Angiogenesis as Prospective Molecular Biology Technique for Cancer Study

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Abstract
Esophageal cancer is a disease with increasing frequency in both esophageal squamous cell carcinoma and especially for esophageal adenocarcinoma, according to statistics representing the sixth leading cause of death from malignancy in the world. The study group included 24 cases of squamous cell carcinoma, from which we collected tissue samples histological stained with hematoxylin–eosin and specific staining technique (Verhoeff–van Gieson and Szekely) and then labeled CD34, immunohistochemically, in order to mark out the neoangiogenesis. At the end, a micro quantitative study was conducted by using the parallel Weibel grid with a distance between points d = 19.39 μm. Vessels and tumor stroma were quantified on a rectangular zone (115.72 μm x 182.45 μm) with 0.0021 mm² area, on 10 consecutive fields on 5 cases of lesions. Micro vessels were counted in areas with the highest density (“hot spot”), after identifying those areas with low magnification. It was found that the tumor vascular morphology system was different from normal esophageal vascular network. Tumoural vessels are small and they have different shapes or may even be distorted. Microscopic and immunohistochemical studies have shown a correlation between the neoangiogenesis and the inflammatory infiltrate that ensures the progression and the extension of esophageal epithelial tumors.

Keywords: esophagus, tumor, neoangiogenesis, quantitative study

1. Introduction
Esophageal cancer is a disease with increasing frequency in both esophageal squamous cell carcinoma and especially for esophageal adenocarcinoma. Worldwide, esophageal cancer mortality and incidence in different geographical areas vary in frequency between the eighth cause of malignancy in the world and the sixth cause of death secondary to malignancy (J. FERLAY & al. [1]) and the 10th leading cause of cancer in the world (S.M. CHUNG & al. [2]) its frequency increases in industrialized countries. The diagnosis is made late when the patient is usually malnourished at diagnosis, with important protein-calorie deficiency. Initially, the squamous cell carcinoma cancer was considered typical and most common form of esophageal cancer but recently it was found an increased incidence of esophageal adenocarcinoma (K.F. TRIVERS & al. [3], A.C. CHANG & al. [4]). The choice of surgical technique and its indication is not different for the two major histological types of esophageal cancer, the only correlation being the frequent location of the esophageal adenocarcinoma in the lower esophagus and of the squamous cell carcinoma in the middle and upper thoracic esophagus (C.E. SCOTT-CONNER [5]). Proliferation, apoptosis and neovascularation are fundamental processes in tumor growth and metastasis (M.K. BRAWER & al. [6], M. SAMOSZUK & al. [7], Z. LIAO & al. [8]) because the tumor cells and blood vessels are forming a highly integrated niche. For this reason intratumoral density of micro vessels has
been proposed as a criterion for prognosis in different cancer types and locations, being used in the assessment of their evolution (D. MILLIARIS & al. [9], J.C. GODDARD & al. [10], Z. DU & al. [11]). Most studies are linking tumor angiogenesis and metastasis, but there are differences caused by immunological marker used to highlight the vessels, vimentin having lower specificity than factor VIII and the last one, lower than CD34. In addition there are significant differences between the three degrees of differentiation of these tumors: low, intermediate and high. It was found a direct correlation between gastro-esophageal reflux disease and the occurrence of esophageal tumors (E. BRÂTUCU & al. [12]).

In this study we aimed to quantify the neoangiogenesis by CD34 immunohistochemistry in esophageal squamous cell carcinoma, given the high frequency and aggressiveness as an effect of vascular architectonics change.

2. Materials and methods

Samples

The study group was represented by 40 cases of esophageal cancer who had surgical cure of the tumor reported in the records of the Department of Pathology from the “St. Spiridon” Hospital from Iasi, Romania. The cases were analyzed in terms of pathology and tumor aggressiveness. The study group included 24 cases of squamous cell carcinomas and 16 esophageal adenocarcinoma, 60% and 40%, respectively.

All the cases of esophageal adenocarcinoma were located in the third lower esophagus, and squamous cell carcinomas were located in the third upper and third mid-esophagus. Most squamous cell carcinomas were located in third middle of the esophagus (14 out of 24 cases of squamous cell carcinoma).

