HOKKAIDO UNIVERSITY

Title	Epigenetic silencing of E- and P-cadherin gene expression in human melanoma cell lines
Author(s)	Tsutsumida, Arata; Hamada, Jun-Ichi; Tada, Mitsuhiro; Aoyama, Tetsuya; Furuuchi, Keiji; Kawai, Yoshiko; Yamamoto, Yuhei; Sugihara, Tsuneki; Moriuchi, Tetsuya
Citation	International Journal of Oncology, 25(5), 1415-1421
Issue Date	2004-11
Doc URL	http://hdl.handle.net/2115/42834
Type	article
File Information	IJO25-5_1415-1421.pdf

Instructions for use

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

ARATA TSUTSUMIDA[1,2], JUN-ICHI HAMADA[1], MITSUHIRO TADA[1], TETSUYA AOYAMA[1],
KEIJI FURUUCHI[1], YOSHIKO KAWAI[3], YUHEI YAMAMOTO[2],
TSUNEKI SUGIHARA[2] and TETSUYA MORIUCHI[1]

[1]Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku,
Sapporo 060-0815 ; [2]Department of Plastic and Reconstructive Surgery, Graduate School of Medicine,
Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638; [3]The 1st Department of Physiology,
Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

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* decreased 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 of 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.

Correspondence to: Dr Jun-Ichi Hamada, Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan
E-mail: jhamada@igm.hokudai.ac.jp

Introduction

Cadherins are a family of transmembrane glycoproteins which function for Ca^{2+}-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 ß 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 de-differentiation, 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 immunohisto-chemically 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 expression in melanoma except for a report indicating that the trans-repressor Snail is involved in the loss of E-cadherin expression 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 melanocyte medium 154S (Kurabo, Osaka, Japan). The cell lines were cultured in a CO_2 incubator (5% CO_2 and 95% air).

RNA preparation and reverse transcriptase-mediated duplex polymerase chain reaction (RT-dPCR). Total cellular RNA was extracted from monolayer cultures of the human melano-cytes and melanoma cells with TRIzol. Total RNA sample (3 µg) was subjected to cDNA synthesis for 2 h at 37°C in 50 µl of a reaction mixture containing 4 U/µl of Molony murine leukemia virus reverse transcriptase, 7.5 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.5 µM dNTP and 2 µM random primer (Gibco BRL, Gaithersburg, MD). PCR amplification of cDNA was performed in 20 µl of reaction mixture containing 2 µl of cDNA sample, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.125 U/µl of *Taq*

polymerase and different primer sets (10 nM each). PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and photographed under UV light. Intensity of the bands observed under a UV illuminator was analyzed by using NIH Image. For prevention of templating possibly contaminated genomic DNA, each primer was designed to encompass an exon junction. The sense/antisense primers for PCR were designed as follows: *E-cadherin*, 5'-AACGCCCCCATACCAGAACC-3'/5'-AACAGCAAGAGC AGCAGAATCAGA-3'; *P-cadherin*, 5'-TACGACACCCTC TTGGTGTTC-3'/5'-CAGACTCATAGCCTGTCTCCG-3'; *snail*, 5'-GCCTTCAACTGCAAATACTGC-3'/5'-CCTGGC ACTGGTACTTTCTTGA-3'; *ß-actin*, 5'-TCTACAATGAGC TGCGTGTGGCTCC-3'/5'-AGGAAGGAAGGCTGGAAGA GTGCCTC-3'.

Immunoblot analysis. Cells ($3x10^6$) were lysed in buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 5 µg/µl pepstain, 5 µg/µl leupeptin) at 4°C for 10 min. The cell lysates were then centrifuged at 20,000 xg 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 nitro-cellulose 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.

