

# Microscopic Invasion Patterns and Epithelial Cell-phenotypes in Early Gastric Cancer with Submucosal Invasion

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#### Abstract

**Background and study aims:** Early gastric cancers show gastric and/or intestinal phenotypes with specific mucin production profiles, and the phenotypes can vary with tumor progression. The aim of this study was to evaluate the correlation between tumor invasion patterns and phenotypes in the mucosa and submucosa of early gastric cancers.

**Methods:** Phenotypic expressions of 44 endoscopically resected gastric cancers with submucosal invasion were evaluated immunohistochemically using MUC5AC and MUC6 as gastric and MUC2 and CD10 as intestinal phenotypic markers.

**Results:** Cancers were classified into two patterns by invasion pattern: 19 collapsing pattern (C-pattern) tumors had cancer cells that invaded to the submucosa with expansive destruction of the muscularis mucosae, while 25 passing-through pattern (P-pattern) tumors formed focal cancer cell aggregations in the submucosa without massive destruction of the muscularis mucosae. Cancers with C-pattern invasion were likely to show similar phenotypes between the mucosa and submucosa, while phenotypes of cancers with P-pattern invasion were likely to differ between the two layers (rate of the same phenotypes: C-pattern 68% vs. P-pattern 28%, p = 0.008). Of 22 cancers with P-pattern invasion that included the intestinal phenotype component in the mucosa, 13 (59%) expressed the gastric phenotype alone in the submucosa.

**Conclusions**: Phenotype presentation in the mucosa and submucosa differ by the invasion pattern in early gastric cancer. Tumors with P-pattern invasion are likely to express the gastric phenotype in the submucosa, regardless of phenotype in the mucosa, suggesting that such cancers might achieve submucosal invasion prior to intestinalization occurring in the mucosa.

**Keywords:** Submucosal gastric cancer; Invasion pattern; Gastric phenotype; Intestinal phenotype

# Abbreviations:

HE: Hematoxylin and eosin; *H. pylori*: *Helicobacter pylori* 

## Introduction

Gastric cancer is one of the leading causes of cancer-related deaths in Japan. With 49,129 deaths in Japan attributed to this cancer in 2012, gastric cancer remains a major health problem [1]. Recently, early gastric cancers, including mucosal and submucosal cancers, have been more frequently found due to progress in endoscopic modalities, and endoscopic resection has been widely performed. Because approximately 20% of gastric cancers with submucosal invasion have lymph node metastases [2], endoscopic resection for these lesions should be judiciously applied. In this context, the clinical processes and basic mechanisms of cancer infiltration into the submucosa appear to be relevant. It is known empirically that gastric cancer invades to the submucosal layer in either of the following two manners, as reported approximately 50 years ago [3,4]: first, tumor cells destroy the muscularis mucosae widely and infiltrate diffusely into the submucosa; and second, tumor cells pass through the muscularis mucosae without destroying it and form focal aggregations in the submucosa. However, the clinical and basic differences between these two types of manners of submucosal invasion are largely unknown. Knowing what accounts for the difference in the manner of invasion may facilitate the elucidation of mechanisms of submucosal invasion of gastric cancer, and it may become useful in determining the indication for endoscopic resection.

Lauren et al. classified human gastric cancers into two major groups, the 'intestinal' and 'diffuse' types [5], which are considered to closely correspond to the 'differentiated' and 'undifferentiated' types, respectively, of Nakamura et al. [6]. In this context, gastric and intestinal phenotypes have been proposed, based on the immunohistochemistry for specific mucin production of gastric cancer cells. It has been considered that differentiated gastric carcinoma arises from atrophic mucosa or intestinal metaplasia, expressing intestinal mucin phenotypes, and that undifferentiated gastric carcinoma arises from non-atrophic gastric mucosa of young persons or women expressing gastric mucin phenotypes [5-8]. However, previous immunohistochemical studies have proven that differentiated gastric cancer usually expresses the gastric 'not intestinal' phenotype in its very early stage, and thereafter, the shift to the intestinal phenotype occurs with cancer progression [9-15]. However, this type of phenotype change has been reported in early gastric cancer confined to the mucosal layer. Few reports indicated the phenotype difference of cancer cells between the mucosal and submucosal layers.

In this study, therefore, the phenotype difference between the mucosa and submucosa was examined in early gastric cancer with submucosal invasion. In particular, the correlation of invasion patterns of cancer cells with the phenotype changes was specifically determined. The present investigation may partly help elucidate the mechanisms of cancer invasion into the submucosa of gastric cancer.

