SPANDIDOS PUBLICATIONS

Activation of the PI3K-AKT pathway in human malignant mesothelioma cells

YUTARO SUZUKI[1,3], HIDEKI MURAKAMI[1], KOJI KAWAGUCHI[1,4], TETSUO TANIGUCHI[1,4], MAKIKO FUJII[1], KEIKO SHINJO[1], YUTAKA KONDO[1], HIROTAKA OSADA[1], KAORU SHIMOKATA[5], YOSHITSUGU HORIO[2], YOSHINORI HASEGAWA[3], TOYOAKI HIDA[2] and YOSHITAKA SEKIDO[1]

[1]Division of Molecular Oncology, Aichi Cancer Center Research Institute; [2]Department of Thoracic Oncology, Aichi Cancer Center Hospital, Nagoya 464-8681; Departments of [3]Respiratory Medicine, and [4]Cardio-Thoracic Surgery, Nagoya University Graduate School of Medicine, Nagoya 466-8550; [5]Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai 487-8501, Japan

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Abstract. Malignant mesothelioma (MM) is a highly aggressive neoplasm, which is associated with asbestos exposure. The dysregulated phosphatidylinositol 3-kinase (PI3K)-AKT pathway plays an important role in cell proliferation, survival and motility in various cancers. In this study, we analyzed the activation status and underlying mechanisms of this pathway in MM cells using 21 cell lines. AKT activation was observed in 13 (62%) of the 21 MM cell lines under serum-starved conditions. Two cell lines, ACC-MESO-1 and Y-MESO-25, showed no expression of PTEN protein, while 7 other cell lines showed low expression of PTEN mRNA and protein compared to expression levels in an immortalized normal mesothelial cell line, MeT-5A. We found that PTEN inactivation in the ACC-MESO-1 and Y-MESO-25 lines was due to a 39.4-kb deletion including *PTEN* exon 2, and to a 7.7-kb deletion including exon 1, respectively. Re-expression of PTEN in these cells reduced the activity of colony formation *in vitro*. In contrast, no mutation of *PIK3CA* or *LKB1* was found in any of the MM cell lines. These findings suggest that AKT is frequently activated in MM cells, in part due to the downregulation of *PTEN*. Thus, the PI3K-AKT signaling pathway is a potential therapeutic target for MM.

Introduction

Malignant mesothelioma (MM), a highly aggressive neoplasm of the pleura, peritoneum or pericardium with a very poor prognosis (1), is presently a worldwide problem due to its increasing incidence (2,3). In Japan, a recent study using an age-cohort model reported that there will be approximately 100,000 deaths due to MM in the next 40 years (4). Patients with MM are usually diagnosed at advanced stages, and the disease is refractory to conventional therapy. Therefore, the survival rate of patients with MM is very poor, with a median survival of 7-11 months following diagnosis, especially in advanced stage patients. This is despite the recent advancement in chemotherapeutic modalities that combines cisplatin and antifolate (5,6).

The long latency period between asbestos exposure and tumor development implies that multiple, and likely diverse, genetic changes are required for the malignant transformation of mesothelial cells. Many studies have been conducted to determine the key underlying genetic and epigenetic events responsible for the development of MM. Karyotyping, allele typing and comparative genomic hybridization analysis have demonstrated that most cases of MM have multiple chromosomal alterations, which include chromosome 9p21 and 22q (7-14). Inactivation of the $p16^{INK4a}/p14^{ARF}$ locus at the 9p21 locus is found in over 70% of MM samples (15-17). The *NF2* gene at the 22q12 locus, which is responsible for a familiar cancer syndrome of neurofibromatosis type II, has also been shown to be inactivated in 40-50% of MMs, mainly with homozygous deletion or nonsense mutation (18,19). In contrast, mutation of the *p53* gene, one of the most frequently mutated genes in human malignancies, is relatively uncommon (20).

The phosphatidylinositol 3-kinase (PI3K)-AKT pathway regulates a number of normal cellular processes, including cell proliferation, survival and motility (21). AKT, also known as protein kinase B (PKB), is a serine/threonine kinase located downstream of PI3K. Activated PI3K generates a lipid second messenger, phosphatidylinositol-3,4,5-tri-phosphate (PIP3), which is essential for the translocation of AKT to the plasma membrane. There, AKT is phosphorylated and activated by phosphoinositide-dependent kinase 1 (PDK1). In tumor cells, AKT can be activated by a variety of mechanisms, including the loss of *PTEN*, mutation of the PI3K catalytic subunit gene *PIK3CA*, and activation of PI3K via autocrine or paracrine

Correspondence to: Dr Yoshitaka Sekido, Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan
E-mail: ysekido@aichi-cc.jp

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stimulation of the receptor tyrosine kinases (22,23). Recently, the *LKB1* tumor suppressor gene encoding a serine/threonine kinase has also been implicated in the regulation of PTEN activity (24).

