RESEARCH ARTICLE

Prognostic significance of *SOX17* and *WNT5a* promoter methylation status in circulating cell-free DNA metastatic colorectal cancer patients

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Abstract

Aim: Carcinogenesis of colorectal cancer is a process involving genetic mutations and epigenetic alterations in its multiple phases. The most considerable epigenetic alteration occurring in colorectal cancer (CRC) tumorigenesis is the methylation-mediated silencing of tumor suppressor genes. The present study aimed to detect the methylation status of *SOX17* and *WNT5a* promoters in cell-free DNA circulating in plasma of metastatic CRC patients and to investigate potential prognostic correlation.

Methods: A methylation-specific real-time polymerase chain reaction was utilized to investigate the methylation status of genes (*SOX17* and *WNT5a*) promoter in the blood of 85 metastatic CRC patients.

Results: We found the *SOX17* promoter methylated in 54/85 (63.5 %) while *WNT5a* was methylated in 39/85 (45.8 %) samples of the advanced CRC. All control samples were negative for *SOX17* and *WNT5a* promoter methylation. Patients with metastatic CRC and methylated *SOX17 and WNT5a* promoter status had a significantly poorer outcome than patients with non-methylated ones.

Conclusions: Plasma *SOX17* and *WNT5a* promoter methylation are frequent epigenetic events in advanced CRC. The reported correlations between the methylation status of genes (SOX17 and Wnt5a) promoter and poorer survival in patients with advanced CRC disease agree with the proposed role of *SOX17* as a tumor suppressor gene. A more considerable CRC patient cohort is required to research these findings' potential further and investigate whether *SOX17* in plasma could serve as a useful prognostic biomarker in metastatic CRC. HIPPOKRATIA 2023, 27 (1):7-11.

Keywords: metastatic colorectal cancer, cell-free DNA, *SOX17, WNT5a* gene, DNA methylation, prognosis

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Introduction

Colorectal cancer (CRC) is, in both genders, the third leading cause of cancer-related death, in part because of significant changes in risk factors, the introduction and dissemination of early detection tests, and improvements in treatment[1,2]. It is a multistep disease resulting from different alterations accumulation in the genome (mutations) and the epigenetic code (DNA methylation, histone modifications, etc.)[3]. DNA methylation plays a critical role as a modulator of main genomic functions such as tumor suppressor gene expression. These cumulative events initially induce adenoma emergence, which, in a proportion of patients, progresses into invasive adenocarcinoma with metastasis potential[4]. An essential and well-characterized event during this progression to adenocarcinoma is the activation of the Wnt-pathway[5].

Wnt-pathway inactivation depicts an early event in CRC tumorigenesis predominantly caused by inactivating mutations in its main component, *APC* gene[6]. The methylation-mediated silencing of additional downstream or upstream Wnt signal-regulating components may propose an important alternative mechanism of constitutive Wnt-pathway activation in CRC, including *AXIN2*[7], *CDH1*[8], *WNT5a* [9], and *SOX17*[10].

As already stated, methylation is fundamental in CRC progression, and many genes frequently modify the methylation levels of tumor cells compared to the normal colon mucosa. The SRY-related high mobility group box (SOX) family of transcription factors regulates stem/precursor cell development and function by repressing the canonical Wnt-signaling pathway. Analysis of CpG island methylation and gene expression in CRC cell lines showed that silencing of the *SOX17* gene is associated with DNA methylation. By suppressing Wnt signaling, *SOX17* plays a tumor suppressor role[11]. *SOX17* has also shown significantly increased methylation in CRC cell lines in polypoid and non-polypoid adenomas and colon carcinomas compared to normal colon mucosa. It has also been shown that there is a functional relation between methylation and *SOX17* silencing[12]. Silva et al, suggested that the methylation frequency of *SOX17* increases during the neoplastic adenoma-carcinoma sequence concerning other Wnt antagonists. In screening studies involving DNA methylation in stool or plasma

samples, authors underline the usefulness of *SOX17* as a biomarker for the early detection of CRC.

