



# Prognostic Role of RASSF1A, SOX17 and Wif-1 Promoter Methylation Status in Cell-Free DNA of Advanced Gastric Cancer Patients

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## Abstract

Epigenetic modification of several genes is a key component in the development of gastric cancer. The methylation status of *RASSF1A*, *SOX17* and *Wif-1* genes was evaluated in the cell free circulating DNA of 70 patients with advanced gastric cancer, using methylation-specific PCR. Patients with higher cell-free DNA concentration seem to have lower PFS, than patients with lower cell-free DNA concentration ( $p = 0.001$ ). *RASSF1A* was the tumor suppressor gene, most frequently methylated in metastatic gastric cancer patients, followed by *SOX17* and *Wif-1* (74.3%, 60.0% and 47.1%, respectively). Patients having the *SOX17* promoter methylated, had lower progression free survival and overall survival, than unmethylated ones ( $p < 0.001$ ). Patients having the *Wif-1* promoter methylated, had lower progression free survival and overall survival, than unmethylated ones ( $p = 0.001$ ). Patients having the *RASSF1A* promoter methylated, had lower progression free survival and overall survival, than unmethylated ones ( $p = 0.004$ ). Promoter methylation of the examined genes was significantly associated with a decrease in progression free survival and overall survival, comparing to that of patients without methylation. Simultaneous methylation of the above genes was associated with even worse progression free survival and overall survival. The methylation of *RASSF1A*, *SOX-17* and *Wif-1* and genes, is a frequent epigenetic event in patients with advanced gastric cancer.

## Keywords

gastric cancer, methylation, *SOX17*, *Wif-1*, *RASSF1A*

## Abbreviations

cfDNA, Cell-free DNA; CTCs, Circulating tumor cells; *H. pylori*, *Helicobacter pylori*; MSP, Methylation specific PCR; OS, Overall survival; PFS, Progression free survival

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## Introduction

Gastric cancer is a very common and fatal cancer worldwide.<sup>1</sup> In 2020, in the United States it is estimated that there will be 27,600 new cases and 11,010 deaths from this disease.<sup>2</sup> At the early stages, in 25% of the cases the tumor is kept to the stomach, in 20% has already metastasized to the regional lymph nodes (locally advanced) and in 34% it has given distant metastases. This tumor occurs most often in the Asian and African-American tribes, more frequently in males. The average age of diagnosis is 69 years, with the highest percentage of new cases being diagnosed between 75-84 years. The overall 5-year survival of gastric cancer patients, is 27.7%. Over the past decades, there is a decline in incidence of the neoplasm, but the causes are not fully explained. The outcomes in advanced gastric cancer remain unfavorable, with a 5-year survival rate of 3.9%.<sup>3</sup> Several studies have shown that consumption of fruits and vegetables can decrease the risk of developing gastric cancer. On the other hand, increased salt intake, and preservatives of processed meats, such as nitrosamines, stimulate the development of gastric cancer.<sup>4</sup> *Helicobacter pylori*, a helical Gram-negative bacterium found in the gastric mucosa, seems to increase the risk of gastric cancer. On the other hand, the risk of gastric cancer is reduced by *H. pylori* eradication.<sup>5,6</sup> Inherited changes in gene expression, other than modifications in the DNA sequence are referred as “epigenetics.” One of the most frequent epigenetic events is DNA methylation. In cancer, although hypomethylation of several genes is common, promoter methylation of tumor suppressor genes, has been correlated with inactivation of micro-RNA encoding genes.<sup>7</sup> Hypermethylation of CpG islands is a critical event in cancer development.<sup>8</sup> It may alter the function of genes involved in DNA repair, and many other cell functions, leading to cancer development.<sup>9</sup> Hypermethylation can be seen at several stages of cancer development, in different cell types and is associated with genetic damage. Each tumor type has specific methylation profiles of CpG islands of the tumor suppressor genes. Moreover, each type of malignant tumor is determined by a specific and defined pattern of DNA hypermethylation.<sup>10</sup> Methylation of DNA has a clinical significance. Methylation markers are under investigation as diagnostic, prognostic and predictive tools.<sup>11</sup> These markers are determined in patient’s blood. In blood of cancer patients, the high concentration of cfDNA can be used as a marker for the determination of tumor origin.<sup>12,13</sup> Several studies have investigated the predictive role of cfDNA methylation in cancer patients.<sup>14</sup> Tumor-related DNA in the bloodstream may be either free or in circulating tumor cells (CTCs).<sup>15</sup> The origin and biologic significance remains largely unknown, cfDNA is a valuable source of biologic material for the development of sensitive and specific markers for the laboratorial diagnosis of several tumors.<sup>16</sup> Studies have shown that cfDNA carry epigenetic modifications similar to those present into the DNA of cancer cells from which they originated. This means that cfDNA is originated from the primary tumor.<sup>17</sup> In gastric cancer, these modifications play a critical role in tumor growth and progression, and include DNA methylation,

