การเกิด ดี เอ็น เอ เมทิลเลชั่นของยีนไมโครอาร์เอ็นเอ 203, ยีนเอเอ็มดี 1 และยีนบีซีเอพี 31 ในโรคสะเก็ดเงิน

นางสาวจีระภา คำเพ็ง

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THE DNA METHYLATION OF MIR-203, AMD1 AND BCAP31 GENE IN PSORIASIS

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Thesis Title	THE DNA METHYLATION OF MIR-203, AMD1 AND BCAP31 GENE IN PSORIASIS
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จีระภา คำเพ็ง : การเกิด ดี เอ็น เอ เมทิลเลชั่นของยีนไมโครอาร์เอ็นเอ 203, ยีนเอเอ็ม ดี 1 และยีนบีซีเอพี 31 ในโรคสะเก็ดเงิน (THE DNA METHYLATION OF MIR-203, AMD1 AND BCAP31 GENE IN PSORIASIS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. พญ.ดร.จงกลนี วงศ์ปิยะบวร, 86 หน้า.

โรคสะเก็ดเงินเป็นโรคผิวหนังอักเสบเรื้อรัง ลักษณะเด่นของโรคคืออาการอักเสบที่ผิวหนังชั้นหนังกำพร้าและชั้นหนังแท้ โดยเกิดความผิดปกติในการเพิ่มจำนวนและการพัฒนาของเซลล์เคอราทิโนไซท์ นอกจากนี้ในโรคสะเก็ดเงินยังพบการเสียหน้าที่ในกระบวนการอะพอพโทซิส โดยพบว่ามีการอะพอพโทซิสของเซลล์เคอราทิโนไซท์บริเวณรอยโรคลดลง ดี เอ็น เอ เมทิลเลชั่น เป็นกระบวนการหลักในการเปลี่ยนแปลงทางอิพิจิเนติก ซึ่งส่งผลกระทบต่อการทำหน้าที่ของเซลล์โดยการทำให้เกิดการเปลี่ยนแปลงการแสดงออกของยีน ที่มีความสำคัญในการเปลี่ยนแปลงการแสดงออกของยีน การเกิด ดี เอ็น เอ เมทิลเลชั่นมักเกิดที่บริเวณ CpG island บนโปรโมเตอร์ของยีน ซึ่งการเกิด ดี เอ็น เอ เมทิลเลชั่นบริเวณนี้จะมีความสัมพันธ์กับการแสดงกดการแสดงออกในการพัฒนาแบบปกติของยีน การเปลี่ยนแปลงไปของการเกิดดี เอ็น เอ เมทิลเลชั่น มีบทบาทสำคัญในการพัฒนาของโรคภูมิคุ้มกันต่อเนื้อเยื่อตนเองรวมทั้งโรคสะเก็ดเงิน จากการศึกษาก่อนหน้าแสดงให้เห็นว่า ความผิดปกติของการเกิดดี เอ็น เอ เมทิลเลชั่นในโรคสะเก็ดเงินพบได้ในหลายยีน เช่น ยีน SHP-1 ยีน p16INKa และยีน p15 ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อ วิเคราะห์แบบแผนของการเกิดดี เอ็น เอ เมทิลเลชั่น ของสามยีน คือ ยีน mir-203, ยีน AMD1 และยีน BCAP31 ในผู้ป่วยโรคสะเก็ดเงิน ด้วยวิธีการ combined bisulfite restriction analysis (COBRA), *methylation specific* PCR (MSP), และ bisulfite sequencing ตามลำดับ จากนั้นจะศึกษาความสัมพันธ์ระหว่างการเกิดดี เอ็น เอ เมทิลเลชั่น และ ระดับการแสดงออกของยีน ผลการศึกษาพบว่า ระดับการเกิดดี เอ็น เอ เมทิลเลชั่นของยีน mir-203 ในเซลล์ผิวหนังชั้นหนังกำพร้า และ ยีน AMD1 ในเซลล์ผิวหนังชั้นหนังกำพร้าและ total leukocyte ของผู้ป่วยโรคสะเก็ดเงิน เมื่อเทียบกับคนปกติพบว่าไม่ต่างกัน ไม่สอดคล้องกับระดับการแสดงออกของยีน อาจสรุปได้ว่า การเพิ่มขึ้นในระดับ เอ็ม อาร์ เอ็น เอ ของยีน mir-203 และ AMD1 ไม่ได้มาจากกลไกการควบคุมด้วย การเกิด ดี เอ็น เอ ดี เมทิลเลชั่น ที่บริเวณโปรโมเตอร์ของยีน การศึกษาต่อมาคือการศึกษาการแสดงออกในระดับ เอ็ม อาร์ เอ็น เอ และการเกิดดี เอ็น เอ เมทิลเลชั่น ของยีน BCAP31 ใน Peripheral Blood Mononuclear Cell (PBMC) ของผู่ป่วยสะเก็ดเงิน ผลการศึกษาเป็นที่น่าสนใจว่า การแสดงออกในระดับ เอ็ม อาร์ เอ็น เอ และการเกิด ดี เอ็น เอ เมทิลเลชั่นของยีนมีความสัมพันธ์แบบผกผัน คือ พบการแสดงออกในระดับเอ็ม อาร์ เอ็น เอ ต่ำลง แต่ระดับการเกิดดี เอ็น เอ เมทิลเลชั่นสูงขึ้นในผู้ป่วยเมื่อเทียบกับคนปกติ โดย BCAP31 นี้จัดเป็นโปรตีนที่พบได้มากบริเวณเยื่อหุ้มผิวของเอนโดพลาสมิกเรติคูลัม ซึ่งเกี่ยวพันกับการควบคุมการเกิดการอะพอพโทซิสของเซลล์ลิมโฟไซต์ และเซลล์เยื่อบุผิวหลายชนิด ดังนั้นจากการศึกษานี้จึงอาจสรุปได้ว่า การลดลงของการแสดงออกของยีน BCAP31 อาจเพิ่มศักยภาพในการต้านทานการเกิดอะพอพโทซิสของเซลล์ลิมโฟไซต์ในโรคสะเก็ดเงินได้ การศึกษานี้เป็นการศึกษาแรกที่แสดงให้เห็นว่าการเกิดเมทิลเลชั่นที่สูงขึ้นนี้ทำให้การแสดงออกในระดับ เอ็ม อาร์ เอ็น เอของยีน BCAP31ลดลงในผู้ป่วยโรคสะเก็ดเงิน

สาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

5087126220 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : PSORIASIS / DNA METHYLATION / MIR-203 / AMD1 / BCAP31

JEERAPA KAMPENG : THE DNA METHYLATION OF MIR-203, AMD1 AND BCAP31 GENE IN PSORIASIS. ADVISOR : ASST.PROF. JONGKONNEE WONGPIYABOVORN, M.D., Ph.D., 86 pp.

Psoriasis is a chronic inflammatory skin disease. Its characteristic features in the skin consist of inflammatory changes in both dermis and epidermis, with abnormal keratinocyte differentiation and proliferation. Dysfunctional apoptotic process occurs in psoriasis. There is a decrease apoptosis of lesional keratinocyte. DNA methylation is a major epigenetic modification, affects cell function by altering gene expression. DNA methylation of promoter region in CpG island, has shown to be associated with transcription silencing of the gene in normal development. Aberrant DNA methylation plays an important role in the development of autoimmune disease including psoriasis. Previous studies show that abnormal DNA methylation was found in many genes, such as SHP-1, P16INK4a, and p15 genes. This study aimed to analyze promoter methylation of mir-203, AMD1 and BCAP31 genes in patients with psoriasis compare to normal using combined bisulfite restriction analysis (COBRA), *methylation specific* PCR (MSP), and bisulfite sequencing, respectively. Then, the correlation between the promoter methylation and their expression level were investigated. We analyzed the methylation level of miR-203 in epidermis and AMD1 in epidermis and total leukocytes. There were no significant differences in methylation level of mir-203 and AMD1 in psoriasis, compared to normal controls. Our results suggest that, mir-203 and AMD1 mRNA expression in psoriasis is not controlled by mir-203 and AMD1 promoter methylation. Then, mRNA expression level and promoter methylation of BCAP31 genes in PBMCs were studied. Interestingly, The expression level of BCAP31 mRNA was significantly decrease and correlated with promoter hypermethylation in psoriasis group compared with healthy control. BCAP31, an ubiquitous ER membrane protein, has been implicated in the regulation of apoptosis in lymphocytes and epithelial cells. Thus, down-regulation of BCAP31 may increase potential to resist apoptosis of lymphocyte in psoriasis. This is the first report showed that hypermethylation of BCAP31 promoter contributes to down-regulation of mRNA expression in psoriasis.

Field of Study : Medical Microbiology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Psoriasis is a chronic inflammatory skin disorder afflicting up to 2% of the world's population. Clinically, psoriasis is distinguished by sharply demarcated erythematous plaques and covered by silvery scale. Psoriasis is characterized by hyperproliferation of basal keratinocytes, a thickened and scaly epidermis, disruption of keratinocyte terminal differentiation (Menter 1998). The extensor surfaces, such as knees and elbows, are commonly involved as well as the lower back, scalp and nails(Luba and Stulberg 2006). Epidermal keratinocytes and mononuclear leukocytes are two fundamentally different cell types interact in the formation of a psoriatic lesion. Keratinocytes are active participants in the recruitment and activation of leukocytes in psoriatic lesions. Chemokines produced by keratinocytes in the epidermis act on both the innate and acquired immune systems, stimulating Dendritic cells (DCs), neutrophils and other innate immune cells as well as T cells. Keratinocytes also release cytokines and growth factors, leading to altered gene expression and regenerative hyperplasia, and also to induce adhesion molecules for T cells on keratinocytes. Immune system derived cytokines, in turn, act on keratinocytes to either induce inflammatory genes or increase proliferation. Thus, there is close interdependence of the epidermis and dermal inflammatory infiltrate, as well as a balance between the innate and acquired immune systems. Furthermore, the genomic imprinting of psoriasis are known to be complex, with ten or more susceptibility loci, and these probably interact with various environmental factors that act on the skin and/or immune system (Lowes, Bowcock et al. 2007).

DNA methylation is the part of epigenetics. DNA methylation is a fundamental mechanism in controlling biological process especially in the regulation of genes and the stability of the genome without changing the DNA sequence. DNA methylation mechanism occurs by add the methyl group to the 5- carbon of CpG dinucleotide by DNA methyltransferase. DNA methylation usually occurs in the CpG islands. Methylation in promoters of DNA usually down regulate of mRNA transcription

either by directly interfering with the binding of transcription factors or by allowing chromatin condensation resulting in loss of gene function (Attwood, Yung et al. 2002; Luczak and Jagodzinski 2006; Rodenhiser and Mann 2006). Aberrant DNA methylation plays an important role in development of autoimmune disease including psoriasis. To date, they have only been four studies in DNA methylation in psoriasis. Ruchusatsawat et al., observed a significant degree of demethylation in SHP-1 promoter2 in psoriasis with reverse correlation of SHP-1 isoform2 mRNA level in psoriatic skin (Ruchusatsawat, Wongpiyabovorn et al. 2006). Other groups reported demethylation of p16 gene in PBMCs from psoriasis patients and hypermethylation of psoriatic epidermis (Zhang, Zhang et al. 2007; Chen, Chen et al. 2008). Finally, Zhang K. et al, found that p15 and p21 promoter hypomethylation related to increased transcription levels in psoriasis comparison to normal volunteers (Zhang, Zhang et al. 2009).

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nt in length that regulate a variety of biological processes by silencing specific target genes (Ambros 2004). The study of Sonkoly et al showed that the up-regulation of miR-203 in psoriatic plaques was concurrent with the down-regulation of an evolutionary conserved target of miR-203, suppressor of cytokine signaling 3 (SOCS-3), which is involved in inflammatory responses and keratinocyte functions. These results suggest that miRNAs contribute to psoriasis pathogenesis by modulating protein expression and cellular functions in keratinocytes (Sonkoly, Wei et al. 2007). mir-203 methylation has been studies in various cancers. Kozaki K. et al., investigated the potential role of methylation in the transcriptional silencing of mir-203 in oral squamous cell carcinoma. They found the aberrant methylation of the mir-203 CpG Island in OSCC and cell lines and are associated with loss of expression of mir-203 (Kozaki, Imoto et al. 2008). We hypothesized that the up-regulation of mir-203 is mediated by the demethylation of mir-203. Thus, we analyzed mir-203 methylation in psoriasis by Combined with Bisulfite Restriction Analysis (COBRA).

S-adenosylmethionine decarboxylase (AdometDC, AMD1) is transcribed by *AMD1* gene on chromosome 6. *S*-Adenosylmethionine Decarboxylase (AdoMetDC) is an initial enzyme in the polyamine synthetic pathway. AdoMetDC catalyzes the removal of the carboxylate group from *S*-adenosylmethionine (AdoMet) to *S*-adenosyl-5′-3-

methylthiopropylamine. The polyamine biosynthetic machinery is upregulated in various types of cancer and parasitic diseases (Bale and Ealick 2010). Recently, Bowcock et al, and Zhou et al, study the expression profiles from uninvolved and lesional psoriatic skin by microarray show that AMD1 mRNA is higher upregulation in lesional psoriatic skin (Zhou, Krueger et al. 2003; Reischl, Schwenke et al. 2007). We hypothesized that the up-regulation of *AMD1* expression is mediated by the demethylation of *AMD1* promoter.

B-cell receptor-associated protein 31 is a multi-pass transmembrane protein of the endoplasmic reticulum (ER) that is involved in the anterograde transport of membrane proteins from the ER to the Golgi. It role of BCAP31 has been reported to participated in the exit of peptide-load MHC class I from the ER. Moreover, it also extended to a role in regulating apoptotic process of lymphocytes and epithelial cells in various diseases such as Cystic fibrosis, human papilloma viral infection. In psoriasis, abnormal apoptosis in lessional keratinocytes and lymphocytes has been described. There is decrease level of caspase-14, downstream signal transducer of cell death. So we hypothesized that down-regulation of BCAP31 may be occurred in psoriasis patients. Promoter methylation of BCAP31 gene has been studied across nine different tissues (white cerebral matter in brain, heart, kidney, liver, prostate, pancreas, lung, skeletal muscle and testis) using bisulfite genomic sequencing. The result shown that the promoter region was found demethylated in all tissues that inverse correlated with expression level (Grunau, Hindermann et al. 2000). We hypothesized that aberrant DNA methylation may be occurred in psoriasis. However, there is no any study about BCAP31 in psoriasis.

In this study, we analyzed promoter methylation of *mir-203*, *AMD1* and *BCAP31* genes in patients with psoriasis compare to normal. Then, the correlation between the promoter methylation and their expression level were determined.

This study might lead to developing new biomarkers, which apply to diagnostic or prognosis of psoriasis.

CHAPTER II
OBJECTIVES

Research Questions

1. Are aberrant DNA methylation of *miR-203, AMD1* and BCAP31 gene detected in psoriasis patients?

2. Do aberrant DNA methylation leading to mediated *miR-203, AMD1* and BCAP31 gene expression in psoriasis patients?

Hypothesis

1. Aberrant DNA methylation leading to irregular of *miR-203, AMD1* and *BCAP31*gene expression in psoriasis patients.

Objectives

1. To analyze DNA methylation pattern within CpG islands around *miR-203* in psoriasis patient and normal epidermis.

2. To detect promoter methylation of *AMD1* gene in leukocyte and epidermis isolated from patients with psoriasis and healthy control.

3. To determine the correlation between the promoter methylation of *AMD1* gene and mRNA expression level in leukocyte and epidermis isolated from patients with psoriasis and healthy control.

4. To detect promoter methylation of *BCAP31* gene in hematopoietic cells isolated from patients with psoriasis and healthy control.

5. To determine the correlation between the promoter methylation of *BCAP31* gene and mRNA expression level in hematopoietic cells, isolated from patients with psoriasis and healthy control.

CHAPTER III

LITERATURE REVIEW

PSORIASIS

Epidermiology

Psoriasis is a chronic inflammatory skin disease. It is an autoimmune disease that triggered by activated cellular immune system. Psoriasis is found worldwide but the prevalence varies among different ethnic groups (Lomholt 1964). The prevalence of psoriasis is low in certain ethnic groups such as the Japanese, and maybe absent in aboriginal Australians and Indians from South America(Green 1984). However, the prevalence of psoriasis still has not been clear in Thailand. Psoriasis can occur at any point in the life span rarely found under 10 years of age and mostly found in the age between 15-30 years. It affects male and female equally.

Clinical features of psoriasis

Clinical feature of psoriasis are raised red skin, covered with silvery white scales. The silvery white scales are the accumulation of the skin cells waiting to be shed, and the redness is due to the increase in blood vessels required to support the increase in cell production (Figure 1). The plaques can appear in a variety of shapes and sizes, varying from a few millimetres to several centimetres in diameter(Lebwohl 2003). Histologically, keratinocytes show abnormal differentiation and hyperproliferation, inflammatory cells invade the dermis and the epidermis, and generate more neo blood vessel (neoangiogenesis) (Ortonne and Ortonne 1999) . Psoriasis vulgaris is the most common form of the disease, occurring more than 80% of cases. Guttate psoriasis occur in about 10% of patients with psoriasis, and erythrodermic and pustular psoriasis each occur in fewer than 3% of patients(Biondi Oriente, Scarpa et al. 1989).

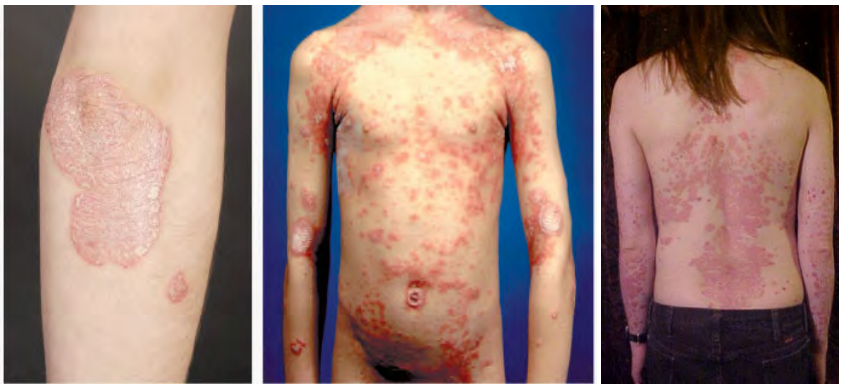


Figure 1 Clinical Features of Psoriasis(Schon and Boehncke 2005).

Psoriasis severity

The severity of psoriasis depends on genetics and environmental factors; some patients have mild disease with isolated scaling erythematous plaques on the knees or elbows while other patients have moderate and severe disease with wildly cutaneous involvement. Psoriasis Area and Severity Index is the clinical score for identification the severity of disease. It defined by the size of the involved area, redness, thickness and scaling(Greaves and Weinstein 1995). The mild, moderate and severe of psoriasis is defined by scores of less than 10, 10 to 15 and greater than 15, respectively.

The onset of psoriasis

On the basis of bimodal distribution of age at onset, psoriasis vulgaris (PV) was classified into early-onset psoriasis (Type I) and late-onset psoriasis (Type II)

Type I psoriasis (Early-onset)

Type I psoriasis, also called early-onset type psoriasis, begins before the age of 40 years (usually at 16-22 years of age)(Greaves and Weinstein 1995).). Type I psoriasis affected more than 75% of patients. The majority of patients with positive strong familial histories reveal positively to human lymphocyte antigen-Cw6 (HLA-Cw6)

(Henseler and Christophers 1985). This is considerable genetic susceptibility and hereditary association in this group of patients. These patients tend to develop more extensive plaques and more severe disease (Greaves and Weinstein 1995).

Type II psoriasis (Late-onset)

Type II psoriasis, also called late-onset type psoriasis, occurred after the age of 40 years (usually at 57-60 years). This late-onset type presents with minor hereditary association and no family history. Compared to the early-onset type, type II psoriasis is considered to be mild and localized. This type has more stable disease (Henseler and Christophers 1985; Greaves and Weinstein 1995)

Genetic study of psoriasis

Evidence of a genetic predisposition for psoriasis is provided by twin studies, showing 35-67% concordance for monozygotic twins versus 12-18% for dizygotic twins(Brandrup, Hauge et al. 1978; Duffy, Spelman et al. 1993). Nowadays, the genetic pattern of psoriasis has still unclear. Nevertheless, several loci for genetic susceptibility to the disease have been reported such as PSORS1-10. The most significant susceptibility locus is thought to be the PSORS1. PSORS1 is estimated to account for 30-50% of the genetic predisposition for psoriasis(Trembath, Clough et al. 1997). In the PSORS1 locus, HLA-CW*06 is the most susceptible allele for psoriasis in many population.

