



# Phenotypic classification of gastric signet ring cell carcinoma and its relationship with clinicopathologic parameters and prognosis

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## Abstract

**AIM:** To distinguish subtypes of gastric signet ring cell (SRC) carcinoma by investigating the expression of gastric and intestinal phenotypic markers, and to study the significance of phenotypic classification in predicting tumor progression and outcome.

**METHODS:** Immunohistochemistry was performed in 66 cases of SRC carcinoma with MUC2, VILLIN, CDX2, Li-cadherin antibodies as intestinal phenotype markers and MUC5AC, HGM, MUC6 antibodies as gastric phenotype markers, and the relationship was analyzed between the phenotypic expression pattern and clinicopathologic parameters, as well as the 3-year survival rate.

**RESULTS:** Expression of intestinal phenotypic markers was positively associated with tumor size, wall invasion, vascular invasion, lymph node metastasis and tumor-node-metastasis (TNM) stage. Cases expressing one or more intestinal markers had a significant lower survival rate than cases expressing none of the intestinal markers.

**CONCLUSION:** The SRC carcinomas expressing intestinal phenotype markers exhibited a high proliferative potential, bad biological behaviors and poor prognosis. Examination of phenotype expression may be useful in distinguishing histological type and in predicting the prognosis of gastric SRC carcinoma.

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**Key words:** Gastric cancer; Signet ring cell; Phenotype; Immunohistochemistry

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## INTRODUCTION

Gastric cancer is the fourth most common cancer and the second cause of cancer-related death worldwide<sup>[1]</sup>. It has been reported that 3.4%-29% of patients with gastric cancer had signet ring cell (SRC) type histology<sup>[2-7]</sup>.

Comparing with other types of gastric cancer, SRC carcinomas have a tendency to appear in young and female patients<sup>[2,6,7]</sup>. Although most researchers believed that gastric SRC carcinoma is characterized by poor differentiation, strong invasive tendency and poor prognosis, the clinicopathologic parameters of this type of malignancy are still controversial<sup>[2,6-8]</sup>. Hyung *et al*<sup>[8]</sup> reported that patients with early gastric SRC carcinoma have higher survival rates than those with other types of early gastric cancer. Similar result can be seen in Kim *et al*<sup>[2]</sup> study showing that the 5-year survival rate of SRC gastric carcinoma is higher than other types. In contrast, Yokota *et al*<sup>[6]</sup> and Theuer *et al*<sup>[7]</sup> respectively found that the 5-year survival rate of gastric SRC carcinoma is significantly lower than other types and the prognosis for gastric carcinomas with SRC components was worse than those without SRC components. The differences in these results suggest that the biological behaviors and prognosis of gastric SRC carcinoma need further investigations.

Gastric carcinomas have been classified into two main subtypes by Lauren *et al*<sup>[9]</sup>: intestinal and diffuse types. The intestinal type of gastric cancer is thought to arise from the metaplastic epithelium, whereas the diffuse type is thought to arise from the proper mucosa that is not metaplastic. Gastric SRC carcinoma belonged to diffuse type in Lauren's classification. However, recent reports have shown that a considerable proportion of gastric SRC carcinoma express intestinal phenotype markers such as CDX2 and MUC2<sup>[10,11]</sup>. Some studies also showed that gastric SRC carcinomas expressing intestinal phenotype had bigger tumor size and deeper wall invasion<sup>[10,12,13]</sup>. These findings gave us clues that there might be different subtypes of gastric SRC carcinoma with distinct clinico-

pathologic parameters and prognosis.

To investigate the phenotypic expression in gastric SRC carcinomas, we chose CDX2, MUC2, VILLIN and Li-cadherin as intestinal phenotype markers. Among them, CDX2 is intestine-specific *caudal* family homeobox transcription factor which regulate intestinal development and differentiation in mouse model. CDX2 is expressed in epithelial cells of intestine in human adult and ectopically expressed in intestinal metaplasia and intestinal type gastric carcinomas<sup>[14]</sup>. MUC2 is characteristically expressed in goblet cells of native intestinal epithelium and intestinal metastasis of gastric mucosa, but not normal gastric epithelium<sup>[15]</sup>. VILLIN is a cytoskeletal protein characterizing the core microfilament bundle of the microvillus of intestinal epithelium<sup>[16]</sup>. Li-cadherin is a novel member of the cadherin family of cell adhesion molecules which specifically expresses in the epithelia of the liver and intestine of rats. Human Li-cadherin specifically expresses in intestinal epithelium<sup>[17]</sup>. We chose MUC5AC, MUC6 and HGM as gastric phenotype markers which were commonly used to identify gastric phenotype. MUC5AC and HGM glycoprotein localizes in the cytoplasm of foveolar and mucopeptic neck epithelial cells of gastric mucosa, while MUC6 glycoprotein localize in the cytoplasm of mucopeptic neck cells and pyloric glands of the gastric mucosa<sup>[13,18]</sup>.

We performed an immunohistochemical study in 66 cases of gastric SRC carcinoma, and analyzed the relationship between the phenotypic expression pattern and clinicopathologic parameters, the 3-year survival rate as well as the existence of intestinal metaplasia in the surrounding mucosa. In addition, Ki67, CD44v6, E-cadherin and  $\beta$ -catenin expressions were detected immunohistochemically and their relationship with phenotypic classification, tumor progression and prognosis was analyzed.

## MATERIALS AND METHODS

### Tissue materials

A consecutive series of 66 patients with gastric SRC carcinoma were studied. All patients were treated by D2 resection in Beijing Cancer Hospital between May 1994 and July 2004. The criteria of the WHO classification for histological typing of gastric carcinomas was followed: a diagnosis of SRC carcinoma of the stomach was made if an adenocarcinoma contained a predominant component (> 50%) of isolated carcinoma cells with intracellular mucin<sup>[19]</sup>. There were 32 males and 34 females with a mean age of 53 (range, 29-91) years. Stage of gastric carcinoma was described according to the 1997 tumor-node-metastasis (TNM) classification of malignant tumors by the International Union against Carcinoma. Among the 66 cases, 20 cases showed intestinal metaplasia in the surrounding mucosa in H.E. staining, 38 cases had a minimum 3 years of follow-ups.

