

Differential methylation of CpG islands within the *dermol* gene promoter in several cancer cell lines

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Abstract. Inactivation of tumor-related genes by promoter hypermethylation is a common epigenetic event in the development of variety of tumors. *Dermol* (also called *twist2*) is a novel cancer-related gene which belongs to the basic helix-loop-helix (bHLH) transcription factor family. Herein, we report that *dermol* expression was sporadically abrogated in human cancer cells by transcriptional silencing associated with CpG island promoter hypermethylation. Direct sequencing of bisulfite-modified DNA from a panel of seven human cancer cell lines (HL60, Molm14, MV4-11, RS4:11, MDM-BA231, H358, and H1299) revealed that CpG dinucleotides in the *dermol* promoter were methylated. RT-PCR results demonstrated that *dermol* CpG island hypermethylation was accompanied by a low basal *dermol* expression level. Our data implicate *dermol* as a tumor suppressor gene and a valuable molecular marker for human cancer.

Introduction

Epigenetic changes such as DNA methylation has important regulatory effects on gene expression, which involves CpG islands, located in the promoter regions of many genes (1,2). Aberrant methylation of CpG islands has been associated with the development of human tumor (3,4). In tumors from different patients, mutations that disrupt gene function often vary in genomic position over a wide region, whereas the

position of CpG island promoter methylation is constant within an individual gene. The changes of methylation status of various gene promoters seem to be a common feature of malignant cells and these changes can occur early in the progression process. Therefore, detection of aberrant promoter hypermethylation of cancer-related genes has become an important tool for cancer diagnosis or detection of cancer recurrence (5-7).

Dermol (also called *Twist2*) and *Twist* (*Twist1*) are highly conserved transcription factors which belong to the basic Helix-loop-helix (b-HLH) protein family, and their structures are highly homologous (8-10). *Twist* can promote malignant tumor development and metastasis, and *Twist* activation in human cancers might both be a prognostic and/or predictive factor as well as a novel therapeutic target (11-13). *Twist* CpG methylation is a useful biomarker in breast cancer diagnosis (14,15). However, very little is known regarding *dermol* function and the regulation model. *dermol* has only been found to be differentially methylated in CLL cells relative to Ig VH mutational statuses (16). The expression of *dermol* has been observed to downregulate p14arf thus antagonizing p53-induced apoptosis (17).

In this study, we investigated methylated regulation profiles of *dermol* gene in leukemia, breast cancer and lung cancer cell lines to testify whether *dermol* methylation exist in different cancers. A pair of primers was designed to amplify a 450 bp fragment, which contains 16 CpG sites in the core promoter region of the *dermol* gene. PCR products of bisulfite-modified CpG islands were analyzed by DNA sequencing. Then two pairs of primers were designed to distinguish methylation from unmethylation DNA taking advantage of the sequence differences resulting from bisulfite modification. The relationship between promoter methylation and gene expression (assessed by reverse transcription PCR) were examined in seven human tumor cell lines (HL60, Molm14, MV4-11, RS4:11, MDM-BA231, H358 and H1299). These observations collectively suggest that promoter methylation of *dermol* might be a regular event in cancer. Our assay might have important implications in early detection and surveillance of cancer.

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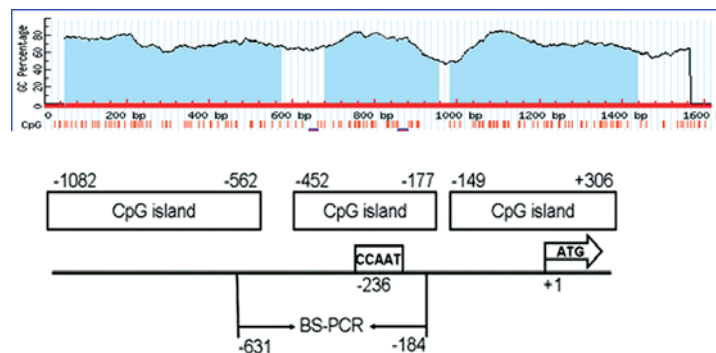


Figure 1. Schematic of *dermo1* promoter-associated CpG island region, primer used for bisulfite polymerase chain reaction (BS-PCR): (criterion: size of CpG island >100 bp, GC content >50.0%, Obs/Exp >0.6).

Materials and methods

Cell lines. Genomic DNA was obtained from seven cell lines: including four leukemia cancer cell lines (MV4-11, HL60, Molm14, and RS4:11), two lung cancer (H358 and H1299), one breast cancer (MDM-BA231), and one cervical cancer cell line (Hela). All cell lines were routinely cultured in RPMI-1640 medium (Gibco) containing with 10% fetal calf serum (Hyclone) at 5% CO₂ and 37°C.