Pathogenesis of psoriasis

Histology of normal skin and psoriatic lesions show in Figure2. The skin has three main layers. First there is the epidermis, which consists mainly of epithelial cells (keratinocytes). Second is the dermis, the bulk of which is made up of an extracellular matrix composed predominantly of collagens. Third is the hypodermis which contains loose connective tissue and fat. In psoriasis, cells of the stratum corneum (the outermost layer of the epidermis) stack abnormally, leading to the formation of scales, and the granular layer of the epidermis is much reduced (Figure 3). Epidermal rete are considerably elongated and blood vessels in the dermis are

enlarged. Although normal skin contains notable numbers of resident and trafficking immune cells but in psoriatic lesions the leukocyte number is significantly increased and many immune-related pathways are activated.

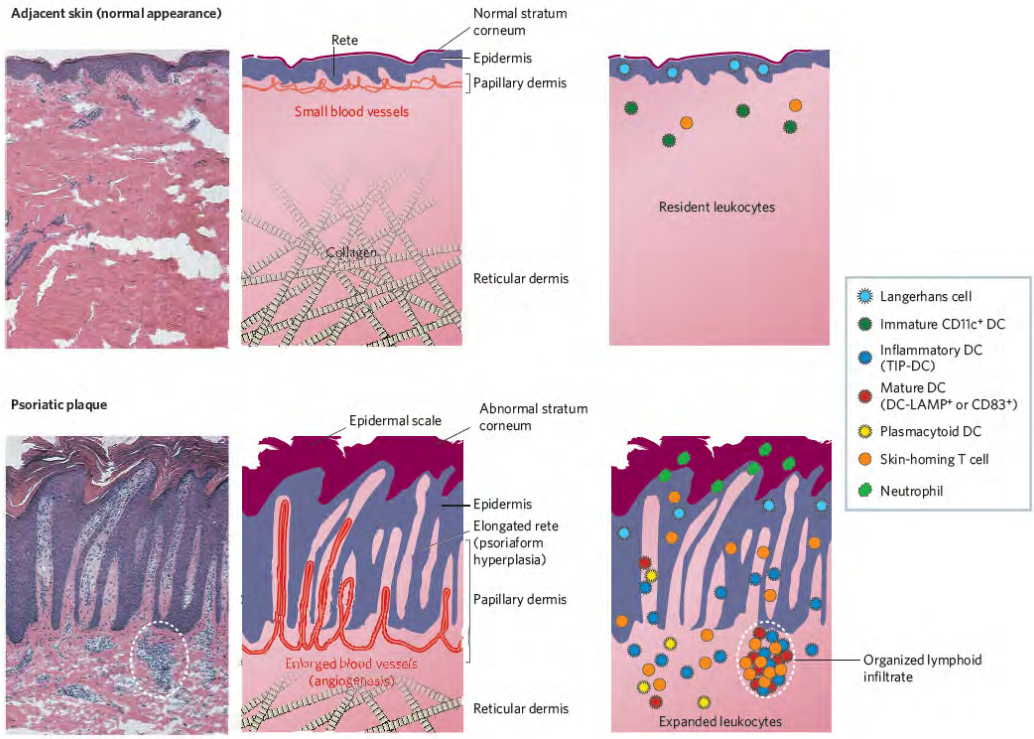


Figure 2 Histological components of a mature psoriatic plaque compared with normal skin (Lowes, Bowcock et al. 2007)

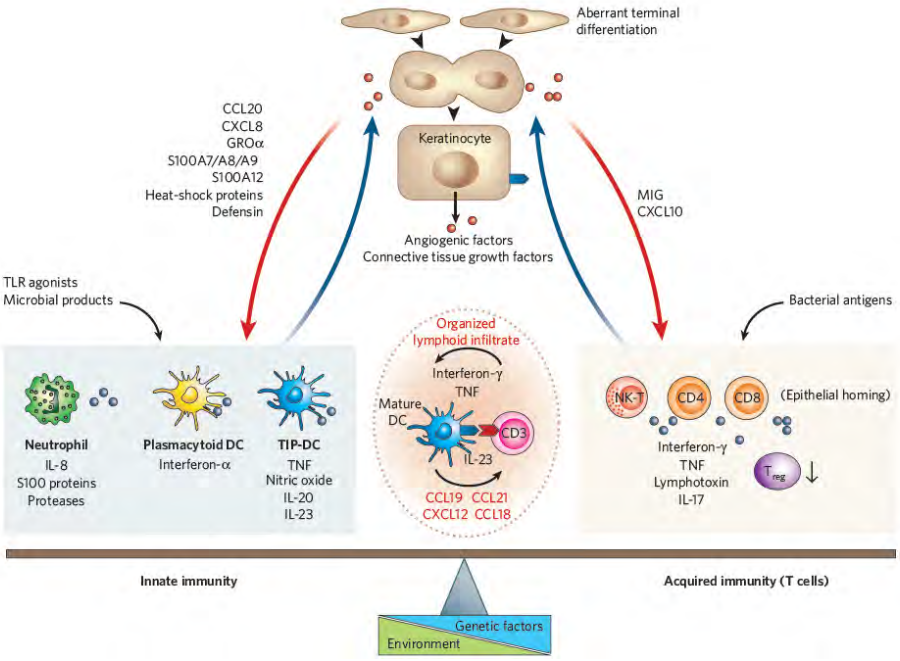


Figure 3 The balance between the activation of innate and acquired immune cell types, and the factors produced by epidermal keratinocytes that directly affect T cells and DCs, and vice versa(Lowes, Bowcock et al. 2007).

Epidermal keratinocytes and mononuclear leukocytes are two fundamentally different cell types interact in the formation of a psoriatic lesion. Keratinocytes are active participants in the recruitment and activation of leukocytes in psoriatic lesions. Chemokines produced by keratinocytes in the epidermis act on both the innate and acquired immune systems, stimulating DCs, neutrophils and other innate mediators as well as T cells. Keratinocytes also release cytokines and growth factors, leading to altered gene expression and regenerative hyperplasia, and also to the induction of adhesion molecules for T cells on keratinocytes. Immune system derived cytokines, in turn, act on keratinocytes to either induce inflammatory genes or increase proliferation. Thus, there is close interdependence of the epidermis and dermal inflammatory infiltrate, as well as a balance between the innate and acquired immune systems (Lowes, Bowcock et al. 2007).

Angiogenesis of psoriasis

Angiogenesis is a natural physiological function. It refers to the process by which new blood vessels form and grow. A characteristic of angiogenesis is the occurrence of increased endothelial cell proliferation. Chronic inflammation of the tissue underlying the epidermis in psoriatic skin creates a strong angiogenic signal. Several studies have shown a high detectable blood flow in the psoriatic plaques. It is now recognized that the keratinocytes of lesional skin are a major source of pro-angiogenic cytokines in psoriasis. Many study have identified numerous angiogenic factors from psoriatic epidermis such as interleukin-8, tumour necrosis factor-α, transforming growth factor-α, endothelial cell stimulating angiogenesis factor, and vascular endothelial growth factor (VEGF) (Ettehadi, Greaves et al. 1994; Nickoloff, Mitra et al. 1994; Bhushan, McLaughlin et al. 1999; Creamer, Sullivan et al. 2002). Several studies have demonstrated that VEGF expression is increased in lesional psoriatic skin that the serum levels of circulating VEGF protein are significantly elevated in patients with severe disease, and that VEGF serum levels were directly correlated with disease activity (Creamer et al., 2002).

Dysfunctional apoptosis in psoriasis

In psoriasis, there is decreased spontaneous keratinocyte apoptosis in lesional skin (Laporte, Galand et al. 2000) which correlates with decreased levels of caspase- 14 (Lippens, Kockx et al. 2000). Keratinocytes in psoriatic plaques exhibit a phenotype reminiscent of keratinocyte senescence, characterized by resistance to apoptosis compared with normal keratinocytes and lack of p53 activation (Qin, Chaturvedi et al. 2002). Interestingly, TNF-α is abnormally elevated in psoriatic skin. IL-15, a potent inhibitor of KC apoptosis *in vitro* that is upregulated in psoriatic skin. In addition, previous studies demonstrated increased levels antiapoptotic, Bcl-xL and Survivin in psoriatic skin (Gilhar, Yaniv et al. 2006; Abdou and Hanout 2008).

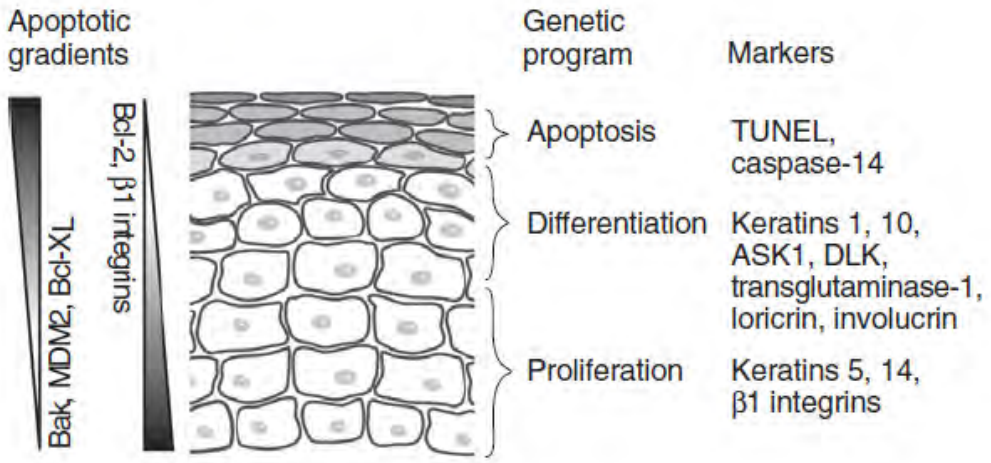


Figure 4 Genetic programs in epidermal development. Apoptosis maintains epidermal homeostasis, balancing cell death in the granular layer with proliferation in the lower layers. The proliferative gene program is characterized by a restricted pattern of keratin gene expression. The differentiation program directs changes in expression of keratin genes, master differentiation genes (apoptosis signal-regulating kinase-1 (ASK1), dual leucine zipper-bearing kinase (DLK)), and genes involved in stratum corneum formation. The apoptotic gene program is manifest throughout the epithelium, maintaining gradients of antiapoptotic and proapoptotic factors that regulate the timing of apoptosis in epidermal development.(Raj, Brash et al. 2006).

EPIGENETICS

Epigenetics is a heritable change in gene expression that occurred without a changing in DNA sequence. Abnormalities in the epigenetic states represent human disease phenotypes, particularly in developmental defects and tumorigenesis(Nakao 2001). Hence, epigenetics will become the focus and a major target for emerging biological and medical discovers. Epigenetic mechanisms regulate gene expression, through DNA methylation, chromatin, and post-translational modification of proteins such as histones.

The human genome contains approximately 120,000 genes, using highly refined and tested algorithms for EST analysis (Liang, Holt et al. 2000) that must be expressed in specific cells at precise times. Cells manage gene expression by wrapping DNA around clusters (octamers) of globular histone proteins to form nucleosomes (Figure 5). These nucleosomes of DNA and histones are organized into chromatin. Changes to the structure of chromatin influence gene expression: genes are inactivated (switched off) when the chromatin is condensed (silent), and they are expressed (switched on) when chromatin is open (active). These dynamic chromatin states are controlled by reversible epigenetic patterns of DNA methylation and histone modifications. Enzymes involved in this process include DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone acetylases, histone methyltransferases and the methyl-binding domain protein MECP2. Alterations in these normal epigenetic patterns can deregulate patterns of gene expression, which results in profound and diverse clinical outcomes (Rodenhiser and Mann 2006). Epigenetic processes are essential for packaging and interpreting the genome, are fundamental to normal development and are increasingly recognized as being involved in human disease. The loss of normal DNA methylation patterns is the best understood epigenetic cause of disease, based on the initial studies during the 1980s that focused on X chromosome in-activation genomic imprinting and cancer.

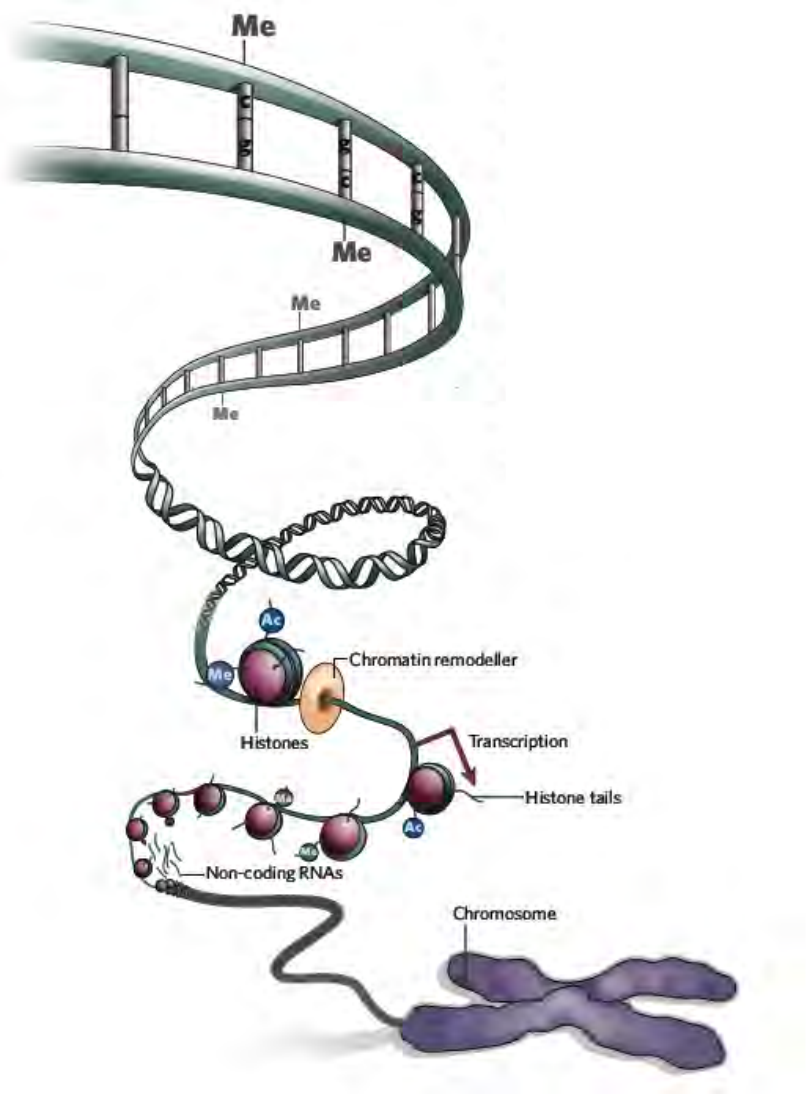


Figure 5 Epigenetic mechanisms. The coding and structural information in the base sequence of DNA is organized in chromatin to form multiple epigenomes. DNA cytosine methylation and covalent modifications of the tails of histones and histone variants contribute information to nucleosomal remodeling to transcription.

DNA methylation

DNA methylation is a fundamental biological process that naturally serves the regulation of genes and the stability of the genome without changing the DNA sequence because both of cytosine and 5-methylcytosine base pair with guanine. DNA methylation is the part of epigenetic. DNA methylation mechanism occurs by add the methyl group to the 5-Carbon of CpG dinucleotide by DNA methyltransferase. DNA methylation in promoter usually shut off gene activity either by directly interfering with the binding of transcription factors or allowing chromatin condensation via recruiting methyl binding protein return attach to chromatin modifier complexs including histones deacetylases (Attwood, Yung et al. 2002; Rodenhiser and Mann 2006).

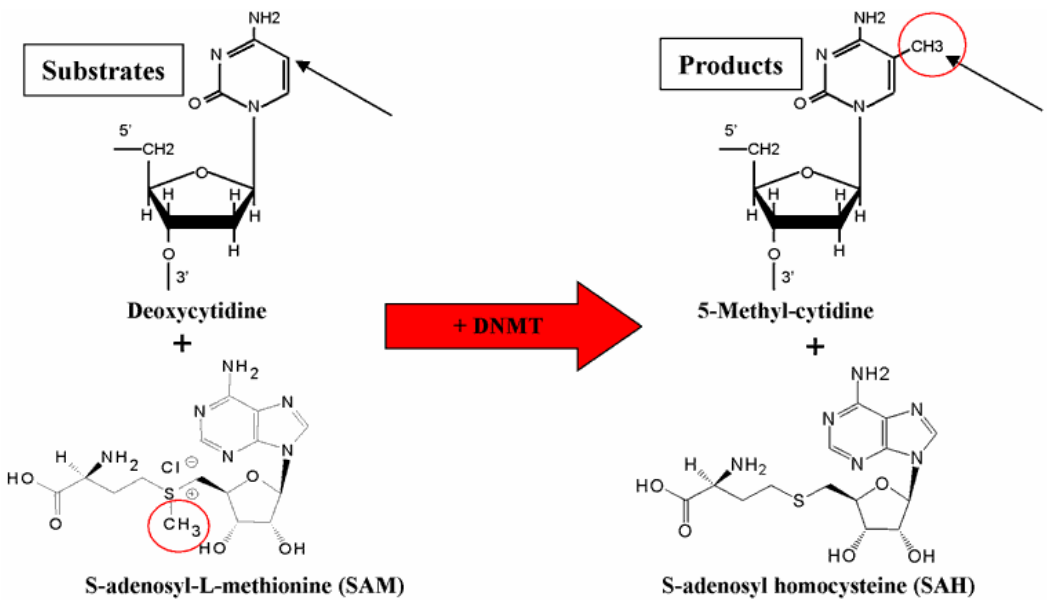


Figure 6 Mechanism of DNA methylation. DNA methylation involves the addition of a methyl group onto the 5 position of a cytosine residue, mediated by the enzymes DNMTs. DNA methylation happens almost exclusively on cytosines in front of a guanine in a CpG dinucleotide.

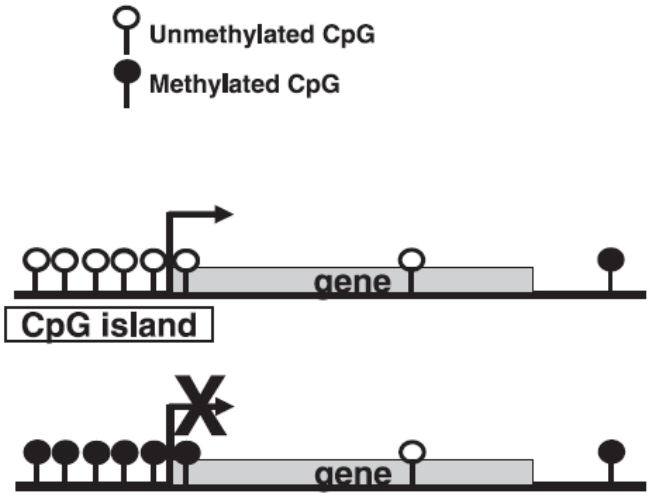


Figure 7.CpG sites in the genome are highly unevenly distributed. CpG islands can be found in the promoter regions of roughly half of the genes and normally remain unmethylated. When they become aberrantly hypermethylated, as can happen in many cancers, they lead to the silencing of downstream genes. (Chuang and Jones 2007)

DNA methyltransferase

DNA methyltransferase is the enzyme, which responsible for the process of DNA methylation. DNA methyltransferase can be divided in to maintenance and de novo DNMTs. DNMT1 is the maintenance methyltransferase responsible for reproducing the parental DNA methylation pattern into daughter cells during DNA replication. The newly synthesized DNA contains hemimethylated sites that provide the signal for DNMT1 to transfer a methyl group to a cytosine residue from its cofactor, S-adenosylmethionine (SAM). But, maintenance methylation does not occur either by decrease in capacity or fidelity of DNMT1 activity or decreased levels of SAM. The daughter DNA will lose a pattern of DNA methylation. DNMT3A and DNMT3B are de novo methyltransferases responsible for adding methyl groups to CpG dinucleotide of unmethylated DNA (Luczak and Jagodzinski 2006). Both enzymes in de novo methylation are necessary for proper development of mammalian embryos by

establishing new methylation patterns, especially DNMT3B for methylation of specific genomic regions such as pericentromic repetitive sequences and CpG islands on the inactive X chromosome (Okano, Xie et al. 1998; Okano, Bell et al. 1999).

