Methylation and mutation of the inhibin-α gene in human melanoma cells and regulation of PTEN expression and AKT/PI3K signaling by a demethylating agent

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Abstract. Inhibin suppresses the pituitary secretion of follicle-stimulating hormone and has been reported to act as a tumor suppressor gene in the gonad in mice. Epigenetic modifications, mutations, changes in the loss of heterozygosity (LOH) of the inhibin-α gene and regulation of gene expression in response to a demethylating agent [5-aza-2'-deoxycytidine (5-Aza-dC)] in human melanoma cells were assessed. In addition, the association between a mutation in the 5'-untranslated region (5'-UTR) of the inhibin-α subunit and the expression of phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2 (PREX2) and phosphatase and tensin homolog (PTEN) as well as AKT/PI3K signaling was determined. The methylation status of the CpG sites of the inhibin-α promoter was analyzed by methylation-specific PCR in bisulfite-treated DNA. Cell viability was counted using the trypan blue assay, mRNA expression was examined via reverse transcription-quantitative PCR, and protein expression was examined via western blot analysis. The inhibin-α promoter was hypermethylated in G361, SK-MEL-3, SK-MEL-24 and SK-MEL-28 cells and moderately methylated in SK-MEL-5 cells. Inhibin-α gene mutations were observed in the 5'-UTR exon 1 of G361, SK-MEL-5, SK-MEL-24 and SK-MEL-28 cells as well as in exon 2 of SK-MEL-3 cells. Allelic imbalance, including LOH, in the inhibin-α gene was detected in human melanoma cells. Treatment with 5-Aza-dC increased inhibin-α mRNA and protein levels, inhibited cell proliferation, and delayed the doubling times of surviving melanoma cells. In 5-Aza-dC-treated cells, PREX2 protein expression was slightly increased in G361 and SK-MEL-24 cells and decreased in SK-MEL3, SK-MEL-5 and SK-MEL-28 cells. However, the protein expression of PTEN was decreased in melanoma cells. In addition, AKT and PI3K protein phosphorylation levels increased in all melanoma cells, except of G361 cells, demonstrating decreased PI3K protein phosphorylation. These data provided evidence that methylation, mutation and LOH are observed in the inhibin α-subunit gene and gene locus in human melanoma cells. Furthermore, the demethylating agent reactivated inhibin-α gene expression and regulated PREX2 expression. AKT/PI3K signaling increased as PTEN expression decreased. In addition, mutations in the tumor suppressor inhibin-α, PTEN and p53 genes were not associated with transcriptional silencing, gene expression and cell growth as analyzed through experiments and literature reviews. These data demonstrated that methylation and mutations were associated with the inhibin-α gene in human melanoma cells and indicated the regulation of PTEN expression and AKT/PI3K signaling by a demethylating agent.

Introduction

Inhibins, which members of the transforming growth factor-β superfamily, are heterodimers consisting of a common α-subunit and one of two homologous β-subunits (βA or βB) (1,2). Inhibin suppresses the production and secretion of follicle-stimulating hormone in classical endocrine function and acts in an autocrine and paracrine manner in reproductive tissues (3,4). Studies in transgenic mice have identified a tumor-suppressor role of inhibin-α (5,6). Inhibin-α gene expression is inhibited in malignant sites of high-grade prostate cancer tissue, but inhibin-α is not expressed in poorly differentiated tumor cells. Accordingly, unlike ovarian granulocyte tumor cells, inhibin-α gene is expressed at low levels in poorly differentiated prostate cancer (7). DNA methylation may be responsible for this transcriptional silencing (8).

Methylation is catalyzed by enzymes and is involved in heavy metal modification, gene expression regulation, protein function regulation and RNA processing. Aberrant methylation of CpG islands that are not normally methylated occurs

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relatively frequently in immortalized and transformed cells, and this phenomenon is associated with inappropriate silencing of human tumor suppressor genes (9,10). This modification is particularly associated with CpG islands in the promoter region and has important regulatory effects on gene expression (11,12). CpG island methylation is an epigenetic change involved in tumorigenesis via transcriptional inactivation (13). Epigenetic changes, including DNA methylation and histone modifications, represent important mechanisms for cancer suppressor gene silencing and affect chromatin structure and genomic stability (14). The DNA methylation characteristics of skin metastatic tumor tissue can provide predictive evidence of therapeutic response to immune checkpoint suppression in patients with stage IV metastatic melanoma (15). Latent methylation component-based detection of DNA methylation has been indicated to be a tool for developing classifiers and evaluating treatment response in patients with cancer undergoing targeted immunotherapy (16). DNA methylation at CpG sites in cancer is an epigenetic change. Hypermethylation limits immune checkpoint blockade therapy by inhibiting the endogenous interferon response in cancer cell recognition (17). Conversely, hypomethylation indicates the expression of programmed death ligand 1 and inhibitory cytokines accompanied by epithelial-mesenchymal changes, which may contribute to immunosuppression (18). Melanoma pathogenesis is facilitated through numerous genomic alterations in various components of the mitogen-activated protein kinase and phosphoinositol-3-kinase (PI3K) pathways (19), and these pathways have been examined for potential use as biomarkers in advanced melanoma treatment and prognosis strategies (20).

Transcriptional silencing of the inhibin-α gene has been confirmed in human prostate cancer, gastric cancer and leukemia (21-23). The primary causes of the silencing were methylation, mutation and heterozygous loss of chromosomal abnormalities in the inhibin-α promoter. The human inhibin α-gene is located at the q33-q36 region of chromosome 2, and a deletion of 2q has been observed in numerous human tumors, including prostate cancer (24-26). Inhibin serves an important role in hormonal regulation; however, there has been no investigation of its methylation, mutations and chromosomal abnormalities in human melanoma cells.

Therefore, the methylation status, mutations and loss of heterozygosity (LOH) of the inhibin-α gene promoter, as well as the regulation of inhibin-α gene expression by an inhibitor of DNA methylation [5-aza-2'-deoxycytidine (5-Aza-dC)] in human melanoma cells were investigated. In addition, the association between a mutation in the inhibin-α 5'-untranslated region (5'-UTR) and the expression of phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2 (PREX2) and phosphatase and tensin homolog (PTEN) as well as AKT/PI3K signaling was examined.

Materials and methods

Cell culture. G361, SK-MEL-3, SK-MEL-5, SK-MEL-24 and SK-MEL-28 human melanoma cell lines were purchased from the American Tissue Culture Collection. SNU-668 cells (inhibin-α positive control cells) were supplied from Korean Cell Line Bank; Korean Cell Line Research Foundation. G361 and SNU-668 cells were cultured in RPMI-1640 medium (Corning, Inc.), and SK-MEL-3, SK-MEL-5, SK-MEL-24 and SK-MEL-28 cells were cultured in Dulbecco's modified Eagle medium (Corning, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2 and 95% air.

Bisulfite modification. Genomic DNA was extracted using Wizard Genomic DNA purification kit (cat. no. A1120; Promega Corporation). DNA (2 μg) methylation was performed using a bisulfite conversion kit (cat. no. D5001; EZ DNA methylation kit; Zymo Research Corp.) according to the manufacturer's protocol. DNA was resuspended in 20 μl water and either used immediately or stored at -20˚C.