There have been a limited number of studies of MM seeking to analyze this signaling cascade. For example, Altomare *et al* reported that elevated phospho-AKT staining was observed in 17 (65%) of 26 MM specimens, and loss of *PTEN* was detected in 1 of 9 human MM cell lines (25). Papp *et al* investigated *PTEN* point mutations in 18 mesothelioma specimens with single-strand polymorphism analysis, but no mutations were detected (26). To the best of our knowledge, there have been no detailed reports analyzing *PIK3CA* and *LKB1* mutation status in MM. Thus, although activation of the PI3K-AKT pathway is likely to be significantly involved in MM development, the molecular mechanism by which AKT is activated in MM remains unclear. In the present study, we carried out mutation and expression analyses of the genes mainly associated with the PI3K-AKT pathway in MM cells. We observed frequent dysregulation of the PI3K-AKT pathway in MMs, with PTEN inactivation being one of the important mechanisms underlying its activation.

Materials and methods

Cell lines. The study employed 21 MM cell lines and 1 non-MM cell line (MeT-5A). In addition to 4 cell lines, including ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D that have previously been reported (27), 11 cell lines, including Y-MESO-9, Y-MESO-12, Y-MESO-14, Y-MESO-21, Y-MESO-22, Y-MESO-25, Y-MESO-26B, Y-MESO-27, Y-MESO-28, Y-MESO-29 and Y-MESO-30, were established in our laboratory and will be described in a future report. Y-MESO-8A and Y-MESO-8D, established from the same patient, showed distinct morphological patterns (27). NCI-H28, NCI-H290, NCI-H2052, NCI-H2373 and NCI-H2452 were gifts from Dr Adi F. Gazdar. MSTO-211H and MeT-5A were purchased from the American Type Culture Collection (Rockville, MD, USA), and MeT-5A was cultured according to their instructions. All MM cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1X antibiotic-antimycotic (Invitrogen) at 37˚C in a humidified incubator with 5% CO_2. MM samples for the establishment of cell lines and clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all patients.

Extraction of DNA and RNA. Genomic DNA was extracted using a standard phenol-chloroform method. Total RNA was prepared using RNeasy Plus RNA Extraction Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. Random-primed, first-strand cDNA was synthesized from 3 μg total RNA using Superscript II according to the manufacturer's instructions (Invitrogen).

Real-time reverse transcription (RT)-PCR. Expression levels of PTEN mRNA were measured by means of quantitative real-time fluorescence detection. Briefly, PTEN-qRTS6, 5'-GGAAGTCTATGTGATCAAGAAACAGT-3' (sense) and PTEN-qRTAS8, 5'-CAGAAGTTGAACTGCTAGCCTCT GGA-3' (antisense) primers, respectively located at PTEN exon 6 and exon 8, were synthesized. Quantitative RT-PCR with the primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was carried out with an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. *GAPDH* served as an endogenous control; the expression levels of PTEN mRNA in each of the samples were normalized on the basis of the corresponding *GAPDH* content and recorded as relative expression levels.

RT-PCR of PTEN exon 1- 5 was carried out using a primer set of PTEN-RT-S1, 5'-ATGACAGCCATCATCAAAGAG ATC-3' (sense) and PTEN-RT-AS1, 5'-AGCTGTGGTGGG TTATGGTCTTCA-3' (antisense).

Mutation analysis. Sequencing of *PIK3CA*, *PTEN* and *LKB1* was carried out using an Applied Biosystems Model 3100 DNA Sequencer with a PCR primer and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) following the PCR amplification of genomic DNA. The primer sets covering the entire coding region of *PIK3CA* and *PTEN* have been previously reported (22,28). The primer sets of *LKB1* are available on request.

Cloning of PTEN homozygous deletion region breakpoints. Genomic PCR of *PTEN* covering exon 1 or exon 2 was carried out using the primer sets PTEN-S1, 5'-GTGACCTCCTT CGGAAAGTA-3' (sense) and PTEN-AS1, 5'-CTTTACTGG ACAGATAAGCTTATT-3' (antisense), or PTEN-S2, 5'-CC TTCTTGTGGGGTGCTAATGAAA-3' (sense) and PTEN-AS2, 5'-CTACTTAAGGGAGTGTAGTAGTGG-3' (antisense), respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gel and sequenced.

