

Prognostic Value of Microvessel Density, Matrix Metalloproteinase-9 and p53 Protein Expression in Esophageal Cancer

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ABSTRACT

Background: Worldwide, esophageal carcinoma is one of the most aggressive cancers. It is relatively common in many countries and characterized by poor prognosis and rapid clinical progression.

Purpose: In this study, we aimed to evaluate the role of CD34, the marker of vascular endothelial cells, MMP-9 (matrix metalloproteinase type 9) and p53 in esophageal carcinoma.

Materials and Methods: Forty-four archival tissue specimens, 38 cases with esophageal carcinoma and six cases with normal mucosa, were immunohistochemically stained with monoclonal antibodies against CD34, MMP-9 and p53. In addition, flow cytometric DNA analysis was carried out for patients and controls.

Results: The results showed that the DNA content was diploid in all normal esophageal mucosa, whereas aneuploidy was detected in twenty cases (52.6%) out of 38. The thirty-eight cases showed positive expression of CD34 antigen. The expression of MMP-9 was identified mainly in the cytoplasm in most of cancer cells in 27 cases (71%) out of 38. On the other hand, 28 (73.7%) out of 38 were positive for p53 expression. There was a statistical significance for CD34, MMP-9 and p53 expressions with tumor stage. Microvessel density in patients with highly positive staining for MMP-9 was higher than in those with negative and weak staining for MMP-9 ($p=0.002$).

Conclusion: Our data suggest that the expression of CD34 and MMP-9 is associated with tumor progression and possibly seems to be valuable markers and could offer additional information about the aggressiveness and activity of esophageal carcinoma lesions.

Key Words: Esophageal cancer - Matrix metalloproteinase-9 (MMP-9) - CD34 - p53 - DNA content.

INTRODUCTION

Esophageal carcinoma is one of the most common malignancies in the world. Since the growth of tumor is relatively fast, patients with esophageal carcinoma generally have a worse prognosis than those with any other gastrointestinal tumors. Intratumoral microvessel count, which represents a measure of tumor angiogenesis, has been associated with the overall survival of patients with a variety of malignancies. Tumor stage, grade, DNA ploidy, p53, and lymph node metastasis have a significant impact on survival. Recently, tumor Angiogenesis was considered as a prognostic factor in many solid tumors [1,2]. Angiogenesis became a recognized field of study after Folkman's observation that tumors are richly supplied with blood [3].

Angiogenesis, which is essential for tumor growth and metastasis, depends on the production of angiogenic factors by tumor cells and normal cells. It involves several pathways including the secretion of angiogenic substances, activation of endothelial cells, degradation of capillary membranes, and endothelial cell migration [4]. Increased vascularity enhances the growth of primary neoplasms and provides a greater chance for hematogenous metastasis.

It has become clear that those angiogenesis regulatory molecules are present in the body on stand-by basis. These pro-angiogenic proteins are maintained in a potentially active state and are releasable by specific enzymes when angiogenesis is required in physiological processes

such as reproduction or repair and when angiogenesis is induced by pathological processes [5].

Patients with submucosal esophageal carcinoma continue to be much worse than those with intramucosal lesions and the reasons are unclear. It is well known that the matrix metalloproteinases (MMPs) are involved in tumor cell invasion and may contribute in such aggressiveness. Expression of these enzymes may have some relation to the difference in survival between patients with esophageal carcinomas. MMP-9 is a member of a class of these enzymes, it cuts through tissue (matrix barriers and collagenolysis) clearing the way for the growth of tumor cells for the development of blood vessels that tumors need to keep growing by degradation of extracellular matrix macromolecules [6]. Alterations of the p53 tumor suppressor gene have been known to be the most common genetic changes in solid tumors including esophageal carcinomas. Recent reports showed that mutations of p53 might be associated with angiogenesis.

The use of a variety of histopathological and biological markers is now offering promising prospects for the future. Vertical tumor invasion, intratumoral microvessel density, antimucin monoclonal antibodies, flow cytometry, telomerase activity, and overexpression of cyclin D1 have been correlated with the staging and prognosis of esophageal carcinomas. By combining these markers with Lugol's staining, a practical new method of staging esophageal tumors may become available in the coming years [7].

