

The aberrant promoter methylation of *BMP3b* and *BMP6* in malignant pleural mesotheliomas

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Abstract. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor- β superfamily. Recent studies have showed that aberrant methylation of *BMP* genes is present in several types of human cancer. We examined the expression and methylation status of *BMP3b* and *BMP6* in malignant pleural mesotheliomas (MPMs). The expression status of *BMP3b*, and *BMP6* mRNAs were examined in seven MPM cell lines by RT-PCR assay. The expression of *BMP3b* was completely suppressed in 2 and partially suppressed in 2 of 7 cell lines and expression of *BMP6* was partially suppressed in 2 cell lines. Methylation status of *BMP3b* in cell lines was determined by methylation-specific assay to find aberrant methylation in 6 cell lines which include 4 cell lines with suppressed *BMP3b* expression. Partial methylation of *BMP6* was found in 2 cell lines whose expression was partially suppressed. Treatment with 5-Aza-dC restored *BMP3b* expression in methylated cell lines. Next, we examined the methylation status in 57 surgically resected MPM cases and found aberrant methylation of *BMP3b* in 9 (53%) out of 17 cases from Japan and 3 (8%) of 40 cases from USA and that of *BMP6* in 4 (24%) cases from Japan and 12 (30%) cases from USA, showing significant difference in frequency of *BMP3b* methylation between MPMs of the two countries ($P=0.0004$). Our study indicated that *BMP3b* and *BMP6* genes were suppressed by DNA methylation and methylation of *BMP3b* is significantly frequent in Japanese

MPMs, suggesting its pathogenic role and the ethnic difference in MPMs.

Introduction

Bone morphogenetic proteins (BMPs) are multifunctional cytokines involved in skeletal development and bone formation (1,2). They are members of the transforming growth factor- β (TGF- β) superfamily and critical mediators of early embryonic patterning. BMPs have been shown to inhibit cellular proliferation and be involved in organogenesis, particularly of the lung, heart and kidney. Inactivation of *BMP* genes has been implicated as important in several cancer types (3-8). Recent studies have shown that *BMP* genes including *BMP3*, *BMP3b* and *BMP6* are epigenetically inactivated in various kinds of cancers, suggesting that inactivation of BMPs may play an important role in carcinogenesis. *BMP3b* is methylated in colorectal neoplasms and non-small cell lung cancers (NSCLCs), while *BMP6* is methylated in breast cancer and NSCLCs.

Malignant pleural mesothelioma (MPM) is an aggressive tumor that develops from the pleural surface. The strong association with exposure to asbestos is known and implication of SV40 infection has been revealed recently (9,10). Regarding molecular alteration of MPM, homozygous deletion of *p16* gene and mutation of neurofibromatosis type 2 gene are well known as genetic alteration of MPM. DNA methylations of several tumor suppressor genes are also known as epigenetic alteration of MPMs (11-13).

In this study, we examined the methylation and expression status of *BMP3b* and *BMP6* genes in primary MPMs and MPM cell lines to investigate the implication of *BMP* genes in MPMs.

Materials and methods

Tumor samples and cell lines. Seven MPM cell lines (NCI-H2452, NCI-H2373, NCI-H2058, NCI-H2052, NCI-H290,

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NCI-H28, HP1) were kindly gifted from Ad.F. Gazdar (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX) or Harvey I. Pass (Department of Cardiothoracic Surgery, NYU School of Medicine). All these cell line samples were maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. Surgically resected specimens of 57 MPMs were obtained after informed consent from each patient. Tumor tissues were obtained from 40 patients with MPMs resected at Karmanos Cancer Center, MI, 6 patients resected at Okayama Rousai Hospital (Okayama, Japan), 5 patients at NHO Sanyo National Hospital, Yamaguchi Japan, and 6 patients at Okayama University Hospital, Okayama Japan. The study was approved by the Institutional Review Board of each institution and informed consent was obtained from the patients.

