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ORIGINAL ARTICLE

Is there a correlation between peripheral blood expression of angiogenic transcriptional factors/receptors and colorectal cancer?

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Summary

Purpose: *The aim of this study was to evaluate whether there is a correlation between peripheral blood expression of angiogenic transcriptional factors/receptors and colorectal cancer (CRC).*

Methods: *Eighty six blood samples collected from patients with CRC (N=42), adenomas and/or hyperplastic polyps (AP, N=30) and individuals without colon pathology (control group/CTR, N=14) were used for this study. Twelve transcription factors and receptors were assessed by qRT-PCR in a case-control study. The molecules with a minimum of 30% differences in gene expression for CRC and AP compared to CTR were then analyzed separately for each sample. Gene expression was evaluated relatively to the CTR after normalization to the large ribosomal protein PO (RPLPO) housekeeping gene, and the differential expression between studied groups was assessed by ANOVA.*

Results: *Seven out of 12 genes presented differences in expression between 10-29% in CRC and/or AP compared to CTR. Considering the selection criteria, we further individually evaluated the levels of expression of 5 genes that had a minimum of 30% expression in the case-control study. Our data showed a significant up-regulation of platelet derived growth factor (PDGF) C in the blood of the patients with CRC compared to CTR (p=0.007). Likewise, clusterin (CLU) was significantly up-regulated both in CRC and AP groups compared to healthy subjects (p=0.01). For VEGFR1, PDGFRA and TGFB1 we didn't find significantly differential expression between any of the studied groups, even if increased levels were observed in both CRC and AP vs CTR.*

Conclusions: *The results of our study indicated that increased blood level of PDGFC mRNA was associated with the presence of CRC (p=0.007). Additionally, high levels of circulating CLU mRNA were observed in both malignant and benign colorectal pathologies.*

Key words: *angiogenesis, blood, colorectal cancer, diagnosis, PDGCF*

Introduction

With more than 1.4 million new cases registered every year, CRC still represents one of leading causes of cancer incidence worldwide [1]. Moreover, morbidity and mortality from CRC is

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regarded as a considerable health issue concerning a malignant disease that is hypothetically preventable via screening and early diagnosis. Screening for CRC permits diagnosis of the disease in an early stage and eventually decreases the mortality of this malignancy [2]. The faecal occult blood test (FOBT) represents a valuable method for screening due to its low price and easiness to be performed, but has low sensitivity and involves dietary limitation that hampers compliance and use. CRC is the only malignancy for which colonoscopy is indicated as a screening test [3]. Even though colonoscopy is a confident screening tool, its invasive character with abdominal pain and high costs has impeded its worldwide application [4]. Early detection would be greatly enhanced if accurate and cost-effective diagnostic biomarkers will be accessible.

Through their progression, tumors disrupt the physiological status of the host as well as the profile of the blood circulating molecules. Therefore, non-invasive blood markers could represent a challenge to improve the stratification of the patients who truly need a colonoscopy. The reason for using blood circulating "sensors" to identify the presence of cancer, is related to the fact that tumor-blood communication involves a large spectrum of signaling molecules that can contribute to cancer progression.

The quest for perfecting non-invasive screening methodologies for CRC screening is subjected to ongoing intensive research. Increasing evidence reported that patients with chronic inflammation have a high risk of developing CRC [5,6]. Different inflammatory mediators generated during chronic inflammation are mobilized through various molecular signaling pathways and determine the formation of a tumor microenvironment [7-9]. On the other hand, angiogenesis, one of the most studied mechanisms in cancer, is a crucial process for tumor formation and growth [10]. Identification of blood mediators of angiogenesis during cancer development is important for a better comprehension of tumor development. Tumor angiogenesis implies the interconnection between tumor and stromal cells, mediated by both growth factors and their membranous receptors [11].

