Original Paper

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/mm^2, 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 tumoral 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

10.12865/CHSJ.44.01.10

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% H_2O_2; 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 25^0C; incubating with primary antibody for 30 minutes at 25^0C; washing in PBS; incubating with EnVision (Dako) 30 minutes at 25^0C; antibody detecting by diaminobenzidine (DAB), 9 minutes at 25^0C; 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 revers 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 95^0C, annealing for 15 seconds at 60^0C and extension for 30 seconds at 72^0C. 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).

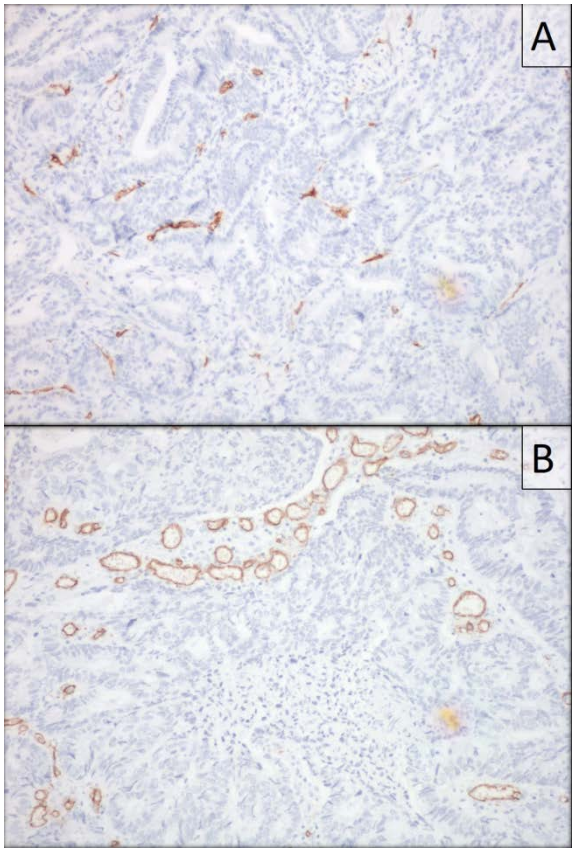

Fig.1. CD34 immunolabelled blood vessels in colon-A and in rectum-B

The study group was divided in two subgroups based on the presence or absence of metastases. From all patients, in 20 cases metastasis were confirmed in the liver and/or in the peritoneal cavity.

The mean CD34 immunolabelled MVD (measured in blood vessels per square millimeter) for the study group was of 307, with a standard deviation of 79.49. For the patients with CRC the mean MVD was 304.6/mm^2 (std. dev.-65.5mm^2), and for the patients with rectum cancer it was 315.2mm^2 (std. dev.-88.45mm^2) (Fig.2). The difference between the two tumor localizations was not statistically meaningful, based on a *Student t test* was applied (p=0.9706).

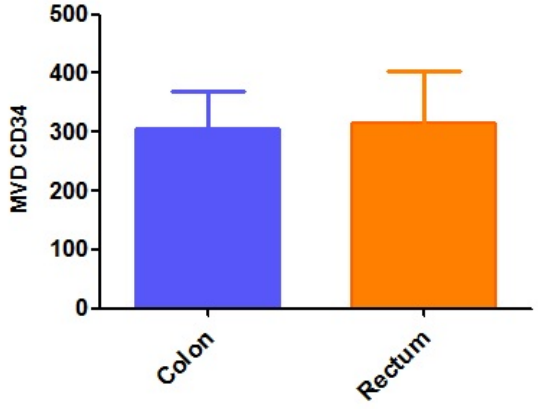

Fig.2. Mean MVD for the patients with colon cancer and rectum cancer

The mean MVD for the patients with confirmed metastasis was 314.1mm^2 (std. dev.-67.74mm^2) in comparison with the subgroup with no confirmed metastasis, were the men MVD was 296.6mm^2 (std. dev.-56.14mm^2) (Fig.3). The difference between the two subgroups was not statistically meaningful, the p value of *Student t test* being 0.6412.

