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Original Article

Correlation between narrow-band imaging endoscopy results and hPTTG expression indicated by angiogenensis and its distribution in the initial phase of colorectal adenoma-carcinoma sequence

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Abstract: The aim of the present study was to investigate the correlation between vascular characteristics under narrow band imaging endoscopy (NBI) and the expression of angiogenic factors of colorectal carcinoma and adenoma, and to evaluate the feasibility and validity of NBI in vivo visualizing angiogenesis. Patients with colorectal polyps, which were pathologically confirmed as early carcinoma, adenoma and hyperplastic polyp, were recruited and examined by NBI. The endoscopic vascular pattern was classified by Showa classification. Immunohistochemical staining was performed by cluster of differentiation (CD34), microvessel density (MVD) and Human Pituitary Tumor-Transforming Gene (hPTTG). The histologic results were compared with the vascular pattern under NBI. Overall, 83 colorectal lesions including 9 intramucosal colorectal carcinomas, 44 adenomas (18 tubular adenomas, 26 tubulovillous adenomas) and 30 hyperplastic polyps were recruited and examined by NBI. A higher proportion (88.6%, 47/53) of intramucosal carcinomas and adenomas were more likely to have the dense pattern (DP) or network pattern (NP), while that of hyperplastic polyps was only 30.0% (9/30). There was an obvious increase in the MVD-CD34 counting from hyperplastic polyps, to adenoma to carcinoma, and a significant difference among the three groups as well. Also, a clear difference can be seen in the expression of hPTTG, which was expressed more in carcinoma than in adenoma and HP group ($P < 0.05$). Conclusion: NBI might be a useful tool as in vivo visualizing angiogenesis. hPTTG expression in colorectal adenoma and carcinoma is related to angiogenesis.

Keywords: Colorectal adenoma, angiogenesis, narrow band imaging, microvessel density, Human Pituitary Tumor-Transforming Gene, cluster of differentiation

Introduction

The incidence and mortality rate of colorectal cancer (CRC) have increased dramatically over the past 3 decades in China due to changes in lifestyle factors [1]. A large body of clinical evidence supports the belief that a majority of CRC arise from precursor lesions, benign adenomatous polyps, that is, the classical adenoma-carcinoma sequence (ACS). The ACS is a series of events whereby colorectal adenomas develop, initially showing low grade dysplasia, from which some will progress to develop areas of high grade dysplasia and eventually invasive carcinoma. Tumor angiogenesis and metastasis are considered key factors for tumor progression, which lead to poor prognosis for patients with CRC [2, 3]. The degree of neovascularization in malignant tissues is a prognostic factor for many human solid tumors including CRC. Angiogenesis inhibitors offer a novel approach for CRC therapy [4]. Although there has been one suggestion that initiation of angiogenesis (the angiogenic switch) occurs simultaneous to invasion [5, 6], another study suggests that the angiogenic switch occurs at the onset of dysplasia in the ACS [7]. Angiogenesis is crucial for the growth of CRC and metastases [8]. In recent years, large quantitative laboratory studies have clarified the molecular mechanism of tumor-related angiogenesis. Vascular endothelial growth factor (VEGF)

is one of the most important proangiogenic factors that can drive tumor angiogenesis [9, 10]. It not only enhances vessel branching and number, but also increases vessel caliber [11]. To date, the widely-used methods to assess angiogenesis are in-vitro means. For example, quantification of microvessel density (MVD) and VEGF expression have been commonly used to assess intratumoral angiogenesis. Human pituitary tumor transforming gene (hPTTG) was recently identified as a protooncogene and a transcriptional activator of several angiogenic factors [12, 13]. It is particularly noteworthy that immunohistochemical double staining indicated co-localization of VEGF in many hPTTG-positive tumor cells. Therefore, higher levels of hPTTG expression contribute to the pathobiology of tumors by promoting angiogenesis [14]. Our previous study has showed that hPTTG expression in ACS tissue and its expression were associated with VEGF in the early stages of CRC development [15]. On the other hand, advances in biomedical optics, especially narrow band imaging endoscopy (NBI), enable the in-vivo observation of microvascular architecture on the mucosal surface of gastrointestinal tract [16, 17]. Generally it is well known that in relation to histological atypia of a colorectal lesion, the tumor vascularized and the diameter and density of microvessels increase. As many studies obtained similar results, microvessels in the superficial layer of a normal mucosa or a hyperplastic lesion are very fine, and are thereby difficult to recognize by NBI. However, neoplastic lesions are recognizable as dark brown because of the increases in diameter and density of microvessels in the superficial layer [18, 19]. There are different appearances under NBI between tubular adenomas and tubulovillous adenomas. However, there are few data to analyze the correlation between angiogenic factors expression and mucosal appearance observed by NBI.

