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Abstract: Parakeratosis refers to incomplete maturation of epidermal keratinocytes, resulting in abnormal retention of nuclei in the stratum corneum. It occurs in many diseases of the skin, particularly in psoriasis. Down-regulation of Inhibitor of Differentiation 4 (ID4) mRNA has been demonstrated in psoriatic skin but the specificity and mechanism for this finding is unknown. In this study, we addressed specificity by immunohistochemical staining for ID4 protein in skin disorders showing parakeratosis, including: psoriasis (n=9), chronic eczema (n=6) and squamous cell carcinoma (n=7). In these conditions, parakeratotic keratinocytes in the upper layers of the skin lacked ID4 protein expression while keratinocytes in the lower layers were densely stained, in contrast to diffuse expression in normal skin. Since promoter hypermethylation of ID4 has been described in several cancers, we determined the methylation pattern the ID4 promoter in psoriasis, and compared this to squamous cell carcinoma. We found a novel methylation pattern of the ID4 promoter in both conditions. ID4 promoter methylation was significantly increased in psoriasis (34.8%) and squamous cell carcinoma (21.8%), compared to normal skin (0%). Moreover, cells in the upper and lower parts of psoriatic epidermis were, respectively, hypermethylated and nonmethylated, at the ID4 promoter. Comparable studies in several cell lines confirmed that hypermethylation of the promoter was associated with loss of ID4 mRNA and protein expression. Our study demonstrates a previously unreported link between gene-specific promoter hypermethylation and abnormal cellular differentiation in several skin diseases. This mechanism might provide clues for novel therapies for skin disorders characterized by parakeratosis.

Parakeratosis in skin is associated with loss of Inhibitor of Differentiation 4 (ID4) via promoter methylation

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Abstract

Parakeratosis refers to incomplete maturation of epidermal keratinocytes, resulting in abnormal retention of nuclei in the stratum corneum. It occurs in many diseases of the skin, particularly in psoriasis. Down-regulation of Inhibitor of Differentiation 4 (*ID4*) mRNA has been demonstrated in psoriatic skin but the specificity and mechanism for this finding is unknown. In this study, we addressed specificity by immunohistochemical staining for ID4 protein in skin disorders showing parakeratosis, including: psoriasis (n=9), chronic eczema (n=6) and squamous cell carcinoma (n=7). In these conditions, parakeratotic keratinocytes in the upper layers of the skin lacked ID4 protein expression while keratinocytes in the lower layers were densely stained, in contrast to diffuse expression in normal skin. Since promoter hypermethylation of *ID4* has been described in several cancers, we determined the methylation pattern the *ID4* promoter in psoriasis, and compared this to squamous cell carcinoma. We found a novel methylation pattern of the *ID4* promoter in both conditions. *ID4* promoter methylation was significantly increased in psoriasis (34.8%) and squamous cell carcinoma (21.8%), compared to normal skin (0%). Moreover, cells in the upper and lower parts of psoriatic epidermis were, respectively, hypermethylated and nonmethylated, at the *ID4* promoter. Comparable studies in several cell lines confirmed that hypermethylation of the promoter was associated with loss of *ID4* mRNA and protein expression. Our study demonstrates a previously unreported link between gene-specific promoter hypermethylation and abnormal cellular differentiation in several skin diseases. This mechanism might provide clues for novel therapies for skin disorders characterized by parakeratosis.

Keywords: ID4, DNA methylation, psoriasis, parakeratosis, immunohistochemistry

Abbreviation: ID4, Inhibitor of Differentiation 4; bHLH, basic helix-loop-helix; SCC, squamous cell carcinoma; DNMT, DNA methyltransferase; Cdc42, Cell division control protein 42; MLK3, Mixed Lineage Kinase3; PAK, p21-activated kinase.

Introduction

The skin is a highly regulated organ maintaining tight control over proliferation, differentiation, and apoptosis. During terminal differentiation in which granular cells turn to cornified cells, keratinocytes lose nuclei and keratohyaline granules, and keratin filaments become compacted. Parakeratosis is an abnormal occurrence in which keratinization of epithelial cells is incomplete, with abnormal retention of keratinocyte nuclei in the stratum corneum. Parakeratosis is found in many skin diseases and is visible clinically as scale. One of the most striking examples is psoriasis, characterized by confluent parakeratosis infiltrated by neutrophils [1]. The precise mechanism of the induction and maintenance of parakeratosis remains unclear. Some insight has come from recent cDNA microarray studies that have identified down-regulation of Inhibitor of Differentiation 4 (*ID4*) mRNA in psoriatic skin [2,3].

ID4 is a member of the basic helix-loop-helix (bHLH) family and is expressed in thyroid, brain, fetal tissue, and some nervous system tumor cell lines [4]. The protein behaves as a dominant negative inhibitor of gene transcription [5]. ID4 is an important factor for cell proliferation and differentiation [6,7], apoptosis, [8] and regulation of oligodendrocyte development and differentiation [9]. Down-regulation of ID4 protein can induce tumor cell dedifferentiation in colorectal cancer. *ID4* may act as a tumor suppressor gene in lymphoma [10] and carcinomas of the stomach [11], breast [12], and colorectum [13]. Conversely, *ID4* can behave as an oncogene in prostate [14] and bladder cancers [15]. Transcriptional silencing of *ID4* by promoter hypermethylation has been reported in many cancers, including cholangiocarcinoma, breast cancer, lymphoma and gastric adenocarcinoma [11,12,16,17].

The particular mechanism by which *ID4* is down-regulated in psoriasis is unknown, as well as the specificity of such a finding. We therefore compared expression of ID4 at the protein level in psoriasis to other skin disorders that show parakeratosis. We also determined the methylation status of the *ID4* promoter in psoriasis and compared this to cutaneous squamous cell carcinoma (SCC) that was characterized by parakeratosis. Lastly, we studied a series of cell lines to correlate promoter methylation, mRNA levels and protein expression for ID4.

Materials and Methods

Patients and controls

Biopsies of 28 subjects seen at King Chulalongkorn Memorial Hospital were used for this study. The biopsies included 9 cases of chronic plaque psoriasis, 7 of SCC, 6 of chronic eczema and 6 of normal skin. All patients were free from systemic skin therapies for at least 4 weeks or topical skin therapies for at least 2 weeks prior to sample collection. Normal skin was obtained from elective plastic surgery cases. No patient had any personal or family history of autoimmune disease. The study was approved by the ethics committee of King Chulalongkorn University and all participants provided informed consent.

Cell lines

Six epithelial cell lines: Hela, HEp2, SiHA, RKO, SW480, and HaCaT and 5 hematopoietic cell lines: Daudi, Jurkat, Molt4, HL-60 and K562 were used in this study. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI1640 (Gibco BRL, Life Technologies, Pairly, UK) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) and antibiotics (50

U/ml penicillin and 50 μg/ml streptomycin). All cells were grown at 37°C in 5% CO_2 under humidified conditions

Immunohistochemistry

Immunohistochemistry was performed on 4-μm sections of formalin-fixed skin specimen from 9 patients with chronic plaque psoriasis, 7 patients with SCC, 6 patients with chronic eczema and 6 healthy volunteers. The tissue sections were mounted on positively charged glass slides and baked overnight at 60°C. Heat-induced epitope retrieval was carried out using Ventana Cell Conditioning 1 solution (Ventana Medical Systems, Tucson, AZ, USA) and microwaving slides for 15 minutes on the high setting in a 900-watt microwave oven. Sections were then blocked for endogenous peroxidase and biotin, followed by incubation with an antibody against ID4 protein (Santa Cruz Biotechnology, CA, USA, at dilution 1:25). Staining was performed using the Ventana Benchmark LT automated immunostainer. Normal colon served as a positive control, and omission of the primary antibody as negative control.

For cell lines, cultured cells were trypsinized to form a suspension and pelleted by centrifugation. The pellet was then fixed in formalin and processed by standard tissue processing procedures. Sections were then immunostained as above.