modification of histones, remodeling in chromatin and non-coding RNA creation. In contrast to classical genetic changes (mutations), epigenetic modifications do not affect the DNA sequence and could be reversible.<sup>18</sup> *SOX17*, *RASSF1A*, and *Wif-1* are 3 major tumor suppressor genes that are frequently methylated in patients with gastric cancer.<sup>19-21</sup> The *RASSF1A* protein correlates with the regulation of microtubules, genome stability, cell cycle, apoptosis, cellular mobility, and tumor infiltration.<sup>22-24</sup> Methylation level of this gene in patients with gastric cancer varies from 30-50%.<sup>25</sup> The *SOX17* gene (SRY-box containing gene 17) regulates the growth of bone marrow progenitor cells by suppressing the wnt signaling pathway. It appears that methylation of this gene activates the wnt signaling pathway. The *Wif-1* gene is one of the most important antagonists of the wnt pathway and is found methylated in human cancers.<sup>26</sup> In our study, we investigated the methylation status of these genes in the cfDNA of patients with metastatic gastric cancer. We also investigated the prognostic role and associations with clinicopathological parameters.

## Methods

70 blood samples obtained from patients suffering from metastatic gastric cancer. Additionally, 25 blood samples were used as a control group and were taken from healthy donors. The majority of them received no medical care at the time of the sample collection. There is a favorable opinion of Scientific Council (No.:EΣ7/ΘEηΔ48/12-10-2016), positive opinion of the Hospital Ethics and Ethics-Audit Committee on Clinical Studies, as well as positive opinion No.:Θ29/ΔΣ29/07.11.2016 of the Administrative Board of the hospital.

### Sample Collection and Isolation of cfDNA

We used plasma collection tubes. Then we centrifuged the whole blood at 3,000 rpm for 10 minutes, in order to obtain plasma and stored it at -80°C. cfDNA from plasma samples was isolated using QIAamp DNA Blood mini kit (Qiagen, Germany) according to the manufacturer’s protocol. We determined the concentration of cfDNA by a real-time PCR method using *GAPDH* gene as an amplifying target. We used 3 μl of DNA elution as a template for the Sybr-green based real time PCR analysis. Concentration of cfDNA was determined according to a reproducible standard dilution curves using a known concentration of MCF-7 genomic DNA.

### Sodium Bisulfite Conversion

We used sodium bisulfite (SB), in order to modify the extracted DNA and convert all unmethylated, but not methylated cytosines to uracils. Bisulfite conversion was carried out up to 500 ng of extracted DNA using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer’s instructions. We stored the converted DNA at -80 °C until use.

**Table 1.** Clinicopathological Characteristics and Correlation With the Methylation Status of Patients With Metastatic Gastric Cancer.

Patient characteristics	n (70)	SOX17 methylation	p value	RASSF1A methylation	p value	Wif-1 methylation	p value
Gender			0.561		0.256		0.356
Females	27	19		22		17	
Males	43	23		30		16	
Age			<b>0.037</b>		<b>0.041</b>		<b>0.049</b>
≤ 60 years	19	9		9		3	
> 60 years	51	33		43		30	
Differentiation			0.687		0.331		0.188
Intermediate	27	18		24		14	
Poor	26	16		20		10	
Good	17	8		8		9	
Histology			0.456		0.651		0.234
Intestinal	32	21		23		20	
Diffuse	23	13		18		9	
Other	15	8		11		4	

### Methylation Specific PCR (MSP)

We detected the methylation status of *SOX17*, *Wif-1* and *RASSF1A* genes in cfDNA samples was detected by real time methylation specific PCR (MSP). We used specific primer pairs for both the methylated and unmethylated promoter sequences. A Rotor Gene Q was used for amplification reactions. We provide the exact primer sequence for the methylated and unmethylated sequences:

Unmethylated

*RASSF1A* Forward 5'–GGT TGT ATT TGG TTG GAG TG 180

Reverse 3'–CTA CAA ACC TTT ACA CAC AAC A

Methylated

*RASSF1A* Forward 5'–GTT GGT ATT CGT TGG GCG C 160

Reverse 3'–GCA CCA CGT ATA CGT AAC G

The methylation status of *SOX17* gene in ctDNA was detected by a more sensitive real time MSP assay was used for the detection of the methylation status of *SOX17* gene in cfDNA, analytically validated in detail previously.<sup>21</sup> We used the same set of MSP specific primer pairs as previously described, while for detection we used the Sybr-Green dye, *SOX17* accession number: NT\_008183.

*SOX17*-M forward, 5'–CAAAAACGAATCCCGTATCC GACG-3';

*SOX17*-M-reverse, 5'–TTGCGTTAGTCGTTTGGCGTTC-3';

*SOX17*-U forward, 5'–CAAACCAAAAACAAATCCCA TATCCAACA-3';

*SOX17*-U reverse, 5'–GATTTTGTGTTAGTTGTT TGTGTTTG-3'

*Wif-1* methylation was detected using the following pair of primers, according to (33):

*Wif-1* unmethylated forward: GGGTGT TTTATTGGG TGTATTGT

Reverse: AAAAAA ACTAACACAAACAAAATACAAAC

*Wif-1* methylated forward: CGTTTTATTGGGCGTATCGT

Reverse: ACTAACGCGAACGAAATACGA

The *Wif1* promoter, which is located at chromosome position 12q14.2, was investigated according to the contig. ENSG00000156076 contained in the Ensembl database.

