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The onset of angiogenesis in a multistep process of esophageal squamous cell carcinoma

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TABLE OF CONTENTS

1. ABSTRACT

Microvessel density (MVD) is an excellent predictive biomarker regarding tumor stage and survival in esophageal squamous cell carcinomas (ESCCs). However, it is obscure when tissues initiate angiogenesis in the malignant transformation of human esophageal squamous epithelium. To investigate the onset of angiogenesis in the multistep progressive process of ESCCs, immunohistochemical staining for CD31, CD105, and vascular endothelial growth factor receptor 2 (VEGFR-2) was performed in normal epithelium, Lugol-unstained lesions with non-dysplastic epithelium (LULs-NDE), low-grade dysplasia (LGD), and high-grade dysplasia (HGD) samples. There were significant differences in the mean MVD for CD31 and CD105 between LULs-NDE and LGD ($p<0.001$, $p<0.001$), and between LGD and HGD ($p<0.001$, $p=0.006$), respectively. Furthermore, a significant difference in MVD for CD105 was seen in normal controls and LULs-NDE ($p=0.002$), while thick vessels (>10μm) stained with anti-CD105 were not present in normal controls and LULs-NDE despite the presence of these thickened vessels in dysplasia. Our results suggest that CD105 is an efficient marker protein to determine MVD, suggesting that the angiogenic switch occurs at the earliest stage of dysplastic transformation in ESCC.

2. INTRODUCTION

The survival rate for advanced stage ESCC is low, mainly due to the late detection of these lesions coupled with the poor efficacy of therapy that generally involves both surgery and chemoradiation. The great majority of esophageal cancers are squamous cell carcinomas although the incidence of adenocarcinoma is increasing in Western countries (1). For instance, 95% of esophageal cancers are ESCCs in Japan (2).

Malignant transformation of human esophageal epithelium is a multistep progressive process (3-13). Precancerous lesions have been classified into dysplastic (i.e. low-grade or high-grade dysplasia, and cancer-in-situ) and non-dysplastic lesions (i.e. basal cell hyperplasia (BCH), chronic esophagitis, and Lugol-unstained lesions with non-dysplastic epithelium (LULs-NDE)). The relationships between these lesions and *p53* gene mutations or *p53* overexpression have been investigated (3-11). The previously reported frequency of *p53* mutations in human ESCC is approximately 50% (10,11). Similar frequencies of *p53* mutations have been reported in LGD (36% to 67%), suggesting that the LGD has already acquired malignant potential (5,10,11).

From the results of a 13-year follow up study, a number of both LGD and HGD were confirmed to transform into ESCC rather than into BCH or chronic esophagitis (14). Therefore, LGD has been widely believed as an initial ESCC lesion. Increasing grades of dysplasia were strongly associated with increasing risk, indicating that the histological grading of esophageal dysplasia was clinically important for the management of precancerous lesions in the esophagus (14). However, the initiation of angiogenesis and its development have been poorly investigated in dysplasia and/or precancerous lesions in ESCC.

Angiogenesis is essential for solid tumor growth. VEGF is one of the most important angiogenic factors, and its expression is associated with tumor angiogenesis (19-20). Two receptors binding VEGF have been identified: Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2). The VEGFR-2 binds VEGF with high affinity, and VEGF expression is correlated with VEGFR-2 expression (21-22). VEGF contributes to the aggressive characteristics of ESCC and possibly correlates with tumor stage, positive lymph nodes, and patient outcome (15-16). The influence of tumor angiogenesis and pro-angiogenic molecules such as VEGF on the progression and recurrence of ESCC has recently been debated (17-18).

