ANGIOGENESIS IN SUPERFICIAL ESOPHAGEAL SQUAMOUS CELL CARCINOMA: MAGNIFYING ENDOSCOPIC OBSERVATION AND MOLECULAR ANALYSIS

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Observations of esophageal squamous cell carcinoma using magnifying endoscopy have now been carried out extensively and, as a result, it has become clear that the morphology of the microvessels evident at the tumor surface reflects the depth of tumor invasion. In M1 and M2 cancer, the surface microvasculature reveals dilation and elongation of the intrapapillary capillary loops (IPCL). However, at this stage, some immature capillaries resembling IPCL also arise inside the tumor and, therefore, the view of the microvasculature should be described as one showing ‘intermixing of modified IPCL and IPCL-like immature capillaries (IPCL-like abnormal capillary)’. As cancer invades into the muscularis mucosa (M3 or deeper), an obviously dilated and irregularly branched tumor-specific vasculature, more accurately described as ‘neovascularature’, can be observed. From our magnifying endoscopy observations and studies of the molecular profile of early esophageal cancer, we conclude that two major angiogenic steps exist in precancerous and M3 lesions in the early phase of cancer progression. In addition, it is now possible to study cell morphology using an endocytoscope with a much higher magnification (>x400–x1000) than magnifying endoscopes currently on the market. The histology revealed in this way may reduce the need for conventional biopsy histology in the future.

Key words: angiogenesis, endocytoscopy system, esophageal cancer, magnifying endoscopy, squamous cell carcinoma, virtual histology.

INTRODUCTION

The most important strategy for successful cure of esophageal cancer is detection at an early stage of cancer progression. Such early-stage cancers are considered to be curable by endoscopic mucosal resection (EMR), which is only minimally invasive, and the resulting clinical prognosis is good. (In the present review, the depth of invasion of superficial esophageal carcinoma is given in accordance with the subclassification criteria of the Japan Esophageal Society [Guidelines for Esophageal Cancer Treatment]1). EMR or endoscopic submucosal dissection (ESD) is unequivocally indicated for the treatment of carcinoma in situ (M1) and tumor invasion to the lamina propria mucosa (M2) esophageal squamous cell carcinoma (ESCC), and it may also be applicable to tumor invasion to the muscularis mucosa (M3) and tumor invasion to the upper third of the submucosal layer (SM1) cancer, depending on whether lymph node metastasis is present.2 However, advanced esophageal cancers require radical surgery that leads to a deterioration in the quality of life and is associated with a poorer prognosis.3,4

Recently, magnifying observation at up to >x80 with narrow-band imaging has proved to be effective for detecting early-stage ESCC.5–8 Furthermore, the morphology of the surface microvessels of a tumor is known to reflect the depth of tumor invasion. Such diagnosis is based on observations of angiogenesis during cancer progression.9 The esophagus is the only organ for which morphological change from a normal microvasculature to that characterizing early-stage or advanced cancer has been demonstrated using magnifying endoscopy. To understand this phenomenon, it is important not only to elucidate the process of angiogenesis in the early phase of cancer progression, but also to consider molecular targeting therapy or chemoprevention.

In the present review, we report the current state of progress of magnifying observation of ESCC, focusing on the morphology of the surface microvasculature, and the correlation between these endoscopic findings and the molecular features reported in the literature.

SEARCH STRATEGY AND SELECTION CRITERIA

Published and unpublished data for this review were identified by searches of PubMed, US National Library of Medicine, National Institute of Health (http://www.ncbi.nlm.nih.gov/pubmed/) and references from relevant articles. We have also cited some articles from our own database.

