DEPENDENCE OF YKL-40 mRNA TISSUE LEVELS ON KRAS MUTATION STATUS IN COLORECTAL CANCER – PRELIMINARY RESULTS

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ABSTRACT

Colorectal cancer (CRC) is one of the most deadly cancers worldwide. Despite the introduction of targeted molecular therapies in the last 10 years, overall survival has not increased substantially. CRC progression is accompanied by numerous genetic and epigenetic alterations and dysregulation of several signaling pathways, among which activation of Wnt and inactivation of TGF-β signaling. The molecular heterogeneity of CRC, however, hinders its molecular subtyping and thus the identification of common biomarkers for this pathology. The only three well established biomarkers for advanced-colorectal-cancer drug treatment are negative biomarkers. These are mutations in the genes *KRAS, NRAS* and *BRAF,* which determine resistance to therapy with anti-EGFR antibodies. YKL-40 is a chitin-binding glycoprotein that has been shown to play a role in extracellular tissue remodeling, angiogenesis, cell migration and inflammation. Increased serum levels of this protein have been detected in patients with CRC, but the role of YKL-40 in this neoplastic disease has not been studied extensively and the precise function of YKL-40 in CRC progression is not known. In the present study, we determined the *KRAS* mutation status and measured the mRNA levels of *YKL-40* of 24 patients with sporadic CRC. Also, we assessed the association between these two parameters by statistical analysis. We are the first to show that in CRC YKL-40 mRNA levels are dependent on the presence of *KRAS* mutations, being prominently elevated in the wild-type background. Our results indicate the potential role of YKL-40 as a target molecule for CRC therapy.

KEYWORDS colorectal cancer (CRC); YKL-40 gene expression; *KRAS* mutations, target therapy

Introduction

Colorectal cancer (CRC) is the third most common neoplastic disease and the world's fourth most deadly cancer [1]. Since 2004 five targeted therapies have been introduced into clinical practice, which have extended overall survival for advanced CRC from 15 to 30-40 months [2-3]. Colorectal cancer results from the gradual accumulation of genetic and epigenetic alterations that lasts 10-15 years [4]. Loss-of-function defects in tumour-suppressor genes *(APC, TP53 and PTEN)* and gain-of-function defects in oncogenes *(KRAS, NRAS, BRAF, PIK3CA)* accompanied by epigenetic changes, such as DNA methylation, are all crucial for CRC initiation and progression [5-6]. Recently, a

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		n	%
Age	<70 years	10	41.7
	>70 years	14	58.3
Mean age		73+/-7 y (61-88)	
Gender	Female	10	41.7
	Male	14	58.3
TNM-stage	T1N0M0	2	8.3
	T2N0M0	8	33.4
	T3N0M0	9	37.5
	T3N1M0	1	4.2
	T3N2M0	2	8.3
	T4N2M0	2	8.3
Cell Differentiation	G1	1	4.2
	G2	21	87.5
	G3	2	8.3
KRAS status	Wild type	16	66.7
	Mutant	8	33.3

Table 1 Clinical parameters and KRAS mutation status

comprehensive molecular characterization of human colon and rectal cancers and a new classification system based on the rate of somatic mutations were proposed [7]. The hyper- and non-hypermutated phenotypes develop through different sequences of genetic events [7]. Nevertheless, Wnt signaling activation and TGF-β signaling inactivation have been shown to be ubiquitous events in CRC [7-8]. Despite the increasing number of gene expression studies, no signature based on mRNA levels has been found useful in clinical practice [6]. Integrating data from gene expression profiles, genetic and epigenetic analyses has led to the identification of distinct molecular subtypes of CRC [9-12]. *KRAS* mutations are found in the different subtypes confirming the notion that *KRAS*-mutated CRC is heterogeneous at the gene expression level. A unified system of molecular classification of CRC has been developed recently [13], but the goal remains – to identify the best treatment for each subtype. The molecular heterogeneity of CRC impedes the search for common biomarkers for this pathology. Presently, only three biomarkers for advanced-colorectal-cancer drug treatment exist, and they are all negative biomarkers. Patients with mutations in the genes *KRAS, NRAS* and *BRAF*, do not respond to treatment with EGFR-antibodies. At the same time, many of the *KRAS* wild-type patients are also resistant to anti-EGFR therapy. Dienstmann et al. (2014) present an excellent summary of the genotype-directed therapy concepts in advanced CRC [8, Table 1]. The co-occurrence of alterations involving the RAS and PI3K pathway in about 30% of tumours indicates that simultaneous inhibition of several signaling pathways is required to achieve therapeutic benefit [7].