DNA methylation and psoriasis

There are many reports about DNA methylation in Cancer. Numerous genes are regulated by DNA methylation mechanism in Cancer. But there are few reports, about DNA methylation in psoriasis. Ruchusatsawat et al., reported the SHP-1 promoter 2 demethylation in psoriasis. They found the SHP-1 promoter 2 hypermethylation was apparent in several epithelial cells lines and tissues while a significant degree of demethylation in psoriasis can be observed. In addition, psoriasis presents a higher level of SHP-1 isoform 2 than normal skin (Ruchusatsawat, Wongpiyabovorn et al. 2006). Zhang, K et al., observed the promoter methylation status and transcriptional activity of the p16 gene in colony-forming cells with high proliferative potential in patients with psoriasis. Their results showed upregulated mRNA transcriptional level and downregulated promoter methylation of p16 (Zhang, Zhang et al. 2007). Chen, M et al, investigated the methylation status of the p16INK4a gene in psoriatic epidermis. They shown that hypermethylation of the p16INK4a gene promoter is found in psoriatic epidermis, which is associated with the mRNA level of p16INK4a expression and activity of the disease. (Chen, Cui et al. 2007).

MICRORNA

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nt in length that regulate a variety of biological processes by silencing specific target genes (Ambros 2004). miRNAs are well conserved during evolution, and it has been estimated that about 250–600 miRNAs have been evolutionarily conserved in vertebrates (Bentwich, Avniel et al. 2005). Additional nonconserved miRNAs have also been reported to contain about 800–1000 miRNAs in human (Bentwich, Avniel et al. 2005; Zamore and Haley 2005). These miRNAs can downregulate various gene products by translational repression when partially complementary sequences are present in the 3' untranslated regions (3'UTR) of the target mRNAs or by directing mRNA degradation. Using these posttranscriptional control mechanisms, mammalian miRNAs appear to target a diversity of cellular functions, including cell proliferation and differentiation (He and Hannon 2004).

MicroRNA genes reside in regions of the genome as distinct transcriptional units as well as in clusters of polycistronic units carrying the information of several microRNAs (Lagos, Baeza et al. 2001; Lau, Lim et al. 2001; Lee, Jeon et al. 2002). Many studies suggest that approximately half of known microRNA reside in non-protein coding RNAs (intron and extron) or within the intron of protein coding genes (Figure 8)

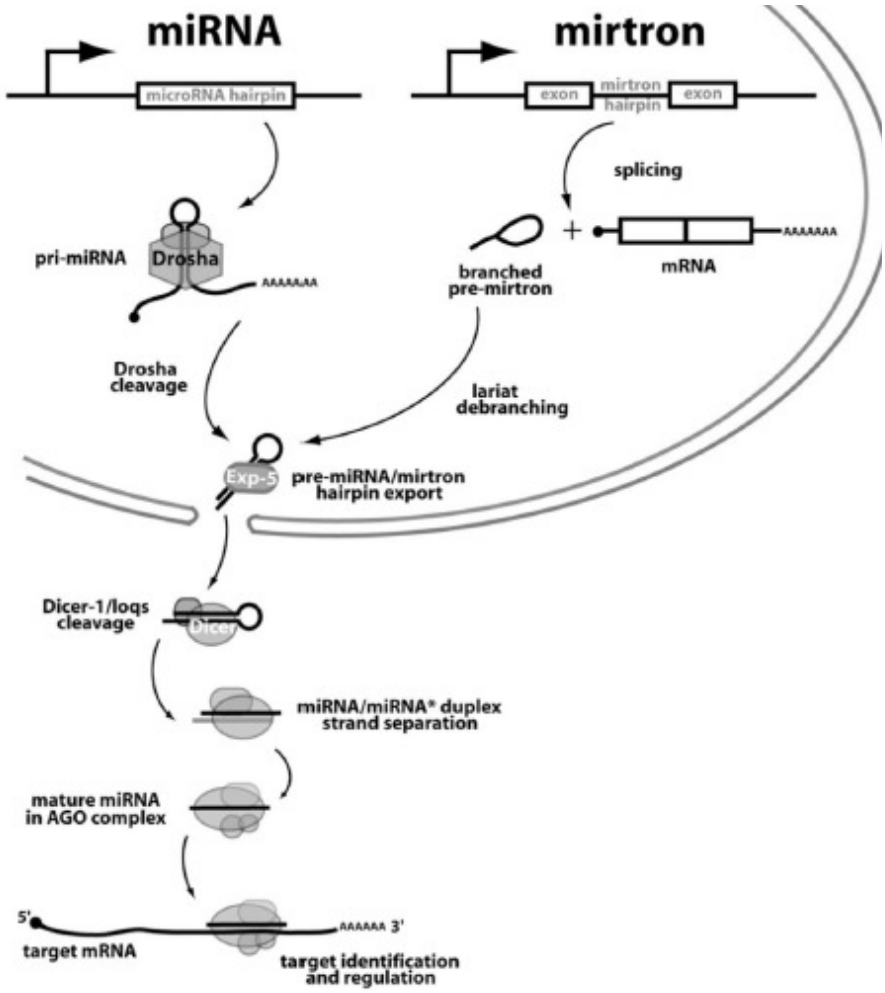


Figure 8 MicroRNA biogenesis (Okamura, Hagen et al. 2007), RNA polymerase II transcribes miRNA genes, generating long primary transcripts (pri-miRNAs) (Kim 2005). Subsequently, the process to yield mature miRNAs involves two steps involving RNase-III enzymes and companion double-stranded RNA-binding domain (dsRBD) proteins. In the nucleus, the RNase III–type enzyme Drosha processes the long primary transcripts (pri-miRNA), yielding a hairpin precursors (pre-miRNA) consisting of approximately 70 nt. The pre-miRNA hairpins are exported to the cytoplasm where they are further processed into unstable, 19-25 nt miRNA duplex structures by the RNase III protein Dicer (Sontheimer and Carthew 2005). The less stable of the two strands in the duplex is incorporated into a multiple-protein nuclease complex, the RNA-induced silencing complex (RISC), which regulates protein expression.

miRNAs have recently been shown to be definitely linked to cancer, and they can act as either oncogenes or tumor-suppressor genes in carcinogenesis. For example, miR-15a and miR-16-1 can target the anti-apoptotic BCL2, and they are often down-regulated in chronic lymphocytic leukemia (Calin, Dumitru et al. 2002; Cimmino, Calin et al. 2005).

Little is known regarding how miRNA expression is regulated in mammalian cells. Knowing that methylation can control the expression of many protein-coding genes, and miRNAs are also generally transcribed by Pol II. It is reasonable to hypothesize that methylation can play fundamental roles in controlling the miRNA expression. An exciting new discovery by Saito, et al. (Saito and Jones 2006) showed that epigenetic mechanisms, such as DNA methylation and histone modifications, can affect the expressions of miRNAs. Many miRNAs have their own promoters. Some studies suggested that CpG islands around miRNAs can act as promoters moreover miRNAs that have CpG islands upstream could be transcribed from their own promoters that are regulated by DNA methylation. Obviously, methylation of miRNA promoters may be key to determining the ability of miRNA transcription. Dys-regulation of methylation may induce the loss of essential miRNAs, and subsequent pathological developments in tissues. This scenario is mostly noted in cancer. Aberrant DNA methylation is found in the genes of mir-9-1, mir-124a3, mir-148a, mir-152, and mir-663 in many human breast cancer tissues (Lehmann, Hasemeier et al. 2008).

MICRORNA-203

miR-203 located at *chromosome 14 (*14q32.33*)*.

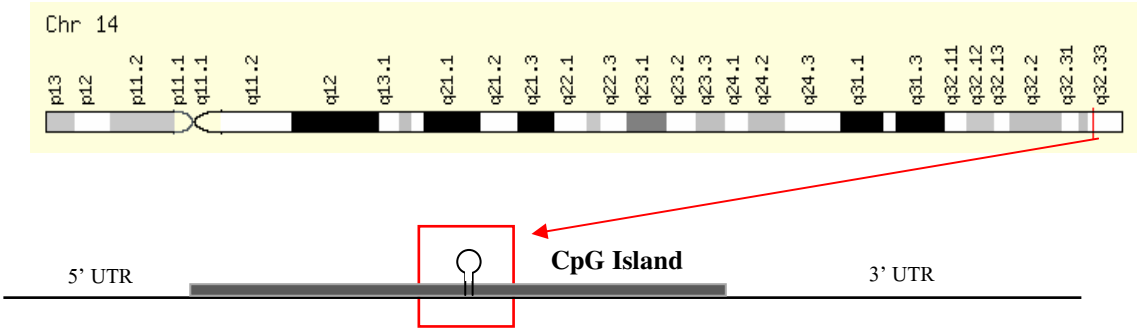


Figure 9 Schematic representation of human miR-203 gene and the pre-miR-203 in hairpin form. Black box shows CpG Island around mir-203 gene.

The study by Sonkoly, et al showed that the psoriasis-associated miRNAs identified in the skin showed different expression profiles among 21 organs studied. miR-203 was expressed more than 100-fold higher in skin compared to other organs, and at lower levels in organs with squamous epithelium. Moreover, in psoriatic plaques, up-regulated miR-203 was shown to be involved in down regulation of an evolutionary conserved target, SOCS3. This protein is involved in inflammatory responses and keratinocyte proliferation and differentiation in psoriasis patients(Sonkoly, Wei et al. 2007) .

Kozaki, et al also showed that miR-203 was decreased by aberrant alterations in DNA methylation in oral squamous cell carcinoma (OSCC) cell lines. The treatment with 5-aza-dCyd restored these expression levels in cells lacking their expressions. The expression level of the miR-203 was inversely correlated with their DNA methylation status in the OSCC lines. In primary tumors of OSCC compared with normal mucosa found that miRNA-203 expression was down-regulated through tumor-specific hypermethylation (Kozaki, Imoto et al. 2008). Other studies, this microRNA is additionally hypermethylated in several tumors, such as chronic myelogenous leukemias

and some acute lymphoblastic leukemia (Bueno, Perez de Castro et al. 2008), hepatocellular carcinoma (Furuta, Kozaki et al. 2009) and epithelial ovarian cancer (Iorio, Visone et al. 2007).

However, there is no report about mechanism which induces abnormal miR-203 expression in psoriasis.

S-adenosylmethionine decarboxylase (AMD1, AdometDC)

AMD1 gene located at chromosome 6 (6q21). AMD1 gene has six exons and five introns. The protein-coding sequence of AMD1 is S-adenosylmethionine decarboxylase (AdometDC)

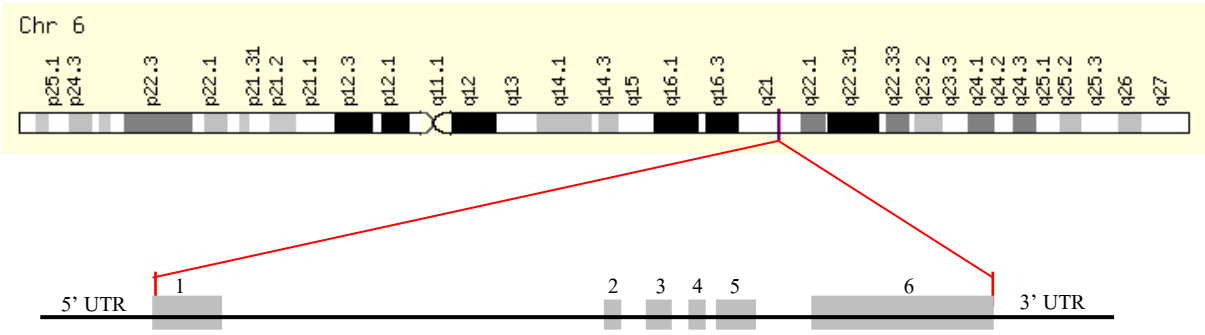


Figure 10 Schematic representation of human AMD1. Gray boxes in the genes denote exons; numbers above each gene are exon numbers; single lines in the genes indicate introns and untranslated.

S-adenosylmethionine decarboxylase (Ado-MetDC) is a critical enzyme in the polyamine biosynthetic pathway and a subject of many structural and biochemical investigations for anti-cancer and anti-parasitic therapy. The enzyme undergoes an internal serinolysis reaction as a post-translational modification to generate the active site pyruvoyl group for the decarboxylation process. AdoMetDC is at a critical initiation point in the polyamine biosynthetic pathway. The polyamines, spermidine and spermine are aliphatic cations critical for cell growth, differentiation and proliferation. AdoMetDC catalyzes the conversion of S-adenosylmethionine (AdoMet) to S-adenosyl-50-(3-methylthiopropylamine) (dcAdoMet) (Figure 11), which is completely committed to polyaminebiosynthesis. Thus, the expression and activity of AdoMetDC are closely related to the cellular concentration of the polyamines. In addition, the activity of

AdoMetDC depletes the cellular pool of AdoMet for other functions such as methylation of DNA and other essential reactions. The polyamine biosynthetic machinery is upregulated in various types of cancer and parasitic diseases(Bale and Ealick 2010).

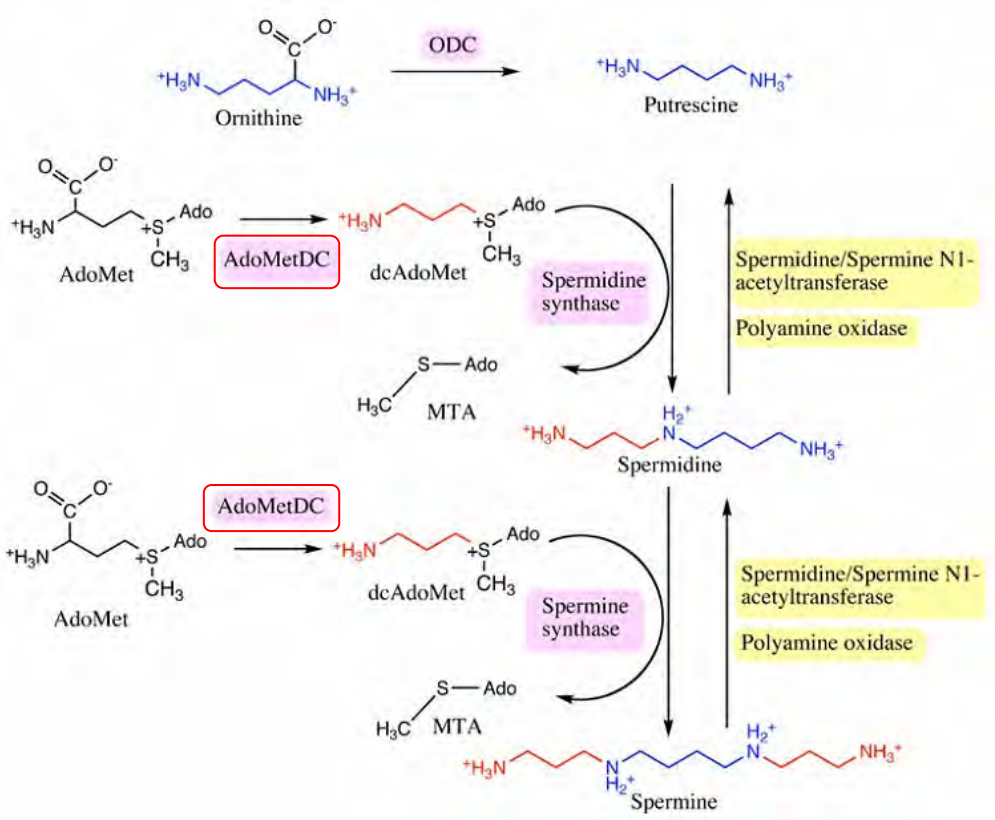


Figure 11 Overview of the polyamine biosynthetic pathway

In psoriasis, Bowcock et al, and Zhou et al, studied the expression profiles from uninvolved and lesional psoriatic skin by microarray show that AMD1 mRNA is higher upregulation in lesional psoriatic skin (Zhou, Krueger et al. 2003; Reischl, Schwenke et al. 2007). Moreover AMD1 gene located at chromosome 6 (6q21) which it's same chromosome with *PSORS1*. The *PSORS1* locus on 6p21.3 is a well-confirmed major susceptibility locus for psoriasis. Even though there are no reports in the literature associating 6q with human psoriasis, Wang et al, showed that PSD1 locus

on chromosome 10 as a verified genetic element contributing to both the psoriasiform skin disease and arthritis in the CD18hypomurine model. The PSD1 on murine chromosome 10 is syntenic to human chromosome 6q16 and 6q21–q24 (Wang, Kess et al. 2008). Moreover, the human chromosome region 6q21 was identified to be one of the major type 1 diabetes mellitus loci, an autoimmune disease (Perez De Nanclares, Bilbao et al. 2000), and it also harbors susceptibility genes for rheumatoid arthritis (Jawaheer, Seldin et al. 2001; MacKay, Eyre et al. 2002; Jawaheer, Seldin et al. 2003).

Hypermethylation of CpG-rich regions (CpG islands) in promoter sequences is an important mechanism for the silencing of gene expression in cancers. The AMD1 promoter region also contains CpG islands. However, there is no study reported a potential correlation between AMD1 expression and promoter methylation in psoriasis.

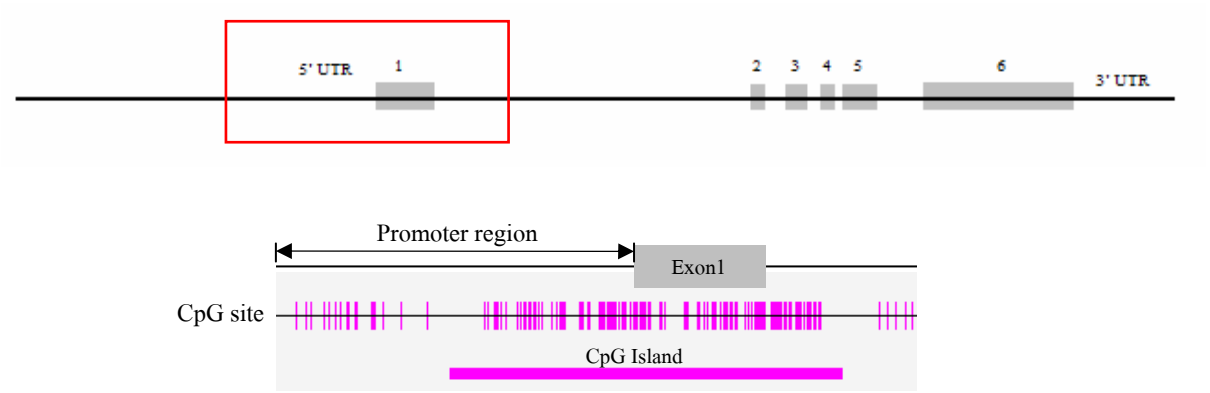


Figure 12 Map of AMD1 gene and CpG Island: Dark pink box indicate CpG islands; vertical tick marks indicate CpG sites; horizontal bars with arrowheads indicate promoter region

We hypothesized that the up-regulation of AMD1 expression is mediated by the demethylation of AMD1 promoter.