Qualitative analysis

After collection, the fragments were processed and included in paraffin and then were used for histological stained with hematoxylin–eosin and specific staining technique (Verhoeff-van Gieson and Szekely) to highlight the micro anatomical qualitative aspects. The plates were examined by microscope Z1 Zeiss (Carl Zeiss, Germany) with 2.5x, 20x lens and after this procedure they have been examined again using the microscopic image analysis system Tissue FAXS. Initially, a preview of the plate sections was made with 2.5x lens and after this procedure some regions on that section were mapped depending on the esophageal tumor.

The parts of interest are placed at the periphery of the tumor in order to study the tumor microvasculature as to avoid a possible tumor necrosis in the center. To identify the blood vessels we used CD34 as a marker for vascular endothelium. The Novolink Polymer Detection System kit was used for our research. It included the following: CD34 [QBEND/10] – AM 236 – 5 M, Peroxidase Block, Protein Block, Post Primary and Novolink™ Polymer. The positive reaction was evidenced by the appearance of dark brown stains on the membrane of endothelial cells. The qualitative immunohistochemical study we used the following grading system: 0 = negative staining; + = positive reaction in less than 10% of tumor vessels/sample; ++ positive reaction ranging between 10 to 33% of tumor vessels/sample and +++ = positive reaction ranging between 10 to 33% of tumor vessels/sample.

Qualitative analysis included the evaluation of staining intensity of the two markers related to the histopathological type of esophageal process and tumor aggressiveness that was assessed by tumor grading (G1/G2/G3) and establishing a direct relationship between tumor growth and the neoangiogenesis process.
Classification of adenocarcinoma was based on tumor grading (G): 46% were well differentiated, 33 were moderate differentiated and 21% were poorly differentiated.

Quantitative analysis

The quantitative measurements have been performed in the Laboratory of quantitative microanatomy of “Ion Iancu” Institute of Anatomy of the University of Medicine and Pharmacy “Gr. T. Popa” Iasi and representative microscopic sections were analyzed by 20 x, 40x or 90x magnification. Images were taken using image acquisition system and analyzed with PRODIT 5.2 software (BMA De Meern, Netherlands). This interactive digital program allowed us to perform numerous measurements by choosing from the menu the desired quantitative method. Weibel parallel grid was used, the distance between two points \( d = 19.39 \, \mu m \). The device quantified the tumor vessels and stroma on a rectangular section \((115.72 \, \mu m / 182.45 \, \mu m)\) with 0.0021112 mm\(^2\) area, on 10 consecutive samples, on five cases for each lesion type. Micro vessels were counted in areas with the highest density ("hot spot"), after identifying those areas by low magnification analyzes.

The quantification presented automatically the micro vessel density/mm\(^2\) on tumor stroma and micro vessel density/mm\(^2\) on tumor at the same time with statistical interpretation of the data.

3. Results and discussion

Fibrosis is a constant aspect that we could see in tumor analysis. The fibrosis degrees vary for well-differentiated squamous cell carcinoma, describing a moderate degree of recent fibrosis, both among the ranges of tumor cells and in tumor periphery.

Fibrosis is accompanied by small caliber vessels (capillary blood), with morphology approaching to normal. No signs of large or medium blood vessels intra or peritumoral.

Tumor blood vessels are small vessels – capillaries that usually can be found in areas presenting inflammatory infiltrate and fibrosis. Capillaries have an elongated shape with apparent endothelial wall and intracapillary blood stasis.

There were changes in the tumor microenvironment and poorly differentiated squamous cell carcinoma. The blood vessels are represented by the tumor capillaries and they are frequent and associated with inflammatory infiltrate and fibrosis of the tumor. The inflammatory infiltrate gather as strips along the collagen fibers.

The fibrosis process presented a high intensity with intratumoral location, dissecting the tumor cell ranges and peritumoral. For both situations there were seen blood capillaries and inflammatory infiltrate. The blood capillaries are oval and long, bounded by a layer of endothelial cells.

Neoangiogenesis study focused on identifying tumor micro vessels inside the tumor tissue and at the periphery of the tumor, and reporting its intensity as histological degree of tumor differentiation.

Well-differentiated squamous cell esophageal carcinoma has a high markup (+ + +) for tumor vessels which are small. The tumor capillaries present morphology similar to the normal mature ones, most of them is having oval or circular shape (Figure 1).
Figure 1. Well-differentiated squamous cell carcinoma. Tumor capillaries located in tumor cells range with almost normal morphology. Immunohistochemistry CD 34 +++ (strongly positive), 200x

In areas with inflammatory infiltrate, CD34 labeling revealed the existence of an abundant capillary network (Figure 2) and their association with peritumoral fibrosis areas is significant.

