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# Time-dependent expression of intestinal phenotype in signet ring cell carcinomas of the human stomach

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**Abstract** Signet ring cell carcinomas of the stomach are thought to arise from the proper gastric mucosa without intestinal metaplasia. It was recently reported that intestinal phenotypes appear along with tumor progression. In this study, we performed several experiments to reconsider the significance of this intestinalization in the growth of signet ring cell carcinoma. We applied mucin histochemistry with monoclonal antibodies MUC2 (Ccp58) and M1 (45M1), and paradoxical concanavalin A staining for class III mucin [PCS(III)] reaction to 29 intramucosal and 25 deeply invasive carcinomas of this type and correlated the phenotypic expression with the size of the mucosal spread and the depth of tumor invasion. It was found that the larger the size of the mucosal lesion, the more frequently the intestinal phenotypes were demonstrated. There was no significant increase in the expression of the intestinal phenotype as the tumor invaded the deeper part of the mucosa or as the intestinal metaplasia increased in the background mucosa. The intestinal expression appeared to be suppressed in the earlier phase of deep invasion. In the mucosal part of the tumor, the intestinal phenotype was often expressed regionally and incompletely, coexisting with gastric phenotypes at the cellular and the tissue levels. These findings indicate that the expression of the intestinal phenotype is a time-dependent and unstable phenomenon probably based on the accumulation of genetic changes and plays a neutral role in progression of signet ring cell carcinomas.

**Keywords** Stomach · Signet ring cell carcinoma · Intestinal phenotype · Time-dependent expression

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## Introduction

Gastric carcinomas have been classified into two main subtypes, so-called intestinal and diffuse types [16]. The intestinal type means that well-differentiated adenocarcinomas generally express an intestinal phenotype and are accompanied with intestinal metaplasia, which has been thought to be a precancerous condition of well-differentiated adenocarcinoma. The diffuse-type carcinomas arise from the proper gastric mucosa without intestinal metaplasia [19, 21]. Because electron microscopy and mucin histochemistry have revealed gastric and intestinal phenotypes in intestinal-type carcinomas and intestinal phenotypes in diffuse-type carcinomas [15, 22, 25], it becomes necessary to clarify the significance of such phenotypic expressions in the tumor and, on this basis, to reconsider the histogenesis of gastric carcinoma.

More recently, the intestinal phenotypes were reported to appear "time dependently" in diffuse-type gastric carcinomas as the depth of tumor invasion proceeded [26, 28]. However, this conclusion has to be reconsidered, taking the following aspects into consideration. Signet ring cell carcinoma, even of the superficially spreading type, has recently been demonstrated to be mostly monoclonal; the size of the tumor cell mass is thought to reflect the time the tumor growth takes from the incipient stage [1]. Accordingly, the spreading fronts in the mucosal part of signet ring cell carcinoma are known to show a characteristic layered structure in which proliferating tumor cells are confined to the middle level of the mucosa consistently from the incipient to the advanced stages [6, 22]. In addition, we have to consider the fact that a growth in the deeper part of the tumor is ten times as fast as that in the superficial part because of relative scarcity of cell loss in the deeper part [7]. After the tumor invades the deeper part of the stomach wall, the growth of the tumor will be considerably accelerated and may scarcely add a time-dependent phenotype. From the viewpoint of growth kinetics, the area of mucosal spread, rather than the depth of tumor invasion, should thus correlate with the expression of intestinal phenotype if it is a time-dependent phenome-

non. However, the relationship between the extent of mucosal spread and the expression of intestinal phenotype in the tumor has not been fully elucidated.

Recently, information on the protein backbone of epithelial mucin, which is generally called apomucin or mucin core protein, has been accumulated. To date, a number of apomucins (MUC1–8) have been identified, and their tissue- and cell-specific expressions were demonstrated, suggesting that each has a distinct role [8, 14]. MUC2 is a major colonic apomucin and is known to be expressed in goblet cells, including metaplastic ones in the stomach and other parts of the alimentary tract [4, 9, 27]. MUC5AC encodes the protein core at least in part of gastric M1 mucin that is expressed in surface mucous cells [2, 3, 10, 20]. Monoclonal antibodies (mAbs) against these apomucins enable us to discriminate between the gastric phenotype and the intestinal one in terms of genetic events more directly than the detection of alteration in sugar residues, which can be affected by various epigenetic events modifying the transferase activity.

In this study, we focused on the intramucosal spreading growth and earlier extension from the mucosa to the deeper part to study whether the mucin phenotype alters in a time-dependent manner or in a close relation with enhanced invasive activity. Thus, we chiefly used signet ring cell carcinomas of earlier stages (stage I and stage II), to which we applied mucin histochemistry with mAbs MUC2 and M1 and paradoxical concanavalin A staining for class III mucin [PCS(III)] [13]. Correlating the phenotypes with the size of mucosal spread and the depth of tumor invasion, we studied the significance of the expression of the intestinal phenotype in growth and progression of signet ring cell carcinoma.