During the same period, Arima microscopic microscope confirmed the validity of magnifying and cancer lesions injected with MICROFIL using a stereo-which was injected to fill the microvessels of the resected reduced MICROFIL (Flow Tech, Inc., Carver, MA, USA), of pooled blood. To solve this problem, Kumagai the resected esophagectomy specimens, in view of their lack of the surface microvasculature of superficial esophageal carcinoma. However, that study used a relatively low-powered magnifying endoscope, and its clinical applicability was therefore limited. Magnifying endoscopic observation of the normal esophageal mucosa and ESCC using the GIF-200UHM (Olympus medical Systems Co. Tokyo, Japan), which had a magnifying capacity of ×150, was first reported by Inoue et al, who succeeded in discovering looped capillary vessels inside the epithelial papillae (intrapapillary capillary loops [IPCL]). The IPCL inside cancer lesions were demonstrated to show abnormal changes, such as ‘dilation, weaving, changes in caliber, and variety of shape’. It also revealed that the morphology of the surface vasculature of superficial esophageal cancers exhibited characteristic changes according to the depth of tumor invasion. However, the area observed by magnifying endoscopy was narrow (approximately 3 × 3 mm) and, thus, there was some concern as to whether the microvasculature that was demonstrated matched the findings of histological observations. In addition, it was considerably disadvantageous that the microvasculature could not be observed in the resected esophagectomy specimens, in view of their lack of pooled blood. To solve this problem, Kumagai et al. introduced MICROFIL (Flow Tech, Inc., Carver, MA, USA), which was injected to fill the microvessels of the resected specimen. Comparison of the normal esophageal mucosa and cancer lesions injected with MICROFIL using a stereoscopic microscope confirmed the validity of magnifying observation. During the same period, Arima et al. reported that the vessels present at the surface of the esophageal mucosa showed characteristic changes according to the degree of atypia or depth of cancer invasion. Thereafter, with an increase in cases observed by magnifying endoscopy, each of the two groups proposed different classifications (Inoue classification and Arima classification), which has resulted in the use of two standards at the present time in Japan.

In 2009, Olympus Medical Systems Co. (Tokyo, Japan) manufactured a novel magnifying endoscope, the GIF-Y002, that has a single lens for which the magnification can be consecutively increased from the conventional endoscopy level to ×380. The GIF-Y002 is designed to allow observation of the surface cells (i.e. as an endocytoscope), as well as visualizing the surface vasculature at higher power, also allowing observation of blood flow in the surface vessels in some cases.

**BACKGROUND TO THE DEVELOPMENT OF MAGNIFYING ENDOSCOPY FOR OBSERVATION OF THE ESOPHAGUS**

Magnifying observation of the esophageal mucosa was first reported by Kozu et al. in 1975. They used a ×25 magnifying endoscope to study various esophageal lesions. However, because of the prevailing clinical circumstances at the time, all of the cases they studied were advanced squamous cell carcinomas. In 1991, Makuuchi et al. reported magnifying observation of the surface microvasculature of superficial esophageal carcinoma. However, that study used a relatively low-powered magnifying endoscope, and its clinical applicability was therefore limited. Magnifying endoscopic observation of the normal esophageal mucosa and ESCC using the GIF-200UHM (Olympus medical Systems Co. Tokyo, Japan), which had a magnifying capacity of ×150, was first reported by Inoue et al, who succeeded in discovering looped capillary vessels inside the epithelial papillae (intrapapillary capillary loops [IPCL]). The IPCL inside cancer lesions were demonstrated to show abnormal changes, such as ‘dilation, weaving, changes in caliber, and variety of shape’. It also revealed that the morphology of the surface vasculature of superficial esophageal cancers exhibited characteristic changes according to the depth of tumor invasion. However, the area observed by magnifying endoscopy was narrow (approximately 3 × 3 mm) and, thus, there was some concern as to whether the microvasculature that was demonstrated matched the findings of histological observations. In addition, it was considerably disadvantageous that the microvasculature could not be observed in the resected esophagectomy specimens, in view of their lack of pooled blood. To solve this problem, Kumagai et al. introduced MICROFIL (Flow Tech, Inc., Carver, MA, USA), which was injected to fill the microvessels of the resected specimen. Comparison of the normal esophageal mucosa and cancer lesions injected with MICROFIL using a stereoscopic microscope confirmed the validity of magnifying observation. During the same period, Arima et al. reported that the vessels present at the surface of the esophageal mucosa showed characteristic changes according to the degree of atypia or depth of cancer invasion. Thereafter, with an increase in cases observed by magnifying endoscopy, each of the two groups proposed different classifications (Inoue classification and Arima classification), which has resulted in the use of two standards at the present time in Japan.

