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Novel insights into transcriptional dysregulation in colorectal cancer

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Colorectal cancer (CRC) is a leading cause of cancer-related mortality worldwide. Although CRC has been comprehensively characterized at the molecular level, the tumor heterogeneity hinders the identification of reliable diagnostic, prognostic and predictive biomarkers. Molecular stratification of CRC is based on prevalent gene mutations and transcription profiles but its significance for clinical practice remains obscure. Indeed, activating mutations in the genes *KRAS*, *NRAS*, and *BRAF* are the only predictive biomarkers for anti-EGFR antibody therapy routinely tested in the clinic for advanced stages of CRC. Gene expression signatures are important for clarifying the molecular mechanisms of CRC development and progression, but only two such tests for predicting recurrence risk are commercially available. The aim of our study was to propose a diagnostic approach based on mutation and gene expression analysis that can be routinely applied in the clinic for defining the most appropriate treatment strategy for each patient. We used qPCR to determine the presence of *KRAS* mutations and measure the transcription levels of a panel of 26 genes in 24 CRC patients. Statistical analyses were applied to check for associations between clinicopathological and molecular parameters. Our results reveal novel data concerning CRC carcinogenesis: almost universal downregulation of *EGFR*; differential role of the pro-inflammatory cytokines TNF-α and IL-6; overexpression of the vitamin B12 transporter transcobalamin 1; tumor-suppressor function of *SETD2*, *CA7*, and *GUCA2B*. The practical application of these findings has yet to be clarified.

Key words: CRC, gene expression, GUCA2B, KRAS

Colorectal cancer (CRC) is the third most frequent cause of cancer-related death worldwide [1, 2]. The improved surgical techniques and the introduction of more effective therapies has led to an incredible decline in CRC incidence and mortality among the population over 50 years of age in the last four decades [3]. Despite this positive trend, an alarming increase in incidence has been reported in younger people [4], necessitating the need for better prevention and early detection strategies. Important steps in this direction are the molecular characterization of CRC and the identification of suitable diagnostic, predictive and prognostic biomarkers. The first model of CRC tumorigenesis, describing it as a progressive accumulation of genetic and epigenetic events [5], was supplemented by more comprehensive description of the molecular events by the Cancer Genome Atlas Network in 2012 [6]. Thirty-two genes were found to be recurrently mutated with eight being most prevalent – *APC, TP53, KRAS, PIK3CA, FBXW7, SMAD4, TCF7L2* and *NRAS*. In addition, copy-number alterations leading to amplification of *ERBB2* and *IGF2* were found to be common events. Tumors were divided into two groups, hypermutated and non-hypermutated with different genetic mutations predominating in the different groups. Thereafter, numerous attempts have been made towards molecular stratification of CRC based on genetic variations and transcriptional profiling [7–13] with the goal of improving personalized treatment approaches [14]. This resulted in the development of a classification system including four consensus molecular subtype (CMS) groups [15], which best represent the heterogeneity of CRC at the gene expression level [16]. While CMS1 is characterized by microsatellite instability, hypermutation and mutations in *MSH6, BRAF* and *PTEN*, the other three subtypes are chromosomally unstable and enriched in mutations in *APC, KRAS, TP53, SMAD4* and *PIK3CA*. CMS2 is marked by upregulation of WNT and MYC downstream targets, overexpression of

