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Molecules and Cells

Hypomethylation of the Thymosin β10 Gene Is Not Associated with Its Overexpression in Non-Small Cell Lung Cancer

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Lung cancer is the leading cause of cancer-related deaths worldwide and is usually associated with a late diagnosis and a poor prognosis. Thymosin β10 (*TMSB10*) is a monomeric actin sequestering protein that regulates actin cytoskeleton organization. The aberrant *TMSB10* expression has been implicated in the pathogenesis of human cancers. However, its role in carcinogenesis is still controversial. To better understand the role of *TMSB10* in lung tumorigenesis and its regulatory mechanism, we examined the methylation status and expression of the *TMSB10* gene in non-small cell lung cancers (NSCLCs) using methylation-specific PCR (MSP) and immunohistochemistry (IHC), respectively. MSP analysis showed that the *TMSB10* promoter was already unmethylated in most tumor tissues and became demethylated in 20 (14.4%) of the 139 NSCLCs. *TMSB10* hypomethylation was not significantly correlated with the clinicopathological features. IHC showed that the TMSB10 protein was strongly expressed in the cytoplasm of malignant cells and its overexpression was detected in 50.0% of the tumor tissues compared to normal tissues. TMSB10 overexpression was frequently observed in squamous cell carcinomas compared to adenocarcinomas with border line significance (*P* = 0.072). However, *TMSB10* methylation status was not linked to its overexpression. Collectively, these results suggest that *TMSB10* hypomethylation may be a frequent event in NSCLCs, but it may not be a common mechanism underlying TMSB10 overexpression. However, further studies with large numbers of patients are needed to confirm our findings.

INTRODUCTION

Epigenetic gene silencing constitutes an alternative or complementary mechanism to mutational events in tumorigenesis (Baylin and Herman, 2000; Jones and Baylin, 2002). An emerging picture of genetic and epigenetic changes and their relationship is unraveling the biological networks responsible for human cancer. Several genes are commonly the target of promoter methylation in lung cancer (Belinsky, 2004; Kim et al., 2007; Tsou et al., 2002). The predisposition of smokers to the acquisition of epigenetic alterations in key cellular regulatory genes (Alberg et al., 2005), suggests that DNA methylation could serve as a biomarker for the earliest stages of preinvasive lung cancer related to tobacco smoking, a major etiological factor. Detailed delineation of methylation alterations are needed for early detection and more effective therapy.

Thymosin β10 (TMSB10), a member of the β-thymosin family, is the main intracellular G-actin-sequestering protein involved in cell motility (Erickson-Viitanen et al., 1983). Importantly, TMSB10 may be correlated with tumor biology such as cell proliferation, apoptosis, angiogenesis, and metastasis behavior (Sribenja et al., 2009). Elevated *TMSB10* expression has been associated with various human tumors such as pancreatic, colon, breast, thyroid, and gastric cancer (Santelli et al., 1999; Sribenja et al., 2009). Similarly, several reports have shown the correlation of *TMSB10* expression level with progression and metastasis as well as poor patient outcome (Califano et al., 1998; Gu et al., 2009; Liu et al., 2004). Unlike its proposed oncogenic role, TMSB10 has been found to be down-regulated in prostate cancer (Cho-Vega et al., 2007) and there are contradicting findings regarding *TMSB10* expression in ovarian and lung cancers (Gu et al., 2009; Lee et al., 2001; McDoniels-Silvers et al., 2002; Santelli et al., 1999). Moreover, it is not clear whether TMSB10 can inhibit or promote tumor growth (Sribenja et al., 2009). Although most studies indicate that TMSB10 levels are mainly regulated at the transcriptional stage (Santelli et al., 1999; Sribenja et al., 2009), the detailed regulatory mechanisms are largely unknown. In order to understand the potential role of the *TMSB10* gene in lung cancer and the molecular mechanisms of gene regulation, we evaluated the methylation status of the promoter region and the expression of the *TMSB10* gene in non-small cell lung cancers (NSCLCs).

