The usefulness of immunohistochemistry in sporadic colorectal cancer

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Abstract

Background: Colorectal cancer is an important disease with a large morbidity and mortality and also with increasing health care costs because of widespread of the multi-modal therapy and of the new drugs that continue to appear. There are 678,000 colorectal cancer cases and 400,000 deaths from the disease worldwide. It is the second commonest cause of cancer death in the European Union but, unlike the commonest cause of cancer related death that is lung cancer, the basis of the disease initiation is currently not understood. At the same time, the incidence increases with age, the carcinomas being rare before the age of 40 years, excepting individuals with genetic predisposition or predisposing conditions such as inflammatory bowel diseases. The early detection of colorectal cancer is potential associated with an important decrease of the cancer related mortality. Aim: Our study proposes to find out the significance of some immunohistochemical markers (VEGF, p53, CK20 and CEA) in sporadic colorectal carcinoma cases and to establish the statistical correlations between molecular markers and tumor grade and stage. Material and Methods: We investigated histopathological 40 inpatients (19 female and 21 males) who undergone surgery for colorectal carcinomas in “Sf. Ioan” Emergency Hospital, Bucharest, between September 2005–September 2006. We proceeded the histopathological examination to establish the grade, stage and the main features of the tumors, and then we analyzed using ABC method for immunohistochemistry the following markers for 20 selected cases: vascular endothelial growth factor (VEGF), carcinoembryonic antigen (CEA), cytokeratin 20 (CK20), and p53 oncoprotein. Finally, we analyzed statistical the results using t–Student test. Results: The distribution of colorectal cancer cases (n = 40) regarding the age has showed the preponderance of patients older than 70 years (22/55%) and a small percentage of younger adults (2/5%). The repartition of colorectal tumors of sex ratio outlines a small difference between males (21/52.5%), and females (19/47.5%). The histopathological analysis of tumor grade in the 40 cases has revealed a high percent of moderate grade tumors (23/57.5%), in comparison with the poor differentiated tumors (11/27.5%) and the well-differentiated cancers (6/15%). The neovascularity within the stroma, the main features of tumor growth, has been noticed in 15 cases (3.75%), and also an important inflammatory lymphocyte infiltrate in nine cases (22.5%). We have noticed positive correlation between VEGF1 and CK20 (r = 0.4, p = 0.05), and between VEGF1 and CEA (r = 0.88, p = 0.001). In addition, our results demonstrate a positive correlation between tumor grade and CEA (r = 0.43, p = 0.009), and no relation among the other markers. Conclusions: Our present study shows that CK20 and CEA are positive immunostaining markers no matter the stage (100%). The oncoprotein p53 has been negative in T1 and T2 stages, but in advanced stages has been positive in a half of cases (50%). Regarding the location, p53 and VEGF showed positively results whatever the topography. We have noticed a direct proportional relation in VEGF expression and CEA, and CEA and tumor grade (r = 0.88, p<0.001).

Keywords: colorectal carcinoma, immunohistochemistry, growth factors, sporadic / hereditary / familial colorectal cancers.

Introduction

Colorectal carcinoma is a malignant epithelial tumor of the colon or rectum. Only tumors that have penetrated through muscularis mucosae into submucosa are considered malignant at this point. The presence of scattered Paneth cells, neuroendocrine cells or small foci of squamous cell differentiation is compatible with the diagnosis of adenocarcinoma [1].

According to the data basis of the United States SEER, the incidence of the colonic adenocarcinoma is 33.7/100,000 and has increased with 18% during 1973–1987, while the incidence of the rectal adenocarcinoma (12.8/100,000) and of the mucinous colonic and rectal carcinoma (0.3 and 0.8, respectively) remained relatively constant [2].

On one hand, during the last decade of the twenty century, the incidence and mortality of the colorectal cancer decreased [2] and, on the other hand, the disease incidence increased with increasing age [3].

The medical history of the patient is extremely useful for the diagnosis of the colorectal cancer and also for the monitoring of the disease [4, 5].