EGFR, ERBB2, IGF2 etc., whereas activation of TGF-β and proteins involved in extracellular matrix remodeling is a key feature of CMS4. *KRAS* mutations are dominant in CMS3 together with metabolic deregulation [16]. The CMS system was challenged in a recent publication by Isella et al. (2017) due to the large contribution of the tumor stromal content towards the transcriptional profiles utilized for the creation of the CMS subgroups. The authors of the study propose CRIS (CRC intrinsic subtypes)-based stratification and provide strong evidence that it can be exploited together with clinical and pathological parameters for better prognostic assessment of CRC [17]. In spite of the accumulating data regarding the molecular mechanisms of CRC and the efforts made towards molecular stratification of this cancer, little of this knowledge has found application in the clinics. Various biomarkers have been suggested for diagnosis, prediction and prognosis (reviewed in [18]), as well as for early CRC detection (reviewed in [19]) but only few of them are actually used in routine practice. Microsatellite instability (MSI) is associated with better prognosis and considering its prevalence in hypermutated cancers, it can be implied that high mutation rate may indicate better prognosis [6]. As far as molecular target therapy is concerned, only three negative biomarkers exist for advanced colorectal cancer drug treatment. Mutations in *KRAS, NRAS, BRAF* predict resistance to anti-EGFR antibodies [20]. Indeed, patients wild type for *KRAS, NRAS, BRAF* and *PIK3CA*, who represent 30% of all cases, are most likely to benefit from this therapy [21] but acquired resistance due to *EGFR* mutations is a major problem [22–26]. No predictive biomarkers for anti-angiogenic treatment have been identified [16]. Gene expression profiling is indispensable for molecular subtyping of CRC but no established genomic signature has been found useful in clinical practice [27]. Gene expression signatures have been proposed for early detection based on blood samples [28, 29]; for prognosis [30]; for estimating the risk of cancer occurrence [31]; for predicting response to chemotherapy in metastatic CRC [32] etc. Two such signatures, the Oncotype SX Colon Cancer Assay and the ColoPrint were validated as predictive markers for recurrence in stage II and III CRC but are not used everywhere. Isella et al. (2017) proposed reduction of the original CRIS 565 gene classifier to a set of 40 gene pairs that can be used for clinical classification of individual patients, which would offer the greatest opportunity until now for personalized treatment. The aim of our study was to propose a diagnostic approach based on *KRAS* mutation and gene expression analysis that can be routinely applied in clinical practice for better evaluation of the patients and for appointing the most appropriate therapy. To this end, we measured the mRNA levels of an original constellation of 26 genes related to tumor growth, invasion and metastasis in CRC and correlated their expression to clinicopathological parameters, as well as to the *KRAS* mutation status of the patients. We did not find any significant associations between the expression levels of the chosen genes and tumor grade/stage or the presence of *KRAS* mutations. Interestingly, the only gene that shows any association to *KRAS* status is *YKL40* [33]. Some of our results are novel and unexpected. First, *SETD2*, a histone methyltransferase, is ubiquitously downregulated in CRC, showing its significance as a tumor-suppressor gene also in this cancer type. Second, *EGFR* mRNA levels are decreased in a high proportion of cases, which can indicate reduced sensitivity to cetuximab even in *KRAS* wild type patients. Third, we show that *TCN1*, a vitamin B12 transporter, is highly overexpressed in CRC. Finally, two genes are universally lost in CRC – *CA7* (carbonic anhydrase VII) and *GUCA2B* (uroguanylin), suggesting them as important tumor suppressor genes in CRC carcinogenesis.

Table 1. List of custom made primers used for qPCR.

Gene	Forward primer [5' → 3']	Reverse primer [5' → 3']
YKL40 (CHI3L1)	GACCACAGGCCATCACAGTCC	TGTACCCCACAGCATAGTCAGTGTT
IL6	ACGCAACCCACGTGTAACTGTC	ACAGCAACAAGCCCGTAGGAAC
MAPK1	TCCCAAATGCTGACTCCAAAGC	TCCTCTTGTGTGGGTTGAATGTC
NFKB1	CCTCCACAAGGCAGCAAATAGACG	AGCTGAGTTTGCGGAAGGATGTC
NRP1	ACAGCAAACGCAAGGCGAAGTC	TGATGAATCGCGTGGAGAGAGC
PIK3CG	AAGTTTCAGGCAGCAGTGGAGAG	ACAAAGGTTGCCACACAGTAGCC
PTEN	TGTACTGGGCACATTCCTCCTC	TCAGAGTGTGGCAGAAGATAGTGG
GAPDH	AGGTCCACCACTGACACGTTG	AGCTGAACGGGAAGCTCACT

Patients and methods

Patients and preparation of tissue samples. The study was approved by the Ethics Committee of the Medical University of Plovdiv, protocol # R-1838/15-07-2013. Patients signed an informed consent in agreement with the requirements of the WMA Declaration of Helsinki. Twenty-four patients who underwent surgery in the years 2013 or 2014 at the two University hospitals "Kaspela" and "St. George" were selected. Tumor tissue and normal colonic mucosa distal to the tumor site were isolated by intraoperative resection and were stored in RNA later (Qiagen, Netherlands) at –80 °C. In addition, formalin-fixed tumor tissue was embedded in paraffin for histological evaluation and DNA isolation.

