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Epigenetic silencing of E- and P-cadherin gene expression in human melanoma cell lines

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Abstract. The degree of E- and P-cadherin expressions inversely correlate with the progression stage of human melanoma. In the present study, we analyzed mechanisms of down-regulation of E- and P-cadherin gene expressions in 8 human melanoma cell lines. In 5 of the 8 melanoma cell lines, E-cadherin expression was lost or markedly decreased compared to that in normal melanocytes, and 4 of the 5 melanoma cell lines lost P-cadherin expression. All of the melanoma cell lines expressed snail, which is known to encode a transcription repressor for E-cadherin, at a higher level than melanocytes whereas expression levels of the snail varied among cell lines. Transduction of snail gene into MMAc cells which expressed a high level of E-cadherin and an extremely low level of snail restored expression of E-cadherin but not P-cadherin. In contrast, transduction of antisense-snail gene into A375M cells which expressed no E-cadherin and a high level of snail restored expression of E-cadherin but not P-cadherin. Methylation-specific PCR analysis revealed CpG methylation in the promoter region of E-cadherin in MeWo and AKI cells. Further, the treatment with a demethylating agent, 5-azacytidine led AKI and A375M cells to re-express both E- and P-cadherin. The results show E-cadherin gene is silenced by at least two distinct mechanisms (methylation and transrepression by Snail) in human melanoma cell lines whereas P-cadherin gene seems to be silenced by methylation but not by snail.

Introduction

Cadherins are a family of transmembrane glycoproteins which function for Ca²⁺-dependent cell-cell adhesion (1). Their subfamily, so called ‘classic’ cadherins including E-cadherin and P-cadherin, are localized in the adhesion junctions (2). The extracellular domain of cadherin plays a role in a molecular zipper mediating homophilic cell-cell adhesion with five cadherin motifs, whereas the cytoplasmic tail is linked to the actin cytoskeleton via a complex of p120ctn, α-catenin and β or γ-catenin (1,3,4). Cell-cell adhesion through the cadherins is a predominant necessity during cell differentiation, tissue development, and tissue homeostasis. Down-regulation of E-cadherin is a frequent event in development and progression of tumors. Immunohistochemical studies have demonstrated the association of E-cadherin down-regulation with dedifferentiation, invasiveness, and metastatic ability in various types of carcinomas (5-10). The functional modification of E-cadherin by blocking antibodies has demonstrated that disruption of E-cadherin-mediated cell-cell adhesion enhances tumor invasion (11,12). In contrast, increased adhesiveness of tumor cells through E-cadherins has resulted in growth retardation and inhibition of the invasive and metastatic phenotypes (12,13). From such evidence, E-cadherin has been regarded as a tumor/invasion-suppressor gene.

Various proposals and attempts have been made to elucidate the mechanism of E-cadherin silencing during tumor development and progression, including genetic and epigenetic mechanisms. Genetic alteration of E-cadherin is represented by gene mutations resulting in exon skipping and/or in-frame and frame-shift deletions and insertions (14,15). Some E-cadherin mutations are found in the coding sequence, which are assumed to abolish the activity of this molecule (14,16). The expression of E-cadherin is known to be reduced by epigenetic mechanisms, not only by the genetic alteration. CpG methylation around the promoter region of E-cadherin gene is demonstrated in a variety of human cancer cell lines and cancer tissues which lack E-cadherin expression (17-21). In addition to hypermethylation of E-cadherin promoter region, a transcription factor, Snail,
has been recognized as a repressor of E-cadherin gene expression in various epithelial tumor cells (22-25).

Melanoma originates from a melanocyte, and is one of the most malignant tumors which frequently metastasize to distant organs. In melanoma as well as other solid tumors, the degree of E-cadherin expression inversely correlates with its progression stage. Analyses using culture cell lines demonstrated that E-cadherin was expressed in cultured normal melanocytes and naevus cells whereas its expression was lost or decreased in melanoma cells (24,26). Down-regulation of E-cadherin expression was immunohistochemically observed in tumor tissues of malignant melanomas compared to benign melanocytes and melanocytic nevi (27-30). P-cadherin is also down-regulated in malignant melanoma (30). So far, little is known about the mechanism of down-regulation of E- and P-cadherin in human melanoma cell lines (24).