Construction of plasmid vectors for transfection. The human *snail* sense expression vector (pCDNA3.1 Hyg(-)-HA*snail*) 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'-CAGGATCCCTATGCCGCGCTCTTTCCT-3'/5'-CTGG ATCCTCAGCGGGGACATCCTGA-3'). The amplified product was then digested with *Bam*HI and cloned into the *Bam*HI cloning site of pCDNA3.1 Hyg(-) with HA tag into upstream of the multicloning site. For *snail* antisense expression vector (pcDNA3.1 Hyg(+)-*snail*AS), a 355 bp cDNA fragment (nt 1-355, nt 1 is A of ATG, start codon) was amplified by PCR using RT-product of RNA from MDA-MB435S cells as template and a set of primers (5'-CACGGCGGATGCCGCGCTCTTTCC-3'/5'-CTGGATCCA GGCTGAAGTAGAGGAGAAG-3'). The amplified product was then double-digested with *Eag*I and *Bam*HI and cloned into the *Bam*HI cloning site of pCDNA3.1 Hyg(+) in the antisense orientation. pCDNA3.1 Hyg(-)-HA*snail* and
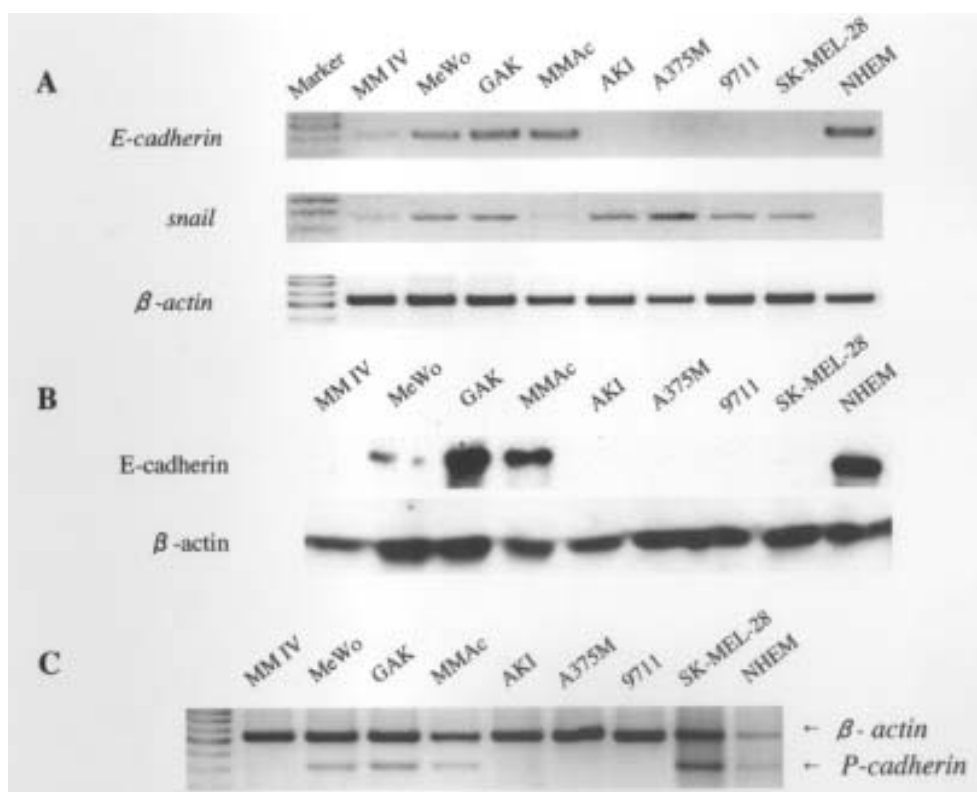
Figure 1. (A), Expressions of *E-cadherin* and *snail* mRNAs in normal human melanocytes (NHEM) and eight human melanoma cell lines assessed by semiquantitative RT-PCR. RNAs extracted from the cells were reverse-transcribed, and cDNAs were amplified by PCR with primers for *E-cadherin*, *snail* and *β-actin*. The primer sequences and detailed experimental conditions are described in Materials and methods. PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed under UV light. Band densities were measured by using the NIH Image software, and ratios of *E-cadherin* and *snail* to *β-actin* control were calculated. (B), E-cadherin protein expression assessed by Western blot analysis. Each 10 μg protein was electrophoresed in a 7.5% SDS-polyacrylamide gel, electrotransferred to a nytrocellulose membrane. Detection of E-cadherin protein was done with anti-human E-cadherin mAb with ECL system. (C), Expressions of P-cadherin in normal melanocytes (NHEM) and melanoma cell lines assessed by semi-quantitative RT-PCR. The primer sequences and detailed experimental conditions are described in Materials and methods.

pCDNA3.1 Hyg(+)-*snail*AS were transfected into competent cells (*Eschrichia 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(-) or 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'-GGT GAATTTTTAGTTAATTAGCGCTAC-3'/5'-CATAACTAA CCGAAAACGCCG-3'; unmethylated DNA, 5'-GGTAGGT GAATTTTTAGTAATTAGTGGTA-3'/5'-ACCCATAACTA ACCAAAAACACCA-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 un-methylated).

