

## RESEARCH ARTICLE

# Correlation between Magnifying Narrow-band Imaging Endoscopy Results and Organoid Differentiation Indicated by Cancer Cell Differentiation and its Distribution in Depressed-Type Early Gastric Carcinoma

Hidezumi Tatematsu<sup>1</sup>, Ryoji Miyahara<sup>1\*</sup>, Yoshie Shimoyama<sup>3</sup>, Kohei Funasaka<sup>1</sup>, Eizaburou Ohno<sup>2</sup>, Masanao Nakamura<sup>1</sup>, Hiroki Kawashima<sup>1</sup>, Akihiro Itoh<sup>1</sup>, Naoki Ohmiya<sup>1</sup>, Yoshiki Hirooka<sup>2</sup>, Osamu Watanabe<sup>1</sup>, Osamu Maeda<sup>1</sup>, Takafumi Ando<sup>1</sup>, Hidemi Goto<sup>1</sup>

### Abstract

**Background:** A close association between patterns identified by magnifying narrow-band imaging (M-NBI) and histological type has been described. M-NBI patterns were also recently reported to be related to the mucin phenotype; however, details remain unclear. **Materials and Methods:** We investigated the cellular differentiation of gastric cancer lesions, along with their mucosal distribution observed by M-NBI. Ninety-seven depressed-type early gastric cancer lesions (74 differentiated and 23 undifferentiated adenocarcinomas) were visualized by M-NBI. Findings were divided into 4 patterns based on abnormal microvascular architecture: a chain loop pattern (CLP), a fine network pattern (FNP), a corkscrew pattern (CSP), and an unclassified pattern. Mucin phenotypes were judged as gastric (G-type), intestinal (I-type), mixed gastric and intestinal (M-type), and null (N-type) based on 4 markers (MAC5AC, MUC6, MUC2, and CD10). The relationship of each pattern of microvascular architecture with organoid differentiation indicated by cancer cell differentiation and its distribution in each histological type of early gastric cancer was investigated. **Results:** All CLP and FNP lesions were differentiated. The cancer cell distribution showed organoid differentiation in 84.2% (16/19) and 61.1% (22/36) of the two types of lesions, respectively, and there was a significant difference from the unclassified pattern with organoid differentiation ( $p < 0.001$ ). Almost all (94.7%; 18/19) CSP lesions were undifferentiated, and organoid differentiation was observed in 72.2% (13/18). There was a significant difference from the unclassified pattern with organoid differentiation ( $p < 0.05$ ). **Conclusions:** Cellular differentiation and distribution are associated with microvascular architecture observed by M-NBI.

**Keywords:** Magnifying endoscopy - early gastric carcinoma - cancer cell differentiation - organoid differentiation

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### Introduction

A close association between the results of magnifying narrow-band imaging (M-NBI) endoscopy and histological type has been noted for depressed-type early gastric carcinomas. Nakayoshi et al. (2004) first reported that M-NBI is capable of predicting histological characteristics: differentiated-type carcinomas are characterized by a fine network pattern (FNP), and undifferentiated-type carcinomas are characterized by a corkscrew pattern (CSP). However, many lesions in their study showed an

unclassified pattern. Therefore Yokoyama et al. (2010) defined a new category for lesions with an unclassified pattern called an intra-lobular loop pattern (ILL). ILL-1 has a papillary or granular micromucosal structure that contains loop-like microvascular architecture, and the lesions with an unclassified pattern without ILL-1 were called ILL-2. Thus, the M-NBI microvascular pattern can be classified as CLP, FNP, CSP, ILL-1 or ILL-2.

These microvascular patterns were closely related to cancer histological type. However, they were observed only in early cancers, and were not found in advanced

<sup>1</sup>Department of Gastroenterology and Hepatology, <sup>3</sup>Department of Pathology and Clinical Laboratories, Nagoya University Graduate School of Medicine, <sup>2</sup>Department of Endoscopy, Nagoya University Hospital, Nagoya, Japan \*For correspondence: myhr@med.nagoya-u.ac.jp

cancers, despite the identical histological type. It is important to investigate why advanced cancers lose the microvascular architecture observed in early-stage cancers.