# **Materials and Methods**

## Samples and tissue collection

Clinical samples were collected from 809 primary solitary gastric cancer specimens that had been resected endoscopically at Wakayama University Hospital between May 2002 and June 2013. The criteria for the collection of samples were early differentiated gastric cancer (tub1, tub2, or pap, according to the Japanese Classification of Gastric Carcinoma [16]) with submucosal invasion, and they were appropriate for histological analysis. There were 52 specimens of submucosal gastric cancers, but 8 were excluded because of the following reasons: i) the part with submucosal invasion was unclear on immunohistochemistry due to inappropriate technique for preparing the pathological sections; ii) the depth of submucosal invasion was unclear due to problems during the endoscopic procedure; and iii) cancers with ulceration or ulcer scars because of difficulties identifying the muscularis mucosae. Finally, 44 specimens were considered eligible and analyzed in this study.

All specimens had been fixed in 10% buffered neutral formalin, embedded in paraffin, and cross-cut into 2- $\mu$ m-thick sections. Hematoxylin and eosin (HE)-stained sections were prepared and observed under a microscope, and the sections in which cancer cells reached the deepest level of the submucosa were subjected to detailed examination. Histological classification was performed by two independent board-certified pathologists in our hospital according to the Japanese Classification of Gastric Carcinoma. When a disagreement arose, it was discussed until a consensus was reached. The phenotypic differences between the mucosal and submucosal components were investigated, along with the patterns of tumor invasion to the submucosal layer.

The study protocol was approved by the ethics committee of Wakayama Medical University. Informed consent was obtained from each patient.

## Immunohistochemistry

MUC5AC and MUC6 were used as gastric phenotypic markers, while MUC2 and CD10 were used as intestinal phenotypic markers. The MUC5AC glycoprotein is known to react with epithelial cells in the surface of gastric foveola. The MUC6 glycoprotein is expressed in mucous cells of the neck zone of oxyntic mucosa and in pyloric gland cells [17]. The MUC2 glycoprotein is expressed in the supranuclear area of goblet cells in mucosal areas showing intestinal metaplasia in the stomach [18,19]. The CD10 glycoprotein is expressed on the brush border of intestinal epithelial cells [20,21]. For immunohistochemistry, the following monoclonal antibodies were used as primary antibodies: MUC5AC (1:40, CLH2; Novocastra, Newcastle, UK), MUC6 (1:100, CLH5; Novocastra), MUC2 (1:40, Ccp58; Novocastra), and CD10 (1:200000, 56C6; DAKO, Glostrup, Denmark). With regard to the epithelial cell markers, the results of immunohistochemical staining were evaluated in terms of the percentage of positive cytoplasmic and luminal stained cancer cells, with 10% and above considered positive.

The immunohistochemical expression of Ki-67 antigen was also examined using the MIB1 monoclonal antibody (1:200, MIB-1, DAKO) to assess the aggressiveness of the gastric carcinomas studied. Any nuclear staining, regardless of the intensity of the reaction, was considered positive for Ki-67. The reaction was quantified through the assessment of a marking index of Ki-67 (MI Ki-67), expressed as a percentage result of the number of Ki-67+ cells reported of 500 cells (Ki-67+ and Ki-67-). MI Ki-67 was evaluated in the submucosal component of the tumor.

## Classification of phenotypic expression of carcinomas

Tumors were classified phenotypically with reference to the expression patterns of a battery of epithelial cell markers. Mucin phenotype expression was judged as follows: i) Gastric phenotype (G-type): MUC5AC- and/or MUC6-positive rate of 10% or more; ii) Intestinal phenotype (I-type): MUC2- and/or CD10-positive rate of 10% or more; iii) Gastric-and-intestinal mixed phenotype (GI-type): MUC5AC- and/or MUC6-positive as well as MUC2- and/or CD10-positive rates of 10% or more; and iv) Null phenotype (N-type): Positive rates for all the four markers less than 10%. The phenotypic expressions of the mucosal and submucosal layers were evaluated individually based on the above classification.



**Figure 1:** A case of moderately differentiated adenocarcinoma with C-pattern. (a) Schema of gastric cancer with C-pattern submucosal invasion. (b) Endoscopic ultrasonography shows diffuse submucosal invasion (white arrowhead). The border between the mucosal and submucosal layers appears obscure. (c) HE staining (×100). Cancer cells invade to the submucosal layer diffusely, and the muscularis mucosae is widely destroyed (black arrow-head).