### Immunohistochemistry

Tumor tissues were fixed in 10% formalin and embedded in paraffin. One paraffin-embedded block of tumorous tissue was selected from each case and was cut into 4  $\mu$ m

Table 1 Antibodies for immunohistochemistry

| Antibody                      | Clone        | Diluent's proportion | Company                       |
|-------------------------------|--------------|----------------------|-------------------------------|
| Gastric phenotypic markers    |              |                      |                               |
| MUC5AC                        | CLH2         | 1:100                | Novocastra, Newcastle, UK     |
| MUC6                          | CLH5         | 1:50                 | Novocastra, Newcastle, UK     |
| HGM                           | 45M1         | 1:50                 | Novocastra, Newcastle, UK     |
| Intestinal phenotypic markers |              |                      |                               |
| MUC2                          | Ccp58        | 1:100                | Novocastra, Newcastle, UK     |
| CDX2                          | CDX2-88      | 1:50                 | BioGenex, San Ramon CA        |
| Li-cadherin                   | Clone 141713 | 1:200                | Neomarker, Fremont, CA, USA   |
| VILLIN                        | CWWB1        | 1:200                | Novocastra, Newcastle, UK     |
| Other antibodies              |              |                      |                               |
| Ki-67                         | K-2          | 1:50                 | Zymed, San Francisco, CA, USA |
| CD44v6                        | VFF-7        | 1:50                 | Zymed, San Francisco, CA, USA |
| E-cadherin                    | 4A2C7        | 1:50                 | Zymed, San Francisco, CA, USA |
| $\beta$ -catenin              | CAT-5H10     | 1:30                 | Zymed, San Francisco, CA, USA |

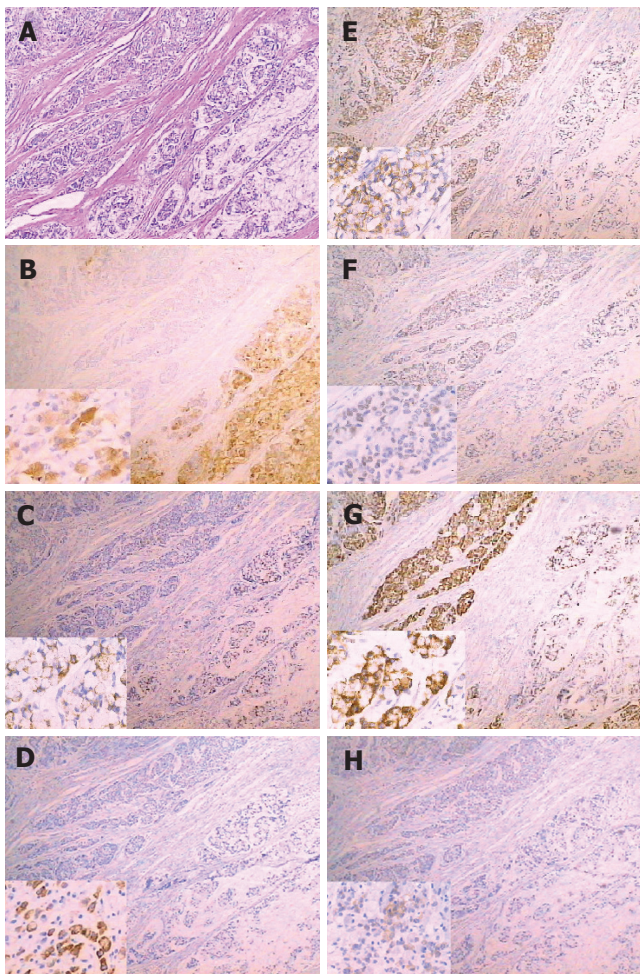
sections. The sections were put in the oven at 60°C for 4 h, deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min. Antigen retrieval was done by microwaving tissues in EDTA buffer (pH 8.0) at over 90°C for 10 min, then cooling at room temperature for 30 min. The sections were then incubated with primary antibodies in appropriate dilutions (Table 1) at 4°C overnight. The primary antibodies were detected using the PowerVision two-step histostaining reagent (PV-6001, Dako, Glostrup, Denmark) as the secondary antibody. Finally, the slides were visualized with 3,3'-diaminobenzidine and counterstained with haematoxylin. Positive controls for gastric phenotype markers were normal gastric mucosa; and for intestinal phenotype markers were normal small intestinal mucosa. Negative controls were performed by replacing the primary antibodies with PBS.

### Immunohistochemical evaluation

Two experienced pathologists independently examined staining, being blind to the clinicopathologic data. At least 10 high-power field at 400  $\times$  magnification were chosen randomly and > 1000 carcinoma cells were counted for each section. The positive expressions for MUC2, VILLIN, MUC5AC, MUC6, HGM and Li-cadherin were located in the cytoplasm and cell membrane. CDX2 was located in nucleus. The cases were defined as positive when > 10% tumor cells were positively stained in each section<sup>[10,14]</sup>.

According to the expression of phenotypic markers, tumors were classified into four differentiated phenotypes. Tumors that were positively stained by one or more gastric phenotypic markers, but no intestinal phenotypic marker,





**Figure 1** A case of signet ring gastric cancer of GI type showed MUC5AC (+), HGM (+), MUC6 (-), Li-cadherin (+), CDX2 (+), MUC2 (+) and VILLIN (-). **A:** HE  $\times 40$ ; **B:** MUC5AC; **C:** HGM; **D:** MUC6; **E:** Li-cadherin; **F:** CDX2; **G:** MUC2; **H:** VILLIN. (**B-H:** Background picture is immunohistochemical staining of this case by each marker,  $\times 40$ ; left and lower pictures show positive staining of each marker,  $\times 400$ ).

were classified as G type; those stained by one or more intestinal phenotypic markers, but no gastric marker, were classified as I type; those positively stained by both gastric and intestinal phenotypic markers were GI (mixed) type; and those stained by none of the phenotypic markers were regarded as carcinomas of the UC (unclassified) type.

Ki67 labeling index was defined as positive nuclear stained cells ratio after counting 1000 cancer cells in 5-10 high power field. E-cadherin and  $\beta$ -catenin showed staining in cell membranes or ectopic staining in cell plasma/nucleus, more than 10% cancer cells stained in one slide was defined as weak positive, and more than 50% cancer cells stained as positive. Positive CD44v6 stain was defined if more than 25% cancer cells were stained in cell plasma and membrane<sup>[20]</sup>.

### Statistical analysis

All data were analysed using SPSS10.0 software. The association of antibody expression with various clinicopathologic parameters was analyzed using the *t* test,  $\chi^2$  test, 2-sided Fisher's test and Spearman's rank correlation analysis. Cumulative survival was estimated

by the Kaplan-Meier method and differences between survival curves were analyzed by the log-rank test. The influence of each variable in survival was analyzed by the multivariate analysis of Cox proportional hazard model (backward, stepwise).  $P \leq 0.05$  was considered statistically significant.

## RESULTS

### Expression of phenotypic markers and phenotypic classification of gastric SRC carcinoma

Both gastric and intestinal markers showed a heterogeneous staining pattern (Figure 1). The positive rates of gastric phenotypic markers MUC5AC, MUC6 and HGM expression in 66 cases were 56.1% (37/66), 10.6% (7/66) and 66.7% (44/66) respectively, while the positive rates of intestinal phenotypic markers MUC2, VILLIN, CDX2 and Li-cadherin were 48.5% (32/66), 7.6% (5/66), 21.2% (14/66) and 36.4% (24/66), respectively. The expression pattern of each case is shown in Table 2.