Molecular analyses. DNA was isolated with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and the *KRAS* mutation status was determined with the Amoy Dx *KRAS* Seven Mutations Detection Kit (Amoy Diagnostics, Haicang, Xiamen, China). RNA from normal and tumor tissue samples was isolated with the RNeasy Mini Kit (Qiagen) and converted to cDNA with the RT^2 First Strand Kit (Qiagen). Gene expression of *YKL40, IL6, MAPK1, NFKB1, NRP1, PIK3CG,* and *PTEN* was measured by qPCR using the Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific, USA) and the primers listed in Table 1 (Integrated DNA Technologies, Leuven, Belgium) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The transcription levels of the other 19

genes, *AKT1, AXIN2, CA7, CD44, CTNNB1, EGFR, FGFR2, GPC1, GPC3, GUCA2B, KRAS, MACC1, MMP9, NOTUM, SETD2, SIRT3, TCN1, TNF* and *VEGF,* were determined with customized RT^2 Profiler PCR Array (SABiosciences, Qiagen). In all cases, samples were run in triplicates according to the MIQE guidelines. PCR reactions were carried out in Rotor Gene Q (Qiagen). The analysis was performed with the $\Delta\Delta C_T$ method individually for each patient. Expression of the gene in the normal tissue served as the calibrator and *GAPDH* was used as a reference gene. *GAPDH* was found to be stably expressed in normal colonic mucosa and tumor tissue and thus proved suitable for normalization of the gene expression data (Suppl. Figure 1).

Statistics. The non-parametric Mann-Whitney U test was applied to compare the values of a continuous variable in two independent groups. To determine the presence of correlation between two continuous variables the non-parametric coefficient Kendall's tau-b was calculated. The Log-rank test (Mantel-Cox) was used to identify the statistical difference between the survival of patients belonging to two different groups. Boxplot diagrams were used for graphical visualization of the continuous variables as well as for distinguishing outliers in the data series. They were created in BoxPlotR (http://shiny.chemgrid.org/boxplotr/). All statistical analyses were performed with SPSS software, version 17.0.

Table 2. Clinical and demographic parameters of the patients.

Parameter		n	%
Sex	Female	10	41.7
	Male	14	58.3
Age	≤70 years	10	41.7
	>70 years	14	58.3
Mean age	Women: 72.5±9.1		
	Men: 74.9±6.5		
	Total: 73.9±7.6		
Localization of the tumor	Caecum	3	12.5
	Transverse colon	4	16.7
	Sigmoid colon	6	25.0
	Rectum	11	45.8
TNM stage			
T stage	T1	2	8.3
	T2	8	33.3
	T3	9	37.5
	T4	5	20.8
Lymph nodes (N) and metastases (M)	T(1–4)N0M0	15	62.5
	T(1–4)N(1–x)M0	6	25.0
	T(1–4)NM1	3	12.5
Cell differentiation	G1	1	4.2
	G2	21	87.5
	G3	2	8.3

Results

Demographic and clinical parameters of the patients. The clinical parameters of the patients included in the study are shown in Table 2. The majority of patients were male; the mean age at the time of diagnosis was 73.9 years with no significant difference between both genders. The majority of tumors were localized in the rectum (45.8%). In most patients, the tumors were staged as T2/T3 (70.8%) and graded as G2 (87.5%); lymph node engagement was observed in 25% of patients, whereas distant organ metastases were detected in 12.5%. Different postoperative therapies were assigned to 62.5% of the patients, including PCT (33.3%), adjuvant chemotherapy (16.7%) or chemotherapy combined with radiation or immunotherapy (12.5%). Within the timeframe of the study (2013–2017), 9 of the patients passed away and 15 are still under surveillance. The statistical analysis (Log-rank test) did not demonstrate any association between overall survival and T-stage (T1–4), age (≥/<70 years) and gender of the patients. The presence of local (lymph nodes) or distant metastases also did not influence the survival of the patients.