## Materials and methods

This study was based on the analysis of 54 surgically resected signet ring cell carcinomas, which were defined as carcinomas with signet ring cells comprising more than 50% of the mucosa and the layered structure at least in part. They were documented between 1982 and 1999 at our affiliated hospitals in Shiga, Kyoto and Fukui, Japan. The stomachs were fixed in 10% formalin, and the entire cancerous lesion with its surrounding tissue was cut into 5-mm thick slices in parallel to the lesser curvature and embedded in paraffin. In each tumor, the paraffin blocks that comprised the tissue slice of the greatest length in the tumor were selected and cut into serial sections. Histologic classification was made using hematoxylin and eosin (H&E) sections according to the classification of the Japanese Research Society for Gastric Cancer [12].

For phenotyping of the tumor, immunohistochemical stainings with the mAbs MUC2 (Ccp58; 1:100, Novocastra, UK) and M1 (45M1; 1:100, Novocastra, UK), and PCS(III) were carried out. The streptavidin–biotin technique was used for immunohistochemistry (LSAB kit, Dako, Carpinteria, Calif.). MUC2 and M1 are known to recognize the intestinal apomucin and the surface gastric mucin, respectively. PCS(III) specifically stains pyloric gland cells and mucous neck cells in the stomach [13].

**Table 1** Intramucosal signet ring cell carcinomas analyzed by means of mucin stainings. The staining results were scored as the percentage of intensely stained carcinoma cells: *(–)* less than 5% of carcinoma cells; *(+)* 5–29% of carcinoma cells; *(++)* 30–59% of carcinoma cells; *(+++)* more than 60% of carcinoma cells. The tumors scored *(–)* were designated as negative and the others as positive for each staining. *IM* intestinal metaplasia; *G* gastric type; *I* intestinal type; *PCS(III)* paradoxical concanavalin A staining for class III mucin

| Case no. | Age (years)/gender | Width (cm×cm) | IM | MUC2 | M1 | PCS(III) | Type | Mosaic pattern of MUC2 expression |
|---|---|---|---|---|---|---|---|---|
| 1 | 50/Female | 1.0×0.5 | (+) | (–) | (+++) | (–) | Gastric | |
| 2 | 65/Male | 1.0×0.8 | (+) | (–) | (+++) | (–) | Gastric | |
| 3 | 70/Female | 1.1×0.8 | (–) | (+) | (+++) | (–) | Mixed(G>I) | (–) |
| 4 | 80/Female | 1.1×0.8 | (–) | (–) | (+++) | (–) | Gastric | |
| 5 | 71/Male | 1.2×0.8 | (+) | (++) | (+++) | (–) | Mixed (G>I) | (+) |
| 6 | 57/Male | 1.4×0.8 | (+) | (–) | (+++) | (++) | Gastric | |
| 7 | 59/Female | 1.4×1.2 | (–) | (+) | (+++) | (+) | Mixed (G>I) | (–) |
| 8 | 50/Male | 1.5×0.8 | (+) | (++) | (+++) | (+) | Mixed (G>I) | (+) |
| 9 | 50/Male | 1.5×1.2 | (+) | (++) | (+++) | (–) | Mixed (G>I) | (–) |
| 10 | 53/Female | 1.6×0.8 | (–) | (+++) | (+) | (+) | Mixed (I>G) | (–) |
| 11 | 44/Male | 1.6×1.4 | (–) | (++) | (+++) | (+) | Mixed (G>I) | (+) |
| 12 | 39/Female | 1.7×1.0 | (+) | (–) | (+++) | (–) | Gastric | |
| 13 | 59/Male | 1.8×1.0 | (+) | (++) | (+++) | (+) | Mixed (G>I) | (+) |
| 14 | 67/Female | 2.0×1.5 | (+) | (+++) | (+++) | (–) | Mixed (G=I) | (+) |
| 15 | 41/Male | 2.2×1.6 | (+) | (–) | (+++) | (++) | Gastric | |
| 16 | 55/Female | 2.5×2.0 | (+) | (–) | (+++) | (–) | Gastric | |
| 17 | 56/Female | 2.5×2.5 | (–) | (+) | (+) | (–) | Mixed (G=I) | (–) |
| 18 | 25/Male | 2.8×2.8 | (+) | (–) | (+++) | (–) | Gastric | |
| 19 | 58/Male | 3.0×2.5 | (+) | (–) | (+++) | (–) | Gastric | |
| 20 | 68/Male | 3.0×3.0 | (+) | (++) | (+++) | (–) | Mixed (G>I) | (–) |
| 21 | 48/Male | 3.5×3.0 | (+) | (++) | (+++) | (–) | Mixed (G>I) | (+) |
| 22 | 44/Female | 3.5×3.5 | (+) | (++) | (–) | (–) | Intestinal | |
| 23 | 49/Female | 4.0×2.5 | (+) | (++) | (+++) | (++) | Mixed (G>I) | (+) |
| 24 | 41/Female | 4.0×3.0 | (+) | (+) | (++) | (–) | Mixed (G>I) | (–) |
| 25 | 76/Female | 6.0×5.0 | (+) | (+) | (+++) | (–) | Mixed (G>I) | (–) |
| 26 | 44/Female | 6.5×6.5 | (+) | (+) | (+++) | (++) | Mixed (G>I) | (+) |
| 27 | 79/Female | 8.5×4.0 | (+) | (++) | (+++) | (++) | Mixed (G>I) | (+) |
| 28 | 31/Female | 8.5×7.5 | (+) | (+) | (+++) | (+) | Mixed (G>I) | (+) |
| 29 | 48/Female | 9.5×5.0 | (+) | (+) | (+++) | (–) | Mixed (G>I) | (–) |