Detection of methylation. Methylation was assessed via polymerase chain reaction (PCR) and sequence analysis of the bisulfite-treated DNA. The bisulfite reaction converted unmethylated cytosine to uracil, whereas methylated cytosine remained unchanged. The inhibin-α 5'-UTR region was amplified using nested PCR with primers designed for the bisulfite-converted sequence, as previously described (26). The primer sequences are listed in Table I. Primer sequences methylation-specific PCR (MSP) 1 and 2 were used for the first round of PCR, whereas primer sequences MSP 3 and 4 were used for the second round. The first round of PCR was performed in a 25-μl reaction mixture consisting of 2 μl bisulfite-converted DNA, 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 10 pmol of each primer (1 and 2) and 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems; Thermo Fisher Scientific, Inc.). The first round of PCR cycle conditions were as follows: 95˚C for 15 min, followed by five cycles at 95˚C for 1 min, 50˚C for 2 min and 72˚C for 3 min and 30 cycles at 95˚C for 1 min, 55˚C for 2 min and 72˚C for 2 min, with a final incubation step at 72˚C for 10 min. A 2-μl sample of the first PCR product was amplified in a 25-μl reaction mixture as aforementioned, except for primers 3 and 4 being used. The second round PCR conditions included 35 cycles at 95˚C for 1 min, 60˚C for 2 min and 72˚C for 2 min. The PCR products were electrophoresed on 1.5% agarose containing 0.5 μg/ml ethidium bromide, gel-purified, ligated into the pCR2.1-TOPO vector, and cloned using the TOPO TA Cloning Kit according to the manufacturer's protocol (cat. no. 45-0641; Invitrogen; Thermo Fisher Scientific, Inc.). For each PCR, 10 clones were sequenced, and the methylation status at each of the seven CpGs was determined. Sequencing analysis was performed by BIONICS Co., Ltd. using a high throughput DNA analyzer (3730XL DNA Analyzer; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the Sanger method.

DNA analysis. DNA was isolated from cultured cells using standard methods. Two regions of the inhibin-α gene were amplified from genomic DNA using PCR with specific oligonucleotide primers as previously described (27). The first region of 240 bp (fragment A), which includes 140 bp of 5'-UTR and 100 bp of exon 1, and the second region of 396 bp (fragment B), which comprises part of exon 2, was amplified using the primers listed in Table I. Genomic DNA (200 ng) was amplified in a 50-μl reaction mixture containing 1X PCR

Table I. Primer sequences.

Type of analysis of inhibin-α gene	Primer name	Orientation	Sequence (5'-3')	Product size (bp)	Annealing temperature (˚C)	GenBank accession number or locus map on the chromosome
Methylation	MSP 1	Forward	GATAAGAGTTTAGATTGGTTTTATTGGTT	681	50, 55	AF272341.1
	MSP 2	Reverse	ACACCATAACTCACCTAACCCTACTAATAA			
	MSP 3	Forward	GAAGGTGTTGTATGTTTGTATGTGTGAGTT	275	60	
	MSP 4	Reverse	ACCCCTTCTACCAAAATCTACCCAAAA			
Mutation	5'-UTR and exon 1	Forward	GACTGGGGAAGACTGGATGA	240	57	NM_002191.2
		Reverse	TCACCTTGGCCAGAACAAGT			
	Exon 2	Forward	AGCAGCCTCCAATAGCTCTG	396	57	NM_002191.2
		Reverse	AGCTCCTGGAAGGAGATGTTC			
LOH	2q32-q33	Forward	TAAAGCCTAGTGGAAGATCATC	198	55	D2S389
		Reverse	GCTGAGTTAACAGTTATCAACAATT			
	2q33-q36	Forward	AAACTGAGATTTGTCTAAGGGG	155	55	D2S128
		Reverse	AGCCAGGAATTTTTGCTATT			
RT-PCR	Inhibin-α	Forward	AGGAAGAGGAGGATGTCTCC	823	50	NM_002191.4
		Reverse	GAGTAACCTCCATCCGAGGT			
	β-actin	Forward	CTTCTACAATGAGCTGCGTG	305	55	NM_001101.3
		Reverse	TCATGAGGTAGTCAGTCAGG			
qPCR	Inhibin-α	Forward	CTCGGATGGAGGTTACTCTTTCAA	88	60	NM_002191.4
		Reverse	GAAGACCCCCCACCCTTAGA			
	β-actin	Forward	GCGAGAAGATGACCCAGATC	77	60	NM_001101.3
		Reverse	GGATAGCACAGCCTGGATAG			

MSP, methylation specific primer; LOH, loss of heterozygosity; RT-PCR, reverse transcription PCR; qPCR, quantitative PCR; UTR, untranslated region.

buffer, 2 mM $MgCl_2$, 2.5% DMSO, 0.2 mM of each dNTP, 20 pmol of each specific primer and 1.5 unit of AmpliTaq Gold DNA polymerase. The conditions for the amplification were as follows: Denaturation at 95˚C for 14 min; followed by denaturation at 95˚C for 40 sec, annealing at 57˚C for 30 sec and extension at 72˚C for 1 min for 35 cycles; and a final extension at 72˚C for 7 min. The polymorphism -16C->T in the 5'-UTR was screened via restriction enzyme analysis with *Spe*I (New England Biolabs, Inc.). Briefly, fragment A was amplified using PCR, and 5 µl of the purified PCR product were digested overnight at 37˚C with 5 units *Spe*I, electrophoresed on 8% acrylamide gels, stained with 0.5 µg/ml ethidium bromide, and visualized using a Gel Doc 1000 Gel Documentation System (Bio-Rad Laboratories, Inc.). The presence of a 240-bp fragment indicated a homozygous wild-type variant, whereas the presence of two 120-bp fragments corresponded to the homozygous variant T. The substitution of 769G->A in exon 2 was analyzed via digestion of fragment B with different restriction enzymes. In brief, 5 µl of the purified PCR product were digested overnight at 37˚C with 5 units of *Bsr*FI (New England Biolabs, Inc.) and analyzed as aforementioned. The restriction site that renders two fragments of 340 and 56 bp was abolished in the variant allele. In addition, 5 ml of the purified PCR product was digested overnight at 37˚C with 5 units of *Fnu4*HI (New England Biolabs, Inc.), electrophoresed on 15% acrylamide gels, stained with 0.5 µg/ml ethidium bromide, and visualized by image analysis. The 396-bp fragment rendered four fragments of 153, 107, 51 and 25 bp, among other fragments of lower molecular weight in the wild-type allele, whereas the allele with a 769G->A substitution rendered four fragments of 153, 107, 76 and 51 bp, among other fragments of lower molecular weight.

LOH analysis. LOH was determined using microsatellite markers on 2q32-q33 and 2q33-q36, as previously described (26). The oligonucleotide primer sequences are listed in Table I. PCR was performed in a 20-µl reaction mixture consisting of 200 ng DNA, 1X PCR buffer, 0.2 mM of each dNTP, 10 pmol of each primer and 1 unit of AmpliTaq Gold DNA polymerase. The conditions for amplification were as follows: Denaturation at 95˚C for 14 min; followed by denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min and extension at 72˚C for 1 min for 35 cycles; and final extension at 72˚C for 10 min. Subsequently, 10 µl PCR products were mixed with 10 ml of stop solution containing 95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene

cyanol FF. The mixture was denatured at 95˚C for 5 min, placed on ice for 5 min, electrophoresed on 12% acrylamide gels containing 10% glycerol with 1X Tris/Borate/EDTA buffer, and stained with 0.5 μg/ml ethidium bromide. LOH was defined as a reduction in the intensity of the signal of a single allele by >50% in the tumor cell DNA as assessed by direct visualization, compared with DNA of peripheral blood lymphocytes (22,23). Allelic imbalance was evaluated when a difference in the running pattern of the products was observed.