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'-GCCCCATCTC CAAAACGAA-3'/5'-TGAACTGACTTCCGCAAGCTC-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'-CAGGATCCCTATGCCGCGCTCTT TCCT-3'/5'-CTGGATCCTCAGCGGGGACATCCTGA-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 human melano-cytes 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 melano-cytes 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 of 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 A375M 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).
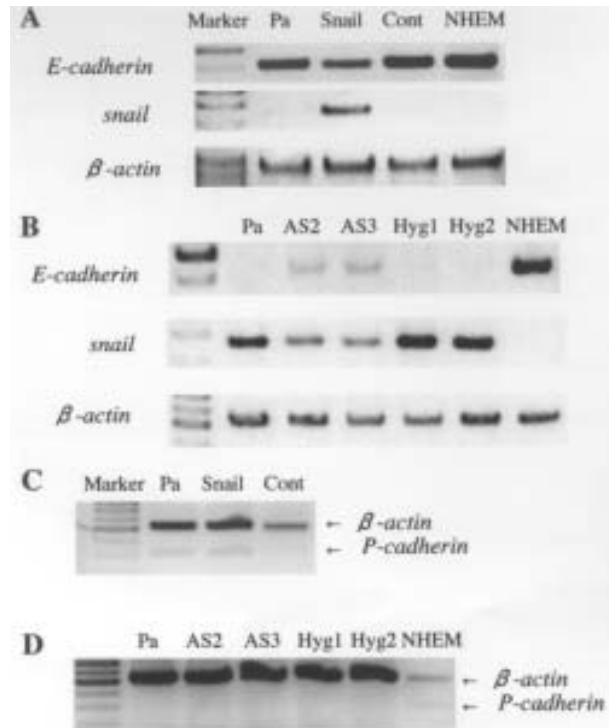

Figure 2. Changes of *E-cadherin* expression, but no change of *P-cadherin* expression, in human melanoma cells by the transduction of *snail* expression vector or *snail* antisense expression vector. (A), Down-regulation of *E-cadherin* gene expression by *snail* transduction in MMAc melanoma cell line. Expressions of *E-cadherin* and *snail* mRNAs were assessed by RT-PCR. Pa, parent MMAc cells; snail, cells transfected with *snail* sense vector; Cont, cells transfected with control vector; NHEM, positive control for *E-cadherin* expression. (B), Up-regulation of *E-cadherin* gene expression by the antisense snail vector in A375M cell line. Pa, parent A375 cells; AS2, clone 2 transfected with antisense *snail* vector; AS3, clone 3 transfected with antisense *snail* vector; Hyg1, clone 1 transfected with vector only; Hyg2, clone 2 transfected with vector only; NHEM, positive control for *E-cadherin* expression. (C), No change of *P-cadherin* expression in MMAc cells by the transduction of the *snail* expression vector. (D), No change of *P-cadherin* expression in A375M cells by the transduction of the *snail* antisense expression vector.