The pan-endothelial markers normally used to assess tumor vascularity, such as CD31, CD34, and von-Willebrand factor, are common factors in both newly formed and pre-existing vasculature. Although these markers were used as tumor prognostic indicators to determine the tumor vascularity, the significance of this determination remains controversial (23-25). In contrast, Endoglin (CD105), a member of transforming growth factor 1 receptor complex, is a useful marker to identify tumor angiogenesis (26-27). Recently, CD105 has been reported as an endothelial marker that appears exclusively associated with the endothelial cells in newly formed vessels and in immature tumor vessels (28-29). In squamous cell carcinoma of the oral cavity, expression of CD105 in tumor-tissue has shown to be associated with clinicopathological parameters for diagnosis and prognosis (30-31). CD105 is a specific and sensitive marker for tumor angiogenesis in ESCC, and also reveals its positive correlation as a prognostic marker with the presence of angiolymphatic invasion, lymph node metastasis, tumor stage, and survival (32-33).

This study aimed to clarify the angiogenic process that occurs in the malignant transformation of human esophageal epithelium. To investigate this multistep process, we performed immunohistochemistry using antibodies against CD31, CD105 and VEGFR-2, analyzed the usefulness of the markers, and attempted to determine the initiation of angiogenesis in the malignant transformation of esophageal epithelium.

3. MATERIALS AND METHODS

3.1. Patients

Recruited subjects were composed of symptom-free individuals who visited our hospital between April 2004 and March 2007. Subjects who had active malignant disease, and who had undergone esophagectomy or chemoradiotherapy for ESCC were excluded. Archival histological specimens were obtained from 65 patients in Showa University Hospital. All specimens were reviewed by M.K. (a consultant histopathologist). This study was approved by the institutional review boards of Showa University Hospital. All subjects were provided according to the authorized protocol with written informed consent.

3.2. Sample collection

After ordinary endoscopic observation, 5 to 10 ml of 2.0% glycerin-free Lugol's iodine solution, a brown liquid consisting of 2.0 g potassium iodine and 4.0 g iodine to 100 ml distilled water, was sprayed from the gastroesophageal junction to the upper esophagus using a plastic spray catheter (washing tube PW-5L; Olympus, Tokyo, Japan) passed through the biopsy channel of the endoscope. The whole esophagus was then observed again and LULs, defined as areas either staining less intensely than normally stained epithelium, or completely unstained were identified. Biopsies of LULs were taken under endoscopic-guidance. We confirmed that each sample was correctly taken from the LUL, with endoscopic findings demonstrating disappearance of a part or whole of the LUL. In contrast, biopsies of normal epithelium were taken from uniformly Lugol stained areas from patients without LULs including dysplastic and non-dysplastic lesions. Samples of normal epithelium were defined as controls.

3.3. Histological analysis

Histological diagnosis of normal epithelium, dysplasia, and carcinoma was made according to the previously described definition (3,34). The grade of dysplasia was defined as follows: lower one-third, two-third, and whole-third from the basal cell layer were defined as mild, moderate, and severe dysplasia, respectively. LGD was defined as mild to moderate dysplasia, and HGD was defined as severe dysplasia and intraepithelial carcinoma.

3.4. Immunohistochemical Examination

Immunohistochemical staining was carried out using the avidin-biotin-peroxidase complex method. Formalin-fixed, paraffin-embedded biopsy materials were cut into 4-μm sections, which were then deparaffinized in xylene, dehydrated in a graded ethanol series, and finally immersed in methanol with 0.3% H_2O_2 for 20 min to inhibit endogenous peroxidase activity. The sections for CD31 and CD105 staining were treated with Proteinase K for 10 min at room temperature, and the VEGFR-2 staining was heated to 95°C by microwave irradiation twice for 10 min in 10 mM citrate buffer solution (pH 6.0). The sections were then cooled for 30 min at room temperature. After washing in TBS, all sections were blocked from nonspecific binding by preincubation with 2% NSS in TBS (150 ml) for 15 min. The sections were then incubated overnight at 4°C with the primary antibodies. The primary antibodies used were mouse antihuman monoclonal antibody (clone JC70A; DAKO, Glostup, Denmark) at a 1:20 dilution for CD31, a mouse monoclonal antibody (clone SN6H; DAKO, Glostup, Denmark) at a 1:50 dilution for CD31, and a rabbit monoclonal antibody (clone 55B11; DAKO, Glostup, Denmark) at a 1:250 dilution for VEGFR-2.