5-Aza-dC treatment. Cells were seeded at a density of $5x10^5$ cells/100 mm culture dish. The cells were allowed to attach for 24 h and then exposed to different concentrations (0, 0.1, 0.5, 2, 5, and 10 μM) of the DNA methylation inhibitor 5-Aza-dC (Sigma-Aldrich; Merck KGaA) for 5 days at 37˚C. The medium and the drug were replaced every 2 days. At the end of the treatment period, the medium was removed, and the cell pellets were subjected to reverse transcription-quantitative PCR (RT-qPCR) and immunoblot analysis.

Cell proliferation assay. For the cell viability assay, cells were seeded at $5x10^4$ cells/well in 6-well plates and exposed to various concentrations (0, 0.1, 0.5, 2, 5 and 10 μM) of 5-Aza-dC for 5 days at 37˚C. To evaluate cell doubling time following exposure to 5 μM 5-Aza-dC, the cells were seeded at $2x10^4$ cells/well in 12-well plates containing culture medium. Cell number was determined daily using the trypan blue exclusion assay (0.4% trypan blue solution; 10 μl diluted cells combined with 10 μl trypan blue solution) for 5 consecutive days. Untreated cells were analyzed under similar conditions as a control. The cell number was counted under a light microscope (magnification, x100). The average cell number from two plates was determined, and the mean cell numbers were plotted to calculate the cell population doubling times.

RNA isolation and RT-qPCR. Total RNA was purified from cultured cells using the TRIzol® reagent method, according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand cDNA synthesis was performed using 1 μg total RNA and $Oligo(dT)_{15}$ primers in a reverse transcription system kit (cat. no. A3500; Promega Corporation), according to the manufacturer's protocol. The primer sequences are listed in Table I.

PCR was performed using 2 μl cDNA in a 50-μl reaction mixture containing 1X PCR buffer, 200 μM of each dNTP, 20 pmol of each inhibin-α primer and 2 units of AmpliTaq Gold DNA polymerase. The reactions were carried out in a thermal cycler with an initial denaturation step at 95˚C for 14 min; followed by 35 cycles (25 cycles for β-actin) of denaturation at 95˚C for 1 min, primer annealing at 50˚C (inhibin-α) and 55˚C (β-actin) for 1 min; and a final extension step at 72˚C for 1 min. The reaction was terminated at 72˚C for 10 min, and samples were stored at 4˚C. Next, 10 μl of PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized using image analysis.

RT-qPCR was performed on a StepOnePlus Real-Time PCR System with the Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The gene-specific primer sequences used are listed in Table I. PCR was performed using 1 μl cDNA in a 20-μl reaction mixture containing 10 μl Power SYBR Green PCR Master Mix, 2 μl primers and 7 μl PCR-grade water. The reaction conditions included denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The crossing points of the target genes with β-actin were obtained, and the relative amounts were quantified using the $2^{-\Delta\Delta Cq}$ method (28).

Fluorescein isothiocyanate (FITC)-flow cytometric analysis of inhibin-α protein. Cultured cells were detached using 0.05% trypsin-EDTA solution. After being washed with cold phosphate-buffered saline (PBS), the cells were incubated with an anti-inhibin-α mouse monoclonal antibody (1:50; cat. no. sc-365439; Santa Cruz Biotechnology, Inc.) or normal mouse serum (1:50; cat. no. sc-45051; Santa Cruz Biotechnology, Inc.) as a negative control for 30 min at 4˚C. After three washes with cold PBS, the cells were stained with a FITC-labeled mouse antibody (1:50; sc-516140; Santa Cruz Biotechnology, Inc.) for 30 min at 4˚C. Washing was repeated in the same manner, and cell-surface immunofluorescence was analyzed using a FACSCalibur instrument with the CellQuest software 6.0 (both from BD Biosciences).

Immunoblot analysis. Cells were washed with cold PBS and then lysed using cell lysis buffer (Cell Signaling Technology, Inc.) containing 1 mM PMSF (Sigma-Aldrich; Merck KGaA). Protein concentration was determined using the BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, 10 μg of protein was fractionated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Amersham; Cytiva). The membranes were blocked with 1% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature and then incubated overnight at 4˚C with antibodies against AKT (cat. no. 4685), phosphorylated (p)-AKT (cat. no. 9271), PTEN (cat. no. 9559) (all from Cell Signaling Technology, Inc.), p-PI3K (cat. no. ab182651), PREX2 (cat. no. ab169027) (both from Abcam), PI3K (cat. no. sc-1637; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. A1978; Sigma-Aldrich; Merck KGaA), which were all diluted at 1:1,000 (1:5,000 for β-actin) with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). After washing with TBS-T for 1 h, the membranes were incubated for 1 h at room temperature with anti-rabbit (cat. no. 7074) and anti-mouse (cat. no. 7076) horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) diluted at 1:2,500 (1:10,000 for β-actin only) in TBS-T. The membranes were subsequently washed with TBS-T for 1 h, and proteins were detected using Amersham ECL Prime Western Blotting Detection Reagent (Cytiva). Protein levels were analyzed using an Amersham Imager 600 (Cytiva). Protein band densities were measured using the ImageJ analysis software (version 1.44; National Institutes of Health).

Statistical analysis. Data are presented as the mean ± SEM of three independent samples. Data were compared using one-way analysis of variance followed by Tukey's post-hoc test. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc.). $^*P<0.05$ and $^{**}P<0.01$ were considered to indicate a statistically significant difference.