Laser capture microdissection

The epidermis from paraffin-embedded tissue sections of 9 psoriatic patients, 7 SCC patients and 6 normal subjects was micro-dissected using The PALM MicroLaser Microdissection System (P.A.L.M. MicroLaser Technologies AG, Burnried, Germany). Samples were first stained with hematoxylin and eosin for

accurate identification of the epidermis. The laser-dissected epidermis from each sample was removed from the slide by laser cutter pulse and collected in a microfuge tube. For three samples of psoriasis, laser capture microdissection was used to separate the upper and the lower regions in psoriatic epidermis using immunohistochemistry for ID4 as a guide (see Results).

DNA extraction and bisulfite modification

Dissected epidermis was digested with 50 µl of proteinase K containing lysis buffer at 50°C for 5 hours, followed by heated deactivation of proteinase K at 95°C for 10 minutes. After extraction, all DNA samples were treated with sodium bisulfite using the EZ DNA methylation Kit™ (Zymo Research, Orange, CA, USA). Briefly, 500 ng of extracted DNA was modified using sodium bisulfite to convert nonmethylated cytosine to uracil but not methylated DNA.

DNA methylation status detection by methylation-specific PCR (MSP) and bisulfite sequencing

The *ID4* promoter region (-615 to +139) was analyzed for methylation using a MSP method and verified by bisulfite cloning and sequencing as previously described [18]. Bisulfite oligonucleotide sequences of *ID4* were derived from Genbank NT 007592. Briefly, this method distinguishes nonmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA. Primers were designed to distinguish methylated from nonmethylated DNA in bisulfite-modified DNA. For MSP, 1µL of modified DNA was amplified using MSP primers that specifically recognized the nonmethylated DNA (U forward 5'-GGT AGT TG GAT TTT TTG TTT TTT AGT ATT-3' U reverse 5'-AAC TAT ATT TAT

AAA ACC ATA CAC CCC A-3', product size 157 bp) or methylated DNA (M forward 5'-TAG TCG GAT TTT TCG TTT TTT AGT ATC-3' M reverse 5'-CTA TAT TTA TAA AAC CGT ACG CCC CG-3', product size 161 bp) [19].

DNA methylation level

Methylation level was assessed by first measuring the intensities of methylated and nonmethylated bands. Band intensities were determined using Quantity One Quantitation Software version 4.2 (BioRad). Methylation level was then calculated as the intensity of methylated band/(combined intensities of the methylated and nonmethylated bands) x100.

Determination of mRNA expression level by Real Time RT-PCR

Total RNA was extracted from cell lines and from blood samples using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two μg of total RNA samples were reversely transcribed with oligo dT primer. For Real Time PCR, 1 μl of cDNA was amplified using *ID4* primers and *GAPDH* primers as previously described. For *ID4* mRNA detection, touchdown PCR was used and cycle threshold (CT) values were normalized in reference to the housekeeping gene *GAPDH*, as previously reported [19,20].

Statistical Analysis

Methylation status was compared between groups by Mann-Whitney U test using SPSS software for Windows 10.0 (SPSS Inc., Chicago, IL, USA). A *P* value of ≤ 0.05 was considered to be significant.

Results

Loss of ID4 protein expression is associated with parakeratotic keratinocytes in psoriasis, SCC and eczema

By immunohistochemistry, normal epidermis was diffusely positive for ID4 protein, whereas only the basal aspect was positive in psoriatic skin, chronic eczema and SCC (Fig 1). There was a marked decrease or absence in ID4 staining in all keratinocytes in the upper part of the epidermis that showed parakeratosis. This was specific to parakeratotic keratinocytes since infiltrating neutrophils (e.g. Munro's abscess) retained ID4 expression.

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Hypermethylation of *ID4* promoter associated with psoriasis and SCC

By MSP, six out of nine (66.7%) microdissected psoriatic epidermis samples showed both methylated and nonmethylated ID4 amplicons, and three out of seven (42.8%) cases of SCC showed both methylated and nonmethylated ID4 amplicons. In contrast, all six microdissected normal skin samples showed only the nonmethylated *ID4* promoter (Fig 2A). The average methylation level in psoriatic epidermis (34.8%) and SCC epidermis (21.8%) was significantly increased compared to normal skin (0%), ($p < 0.01$) and ($p = 0.05$), respectively (Fig 2B). To verify the MSP results, bisulfite cloning and sequencing of the *ID4* promoter region, which represented 26 CpG sites, was performed using DNA from microdissected epidermis from one healthy control with nonmethylated *ID4* and two psoriasis patients with semihypermethylated *ID4*. Nearly all clones from healthy control (9/10 clones) showed nonmethylated CpG. In contrast, almost half of all clones from both psoriatic patients show two distinct epigenetically modified populations, namely heavily

methylated and almost completely nonmethylated CpGs (Fig 2C). The relationship between *ID4* methylation status and ID4 protein expression in various skin lesions is summarized in Table 1. In general, methylation tended to be seen in biopsies with a greater degree of parakeratosis but the latter was not quantitated precisely. Methylation was never seen in the absence of parakeratosis.

To explore more precisely the role of DNA methylation controlling ID4 protein expression in psoriatic epidermis, laser capture microdissection was used to separate the upper (ID4-non-expressing) and the lower (ID4-expressing) regions in three cases of psoriatic epidermis using immunohistochemistry as a guide (Fig 3A, B and C). From all three psoriatic patients, the upper layers of the epidermis showed only methylated bands while the lower layers showed only nonmethylated bands (Fig 3D). This finding supports the concept that reduced expression of ID4 protein expression is related to methylation of the *ID4* promoter.

Inverse correlation between DNA methylation status and mRNA expression level in cell lines

Whether promoter methylation was the basis for decreased expression of ID4 protein in psoriatic skin and SCC was explored further using cell lines. *ID4* promoter methylation was determined in 11 cell lines by MSP (Fig 4A). Most of the epithelial cell lines including Hela, HEp2, SiHA and RKO showed only a band for methylated DNA. SW480 showed bands for both methylated and nonmethylated DNA. Conversely, HaCaT showed only a band for nonmethylated DNA. In hematopoietic cell lines, Daudi, Jurkat, Molt4, HL-60 showed only a band for methylated DNA, whereas, K562 showed bands for both methylated and nonmethylated DNA. The results of bisulfite cloning and sequencing of the *ID4* promoter of HEp2, SW480 and

HaCaT are shown in Figure 4B. Most CpG dinucleotides of HEp2 were methylated whereas most of HaCaT were nonmethylated. However, variable numbers of methylated CpGs were discovered in SW480 alleles. This partial methylation may explain how SW480 MSP yielded both methylated and nonmethylated amplicons.

Real-time RT-PCR revealed an inverse correlation between *ID4* mRNA expression and DNA methylation status in all cell lines (Table 2). Hela, HEp2, SiHA, RKO, Daudi, Jurkat, Molt4, HL-60, K562 lacked *ID4* mRNA expression. In contrast, only cell lines with nonmethylated promoters expressed *ID4,* with the highest mRNA level seen in HaCaT (19.4).

HaCaT, an nonmethylated *ID4* promoter cell line, and HEp2, a methylated *ID4* promoter cell line, were investigated for ID4 protein expression by immunohistochemistry. HaCaT showed diffuse expression of ID4 protein, whereas HEp2 was negative (Fig 5). This finding confirms that reduced expression of ID4 protein is related to methylation of the *ID4* promoter.

Discussion

Parakeratosis, or retention of keratinocyte nuclei in stratum corneum, is a common occurrence in skin disorders manifesting clinically as scale, for which patients often seek medical intervention. Although it is one of the key features in psoriasis, parakeratosis can be seen in other benign skin diseases including chronic eczema and even in cutaneous malignancy, such as squamous cell carcinoma. Treatment of parakeratosis is essentially symptomatic rather than based on an understanding of pathogenesis, since this is poorly understood.