Table 1. MVD for CD31 and CD105, and VEGFR-2 expression

	CD31		CD105		VEGFR-2	
	MVD	**p-value**	**MVD**	**p-value**	**MVD**	**p-value**
Normal (n=10)	6.2		0.0		-	
		0.458		0.002		-
LULs-NDE (n=23)	7.1		2.2		-	
		< 0.001		< 0.001		-
LGD (n=15)	10.9		7.1		-	
		< 0.001		0.006		-
HGD (n=17)	16.9		12.1		-	

Normal, normal controls; VEGFR-2, vascular endothelial growth factor receptor 2; MVD, microvessel density; LULs-NDE, Lugol-unstained lesion with non-dysplastic epithelium; LGD, low-grade dysplasia; HGD, high-grade dysplasia. Statistical analyses were performed among normal controls and LUL-NDE, LUL-NDE and LGD, and LGD and HGD, respectively.

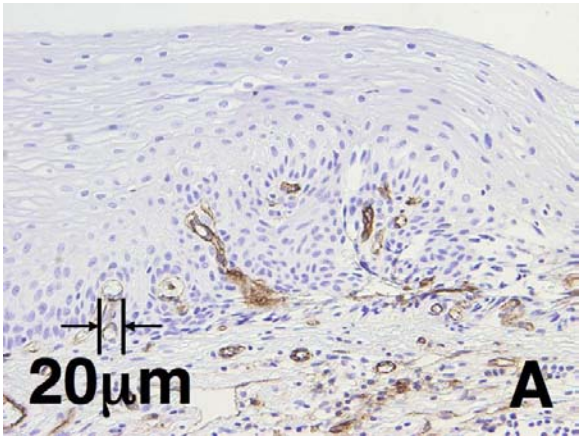

Figure 1. Immunohistochemical analysis of CD31, CD105, and VEGFR-2 expression in serial sections of LGD (x200). Figure 1: As shown by brown staining, there are many microvessels defined as completely stained areas surrounding the inner space of a vessel when CD31-antibodies were used. Diameter of all vessels is less than 10 μm.

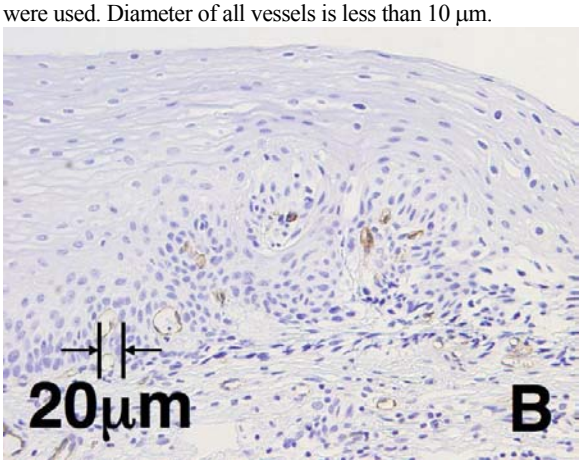

Figure 2. CD105-antibodies stained a few areas, which are a portion of the microvessels stained with CD31-antibodies as seen in Figure 1.

After washing five times in TBS, slides were incubated with secondary antibody (biotinylated rabbit anti-mouse IgG (DAKO); dilution 1:200). After being washed five times with washing buffer, the sections were incubated with avidin-biotin complex (ABC) reagent (DAKO, Glostrup, Denmark), and a color reaction was developed using 2% 3,3'-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen peroxide for 5–10 min. The sections were counterstained with Meyer's hematoxylin. For negative controls, the primary antibody solutions were replaced by the blocking buffer.