In the present study, we analyzed the mechanisms of down-regulation of E- and P-cadherin in 8 human melanoma cell lines, focusing on the promoter methylation and over-expression of the transcription repressor Snail.

Materials and methods

Cells and culture condition. Human melanoma MeWo cells were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan); AKI and MMAc cells were from Riken Cell Bank (Tsukuba, Japan); GAK cells were from Institute for Fermentation (Osaka, Japan); SK-MEL-28 cells were from ATCC (Manassas, VA). Human melanoma A375M and 9711 cells were kindly provided by Dr I. Saiki (Research Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan) and Dr M. Shindo (Hokkaido University School of Dentistry), respectively. We established MMIV cells from a metastatic lymph node of a patient with malignant melanoma (31). The melanoma cells were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 5% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD). Human melanocytes were isolated from neonatal foreskin (obtained from Clonetics, San Diego, CA). The melanocytes were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 5% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD).

Human melanocytes were isolated from neonatal foreskin (obtained from Clonetics, San Diego, CA). The melanocytes were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 5% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD). Human melanocytes were isolated from neonatal foreskin (obtained from Clonetics, San Diego, CA). The melanocytes were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 5% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD).

RNA preparation and reverse transcriptase-mediated duplex polymerase chain reaction (RT-dPCR). Total cellular RNA was extracted from monolayer cultures of the human melanocytes and melanoma cells with TRIzol. Total RNA sample (3 µg) was subjected to cDNA synthesis for 2 h at 37°C in 40 µl of a reaction mixture containing 200 µM dNTPs, 0.5 mM MgCl₂, 0.5 µM random primer (Gibco BRL, Gaithersburg, MD). The amplified product was then digested with EcoRI and cloned into the BamHI cloning site of pCDNA3.1 Hyg(+) (Promega, Madison, WI). The amplified product was then digested with BamHI and cloned into the BamHI cloning site of pCDNA3.1 Hyg(+) with HA tag into upstream of the mult cloning site. For snail antisense expression vector (pCDNA3.1 Hyg(-)HA-snaill) was generated as follows: full length cDNA was amplified by PCR using RT-product of RNA from human breast cancer MDA-MB435S cells as template and a set of primers (5'-CAGGATCCTTGTGCGACTCTTTCCCT-3' and 5'-CTGGATTCGTGGGATCCAGGAAAAAGAAG-3'). The amplified product was then digested with BamHI and cloned into the BamHI cloning site of pCDNA3.1 Hyg(+) in the similar manner as in the detection for E-cadherin.

Immunoblot analysis. Cells (3x10⁶) were lysed in buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM Na₂VO₄, 50 mM NaF, 1% Triton X-100, 5 µg/µl pepstatin, 5 µg/µl leupeptin) at 4°C for 10 min. The cell lysates were then centrifuged at 20,000 g for 10 min. The supernatants were collected and protein concentration was determined with DC Protein Assay kit (Bio-Rad, Hercules, CA). Each protein (10 µg) was separated by SDS-polyacrylamide gel electrophoresis and subsequently blotted onto a nitrocellulose membrane. After blocking for 1 h in PBS with 5% skim milk, the membrane was incubated for 1 h with a 1:2500 dilution of a mouse monoclonal antibody to human E-cadherin (Transduction Laboratory, Lexington, KY). The membrane was washed three times in PBS containing 0.1% Tween-20 (PBS-T), and then incubated for 1 h with a 1:2000 dilution of an horseradish peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA). The membrane was washed three times in PBS-T. Finally immunoreactions were visualized with reagents from the ECL Detection System (Amersham Pharmacia, Little Chalfont, UK). Next, the same membrane was reprobed with using a 1:5000 dilution of an actin antibody as a primary antibody (Clone 4; Boehringer-Mannheim, Mannheim, Germany) in the similar manner as in the detection for E-cadherin.
pCDNA3.1 Hyg(-)-snail AS were transfected into competent cells (Escherichia coli, XL-1), respectively. The plasmid DNA was then amplified and purified. The accuracy of the construct was confirmed by DNA sequence.

