The bone morphogenetic protein antagonist Gremlin is overexpressed in human malignant mesothelioma

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Abstract. Gremlin is a member of the bone morphogenetic protein (BMP) antagonist family and its antagonistic effect is likely through direct binding to BMP proteins. As an antagonist of BMP, Gremlin plays a role in regulating organogenesis, body patterning and tissue differentiation. Recent studies have shown a deregulation of Gremlin in several types of human cancers. However, the role of Gremlin in human malignant mesothelioma (MM) is still unknown. In this study, we investigated the expression of Gremlin in human MM. We found that Gremlin mRNA and protein were both overexpressed in the majority of primary MM tissue samples that we examined. We also observed high level expression of the Gremlin gene in 4 of the 6 MM cell lines. Consistently, we found that the Gremlin promoter activity was significantly elevated in those MM cell lines expressing the Gremlin gene. On the other hand, no activity of the Gremlin promoter was detected in the two MM cell lines lacking Gremlin expression. Moreover, to examine the functional significance of the Gremlin overexpression in MM, we used shRNA to knock down Gremlin expression in MM cell lines expressing Gremlin and found that inhibition of Gremlin expression significantly suppressed proliferation of those MM cells. Taken together, our results suggest that the BMP antagonist Gremlin is overexpressed in MM and that aberrant activation of Gremlin may play a critical role in the tumorigenesis of human MM.

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Introduction

Malignant mesothelioma (MM) is associated with asbestos and it is characterized by rapidly progressive and diffuse growth, late metastases, and very poor prognosis (1,2). The disease is of rapid progression and most patients die within 12-15 months of the first symptoms if untreated (1,3). It is critical to improve understanding of the molecular mechanism of MM in order to find much needed effective new therapies for the treatment of this disease. Gremlin encodes a member of the Dan family of secreted glycosylated proteins [also known as Down-regulated by v-mos (Drm)] (4,5). Gremlin has been reported to be an antagonist of the bone morphogenetic protein (BMP) family, likely through direct binding to BMP proteins (6). BMP family proteins are members of the transforming growth factor-β (TGF-β) superfamily and critical mediators of early embryonic patterning involved in skeletal development and bone formation (7,8). Inactivation of the BMP genes has been reported in several cancer types, including lung, breast, prostate and colon cancers, lymphomas and mesotheliomas (9-15), suggesting the importance of BMPs in tumorigenesis of these cancers. As an antagonist of BMP proteins, Gremlin also plays important roles during early development (16-19), as well as tumorigenesis of several types of cancers (20-25). However, the role of Gremlin in MM has not been reported before and still remains unknown. Therefore, in this study, our goal was to examine the expression of the Gremlin gene in human MM cell lines as well as primary tissue samples, and to investigate the possible functional roles that Gremlin plays in MM.

Materials and methods

Cell lines and tissue samples. Human mesothelioma cell lines were obtained from the following sources: H290, H2450, H28, and H2052 from American Type Culture Collection (Manassas, VA, USA), MS-1 from NIH (Frederick, MD, USA), and REN originally developed by Dr Steven Albelda (University of Pennsylvania, PA, USA). All cell lines were

Table I. Primer sequences for cloning the Gremlin promoter-luciferase constructs.

Construct	Primer sequences	Size (bp)
1	S: 5'-CAGGAGATCTAAACCACAGTGTCAGGGCTC-3' A: 5'-CAGGAAGCTTCTCTTTAGTCCGGCGTTAGG-3'	601
2	S: 5'-CAGGAGATCTAAACCACAGTGTCAGGGCTC-3' A: 5'-CAGGAAGCTTGTCTGTATTGTCTAACGCGA-3'	729
3	S: 5'-CAGGAGATCTAAACCACAGTGTCAGGGCTC-3' A: 5'-CAGGAAGCTTCGAGAGGGGTTTTCTGTAGC-3'	1074
4	S: 5'-CAGGAGATCTGCTACAGAAAACCCCTCTCG-3' A: 5'-CAGGAAGCTTGAGGGAAGAGCGGGAGGAAA-3'	530
5	S: 5'-CAGGAGATCTTTTCCTCCCGCTCTTCCCTC-3' A: 5'-CAGGAAGCTTTACGTTTCCCTGCAGACCCA-3'	681
6	S: 5'-CAGGAGATCTGCTACAGAAAACCCCTCTCG-3' A: 5'-CAGGAAGCTTTACGTTTCCCTGCAGACCCA-3'	1190

S, sense primer; A, antisense primer. The underlined sequences show the restriction enzyme sites (AGATCT and AAGCTT for *Bgl*II and *Hin*dIII, respectively).

cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37˚C in a humidified incubator with 5% CO_2. Fresh MM tissue and adjacent normal tissue samples were obtained at the time of surgical resection of MM patient tumors at local hospitals (all MM patients were consented to tissue specimen collection and research and approval by Institutional Review Board) and were immediately snap-frozen in liquid nitrogen. They were stored at -80˚C before use.