Western blot analysis. Preparation of total cell lysates and Western blotting were carried out as described previously (27). In brief, cells growing subconfluently were rinsed twice with PBS, lysed in SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol) and homogenized. Total cell lysate (30 μg) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Following blocking with 3% non-fat dry milk, the filters were incubated with the primary antibody, washed with PBS with 0.5% Tween-20, reacted with the secondary antibody, and then detected with ECL (Amersham Biosciences, Buckinghamshire, UK). Anti-AKT, anti-phosphorylated-AKT (Ser473) and anti-PTEN antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and anti-ß-actin antibody from Sigma (St. Louis, MO, USA).

Constructs. A 1.2-kilobase (kb) fragment covering the entire human *PTEN* coding region was amplified using RT-PCR and introduced into the pcDNA3.1-V5/HIS expression vector (Invitrogen) to generate a wild-type PTEN expression construct (pcDNA-PTENwt-V5/HIS). A *PTEN* mutant-type (H123Y) expression construct (pcDNA-PTENmt123Y-V5/HIS) was synthesized using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according

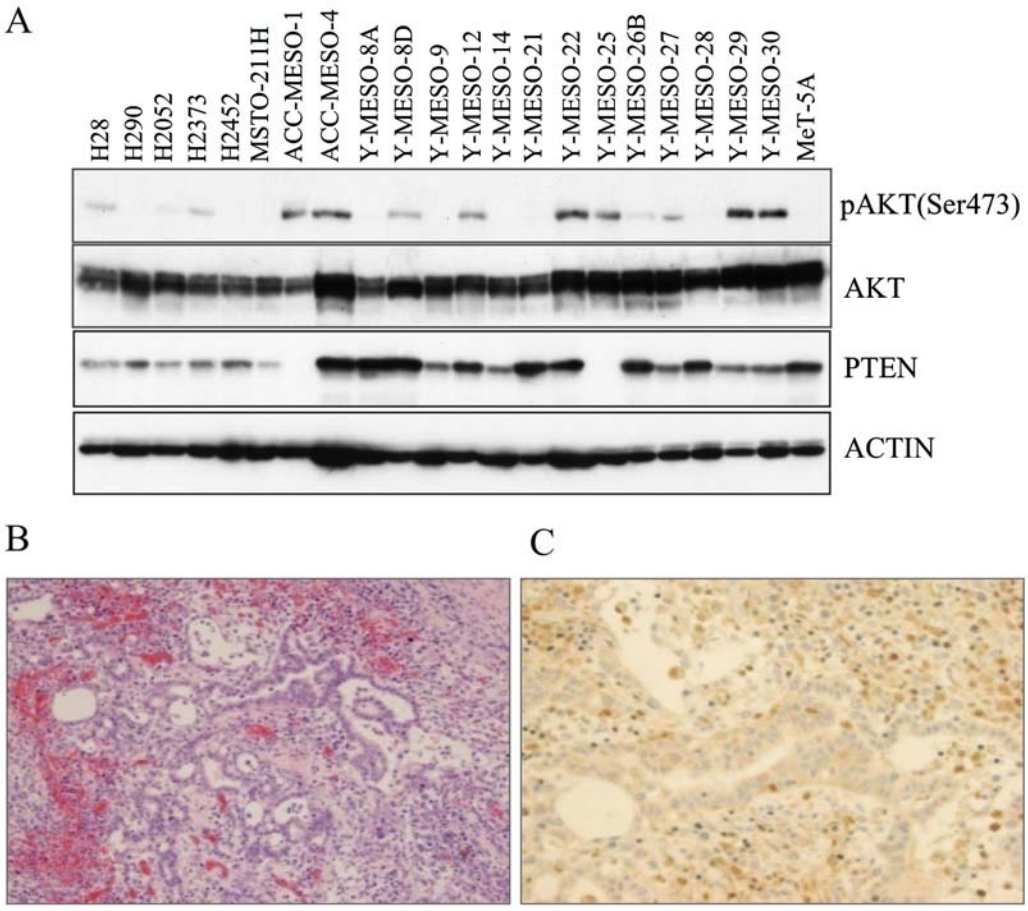

Figure 1. Expression of AKT and PTEN in MM cells. (A) Western blot analysis of AKT and PTEN in 21 MM cell lines and normal mesothelial cell line MeT-5A. Activation of AKT was studied with anti-phospho-AKT (Ser473) antibody. Expression of ß-actin was used as the control. (B) The primary tumor cells of the Y-MESO-25 cell line showed a papillotubular pattern with hematoxylin and eosin staining. (C) Immunohistochemical staining of PTEN showed no expression in the tumor cells, but was positive in stromal cells (original magnification, x200).

to the manufacturer's instructions. The sequences of all constructs were confirmed. The expression vector of the dominant negative form of AKT (AKT DN) has been described previously (29).