The rationale of the present study was to assess the role of CD34, matrix metalloproteinase 9 and p53 expression as possibly better prognostic markers in esophageal carcinomas correlating them with DNA ploidy and clinicopathological findings.

PATIENTS AND METHODS

Patients:

This study included thirty-eight patients (26 men and 12 women) with esophageal cancer in addition to six with normal esophageal mucosa. They were hospitalized at the Gastroenterology Surgical Center, Mansoura University, Mansou-

ra, Egypt. The patients' ages ranged from 34 to 67 years. Two biopsy specimens were obtained as close as possible from the lower esophageal mucosa for each case, one for DNA flow cytometric analysis and the other for histopathologic and immunohistochemical examinations. The clinicopathological factors of these patients were estimated according to the TNM classification proposed by the International Union against Cancer (UICC) in 1987 [8]. The disease type, grade and stage in different patients were as described in table (1).

DNA flow cytometric analysis:

Analysis of DNA content was performed on archival tissue specimens according to the method of Hedley [9]. From each case, two 20 μ m-thick sections were used for the DNA flow cytometric technique. The DNA histogram derived from each specimen was analyzed using DNA analysis software (Cytological software, Coulter Co. Hialeah, FL, USA). The DNA profile was divided into two groups, diploid and aneuploid.

Immunohistochemical staining:

Formalin-fixed paraffin-embedded tissue blocks were sectioned at 4- μ m thickness and the immunostaining was performed using the avidin-biotin peroxidase method. Sections were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in PBS (phosphate buffer saline) [10 mM sodium phosphate, 140 mM sodium chloride, pH 7.2]. The slides were washed three times, 2 minutes each and then incubated with blocking serum for 10 minutes to block the nonspecific binding. The excess of blocking serum was removed, and then the sections were incubated with primary monoclonal antibodies anti-CD34 [CD34 Ab-2 (clone 34CO2)] or anti-MMP-9 [MMP-9 (92Kd Collagenase IV) Ab-9] [NeoMarkers, LABVISION, USA.] and anti-p53 [DO-1] (Immunotech., France) diluted at 15 μ g/ml, for one hour at room temperature. After washing, the biotinylated secondary antibody was used as a second layer for 10 minutes. After that, the sections were washed three times, 5 minutes each. Streptavidin-peroxidase complex was applied for 10 minutes. The sections were washed in PBS and the peroxidase signal was developed in 0.05% diaminobenzidine and

0.01% hydrogen peroxide in PBS. The sections were lightly counterstained with haematoxylin. Brown diffuse cytoplasmic staining was seen in positive cases of MMP-9 and brown staining of vascular endothelial cells was observed in positive cases of CD34.

Vessel counting was assessed by light microscopy in areas with maximal neovascularization within the tumors away from any areas of artifact, necrosis, or inflammation. The highly vascular areas were identified by scanning tumor sections at low power (x40 and x100) to determine three hot spots. Microvessels were counted within areas defined in each of the three hot spots at x200 magnification. Microvessel density (MVD) was estimated as the mean of the highest three counts. According to the MVD count, the results were classified into four categories (I, II, III and IV) where the MVD was 0, 5-10, 10-25, and more than 25, respectively.

Statistical analysis:

Univariate comparisons were assessed using either Fisher's probability exact or chi-square tests, as appropriate. The correlation between different variables was estimated using Spearman's correlation. The minimal level considered significant was $p \leq 0.05$. All analyses were performed using commercial statistical program software, SPSS 9.05 for Windows.

RESULTS

Tumor characteristics of these patients and the obtained data are summarized in table (1).

Analysis of DNA content:

The DNA profile was diploid in six cases (histopathologically diagnosed as normal esophageal mucosa) and abnormal DNA content (aneuploidy) was detected in 20 (52.6%) cases out of 38 (histopathologically diagnosed as esophageal carcinoma) (Figs. 1,2). There was statistical insignificance between DNA content and tumor grade.