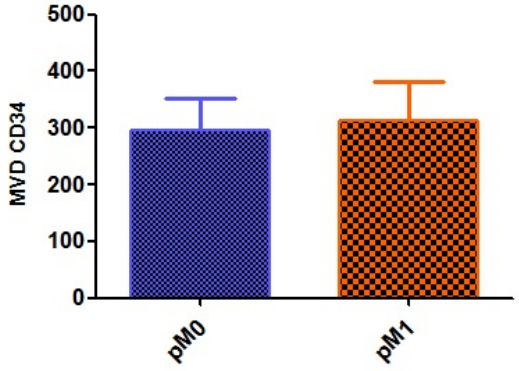

Fig.3. Mean MVD for the two subgroups (left-pM0=no metastasis confirmed; right-pM1=metastasis confirmed)

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 (ΔCT 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 ΔΔCT value was obtained by subtracting the ΔCT value for normal epithelium (ΔCT_{Normal}) from the Delta CT value of the tumoral epithelium (ΔCT_{Tumor}). In order for the results to be interpreted, the next formula was applied: $2^{-\Delta\Delta CT}=2^{-(\Delta CT_{Tumor} - \Delta CT_{Normal})}$

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 were the men CD34 gene expression value was 21.851 (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).

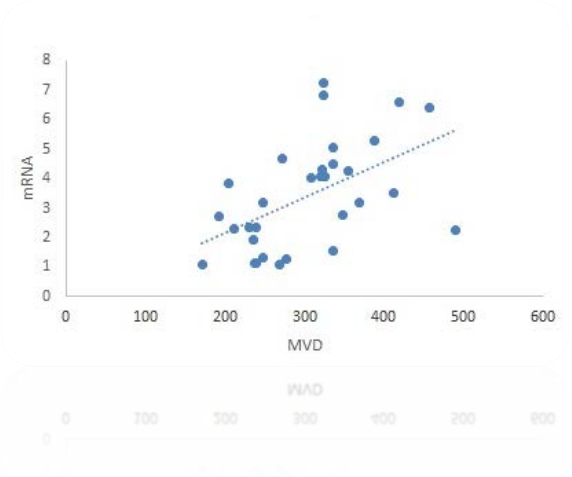

Fig.4. Correlation between CD34 MVD and CD34 gene expression

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,

10.12865/CHSJ.44.01.10

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.

References

1. Tabung FK, Brown LS, Fung TT. Dietary Patterns and Colorectal Cancer Risk: A Review of 17 Years of Evidence (2000-2016). Curr Colorectal Cancer Rep, 2017, 13(6):440-454.
2. Bénard F, Barkun AN, Martel M, von Renteln D. Systematic review of colorectal cancer screening guidelines for average-risk adults: Summarizing the current global recommendations. World J Gastroenterol, 2018, 24(1):124-138.
3. Battaglin F, Puccini A, Intini R, Schirripa M, Ferro A, Bergamo F, Lonardi S, Zagonel V, Lenz HJ, Loupakis F. The role of tumor angiogenesis as a therapeutic target in colorectal cancer. Expert Rev Anticancer Ther, 2018,18(3):251-266.
4. Alwers E, Jia M, Kloor M, Bläker H, Brenner H, Hoffmeister M. Associations Between Molecular Classifications of Colorectal Cancer and Patient Survival: a Systematic Review. Clin Gastroenterol Hepatol, 2018, pii: S1542-3565(17)31536-7.
5. Ma YL, Peng JY, Zhang P, Liu WJ, Huang L, Qin HL. Immunohistochemical analysis revealed CD34 and Ki67 protein expression as significant prognostic factors in colorectal cancer. Med Oncol, 2010, 27(2):304-309.
6. Des Guetz G, Uzzan B, Nicolas P, Cucherat M, Morere J-F, Benamouzig R, Breau J-L, Perret G-Y. Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature, 2006, 94(12):1823-1832.
7. Tanigawa N, Amaya H, Matsumura M, Lu C, Kitaoka A, Matsuyama K, Muraoka R. Tumor angiogenesis and mode of metastasis in patients with colorectal cancer. Cancer Res, 1997, 57(6):1043-1046.
8. Tomisaki S, Ohno S, Ichiyoshi Y, Kuwano H, Maehara Y, Sugimachi K. Microvessel quantification and its possible relation with liver metastasis in colorectal cancer. Cancer, 1996, 77(8 Suppl):1722-1728.
9. Mousa L, Salem ME, Mikhail S. Biomarkers of Angiogenesis in Colorectal Cancer. Biomark Cancer, 2015, 7(Suppl 1):13–19.
10. Rmali KA, Puntis MC, Jiang WG. Tumour-associated angiogenesis in human colorectal cancer. Colorectal Dis, 2007, 9(1):3-14.
11. Deliu IC, Neagoe CD, Beznă M, Genunche-Dumitrescu AV, Toma SC, Ungureanu BS, Uscatu CD, Beznă MC, Lungulescu CV, Pădureanu V, Gheonea DI, Ciurea T, Forțofoiu M. Correlations between endothelial cell markers CD31, CD34 and CD105 in colorectal carcinoma. Rom J Morphol Embryol, 2016, 57(3):1025-1030.

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