### Statistical Analysis

We used SPSS, version 21.0 (IBM) for the statistical analysis of our data. We expressed the methylation status of *SOX17*, *Wif-1* and *RASSF1A* genes, as well as all other qualitative variables as frequencies and percentages (%). We used chi-square test in order to evaluate any potential association of methylation status of the above genes with patients' demographic and clinicopathological characteristics. The Kaplan–Meier method, was used in order to calculate the survival rates. Log-rank and Breslow tests were used for the determination of the statistical difference between survival curves. The independent effect of *SOX17*, *Wif-1* and *RASSF1A* methylation status on overall survival, was determined with both multivariate Cox proportional hazards regression analysis. We also included patients' gender, age, differentiation, and histologic subtype in the multivariate model, as potential confounders. All tests were 2 tailed and statistical significance was considered for p values <0.05.

### Results

We evaluated the methylation status of tumor suppressor genes *RASSF1A*, *SOX17* and *Wif-1* in the cfDNA of 70 patients with advanced gastric cancer. The clinicopathological characteristics of patients are reported in Table 1.

### Correlation of cfDNA Concentration with PFS

The mean concentration of cfDNA in patient group was estimated to be 67.0 ng/ $\mu$ L, whereas the mean cfDNA concentration in the healthy control group (n = 25) was estimated to be 38.0 ng/ $\mu$ L. In patient group with cfDNA concentration  $\leq 50$  ng/ $\mu$ L, 16 subjects (22.8%) had PFS >6 months, while 2 subjects had PFS < 6 months (p = 0.001). In contrast, in patient group with cfDNA concentration >50 ng/ $\mu$ L, 27 subjects had PFS < 6 months, while 10 subjects had PFS >6 months (p = 0.001).

Correlation of promoter methylation status of *SOX17*, *Wif-1* and *RASSF1A* genes with clinicopathological parameters.

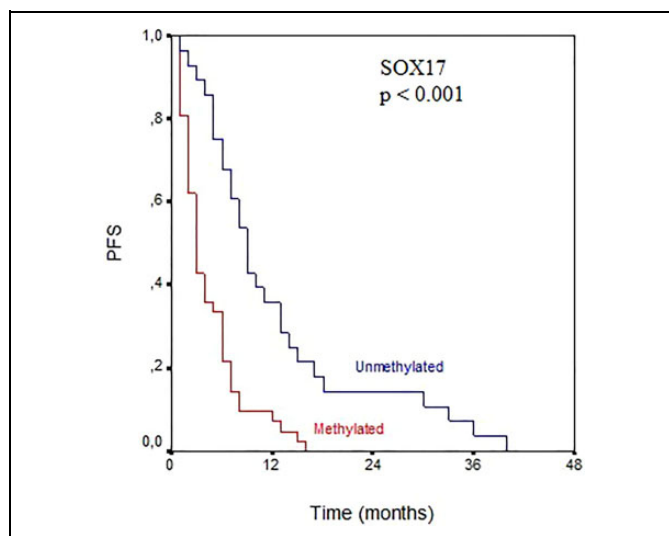
Most patients (51/70, 72.8%) were >60 years old and in the majority of them, the *SOX-17*, *Wif-1* and *RASSF1A* gene promoters were methylated (p = 0.037, p = 0.049, and p = 0.041, respectively). In particular, *SOX-17* gene promoter was found to be methylated in 33/51 patients >60 years old (64.7%), *Wif-1* gene promoter was methylated in 30/51 patients >60 years old (58.8%) and *RASSF1A* gene promoter was methylated in 43/51 patients (84.3%) >60 years old. Nineteen out of 70 patients (27.1%), were  $\leq 60$  years old. In these patients, the *SOX-17*, *Wif-1* and *RASSF1A* gene promoters were methylated in 9/19 (47.4%), 3/19 (15.8%) and 9/19 (47.4%), respectively. No statistically significant correlation of the methylation status of the above genes, was correlated with gender, histological type and differentiation of gastric cancer.

Methylation status of *SOX17*, *RASSF1A* and *Wif-1* gene promoters in patients with advanced gastric cancer.

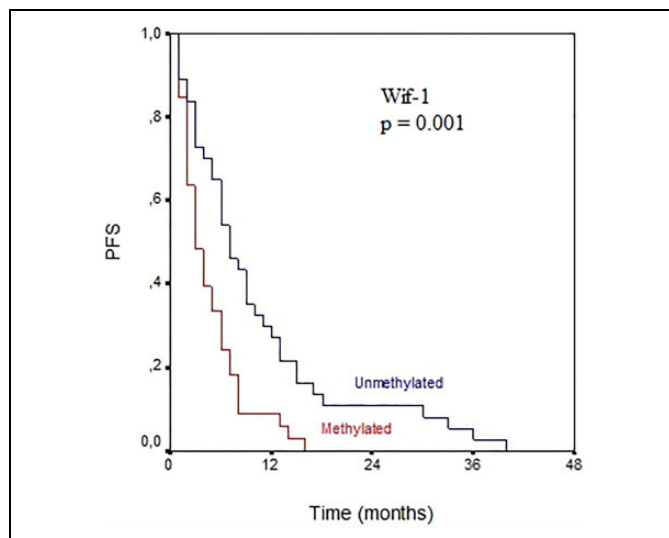
The promoter of *RASSF1A* gene was found to be methylated in 52 out of 70 patients (74.3%), the *SOX17* gene promoter was methylated in 42 out of 70 patients (60.0%), while *Wif-1* gene promoter was methylated in 33 out of 70 patients (47.1%). In healthy control group, no promoters from the 3 genes studied were found to be methylated (0.00%). Concomitant promoter methylation status, was also determined. The promoters of *SOX17+Wif-1* genes were methylated in 23 out of 70 patients (32.9%), the promoters of *SOX17+RASSF1A* genes were methylated in 38 out of 70 patients (54.3%) and the promoters of *Wif-1+RASSF1A* genes methylated in 27 out of 70 patients (38.6%). Finally, the promoters of all 3 *SOX17+Wif-1+RASSF1A* genes were methylated in 21 out of 70 patients (30.0%).