RNA extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total-RNA of MM cell lines and tissue samples were extracted using TRIzol reagent (Tiangen, Beijing, China) according to the manufacturer's protocol. RT-PCR was performed as follows: cDNA was first produced by using AMV reverse transcriptase [Promega (Beijing) Biotech Co., Ltd., Beijing, China] and N9 random primers; PCR was performed in a GeneAmp 2700 system (Applied Biosystems, CA, USA) using cDNA as template. Taq enzyme and PCR reagents were purchased from Tiangen Corp. Primers for amplifying the Gremlin mRNA in RT-PCR were purchased from Sangon Corp. (Shanghai, China) and their primer sequences were: 5'-AACAGTCGCACCATCATCAA-3' (forward) and 5'-CGATGGATATGCAACGACAC-3' (reverse), and the amplicon size was 220 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for RNA quality and loading. The primer sequences for GAPDH were: 5'-AGTCAACGGATTTGGTCGT-3' (forward) and 5'-TTGATTTTGGAGGGATCTCG-3' (reverse), and the amplicon size was 238 bp. The PCR condition were: 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec for 35 cycles, followed by a final extension at 72˚C for 10 min. Semi-quantitative RT-PCR products were analyzed on 1% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time PCR. Quantitative real-time PCR was performed on Mx3000P™ (Stratagene, La Jolla, CA, USA). Each reaction mixture (20 μl) contained 1X reaction buffer, 60 μmol $MgCl_2$, 4 μmol of each dNTP, 80 units TaqDNA polymerase (Tiangen Biotech Corp., Beijing, China), 3 μl of diluted cDNA, 10 pmol forward and reverse primers, 4 pmol probe modified with FAM and TAMRA at 5' and 3', respectively. The cycling condition used is: 95˚C for 10 min; 35 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 20 sec. The fluorescence intensity of amplicons was analyzed during the extension step of the cycling. 18S rRNA was used as an internal control. The primers and probes were synthesized at Sangon Corp. The sequences of the primers and probes were: for *Gremlin*, forward, 5'-ATCAACCGCTTCTGTTACGG-3'; reverse, 5'-CGATGGATATGCAACGACAC-3'; probe, 5'-GTCACACTCAACTGCCCTGA-3'; for 18S rRNA, forward, 5'-ACATCCAAGGAAGGCAGCAG-3'; reverse, 5'-TTCGTCACTACCTCCCCGG-3'; probe, 5'-CGCGCAAATTACCCACTCCCGA-3'. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression levels of Gremlin by normalizing to a normal control sample made by mixing an equal amount RNA of the eight matched normal pleural tissues from MM patients (26).

Western blotting. Whole cell lysates of MM primary tissue cultures were obtained using CytoBuster protein extraction reagent (Novagen, Madison, WI, USA). Samples containing 40 μg of proteins were electrophoresed on a 10% SDS-polyacrylamide gel. A standard protocol for Western blotting was used. Antigen-antibody complexes were detected by the ECL blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Anti-Gremlin polyclonal and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cloning and construction of the Gremlin promoter region. PCR was used to amplify the Gremlin promoter region. Sense and antisense primers were chosen based on the 5' flanking genomic sequence upstream the Gremlin gene (the first nucleotide of the first exon of the Gremlin gene is defined as +1). Two adaptors, 5'-CAGGAGATCT-3' (*Bgl*II site underlined) and