With recent progress in studies of cell differentiation markers, many studies, including the example conducted by the World Health Organization (WHO, 2010), have indicated that phenotypic expression of gastric cancer cells can be classified using 4 markers. The gastric surface mucous cell and pyloric gland cell types are gastric, and the goblet and absorptive epithelial cell types are intestinal (Saito et al., 2001; Shiroshita et al., 2004). In intramucosal cancers, cancer cells of the surface mucous cell type and the pyloric gland cell type show a laminar distribution resembling normal pyloric gastric mucosa. Microvascular architecture observed by M-NBI should be related not only to histological type but also to phenotypic expression and distribution of cancer cells in the mucosa.

Thus, we investigated the cellular differentiation of gastric cancer cells, along with their distribution in the mucosa observed by M-NBI.

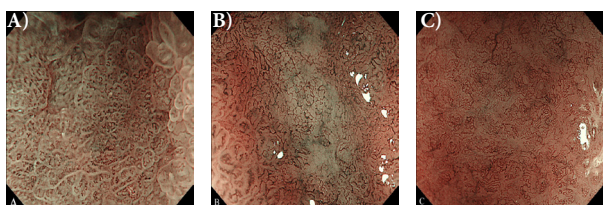
## Materials and Methods

### Patients

NBI-combined magnifying observation was performed following conventional endoscopy as a part of detailed preoperative examinations between June 2007 and March 2011. Since it is very difficult to differentiate precisely between gastric adenomas and elevated-type gastric cancers, only depressed-type early gastric cancers were included. We examined 110 lesions in 106 cases. A total of 13 lesions were excluded for the following reasons: microvascular architecture could not be evaluated due to erosion or ulcer for 6; identification of the vascular architecture was difficult due to hemorrhage for 4; and image quality was insufficient for 3. As a result, 97 depressed-type early gastric cancer lesions (74 differentiated adenocarcinomas and 23 undifferentiated adenocarcinomas) were analyzed. Magnified endoscopic images and histopathological findings were investigated retrospectively with regard to the association with phenotype expression. Clinicopathological data are summarized in Table 1. The study protocol was reviewed and approved by the ethics committee in our hospital.

### Examination and Diagnostic criteria for magnified endoscopic findings (Figure 1)

All endoscopic examinations were performed with a



**Figure 1. Magnifying Endoscopic Images of Depressed-type Early Gastric Adenocarcinoma.** (A) Chain loop pattern: Chain loop-like microvascular architectures enclosed in papillary or granular micromucosal structures. (B) Fine network pattern. (C) Corkscrew pattern

magnifying endoscope (GIF-H260Z; Olympus Medical Systems, Tokyo, Japan), a video system (EVIS LUCERA CV-260; Olympus Medical Systems, Tokyo, Japan), and a high-intensity light source (EVIS LUCERA CLV-260NBI; Olympus Medical Systems, Tokyo, Japan). A black soft hood (MB-162; Olympus Medical Systems, Tokyo, Japan) was mounted on the tip of the endoscope to fix the optimal distance between the lens of the endoscope and the mucosal surface under high-power magnification (Yao et al., 2002). Written informed consent was obtained from all patients who underwent an endoscopic examination with the NBI system. Endoscopy was performed by five endoscopists with broad experience in using a magnifying endoscope. Initially, the lesions were carefully observed without magnification, and then examined sequentially by magnified endoscopy with the NBI system. As a rule, a careful search for lesions was first performed by conventional observation, and then the device was switched to NBI for magnifying observation. Lesions were localized at moderate power magnification based on the demarcation line proposed by Yao (2002), and then the magnification was increased to high power by focusing on the white zone proposed by Yagi (2008). Stored magnified endoscopic images were classified by the criteria of Nakayoshi et al. (2004) as FNP, CSP, and unclassified pattern. Then, according to Yokoyama et al. (2010), the unclassified pattern lesions were further classified into lesions with papillary or granular micromucosal structures containing loop-like microvessels as chain loop pattern (CLP) and unclassified pattern. This CLP was first reported by Yokoyama et al. (2010) as ILL-1. ILL-2 should be included in the unclassified lesions related to FNP and/or CSP. Therefore, we replaced ILL-1 with CLP, and ILL-2 with unclassified pattern. So, in this study, we classified the M-NBI microvascular patterns as CLP, FNP, CSP or unclassified pattern.

