Correlations between CD34 Immunolabelled Blood Vessels and CD34 mRNA Expression in Colorectal Cancer

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ABSTRACT: Purpose: This study aims to determine the correlation between microvessel density of CD34 immunolabelled blood vessels and CD34 mRNA gene expression in colorectal cancer tissue. Material/Methods: Standard immunohistochemistry and gene expression was perform on samples collected from 76 patients with colorectal cancer in order to determinate the number of CD34 immunolabelled blood vessels and the relative quantity of CD34 mRNA. Results: For the study group, the mean CD34 immunolabelled microvascular density (MVD) was of 307/mm², and the mean CD34 gene expression value for colon cancer was 2.303. The low p value (<0.001) of the Spearman correlation test showed a significant direct correlation between CD34 MVD and CD34 gene expression for the entire study group. Conclusions: CD34 gene’s expression can be looked at as a prognostic factor in colorectal cancer.

KEYWORDS: colorectal cancer, CD34, vascularization

Introduction

Colorectal cancer (CRC) continues to be one of the most important cause of morbidity and mortality worldwide. According to World Health Organization, colorectal cancer is the second cause of cancer in women and the third in men [1]. Also, it is seen as the fourth most common cause of cancer death [2].

The five-year survival rate for CRC is less than 60% in Europe, and about a third of people who suffer from this type of cancer die from it in the developed world [3]. The survival rate is influenced by the pathological type, stage and detection rate [4].

Colorectal cancer’s prognostic depends on many factors, one of them being tumor vascularization. Immunohistochemistry studies on CD34 immunolabelled tumors demonstrated that the microvessel density (MVD) of CD34 positive blood vessels is linked with CRC progression and prognostic [5]. A higher number of CD34 positive blood vessels was inversely related to survival [6], and directly proportional with the presence of metastasis [7,8].

The aim of this study is to determine CD34 gene’s expression probability as a prognostic factor in colorectal cancer by establishing a correlation between CD34 MVD and CD34 mRNA in tumoral tissue.

Material and Methods

This study is conducted on 72 patients that were diagnosed with CRC and underwent surgery at Emergency County Hospital, Craiova, Romania, from whom tissue samples were collected. A written informed consent was provided by every patient that was enrolled in this study.

Three tissue samples were taken from every patient: one for immunohistochemistry and two for gene expression. The sample for immunohistochemistry was taken from tumor al tissue, which was carefully removed in such a way that would contain all the layers of the colon or rectum. For gene expression, the first sample was collected from tumoral epithelial tissue, avoiding necrotic tissue, and a second sample from normal epithelial tissue from the surgical resection margin.

The samples used to determine MVD of CD34 immunolabelled blood vessels were processed using the classical histopathological technique (fixation in 10% buffered neutral...
formalin and embedded in paraffin). From each paraffin block one 3µm thick section was cut and stained for CD34 using the Novocastra Lyophilized Mouse Monoclonal Antibody Endothelial Cell Marker (CD34), diluted as 1:70. Standard immunohistochemistry technique was applied: dewaxing in xylene; rehydration using graded ethanol solutions; blocking of endogenous peroxidase with 1% H2O2; antigen retrieving by microwaving the slides in citrate buffer-pH 6.0 for 20 minutes at 650W; washing in PBS (pH 7.0); blocking nonspecific binding sites with 3% Bovine nonfat-Dried Milk (Sigma-Aldrich) for 30 minutes at 25°C; incubating with primary antibody for 30 minutes at 25°C; washing in PBS; incubating with EnVision (Dako) 30 minutes at 25°C; antibody detecting by diaminobenzidine (DAB), 9 minutes at 25°C; counterstaining with Hematoxylin-Eosin. After staining, all tissue slides were analyzed with the help of an Olympus CX 31 microscope equipped with a ColorView II camera. Using the 40x objective of the microscope, ten photographs were randomly selected for each slide in order to approximate the MVD of CD34 immunolabelled blood vessels. All measurements were done with Analysis Pro 5.0 and exported in Excel (Microsoft Office, Microsoft Corporation). Statistical analysis of the data were done with GraphPad Prism version 6.