Seventeen (85%) out 20 cases with high MVD count were aneuploid. In addition, there were 18 cases (66.7%) with aneuploid DNA pattern out of 27 cases with positive MMP-9. These data may correlate between both tumor vascularity and matrix metalloproteinase 9 with DNA aneuploidy.

Immunohistochemical findings:

Thirty-eight cases were evaluated for microvessel density (MVD). All cases showed a positive expression for CD34 antigen as shown in Fig. (3-A). The range of MVD in esophageal cancer was 5 to 45 with median of 25. There was a significant correlation between MVD count with tumor grade and stage. High-grade and stage tumors had a significantly higher MVD than did low-grade and stage tumors.

The expression of MMP-9 was identified mainly in the cytoplasm of cancer cells as shown in Fig. (3-B). 27 (71%) cases out of the 38 cancerous cases were MMP-9 positive. In respect to tumor grade, the 7 cases (70%) in grade I, 13 (81.3%) cases in grade II, and 7 (58.3%) cases in grade III were positive for MMP-9; this difference was statistically insignificant ($p = 0.39$). In respect to tumor stage, 4 (36.4%) cases in stage I, 9 (75%) cases in stage II, 9 (90%) cases in stage III, and 5 (100%) cases in stage IV were positive for MMP-9 ($p = 0.02$). Heterogeneous intensity of the staining was observed in most of the cases. The counting of MVD in patients with highly positive staining for MMP-9 was higher than in those with negative or weak staining for MMP-9 and this correlation was highly significant ($p = 0.002$).

The p53 expression was evaluated in all cases. Twenty-eight cases (73.7%) were p53 positive. There were significant differences with tumor stage ($p < 0.001$). Nuclear staining for p53 was positive in 20 cases with high microvessel count (25-45), and in 23 cases out of 27 positive for MMP-9. There was a positive correlation between the CD34, MMP-9 and p53 obtained by Spearman's correlation statistical analysis. MMP-9 was correlated with CD34 ($r = 0.352$, $p = 0.03$). In addition, p53 was positively correlated with MMP-9 and CD34 ($r = 0.448$, $p = 0.002$; $r = 0.748$, $p < 0.001$, respectively).

DISCUSSION

A significant increase in the incidence of esophageal adenocarcinoma in several countries such as Australia, New Zealand, Norway, Scotland, Sweden, Switzerland, USA and England was reported [10-15].

Table (1): Tumor characteristics and the obtained results.

Variable	CD34				p-value	MMP-9		p-value	p53		p-value	DNA ploidy		p-value
	I	II	III	IV		Negative	Positive		Negative	Positive		Diploid	Aneuploid	
<i>Type:</i>														
Squamous	—	3	8	13	=0.31	8	16	=0.59	6	18	=0.76	16	8	=0.2
Adenocarcinoma	—	2	4	2		1	7		3	5		—	8	
Undifferentiated	—	—	1	5		2	4		1	5		2	4	
<i>Grade:</i>														
GI	—	3	4	3	=0.07	3	7	=0.39	4	6	=0.52	5	5	=0.92
GII	—	2	7	7		3	13		4	12		8	8	
GIII	—	—	2	10		5	7		2	10		5	7	
<i>Stage:</i>														
I	—	4	4	3	<0.001	7	4	=0.02	8	3	<0.001	8	3	<0.24
II	—	1	8	3		3	9		2	10		5	7	
III	—	—	1	9		1	9		—	10		3	7	
IV	—	—	—	5		—	5		—	5		2	3	
Total	—	5	13	20		11	27		10	28		18	20	

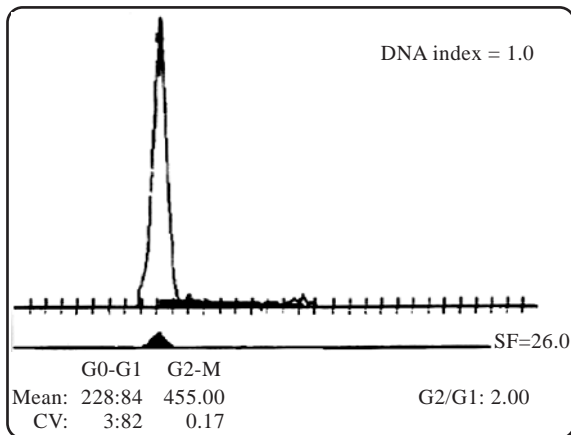


Fig. (1): Normal diploid pattern with DNA index DI = 1.