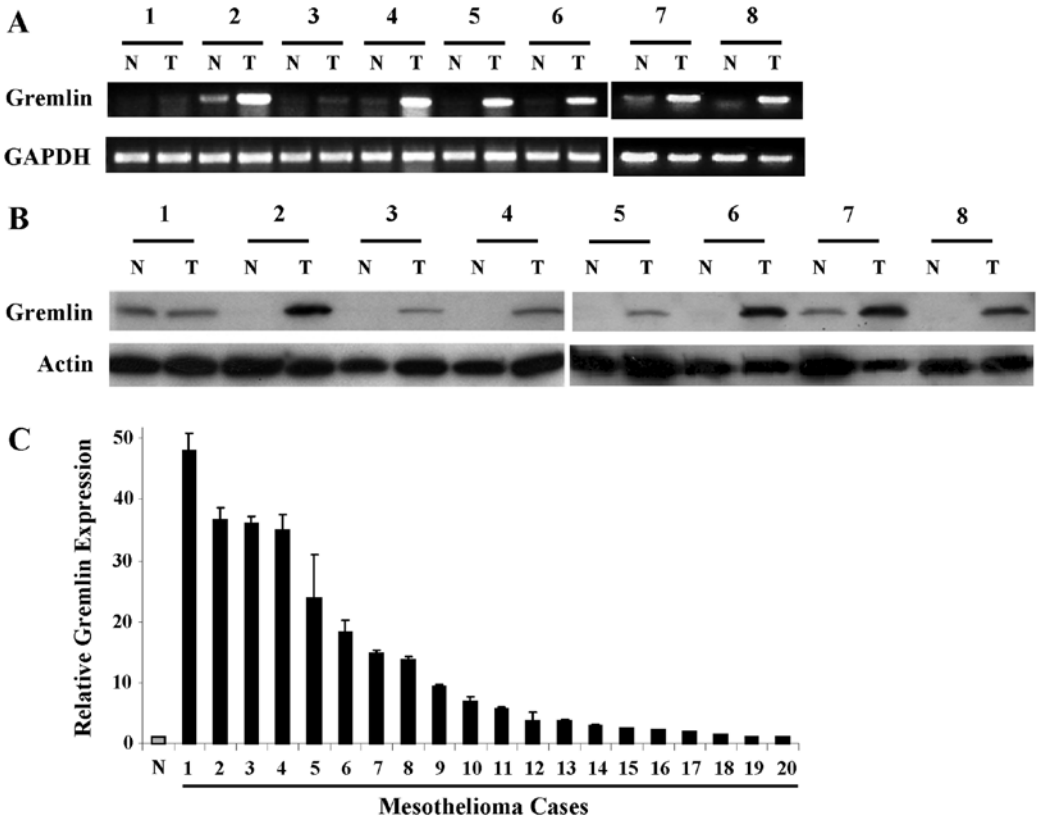

Figure 1. Gremlin mRNA and protein expression is up-regulated in human mesothelioma tissues, (A) Semi-quantitative RT-PCR analysis of eight pairs of MM tissues (T) and their matched normal pleural tissues (N). GAPDH served as a loading control; (B) Western blotting of the eight pairs of MM tissues (T) and their matched normal pleural tissues (N) analyzed in (A). Whole-cell lysate was used. Actin served as a loading control; (C) Real-time RT-PCR analysis of 20 additional MM tissue samples. N, normal pleural tissue.

5'-CAGGAAGCTT-3' (*Hin*dIII site underlined) were added to each sense and antisense primers, respectively in order to clone the promoter fragments into a luciferase reporter vector pGL3Basic (Promega) for functional analysis. The primers used to generate different constructs are listed in Table I. The relative positions of all amplicons are also illustrated in Fig. 3. Forty cycles of amplification were performed using human genomic DNA isolated from cultured 293T cells as the template. The PCR products were then extracted from the agarose gel using the TIANgel Midi Purification kit (Tiangen), digested with *Bgl*II and *Hin*dIII (Takara), and subcloned into the *Bgl*II (5') and *Hin*dIII (3') sites of the pGL3Basic vector. GLprimer2 (Promega) was used as a sequencing primer to ensure the correct orientation and sequence of each construct at Shanghai Invitrogen Biotechnology Co., Ltd.

Transfection and promoter activity analysis. Cells ($1x10^4$) were plated in 12-well plates one day before transfection. When cells reached 60-80% confluence, 2.5 μg of each construct were co-transfected with 0.05 μg pRL-TK vector (Promega), which contains *Renilla* luciferase as an internal control for the transfection efficiency. Vigofect (Vigorous, Beijing, China) was used to mediate all transfections according to the manufacturer's protocol. The transfected cells were cultured for an additional day before being lysed for luciferase assays. Briefly, cells were lysed in passive lysis buffer and then firefly and *Renilla* luciferase activities of each well were measured using the Dual-Luciferase Assay system (Promega).

