Methylation status of the SOCS3 gene in human malignant melanomas

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Received October 13, 2006; Accepted December 4, 2006

Abstract. The suppressors of cytokine signaling (SOCS) family inhibits not only Janus kinase (JAK)/signal transducers and activators of transcription (STAT) but also focal adhesion kinase (FAK) signaling pathways, and has tumor suppressor activity. Aberrant methylation in the promoter region of the SOCS3 gene frequently occurs in several types of human malignancy, and its transcriptional silencing is associated with malignant tumor behavior. In malignant melanomas, the expression and methylation status of the SOCS3 gene have not been elucidated. We therefore examined the methylation status and/or protein expression of the SOCS3 gene in 5 human malignant melanoma cell lines, 2 primary cultures of normal melanocytes, and surgically resected tumors (5 malignant melanomas and 2 melanocytic nevi). Four of the 5 melanoma cell lines and the 2 primary cultures of normal melanocytes expressed SOCS3 protein to various degrees, and only one melanoma cell line was negative. Expression of SOCS3 protein was inversely correlated with methylation status in the SOCS3 promoter region, and treatment with a demethylating agent (5-aza-2'-deoxycytidine) was able to induce expression of the protein in one melanoma cell line that was SOCS3-negative and another that was weakly positive. Three of the 5 primary malignant melanomas and one of the 2 melanocytic nevi showed aberrant methylation. These results suggest that inactivation of the SOCS3 gene by hypermethylation may be involved in the promotion of malignant behavior of melanomas.

Introduction

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor protein tyrosine kinase that has emerged as a crucial molecule in integrating signals from integrins and receptor tyrosine kinases in processes such as cell survival, proliferation and motility (1,2). Enhanced FAK signaling increases cell motility and promotes cell survival in an anchorage-independent manner (3,4). Along these lines, a number of groups have reported that the protein level and/or activity of FAK are up-regulated in invasive cancer cells including malignant melanomas (5-9). Akasaka *et al* (5) demonstrated that phosphorylated FAK protein in melanoma cell lines modulates cytoskeletal function and is correlated significantly with their migration rate. *In vitro* and *in vivo* studies have demonstrated that activation of the FAK signaling pathways contributes to aggressive melanoma phenotypes (cell invasion, migration, and vasculogenic mimicry) (5-9).

Ligand binding of integrins results in catalytic activation of FAK protein through the SH2 (src homology 2) domain. Several studies have demonstrated that the SOCS (suppressors of cytokine signaling) family is a negative regulator of FAK signaling (10,11). Interactions of SOCS1 and SOCS3 with FAK through the SH2 domain have been reported to promote polyubiquitination and subsequent degradation of FAK proteins (10). Moreover, the SOCS family acts via a feedback loop to inhibit cytokine responses and activation of the JAK (Janus kinase)/STAT (signal transducers and activators of transcription) pathway (12-14). Acting through the JAK/STAT and FAK signaling pathways, SOCS1 and SOCS3 have multiple molecular targets associated with tumorigenesis in diverse human malignancies.

Several groups have shown that aberrant methylation of the CpG islands (CGIs) within the SOCS1 and SOCS3 promoter regions frequently occurs in hepatocellular carcinoma (11,15,16), head and neck squamous cell carcinoma (17,18), and lung (19), gastric (20) colon (21,22) and urinary bladder cancers (23). In these studies, both epigenetic modifications were shown to be well correlated with transcriptional gene silencing and aggressive phenotypes of each tumor (11,15-23). In malignant melanomas, expression and methylation status have been examined in the SOCS1 gene (24,25), whereas those in SOCS3 are still unclear. In the present study, therefore, we examined the methylation status and protein expression of the SOCS3 gene in human malignant melanoma cell lines, primary cultures of normal melanocytes, and samples of primary tumors obtained from patients with malignant melanomas and melanocytic nevi.