Moreover, tumors release some autocrine and paracrine factors that activate or facilitate this process [12]. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) pathways have been reported to play an important role in tumor angiogenesis [13]. VEGF ligands (VEGFA, VEGFB, VEGFC, and VEGFD), VEGF receptors (VEGFR1, VEGFR2 and VEGFR3) and PDGF family have different roles in supporting vascularization and angiogenesis in malignant tissue [14-16]. In this line, Petersen et al. [17] reported that PDGF and VEGF blood proteins can become independent predictors of CRC.

A general feature of tumor development is represented by the mutual interaction of tumor cells with normal cells, inflammatory cytokines and constituents of the extracellular matrix. Cysteine-rich 61 (CYR61), a matricellular protein, has an important role in the processes of angiogenesis and neovascularization [18]. Previous data highlighted that abnormal CYR61 expression is linked to the development of different cancers and diseases related to chronic inflammation [19-21]. Likewise, the circulating inflammatory cytokine growth differentiation factor 15 (GDF15), a member of the transforming growth factor-beta1 (TGFB1) superfamily, has been associated to the development of cancers, such as those of the prostate, thyroid, pancreas and colon [22,23]. CLU, a molecule with a dual face, is also associated with cancer promotion and metastasis [24] and represents a valuable target that could be tested for noninvasive diagnosis of CRC.

Therefore, the development of novel biomarkers of CRC diagnosis may be lightened by characterization of blood angiogenic modulators, and this can be achieved by conducting studies on various proangiogenic molecules in patients with CRC.

The aim of this study was to evaluate whether there is a correlation between mRNA expression of peripheral blood expression of 12 genes including tumor necrosis factor alpha (TNF-α), TGFB1, PDGFB, PDGFC, PDGFRA, PDGFRB, VEGFA, VEGFR1 or Fms-like tyrosine kinase 1 (FLT1), VEGFR2, CYR61, GDF15 and CLU, and colorectal cancer.

Methods

Patients

Eighty-six patients of which 42 CRC (stage I-II), 30 with benign lesions (AP) (adenomas and/or hyperplastic polyps) and 14 without colonic pathological lesions (CTR) were included between April 2014 and February 2015 in this prospective study. The study inclusion criteria were: age >30 years, no medical history of inflammatory bowel disease, colon cancer or familial adenomatous polyposis. The patients underwent colonoscopy, and the histopathology exam of their biopsy was used to define the groups of this study (Table 1). The study was approved by the Board of Ethics of University of Medicine and Pharmacy "Iuliu Hatieganu"

Table 1. General characteristics of the study population

Characteristics	*Colorectal cancer (N=42)*	*Benign lesions (N=30)*	*Control (N=14)*
Age (years)			
Median	66.5	59	64
Sex, N (%)			
Male	26 (59.1)	18 (60)	5 (35.7)
Female	18 (40.9)	12 (40)	9 (64.3)
Histopathological type			
Adenocarcinoma	44	-	-
Adenoma polyp	-	27	-
Hyperplastic polyp	-	2	-
Stage grouping			
Stage I (T_{1-2}, $N_{0,}$ M_0)	24	-	-
Stage II (T_{3-4}, N_0, M_0)	18	-	-

Cluj-Napoca (No 160/05.05.2014). All patients signed informed consent before entering into the study.

Sample collection and processing

Four ml of blood specimen were collected in an EDTA vacutainer from each patient before any treatment or colonoscopy. After collection, all samples were processed according to standard protocols. Total RNA from nucleated blood cells was extracted with phenol-chloroform method and further purified with RNeasy mini silica-gel columns (Qiagen, Hilden, Germany), after plasma and red blood cell (RBC) removal. RNA integrity was assessed by RNA Integrity Number (RIN) values obtained with Lab-on-a-chip Technology (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA, USA), while RNA quantification was performed with Nanodrop-ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNAs with RIN ≥7 were considered for further analysis and were stored to -80°C until qRT-PCR analysis.