B-cell receptor associated protein 31 (BCAP31)

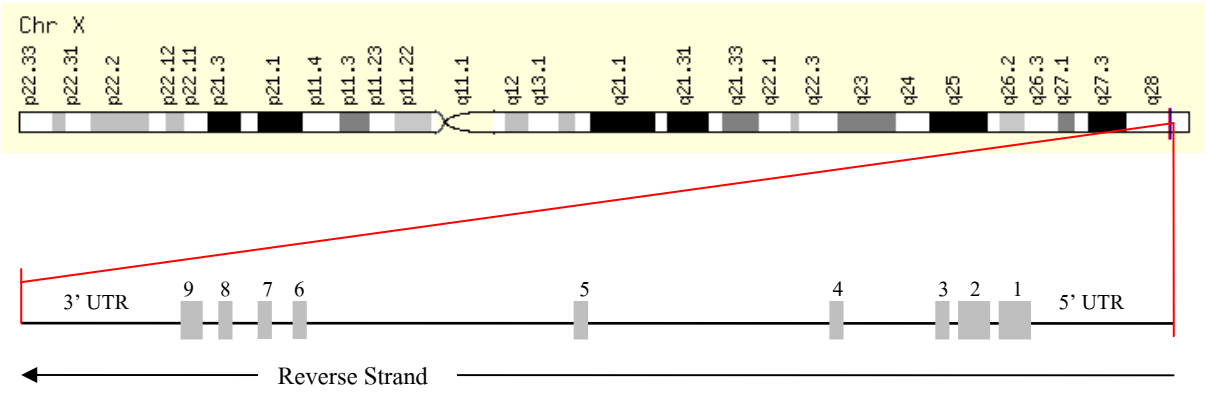


Figure 13 Schematic representation of human BCAP31. Gray boxes in the genes denote exons; numbers above each gene are exon numbers; single lines in the genes indicate introns and untranslated; horizontal bars with arrowheads indicate reverse direction to transcription.

By somatic cell hybrid analysis (Mosser, Sarde et al. 1994) the BCAP31 gene mapped near the ALD gene on chromosome Xq28. The BCAP31 genes show transcribed from CpG island in reverse direction. Microdeletions in this gene are associated with the contiguous ABCD1/DXS1375E deletion syndrome (Corzo, Gibson et al. 2002). Alternatively spliced transcript variants encoding distinct four isoform have been described, although, the biological validity of some of the variants has not been determined. BCAP31 is a multi-pass transmembrane protein of the endoplasmic reticulum that is involved in the anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi (Nguyen, Breckenridge et al. 2000; Wang, Nguyen et al. 2003). BCAP31 is associated with human class I MHC molecules, and that overexpression of BCAP31 increases the amount of class I on the cell surface, in a dose-dependent manner (Ladasky, Boyle et al. 2006; Zhang and Williams 2006). Furthermore Spiliotis, et al showed that mouse class I molecules can bind BCAP31 that

it is the putative cargo receptor (Spiliotis, Manley et al. 2000). Previous findings also suggest that BCAP31 may take part in the control of programmed cell death. Human BCAP31 can bind Bcl-2/Bcl-xL, caspase-8 pro-enzyme, and Ced-4 (Ng, Nguyen et al. 1997). Psoriatic keratinocytes and lymphocyte possess an enhanced ability to resist apoptosis, which might be one of the key pathogenetic mechanisms in psoriasis. However, up to now limited data exist regarding the underlying mechanisms of this defect in the apoptosis control mechanisms of psoriatic keratinocytes and lymphocyte. Previous studies showed increased levels antiapoptotic, Bcl-xL and Survivin in psoriatic skin (Gilhar, Yaniv et al. 2006; Abdou and Hanout 2008). Meanwhile, Yildiz et al, observed an over-expression of Bcl-2 in the lymphocytes of psoriatic skin that related to the prolonged survival of lymphocytes resulting in the relapsing and chronic characters of psoriasis (Yildiz, Baris et al. 2003).. Su et al, studied the expression profiles from various cell types by microarray showed that BCAP31 mRNA is higher up-regulation in most of normal hematopoietic cells (Su, Wiltshire et al. 2004).A major clinical feature of patients with psoriasis is migration of leukocyte into the skin. To date, there are no any reports about BCAP31 psoriasis.

The BCAP31 promoter region contains also CpG islands.

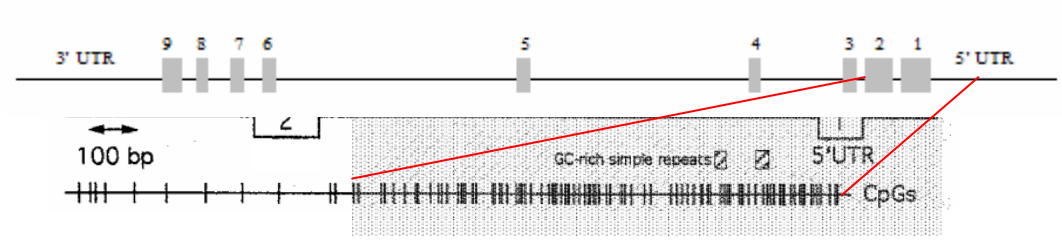


Figure 14 Map of BCAP31 gene and CpG Island: The BCAP31 gene is schematically represented and corresponds to the x-axes. Boxes indicate exons with numbers in order of transcription. On the x-axes of the panels the genomic sequence of BCAP31 is given in base pairs. The distribution of CpG pairs is represented by vertical lines. The shaded box in the background depicts a predicted CpG island (Grunau, Hindermann et al. 2000).

Grunau, et al, study the methylation patterns of X-linked genes in two adult individuals and across nine different tissues (white cerebral matter in brain, heart, kidney, liver, prostate, pancreas, lung, skeletal muscle and testis) using bisulfite genomic sequencing. The methylation profiles of the BCAP31 gene are tissue specific. The level of expression and the degree of methylation appear to be correlated (Grunau, Hindermann et al. 2000).

However, there is no study reported BCAP31 promoter methylation in psoriasis. So we investigate correlation between BCAP31 expression and BCAP31 promoter methylation.

CHAPTER IV

METERIALS AND METHODS

Materials

1. Cell lines

   Hematopoietic cell lines: Duadi, Jurkat, HL-60, Molt4, and K562

   Epithelial cell lines: Hela, SW480, Hep2, HepG2, UACC903, HaCaT, RKO Caski, SIHA, TSU, HSC2, HSC7, HCT16, OM1, and OM2
2. E.coli DH5α competent cells
3. pGEM-T easy vector (Promega)
4. GeneJET™ Plasmid Miniprep Kit (Fermentas)
5. DNA Extraction kit (QIAGEN)
6. QIAquick Gel Extraction Kit (QIAGEN)
7. ImProm-II™ Reverse Transcription System (Promega)
8. EZ DNA Methylation-Gold™ Kit (Zymo Research)
9. MicroAmp PCR Tube (AxyGen®Scientific, USA)
10. Microcentrifuge tube: 0.5 and 1.5 ml (AxyGen®Scientific, USA)
11. LightCycler Capillaries: 20 µl. (Roche)
12. Polypropylene conical tube 15 and 50 ml (AxyGen®Scientific, USA)
13. Pipette tip: 10, 100, 200 and 1000 µl (AxyGen®Scientific, USA)
14. Cryotube (Nunc, USA)
15. Glassware: Beaker, Flask, Cylinder and reagent bottles (Pyrex)
16. Tissue Culture Flask, Culture plate, Sterile serological pipette 1,5 and 10 ml (Costar, USA)
17. Glass slides
18. Blood collection tube with sodium heparin 6 ml and K2EDTA 4 ml (Vacuette, Greiner Bio-One)
19. Hematocytometer

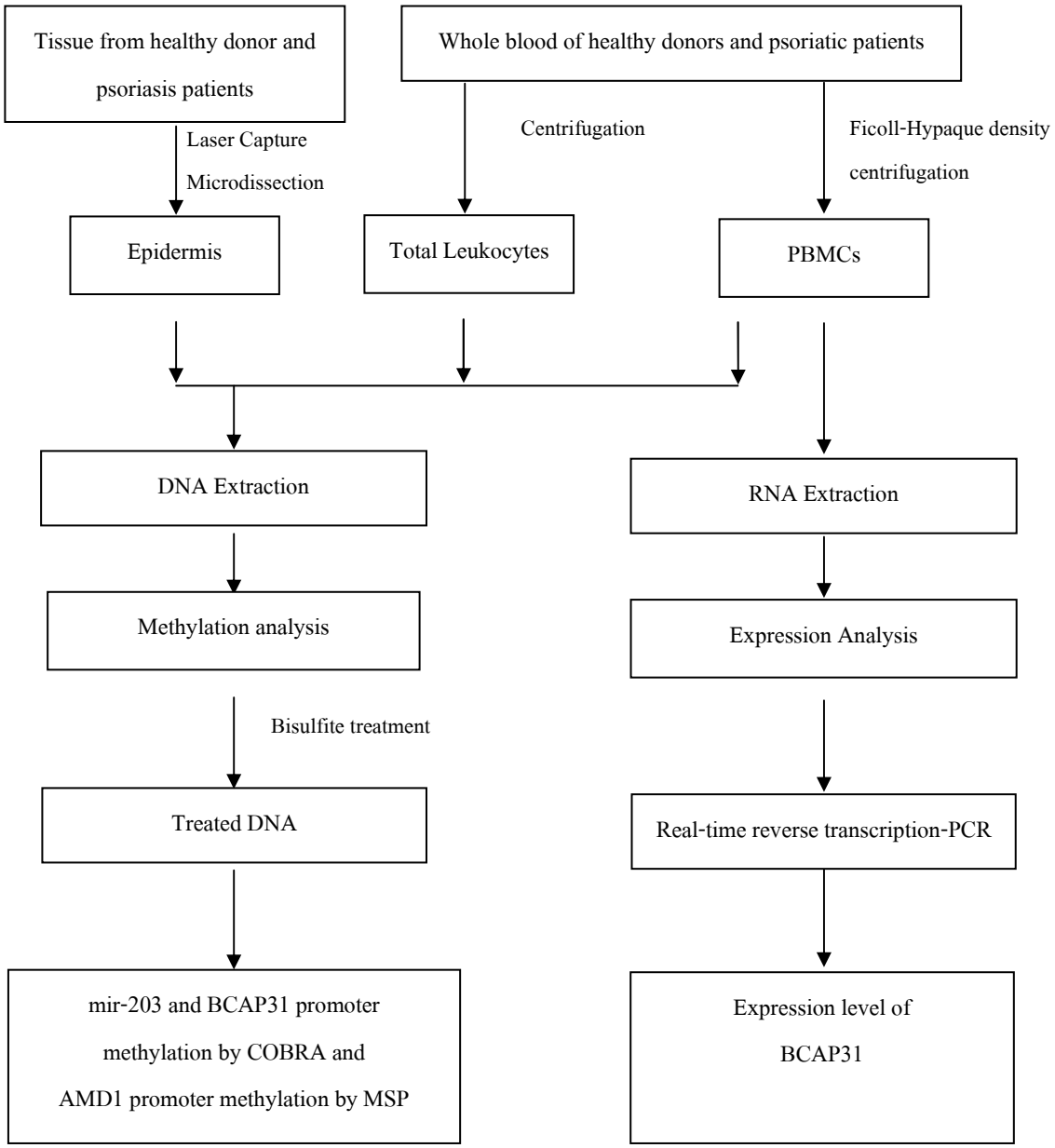
Equipment

1. Laser Capture Microdisecsion (LCM)
2. Centrifuge
3. Refrigerated microcentrifuge
4. -80°C freezer
5. -20°C freezer
6. Light microscopy
7. DNA thermal cycler
8. LightCycler 2.0 Real-time PCR Instrument
9. Spectrophotometry
10. CO_2 humidified incubator
11. Autoclave
12. Hot air oven
13. Multi-block heater
14. Microwave oven
15. Water bath (Mammert, West Germany)
16. Gel documentation Bio-Rad (Gel doc 1000)
17. PhosphoImager using Image Quant software (Molecular Dynamics)

Reagent

1. Agarose gel(FMC Bioproduct, USA)
2. Ethidium bromide (Sigma, USA)
3. Powder LB media (Lennox)
4. Ampicillin antibiotic
5. 5-Bromo-4-Chloro-3-Indolyl-β-D-galactopyraniside (X-Gal) (USB)
6. Isopropyl-β-D-Thiogalactopyranoside (IPTG) (USB)
7. TRIzol Reagent (Invitrogen)
8. Chloroform
9. Isopropyl alcohol
10. 75% Ethanol (in DEPC-treated water)
11. RNase-free water
12. Isoprep (Robbins Scientific Corporation)
13. Polymorph Prep (Nycomed, Oslo, Norway)
14. Dimethyl sulfoxide (DMSO) ()
15. Phosphate-buffered saline (PBS) (Sigma, USA)
16. RPMI culture medium (Gibco)
17. Fetal Bovine Serum (Gibco)
18. Trypan Blue (0.8 mM in PBS)
19. Fast start Taq DNA polymerase (Roche) for all PCR in this study
20. BSTU I restriction enzyme (NEB)

Methodology



Subject

Inclusion criteria

The following inclusion criteria will be used to determine eligibility for this study:

1. Aged 18 years or older
2. Patients were diagnosed clinically and pathologically by an experienced dermatologist.
3. Ability to understand and sign an informed consent form

Exclusion criteria

The following exclusion criteria will be used to determine ineligibility for this study:

1. Patients receive any skin therapies 4 week prior to taking biopsy sample.
2. Immunocompromised patients
3. Patients receive Corticosteroids or immunosuppressive drugs.
4. Patients who had been diagnosed other autoimmune diseases.
5. Patients who had been diagnosed Cancers.

Healthy controls

Inclusion criteria

The following inclusion criteria will be used to determine eligibility for this study:

1. Donor healthy controls
2. Aged 18 years or older
3. Ability to understand and sign an informed consent form

Exclusion criteria

The following exclusion criteria will be used to determine ineligibility for this study:

1. Donor healthy controls who had been diagnosed other autoimmune diseases.
2. Donor healthy controls who had been diagnosed Cancers.
3. Donor healthy controls with any skin treatments

This study was approved by the ethics committee of the King Chulalongkorn University and the subjects gave their informed consent.

Sample Size

<u>miR-203 promoter methylation analysis by COBRA</u>

To date, methylation of microRNA in psoriasis miR-203 promoter methylation study in Oral squamous cell carcinoma (OSCC) showed the difference of mir-203 promoter methylation in primary OSCC tumors (n=11) and corresponding noncancerous oral mucosa (n=11). In total miR-203 promoter methylation was found in 18.18% (2/11) of primary OSCC tumors and 0% (0/11) of corresponding noncancerous oral mucosa. Thus the sample size was calculated by

$$n/group = \left(Z_{\alpha/2} \sqrt{2P_cQ_c} + Z_{\beta} \sqrt{(P_tQ_t + P_cQ)} \right)^2 \Big/ (P_t-P_c)^2$$

When

α = 0.05

β = 0.10

$Z_{\alpha/2}$ = 1.96 (two-tails)

Z_{β} = 1.28

Pt = Risk of miR-203 promoter methylation in primary OSCC tumors (2/11) = 0.1818

Q_t = 1- P_t = 1- 0.1818= 0.8182

Pc = Risk of mir-203promoter methylation in corresponding noncancerous oral mucosa (0/11) = 0

Q_c = 1- P_c = 1-0 = 1

n/group = 6.381 ~6

Since we calculated the sample size from Oral squamous cell carcinoma study that showed a strong significant between primary OSCC tumors and corresponding noncancerous oral mucosa. Sample size calculation showing to samples number in each group (n=6).

Sample preparation

1. Total leukocytes

Buffy coat were prepared from EDTA blood collection tube (4 ml). The blood was centrifuged for 10 min at 1,500 g. The interface between the plasma and red blood cells, which contains the leukocytes, was collected to a new polypropylene tube. The buffy coat was added with 10 volumes of cold red cell lysis buffer. The tube was centrifuged for 10 min at 1000g, then remove supernatant.

2. Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were prepared by Ficoll-Hypaque density centrifugation using IsoPrep (Robbins Scientific Corporation). Briefly, whole blood sample carry in sodium heparin tube was diluted by an equal volume of RPMI 1640 medium. The diluted blood was carefully layered over IsoPrep and centrifuged at 2000 rpm for 30 min. The PBMCs at the sample and IsoPrep interface were carefully transferred to a new polypropylene tube and diluted with medium. After centrifugation at 1500 rpm for 10 min, the cell pellets were resuspended with medium.

3. Cell Culture

Hela (cervical cancer), K-562 (erythroleukemia), SW480 (colorectal adenocarcinoma), Hep2 (human, Caucasian, larynx, carcinoma), HepG2 (human hepatocellular liver carcinoma), RKO (human colorectal), Caski (Human cervical carcinoma), SIHA (human cervical tumor), UACC903 (parental malignant melanoma), HaCaT (Human keratinocyte) and oral squamous cell carcinoma (TSU, HSC-2, HSC-7, HCT16, OM1 and OM2) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Paisly, UK) (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Sigma, St Louis, MO) and antibiotics (50U/ml penicillin, 50µg/ml streptomycin). Daudi (B lymphoblast), Jurkat (T cell leukemia), HL-60 (human promyelocytic leukemia) and Molt4 (T lymphoblast) cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Sigma,

St Louis, MO) and antibiotics (50U/ml penicillin, 50µg/ml streptomycin). All cells were grown in a humidified atmosphere at 37°C with 5% CO_2.

4. Paraffin embedded tissue samples

Paraffin embedded tissues from patients with psoriasis and normal control were received from Chulalongkorn Hospital. These tissues were obtained from psoriasis vulgaris (9 cases) and normal skin from plastic surgery (6 cases). All paraffin embedded tissue will be separated the area of epidermis using Laser Capture Microdissection (LCM). The paraffin was removed with xylene at room temperature (23-25 °C) for 5 min twice times, then washing them with (100%) absolute ethanol for 5 min twice times, 90% ethanol and 70% ethanol for 5 min each. After being air dried, the areas of interest were micro-dissected using Laser Capture Microdissection (LCM). The microdissected tissue fragments were kept in sterile tube.

The area of epidermal tissues (microdissected tissues), PBMCs, Total leukocytes and cell lines were extracted the DNA using QIAgen DNA mini kit.

DNA extraction

The procedure is follow by QIAamp® DNA mini and Blood mini Handbook. (In brief)

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 cells in 200 µl PBS.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15s
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6. Add 200 µl of ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Bisulfite treatment DNA

Extracted DNA amount 500 ng was modified by sodium bisulfite using the EZ DNA methylation Kit™ (Zymo Research, Orange, CA, USA). Bisulfite treatment converts unmethylated but not methylated, cytosines to uracil.

Procedure is follow by EZ DNA methylation Kit™ Handbook (In brief)

1. Preparation of CT Conversion Reagent.

The CT Conversion reagent supplied with this kit is a solid chemical mixture; therefore it is necessary to prepare it before use. Prepare the CT Conversion Reagent by adding 750 µl of water and 210 µl of M-Dilution Buffer to one tube of CT Conversion Reagent and then mix by vortexing 1 – 2 minutes for a total of 10 minutes. The CT Conversion Reagent is saturated, so it is normal for not all of to be dissolved. Store the prepared CT Conversion Reagent away from light at room temperature (20 °C – 30 °C) until use. Each tube of CT Conversion Reagent is designed to treat 10 DNA samples. The amount of DNA per treatment can range from500 pg to 2 µg. For best results the prepared CT Conversion Reagent should be used immediately. If the prepared CT Conversion reagent will not be used for over one hour then the prepared CT Conversion reagent can be stored for up to one week at – 20 °C, but the CT Conversion reagent must be thawed at room temperature and vortexed for 2 minutes prior to use.

2. Preparation of M-Wash Buffer.

Add 24 ml (96 ml for EZ DNA Methylation Kit 200 reaction size (D5002)) of 100% ethanol to the M-Wash Buffer Concentrate to make final M-Wash Buffer.

Protocol

1. Add 5 µl of M-Dilution Buffer to the DNA Sample and adjust the total volume to 50 µl with sterile H2O. Mix the sample by flicking or pipetting up and down.

2. Incubate the Sample at 37 °C for 15 minutes.

3. After the above15 minute incubation, add 100 µl of the prepared CT Conversion Reagent to each sample and lightly vortex.

4. Incubate the sample in the dark at 50 °C for 12 – 16 hours. (The CT Conversion reagent is light sensitive, so try to minimize the reaction's exposure to light whenever possible.)

5. Incubate the sample on ice for 10 minutes.

6. Add 400 µl of M-Binding Buffer to the sample and mix by pipetting up and down.

7. Load sample into a Zymo-Spin I Column and place column into a 2 ml collection tube.

8. Centrifuge at full speed (>10,000 g) for 15 – 30 seconds. Discard the flow-through.

9. Add 200 µl of M-Wash Buffer to the column. Spin at full speed for 15 – 30 seconds.

10. Add 200 µl of M-Desulphonation Buffer to the column and let column stand at room temperature (20 °C – 30 °C) for 15 minutes. After the incubation, spin at full speed for 15 – 30 seconds.

