5)	19 (40.4)	35 (30.2)
T4	18	10 (13.2)	8 (9.2)	6 (9.4)	12 (12.1)	4 (11.8)	14 (10.8)	13 (10.8)	5 (11.6)	3 (6.4)	15 (12.9)
Vascular invasion											
Positive	32	18 (23.7)	14 (16.1)	15 (23.4)	17 (17.2)	7 (20.6)	25 (19.4)	23 (19.2)	9 (20.9)	12 (25.5)	20 (17.2)
Negative	131	58 (76.3)	73 (83.9)	49 (76.6)	82 (82.8)	27 (79.4)	104 (80.6)	97 (80.8)	34 (79.1)	35 (74.5)	96 (82.8)
Neural invasion											
Positive	52	27 (35.5)	25 (28.7)	26 (40.6)	26 (26.2)	10 (29.4)	42 (32.6)	37 (30.8)	15 (34.8)	17 (36.2)	35 (30.2)
Negative	111	49 (64.5)	62 (71.3)	38 (59.4)	73 (73.8)	24 (70.6)	87 (67.4)	83 (69.2)	28 (65.2)	30 (63.8)	81 (69.8)
Lymph node metastasis											
Positive	69	42 (55.3)[e]	27 (31.0) [e]	31 (48.4)	38 (38.4)	19 (55.9)	50 (38.8)	46 (38.3)	23 (53.5)	24 (51.1)	45 (38.8)
Negative	94	34 (44.7)	60 (69.0)	33 (51.6)	61 (61.6)	15 (44.1)	79 (61.2)	74 (61.7)	20 (46.5)	23 (48.9)	71 (61.2)
TNM stage											
I+II	94	43 (56.6)	51 (58.6)	34 (53.1)	60 (60.6)	17 (50.0)	77 (59.7)	77 (64.2)	17 (39.5)	27 (57.4)	67 (57.7)
III+IV	69	33 (43.4)	36 (41.4)	30 (46.9)[f]	39 (39.4)[f]	17 (50.0)	52 (40.3)	43 (35.8)	26 (60.5)	20 (42.6)	49 (42.3)

TNM = tumor-node-metastasis. [a]P = 0.004 (CDX2 expression); [b]P = 0.001 (MUC2 expression); [c]P = 0.001 (CDX2 expression); [d]P = 0.001(MUC5AC expression); [e]P = 0.002 (MUC2 expression); [f]P = 0.002 (CDX2 expression). [b,c,d]T1 *vs* T2+T3+T4.

Relationship between phenotypic classification and clinicopathologic parameters

Associations between tumor phenotypes and clinicopathologic parameters are shown in Table 3. The proportion of patients with lymph node metastasis was 34.9 (22/63), 40.8 (29/71), and 62.1% (18/29), in the G-, GI-, and I-phenotype groups, respectively. The G phenotype was associated with a significantly lower rate of lymph node metastasis than the other phenotypes identified ($P < 0.01$). Compared to those with tumors of the G phenotype, a greater number of patients with I-phenotype tumors exhibited wall invasion deeper than the submucosal layer (46.0 *vs* 86.2%, respectively, $P < 0.01$) and high TNM stage (III+IV; 14.3 *vs* 44.8%, respectively, $P < 0.01$). Significantly more GI- than G-phenotype tumors were in the larger size category (diameter > 5.0 cm; 54.9 *vs* 46.0%, respectively, $P < 0.01$). Phenotypic classification did not differ according to patient sex, tumor diameter, or vascular invasion.

K-ras gene mutation and its relationship with clinicopathologic findings

Our results concerning K-ras mutations are summarized in Table 3, Table 4, and Table 5. K-ras aberrations, all of which were present in codon 12, were detected in 20 (12.27%) of the 163 specimens. Of these, 40% (8/20) carried the mutation GGT→GTT, 50% (10/20) GGT→GAT, 5% (1/20) GGT→AGT, and 5% (1/20) GGT→TGT (Figure 2). No sequence variations in codons 13 or 61 were detected. Moreover, no significant relationships were noted between clinicopathologic findings and K-ras mutation.

Relationship between phenotypic markers, phenotypic classification, and K-ras mutation

Relationships between the expression of phenotypic markers and K-ras mutation are shown in Table 6. The frequency of K-ras mutation was 6.3 (4/63), 9.9 (7/71), and 31.0%

Table 3. Associations between phenotypic classification, K-ras mutation [N (%)], and clinicopathologic features.