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MATERIALS AND METHODS

Patients and tissue samples

Tumor and corresponding nonmalignant lung tissue specimens were provided by the National Biobank of Korea - Kyungpook National University Hospital (KNUH), which is supported by the Ministry of Health, Welfare and Family Affairs. All materials derived from the National Biobank of Korea-KNUH were obtained under Institutional Review Board approved protocols. The clinical and pathological characteristics of the patients were previously described (Kim et al., 2009). All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and rapidly frozen in liquid nitrogen and stored at -80°C until genomic DNA preparation. Only samples that were macroscopically > 80% tumorous were sent for DNA extraction and methylation analysis. Macroscopically normal lung tissues were confirmed to be normal by hematoxylin-eosin staining.

Cell culture and 5-aza-2′-deoxycytidine (5-AzadC) treatment

A normal human lung epithelial cell line (BEAS2) and 6 human NSCLC cell lines, 3 adenocarcinomas (AC) (H522, H1793, and H2009) and 3 squamous cell carcinomas (SCC) (H157, H226, and H1703), were obtained from the American Type Culture Collection (ATCC, USA). All cells were propagated with the instructions from the ATCC. H226 cells were treated with 20 μM 5-AzadC for 3 days and the culture media was changed daily.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cultured cells using TRIzol (Invitrogen, USA) according to the manufacture's instructions. The structural integrity of the total RNA was confirmed by electrophoresis on 1.2% agarose-formaldehyde gels. Residual genomic DNA was digested with RNase-free DNase (Invitrogen). First strand cDNA was reverse-transcribed from 2 μg of total RNA in a total volume of 20 μl using oligo(dT) and a SuperScript preamplification kit (Invitrogen). The resulting cDNA was amplified by forward (5′-GCTCGGAACGAGACTGCACGG-3′) and reverse (5′-CAGTGCAGCTTGTGGCTCGT-3′) primers. Amplified products were separated on 2% agarose gels, visualized using ethidium bromide, and photographed.

Genomic DNA isolation and methylation analysis

Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, USA). After the treatment of the genomic DNA with sodium bisulfite, the methylation status of the *TMSB10* gene was analyzed by using a methylation-specific PCR (MSP) with primers specific for either unmethylated or methylated alleles (Kee et al., 2007). The primer sequences of the *TMSB10* were for the methylated reaction 5′-GAGTTGGGGGTGTTCGGC GC-3′ (forward) and 5′-CCCTAACCTTATATACGCCGCG-3′ (reverse), and for the unmethylated reaction 5′-GAGTTGGG GGTGTTTGGTGT-3′ (forward) and 5′-CCCTAACCTTATATA CACCACA-3′ (reverse). All PCR amplifications were carried out using reagents supplied in a GeneAmp DNA Amplification Kit with AmpliTaq Gold as the polymerase (PE Applied Biosystems, USA) on PTC-100 (MJ Research, USA). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, USA) were used as a positive control for the methylated and unmethylated genes, respectively. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Each MSP was repeated at least once to confirm the results.

Immunohistochemistry (IHC)

Tissue sections (3 μm) were cut from the paraffin block and mounted on gelatin-coated slides. The sections were deparaffinized in xylene and rehydrated by passing through a graded series of alcohol, and then endogenous peroxidase was blocked by incubating them with 0.3% H_2O_2 for 10 min. Antigen retrieval was carried out by microwave-heating for 10 min in citrate buffer (pH 6.0). After blocking with a protein blocking agent, sections were incubated with a TMSB10 primary antibody (gift from Evangelia Livaniou, 1:100 dilution) against the synthetic C-terminal fragment [38-43] of TMSB10 at 4°C overnight (Leondiadis et al., 1996). An UltraTech HRP Streptavidin-Biotin Detection System (Beckman Coulter, USA) was used to visualize the antibody binding, and the sections were counterstained with hematoxylin. A percentage of nuclei staining and the staining intensity in each section were estimated by a pathologist unaware of the clinical data.

