SMAD4 Gene Methylation May Be Effective in Adenocarcinoma Lung Cancers

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ABSTRACT

Objective: In this study, we aimed to investigate the effect of promoter region methylation of *small mothers against decapentaplegic 4 (SMAD4)* gene in adenoma type lung cancer cases. Adenocarcinoma and squamous type carcinomas are the most common types of lung cancer. *SMAD4* gene is an intracellular signal protein. The protein of this gene, which is one of the transcription factors, functions in tissue homeostasis during embryonic development and has effects in the cancer process.

Methods: In this retrospective study, a total of 40 samples including 20 paraffin-embedded tumor tissues of 20 patients with adenocarcinoma lung cancer and normal lung tissue of the same patients were included. After DNA isolation from this paraffin-embedded adenocarcinoma lung tumor tissue and its normal counterparts, methylation specific polymerase chain reaction followed by agarose gel imaging methods were applied to investigate the *SMAD4* promoter methylation after bisulfite modification.

Results: As a result of our study, an increased presence of methylation in the promoter region of the *SMAD4* gene in the tumor tissue of a total of 12 (60%) of 20 adenocarcinoma cases compared to normal tissue was detected. A statistically significant increase in methylation rate of approximately 25-45% was found in tumor tissues of these cases compared to normal tissues ($p < 0.05$).

Conclusion: As a result of our study, we suggested that *SMAD4* gene methylation may be a tumor marker for lung cancers and may contribute to the development of cancer by inhibiting *SMAD4* protein expression by gene methylation and disrupting the intracellular signal pathway.

Keywords: *SMAD4*, DNA methylation, lung cancer, adenocarcinoma

INTRODUCTION

Lung cancer is the first cancer to cause death in men and the second cancer in women among all types of cancer. Approximately 1.3 million people die from lung cancer each year in the world. However, with the newly developed lung cancer treatment methods, the average life span and quality have started to increase relatively (1). Approximately 80% of lung cancers with a high mortality rate worldwide are reported as non-small-cell lung cancer (NSCLC). NSCLC have two subtypes, adenocarcinoma and squamous,

and are classified histopathologically as adenocarcinoma and squamous cellular carcinoma. Adenocarcinomas and squamous carcinomas represent approximately 50% of all NSCLC (1,2).

Small mothers against decapentaplegic 4 (*SMAD4*) is a gene that synthesizes a protein product that carries chemical signals from the cell surface towards the nucleus. This signaling pathway works through the transforming growth factor (TGF)-pathway and can be affected by the cell's peripheral environment. Signal formation begins with the binding of TGF-beta (TGF- β) protein to the corresponding receptor on the cell surface, and this event provides

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the activation of SMAD group proteins (3). SMAD proteins bind to SMAD4 to form a protein complex, and this new complex that is formed transmits the signal by moving towards the cell nucleus. It also controls the growth and activation of tumor suppressor genes in the nucleus (4).

Methylation is a process characterized by the addition of a methyl group to the 5-carbon cytosine in the CpG nucleotide sequences, particularly in the promoter regions of genes. Studies have shown that a significant portion of mammalian genomes are methylated (5,6). Methylation reactions in genes affect gene expression and therefore genes can be inactivated or activated. In this study, in order to determine the contribution of the *SMAD4* gene to the formation of lung cancers, we aimed to show the changes in the stage of transformation from normal tissue to tumor by determining the potential cellular methylation in the promoter region of normal and tumor tissues in adenoma type lung cancers with methylation specific polymerase chain reaction (MSP) method. In this respect, our study is the first study in the literature.