Bisulfite modification. DNA (2.0 µg) in a volume of 50 µl was denatured by NaOH (final concentration, 0.3 M) for 20 min at 42°C. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 µl of 4.2 M sodium bisulfite (Sigma) at pH 5.0, both freshly prepared, were added, and were incubated under mineral oil at 55°C for 16 h. Modified DNA was purified using the Wizard clean-up system (Promega) and eluted into 180 µl of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 15 min at 37°C, followed by ethanol precipitation. DNA was resuspended in 40 µl TE and stored at -20°C for use.

Bisulfite genomic sequencing and combined bisulfite restriction analysis. Primers were designed according to CpG island map of *dermo1* gene. The forward primer was 5'-ggg cgcggtagattagttg-3' and the reverse primer was 5'-cta aactaaattactaaataattatc-3'. The PCR mixture contained BSP amplifications were performed in 50 µl reaction mixtures containing 4 µl bisulfite-treated DNA, 200 µM dNTPs, 100 nM primers, 2 mM MgCl₂ and 1 unit of FastTaq DNA polymerase (Roche). PCR conditions were: 95°C for 2 min, 40 cycles of 95°C for 50 sec, 60°C for 50 sec, 72°C for 1 min, and finally 5 min at 72°C. The PCR products were purified from 1.5% agarose gels using the QIAquick PCR purified kit (Qiagen). For restriction analysis, PCR product was digested by BstUI restriction enzyme for 4 h at 60°C and separated on a 3% agarose gel.

Methylation-specific PCR (MSP). Methylation-specific PCR procedures were used to amplify bisulfite-modified genomic DNA. MSP primers directed against methylated and unmethylated alleles of *dermo1* as follows: methylated primer (M-primer), forward: 5'-gttaagatttataggtcg-3', reverse: 5'-cgcctcaavgtaaaaatcg-3'; unmethylated primer (U-primer),

forward: 5'-gttaagatttataggttg-3', reverse: 5'-cacctcaacataaa aatca-3'. PCR conditions were: 94°C denaturing for 5 min; 94°C for 45 sec, annealing at 60°C for 45 sec, 72°C 1 min. After purification from 2.0% agarose gels, PCR products were sequenced and analyzed to identify the methylated and unmethylated sequences.

Reverse transcription PCR assay. Total cellular RNA was extracted, isolated, and purified using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The GAPDH transcripts in each sample were also amplified as internal controls to normalize the amount of *dermo1*-specific products. The primer sequences utilized were: GAPDH primer, forward: 5'-gtattggcgctggtcac-3', reverse: 5'-ctcctggaa gatggtgatgg-3'; *dermo1* primer, forward: 5'-tcgagaggcagcc caagcg-3', reverse: 5'-aggatccctagtgggaggcgga-3'. All of the reverse-transcriptase reactions were performed using SuperScript™ First-Strand synthesis system (Invitrogen). The PCR reaction mixture consisted of cDNA from 250 ng of total RNA, 200 pmol each primer, 0.2 mM dNTP, 1.5 mM Mg²⁺, 2.0 units of Taq DNA polymerase (Promega). Thermal cycles were: at 95°C for 2 min, then 30 cycles at 95°C for 30 sec, at 62°C for 30 sec, at 72°C for 1 min followed by extension at 72°C for 7 min.

Results

The group of Plass has reported that *dermo1* is methylated in chronic lymphocytic leukemia (CLL) patient samples (16), thus we hypothesis that *dermo1* might be regulated through methylation generally. Herein, we assessed methylation status within promoter region of *dermo1* in various cancer cell lines by using bisulfite PCR. We mapped the location of three big CpG island (Fig. 1) (criterion: size of CpG island >100 bp, GC content >50.0%, Obs/Exp >0.6) within the *dermo1* gene, and the location of the probe (bisulfite PCR) designed to amplify the -631 to -184 bp region of the promoter. BS-PCR analysis of DNA from the selected cancer cell lines (HL60, RS4:11, MDM-BA231, H358 and H1299) indicated that the 430 bp fragment was detectable, and the primer we designed was feasible (Fig. 2).

If the original DNA is methylated, BS-PCR products could be digested to small fragments by BstUI (enzyme specially targeted at methylated CG¹CG).



Figure 2. The *dermo1* gene BS-PCR analysis for 5 different cancer cell lines including HL60, RS4:11, MDM-BA231, H358 and H1299.