The *WNT5a* gene, located at 3p14, is a tumor suppressor locus commonly deleted in multiple tumors. The WNT5a protein is classified as a non-canonical Wnt protein, and its role in tumorigenesis is still unclarified[13]. Studies have shown that increased WNT5a expression is essential for tumor progression and have nominated it as proto-oncogene[14]. On the other hand, *WNT5a* also inhibits tumor cell proliferation, whereas WNT5a expression is considered a good prognostic factor in patients with breast and colon cancer. Recent studies have confirmed that the most frequent mode of its inactivation in CRC is by tumor-specific methylation[15].

Plasma cell-free DNA (cfDNA) is a principal component of Liquid biopsy that contains large amount of information that reflects the tumor's molecular profile and can lead to its decoding in a less invasive way. Despite the preanalytical obstacles and the need for standardization, cfDNA circulating in plasma still remains a great source of novel molecular tumor biomarkers[16,17].

Numerous studies have described in plasma samples and in the corresponding primary tumors, similar methylation patterns of tumor suppressor genes[18]. Methylation in peripheral blood usually reflects the methylation status of the primary tumor as numerous studies have already reported[19]. A blood-based test is noninvasive and versatile both for primary detection and postoperative follow-up for local or distant relapse[20].

This study focuses on detecting the methylation status of the *SOX17* and *WNT5a* genes in the cfDNA circulating in the plasma of metastatic CRC patients. In particular, we examined the dynamic of the methylation frequency between *SOX17* and *WNT5a* in metastatic CRC (mCRC) patients. In plasma cfDNA samples from mCRC patients, we aimed to estimate the DNA methylation status prognostic significance regarding these two gene promoters.

Materials and Methods

Study design

Eighty-five samples collected at the beginning of the first-line therapy from mCRC patients comprised our study's material. The methylation status of *SOX17* and *WNT5a* genes in the cfDNA circulating in those plasma samples obtained were examined. All mCRC patients had a performance status of 0-1 and provided informed consent to participate in the study. Plasma circulating cfDNA was isolated from those CRC patients and 40 healthy individuals comprising the healthy volunteer group, totally independent regarding age but the gender-matched group who had not received any medical care and had no relation with the patients. The study was approved by the Scientific Committee of our Institution (Alexandroupolis University hospital, decision No EΣ6, date 28/09/2017).

Collection of samples and plasma circulating cfDNA isolation

We collected peripheral blood in EDTA tubes, and samples were subsequently centrifuged for 10 min at 1500 x g, and the plasma was stored until further analysis at -80 ^{o}C. We extracted cfDNA from 1,200 mL of plasma using the MagCore automated nucleic acid Extractor (MagCore Plasma DNA Extraction kit) according to the manufacturer's instructions. The DNA concentration was determined in the Qubit fluometer (Thermo Fisher Scientific).

Sodium bisulfite conversion

According to the manufacturer's protocol, we performed sodium bisulfite conversion of up to 0.5 g cfDNA utilizing the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), using as the 100 % methylated control, the Universal Methylated Human DNA Standard (Zymo Research Corp., USA). We employed the dH_2O and gDNA from the 100 % methylated control as negative and positive controls in each conversion reaction, respectively. We stored the converted DNA at -70 °C until it was used.