Assessment of the study

The study was designed on two steps: a prospective study including case-control cohort analysis, followed by individual assessment of genes of interest with a minimum of 30% differences in gene expression for CRC and AP compared to CTR.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to evaluate the expression of genes of interest. One µg of input RNA was reversed-transcribed both for case-control study as well as for individual assessment using First Strand cDNA Synthesis Kit (Roche Applied Science Penzberg, Germany). For the case-control study we generated three pools of RNA aliquots, one for each group (CRC, AP and CTR) derived from 1 µg of RNA from each sample included in every group. The qRT-PCR reactions were set up in a final volume of 10 µl, using 2.5 µl of cDNAs (diluted 1:20 with nuclease-free water), 0.5 µM of specific primers and 0.2 µM of Universal Probe Library (UPL) and Light Cycler Taqman Master Kit (Roche Applied Science, Penzberg, Germany). The structure of primers and UPL probes were designed with Roche Applied Science software as follows: TNF-α (NM_000594.2): F-cagcctcttctcct-tcctgat, R-gccagagggctgattagaga, UPL (#29); TGFB1 (NM_000660.4) F-cacgtggagctgtaccagaa, R-cagc-cggttgctgaggta UPL (#72); PDGFB (NM_002608.2), F-tgatctccaacgcctgct, R-tcatgttcaggtccaactcg, UPL (#55); PDGFC (NM_016205.2), F-tccagcaacaaggaa-cagaa, R-ttgggctgtgaatacttccat, UPL (#73); PDGFRA (NM_006206.4), F-tgcctgacattgaccctgt, R-tcagaggtctg-cgagctg, UPL (#63); PDGFRB (NM_002609.3), F-gact-gttgggcgaaggttac, R-gggtggtcactcctcagaaa, UPL (#62); VEGFA (NM_001025366.2), F-ccacttcgtgatgattctgc, R-tacctccaccatgccaagt, UPL (#29); VEGFR1 (FLT1) (NM_0020109.4) F-ccactcccttgaacacgag, R-gtcgccttacg-gaagctct, UPL (#85); VEGFR2 (NM_002253.2) F-gaa-catttgggaaatctcttgc, R-cggaagaacaatgtagtctttgc UPL (#18); CYR61 (NM_001554.4): F-aagaaacccggatttgtgag, R-gctgcatttcttgcccttt UPL (#66); GDF15 (NM_004864.2) F-ccggatactcacgccaga, R-agagatacgcaggtgcaggt, UPL (#28); CLU (NM_001831.1) F-gggaccagacggtctcag, R-cg-tacttacttccctgattggac, UPL (#1); RPLP0 (NM-001002.3): F-gatgcccagggaagacag, R-tctgctcccacaatgaaacat, UPL (#85). The cDNA amplification was perfomed in a 96-well plate using the LightCycler 480 instrument (Roche Applied Science, Penzberg, Germany) under the following conditions: 10 min at 95°C followed by 40

cycles of 15 sec at 95°C, 20 sec at 55°C and 1 sec at 72°C, followed by a final cooling step at 40°C for 30 sec. The relative gene expression levels were quantified by ΔΔCt method after normalisation to RPLPO housekeeping gene.

Statistics

Statistical analysis was done in GraphPad Prism v. 5.00. The differences in expression between CRC, AP and CTR groups were assessed by one-way ANOVA of the log-transformed data, followed by Tukey's *post-hoc* test. P values less than 0.05 were considered statistically significant.

Results

We established a list of 12 molecules including TNF-α, TGFB1, PDGFB, PDGFC, PDGFRA, PDGFRB, VEGFA, VEGFR1 (FLT1), VEGFR2, CYR61, GDF15 and CLU with role in the angiogenesis modulation, to be evaluated in the blood of patients with colorectal pathology.