3.5. Evaluation of Immunohistochemical images

The microvessel count was assessed by light microscopy in the most extensive areas of neovascularization (termed "hot spots") at a high-power view (x200; 0.15 mm^2/field). As shown in Figure 1-3, microvessels were defined as completely stained areas surrounding the inner space of a vessel. Stained regions, which were not surrounded by a closed area with an inner space or were formed as a mass area without an inner space, were not defined as microvessels.

3.6. Immunohistochemical quantification

The MVD was defined as described in previous reports (35-36). The pathologist (M.K.) scanned each section under a high power view (x200 magnification) after checking with a lower power view (x40 magnification) to identify the stained "hot spots". Furthermore, the vessel diameter and the number of vessels under immunohistochemical examination using CD31, CD105, and VEGFR-2 antibodies were also evaluated. Vessel diameter was measured using a unit of 20 μm. Vessels measuring ≤10 μm, and >10 μm in diameter were defined as thin, and thick vessels, respectively. No upper limit was placed on vessel size.

3.7. Statistical Analysis

Statistical analyses were performed between normal controls and LULs-NDE, LULs-NDE and LGD, and LGD and HGD, respectively. The significance of differences between any two groups was assessed by the Mann–Whitney U test. P value of less than 0.05 was considered significant.

4. RESULTS

4.1. Characteristics of esophageal precancerous lesions

The mean age of all patients was 61 years, ranging from 43 to 80 years, and the male to female ratio was 43/22. A total of 65 samples were taken from 65 patients without invasive ESCC. All specimens consisted of normal epithelium (n = 10), LULs-NDE (n = 23), LGD (n = 15), and HGD (n = 17).

4.2. Immunohistochemical analysis for CD31, CD105, and VEGFR-2

As shown in Table 1, there were significant

Table 2. MVD according to vessel diameter in CD31 stained vessels

	Diameter of vessels			
	≤ 10 μm	**p-value**	**> 10 μm**	**p-value**
Normal (n=10)	6.0 (2-10)		0.4 (0-3)	
		0.445		0.182
LULs-NDE (n=23)	6.9 (0-13)		0.2 (0-5)	
		0.005		0.078
LGD (n=15)	10.1 (6-15)		1.0 (0-4)	
		0.214		< 0.001
HGD (n=17)	12.2 (2-24)		4.9 (0-13)	

(), range of the number of vessels; Normal, normal control; LULs-NDE, Lugol-unstained lesion with non-dysplastic epithelium; LGD, low-grade dysplasia; HGD, high-grade dysplasia. Statistical analyses were performed among normal controls and LUL-NDE, LUL-NDE and LGD, and LGD and HGD, respectively.

Table 3. MVD according to vessel diameter in CD105 stained vessels

	Diameter of vessels			
	≤ 10 μm	**p-value**	**> 10 μm**	**p-value**
Normal (n=10)	0.0 (0-0)		0.0 (0-0)	
		0.002		-
LULs-NDE (n=23)	2.2 (0-7)		0.0 (0-0)	
		< 0.001		0.026
LGD (n=15)	6.1 (4-11)		1.0 (0-4)	
		0.254		0.002
HGD (n=17)	7.6 (0-16)		4.4 (0-13)	

(), range of the number of vessels; Normal, normal control; LULs-NDE, Lugol-unstained lesion with non-dysplastic epithelium; LGD, low-grade dysplasia; HGD, high-grade dysplasia. Statistical analyses were performed among normal controls and LUL-NDE, LUL-NDE and LGD, and LGD and HGD, respectively.

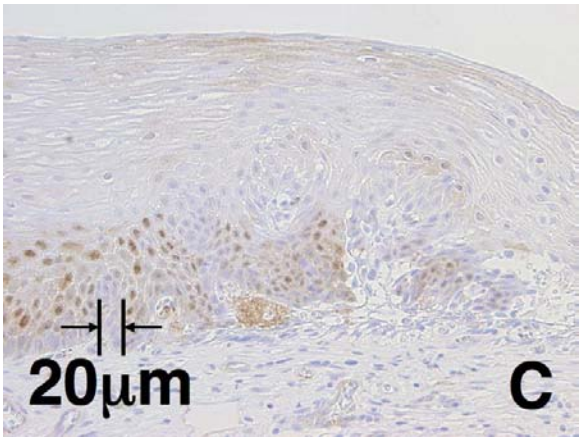

Figure 3. Overexpression of VEGFR-2 is not seen in microvessels.