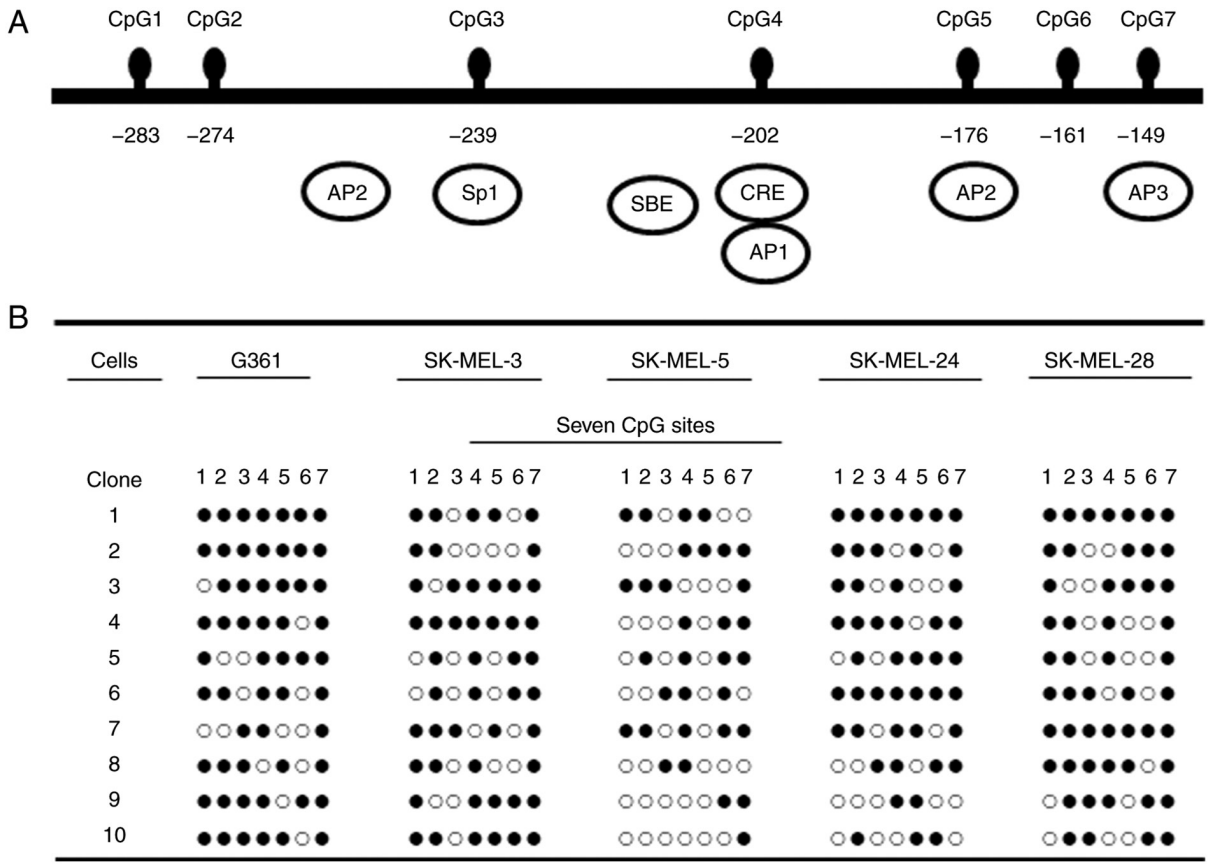

Figure 1. Methylation status of the inhibin-α promoter region in human melanoma cells. (A) Map of CpG sites in the inhibin-α promoter, in the 135 bp region from -149 to -284 of the ATG start site (26). (B) Methylation profile of the inhibin-α promoter region in human melanoma cells. Methylation levels were determined via sequencing 10 independent clones derived from amplified bisulfite-treated DNA isolated from human melanoma cells. Unmethylated and methylated CpG dinucleotides are represented by open (○) and closed circles (●), respectively. AP, activator protein; CRE, cAMP response element; SP1, specificity protein 1; SBE, Smad-binding element.

Results

Methylation status of the inhibin-α gene promoter in human melanoma cells. The methylation state of the inhibin-α gene was investigated in human melanoma cells. The methylation status of the CpG sites of the inhibin-α promoter was analyzed via MSP of bisulfite-treated DNA, and clones were analyzed by sequencing. Methylation was determined for the seven CpG sites in the 135 bp regions from -149 to -284 of the ATG site in the human inhibin-α gene promoter via bisulfite DNA sequencing (Fig. 1A). The inhibin-α promoter was hypermethylated in G361, SK-MEL-3, SK-MEL-24 and SK-MEL-28 cells and moderately methylated in SK-MEL-5 cells (Fig. 1B).

Mutations and LOH in the inhibin-α gene of human melanoma cells. Two polymorphic sites in the inhibin-α gene of human melanoma cells, namely -16C->T in the 5'-UTR and 769G->A in exon 2, were assessed for mutations. Genomic DNA was amplified by PCR. PCR products were digested with restriction enzyme, and electrophoresed on acrylamide gels, stained with ethidium bromide and photographed. The PCR product (fragment A) including nucleotide-16 was digested using *Spe*I enzyme (Fig. 2A). Polymorphisms were examined within the 5'-UTR and exon 1 and were used to divide the cell lines into the following two groups: i) The CC genotype (SK-MEL-3 cells); and ii) the CT genotype (G361, SK-MEL-5, SK-MEL-24, and SK-MEL-28 cells). Substitution of 769G->A in exon 2 was detected via digestion by a restriction enzyme. A PCR product comprising nucleotide 769, fragment B, was digested with *Bsr*FI enzyme, but was not detected in human melanoma cells (Fig. 2B). Fragment B was digested using *Fnu4*HI enzyme, and a single base change at 769G->A of exon 2 was observed in SK-MEL-3 cells (Fig. 2C). Analysis of the 2q chromosome arm investigated microsatellite markers on 2q32-33 (D2S389) and 2q33-36 (D2S128). Furthermore, 2q32-33 was revealed to display allelic imbalance in G361, SK-MEL-3, SK-MEL-24 and SK-MEL-28 cells. LOH at 2q33-36 was observed in G361 and SK-MEL-3 cells, and allelic imbalance was identified in SK-MEL-5 cells (Fig. 2D).

Gene mutations in human melanoma cells. The BRAF gene was mutated in G361, SK-MEL-28 (V599E), SK-MEL-3, SK-MEL-5 and SK-MEL-24 (V600E) cells. The p53 gene was mutated in SK-MEL-3 (exon 8) and SK-MEL-28 (exon 5) cells; however, the wild-type gene was identified in G361, SK-MEL-5 and SK-MEL-24 cells. The N-ras gene was not mutated in any melanoma cells (Table II).

Effects of 5-Aza-dC on the cell growth suppression and doubling time of human melanoma cells. Human melanoma cells were treated with various concentrations of 5-Aza-dC for 5 days. Cell viability was determined using an automatic