METHODS

Our study is a cross-sectional retrospective study and patient consent was not obtained. For this study, 10 histologically confirmed advanced stages (stage 3); 5 sections from early stage (stage 1) and middle stage (stage 2) lung tumor tissues with adenocarcinoma and 5 sections from the outermost parts of the surgical margins as normal lung tissue of the same cases, (10 pieces per case) a total of 200 slice slide tissue samples belonging to 20 cases were prepared in formalin-fixed paraffin-embedded tissue blocks (7). The clinical characteristics of the patients are shown in Table 1. The number of samples to be studied was determined by power analysis, and all cases used in this study were used in the paraffin tissue archive of patients diagnosed with lung cancer between 2007-2018 in Trakya University Pathology Department. Pathological evaluation of all tissues was made in pathology department. This study was conducted with the permission of the Trakya University Local Ethics Committee (approval number: 05/21, approval date: 27.02.2013).

Genomic DNA Isolation and Bisulfite Modification from Paraffin Embedded Tissues

As stated before, sections were obtained from selected tissues and were scraped from the slides into tubes and their DNAs were isolated as stated in the literature (8). These DNAs were then analyzed by spectrophotometric method after determining the amount of DNA at 280 nm and 260 nm wavelengths; and samples were stored at +4 °C for further analysis. Bisulfite modifications of DNAs were made in accordance with the EpiJET Bisulfite Conversion Kit and Protocol (Thermo Fisher Scientific-USA) (7,8).

Methylation Analysis of the SMAD4 Promoter

With the sodium bisulfite modification of genomic DNA, all unmethylated cytosines in DNA are converted to thymine, but this reaction does not affect cytosines in methylated state, creating a potential sequence difference. After this chemical modification of

DNA, DNA samples were used for methylation analysis of *SMAD4* gene. The MethPrimer V1.1 beta program was used (available at www.urogene.org) to identify potential methylation sites and methylation-specific primer sequences for the *SMAD4* gene sequence (9).

Methylation Specific Polymerase Chain Reaction Method

The methylation specific primers for the promoter of the *SMAD4* gene region are as follows: Forward 5': GTAATAATACGGTTTGGTCGTC-3', Reverse: 5'-TCCCACCCCC TAAACGACCGCG-3', product size: 164 base pair (bp), Tm: 77.4 °C, *SMAD4* gene specific primers of unmethylated used for the promoter region were as follows: Forward: 5'- GTAATAATATGGTTTGGTTGTT-3', reverse: 5'-CTCCCACCCCCTAAACAACCACA-3', product size: 163 bp, Tm: 72.3 °C. The polymerase chain reaction (PCR) conditions have been created from 95 °C 45', 55 °C 30', 72 °C 30'x35 cycles following initiation for 10' at 95 °C and finally 5' termination at 72 °C. Methylated and unmethylated PCR media; PCR buffer 1x, MgCl₂: 2 mM, DMSO: 5% (v/v), dNTP: 12.5 mM, primer forward: 10 nM, primer reverse: 10 nM, taq polymerase: 1U (5U/μL). Template DNA 100 ng was completed with up to 50 μL of dH₂O. Methylated and unmethylated human DNAs were used as positive and negative control DNAs (S8001 | CpGenome™ Human Methylated and Unmethylated DNA Standard Set). These DNAs were bisulfite modified before PCR. PCR with these modified DNA samples was performed using methylation-specific and non-methylation-specific primers. Later, PCR products were evaluated for 2% agarose gel under ultraviolet light (9), tumors and normal tissues were compared for methylation, and methylation rates were determined.

Statistical Analysis

Statistical analysis of the study was performed using the SPSS 20 program (IBM Corp, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation and qualitative variables as percentages. Statistical analysis of methylation specific PCR results was performed and the results of the experimental and control groups were compared with the χ^2 test, $p < 0.05$ was considered statistically significant (10).

RESULTS

1-3 μL of bisulfite modified DNA was used for each methylated and un-MSP. MSP and un-MSP products were stained with ethidium bromide and evaluated on a 2 percent agarose gel (Figure 1 A-B). The expected band size in the promoter of the *SMAD4* gene was 163-164 bp for MSP and un-MSP (Figure 1) (11). As a result, *SMAD4* promoter methylation was present in approximately 12 (60%) of adenocarcinoma tumors and un-methylation was 3-10%, whereas in normal lung tissues, 70% un-methylation and approximately 20% methylation were observed ($p < 0.05$). When evaluated according to tumor stages, 40% of the first stage tumors (in 4 samples), 87.5% of the second stage tumors (6 samples) and 66%

of the third stage tumors (in 2 samples) were observed to increase in methylation (Table 1).