According to the expression of phenotype markers, we classified the 66 cases of SRC carcinomas into four phenotypes: 17 cases (25.8%) were G type, 10 cases (15.2%) were I type, 31 cases (47.0%) were GI type and 8 cases (12.1%) were UC type.

Based on the number of positive markers, G type cases can be divided into three subgroups G1-G3 (Table 3). Five cases (29.4%) showed only one positive marker among MUC5AC, MUC6 and HGM, 9 cases (52.9%) showed two positive markers and 3 cases (17.6%) expressed all three gastric markers.

I type cases also can be divided into three subgroups I1-I3 based on the number of positive markers. Four cases (40%) expressed only one of the four intestinal phenotypic markers MUC2, VILLIN, CDX2 and Li-cadherin, 3 cases (30%) showed two positive markers and 3 (30%) expressed three markers, and no case expressed all intestinal markers in I type.

There were nine expression patterns GI1-GI9 in GI type cases according to the expression of seven phenotypic markers. Among these subgroups, the cases expressing one gastric marker with one intestinal marker and the cases expressing two gastric markers together with one intestinal marker were most frequently seen, both were 19.4% (6/31). The second common situations were two positive gastric markers coexisting with two or three positive intestinal markers, both being 16.1% (5/31). The other five expression patterns were less common (9 cases or 29.0%). No case expressed three gastric markers together with more than three intestinal markers or one gastric marker together with four intestinal markers.

### Ki67 labeling index and expression of adhesive molecules in gastric SRC carcinomas

Among 66 cases, 22 (33.3%) showed Ki67 labeling index of less than 5%, 18 (27.3%) between 6%-25%, 15 (22.7%) between 26%-50% and 11 (16.7%)  $> 50\%$ . The proportion of cases with Ki67 labeling index  $> 25\%$  was 11.8% (2/17) in G type, 50.0% (5/10) in I type, 48.4% (15/31) in GI type and 37.5% (3/8) in UC type (Table 3).  $\chi^2$  test showed that the proportion of cases with Ki67 labeling index  $>$

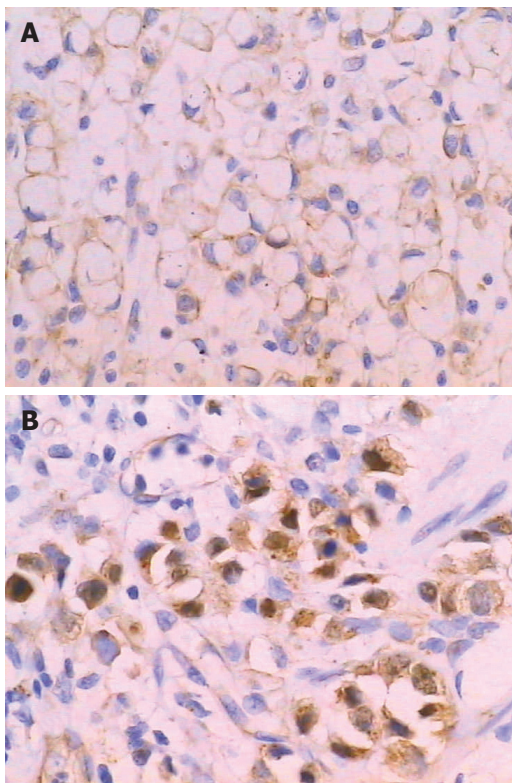
Table 2 Gastric and intestinal phenotypic marker expression in gastric SRC carcinoma

| Phenotype                | Age/Gender | MUC2 | Li-cadherin | CDX2 | VILLIN | HGM | MUC5AC | MUC6 |
|--------------------------|------------|------|-------------|------|--------|-----|--------|------|
| G type ( <i>n</i> = 17)  |            |      |             |      |        |     |        |      |
| 1                        | 65/F       | -    | -           | -    | -      | +   | +      | +    |
| 2                        | 60/M       | -    | -           | -    | -      | +   | +      | -    |
| 3                        | 67/F       | -    | -           | -    | -      | +   | +      | -    |
| 4                        | 29/F       | -    | -           | -    | -      | +   | +      | +    |
| 5                        | 66/F       | -    | -           | -    | -      | +   | +      | +    |
| 6                        | 49/M       | -    | -           | -    | -      | -   | +      | -    |
| 7                        | 49/M       | -    | -           | -    | -      | +   | +      | -    |
| 8                        | 69/F       | -    | -           | -    | -      | +   | +      | -    |
| 9                        | 33/F       | -    | -           | -    | -      | +   | +      | -    |
| 10                       | 38/F       | -    | -           | -    | -      | -   | +      | -    |
| 11                       | 35/F       | -    | -           | -    | -      | +   | +      | -    |
| 12                       | 56/M       | -    | -           | -    | -      | +   | +      | -    |
| 13                       | 39/M       | -    | -           | -    | -      | -   | +      | -    |
| 14                       | 70/F       | -    | -           | -    | -      | +   | +      | -    |
| 15                       | 42/M       | -    | -           | -    | -      | +   | +      | -    |
| 16                       | 42/M       | -    | -           | -    | -      | -   | +      | -    |
| 17                       | 66/M       | -    | -           | -    | -      | +   | -      | -    |
| I type ( <i>n</i> = 10)  |            |      |             |      |        |     |        |      |
| 1                        | 63/M       | +    | +           | +    | -      | -   | -      | -    |
| 2                        | 75/M       | +    | +           | +    | -      | -   | -      | -    |
| 3                        | 59/F       | +    | +           | +    | -      | -   | -      | -    |
| 4                        | 60/M       | +    | -           | -    | -      | -   | -      | -    |
| 5                        | 31/F       | +    | -           | -    | -      | -   | -      | -    |
| 6                        | 76/F       | +    | -           | -    | -      | -   | -      | -    |
| 7                        | 73/F       | +    | -           | -    | +      | -   | -      | -    |
| 8                        | 46/F       | +    | +           | -    | -      | -   | -      | -    |
| 9                        | 62/M       | -    | +           | -    | +      | -   | -      | -    |
| 10                       | 41/M       | -    | +           | -    | -      | -   | -      | -    |
| GI type ( <i>n</i> = 31) |            |      |             |      |        |     |        |      |
| 1                        | 31/M       | +    | -           | -    | -      | +   | +      | -    |
| 2                        | 69/F       | +    | -           | -    | -      | +   | +      | -    |
| 3                        | 61/M       | +    | -           | +    | -      | +   | +      | +    |
| 4                        | 67/M       | +    | -           | -    | -      | +   | +      | +    |
| 5                        | 56/M       | +    | +           | +    | -      | +   | +      | -    |
| 6                        | 47/F       | +    | +           | -    | -      | +   | +      | -    |
| 7                        | 65/M       | +    | +           | -    | -      | +   | +      | -    |
| 8                        | 64/M       | +    | -           | -    | -      | +   | +      | -    |
| 9                        | 63/M       | +    | +           | +    | -      | +   | +      | -    |
| 10                       | 48/F       | +    | +           | -    | -      | +   | +      | +    |
| 11                       | 52/F       | +    | -           | -    | -      | +   | +      | -    |
| 12                       | 65/M       | +    | +           | +    | -      | +   | +      | -    |
| 13                       | 72/M       | +    | +           | -    | -      | +   | +      | -    |
| 14                       | 39/F       | +    | +           | +    | -      | +   | +      | -    |
| 15                       | 36/M       | +    | +           | +    | +      | +   | +      | -    |
| 16                       | 51/M       | +    | +           | -    | -      | +   | +      | -    |
| 17                       | 60/F       | +    | +           | -    | -      | +   | -      | -    |
| 18                       | 46/F       | +    | -           | -    | -      | +   | -      | -    |
| 19                       | 45/F       | +    | -           | -    | -      | +   | -      | -    |
| 20                       | 45/F       | +    | -           | +    | -      | +   | -      | -    |
| 21                       | 55/M       | +    | +           | +    | -      | +   | -      | -    |
| 22                       | 65/F       | +    | -           | -    | -      | +   | -      | -    |
| 23                       | 47/F       | +    | -           | -    | -      | +   | -      | -    |
| 24                       | 60/F       | +    | +           | -    | -      | +   | -      | -    |
| 25                       | 46/F       | -    | -           | +    | -      | +   | +      | +    |
| 26                       | 50/F       | -    | +           | -    | -      | +   | +      | -    |
| 27                       | 54/F       | -    | +           | +    | -      | +   | +      | -    |
| 28                       | 32/M       | -    | -           | -    | +      | +   | +      | -    |
| 29                       | 55/M       | -    | +           | +    | +      | +   | +      | -    |
| 30                       | 38/F       | -    | +           | -    | -      | +   | -      | -    |
| 31                       | 59/M       | -    | +           | -    | -      | +   | -      | -    |
| U type ( <i>n</i> = 8)   |            |      |             |      |        |     |        |      |
| 1                        | 36/M       | -    | -           | -    | -      | -   | -      | -    |
| 2                        | 49/F       | -    | -           | -    | -      | -   | -      | -    |
| 3                        | 39/M       | -    | -           | -    | -      | -   | -      | -    |
| 4                        | 61/F       | -    | -           | -    | -      | -   | -      | -    |
| 5                        | 70/F       | -    | -           | -    | -      | -   | -      | -    |
| 6                        | 67/M       | -    | -           | -    | -      | -   | -      | -    |
| 7                        | 64/M       | -    | -           | -    | -      | -   | -      | -    |
| 8                        | 34/F       | -    | -           | -    | -      | -   | -      | -    |