Colony formation assay. Cells were cultured to 70% confluence on 6-well plates and transfected with the wild-type PTEN, mutant-type PTEN, or pcDNA3.1-V5/HIS vector along with FuGENE 6 reagent following the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). After 24 h, G418 was added to the medium for a final concentration of 400 μg/ml. After 16-18 days, the cells were stained with methylene blue.

Results

Activation of the PI3K-AKT pathway in malignant mesothelioma cell lines. Dysregulated activation of the PI3K-AKT signaling pathway has been demonstrated in a variety of human malignancies. To determine the frequency and mechanisms underlying the activation of this pathway in MM, we analyzed 21 MM cell lines as well as the MeT-5A cell line, an immortalized cell line from normal mesothelial cells. Western blot analysis was performed for AKT and PTEN, a tumor suppressor involved in the PI3K-AKT pathway. Under serum-starved conditions, we found that AKT was phosphorylated (activated) at a high level in 6 cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-22, Y-MESO-25, Y-MESO-29 and Y-MESO-30) and at mid-level in 7 cell lines (NCI-H28, NCI-H2052, NCI-H2373, Y-MESO-8D, Y-MESO-12, Y-MESO-26B and Y-MESO-27), but was undetectable in another 8 cell lines and MeT-5A (Fig. 1A). Thus, overall elevated AKT activation was shown in 13 (62%) MM cell lines.

Mutational analyses of PIK3CA, LKB1 and PTEN in malignant pleural mesothelioma cell lines. We performed mutation analyses for the *PIK3CA* and *LKB1* genes, since their activating mutations are known to be responsible for AKT activation. All 20 exons of the *PIK3CA* coding region and 9 exons of *LKB1* were sequenced, but no mutations were found (data not shown).

Next, we studied whether PTEN can be altered in malignant pleural mesothelioma (MPM), since PTEN inactivation is also known to be one of the major causes of AKT activation. Western blot analysis revealed that PTEN expression was completely lost in two cell lines, ACC-MESO-1 and Y-MESO-25 (Fig. 1A). Furthermore, compared to the Met-5A level, 11 other cell lines clearly exhibited a relatively low level of PTEN, including NCI-H28, H290, H2052, H2373, H2452, MSTO-211H, Y-MESO-9, Y-MESO-14, Y-MESO-27, Y-MESO-29 and Y-MESO-30. Since the original pathological specimen of the Y-MESO-25 cell line was available, we performed