**Transfection and cell cloning.** The transfection of pCDNA3.1 Hyg(-)-HA snail, pCDNA3.1 Hyg(+)-snail AS, pCDNA3.1 Hyg(+) into melanoma cells was performed with Lipofectamine PLUS (Life Technologies, Rockville, MD) according to the manufacturer's instruction. The cells stably transfected with the expression plasmid vectors were selected by their resistance to 100 units/ml of Hygromycin B. Cell cloning of the stable transfectants was performed by the limiting dilution method.

**DNA preparation, bisulfite modification and methylation-specific PCR analysis.** Genomic DNA was extracted from monolayer cultures of human melanoma cells with DNAzol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instruction. The DNA (1 µg) was denatured with 0.2 M NaOH and treated with 2.5 M sodium bisulfite in the presence of 0.5 mM hydroquinone for 18 h at 50°C. And then, the DNA was purified by using Cpg Genome™ DNA Modification Kit (Intergen, New York, NY). Modified DNA (100 ng) was used as a template in the PCR amplification. The sense/antisense primers to amplify methylated or unmethylated DNA in CpG island 3 region of E-cadherin (19) were as follows: methylated DNA, 5'-GGTGAATTTTTAGTTAATTAGCGCTAC-3'/5'-CATAACTAACCAGAAAACGCCG-3'; unmethylated DNA, 5'-GGTAGGTGAATTTTTAGTAATTAGTGGTA-3'/5'-ACCCATAACTACCACAAAACACCA-3'. PCR was performed for 35 cycles consisting of denaturation at 95°C for 20 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec. PCR products were electrophoresed in 3% NuSieve (FMC BioProducts, Rockland, ME) combined with 1% agarose gels, stained with ethidium bromide and photographed under UV light (204 bp for methylated and 211 bp for unmethylated).

**Treatment of melanoma cells with a demethylating agent, 5-azacytidine.** The demethylating agent 5-azacytidine (Sigma, St. Louis, MO) was freshly prepared in DME/F12 and filter-sterilized. Cells were plated in 100-mm tissue culture dishes in DME/F12 supplemented with 10% FBS. After 24 h, the cells were treated with 1, 2, 3, 5, 10 µM 5-azacytidine. The medium was daily changed during the indicated incubation period.
DNA sequencing. For sequencing of promoter region (nt -347 - +48, +1 is A of ATG, translation start codon) of E-cadherin, DNA extracted from melanoma cells was used as a template in the PCR amplification with a primer pair (5'-GCCCATCTCACAAACGAA-3'/5'-TGAACGTCTCCGCAAGCTC-3'). The PCR product was TA-cloned with TOPO Cloning kit (Invitrogen, Carlsbad, CA), and sequenced by using ABI377A (Applied Biosystems Japan, Tokyo, Japan). For sequencing open reading frame of snail gene, total RNA extracted from melanoma cells was subjected to cDNA synthesis. The cDNA was used as a template in the PCR amplification with a primer pair (5'-CAGGATCCCTAATGCGCGGCCTCTTTCCT-3'/5'-CTGGATCTCTAGCGGGACATCCTGA-3'). The PCR product was TA-cloned, and sequenced in the same manner as E-cadherin promoter region.

Results

Expressions of E- and P-cadherin in normal melanocytes and melanoma cells. As shown in Fig. 1A, RT-PCR analyses revealed that E-cadherin expression was down-regulated in five of eight melanoma cells (MMIV, AKI, A375M, 9711, SK-MEL-28) compared to normal human melanocytes (NHEM). Western blot analysis demonstrated that these five cell lines expressed lower levels of E-cadherin protein than NHEM (Fig. 1B). Normal melanocytes and the three melanoma cell lines (MeWo, GAK and MMAc) which expressed E-cadherin showed high expression of P-cadherin whereas the other four melanoma cell lines (MMIV, AKI, A375M and 9711) had neither expression (Fig. 1C). SK-MEL-28 cells expressed P-cadherin but not E-cadherin.