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Key words: suppressors of cytokine signaling 3, methylation, epigenetics, malignant melanoma, tumor suppressor

Materials and methods

Cell lines, tissue samples and DNA extraction. We purchased 4 human melanoma cell lines (SK-MEL-28, HMV-I, CRL1579, and G361; Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) and two primary cultures of normal human epidermal melanocytes (NHEM-Lightly and NHEM-Darkly, Cascade Biologics Inc., Portland, OR, USA). A melanoma cell line (MM-AN) was also kindly donated by Professor Mihm (Department of Dermatology, Harvard Medical University, Boston, MA, USA). The cells were maintained under the recommended conditions. Five primary malignant melanomas (male/female = 2/3; mean age: 68 years, range 56-80 years) and 2 melanocytic nevi (male/female = 1/1; age 26 and 36 years, respectively) were also examined. Permission for the study was obtained from the Institutional Review Board (IRB) of Iwate Medical University School of Medicine, Morioka, Japan. Genomic DNA was isolated with a Puregene™ DNA purification system, cell and tissue kit (Gentra Systems Inc., Minneapolis, MN, USA).

Bisulfite genome sequencing. DNAs obtained from cell lines were treated by bisulfite modification and examined for the methylation status of 44 CpG dinucleotides within the SOCS promoter region (-706 to -188) (Fig. 1) (11). Bisulfite modification was done using a Methylamp™ DNA modification kit (Epigenetek Inc., Broadway, NY, USA). Modified DNA was PCR-amplified using previously described primer sets; SF: GGG ATT YGA GGG GGT TTA GTT TTA AGG A (Y means C or T), and SR: CAC TAC CCC RAA AAC CCT CTC CTA (R means A or G) (Fig. 1) (11). The final reaction volume for all PCRs was 50 μl, and the mixture contained 0.2 μM each primer, 1 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 30 μl of distilled water. PCR conditions were 95˚C for 2 min followed by 5 cycles of 95˚C for 30 sec and 64˚C for 1 min, 5 cycles of 95˚C for 30 sec and 62˚C for 1 min, 5 cycles of 95˚C for 30 sec and 60˚C for 1 min, and then 35 cycles of 95˚C for 30 sec and 58˚C for 1 min. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR products were purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). The PCR fragments were ligated to pGEM-T Easy Vectors (Promega Co., Madison, WI, USA) and transformed into DH5α competent cells (Toyobo, Tokyo, Japan). Ten subcloned colonies were chosen at random from each melanoma cell line. Plasmid DNA was purified by a PI-200 DNA automatic isolation system (Kurabo, Osaka, Japan). Cycle sequencing was done using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems; ABI, Foster City, CA, USA) and an ABI PRISM 3100 DNA sequencer (ABI).

Methylation-specific PCR (MSP). For analysis of the methylation status of surgical specimens, we used the MSP method. DNAs extracted from primary tumors were treated with a Methylamp DNA modification kit (Epigenetek). Two sets of primers were previously designed to specifically amplify either the methylated (M, -525 to -384) (MF: GGA GAT TTT AGG TTT TCG GAA TAT TTC/ MR: CCC CCG AAA CTA CCT AAA CGC CG) or unmethylated (U, -529 to -379) (UF: GTT GGA GAT TTT AGG TTT TTG GAA TAT TTT/ UR: AAA CCC CCA AAA CTA CCT AAA CAC CA) samples (11). PCR mixtures contained 25 μl of Aplitic Gold PCR Master mix (ABI), and 200 nmol/l each primer each in a final volume of 50 μl. PCR amplification was performed under the following conditions: 95˚C for 5 min followed by 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min, and ending with a final extension of 72˚C for 5 min.