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#### Patterns of submucosal invasion

The pattern of tumor invasion to the submucosal layer was classified into two categories: collapsing pattern (C-pattern) and passingthrough pattern (P-pattern). C-pattern was defined as a pattern in which cancer cells invaded to the submucosal layer with expansive destruction of a wide range of muscularis mucosae. In cancers with Cpattern, the muscularis mucosae beneath the cancer was pressed down, and the histological border between the mucosal and submucosal layers appeared obscure (Figures 1a-1c). In contrast, in P-pattern invasion, the cancer cells penetrate the muscularis mucosae and form focal cancer cell aggregations in the submucosal layer, with little or no destruction of the muscularis mucosae. The histological structure of the muscularis mucosae is spared except for the site of penetration, and cancer cells form a neck-like appearance between the mucosal and submucosal layers (Figures 2a-2c).



**Figure 2:** A case of moderately differentiated adenocarcinoma with P-pattern. (a) Schema of gastric cancer with P-pattern submucosal invasion. (b) Endoscopic ultrasonography shows hypoechoic lesions in the third layer, which indicate focal submucosal invasion (white arrowhead). (c) HE staining (×100). Cancer cells focally invade to the submucosal layer like passing through the muscularis mucosae, which is maintained (black arrow-head).

## Statistical analyses

Statistical analyses were performed using the  $\chi^2$  test, Fisher's exact test, and the Mann-Whitney U test as appropriate. The values were considered significantly different when the p value was less than 0.05. SPSS version 11.0 (Chicago, IL) was used for all calculations.

## Results

Of the 44 analyzed submucosal gastric cancers, 19 were categorized as C-pattern, while 25 were P-pattern. Table 1 shows the clinicopathological features of the cancers in each pattern. Most clinicopathological features showed no differences between the two patterns of cancers. In terms of tumor location, however, P-pattern cancers were more frequent in the upper or middle portion of the stomach. Cancers with deep (more than 500  $\mu$ m) submucosal invasion and/or with vessel invasion were similarly observed in both groups, and one P-pattern case was found to have lymphatic metastases with additional surgery. Epithelial cell phenotypes in the mucosal layer of C-

	C-pattern (n=19)	P-pattern (n=25)	P value
Age, median (range), y	68 (58-90)	67 (54-84)	0.38
Sex (Male/Female)	17/2	24/1	0.4
Location (Upper or Middle/Lower)	11-Aug	18/7	0.04
Size, median (range), mm	17 (8-60)	17 (7-55)	0.23
Macroscopic type (elevated, depressed)	10-Sep	17-Aug	0.3
Histological type (tub1,tub2,pap)	11/6/2002	16/9/0	0.25
Lymphatic or venous invasion (+/-)	13-Jun	18-Jul	0.79
Depth of invasion (sm1/sm2)	9-Oct	15-Oct	0.41
MI Ki-67, median (range)	62 (46-72)	58 (44-76)	0.47
Phenotype of mucosal layer (G/GI/I)	4/6/2009	3/11/2011	0.6

Upper and middle, upper and middle thirds of the stomach; Lower, lower third of the stomach; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; pap, papillary adenocarcinoma; sm1, submucosal invasion of less than 500 mm; sm2, submucosal invasion of 500 mm or more.

Table 1: Clinicopathological features of analyzed gastric cancers.



**Figure 3:** Epithelial cell-phenotype differences between the mucosa and submucosa. Cancers with C-pattern invasion are likely to show similar phenotypes between the mucosa and submucosa, while phenotypes of cancers with P-pattern invasion are likely to differ between the two layers (rate of the same phenotypes: C-pattern 68% vs. P-pattern 28%, p = 0.008). In the submucosal layer, in particular, G-type is more frequently observed in cancers with P-pattern invasion than in those with C-pattern invasion (60% vs. 26%, p = 0.026). (a) C-pattern. (b) P-pattern.