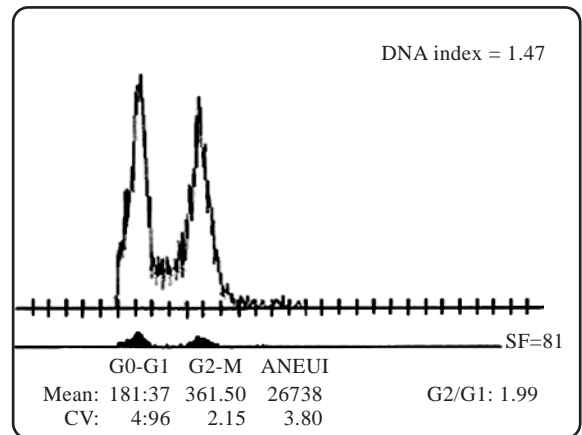
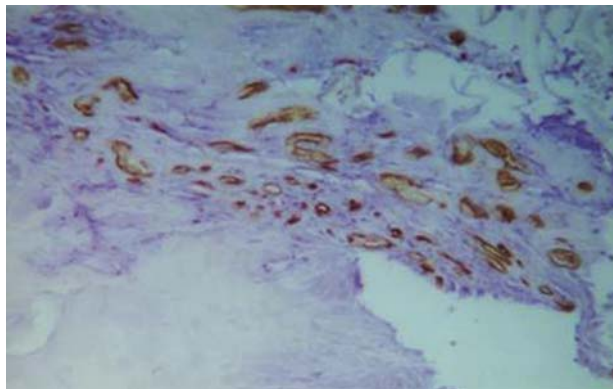
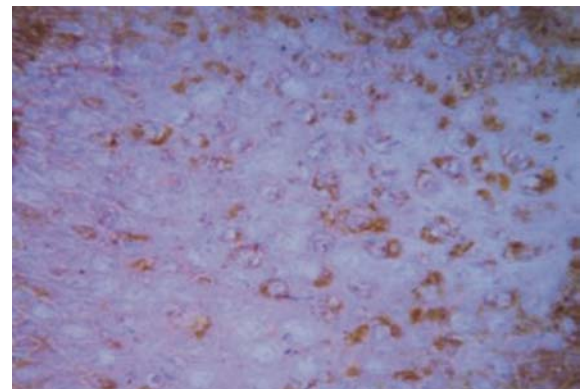


Fig. (2): Aneuploid pattern with DI = 1.5.



(A)



(B)

Fig. (3): Immunohistochemical staining for CD34 (A) and MMP-9 (B) in human esophageal carcinoma. (A) CD34 antigen is expressed on many of endothelial cells. (B) MMP-9 immunoreactivity is observed in the cytoplasm of the tumor cells x400.

Carcinoma of the esophagus has one of the worst prognoses among digestive carcinomas because more than 90% of patients are detected in an advanced stage of the disease [16], and more than half undergo noncurative surgery due to either local tumor invasion of the surrounding tissue or distant metastasis at the time of operation [17].

In the present study, aneuploidy was detected in 52.6% of patients with esophageal malignancies. These results are in agreement with previous data that mentioned 47.3% of cases were aneuploid [18]. In another study [19], the investigators reported 79% and 71.4% of aneuploidy in two different patient groups with esophageal carcinoma.

Aneuploidy in patients with squamous cell carcinoma was 33.3%, which is in agreement with another study that demonstrated a similar percent (31%) of aneuploidy in patients with squamous cell carcinoma [20]. Kimura et al [21] found that the aneuploidy in the esophageal carcinoma patients was 55.9%, while Robaszkiewicz et al. [22] reported that the aneuploidy in the same type of cancer was 80.7%.