**Table 3** Different expression patterns of phenotype markers and Ki67 and  $\beta$ -catenin expression in gastric SRC carcinoma

| Phenotype of gastric SRC carcinomas | Number of positive GPM | Number of positive IPM | Cases | Ki67 index > 25%        | $\beta$ -catenin membrane expression | Plasma/Nucleus $\beta$ -catenin expression |
|-------------------------------------|------------------------|------------------------|-------|-------------------------|--------------------------------------|--|
| G type                              |                        |                        | 17    | 2 (11.8%)               | 11 (64.7%)                           | 2 (11.8%)                                  |
| G <sub>1</sub>                      | 1                      | 0                      | 5     | 0                       | 3 (60.0%)                            | 0  |
| G <sub>2</sub>                      | 2                      | 0                      | 9     | 2 (22.2%)               | 6 (66.7%)                            | 1 (11.1%)                                  |
| G <sub>3</sub>                      | 3                      | 0                      | 3     | 0                       | 2 (66.7%)                            | 1 (33.3%)                                  |
| I type                              |                        |                        | 10    | 5 (50.0%)               | 1 (10.0%) <sup>b</sup>               | 4 (40.0%)                                  |
| I <sub>1</sub>                      | 0                      | 1                      | 4     | 1 (25.0%)               | 0                                    | 1 (25.0%)                                  |
| I <sub>2</sub>                      | 0                      | 2                      | 3     | 3 (100%)                | 0                                    | 2 (66.7%)                                  |
| I <sub>3</sub>                      | 0                      | 3                      | 3     | 1 (33.3%)               | 1 (33.3%)                            | 1 (33.3%)                                  |
| GI type                             |                        |                        | 31    | 15 (48.4%) <sup>a</sup> | 12 (36.4%)                           | 14 (45.2%) <sup>a</sup>                    |
| GI <sub>1</sub>                     | 1                      | 1                      | 6     | 3 (50.0%)               | 2 (33.3%)                            | 3 (50.0%)                                  |
| GI <sub>2</sub>                     | 1                      | 2                      | 3     | 1 (33.3%)               | 1 (33.3%)                            | 1 (33.3%)                                  |
| GI <sub>3</sub>                     | 1                      | 3                      | 1     | 0                       | 0                                    | 1 (100%)                                   |
| GI <sub>4</sub>                     | 2                      | 1                      | 6     | 3 (50.0%)               | 3 (50.0%)                            | 3 (50.0%)                                  |
| GI <sub>5</sub>                     | 2                      | 2                      | 5     | 4 (80.0%)               | 4 (80.0%)                            | 2 (40.0%)                                  |
| GI <sub>6</sub>                     | 2                      | 3                      | 5     | 2 (40.0%)               | 1 (20.0%)                            | 1 (20.0%)                                  |
| GI <sub>7</sub>                     | 2                      | 4                      | 1     | 0                       | 0                                    | 1 (100%)                                   |
| GI <sub>8</sub>                     | 3                      | 1                      | 2     | 1 (50.0%)               | 1 (50.0%)                            | 1 (50.0%)                                  |
| GI <sub>9</sub>                     | 3                      | 2                      | 2     | 1 (50.0%)               | 0                                    | 1 (50.0%)                                  |
| U type                              | 0                      | 0                      | 8     | 3 (37.5%)               | 4 (50.0%)                            | 3 (37.5%)                                  |

GPM: Gastric phenotype marker. IPM: Intestinal phenotype marker. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.05$  vs G type.



**Figure 2** A:  $\beta$ -catenin membrane expression in a G type case ( $\beta$ -catenin staining,  $\times 400$ ); B:  $\beta$ -catenin plasma/nucleus expression in a GI type case ( $\beta$ -catenin staining,  $\times 400$ ).