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disorder, characterised by numerous colorectal polyps, with an intrinsic tendency of progression through adenocarcinoma [1]. APC (adenomatous polyposis coli) gene is located on the long arm of the fifth chromosome (5q21-22) [6]. Gardner syndrome is a variant of FAP that includes the presence of epidermoid cysts, osteomas, dental abnormalities and desmoids tumors, together with the
syndrome of flat adenomas" and the associated lesions in the colonic area [8, 9].

The attenuated form, also known as “hereditary syndrome of flat adenomas” and the associated lesions (retinal lesions, desmoids tumors) is extremely rare. Gardner syndrome, is characterized, besides the colonic adenomatous polyps, by osteomas and soft tissue tumors, while Turcot syndrome includes central nervous system tumors (medullo-blastomas) [7].

FAP is caused by germline mutations of the APC gene, the gene that contains 150 Kb pairs and 15 exons that represents only 8 Kb [10]. The codified protein has 2843 amino acids and it is expressed in numerous adult tissues. The majority of mutations are localized on the first 2000 codons [11].

**Hereditary Non-polyposis Colorectal Cancer (HNPCC) or Lynch syndrome** is an autosomal dominant disease characterized by the development of colorectal cancer, endometrial cancer and small bowel cancer, ureter or renal pelvis [12, 13]. Recently, new diagnosis criteria have been stated, in order to identify the families at risk for developing colorectal cancer during their lifetime (diagnostic criteria ACII) [14].

Gathering the data about non-polyposis colorectal cancer/Lynch syndrome brought an important contribution for completing the national registers of familial cancer syndromes and also for genetic cancers [15]. The discovery of the DNA mismatch repair genes, and their mutations, that lead to the development of HNPCC defined a certain clinical, pathological and molecular features of this condition [16, 17]. Thus, it became possible to demonstrate the presence of microsatellite instability in adenomas and carcinomas of the affected families and also in the extracolonic neoplasms [18, 19].

Bethesda criteria have been reviewed and the main histological features of the colorectal cancers with high microsatellite instability have been stressed as following: tumor infiltrating lymphocytes, Crohn disease-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern. These aspects are easy to recognize for the pathologist and also important because they provide important data for diagnosis of HNPCC with later onset [20, 21].

Molecular markers like transforming growth factor (TGF), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), adhesion molecules (CD44, E-cadherin) and other cell proteins (CK20) should be analyzed as individual factors and should be evaluated in large studies as prognostic factors, using multivariate analysis [22–24].

The Consensus Statement of the American Pathologists has established the value of the colorectal cancer prognostic factors [22]. Preoperatively evaluation of the carcinoembryonic antigen proved to have prognostic significance. The values higher than 5 ng/mL should be considered as an independent factor in multivariate analysis [22].

**P53** gene presents numerous mutations in human solid tumors. The wild type is localized on chromosome 17p and synthesizes a protein that maintains the genetic integrity by stopping the cellular cycle and by inducing apoptosis after detecting DNA lesions [22, 25]. Gene inactivation by allele losing, genetic mutation or protein retention leads to increase the genetic instability and the injured cell survival. The wild type of p53 gene has a short half lifetime and is not detectable through IHC. However, there are many discrepancies in literature regarding the correlation type between the gene mutations and the protein immunopositivity [26, 27].

Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis initiation [28, 29]. Studies performed on limited number of patients with all stages of colorectal cancer outlined that patients with VEGF-positive tumors had a poor prognosis comparative with VEGF negative tumors [30].

It has been suggested that p53 oncogene might be implicated in regulating VEGF expression with angiogenic phenotype modulation, depending on p53 injury [31, 32]. The research works revealed that IHC detection of p53 (that reflect indirectly the gene status) is associated with VEGF overexpression; in addition, the staining intensity is correlated with vascular status and also with the number of vessels. All these data sustains the strong association between p53 mutations, VEGF expression, and angiogenesis in human colorectal cancer [33–35].