Correlation of methylation status of *RASSF1A*, *SOX17* and *Wif-1* gene promoters with PFS.

In patient group, those who had methylated *SOX17* gene promoter, showed significantly lower PFS (15.1 months) as compared to patients with unmethylated promoter (40 months, p < 0.001) (Figure 1). Patients with methylated *Wif-1* gene promoter, also had lower PFS (13.8 months) as compared to patients with unmethylated gene promoter (40 months, p = 0.001) (Figure 2). Patients with methylated *RASSF1A* gene promoter, also showed significantly lower PFS (17.9 months) as compared to patients in whom the gene promoter was unmethylated (40 months, p = 0.026) (Figure 3). Patients who had simultaneously methylated promoters of the

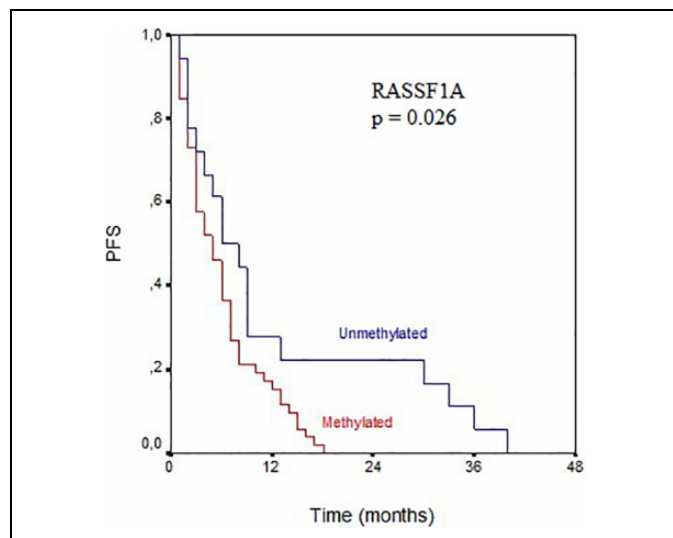


**Figure 1.** Correlation of *SOX17* promoter methylation with PFS in patients with advanced gastric cancer.

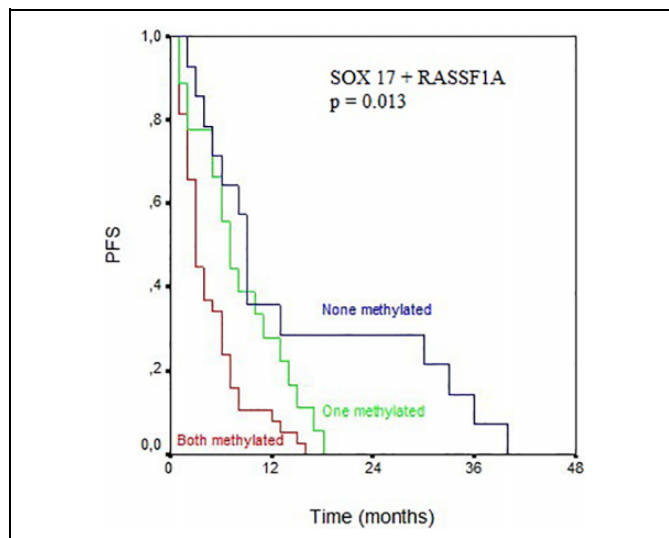


**Figure 2.** Correlation of *Wif-1* promoter methylation with PFS in patients with advanced gastric cancer.

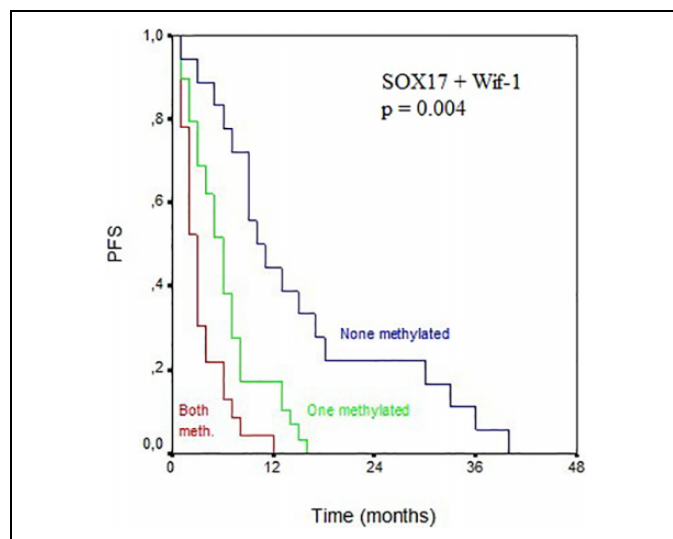
*SOX17+Wif-1* genes, had even lower PFS (12 months) compared to patients unmethylated promoters (40 months, p = 0.004) (Figure 4). Patients who had simultaneously methylated promoters of the *SOX17+RASSF1A* genes, also had lower PFS (14.8 months) compared to patients with unmethylated promoters (40 months, p = 0.013) (Figure 5). Patients who had simultaneously methylated promoters of the *Wif-1+RASSF1A* genes, also had lower PFS (14.5 months) compared to patients with unmethylated promoters (40 months, p = 0.144), but this was not statistically significant (Figure 6). Patients who had simultaneously methylated promoters of the *SOX17+Wif-1+RASSF1A* genes, had even lower PFS (11.9 months) compared to patients with unmethylated promoters, and this was statistically significant (40 months, p = 0.011) (Figure 7).