Expression of transcription repressor snail in normal melanocytes and melanoma cells. We next examined the expression of snail gene which encodes a transcription repressor of E-cadherin by RT-PCR analysis (Fig. 1A). The five cell lines (MMIV, AKI, A375M, 9711, SK-MEL-28) which had no expression of E-cadherin expressed snail whereas normal human melanocytes did not. Of the three cell lines which expressed E-cadherin (MeWo, GAK, MMAc), one (MMAc) did not express snail at all whereas the other two cell lines highly expressed it.

Effects of transduction of snail sense or antisense expression vector on E- or P-cadherin expression in human melanoma cells. To investigate the role of Snail in E- or P-cadherin expression in melanoma cells, we transfected the snail sense expression vector into MMAc cells which did not express snail at all. Transient transfection of snail decreased the expression of E-cadherin compared to parental or control cells (Fig. 2A). We next transfected the snail antisense expression vector into MMAc cells which highly expressed snail mRNA. Stably transfected clones (AS2, AS3) showed increased expression of E-cadherin compared to parental (Pa) or control (Hyg1, Hyg2) cells although the expression level did not reach the expression level of normal melanocytes (Fig. 2B). P-cadherin expression of MMAc cells did not decrease by the transduction with the snail expression vector, and that of A375M cells did not increase by the transduction with the snail antisense expression vector (Fig. 2C and D).

Methylation status of CpG islands of E-cadherin promoter region. It is known that there are 6 CpG islands from the promoter region to the exon 2 in E-cadherin gene and that especially the methylation of island 3 is closely related to the down-regulation of transcription of E-cadherin (19). To determine the effects of CpG island methylation on E-cadherin expression, the methylation status of all melanoma cell lines on island 3 was analyzed by methylation-specific PCR (MSP) method. Two (AKI and A375M) of the five cell lines lacking or decreasing E-cadherin expression showed a methylated band in island 3, and AKI cells were particularly heavily methylated (Fig. 3). Two (GAK and MMAc) of the remaining three cell lines that expressed E-cadherin showed no evidence of methylation at island 3 whereas one (MeWo) of the three showed a weak methylation band (Fig. 3). Both methylated and unmethylated bands were observed in MeWo and A375M cells.

Recovery of E- and P-cadherin expressions by treatment with 5-azacytidine. E-cadherin-negative AKI and A375M cells...
were treated with the demethylating agent 5-azacytidine. AKI cells re-expressed E-cadherin when they were treated with more than 5 μM 5-azacytidine for more than 5 days (Fig. 4A and B). Re-expression of E-cadherin was observed in A375M cells treated with 1 μM 5-azacytidine for more than 5 days (Fig. 4C). The E-cadherin expression levels of these cells treated with 5-azacytidine were low compared to those of normal melanocytes (Fig. 4A, B and C). P-cadherin expression of the two cell lines was recovered in the similar manner to that of the recovery of E-cadherin, and the expression levels were not so high as normal melanocytes (Fig. 4D and E).

Sequence of E-cadherin promoter region and snail cDNA. GAK and MeWo cell lines expressed both E-cadherin and snail. We assumed a failure in the interaction between Snail and...
E-cadherin promoter region and performed sequence analysis of the Snail binding sites in E-cadherin promoter region and full-length of coding sequence of snail. The E-cadherin promoter region was sequenced from nt. -347 to +48 (nt. +1 is A of ATG, translation start codon). The analysis revealed that the sequence of the E-cadherin promoter region and snail were identical with the sequences which had previously been reported (EMBL/GenBank/DDBJ: Accession no.: E-cadherin, L34545; snail, O95863) (data not shown).

Discussion

Down-regulation of E- or P-cadherin expression is frequently observed in human melanoma cell lines and tissues (24,26-30). The present study confirmed the down-regulation of E- or P-cadherin expression in melanoma: 6 of 8 melanoma cell lines examined here lacked or decreased E-cadherin expression and 5 cell lines lacked P-cadherin expression. And we investigated the mechanism by which E- or P-cadherin expression was down-regulated in these melanoma cell lines.