25% was significantly lower in G type than in GI type,  $P = 0.011$ . Compared with G type, the proportion of cases with Ki67 labeling index > 25% was higher in I type, but the difference was not significant after 2-sided Fisher's test,  $P = 0.065$ . No difference was found between I type

and GI type or UC type and other three types. Compared with G + UC type (cases expressed none of the intestinal markers), GI + I type (cases expressed one or more intestinal markers) had significantly higher proportion of cases with Ki67 labeling index > 25%,  $P = 0.012$ .

$\beta$ -catenin stained extensively in positive cases of gastric SRC carcinomas. In 28 cases (42.4%),  $\beta$ -catenin mainly expressed on membrane, and 23 cases (34.8%) were mainly stained in plasma/nucleus (Figure 2). Both membrane and plasma/nucleus stained cases accounted for 13.6% (9/66) while negative stained cases comprised 36.4% (24/66) (Table 3). Significant difference of  $\beta$ -catenin plasma/nucleus staining was found between GI + I type and G + UC type (43.9% vs 20.0%  $P = 0.048$ ). The proportion of cases with Ki67 labeling index > 25% was significantly higher in  $\beta$ -catenin plasma/nucleus positive cases than in plasma/nucleus negative cases (56.5% vs 30.2%,  $P = 0.037$ ).

The positive rate of CD44v6 in 66 gastric SRC carcinomas was 7.6% (5/66), two CD44v6 positive cases were G type, three were GI type and one belonged to I type. Only 2 cases (3.0%) in this study showed weakly stained E-cadherin, including one G type case and one GI type case. No difference was found between CD44v6, E-cadherin expression and phenotypes.

#### **Correlation between expression of phenotypic markers, Ki67 labeling index, adhesive molecules and clinicopathologic parameters**

MUC2 (+) cases had a significantly higher lymph node metastasis rate (75.0% vs 38.2%) and vascular invasion rate (59.4% vs 20.6%) than MUC2 (-) cases ( $P = 0.003$  and  $0.001$  respectively), (Table 4). The expression of MUC2 was also significantly higher in cases with larger tumor diameter ( $\geq 5$  cm), deeper wall invasion (T2 + T3 + T4) and higher TNM stage (III + IV) ( $P = 0.001$ ,  $0.008$  and  $0.007$ ,



Table 4 Correlation between muc2, cdx2, Li-cadherin, Ki-67 index and clinicopathologic features *n* (%)

| Factors                | Cases | MUC2 expression           |                           |                     | CDX2 expression           |                           |                     | Li-cadherin expression      |                             |                     | Ki-67 index               |                           |                     |
|------------------------|-------|---------------------------|---------------------------|---------------------|---------------------------|---------------------------|---------------------|-----------------------------|-----------------------------|---------------------|---------------------------|---------------------------|---------------------|
|                        |       | MUC2-<br>( <i>n</i> = 34) | MUC2+<br>( <i>n</i> = 32) | <i>P</i>            | CDX2-<br>( <i>n</i> = 52) | CDX2+<br>( <i>n</i> = 14) | <i>P</i>            | Li-cad-<br>( <i>n</i> = 42) | Li-cad+<br>( <i>n</i> = 24) | <i>P</i>            | ≤ 25%<br>( <i>n</i> = 40) | > 25%<br>( <i>n</i> = 26) | <i>P</i>            |
| Gender                 |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| Male                   | 32    | 17 (50.0)                 | 15 (46.9)                 | > 0.05              | 23 (44.2)                 | 9 (64.3)                  | > 0.05              | 18 (42.9)                   | 14 (58.3)                   | > 0.05              | 18 (45.0)                 | 14 (53.8)                 | > 0.05              |
| Female                 | 34    | 17 (50.0)                 | 17 (53.1)                 |                     | 29 (55.8)                 | 5 (35.7)                  |                     | 24 (57.1)                   | 10 (41.7)                   |                     | 22 (55.0)                 | 12 (46.2)                 |                     |
| Age (yr)               |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| Mean ± SE              | 66    | 50.9 ± 2.2                | 56.0 ± 2.2                | > 0.05 <sup>1</sup> | 53.5 ± 1.9                | 52.9 ± 3.1                | > 0.05 <sup>1</sup> | 51.9 ± 3.0                  | 54.6 ± 2.6                  | > 0.05 <sup>1</sup> | 53.9 ± 2.2                | 51.0 ± 2.3                | > 0.05 <sup>1</sup> |
| Tumor diameter         |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| < 5.0 cm               | 32    | 23 (67.6)                 | 9 (28.1)                  | 0.001               | 30 (57.7)                 | 2 (14.3)                  | 0.004               | 24 (57.1)                   | 8 (33.3)                    | > 0.05              | 24 (60.0)                 | 8 (30.8)                  | 0.02                |
| ≥ 5.0 cm               | 34    | 11 (32.4)                 | 23 (71.9)                 |                     | 22 (42.3)                 | 12 (85.7)                 |                     | 18 (42.9)                   | 16 (66.7)                   |                     | 16 (40.0)                 | 18 (69.2)                 |                     |
| Depth of wall invasion |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| T <sub>1</sub>         | 13    | 11 (32.4)                 | 2 (6.3)                   | 0.008 <sup>2</sup>  | 13 (25.0)                 | 0                         | > 0.05              | 10 (23.8)                   | 3 (12.5)                    | > 0.05              | 11 (27.5)                 | 2 (7.7)                   | 0.048 <sup>2</sup>  |
| T <sub>2</sub>         | 22    | 10 (29.4)                 | 12 (37.5)                 |                     | 16 (30.8)                 | 6 (42.9)                  |                     | 15 (35.7)                   | 7 (29.2)                    |                     | 14 (35.0)                 | 8 (30.8)                  |                     |
| T <sub>3</sub>         | 22    | 10 (29.4)                 | 12 (37.5)                 |                     | 15 (28.8)                 | 7 (50.0)                  |                     | 11 (26.2)                   | 11 (45.8)                   |                     | 10 (25.0)                 | 12 (46.2)                 |                     |
| T <sub>4</sub>         | 9     | 3 (8.8)                   | 6 (18.8)                  |                     | 8 (15.4)                  | 1 (7.1)                   |                     | 6 (14.3)                    | 3 (12.5)                    |                     | 5 (12.5)                  | 4 (15.4)                  |                     |
| Lymph node metastasis  |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| LN (-)                 | 29    | 21 (61.8)                 | 8 (25.0)                  | 0.003               | 25 (48.1)                 | 4 (28.6)                  | > 0.05              | 20 (47.6)                   | 9 (37.5)                    | > 0.05              | 22 (55.0)                 | 7 (26.9)                  | 0.025               |
| LN (+)                 | 37    | 13 (38.2)                 | 24 (75.0)                 |                     | 27 (51.9)                 | 10 (71.4)                 |                     | 22 (52.4)                   | 15 (62.5)                   |                     | 18 (45.0)                 | 19 (70.1)                 |                     |
| Distant metastasis     |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| M <sub>0</sub>         | 60    | 32 (94.1)                 | 28 (87.5)                 | > 0.05              | 48 (92.3)                 | 12 (85.7)                 | > 0.05              | 39 (92.9)                   | 21 (87.5)                   | > 0.05              | 36 (90.0)                 | 24 (92.3)                 | > 0.05              |
| M <sub>1</sub>         | 6     | 2 (5.9)                   | 4 (12.5)                  |                     | 4 (7.7)                   | 2 (14.3)                  |                     | 3 (7.1)                     | 3 (12.5)                    |                     | 4 (10.0)                  | 2 (7.7)                   |                     |
| Vascular invasion      |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| V (-)                  | 40    | 27 (79.4)                 | 13 (40.6)                 | 0.001               | 34 (65.4)                 | 6 (42.9)                  | > 0.05              | 31 (73.8)                   | 9 (37.5)                    | 0.004               | 28 (70.0)                 | 12 (46.2)                 | > 0.05              |
| V (+)                  | 26    | 7 (20.6)                  | 19 (59.4)                 |                     | 18 (34.6)                 | 8 (57.1)                  |                     | 11 (26.2)                   | 15 (62.5)                   |                     | 12 (30.0)                 | 14 (53.8)                 |                     |
| TNM stage              |       |                           |                           |                     |                           |                           |                     |                             |                             |                     |                           |                           |                     |
| I + II                 | 32    | 22 (64.7)                 | 10 (31.2)                 | 0.007               | 27 (51.9)                 | 5 (35.7)                  | > 0.05              | 21 (50.0)                   | 11 (45.8)                   | > 0.05              | 24 (60.0)                 | 8 (30.8)                  | 0.02                |
| III + IV               | 34    | 12 (35.3)                 | 22 (68.8)                 |                     | 25 (48.1)                 | 9 (64.3)                  |                     | 21 (50.0)                   | 13 (54.2)                   |                     | 16 (40.0)                 | 18 (69.2)                 |                     |