We sought to evaluate the expression of these transcriptional factors and receptors in blood because tumor-blood cooperation plays an important role in cancer development. Due to the high number of molecules as well as the samples included in the study, we have chosen first to evaluate the candidate genes of patients with CRC, AP and CTR groups in a case-control cohort study. The results of this study indicated that FLT1, PDGFC1, CLU, PDGFRA and TGFB1 presented a minimum of 30% differences of expression in the RNAs blood pool of CRC and/or RNAs blood pool of AP compared with the RNAs blood pool of CTR group (Table 2).

Because we evaluated the expression of genes of interest in blood, we considered that a minimum threshold of 30% can be acceptable to register the differences between gene expressions.

Considering the selection criteria, we further individually evaluated the levels of expression of FLT1, PDGFC, CLU, PDGFRA, and TGFB1 genes in the CRC, AP and CTR patients. Fold regulation (FR) for each group was calculated relative to the CTR. The genes with FR >±1.3 and p<0.05 were considered significantly differentially expressed between groups. The data are presented as mean ± standard error of mean (SEM).

Our data showed a significantly up-regulation of CLU mRNA in the blood of the patients with CRC (FR=2.83) and AP (FR=2.47) compared to healthy subjects (p=0.01) (Figure 1).

We also found a significantly increased expression of PDGFC in the blood of CRC patients compared to healthy subjects (FR=1.94, p=0.007) but no significant expression changes were observed in the blood of AP patients when compared to CTR (Figure 2).

For the other investigated genes, we didn't find significantly differential expression between any of the studied groups even if increased levels of TGFB1, FLT1, and PDGFRA genes were observed in CRC and AP vs CTR (Table 3).

Discussion

A relative new approach to detect a pathological signal created by cancer is by monitoring the changes of gene expression that occur in the whole blood. In this study, we evaluated the whole blood mRNA expression of 12 genes involved in modulation of angiogenesis. In CRC, angiogenesis starts in an early stage of tumor development, compared with other types of cancer [25]. Specif-

Table 2. The qRT-PCR values obtained for every proposed gene in the case-control cohort study. The bolded genes, with a minimum of 30% expression for CRC and/or AP compared to control group, were selected for individual assessment

Gene	*Stage*	*FR*	*Gene*	*Stage*	*FR*	*Gene*	*Stage*	*FR*
VEGFA	CRC	-1.19	TNF-α	CRC	-1.04	PDGFB	CRC	-1.03
	AP	-1.26		AP	1.05		AP	1.13
VEGFR1	CRC	**1.79**	**TGFB1**	CRC	**1.3**	**PDGFC**	CRC	**1.56**
	AP	**1.78**		AP	1.04		AP	**1.39**
VEGFR2	CRC	-1.07	GDF15	CRC	1.16	**PDGFRA**	CRC	**1.31**
	AP	-1.26		AP	1		AP	1.29
CYR61	CRC	NE	**CLU**	CRC	**1.46**	PDGFRB	CRC	-1.24
	AP	NE		AP	**1.48**		AP	-1.23

CRC: colorectal cancer, AP: adenomas and/or hyperplastic polyps, FR: fold regulation, NE: not expressed

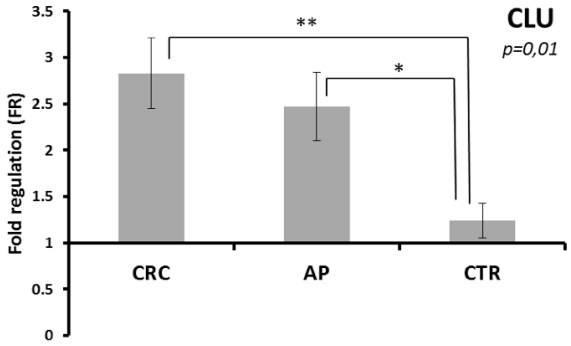

Figure 1. Fold regulation of CLU expression in CRC, and AP samples relative to CTR. FR was calculated by ΔΔCt method after normalization to RPLPO housekeeping gene. The bars represent the mean FR values of each group (± SEM). The differences between groups means were tested with ANOVA (p=0.01). The p values returned by Tukey's *post-hoc* test are marked with * (p<0.05) and ** (p<0.01).