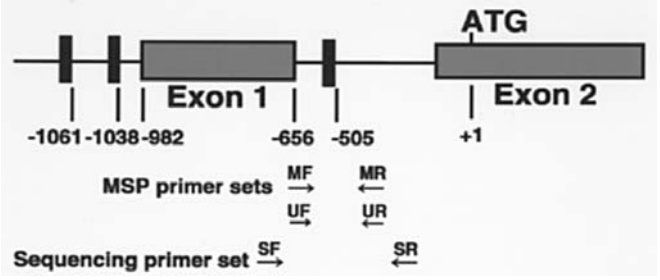

Figure 1. Schematic representation of the SOCS3 gene. The translational start site for the SOCS3 gene is defined as +1. Shaded boxes are the exons of the SOCS3 gene, and STAT binding consensus sequences are depicted as black boxes. Primers used for bisulfite genome sequencing and methylation-specific PCR are indicated by arrows.

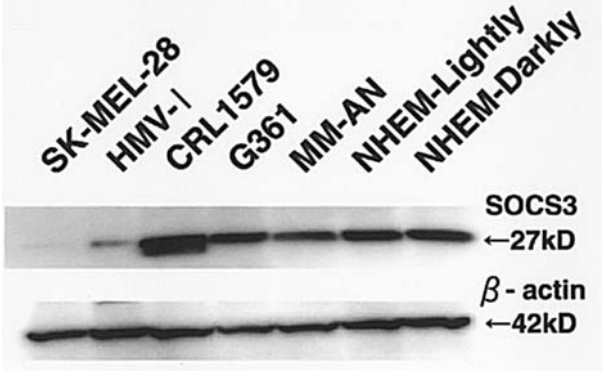

Figure 2. Western blot analysis of SOCS3 protein in 5 melanoma cell lines (SK-MEL-28, HMV-I, CRL1579, G361, and MM-AN) and two primary cultures of normal human epidermal melanocytes (NHEM-Lightly and NHEM-Darkly). One melanoma cell line (SK-MEL-28) was negative for SOCS3. The remaining cultured cells expressed SOCS3 protein to various degrees.

Western blotting. The 5 malignant melanoma cell lines and 2 primary cultures of normal melanocytes were cultured to 70-80% confluence on a 10-cm Petri dish, 1 ml of cold phosphate-buffered saline (PBS) was added, and the cells were removed from the dishes by scraping. The cell suspension was then centrifuged. After removal of the supernatant, the cell pellet was dissolved in 1.0% NP-40 lysis buffer [50 mM HEPES (pH 7.5)/1 mM EDTA/150 mM NaCl/2.5 mM EGTA/1.0% NP-40] and spun at 4˚C for 30 min. Insoluble material was precipitated and the clear supernatant was collected. The protein concentration of the lysates was measured using a Quick Start™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were mixed with 6X concentrated loading dye, heated for 4 min at 95˚C,

and subjected to SDS-PAGE on a 10% polyacrylamide gel (Ready Gels J, Bio-Rad). The proteins were then transferred to a PVDF membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The membrane was washed twice in 0.05% Tween-20-PBS (PBST) for 10 min, and then blocked with 5% blocking reagent (Amersham Biosciences) in PBST for 1 h at room temperature, followed by two washes in PBST for 10 min. The primary mouse monoclonal antibodies against SOCS3 (SO1; Abcam, Cambridge, UK) and anti-ß-actin (Sigma Chemical Co., St. Louis, MO, USA) were diluted 1:700 and 1:1000 in immunoreaction enhancer solution (Can Get Signal Solution 1; Toyobo Co., Ltd., Osaka, Japan). The membrane was incubated for 1 h at room temperature and washed as described. The second antibody, anti-mouse IgG (Amersham Biosciences), was diluted 1:10000 in immunoreaction enhancer solution (Can Get Signal Solution 2, Toyobo). The membrane was incubated for 45 min at room temperature and washed. Signals were detected with ECL Plus (Amersham Biosciences) and ChemiDoc XRS (Bio-Rad).