KRAS mutation status. Mutations in the genes *KRAS, NRAS* and *BRAF* are the only predictive biomarkers in advanced CRC, which determine resistance to targeted therapy with anti-EGFR antibodies. With qPCR as the method of choice in the current study, we detected mutations in the gene *KRAS* in 8 of the 24 patients, which is in agreement with the reported average prevalence of these mutations in 30–40% of CRC patients. We did not observe any difference in the overall survival between the two groups – *KRAS*-mutant and *KRAS*-wild type.

Gene expression. We selected an original panel of 26 genes whose products participate in different signaling pathways or processes in CRC carcinogenesis (Figure 1). We used qPCR to measure the transcription levels of all genes and applied relative quantification individually for each patient using normal tissue as the calibrator. Despite the great heterogeneity among the studied samples, we grouped the genes in three different categories based on their differential expression in tumor versus normal tissue: (i) overexpressed genes (fold change >2, Figure 2A); (ii) genes with unchanged expression (fold change between 1 and 2, Figure 2B) and (iii) underexpressed genes (fold change <1, Figure 2C). The overexpressed genes were: *NOTUM, TCN1, MACC1, YKL40, GPC3, AXIN2* and *IL6* (Figure 2A, a1 insert). The most highly expressed gene in CRC tissue is *NOTUM*, a negative regulator of the Wnt signaling pathway, which was recently shown to be the only known extracellular deacylase that removes palmitoleate from Wnt proteins and inactivates them [34]. We recently demonstrated enhanced expression of *NOTUM* and deregulated expression of glypicans (*GPC1* and *GPC3*) in CRC tissue [35] and confirmed some of these findings here. Genes that showed on average unchanged expression between the tumor and the normal tissue were: *GPC1, CTNNB1, VEGF, CD44* and *AKT1*

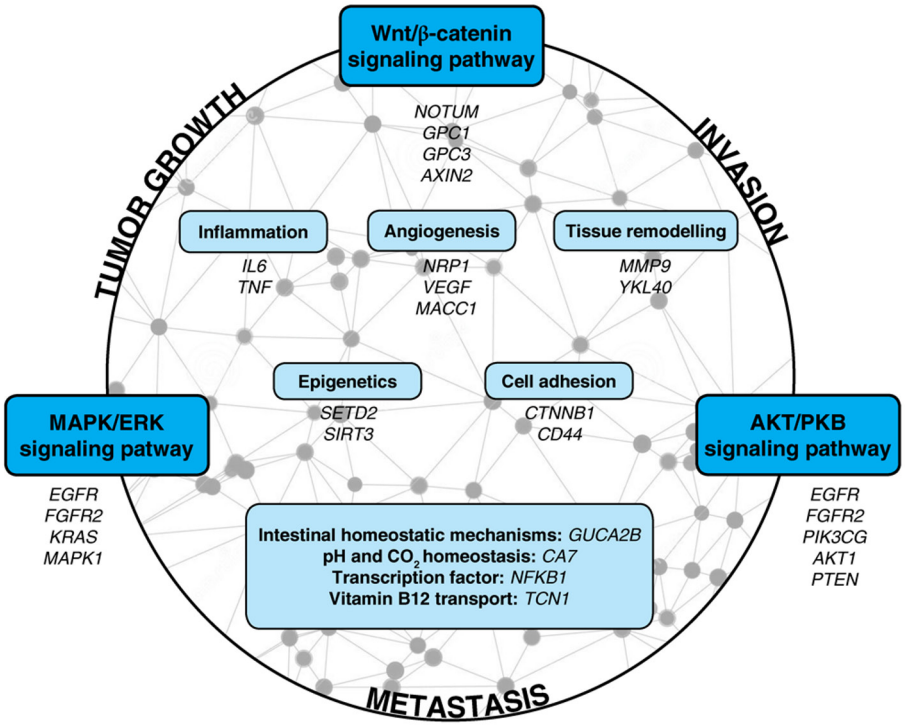

Figure 1. The panel of 26 genes whose transcription levels were measured in the study. The genes are roughly ordered according to the different signaling pathways they belong to or processes involved in CRC carcinogenesis. Receptor tyrosine kinases, e.g. EGFR and FGFR2 activate both MAPK/ERK and AKT/PKB signaling. The circle is filled in with a pattern pointing to the fact that the products of these genes are all part of a network regulating tumor growth, invasion and metastasis.