Metastasis lesions with a diameter over 2.0 mm are defined as macrometastasis; whereas the lesions under 2.0 mm but exceeding 0.2 mm are defined as micrometastasis; ITC (Isolated Tumor Cells) are tumor deposits under 0.2 mm [36, 37]. The research works concerning gastric cancer but also colorectal cancer proved that the tumor cells from micrometastasis or ITC localized in the marginal sinuses of lymph nodes will lose the VEGF-C expression, while the macrometastasis localized in the marginal area have a marked expression of VEGF-C [38, 39].

**CEA** is a glycoprotein that belongs to CEA family. Monoclonal or polyclonal antibodies may identify several antigens belonging to glycoproteins of CEA family. Inflammatory or necrotic tissues react with CEA (carcinoembrionic antigen) [30]. The new variants of monoclonal CEA antibodies react only with epitopes of “true” CEA 80–100% of colorectal cancers, making a strong and diffuse positive staining [42].

**Cytokeratins** belong to intermediate filaments family, together with gylial fibrillary acid protein, desmin, vimentin and neurofilaments. A cytokeratin
antibody binds to an epitope of acid or basic proteins, unique or selected, and identifies a subset of epithelial cells. CK20 is positive in 85–100% of colorectal cancer cases, and immunostaining does not diminish with tumoral degree. CK7-/CK20+ is the standard combination for confirming or infirming a colorectal tumoral degree. CK7-/CK20+ is the standard cases, and immunostainig does not diminish with cells. CK20 is positive in 85–100% of colorectal cancer antibodies.

Material and Methods

The aim of the paper is to establish working protocols for researching the role of some important protein markers for colorectal cancer investigation. Immunohistochemistry evaluation became recently the cornerstone for patients with colorectal cancer, and in the last ten years the targeted therapy of colorectal cancer has gained an important role [20, 35, 43].

We analyzed a group of 40 inpatients from “Sf. Ioan” Emergency Hospital, Bucharest, who underwent surgery for colorectal cancer, between September 2005–September 2006. Simultaneous with the admission, into the Pathology Department we selected 20 cases with sporadic colorectal cancer for immunohistochemistry, based on disease history, the histopathological diagnosis (HE stain) and on the most important histological features that can provide useful data for the final assessment of the applied methods (Figure 1).

The tumor tissues were fixed in 10% formalin and paraffin-embedded. Histological sections were cut at 3-µm thickness and stained with Hematoxylin and Eosin.

We considered that immunohistochemistry tests (IHC) should use specific antibodies for epithelial malignant tumors and especially for colorectal neoplasia [44].

We used four markers from the antibodies panel presented before: VEGF1 (vascular endothelial growth factor 1), p53 (oncoprotein p53), CK20 (cytokeratin 20) and CEA (carcinoembrionic antigen). After the analysis of the selected patients regarding the antibodies distribution, we also performed a statistic analysis, considering it will be extremely useful during the research work.

The 20 cases of paraffin-embedded samples were processed for the immunohistochemistry examination. The immunohistochemistry (IHC) was performed on 3-µm thick sections from 10% formalin-fixed paraffin-embedded tissues.

To ensure the reliability of the experimental study, internal quality control of immunohistochemical techniques was performed as a part of an implemented and certified quality assurance system (ISO 9001/2001). Finally, we statistically analyzed the 20 cases selected for IHC, applying the standard methods, while the results were presented as abstracts at scientifically meetings.

The data were statistically analyzed using the program Analysis Tool Pack of Microsoft Excel 2003 under Window XP Professional. We have used statistic descriptive tests as the parametric t-Student test and also tests of correlation and regression.

Results

The distribution of the 40 cases of colorectal carcinomas regarding the age has showed the following results (Figure 1).

The analysis of the distribution of the tumors regarding the sex ratio provided the data from Figure 2.

The analysis of the 40 cases concerning the tumor grade has released the data from the Figure 3 (according to histopathological analysis in Figure 6).