Recently zinc finger type transcription factor Snail has been reported to repress E-cadherin expression in various types of cancer including melanoma (22-25). Poser et al described that all the human melanoma cell lines used in their study expressed snail and no E-cadherin (24). Furthermore, they observed the appearance of E-cadherin expression by transduction of snail antisense expression vector into these cells (24). We also transfected the snail antisense expression vector into A375M and SK-MEL-28 cells which expressed snail but not E-cadherin, as a result, E-cadherin expression was recovered by the snail antisense gene transfection. When transfected with snail expression vector, MMAc cells expressing E-cadherin but not snail would decrease their E-cadherin expression. These results indicate that the up-regulation of transrepressor Snail was involved in the decrease of E-cadherin expression in these melanoma cell lines. However, unlike the report by Poser et al. we did not observe the loss of E-cadherin in any of the cell lines that expressed snail gene: MeWo and GAK cells expressed both snail and E-cadherin. Hence we suspected the possibility that these cells had mutations on coding sequences of snail and/or promoter of E-cadherin. However, we could not find any mutation of the snail or E-cadherin genes. These results indicate that the E-cadherin expression is not necessarily repressed in melanoma cells just because snail is expressed, and that Snail may need other factor(s) to function as a transrepressor of the E-cadherin gene and MeWo and GAK cells may cause dysfunction of the factor(s).

Methylation of CpG islands in the E-cadherin promoter region is known as one of the mechanisms responsible for the down-regulation of E-cadherin expression in stomach, liver, breast, thyroid, and prostate cancers (17-21,32). There are 6 CpG islands between the promoter region and exon 2 in E-cadherin gene (19). Of the 6 CpG islands, the methylation of island 3 is closely related to the down-regulation of transcription of E-cadherin (19). We tried to determine the methylation status of the CpG island 3 by the methylation-specific PCR and found the methylation-specific band in 2 cell lines (AKI and A375M) of the five cell lines lacking or with decreased E-cadherin. When the two cell lines were treated with the demethylating reagent 5-azacytidine, E-cadherin was re-expressed. These results indicate that the methylation of CpG islands is a possible cause by which E-cadherin is down-regulated in melanoma as well as other solid tumors. Both methylated and unmethylated bands were detected in MeWo cells expressing E-cadherin and A375M cells not expressing E-cadherin. As E-cadherin appeared in A375M cells lacking E-cadherin expression by the treatment with 5-azacytidine or the transfection with antisense snail expression vector, it is likely that one allele of E-cadherin gene is methylated and the other one is repressed by Snail in the cells. Otherwise, A375M cell line may be composed of at least two populations: in one the E-cadherin gene is methylated and in the other it is repressed by Snail. It is speculated that either of two alleles of E-cadherin gene is transcribed and the other is inactivated by methylation in MeWo cells; MeWo cell line may also consist of multi-population and some populations express E-cadherin whereas others do not express E-cadherin due to methylation.

The cell lines expressing P-cadherin (MeWo, GAK, MMAc and SK-MEL-28), except SK-MEL-28 cells, expressed E-cadherin. MMAc cells transduced with the snail expression vector or A375M cells transduced with the snail antisense expression vector did not show an alteration of P-cadherin expression. These findings suggest that Snail functioned as a repressor for E-cadherin but not for P-cadherin in some human melanoma cell lines. When AKI and A375M cells which expressed neither E- nor P-cadherin were treated with 5-azacytidine, they showed the expressions of both cadherins. From these data, methylation of P-cadherin gene should be considered as a mechanism of silencing the P-cadherin gene although no previous report has indicated this. Human P-cadherin gene is located 32 kbp upstream of the E-cadherin gene, mapping it to chromosome 16q22.1 (33). The genomic region including E- and P-cadherin genes may be susceptible to methylation although further analysis of the methylation status of P-cadherin gene is required.

In conclusion, the results presented here show that E-cadherin gene is silenced by at least two distinct mechanisms (methylation and transrepression by Snail) in human melanoma cell lines whereas P-cadherin gene seems to be silenced by methylation, but not Snail. In some of the cell lines the silencing of E-cadherin expression is caused through both of these mechanisms.

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