**Table 2** Deeply invasive signet ring cell carcinomas analyzed by means of mucin stainings. Staining results of mucosa part/submucosa or deeper part: *(–)* less than 5% of carcinoma cells; *(+)* 5–29% of carcinoma cells; *(++)* 30–59% of carcinoma cells; *(+++)* more than 60% of carcinoma cells. *M* mucosa; *SM* submucosa; *MP* muscularis propria; *SS* subserosa; *SE* serosal exposure; *IM* intestinal metaplasia; *G* gastric type; *I* intestinal type; *PCS(III)* paradoxical concanavalin A staining for class III mucin; *T1* tumors with submucosal invasion; *T2* invasion to the muscularis propria or the subserosa; *T3* tumors with serosal exposure

| Case no. | Age (years)/Gender | Mucosal spread (cm×cm) | Depth | IM | MUC2 | M1 | PCS(III) | Type of intramucosal lesion | Type of SM or deeper lesion | Mosaic pattern of MUC2 expression |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 62/Male | 1.0×0.8 | T1 (SM) | (+) | (–)/(–) | (+++)/(+++) | (–)/(–) | Gastric | Gastric | |
| 2 | 50/Male | 1.5×1.0 | T1 (SM) | (+) | (+++)/(+) | (+)/(+) | (–)/(–) | Mixed (I>G) | Mixed (G=I) | (–) |
| 3 | 48/Female | 1.5×1.2 | T1 (SM) | (–) | (++)/(++) | (++)/(+) | (+)/(+) | Mixed (G>I) | Mixed (G=I) | (+) |
| 4 | 28/Female | 1.8×0.7 | T1 (SM) | (–) | (+++)/(+) | (+)/(–) | (+)/(–) | Mixed (I>G) | Mixed (I>G) | (–) |
| 5 | 48/Male | 2.0×1.8 | T1 (SM) | (+) | (–)/(–) | (+++)/(++) | (–)/(–) | Gastric | Gastric | |
| 6 | 28/Female | 2.3×0.6 | T2 (SS) | (–) | (–)/(–) | (+++)/(++) | (+)/(++) | Gastric | Gastric | |
| 7 | 39/Male | 2.6×1.8 | T2 (MP) | (+) | (+)/(–) | (+++)/(+++) | (–)/(–) | Mixed (G>I) | Gastric | (–) |
| 8 | 54/Female | 3.0×1.8 | T1 (SM) | (–) | (–)/(–) | (+++)/(–) | (–)/(+) | Gastric | Gastric | |
| 9 | 54/Female | 3.0×2.0 | T2 (SS) | (–) | (–)/(–) | (+++)/(+++) | (–)/(–) | Gastric | Gastric | |
| 10 | 58/Male | 3.0×2.0 | T3 (SE) | (+) | (+)/(+) | (+)/(++) | (–)/(–) | Mixed (G=I) | Mixed (G>I) | (+) |
| 11 | 59/Male | 3.3×2.4 | T3 (SE) | (+) | (++)/(++) | (+++)/(+++) | (++)/(++) | Mixed (G>I) | Mixed (G>I) | (+) |
| 12 | 49/Female | 3.5×3.2 | T1 (SM) | (+) | (+)/(–) | (+++)/(+) | (–)/(–) | Mixed (G>I) | Gastric | (–) |
| 13 | 55/Male | 3.8×3.3 | T1 (SM) | (+) | (++)/(–) | (++)/(++) | (+)/(++) | Mixed (G>I) | Gastric | (+) |
| 14 | 46/Male | 4.0×2.8 | T2 (MP) | (+) | (+)/(–) | (++)/(–) | (+)/(++) | Mixed (G>I) | Gastric | (+) |
| 15 | 60/Male | 4.0×3.8 | T2 (MP) | (+) | (++)/(++) | (–)/(–) | (–)/(–) | Intestinal | Intestinal | |
| 16 | 47/Male | 4.5×2.0 | T1 (SM) | (+) | (+)/(–) | (++)/(+) | (–)/(–) | Mixed (G>I) | Gastric | (–) |
| 17 | 72/Female | 5.0×3.0 | T2 (MP) | (+) | (+++)/(+++) | (+++)/(+++) | (+)/(–) | Mixed (G>I) | Mixed (G=I) | (+) |
| 18 | 67/Female | 5.0×4.5 | T3 (SE) | (–) | (++)/(++) | (++)/(++) | (++)/(++) | Mixed (G>I) | Mixed (G>I) | (+) |
| 19 | 57/Male | 5.5×2.2 | T2 (MP) | (+) | (++)/(++) | (++)/(–) | (–)/(–) | Mixed (G>I) | Intestinal | (+) |
| 20 | 35/Female | 5.5×4.0 | T1 (SM) | (+) | (+)/(–) | (++)/(++) | (+)/(–) | Mixed (G>I) | Mixed (G>I) | (+) |
| 21 | 61/Male | 5.5×4.0 | T1 (SM) | (+) | (+)/(–) | (+++)/(+) | (++)/(–) | Mixed (G>I) | Gastric | (–) |
| 22 | 71/Female | 6.0×2.0 | T3 (SE) | (+) | (–)/(–) | (+++)/(+++) | (+)/(–) | Gastric | Gastric | |
| 23 | 69/Female | 6.0×5.0 | T1 (SM) | (+) | (++)/(–) | (+++)/(++) | (++)/(++) | Mixed (G>I) | Gastric | (+) |
| 24 | 64/Male | 10.5×7.5 | T2 (MP) | (+) | (++)/(+) | (+++)/(++) | (+)/(+) | Mixed (G>I) | Mixed (G>I) | (+) |
| 25 | 48/Female | 12.0×6.5 | T3 (SE) | (+) | (+)/(+) | (+++)/(++) | (+)/(+) | Mixed (G>I) | Mixed (G>I) | (+) |