The tumor stage, that means the tumor invasiveness within the adjacent histological structures is showed in Table 2.

Table 1 – Antibodies used for IHC analysis of the malignant colorectal tumors

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Source</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF1</td>
<td>Vascular endothelium</td>
<td>1/50</td>
<td>Santa Cruz</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>P53</td>
<td>Oncoprotein P53</td>
<td>1/50</td>
<td>DAKO</td>
<td>DO-7</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembrionic antigen of epithelial cell</td>
<td>1/500</td>
<td>NeoMarkers</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>CK20</td>
<td>Cytokeratin 20: proteins of intermediate filaments</td>
<td>1/50</td>
<td>Novocastra</td>
<td>Ks20.8</td>
</tr>
</tbody>
</table>

Table 2 – The distribution of cases regarding the stage (T)

<table>
<thead>
<tr>
<th>Stage (T)</th>
<th>No. of patients (n = 40)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>T2</td>
<td>13</td>
<td>32.5</td>
</tr>
<tr>
<td>T3</td>
<td>19</td>
<td>47.5</td>
</tr>
<tr>
<td>T4</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>
We analyzed immunohistochemical 20 cases (n = 20) from the 40 cases of the group (n = 40). Here is shown the tumor grade (Figure 4) and stage (Figure 5) distribution of the colorectal tumors for immunohistochemical analysis. Therefore, the majority of the cases were in the advanced grade (G3/60%) and stages (T3/60%) with perirectal adipose tissue invasion. The immunohistochemical technique applied on paraffin-embedded tumoral samples for VEGF1 (Figure 7), p53 (Figure 8), CK20 (Figure 9) and CEA (Figure 10) antibodies investigation, provided the following results. We used a random scale for the staining intensity (-, -/+, +, ++, ++++) as negative, slight, moderate and intense positive.

Figure 1 – The distribution of colorectal tumors regarding the age

Figure 2 – The repartition of colorectal tumors regarding the sex ratio

Figure 3 – The distribution of colorectal tumors regarding the grading (G)

Figure 4 – The immunopositivity of cases regarding the grading

Figure 5 – The immunopositivity according the staging of the analyzed cases: T1 – limited at submucosa; T2 – limited at muscularis; T3 – limited at submucosa/muscularis + adventiceal nodules, limited at subserosa/invading the perirectal adipose tissue
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Figure 6 – Moderate differentiated colon adenocarcinoma, muscular layer invasion, inflammatory infiltrate (HE stain, ob. 10×)

Figure 7 – Well-differentiated colon adenocarcinoma, strong immunostaining for VEGF in tumor cells, ob. 10×, +++

Figure 8 – Well-differentiated colon adenocarcinoma, strong immunostaining for p53 in tumor cells nuclei, ob. 4×, +++

Figure 9 – Well-differentiated colon adenocarcinoma, strong immunostaining for CK20 in tumor cells perimembrane, ob. 10×, ++++

Figure 10 – Well-differentiated colon adenocarcinoma, cytoplasmic and mostly perimembrane immunostaining for CEA, ob. 10×, ++++

The distribution of the immunostaining regarding the tumor grade is reflected in Table 3.

The antibodies distribution regarding the tumor stage is reflected in the Table 4.

One single case with mucinous/colloid aspects was characterized by an intense staining for VEGF (+) and negative for p53. The antibodies distribution regarding the tumor location is presented in Table 5.
The two groups. We also found the same positive correlation of VEGF with CK20, with statistical significance (\( p = 0.05 \)).

The null hypothesis is the following: there is no relationship between the variables. The correlation between two variables reflects the degree to which the variables are related. The most common measure of correlation is the Pearson correlation. We found no relation between the other markers (independent parameters).

### Discussion

Colorectal cancer is a malignant epithelial tumor of colon or rect. Only tumors that have penetrated through muscularis mucosae into submucosa are considered malignant. The presence of scattered Paneth cells, neuroendocrine cells or small foci of squamous cell differentiation is compatible with the diagnosis of adenocarcinoma [1].