When only an irregular mucosal pattern was observed with no abnormal blood vessels, it was excluded. When 2 or more types were observed, the quantitatively dominant type was assigned. The M-NBI and histopathological findings, phenotype expression of cancer cells, and distribution of each type of cancer cells in cancer tissues were carefully analyzed.

### Phenotypic classification by expression of cell differentiation markers

Endoscopically or surgically resected lesions were fixed in 10% buffered neutral formalin, and cross-cut into 2.5-mm slices. Hematoxylin/eosin (HE) preparations were prepared and observed under a microscope, and blocks containing sections in which the lesions reached the deepest sites were subjected to detailed examination. MUC5AC, MUC6, MUC2, and CD10 were specifically expressed in gastric crypt epithelium, gastric pyloric glands, goblet cells, and brush borders, respectively. For all markers, antibodies from Novocastra Co (Newcastle, United Kingdom) were used. After specimens were autoclaved, staining was performed employing a standard method with VECTASTAIN ABC Mouse IgG Kits from Vector Laboratories (Burlingame, CA). Following the Japanese Classification of Gastric Carcinomas

(second English edition, 1998), lesions were classified as differentiated (tub1, tub2, pap) and undifferentiated (por, sig), based on histological type. The histological diagnosis and judgment of cellular differentiation of cancer cells was accomplished by a pathologist who was blinded to the endoscopic findings. 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) Mixed phenotype (M-type): MUC5AC- and/or MUC6- as well as MUC2- and/or CD10-positive rates of 10% or more. iv) Null phenotype (N-type): All positive rates lower than 10%.

#### Judgment of organoid differentiation

In G-type and M-type intramucosal cancers, cancer cells of the surface mucous cell type (MUC5AC-positive) were found in the upper layer, and cancer cells of the pyloric gland cell type (MUC6-positive) were observed in the lower layers of the mucosa. This distribution of cancer cells resembled normal pyloric mucosa, first reported as a laminated structure and then called organoid differentiation (Akamatsu et al., 1990; Fujimori et al.,

1995). G and M-types of differentiated and undifferentiated adenocarcinomas with laminated structure were defined as organoid differentiation (Figure 2, 3). In the case of I-type differentiated cancers, cancers consisting of glands resembling intestinal crypts lined with a single layer of CD10-positive cells with or without MUC2-positive cells were included in the organoid differentiation category (Figure 4). Cancers showing an alveolar structure were excluded from the organoid differentiation category. No I-type undifferentiated carcinomas were found in this study.