The staining results were scored as the percentage of intensely stained carcinoma cells: (–), less than 5% of carcinoma cells; (+), 5% to 29% of carcinoma cells; (++), 30% to 59% of carcinoma cells; (+++), more than 60% of carcinoma cells. The tumors scored (–) were designated as negative and the others as positive for each staining. Because we found double-positive cells of MUC2 and M1, we made some "mirror-image" sections for all double-positive cases.

After we confirmed that there was no significant difference in staining intensity of the normal mucosa between the oldest and most recent cases, we classified 54 cancers (29 intramucosal carcinomas and 25 deeply invasive carcinomas) according to the following histochemical criteria:

1. Gastric type. M1 (++ or +++) and/or PCS(III) (++ or +++) and MUC2 (–)
2. Intestinal type. M1 (–), PCS(III) (–), and MUC2 (++ or +++)
3. Mixed gastric and intestinal type. Carcinomas other than (1) or (2)

We analyzed the phenotypic expression of deeply invasive carcinomas in the intramucosal and deeper parts separately in each case in order to correlate the phenotype not only with the size of the mucosal spread but also with the depth of tumor invasion. For the statistical analysis of these correlations, Spearman's rank correlation test was used. Differences of $P<0.05$ were considered significant. The distribution of MUC2-positive cells was described as to whether they were regionally distributed to exhibit a "mosaic pattern" of MUC2-positive and -negative areas. The surrounding non-neoplastic gastric mucosa within 5 mm from the outer margin of tumor and residual non-neoplastic glands in the cancer area of each case were examined to assess the presence or absence of intestinal metaplasia.

## Results

### Incidence of mucin phenotypes

Mucin histochemical results are summarized in Table 1 and Table 2, which present intramucosal and deeply invasive signet ring cell carcinomas, respectively, in increasing