Clinical, experimental and epidemiological studies showed that colorectal cancer incidence and mortality are lower in females comparing with males and suggested that replacement estrogen therapy in postmenopausal women may reduce the risk of cancer development [45]. Two estrogen receptor subtypes, alpha and beta ER, have been investigated using PCR, through reverstranscription and then Southern blot. The results were promising, suggesting that activation of ERbeta-mediated processes in superficial colonic epithelium may have a role in colorectal cancer prevention [45, 46].

Molecular markers like transforming growth factor (TGF), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), adhesion molecules (CD44, E-cadherin) and other cell proteins (CK20) should be analyzed as individual factors and should be evaluated in large studies as prognostic factors, using multivariate analysis [22, 47, 48].

CEA is a glycoprotein that belongs to CEA family. Monoclonal or polyclonal antibodies may identify several antigens belonging to glycoproteins of CEA family. Inflammed or necrotic tissues react with CEA antigens. The new variants of monoclonal CEA antibodies react only with epitopes of “true” CEA 80–100% of colorectal cancers make a strong and diffuse positive staining.

The studies were directed upon the discovery of a predictive marker for liver metastases or micrometastasis in colorectal cancer. Seventy-fifth percent of liver metastases determine a raise in CEA blood level, whereas 33% of non-metastatic disease showed that colorectal cancer incidence and mortality are lower in females comparing with males and suggested that replacement estrogen therapy in postmenopausal women may reduce the risk of cancer development [45]. Two estrogen receptor subtypes, alpha and beta ER, have been investigated using PCR, through reverstranscription and then Southern blot. The results were promising, suggesting that activation of ERbeta-mediated processes in superficial colonic epithelium may have a role in colorectal cancer prevention [45, 46].

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Student t-test deals with the problem associated with inference based on “small” samples: the calculated mean and standard deviation.

The null hypothesis is the following: there is no difference between the two studied groups.

Alternate hypothesis: there is a significance between the two groups.

Compared the value of the calculated \( t \) with the \( t \)-tabular it can be decided if the null hypothesis is or not accepted. If the null hypothesis is rejected the difference between the two groups is statistical significant. The value returned by \( t \)-test should be smaller then \( p = 0.05 \) for a stronger significance.

The correlation between two variables reflects the degree to which the variables are related. The most common measure of correlation is the Pearson correlation, and it reflects the degree of linear relationship between two variables.

In the present paper, the data for the statistical analysis are normally distributed. We use the paired samples \( t \)-test because the sample size is \( n=30 \).

We obtained a positive correlation between VEGF1 and CK20, with statistical significance \( (r = 0.4, p = 0.05) \) that implies a direct proportional correlation. We also found the same positive correlation of VEGF with CEA with statistical significance \( (r = 0.88, p<0.001) \), that implies the same direct proportional

### Table 3 – Antibodies distribution regarding the colorectal tumor grade

<table>
<thead>
<tr>
<th>Antibody</th>
<th>G1 (n = 4)</th>
<th>G2 (n = 4)</th>
<th>G3 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF1</td>
<td>2 (-) 50%</td>
<td>1 (+/-) 25%</td>
<td>4 (+/-) 100%</td>
</tr>
<tr>
<td></td>
<td>1 (+++) 25%</td>
<td>12 (+++) 100%</td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>3 (-) 75%</td>
<td>1 (-) 25%</td>
<td>3 (+++ 25%</td>
</tr>
<tr>
<td></td>
<td>1 (+/-) 25%</td>
<td>9 (-) 75%</td>
<td></td>
</tr>
<tr>
<td>CK20</td>
<td>4 (+++) 100%</td>
<td>4 (+++) 100%</td>
<td>12 (+++) 100%</td>
</tr>
<tr>
<td>CEA</td>
<td>4 (+++) 100%</td>
<td>4 (+++) 100%</td>
<td>12 (+++) 100%</td>
</tr>
</tbody>
</table>