Factor	Cases	Phenotypic classification			K-ras mutation	
		G (N = 63)	GI (N = 71)	I (N = 29)	Positive (N = 20)	P
Sex						
Female	95	36 (57.1)	45 (63.4)	14 (48.3)	12 (12.6)	>0.05
Male	68	27 (42.9)	26 (36.6)	15 (51.7)	8 (11.7)	
Age (years)						
≤50	62	16 (25.4)	34 (47.9)	12 (41.4)	7 (11.3)	>0.05
>50	101	47 (74.6)	37 (52.1)	17 (58.6)	13 (12.9)	
Tumor diameter						
≤5.0 cm	81	34 (54.0)	32 (45.1)[a]	15 (51.7)	7 (8.6)	>0.05
>5.0 cm	82	29 (46.0)	39 (54.9)[a]	14 (48.3)	13 (15.9)	
Depth of wall invasion						
T1	57	34 (54.0)[b]	19 (26.8)	4 (13.8)	5 (8.8)	>0.05
T2	34	4 (6.3)[b]	19 (26.8)	11 (37.9)	3 (8.8)	
T3	54	18 (28.6)[b]	25 (35.2)	11 (37.9)	8 (14.8)	
T4	18	7 (11.1)[b]	8 (11.3)	3 (10.3)	4 (22.2)	
Vascular invasion						
Positive	32	11 (17.5)	16 (22.5)	5 (17.2)	3 (9.4)	>0.05
Negative	131	52 (82.5)	55 (77.5)	24 (82.8)	17 (12.9)	
Neural invasion						
Positive	52	18 (28.6)	24 (33.8)	10 (34.5)	7 (13.7)	>0.05
Negative	111	45 (71.4)	47 (66.2)	19 (65.5)	13 (11.7)	
Lymph node metastasis						
Positive	69	22 (34.9)[c]	29 (40.8)	18 (62.1)	7 (10.1)	>0.05
Negative	94	41 (65.1)[c]	42 (59.2)	11 (37.9)	13 (13.8)	
TNM stage						
I+II	94	54 (85.7)[d]	24 (33.8)	16 (55.2)	10 (10.6)	>0.05
III+IV	69	9 (14.3)[d]	47 (66.2)	13 (44.8)	10 (14.5)	

G = gastric; GI = gastrointestinal; I = intestinal; TNM = tumor-node-metastasis. [a]$P < 0.01$ *vs* GI phenotype; [b,c,d]$P < 0.01$ *vs* I phenotype. [b]T1 *vs* T2+T3+T4.

Table 4. Gastric signet ring cell carcinoma cases for which K-ras mutations were identified.

Case No.	Age (years)/sex	Tumor size	Invasion depth	Vascular invasion	Neural invasion	Lymph node metastasis	MUC2	CDX2	Phenotype	Codon	Mutation
1	47/M	1 x 1	T1	-	+	-	+	-	I	12	GGT→GTT
2	72/F	3 x 2.5	T1	-	-	-	-	-	G	12	GGT→GAT
3	42/M	1.5 x 1.5	T1	-	-	+	+	-	GI	12	GGT→GTT
4	45/F	6 x 4	T1	-	-	+	+	+	I	12	GGT→GTT
5	37/M	3.5 x 3	T1	-	-	-	-	+	GI	12	GGT→GTT
6	54/M	2 x 1	T2	-	-	-	+	+	I	12	GGT→AGT
7	61/M	1 x 1	T2	-	-	-	-	+	GI	12	GGT→TGT
8	63/F	3 x 2	T2	-	-	-	+	-	I	12	GGT→GAT
9	60/F	3 x 2.5	T2	-	-	-	+	-	GI	12	GGT→GAT
10	77/M	x 4.5	T2	+	+	-	+	+	GI	12	GGT→GAT
11	21/F	2 x 1	T2	-	-	-	+	+	I	12	GGT→GAT
12	52/M	3.5 x 3	T2	-	+	-	-	-	G	12	GGT→GTT
13	81/M	8 x 6	T3	-	-	-	-	-	G	12	GGT→GTT
14	56/M	5 x 4	T3	-	+	+	+	+	I	12	GGT→GAT
15	71/M	2 x 1	T3	-	+	-	+	+	I	12	GGT→GTT
16	51/M	1.5 x 1.5	T3	+	+	-	-	+	GI	12	GGT→GTT
17	48/M	3 x 2	T4	-	-	-	+	+	GI	12	GGT→GAT
18	78/M	3 x 3	T4	+	+	+	-	-	G	12	GGT→GAT
19	76/F	1.5 x 1.5	T4	-	-	+	+	+	I	12	GGT→GAT
20	54/F	7.5 x 7	T4	-	-	+	+	-	I	12	GGT→GAT

M = male; F = female; I = intestinal; G = gastric; GI =gastrointestinal.

Table 5. K-ras mutations identified among the signet ring cell carcinoma cases in this study.