5-aza-2'-deoxycytidine treatment. Cells were seeded at a density of $5x10^5$ cells/10 cm plate on day 1. Twenty-four hours later, 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma) was added to a final concentration of 10 μM. Three days (72 h) after 5-Aza-dC treatment, the cells were harvested for Western blotting.

Results

SOCS3 protein expression in malignant melanoma cell lines and primary cultures of normal melanocytes. Expression of SOCS3 protein was detected in 4 of the malignant melanoma cell lines (HMV-I, CRL1579, G361, and MM-AN) and the two primary cultures of normal melanocytes (NHEM-Lightly and -Darkly) by Western blotting (Fig. 2). One cell line (CRL1579) expressed SOCS3 protein strongly whereas another (HMV-I) exhibited only a faint SOCS3 signal (Fig. 2). The SOCS3 expression levels of the other two malignant melanoma cell lines (G361 and MM-AN) and primary cultures of normal melanocytes (NHEM-Lightly and -Darkly) were equal (Fig. 2). No SOCS3 protein expression was detected in the fifth malignant melanoma cell line (SK-MEL-28).

Methylation status of the SOCS3 promoter region in malignant melanoma cell lines and primary cultures of normal melanocytes. We examined the methylation status of the SOCS3 gene promoter region in cell cultures by the bisulfite genome sequencing method (Fig. 3). Two parts of the SOCS3 promoter region were extensively methylated (M1, -538 to -500; and M2, -337 to -216) in the SOCS3-negative cell line (SK-MEL-28) (Fig. 3). Of 18 CpG sites within the M2 region, 16 (89%) were >50% methylated, and 4 (100%) of 4 CpG sites within the M1 region were also methylated. The M2 region was also frequently methylated in the weakly SOCS3-positive melanoma cell line (HMV-I), but the M1 region was not frequently methylated (Fig. 3). Cytosine methylation occurred randomly in three SOCS3-positive melanoma cell lines and two normal melanocyte primary cultures, being <30% at any of the sites (Fig. 3).

5-Aza-dC treatment. To test whether cytosine methylation in the SOCS3 promoter is functionally linked to silencing of its transcripts in malignant melanoma cell lines, we treated the SOCS-negative (SK-MEL-28) and weakly positive (HMV-I) cell lines with 5-Aza-dC, a demethylating agent, and observed a subsequent gain of SOCS3 protein expression in both cell lines (Fig. 4).

MSP analysis of primary tumors. We next evaluated the promoter status at the SOCS3 gene in tumors obtained from 5 patients with malignant melanomas and two with melanocytic nevi. We failed to set primers in the M2 region because of its repetitive sequence. Therefore, we used primer sets reported previously in a study of hepatocellular carcinomas (11). We found that the SOCS3 promoter was aberrantly methylated in 3 (60%) of the 5 malignant melanomas. A weak band was detected in one of the two melanocytic nevi (Fig. 5). In this nevus, both the methylated (M)- and unmethylated (U)-primer sets produced a positive signal (Fig. 5).

Discussion

Methylation of promoter CGIs leads to transcriptional silencing of their downstream genes, and plays an important role in embryonic development and tissue differentiation (26,27). In cancers, tumor suppressor genes such as p16, CDH1 and MLH1 are inactivated by methylation of their promoter, along with mutations and loss of heterozygosity (26-30). At the same time, overall genome-wide hypomethylation is consistently observed in tumors (31-33). Some tumors display aberrant demethylation of normally methylated CGIs and expression of their downstream product, such as melanoma antigen genes (MAGEs), cancer-testis antigen, and maspin (34-40). Genome-wide search for epigenetic modifications (hyper- and hypo-methylation) in cancers has been attempted for several human malignancies.