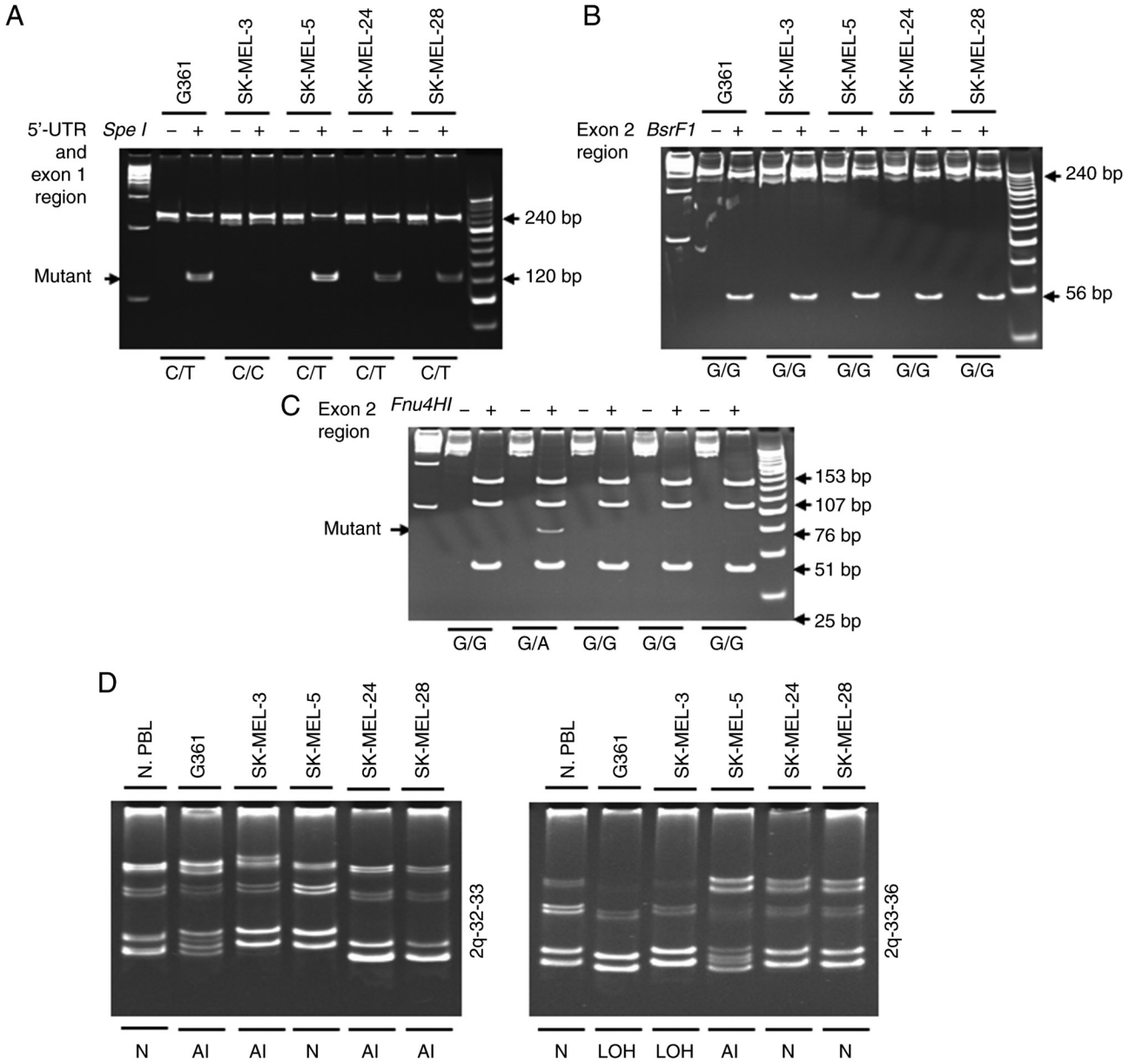

Figure 2. Analysis of the -16C>T polymorphism in the 5'-UTR region and the 769G->A substitution in exon 2 via restriction enzyme digestion. (A) Fragment A was digested using *Spe*I. The presence of a 240-bp fragment indicated a variant homozygous for C, whereas the presence of two fragments of 120 bp each corresponded to a variant homozygous for T. (B) Fragment B was digested using *Bsr*FI. Digestion of the 396-bp PCR product yielded two fragments of 340 and 56 bp in the wild-type allele G, whereas the mutated A allele remained non-cleaved. (C) Fragment B was digested using *Fnu*HI. Digestion of the 396-bp PCR product yielded four fragments of 153, 107, 51 and 25 bp (among others of lower molecular weight) in the wild-type allele, whereas the allele with the 769G->A substitution yielded four fragments of 153, 107, 76 and 51 bp (among others of lower molecular weight). PCR products were incubated with (+) or without (-) restriction enzymes. (D) LOH analysis of 2q (2q32-33 and 2q33-36) in human melanoma cells. Genomic DNA was amplified using PCR for the analysis of the 2q chromosomal region. N, normal; LOH, loss of heterozygosity; AI, allelic imbalance; N. PBL; normal peripheral blood leucocytes; UTR, untranslated region.

counter. 5-Aza-dC notably inhibited the proliferation of melanoma cells in a dose-dependent manner, compared with that of the control. The doubling time of human melanoma cells was increased by 1.07-1.75-fold (Table III).

Effect of 5-Aza-dC treatment on inhibin-α mRNA and protein levels in human melanoma cells. RT-qPCR analysis revealed low or undetectable mRNA expression levels of inhibin-α in all melanoma cells (Fig. 3A). To evaluate the role of methylation in the inactivation of the inhibin-α gene promoter in human melanoma cells, a DNA methyltransferase inhibitor, 5-Aza-dC, was used. Human melanoma cells were treated with 2 and 5 μM of 5-Aza-dC, and inhibin-α mRNA and protein levels were evaluated using RT-qPCR and flow cytometric analysis with FITC staining, respectively. 5-Aza-dC increased the mRNA levels of inhibin-α by 3.6-37.9-fold (Fig. 3B) and its protein levels by 1.5-3.3-fold (Fig. 3C).

Effect of 5-Aza-dC treatment on PREX2 and PTEN expression in human melanoma cells. After treatment with 5-Aza-dC, protein expression was determined using immunoblot analysis. PREX2 protein expression increased in G361 and

Table II. Gene mutations in human melanoma cells.

Human melanoma cells	Gene mutations						
	Inhibin-α		BRAF	PTEN	p53	N-ras	(Refs.)
	Exon 1	Exon 2					
G361	Mutant	Wild-type	V599E	Wild-type	Wild-type	Wild-type	(53,54)
SK-MEL-3	Wild	Mutant	V600E	Wild-type	R267W	Wild-type	(55)
SK-MEL-5	Mutant	Wild-type	V600E	Not known	Wild-type	Wild-type	(55)
SK-MEL-24	Mutant	Wild-type	V600E	del	Wild-type	Wild-type	(56)
SK-MEL-28	Mutant	Wild-type	V599E	A499G	L145R	Wild-type	(53,54)

Inhibin-α mutations correspond to results obtained in the present study, while BRAF, PTEN, p53 and N-ras mutations are results obtained from the indicated references. PTEN mutant, del (164del85); A499G (T167A, GTG>GAG). p53 mutant, R267W (exon 8, GCC→TGG); L145R (exon 5, TGT→GTT). BRAF, B-raf proto-oncogene serine/threonine-protein kinase; PTEN, phosphatase and tensin homolog; del, deletion.

Table III. Effects of 5-Aza-dC on cell proliferation in human melanoma cells.

Human melanoma cell lines	IC_{50}	5-Aza-dC treatment								
		Viability, %						Doubling time, h		Fold growth suppression
		0 μM	0.1 μM	0.5 μM	2 μM	5 μM	10 μM	0 μM	5 μM	
G361	0.60	100±0.0	92.4±3.1	75.0±3.0	59.1±2.6	54.7±2.3	47.9±2.9	22.4	34.4	1.54
SK-MEL-3	0.78	100±0.0	94.2±2.0	78.7±3.8	66.9±2.4	58.0±3.0	54.0±4.1	68.0	72.7	1.07
SK-MEL-5	0.24	100±0.0	84.0±2.7	53.0±2.2	42.6±2.3	33.4±1.9	29.5±1.3	37.9	41.6	1.10
SK-MEL-24	0.19	100±0.0	84.2±1.6	48.1±2.8	33.9±4.7	30.3±3.0	28.7±4.1	28.8	50.5	1.75
SK-MEL-28	0.17	100±0.0	83.4±3.8	46.0±3.2	29.0±2.3	23.2±2.1	23.1±1.5	30.7	53.0	1.73

5-Aza-dC, 5-aza-2'-deoxycytidine.

SK-MEL-24 cells, but decreased in SK-MEL-3, SK-MEL-5 and SK-MEL-28 cells. PTEN protein expression was mostly decreased in melanoma cells (Fig. 4A and B).

Effect of 5-Aza-dC treatment on AKT and PI3K phosphorylation levels in human melanoma cells. The protein expression of AKT and PI3K in 5-Aza-dC-treated cells was evaluated via immunoblot analysis. AKT phosphorylation was increased in all melanoma cells, and PI3K phosphorylation was enhanced in melanoma cells except for G361 cells (Fig. 5A and B).