Down-regulation of *ID4* mRNA in psoriasis, based on the large-scale gene expression studies, [2,3] prompted us to evaluate ID4 protein expression in psoriasis,

and we found that ID4 protein expression was absent in the layers of parakeratosis whereas normal skin showed expression in all layer of the epidermis. In order to establish whether this was specific to psoriasis, we studied chronic eczema, another skin disorder in which parakeratosis is a feature, and also found loss of ID4 protein in the parakeratotic layer. This finding extended to neoplastic diseases as well, since SCC showed similar results. Thus, loss of ID4 protein expression would appear to be a phenomenon of parakeratosis rather than psoriasis specifically. We postulate that *ID4* down-regulation was linked to psoriasis in other studies because of the marked degree of parakeratosis seen in this condition, compared to other skin diseases.

We then investigated the mechanism of ID4 protein loss in the setting of psoriasis and found that hypermethylation of *ID4* promoter is responsible for silencing the *ID4* gene in psoriasis. Aberrant CpG methylation in the 5' promoter regions of tumor suppressor genes has been demonstrated in various diseases [21]. We found that aberrant methylation of *ID4* was not specific to psoriasis, as similar results were found in SCC and in several cell lines. Thus, our hypothesis is that *ID4* down-regulation is associated with parakeratosis rather than with any specific skin disease. Decreased expression of ID4 protein in parakeratotic keratinocytes is consistent with many previous studies in a variety of malignancies that show down-regulation of ID4 protein promotes dedifferentiation and proliferation [11,12,16,22]. Since keratinocyte differentiation is a multi-step molecular event, it is postulated that ID4 methylation inhibits cellular differentiation of the keratinocyte, and leads to parakeratosis.

In support of this hypothesis, and similar to a prior study [23], we verified *ID4* promoter methylation as an epigenetic mechanism that controls *ID4* mRNA expression in various cell lines, including HaCaT, Hela, SiHA and Jurkat. Cell lines

with methylated promoters showed low or absent levels of mRNA and no ID4 protein expression, whereas cell lines with nonmethylated promoters showed mRNA and ID4 protein expression. Although we could not do comparable mRNA studies in skin biopsies, the correlation between *ID4* promoter methylation and mRNA levels in cell lines, provides support for the concept that promoter methylation leads to loss of ID4 protein expression in regions of parakeratosis in psoriasis. Given that similar results were found in eczema and the cutaneous malignancy SCC, we extrapolate that methylation of *ID4* might underlie parakeratosis in multiple skin diseases.

The underlying defects that induce *ID4* promoter methylation in psoriasis and SCC remain unknown. Cell division control protein 42 (Cdc42) has been reported to induce *ID4* promoter methylation in colorectal adenocarcinoma by modulation of DNMTs levels [24], and has been shown to influence expression of DNMTs in several cancers [21]. Cdc42 also activates the Mixed Lineage Kinase3 (MLK3) and p21-activated kinase (PAK) [25] pathways that are involved in the JNK pathway, thereby increasing phosphorylated p38 [26]. Elevated phosphorylated p38 in the skin is considered an important pathogenetic step in psoriasis [27,28]. Hence, it is tempting to hypothesize that there might be a relationship between Cdc42 and *ID4* promoter methylation.

In conclusion, this study has documented that DNA methylation may be a key mechanism that downregulates *ID4*, and this epigenetic change may be closely related to cellular differentiation. Specifically, *ID4* promoter hypermethylation correlates with parakeratosis of the epidermis in psoriasis, chronic eczema and SCC, and possibly parakeratosis in general. Thus, ID4 becomes a candidate for understanding the molecular pathogenesis of parakeratosis, although the exact function of ID4 in keratinocyte differentiation still needs further elucidation. Determining the molecular

events of this condition might also provide clues for novel therapies for skin conditions characterized by parakeratosis.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Immunohistochemical detection of ID4 in skin from psoriasis, SCC, chronic eczema and normal subjects using a polyclonal antibody to ID4 with (A-C) normal skin, (D-F) psoriasis, (G-I) chronic eczema and (J-L) SCC. A, D, G and J show the light microscopic appearances (hematoxylin and eosin, x100). Normal skin displays nuclear staining of ID4 in all layers of keratinocytes (Bx100 and Cx200). Keratinocytes in psoriasis, chronic eczema and SCC display staining of ID4 only in the lower part of the epidermis. Parakeratotic keratinocytes in psoriasis, chronic eczema and SCC lack staining for ID4. In contrast, a Munro's microabscess (a collection of neutrophils in the stratum corneum of a psoriatic lesion) displays positive staining for ID4 (E, H and K, x100 and F, I and L x200)

Figure 2. *ID4* promoter methylation in normal skin, psoriasis and SCC. (A) *ID4* promoter methylation status of microdissected epidermis from 9 psoriatic patients, 7 SCC patients and 6 normal subjects was analyzed using the MSP technique. The 161 and 157 bp PCR products reflecting nonmethylation and methylation, are indicated by *U* and *M*, respectively. Sample sources of genomic DNA are listed above each lane. HEp2, HaCaT, SW480 were used as positive controls for methylation, nonmethylation and semi-methylation, respectively. The asterisk indicates the standard size DNA marker. (B) The percentage of ID4 methylation was compared between normal skin, SCC and psoriasis. *T* indicates standard deviation (SD). The white star indicates significant differences of $p < 0.01$ comparing normal skin to psoriasis and $p = 0.05$ comparing normal epidermis to SCC. (C) Bisulfite cloning and sequencing using primers covering all 26 CpG sites in MSP region was performed to

verify the MSP results. Microdissected epidermis from two psoriatic patients (P6 and P7) and one normal subject (N1) that represented methylation, semi-methylation and nonmethylation, respectively, were analyzed to identify methylation patterns. The upper most *horizontal bar* identifies the relevant nucleotide sequence in GenBank (Accession NT007592). The *numbers* and *arrows* indicate the start and end nucleotide in relation to the transcriptional start codon, ATG (nucleotide 1). The *numbers* of the line below indicate CpG nucleotides. *Each circle* indicates the methylation status of each selected clone. *Black* and *white circles* are methylated and nonmethylated CpG dinucleotides, respectively.

Figure 3. *ID4* promoter methylation analysis of psoriatic epidermis comparing microdissected upper and lower epidermis. Hematoxylin and eosin staining of paraffin-embedded psoriatic skin (A). Laser capture microdissection was use to separate keratinocytes from the upper (ID4-expressing) (B) and lower parts of the epidermis (ID4-non-expressing) (C). Munro's microabscesses were removed from the slide by cutter pulse before capturing upper keratinocytes. Specimens from three psoriatic patients (P6, P7 and P8) were analyzed for ID4 promoter methylation status by the MSP technique. The 161 and 157 bp PCR products, reflecting nonmethylation and methylation, are indicated by *U* and *M,* respectively. Sample sources of genomic DNA are listed above each lane. HEp2, HaCaT, and SW480 were used as positive controls for methylation, nonmethylation and semi-methylation, respectively (not shown). The asterisk indicates standard size DNA markers.

Figure 4. *ID4* promoter methylation in 11 cell lines. (A) *ID4* promoter methylation of epithelial and hematopoietic cell lines as detected by MSP. The 161 and 157 bp

PCR products represent nonmethylation and methylation, and are indicated by *U* and *M*, respectively. Cell sources of genomic DNA are listed above each lane. The asterisk indicates standard size DNA markers. (B) Methylation status of CpG nucleotides in the promoter of HEp2, SW 480 and HaCaT was confirmed by bisulfite DNA sequencing. This Figure follows the same format as Figure 2C. *Black* and *white circles* are methylated and nonmethylated CpG dinucleotides, respectively.

Figure 5.