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**TECHNIQUE OF OBSERVATION USING MAGNIFYING ENDOSCOPY AND ITS RESULTS FOR ESCC**

The preparations for magnifying observation, including pharyngeal anesthesia, are similar to those for conventional endoscopy. Sometimes, however, it is difficult to obtain detailed pictures because of mucosal movement caused by heartbeat, respiration and peristalsis. Therefore, we have devised some methods for limiting such technical failure. First, a soft plastic hood 2-mm thick is attached to the distal end of the magnifying endoscope to maintain an appropriate distance from the target tissue and to allow observation at the maximum magnification of ×80. We have also tried to exclude the influence of peristalsis and swallowing by using i.v. midazolam and i.m. scopolamine butylbromide.

Magnifying observation of ESCC is useful for pretreatment diagnosis of depth of tumor invasion. Based on a study of 263 esophageal cancers, Arima et al. reported that the diagnostic accuracy of magnifying endoscopy for depth of invasion was 98.8% for M1 and M2 cancers, 68% for M3 and SM1 cancers, and 84.0% for tumor invasion to the middle third (SM2) and lower third (SM3) of the submucosal layer cancers. They concluded that magnifying observation facilitated the identification of lesion microinvasion, thereby increasing the proportion of correctly diagnosed M3 or SM1 cancers in comparison with conventional endoscopic diagnosis.

Kumagai et al. also reported that the rate of accurate diagnosis of depth of invasion was 83.3% in cases where detailed pictures were obtainable using magnifying endoscopy. They mentioned that it was possible to clearly distinguish the depth of invasion of the tumor as shallower than M2 or deeper than M3, considering the superficial microvasculature of the esophageal cancer.

**MICROVASCULAR ARCHITECTURE OF THE NORMAL ESOPHAGEAL MUCOSA**

Several groups have described the microvascular architecture of the normal esophageal mucosa, in relation to analysis of esophageal varices. Aharinejad et al. reported a detailed three-dimensional analysis of the esophageal microvasculature based on a study of microvascular corrosion casts of the esophagus using scanning electron microscopy. They noted the IPCL as ‘capillary loops protruding towards the lumen’ in observations of guinea-pigs and rats. However, they did not mention the presence of these capillary loops inside the epithelial papillae.

Based on observations in humans using ×120 magnifying endoscopy, Inoue et al. reported that submucosal vessels connected to the arborescent vascular network could be observed at lower magnification. These arborescent vessels are recognizable as being interconnected, usually at the level of the fourth branch of the fine network. At higher magnification, capillary vessels in the papillae after the arborescent vessels can be observed. Furthermore, every intrapapillary capillary was observed as a single capillary loop. These were referred to as ‘intrapapillary capillary loops’ (IPCL).

Kumagai et al. clarified the relationship between this vascular structure and the esophageal wall using stereoscopic
microscopy after MICROFIL injection. They mentioned that the vessels pierced the muscle layer, forming an arborescent vascular network. This network was relatively sparse in the submucosal layer, but became dense both above and below the muscularis mucosae. In addition, these arborescent vessels gradually formed a thin net as they rose to the surface of the esophageal mucosa, and finally formed the IPCL (Fig. 1b). Furthermore, the IPCL were arranged regularly at intervals of approximately 100 μm, which corresponds to the maximum distance oxygen can diffuse from a vessel.

Use of the GIF-Y0002 has made it possible to clearly distinguish arterioles and venules based on observation of blood flow. We were able to visualize the relationship between the subepithelial capillary network of the arterioles, IPCL and subepithelial drainage venules. The arterioles arising in the lamina propria mucosae formed a subepithelial capillary network, forming an arborescent vascular network, and eventually formed the IPCL at the surface of the mucosa (Fig. 1b). Furthermore, the IPCL were arranged regularly at intervals of approximately 100 μm, which corresponds to the maximum distance oxygen can diffuse from a vessel.