<sup>1</sup>*t* test; <sup>2</sup>T<sub>1</sub> vs T<sub>2</sub> + T<sub>3</sub> + T<sub>4</sub>.

respectively). No significant correlation was found between MUC2 expression and gender, age and distant metastasis. There was significantly higher CDX2 expression in cases with larger tumor diameter ( $\geq 5$  cm) ( $P < 0.01$ ), and higher Li-cadherin expression in vascular invaded cases ( $P < 0.01$ ). The expression of CDX2 and Li-cadherin was not found to correlate with other clinicopathologic parameters. No significant association was observed between VILLIN, MUC5, MUC6, HGM expression and any of the clinicopathologic parameters.

The relationship between phenotypic classification and clinicopathologic parameters is shown in Table 5. The proportions of positive lymph node metastatic cases in four phenotypes were 23.5% (4/17) in G type, 60.0% (6/10) in I type, 74.2% (23/31) in GI type and 50.0% (4/8) in UC type, respectively. The frequency of vascular invasion was 11.8% (2/17) in G type, 30.3% (3/10) in I type, 61.3% (19/31) in GI type and 25.0% (2/8) in UC type. G phenotype cases had significantly lower rates of lymph node metastasis and vascular invasion than other phenotypes (both  $P < 0.01$ ). More cases of GI type SRC carcinomas had tumor diameters  $\geq 5$  cm (67.7% vs 11.8%,  $P < 0.001$ ), wall invasion deeper than submucosa layer (96.8% vs 58.8%,  $P = 0.002$ ) and higher (III + IV) TNM stage (63.1% vs 29.1%,  $P = 0.035$ ) than G type cases. I type

also had more cases with tumor diameters  $\geq 5$  cm than G type (70.0% vs 11.8%,  $P = 0.002$ ). No difference was found between UC type and other three types. Phenotypic classification had no correlation with patient's gender, age and distant metastasis.

Differences in biological behaviors were observed between G + UC type (cases expressed none of the intestinal markers) and GI + I type (cases expressed one or more intestinal markers), the latter had higher proportion of vascular invasion (53.7% vs 16.0%,  $P = 0.002$ ) and lymph node metastasis (70.0% vs 32.0%,  $P = 0.002$ ), larger tumor diameters (68.3% vs 24.0%,  $P < 0.001$ ), deeper wall invasion (90.2% vs 64.0%,  $P = 0.023$ ) and higher TNM stage (61.0% vs 36.0%,  $P = 0.048$ ). Spearman's rank correlation analysis showed that the number of positive intestinal phenotype markers in one case was positively correlated with higher rates of lymph node metastasis ( $P < 0.01$ ) and vascular invasion ( $P < 0.01$ ), larger tumor diameter ( $P < 0.01$ ) and deeper wall invasion ( $P < 0.05$ ), (Figure 3).

$\chi^2$  test showed that, comparing with the cases with Ki67 labeling index  $\leq 25\%$ , the cases with Ki67 labeling index  $> 25\%$  had a higher rate of lymph node metastasis (70.1% vs 45.0%,  $P = 0.025$ ), larger tumor diameters (69.2% vs 40.0%,  $P = 0.020$ ), deeper wall invasion (92.3%

Table 5 Correlation between four phenotypes of gastric SRC carcinoma and clinicopathologic features *n* (%)

