Neoangiogenesis. Assessment in Esophageal Adenocarcinomas

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Abstract Esophageal cancer has always been subject of research for various studies. According to some authors, esophageal cancer represents the 10th leading cause of cancer in the world with a 5-year survival of 10 %. In terms of anatomopathological form of the esophageal neoplasia, the literature mainly describes two major pathological types: adenocarcinoma and esophageal squamous cell carcinoma. Lately there has been an increased incidence of esophageal adenocarcinoma. The aim of the present work was to study neoangiogenesis in esophageal adenocarcinomas. The study was conducted on 40 cases diagnosed and surgically treated. Subsequently, fragment processing was performed using various immunohistochemical staining and marking with CD34 and p53 antigen. Later, quantitative measurements were performed, and images were taken using a microscope imaging system. In the end of the procedures, the professional program PRODIT 5.2. was applied. The study of the vascular system in the esophageal epithelial tumors revealed an axis consisting of three elements which have a mutual induction process: inflammatory infiltrate—neoangiogenesis—fibrosis, with significant differences between the three degrees of differentiation. A significant increase in tumor micro vascular density was present together with the increasing of the histological grading, with an inverse correlation with the degree of differentiation and directly proportional to the risk of malignancy.

Keywords Esophagus · Tumor · Neoangiogenesis · Quantitative

Introduction

Esophageal cancer has always made a subject for research in various studies given the increasing in incidence and a low rate of survival up to 5 years. Chung [1] stated that esophageal malignancies are the 10th leading cause of cancer in the world with a 5-year survival rate of 10 %. A more recent study in 2010 included a meta-analysis of 27 types of cancer mortality and incidence in different geographical areas in 128 countries [2]. According to this study, esophageal cancer is the eighth leading cause of malignancy in the world and the sixth cause of death secondary to malignancy. This study makes no reference to histopathology types of esophageal malignancy.

In terms of anatomopathological forms of esophageal neoplasia, the literature mainly describes two major pathological types: adenocarcinoma and esophageal squamous cell carcinoma. The incidence of the two types of cancer is in a continuous change. Thus, if initially the most common and typical form of esophageal cancer was considered the squamous cell carcinoma, it seems that lately, there is an increase in incidence of the esophageal adenocarcinoma [3]. A recent study [4] that included 4,752 cases of esophageal cancer revealed that 56 % were adenocarcinomas and 46 % squamous cell carcinomas.

The mechanism of carcinogenesis and neoangiogenesis is complex and not understood completely which is why we have focused only on this direction: neoangiogenesis and its relationship with the tumor virulence. To achieve this goal, we have used immunohistochemical examination which consisted of two markers: CD34, p53. Both markers had been used in the evaluation of esophageal adenocarcinomas.

Material and Method

In order to reveal the vasculo-esophageal system in pathological situations, we chose to study this form of esophageal

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tumor due to its high pathological rate as well as its virulence that we consider to be related to changes in vascular system providing favorable conditions for expansion and remote metastasis. We organized a sample case group to show these aspects on which we applied selected techniques and methods.

The sample group is formed by 40 patients with esophageal cancer who underwent surgical treatment of the tumor. All cases were reported by the Department of Pathology of “St. Spiridon” Hospital in Iasi. The cases were analyzed anatomopathologically and in terms of aggressiveness of the tumor. The study group included 16 cases of esophageal adenocarcinomas and 24 cases of squamous cell carcinomas, a 40 to 60 % rate.

After sampling, the fragments were processed by paraffin-embedded technique, and histological techniques had been used to point out the quantitative micro-anatomical aspects: normal stains (hematoxylin-eosin) and special stains (Szekely, van Gieson, Verhoeff).

The slides were examined with Z1 Zeiss microscope with $\times 2.5$, $\times 20$ lenses and afterwards being reexamined using the image analysis software Tissue FAXS in the Laboratory of Genetics and Immunology (Prof. Dr. Carasevici E.).

Initially, a preview of sections on the slides was made with $\times 2.5$ lens and afterwards, some regions were mapped depending on the area of the esophageal tumor. Images of the regions were achieved with $\times 20$ microscope objective, $\times 10$, or $\times 20$ ocular lens.

In order to study the microvasculature of the tumor, the area to be sampled is from the periphery of the tumor so that a possible necrotic area to be avoided.

Immunohistochemical study is based on the method of indirect binding the antigen-antibody complex that is useful for marking the microvasculature of the tumor and its virulence.