(Figure 2B). The group of genes with decreased mRNA levels included: *PTEN, SIRT3, MMP9, NRP1, NFKB1, TNF, MAPK1, SETD2, EGFR, KRAS, FGFR2, PIK3CG* (Figure 2C) with *CA7* and *GUCA2B* showing the most prominent reduction in transcription (Figure 2, c1 insert). We applied statistical tests (Mann Whitney U) to determine if any association exists between the expression levels of the studied genes and the *KRAS* mutation status of the patients, as well as various clinical and demographic parameters of the patients. This analysis showed that the transcription levels of the genes are not related to the presence of mutations in *KRAS*. The only exception is *YKL40* whose expression is upregulated 5 times in the *KRAS*-wild type background [33]. Despite the lack of statistical significance, two other genes, *SETD2* and *MMP9,* show a trend to be differentially regulated in the two patient groups. *SETD2* expression, albeit generally low, is higher in patients bearing *KRAS* mutations (p=0.098). The opposite is true for *MMP9,* which shows slightly elevated levels in *KRAS*-wild type patients than in those with mutations in the gene (p=0.066). In addition, the Kendall tau test showed significant correlation between the expression of *SETD2* and that of several other genes – *EGFR, VEGF, CD44, PTEN, NRP1, YKL40, AKT1, FGFR2* and *SIRT3*. This result might point to the important role of *SETD2* in CRC development/progression, as well as its possible function in the regulation of different cellular processes. The transcription levels of the 26 genes are affected neither by the age or the sex of the patients, nor by the localization, T stage or the grade of the tumor. There are two possible explanations for this phenomenon: the heterogeneity of CRC and the small sample size which do not allow very definitive interpretation of the results. The most extremely downregulated gene was *GUCA2B*. It was observed that the group with higher expression levels (≥ mean = 0.05) had a worse overall survival than the group with lower expression levels (< mean = 0.05) (Figure 3).

Despite the difficulty in extrapolating the results from such a small patient cohort and making general conclusions, some of the findings of our study are novel and deserve attention. First, in 46% of patients the two pro-inflammatory cytokines, IL-6 and TNF-α, were not differentially expressed in the tumor compared to the normal tissue. In 50% of the cases, *IL6* displayed moderately elevated levels (2–6 fold), whereas *TNF* was underexpressed. Second, we did not observe upregulation of *EGFR* in any of the studied patients. On the contrary, the gene was underexpressed in 71% of the cases and showed normal regulation in the rest. The same is true for the gene *KRAS*. Third, we obtained information about the expression of genes that have not been previously discussed in the context of CRC. Thus, *SETD2* encoding a histone H3K36 methyltransferase is downregulated in 67%