Therefore, this study quantified the MVD, the expression of hPTTG in the initial phase of the ACS, and evaluated its correlation with mucosal microvasculature observed by NBI, and evaluated the validity of NBI in-vivo visualizing angiogenesis of ACS.

Materials and methods

Patients

Consecutive patients who had colonoscopy examination and were diagnosed with colorectal polyps from November 2013 to February 2014 were enrolled in the study at Beijing Shijitan Hospital. Some patients were excluded from the study according to the following exclusion criteria: the patients with advanced colorectal cancer, inflammatory bowel disease, familial adenomatous polyposis, serrated adenoma, acute gastrointestinal bleeding, coagulopathy, impaired renal function, inability to provide informed consent. Written informed consent was obtained from all participating patients before the procedure. This protocol has been approved by the ethics committee of the Beijing Shijitan Hospital.

Narrow band imaging (NBI) system

Colonoscopy was performed using the Lucera system with sequential red-green-blue illumination (CV-H260, Olympus, Tokyo, Japan) incorporating NBI. The NBI filter is activated by pushing a button on the handle of the colonoscope (CF-H260, Olympus, Tokyo, Japan). It takes approximately 1 to 2 seconds for the image to change on the monitor.

Endoscopic procedure and vascular evaluation

Routine endoscopy was first performed using the non-NBI function. If polyps or polypoid lesions present, the location, size, and shape were recorded. Then, we pressed the button for NBI, and microvascular pattern was observed carefully using the NBI system. All the lesions were photographed under NBI during colonscopy and removed by endoscopic submucosal dissection (ESD), endoscopic mucosal resection (EMR), or a biopsy forceps. Retrospectively analysis was performed on the microvascular pattern of all the lesions. All of the lesions were carefully observed by two experienced endoscopists (HL and JW).

In the present study, the Showa classification of NBI endoscopic characteristics proposed by Showa University Northern Yokohama Hospital was used to evaluate microvascular architecture [20]. The classification and evaluation methods are provided as follows:

Faint pattern: vessels were rather thin and obscure in NBI, usually indicate hyperplastic polyps; Network pattern: tubular adenomas showed a regular vessel; Dense pattern: in villous and tubulovillous adenomas, the vessels were well developed and rather thick; Irregular

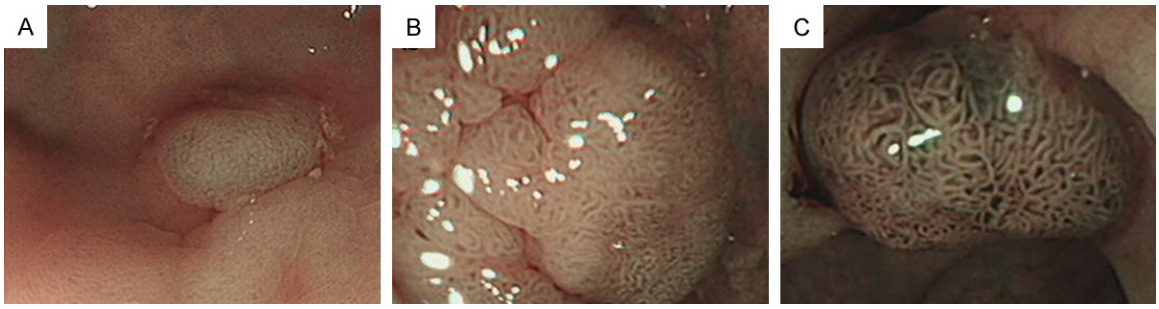

Figure 1. The Showa classification of NBI. A. Faint pattern: vessels were rather thin and obscure in NBI, and usually indicate hyperplastic polyps; B. Network pattern: tubular adenomas showed regular vessels; C. Dense pattern: in villous and tubulovillous adenomas, the vessels were well developed and rather thick.

pattern: the vessels were thick and irregular, interruption of the network, a tortuous course of vessels, and unusually large caliber of vessels, often observed in protruded submucosally invasive cancers; Sparse pattern: depressed invasive cancers showed decreased vessels.

Examples of all above pattern polyps are shown in **Figure 1**. In this study, the microvascular type of each lesion was recorded and compared with the histopathologic and immunohistochemical findings accordingly.