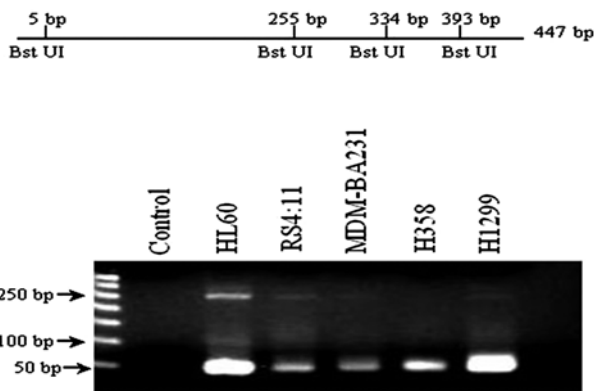


Figure 3. BS-PCR products from the indicated cancer cell lines were restricted with BstUI. Methylated CG sites are indicated by BstUI fragments.

products from the selected cell lines (HL60, RS4:11, MDM-BA231, H358 and H1299) were completely cleaved by BstUI (Fig. 3), only 250 and 50 bp fragments were visualized by agarose gel electrophoresis. This result demonstrated that promoter region of the *dermo1* gene was methylated in the analyzed cancer cells.

To further confirm that *dermo1* was aberrantly methylated in the above cells, we performed BS-PCR sequencing, and verified that the *dermo1* gene promoter was extensively methylated in five different cancer cell lines: HL60, RS4:11, MDM-BA231, H358 and H1299 (Fig. 4).

We designed MSP primer to discriminate between methylated and unmethylated alleles following bisulfite treatment for future sample use due to faster detection. The specificity of two pairs of MS-PCR primers were verified by sequencing the MSP products from HL60 and RS4:11 (Fig. 5).

We also examined the promoter methylation status of *dermo1* in seven human tumor cell lines (HL60, Molm14, MV4-11, RS4:11, MDM-BA231, H358 and H1299) with MSP analysis. We observed that six cell lines (85.7%) had partially methylation, and one cell line (Molm14) had total methylation (14.3%) (Fig. 6A). We next examined these samples to determine whether *dermo1* promoter methylation regulates its expression. Reverse transcription PCR shows that Molm14 cell line did not express *dermo1*, MV4-11 and RS4:11 cells expressed low basal level of *dermo1*. However, four cell lines (HL60, MDM-BA231, H358 and H1299) expressed some *dermo1* mRNA (Fig. 6B).

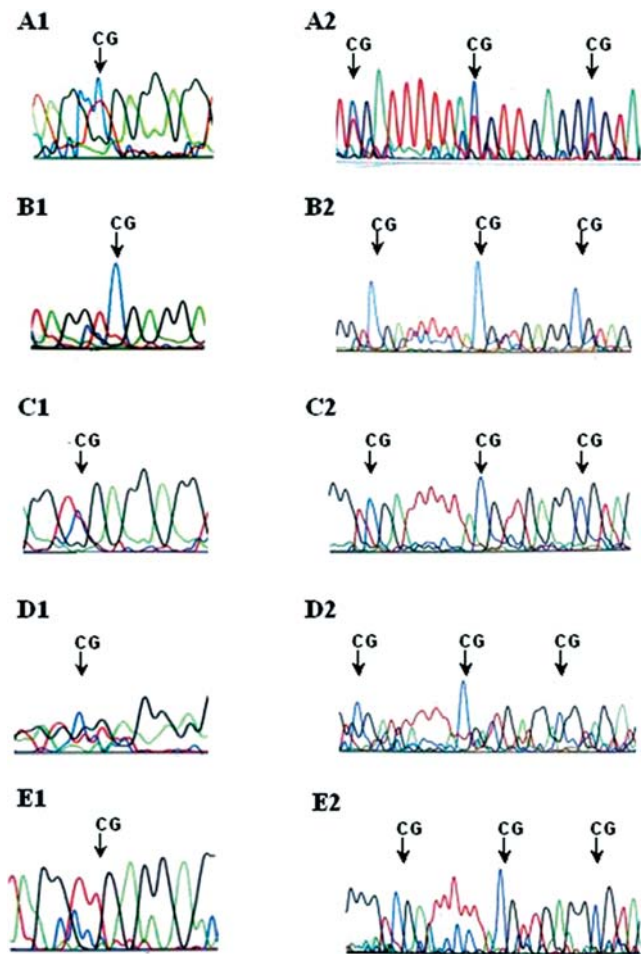


Figure 4. BS-PCR sequencing of cancer cell lines. *Dermo1* gene promoter methylation status for the same forward (1) and reverse (2) sites, in which CG dinucleotide residues are shown. (A) HL60, (B) RS4:11, (C) MDM-BA231, (D) H358, (E) H1299.