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The phenotypic differences between the mucosal and submucosal components in each cancer by invasion pattern are shown in Figure 3. Cancers with C-pattern invasion were likely to show similar phenotypes between the mucosa and submucosa, while phenotypes of cancers with P-pattern invasion were likely to be different between the two layers (rate of the same phenotypes: C-pattern 68% vs. P-pattern 28%, p = 0.008). In the submucosal layer, in particular, G-type was more frequently observed in cancers with P-pattern invasion than in those with C-pattern invasion (60% vs. 26 %, p = 0.026). Of 22 cancers with P-pattern invasion with an intestinal phenotype component in the mucosa, 15 (68%) lost the intestinal phenotype component in the submucosa, and 13 (59%) showed G-type alone in the submucosa. In contrast, of 15 C-pattern cancers with an intestinal phenotype component, only 1 (6.7%) showed G-type alone in the submucosa. Null type was seen in the submucosal layer alone in each invasion group. These results suggest that the phenotype presentation in the mucosa and submucosa differs according to invasion pattern, and that tumors with P-pattern invasion are likely to express the gastric phenotype in the submucosa regardless of the phenotype in the mucosa.



**Figure 4:** Histology and immunohistochemistry of gastric cancer with C-pattern submucosal invasion. A case of moderately differentiated adenocarcinoma of type 0-IIc, measuring 12 mm in diameter, with submucosal invasion of >500 µm is shown. Both mucosal and submucosal layers are I-type. (a) HE staining (×100) (b) MUC5AC-negative (×100) (c) MUC6-negative (×100) (d) MUC2-negative (×100 (e) CD10-positive both in the mucosal and submucosal layers (×100) (f) MI Ki-67 is 61%.

Figures 4 and 5 show HE staining and immunohistochemical expressions of C-pattern and P-pattern cancers, respectively. In Figure 4, tumor cells in both the mucosal and submucosal layers were positive for CD10 and negative for MUC2, MUC5AC, and MUC6, indicating that the tumor was classified as I-type both in the mucosa and in the

submucosa. In Figure 5, the phenotype of the mucosal layer was I-type with positive CD10 expression and with negative expressions of MUC2, MUC5AC, and MUC6. In contrast, the phenotype of the submucosal layer was G-type with positive expression of MUC5AC alone.



**Figure 5:** Histology and immunohistochemistry of gastric cancer with P-pattern submucosal invasion. A case of moderately differentiated adenocarcinoma of type 0-IIc, measuring 8 mm in diameter, with submucosal invasion of >500  $\mu$ m. The mucosal layer is I-type, while the submucosal layer is G-type. (a) HE staining (×100). (b) MUC5AC-positive only in the submucosal layer (black arrow-head) (×100). (c) MUC6-negative (×100). (d) MUC2-negative (×100). (e) CD10-positive only in the mucosal layer (black arrow-head) (×100). (f) MI Ki-67 is 70%.

# Discussion

In the present study, the difference in the phenotypic expression between the mucosal and submucosal layers of early gastric cancer was evaluated with reference to the pattern of tumor invasion to the submucosal layer. In cancers showing C-pattern invasion, the phenotypes of the mucosa and submucosa are likely to be similar, while in cancers with P-pattern invasion, the phenotypes are likely to differ between the two layers. In particular, cancer cells in the submucosa of the tumor with P-pattern invasion are more likely to be G-type, and the I-type component in the mucosa of the tumors was frequently lost in the submucosa. The specific correlations between

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invasion pattern and phenotype presentation suggest the presence of unique mechanisms of gastric cancer progression.

Most gastric cancers, independent of the histological type, are considered to occur with a complete gastric phenotype in the early stage and acquire intestinal phenotypic expression in the proliferating zone as they grow. Components of the intestinal phenotype then spread outside the proliferating zone and the whole tumor shifts to an intestinal phenotype [9-12]. Intestinalization is at least partly caused by Helicobacter pylori (H. pylori) infection, which is strongly associated with intestinal-specific gene expression, such as Caudal-related homeobox gene (Cdx) 1 and Cdx 2 [22-24]. In this context, Yamamoto et al. reported that approximately 70% of gastric cancers found after eradication of H. pylori were complete gastric type or gastricpredominant mixed type [25,26]. In addition, Kato et al. reported that most H. pylori infection-negative gastric cancers were complete gastric type or gastric-predominant mixed type [27].