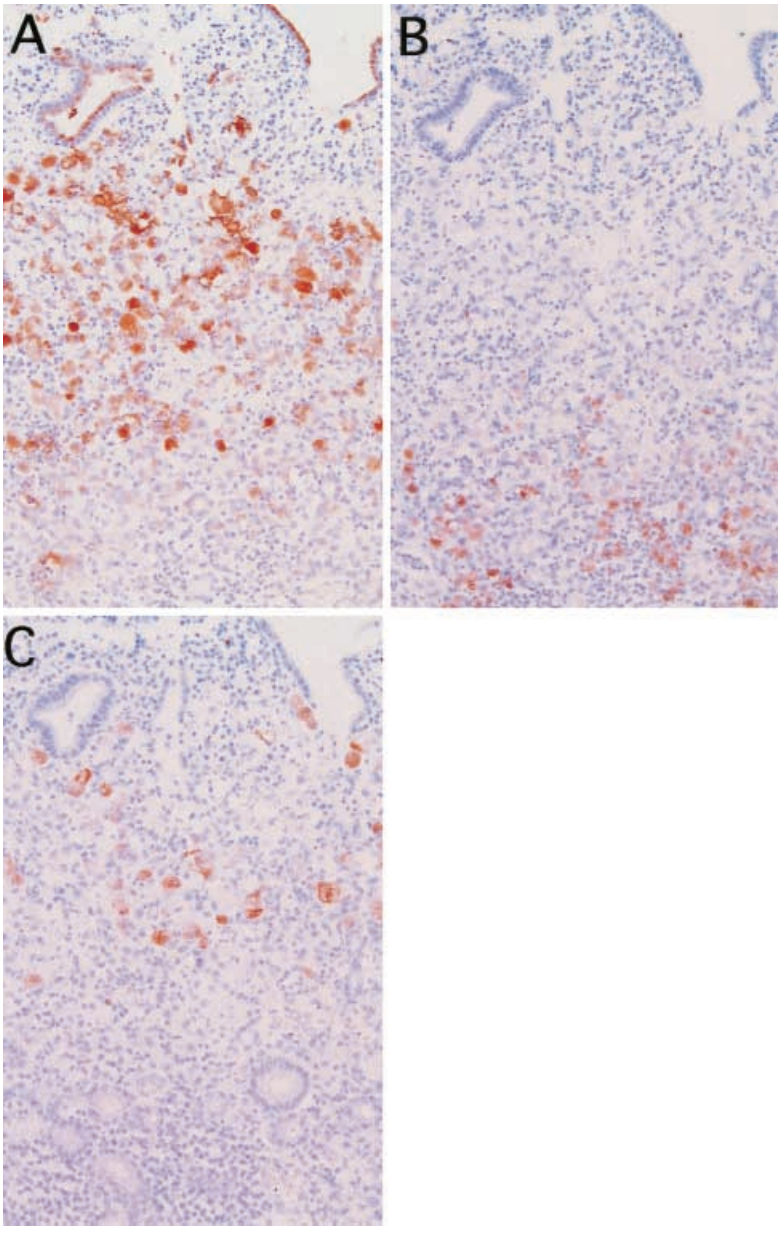


**Fig. 1** Mucin stainings in an intramucosal signet ring cell carcinoma (case no. 23). **A** M1-positive tumor cells are distributed in the superficial parts of the mucosa. Counterstained with hematoxylin; ×40. **B** Paradoxical concanavalin A staining for class III mucin-positive tumor cells are distributed in the deeper parts of the mucosa. Counterstained with hematoxylin; ×40. **C** MUC2-positive cells are distributed sporadically. Counterstained with hematoxylin; ×40

order of the size of the mucosal spread. As a marker of gastric phenotype, M1 was more sensitive than PCS(III); all of the tumors positive for PCS(III) were also M1 positive. Of the 29 intramucosal carcinomas, 9 (31.0%) were classified as the gastric type. They were smaller than 3.0 cm×2.5 cm in dimension. In the mucosal lesions of 25 deeply invasive carcinomas, 6 (24.0%) were of the gastric type. Only 2 (3.7%) of all the 54 tumors were of the intestinal type. All the others expressed intestinal and gastric phenotypes more or less (the mixed type).

The incidence of the gastric type in the intramucosal tumors was much lower than that previously reported, which was 92.6% [28]. This discrepancy can be explained by the difference in the definition of the gastric type; in the previous report, gastric type corresponded to the tumor that consisted of 90% or more of gastric-type cells irrespective of the presence of intestinal-type cells, while we simply regarded such a tumor as the mixed type when the intestinal-type cells comprised 5% or more.

Frequencies of the tumors that had a gastric phenotype in greater than 60% of the tumor cells were 86.2% (25/29) and 56.0% (14/25) in the intramucosal carcinomas and the mucosal parts of deeply invasive carcinomas, respectively. This roughly corresponds to the gastric type of the previous report. As these data are quite similar to those from the previous report, the above discrepancy is not thought to result from the difference in material composition.

## Expression pattern of phenotypes in tissues and cells

As shown in Fig. 1, M1-positive and PCS(III)-positive tumor cells tended to be distributed in the superficial and deeper parts of the mucosa, respectively. While MUC2-positive cells were often distributed sporadically, being intermingled with gastric-type cells within regionally restricted parts of the tumor (mosaic pattern; Fig. 1, Fig. 2, and Fig. 3). This regional distribution was clearly seen in 22 of the 37 tumors of the mixed type (Table 1 and Table 2).

At the cellular level, there were some hybrid signet ring cells that were positive for both M1 and MUC2, as demonstrated in a pair of “mirror-image” sections (Fig. 4).

## Mucin phenotypes and the background mucosa

There was no significant difference in the incidence of intestinal metaplasia in the surrounding non-neoplastic mucosa and/or the residual non-neoplastic glands within the cancer area between intramucosal and deeply invasive tumors (79.3% and 76.0%, respectively).

## Mucin phenotypes and size of tumor spread in the mucosa

Table 3 shows a relationship between the mucin phenotype and the size of the mucosal spread in intramucosal and deeply invasive carcinomas. The larger the size of a mucosal part, the more the intestinal-type tumor cells were encountered in the mucosa in both intramucosal and deeply invasive tumors, while there was no significant correlation between the tumor size and the phenotype in the submucosa or deeper.