Statistical analysis

The relationships between the methylation and expression of the *TMSB10* gene and the clinicopathological characteristics were analyzed using a chi-square test for categorical variables. Overall survival (OS) was measured from the day of surgery until the date of death or to the date of the last follow-up. The survival estimates were calculated using the Kaplan-Meier method. The differences in OS across different groups were compared using the log-rank test. The values of $P < .05$ were considered statistically significant. All analysis were performed using the Statistical Analysis System for Windows, version 9.1 (SAS institute, USA).

RESULTS

Hypomethylation of *TMSB10* gene in NSCLC and its correlation with clinicopathological features

We determined the methylation frequency of the *TMSB10* gene in 139 resected NSCLCs and their corresponding nonmalignant lung tissues. A long CpG island (CGI) was found in the 850-bp 5′ flanking region of the *TMSB10* gene including the first exon (ENSG 34510) (Fig. 1A). We designed the MSP primer pairs to target a region covering the transcription start site and minimal promoter linked with expression by using a web-based MethPrimer program. The allele-specific primer set yielded a single band at the expected size, and representative examples of the MSP analysis are illustrated in Fig. 1B. Bisulfite sequencing of PCR products confirmed their methylation statuses and showed that all cytosines at the non-CpG sites were converted to thymine (data not shown). Unmethylated (U) bands were detected in both nonmalignant and malignant tissues, thus confirming the integrity of the DNA in these samples. In addition, methylated (M) bands were detected in 20 (14.4%) and 51 (36.7%) samples of the malignant and matching normal tissues, respectively (Table 1), indicating *TMSB10* gene to be highly hypomethylated in NSCLCs. However, there is no significant association of *TMSB10* hypomethylation with clinicopathological features such as age, smoking status, histologic type, pathologic stage, and *EGFR* mutation status in the NSCLC (data now shown). In addition, *TMSB10* hypomethylation did not significantly associate with the OS of the patients (Fig. 2A). However, despite the lack of statistical significance due to the small number of samples, there was a trend that patients with hypomethylated *TMSB10* showed worse survival than those

Table 1. Summary of methylation status change of *TMSB10* gene in NSCLC

Normal tissue	Tumor tissue	No. of cases
Methylation	Methylation	20 (14.4%)
Methylation	Unmethylation	31 (22.3%)
Unmethylation	Unmethylation	88 (63.3%)

with methylated one (Fig. 2B).

Overexpression of *TMSB10* protein in NSCLC and no association with its methylation status

We examined the expression levels of the TMSB10 protein in 30 representative tissue samples using the IHC method. A strong cytoplasmic staining of TMSB10 was observed in most tumor tissues, while no or weak staining of TMSB10 was observed in the cytoplasm of normal tissues (Fig. 3). Consequently, in 15 cases TMSB10 expression was higher in tumor cells compared to the corresponding normal tissues (Fig. 3 and Table 2). In addition, although no significant correlations were