| Factors                | <i>n</i> | Four phenotypes of gastric SRC carcinoma |                         |                          |                         |
|------------------------|----------|--|-------------------------|--------------------------|-------------------------|
|                        |          | G type ( <i>n</i> = 17)                  | I type ( <i>n</i> = 10) | GI type ( <i>n</i> = 31) | UC type ( <i>n</i> = 8) |
| Gender                 |          |  |                         |                          |                         |
| Male                   | 32       | 8 (47.1)                                 | 5 (50.0)                | 15 (48.4)                | 4 (50.0)                |
| Female                 | 34       | 9 (52.9)                                 | 5 (50.0)                | 16 (51.6)                | 4 (50.0)                |
| Age (yr)               |          |  |                         |                          |                         |
| Mean ± SE              | 66       | 53.7 ± 2.9                               | 49.1 ± 4.0              | 54.7 ± 2.5               | 52.8 ± 4.3              |
| Tumor diameter         |          |  |                         |                          |                         |
| < 5.0 cm               | 32       | 15 (88.2) <sup>a</sup>                   | 3 (30.0) <sup>b</sup>   | 10 (32.3)                | 4 (50.0)                |
| ≥ 5.0 cm               | 34       | 2 (11.8) <sup>a</sup>                    | 7 (70.0) <sup>b</sup>   | 21 (67.7)                | 4 (50.0)                |
| Depth of wall invasion |          |  |                         |                          |                         |
| T <sub>1</sub>         | 13       | 7 (41.2) <sup>c</sup>                    | 3 (30.0)                | 1 (3.2)                  | 2 (25.0)                |
| T <sub>2</sub>         | 22       | 5 (29.1) <sup>c</sup>                    | 2 (20.0)                | 12 (38.7)                | 3 (37.5)                |
| T <sub>3</sub>         | 22       | 4 (23.5) <sup>c</sup>                    | 2 (20.0)                | 13 (41.9)                | 3 (37.5)                |
| T <sub>4</sub>         | 9        | 1 (5.9) <sup>c</sup>                     | 3 (30.0)                | 5 (16.1)                 | 0                       |
| Lymph node metastasis  |          |  |                         |                          |                         |
| LN (-)                 | 29       | 13 (76.5) <sup>c</sup>                   | 4 (40.0)                | 8 (25.8)                 | 4 (50.0)                |
| LN (+)                 | 37       | 4 (23.5) <sup>c</sup>                    | 6 (60.0)                | 23 (74.2)                | 4 (50.0)                |
| Distant metastasis     |          |  |                         |                          |                         |
| M <sub>0</sub>         | 60       | 16 (94.1)                                | 8 (80.0)                | 28 (90.3)                | 8 (100)                 |
| M <sub>1</sub>         | 6        | 1 (5.9)                                  | 2 (20.0)                | 3 (9.7)                  | 0                       |
| Vascular invasion      |          |  |                         |                          |                         |
| V (-)                  | 40       | 15 (88.2) <sup>c</sup>                   | 7 (70.0)                | 12 (38.7)                | 6 (75.0)                |
| V (+)                  | 26       | 2 (11.8) <sup>c</sup>                    | 3 (30.0)                | 19 (61.3)                | 2 (25.0)                |
| TNM stage              |          |  |                         |                          |                         |
| I + II                 | 32       | 12 (70.6) <sup>d</sup>                   | 4 (40.0)                | 12 (38.7)                | 4 (50.0)                |
| III + IV               | 34       | 5 (29.1) <sup>d</sup>                    | 6 (60.0)                | 19 (61.3)                | 4 (50.0)                |
| IM                     |          |  |                         |                          |                         |
| IM (-)                 | 46       | 11 (64.7)                                | 6 (60.0)                | 23 (74.2)                | 6 (75.0)                |
| IM (+)                 | 20       | 6 (35.3)                                 | 4 (40.0)                | 8 (25.8)                 | 2 (25.0)                |

IM: Intestinal metaplasia in the surrounding mucosa. <sup>a</sup>*P* < 0.001 vs GI type; <sup>b</sup>*P* < 0.01 vs I type; <sup>c</sup>*P* < 0.01 vs GI type; <sup>d</sup>*P* < 0.05 vs GI type.

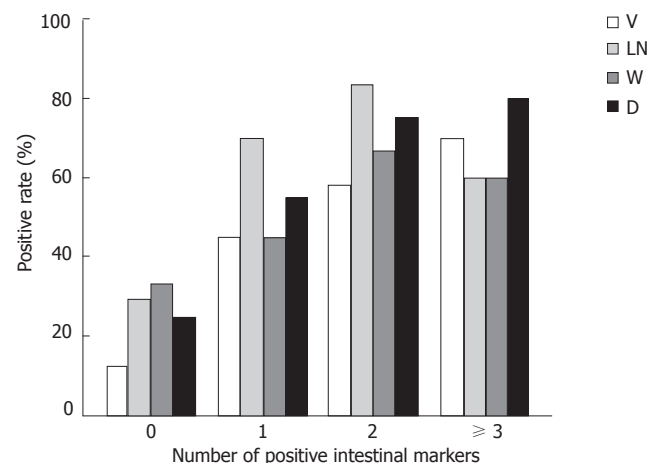
vs 72.5%, *P* = 0.048) and higher TNM stage (69.2% vs 40.0%, *P* = 0.020), (Table 4). No correlation was found between expression of β-catenin, CD44v6, E-cadherin and clinicopathologic parameters.

#### Correlation between expression of phenotypic markers, Ki67 labeling index, adhesive molecules and prognosis

In the 38 cases with 3-year follow-up, vascular invasion, lymph node metastasis, TNM stage and Ki67 labeling index > 25% were found correlated with poor prognosis (*P* = 0.0001, 0.0010, 0.0012 and 0.0141, respectively). GI + I type had significantly a lower overall survival rate than G + UC type (31.82% vs 68.75%, *P* = 0.0146 (Figure 4). Cox proportional hazard model (backward, stepwise) showed that vascular invasion and TNM stage were independent prognosis factors (*P* = 0.017 and 0.037, respectively), while GI + I type, lymph node metastasis and Ki67 labeling index were not independent prognosis factors.

#### Correlation between expression of phenotypic markers and intestinal metaplasia in surrounding mucosa

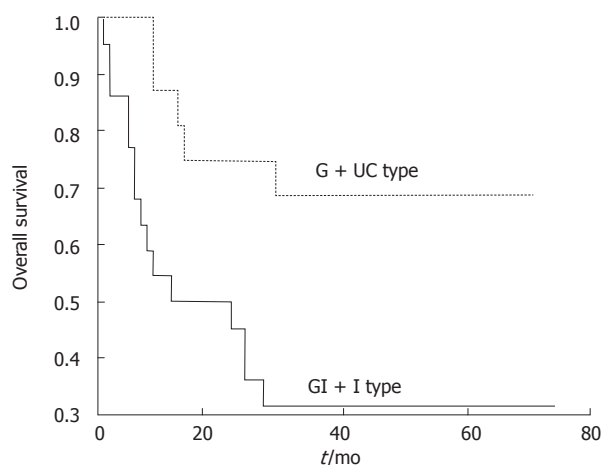
Among 66 SRC carcinomas, 20 cases (30.3%) possessed intestinal metaplasia in the surrounding mucosa in H.E. staining slides. The proportions of metaplastic cases in four phenotypes were between 25%-40%, no significant difference was found (Table 5). Expression of seven intestinal and gastric phenotypic markers was not correlated with metaplasia in the surrounding mucosa (data not shown).



**Figure 3** Clinicopathological features of different intestinal marker expression patterns. V: vascular invasion; LN: lymph node metastasis; W: wall invasion deeper than submucosa layer; D: tumor diameter larger than 5 cm. The number of positive intestinal phenotypic markers in one case was positively correlated with higher rates of lymph node metastasis (*P* < 0.01), vascular invasion (*P* < 0.01), larger tumor diameter (*P* < 0.01) and deeper wall invasion (*P* < 0.05). (Spearman's rank correlation analysis).