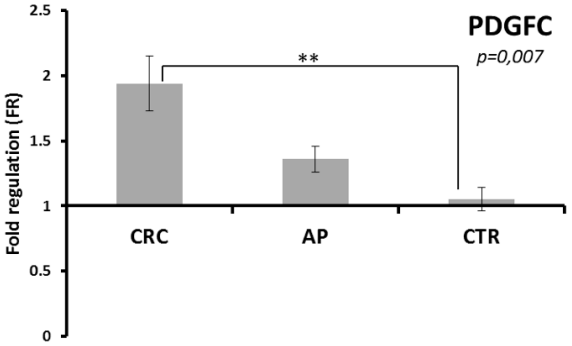

Figure 2. Fold regulation of PDGFC expression in CRC, AP samples relative to CTR. FR was calculated by ΔΔCt method after normalization to RPLPO housekeeping gene. The bars represent the mean FR values of each group (± SEM). The differences between groups means were tested with ANOVA (p=0.007). The p values returned by Tukey's *post-hoc* test are marked with ** (p<0.01).

ic pathways of angiogenic transcriptional family factors including VEGF and PDGF have been attracted important attention due to their possible clinical use [26,27].

We considered performing qRT-PCR with specific fluorescence probe (UPL) for every gene of interest, due to its detection sensitivity and because the levels of mRNA transcripts are quite low in the blood. Our preliminary case-control data revealed that 2 receptors, including VEGFR1 (FLT1) and PDGFRA as well as 3 transcriptional factors including PDGFC, CLU and TGFB1, had more than 30% expression in the blood of CRC and/or AP compared with the control group.

VEGFR1 (FLT1), one of the receptors of VEGF, has a significant contribution of angiogenesis modulation [28]. Moreover, VEGFR1 (FLT1) signaling plays a considerable role in the inflammatory process by recruiting monocytes and macrophages [29]. In a recent study, Wei et al. [30] demonstrated that high expression of FLT1 leads to increasing CRC cells invasive capability and is correlated with poor prognosis. Tumor cells have the capacity to induce autocrine and paracrine VEGFA-VEGFR1/2 signaling [31]. Thereby, in a paracrine signaling VEGF, bFGF and IGF-1 can induce the development of the endothelial-like cell (ELCs) from immature dendritic cells derived from monocytes [32]. ELCs are characterized by increased expression of VEGF receptors, especially VEGFR2 and VEGRF3. In our study, we didn't find significantly differential expression between the study groups concerning VEGFA, VEGFR1 (FLT1), and VEGFR2, even if increased levels of FLT1 were observed in CRC (FR=2.05) and AP (FR=2.22) vs the control group (Table 3). Previous studies have shown that inflammatory cytokines such as TNF-α and TGFB1 have an important effect on the inflammatory neovascularization, VEGFR1 being also involved in this process [33]. In our study, we didn't find any significant differences between TNF-α and TGFB1 mRNAs in the blood of the studied groups.

The knowledge of the implication of various angiogenic factors is essential for the understanding of the adenoma-carcinoma sequence in intestinal epithelia. In this context, there are some data supporting the transcriptional factors and receptors in peripheral blood as being potential diagnostic biomarkers for CRC and other cancers. Several studies demonstrated the involvement of the PDGFs and their receptors in carcinogenesis [8,34]. PDGF receptors play an important role in signaling in pericytes, which in turn contribute to blood vessel development that sustain the tumor angiogenesis processes [35]. The PDGFRA has been reported to stimulate the growth of gastrointestinal stromal tumors (GISTs) [36]. Even if we found a minimum of 30% expression of PDGFRA in the case-control study, the individual evalua-