DISCUSSION

The *SMAD4* gene, located on chromosome 18q21, is known somatically as a candidate tumor suppressor gene in many pancreatic and colorectal tumors. It has been shown that changes in the *SMAD4* gene that cause it to inactivate by mutation mechanisms such as deletion have been shown to be effective in pancreatic and colorectal cancers (11). For these reasons, *SMAD4* methylation has been studied primarily in colorectal and pancreatic cancers. MSP is the main method used to investigate

gene methylations and has been used successfully in many cancer and other disease groups (12). Since there is no study in the literature showing the *SMAD4* methylation status in lung cancers, we examined the *SMAD4* promoter region using the MSP method. *SMAD4* may be a good chemotherapeutic option, especially because of its role in transcriptional function (13). There is a relationship between *SMAD4* expression and associated TGF- β and increased functional gene expression and activation of oncogenesis signaling pathways, especially in cancer development (10,11). Our research has shown that *SMAD4* methylations can be seen in lung adenocarcinoma. Perhaps in this way, it could be one of the reasons for *SMAD4* gene inactivation and, in conjunction with other active genes, reduced life expectancy in lung cancers. There is limited information on this subject in the literature. In the light of this information, it shows the potential importance of activating or inactivating oncogenesis pathways in cancer treatments. Studies examining both epigenetic properties of this gene and explaining epigenetic inhibition mechanisms may be interesting in the search for new cancer chemotherapy agents (10,11,14).

However, even from this aspect alone, our study showed that *SMAD4* methylation occurs in one of the most common lung cancer types, namely adenocarcinoma lung cancers. Our study is a rare study showing that *SMAD4* promoter methylation may be associated with adenocarcinoma lung cancers.

Study Limitations

The fact that *SMAD4* gene methylation and *SMAD4* gene expression in RNA and protein levels could not be studied in tumor and normal lung tissues in our study is a limitation.

CONCLUSION

More research is needed on gene expression and pathways for *SMAD4* in lung cancers. In the near future, the methylation sites of *SMAD4* promoters promise to be therapeutic targets in lung cancers and other types of cancer where this gene is known to play a role. We believe that this may increase the effectiveness of available cancer treatments and have positive effects on life expectancy/quality of life for cancer patients.

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Ethics Committee Approval: This study was conducted with the permission of the Trakya University Local Ethics Committee (approval no: 05/21, approval date: 27.02.2013).

Informed Consent: Our study is a cross-sectional retrospective study and patient consent was not obtained.

Peer-review: Externally peer-reviewed.

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Figure 1. A. Un-methylation specific polymerase chain reaction (PCR) result for *SMAD4* gene, line 1: M: 100 bp marker, line 2: (+) control DNA, normal and tumor pairs for each case line 3-11. B. Specific PCR results for *SMAD4* gene methylation, line 1: M: 100 bp marker, line 2: (+) control DNA, normal and tumor pairs for each case line 3-11
N: normal, T: tumor

Table 1. Clinical characteristics of patients with adenocarcinoma

	Normal case (n=20)	Methylation normal tissue	Tumor case (n=20)		Methylation tumor tissue
	n (%)	n (%)	n (%)	%	n (%)
Age, year (Mean $\pm$ SD)	58 $\pm$ 7	-	-	-	-
$\leq$ 53	9 (45)	-	9 (45)	45	-
>59	11 (55)	-	11 (55)	55	-
Gender					
Female	5 (25)	-	5 (25)	25	-
Male	15 (75)	-	15 (75)	75	-
Phase					
1	-	-	10	50	4 (40)
2	-	-	7	35	6 (85.7)
3	-	-	3	15	2 (66.6)

SD: standard deviation

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