Reverse transcription polymerase chain reaction (RT-PCR). The expression status of *BMP3b* and *BMP6* mRNAs were examined in seven MPM cell lines by RT-PCR assay. Total-RNA was isolated using RNeasy mini kitTM (Qiagen, Valencia, CA). cDNA was synthesized from the RNA using the SuperScript II kit (Invitrogen, Carlsbad, CA). RT-PCR amplification of *BMP3b* and *BMP6* cDNA was performed using primers designed with Primer 3. *BMP3b* primers used were: 5'-GGTGGACTTCGACAGACATCG-3' (sense) and 5'-GATGGTGGCATGGTTGGATG-3' (antisense). *BMP6* primers were: 5'-ACAGCATAACATGGGGCTTC-3' (sense) and 5'-CTCGGGGTTTCATAAGGTGAA-3' (antisense). *GAPDH* was used as an internal control to confirm the success of the RT reaction (sense primer, 5'-ACAGTCCATGCCA TCACTGCC-3' and antisense primer, 5'-GCCTGCTTCA CCACCTTCTTG-3') (14). The PCR mixture contained 10X PCR buffer, dNTPs (200 μ M of each), primers (1 μ M of each) and 0.25 μ l TaqGold. cDNA was amplified using an annealing temperature at 60°C and two different cycles of 17 and 35 cycles. PCR product was separated by electrophoresis and visualized on an ethidium bromide-stained agarose gel.

DNA extraction and bisulfite treatment. Genomic DNA was isolated from cell lines and frozen tissues of primary tumors by homogenization by digestion with 100 μ g/ml, SDS/proteinase K followed by standard phenol-chloroform (1:1) extraction, and ethanol precipitation. One microgram of extracted DNA was modified with bisulfate treatment using the EZ DNA Methylation kitTM (Zymo Research, Orange, CA) according to the manufacturer's instructions.

Methylation-specific PCR assay. Aberrant methylation of *BMP3b* and *BMP6* genes was determined by MSP assay using DNAs treated with sodium bisulfite as described previously. The primer sets for both methylated and unmethylated forms of each gene were prepared. The primers to *BMP3b* promoter region were: 5'-CGGCGTCGATATATAGGAGTC-3' (sense) and 5'-AAATCGTCCCTAACCCGACT-3' (antisense) for methylated form and 5'-TGGTGTGATATA TAGGAGTT-3' (sense) and 5'-AAATCATCCCTAACCCA ACT-3' (antisense) for unmethylated form. The primers for *BMP6* were: 5'-GGTTTGTGGGTAGTCGGG-3' (sense) and 5'-GCCCCCTCCCCAAATCG-3' (antisense) for

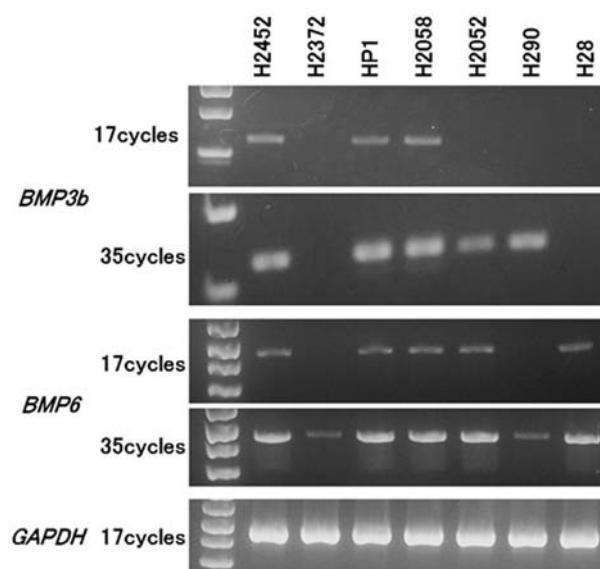


Figure 1. mRNA expression of *BMP3b* and *BMP6* in MPM cell lines. RT-PCR for each gene was carried out using two cycles, 17 and 35. The expression of *BMP3b* was completely suppressed in NCI-H2372 and NCI-H28. But in two weak expressing cell lines; NCI-H2052 and H290, *BMP3b* amplicon was not detected using 17 cycles but observed using 35 cycles. *BMP6* amplicon in NCI-H2372 and NCI-H290 was not detected using 17-cycle amplification. *GAPDH* was used as internal control.

methyated form and 5'-TTGGGTAGTTGGGTGATTGTT-3' (sense) and 5'-ACACCCCTCCCCAAATCA-3' (antisense) for unmethylated form. Bisulfite-modified DNAs were mixed with 10X PCR Buffer, 150 μ M of deoxynucleotide triphosphates, 0.4 μ M of primers and 1 unit of HotStar Taq (Qiagen). The PCR condition for methylated alleles of *BMP3b* consisted of 12 min at 95°C, 40 cycles of 30 sec of 95°C, 60 sec of 61°C and 60 sec of 72°C followed 7 min elongation. The PCR condition for unmethylated alleles of *BMP3b* was similar to that for methylated allele: 12 min at 95°C, 40 cycles of 30 sec of 95°C, 60 sec of 56°C and 60 sec of 72°C followed 7 min elongation. The PCR condition for methylated alleles of *BMP6* consisted of 12 min at 95°C, 40 cycles of 30 sec of 95°C, 60 sec of 62°C and 60 sec of 72°C, followed 7 min elongation. The PCR condition for unmethylated alleles of *BMP6* was 12 min at 95°C, 40 cycles of 30 sec of 95°C, 60 sec of 60°C and 60 sec of 72°C, followed 7 min elongation. PCR products were separated by electrophoresis and visualized on an ethidium bromide-stained 2.5% agarose gel. DNA from non-malignant lung tissue was treated with SssI methyltransferase (New England BioLabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles.