To identify the blood vessels, we used CD34 marker for vascular endothelium, and to identify the tumor’s aggressiveness, we used the p53 marker.

Given the virulence of the tumor and also because it is related to the neoangiogenesis process, we chose to study the peripheral microvasculature of the tumor, at the limit of the normal esophageal tissue.

The Novolink Polymer Detection System kit was used for our research and it included the following: CD34 [QBEND/10]—AM 236—5 M, Peroxidase Block, Protein Block, Post Primary and Novolink™ Polymer.

Positive reaction was evidenced by the appearance of dark brown stains on the membrane of endothelial cells. For the qualitative immunohistochemical examination, 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
- +++=positive reaction over 33 % of tumor vessels/sample

For p53 [BP 53/12]—AM 195—5 M, we used the Novolink Polymer Detection System kit that included the following: Peroxidase Block, Protein Block, Post Primary and Novolink™ Polymer.

Positive reaction is evidenced by the appearance of dark brown stains on the tumor cell nuclei. For the qualitative immunohistochemical study, we used the following grading system:

- 0=negative staining
- +=positive reaction in less than 10 % of tumor cell nuclei/sample
- ++=positive reaction ranging between 10 to 33 % of tumor cell nuclei/sample
- +++=positive reaction over 33 % of tumor cell nuclei/sample

The immunohistochemical slides were examined under the same conditions as described above for the current histopathology.

Initially, a preview of sections on the slides was made with $\times 2.5$ lens and afterwards, some regions on the peripheral tumor were mapped. Images of the regions were achieved with $\times 20$ microscope objective, $\times 10$, or $\times 20$ ocular lens.

Qualitative analysis included the evaluation of stain intensity of the two markers related to the anatomopathological type of esophageal process, as well as tumor aggressiveness that was evaluated on tumor grading (G1/G2/G3) thus establishing a direct relationship between tumor virulence and the neoangiogenesis process.

The quantitative measurements of representative microscopic sections 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” of Iasi, with object lens of $\times 20$, $\times 40$, or $\times 90$ imaging. Images were processed using a microscopical acquisition system, after which we used the PRODIT 5.2 digital program. This interactive digital program enhanced performing numerous measurements by choosing from the menu the desired quantitative method. Weibel grid was used, the distance between two points being $d=19.39 \mu\text{m}$. The device quantified the tumor vessels and stroma on a rectangular section ($115.72 \mu\text{m}/182.45 \mu\text{m}$) with 0.0021112 mm^2 area, on 10 consecutive samples, on 5 cases of each lesion type. Microvessels were counted in areas with the highest density (“hot spot”), after identifying those areas by low magnification rate.

The report of stereological quantification presented automatically the microvessel density/ mm^2 of tumor stroma and microvessel density/ mm^2 of tumor in the same time as statistical verification of data.

Results

All 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).

Classification of adenocarcinoma was based on tumor grading (G): 19 % were poorly differentiated, 50 % were poorly differentiated, and 31 % were well differentiated.

Esophageal adenocarcinomas show a marked microvasculature surrounded by inflammatory infiltration. Tumor vessels are small and they are set in spans longitudinally on the ranges of the tumor cells.

For the *poorly differentiated adenocarcinoma*, the vessel morphology is distorted (1). Neoangiogenesis association with inflammatory infiltrate is marked in all cases (Fig. 1).

The *moderate differentiated adenocarcinoma* presents a positive marking (+++) for endothelial cells indicating tumor microvasculature between intestinal metaplasia and epithelium (Fig. 2).

The *well differentiated adenocarcinoma* presents a distinctive positive marking with CD 34 (+++) for both stroma and intraepithelial tumor or associated with inflammatory infiltrate (Fig. 2).

The neoangiogenesis process is well highlighted by immunohistochemical examination for CD34 marker, and the positive marking is high for all studied adenocarcinoma (G1/G2/G3), which is well represented as compared with esophageal epidermoid carcinoma (Fig. 3). Given that the most common esophageal adenocarcinoma develops in Barrett's esophagus, we conclude that there is a correlation between the inflammatory process and the process of neoangiogenesis. Barrett's esophagus develops as a result of gastroesophageal reflux which entails a chronic inflammation of the distal esophagus.

Fig. 1 Poorly differentiated esophageal adenocarcinoma. HE staining, $\times 200$

Fig. 2 Well differentiated adenocarcinoma. Tumor capillaries associated with inflammatory infiltrate. IHC-CD 34 +++ examination, $\times 200$

We have studied the uncontrolled proliferation capacity by avoiding the process of programmed cell death in esophageal adenocarcinoma using the p53 marking.