Methylation Specific PCR (Real-time MSP)

We detected the methylation status of *SOX17* and *WNT5a* in cfDNA by real-time MSP using specific primer pairs for the unmethylated and methylated promoter sequences. In this study, both real-time MSP assays designed and used have been evaluated for their specificity and sensitivity. In the presence of 99.9 % of non-methylated sequences, they detected down to 0.1 % of the methylated. The assays are not quantitative, so a cut-off has not been established, but when an amplification signal is detected (0-40 cycles), the sample is reported positive. We performed the MSP reactions in the Qiagen Rotor-Gene instrument of a total of 20μl. We used as fully methylated (100 %) MSP positive control, human placental genomic DNA (gDNA; Sigma Aldrich) methylated *in vitro* with SssI methylase (NEB, Ipswich, MA), after sodium bisulfite conversion, while as a negative MSP control, we used the same unmethylated placental gDNA after sodium bisulfite conversion. To prepare serial dilutions of known concentrations, we mixed the unmethylated DNA control with 100 % methylated DNA to evaluate the analytical sensitivity. After optimizing for real-time MSP, the MSP primers from previous studies[20] were used to detect *SOX17* methylation, while the MSP primers described previously[21] were used to detect the *WNT5a* gene.

Statistical analysis

We performed data statistical analysis utilizing IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA). We report the methylation status of *SOX17*, *WNT5a*, and all other qualitative variables as frequencies and percentages (%). We calculated survival rates using the Kaplan-Meier method and determined the statistical difference between the survival curves with the log-rank test.

Results

Study's population characteristics

Our study's population consisted of 85 mCRC patients with a mean age 68.35 (±9.6) years, a median age of 70, and a range of 47-76 years. More than half (54.7 %) were males.

Correlation between tumor parameters and SOX17 promoter methylation

We examined the methylation status of *SOX17* in cfDNA obtained from 85 mCRC patients and observed a high *SOX17* promoter methylation status in this group of patients (54/85, 63.5 %), while no promoter methylation was documented in the control group. A statistically significant correlation was seen between patients with methylated *SOX17* status and poor differentiation (p =0.01).

Correlation between tumor parameters and WNT5a promoter methylation

We found the *WNT5a* promoter to be unmethylated in the control group, while it was methylated in 39/85 (45.8 %) of the patients, and its methylation status was associated with poor differentiation and right-sided CRC (p =0.01 and p =0.021, respectively).

Survival analysis

After a 48-month median follow-up period, 81 of the 85 (95.2 %) patients presented disease progression, and 78 of the 81 (96.2 %) patients died.

Correlation between survival and SOX17 promoter methylation

We evaluated the Kaplan-Meier estimates for the independent cohort of 85 advanced CRC patients concerning the methylation status of *SOX17* promoter in cfDNA circulating in plasma. A significantly shorter progression-free survival period was observed in patients with methylated *SOX17* gene in correlation with those with an unmethylated one (p <0.001). Additionally, patients with mCRC and a methylated *SOX17* promoter status had statistically significantly poorer survival than those with an unmethylated *SOX17* promoter status (p =0.001). Figure 1a and Figure 1b give Kaplan-Meier survival estimates according to the methylation status in mCRC.

Correlation between survival and Wnt5a promoter methylation

The Kaplan-Meier estimates for the entire cohort of patients have shown that patients with methylated *Wnt5a* gene presented a statistically shorter progression-free survival period in correlation with those with an unmethylated one (p <0.001). Additionally, as shown in Figure 2a and Figure 2b, patients with mCRC and a methylated *WNT5a* promoter status had statistically significantly poorer survival than those having an unmethylated *WNT5a* promoter status (p =0.001).