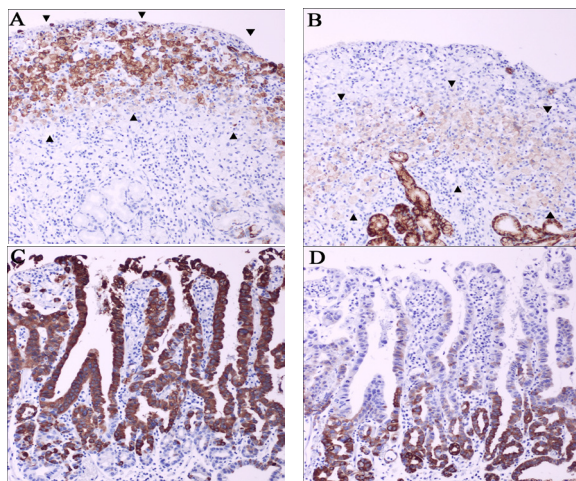
## Results

#### Correlation between histological findings and phenotype of cancer cells

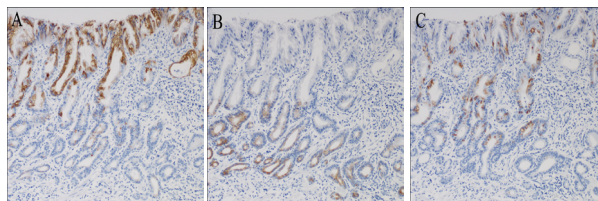
The details for the 74 differentiated early gastric cancers were: G-type, 25.7% (19/74); M-type, 39.2% (29/74); I-type, 35.1% (26/74); and N-type, 0% (0/74). There was no significant difference in the proportion of mucin phenotype expression. The details for the 23 undifferentiated early gastric cancers were: G-type, 52.2% (12/23); M-type, 43.5% (10/23); I-type, 0% (0/23); and N-type, 4.3% (1/23). Twenty-two (95.7%) of the 23 undifferentiated early gastric cancers were the G-type or M-type, and none was the I-type.

#### Correlation between M-NBI findings and organoid differentiation (Table 2)

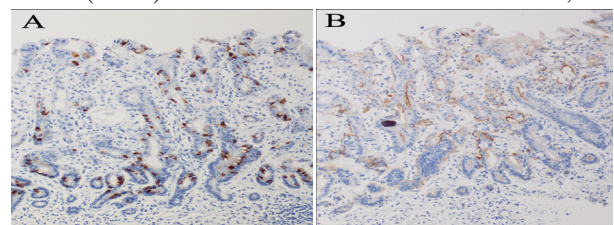
All 19 cases with the CLP and 36 cases with the FNP were differentiated adenocarcinoma, and no bias was noted in the frequency of the G, M or I-type. CLP and FNP lesions showed an organoid differentiation in 84.2% (16/19) and 61.1% (22/36), respectively. In 19 cases with the CSP, 94.7% (18/19) were undifferentiated adenocarcinoma, and



**Figure 2. Histochemical Demonstration of Organoid Differentiation in G-type Intramucosal Carcinomas.** (A) MUC5AC-positive cancer cells only in the upper layer of a signet ring cell carcinoma (black arrow-head) (mag. x100). (B) MUC6-positive cancer cells only in the lower layers (black arrow-head) (mag. x100). (C) MUC5AC-positive cancer cells only in the upper layer of a differentiated adenocarcinoma (mag. x100). (D) MUC6-positive cancer cells only in the lower layer of the cancer (mag. x100)



**Figure 3. Histochemical Demonstration of Organoid Differentiation in M-type Intramucosal Carcinomas.** (A) MUC5AC-positive cancer cells only in the upper layer of a differentiated adenocarcinoma (mag. x100). (B) MUC6-positive cancer cells only in the lower layer of the cancer (mag. x100). (C) MUC2 stained in spots only in the two-thirds of upper layer of the cancer (mag. x100)



**Figure 4. Histochemical Demonstration of Organoid Differentiation in I-type Intramucosal Carcinomas.** (A) MUC2 stained in spots only in the two-thirds of upper layer of the cancer (mag. x100). (B) Cancers consisted of single or bifurcated glands resembling an intestinal crypt line with a single layer of CD10-positive cells

**Table 1. Clinicopathological Features of Depressed-Type Early Gastric Cancer**

Number of lesions	97
Age (Mean±SD), y	70.2±7.9
Size (Mean±SD), mm	20.1±13.5
Histological type:	
Differentiated (tub1, tub2, pap)	53, 17, 4
Undifferentiated (por, sig)	19, 4
Location:	L, M, U
Depth:	M, SM
	81, 16