The highest marking intensity (+++) in poorly differentiated adenocarcinomas.

For the moderate differentiated adenocarcinoma, the p53 intensity was evaluated most frequently with ++/+++ (Fig. 4), and for well differentiated adenocarcinoma, p53 presented a diffuse marking, with areas where the intensity of the marking is clearly marked, but with lower intensity in the individual cells, as a whole was evaluated by ++ (Fig. 4).

Fig. 3 Poorly differentiated adenocarcinoma. Distorted tumor capillaries surrounded by inflammatory infiltrate. IHC-CD 34 +++ examination, $\times 200$

Fig. 4 Well differentiated adenocarcinoma. p53 (++) marking, $\times 200$

Quantitative Immunohistochemical Study

The results of measurements performed separately for 10 consecutive samples on 5 cases of each tumor type were presented in the stereological quantification report showing the automatic calculation of density of micro vessels/ mm^2 of the tumor stroma and microvessel density/ mm^2 of tumor (Fig. 5) for esophageal adenocarcinomas we have studied. All have affected the lower third of the organ.

Depending on the degree of differentiation in adenocarcinoma, ranging from well-differentiated to poorly differentiated, the density of microvessels varied as follows: density of microvessels/ mm^2 of tumor stroma progressively increased from 493.60/ mm^2 to 855.82/ mm^2 , respectively, 1,520, 82/ mm^2 and the density of microvessels/ mm^2 tumor showed the same change, ranging from 671.54/ mm^2 to 1,073.84/ mm^2 , respectively, to 2,260.67/ mm^2 of tumor (Fig. 5).

The graphic representation of changes (Fig. 5) emphasizes the significant increase in tumor microvascular density with inverse increase in differentiation grading. In general, for

tumor metastasis, the microvascular density is higher than in nonmetastatic tumors. In addition, there are large variations in vascular density between different areas of the same tumor in poorly differentiated forms, whereas in well-differentiated carcinomas, the situation is more constant. In the central area, perinecrotic, and remote extratumoral areas, the microvascular densities are similar.

Discussions

The association of chronic inflammation with carcinogenesis has been reported in the literature [5] as well as the association of risk factors to neoangiogenesis for Barrett's esophagus [6]. The relation between neoangiogenesis and inflammatory process may be considered a prominent aspect of esophageal adenocarcinoma.

The growth and tumor progression have been described in six major steps: the ability of uncontrolled growth, lack of response to antigrowth factors, avoidance of controlled apoptosis, replication capacity, sustained angiogenesis, invasion, and dissemination capacity [7].

p53 is required for the initiation of carcinogenesis in esophageal adenocarcinoma, its accumulation being demonstrated by immunohistochemical examination supporting this theory. It is interesting to study whether accumulation and mutations of p53 are found in Barrett's esophagus without obvious adenocarcinoma lesions. For this purpose, we used specialized studies demonstrating that p53 accumulation and genetic mutations may be related with the degree of dysplasia on esophageal intestinal metaplasia. The same study demonstrates that intranuclear accumulation of p53 was not found in the lining of non-dysplastic Barrett's esophagus, and this accumulation increased in intensity when moving from low to high degrees of dysplasia [8].

Fig. 5 Graphic representation for microvessel density/ mm^2 for tumor stroma (MD/TS) and microvessel density/ mm^2 for tumor (MD/T) in esophageal adenocarcinoma we studied

Chung (2007) supports the same opinion, reporting that p53 can be considered a marker of transformation from Barrett's esophagus into esophageal adenocarcinoma [1]. P53 mutations were associated with low histological tumor differentiation and low survival rate with esophageal adenocarcinoma [7, 9, 10], these results coming to support the results of our study—intensity marking (+++) of p53 in poorly differentiated esophageal adenocarcinoma.

Angiogenesis is essential for the tumor to grow and can be considered a mark of the neoplastic process. Tumor neoangiogenesis process supposes several stages and taking these steps is a phenomenon that occurs early in the development of esophageal adenocarcinoma neoplastic process. The microanatomic qualitative study of esophageal neoplasia revealed several important aspects. Both in esophageal adenocarcinoma and squamous cell carcinoma, the tumor microenvironment with its two components (cellular and non-cellular component) plays an important role in the development and tumor progression, given the constant changes seen at this level. Due to the carcinogenesis, a specialized tumor stroma is produced, favoring the persistence and metastasing of the neoplastic process.