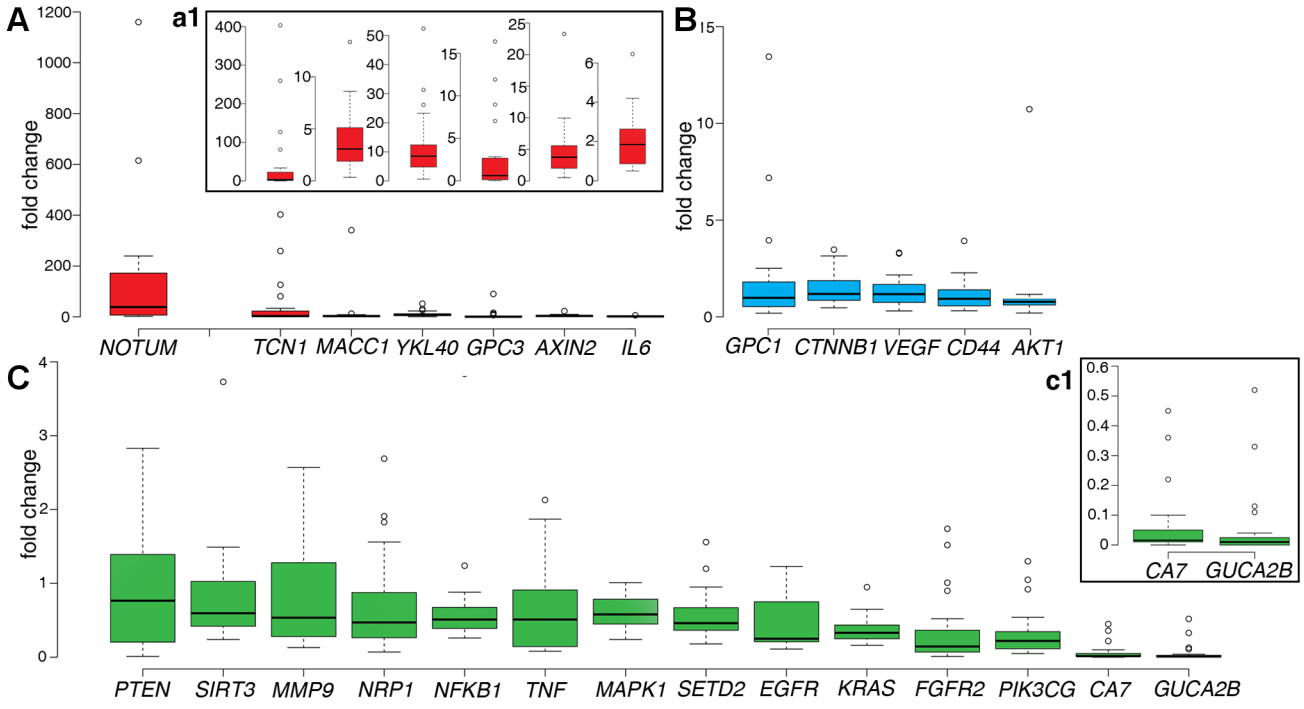

Figure 2. Transcription levels of the studied genes. A. Overexpressed genes (fold change >2). The most significantly upregulated gene is *NOTUM*. For clarity, the mRNA levels of *TCN1, MACC1, YKL40, GPC3, AXIN2* and *IL6* are shown on separate scales in a1. The genes which do not show significant changes in transcription levels in comparison to the normal mucosa are depicted in B. Downregulated genes (fold change <1) are depicted in C. *CA7* and *GUCA2B* show the lowest levels of expression in CRC tissue (c1). Genes are classified as belonging to one of the three groups based on mean values of expression (n=24). Boxplots are created in BoxPlotR.

of our patients. *TCN1,* whose product transcobalamin is involved in vitamin B12 transport, is strongly overexpressed in the tumor tissue. Carbonic anhydrase VII (CA7) is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide and has multiple functions, among which is the regulation of the acid-base balance. Our results demonstrate extreme underexpression of the gene in 100% of the samples (Figure 2, c1 insert). Furthermore, a correlation was found between the expression levels of this gene and *GUCA2B,* the product of which (uroguanylin) is thought to participate in the regulation of salt and water homeostasis in the intestine and kidneys.

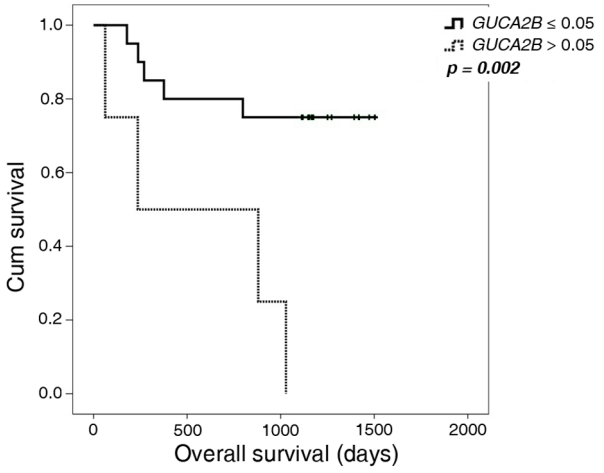

Figure 3. Kaplan-Meier estimates of overall survival in patients with colorectal cancer according to the expression level of *GUCA2B*.