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-cadhrin* 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
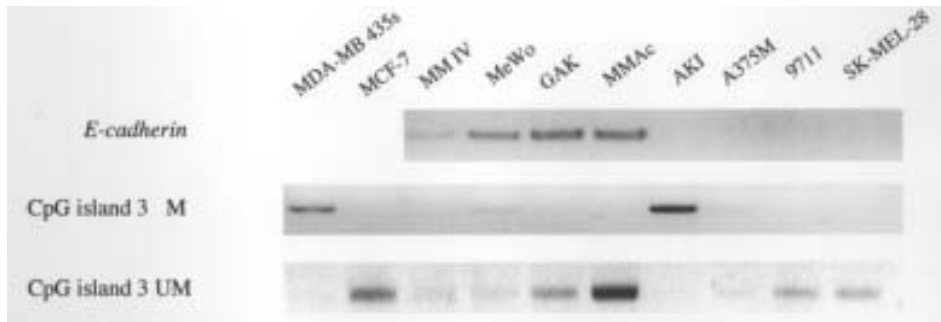
Figure 3. Methylation status of the CpG island 3 in *E-cadherin* promoter of the eight melanoma cell lines assessed by methylation-specific PCR (M, methylated; UM, unmethylated). MDA-MB435s and MCF-7 were control cell lines respectively with and without methylation.
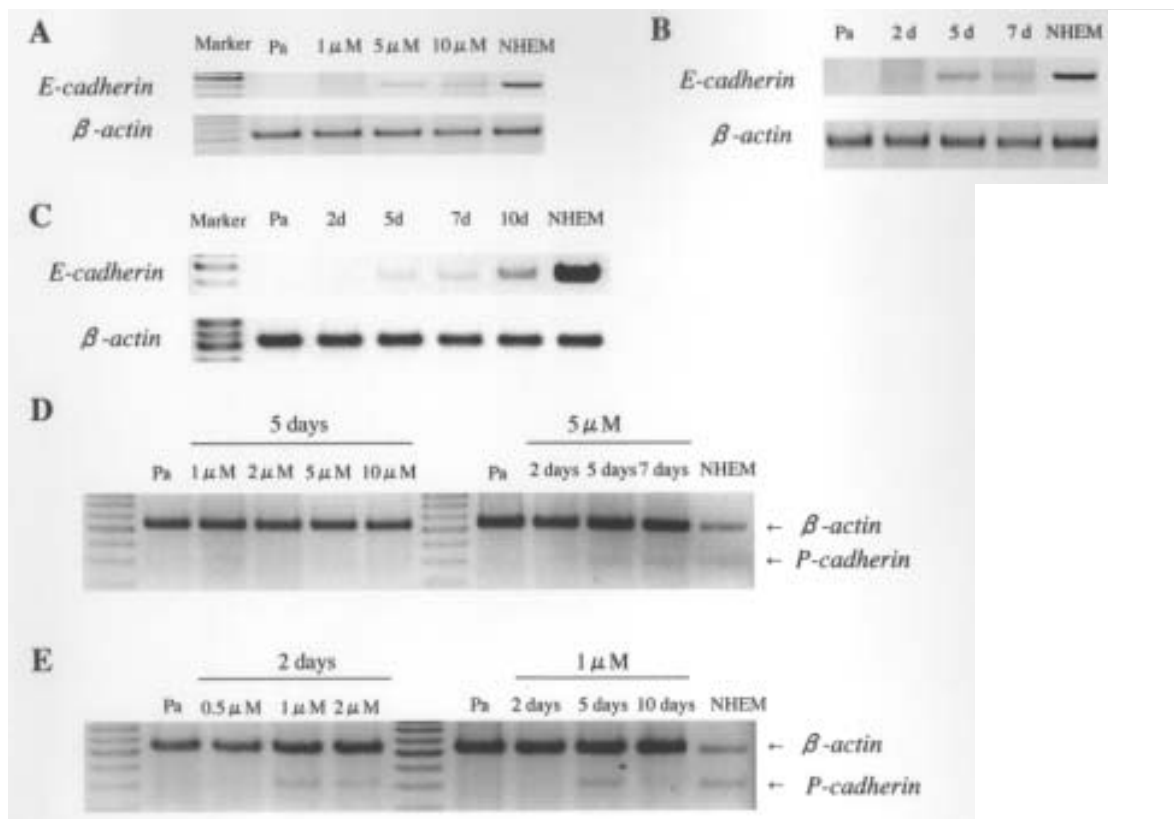

Figure 4. Re-expressions of *E-* and *P-cadherins* of human melanoma cells by treatment with demethylating agent, 5-azacytidine (5-aza). (A), Re-expression of *E-cadherin* in AKI cells by the 5-aza-treatment. The cells were treated with the indicated concentrations of 5-aza for 5 days. (B), Re-expression of *E-cadherin* in AKI cells by the 5-aza-treatment. The cells were treated with 5 μM 5-aza for the indicated period. (C), Re-expression of *E-cadherin* in A375M cells by the 5-aza-treatment. The cells were treated with 1 μM 5-aza for the indicated period. (D and E), Re-expression of *P-cadherin* in AKI cells (D) and A375M cells (E), respectively, by the 5-aza-treatment. The cells were treated with the indicated concentration of 5-aza for the indicated period.

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* expression 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.

Acknowledgements

References

1. Takeichi M: Cadherin cell adhesion receptors as a morpho-genetic regulator. Science 251: 1451-1455, 1991.
2. Kemler R: From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet 9: 317-321, 1993.

3. Cowin P: Unraveling the cytoplasmic interactions of the cadherin superfamily. Proc Natl Acad Sci USA 91: 10759-10761, 1994.

4. Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand JF, Als-Nielsen J, Colman DR and Hendrickson WA: Structural basis of cell-cell adhesion by cadherins. Nature 374: 327-337, 1995.

5. Tamura S, Shiozaki H, Miyata M, Kadowaki T, Inoue M, Matsui S, Iwazawa T, Takayama T, Takeichi M and Monden M: Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br J Surg 83: 1608-1614, 1996.

6. Hunt NC, Douglas-Jones AG, Jasani B, Morgan JM and Pignatelli M: Loss of E-cadherin expression associated with lymph node metastases in small breast carcinomas. Virchows Arch 430: 285-289, 1997.

7. Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, Debruyne FM and Schalken JA: Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res 54: 3929-3933, 1994.

8. Otto T, Birchmeier W, Schmidt U, Hinke A, Schipper J, Rubben H and Raz A: Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas. Cancer Res 54: 3120-3123, 1994.