11. Add 200 µl of Wash Buffer to the column. Spin at full speed for 15 – 30 seconds. Add another 200 µl of M-Wash Buffer and spin at top speed for 30 seconds.

RNA extraction

RNA was extracted from cells by TRIzol Reagent (Invitrogen).

1. Lyse pellet cells in TRIZOL Reagent by repetitive pipetting to fully homogenesis the lysate. Use 1 ml of the reagent per 5-10 × 10^6 cells.

2. Incubate the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

3. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at room temperature for 2 to 3 minutes.

4. Centrifuge the samples at 12,000 × g for 15 minutes at 4°C.

5. The mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

6. Transfer the aqueous phase to a fresh tube.

7. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent.

8. Incubate samples at room temperature for 10 minutes and centrifuge at 12,000 × g for 10 minutes at 4°C.

9. Wash the RNA pellet once with 1 ml of 70% ethanol.

10. Centrifuge at 7,500 × g for 5 minutes at 4°C.

11. Briefly dry the RNA pellet by air-dry and dissolve RNA in RNase-free water.

Reverse transcription reaction and Real-time quantitative PCR assay

The single stranded cDNA synthesis by the reverse transcription. The reaction was performed in the final volume of 20 μl, at 25°C for 5 minutes, 42 °C for 60 minutes and 70°C 15 minutes using Improm-II reverse transcription kit (Promega) (1 U of Improm-II reverse transcription, 2 μl of 1x Improm-II reaction buffer, 25 mM $MgCl_2$, 10mM dNTP mix, and 0.5 μg Oligo dT).

Real-time quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen) on LightCycler 2.0 Real-time PCR (Roche) according to manufacturer's instruction. The primers for BCAP31 in q PCR are listed in Table 1. Briefly, 1 ng of cDNA was added to the reaction mixtures (20 μl) containing 1x QuantiTect SYBR Green PCR Master Mix (dNTP, HotStarTaq DNA Polymerase, and 5 mM $MgCl_2$), 0.3μM forward and reverse primers. Reaction mixtures were loaded in glass capillary cuvets (Roche) and were centrifuged to place the sample at the capillary tip. After an initial denaturation step at 95°C for 15 min, amplification was performed by using 40 cycles of denaturation (95°C for 30 sec), annealing (605°C for 60 sec), and

extension (72°C for 60 sec). The temperature transition rates were 20°C/sec from denaturation to annealing, annealing to extension, and extension to denaturation. Fluorescence was measured at the end of the extension period of each cycle to monitor the concentration of amplicon. After amplification was complete, a final melting curve was recorded by heating to 95°C for <1 sec and then cooling to 65°C at 0.1°C/sec, followed by a 60 sec hold before heating slowly at 0.1°C/sec until a temperature of 95°C was attained. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of SYBR Green I Reporter Dye. Copy numbers were quantitated by generating a standard curve. Housekeeping gene (GAPDH) was measured to normalize expression of the target gene (BCAP31).

Table 1 Primers used for BCAP31 (Devauchelle, Marion et al. 2004) and 18s Real-time quantitative PCR.

Gene	Product size (bp)	Tm °C	Name of Primer	Sequence
18s	150	60	18s sense	5-GCC CGA AGC GTT TAC TTT GA-3
			18s antisense	5-TCC ATT ATT CCT AGC TGC GGT ATC-3
			18s probe	5-FAM AAA GCA GGC CCG AGC CGC C TAMRA-3
BCAP31	190	60	BCAP31_F	5-GAAGAGAACAGGAGCCTGAA-3
			BCAP31_R	5-CCATGGGACCATCTACTGCA-3

Bisulfite-modification and Combined Bisulfite -Restriction analysis (COBRA) Method

miR-203, bisulfite oligonucleotide sequences was derived from Genbank. Genomic DNA was treated with sodium bisulfite and subjected to PCR using primer sets designed by Kozaki's study(Kozaki, Imoto et al. 2008). In brief, Bisulfite-modification and Combined Bisulfite Restriction analysis (COBRA) were performed as 500ng of genomic DNA was bisulfite-treated using the EZ DNA methylation Kit™ (Zymo Research, USA) according to the manufacturer's specifications. For COBRA, 2μL of modified DNA was amplified using primers list in table2. After a hot start, 40 cycles of 94°C for 60 s,60°C for 60 s, and 72°C for 60 s, and 72°C for 7 min. After amplification, 8.5 μL of PCR products were digested with the restriction enzyme *BstUI* (NEB), which recognize sequences unique to the methylated and bisulfite-converted alleles. Each reaction will be incubated overnight at 60°C and then DNA fragments will be separated in 8% polyacrylamide gels. The intensity of DNA fragments was measured with PhosphoImager using Image Quant software (Molecular Dynamics).

BCAP31, 500 ng genomic DNA Genomic DNA was treated with sodium bisulfite (Zymo Research, USA) and subjected to PCR using primer sets designed to amplify regions of interest by Grunau's study (Table 1) (Grunau, Hindermann et al. 2000). For the combined bisulfite restriction analysis (COBRA) 2.5μL of bisulfite DNA conversion was performed using the following first touchdown PCR conditions then 2.5μL of first PCR product were used as template for the second touchdown PCR. First touchdown PCR conditions was 94 °C for 2 min followed by 5 cycles (94°C for 1 min, 50°C for 2 min, 72°C for 3 min), 25 cycles (94°C for 1 min, 50°C for 2 min, 72°C for 1.5 min) and 72 °C for 10 min. Second touchdown PCR conditions was 94 °C for 2 min followed by 5 cycles (94°C for 1 min, 40°C for 2 min, 72°C for 3 min), 25 cycles (94°C for 1 min, 50°C for 2 min, 72°C for 1.5 min) and 72 °C for 10 min.After amplification, 8.5 μL of PCR products were digested with the restriction enzyme *BstUI* (NEB), which recognize sequences unique to the methylated and bisulfite-converted alleles. Each reaction will be incubated overnight at 60°C and then DNA fragments will be separated in 8% polyacrylamide gels. The intensity of DNA fragments was measured with PhosphoImager using Image Quant software (Molecular Dynamics).

Methylation analysis of the promoter region by MSP method

AMD1, bisulfite oligonucleotide sequences was derived from Genbank. DNA methylation patterns in the AMD1 promoter were determined by methylation-specific-PCR. In brief, this method distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA. Primers are designed to distinguish methylated from unmethylated DNA in bisulfite-modified DNA. After extraction, all genomic DNA samples were treated with sodium bisulfite (Zymo Research, Orange, CA, USA), according to the manufacturer's specifications. The methylation status of AMD1 promoter was determined by methylation specific PCR (MSP) which MSP primers specifically recognized the methylated or unmethylated DNA after bisulfite conversion. Methylation-specific primer sequences were 5' -GAG-TCG-GTT-AGA-GTT-CGA-GTC- 3' and 5'- CCA-AAA-AAT-AAC-GCA-TCG-T- 3' , and unmethylation-specific primer sequences were 5' -TAG-GAG-TTG-GTT-AGA-GTT-TGA-GTT-3' and 5' -CCA-AAA-AAT-AAC-ACA-TCA-TCC-C- 3'. Both of amplification products, 149 bp were visualized by UV illumination on 2% agarose gel that contained ethidium bromide.

Table 2 Primers used for analysis miR-203 (Kozaki, Imoto et al. 2008), AMD1 and BCAP31 promoter methylation (Grunau, Hindermann et al. 2000).

Gene	Product size (bp)	Tm °C	Name of primer	Sequence
miR-203	287	60	MIR203F3	5- GTTGTAGTAGGGTAGGGGGT-3
			MIR203R3	5- ACCCCTAACTATAACTCTAACTCCA-3
BCAP31	987	50/50	BCAP31COF	5-GTAGGGTTTTTTGGTTAGTAG-3
			BCAP31COR	5-ACTACTATAAAAAAATTCATTAC-3
	494	40/50	BCAP31CIF	5-ATTTTTAGAGGGTAGGATT-3
			BCAP31CIR	5-ACTACTATAAAAAAATTCATTAC-3
AMD1	149	60	AMD1FM2	5-GAGTCGGTTAGAGTTCGAGTC-3
			AMD1RM2	5-CCAAAAAATAACGCATCGT-3
	149	60	AMD1FU2	5-TAGGAGTTGGTTAGAGTTTGAGTT-3
			AMD1RU2	5-CCAAAAAATAACACATCATCCC-3
	444	56	AMD1SEF	5-ATGTTTTTTGTTAATTATTTTTTTTTTT-3
			AMD1SER	5-ATAAAAACAAATACAATTCAATCTCT-3

Cloning and Sequencing

The different band of amplicons was cut and extracted from agarose gel using Qiagen Gel Extraction Kit (Qiagen) then ligated to pGEM-T easy cloning vector (promega) and transformed to E.coli DH5α competent cells. Plate the transformation reaction onto ampicillin LB agar plate containing X-Gal and IPTG, incubated at 37°C overnight. The white bacterial colonies were selected and colonies were then cultured in LB broth containing ampicillin overnight and extracted plasmid by miniprep using GeneJET™ Plasmid Miniprep Kit (Fermentas). For sequencing Analysis, the purified recombinant plasmid (pGEM-T easy containing interested insert PCR products were used for the sequencing reaction using Prism Ready Reaction DyeDeoxy Terminator FS Cycle Sequencing Kit (Applied Biosystem, Inc., USA) according manufacturer's instruction. The DNA template was mixed with 8 µL of Prism Terminator Mix, 3.2 pmol of

primer M13 and distilled water was added to bring the final volume to 20 µL per reaction. The sequencing reaction was subjected to 25 PCR cycles, each consisted of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 min in a thermalcycler (GeneAmp PCR system 24000).

Then sequencing reaction was purified using simplified ethanol precipitation. Then microliters of sequencing reaction was added with 2 µL of 3M Sodium acetate (NaOAC), 50µL of 95% ethanol, vortexed briefly, and stood at room temperature in the dark for 15 min. The precipitate of sequencing reaction was centrifuged at 13,000 g at room temperature in the dark for 15 min, and the pellet was washed with 250 µL of 70% ethanol. The suspension was centrifuged at 13,000 g at room temperature for 5 min, and the pellet was dried for 1 min at 90°C for 1 min in a Dri-bath. The pellet could be kept at -20°C for 1 week.

The pellet was subjected to sequence analysis by ABI Prism 310 Genetic Analyzer (PE-Applied Biosystems). Regarding the rest of subsequent steps, we referred to the ABI Prism 310 Genetic Analyzer user's manual. The nucleotide sequences were analyzed with Sequence Analysis Software, which analyzed the electrophroregram pattern in comparision with the matrix file.

Statistical analysis

Student's t-test were used to determine the statistical significance of data.

CHAPTER V

RESULT

Mir-203 methylation in psoriasis by COBRA

In the present study, the level of *mir-203* methylation was analyzed by COBRA as previously described in method. Maps of miRNAs, CpG islands, CpG sites, and PCR products used for COBRA are shown in Figure 15 We examined methylation pattern in OSCC cell lines to indentify positive control (Figure 16). Next, we determined the methylation profile of miR-203 in microdisected keratinocytes from psoriasis patients (n=9) and microdisected keratinocytes from normal (n=6). There are no statistically significant in level of *mir-203* methylation in psoriasis, compared to normal controls. (Figure 17 and 18)

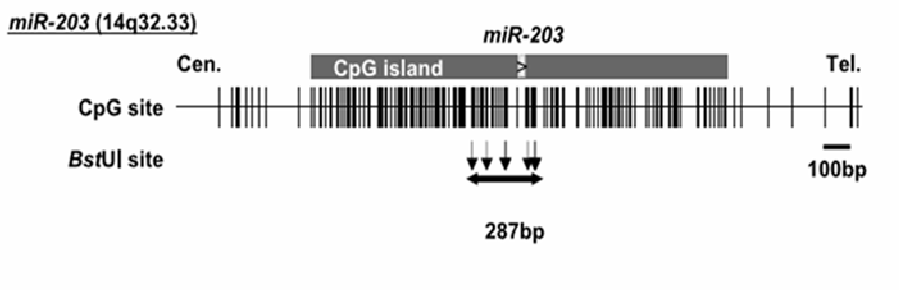


Figure 15 Maps of miRNAs, CpG islands, CpG sites, and PCR products used for COBRA *Dark gray box*, CpG islands; *light gray box*, miRNAs; *vertical tick marks*, CpG sites; *horizontal bars with arrowheads*, PCR product; *vertical arrows*, restriction enzyme sites. PCR product sizes (*horizontal arrows*) are as follows miR-203 region, 287 bp (restricted by BstUI)

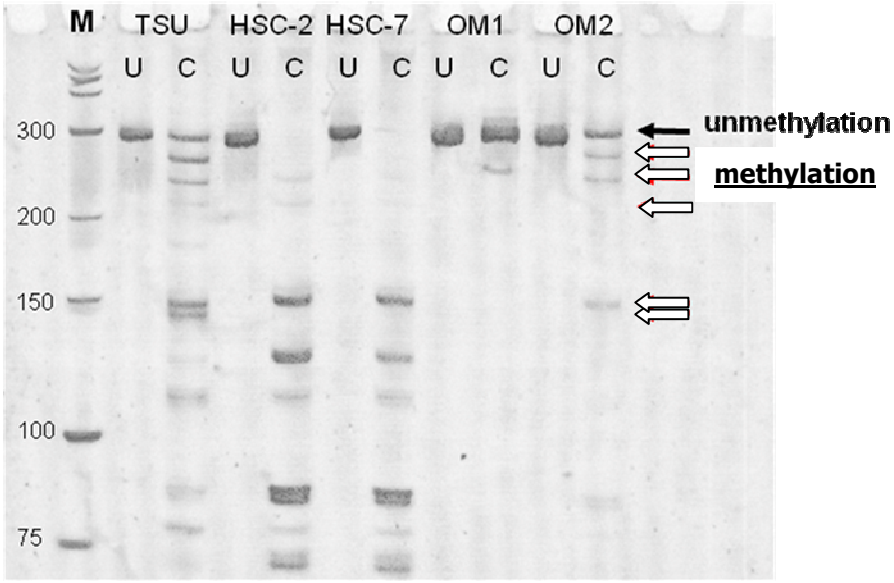


Figure 16 the results of COBRA in OSCC cell lines. *Black arrows*, unmethylated alleles; *white arrow*, methylated alleles; *U* , uncut PCR products by restriction enzyme; *C* , cut PCR product by restriction enzyme; *M*, DNA marker, Unmethylated band; 287 bp, Methylated band; 265 bp, 230 bp, 145 bp, 132 bp

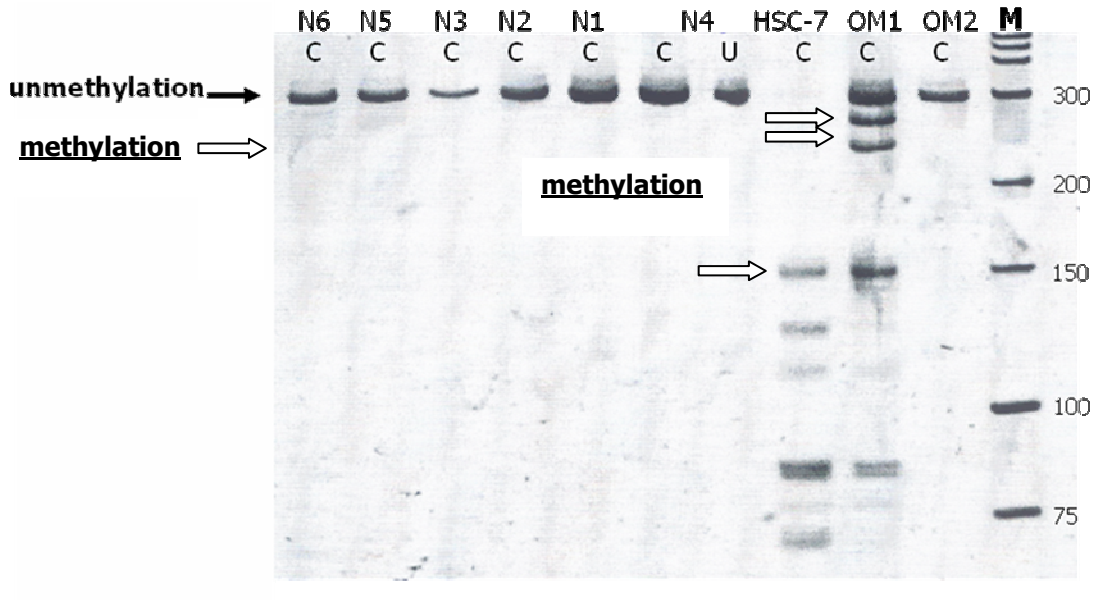


Figure 17 the results of COBRA in normal keratinocytes (n=6). *Black arrows*, unmethylated alleles; *white arrow*, methylated alleles; *U* , uncut PCR products by restriction enzyme; *C* , cut PCR product by restriction enzyme; *M*, DNA marker. , Unmethylated band; 287 bp, Methylated band; 265 bp, 230 bp, 145 bp, 132 bp

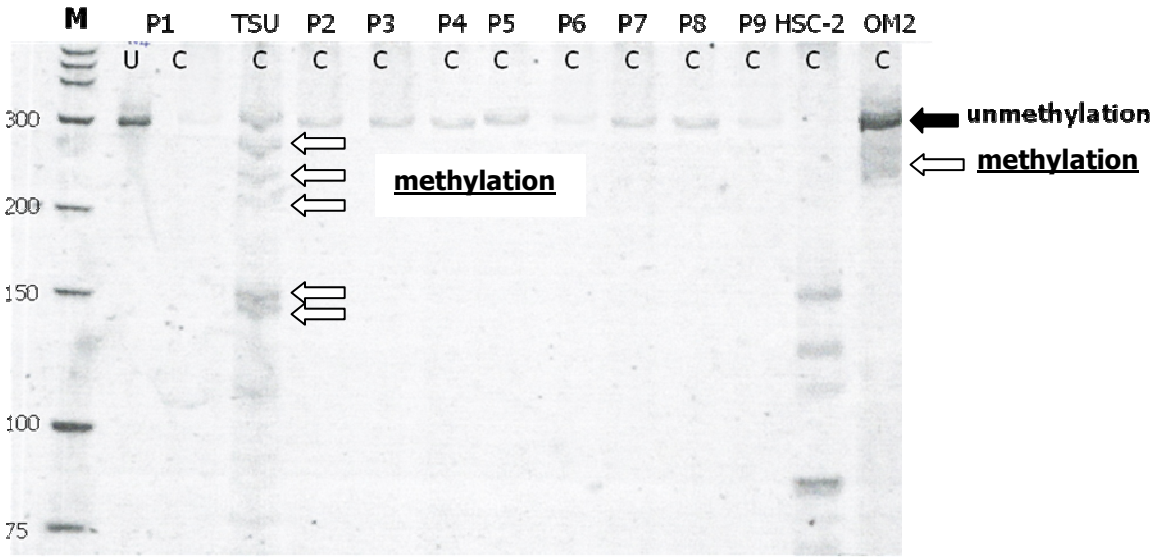


Figure 18 the results of COBRA in psoriasis keratinocytes (n=9). *Black arrows*, unmethylated alleles; *Red arrow*, methylated alleles; *U* , uncut PCR products by restriction enzyme; *C* , cut PCR product by restriction enzyme; *M*, DNA marker. , Unmethylated band; 287 bp, Methylated band; 265 bp, 230 bp, 145 bp, 132 bp

AMD1 promoter methylation in psoriasis by MSP

The previous study showed AMD1 mRNA was up-regulated in lesional psoriatic skin. We hypothesize that AMD1 expression is controlled by promoter methylation. Then, we investigated the methylation level of AMD1 promoter in several cell lines by MSP technique. Almost of cell lines (HCT16, HSC2, OM2, TSU, Hela, Caski, RKO, SW480, HEp2, HepG2, Daudi, Jurkat, Molt4, HL-60, K562 and UACC903) revealed only unmethylatated band. The OM1 and HaCat cell lines revealed both methylated and unmethylated bands as shown in Figure 19a. To verify MSP technique, bisulfite cloning and sequencing of AMD1 promoter methylation of TSU and Hacat was done as shown in Figure 19b.