Histopathologic analysis

In the study, we performed EUS on giant polyps or lateral spreading tumor after NBI colonscopy to obtain a rough estimation of tumor invasion depth. Then EMR, ESD biopsy, or surgery was performed and the final diagnosis rested on histopathological examination of the resection specimen.

The specimens were fixed with 10% formalin, embedded in paraffin, and then sectioned. One of three consecutive sections at the center of the tumor was stained with hematoxylin-eosin (HE) to determine the histopathological type, while the others was immunostained by anti-CD34 and anti-hPTTG monoclonal antibody (Santa Cruz Biotechnology, Inc., US).

We adopted the WHO histologic diagnostic criteria. The cases were classified as adenomatous polyps (tubular adenomas, villous adenomas, tubulovillous adenomas), or hyperplastic polyps. The histopathologic diagnosis was performed by two pathologists independently and blindly to the endoscopic results, and the consensus diagnoses were used in the subsequent analysis.

MVD assessment and quantification of PTTG staining

MVD was assessed in areas of polyps containing the greatest number of microvessels and small venules at the superficial mucosa of highest atypia. When the five most highly vascularized areas, or hot spots, labeled by CD34 were initially selected under 40× field, a 200× field was used to count microvessels in each hot spot, and the average count of the 5 fields was calculated. hPTTG staining was assessed using a semi-quantitative grading system which reflected the intensity of staining present within the specimen. hPTTG protein positive staining was defined as when brown granular appeared in the cytoplasm. hPTTG protein staining intensity classification: non-colored or colored cells less than 10% was scored zero, color tinted or colored cells in 10% to 25% was scored 1, moderate colored or colored cells in 26% to 50% of cells was scored 2, and deep color or colored cells in 51% to 75% of cells was scored 3, colored cells more than 75% of cells was scored 4. The sum of the degree of color score and color cells score was defined as color index score.

Statistical analysis

All the statistical analysis was performed using the statistical software package SAS (9.12; SAS Institute Inc., Cary, NC).

All data are presented as mean ± standard deviation. Significance level was set at $P < 0.05$. Appropriate non-parametric tests were used to investigate microvascular architecture observed by NBI and angiogenesis parameters. Spearman's correlation coefficient was used firstly, and then ANOVA analysis of variance was

Table 1. Histologic type and endoscopic findings of the lesions

Total number of lesions	83
Mean size SD, cm	
Carcinoma	1.80 ± 0.21
Adenoma	1.67 ± 0.25
HP	0.94 ± 0.19
Location, no.	
Right side	24
Left side	40
Rectum	19
Morphology, no.	
Protruded	55
Elevated	26
Depressed	2

HP: hyperplastic polyp.

Table 2. Correlation between NBI type and histologic findings

NBI type	No.	HP	TA	VA	Intramucosal carcinoma
F type	23	21	1	1	0
N type	30	9	14	7	0
D type	26	0	3	16	7
I type	4	0	0	2	2
	83	30	18	26	9

F type: Faint pattern; N type: Network pattern; D type: Dense pattern; I type: Irregular pattern.

used to analysis the difference of MVD/PTTG among different NBI types.

Results

A total of 83 polyps of 69 patients were enrolled in the study. The mean age of the patients was 53.8 years (range: 32-75). Among the 83 polyps, 9 cases were intramucosal carcinomas, 44 cases were adenomas and 30 cases were hyperplastic polyps. In the 44 adenomas, 18 were tubular adenoma, 26 were tubulovillous adenoma. The histologic diagnoses of polyps, as well as polyp sizes and locations as detected and viewed with WL, are shown in **Table 1**.

Correlation between NBI type and histological findings

The vascular pattern observed by NBI wis shown in **Table 2**. Specifically, out of the total 83 polyps, no polyp is sparse pattern, only 4 is irregular pattern, 26 are dense pattern, 30 are network pattern, 23 are faint pattern. Overall, intramucosal carcinoma and adenomatous polyps were more likely to have a dense or network pattern of vasculature compared with hyperplastic polyps (88.6% vs 30.0%, P < 0.001). 7 out of 9 intramucosal carcinomas, but no hyperplastic polyps, had a dense pattern. In the 26 villous adenomas, 16 were dense pattern. In the 18 tubular adenomas, 14 were network pattern. Of the dense pattern of adenoma, the most common histology was tubulovillous adenoma (61.5%, 16/26). In the network pattern of adenoma, the most common histology was tubular adenoma (46.7%, 14/30) (**Table 2**).