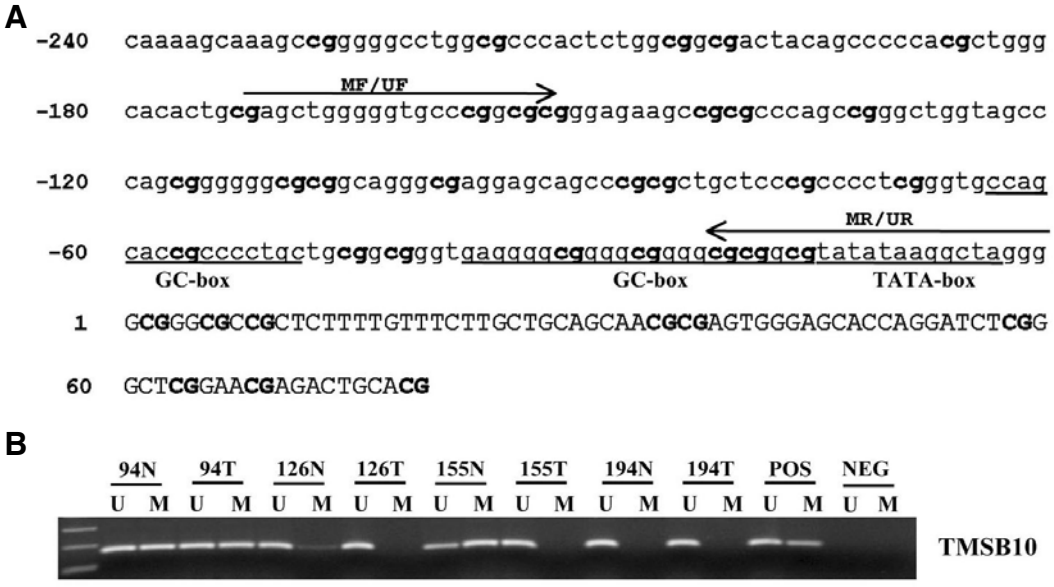

Fig. 1. Promoter sequence of *TMSB10* gene spanning positions -240 to +80 with respect to the transcription start site (A) and representative results of MSP analysis of *TMSB10* gene in NSCLC patients (B). (A) The promoter sequence and 5′-untranlated region are shown in small and capital letters, respectively. Transcription start site is numbered +1. TATA and GC box are underlined. Thirty-seven CpG sites are indicated in bold. The primers used for MSP are indicated by arrows. MF, forward, methylation-specific primer; MR, reverse, methylation-specific primer; UF, forward, unmethylation-specific primer; UR, reverse, unmethylation-specific primer. (B) The methylation status of *TMSB10* gene was analyzed by a MSP. CpGenome™ Universal methylated or unmethylated DNA was used as a positive control for the methylated or unmethylated products, respectively. N, adjacent normal tissues of lung cancer; T, tumor tissues of lung cancer.

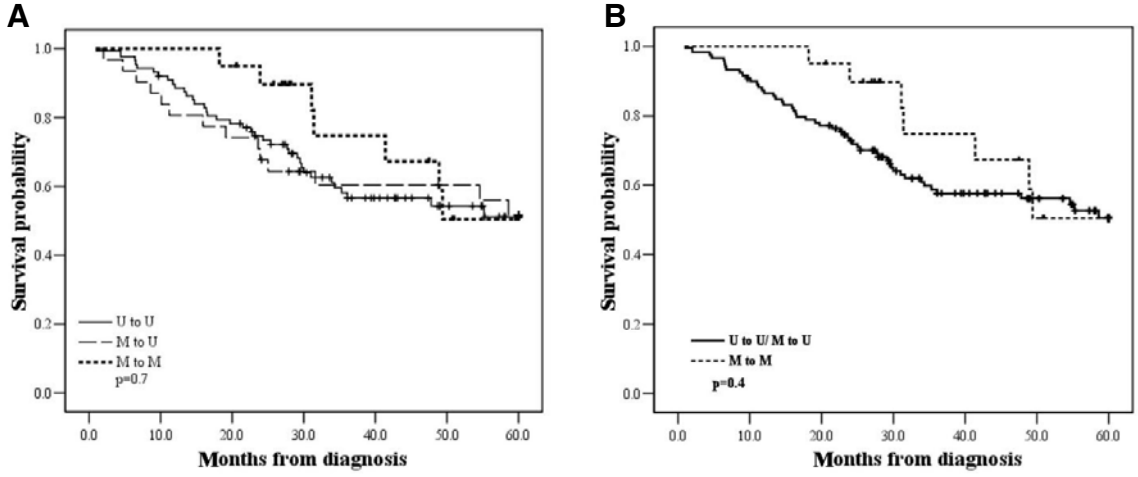

Fig. 2. Overall survival (OS) of 139 NSCLC patients according to *TMSB10* methylation status. (A) Survival curve in three groups. U to U, unmethylation status in the matching normal and malignant tissues. M to M, methylation status in the matching normal and malignant tissues. M to U, methylation in the matching normal shifted to unmethylation status in the malignant tissues. (B) Survival curve in two groups. U to U and M to U were combined into one group.