Fig. 1.  (a) Normal squamous epithelium observed by magnifying endoscopy at ×80 (narrow band imaging). Arborescent vascular network and normal intrapapillary capillary loops (IPCL) are evident. (b) Cross-section of the esophageal wall after injection of MICROFIL (Flow Tech, Inc., Carver, MA, USA). The relationship between the arborescent vascular network and IPCL is clearly visualized. *, squamous epithelium; **, lamina mucosal propria; ***, muscularis mucosae; ****, submucosa; ***** muscularis propria. Small arrow, arborescent vascular network; large arrow, IPCL at the surface of the mucosa. (This figure is quoted from ref. no. 7.) (c) Microvasculature of the normal esophageal mucosa using the highest magnification of the GIF-Y0002 (Olympus Medical Systems Co., Tokyo, Japan) (narrow band imaging). We were able to visualize the relationship between the subepithelial capillary network of the arterioles, IPCL and subepithelial drainage venules. Black arrow, subepithelial capillary network of the arterioles; white arrow, IPCL; red arrow, subepithelial drainage venules. (This figure is quoted from ref. no. 15.) (d) Histological section of the normal esophageal wall after MICROFIL injection. Epithelial papillae and IPCL are evident. (e) Schema of the superficial vascular network of the normal esophageal mucosa. SA, submucosal artery; SCN, subepithelial capillary network; SDV, subepithelial drainage vein; SV, submucosal vein. (This figure is quoted from ref. no. 15.)
network beneath the epithelium from which IPCL in the epithelial papillae arose. IPCL were recognized as the terminal capillaries of the squamous epithelium, and then drained into the venules (Fig. 1c–e). Because these venules were thicker than arterioles, the arborescent vascular network that was evident using conventional endoscopy was considered to consist mainly of veins.

**MICROVASCULAR ARCHITECTURE OF ESOPHAGEAL NEOPLASMS**

Using ×120 magnifying endoscopy, Inoue et al. demonstrated that the IPCL of carcinoma in situ (M1) had characteristic changes such as ‘dilatation, weaving, changes in caliber and variations in shape’, compared with normal esophageal mucosa.6 As cancer invaded the lamina propria mucosae (M2), the papillae became longer, and the IPCL revealed elongation as the tumor thickness increased (Fig. 2a–c).7,14,16 However, there is some concern that the number of IPCL in a mucosal cancer increases in comparison with the normal squamous epithelium (Fig. 2d,e). Using CD105 antibody (endoglin), Kubota et al. counted the microvessel density (MVD) of lugol-unstained lesions with non-dysplastic epithelium (esophagitis), and low-grade and high-grade dysplasia of the squamous epithelium.22 Endoglin/CD105 is well acknowledged as being the most reliable marker of endothelial cell proliferation, and is overexpressed on tumor vessels.23 Kubota et al. mentioned that CD105-positive vessels were already present in esophagitis and low-grade dysplasia, and that CD105-positive MVD in high-grade dysplasia was significantly increased in comparison with low-grade dysplasia. These newly recruited capillaries are similar to adjacent modified IPCL in shape. However, in general, these capillaries may reveal hyper-permeability characteristics and have a fragile structure. We therefore, favour, the descriptive term ‘intermixing of modified IPCL and immature IPCL-like capillaries (IPCL-like abnormal capillary)’ for the vasculature of mucosal cancer (M1, M2) and dysplasia. Furthermore, the arborescent vascular network of M1 or M2 cancer that exists in the lamina propria mucosae and submucosa (beneath the tumor) is densely arranged in comparison with the normal squamous epithelium, based on stereoscopic microscopy observations after MICROFIL injection (Fig. 2f). In this connection, Kuwano et al. have reported that the MVD of M1 and M2 cancer determined on the basis of positivity for factor VIII-related antigen was significantly higher than that of adjacent normal squamous epithelium.24 This phenomenon reflects the fact that, under magnifying endoscopic observations, the background mucosa without IPCL appears homogeneously reddish in comparison with normal squamous epithelium. As cancer invades to the muscularis mucosae (M3), the tumor becomes thicker, and papillae begin to be destroyed. At the surface of the tumor, the newly developed vessels appear dilated and irregularly branched, with a shape that obviously differs in comparison with the IPCL-like capillaries of M1 or M2 cancer. When cancer has invaded to the submucosa (SM), the papillae are destroyed and the IPCL-like capillaries disappear completely. The newly developed vessels rearranged at the surface of the tumor (Fig. 2g–i)7,14–16 are cancer specific and are not evident in benign lesions, except in special circumstances (e.g. after a biopsy procedure). Accordingly, we refer to these vessels as ‘neovasculature’. As described above, the presence of neovasculature suggests that the depth of invasion must be greater than M3 and, therefore, this feature is a good indicator when considering the propriety of endoscopic mucosal resection.