### Table 4 – Antibodies distribution regarding the tumor stage

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>VEGF</th>
<th>p53</th>
<th>CK20</th>
<th>CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (n = 2)</td>
<td>1 (-) 50%</td>
<td>2 (-) 100%</td>
<td>2 (+++) 100%</td>
<td>2 (+++) 100%</td>
</tr>
<tr>
<td>T2 (n = 6)</td>
<td>2 (+/-) 33.33%</td>
<td>6 (-) 100%</td>
<td>6 (+++) 100%</td>
<td>6 (+++) 100%</td>
</tr>
<tr>
<td>T3 (n = 12)</td>
<td>3 (+) 25%</td>
<td>6 (+++ 50%</td>
<td>12 (+++) 100%</td>
<td>12 (+++) 100%</td>
</tr>
</tbody>
</table>

### Table 5 – Antibodies distribution regarding the colorectal tumor location

<table>
<thead>
<tr>
<th>Topography</th>
<th>VEGF</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right colon (n = 6)</td>
<td>3 (+++) 50%</td>
<td>4 (+) 66.66%</td>
</tr>
<tr>
<td>Left colon (n = 10)</td>
<td>2 (+/-) 33%</td>
<td>2 (+/-) 33.34%</td>
</tr>
<tr>
<td>Rectum (n = 4)</td>
<td>2 (+) 50%</td>
<td>1 (+/-) 25%</td>
</tr>
<tr>
<td></td>
<td>1 (+++) 25%</td>
<td>3 (+) 75%</td>
</tr>
</tbody>
</table>
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The wild type is localized on chromosome 17p and synthesizes a protein that maintains the genetic integrity by stopping the cellular cycle and by inducing apoptosis after detecting DNA lesions [22, 25]. Gene inactivation by allele losing, genetic mutation or protein retention leads to increase the genetic instability and the injured cell survival. The wild type of p53 gene has a short half lifetime and is not detectable through IHC. However, there are many discrepancies in literature regarding the correlation type between the gene mutations and the protein immunopositivity [26, 27]. Generally, the studies, which report a weak survival in p53 positive cases, are equal to those, which report no association between the overexpression of p53 and prognosis [27].

Other authors have tried to evaluate the p53 expression as a determinant of therapy response. It has been suggested that, pre-therapy, especially in rectal cancers, the p53 overexpression has an inverse relation with chemo-/radiation therapy response and a direct relation with the presence of residual tumor in lymph nodes during surgery. These studies suggest that p53 detection is probably a predictor of therapy response [51].

Angiogenesis is essential for development, progression and metastasis of malignant tumors [52]. The studies proved that VEGF, besides modulation of tumor angiogenesis, has a major role in vascular phenotype determination for the majority of human cancers, including colorectal cancer; antibodies anti VEGF given to mice together with xenografts of malignant cells of colorectal cancer led, experimentally, to decrease the tumor growth and to inhibit the metastasis [31, 53].

The evaluation of VEGF expression using IHC techniques in patients with colorectal cancer either preoperatively, only if a prior biopsy was taken or in patients that did not respond to therapy, has indicated that VEGF might be preoperatively, a marker used for evaluation the response to radiation therapy [38, 54].

A main topic in the third Gastrointestinal Cancers Symposium, which took place in Orlando, Florida, in January 2007, was the targeted therapy in gastrointestinal cancers, especially in colorectal cancers; many studies, based on large trials were presented and the data suggests that the VEGF inhibitor bevacizumab is efficient in colorectal cancers [6, 55–57].

Studies that used VEGF type C, involved in lymphangiogenesis, especially in immunodetection on tumor tissue, but also in lymph-nodes metastases, difficult to be detected at standard Hematoxylin–Eosin staining (especially micrometastases and isolated tumor cells/ITC), highlighted that this marker could not be reliable regarding the treatment options; it is not essential for lymphatic transport, but only in the development of the macronodular lymph nodes metastases [58].