Discussion

Epigenetic changes, represented by promoter CpG island hypermethylation and histone alterations, are important carcinogenic mechanisms observed in almost all types of cancer (13,14). A total of ~60-70% of human genes have CpG islands in their promoters, and certain of these genes are hypermethylated to block the expression of the corresponding genes (29). Accordingly, the tumor suppressor function is lost, thereby promoting the growth of tumor cells. In the majority of cancers, in addition to local changes, such as promoter CpG island hypermethylation, genome-wide demethylation is often simultaneously observed. Such genomic hypomethylation is closely related to chromosomal instability (30,31). Melanoma was analyzed in a tumor model to investigate the association between DNA methylation of the MMP-9 gene and its overexpression at the transcription and protein levels, and hypermethylation was observed in the CpG-2 region of the MMP-9 gene (32). Aberrant DNA methylation is important for epigenomic regulation in melanoma formation and progression (33). Research on DNA methylation has advanced its potential use as a diagnostic, prognostic and therapeutic biomarker for cancer (34). In the present study, the degree of methylation was assessed in seven CpG sites in the inhibin-α gene promoter of human melanoma cells. A total of seven CpG sites in the 135 bp region in the inhibin-α gene promoter revealed significant hypermethylation (G361, SK-MEL-3, SK-MEL-24 and SK-MEL-28 cells) or moderate methylation (SK-MEL-5 cells) in human melanoma cells. Previously, primary prostate carcinoma has been revealed to present hypermethylation at the CpG1-4 and CpG7 sites, compared with non-malignant cells. The CpG6 site exhibited lower

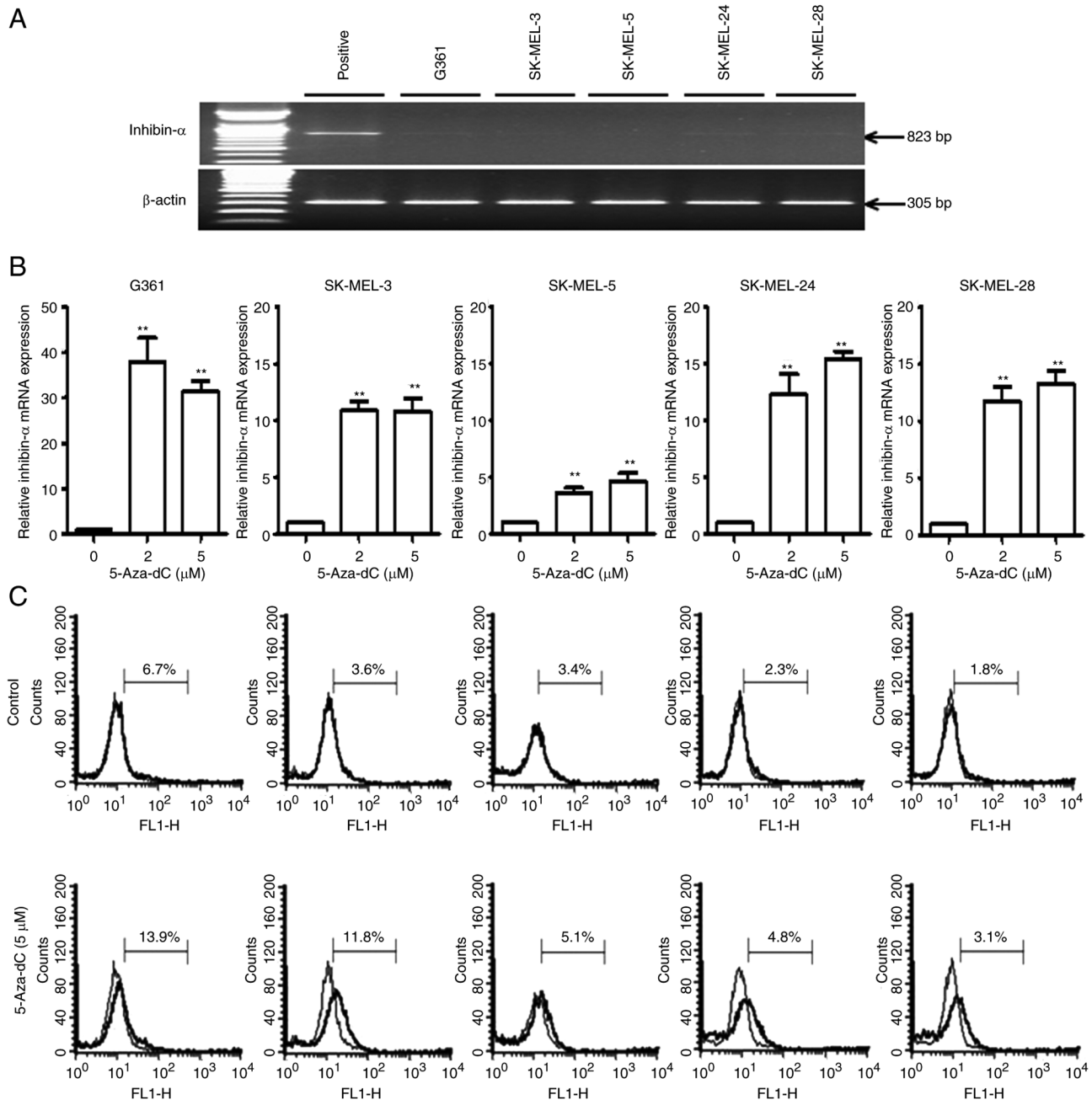

Figure 3. mRNA and protein expression levels of inhibin-α in 5-Aza-dC-treated human melanoma cells. Cells were exposed to 2 or 5 μM 5-Aza-dC for 5 days. The medium and the drug were replaced every 2 days. mRNA levels were determined using RT-PCR. (A) PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and images were captured. A positive control from human gastric cancer SNU-668 cells was used (22). (B) For RT-qPCR, the relative expression of inhibin-α was quantified using the $2^{-\Delta\Delta Cq}$ method with β-actin as the normalization control. Data are expressed as the mean ± SEM of three independent samples. (C) Inhibin-α protein expression was quantified using flow cytometric analysis. The cells were incubated with an anti-inhibin-α polyclonal antibody and FITC-conjugated secondary antibody. Diluted normal goat serum was used as a negative control. **P<0.01 compared with 0 μM. RT-qPCR, reverse transcription-quantitative PCR; 5-Aza-dC, 5-aza-2'-deoxycytidine.

methylation levels, whereas the CpG5 site was unmethylated in non-malignant and malignant samples (26). The findings of the present study suggested that methylation of the CpG sites occurred in human melanoma cells and may be related to the cause of transcriptional silencing.

Mutations are frequently involved in the transcriptional silencing of cancer suppressor genes (35). A mutation at the -16-bp site of the 5'-UTR was found to be heterozygous in melanoma (G361, SK-MEL-5, SK-MEL-24 and SK-MEL-28) cells, and a mutation at 769G->A in exon 2 was identified in SK-MEL-3 cells, suggesting that human melanoma cells may be mostly affected by the -16>T allele variant. The present study revealed the occurrence of LOH in the 2q chromosome arm with one microsatellite marker at 2q32-36 in human melanoma cells. The 2q32-33 region displayed allelic imbalance in G361, SK-MEL-3, SK-MEL-24 and SK-MEL-28 cells. LOH at 2q33-36 was observed in G361 and SK-MEL-3 cells, and allelic imbalance was revealed in SK-MEL-5 cells; there were no cells with LOH both at 2q32-33 and 2q33-36. Chromosome 2q has been indicated to exhibit changes in prostate carcinoma