**MOLECULAR ANALYSIS OF ANGIOGENESIS DURING ESCC: CORRELATION WITH ENDOSCOPIC FINDINGS**

The concept of a tumor angiogenesis factor that diffuses into the microenvironment and initiates the generation of tumor blood vessels was first proposed in 1971 by Folkman.25,26 He considered that neovascularization is an event that separates the development of any solid tumor into two stages: (i) the avascular stage; and (ii) the vascular stage. In the avascular stage, nutrients and wastes are exchanged by simple diffusion from the host vessels. Capillary proliferation from the vascular system of the host can begin while the tumor colony is still very small. However, in the vascular stage, once capillaries have penetrated along the edge of a tumor, rapid exponential tumor growth begins. The morphological characteristics of tumor vessels have been described as tortuous, dilated, or irregular in shape.27 Such vessels are also dead-ended and leaky. Because of these structural differences from normal vessels, the blood supply to tumors is often slow and may be intermittent.

Using the GIF-Y0002, we have been able to observe blood flow in the tumor vasculature in some cases.14,15 Blood flow to mucosal cancer (modified IPCL with immature IPCL-like capillaries) seems to be maintained at a level similar to that provided by normal IPCL (Fig. 3a). In advanced cancer (with neovasculature), the surface vasculature is irregularly branched, and the blood flow is slow or often intermittent (Fig. 3b). The tumor vessels present at this point are consistent with those observed in M3 or deeper ESCC. Neovascularization is controlled and maintained by various angiogenic regulators produced by tumor and host cells.

Kitadai et al. have reported the molecular angiogenic profile of esophageal ESCC at the precancerous and cancerous stages. They carried out an immunohistochemical analysis of precancerous and cancerous lesions using antibodies against vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP), basic fibroblast growth factor (bFGF) and interleukin (IL)-8,28 and observed initially enhanced expression of PD-ECGF/TP and VEGF in dysplastic lesions. bFGF and IL-8 were not expressed in dysplasias and mucosal carcinomas, but their expression increased in the late stage of cancer progression. Kitadai et al. concluded that angiogenic switching is a very early event in the development of ESCC. However, other reports have indicated that overexpression of VEGF is observed in cancer that has invaded beyond the submucosal layer.29–32

Similar to cyclooxygenase (COX)2, inducible nitric oxide synthase (iNOS) is overexpressed in both mucosal and invasive esophageal cancer.33–35 Production of these two enzymes occurs in chronically inflamed tissues, including precancerous lesions, and they are thought to have several functions. COX2 inhibits apoptosis and promotes angiogenesis,
whereas iNOS is also involved in various stages of neovascularization from vasodilatation to vascular remodeling. Hypoxia-inducible factor (HIF-1) is recognized as an important protein that regulates transcription of several genes related to angiogenesis, including VEGF, plasminogen activator inhibitor-1 and VEGF receptor-1. In the ESCC, HIF-1-alpha is overexpressed in submucosal cancer, however, examination concerning mucosal cancer is undetermined.

Several oncogenes and tumor-suppressor genes, including HER2 and p53, play a significant role in promoting or suppressing neovascularization. For example, abnormalities of p53 are thought to facilitate neovascularization by downregulating the production of TSP-1 (a potent negative
endothelial regulator). In ESCC, p53 mutations have been detected at all stages of carcinogenesis, from precancerous lesions, such as hyperplasia or dysplasia, to invasive cancer. However, thrombospondin (TSP)-1 is overexpressed at the late stage of cancer progression.

Transforming growth factor-alpha (TGF-α), known to be an endothelial mitogen and angiogenesis inducer of VEGF expression, is not expressed in cancer in situ, but is overexpressed in T1 cancer.