VEGF-C binds VEGFR-3 (receptor of vascular endothelial growth factor type 3), is specifically expressed on lymph vessels, and stimulates lymph angiogenesis. Some studies tried to find a positive correlation between VEGF-C expression and the risk of lymphatic metastasis in many cancers (breast, lung, colorectal, pancreatic, prostate, esophageal, head and neck cancer) [59, 60].

The studies proved that anti-VEGF treatment with bevacizumab significantly decreases angiogenesis (\textit{in vivo} assessed, at video-microscope) and a slight decrease in tumor size. The decrease of tumor size is obtained with doxorubicin, with no effect on angiogenesis. The combination of the two drugs was more efficient, with more significant decrease of tumor size, and a few complete responses [3, 43, 61]. The intrinsic mechanism of anti-VEGF therapy, responsible of increasing chemotherapy results is not fully understood. Angiogenesis inhibition may increase the rate of tumor apoptosis. The same results were obtained when anti VEGF therapy has been combined with radiation therapy. The hypoxia is associated with response failure at chemo/radiotherapy [29, 62, 63].

Targeting the tumor expression of VEGF is an attractive approach of cancer management. Bevacizumab, a human monoclonal antibody, targeted on VEGF, proved to be of value in colorectal cancer treatment [43].

VEGF, also known as VEGF-A, is a key mediator of angiogenesis is a membrane glycoprotein that belongs to a family of vascular endothelial growth factors, which currently includes: VEGF-B, -C, -D and -E and also placenta growth factor (PIGF-1 and PIGF-2) [29, 38, 64]. Currently, it is involved in embryologic development of vascular system, ovulation, menstruation, pregnancy, wound healing, blood pressure regulation, maintaining of vascular endothelium. The most efficient VEGF inhibitor is bevacizumab, a human monoclonal antibody. The adverse events should not be neglected: hypertension, proteinuria, thrombosis, bleedings, non healing wounds, even gastrointestinal perforations; but above all these, in advanced colorectal cancer, bevacizumab effects are really beneficial and we should focus on preventing the clinical toxicity [33, 60, 65].

There is a strong need for more efficient and well-tolerated medical treatments. One trend is to find out new combinations of the existing drugs. In colorectal cancer there are several chemotherapy combinations: capcitabine and oxaliplatin (XELOX); irinotecan, 5-fluorouracil and leucovorin (S-FU/LV); oxaliplatin and S-FU/LV (FOLFOX-4) [66]. The other trend is focused on discovering new therapies, targeted on tumor growth and differentiation. Thus angiogenesis has became an important target in cancer management, knowing that neoangiogenesis is essential for tumor development [67, 43].

Many tumors may be destroyed by the cell-mediated immune response, usually through cytotoxic
lymphocytes (CD8). However, colorectal cancers have low immunogenity and may escape the immune response, through variants mechanisms, so called “immune tolerance”. There have been several attempts to increase the antitumor immune response. At the beginning, vaccines with autologous tumor cells were used. In order to increase the BCG efficiency, variants combinations have been used, but showed a relative low benefit. At the same time, it has been tried the vaccination against tumor associated antigens, like CEA, and the results were encouraging.

The benefit of targeted therapy using monoclonal antibodies was encouraged by the success of herceptin in breast cancer. AntiEGFR antibodies therapy is the beginning of a new era for colorectal cancer management, with promising, but still discordant results [3, 44].

Conclusions

Colorectal cancer is an important disease, with high morbidity and mortality, and also with increasing costs, concomitant with multi modal therapy development and discovery of new drugs.

The group selection in our study, has followed some clinical and histopathological criteria analysed in the previous step (the histopathological analysis).

The selected group for immunohistochemistry analysis represents 50% out the large group of patients with colorectal cancers. The methods used here were applied on the same histological type of colorectal cancers, adenocarcinoma without dedifferentiation.

Poor differentiated tumors represent more than half of them (G3/60%), while moderate and well differentiated tumors represents 20% each.

Regarding the tumor stage, we obtained that more than half of patients were in advanced stages of disease (T3/60%), and a few patients exhibit early stages (T1/10%; T2/30%).