\*tub1 Well-differentiated tubular adenocarcinoma, tub2 Moderately differentiated tubular adenocarcinoma, pap Papillary adenocarcinoma, por Poorly differentiated adenocarcinoma, sig Signet-ring cell carcinoma, L lower third of the stomach, M middle third of the stomach, U upper third of the stomach, M Tumor invasion of mucosa and/or muscularis mucosa, SM Tumor invasion of submucosa



**Table 2. Correlation between Magnifying Narrow-Band Imaging Findings and Organoid Differentiation**

M-NBI		Phenotype expression				Total
		G	M	I	N	
Diff.	Chain loop (ILL-1)	6 (6)	7 (5)	6 (5)	0	19 (16)*
	Fine network	9 (5)	13 (8)	14 (9)	0	36 (22)*
	Corkscrew	0	1 (1)	0	0	1 (1)
	Unclassified	4 (1)	8 (2)	6 (1)	0	18 (4)
Undif.	Chain loop (ILL-1)	0	0	0	0	0
	Fine network	0	0	0	0	0
	Corkscrew	10 (8)	7 (5)	0	1	18 (13)**
	Unclassified	2 (0)	3 (1)	0	0	5 (1)
		31 (17)	36	29	1	97

Significant difference from unclassified pattern with organoid structure \*(p<0.01).

\*\* (p<0.05). ( ): number of organoid structures

all undifferentiated adenocarcinomas showing the CSP had a gastric component. Of undifferentiated adenocarcinomas showing the CSP, organoid differentiation was observed in 72.2% (13/18). In the 23 cases with an unclassified pattern, 78.3% (18/23) were differentiated, and 21.7% (5/23) were undifferentiated. Organoid differentiation was observed in 22.2% (4/18) and 20% (1/5), respectively.

For differentiated adenocarcinoma, tumors with the CLP and the FNP showed a significantly higher frequency of organoid differentiation than unclassified pattern tumors (p<0.001, Chi-square test). In contrast, for undifferentiated adenocarcinoma, tumors with the CSP showed a significantly higher frequency of organoid differentiation than unclassified pattern tumors (p<0.05, Fisher's exact test).

## Discussion

The aim of this study was to indicate clearly why advanced gastric cancers lost the microvascular architectures observed in early-stage gastric cancers, despite their identical histological type.

M-NBI can yield very clear images of microvessels and of the micromucosal structure of cancer tissues. Nakayoshi et al. (2004) have demonstrated that the FNP is typical of differentiated-type carcinoma and the CSP is typical of undifferentiated-type carcinoma. Yokoyama et al. (2010) also demonstrated that ILL-1 (CLP in this study) is related to differentiated adenocarcinoma. In this study, all cases with the CLP and the FNP were differentiated adenocarcinoma, and 18 of 19 cases with the CSP were undifferentiated adenocarcinoma. It is clear that each M-NBI pattern is closely related to each histological type of cancer. However, it is hard to explain why advanced cancers lost their specific micromucosal structure observed in early-stage cancers, despite their identical histology.

Phenotype expression of cancer cells might be involved in morphogenetic differences visualized by M-NBI. Several authors have reported that clinical findings vary corresponding to cancer cell differentiation. Kobayashi et al. (2011) investigated the phenotype expression in the morphogenetic differences between the FNP and the ILL (CLP in this study). They reported that the ILL (CLP in this study) was associated with the G-type and the FNP was associated with the I-type. However, in this study, all cases with the CLP and the FNP were differentiated

adenocarcinoma, and no bias was noted in the frequency of the G, M, or I-types. Therefore, these vascular patterns are characteristic of differentiated adenocarcinoma regardless of the phenotype expression. On the other hand, 18 of 19 cases with the CSP were undifferentiated adenocarcinoma, and 17 of these had a gastric component, and no I-type was found in this study. Since I-type early undifferentiated adenocarcinoma is very rare (Yamachika et al., 1997), its vascular construction is unclear. Further accumulation of cases of I-type early undifferentiated adenocarcinoma in the future may clarify whether the CSP is characteristic of G-type undifferentiated adenocarcinoma or of all types including I-types of undifferentiated adenocarcinoma.