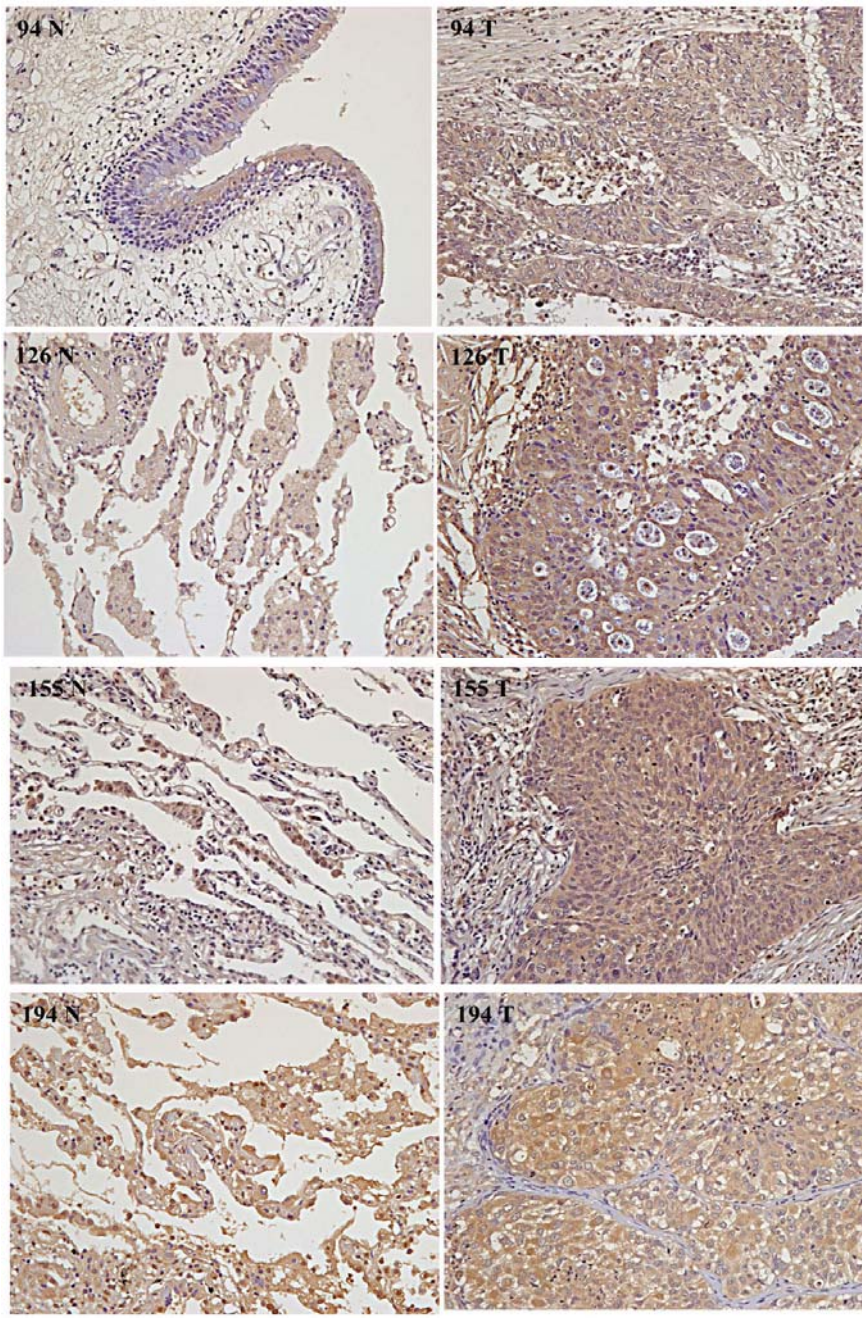

Fig. 3. Expression of TMSB10 protein in NSCLC patients tissues with immunohistochemical staining. No or weak staining was observed in the cytoplasm of normal tissues (94N, 126N, 155N). A strong cytoplasmic staining can be observed in normal (194N) and neoplastic tissues (94T, 126T, 155T, 194T).

observed between TMSB10 overexpression and clinicopathological factors, a trend was shown toward its overexpression being associated with histologic types ($p = 0.072$) (Table 2).