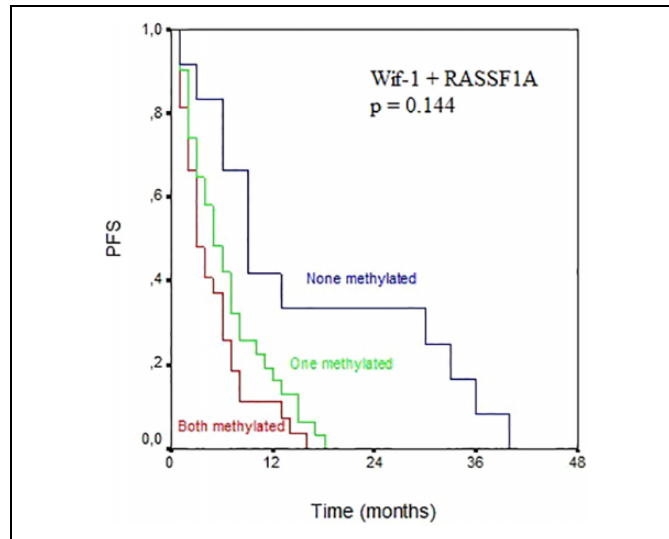
**Figure 3.** Correlation of *RASSF1A* promoter methylation with PFS in patients with advanced gastric cancer.



**Figure 5.** Correlation of *SOX17* + *RASSF1A* promoter methylation with PFS in patients with advanced gastric cancer.



**Figure 4.** Correlation of *SOX17* + *Wif-1* promoter methylation with PFS in patients with advanced gastric cancer.

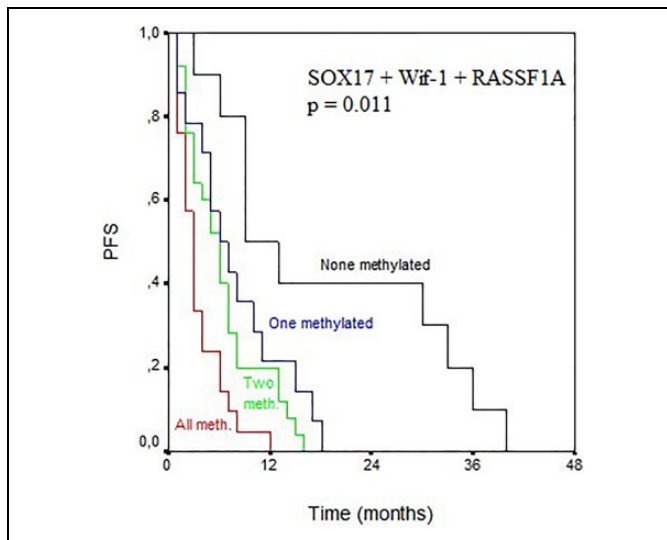


**Figure 6.** Correlation of *Wif-1* + *RASSF1A* promoter methylation with PFS in patients with advanced gastric cancer.

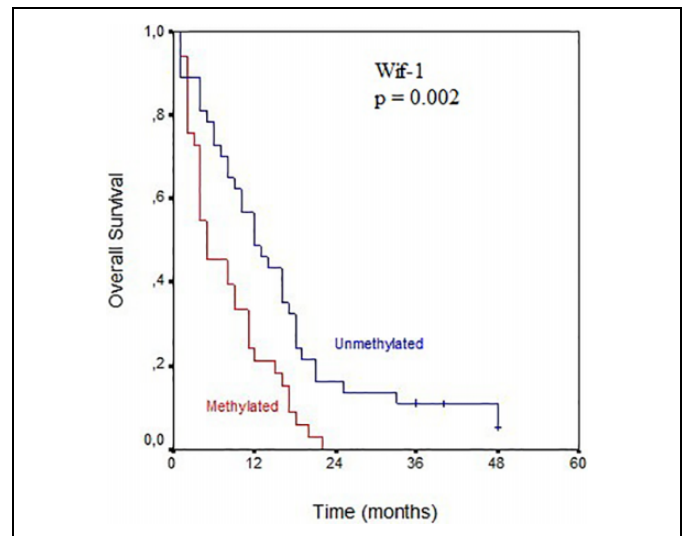
Correlation of methylation status of *RASSF1A*, *SOX17* and *Wif-1* gene promoters with OS.

