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SMAD4 Gene Analysis in Patients with Early Onset Colorectal Cancer: A Pilot Study

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Abstract—In colorectal cancer (CRC), inactivation of SMAD4 occurs early in the disease development and *SMAD4* represents one of key driver genes in progression and metastasis. Loss of SMAD4 protein expression is a relatively common feature of sporadic colorectal cancers, and it was observed to be even more frequent in tumors of patients with early onset disease and also more frequent in microsatellite stable tumors. Pathogenic variants in the *SMAD4* gene are usually missense or nonsense mutations, and they are more frequent in the C-terminal domain. The aim of this study was to perform genetic analysis of SMAD4 C-terminal domain in colorectal cancer patients with early onset disease and microsatellite stable tumors. This pilot study was conducted with a purpose of investigating if such genetic screening strategy would be useful for diagnostic purposes in this specific subgroup of CRC patients. The study was conducted in a selected set of DNA samples extracted from the tumors of CRC patients who had less than 50 years at the time of diagnosis. Genetic analysis of C-terminal domain has encompassed analysis of exons 9, 10, 11 and 12 of the *SMAD4* gene by PCR and direct DNA sequencing. Among the twenty analyzed tumor DNAs, one sample was found to harbor a SMAD4 variant: NC_000018.9:g.48591918C > T; (NM005359.5: c.1081C > T; Arg361Cys). The variant was discovered in exon 9, affecting the codon 361, which represents a mutational hot spot within the SMAD4 gene. This variant was discovered in homozygous state in the tumor of a 47 yr old female with T3 stage carcinoma of the right colon. Considering the incidence and functional consequences of SMAD4 exon 9 variants, the screening of this region could be a useful low cost strategy for the genetic analysis of colorectal tumors from patients with early onset disease, as well as for susceptibility testing.

Keywords: colorectal cancer, early onset disease, genetic testing, pathogenic variant, SMAD4

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INTRODUCTION

SMAD family member 4 (SMAD4, DPC4) is the central mediator of the transforming growth factor beta (TGFB) family of signal transduction proteins involved in many biological processes, including cell growth, differentiation, apoptosis and migration (McCarthy and Chetty, 2018). Gene *SMAD4* acts as a tumor suppressor and is commonly inactivated due to somatic variants in human cancers, most commonly in pancreatic and colorectal tumors (Zhao et al., 2018). In colorectal cancer (CRC), inactivation of SMAD4 occurs early in the disease development and *SMAD4* represents one of key driver genes in progression and metastasis (Huang et al., 2018). Germline *SMAD4* variants cause two non-malignant hereditary conditions, juvenile polyposis (JP) and hereditary hemorrhagic telangiectasia (HHT) (Schwenter et al., 2012). While JP is characterized by the presence of benign polyps in the digestive tract of young individuals, HHT leads to abnormal blood vessel formation. In both conditions, risk of developing malignancy is high and these patients often develop cancers at young age.

Loss of SMAD4 protein expression is a relatively common feature of sporadic colorectal cancers, and it was observed to be even more frequent in tumors of patients with early onset disease and also more frequent in microsatellite stable tumors (Perea et al., 2010; Royce et al., 2010). Most common mechanism of SMAD4 inactivation is acquired loss of chromosome 18 or its q21 region accompanied by an inactivating somatic mutation in the *SMAD4* gene, which leads

to loss of SMAD4 protein. However, loss of SMAD4 functionality can also happen due to pathogenic variants that do not lead to loss of protein. Increasing number of such variants has been discovered and evidenced in various databases, listed at https://grenada.lumc.nl/LSDB_list/lsdbs/SMAD4.

Pathogenic variants in the *SMAD4* gene are usually missense or nonsense mutations, and they are more frequent in the C-terminal domain (De Bosscher et al., 2004). While nonsense mutations result in protein truncation, missense mutations have been reported to affect the ability of SMAD4 to form complexes with other SMAD proteins (Shi et al., 1997). Variants located in the N-terminal domain can affect protein stability, DNA binding or nuclear translocation. In general, their functional consequences are less severe in comparison to C-terminal domain variants.

The aim of this study was to perform genetic analysis of *SMAD4* C-terminal domain in colorectal cancer patients with early onset disease and microsatellite stable tumors. This pilot study was conducted with a purpose of investigating if such genetic screening strategy would be useful for diagnostic purposes in this specific subgroup of CRC patients.