Recent literature focused on the relationship between tumor stroma and tumor epithelial cells, many authors believing that there is a process of mutual induction between the epithelial and stroma component, describing the phenomenon of EMT—epithelial-mesenchymal transformation [11, 12].

During our research, we constantly met a combination of three key elements: inflammatory infiltrate, tumor fibrosis, and neoangiogenesis represented by blood capillaries. This combination led us to the conclusion that mutual induction is a link between all of them. According to extensive studies [13], the fibroblasts have the main role in the induction and transformation of the stroma and tumor epithelial components. They also may lead to formation of collagen fibers by tumor stroma remodeling, and this can be found in epithelial tumors too.

In our research, the overall fibrosis process is more evident in the case of squamous carcinomas than in the adenocarcinoma ones. It seems that fibroblasts are cells with a dominant role in tumor stroma, having an activation status [14, 15] that allows interaction with other resident stroma cells and other tumor epithelial components. Thus, fibroblasts secrete matrix metalloproteinases leading to the reorganization of stroma with synthesized collagen type I and II. The decrease of type IV collagen synthesis leads to a qualitative and quantitative depreciation in extracellular matrix.

It is well known that neoangiogenesis is directly related to tumor hypoxia [16], leading to the secretion of VEGF fibroblasts with chemoattractism for macrophages and triggering the process of neoangiogenesis [17]. Moreover, hypoxia causes activation of fibroblasts by transforming them into myofibroblasts that secrete SDF1 and VEGF. SDF1 allows the release of endothelial precursor cells

from the hematogene bone marrow and increased VEGF synthesis allows the transformation of precursor endothelial cells into endothelial cell [17].

According to recent data, the microenvironment of tumor stroma has not only a supportive role, but can also lead to the initiation of carcinogenesis and the mutations in the stroma cells that can cause the secretion of growth factors that initiate epithelial cancers. There is a mutual induction between the two elements and finding therapeutic methods to break this vicious circle could be a target to reach.

The tumor heterogeneity and vascularity is another aspect which we have correlated with the histological differentiation in esophageal squamous cell carcinoma. This is more evident in poorly differentiated tumors with or without histological differentiation. Tumor heterogeneity is the existence of several types of tumor cells showing that a specific tumor tissue consists of more malignant clones.

Nowadays, tumor heterogeneity is an important topic of study in an attempt to explain and understand tumor virulence and metastasis development. Papers which were based on the study of the DNA of a tumor showed that there is an intratumoral genetic heterogeneity in different areas of the same tumor, and this may be related to the degree of histological differentiation [18].

The fact that a poorly differentiated tumor is more aggressive may be connected with this phenomenon of genetic and histological heterogeneity that prints into each malignant clone an own survival and expansion rhythm, independent or partially dependent to other malignant clones within the same tumor.

The above mentioned aspects led us to the idea of investigating an immunohistochemical marker of tumor virulence for esophageal squamous cell carcinoma, choosing the p53 tumor suppressor protein that plays an important role in cell cycle regulation. It is called the guardian of the human genome, ensuring the stability of the human genome by preventing genetic mutations, i.e., carcinogenesis [19].

Typically, p53 acts directly by its intercellular signaling ways that lead to the initiation of apoptosis, inhibition of neoangiogenesis, or block of cells in phase G1/S of the cell cycle, thereby allowing DNA repair for certain enzymes [20]. In the normal cell cycle, p53 increases under stress or any other conditions that cause DNA damage, after which it is degraded by a number of control mechanisms to such a degree that its immunohistochemical expression becomes not visible.

In the case of neoplastic process, mutations in the gene encoding the p53 lead to changes in structure. Thereby, cells escape apoptosis and the DNA is not repaired initiating the process of carcinogenesis [21]. On the other hand, some mutations in p53 lead to its accumulation in the cells, and its expression becomes visible by immunohistochemical examination.

Angiogenesis is an essential condition for the tumor to grow and can be considered a marker in the neoplastic process. This was demonstrated by the fact that the process of neoangiogenesis is intense in both histological types of the studied tumor (adenocarcinoma and esophageal squamous cell carcinoma).

In esophageal epithelial neoplasm, the neoangiogenesis is an intense process of tumor progression and remote metastasis, so that one of the following lines of research should be directed toward finding therapeutic ways to interfere with this process.

Conclusions

The study of vascular system related to esophageal epithelial tumors revealed a formation of three lesion types which have a mutual induction process: the inflammatory infiltrate—neoangiogenesis—fibrosis.