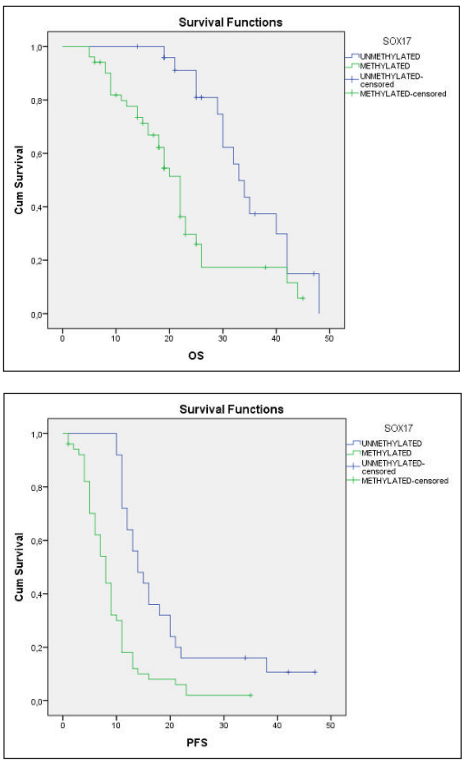

Figure 1: a) Overall survival and b) Progression-free survival of patients with metastatic colorectal cancer in relation to *SOX17* methylation status.
OS: overall survival, PFS: progression-free survival.

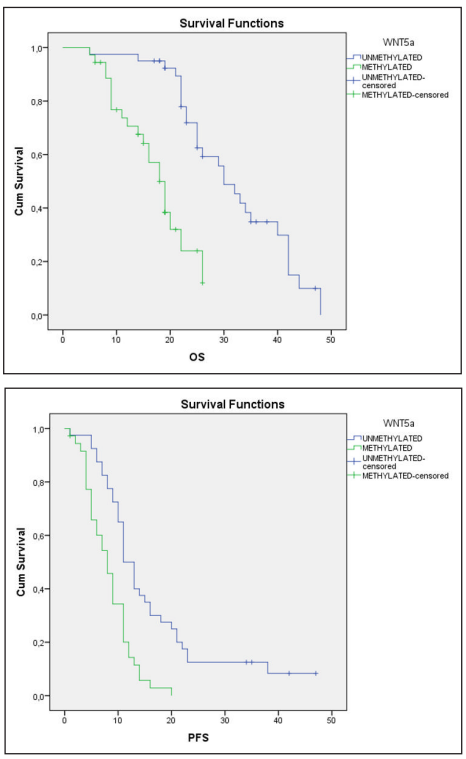

Figure 2: a) Overall survival and b) Progression-free survival of patients with metastatic colorectal cancer in relation to *WNT5a* methylation status.
OS: overall survival, PFS: progression-free survival.

Correlation between survival and both genes' promoter methylation

Thirty patients from the entire cohort presented simultaneous methylation of both genes. As demonstrated in shown in Figure 3a and Figure 3b, these patients presented a shorter progression-free survival and overall survival than those with both genes unmethylated and those with at least one gene unmethylated (p =0.001 and p =0.001, respectively).

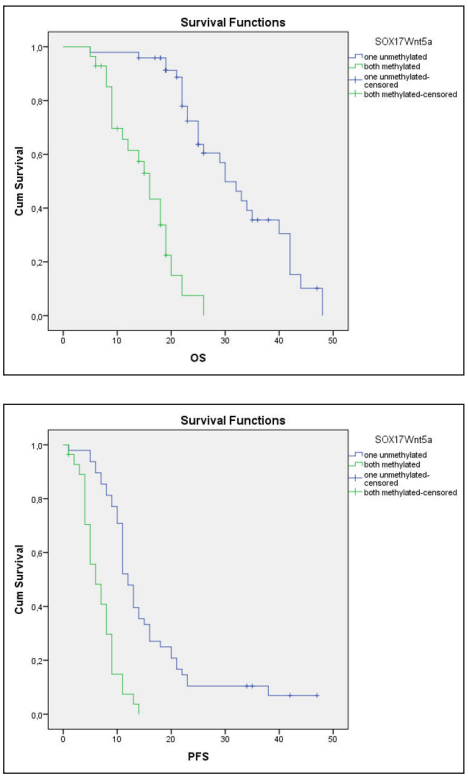

Figure 3: a) Overall survival and b) Progression-free survival of patients with metastatic colorectal cancer in relation of both *SOX17* and *WNT5a* methylation status.
OS: overall survival, PFS: progression-free survival.