Firefly luciferase activities were all normalized to *Renilla* luciferase activities in the same well and were expressed relative to the basal activity of the empty pGL3Basic vector, which was set to unity. The data represent the mean values ± SD. All measurements in each experiment were performed in triplicate and at least three independent experiments were repeated to ensure consistency.

Gremlin shRNA transfection and cell proliferation assay. Gremlin shRNA plasmid (the targeted Gremlin sequence was: 5'-CCGCTTCTGTTACGGCCAGTGCAACTCTT-3', cat. no. TR312620) was purchased from Origene (Rockville, MD, USA). The control scrambled shRNA (Cat. no. TR30012) was also from Origene. Vigofect was used to mediate all transfections according to the manufacturer's protocol. Cell proliferation was determined by the CellTiter 96 AQueous Non-Radiotive Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol. Briefly, transfected cell lines were plated into 96-well tissue culture plates at $5x10^2$ cells/well. The MTS solution was added to the medium and incubated for 1.5 h at 37˚C at different time points post cell plating. The absorbance at 490 nm was measured using the microplate reader model 680 (Bio-Rad, Hercules, CA, USA).

Statistical analysis. The data presented represent the means ± SD. Comparisons were conducted using the unpaired t-test. A $P<0.05$ was considered to be significant.

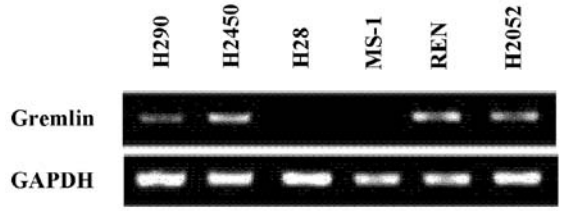

Figure 2. Semi-quantitative RT-PCR analysis of Gremlin mRNA expression in human mesothelioma cell lines. GAPDH served as a loading control.

Results

Expression of Gremlin is up-regulated in primary MM tissue samples. To investigate the possible role of Gremlin in MM, we first examined the expression level of the Gremlin gene in primary MM tissue samples (Fig. 1). We found that the mRNA level of the Gremlin gene was significantly up-regulated in 7 of the 8 MM tissue samples that we examined when compared to their paired normal tissue samples from same patients (Fig. 1A). Next, we performed Western blotting on the same 8 MM tissue samples and we found that the Gremlin protein expression was also up-regulated in the same 7 MM tissue samples compared to their paired normal tissues, consistent with their mRNA expression levels (Fig. 1B). We also examined additional 20 primary MM tissue samples by using real-time RT-PCR and found that 85% (17/20) showed the up-regulated Gremlin mRNA expression (Fig. 1C) ($P<0.05$). Taken together, these results suggest that the Gremlin is overexpressed in human MM.

Expression of Gremlin in MM cell lines. To confirm our observation in MM tissue samples, we next examined the Gremlin expression in MM cell lines (Fig. 2). Of the 6 MM cell lines examined, 4 cell lines (H920, H2450, REN and H2052) showed high level of the Gremlin mRNA expression. On the other hand, little or no mRNA expression of Gremlin was found in cell lines H28 and MS-1.

Activity of the Gremlin promoter in MM cell lines. In order to investigate the transcriptional regulation of the Gremlin expression in MM, we seek to clone and analyze the Gremlin promoter. A BLAST search was first performed using the Gremlin coding sequence as a probe against the human genomic database at the UCSC server (http://genome.ucsc.edu/) to identify the 5' flanking region of the Gremlin gene (Fig. 3A). We confirmed classical features of a promoter in