Discussion

The results of our study should be interpreted with caution due to the small number of patients included in it. Though, they reveal several interesting aspects of CRC carcinogenesis, which we hereby discuss in more detail.

EGFR signaling. Blocking of EGFR signaling with anti-EGFR monoclonal antibodies has emerged as a successful target therapy that has contributed to the increase in overall survival in CRC patients. Mutations in four genes are considered as negative predictive markers for this therapy – *KRAS, NRAS, BRAF* and *PIK3CA,* with quadruple wild type CRC patients benefiting most from cetuximab treatment [16]. EGFR overexpression is a hallmark of numerous human cancers, including CRC, and a few studies have looked into its effects on target therapy efficiency and prognosis. Some authors showed significant association between high EGFR expression and advanced tumor stage [36, 37], lymph node

metastasis [38] and poorer overall survival [39], whereas others did not find it to be a useful prognostic marker [37, 40]. Cetuximab was also shown to be more effective in patients with high protein expression of EGFR [41] or increased *EGFR* gene copy number [42]. Furthermore, mutations in the gene are associated with acquired resistance to anti-EGFR mAbs [26, 43]. Surprisingly, our results showed moderately decreased mRNA levels of *EGFR* in 71% of the patients in the study but this low expression did not show any association with clinicopathological features. We can only speculate that a combination of *KRAS* mutations and *EGFR* downregulation might be indicative of poor response to anti-EGFR therapy.

Inflammation – IL-6 and TNF-α. Despite the tight link between chronic inflammation and different types of human cancers [44], studies concerning the levels of pro-inflammatory cytokines in the CRC tumor environment are contradictory. Furthermore, the role of pro-inflammatory cytokines for CRC progression from adenoma has been disputed [45]. Tumor Necrosis Factor Alpha (TNF-α) activates the transcription factor NFκB, thus promoting proliferation and metastasis of tumor cells [44,46]. Several studies have demonstrated the link between increased *TNF* mRNA levels in the tumor tissue and advanced stages of CRC [47–49], suggesting that targeting TNF-α can be applied in treatment of CRC, especially when it has progressed from ulcerative colitis [50, 51]. However, some authors report suppression of this cytokine in tumor, attributed to promoter-methylation of the gene [52]. Data on TNF-α serum levels are not less contradictory. While some authors claim significantly higher levels of the cytokine in serum of CRC patients and link this to the tumor development and progression [53], others do not detect it at all [54, 55]. In contrast, studies investigating the role of IL-6 in CRC are much more definitive. The elevated levels of the cytokine in CRC tissue [56] and serum [57] have been shown to be significantly associated with higher risk of relapse and survival of the patients. A monoclonal antibody binding to the IL-6 receptor was shown effective in targeting colon cancer-stem like cells [58]. Despite the proposed downregulation of p53 by IL-6, which offers a link between inflammation and carcinogenesis [59], a meta-analysis by Zhou et al. (2014) did not find any significant association between IL-6 and the risk of CRC [60]. Our results show slightly increased mRNA level of *IL6* and decreased level of *TNF* in 50% of the patients. We did not find any association between the levels of these two cytokines and tumor stage or patients' age. The Kendal tau test showed significant correlation of *IL6* expression with *VEGF, NFKB1, PIK3CG, MAPK1, CTNNB1, AXIN2, GPC1, AKT1* and *SIRT,* suggesting a possible role of this pro-inflammatory cytokine in activating/regulating a variety of signaling pathways.

Histone modifications – SETD2. Proper functioning of the epigenetic regulatory machinery is crucial for maintaining genome integrity and cellular function. *SETD2* is a single gene responsible for trimethylation of H3K36, a histone modification implicated in transcription elongation, splicing and DNA repair [61]. It has been also proposed that this methyltransferase interacts with p53 and selectively regulates the transcription of the transcription factors' target genes [62] but the significance of this interaction remains unknown. Inactivating mutations in the genes are most frequent in the clear cell renal cell cancer [61] but are also associated with high-grade gliomas [63]. Decreased mRNA levels of *SETD2* have been associated with increasing tumor stage in breast cancer pointing to a tumor-suppressor role of this gene [64]. Our results of downregulated *SETD2* expression in CRC tissue (67% of patients) lead to a similar conclusion, although we did not find a link between the expression of this gene and the clinicopathological parameters. Although not significant, an association between *KRAS* mutation status and *SETD2* expression was noted, the functional importance of which remains to be elucidated.