In the patient group, those who had a methylated *SOX17* gene promoter showed significantly lower OS (25.1 months) as compared to patients with unmethylated gene promoter (48 months,  $p < 0.001$ ) (Figure 8). Patients with methylated promoter of the *Wif-1* gene showed significantly lower OS (21.9 months) as compared to patients with unmethylated promoter (48 months,  $p = 0.002$ ) (Figure 9). Patients with methylated promoter of the *RASSF1A* gene showed significantly lower OS (25.2 months) compared to patients with unmethylated promoters (48 months,  $p = 0.009$ ) (Figure 10). Patients who had simultaneously methylated promoters of the *SOX17*+*Wif-1* genes, had an even lower

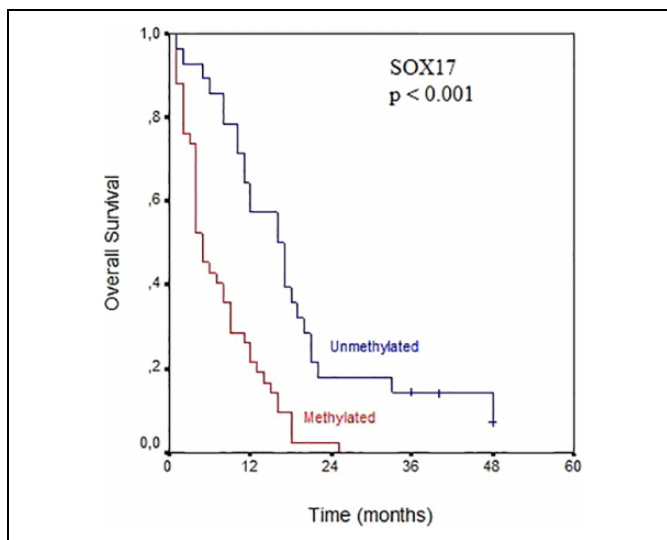
OS (15.7 months) compared to patients with unmethylated promoters (48 months,  $p = 0.005$ ) (Figure 11). Patients who had simultaneously methylated promoters of the *SOX17*+*RASSF1A* genes, had also lower OS (25.3 months) compared to patients with unmethylated promoters (48 months,  $p = 0.055$ ), although these results were not statistically significant (Figure 12). Patients who had simultaneously methylated promoters of the *Wif-1*+*RASSF1A* genes, had lower OS (19.2 months) compared to patients with unmethylated promoters (48 months,  $p = 0.037$ ) (Figure 13). Patients who had simultaneously methylated promoters of all 3 *SOX17*+*Wif-1*+*RASSF1A* genes, had lower OS (14.9 months) compared to patients with unmethylated promoters (48 months,  $p = 0.004$ ) (Figure 14).



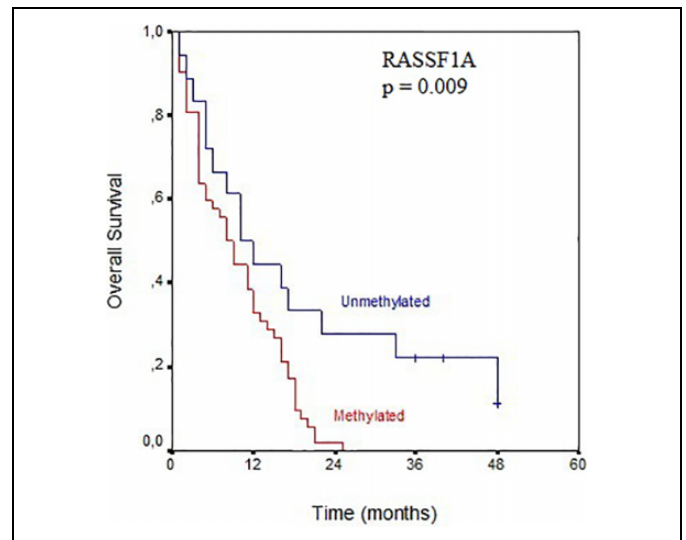
**Figure 7.** Correlation of *SOX17* + *Wif-1* + *RASSF1A* promoter methylation with PFS in patients with advanced gastric cancer.



**Figure 9.** Correlation of *Wif-1* promoter methylation with OS in patients with advanced gastric cancer.



**Figure 8.** Correlation of *SOX17* promoter methylation with OS in patients with advanced gastric cancer.



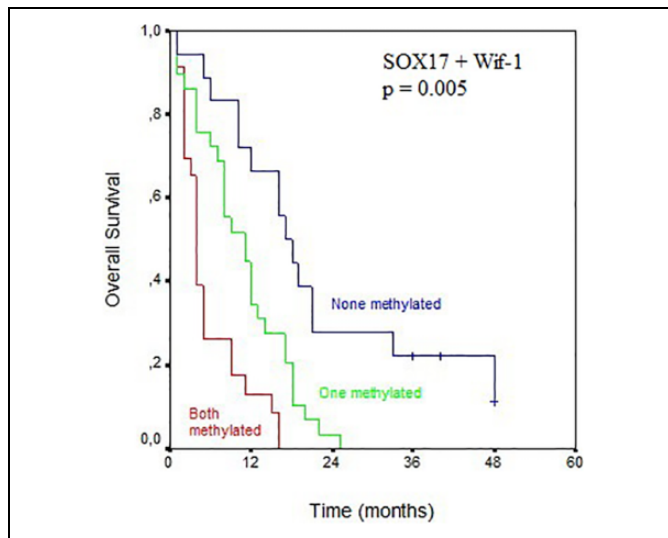
**Figure 10.** Correlation of *RASSF1A* promoter methylation with OS in patients with advanced gastric cancer.