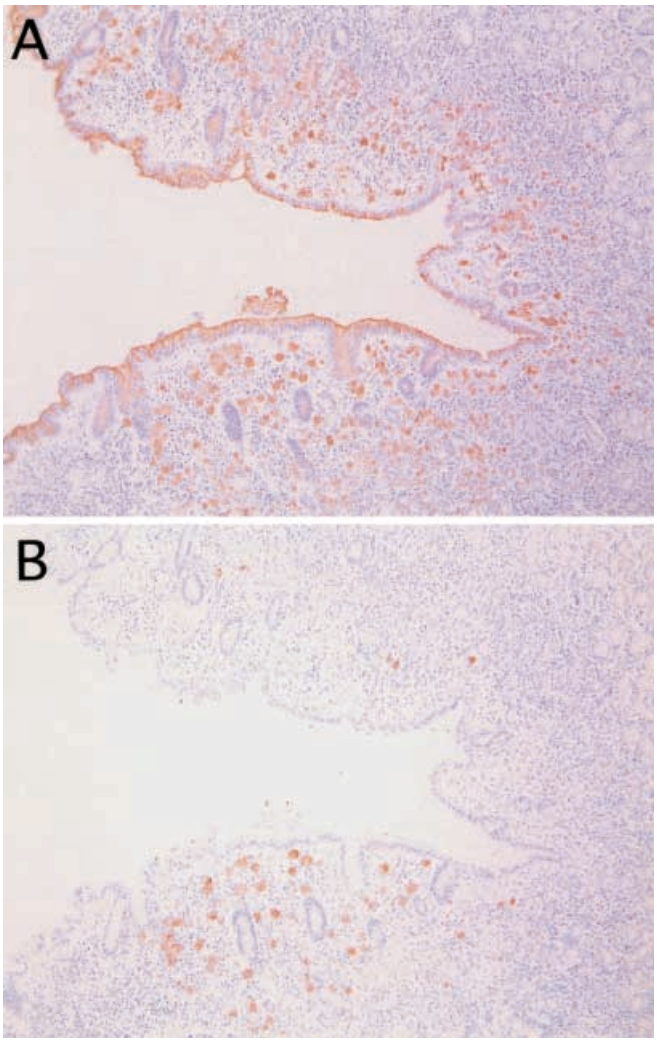


**Fig. 2** Mucin stainings in an intramucosal signet ring cell carcinoma (case no. 5). **A** M1-positive cells are diffusely seen. Counterstained with hematoxylin; ×16. **B** MUC2-positive cells are regionally distributed to exhibit a mosaic pattern. Counterstained with hematoxylin; ×16

## Mucin phenotypes and depth of tumor invasion

In the deeply invasive tumors, we analyzed the intramucosal and deeply invasive parts separately. In the mucosa, the incidence of intestinal phenotype in the mucosal lesion was 69.0% in 29 intramucosal tumors, 75.0% in 12 tumors with submucosal invasion (T1), 75.0% in 8 tumors with invasion to the muscularis propria or the subserosa (T2), and 80% in 5 tumors with serosal exposure (T3), showing no significant difference among these

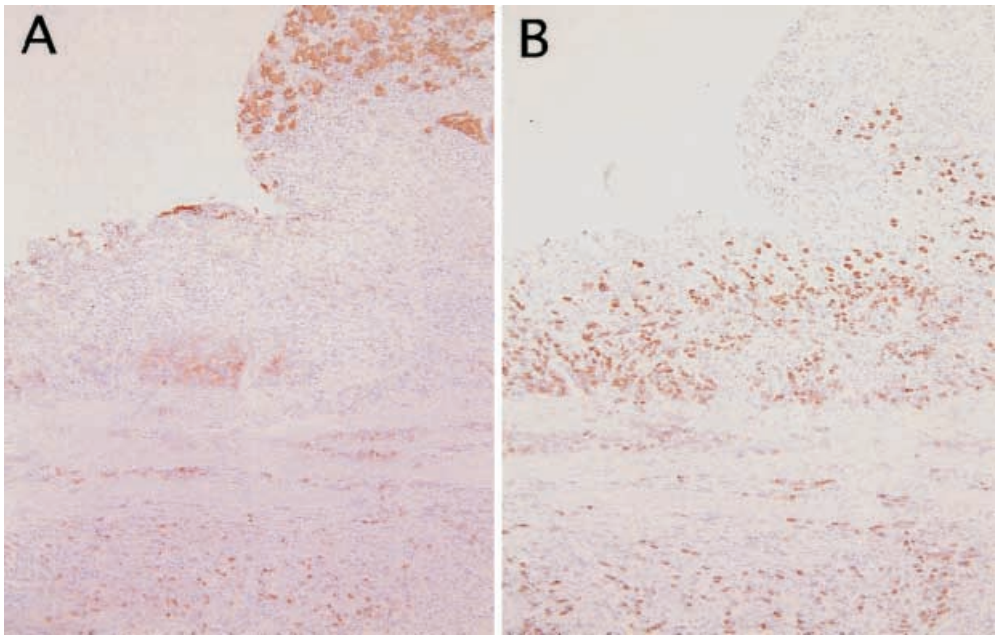


**Fig. 3** Mucin stainings in a deeply invasive signet ring cell carcinoma (case no. 18). **A** M1-positive cells are regionally distributed to exhibit a mosaic pattern in the mucosa and are diffusely seen in the submucosa. Counterstained with hematoxylin; ×16. **B** MUC2-positive cells are regionally distributed to exhibit a mosaic pattern in the mucosa and are diffusely seen in the submucosa. Counterstained with hematoxylin; ×16