Correlation between NBI microvasculature pattern and MVD counts

The microvessels within lesion tissues were clearly identified by CD34 antibody (**Figure 2**). The MVD-CD34 counting of 9 cases carcinoma was 26.50 ± 6.27, that of adenoma was 22.86 ± 4.85, and that of hyperplastic polyps was 9.61 ± 3.77. From these figures, we can see a clear increase of MVD-CD34 from HP, and adenoma to carcinoma, and a significant difference among the three groups (F = 69.70, P < 0.0001) (See **Table 3**).

The MVD counting of dense pattern (DP) was 25.27 ± 5.82, that of network pattern (NP) was 21.70 ± 5.00, and of faint pattern (FP) was 10.81 ± 5.41 (See **Table 4**). In the study of the correlation between NBI microvascular pattern and MVD counting, we can figure that the Spearman's rank correlation coefficient is 0.6730, P < 0.001, which indicates a definite correlation between the two aspects. The MVD counting presents an increasing trend along with the microvascular pattern going from light faint pattern to dark dense pattern. An apparent difference can be found in the MVD counting in the three endoscopic microvascular patterns with further analysis (F = 46.48, P < 0.0001).

Correlation between NBI microvasculature pattern and hPTTG expression

As a newly found histologic marker, hPTTG has an advantage to identify neo-vasculature in the process of ACS. In contrast to CD34 experimental results, the microvessels within the lesion tissues identified by PTTG antibody are shown in **Figure 3**. The mean value of PTTG is 5.89 ± 1.54 in the carcinoma group, is 4.51 ± 1.42 in the adenoma group, and 1.77 ± 1.15 in the HP

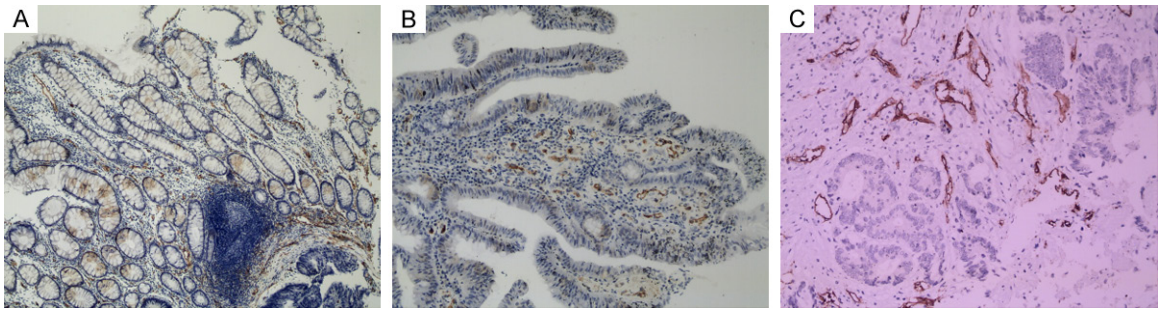

Figure 2. Expression of CD34: (A) The expression of CD34 in hyperplastic polyp (200×); (B) The expression of CD34 in adenoma (200×); (C) The expression of CD34 in intramucosal colorectal cancer (200×).

Table 3. Correlation between histologic type and MVD and hPTTG expression

Histology	No.	MVD	*P* value	hPTTG	*P* value
HP	18	9.61 ± 3.77	< 0.0001	1.77 ± 1.15	< 0.0001
Adenoma	51	22.86 ± 4.85		4.51 ± 1.42	
Carcinoma	11	26.50 ± 6.27		5.89 ± 1.54	

MVD: Microvessel Density.

Table 4. Correlation between NBI vascular type and immunohistochemical findings

NBI	Area (No.)	MVD	*r* value	PTTG	*r* value
DP	24	25.27 ± 5.82	0.6730	5.46 ± 1.59	0.7295
NP	32	21.70 ± 5.00		4.28 ± 1.20	
FP	23	10.81 ± 5.41		1.78 ± 1.13	

F type: Faint pattern; N type: Network pattern; D type: Dense pattern; I type: Irregular pattern.

group. Compared with adenoma and HP, hPTTG was significantly increased in carcinoma ($P < 0.05$). There is no significant difference between adenoma and HP ($P > 0.05$) (See **Table 3**). The mean value of hPTTG in dense pattern (DP) was 5.46 ± 1.59, that of network pattern (NP) was 4.28 ± 1.20, and that of faint pattern (FP) was 1.78 ± 1.13 (See **Table 4**). Through the analysis of the Spearman's rank correlation coefficient, which is 0.6730 ($P < 0.001$), between the NBI microvasculature pattern and hPTTG expression, we see that a correlation between the two points and the correlation coefficient was even higher than that of NBI microvasculature pattern and MVD counts.