## Discussion

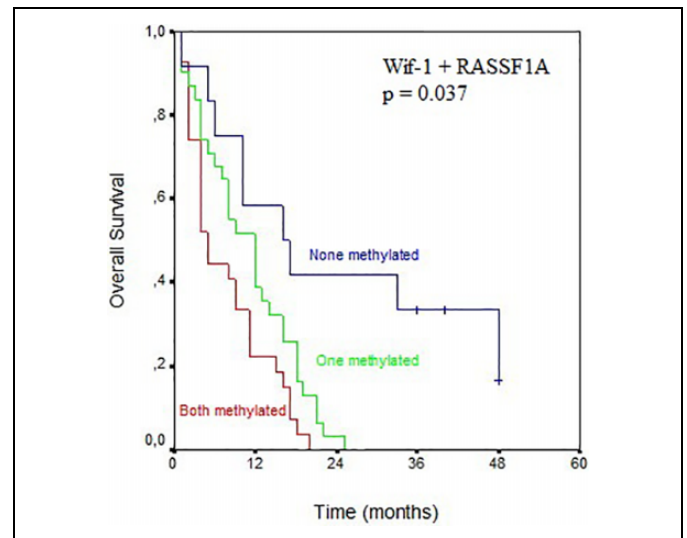
Gastric cancer represents a frequent and aggressive tumor. Although the incidence of the disease varies considerably across countries, it does not cease to be a public health problem worldwide. Its etiology has not yet been sufficiently elucidated. However, it is commonly accepted that this disease is a multi-stage process involving not only genetic, but also epigenetic modifications such as activation of oncogenes, growth factors and their receptors overexpression as well as the inactivation of tumor suppressor genes. Methylation of tumor suppressor genes, is an important mechanism in gastric cancer development, which involves several factors. Indeed, this procedure is

mediated by hypermethylation of their promoter region rather than through mutations. It is therefore understood that this frequent molecular event could be developed as a biomarker, for the early diagnosis and prognosis of gastric cancer.<sup>27</sup> cfDNA has recently evolved as a useful tool for the screening and follow-up of patients with different solid tumors, including gastric cancer.<sup>21</sup>

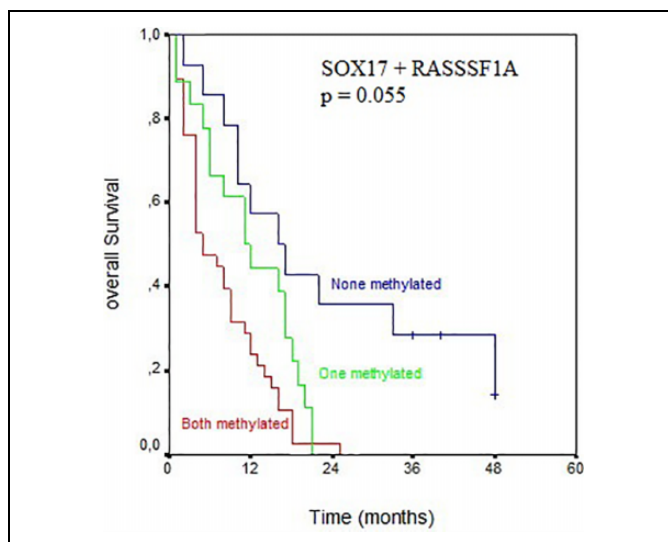
In this study, concentration of cfDNA was significantly higher (67.0 ng/ $\mu$ L) in patients than in healthy controls (38.0 ng/ $\mu$ L). It seems that cfDNA in healthy individuals reflects the amount of DNA entering the bloodstream after the normal procedure of apoptosis of several types of cells. The increased cfDNA concentration in patients with inoperable



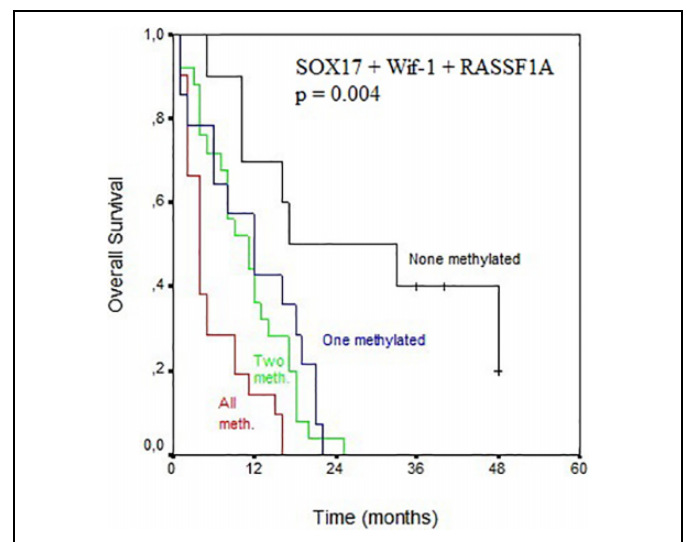
**Figure 11.** Correlation of *SOX17* + *Wif-1* promoter methylation with OS in patients with advanced gastric cancer.