MATERIALS AND METHODS

Study Samples

Twenty DNA samples extracted from the tumor tissue of patients with colorectal cancer were included in the study. All patients were diagnosed and underwent surgical resection of tumor at the First Surgical Clinic, Clinical Centre of Serbia. The samples were selected for the study from a larger cohort of patients based on results of microsatellite instability (MSI) analysis for the following loci: BAT25, BAT26, NR21, NR22 and NR24. Criteria for selection were no MSI and patient age at diagnosis of ≤50 yr. Among the subjects there were no patients treated by preoperative radiotherapy or chemotherapy, those with inflammatory bowel disease or with known history of familial adenomatous polyposis.

Genetic Analysis

Polymerase chain reaction (PCR) was used to amplify exons 9, 10, 11 and 12 of the *SMAD4* gene using previously published primers: 5'-GGATGTTCTTTCCCATTTAT-3' and 5'-ACAATCAATACCTTGCTCTC-32' for exon 9, 5'-TATTAAGCATGCTATACAATCTG-3' and 5'-CTTCCACCCAGATTTCAATTC-3' for exon 10, 5'-AGGCATTGGTTTTTAATGTATG-3' and 5'-CTGCTCAAAGAAACTAATCAAC-3' for exon 11 and 5'-CCAAAAGTGTGCAGCTTGTTG-3' and 5'-ATTGTATTTTGTAGTCCACC-3' for exon 12 (Houlston et al., 1998). The amplification was carried out in 50 μL total volume containing genomic DNA (20–100 ng/μL), FireTaq (Solis BioDyne), 10× FireTaq buffer (Solis BioDyne), 25 mM $MgCl_2$, 2 mM of each dNTP and 10 pmol of each primer. The amplification was performed under the following conditions: 94°C for 5 min; 30 cycles: 94°C for 30 s, 55°C for 45 s, 72°C for 30 s; 72°C for 10 min. Amplified fragments encompass complete exons and noncoding sequences at the exon/intron junctions. The obtained PCR products were subjected to direct DNA sequencing with one of the primers used for amplification using BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). Sequencing products were analyzed on a 3130 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed using the Sequencing Analysis Software (Applied Biosystems).

RESULTS

In this study, genetic screening of the *SMAD4* C-terminal coding exons was conducted in a selected subgroup of CRC patients with early onset disease and MSS tumors. Demographic and clinical characteristics of patients are given in Table 1.

Among twenty patients included in the study there were 11 males and 9 females, and average age was 44 yr. Most patients (70%) were diagnosed with rectal cancer, while the others were diagnosed with colon cancer. Tumors of most patients (60%) were characterized by high mucus production (≥50%).

The tumor of one patient was found to harbor a *SMAD4* variant in exon 9: NC_000018.9:g.48591918C > T (NM 005359.5: c.1081C > T; Arg361Cys) (Fig. 1). This variant was discovered in a 47-yr old female with T3 stage carcinoma of the right colon characterized by low mucus production (≤25%).

DISCUSSION

The study was aimed at genetic screening of the *SMAD4* C-terminal coding exons in a selected subgroup of CRC patients. The *SMAD4* genetic analysis was focused on C-terminal domain due to the fact that it is responsible for the interaction of SMAD4 with other molecules, both proteins and DNA. It is the most likely candidate region within *SMAD4* gene to harbor pathological variants and it was found to be most commonly affected by mutations in various human pathologies. These variants often abolish the function of the SMAD4 protein, usually lead to severe clinical consequences and are mostly found in malignant diseases (Yang and Yang, 2010). Variants in the N-terminal domain and linker region are less common and lead to less severe SMAD4 protein defects, considering that they alter protein conformation and activity rather than abolish its function. Variants outside C-terminal domain may act as potential risk factors and/or phenotype modulators rather than being the cause of the disease. Regulatory region of the SMAD4 gene may harbor genetic variants associated

with haploinsufficiency that could affect the amount of the synthesized protein, but their clinical relevance remains uncertain (Nikolic et al., 2011).

The study was focused on a specific subgroup of CRC patients—individuals with MSS tumors who developed disease before the age of 50. Loss of SMAD4 protein expression was found to be more frequent in early onset colorectal cancers and majority of tumors that displayed loss of SMAD4 were found to be MSS (Royce et al., 2010). Loss of SMAD4 appears to be linked to the worse behavior of early onset CRC, particularly in the MSS subgroup of patients (Perea et al., 2010). The *SMAD4* missense variant detected in our study was found in a patient that was the only one in the study group presenting with a right-sided colon cancer and with highest value of MsPath score (3.4), which is usually indicative of the presence of mismatch repair deficiencies that result in MSI (Bessa et al., 2011). As right-sided colon cancers tend to produce symptoms only when they are relatively advanced, it is probable that the disease onset in this patient happened some time before she was diagnosed at the age of 47 (Venook, 2017).