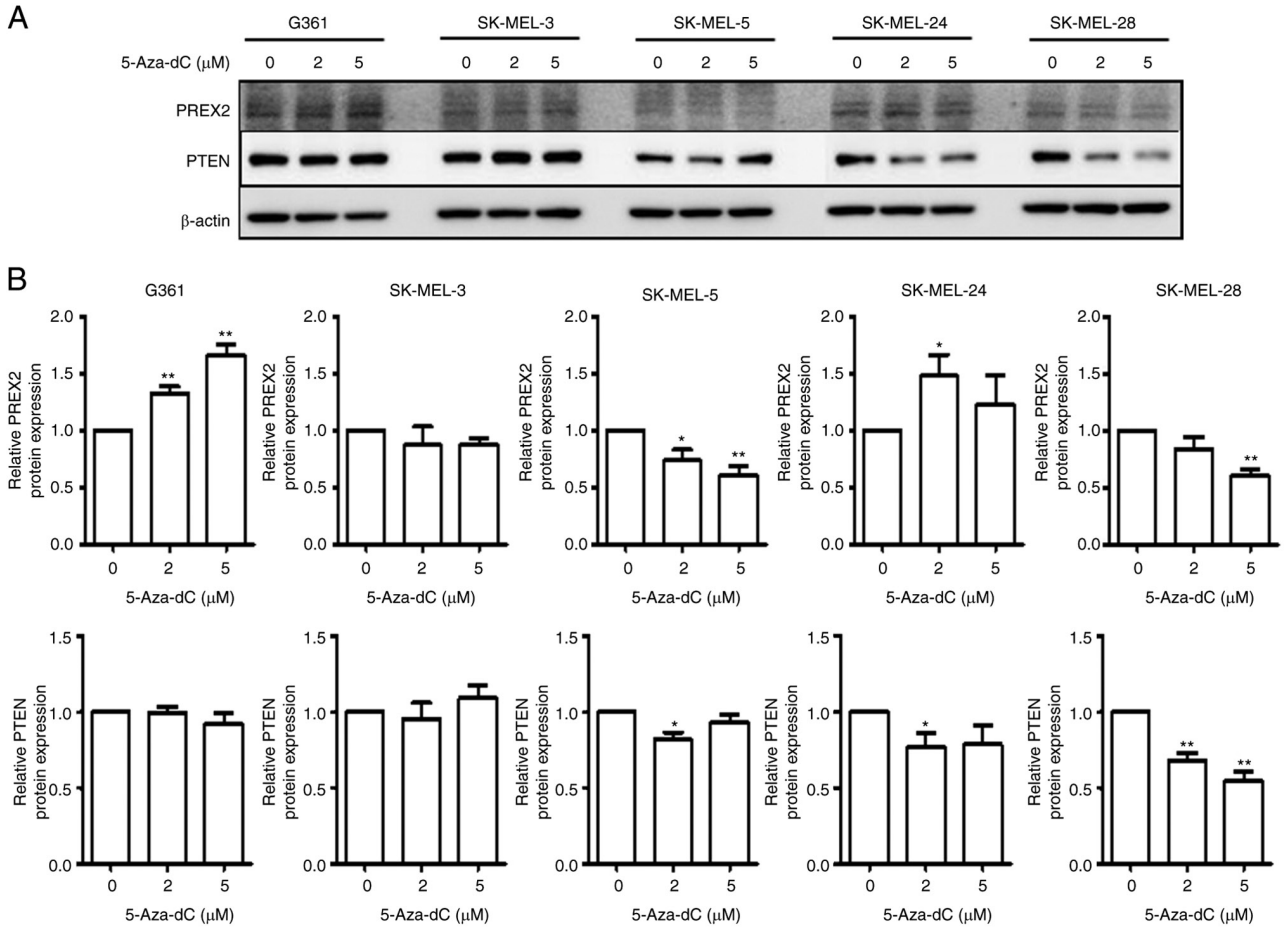

Figure 4. PREX2 and PTEN protein expression in 5-Aza-dC-treated human melanoma cells. Cells were exposed to 2 or 5 μM 5-Aza-dC for 5 days. The medium and the drug were replaced every 2 days. (A) Protein levels were evaluated using immunoblot analysis. (B) Densitometry results are presented as the relative ratios of PREX2 and PTEN to β-actin. Data are expressed as the mean ± SEM of three independent samples. *P<0.05 and **P<0.01 compared with 0 μM. 5-Aza-dC, 5-aza-2'-deoxycytidine; PREX2, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2; PTEN, phosphatase and tensin homolog.

and pediatric adrenocortical tumors (36-38). In addition, a 2q deletion has been revealed to indicate poor prognosis in the advanced stage of bladder carcinoma and head-and-neck squamous cell carcinoma (39,40). Taken together, the results of the present study indicated that hypermethylation and mutations in the inhibin-α gene and changes in its LOH may be associated with transcriptional silencing in human melanoma cells.

In the experiments of the present study, after treatment with the demethylating agent 5-Aza-dC, it was determined whether inhibin-α gene expression was reactivated. The mRNA and protein expression of inhibin-α in melanoma cells was increased after 5-Aza-dC treatment. Furthermore, an association was observed between inhibin-α mRNA and protein levels in human melanoma cells. In human cancer cells, inhibin-α mRNA expression has been demonstrated to be reactivated following treatment with 5-Aza-dC (21-23). Collectively, the results of the present study revealed that inhibin-α gene expression was increased by 5-Aza-dC in a dose-dependent manner. Interestingly, cells with a hypermethylated inhibin-α promoter exhibited a high mRNA and protein expression following 5-Aza-dC treatment, whereas cells that were moderately methylated exhibited low 5-Aza-dC gene expression. Among the examined melanoma cells, G361 and SK-MEL-3 cells, which exhibited LOH, presented higher mRNA and protein expression levels of inhibin-α than SK-MEL-5, SK-MEL-24 and SK-MEL-28 cells. This finding suggests that LOH is involved in demethylation.

Treatment with 5-Aza-dC decreased cell proliferation in a dose-dependent manner and substantially delayed the doubling time of surviving melanoma cells. These results suggested that inhibin-α has an important cellular function. Additionally, 5-Aza-dC affects the expression levels of several genes involved in cell cycle regulation, apoptosis and survival in hepatocellular carcinoma and leukemia cells (41,42).

PREX2 binds to PTEN through the guanine nucleotide exchange factor domain, thereby inhibiting PTEN activity. When PTEN activity is inhibited, PREX2 activates the downstream PI3K signaling pathway (43,44). PREX2 regulates pancreatic cancer cell proliferation, invasion and migration, presumably at least through modulation of the activity of PTEN and the PI3K signaling pathway (45). PTEN is a tumor suppressor gene that is frequently mutated or deleted. PTEN has been identified to inhibit cell proliferation and promote apoptosis in numerous cancer cell types, including breast cancer cells and HepG2 cells (46,47). Downregulation, inhibition or inactivation of PTEN increases mitochondrial ATP production. In addition, PTEN induces activation of the proteolytic cell apoptosis cascade by inhibiting the AKT/PI3K signaling pathway (48,49).