differences in the mean MVD for CD31 between LULs-NDE and LGD (p<0.001), and between LGD and HGD (p<0.001), respectively. Furthermore, there were significant differences in the mean MVD for CD105 between controls and LULs-NDE (p=0.002), between LULs-NDE and LGD (p<0.001), and between LGD and HGD (p=0.006), respectively. In contrast, overexpression of VEGFR-2 was indistinguishable among individual groups. In the mean MVD based on the expression of both CD31 and CD105, there were significant differences among LULs-NDE and LGD, and LGD and HGD, respectively.

4.3. MVD of thin and thick vessels for CD31 or CD105

The number of thin vessels (≤10 μm) analyzed by the expression of CD31 was less in LULs-NDE than in LGD (p=0.005). Furthermore, the number of thick vessels (>10 μm) determined with the expression of CD31 was increased in HGD compared with in LGD (p<0.001, Table 2).

Thin vessels expressing CD105 were found in none of 10 controls (0%), 14 of 23 LULs-NDE specimens (61%), and all LGD and HGD specimens.

Thick vessels expressing CD105 were found in none of 10 controls (0%), none of 23 LULs-NDE specimens (0%), 5 of 15 LGD specimens (33%), and 14 of 17 HGD specimens (82%). In regard to the number of thin vessels (≤10 μm) expressing CD105, there was a significant difference between controls and LULs-NDE (p=0.002), and between LULs-NDE and LGD (p<0.001), while no significant difference was seen in LGD and HGD (p=0.254, Table 3). In contrast, there was a significant difference in the number of thick vessels (>10 μm) expressing CD105 seen between LULs-NDE and LGD (p=0.026), and between LGD and HGD (p=0.002), respectively. However, no thick vessels expressing CD105 were found in specimens of LULs-NDE, and only a few thick vessels expressing CD31 were seen in LULs-NDE. Therefore, thick vessels expressing CD105 were found in dysplastic lesions, but not in non-dysplastic lesions, such as controls and LULs-NDE.

5. DISCUSSION

Malignant transformation of human esophageal epithelium is a multistep progressive process (3-13). Genetic abnormalities, such as *p53* gene mutations, are acquired even in precursor lesions of ESCC. However, there have been no reports with regard to angiogenesis occurring in ESCC precursors. To investigate angiogenesis in precursor lesions of ESCCs, immunohistochemical staining for CD31, CD105, and VEGFR-2 was performed in normal epithelium, LULs-NDE, LGD, and HGD

samples. From our results of CD31 and CD105 expression, MVD was increased at the time of histological transformation from the non-dysplasic to dysplastic state. Furthermore, a increase in MVD was demonstrated to occur with increasing grade of dysplasia. The greatest increase in angiogenesis occurs at the earliest stage of dysplastic transformation, suggesting that the angiogenic switch may turn on at this point in the multistep progression process of ESCC. Conversely, although VEGF expression is reported to reflect tumor stage of ESCC, there was no association between overexpression of VEGFR-2 and angiogenesis of ESCC precursor lesions in our study.

One important step in correctly evaluating angiogenesis of ESCC precursor lesions is the identification of valid histological precursors. Identification and validation of such precursors requires classification of lesions by well-defined histological categories. Therefore, histological diagnosis was made according to previously described definitions (3,34). In our study, all 65 samples were taken from 65 individual patients without invasive ESCC, in order to avoid any potential problems that may arise due to the possibility that angiogenesis has already had an effect on the background epithelium of ESCC patients. Furthermore, control samples of normal epithelium were taken from uniformly Lugol staining epithelium in patients without LULs-NDE, dysplasia, and carcinoma.