Codon	Mutation	Amino acid change	Cases	%
12	GGT→GTT	Gly→Val	8	40 (8/20)
12	GGT→GAT	Gly→Asp	10	50 (10/20)
12	GGT→AGT	Gly→Ser	1	5 (1/20)
12	GGT→TGT	Gly→Sys	1	5 (1/20)

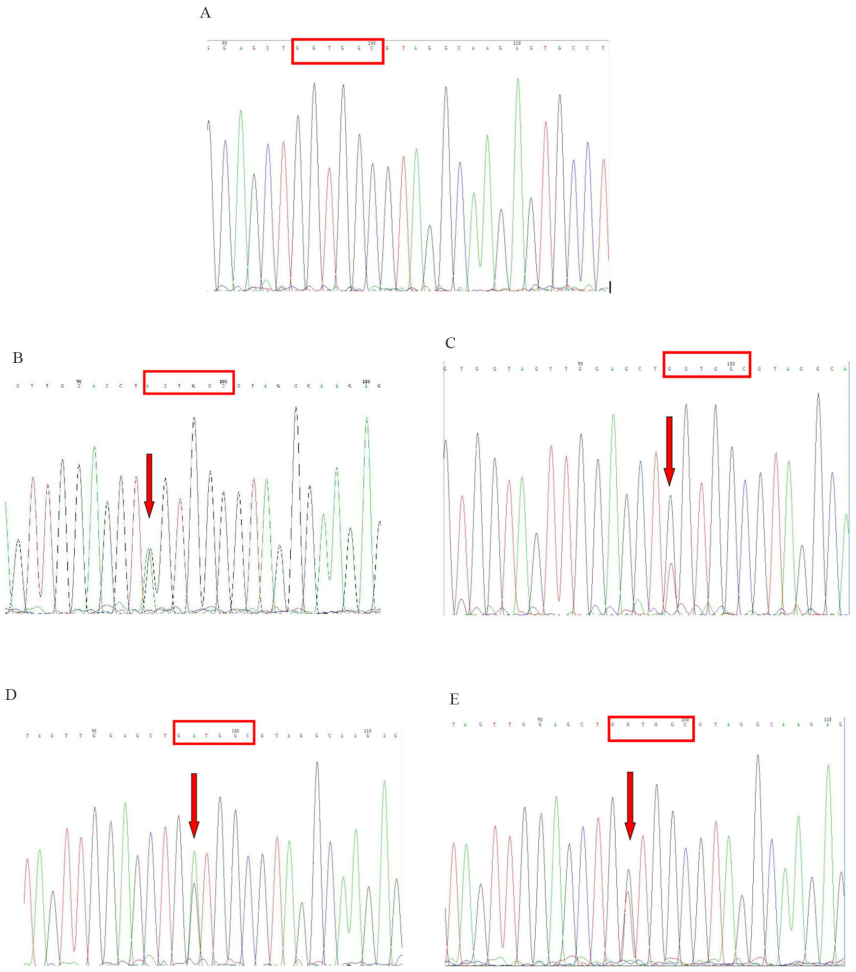

Figure 2. DNA sequencing analysis revealed mutations in codon 12 of the K-ras gene. **A.** Wildtype sequence of codons 12 and 13 (GGTGGC) on the sense strand; **B.** and **C.** GGT→AGT (B) and GGT→TGT (C) mutations of the first base of codon 12 on the sense strand; **D.** and **E.** GGT→GAT (D) and GGT→GTT (E) mutations of the second base of codon 12 on the sense strand.

(9/29) among gastric SRC carcinomas of the G, GI, and I phenotype, respectively. Considering all 163 cases, the frequency of K-ras mutation was significantly lower among MUC5AC-positive tumors than MUC5AC-negative malignancies (8.3 *vs* 23.3%, respectively, P = 0.01). Similarly, tumors positive for MUC6 demonstrated a significantly lower K-ras mutation rate than those negative for this marker (2.1 *vs* 16.4%, respectively, P = 0.012). No significant differences in the incidence of K-ras mutation were observed in relation to MUC1, MUC2, or CDX2 expression. With respect to the relationship between phenotypic classification and K-ras aberration, mutations were observed significantly more often in tumors of the I phenotype (31.0%) than in those of the G (6.3%, P = 0.002) and GI (9.9%, P = 0.009) phenotypes.

Table 6. Associations between phenotypic marker expression, phenotypic classification, and K-ras mutation [N (%)].