Discussion

Angiogenesis is important in the diagnosis and treatment of tumors. A number of studies have indicated that angiogenesis increases at the stage of precancerous lesions. NBI endoscopy enables the observation of angiogenesis in vivo [6, 7]. In the present study, the microvascular morphologic changes of colorectal polyps were observed to be positively correlated with angiogenesis indexes in histologic examination under NBI endoscopy.

In this program we preferred to adopt the Showa classification standard to identify the NBI endoscopic characteristics, because this method has nothing to do with the endoscopic magnifying technique. Almost all of the Olympus 260 series endoscopes are equipped with NBI technique, but not the magnifying technique. In some western countries, the magnifying endoscope is much less used for the clinical practice than that in China and Japan. So we determined to choose the Showa classification to category the NBI images, this classified system shows much advantages for observing the microvascular morphology changes in colorectal lesions in medical practice.

In this study, we can see the correlation between the NBI classification of colorectal lesion and its pathologic result, which is concordant with our previous study and other similar published results [21, 22].

The microvacular features observes by NBI are correlated with the expression of MVD and hPTTG, which indicating that the angiogenesis changes in early phage in ACS (from normal mucosa, precursor lesions to benign adenomatous polyps) can be observed in-vivo by NBI. As the number of microvessels increased and the color deepened, the angiogenesis factor expression increased in the tissues, which in-

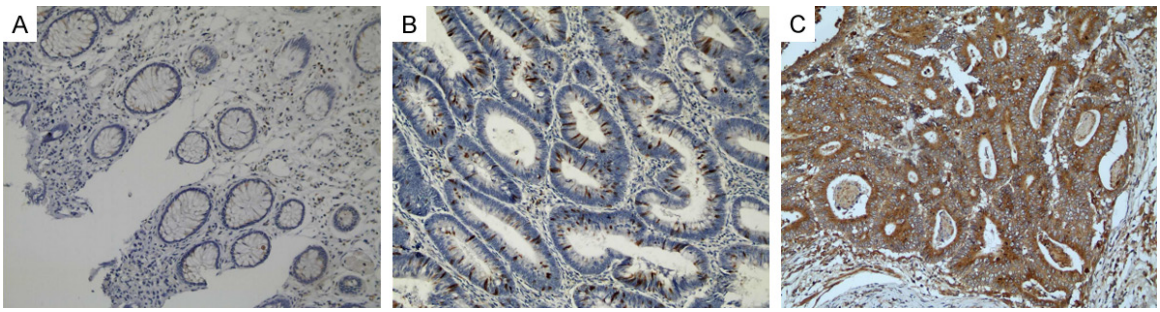

Figure 3. The expression of hPTTG: (A) The expression of hPTTG in hyperplastic polyp (200×); (B) The expression of hPTTG in adenoma (200×); (C) The expression of hPTTG in intramucosal colorectal cancer (200×).

dicated the feasibility of observing angiogenesis under endoscopy. There was a correlation between the endoscopic classification and histologic results. In the present study, the expression of MVD was examined by labeling vascular endothelial with CD34 by immunohistochemistry. The results indicated that MVD in adenoma and intramucosal carcinoma was higher than in hyperplastic polyps, and that MVD increased markedly in adenoma. Previous research has demonstrated that the increase of MVD depends on the expression level of angiogenesis factors [23]. The current study indicated that there was a similar tendency between MVD and the expression of hPTTG, increasing gradually in hyperplastic polyps, adenomas and intramucosal carcinoma.

In the present study, consecutive patients were not chosen as study subjects, because in conventional endoscopic examination there are a large number of hyperplastic polyps and low-grade tubular polyps. In order to observe a relationship between microvessel pattern and histological result in carcinogenesis, adenomas were more chosen as targets.

In conclusion, NBI might be a useful tool for in vivo visualizing of angiogenesis. hPTTG expression in colorectal adenoma and carcinoma is related to angiogenesis, which could be a potential promising histologic factor compared with the MVD.

Disclosure of conflict of interest

None.

Abbreviations

CRC, colorectal cancer; ACS, adenoma carcinoma sequence; NBI, Narrow Band Imaging; MVD, Micro Vessel Density.

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