The detected variant Arg361Cys is located in a hot spot of missense mutations, which disrupts binding of SMAD4 to the other SMAD proteins (Miyaki and Kuroki, 2003). According to publicly available databases (dbSNP and 1000 Genomes), this nucleotide is affected by alteration with a frequency of 0.2–0.3 (rs80338963), so it is relatively common in the general population. Nucleotide substitutions C > A, C > G and C > T at codon 361 were previously detected in patients with JP and HHT as germline variants, and they were also found as somatic alterations in different malignancies, including CRC (Houlston et al., 1998; Gallione et al., 2006; Chang et al., 2016). When SMAD4 variant Arg361Cys was detected in our study, no other allele was found at this position. Considering the early onset of disease, it is possible that in this case the variant was present in the germline and that either the loss of chromosome carrying the wild type allele or its mutation occurred in the tumor. However, this could not be confirmed as non-tumor tissue or blood sample were not available from this patient. Two other pathogenic variants affecting the same amino acid, Arg361Gly and Arg361Ser, were found in patients with JP (Howe et al., 2004). Variant Arg361Gly was also detected in a combined JP/HHT syndrome (Gallione et al., 2004).

Considering the number, frequency and clinical relevance of missense *SMAD4* variants that do not lead to loss of SMAD4 protein, genetic testing remains superior to protein expression analysis by immunohistochemistry in diseases where these aberrations may be among underlying causes. On the other hand, other relevant pathogenic variants, especially in the non-coding regions, can be missed when applying such approach. The future research should resolve the clinical relevance of variants in *SMAD4* non-coding regions, including regulatory elements, and indicate if they should be included in diagnostic testing. Current strategies rely on next generation sequencing platforms and aim for the analysis of the entire coding region of the *SMAD4* gene. However, when two com-

Table 1. Demographic and clinical characteristics of early onset CRC patients

Average age (years)	44.0
Age range (years)	35–50
Male gender, %	55
Average MsPath score	1.6
Tumor localization, %	
rectum	70
left colon	25
right colon	5
Dukes, %	
A	15
B	25
C	40
D	20
Tumor stage, %	
T2	20
T3	60
T4	20
Regional lymph node metastases	
no	40
yes	60
Tumor differentiation grade, %	
G1	5
G2	35
G3	60
Mucus production, %	
Low (≤25%)	5
Medium (25–50%)	35
High (≥50%)	60

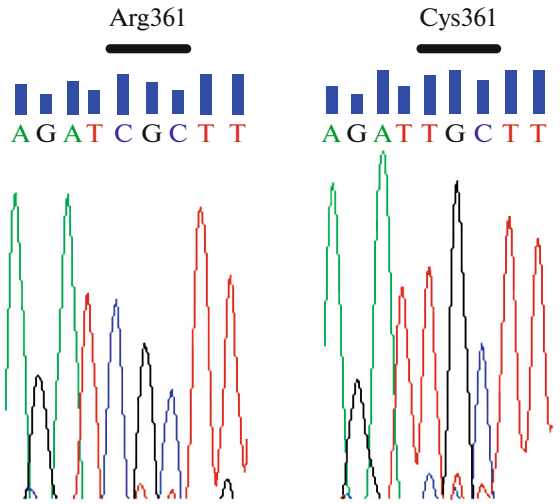

Fig. 1. DNA sequence of the part of *SMAD4* exon 9 from tumor carrying wild type allele (Arg361) and mutant allele (Cys361).

mercial human whole-exome capture systems were compared using formalin-fixed paraffin-embedded lung adenocarcinoma samples as a starting material, the variant detected in our study was missed by Agilent's platform (Bonfiglio et al., 2016). Therefore, direct DNA sequencing remains the most suitable method for screening purposes in patients with strong indications of the presence of *SMAD4* mutations.

This study supports the frequent findings of pathogenic variants in *SMAD4* C-terminal domain in CRC. Therefore, the screening of this region, especially exon 9 harboring mutational hot spot, could be a useful low cost strategy in the genetic analysis of colorectal tumors from patients with early onset disease. Absence of MSI in the tumors and their presentation as right-sided lesions are additional factors to be considered as parameters for selection of patients for this genetic testing approach.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement of compliance with standards of research involving humans as subjects. The study was conducted in compliance with principles of ethical research involving humans. The study was approved by the Ethics Committee of the Clinical Center of Serbia and cataloged under number 1856/6. Informed consent was obtained from all study subjects.

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