In terms of *H. pylori* infection, the following explanations may be plausible for our findings. In P-pattern invasion, the tumor cells pass through the space of the muscularis mucosae and invade to the submucosal layer at an early stage while the intestinalization does not yet occur. Thereafter, they grow individually in the mucosal and submucosal layers. Tumor cells in the mucosal layer acquire intestinal phenotype by exposure to H. pylori, whereas those in the submucosal layer maintain the gastric phenotype because of the lack of H. pylori and presumably related factors. The alternative scenario is the reversion to the gastric phenotype from the intestinal phenotype along with invasion to the submucosa possibly due to the decrease of factors correlated with intestinalization present only in the mucosa. On the other hand, in C-pattern invasion, the tumor cells destroy the muscularis mucosae and invade to the submucosal layer diffusely. During such a process, tumor cell phenotype is unlikely to change, because factors that determine the phenotypes also appear to infiltrate to the submucosa.

In the present study, cancers with P-pattern invasion were more frequently located in the middle or upper portion of the stomach. This result may be attributed to the difference in the structure of the muscularis mucosae among gastric portions. It is known that vessels penetrating the muscularis mucosae are more frequently observed in the upper or middle portion of the stomach than in the lower portion. In addition, the thickness of the muscularis mucosae of the middle or upper body is thinner than that of the lower body. Thus, the thin and gappy structure of the muscularis mucosae may facilitate P-pattern submucosal invasion. The present study cohort included more Ppattern than C-pattern cancers, although the depth of invasion had been fully evaluated in all cases prior to treatment, and the diagnosis of submucosal cancer usually prohibited endoscopic resection. This suggests that precise diagnosis of P-pattern submucosal invasion prior to resection is difficult. Therefore, the indication for endoscopic resection of cancer in the upper or middle portion of the stomach should be carefully determined.

Nakamura et al. previously reported that there were no combinations of a gastric phenotype in the mucosal layer and an intestinal phenotype in the submucosal layer, or an intestinal phenotype in the mucosal layer and a gastric phenotype in the submucosal layer in surgically resected gastric cancer with submucosal invasion [28]. Their findings were not consistent with the present findings regarding the latter, in particular, in cancers with P-pattern invasion. This difference may be attributed to the depth of cancer invasion. Surgically resected cancer is considered to harbor deeper

submucosal invasion than endoscopically resected submucosal cancer. Cancers showing P-pattern submucosal invasion with I-type in the mucosa and G-type in the submucosa might lose the gastric phenotype component of the submucosa along with the deeper advance of submucosal invasion, because, even in cancers showing the P-pattern, massive submucosal invasion would destroy the muscularis mucosae and factors associated with intestinalization would penetrate into the submucosa.

It has been reported that differentiated adenocarcinoma with gastric phenotype has a high malignant potential [29-31], and Koseki et al. reported that cancer with the gastric phenotype is significantly more likely to invade to vessels or metastasize to lymph nodes than that with the intestinal phenotype or mixed type [32]. Cancer with P-pattern invasion, therefore, may have high malignant potential because G-type is more frequently observed in the deepest region of those cancers. In the present study, in fact, one case of submucosal invasive cancer with lymphatic metastasis showed P-pattern invasion, and cells in the submucosal layer of the case showed a completely gastric phenotype. However, the difference in prognosis between cancers with the gastric and intestinal phenotypes is controversial [33], and the Ki-67 index was not significantly different between the two patterns of cancers in the present study. Further studies are needed to clarify the malignant potential of gastric cancer according to cell phenotypes and patterns of submucosal invasion.

Null type was seen only in the submucosal layer in the present study. The result is consistent with that of the previous report in which the authors indicated that loss of phenotypic expression occurred during the course of invading the submucosa or creating metastases, and that the loss was associated with dedifferentiation of cancer histology [28]. The null type cancer seen in the present study, therefore, might advance into undifferentiated cancer during progression.

This study has limitations. In particular, the number of analyzed cancers was relatively small, and all were differentiated and endoscopically resected cancers. Analysis of a larger number of cancers including other histological types and various depths of invasion may reveal more detailed correlations between gastric cancer invasion and epithelial cell phenotypes. It is also known that E-cadherin and Matrix metalloproteinases (MMPs) are associated with tumor invasion and metastasis in gastric cancer [34,35], and further studies are needed in order to examine the relationship between such factors and invasion pattens.

In conclusion, the results of the present study showed that cancer cell phenotype presentations in the mucosa and submucosa are correlated with the patterns of tumor invasion into the submucosal layer in early gastric cancer. Cancers with P-pattern invasion are likely to express the gastric phenotype in the submucosa even when the phenotype of the mucosa showed the intestinal type, suggesting the presence of unique invasion mechanisms in this type of cancers. The present findings may help, at least in part, to elucidate the mechanisms of gastric cancer invasion.

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