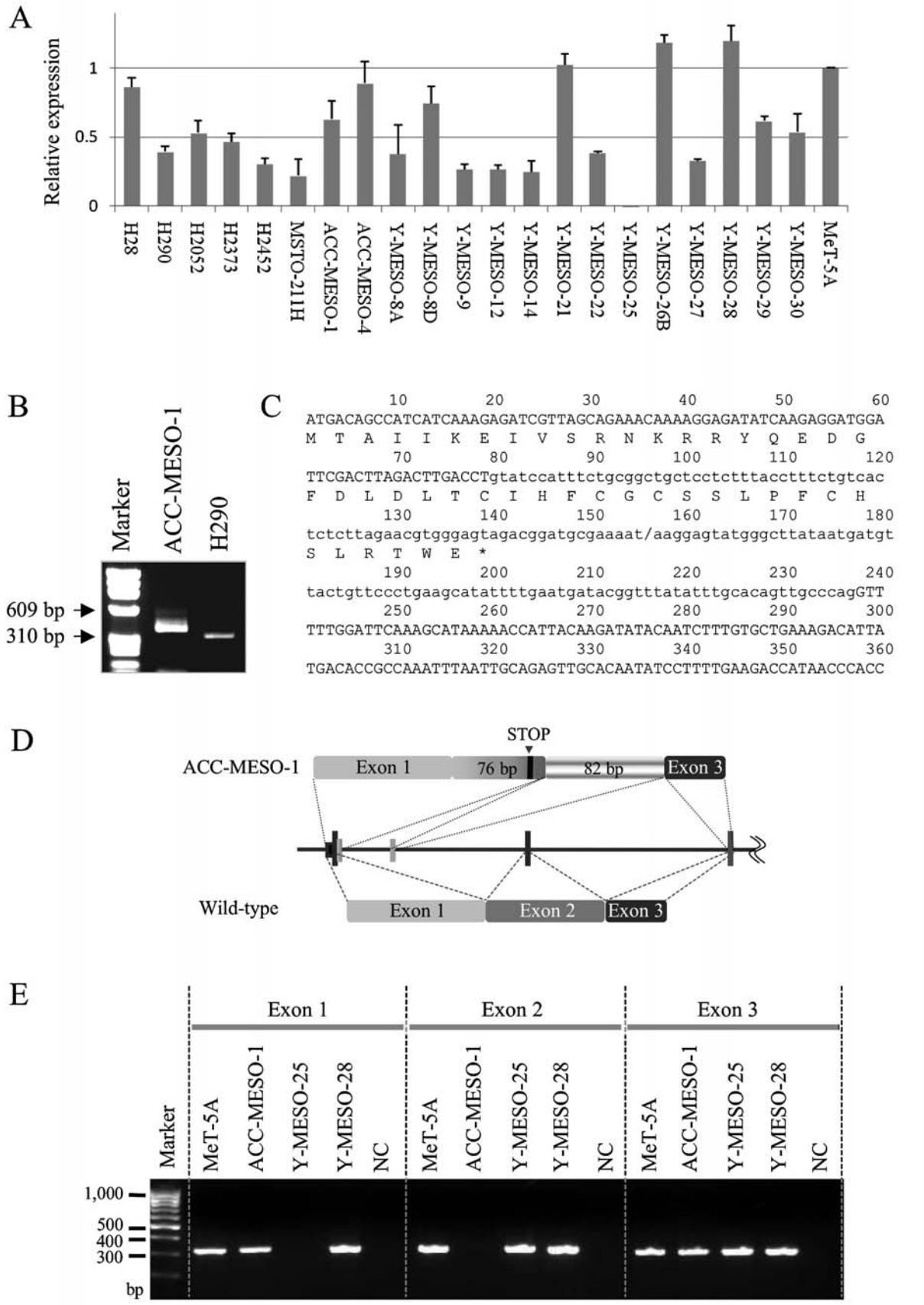

Figure 2. Expression analysis of PTEN mRNA in MM cell lines. (A) Quantitative RT-PCR analysis of PTEN with a primer set covering exon 6 to 8. The relative expression of MeT-5A was arbitrarily set to 1.0. Data represent mean ± SD. Y-MESO-25 showed an undetectable level of PTEN mRNA expression and 10 other cell lines exhibited lower expression under 0.5. (B) RT-PCR analysis with a *PTEN* primer set covering exon 1 to 6 showed an aberrant sized band in ACC-MESO-1. (C) Sequence analysis of the amplified PTEN PCR product of ACC-MESO-1. Seventy-six nucleotides (indicated by lowercase letters from 80 to 155) were intron 1 sequence which followed the exon 1. Eighty-two nucleotides (lowercase letters from 156 to 237) were also from intron 1. (D) Schematic representation of the aberrant transcript of PTEN in ACC-MESO-1 (above) and the wild-type (below). (E) Genomic PCR analysis of Y-MESO-25 and ACC-MESO-1 cells detected no PCR products of PTEN exon 1 and exon 2, respectively. NC, negative control (water blank).