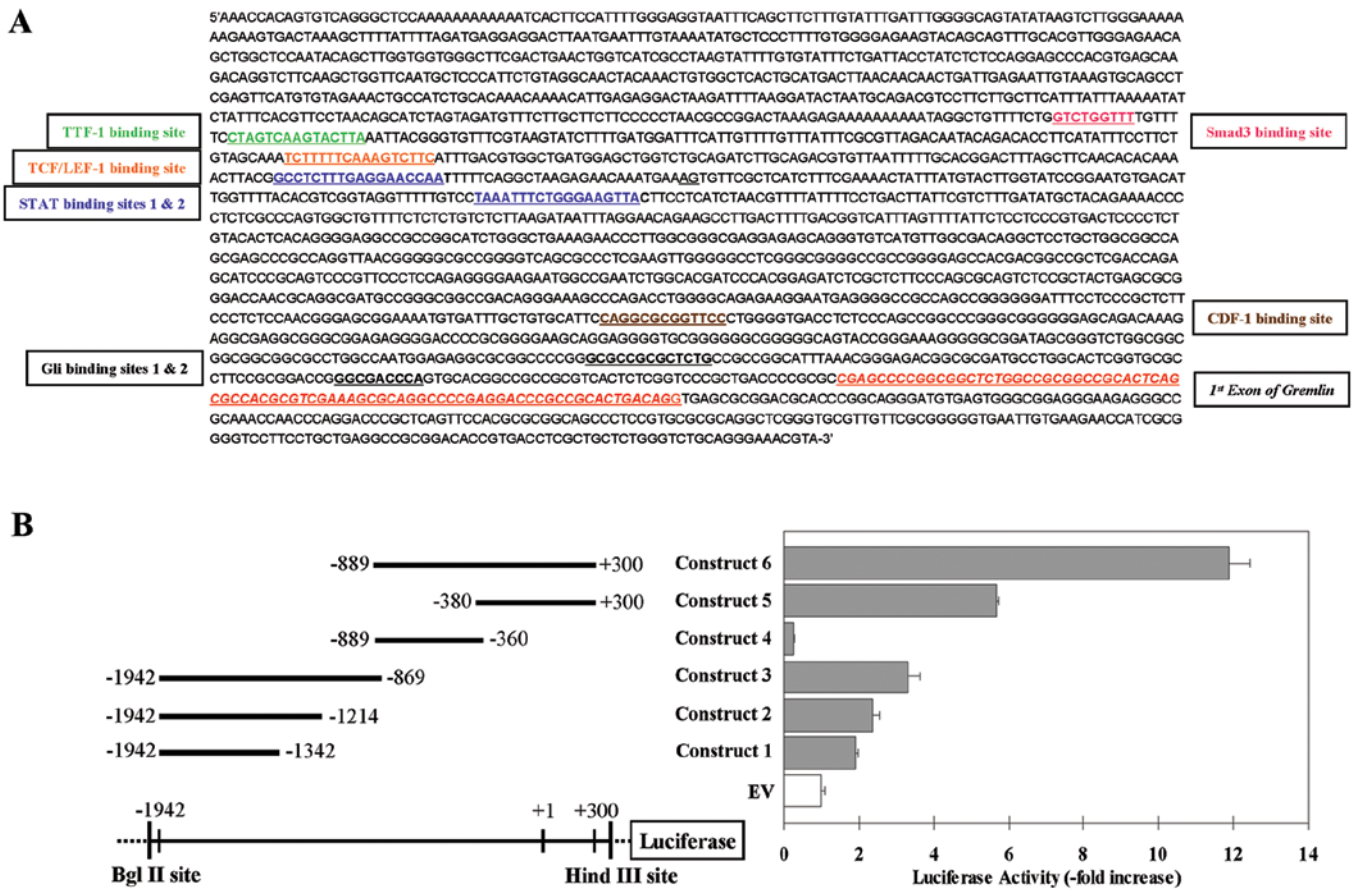

Figure 3. Cloning of the human Gremlin gene promoter. (A) The nucleotide sequence of the 5'-flanking region of the human Gremlin gene. Several important transcription factors and the first exon of the Gremlin gene are highlighted and indicated on the side; (B) Luciferase activity of different constructs of the human Gremlin promoter region. The diagram on the left represents the different pGL3Basic-derived constructs obtained as described in Materials and methods. Luciferase activity was measured after transient transfection of each construct into 293T cells. All relative luciferase activities were normalized to the activity of the empty pGL3Basic vector alone in 293T cells (defined as 1.0). Results are the means ± SD (error bars) of three individual experiments.