YKL-40 is a chitin-binding glycoprotein, belonging to the family of chitinase-like proteins [14]. YKL-40 is produced by restricted cell types, including colonic epithelial cells and macrophages [14-15] and it mainly acts extracellularly [16]. The functions of this protein are still not precisely elucidated, but it has been suggested to play a role in extracellular tissue remodeling, migration, angiogenesis and inflammation [17]. Elevated serum YKL-40 levels have been observed in patients with inflammatory and malignant diseases [16], including colorectal cancer [18-19]. We have previously studied the protein and mRNA levels of YKL-40 in high-grade glioma and shown that YKL-40 expression is related to the malignancy of the tumours [20]. Very few studies address the presence of YKL-40 in CRC and its role in this type of cancer is entirely unknown.

The aim of the current study was to determine *KRAS* mutation status and to measure mRNA levels of *YKL-40* in patients with CRC. We were also interested to find out if there is any association between these two parameters which might suggest a potential role of YKL-40 as a novel target molecule for CRC therapy. We are the first to provide evidence for the dependence of YKL-40 mRNA levels on *KRAS* mutation status in CRC.

Materials & Methods

Patients and preparation of tissue samples

The study was approved by the Ethics Committee of Medical University of Plovdiv, protocol # R-1838/15-07-2013. Twenty-four patients were included in the study after signing an informed consent in agreement with the requirements of the WMA Declaration of Helsinki. Tumour tissue and normal colonic mucosa (internal control) distal to the tumour site were isolated from the patients by intraoperative resection and were stored in RNA later (Qiagen, Netherlands) at - 80 ºC. Part of the tumour tissue was fixed in 10% formalin and embedded in paraffin for histological evaluation and DNA isolation.

Molecular analyses

DNA was isolated with the QIAamp DNA FFPE Tissue Kit (Qiagen), 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 tumour tissue samples was isolated with the RNeasy Mini Kit (Qiagen) and converted to cDNA with the RT^2 First Strand Kit (Qiagen). YKL-40 gene expression was measured by RT-qPCR using the Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific, USA) and the following primers: F: GACCACAGGCCATCACAGTCC and R: TGTACCCCACAGCATAGTCAGTGTT. Samples were run in triplicates according to MIQE guidelines. The analysis was performed with the $\Delta\Delta C_T$ method individually for each patient. The expression of the gene in the normal tissue served as the calibrator and GAPDH were used as a reference gene.

Statistical analysis

The Mann-Whitney U test was applied to compare the values of a continuous variable in two independent groups. Boxplot diagrams were used for graphical visualization of continuous variables as well as for distinguishing outliers in the data series (the criteria 1.5 of the interquartile ranger have been applied).