Immunohistochemical detection of ID4 in cell lines using a polyclonal antibody to ID4. (A-B) HaCaT cell line and (C-D) HEp2 cell line. A and C show the light microscopic appearance of the cell lines (hematoxylin and eosin, x600). HaCaT displays dense nuclear staining of ID4 (Bx600) whereas HEp2 lacks staining for ID4 (Dx600).

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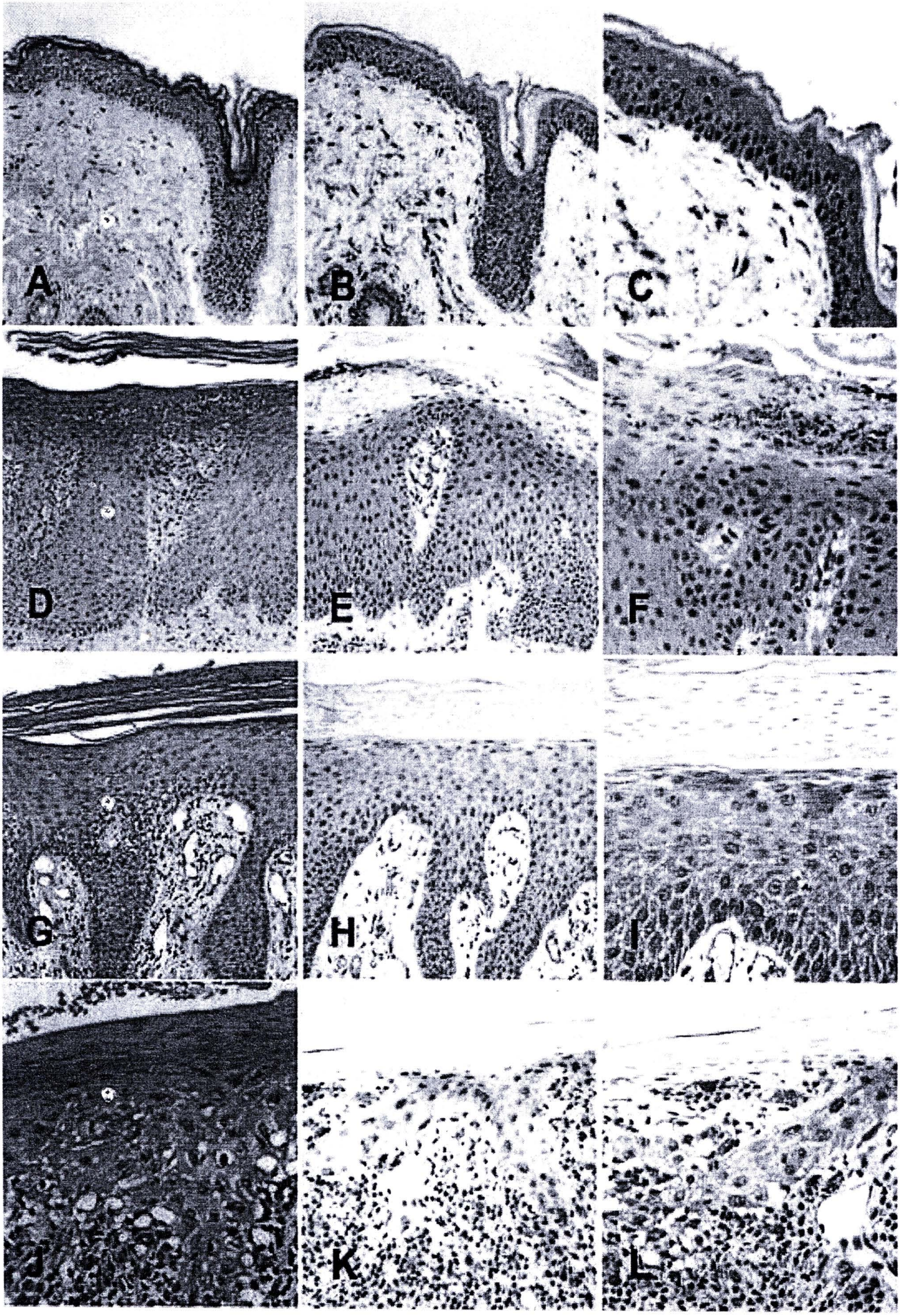

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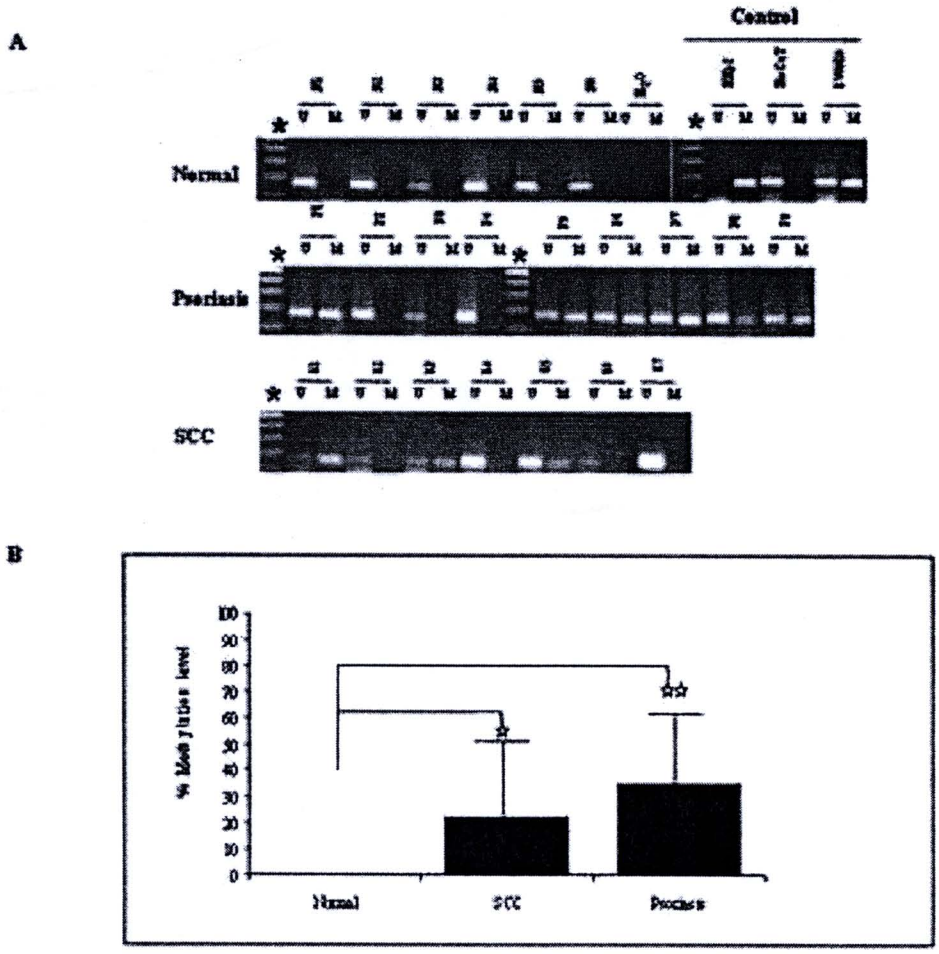

Table 1. Correlation of ID4 promoter methylation and protein expression in skin

Disease	No. of cases	ID4 promoter methylation status	Immunohistochemical staining of ID4	
			Upper epidermis	Lower epidermis
Psoriasis (n=9)	6	Nonmethylated /methylated	-	+
	3	Nonmethylated	-	+
SCC (n=7)	3	Nonmethylated /methylated	-	+
	4	Nonmethylated	-	+
Normal (n=6)	6	Nonmethylated	+	+