	Cases	K-ras mutation (N = 20)	P
MUC1			
Positive	34	3 (8.8)	>0.05
Negative	129	17 (13.2)	
MUC5AC			
Positive	120	10 (8.3)	0.01
Negative	43	10 (23.3)	
MUC6			
Positive	47	1 (2.1)	0.012
Negative	116	19 (16.4)	
MUC2			
Positive	76	13 (17.1)	>0.05
Negative	87	7 (8.0)	
CDX2			
Positive	64	9 (14.1)	>0.05
Negative	99	11 (11.1)	
Phenotypic classification			
G[a]	63	4 (6.3)	0.002
GI[b]	71	7 (9.9)	0.009
I	29	9 (31.0)	

G = gastric; GI = gastrointestinal; I = intestinal. [a]P = 0.002 *vs* I-type; [b]P = 0.009 *vs* I-type.

DISCUSSION

In the present study, 163 gastric SRC carcinomas were classified into G (63; 38.6%), GI (71; 43.5%), and I (29; 17.9%) phenotypes. Previous investigations have reported the frequency of these tumor phenotypes as 17.7-41.1, 28.6-60.1, and 18.5-46.6%, respectively (Sasaki et al., 1999; Tajima et al., 2001), consistent with the present results. This indicates that both the G and I phenotype are frequently observed among gastric SRC carcinomas.

Concerning correlations between clinicopathologic findings and phenotypic markers, our data showed that expression of MUC2 was associated with submucosal invasion and lymph node metastasis, and that of CDX2 was connected to tumor size and depth of invasion. MUC5AC expression was found to be inversely associated with invasion of the submucosa. Combined analysis of gastric and intestinal phenotypic markers showed that tumors expressing only the latter had greater malignant potential in terms of invasion and metastasis compared with tumors of other phenotypes. These findings revealed distinct differences in gastric SRC carcinoma aggressiveness according to phenotypic marker expression. Similar results have been described in several Japanese studies. Yamachika et al. (1997) classified 203 gastric SRC carcinomas into G and I phenotypes with paradoxical concanavalin A (PCS), galactose oxidase-Schiff (GOS), and sialidase-GOS staining, and immunohistochemistry using PGII, SH-9, and TKH-2 antibodies. Their results showed that the proportion of G-phenotype carcinoma cells decreases with invasion depth. Moreover, to define the phenotypes of 54 gastric SRC carcinomas, Bamba et al. (2001) conducted immunohistochemistry to detect MUC2 and M1 expression, and PCS of class III mucin. They found that the larger the mucosal lesion, the more frequently the I phenotype is observed. Aihara et al. (2004) employed MUC2, M1, and MUC6 staining to classify 69 early gastric SRC carcinomas into G and GI phenotypes, finding that the latter correlates with depth of wall invasion. Although there are certain minor differences between their results, these studies demonstrate a tumor phenotypic shift during gastric SRC carcinoma progression. Such carcinomas expressing intestinal phenotypic markers exhibit greater aggressiveness.

In our study, 12.27% of the gastric SRC carcinomas tested showed mutational activation of K-ras. To the best of our knowledge, this is the first detailed description of the presence of activating point mutations in the K-ras oncogene in this disease. Notably, Liu et al. (2015) reported that the K-ras mutation rate is higher in SRC carcinoma than other gastric cancer types. Gastric malignancies harboring such oncogenic K-ras mutations might be treated with targeted MEK inhibitor therapy. Moreover, our present work revealed significant associations between phenotypic classification and K-ras mutation in gastric SRC carcinoma. K-ras aberrations were significantly more common among tumors of the I phenotype, and their presence was inversely associated with expression of MUC5AC and MUC6. These findings suggest that phenotypic marker expression is closely related to K-ras mutation in the tumorigenic phase of this malignancy. The divergent behavior of tumors with different phenotypic marker expression patterns indicates the effects of distinct genetic alterations. Morohara et al. (2006) reported that chromosomal changes detected by a comparative genomic hybridization technique considerably differ according to phenotypic marker expression patterns of differentiated-type gastric carcinomas. Yamazaki et al. (2006) demonstrated that APC gene mutation is relatively common in tumors of the I phenotype, but rather rare in those of the G phenotype, although such aberrations are generally considered to be involved in differentiated-, not diffuse-type carcinomas. Shibata et al. (2003) reported the apoptotic/proliferative index ratio to be significantly lower in G-phenotype tumors than I-phenotype malignancies. Previous molecular genetic studies have shown that gastric tumorigenesis is a multistep process involving the accumulation of genetic alterations (Stadtländer and Waterbor, 1999). Therefore, prior data and our present findings suggest that different genetic pathways associated with phenotypic marker expression patterns might play a role in the tumorigenesis of gastric SRC carcinoma, leading to variations in tumor behavior.

In conclusion, our present investigation showed that phenotypic classification reflected the behavior of gastric SRC carcinoma. Differences in the biological characteristics of tumors with distinct phenotypes might result from genetic dissimilarities during tumorigenesis. The current analysis was limited by its small sample size; therefore, a larger study population will be included in future work to confirm our results.

Conflicts of interest

The authors declare no conflict of interest.

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