In malignant melanomas, Furuta *et al* (41) performed a genome-wide screening for CGIs using methylation-sensitive representational difference analysis, and 34 putative promoter regions that are affected by aberrant cytosine methylation. However, neither SOCS1 nor SOCS3 was nominated as an affected region in their study. Li *et al* (24) demonstrated that SOCS1 was undetectable in normal human melanocytes whereas it was uniformly expressed in all tested cell lines, although they did not examine methylation status at the SOCS1 promoter. Moreover, SOCS1 immunoreactivity was found to be closely associated with tumor invasion, tumor thickness, and disease stage. This study asserted that aberrant SOCS1 expression was well correlated with stepwise progression of melanomagenesis and the aggressive behavior of malignant melanomas. However, this is contradictory to the theory derived from numerous studies on other types of human malignancy (11,15-23). Both SOCS1 and SOCS3 are negative regulators of FAK signaling, promoting aggressive behavior such as invasion and metastasis. However, Marini *et al* (25) recently demonstrated that CGIs of the SOCS1 promoter region are frequently hypermethylated, and confirmed transcriptional silencing of the SOCS1 gene in primary and/or metastatic tumors. Herein, we also demonstrated that SOCS3 was frequently affected by aberrant cytosine methylation, resulting

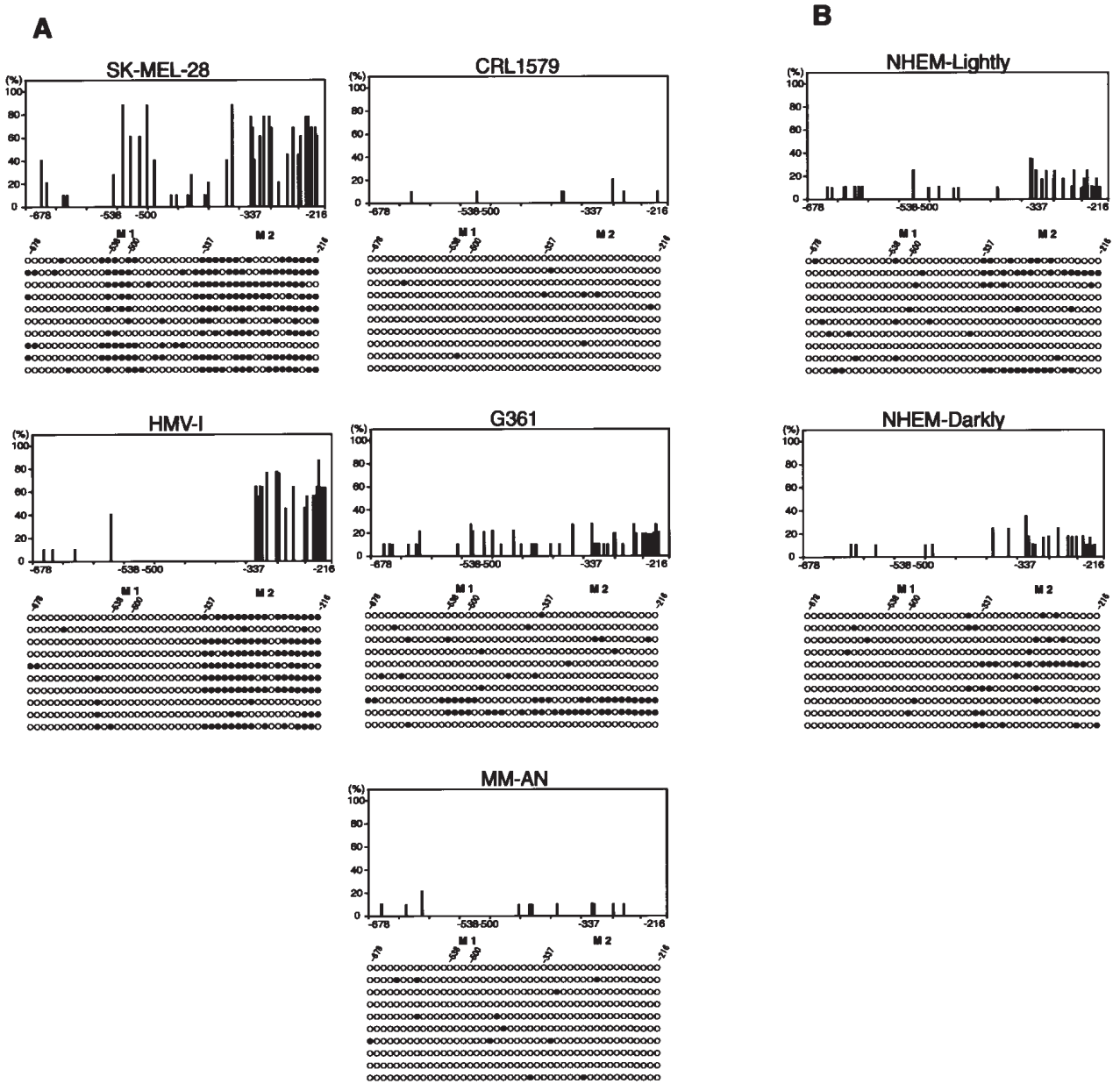

Figure 3. Summary of 5-methylcytosine levels obtained by bisulfite-modified genome sequencing of the SOCS3 promoter in 5 melanoma cell lines (A) (SK-MEL-28, HMV-I, CRL1579, G361, and MM-AN) and two primary cultures of normal human epidermal melanocytes (B) (NHEM-Lightly and NHEM-Darkly). Ten cloned PCR products were sequenced to determine the percentage methylation of the 44 CpG sites in the region analyzed. The y-axis represents percentage cytosine methylation and the x-axis shows the nucleotide