Figure 2. Well-differentiated squamous cell carcinoma. Tumor capillaries associated with inflammatory infiltrate and fibrosis. Immunohistochemistry CD 34 +++ (strongly positive), 200x
Moderately differentiated squamous cell carcinoma presents more frequent intratumoral capillaries than the well differentiated one, with their arrangement in the areas of fibrosis and inflammatory infiltrate (Figure 3).

**Figure 3.** Moderately differentiated squamous cell carcinoma. Tumor sanguine vessels of different sizes, intratumoral, transversely and obliquely sectioned. Immunohistochemistry: CD 34 +++ (strongly positive), 200x

The neoangiogenesis capillaries have a more heterogeneous morphology with different shapes and sizes being surrounded by inflammatory infiltrate (Figure 4).

**Figure 4.** Moderately differentiated squamous cell carcinoma. Suppression of tumor capillaries associated with tumor inflammatory infiltrate and fibrosis. Immunohistochemistry: CD 34 +++ (strongly positive) 200x
Poorly differentiated squamous cell carcinoma has a higher vascularization compared to anterior histological types. The significant difference found is the vascular morphology (Figure 5).

Figure 5. Poorly differentiated squamous cell carcinoma. Oblong and oval tumor capillaries with different orientations around the tumor. Flatten and reduced lumen by compression of tumor cells. Small distances between cell clusters, wider in the tumor cell ranges inside the stromal connective tissue. Immunohistochemistry: CD 34 +++ (strongly positive), 200x

Capillaries are immature, showing a varied morphology and images showing neoformation vessels with specific parietal histological features (figure 6).

Figure 6. Poorly differentiated squamous cell carcinoma. Suppressed tumor capillaries, uneven and reduced lumens by compression of near cell clusters. Immunohistochemistry: CD 34 +++ (strongly positive), 200x
Quantification of neovascularization was performed on sections stained with CD-34 antigen with intense markings inside the neoformation vessels in the endothelium considered a direct marker for neoangionesis degree.

As stated previously, panendotelial marker CD34 shows the status of vascular endothelia in the esophageal carcinoma and marks both newly formed vessels and the normal ones. Screen captures from the analysis software (Figure 7, 8 and 9) highlight the significant increase in tumor micro vascular density with increasing grading, inversely proportional to the degree of differentiation. In general, in tumor metastasis the micro vascular density is higher than in the non-metastatic. In addition, there are large variations in vascular density between different areas of the same tumor in poorly differentiated forms, while in well-differentiated carcinomas topography is constantly available. Micro vascular densities are similar for central area, perinecrotic area and the remote extratumoral area.
Depending on the degree of differentiation, ranging from the lowest form of malignancy to invasive squamous cell carcinoma, micro vascular density ranged as follows: density of micro vessels / mm² tumor stroma has a relatively moderate density in G1 (467.80), maintain a close value, but significant in squamous G2 (479.55) and reaches the maximum value in squamous cell carcinoma G3 (519.63). The density of micro vessels / mm² tumor showed the same direction of change and differences were showing a greater significance (figures 7, 8 and 9).

![Figure 9](image-url)  
**Figure 9.** Quantification report for neoangiogenesis in poorly differentiated squamous esophageal carcinoma

![Figure 10](image-url)  
**Figure 10.** Graphical representation of micro vessels/mm² density of the tumor stroma (DM / ST) and micro vessels/mm² density of the tumor (DM / T) in esophageal squamous cell carcinoma
Graphical representation of related changes (Figure 10) highlights the significant increase in tumor microvascular density with increased risk. Compared with squamous cell carcinoma G1 (G- grading / degree of tumor differentiation; G1- well differentiated tumor, G2- moderately differentiated tumor, G3- poorly differentiated tumors), increased vascular density in squamous cell carcinoma G2 is moderate, but significant, and the highest density of disseminated carcinoma G3 maintains its capacity, supporting directly the risk of aggressiveness and metastasis.

In general, the values for quantified densities are lower than in the studied adenocarcinoma mainly due to the high density of specific structures, keratotic pearls, excepting the poorly differentiated adenocarcinoma.

Comparative graphical representation of vascular densities (Figure 11) shows the highest density values for micro vessels/mm² in tumor stroma inside the squamous cell carcinoma of the esophagus, located in the upper third or in the middle third of the esophagus.