Interestingly, IHC analysis showed that there was no complete concordance between TMSB10 expression and its methylation status (Figs. 1B and 3). For example, no or weak staining was observed in the cytoplasm of normal tissues containing methylated promoter (94N and 155N). However, tumor cells with methylated promoter (94T) were also accompanied by significant expression of TMSB10 protein and there was no apparent methylation despite loss of expression (126N). Furthermore, RT-PCR and MSP analyses in 7 human NSCLC cell lines showed that *TMSB10* mRNA was detected in all examined cell lines with unmethylated alleles, but this mRNA was also present in H226 cell lines containing the methylated promoter (Figs. 4A and 4B). H226 cells treated with the demethylating agent 5′-AzadC for 3 days resulted in the disappearance of methylated allele of *TMSB10* gene, but failed to dramatically induce *TMSB10* mRNA transcripts (Fig. 4C). Taken together, these results suggest that *TMSB10* overexpression may be not closely associated with its promoter hypomethylation.

DISCUSSION

The present study has shown that *TMSB10* promoter was highly hypomethylated in NSCLCs and its protein is also over-

Table 2. Comparison between TMSB10 overexpression and clinicopathologic features

Variables	Overexpression	P
All subjects (n = 30)	15 (50.0)	
Age (years)		
≤ 63 (n = 12)	6 (50.0)	.645
> 63 (n = 18)	9 (50.0)	
Gender		
Female (n=5)	4 (80.0)	.165
Male (n = 25)	11 (44.0)	
Smoking status		
Never (n =6)	4 (66.7)	.326
Ever (n = 24)	11 (45.8)	
Histologic types*		
SCC (n = 15)	5 (33.3)	.072
AC (n = 15)	10 (66.7)	
Pathologic stage		
Stage I (n = 20)	10 (50.0)	.650
Stage II-IIIA (n = 10)	5 (50.0)	
TP53 mutations		
Negative (n = 12)	8 (66.7)	.109
Positive (n = 18)	7 (38.8)	
EGFR mutations		
Negative (n = 27)	12 (44.4)	.122
Positive (n = 3)	3 (100)	

*SCC, squamous cell carcinoma; AC, adenocarcinoma

expressed in tumor tissues regardless of promoter methylation. These data suggest an oncogenic function of TMSB10 during lung carcinogenesis and also implicate *TMSB10* gene as the potential marker for NSCLC. Although the current investigation was limited by the small number of samples, it herein is the first to examine the relationship between *TMSB10* expression and methylation status in NSCLC. TMSB10 is an acting-sequestering protein found in many tissues and has several biological activities. However, the detailed molecular mechanisms of TMSB10 are not fully understood. In some cancer types, *TMSB10* expression could be increased or decreased. It is also ambiguous whether TMSB10 can inhibit or promote tumor growth. Thus, elucidating the functions of TMSB10 in cancer remains a significant scientific and therapeutic challenge.

TMSB10 induction is a general event in a wide variety of human carcinomas (Santelli et al., 1999; Sribenja et al., 2009). In contrast, some reports have described that retinoic acid responsive TMSB10 accelerates apoptosis and acts as a tumor suppressor by disrupting the actin structure and inhibiting Ras signal transduction (Hall, 1995; Lee et al., 2005). Recent reports regarding the expression of *TMSB10* in lung cancer are contradictory (Gu et al., 2009; McDoniels-Silvers et al., 2002). Gu et al. have shown the overexpression of TMSB10 protein promoting the distant lymphnode metastasis, while McDoniels-Silvers et al has found the tumor underexpression of *TMSB10* mRNA compared with normal lung tissues. In this regard, the present study suggests that the *TMSB10* gene may be involved in lung cancer through tumor-promoting pathways. Similarly, patients with hypomethylated *TMSB10* had a tendency to be unfavorable prognosis than those with methylated one, despite the lack of statistical significance, faithfully reflecting recent finding that TMSB10 high expression is correlated with poor postoperative survival (Gu et al., 2009). However, further studies with a larger number of patients are warranted to confirm our findings.