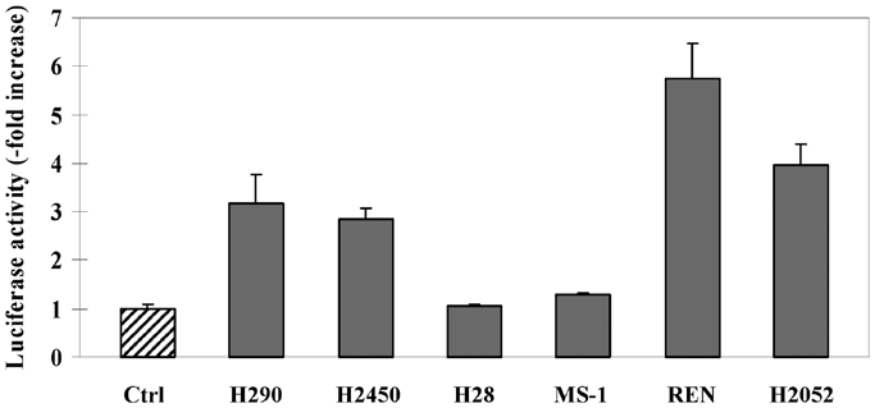

Figure 4. Activity analysis of the human Gremlin promoter in mesothelioma cell lines. Construct 6 (Fig. 3B) was transfected in different MM cell lines. All relative luciferase activities in different MM cell lines were normalized to the average activity of the empty pGL3Basic vector alone in these cells. Results are the means ± SD (error bars) of three individual experiments.

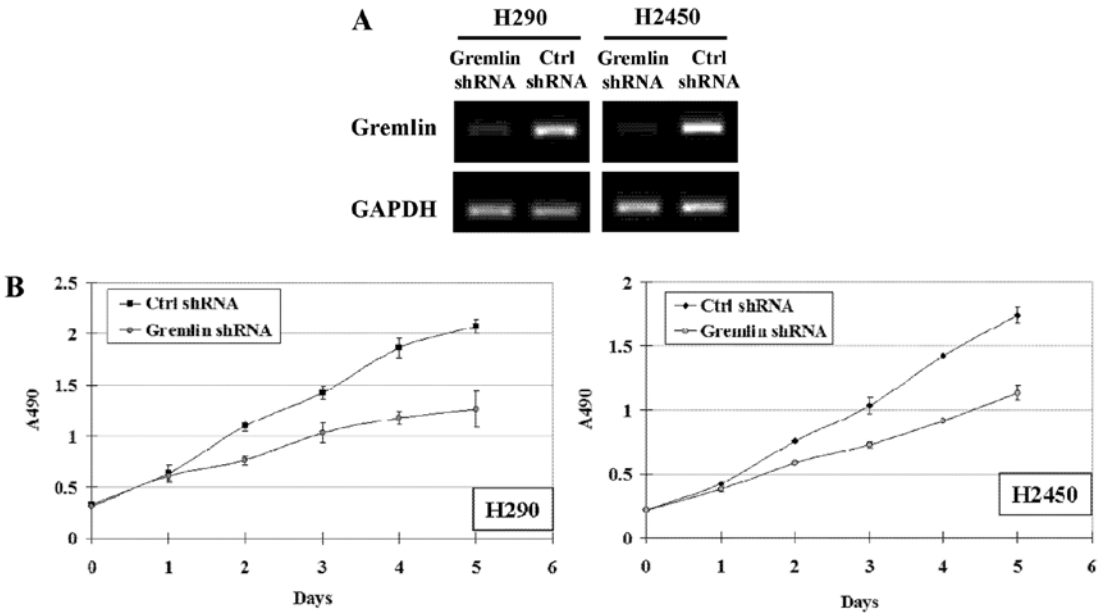

Figure 5. Knock-down of Gremlin mRNA expression by shRNA suppresses proliferation of mesothelioma cells expressing Gremlin. (A) Semi-quantitative RT-PCR analysis in the MM cell lines H290 and H2450 after Gremlin shRNA transcfection. GAPDH served as a loading control; (B) Cell proliferation (MTS) assay of MM cell lines H290 and H2450 after Gremlin shRNA transcfection. Experiments were performed in triplicate. Data are the means ± SD.