Other micro vascular density values are almost equal for both forms of the studied esophageal tumors, increasing progressively with increasing grading, therefore with the malignant risk.

![Figure 11. Comparative graphical representation for micro vessels/mm² density of the tumor stroma (DM / ST) and micro vessels/mm² density of the tumor (DM / T) in squamous cell carcinoma (carcinoma) and esophageal adenocarcinoma (Adeno)](image)

Esophageal neoplasia micro anatomic qualitative study revealed several important aspects. Both in esophageal squamous cell carcinoma and for adenocarcinoma the tumor microenvironment with its two components (cellular and non-cellular component) has an important role to facilitate development and tumor progression, given the constant changes seen at this level. Following the initiation of carcinogenesis process, a specialized tumor stroma can facilitate metastasis and persistence of the neoplastic process in time.

Esophageal squamous cell carcinoma neoangiogenesis process is encountered in all three histological types, being highlighted by CD34 immunohistochemical staining.
Neoangiogenesis is marked by the presence of small blood vessels that have a more distorted morphology as the epidermoid carcinoma is poorly differentiated. This aspect that was demonstrated by our study, leads us to the idea that tumor progression is based on neoangiogenesis process and this process is more aggressive once the tumor is histological virulent. In this context we can conclude that for esophageal squamous cell carcinoma we can establish a direct relation between the endothelial marker CD34 and tumor grading.

Many authors consider that there is a phenomenon of mutual induction between stroma and tumor epithelium describing the phenomenon of epithelial-mesenchymal transformation (T.D. TLSTY [13], N.A. BHOWMICK & al. [14], J.R. SIERRA & al. [15]).

In esophageal squamous cell carcinoma there was a constant association between inflammatory infiltrate, tumor fibrosis and capillary neoangiogenesis with a mutual induction. Advanced studies (R. KALLURI & al. [16], K. PIETRAS & al. [17]) reported that the fibroblasts have the main role that remodel the tumor stroma allowing to precursor endothelial cells to be transformed into endothelial cells (O. DE WEVER [18]).

Association of neovascularization and inflammatory infiltrate shows the importance of tumor microenvironment in neoplastic progression and this aspect was evidenced by other authors that quantified by specific methods the relationship between the two pathological processes (S. ISHIBASHI & al. [19]).

Vascular and tumor heterogeneity is another area in esophageal squamous cell carcinoma. This is more evident in poorly differentiated tumors with or without histological differentiation. Tumor heterogeneity shows that tumor tissue is composed of a number of malignant clones. Papers which were based on the study of tumor DNA showed that there is genetic intratumoral heterogeneity in different areas of the same tumor and this may be related to the degree of histological differentiation (X. WANG 7 al. [20]).

Quantification of angiogenesis together with other prognostic factors, commonly used in the evaluation of patients with colorectal cancer may be an important indicator of tumor biological behavior by identifying a subgroup of tumors with high malignant potential, as demonstrated by deep invasion of tumor associated with an increase in micro vessels density (D. ENE & al.[21], S. CONSTANTINOIU & al. [22]).

Angiogenesis is essential for the tumor to grow and it can be considered as a specific mark of the neoplastic process, as demonstrated by the fact that the process of neoangiogenesis is intense for both histological types of tumor we studied (adenocarcinoma and esophageal squamous cell carcinoma).

Neoangiogenesis is a process that involves several steps. This process depends on the relationship between the tumor microenvironment and epithelial tumor component.

There are significant differences between the three degrees of differentiation (low, moderate and high) of esophageal adenocarcinoma and squamous carcinomas and spatial variations are predictable. In low-risk lesions for well-differentiated forms, the blood vessels are uniform and regular compared with high-risk forms where vessels are irregular, tortuous, and dilated.

4. Conclusions

Microscopic and immunohistochemical studies have shown a correlation between the neoangiogenesis and inflammatory infiltrate that ensure progression and extension of esophageal epithelial tumors.

Study of the vascular system in the esophageal epithelial tumors revealed an axis formation consisting of three elements which have a mutual relationship: inflammatory infiltrate - neoangiogenesis - fibrosis.
The significant increase in tumor microvascular density occurs with increasing histological grading, with an inverse correlation with the degree of differentiation and directly proportional to the risk of malignancy. Evaluation of tumor density in micro vessels/mm² has a level of exactness and accuracy higher than micro vessels/mm² density of tumor stroma.

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