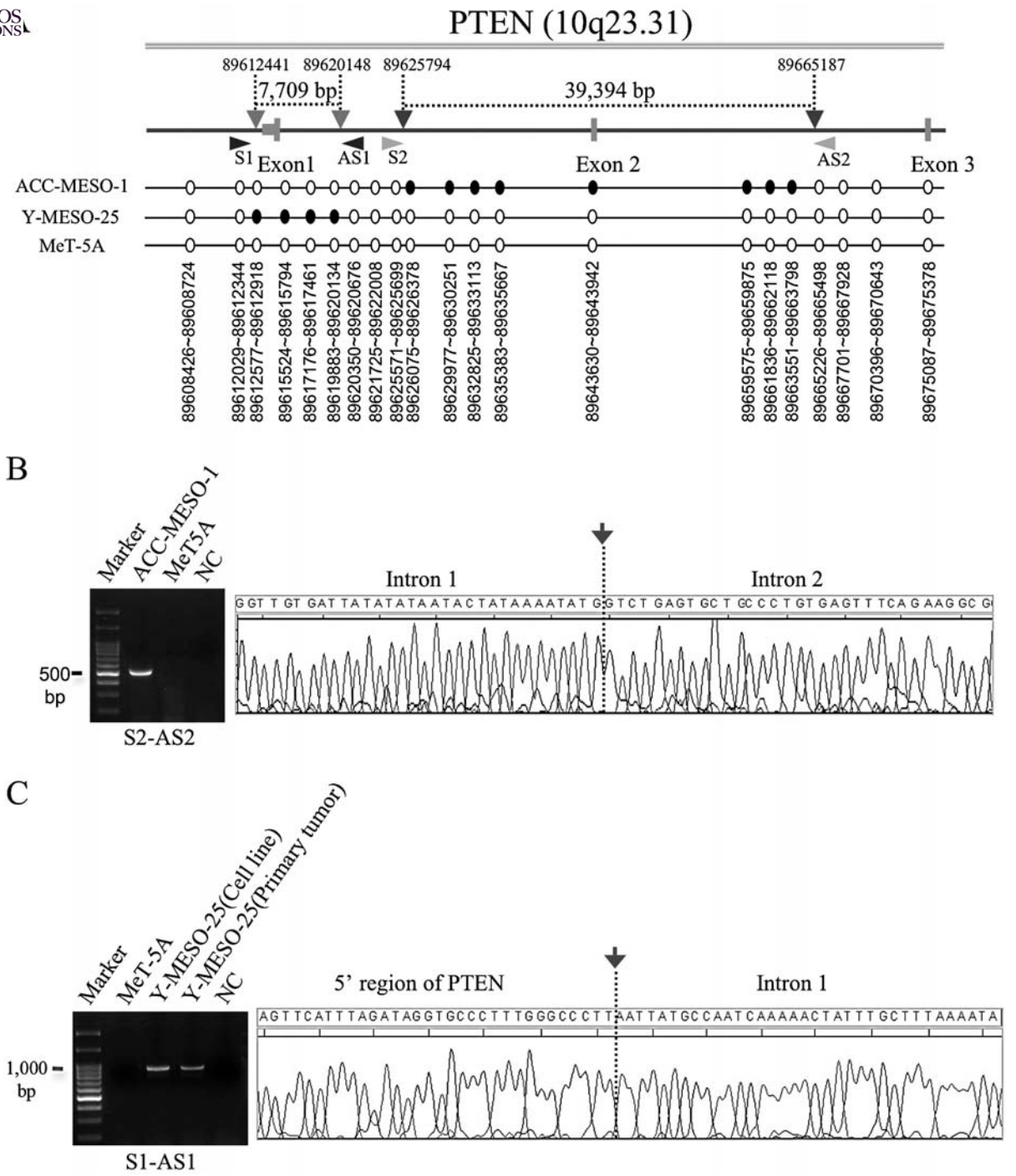

Figure 3. Cloning of the homozygous deletion breakpoints of the PTEN locus in ACC-MESO-1 and Y-MESO-25. (A) Results of PCR analysis for each locus are shown by open ovals (retention) and closed ovals (homozygous deletion). Nucleotide numbers of each location are indicated according to the March 2006 human reference sequence (NCBI Build 36.1). (B) PCR amplification with the S2 and AS2 primers yielded an aberrant 534-bp product with genomic DNA from ACC-MESO-1, but not from MeT-5A (left). The sequence of the PCR product detected the breakpoint indicated by an arrow (right). (C) PCR amplification with the S1 and AS1 primers yielded an aberrant 940-bp product with genomic DNA from Y-MESO-25 and its primary tumor, but not from MeT-5A (left). The nucleotide sequence of the PCR product detected the breakpoint indicated by the arrow (right).

immunohistochemical analysis and found additional defective expression of PTEN in the primary tumor cells (Fig. 1B and C).

PTEN expression was examined by quantitative real-time PCR (qRT-PCR) analysis using a primer set covering exons 6-8. While, as in other cell lines, the PTEN mRNA transcript in ACC-MESO-1 was detectable, a complete loss of PTEN expression was observed in Y-MESO-25 (Fig. 2A). Ten other cell lines also showed low expression of PTEN mRNA (less than half of the MeT-5A level), including NCI-H290, H2373, H2452, MSTO-211H, Y-MESO-8A, Y-MESO-9, Y-MESO-12, Y-MESO-14, Y-MESO-22 and Y-MESO-27 (Fig. 2A). RT-PCR of PTEN was then performed using various primer sets. When a primer set encompassing exons 1-5 was used, an aberrant-sized PTEN transcript (~370 bp) was detected in ACC-MESO-1 (Fig. 2B). Sequence analysis of this cDNA fragment revealed an abnormal splicing pattern with the addition of a 76-bp intron 1-fragment following exon 1 and another 82-bp intron 1-fragment between exons 1 and 3, while