Marking intensity of p53 can be correlated with the degree of tumor differentiation, becoming more intense when the degree of histological differentiation is weaker. Mutations in genes encoding the p53 lead to intranuclear accumulation of p53 which enhances uncontrolled growth of tumor cells by preventing the controlled apoptosis process.

There were significant differences between the three degrees of differentiation (low, moderate, and well-differentiated) of esophageal adenocarcinomas, with space variations becoming predictable. In well-differentiated forms with lesions with a lower risk, vessels are uniform and regular compared with high-risk forms where vessels are irregular, tortuous, sinuous, and dilated.

The significant increase in microvessel tumor density occurs with the increasing of histological grading, with an inverse proportion with the degree of differentiation and directly proportional to the malignancy risk.

References

1. Chung SM, Kao J, Hyjek E, Chen YT (2007) p53 in esophageal adenocarcinoma: a critical reassessment of mutation frequency and identification of 72Arg as the dominant allele. *Int J Oncol* 31(6): 1351–1355
2. Ferlay J, Parkin DM, Steliarova-Foucher E (2010) Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 46: 765–781
3. Trivers KF, Sabatino SA, Stewart SL (2008) Trends in esophageal cancer incidence by histology, United States, 1998–2003. *Int J Cancer* 123(6):1422–1428
4. Chang AC, Lee JS, Sawicki KT, Pickens A, Orringer MB (2010) Outcomes following esophagectomy in patients with prior antireflux or hiatal hernia surgery. *Ann Thorac Surg* 89:1015–1023
5. Keibel A, Singh V, Sharma MC (2009) Inflammation, microenvironment, and the immune system in cancer progression. *Curr Pharm Des* 15(17):1949–1955
6. Zhai R, Liu G, Monica Ter-Minassian M, Wu I-C, Zhaoxi Wang Z, Su L, Asomaning K, Chen F, Kulke MH, Lin X, Heist RS, Wain JC, Christi DC (2012) Interactions between environmental factors and polymorphisms in angiogenesis pathway genes in esophageal adenocarcinoma risk: a case-only study. *Cancer* 118:804–811
7. Schneider K (2011) Counseling about cancer: strategies for genetic counseling. John Wiley and Sons, New Jersey
8. Bian YS, Osterheld MC, Bosman FT, Benhattar J, Fontollet C (2001) p53 gene mutation and protein accumulation during neoplastic progression in Barrett's esophagus. *Mod Pathol* 14(5):397–403
9. Ribeiro KB, Levi JE, Pawlita M (2011) Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions. *Int J Epidemiol* 40:489–502
10. Casson AG, Evans SC, Gillis A et al (2003) Clinical implications of p53 tumor suppressor gene mutation and protein expression in esophageal adenocarcinomas: results of a ten year prospective study. *J Thorac Cardiovasc Surg* 125:1121–1131
11. Tlsty TD (2001) Stromal cells can contribute oncogenic signals. *Semin Cancer Biol* 11(2):97–104
12. Sierra JR, Tsao MS (2011) c-MET as a potential therapeutic target and biomarker in cancer. *Ther Adv Med Oncol* 3(1 Suppl):S21–S35
13. Kunz G Jr, Chung J, Syed ZA (2002) Hepatocellular carcinoma-fibrolamellar variant: cytopathology of an unusual case. *Diagn Cytopathol* 26(4):257–261
14. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6(5):392–401
15. Pietras K, Ostman A (2010) Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 316(8):1324–1331
16. Heil M, Clauss M, Suzuki K, Buschmann IR, Willuweit A, Fischer S, Schaper W (2000) Vascular endothelial growth factor (VEGF) stimulates monocyte migration through endothelial monolayers via increased integrin expression. *Eur J Cell Biol* 79(11):850–857
17. De Wever O (2008) Stroma reactions and cancer invasion. *Exp Dermatol* 17(10):891–892
18. Wang X, Fan M, Chen X, Wang S, Alsharif MJ, Wang L, Liu L, Deng H (2006) Intratumor genomic heterogeneity correlates with histological grade of advanced oral squamous cell carcinoma. *Oral Oncol* 42(7):740–744
19. Harris SL, Levine AJ (2005) The p53 pathway: positive and negative feedback loops. *Oncogene* 24(17):2899–2908
20. Levine AJ, Hu W, Feng Z (2006) The P53 pathway: what questions remain to be explored? *Cell Death Differ* 13(6):1027–1036
21. Scoumanne A, Che X (2008) Protein methylation: a new regulator of the p53 tumor suppressor. *Histol Histopathol* 23(9):1143–1149