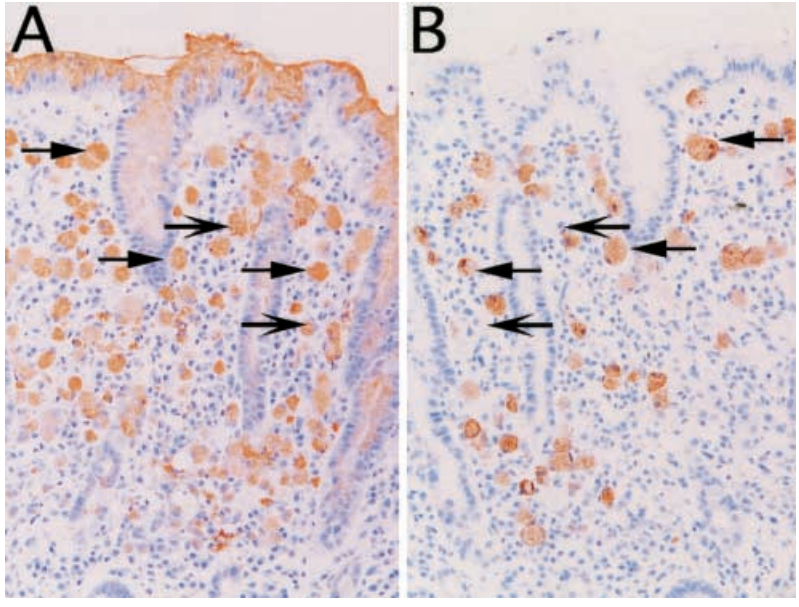


**Fig. 4** A pair of "mirror-image" sections of Fig. 2. The hybrid signet ring cells are indicated by *thick head arrows*, and non-hybrid cells are indicated by *thin head arrows*. **A** M1-positive cells are seen. Counterstained with hematoxylin; ×40. **B** MUC2-positive cells are seen. Counterstained with hematoxylin; ×40

**Table 3** Expression of intestinal phenotypes in both intramucosal and deeply invasive signet ring cell carcinomas.; *M* mucosal lesions; *SM or deeper* submucosal or deeper lesions

| | Size (cm×cm) | | | Spearman's rank correlation |
|---|---|---|---|---|
| | <1×1 | 1×1≦<3×3 | 3×3≦ | |
| Intramucosal | 2/5 (40.0%) | 8/14 (57.1%) | 10/10 (100%) | *P*=0.0088 |
| Deeply invasive (M) | 0/1 (0%) | 6/10 (60.0%) | 13/14 (92.9%) | *P*=0.0178 |
| Deeply invasive (SM or deeper) | 0/1 (0%) | 5/10 (50.0%) | 7/14 (50.0%) | *P*=0.7084 |

groups (Table 4). In the extramucosal part, the incidence of the intestinal phenotype tended to increase as the tumor invaded the deeper part: 33%, 50%, and 80% in T1, T2, and T3, respectively (Table 4). This tendency was not statistically significant, probably because of smaller case numbers, especially of T2 and T3. In each deeply invasive tumor, however, there was no difference in the phenotype among the submucosa, the proper muscle layer, and the subserosa.

In 13 (52.0%) of the 25 tumors, the intramucosal phenotype was similar to that of deeply invasive parts, including six purely gastric-type tumors and one purely intestinal-type tumor. In the other 12 tumors, the phenotype in the mucosa was different from that in the submu-

**Table 4** Expression of intestinal phenotypes in deeply invasive signet ring cell carcinomas. *SM* submucosa; *MP* muscularis propria; *SS* subserosa; *SE* serosal exposure; *T1* tumors with submucosal invasion; *T2* invasion to the muscularis propria or the subserosa; *T3* tumors with serosal exposure

| | Depth | | | Spearman's rank correlation |
|---|---|---|---|---|
| | T1 (SM) | T2 (MP, SS) | T3 (SE) | |
| Mucosal lesions | 9/12 (75.0%) | 6/8 (75.0%) | 4/5 (80.0%) | *P*=0.8630 |
| SM or deeper lesions | 4/12 (33.3%) | 4/8 (50.0%) | 4/5 (80.0%) | *P*=0.0985 |

cosa or deeper parts; the deeper part of the tumor had a greater proportion of gastric and intestinal phenotypes than the mucosal part in 9 (75.0%) and 3 (25.0%) tumors, respectively (Table 4).

## Discussion

In the stomach, signet ring cell carcinoma is classified as the undifferentiated type because of a poor tendency for glandular formation [21]. Cellularly, however, this type of carcinoma is well differentiated to mucin-producing cells. It is thought to arise from the proper gastric mucosa without intestinal metaplasia, because minute carcinomas of this type were commonly detected in such mucosa while, at the cellular level, intestinal phenotypes were demonstrated using electron microscopy and mucin histochemistry [15, 22, 25]. In the present study, we demonstrated that the intestinal phenotypes were rare in the tumors smaller than 1.0 cm×1.0 cm in dimension and became significantly more common as the mucosal spread proceeded in this type of carcinoma.