5-Aza-2'-deoxycytidine treatment. Cell lines were treated with 5-aza-2'-deoxycytidine (5-Aza-CdR) (Sigma-Aldrich, St. Louis, MO) at a concentration of 1-2 μ g/ml for 6 days with medium changes on days 1, 3 and 5. Treated or untreated cell from individual triplicate flasks were harvested to detect expression level using RT-PCR as described earlier. Two MPM cell lines (NCI-H290, NCI-H28) with weak and negative *BMP3b* expression were used for this assay.

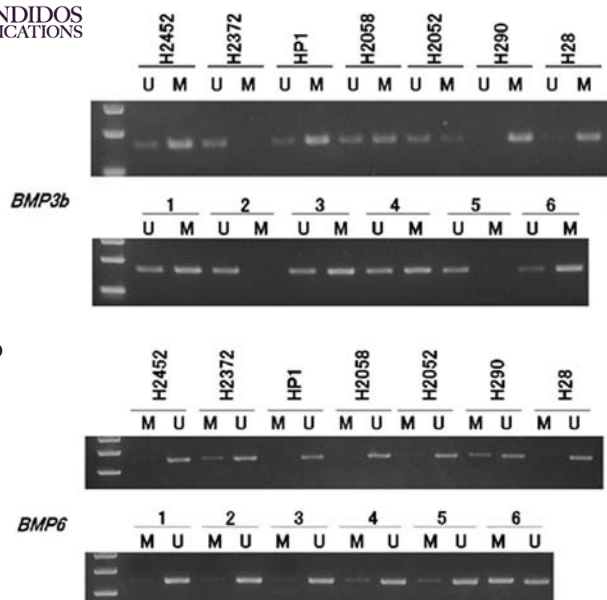


Figure 2. Methylation specific PCR for *BMP3b* (a) and *BMP6* (b) in MPM cell lines and representative examples of primary tumors. Result of testing for the methylated (M) and unmethylated (U) forms for each sample are illustrated. Samples from 1 to 7 are primary MPMs.

Data analysis. A comparison of the proportion of gene methylation was done using Fisher's exact test. A P-value <0.05 was defined as being statistically significant. All data were analyzed with the use of Survival Tools for StatView (Adept Scientific Inc., Acton, MA).

Results

The expression of *BMP3b* and *BMP6* was examined in 7 MPM cell lines by RT-PCR (Fig. 1). RT-PCR is not a quantitative assay for expression, but 2 different amplification cycles, 17 and 35 cycles; of PCR distinguished complete suppression or partial suppression of these genes. The expression of *BMP3b* was completely suppressed in 2 cell lines; NCI-H2372 and NCI-H28; and partially suppressed in 2 cell line; NCI-H2052 and NCI-H290, while expression of *BMP6* was partially suppressed only in 2 cell lines. Methylation status of *BMP3b* in cell lines was determined by MSP assay (Fig. 2). DNAs which were amplified with primers for methylated alleles but not with primers with unmethylated alleles were determined to be heavily methylated. DNAs which were amplified with both methylated and unmethylated primer sets were

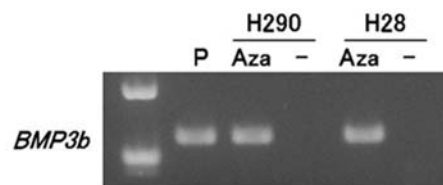


Figure 3. The restoration of *BMP3b* expression by 5-Aza-CdR in MPM cell lines. *BMP3b* amplicon was detected using 17 cycles in both H290 and H28 treated with 5-Aza-CdR. Aza, 5-Aza-CdR treated; -, untreated; P, positive control.

considered to be partially methylated. In 7 MPM cell lines, 2 cell lines (NCI-H290 and NCI-H28) were strongly methylated and 4 cell lines (NCI-H2452, HP1, NCI-H2058 and NCI-H2052) were partially methylated in the *BMP3b* gene. NCI-H28 in which *BMP3b* expression was not present was strongly methylated, but NCI-H2372 with negative expression was not methylated. Two cell lines with weak expression of *BMP3b* were heavily (H290) and partially methylated (H2052). By contrast, expression of *BMP3b* was not suppressed in 3 cell lines with partial methylation (NCI-H2452, HP1 and NCI-H2058). In the *BMP6* gene, only two cell lines (NCI-H2373 and NCI-H290) were partially methylated and no cell lines were heavily methylated. The expression of these two cell lines was partially suppressed.