To explore methylation status of AMD1 promoter in psoriatic skins, lesional skin from 5 psoriatic patients and normal skin from 5 healthy subjects was compared using MSP. The methylation status of AMD1 promoter was not significantly difference between psoriasis and normal controls (Fig 20a). We also compared AMD1 promoter methylation in total leukocytes from psoriatic patients and healthy subjects (n=5). The AMD1 promoter methylation was not different in psoriasis compared to normal controls. Four of 5 psoriatic patients and normal controls showed only unmethylated bands. One of 5 psoriatic patients and normal controls showed both methylated and unmethylated bands (Fig 20b).

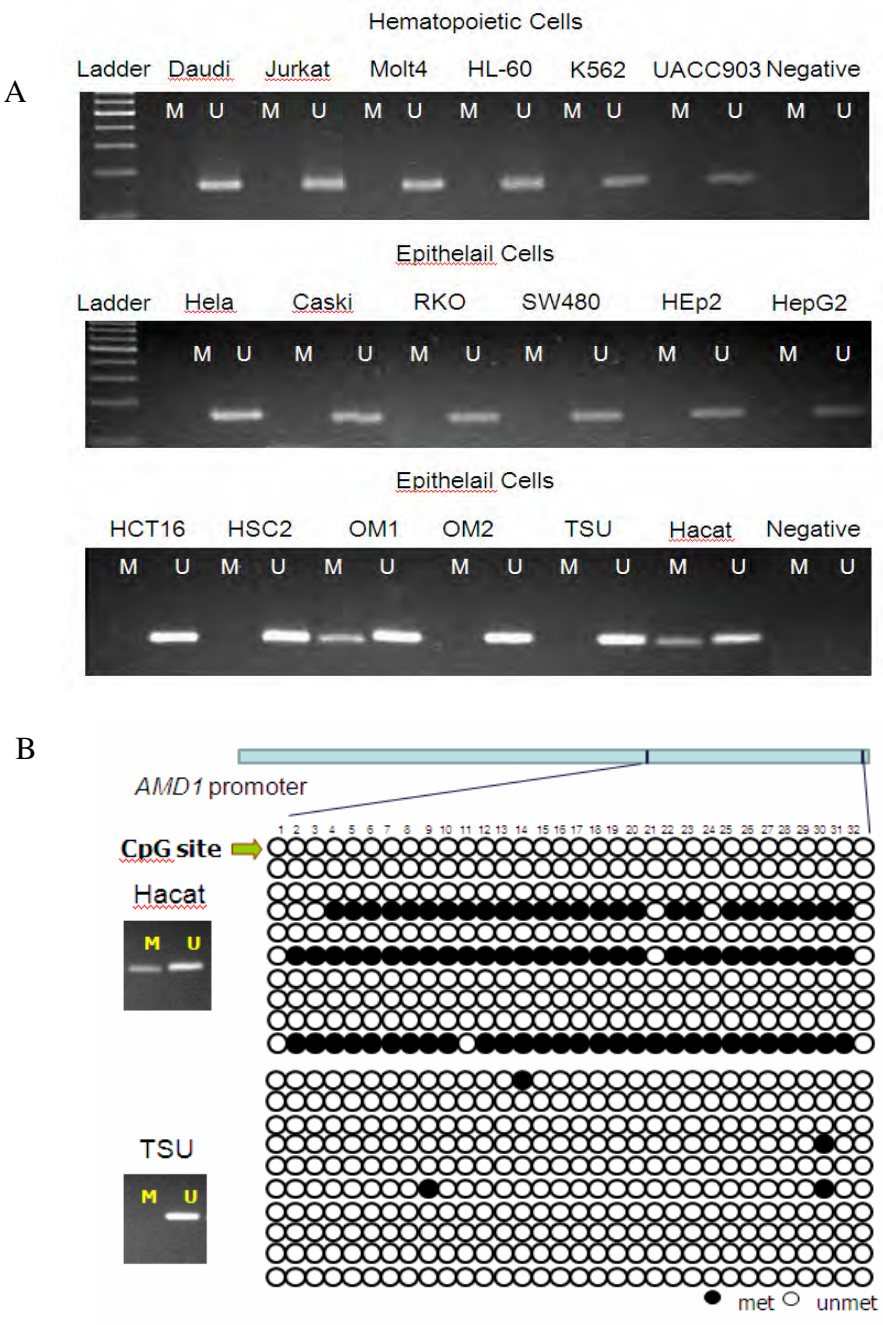


Figure 19 *AMD1* promoter methylation in various cell lines.

a) Promoter methylation is detectable by MSP. U is unmethylated amplicons, and M is methylated amplicons. Both of amplification products are 149 bp. Cell sources of genomic DNA are listed above each lane.

b) Methylation status of CpG nucleotides at promoter of TSU and HaCat by bisulfite sequencing. The numbers of circle indicate CpG nucleotides. Each circle represents the methylation status of each selected clone. Black and white circles are methylated and nonmethylated CpG dinucleotides, respectively.

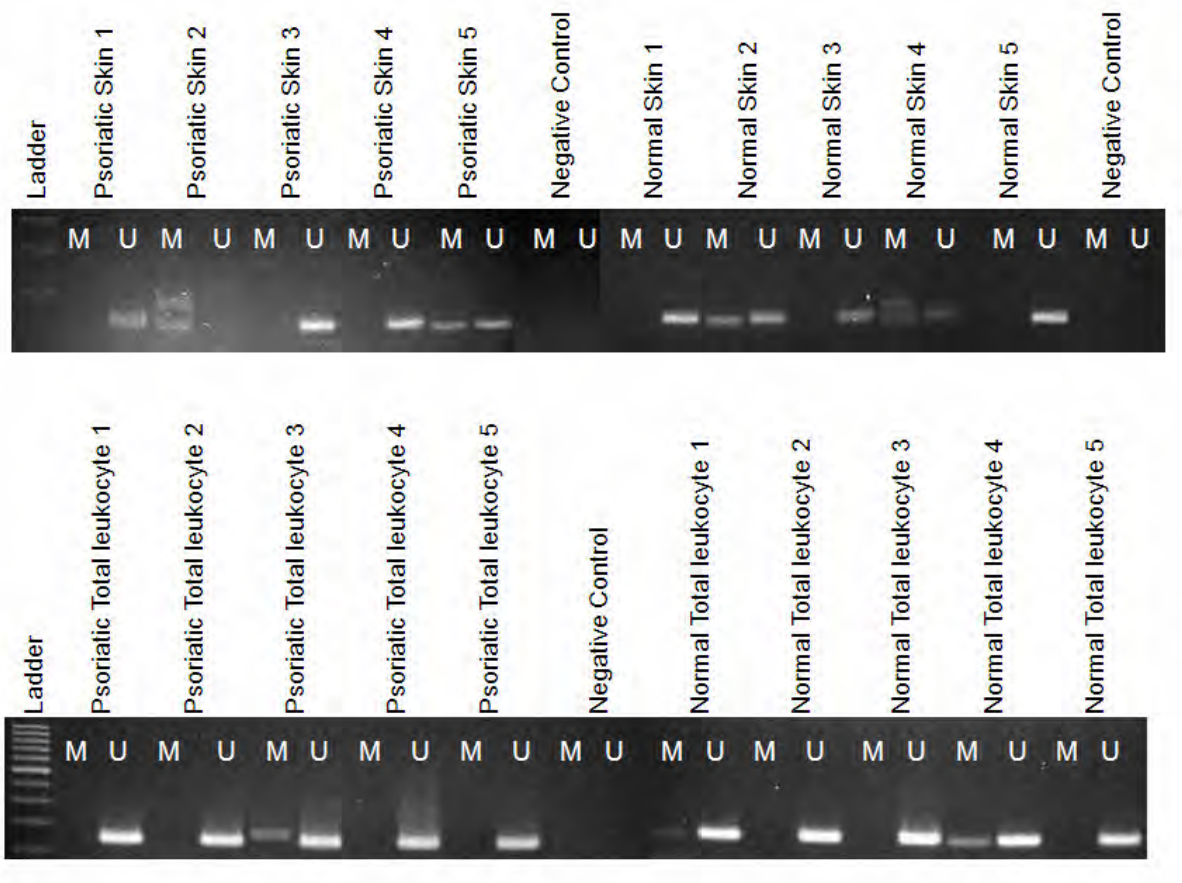


Figure 20 *AMD1* promoter methylation of psoriatic skins and normal skins (a.) and total leukocytes (b.) are detectable by MSP. U is unmethylated amplicons, and M is methylated amplicons.

BCAP31 expression is signficantly decreased in PBMCs of psoriasis

We performed quantitative real-time PCR analysis of BCAP31expression in several cell lines (Figure 21). At present study we show that in hematopoietic cancer cell lines, HL-60 Molt4, K562 and Daudi cell lines were expressed lower levels. Next, we measured BCAP31 mRNA levels in PBMCs by real-time RT-PCR. The mean expression level of BCAP31 relative to 18s rRNA was signficantly decreased in the psoriasis group compared with controls (0.0565 ± 0.0055 vs. 0.00661 ± 0.0013 $p< 0.0001$) (Figure 22 and Figure 23).

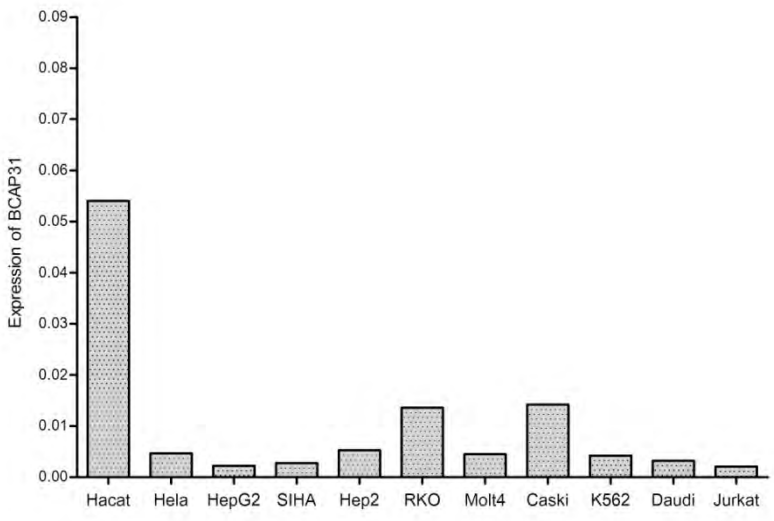


Figure 21 Expression level of BCAP31 in the several cell lines. Relative BCAP31 mRNA levels in several cell lines measured by quantitative real-time PCR. Results are presented as expression levels normalized to 18sRNA.

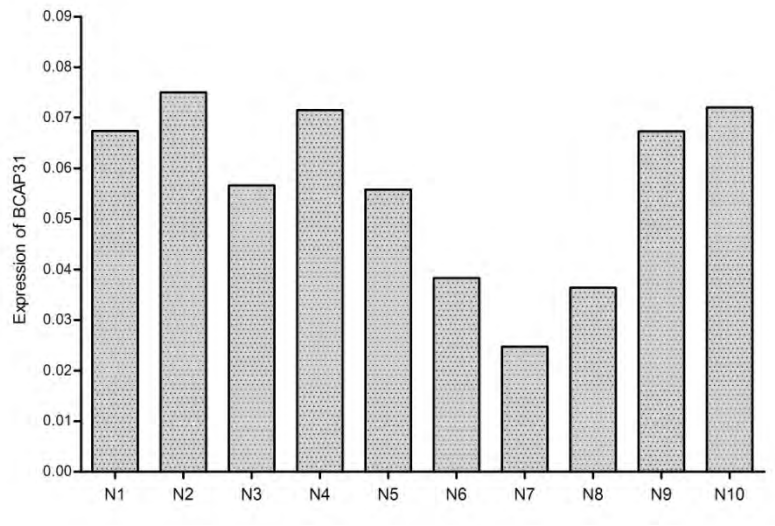


Figure 22 Expression level of BCAP31 in PBMCs from healthy control (n=10). Relative BCAP31 mRNA levels in healthy control PBMCs measured by quantitative real-time PCR. Results are presented as expression levels normalized to 18sRNA.

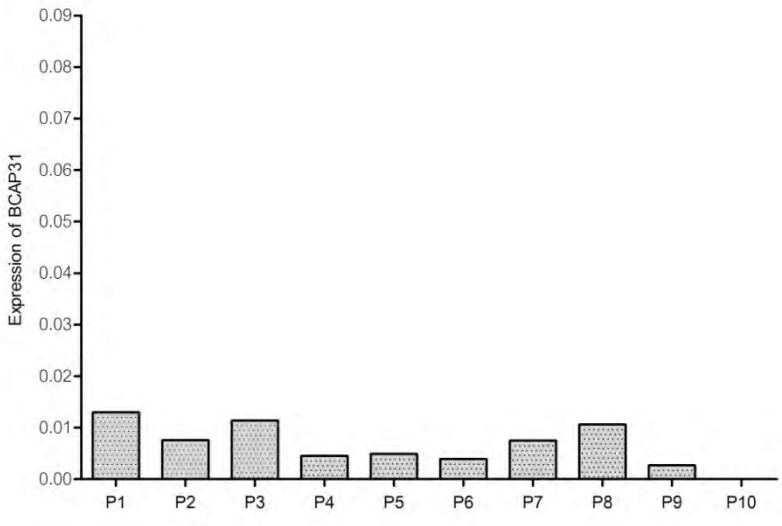


Figure 23 Expression level of BCAP31 in PBMCs from patients with psoriasis (n=10). Relative BCAP31 mRNA levels in psoriasis PBMCs measured by quantitative real-time PCR. Results are presented as expression levels normalized to 18sRNA.

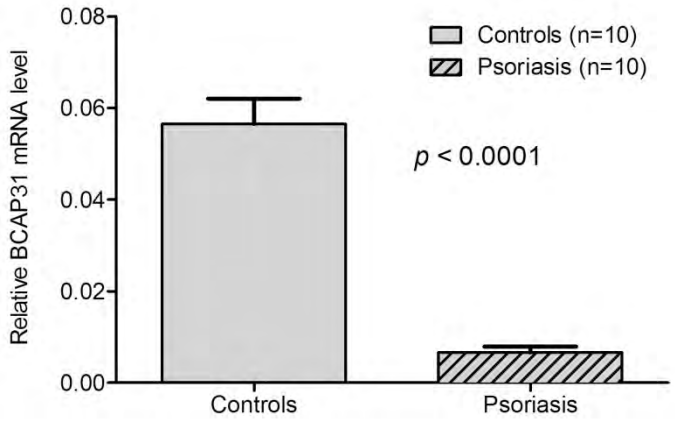


Figure 24 Expression level of BCAP31 in PBMCs from patients with psoriasis (n=10) and healthy controls (n=10). Relative BCAP31 mRNA levels in PBMCs measured by quantitative real-time PCR. Results are presented as expression levels normalized to 18sRNA. Mean BCAP31 mRNA expression level was significantly decreased in psoriasis samples compared with healthy controls (0.0565 ± 0.0055 vs. 0.00661 ± 0.0013 $p < 0.0001$)

The BCAP31 promoter is hypermethylated in PBMCs of psoriasis patients

To investigate the methylation status of BCAP31 promoter in psoriasis PBMCs, we analyzed the methylation status of 48 CpG pairs by bisulfite sequencing. The fragment of promoter region, between 36 167 to 37 432 of BCAP31 database (U52111.3), was amplified by nested PCR and amplicons were cloned. Subsequently, ten clones from each amplicon were sequenced. Representative sequence diagrams of psoriasis patients (n=10) and healthy controls (n=10) PBMCs are shown in Figure 28. The promoter of BCAP31 of 10 psoriasis PBMCs were mostly methylated while 10 healthy controls PBMCs were unmethylated.

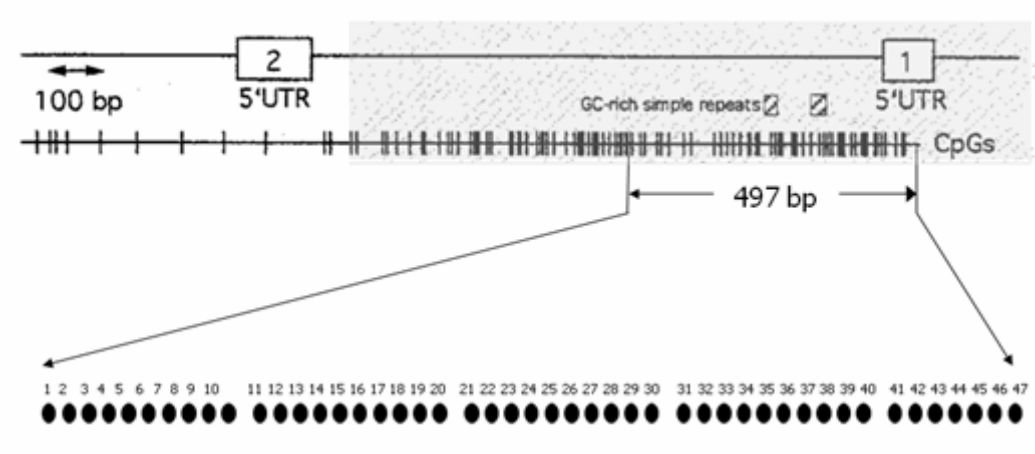


Figure 25 Maps of BCAP31 gene, CpG islands, CpG sites, and PCR products used for bisulfite sequencing. Closed circle represent each CpG sites.

The mean methylation of each of 48 CpG pairs in this 497 bp region was analyzed using bisulfite sequencing as shown in Figure 29. The mean methylation frequency was induced in patients DNA (n = 10) compared with control DNA (n = 10) The difference in the overall average mean methylation frequency of all 48 pairs was also significant (6.844 ± 0.4365 vs. 0.5563 ± 0.04395 $p < 0.0001$ (Fig. 30)

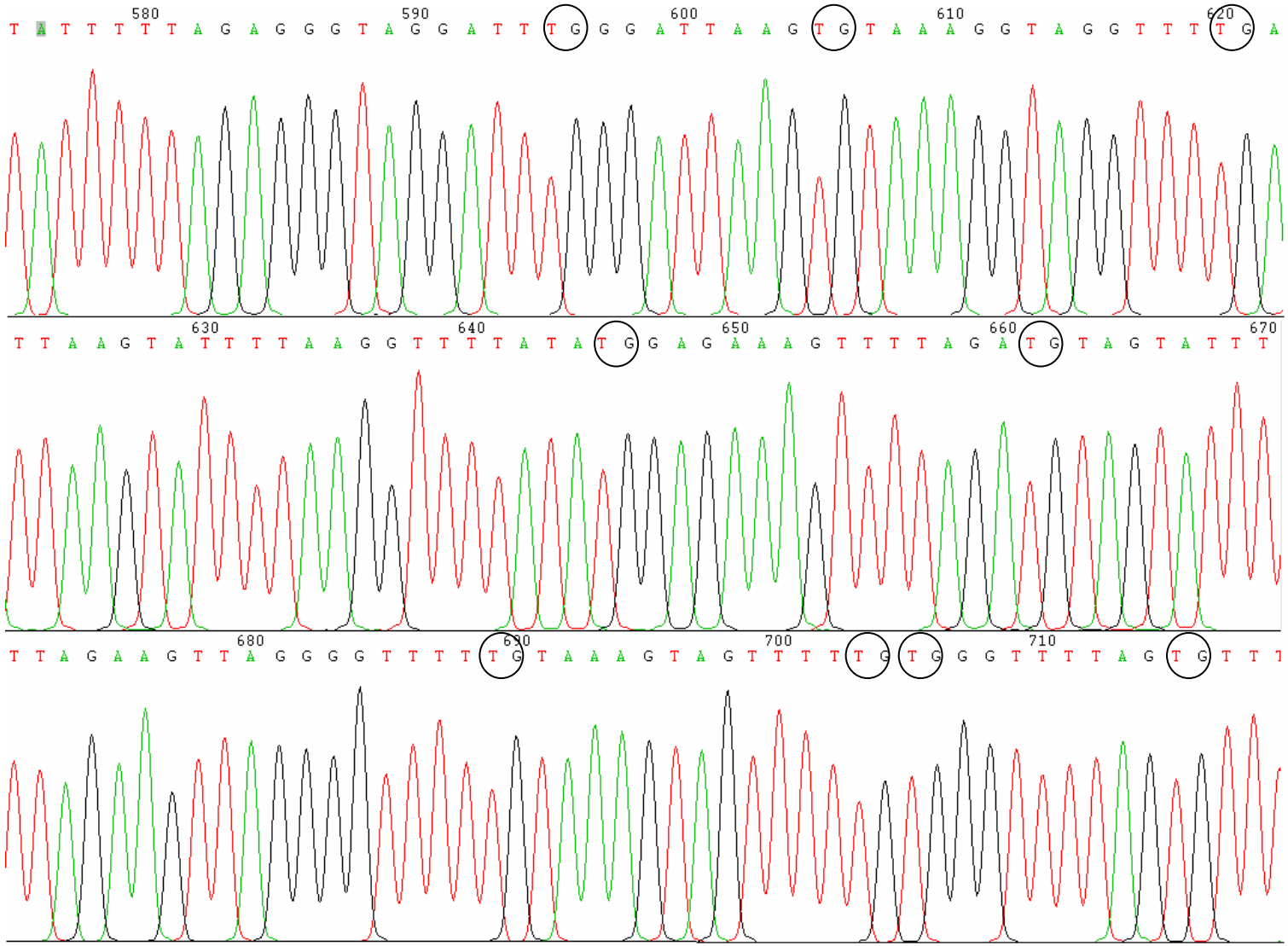


Figure 26 Examples of unmethylated CpG site of recombinant pGEM-T of normal PBMC, N1 clone 1 war shown. Black circles are unmethylated CpG dinucleotides.