**Figure 13.** Correlation of *Wif-1* + *RASSF1A* promoter methylation with OS in patients with advanced gastric cancer.



**Figure 12.** Correlation of *SOX17* + *RASSF1A* promoter methylation with OS in patients with advanced gastric cancer.



**Figure 14.** Correlation of *SOX17* + *Wif-1* + *RASSF1A* promoter methylation with OS in patients with advanced gastric cancer.

gastric cancer indicates that the amount of cfDNA is greater due to the additional destruction of cancer cells in this case, mostly due to progression of disease, resulting in additional amount of cfDNA to the bloodstream. Several studies have postulated that the release of cfDNA in this case can occur either by apoptosis, by necrosis or by active release of DNA from cancer cells or macrophages.<sup>28</sup>

We divided patients into 2 groups, based on the concentration of cfDNA detected in their plasma. In patients with a cfDNA concentration of  $\leq 50$  ng/ $\mu$ L, the PFS was  $>6$  months in 16 of them, and  $\leq 6$  months in 2 of them. In patients with a cfDNA concentration of  $>50$  ng/ $\mu$ L, PFS was  $\leq 6$  months in 27 of them, and  $>6$  months in 10 of them. These results indicate

that the amount of cfDNA in the plasma is directly correlated with PFS. Elevated concentration of cfDNA is correlated with a decrease in PFS. Thus, the measurement of cfDNA could serve as a potential prognostic biomarker for the disease.

In healthy controls, none of the studied genes were found to be methylated, as opposed to gastric cancer patients, where methylation of the gene promoter varied. This shows that methylation status of tumor suppressor genes is only seen in patients with cancer, suggesting that hypermethylation of the promoter region of the aforementioned genes could serve as a potential biomarker for the early diagnosis of advanced gastric cancer. In our study, *RASSF1A* gene was methylated in 52 out of 70 patients (74.3%). It seems that there is a high degree of

methylation of this gene in advanced gastric cancer. This suggests a crucial role of this gene in early and late stages of gastric carcinogenesis. A protein similar to the RAS family of effector proteins is encoded by this gene, acting as tumor suppressor and its methylation is correlated with the development of gastric cancer. A more aggressive tumor phenotype is probably associated by methylation of the gene. This finding is in agreement with similar findings from other studies.<sup>29</sup> An important correlation between *RASSF1A* hypermethylation and OS as well as PFS of patients, was seen. The findings are also in agreement with other studies.<sup>30,31</sup> In these studies, the incidence of hypermethylation of the *RASSF1A* gene promoter in the serum of gastric cancer patients was 25% and 34% respectively, far below the level observed in our study (74.3%). It seems that gastric cancer is a different disease in Europe and Asia, and this could explain the variations between studies, due to national, environmental and/or geographic factors. In addition, the number of 70 samples in our study, is relatively small in order to draw safe conclusions. Regarding *SOX17* gene, it was found to be methylated in 42 out of 70 patients (60.0%), indicating its key role in gastric carcinogenesis. There were no significant correlations with other oncological parameters for this particular gene. Survival analysis showed that methylated promoter of this gene is correlated with a worse prognosis. Indeed, overall survival in patients with methylated gene promoter was 25.1 months, whereas OS in patients with unmethylated gene promoter was 48 months. These differences in OS are most likely correlated with the inactivation of the *SOX17* gene that occurs through methylation. Similar conclusions can be drawn for the *Wif-1* gene that was found to be methylated in 33 out of 70 patients (47.1%). In this case, OS in patients with the methylated gene promoter was 21.9 months, while OS in patients with an unmethylated promoter was 48 months. Patients who had simultaneously methylated promoters of the above genes (in pairs), had lower PFS and overall survival compared to patients with unmethylated genes. Patients who had simultaneously all the above methylated promoters had even lower PFS and OS. It seems that all these genes are involved in gastric carcinogenesis each one to a significant extent and their simultaneous inactivation by methylation in the final stages of cancer is a frequent event, which associates with a worse prognosis. The exact mechanism by which the methylation of the above genes, is associated with a decrease in OS and PFS, should be further investigated.

We have tried to correlate the methylation status of these genes with all the clinical characteristic of the patients. As it is known from the literature, increasing evidence suggests that most epigenetic alterations are generated in a programmed manner and occur in a subpopulation of tissue cells during normal aging, probably predisposing them for tumorigenesis.<sup>32</sup>

Hypermethylation of tumor suppressor genes *SOX17*, *RASSF1A*, and *Wif-1* is a frequent finding in metastatic gastric cancer patients. In the study, there was also an important correlation between methylation of these genes and the PFS and OS of the disease. The sample size of our study is relatively small, so it is difficult to draw general conclusions. However,

there is a tendency for the correlation of the methylation status of these genes and the prognostic significance, which has been described in the manuscript. Further studies are needed so that methylation of tumor suppressor genes becomes a potential biomarker in patients with advanced gastric cancer.

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