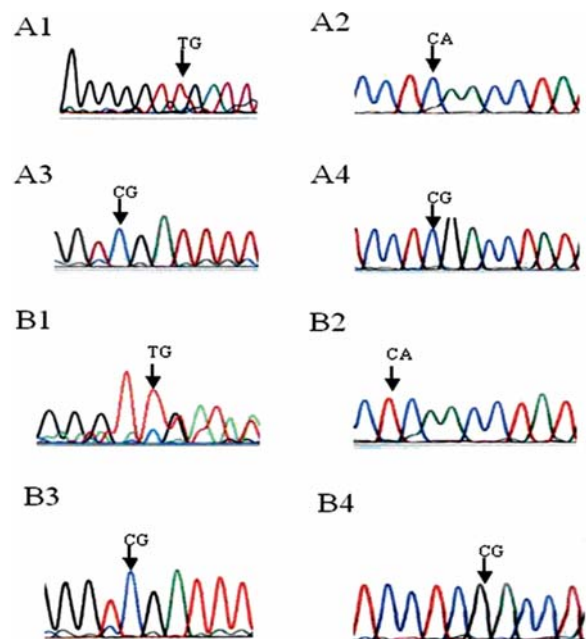


Figure 5. MSP product sequencing to test the two pairs of *dermo1* MSP primers by the unmethylated forward (1) and reverse (2) primer, the methylated forward (3) and reverse (4) primer in two different cancer cell lines. (A) HL60, (B) RS4:11.

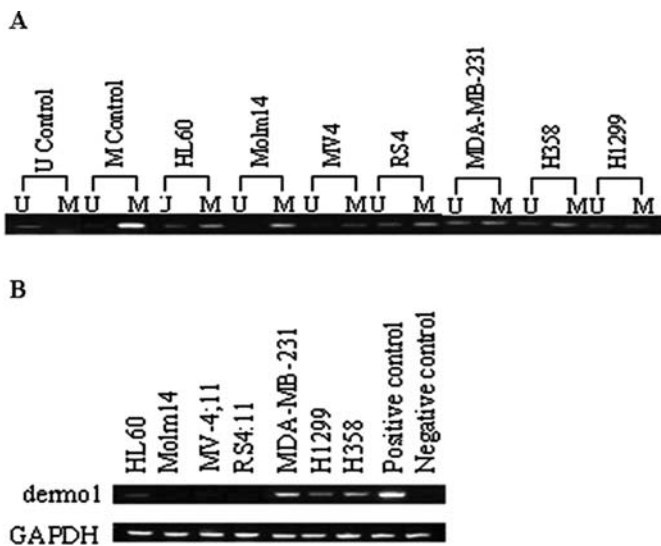


Figure 6. *Dermo1* expression and methylation in different cancer cell lines. (A) Methylation status of the *dermo1* gene, determined by methylation-specific PCR (MSP), is reported for each sample for direct comparison as methylated (M) or unmethylated (U). Peripheral WBC (WBC) DNA served as control for unmethylated (U), normal semen DNA served as control for methylated (M). (B) *Dermo1* expression by reverse transcription polymerase chain reaction in seven different tumor cell lines, including four leukemia cell lines, HL60, MOLM14, MV4-11, RS4-11; one breast cancer cell line: MDM-BA231; two lung cancer cell lines: H358, H1299. Water and *dermo1* expression plasmid was used as negative and positive controls, respectively. GAPDH was used as the internal control.

Discussion

The *dermo1* gene is highly conserved in several vertebrates such as humans, mouse, rats, and chicks (8,18). It has been shown that *dermo1* functions as a potent transcriptional repressor for MyoD and as an anti-apoptotic agent for Myc- and p53-dependent cell death (10,18), and its methylation is associated with mutated Ig VH genes in CLL cases (16). *dermo1* can also cooperate with activated oncoproteins such as Ras in promoting EMT, which indicates that reactivation of Twist proteins *in vivo* promotes malignant conversion concomitantly with metastatic dissemination (19,20).

However, the precise biological roles of *dermo1* during tumorigenesis and its molecular mechanisms of action remain largely unknown. Considering its potential role in development and tumorigenesis, unraveling *dermo1* methylation status holds important implications for cancer diagnosis, identification of new targets for therapy, and development of new strategies for clinical management (21).

The CpG promoter area we investigated is much larger than that used by the Plass group (16), thus providing more information. Our finding shows *dermo1* methylation regulation extensively existed in different cancer cells except for CLL. Our data suggest that the methylation of *dermo1* promoter might suppress *dermo1* gene expression, therefore demethylation therapy might be a promising approach to treat cancer. The MS-PCR analysis of *dermo1* also provides an easy way for cancer diagnosis.

Four types of cancer cells in which promoter methylation was detected by MSP partially expressed *dermo1* mRNA, indicating that although promoter methylation could be one of

the mechanisms regulating expression in cancer cells, there are additional regulatory pathways involved in *dermo1* silencing. While more research needs to be done to fully understand the implication of methylation *dermo1* in tumorigenesis, the existence of cancer cells with aberrant *dermo1* methylation is potentially promising for diagnosis.

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