Next, methylation status in 57 surgically resected MPM cases was tested by MSP assay (Fig. 2). Because non-malignant cells were contained in surgically resected samples, all primary samples were amplified with primers for unmethylated alleles. Aberrant methylation of *BMP3b* were found in 9 (53%) out of 17 cases from Japan and 3 (8%) of 40 cases from USA and that of *BMP6* were in 4 (24%) cases from Japan and 12 (30%) cases from USA (Table I). While the frequency of *BMP6* methylation was similar in MPMs in Japan and USA, but *BMP3b* methylation was significantly frequent in Japanese patients (P=0.0004).

To confirm the responsibility of DNA methylation for *BMP3b* silencing, we treated two methylated cell lines (NCI-H290, NCI-H28) with 5-Aza-CdR. *BMP3b* expression was significantly up-regulated by 5-Aza-CdR treatment in methylated cell lines (Fig. 3).

Discussion

Bone Morphogenetic Proteins (BMPs) are multifunctional cytokines involved in skeletal development and bone

Table I. The frequency of *BM3b* and *BMP6* methylation in MPM cell lines and tumors.

	<i>BMP3b</i> methylation (%)	P-value	<i>BMP6</i> methylation (%)	P-value
Cell line (n=7)	6 (86)		2 (29)	
Primary tumor				
USA (n=40)	3 (8)	0.0004	12 (30)	0.75
Japan (n=17)	9 (53)		4 (24)	

formation. They are members of the transforming growth factor- β (TGF- β) superfamily and critical mediators of early embryonic patterning. BMPs have shown to inhibit cellular proliferation and involved in organogenesis, particularly of the lung, heart and kidney. Recent work has shown that *BMP3b* and *BMP6* are epigenetically inactivated in several malignancies. *BMP3b* has been demonstrated to be methylated in NSCLCs, and colorectal cancer, and *BMP6* gene in NSCLCs, prostate cancer, malignant lymphomas and breast cancer (9-13).

We have screened for aberrant methylation of *BMP* genes in MPMs. In spite of the limitation of the number of cell lines and primary samples, we showed the suppression of *BMP3b* and *BMP6* expression in MPM cell lines and DNA methylation was one of the mechanisms of gene suppression. Aberrant methylation of *BMP3b* and *BMP6* was also detected in primary MPMs. The frequency of methylation of *BMP6* was quite similar in cell lines, primary tumors in USA and those in Japan, but methylation of *BMP3b* gene was significantly more frequent in MPMs of Japan than those of USA. These results indicate that the methylation of *BMP3b* may be influenced by ethnic or pathogenic differences. Of note, we have reported that the methylation of insulin-like growth factor binding protein-3 was significantly more frequent in Japanese MPMs than in those of USA (15).

As shown in Fig. 1, methylated and unmethylated bands were observed in 4 cell lines, indicating partial methylation. In addition, even partial methylated cell lines, expression status was not identical. These findings suggest heterogeneity of methylation among individual cells even in the same cell lines. The importance of partial methylation of *BMP3b* is not clear and further study is necessary. Of note, the seven cell lines examined were established in USA, but the frequency of methylation in primary tumors in USA is not frequent. Thus, partial methylation in cell lines might be artificial phenomenon in the process of establishing cell lines. Furthermore, there must be other mechanisms for gene suppression of *BMP3b* because it was completely suppressed in NCI-H2372, in which DNA methylation was absent.

Recent work of others and our study strongly support that *BMP3b* and *BMP6* have a role as tumor suppressor genes in several malignancies. Other members of BMPs are shown to be involved in malignancies but their roles in carcinogenesis are still controversial. Methylation and loss of expression of *BMP2* was observed in gastric carcinomas, but it was also shown to promote tumor growth in A549, NSCLC cell lines (16). The roles of BMP proteins in carcinogenesis may be different according to the organ and further studies are necessary.

In conclusion, we showed that DNA methylation causes the suppression of *BMP3b* and *BMP6* expression in MPMs. In addition, there was an ethnic difference of frequency of the methylation in *BMP3b*, suggesting the etiology of MPM may be different between Japanese and USA.

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