Table 3. Mean FR values of genes of interest in CRC, AP and CTR groups with associated p values obtained by ANOVA. FR values were calculated relative to control group. FR values with asterisk represent statistically significant differences in gene expression obtained after Turkey's *post hoc* test (*p<0.05, **p<0.01)

Genes	*CRC*		*AP*		*CTR*		*p value*
	FR	*SEM*	*FR*	*SEM*	*FR*	*SEM*	
FLT1	2.05	0.33	2.22	0.33	1.38	0.32	0.18
PDGFC	1.94**	0.21	1.36	0.1	1.05	0.09	0.007
CLU	2.83**	0.38	1.36*	0.1	1.05	0.09	0.01
PDGFRA	1.99	0.28	1.47	0.15	1.05	0.21	0.11
TGFB1	1.46	0.13	1.25	0.12	1.04	0.08	0.13

For abbreviations see text

tion didn't reveal significant differences, although an FR=1.99 and FR=1.47 were observed in the blood of CRC and AP when compared to control group. Another molecule of interest identified in our study, PDGFC, represents an important angiogenic factor with a VEGF-like structure [37,38]. In a similar manner as PDGF-AB, PDGFC modulates through activation of PDGFRA and PDGFRB receptors the tyrosine kinases ras/MAPK pathway. The activation of MAPK pathway leads to cellular proliferation and survival activation by PI3K/Akt pathway [39]. In addition, by both autocrine and paracrine ways, PDGFC can promote tumor progression through modulation of the tumor microenvironment by recruiting cancer-associated fibroblasts (CAFs) [40]. Being a key factor of the PDGFRA signaling pathway, PDGFC is implicated in inflammation, proliferation, and remodeling of angiogenic vessels [41]. Recently, Yamauchi et al. [42] reported that both mRNA and protein levels of PDGFC are highly expressed in CRC cells but not in normal epithelial cells. Likewise, higher levels of PDGFC were significantly related to metastatic stages compared with non-metastatic stages, suggesting that PDGFC could contribute to cancer metastasis. Moreover, PDGFC expression in CRC is related to prognosis prediction, and high protein level of PDGFC suggests CRC recurrence after curative surgery. However, the contribution of PDGFC in early diagnosis of CRC is less well studied, and the characterization of the level of peripheral blood PDGFC involved in colorectal carcinogenesis is still an uncharted territory. In the present study, one important finding concerns the significant overexpression of mRNA PDGFC in the blood of CRC patients compared to healthy subjects (FR=1.94, p=0.007) (Figure 2), suggesting its use as possible noninvasive marker for CRC diagnosis. To our knowledge, this is the first study that suggests the importance of assessing PDGFC mRNA in the blood of patients with CRC as a new possible tool for early diagnosis.

CLU represents another important modulator of angiogenesis with a role in cancer promotion and progression. Two different isoforms of CLU protein are described, a glycosylated (the secreted form, sCLU) and an apparently non-glycosylated form (the nuclear clusterin, nCLU) [43]. The sCLU isoform has been described as a sensitive and stable biomarker for intestinal tumors [44]. Mazzarelli et al. [24] reported a significant increase of the CLU in serum and stool of patients with CRC, suggesting a possible role of sCLU as a diagnostic biomarker for CRC screening. Our results are consistent with those of Mazzarelli et al. and indicate the significant up-regulation of sCLU in the blood of patients with CRC compared to CTR (FR=2.83). We have also found overexpression of sCLU in AP (FR=2.47) (Figure 1). This finding suggests that sCLU is involved in the adenoma-carcinoma transformation, and this is the reason we believe that CLU could be considered as a potential blood biomarker that will help in the screening of patients who need to undergo a colonoscopy.

Conclusions

The results of our study indicated that increased blood level of PDGFC mRNA (FR=1.94) was associated with the presence of CRC (p=0.007). Additionally, high levels of circulating CLU mRNA were observed in both malignant and benign colorectal pathologies. These results could be pursued prospectively on a larger cohort of patients to bring additional information for early detection of CRC.

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