position relative to the transcription start site. Each row of circles represents the methylation pattern obtained from individual clones of the SOCS3 gene promoter. Each circle represents a CpG dinucleotide site. The black circles are methylation-positive and the white circles are methylation-negative. The number above the circle indicates the position of each CpG site from the major transcription start site. Two parts of the SOCS3 promoter region were extensively methylated (M1, -538 to -500; and M2, -337 to -216) in a SOCS3-negative cell line (SK-MEL-28).

in transcriptional silencing. Inactivation of both SOCS1 and SOCS3 by aberrant methylation might contribute to gain of function of FAK signaling.

There are several pathways other than FAK downstream of SOCS3 inactivation. Both SOCS1 and SOCS3 have an N-terminal kinase-inhibitory region and inhibit JAK activity. However, SOCS3 inhibits JAK by binding to cytokine-receptor tyrosine residues, whereas SOCS1 binds directly to JAK. The high-affinity interaction of SOCS3 with cytokine receptors through its SH2 domain probably ensures relatively specific inhibition of particular cytokine signaling (42). Because the binding of the SH2 domain of SOCS3 is relatively specific to receptors for STAT3-activating cytokines, such as IL-6, granulocyte-colony stimulating factor, and leptin, the effect of SOCS3 may be relatively restricted to STAT3 (43-47). The relationship between SOCS3 inactivation and its downstream SOCS3-JAK/STAT pathway has been intensively examined in relation to tumorigenesis of hepatocellular carcinoma.

Ogata *et al* (16) showed that the expression of SOCS3 was reduced in regions of hepatocellular carcinoma in comparison with surrounded normal hepatic tissues. In addition, by using liver-specific SOCS3-deficient mice with T-cell-mediated hepatitis and carcinogen-induced hepatocarcinogenesis as models, they also demonstrated that deletion of the SOCS3