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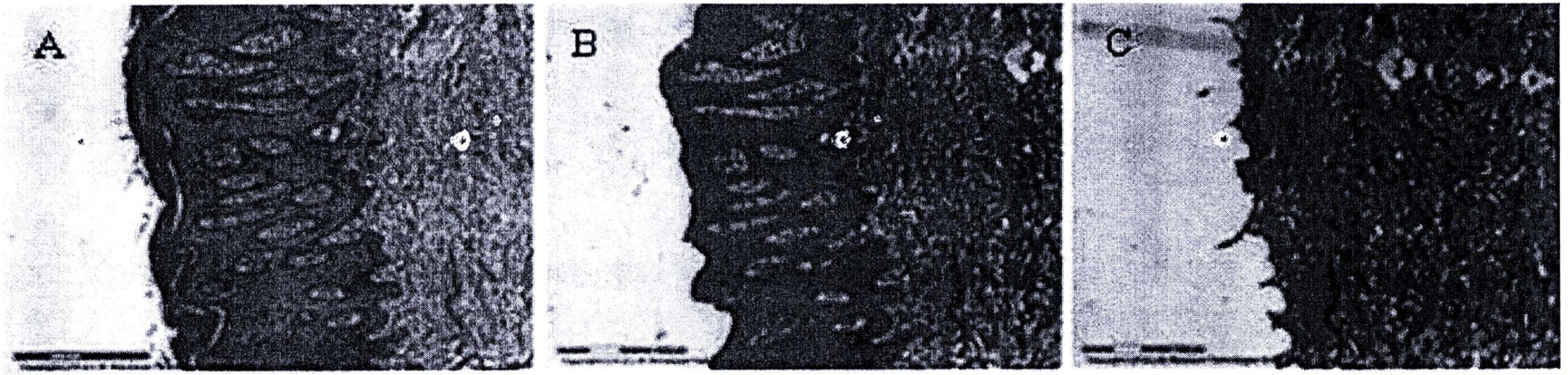

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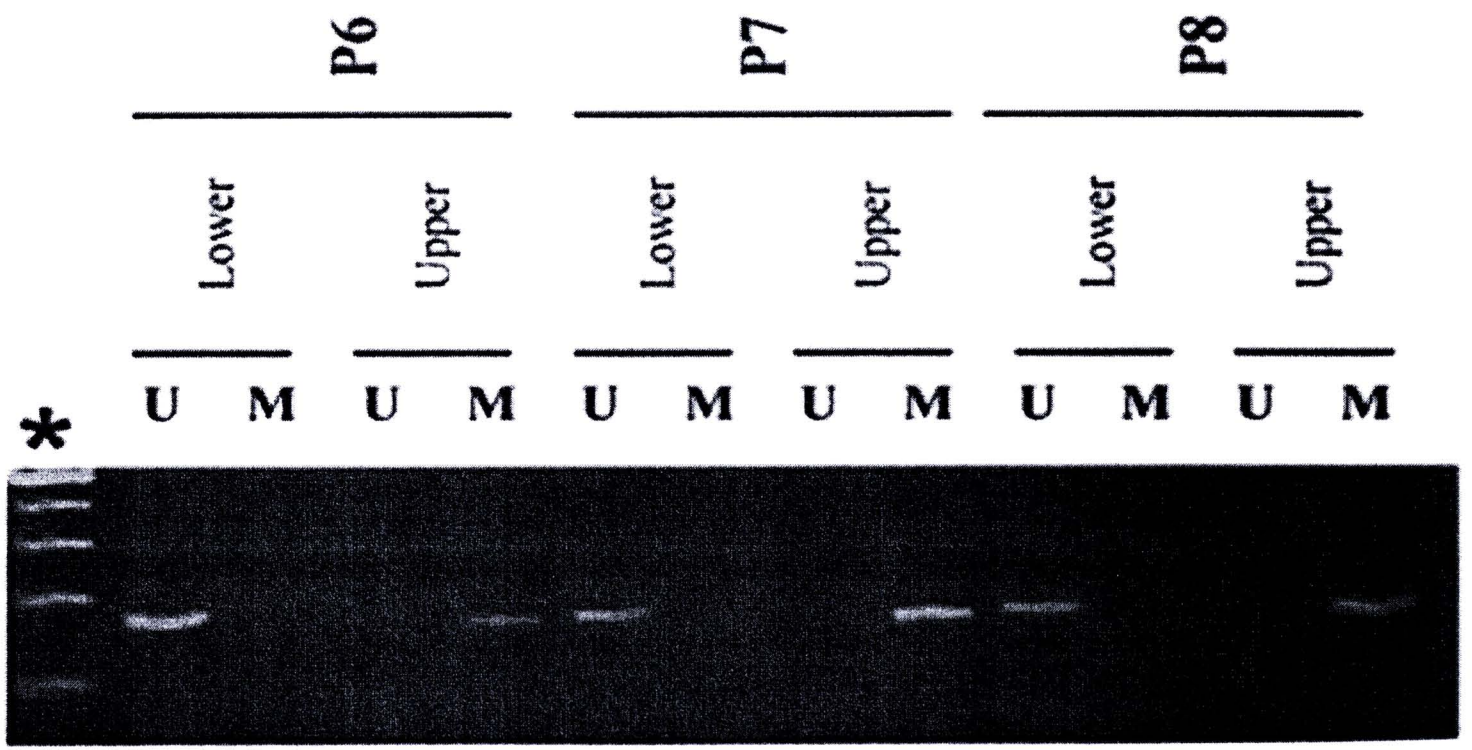

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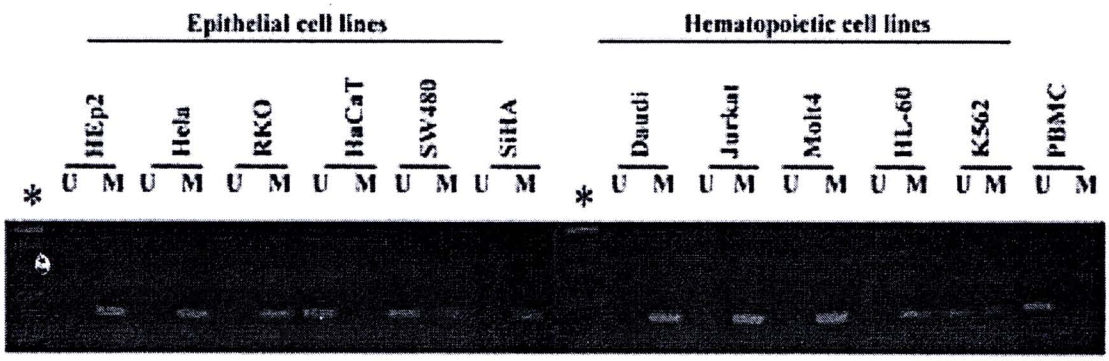

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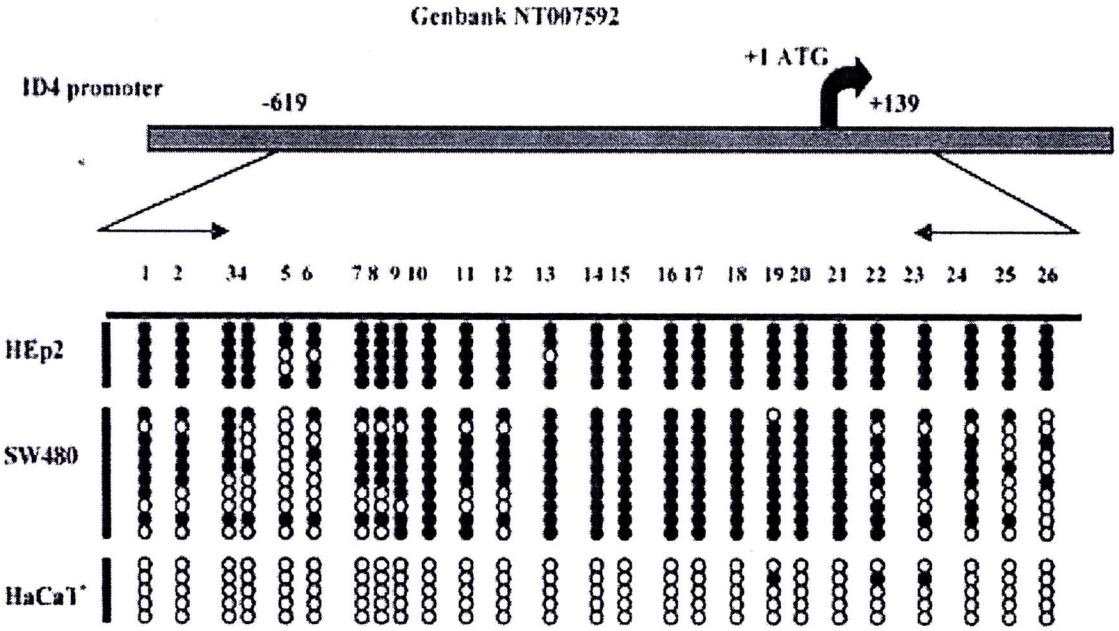