## DISCUSSION

The phenotypic marker expression of tumors is conventionally thought to imitate that of the tissue of origin. In this study, immunohistochemistry showed that three gastric phenotypic markers were positively stained in



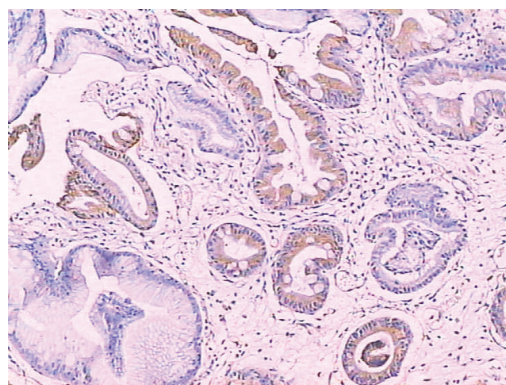
**Figure 4** Gastric SRC carcinomas expressing intestinal phenotype markers (GI+I type cases) had significantly lower survival rates than those with no expression of intestinal phenotype markers (G + UC type cases),  $P = 0.0146$  by Kaplan-Meier method.

normal gastric mucosa, but negatively in intestinal mucosa, while four intestinal phenotypic markers were positive in intestinal mucosa and metaplasia of stomach, but negative in normal gastric mucosa. These results indicated that the phenotypic markers in our study possessed tissue specificity.

The expression of MUC2, CDX2 and Li-cadherin was positively associated with tumor growth or invasion, and cases expressing more positive intestinal markers showed higher invasive potential, and cases expressing one or more intestinal markers had a lower survival rate than cases with none of intestinal markers positive. Combined analysis of gastric and intestinal phenotypic markers displayed that cases only expressing gastric markers had better biologic behaviors than the cases expressing both gastric and intestinal phenotype. These results suggest that different expression patterns of phenotype are associated with clinicopathologic parameters and prognosis.

In our study, Ki67 was positively associated with vascular invasion, lymph node metastasis, tumor size and TNM stage. Ki67 is well known to express in all cell cycle phases except in the resting cell (G0 phase). Ki67 immunohistochemistry has been substituted extensively for mitotic counting in assessing tumor cell proliferation<sup>[21]</sup>. A great deal of studies have demonstrated that Ki67 is correlated with poor differentiation and worse biological behaviors in many malignancies<sup>[22-24]</sup>. In gastric SRC carcinomas of this study, the intestinal phenotype positive cases (GI + I type) had higher Ki67 labeling index, indicating that these cases had a higher proliferative potential which was concordant with the worse biological behaviors shown by this kind of gastric SRC carcinomas.

$\beta$ -catenin is a multifunctional protein, which takes part in intercellular adhesion on cell membrane and also plays an important role in Wnt signal pathway. Most  $\beta$ -catenins are located on cell membrane in normal mature cells and mediate cell adhesion by connecting with E-cadherin. If  $\beta$ -catenin accumulates in cytoplasm and translocates to the nucleus, it binds to the Tcf/Lef family of transcription factors, resulting in increased transcription of numerous



**Figure 5** Li-cadherin expression in intestinal metaplasia of gastric mucosa (Li-cadherin staining,  $\times 100$ ).

genes, including c-myc and cyclin D1, and abnormal proliferation<sup>[25]</sup>. Our finding is consistent with previous reports that plasma/nucleus staining of  $\beta$ -catenin is positively correlated with higher Ki67 labeling index. The intestinal phenotype positive cases with high plasma/nucleus  $\beta$ -catenin staining rates indicated that the high proliferative activity of these cases is associated with activation of Wnt signal pathway. GI type SRC carcinomas had lower  $\beta$ -catenin membrane staining rates than G type, indicating that the higher metastasis ability of GI type may be associated with low cellular adhesion due to reduction of membrane  $\beta$ -catenin.

Li-cadherin is a novel member of the cadherin family specifically expressed in polarized epithelia of the liver and intestine of rats which was discovered in 1994<sup>[26]</sup>. Human Li-cadherin is an intestine specific cell adhesive molecule that also ectopically expressed in the metaplastic mucosa of the stomach as well as in gastric adenocarcinomas of the intestinal type<sup>[27]</sup>. Ko *et al*<sup>[28]</sup> reported that lymph node metastasis was significantly associated with the expression of Li-cadherin. Ito *et al*<sup>[29]</sup> reported Li-cadherin had higher expression in advanced gastric carcinoma than early gastric cancer, and associated with poor prognosis. Oue *et al*<sup>[30]</sup> discovered that Li-cadherin is positively correlated with tumor stages. Our result is concordant with these reports, which indicates that the intestinal phenotype positive cases possessed worse biological behavior possibly due to the expression of intestinal specific adhesive molecule Li-cadherin. However, the mechanism of Li-cadherin expression associated with worse biological behavior remains to be investigated<sup>[30]</sup>. In addition, we studied Li-cadherin immunohistochemically in 20 gastric biopsies with intestinal metaplasia and found that Li-cadherin was positively detected in all cases (Figure 5). Taken together, ours and previous studies suggest that Li-cadherin may be a novel specific marker for intestinal metaplasia.

In this study, we found an increased intestinal phenotype expression along with the progression of gastric SRC carcinoma. This phenomenon may be an appearance of abnormal differentiation and increased heterogeneity. Similar results can be seen in several Japanese reports: Yamachika *et al*<sup>[12]</sup> classified 203 gastric signet ring cell carcinomas into gastric phenotype and intestinal phenotype with PCS, GOS, S-GOS, anti-PgII,



SH-9 and TKH-2 immunohistochemical staining. Their results showed that the proportion of gastric phenotype carcinoma cells decreases with the depth of invasion and the intestinal phenotype in four carcinomas involved the serosa. Bamba *et al*<sup>[10]</sup> applied immunohistochemistry with MUC2, M1 and PCS (III) to define the phenotype of 54 gastric signet ring cell carcinomas. They found that the larger size of the mucosal lesion was, the more frequently the intestinal phenotype was demonstrated. Aihara *et al*<sup>[13]</sup> examined MUC2, M1 and MUC6 staining of 69 early gastric SRC carcinomas; they classified these cases into G type and GI type and found the GI type was correlated with the depth of wall invasion. The findings of these studies showed that there is a phenotypic shift of cancer cell during gastric SRC carcinoma progression. This phenotypic shift may be a comitant phenomenon of tumor progression due to increasing heterogeneity.

A number of clinical studies revealed the difference of biological behaviors and prognosis among patients with gastric SRC carcinoma, indicating that morphologic classification is not enough for us to predict the progression and outcome of this kind of gastric carcinoma, and subtype classification needs further investigations. Our study showed that different phenotypic expression patterns were significantly associated with clinicopathologic parameters and prognosis of SRC carcinomas of stomach. Examination of phenotype expression may be a useful evidence for further classification and prognostic prediction in gastric SRC carcinomas.

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