Discussion

CRC remains a leading cancer-associated morbidity and mortality cause worldwide. The overall survival of CRC patients depends highly on the disease stage at diagnosis. The five-year survival estimates range from 85-90 % for patients with stage I to less than 10 % for patients with stage IV disease. Around half of all patients with CRC develop local recurrence or distant metastasis during their disease[22].

In recent times, abnormal DNA methylation patterns have emerged as potential candidate cancer biomarkers[23] to explore the molecular profile of tumorigenesis. Most of these studies reveal promising aspects that could impact the treatment of CRC patients in the future.

A tumor suppressor role is attributed to the *SOX17* gene through modulation of Wnt signaling. In the early stages of gastrointestinal tumorigenesis, *SOX17* is induced by Wnt activation; however, progressively, during malignant progression, *SOX17* is downregulated by methylation. Hence, *SOX17* appears to prevent the malignant progression at the early stage of tumorigenesis. Its methylation-dependent silencing is additionally supported by the fact that *SOX17* is commonly unmethylated in normal human tissues but highly methylated in various primary tumors, including CRC. Detection of aberrant promoter methylation of several Wnt inhibitors, including *SOX17* and *WNT5a*, at different stages and time points along CRC progression, supplies a significant increment of methylation from adenoma to carcinoma. Therefore, Wnt signaling levels may alter during the evolution of cancer due to simultaneous or alternative methylation of the genes involved in the pathway. Literature on *SOX17* promoter methylation status mainly includes studies performed at the primary tumor level. A previous study demonstrated that *SOX17* promoter methylation in cfDNA obtained from operable gastric cancer patients is associated with the clinical outcome[19]. Similarly, in other types of cancer, including breast cancer, *SOX17* has been found highly methylated in circulating tumor cells isolated from breast cancer patients and in corresponding cfDNA. Additionally, *WNT5A*, which is another canonical WNT inhibitor and noncanonical WNT member, was repeatedly methylated in the promoter region[24,25]. Consequently, expression of the *WNT5A* gene was downregulated or silenced, deteriorating or losing its antagonistic action against the anomalous activation of canonical WNT signaling.

The present study evaluated the frequency and potential prognostic significance of *SOX17* and *WNT5a* promoter methylation in circulating cfDNA from mCRC patients. *SOX17 and WNT5a* methylation was detected in 63.5 % and 45.8 % of the mCRC patients, respectively. These observations indicate that *SOX17* promoter methylation represents a frequent event in advanced disease. The observed high incidence of *SOX17* methylation may also be relevant to the critical role of Wnt/β catenin signaling pathway in CRC progression. We suggest that *SOX17* methylation and inactivation, along with a subsequent excessive Wnt/β catenin signaling, is a frequent but gradually occurring event that is noticeable at later disease stages, associating with a more aggressive tumor phenotype and a higher metastatic potential. This agrees with the observed significant association between unmethylated *SOX17* promoter status and better survival in mCRC. This survival difference was also seen in patients with unmethylated *WNT5a* gene. Patients with methylated *WNT5a* had a poorer prognosis than those with unmethylated one.

These data, in sum, demonstrate that *SOX17* and *WNT5a* are critical in controlling the signaling of the Wnt/β catenin pathway, and *SOX17* inactivation as a result of DNA methylation up-regulates this critical pathway, resulting in an accelerated disease progression. It is also demonstrated that the methylation of both genes gives prognostic solid results. Nevertheless, "liquid biopsy" could also be a potential surrogate for biomarker discov-

ery through the epigenetic approach.

We conclude that *SOX17* and *WNT5a* methylation is a frequent event in mCRC and, when present, is associated with bad prognosis, particularly in this group of mCRC patients. Further studies in a larger cohort of patients are required to explore and establish its prognostic significance further.

Conflict of interest

Authors declare no conflicts of interest.

In memoriam

This paper is dedicated to the memory of Professor Nikolaos Xenidis (1966-2022).

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