Table 2 Correlation of ID4 promoter methylation status and mRNA level in cell lines

Epithelial cell lines	ID4 promoter methylation status	*ID4* mRNA level
HEp2	Methylated	0
Hela	Methylated	0
RKO	Methylated	0
HaCaT	Nonmethylated	19.4
SW480	Methylated/Nonmethylated	0.14
SiHA	Methylated	0
Hematopoietic cell lines		
Daudi	Methylated	0
Jurkat	Methylated	0
Molt4	Methylated	0
HL-60	Methylated	0
K562	Methylated/Nonmethylated	0

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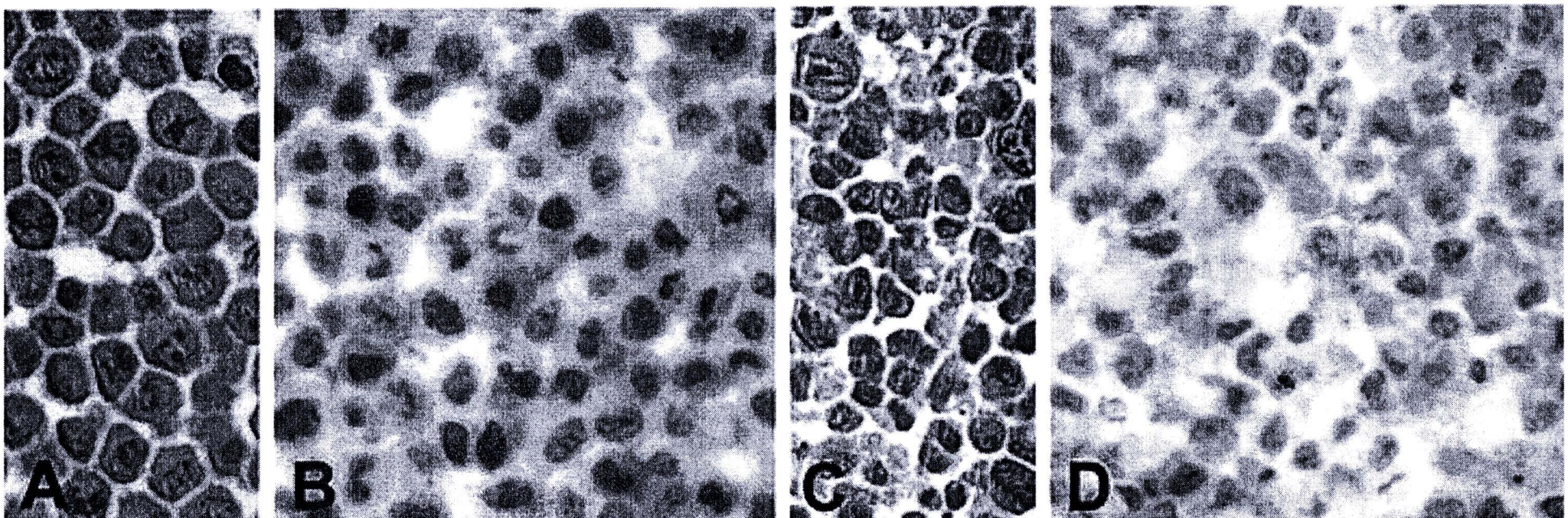

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Effect of methotrexate on serum levels of IL-22 in patients with psoriasis

A short title: Effect of methotrexate on IL-22 in psoriasis

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ABSTRACT

Background: Interleukin-22 (IL-22) is the effector molecule of T-helper subset 22 (Th-22) lineage that promotes keratinocyte proliferation and dermal inflammation in psoriasis. Methotrexate is widely used as a first-line treatment in moderate to severe psoriasis. Methotrexate inhibits inflammatory and cytokinetic processes via various mechanisms, but the relevance of these to psoriasis is limited and whether methotrexate is specifically able to down-regulate Th22 cytokines is unknown.

Objective: To determine if methotrexate reduces IL-22 in cases of psoriasis.

Methods: Nineteen patients with moderate to severe psoriasis were given methotrexate 15 mg per week for up to 12 weeks. Serum levels of IL-22 were determined by enzyme-linked immunosorbent assay (ELISA) before and after treatment.

Results: Eleven of 19 patients (57.8%) achieved a 75% PASI score reduction. IL-22 levels were significantly higher in untreated psoriasis patients (56.63±60.73 pg/ml) than in controls (12.58±12.59 pg/ml). Methotrexate significantly reduced serum levels of IL-22 in psoriasis patients to 5.91± 7.97 pg/ml ($p<0.001$). Moreover, there was a significant positive correlation between IL-22 levels and PASI ($r=0.63$, $p=0.004$).

Conclusion: Methotrexate significantly reduces serum IL-22 levels in cases of psoriasis. This is a novel mechanism by which methotrexate acts in the treatment of this disease.

Keywords: Interleukin-22, methotrexate, psoriasis

INTRODUCTION

Psoriasis is a common autoimmune skin disease that occurs worldwide. Dysregulation of the immune system results in abnormal proliferation and differentiation of keratinocytes. Pro-inflammatory, T helper-1 (Th1) cytokines and chemokines such as tumor necrotic factor-α (TNF-α) and interferon-γ (IFN-γ) are key cytokines in development of psoriatic skin lesions [1, 2]. More recently, interleukin-22 (IL-22) has been shown to play an important role in psoriasis [3, 4]. IL-22, a member of the interleukin-10 (IL-10) family, is a major cytokine involved in the distinctive epidermal alteration and production of antimicrobial peptides in psoriatic skin lesions [5, 6]. IL-22 is a downstream effector molecule produced by various cell types such as T helper-1 (Th1), T helper-17 (Th-17), natural killer cells, dendritic cells and the more recently identified distinctive T helper-22 (Th-22) subset [7].

Treatment of psoriasis has changed over the past decade to include many biologic agents. Some of these (e.g. Etanercept) have validated the role of IL-22 in psoriasis by rapid down-regulation of IL-22 production after treatment [8, 9]. Methotrexate (MTX) is still used as a cost-effective treatment for moderate to severe psoriasis [10]. MTX is known to inhibit DNA synthesis by competing as a substrate for dihydrofolate reductase, resulting in an anti-proliferative effect. However, this does not fully explain the effectiveness of MTX in psoriasis. In vitro studies have suggested that MTX has an anti-inflammatory effects through induction of immune cell apoptosis and inhibition of T cell activation [11, 12]. MTX has been shown to reduce expression of adhesion molecules such as cutaneous lymphocyte-associated antigen (CLA), intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-3 (ICAM-3), and E-selectin in psoriasis and rheumatoid arthritis [11, 13-17]. A number of studies in rheumatoid arthritis have illustrated that MTX can downregulate IL-4, IL-13, TNF-

and IFN- [13, 18]. However, such inhibitory effects have not be detected in psoriasis [19, 20]. Moreover, the effect of MTX on IL-22 has not been reported. We therefore studied a series of psoriasis patients and found that down regulation of serum IL-22 occurred after treatment with MTX and correlated with clinical improvement.