Various metalloproteinases (MMP) and other proteases, such as trypsin, are involved in neovascularization and tumor-induced proteolysis. These enzymes tend to be expressed at higher levels in tumors that have invaded to the submucosa or deeper.

Considering these findings as a whole, it seems that the initial angiogenic switch occurs at the precancerous stage or in mucosal cancer. However, at this stage, as the vascularity is present mainly in the lamina propria mucosae or epithelial papillae and has not yet penetrated the tumor, we should consider this to be the ‘avascular’ stage. In addition, at this stage, the morphology of the surface vasculature is similar to that of the vasculature of the inflammation from the magnifying endoscopic findings at the point of dilation and elongation of the IPCL, with recruitment of the IPCL-like immature capillaries. Various endothelial regulators do not become upregulated during localized cancer growth in the mucosal layer, but their production increases as the cancer invades the submucosa or deeper layer. From the magnifying endoscopic

Fig. 3. (a) Microvasculature of M2 esophageal cancer at the highest magnification of the GIF-Y0002 (narrow band imaging). Dilated intrapapillary capillary loops (IPCL) compared with normal IPCL and moving blood cells inside the tumor vasculature can be seen. In this case, the speed of the blood flow is maintained in the superficial lesions in comparison with the normal vasculature. (b) Microvasculature of advanced esophageal cancer at the highest magnification of the Y0002 (narrow band imaging). Significantly dilated neovascularization of the advanced tumor can be seen. In this case, the blood flow inside the vasculature was significantly slow. The shape of the red blood cells is also recognizable. (c) Hypothesis of ‘multi-step angiogenesis’ in the early stage of esophageal squamous cell carcinoma (ESCC). ep, epithelium; lpm, lamina propria mucosae; mm, muscularis mucosae; mp, muscularis propria; sm, submucosa. (This figure is partially quoted from ref. no. 15.)
Neovascularization could be observed when the cancer invades into the muscularis mucosa (M3). It is at this stage that growth of the tumor vasculature starts to become apparent (‘vascular’ stage). A second angiogenic step exists in ESCC when the cancer invades the muscularis mucosa (Fig. 3c). However, the mechanism of formation of the neovasculature is still unclear. Further investigation is necessary.

**FUTURE APPLICATIONS OF MAGNIFYING ENDOSCOPY FOR ESCC: ENDOCYTOSCOPIC HISTOLOGICAL DIAGNOSIS**

Microvasculature can be formed secondarily as a result of histological abnormalities, such as esophagitis, dysplasia or ESCC; biopsy histology cannot be omitted only on the basis of microvascular architecture. In order to conclude whether target mucosa reveals malignancy, observation of nuclear abnormality is necessary using biopsy histology or other methods.

In 2003, Olympus Medical Systems Corporation introduced a probe-type endocytoscopy system (ECS). ECS is based on the technology of light-contact microscopy. The most evident use of ECS is for real-time, high-resolution diagnosis of nuclear abnormalities (Fig. 4). Kumagai et al. were the first to report in vivo observations of squamous epithelium and ESCC using ECS, and proposed a classification of the esophageal mucosa based on a flow chart of features observed using this system. They also worked in consultation with a pathologist to evaluate whether ECS observation might be able to replace biopsy histology. It was concluded that ECS would be able to replace histological examination of biopsy samples in approximately 84% of ESCC if a magnification of ×1125 was used, considering that an increase of nuclear density and nuclear abnormalities would be sufficiently convincing proof of malignancy.

From this viewpoint, endocytoscopic histological diagnosis and endoscopic therapy may be carried out simultaneously in a limited area of squamous mucosa. However, histological examination of EMR and ESD specimens remains very important for estimating the tumor-free resection margin, lymph node metastasis and patient outcome. GIF-Y0002 can visualize the surface cells, and is an excellent ECS instrument in terms of ease of use, ready access to lesions, and durability of the endoscope, and we consider that ECS has potential for screening use. It is anticipated that, eventually, the power of the magnifying endoscope will be improved to allow observations at the cellular level, thus dispensing with the need for biopsy histology.

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