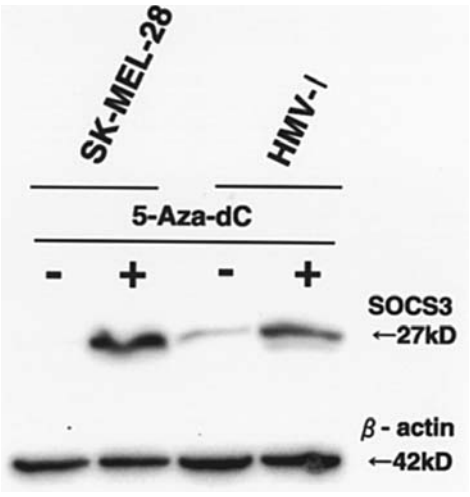

Figure 4. Western blot analysis of SOCS3 protein before and after treatment with 5-Aza-dC in two malignant melanoma cell lines (SK-MEL-28 and HMV-I), which were aberrantly methylated in the SOCS3 promoter region. Expression of the SOCS3 protein was observed in both cell lines after treatment with 5-Aza-dC. Equal loading was confirmed by incubation with ß-actin antibody.

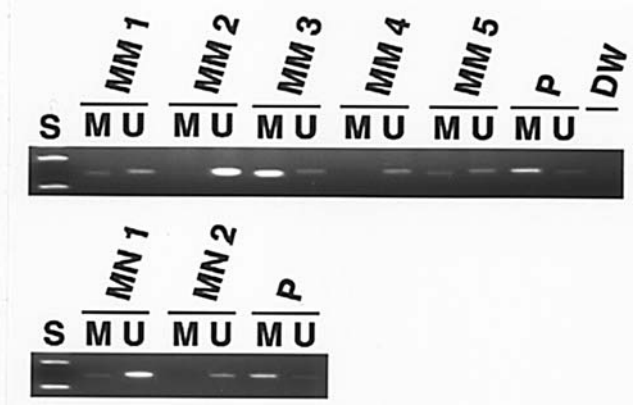

Figure 5. Methylation-specific PCR analysis of the SOCS3 gene in primary tumors obtained from 5 patients with malignant melanomas and two with melanocytic nevi. Aberrant methylation was observed in 3 of the malignant melanomas (MM) (MM1, MM3, and MM5), and a weak band was detected in one (MN1) of the melanocytic nevi (MN). S, size marker; M, methylated primer set; U, unmethylated primer set; P, positive control; DW, distilled water.

gene protected hepatocytes from concanavalin A-mediated apoptosis and promoted chemical compound-induced hepatocarcinogenesis. Inactivation of the SOCS gene in hepatocytes promotes the activation of STAT3, resistance to apoptosis (through B-cell lymphoma XL, Bcl-XL) and acceleration of proliferation (through cyclin D1) (16). Repression of SOCS3 in malignant melanoma cells may share these pathways, and participate in anti-apoptosis and/or acceleration of cell proliferation.

Interferons (IFNs) are used for the treatment of renal cell carcinoma and malignant melanoma through their apoptosis-inducing effects (48,49). It is well known that treatment with a demethylating agent (DNMT1 inhibitor, 5-Aza-dC) can overcome resistance to apoptosis induction by IFNs in these tumor cell types (48,49). Several candidate genes (such as MAGEs and RASAAF1A) targeted by DNMT1 inhibitors have been nominated. The SOCS-JAK/STAT pathway is a major pathway regulated by IFN induction. We expect that transcriptional silencing of the SOCS3 gene by aberrant methylation might contribute to resistance of malignant melanoma cells to IFN treatment. Recently, a phase I trial of low-dose 5-Aza-dC plus interleukin-2 was performed in patients with malignant melanoma (50). Among 16 patients, the response rate was 31% (50). Although global methylation was evaluated using repetitive sequences in this study, the methylation status of specific genes affected by 5-Aza-dC treatment were not examined. If our hypothesis is valid, i.e. that inactivation of the SOCS3 gene by aberrant methylation involves the mechanism of resistance to IFN treatment in malignant melanomas, then examination of SOCS3 promoter methylation would be a good biomarker for evaluating and predicting the efficacy of combination therapy with IFN and DNMT1 inhibitor. Recent studies have shown that the methylation status of circulating melanoma cells can be evaluated in serum samples (25,51). We intend to study further the relationship between SOCS3 promoter methylation and resistance to IFN treatment in malignant melanomas.

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