Results

Clinical parameters and KRAS mutation status

Twenty-four patients with sporadic CRC were included in the study (Table 1). None of them had been subjected to radio- or

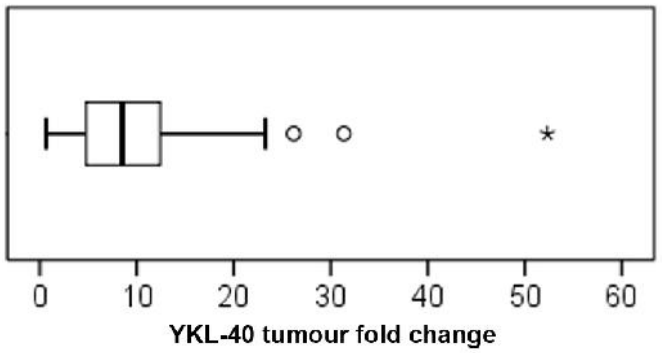

Fig. 1: Fold change of *YKL-40* mRNA levels in tumour tissue. The change in expression level was determined by the $\Delta\Delta C_T$ method where normal tissue served as the calibrator and *GAPDH* as the reference gene.

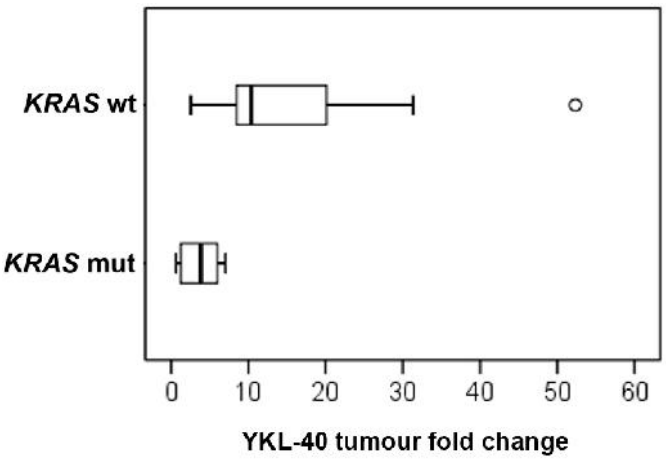

Fig. 2: Fold change of *YKL-40* mRNA levels in *KRAS* wild-type (*KRAS* wt) and *KRAS* mutant patients (*KRAS* mut). Gene expression of YKL-40 is significantly dependent ($p<0.001$) on the presence of mutations in the gene *KRAS* as determined by the Mann-Whitney U test.

chemotherapy prior to the surgical removal of the tumour. Individuals with recurrent CRC were excluded from the study. Tissue samples were collected intraoperatively in the two university hospitals – "St. George" and "Kaspela" and sent for pathohistological assessment. 50% of the patients were staged as T3 according to the TNM system and in 87.5% of the cases the degree of cell differentiation was evaluated as G2. From each paraffin block, 6 sections of 10 microns of thickness were prepared for subsequent DNA isolation. The presence of mutations in the *KRAS* gene was determined with the Amoy Dx KRAS Seven Mutations Detection Kit. Sixteen of the patients were defined as wild type and in 8 a *KRAS* mutation was identified, predominantly 12ASP (50%).

YKL-40 is strongly upregulated in tumour tissue of CRC patients

We measured the transcription levels of the gene *YKL-40* in normal and tumour tissue of 24 patients with sporadic CRC. Unyielding up-regulation of the gene *YKL-40* in tumour tissue was observed in 87.5% of the patients with a fold change varying between +2.6 and +52.4 and a median value of +8.5 (Figure 1). Since most of the patients were staged either T2 (8) or T3 (12) we were able to assess the relationship between *YKL-40* expression and tumour grade only in these two groups of patients. We did not find any statistically significant difference between mRNA levels of the gene and tumour malignancy as determined by the Mann-Whitney test ($p=0.405$). Gene expression was also not related to the sex of the patients ($p=0.519$).

Gene expression of YKL-40 is significantly associated with the presence of KRAS mutations

We conducted a statistical analysis to determine if mutations in the *KRAS* gene affect the expression of the *YKL-40* gene. We applied Mann-Whitney test and found that *YKL-40* transcription levels are significantly dependent on the presence of mutations in the *KRAS* oncogene ($p < 0.001$). Although *YKL-40* was significantly upregulated in all tumour samples, its levels were approximately 5 times higher in KRAS wild-type than in *KRAS* mutant patients (Figure 2).