Vitamin B12 metabolism. Transcobalamin 1 (TCN1) is a member of the vitamin B12-binding protein family. It is expressed in various tissues and facilitates the transport of cobalamin into cells. The role of vitamin B12 metabolism has been studied in gastric cancer [65, 66], breast phyllodes cancer [67] and prostate cancer [68]. A recent meta-analysis of gene expression data obtained by microarrays showed that *TNC1* is overexpressed in CRC tissue thus acting as an oncogene [69]. Although the role of vitamins has been extensively studied in gastrointestinal diseases (reviewed in [70]), the function of genetic variants and mRNA levels of *TCN1* in CRC development and progression have not been thoroughly addressed. Our results demonstrate highly an increased transcription level of the gene in tumor tissue in 67% of CRC patients, in agreement with the results obtained from Chu et al. (2014).

Carbonic anhydrase VII and CRC. The meta-analysis of Chu et al. (2014) showed that *CA7* and *GUCA2B* are also among the top eight differential genes in CRC, which act as tumor suppressors. Our results corroborated this view by showing decreased mRNA levels of both genes in 100% of the patients. Actually, these two genes were the most prominently downregulated genes within the gene panel. Carbonic anhydrase VII (CA7*)* belongs to a group of metalloenzymes that catalyze the reversible hydration of carbon dioxide and are involved in many physiological and pathological processes. Inhibiting CAs represents a potential novel treatment for obesity, infections and cancer [71]. Expression of CA IX is highly elevated in many tumors as a consequence of hypoxia [71] and is linked to worse prognosis in CRC [72, 73]. Only few studies address the role of CA VII in cancer. Upregulation of the gene is associated with poor prognosis in astrocytoma patients [74], whereas decreased *CA7* mRNA levels significantly correlated with poor differentiation, positive lymph node metastasis, advanced TNM stage and increased death rate in CRC patients [75]. Despite accumulating data, the differential role of CA isoenzymes in CRC remains to be elucidated.

Guanylyl cyclase C signaling in CRC carcinogenesis. The gene *GUCA2B* codes for the hormone uroguanylin, one of the two ligands that activate guanylyl cyclase C (GUCY2C) signaling axis controlling fluid and electrolyte homeostasis [76]. Loss of uroguanylin (GUCA2B) and guanylin (GUCA2A) abrogates GUCY2C signaling, which is a universal feature of CRC [77, 78], strongly suggesting a role of *GUCY2C* as a tumor suppressor. In addition, GUCY2C signaling is downregulated in inflammatory bowel disease (IBD) [79,80] providing a novel link between chronic inflammation and CRC carcinogenesis and suggesting a target for prevention of CRC by hormone replacement therapy [76]. The extreme downregulation of *GUCA2B* in all our patients conforms to previous studies underscoring the important role of uroguanylin-mediated signaling in CRC. Although ubiquitously lost, the levels of the gene are lower in patients with longer survival time. Also, *GUCA2B* expression showed no significant correlation with the expression of *IL6* and *TNF*, which questions a direct link between GUCY2C signaling and inflammation. We have to note, however, that we did not study the expression of the other GUCY2C ligand, guanylin, and thus cannot make definitive conclusions since both hormones might have redundancy in function. In general, our results suggest that uroguanylin, as a secreted protein, can be indeed used as a biomarker for early detection of CRC [81], but hormone replacement therapy (linaclotide) should be applied rather for protection/prevention of inflammation-induced CRC than for treatment of advanced CRC.

Our study provides novel data on the transcriptional activity of a panel of 26 genes related to the tumor growth, invasion and metastasis in CRC. It offers new insights into a variety of signaling pathways involved in colorectal carcinogenesis, some of which are still waiting for their clinical implications.

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