Akamatsu et al. (1990) reported that derangement of intramucosal laminar distribution triggers invasion of submucosal tissues. In addition, Takizawa et al. (2006) reported that preservation of laminar differentiation of undifferentiated type intramucosal carcinoma has little risk of lymph node metastasis. These reports suggest that destruction of organoid differentiation of cancers is closely related to the progression of cancer from early to advanced stages. Tumors with the CLP and the FNP showed a significantly higher frequency of organoid differentiation than of the unclassified pattern (p<0.001, Chi-square test). Advanced cancers show no organoid differentiation. The lower incidences of organoid differentiation in tumors with an unclassified pattern suggest that this is more dedifferentiated than the CLP and FNP. From the viewpoint of cellular differentiation, an unclassified pattern represents the vascular view of depressed-type early gastric carcinoma unable to form the characteristic vascular pattern.

Eighteen of 19 cases with the CSP were undifferentiated adenocarcinoma, similar to the findings reported by Nakayoshi et al. (2004), and 13 of the 18 cases (72.2%) showed organoid differentiation. Undifferentiated adenocarcinomas with the CSP also showed a significantly higher frequency of organoid differentiation than tumors of the unclassified pattern (p<0.05, Fisher's exact test). Tumor cells were functionally (mucus productivity) well differentiated, although the glandular duct structure was undifferentiated. Early undifferentiated gastric cancer is comprised of the G-type and I-type cancer cells, which increase with progression (Yamachika et al., 1997) while organoid differentiation may be lost. Therefore, the CSP is characteristic of the early stage of G-type undifferentiated adenocarcinoma. Advanced cancers showed various unclassifiable vascular presentations without a characteristic vascular pattern.

There are several limitations to our study. First, it was retrospective, so the possibility of evaluation bias could not be ruled out, because cases with insufficient evaluation due to ulceration or bleeding were excluded. Second, the micromucosal structure was evaluated employing only NBI-combined magnifying observation. We did not include a dye-based imaging method, such as indigocarmine or acetic acid, both of which are useful. The visibility of micromucosal structures may be increased by spraying, especially with acetic acid (Yagi et al., 2005). Third, since the range of M-NBI covers only a part of the tumor, it is difficult to quantitatively analyze the composition of the

overall tumor pattern. Since the CLP, FNP, and CSP are assumed to represent early gastric cancer, a quantitative analysis method should be established employing them. Despite these limitations, we believe that NBI has potential for the evaluation of the degree of differentiation and is useful for understanding the biological behavior of depressed-type early gastric carcinoma.

Endoscopic submucosal dissection (ESD) is a useful treatment method for intramucosal differentiated carcinoma, and expansion of the indication for undifferentiated intramucosal cancer smaller than 2 cm is being investigated. Loss of organoid differentiation has been pointed out as a risk factor for lymph node metastasis-positive undifferentiated intramucosal cancer (Takizawa et al., 2006). However it is difficult to diagnose organoid differentiation with only endoscopic biopsy specimens due to the small size of sample. The presence of each type of characteristic microvascular architecture visualized by M-NBI might be indicated in gastric cancers with organoid structure. For example, if loss of the CSP and organoid differentiation can be revealed by M-NBI, this may be very useful for expanding the indication of endoscopic treatment in the future.

In conclusion, for depressed-type early gastric carcinoma, microvascular architectures observed by M-NBI are associated with cancer cell distribution regardless of phenotypic expression.

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