Discussion

In contrast to other types of cancer, e.g. breast cancer, CRC is highly heterogeneous at the molecular level, and this fact hinders the identification of common biomarkers for this pathology. Reimers at al. (2013) offer a nice overview of all existing prognostic and predictive biomarkers in CRC [21]. It is now known that patients with mutated *KRAS, NRAS* and *BRAF* genes do not respond to treatment with the EGFR-antibodies cetuximab and panitumumab. Also, many of the *KRAS* wild-type patients are also resistant to anti-EGFR therapy. The dysregulation of several signaling pathways in many of the tumours suggests that simultaneous inhibition might be required to achieve better therapeutic effect [7]. Thus, identifying not only biomarkers for the disease but also new targets for molecular therapy is of crucial significance for increasing the lifespan of CRC patients.

In this study, we showed that the mRNA levels of the gene *YKL-40* are significantly increased in the tumour tissue of CRC patients. The elevation was in the range 3-52 fold as compared to the normal colonic tissue isolated from the same patients. Furthermore, transcription levels of *YKL-40* were related to the presence of mutations in the gene *KRAS* with upregulation being more prominent in the wild type phenotype. Our previous investigations have demonstrated significantly elevated levels of serum and synovial *YKL-40* in inflammatory joint diseases [22] and high-grade glioma [20]. *YKL-40* expression at the mRNA and protein levels in the tumour site and in the serum of glioma patients parallels with the malignancy grade of the tumours, suggesting a potential role of the protein in neoplastic development [20]. Our observations were supported by previous studies showing increased mRNA and serum levels in human glioma correlating with tumour grade and tumour burden in glioblastoma multiforme [23]. Downregulation of *YKL-40* has been also shown to be an important factor to overcome temozolomide resistance in the glioblastoma cell line U87 [24]. Elevated serum *YKL-40* levels have been detected in patients with breast, ovarian and endometrial cancer and are associated with disease progression, histologic grade and survival time [25-27]. Transcription levels of *YKL-40* are related to tumour grade, poor differentiation, and other breast cancer markers, highlighting that tissue levels of *YKL-40* serve as a valuable biomarker for breast cancer diagnosis and prognosis [28]. Serum and tissue levels of *YKL-40* are also increased in bladder cancer and are associated with poor patients' survival [29], but it is clear that secretion of the protein from the tumour tissue is not the only source of serum *YKL-40*

[27, 29]. Colorectal cancer patients with high serum *YKL-40* levels had significantly shorter survival than patients with serum *YKL-40* below the median [18]. Thus, determination of serum *YKL-40* may be useful in combination with other biomarkers in risk assessment for colorectal cancer [19]. A recent study demonstrated that *c-Met/YKL-40* expression reliably predicts poor or absent response to neoadjuvant chemoradiotherapy in rectal cancer [30]. Our results of elevated *YKL-40* mRNA levels in the tumour site of CRC patients are in agreement with previous studies showing that expression of this gene in the primary tumour may be positively associated with the invasiveness and metastatic ability of tumour cells [15] confirming the notion that *YKL-40* can be used as a biomarker for neoplastic changes. We did not find any relationship between *YKL-40* expression and tumour stage but an extremely significant link between the mRNA levels and the *KRAS* mutation status. The precise meaning of this association remains to be elucidated. However, this data offers a ground for the development of novel molecularly-based therapies for CRC.

Conclusion

Gene expression of *YKL-40* is significantly upregulated in tumour tissue of CRC patients confirming the role of the protein in neoplastic progression. The mRNA levels of *YKL-40* are dependent on the presence of mutations in the gene KRAS and are more prominently increased in the non-mutated background. This association implies the potential of *YKL-40* as a future target for molecular CRC therapies.

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Authors' Statements

Competing Interests

The authors declare no conflict of interest.

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