The antibodies panel has included the antibodies used for the diagnosis of presumed digestive metastases (CEA, CK20), and also those antibodies with a possible prognosis role (p53, VEGF).

CEA (carcinoembrionic antigen) is associated with metastasis progression. Regarding the tumor grade, we have noticed the positivity of CEA in all selected tumors (100%), irrespective the tumor stage.

We have any data concerning the possible predictive role of CEA in the development of liver metastasis and we did not make correlations between liver metastasis and serum level of CEA, because in the hospitals, CEA measurements are made after surgery, in order to monitorize the postoperative evolution of the disease and the possible metastases.

At the same time, cytokeratin (CK20) was positive in all cases (100%), irrespective the tumor grade. Our study sustains the data published in literature concerning the immunostaining, which did not decrease with tumor grading.

Our study confirms the importance of CK20 sustaining or infirming a colorectal cancer, especially in combination with cytokeratin 7.

VEGF1 immunostaining in colorectal cancers was 100% positive, particularly in advanced, non-differentiated tumors and variable in well-differentiated. This marker could also be negative in well-differentiated tumors (50%), but also expressed in extremes (25%).

Regarding VEGF1, our data are concordant with literature findings, that is no or slight expression of VEGF1 in colonic adenoma and in normal colonic mucosa and also positive correlations with recurrences, metastasis and survival.

Whereas VEGF became positive in advanced stages (T3/75%; T2/66.66%) and T1/50%, it showed immunonegativity in one case in T1 (50%) and in three cases in T3 (25%). There is discordant data concerning p53 immunostaining in colorectal cancers.

Concerning the tumor degree, p53 was negative in 75% of well-differentiated tumors and intense positive in 25% of cases. P53 oncoprotein was positive in 75% out of moderate differentiated cancers and negative in 25% of cases. Concerning the location of the cancer, the oncoprotein p53 was negative in 75% of cases and positive in 25% in those tumors located in rectum.

P53 protein was negative in T1 and T2 stages in all patients, while in advanced stages it became positive in half of them (50%), the other half remaining also negative (50%).

The high variability of p53 staining probably derived from the observation of the wild type, which had a short half-lifetime and was difficult to detect it immunohistochemically. The detection may be easier due to mutations development, which may prolongs the protein lifetime.

Both CK20 and CEA had an intense immunostaining in all cases, no matter the stage (100%). The results suggested that the two antibodies investigate and diagnose certain subgroups of epithelial cells and also the disease progression and offer important data for subsequently follow-up of the patients.

Regarding the topography, p53 and VEGF showed on one hand VEGF immunopositivity irrespective the tumor localization, and on the other hand p53 positivity strong correlated with one rectal tumor (25%). In addition, irrespective the localization, all colorectal malignant tumors express VEGF, in order to promote angiogenesis. P53 immunopositivity regarding tumor topography is accidentally. From statistical point of view, the methods applied have showed positive correlation between VEGF1 and CK20, with statistical significance ($r = 0.4, p = 0.05$), which express a direct proportional relation.

We found the same positive correlation of VEGF with CEA, with statistical significance ($r = 0.88, p<0.001$) which express the same direct proportional relation. At the same time, the positive correlation between the tumor grading and CEA, statistical significant ($r = 0.43, p = 0.009$) sustained the same direct proportional relation.

In conclusion, our study supports that CK20 and CEA are positive immunostaining markers no matter the stage. The oncoprotein p53 has been negative in T1 and T2 stages but in advanced stages has been positive in
most of cases. Regarding the location, p53 and VEGF showed positively results whatever the topography. We have noticed a direct proportional relation in VEGF expression and CEA, and CEA and tumor grade ($r = 0.88, p < 0.001$). Molecular markers that we have chosen should be analyzed as individual factors and should be evaluated in large studies as prognostic factors, using multivariate analysis studies.

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References


The usefulness of immunohistochemistry in sporadic colorectal cancer


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