METHODS

Patients and samples

Nineteen patients with moderate to severe chronic plaque type psoriasis (14 males, 5 females, mean age 44.2 ± 9.7 years) at King Chulalongkorn Memorial Hospital were enrolled. The severity of psoriasis was classified according to the Psoriasis Area and Severity Index (PASI) (<10=mild, 10-15=moderate, >15=severe). Patients with psoriatic arthritis, other autoimmune diseases, cancer, liver or renal disease were excluded from the study. All patients were treated with MTX 15 mg once a week orally up to 12 weeks. At that start of the study, all patients were free from systemic therapy and phototherapy for at least four weeks, and topical anti-psoriatic therapies for at least 2 weeks. Patients received no other psoriasis treatments during the course of the study. The control subjects were 19 normal individuals recruited from blood donors at the National Blood Center, Thai Red Cross Society. The study was approved by the Ethics Committee of King Chulalongkorn University and all participants signed an informed consent.

Determination of IL-22 serum level

Serum samples were collected from nineteen patients and nineteen controls at baseline and when PASI score was improved more than 75%. The level of serum IL-22 was quantified using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis,

MN, USA) according to manufacturer's instructions. All samples and standards were measured in duplicate.

Statistical analysis

IL-22 serum level was compared between patients and normal subjects and, for psoriasis patients, before and after treatment using the nonparametric test Wilcoxon signed rank test. Correlation between PASI score and serum IL-22 was performed by Spearman's correlation. Testing for associations between IL-22 and other factors (disease severity, clinical response and psoriasis aggravating factors) was performed using the Mann Whitney test and Krauskal Wallis test. A p value of <0.05 was considered to be significant.

RESULTS

Serum levels of IL-22 were significantly reduced after treatment with MTX

IL-22 serum levels were detectable in 17 patients (89.47%) and 13 normal subjects (68.42%). IL-22 serum level of psoriasis patients was significantly higher than in normal subjects (56.63± 60.73 pg/ml vs. 12.58±12.59 pg/ml, $p<0.001$) (Fig 1A). Moreover, at baseline, serum levels of IL-22 were significantly higher in patients with severe psoriasis (PASI>15) compared to moderate psoriasis (PASI=10-15) (92.81±64.47pg/ml vs. 16.43±12.88 pg/ml, $p=0.001$) (Fig 1B).

After treatment with MTX, 11 of 19 patients (57.8%) achieved 75% PASI score reduction. There was a significant reduction in the mean PASI score of the 19 psoriasis patients compared to pre-treatment levels (5.98±4.69 vs. 20.09±10.58, $p<0.001$). The mean serum level of IL-22 was also significantly reduced after treatment compared to before treatment (5.91± 7.97 pg/ml vs. 56.63± 60.73 pg/ml) ($p<0.001$) (Fig 1A).

Correlation between IL-22 serum level and PASI score

The PASI scores and IL-22 serum levels before and after treatment for the 19 psoriasis patients are shown in Table 1. Before treatment with methotrexate, there was a significant correlation between IL-22 serum levels and PASI score. ($r=0.63$, $p=0.004$) (Fig 2A). Patients were then divided into two groups according to pre treatment serum level of IL-22: high serum level group for IL-22 >50 pg/ml (n=6) and low serum level group for IL-22 ≤50 pg/ml) (n=13). The high serum IL-22 level group showed a significantly greater reduction in PASI score after treatment compared to the low serum IL-22 level group. The mean reduction in PASI score reduction ± SD for the high serum level group was 22.92±10.67 compared to 10.05±5.83 for the low serum level group ($Z=2.68$, $p=0.007$) (Fig 2B). Serum IL-22 level was not associated with any aggravating factors of psoriasis (age of onset, sex, smoking, sun-exposure and body mass index) (data not shown).

DISCUSSION

Recent studies have demonstrated that IL-22 is a key cytokine involved in the epidermal alteration and dermal inflammation of psoriasis [6, 21]. Serum level of IL-22 has been reported to correlate with clinical severity of psoriasis [22]. Moreover, IL-22 production is rapidly reduced after treatment with several biological agents, suggesting this is one mechanism of action to account for the efficacy of these agents in psoriasis [8, 9]. Although MTX is an effective first-line treatment for psoriasis, the exact mechanism for the efficacy of MTX is equivocal, and the effect of MTX on IL-22 in psoriasis patients has never been reported. Our study demonstrates a significant reduction in IL-22 serum levels in patients with moderate to severe psoriasis after treatment with MTX. There were 2/19

psoriasis patients with a zero level of IL-22 before treatment and both of these had a moderate rather than severe PASI score. Undetectable levels of IL-22 in psoriasis have been reported in 11/33 cases in another study [23]. This study postulated that the source of IL-22 in psoriasis might be from inflammatory cells in the skin rather than circulating cells, to account for a zero serum level in some patients. To confirm this would require direct analysis of the skin specimens for IL-22, something that was beyond the scope of our study.

The mechanism by which MTX reduces IL-22 is speculative at this point. IL-22 is produced by several T cell types including Th-22, Th-17, T helper 1, activated γδ T cells, and CD8+ T cells as well as monocytes and dendritic cells [21, 24]. The anti-proliferative effect of MTX was originally considered to be the fundamental therapeutic mechanism in psoriasis. In this regard, reduced levels of IL-22 could be the consequence of an anti-proliferative effect of MTX on dermal dendritic cells and varieties of T-cells. However, studies have not found reduced proliferation of these cell types to be a consistent finding in psoriasis [18-20]. Both vivo and vitro studies reported that MTX could reduce T cell numbers in psoriatic skin and in the circulation but had no effect on interferon-γ and IL-4 production [19, 20]. MTX can suppress inflammation in psoriasis by reducing the numbers of T cell and monocytes in the skin along with reduced expression of adhesion molecules [11, 13-16] . Thus, it is possible that reduced levels of IL-22 may result from the anti-inflammatory effects of MTX.

MTX was more effective in reducing serum IL-22 levels, than has been reported for other therapeutic agents such as etanercept and acitretin [9]. This may be explained by the fact that MTX acts on immune cells as well as keratinocytes, and both these cell types play important roles in the pathogenesis of psoriasis, serving as potent sources of proinflammatory cytokines, chemokines, and growth factors. Acitretin, a vitamin A derivate, has a direct effect on keratinocyte proliferation and differentiation but no effect on immune pathways. Etanercept, a tumour necrosis factor-α inhibitor, inhibits dendritic cell activation and

maturation, and subsequently suppresses T cell activation, but has no direct effect on keratinocyte proliferation [25].

In summary, the present study presents evidence for a novel therapeutic mechanism of methotrexate in psoriasis via reduction of serum IL-22. One other noteworthy finding in our study was the significant correlation between IL-22 serum level and clinical severity as determined by the PASI score. The clinical response to MTX treatment was greater in those patients with high serum IL-22 levels compared to those with lower serum IL-22 levels. Thus, IL-22 serum level may serve as a potential biomarker for prediction of prognosis and therapeutic response when using MTX to treat psoriasis. Additional clinical studies would be of value to confirm this finding.

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FIGURE LEGENDS

Figure 1 IL-22 serum level in healthy controls and in psoriasis patients at baseline and after treatment. Each dot represents the IL-22 serum level of a sample. Horizontal bars represent the mean. (A) Comparison between controls and psoriasis patients, at baseline and after treatment. (B) Comparison between psoriasis patients divided into high and low PASI groups, at baseline and after treatment.

Figure 2 IL-22 serum level and PASI score in psoriasis patients. (A) Correlation between IL-22 serum level and PASI score at baseline ($r=0.63$, $p=0.004$). Each dot represents the IL-22 serum level and PASI score of each patient at baseline. (B) The clinical response to MTX was significant different between the group with high and low IL-22 serum level ($Z=2.68$, $p=0.007$). Each dot represents PASI difference between baseline and after treatment of each patient. Horizontal bars represent the mean of PASI difference. 'High' indicates the high serum level group (IL-22 >50 pg/ml) (n=6) and 'Low' indicates the low serum level group (IL-22 $\leq$50 pg/ml) (n=13).