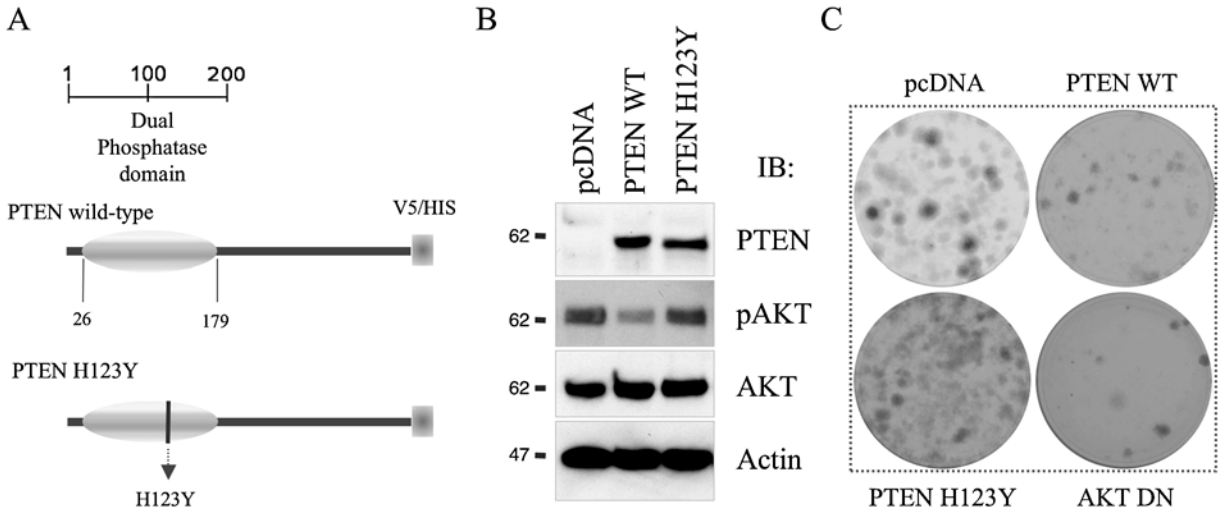

Figure 4. Re-expression of PTEN suppresses AKT activation and reduces the colony formation of malignant pleural mesothelioma cells. (A) Schematic representation of V5-His tagged wild-type and mutant-type (H123Y) PTEN constructs. (B) Western blot analyses of PTEN, pAKT and AKT after transfection of wild-type and mutant-type PTEN into Y-MESO-25 cells. ß-actin antibody was used as the control. (C) G418-resistant colonies of ACC-MESO-1 were detected with methylene blue staining 16-18 days after cells were transfected with control (pcDNA3.1), wild-type PTEN (pcDNA-PTENwt-V5/HIS), mutant-type PTEN (pcDNA-PTENmt123Y-V5/HIS) or AKT dominant negative (AKT DN) construct.

skipping exon 2 (Fig. 2C and D). This splicing alteration caused the addition of an extra 158 bp with the deletion of exon 2, resulting in a premature stop (Fig. 2C and D).

To determine whether a genetic mutation was responsible for PTEN inactivation, we sequenced 9 exons covering the entire coding region of the *PTEN* gene. Although no mutations were detected in the other 19 cell lines, no amplification of exon 1 in Y-MESO-25 or exon 2 in ACC-MESO-1 was found, indicating that these cell lines harbored a homozygous deletion of *PTEN* (Fig. 2E).

Homozygous deletion on the PTEN locus. We further carried out a genomic PCR analysis for these two cell lines using multiple primer sets to determine the break-points of the homozygous deletion regions in more detail. The results of the PCR analysis are summarized in Fig. 3A. Genomic PCR with the S2 and AS2 primer demonstrated a 530-bp PCR product in ACC-MESO-1 (Fig. 3B), which resulted from an ~40-kb deletion including exon 2. Meanwhile, in Y-MESO-25, genomic PCR with the S1 and AS1 primers detected an ~900-bp PCR product in DNA derived from the cell line as well as from its primary tumor (Fig. 3C). The Y-MESO-25 cell line was demonstrated to have a 7.7-kb homozygous deletion including the promoter region and exon 1 of *PTEN*.

Re-expression of PTEN or AKT dominant negative constructs reduces colony formation of MM cells. To determine whether the loss of *PTEN* plays a pro-oncogenic role for ACC-MESO-1 and Y-MESO-25 cell lines, we transfected a wild-type PTEN expression vector into these cell lines. In addition to the wild-type *PTEN*, a mutant-type construct with a tyrosine for histidine substitution at amino acid position 123 was also synthesized. This mutation has been shown to abrogate the phosphatase function of PTEN (30) (Fig. 4A). Western blot analysis showed that the wild-type but not the H123Y mutant-type of *PTEN* suppressed the phosphorylation status of AKT (Fig. 4B). Colony formation assay showed that wild-type, but not mutant-type, *PTEN* reduced the number of colonies, indicating that PTEN inactivation is critical for the cell survival or proliferation of these cell lines (Fig. 4C). AKT dominant negative construct also significantly inhibited colony formation (Fig. 4C).

Discussion

In this study, we examined the frequency and underlying mechanisms of the dysregulated PI3K-AKT pathway in MM cells. Twenty one MM cell lines were used, including 15 cell lines established in our laboratory from Japanese patients. Our results indicate that AKT was highly phosphorylated in 13 (62%) of 21 MM cell lines under serum-starved conditions, suggesting that constitutively activated AKT may be observed in most MMs. Two cell lines harbored a homozygous deletion of *PTEN*, and 11 cell lines showed low expression of PTEN protein, although no mutation of *PIK3CA* or *LKB1* was detected. Re-expression of *PTEN* in the cell lines with *PTEN* homozygous deletion suppressed colony formation, indicating that *PTEN* inactivation was critical for cell survival or proliferation in these cell lines.