Gastric carcinoma is known to have a long natural history. This concept was supported by the recent clonal analysis [1], demonstrating that most of the superficial depressed, diffuse-type gastric carcinomas were of monoclonal constitution. The size of intramucosal lesion is thus considered to reflect the length of the natural history of the tumor from an incipient carcinoma. From this point of view, the above-mentioned results suggest that the expression of intestinal phenotype is a time-dependent phenomenon. The intestinal phenotype appears to occur spontaneously in the tumor, being independent of the intestinal metaplasia in the background mucosa of the tumor, as demonstrated in the present and in the previous studies [22, 28].

In this study, we used only MUC2 apomucin as an intestinal marker, because it was reported to show high sensitivity and high specificity to goblet cells [4, 9, 27] and because the other intestinal expression of the brush border type that is detectable by a mAb recognizing CD10 [17] is uncommon in our materials of signet ring cell carcinomas of the stomach (data not shown).

The expression of the intestinal phenotype was often unstable in the tumor; the intestinal-type cells were often observed sporadically in the tumor and occasionally as hybrid cells with both gastric and intestinal phenotypes. However, such unstable expression of intestinal phenotype was often regionally restricted, sharply demarcated from the non-expressed part. This pattern may reflect a subclonal growth of the cells with an unstable intestinal phenotype. This kind of subclone may be of a neutral significance in tumor progression, because it accumulated time dependently to form a mosaic pattern within the mucosa and, because the incidence of the intestinal phenotype in the submucosa or deeper was rather lower than that in the mucosa.

Between the mucosal and the extramucosal parts in each of the 25 tumors with the extramucosal invasion, there was no overt difference in phenotype in 13 tumors (52.0%). And, within each extramucosal invasive lesion, there was no difference in the phenotypic expression among the submucosa, the proper muscle layer, and the subserosa. Furthermore, 9 and 3 of the remaining 12 tumors showed a phenotypic shift to the gastric and the intestinal types, respectively, as the tumor progressed from the mucosa to the extramucosal part in each tumor. In these 9 tumors, the cancer cells with the gastric phenotype appear to invade the deeper layer more readily than those with the intestinal phenotype, as pointed out previously [22]. Some authors [15, 28] reported, on the contrary, that intestinal phenotype, recognized by syalyl Tn antigen (STN), became more common as the depth of tumor invasion proceeded.

To study the cause of this discrepancy, we correlated the mucin phenotype with the tumor depth as they did. Our data confirmed the tendency of an increase of intestinal expression in the extramucosal parts as the depth proceeded. This suggests that the difference in the intestinal marker, MUC2 versus STN, cannot explain that discrepancy. It was also found that the incidence of the intestinal expression in the mucosa was constantly as high as that in the extramucosal parts of T3 tumors. The depth-related increase of intestinal expression we confirmed is thus considered to result not from the increase of intestinal expression in deeper parts of T3 tumors but from the lower expression of intestinal phenotype in the submucosal parts than in the mucosal parts of T1 tumors. Why the intestinal expression appeared to be suppressed in deeply invasive parts of T1 but not in T3 tumors remains to be clarified.

It is generally accepted that the accumulation of multistep genetic changes in the tumor cell is necessary for the occurrence and progression of tumors [5, 12, 18, 29], and it is a clonal growth that enables the tumor cell to accumulate the multi-step genetic events. The time-dependent expression of the intestinal phenotype, basically as a mosaic pattern demonstrated in this study, may reflect the appear-

ance of clonal populations elicited by a genetic change that is not related to the growth advantage. Such neutral subclones are unlikely to expand excessively or to invade the deeper part unless additional genetic changes that give a growth advantage to the cells are accumulated. In signet ring cell carcinoma, such additional changes may be related to the ploidy alteration in the tumor. The mapping of DNA ploidy in individual tumors demonstrated that intramucosal spreading growth is characteristic of DNA-diploid tumor cells of this type and that the appearance of a DNA-aneuploid subclone is closely related to enhanced chromosomal instability and deeply invasive growth [23]. Such subclones are reported to occur in a stochastic rather than a time-dependent manner [24]. It may be because the occurrence of a subclone with an enhanced growth advantage is a much rarer event than the occurrence of a neutral subclone with changes in mucin phenotype.

Of course, we cannot exclude the effects of variable vascularization, cytokine, or growth factor on the intestinal expression of the tumor. However, it may be difficult to explain the time-dependence and the distribution pattern of the intestinal phenotype by these extracellular environmental factors alone. It remains unanswered whether there are any concrete changes in genome or methylation pattern that are closely linked to the expression of the intestinal phenotype.

In this study, the neutral nature of the acquisition of intestinal phenotype was disclosed by the individual tumor-based approach, comparing the mucin phenotype between the mucosal and the extramucosal parts in each tumor. Individualized as well as statistical approaches using the averaged features of tumors should be taken into consideration when assessing the correlation between the expression of a certain phenotype and the biological behavior of tumor cells.

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