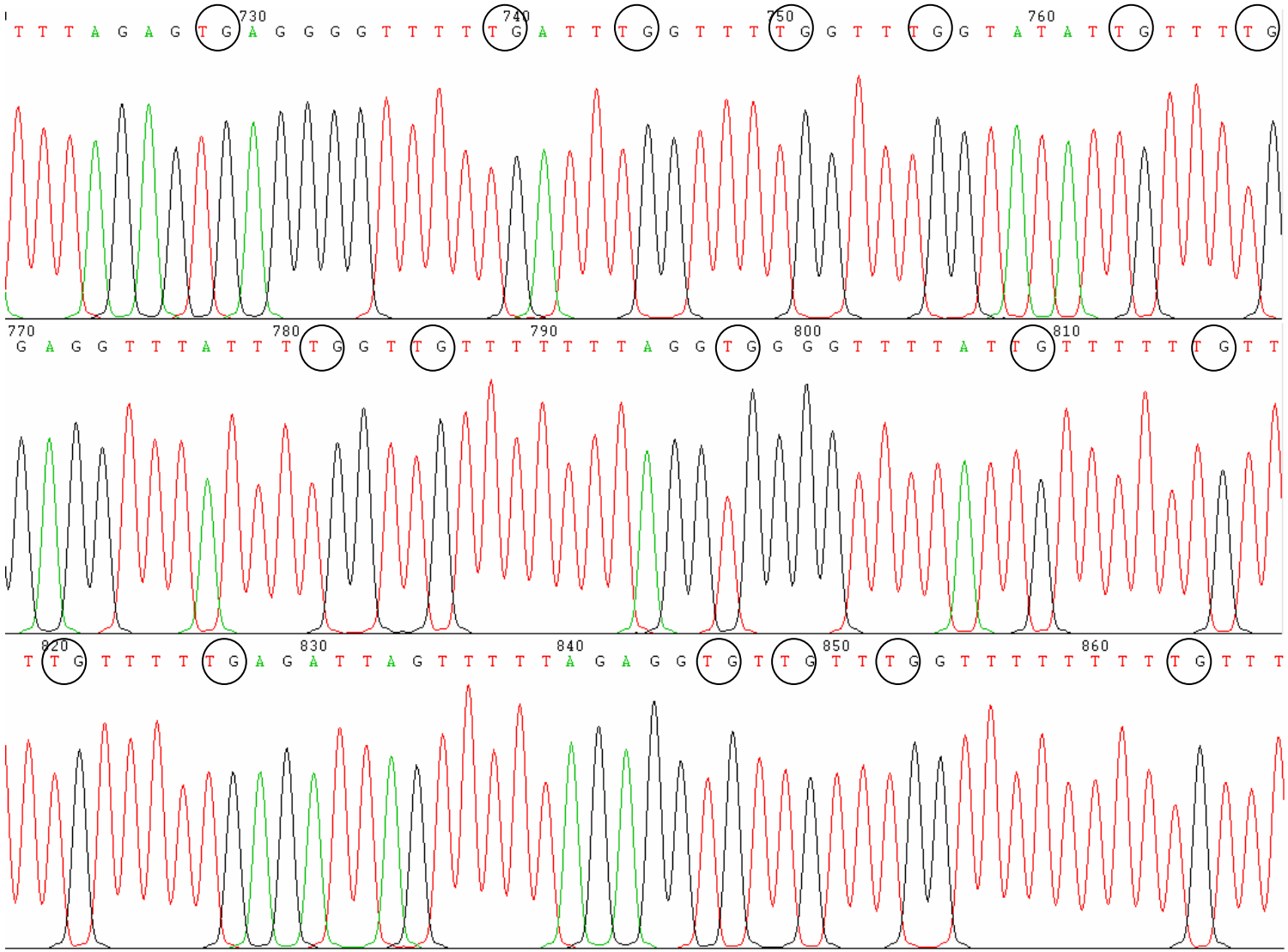


Figure 26 Examples of unmethylated CpG site of recombinant pGEM-T of normal PBMC, N1 clone 1 war shown. Black circles are unmethylated CpG dinucleotides.

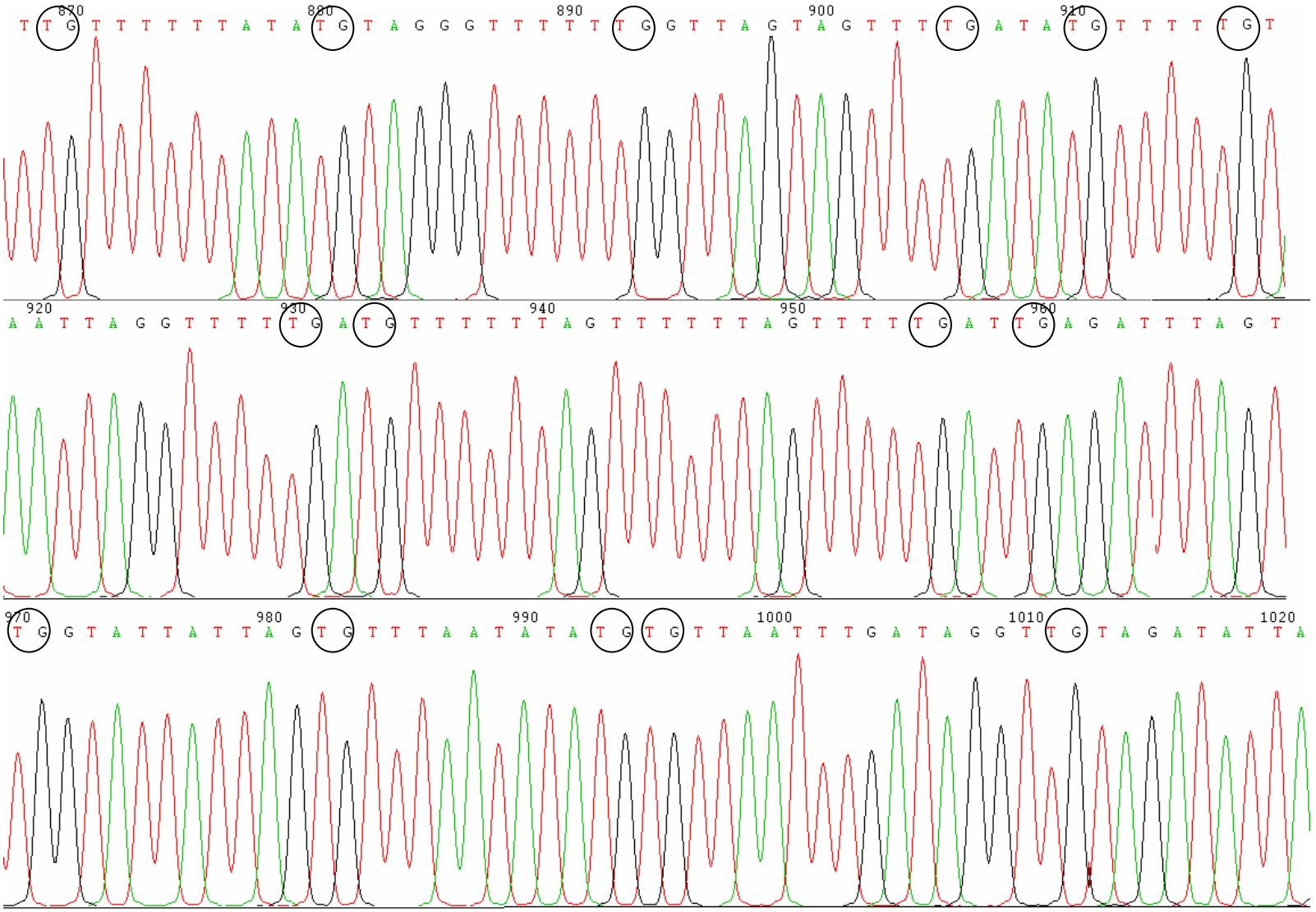


Figure 26 Examples of unmethylated CpG site of recombinant pGEM-T of normal PBMC, N1 clone 1 war shown. Black circles are unmethylated CpG dinucleotides.

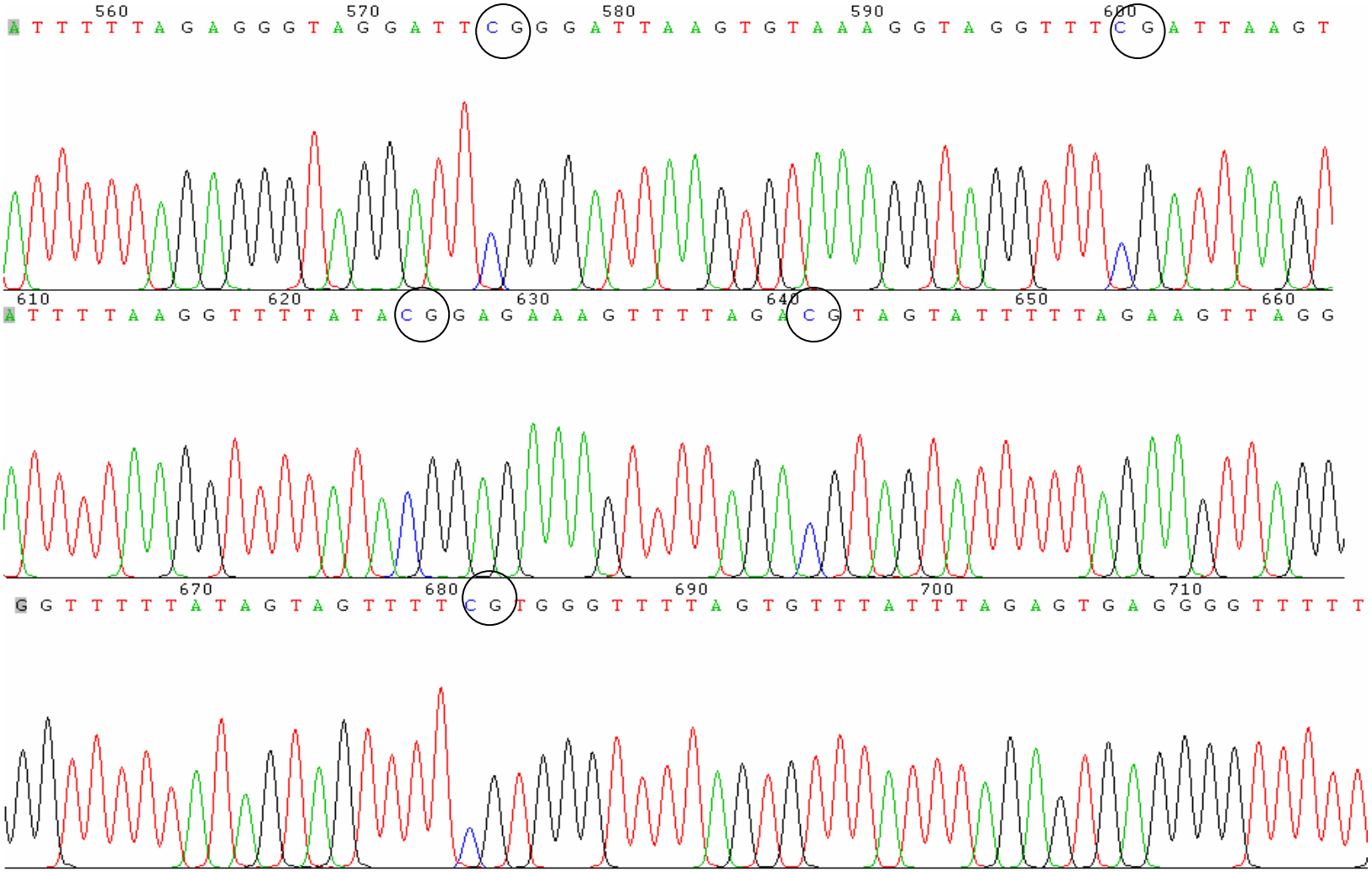


Figure 27 Examples of methylated CpG site of recombinant pGEM-T in PBMC of psoriasis, P2 clone 1 was shown. Black circles are unmethylated CpG dinucleotides

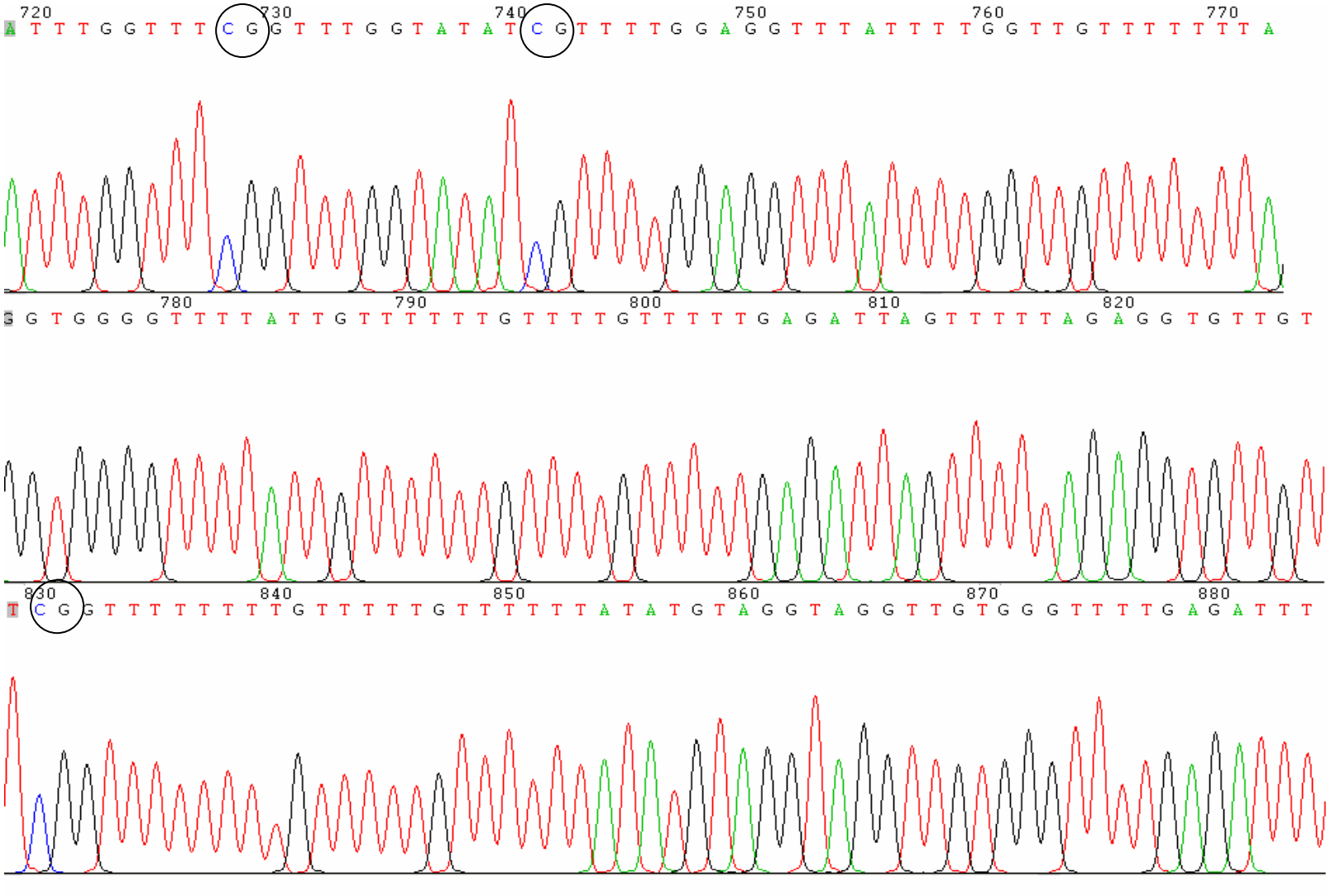


Figure 27 Examples of methylated CpG site of recombinant pGEM-T in PBMC of psoriasis, P clone 1 war shown. Black circles are unmethylated CpG dinucleotides

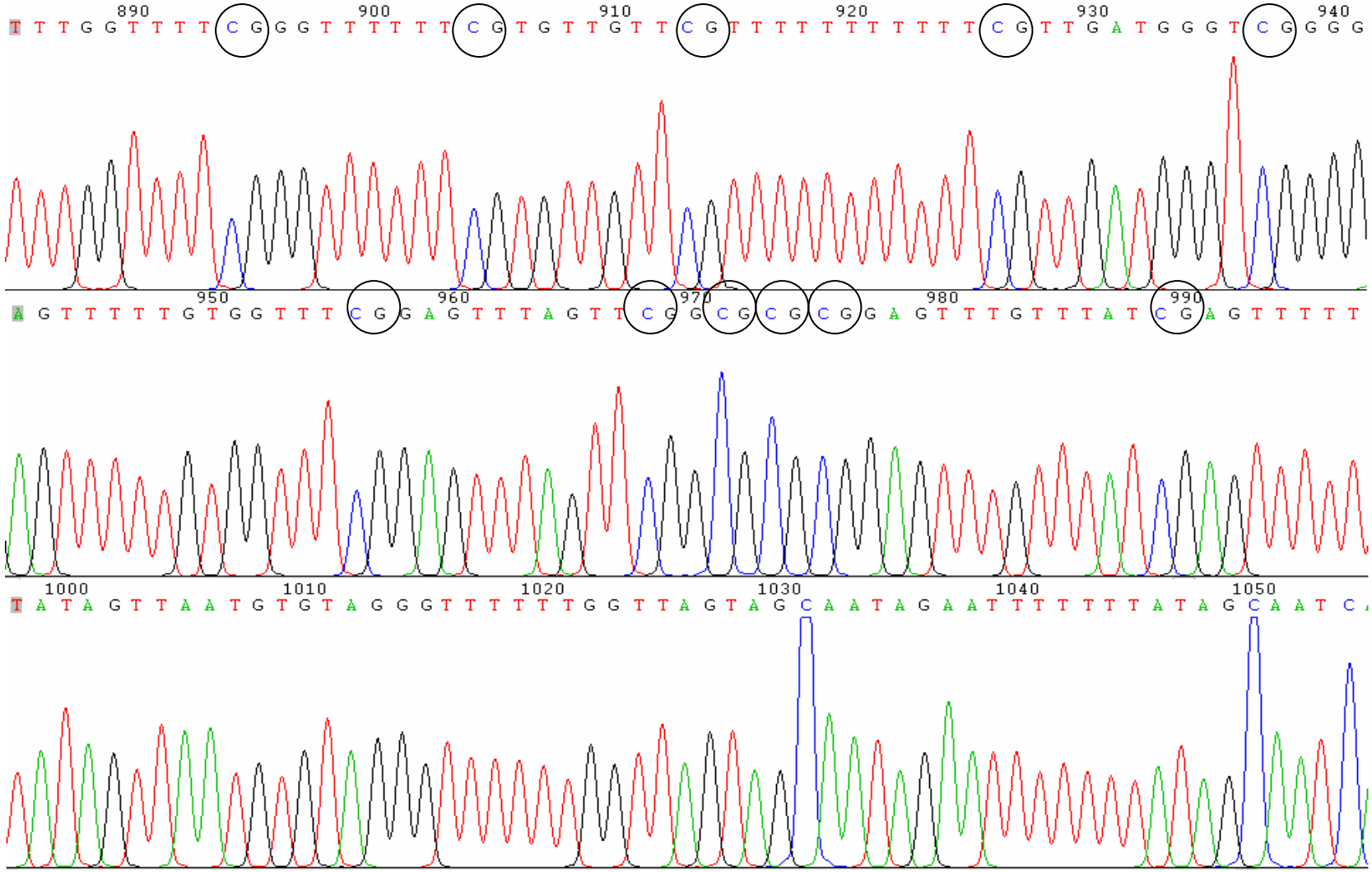


Figure 27 Examples of methylated CpG site of recombinant pGEM-T in PBMC of psoriasis, P clone 1 war shown. Black circles are unmethylated CpG dinucleotides