It was interesting to note that TMSB10 overexpression was tended to be more frequent in ACs than in SCCs. Therefore, considering that *EGFR* mutations are found more frequently in patients with ACs (Janne et al., 2005), it could be speculated that TMSB10 overexpression might be closely related with

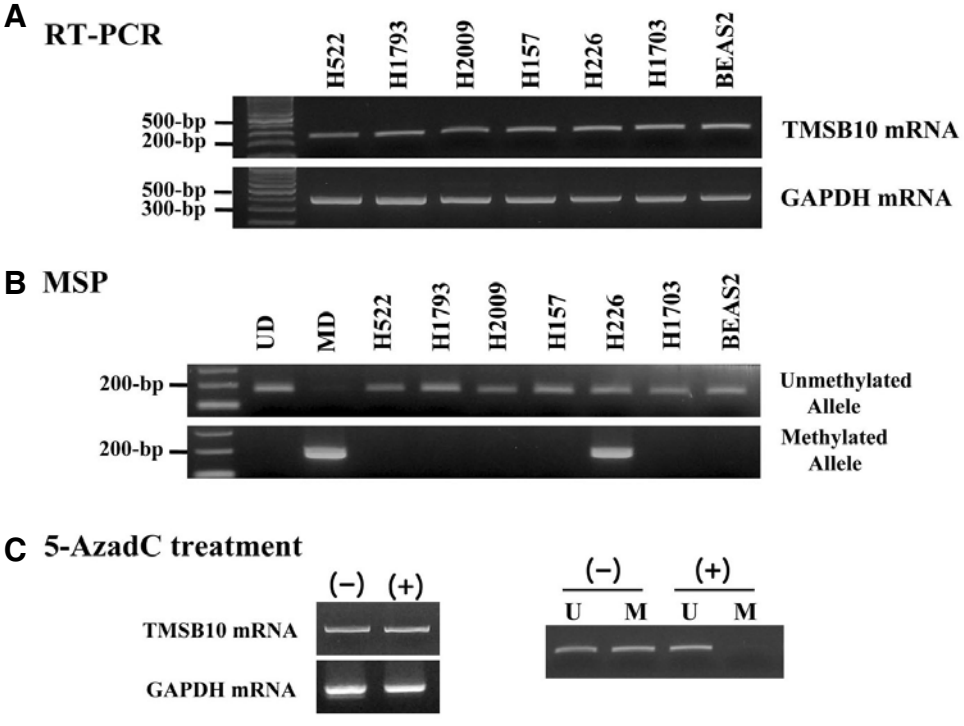

Fig. 4. RT-PCR and MSP analysis of *TMSB10* gene in human NSCLC cell lines. (A) Expression of *TMSB10* mRNA was performed by RT-PCR. Amplification of GAPDH was used as an internal loading control. (B) The methylation status of *TMSB10* gene was analyzed by MSP. (C) Expression and methylation status of *TMSB10* in H226 cells was analyzed after 5-AzadC treatment. (-) indicated vehicle alone treatment, (+) indicated the 5-AzadC treatment for 3 days. U, MSP with unmethylation-specific primer; M, MSP with methylation-specific primer.

somatic mutations in *EGFR*. This could be supported by a previous report that TMSB10 is repressed in lung cancer cell lines stably transfected with EGFR type III, which is the most common in NSCLC (Pedersen et al., 2001). Although it has recently demonstrated that TMSB10 expression level is not correlated with the histologic type of the cancer (Gu et al., 2009), this discrepancy might be due to genetic or environmental differences of the study population. Alternatively, these divergent results might be due to chance as a result of relatively small number of the study subjects examined or detailed characteristics of investigated specimens. Also, we have to take into consideration the possibility that our study did not have fully objective viewpoint because of the small number of patients studied for IHC analysis.

Intriguingly, most of examined tissues with unmethylated alleles abundantly expressed TMSB10 proteins, however, some discordant values were recorded between the methylation status of *TMSB10* gene and its expression by IHC and MSP analysis. It could be explained by different status in both alleles of the *TMSB10* gene. Actually, there was the presence of the *TMSB10* mRNAs in the H226 cells which one allele was unmethylated but the other was methylated. However, a demethylating agent 5-AzadC treatment did not significantly increase the *TMSB10* mRNAs. Therefore, our data suggested that promoter hypomethylation may not be a common mechanism underlying TMSB10 overexpression.

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