this 5' flanking region using a promoter search program (http://www.genomatix.de/). Several important putative binding sites of transcription factors such as Smad3, Gli, TCF/LEF-1, TTF-1 and STAT were observed in this region (Fig. 3A). These transcription factors themselves have been shown to play important oncogenic roles in tumorigenesis of many cancer types, including MM (27-31). We next cloned several fragments of the Gremlin promoter region into a promoterless luciferase expression vector pGL3Basic and transfected 293T cells with these constructs to examine luciferase activities (Fig. 3B). We confirmed that this region had significant promoter activity compared to the empty vector control. Next, we transfected the fragment of the Gremlin promoter construct with the highest activity in 293T cells (construct no. 6) into different MM cell lines and examined the luciferase activity after 24 h (Fig. 4). We found that this promoter construct displayed significantly high luciferase activities ($P<0.04$) in the 4 MM cell lines (H920, H2450, REN, H2052) which strongly expressed Gremlin mRNA (Figs. 2 and 4). Consistently, no Gremlin promoter activity was observed in MS-1 and H28 that lacked endogenous Gremlin expression (Figs. 2 and 4).

shRNA knock-down of Gremlin mRNA expression suppressed proliferation of MM cell lines. To study the functional role of the Gremlin overexpression in MM, we used shRNA to knock down Gremlin expression in the MM cell lines H290 and H2450 that expressed high levels of Gremlin. We confirmed the silencing of Gremlin expression after Gremlin shRNA treatment (100 nM for 48 h) by semi-quantitative RT-PCR analysis (Fig. 5A). We found that knock-down of Gremlin expression led to significant proliferative suppression in both cell lines (Fig. 5B) ($P<0.002$), suggesting that Gremlin might be necessary for proliferation of these MM cells.

Discussion

Progression of malignant mesothelioma (MM) is rapid and if untreated, most patients die within 12-15 months of the first symptoms (1,3). However, no current chemotherapy regimen for mesothelioma has yet proven wholly curative. Clinical trials show objective response rates of 41-48%, and the therapies themselves are still controversial (32-35). Recently, molecular target therapies such as imanitib and gefinitib have also been studied in MM patients. However, these drugs do not appear to be effective against MM according to early studies (36,37). Therefore, it is critical to improve our understanding of the mechanisms of MM in order to find much needed effective new therapies for the treatment of this disease.

It has been reported that the BMP gene family is inactivated in a number of cancer types, including lung cancer, breast cancer, prostate cancer, colon cancer, lymphoma, and mesothelioma and BMP proteins are of great importance in tumorigenesis of these cancers (9-15). As an antagonist of BMP proteins likely through direct binding to BMP proteins (6), Gremlin has recently been shown to be deregulated during tumorigenesis of several types of cancers such as lung, breast, ovarian and cervical cancers (20-25). However, the role of Gremlin in malignant mesothelioma has not been studied before and still remains unknown. In this study, we reported, for the first time, that Gremlin was overexpressed in the majority of primary human MM tissue samples and cell lines examined. As a confirmation study of the Gremlin overexpression in MM, we cloned the Gremlin promoter region and examined its activity in MM cell lines. Consistently, we found that these MM cell lines with high level of Gremlin expression displayed significantly elevated Gremlin promoter activity and that these MM cell lines lacking Gremlin expression did not show Gremlin promoter activity. Although further investigation with larger number of MM tissue samples will be needed, our findings strongly suggest that increased expression of the Gremlin gene may be associated with tumorigenesis of human MM.

Moreover, to examine the functional significance of the Gremlin overexpression in MM, we used shRNA to knockdown the Gremlin expression in MM cell lines expressing Gremlin and found that inhibition of the Gremlin expression significantly suppressed proliferation of those MM cells. A recent study by Kimura *et al* (15) reported aberrant methylation silencing of BMP genes BMP3b and BMP6 in human MM, suggesting that these BMP genes may have a role as tumor suppressor genes in MM. Taken together, our results are consistent with these previous findings in MM and support the importance of aberration of BMP signaling in tumorigenesis of human MM. While further studies are needed to characterize cellular functions and regulatory mechanisms, such as the interaction between Gremlin and BMP family members and downstream signaling events, the BMP antagonist Gremlin seems to be a candidate oncoprotein in the development of human MM, and could be a good target for developing diagnostic and therapeutic strategies against human MM.

In summary, we have shown that Gremlin is overexpressed in human MM cell lines and tissue samples. Overexpression of Gremlin may be important for the aberrant BMP signaling pathway in MM and for the consequent proliferation of MM cells. This is the first demonstration of the importance of Gremlin as a putative oncogene in human mesothelioma. Our results support Gremlin as a therapeutic target for this highly aggressive cancer.

Acknowledgements

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