Figure 1

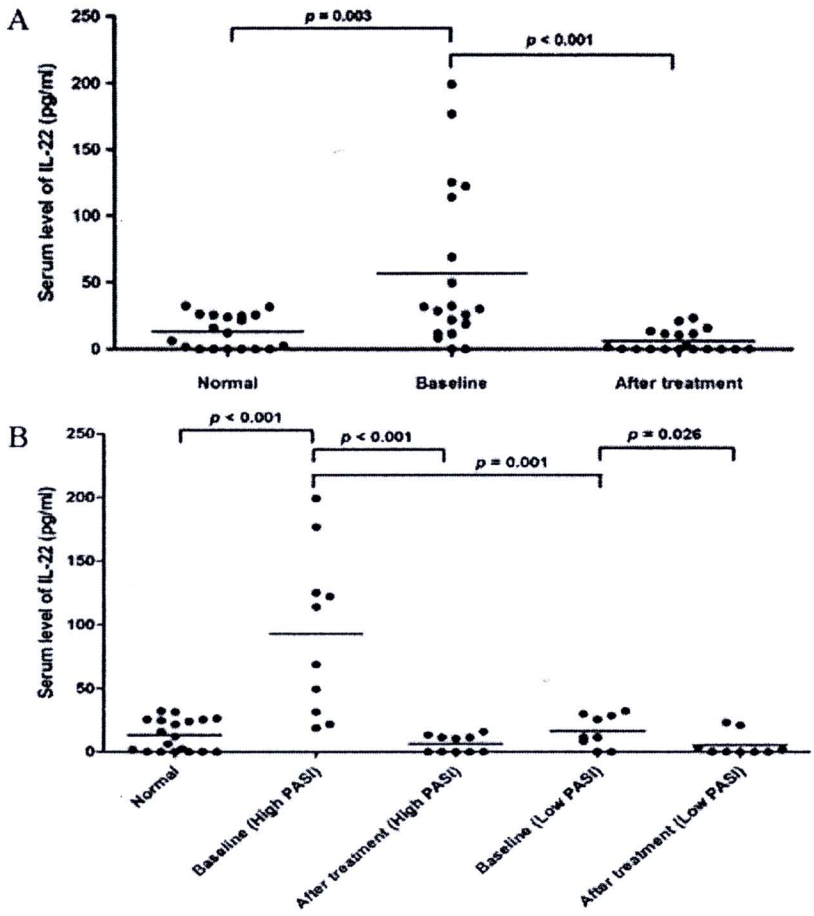
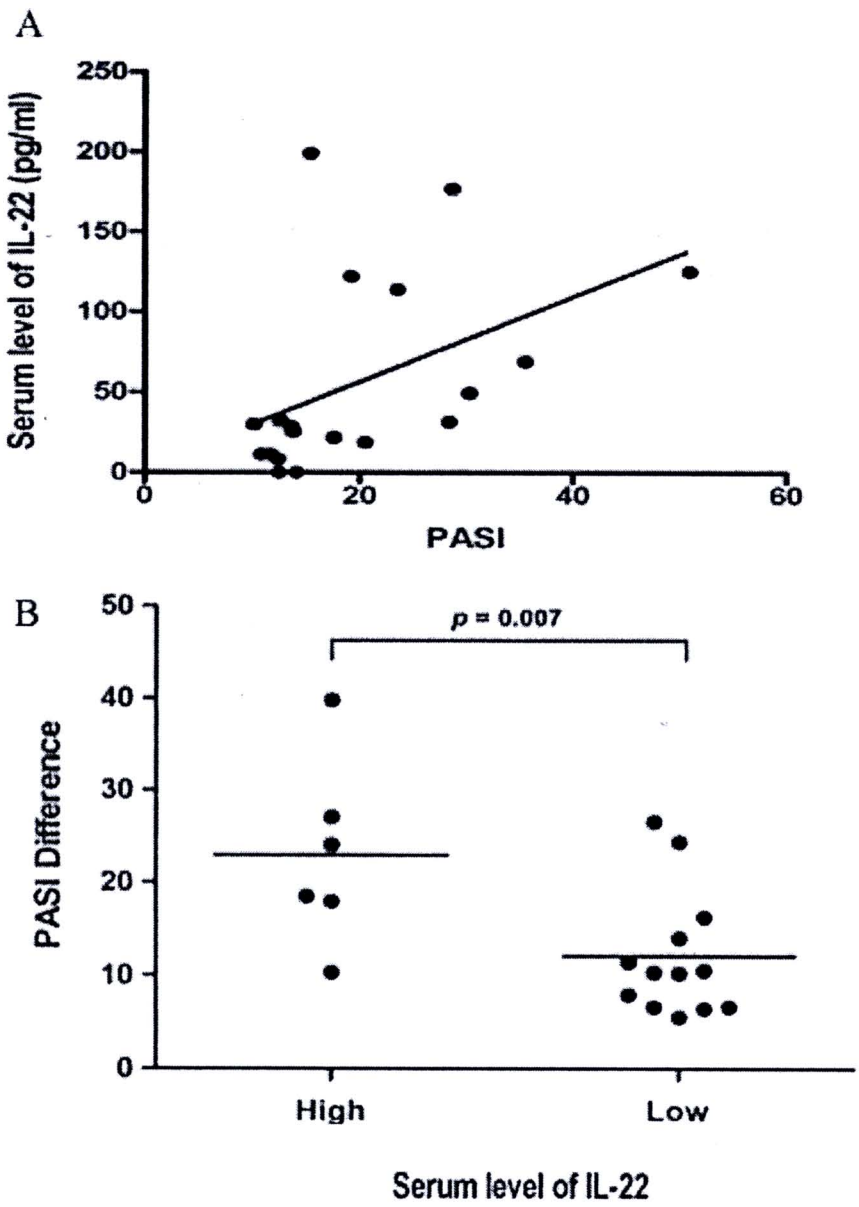

A
Serum level of IL-22 (pg/ml)
250
200
150
100
50
0
p = 0.003
p < 0.001
Normal
Baseline
After treatment
B
Serum level of IL-22 (pg/ml)
250
200
150
100
50
0
p < 0.001
p < 0.001
p = 0.001
p = 0.026
Normal
Baseline (High PASI)
After treatment (High PASI)
Baseline (Low PASI)
After treatment (Low PASI)

Figure 2


A
Serum level of IL-22 (pg/ml)
250
200
150
100
50
0
0
20
40
60
PASI
B
PASI Difference
50
40
30
20
10
0
p = 0.007
High
Low
Serum level of IL-22

สถาบันวิจัยแห่งชาติ คณะกรรมการวิจัยแห่งชาติ ห้องสมุดงานวิจัย

TABLES

Table 1. PASI scores and IL-22 serum levels for each psoriasis patient.

Patient No.	PASI score		Serum IL-22 (pg/ml)	
	Before treatment	After treatment	Before treatment	After treatment
1	11.7	5.1	11.39	0
2	10.2	0	30.1	0
3	13.6	12.9	28.6	23.36
4	12.6	4.7	32.34	0
5	12.4	2.1	8.4	0
6	13.8	2.4	25.61	3.16
7	10.9	0.4	11.39	21.12
8	12.5	6.1	0	1.66
9	14.1	7.5	0	0
10	28.6	1.6	176.78	0.16
11	50.9	11.1	125.14	13.33
12	23.5	5	113.92	0
13	15.4	5.1	199.23	11.39
14	19.2	1.3	122.15	10.64
15	28.4	4.1	31.6	0
16	17.6	12.1	21.87	0.16
17	30.3	16.3	49.56	15.88
18	20.5	4.3	18.87	0
19	35.5	11.5	69.01	11.39