Previous immunohistochemical studies demonstrated the elevated activation of AKT in 65-84% of MM samples (25,31). Thus the frequency of AKT activation in MMs in our study using cell lines seems to be very similar to that of previous studies. Whereas one previous study reported that no *PTEN* mutation was detected in 18 MM specimens, another study demonstrated that a homozygous deletion of *PTEN* was detected in 1 of 9 MM cell lines (25,26). Thus, our finding that a *PTEN* homozygous deletion, but not point mutation, was present in 2 (9.5%) of 21 MM cell lines is compatible with the results of previous studies.

The loss of PTEN expression has been attributed to allelic loss or promoter hypermethylation in some malignancies (32,33). In non-small cell lung cancer, the homozygous deletion and mutation of *PTEN* occur rarely, although loss of hetero-

(LOH) has been reported to occur in 26% to more of tumors (34). While we did not study the LOH or methylation status of the *PTEN* gene, among another 19 cell lines excluding ACC-MESO-1 and Y-MESO-25, over half the lines also exhibited low PTEN mRNA and protein expression levels. Ten cell lines expressed PTEN mRNA at less than half the level observed in MeT-5A, and 11 cell lines showed significantly low expression of PTEN, with 7 cell lines (NCI-H290, H2373, H2452, MSTO-211H, Y-MESO-9, Y-MESO-14 and Y-MESO-27) showing simultaneous suppression at the mRNA and protein level. This suggests that these cell lines had only one normal allele or suffered from hypermethylation of the other allele of the *PTEN* gene, resulting in low levels of PTEN mRNA and protein expression. From this standpoint, Garland *et al* reported that, while no PTEN expression was noted in 3 (16%) of 19 primary MMs, 15 (79%) showed only a mild intensity of staining (31). Opitz *et al* also reported negative PTEN expression in 62% of MPMs from an immunohistochemical study using tissue microarray containing 341 cases, with 14% being weak, 9.5% moderate, and 14.5% strong (35). Taken together with other studies, our results suggest that, as one of the underlying mechanisms, activation of the PI3K-AKT pathway is due to a *PTEN* homozygous deletion in ~10% of MMs, and to the suppression of PTEN expression in a significant proportion of MM cases, probably caused either by the allelic loss or epigenetic silencing of the *PTEN* gene.

Meanwhile, several cell lines showed a distinct difference between the expression of PTEN mRNA/protein and AKT phosphorylation status in each MPM cell line. For example, although NCI-H290, H2452, MSTO-H211 showed low expression of PTEN at both the mRNA and protein level, AKT phosphorylation was not very obvious. Thus, significant down-regulation of PTEN does not necessarily lead directly to AKT activation. In this regard, recent evidence suggests that PTEN has other functions as a tumor suppressor, such as chromosomal instability (36). Thus, other functions of PTEN that are not involved in the dephosphorylation of PIP3 may be responsible for a pro-oncogenic role of MM cells when PTEN is suppressed.

For PI3K-AKT pathway activation, it should also be considered that the activation of upstream molecules, including receptor tyrosine kinases, may play an important role. For example, hepatocyte growth factor-MET receptor signaling activation has been demonstrated to be accompanied by AKT activation in MPM cell lines (25,37). Activation of HGF/MET signaling has also been shown to induce the proliferation of MM cells via a PI3K/MEK5/Fra-1 pathway (38). Meanwhile, overexpression of EGFR and PDGFR ß has been implicated in the tumorigenesis of mesothelioma (39-42). Thus, more detailed investigation of the activation status and underlying genetic abnormalities of these receptor tyrosine kinases is warranted in future, in order to reveal other possible underlying mechanisms of PI3K-AKT activation in MM cells.

Finally, exogenous expression of wild-type PTEN in the MM cell lines with a PTEN homozygous deletion clearly triggered significant cell-growth suppression, while the H123Y mutant lacking both dual-phosphatase activities was ineffective. Expression of the AKT dominant negative form also exhibited growth suppression. These results suggest that the protein phosphatase activity of PTEN likely contributes to its tumor suppressor function in a subset of MMs. This is supported by a previous study, which reported that PTEN overexpression transfected with PTEN adenoviral vectors engendered apoptotic cell death in two human MM cell lines by AKT hypophosphorylation (43).

In conclusion, our data strongly suggest that the PI3K-AKT pathway is significantly involved in MM carcinogenesis, and that elucidation of the downstream targets that dictate cellular response to this signaling pathway may have important implications for the development of future MM treatment strategies.

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