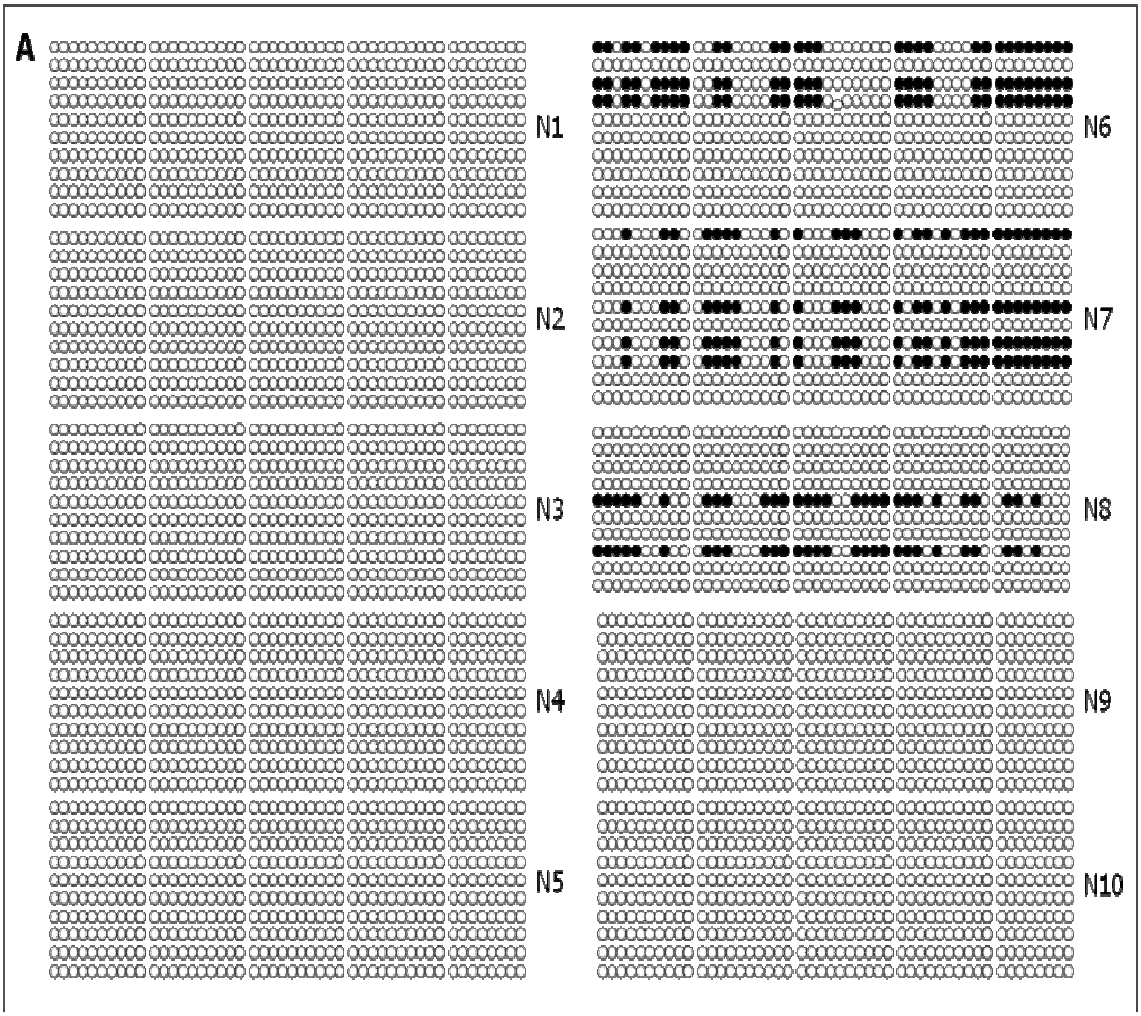


Figure 28a Analysis of methylation status of promoter CpG islands of BCAP31 by bisulfite sequencing from healthy controls Circle represents CpG dinucleotides; closed circle: Methylation; Open circles: Unmethylation.

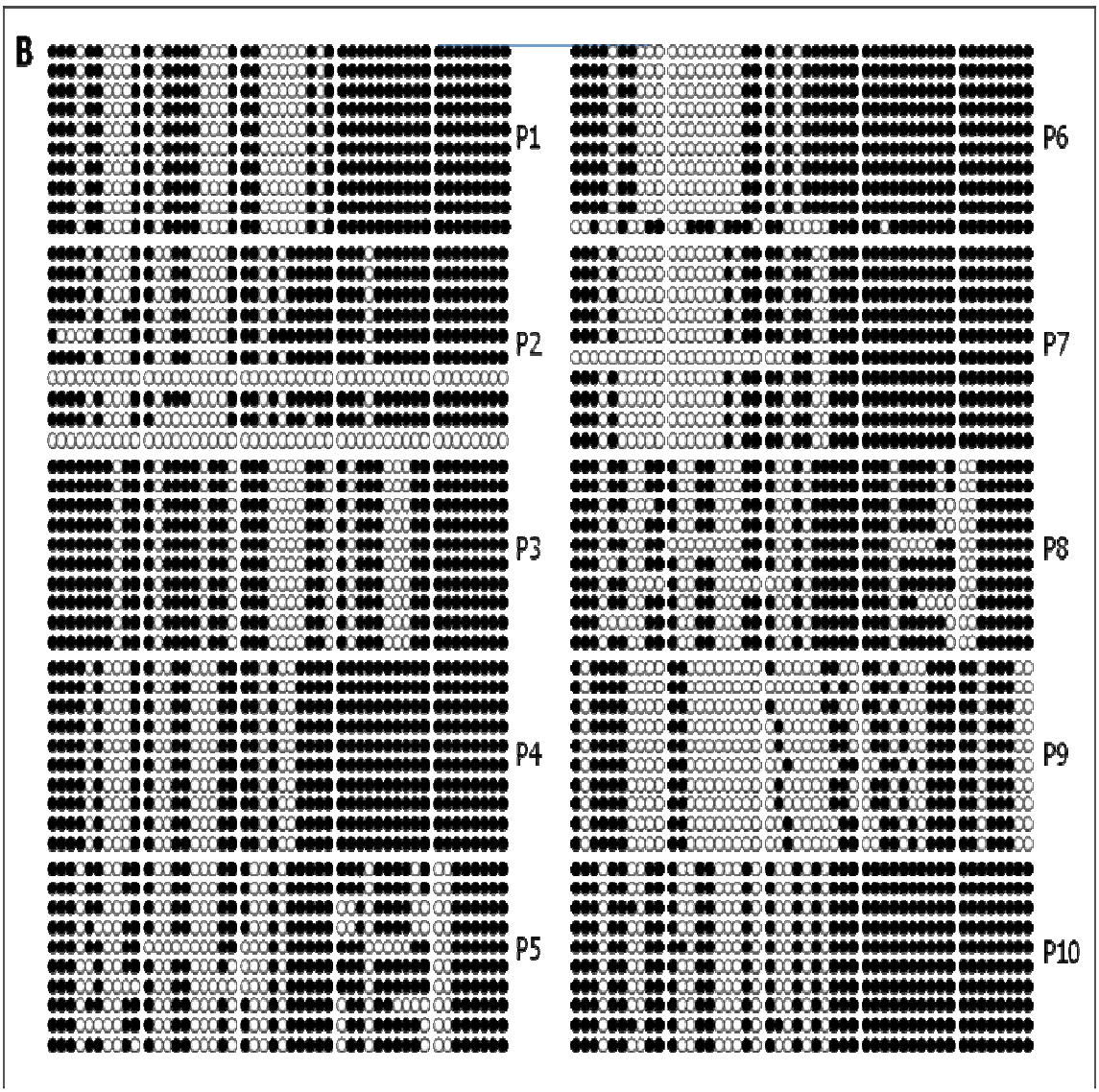


Figure 28b Analysis of methylation status of promoter CpG islands of BCAP31 by bisulfite sequencing from psoriasis patients Circle represents CpG dinucleotides; closed circle: Methylation; Open circles: Unmethylation.

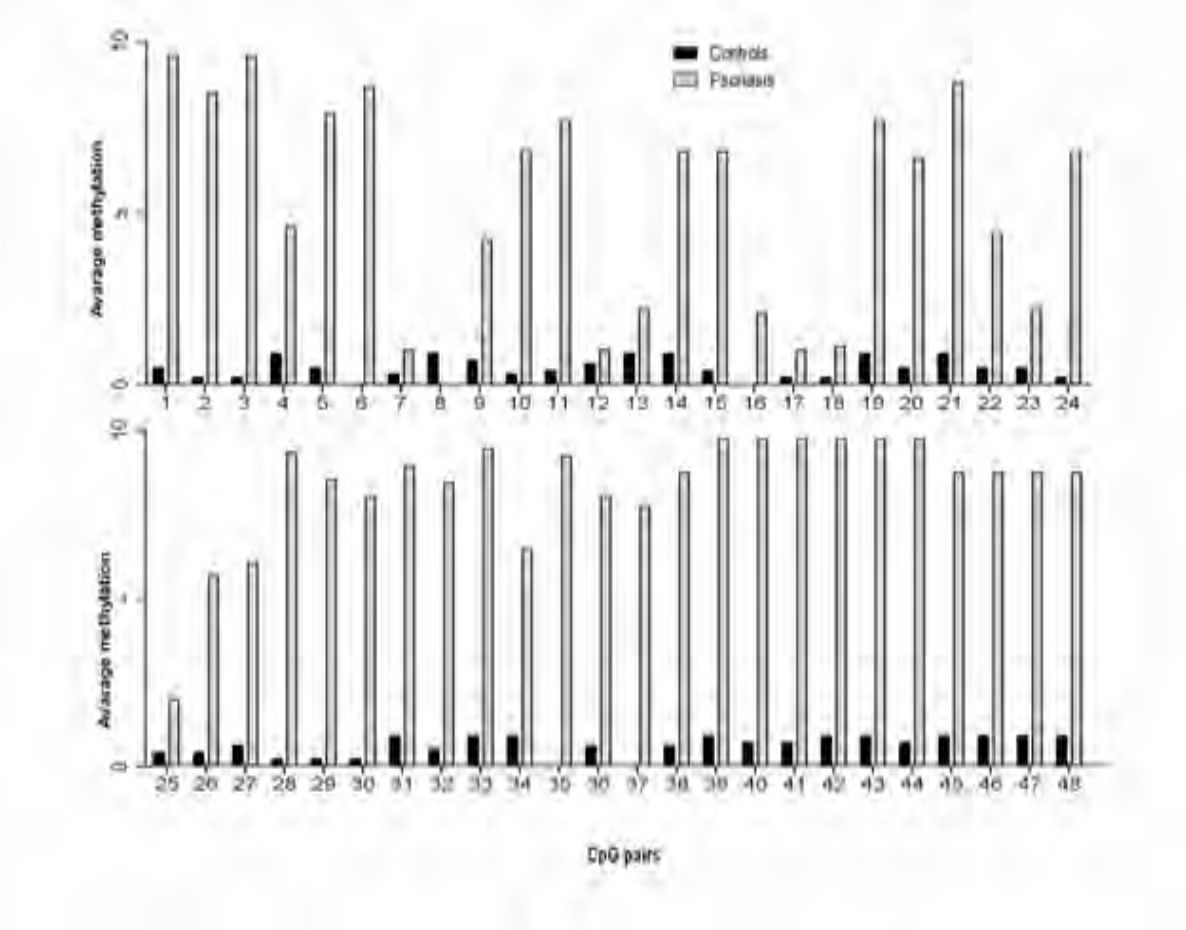


Figure 29 BCAP31 promoter methylation patterns in PBMCs from patients with psoriasis (n = 10) and healthy controls (n = 10). Ten clones from each amplfied fragment were sequenced and the methylation status of the 48 CG sites (X-axis) within the region was then assessed and expressed as the mean methylation status (Y-axis). The average methylation of 48 CG pairs was significantly increased in patients with psoriasis relative to healthy controls (6.844 ± 0.4365 vs. 0.5563 ± 0.04395 $p < 0.0001$).

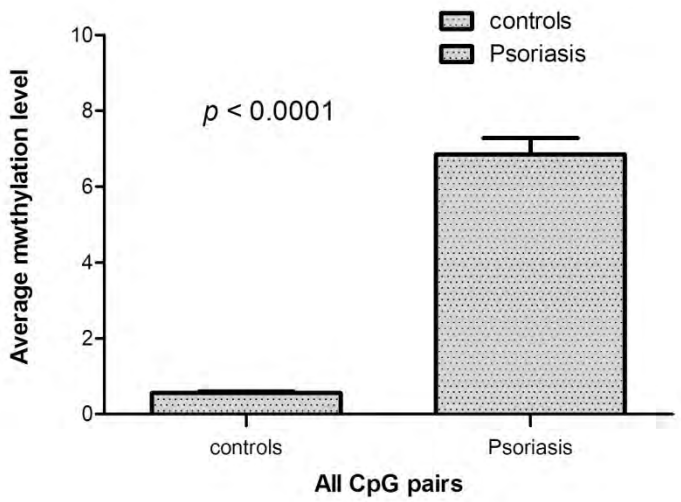


Figure 30 Significant difference in the average mean methylation status of all 48 CG pairs between the psoriasis (n = 10) and control group (n = 10) is observed (6.844 ± 0.4365 vs. 0.5563 ± 0.04395 $p < 0.0001$).

CHAPTER VI
DISCUSSION

Little is known regarding how miRNA expression is regulated in mammalian cells. DNA methylation has been shown to control the expression of many protein-coding genes. Recent studies also showed that DNA methylation to be an important molecular mechanism for regulation of miRNA expression. In the present study, the level of *mir-203* methylation in psoriatic skin was analyzed by COBRA. Our result showed that there was no significant difference in methylation status of *miR-203* in psoriasis compared to normal controls. Thus, up-regulation of *mir-203* mRNA in psoriatic epidermis could not explain by *mir-203* demethylation. There are other mechanisms that can control *miR-203* expression. Negative feedback of their miRNA target can regulate the expression of miRNA, for example in miR-146 and miR-200 family. The study by Sonkoly showed that the up-regulation of *miR-203* in psoriasis plaques was concurrent with down-regulation conserved target of *miR-203*, suppressor of cytokine signaling 3 (socs-3). Socs-3 deficiency leaded to sustained activation of STAT3 and STAT1. Probably, up-regulation of miRNA may control by socs-3 through a negative feedback regulation loop. In psoriasis, STAT1, STAT3, and nuclear factor –kB transcription factors are up-regulated. Analysis of the putative promoter region of *miR-203* gene showed transcription binding sites of STAT1 and NF-kB. It suggested that STAT1 and NF-kB may are directly involved in transcriptional regulation of *miR-203*. However, the further clarification of the mechanism of miR-203 expression will be elucidated.

S-Adenosylmethionine decarboxylase (AdoMetDC) is one of the key enzymes involved in the biosynthesis of spermidine and spermine, which are essential for normal cell growth. Previous study shows up-regulation of *AMD1* mRNA in psoriatic epidermis. To explore methylation status of AMD1 promoter in psoriatic skins, lesional skin from psoriatic patients and normal skin from healthy subjects were compared using MSP. The methylation status of AMD1 promoter was not significantly difference between psoriasis and normal controls. We also compared AMD1 promoter methylation in total leukocytes from psoriatic patients and healthy subjects. The AMD1 promoter methylation

was not different in psoriasis compared to normal controls. Four of five psoriatic patients and normal controls showed only unmethylated bands. One of psoriatic patients and normal controls showed both methylated and unmethylated bands. The present study demonstrated promoter methylation of AMD1 was not different between psoriasis patients compared to normal healthy subjects. The mechanism controlled up-regulation of *AMD1* mRNA in psoriatic epidermis, should not be DNA methylation. The mechanisms that control the mRNA expression of AMD1 gene are still unknown. Other evidences that described up-regulation of AMD1 in various cells, not explained the mechanism controlled expression. It has been known that the mRNA levels of AMD1 are double during the cell cycle. Elevated levels of AMD1 activity was found in abnormal proliferation cell and various cancers such as prostate, breast, and colorectal cancer (Koomoa, Borsics et al. 2009). Mechanism of the control of AMD1 expression needs to further investigate.

BAP31 is a 28-kDa integral membrane protein that is ubiquitously expressed and highly enriched in the ER. It is involved in the interrogated transport of membrane proteins from ER to Golgi. To date, there is no any study about BCAP31 in psoriasis. Our study demons traded that mRNA level of BCAP31 was significantly decreased in psoriasis compared with healthy controls. Psoriatic keratinocytes and lymphocytes possess an enhanced ability to resist apoptosis which which might be one of the key pathogenetic mechanisms in psoriasis. As mentioned before, the process of apoptosis is controlled by Bcl-2 family proteins including several pro-apoptotic and anti-apoptotic proteins. BCAP31 is a member of Bcl-2 family that plays an important role in stimulated apoptotic process. It was involved in a complex that includes Bcl-2/BcL-xL and procaspase-8. Our data suggested that down-regulation of BCAP31 may improve ability of lymphocytes to resist apoptosis. A previous published data demonstrated that a 497 bp (between 36 167 to 37 432 of BCAP31 database: U52111.3) fragment upstream of the BCAP31 gene own promoter activity and is essential for BCAP31 expression. The region contains binding sites for several transcription factors that are down-regulated in psoriasis, such as c-Jun, c-Fos, and AP-1 transcription factors. Protein-1 (AP-1), a transcription factor consisting of homodimers or heterodimers of the Jun and Fos families of nuclear proteins, is a downstream target for protein kinase C

(PKC) pathway. AP-1 proteins have essential roles in the normal regulation of keratinocyte growth and differentiation. c-Jun is primarily a positive regulator of proliferation (Shaulian and Karin, 2001; Zenz and Wagner, 2006; Ikebe et al., 2007). c-Fos could be involved in an early and common pathway shared by differentiation and apoptosis in keratinocytes (V Mils et al, 1997). In psoriasis, C-Jun and AP-1 transcription factor were reduced. Hypermethylation of regulatory regions is characteristic of silencing transcription. When promoter hypermethylated, the active transcription complex is dispersed and not access to DNA. A repressor complex, methyl-cystosine binding protein (MBP) was bind to the DNA which results in silencing expression. We analyzed the methylation status of 48 CpG sites within 497 bp promoter region. We found that the methylation status of most of these CpG sites were significantly increased in PBMCs of patients with psoriasis compared with controls. This data suggested that the hypermethylation of BCAP31 may trigger BCAP31 down-regulation. However, the function of BCAP31 and the underlying defects that induce BCAP31 promoter hypermethylation in psoriasis remain unknown and need to be elucidated.

CHAPTER VII
CONCLUSION

The present study demonstrated demethylation of *miR-203* and AMD1 promoter in psoriatic epidermis. Previous study demonstrated up-regulation of *miR-203* and AMD1 mRNA in psoriatic epidermis. Thus, the mechanism controlled up-regulation of *miR-203* and AMD1 mRNA in psoriatic epidermis should not be DNA methylation. This is the first study demonstrated down-regulation of BCAP31 mRNA expression via promoter hypermethylation in psoriasis. This finding provides new insight of pathogenesis of psoriasis.

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