9. Katagiri A, Watanabe R and Tomita Y: E-cadherin expression in renal cell cancer and its significance in metastasis and survival. Br J Cancer 71: 376-379, 1995.

10. Shimoyama Y and Hirohashi S: Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. Cancer Lett 57: 131-135, 1991.

11. Behrens J, Mareel MM, van Roy FM and Birchmeier W: Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 108: 2435-2447, 1989.

12. Vleminckx K, Vakaet L Jr, Mareel M, Fiers W and van Roy F: Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 66: 107-119, 1991.

13. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D and Birchmeier W: E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 113: 173-185, 1991.

14. Oda T, Kanai Y, Oyama T, Yoshiura K, Shimoyama Y, Birchmeier W, Sugimura T and Hirohashi S: E-cadherin gene mutations in human gastric carcinoma cell lines. Proc Natl Acad Sci USA 91: 1858-1862, 1994.

15. Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR and Hofler H: E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res 54: 3845-3852, 1994.

16. Kanai Y, Oda T, Tsuda H, Ochiai A and Hirohashi S: Point mutation of the E-cadherin gene in invasive lobular carcinoma of the breast. Jpn J Cancer Res 85: 1035-1039, 1994.

17. Yoshimura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T and Hirohashi S: Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci USA 92: 7416-7419, 1995.

18. Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE and Baylin SB: E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55: 5195-5199, 1995.

19. Graff JR, Herman JG, Myohanen S, Baylin SB and Vertino PM: Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. J Biol Chem 272: 22322-22329, 1997.

20. Kanai Y, Ushijima S, Hui AM, Ochiai A, Tsuda H, Sakamoto M and Hirohashi S: The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. Int J Cancer 71: 355-359, 1997.

21 Hiraguri S, Godfrey T, Nakamura H, Graff J, Collins C, Shayesteh L, Doggett N, Johnson K, Wheelock M, Herman J, Baylin S, Pinkel D and Gray J: Mechanisms of inactivation of E-cadherin in breast cancer cell lines. Cancer Res 58: 1972-1977, 1998.

22. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J and Garcia De Herreros A: The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2: 84-89, 2000.

23. Yokoyama K, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R and Nagayama M: Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. Oral Oncol 37: 65-71, 2001.

24. Poser I, Dominguez D, De Herreros AG, Varnai A, Buettner R and Bosserhoff AK: Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. J Biol Chem 276: 24661-24666, 2001.

25. Jiao W, Miyazaki K and Kitajima Y: Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo. Br J Cancer 86: 98-101, 2002.

26. Danen EH, De Vries TJ, Morandini R, Ghanem GG, Ruiter DJ and van Muijen GN: E-cadherin expression in human melanoma. Melanoma Res 6: 127-131, 1996.

27. Hsu MY, Wheelock MJ, Johnson KR and Herlyn M: Shifts in cadherin profiles between human normal melanocytes and melanomas. J Investig Dermatol Symp Proc 1: 188-194, 1996.

28. Silye R, Karayiannakis AJ, Syrigos KN, Poole S, van Noorden S, Batchelor W, Regele H, Sega W, Boesmueller H, Krausz T and Pignatelli M: E-cadherin/catenin complex in benign and malignant melanocytic lesions. J Pathol 186: 350-355, 1998.

29. Sanders DS, Blessing K, Hassan GA, Bruton R, Marsden JR and Jankowski J: Alterations in cadherin and catenin expression during the biological progression of melanocytic tumours. Mol Pathol 52: 151-157, 1999.

30. Seline PC, Norris DA, Horikawa T, Fujita M, Middleton MH and Morelli JG: Expression of E and P-cadherin by melanoma cells decreases in progressive melanomas and following ultraviolet radiation. J Invest Dermatol 106: 1320-1324, 1996.

31. Okubo Y, Hamada J, Takahashi Y, Tada M, Tsutsumida A, Furuuchi K, Aoyama T, Sugihara T and Moriuchi T: Transduction of HOXD3-antisense into human melanoma cells results in decreased invasive and motile activities. Clin Exp Metastasis 19: 503-511, 2002.

32. Graff JR, Greenberg VE, Herman JG, Westra WH, Boghaert ER, Ain KB, Saji M, Zeiger MA, Zimmer SG and Baylin SB: Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. Cancer Res 58: 2063-2066, 1998.

33. Bussemakers MJ, van Bokhoven A, Voller M, Smit FP and Schalken JA: The genes for the calcium-dependent cell adhesion molecules P- and E-cadherin are tandemly arranged in the human genome. Biochem Biophys Res Commun 203: 1291-1294, 1994.