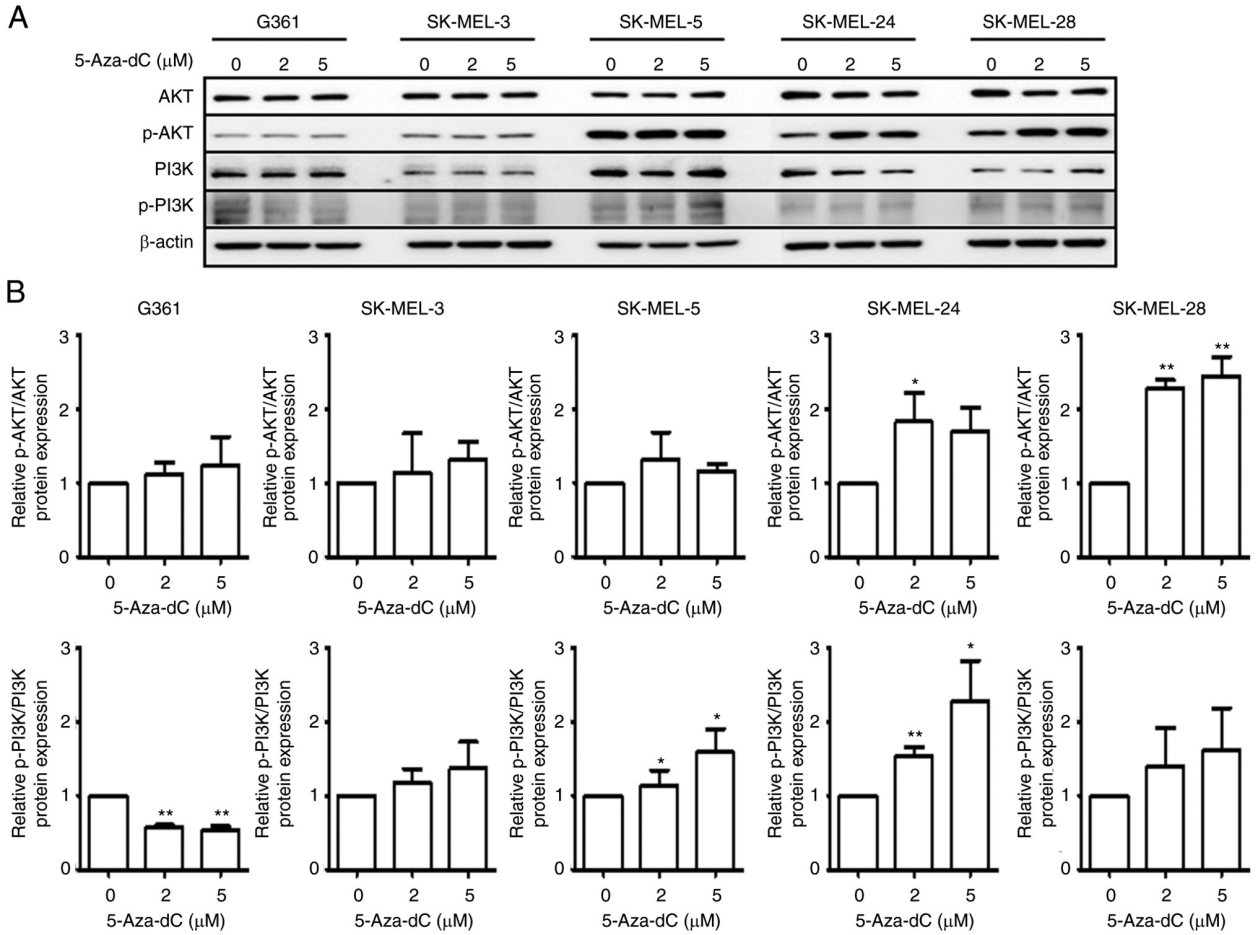

Figure 5. AKT and PI3K protein expression in 5-Aza-dC-treated human melanoma cells. Cells were exposed to 2 or 5 μM 5-Aza-dC for 5 days. The medium and the drug were replaced every 2 days. (A) Protein levels were determined using immunoblot analysis. (B) Densitometric results are presented as the relative ratios of AKT and PI3K to β-actin. Data are expressed as the mean ± SEM. *P<0.05 and **P<0.01 compared with 0 μM. 5-Aza-dC, 5-aza-2'-deoxycytidine; p, phosphorylated.

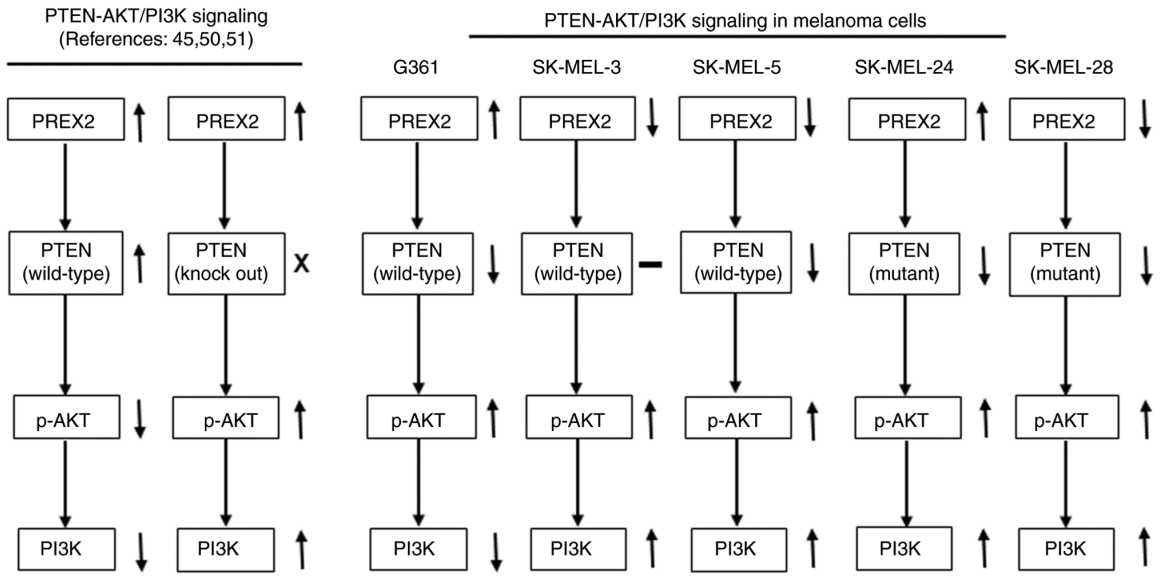

Figure 6. Summary of the PREX2, PTEN and AKT/PI3K signaling pathways in human melanoma cells. PREX2, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2; PTEN, phosphatase and tensin homolog; p, phosphorylated.

In the present study, PREX2 protein expression increased in G361 and SK-MEL-24 cells and decreased in SK-MEL-3, SK-MEL-5 and SK-MEL-28 cells after 5-Aza-dC treatment. PTEN protein expression mostly decreased in all melanoma

cells. However, AKT and PI3K phosphorylation increased in most melanoma cells. These findings suggested that the AKT/PI3K signaling pathway was possibly affected by the PTEN expression pattern. Additionally, PREX2 overexpression has been indicated to decrease PTEN activity and increase AKT phosphorylation and PI3K signaling pathway activity. By contrast, knockdown of PREX2 enhanced PTEN activity and inhibited PI3K signaling pathway activity (45,50). A previous study indicated that wild-type mouse embryonic fibroblasts exhibited increased PTEN expression and decreased AKT phosphorylation. When PTEN was knocked out, PREX2 expression was slightly increased, PTEN was not expressed, and AKT phosphorylation was increased. However, when PREX2 was knocked out, PREX2 was not expressed, and AKT phosphorylation was decreased (51). PTEN is methylated by protein arginine N-methyltransferase 6 in the cytoplasm, and methylated PTEN can migrate from the cytoplasm to the nucleus, suggesting that it is difficult for PTEN to inhibit AKT/PI3K signaling (52). In the present study, the expression of PTEN and that of genes involved in AKT/PI3K signaling were compared following treatment with a demethylating agent. Based on the findings of the present study, it remains difficult to establish a direct association between inhibin-α methylation and PTEN expression as well as AKT/PI3K signaling.

In conclusion, the present study revealed the methylation, mutation and LOH of the inhibin-α gene in human melanoma cells. Inhibin-α gene expression was reactivated by a demethylating agent, and cell proliferation was inhibited. However, PREX2 expression either weakly increased or decreased, whereas AKT/PI3K signaling increased potentially owing to the decreased PTEN expression (Fig. 6). In addition, mutations in the tumor suppressor genes inhibin-α, PTEN and p53 were not associated with transcriptional silencing, gene expression and cell growth. Overall, mutations in the inhibin-α and BRAF genes appear to be frequent in melanoma cells. Therefore, further studies are required to determine the importance of biomarkers for the tumor suppressor gene inhibin-α.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

HK and YIK performed the experiments. HK, HJA and YIK analyzed the data and wrote the manuscript. HJA and YIK contributed to the study concept and design of the project. HJA and YIK confirm the authenticity of all the raw data. All authors reviewed the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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