The samples used for gene expression were kept in RNA later for 24 hours and then processed with TRizol (Ambion) and Chloroform for RNA extraction. The RNA was purified with the help of QIAGEN RNeasy Mini Kit, after which it’s concentration was measured using NanoDrop 2000c UV-Vis (Thermo Scientific) spectrophotometer. All RNA samples used in this study were standardized to a 500ng/µl concentration prior to reverse transcription. The complementary DNA was obtained with the use of High Capacity cDNA Revers Transcription kit (Applied Biosystems) that has a 99.9% conversion rate using the protocol included in the kit. CD34 mRNA was quantified using the Real Time Polymerized Chain Reaction system ViiA 7 from Applied Biosystems and TaqMan probes. A total number of 40 RT-PCR cycles were used for amplification, each cycle having three steps: denaturation for 10 seconds at 95°C, annealing for 15 seconds at 60°C and extension for 30 seconds at 72°C. Each sample was amplified in triplicate for a more accurate evaluation of CD34 gene expression rate. The results were processed in Excel (Microsoft Office, Microsoft Corporation) using the Double Delta CT Analysis method. Statistical analysis of the data was done with GraphPad Prism version 6.

Results

The study group was composed of 48 males and 24 females, age ranging from 45 to 85 years old. At 44 of them the tumor was located in the colon, and for the rest in the rectum (Fig.1 contains examples for CD34 immunolabelled blood vessels in colon-A; rectum-B).
The mean MVD for the patients with confirmed metastasis was 314.1 mm$^2$ (std. dev. -67.74 mm$^2$) in comparison with the subgroup with no confirmed metastasis, where the mean MVD was 296.6 mm$^2$ (std. dev. -56.14 mm$^2$) (Fig.3). The difference between the two subgroups was not statistically meaningful, the $p$ value of Student t test being 0.6412.

Gene expression for the study group was evaluated on both normal and tumoral epithelium using CD34 and GAPDH probes. The level of CD34 gene expression ($\Delta CT$ value) for normal and tumoral epithelium was obtained by subtracting the CT value of the housekeeping gene (GAPDH) from CD34 gene’s CT value. The $\Delta \Delta CT$ value was obtained by subtracting the $\Delta CT$ value for normal epithelium ($\Delta CT_{\text{Normal}}$) from the Delta CT value of the tumoral epithelium ($\Delta CT_{\text{Tumor}}$). In order for the results to be interpreted, the next formula was applied: 

$$2^{-\Delta \Delta CT} = 2^{-(\Delta CT\_{\text{Tumor}} - \Delta CT\_{\text{Normal}})}$$

The mean CD34 gene expression value for colon cancer was 2.303 (std. dev. -2.577), and for the rectum cancer was 2.097 (std. dev. -1.885). The difference between the two tumor localizations was not statistically meaningful for CD34 gene expression when Student t test was applied ($p=0.54$).

The mean CD34 gene expression value for the patients with confirmed metastasis was 3.218 (std. dev. -3.280) in comparison with the subgroup with no confirmed metastasis where the men CD34 gene expression value was 2.1851 (std. dev. -1.674). The difference between the two subgroups was not statistically meaningful, the $p$ value of Student t being 0.1791.

Next, we correlated the CD34 MVD with the CD34 gene expression for the entire study group. The $p$ value from the Pearson correlation test ($r=0.5189$) was used to evaluate the degree of correlation between the two strings of values. $P$ value ($<0.0001$) showed a direct correlation between them (Fig.4).

**Discussion**

CRC still represents a major health problem worldwide, even though survival in a metastatic stage has improved from nine to more than 30 months over the last years. Angiogenesis process is considered to have a key role in the development or relapse of CRC, with still unclear mechanisms and has become a benchmark for anticancer drug development,
targeting the signaling pathways [9]. The new blood vessels formation may be expressed using markers expressed by endothelial cells such as CD 31, CD 34, CD 105 etc [10,11]. Microvascular density may be assessed based on immunohistochemical techniques and also may be used as prognostic indicator for increase metastatic risk.

Our study tried to highlight the CD34 gene’s expression probability as a prognostic factor in CRC. A link between CD34 MVD and CD34 mRNA in harvested tumor tissue was taking into consideration. We tried to sum the relationship of these two markers and by comparing the location of tumors.

The results obtained revealed that CD34 MVD and gene expression had a better vascularization in rectum cancers compared to colon ones.

The same higher, although statistically insignificant, mean values for CD34 MVD and gene expression were found at patients with confirmed metastasis compared with unconfirmed ones, findings that affirm other researches [7,8] in which rich vasculature was directly linked with a higher risk for metastasis.

We found that CD34 MVD and the CD34 gene expression are directly related, for the entire study group, which translates in a scenario where a higher number of vessels corresponds to a higher quantity of mRNA in the same tumor.

Conclusion
CRC angiogenesis drugs still require a larger array of therapeutic agents and this translates in a continuous research. Our results obtained in this study confirm the original aim that CD34 gene’s expression probability is as a prognostic factor for CRC.

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