We previously reported that some LULs-NDE represent the earliest state of esophageal squamous cell carcinoma, since the *p53* missense mutations at hotspots were found in not only dysplasia or carcinoma but also Lugol unstained lesions with non-dysplastic epithelium (3,37). The *p53* hotspot mutations have high transforming activities resulting in carcinomas (38-40). We proposed that LULs-NDE acquired malignant potential before morphologically manifested cell proliferation occurs at an early molecular level of carcinogenesis (3). In our present study, significant differences in the number of both thin and thick vessels for CD105 were seen in LULs-NDE and LGD, respectively. We strongly suggest that the angiogenic switch may turn on at the point of LGD in the multistep progressive process during carcinogenesis. In contrast, the thin vessels (≤10 μm) for CD105 were found in LULs-NDE, while there were no vessels stained for CD105 in controls. Although the grade of angiogenesis was lower in LULs-NDE than in dysplasia, our data clearly demonstrated a significant increase in the number of thin vessels in LULs-NDE specimens compared with controls. Angiogenic alterations that provide a selective growth advantage to the region would have already occurred in LULs-NDE before histologic transformation into a dysplastic region. Therefore, we suggest that some LULs-NDE may acquire angiogenic alterations.

Our results for CD31 staining in regard to dysplasia were similar to those for CD105 staining. In previously reported results, the pan-endothelial markers, such as CD31, and CD34, normally used to assess tumor vascularity do not distinguish between newly formed and pre-existing vasculature (23-25). In contrast, CD105 is an endothelial marker that appears exclusively associated with the endothelial cells in the newly formed vessels and in the immature tumor vessels (28-29). In our study, significant increase in MVD for CD105 was demonstrated between controls and LULs-NDE ($p=0.002$), while no significant difference in MVD for CD31 was seen in controls and LULs-NDE ($p=0.45$). In the analysis of thin vessels from normal control tissue, CD31 was expressed while overexpression for CD105 was not found. Our data clearly demonstrate a significant increase in MVD of LULs-NDE rather than control MVD, in agreement with previous studies in which CD105 is either absent or weakly expressed in vascular endothelium of normal tissue, but strongly expressed in malignant tumor cells. Furthermore, thick vessels stained for CD105 were newly present in LGD, with one piece of a thick vessel found in individual LGD at a high-power view (x200; 0.15 mm^2/field). We propose that the presence of thick vessels stained for CD105 is associated with angiogenesis at the earliest stage of dysplastic transformation. Thus we believe that endoglin (CD105) is a specific and sensitive marker for angiogenesis of ESCC precursor lesions, normal tissue via HGD. We propose that angiogenic alterations occurring in ESCC precursor lesions may develop as follows: Firstly, thin vessels are present in normal tissue. At an early stage of dysplasia, thick vessels develop increasing the total number of vessels, including thin vessels. The number of thick vessels then further increases during the stage of high-grade dysplasia. Finally, dysplasia will transform into invasive cancer.

In conclusion, this study is the first example that evaluates the expression of CD105 in ESCC precursors containing dysplastic (LGD, HGD) and non-dysplastic lesions (LULs-NDE, normal epithelium) in a multistep progressive process. We strongly suggest that the angiogenic switch occurs at the point of LGD. Furthermore, we propose that angiogenic alterations providing a selective growth advantage would have already occurred in LULs-NDE before histologic transformation into a dysplastic region. The relation between genetic or epigenetic alterations and angiogenesis in precursor lesions of ESCC requires further investigation.

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Abbreviations: MVD**:** Microvessel density; ESCCs: esophageal squamous cell carcinomas; VEGFR-2: vascular endothelial growth factor receptor 2; LULs-NDE: Lugol-unstained lesions with non-dysplastic epithelium; LGD: low-grade dysplasia; HGD: high-grade dysplasia; BCH: basal cell hyperplasia

Key Words: Esophageal squamous dysplasia, Angiogenesis, CD105, Immunohistochemical staining

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