DNA aneuploidy was found in eight cases (100%) of patients with adenocarcinoma. These results are in concordance with those obtained by Reid et al. [23], where they found DNA aneuploidy in seven cases (100%) with adenocarcinoma. In contrast, aneuploidy was found in a low percent of patients with the same type of carcinoma in another study [24].

The established histological criteria for prediction of the malignant potential of esophageal carcinoma are not satisfactory. Pathologists investigated a series of factors as trials to predict the disease prognosis accurately. Among these, p53, angiogenesis, MMP-9 and DNA content have been especially emphasized.

The wild-type p53 protein can suppress cell proliferation and usually is not detected by immunohistochemical methods because it has a short half-life of 6 to 20 minutes. The mutant form of p53 that has lost the ability to suppress cell proliferation can be detected by immunostaining, however, because the product has a longer half-life (6 hours) and thus can accumulate to be readily detected [25]. The p53 expression was detected in 73.7% of our studied cases.

There were significant differences with tumor stage ($p < 0.001$). In other studies, p53 positivity was shown in a comparable ratio in patients with oesophageal cancer [26-29]. Therefore, from the clinical standpoint of using p53 as a prognostic marker in esophageal cancer, currently, the simplest and most practical approach is the use of immunohistochemistry alone to detect p53 protein accumulation. This strategy was also suggested for prognostic studies on p53 in breast cancer [29]. In the present study, nuclear staining for p53 was positive in twenty cases with high microvessel counts (25-45), and in 23 cases out of 27 positive for MMP-9 and these data may reflect that p53 mutations may be associated with angiogenesis.

Angiogenesis has been proposed as a prognostic factor in tumors in which it is a prerequisite for tumor growth. In this study, we analyzed the relationships among angiogenesis factor expression, matrix metalloproteinase 9, flow cytometric DNA analysis and clinicopathological features in esophageal carcinomas. It was found that the angiogenic activity (positive expression of CD34) was noted in all cases and these data are consistent with findings of a previous study [30]. Furthermore, we found higher MVD counts in tumors invading the muscularis propria than in submucosal and mucosal tumors. There is a correlation between CD34 and tumor stage and grade. However, the association between CD34 expression and clinicopathological findings is controversial, for example some reports showed that CD34 expression was correlated with the tumor stage only [31-33].

We argue that the high-grade tumors might be associated with increased MVD counts due to increased activity of angiogenic stimulatory factors such as thymidine phosphorylase, which indicates that CD34 has the potential to contribute to tumor growth [34].

Concerning MMP-9, in the present study we found that 71% of esophageal carcinoma cases exhibited intense MMP-9 immunoreactivity in tumor tissues and no expression in normal tissues, and it was significantly correlated with tumor stage; this is agreement with previous studies [6,33]. Our study illustrates that esophageal carcinoma produces MMP-9, suggesting that the ability of MMP-9 production by the tumor may play an important role in its malig-

nant behavior and that it is closely associated with invasion depth in esophageal carcinoma. However, our data are controversial with other data demonstrating that the intense MMP-9 expression appears in early stage of tumorigenesis [35, 36].

The initiation of new blood vessels through angiogenesis is critical to tumor growth. Tumor cells release soluble angiogenic factors that induce neovascularization, without which nutrients and oxygen would not be available to allow tumors to grow more than 2-3mm in diameter. This "angiogenic switch", or angiogenic phenotype, requires an imbalance between proangiogenic and antiangiogenic factors since the formation of new blood vessels is highly regulated [37]. Thus, the process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by both tumor cells and normal cells. Numerous angiogenic factors have been described; however, the ones responsible for angiogenesis in esophageal carcinoma remain unknown [32].

In conclusion, CD34 and MMP-9 are highly expressed in human esophageal carcinomas and a significant relationship was noted between MVD count and expression of MMP-9. Therefore, MVD count and MMP-9 may influence esophageal tumorigenesis.

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