เพื่อศึกษาความสัมพันธ์ของการเกิดดีเอ็นเอเมทิลเลชันที่โปรโมเตอร์ของยีน SHP-1 ในเยื่อบุผิวกับโรคสะเก็ดเงิน

นายเกรียงศักดิ์ ฤชุศาศวัต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549
ISBN 974-14-3534-7

TO STUDY THE ASSOCIATIONS BETWEEN SHP-1 METHYLATION IN NORMAL EPITHELIAL TISSUES AND DEMETHYLATION IN PSORIASIS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2006

ISBN 974-14-3534-7

Thesis Title	TO STUDY THE ASSOCIATIONS BETWEEN SHP-1 METYLATION IN NORMAL EPITHELIAL TISSUES AND DEMETHYLATON IN PSORIASIS
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เกรียงศักดิ์ ฤทุศาศวัต : เพื่อศึกษาความสัมพันธ์ของการเกิดดีเอ็นเอเมทิลเลชั่นที่โปรโมเตอร์ของยีน SHP-1 ในเนื้อเยื่อบุผิวกับโรคสะเก็ดเงิน. (TO STUDY THE ASSOCIATIONS BETWEEN SHP-1 METHYLATION IN NORMAL EPITHELIAL TISSUES AND DEMETHYLATION IN PSORIASIS) อ. ที่ปรึกษา: ศ. นพ. อภิวัฒน์ มุทิรางกูร, อ.ที่ปรึกษาร่วม: รศ. พญ. ณัฏฐิยา นิรัญกาญจน์, 91 หน้า. ISBN 974-14-3534-7.

ยีนของ SHP-1 อยู่บนโครโมโซม 12p13 มี mRNA 2 แบบ โดยเริ่มต้นจากตำแหน่งของโปรโมเตอร์ที่ใน exon 1 (โปรโมเตอร์ 1) และ exon 2 (โปรโมเตอร์ 2) ทำให้ได้ *SHP-1* 2 แบบ คือ ไอโซฟอร์ม1 และ ไอโซฟอร์ม2 ซึ่งมีความแตกต่างกันตามชนิดของ เซลล์ โดยพบว่า ในกลุ่มเซลล์เยื่อบุผิว พบไอโซฟอร์ม 1 และพบมากที่นิวเคลียส ในขณะที่ กลุ่มของเซลล์เม็ดเลือด มี เฉพาะแต่ไอโซฟอร์ม2 เท่านั้น นอกจากนี้พบว่า การลดลงของ SHP- 1 ทั้ง mRNA และ โปรตีน มีความสัมพันธ์กับโรคมะเร็งเม็ดเลือด เช่น มะเร็งเม็ดเลือดลิมโฟร์มาและลิวคีเมีย ซึ่งเป็นผลที่เกิดจากการเกิด เมทิลเลชั่นที่ส่วนโปรโมเตอร์ 2 แต่ในการศึกษาครั้งนี้ กลับพบว่าการเกิดเมทิลเลชั่นนี้ในส่วนเนื้อเยื่อบุผิวสความัส เป็นลักษณะที่จำเพาะ และยังมีความสัมพันธ์ต่อการก่อโรคสะเก็ดเงิน โดยการวิจัยกำหนดตัวอย่าง คือ 1) กลุ่มCell line ชนิดเซลล์เม็ดเลือด 6 ชนิด เช่น Daudi, Jurket, BLCL, Molt4, K562 และ U937 2) กลุ่ม Cell line ชนิดเซลล์เยื่อบุผิว 5 ชนิด เช่น Hela, HaCaT, Hep2, SW480 และ HepG2 3) ตัวอย่างเนื้อเยื่อปกติ ที่ทำพาราฟิน ที่มาจาก ตับ ปอด เต้านม กระเพาะปัสสาวะ ไต และ ผิวหนัง รวม 27 ตัวอย่าง 4) ตัวอย่างชิ้นเนื้อของโรคผิวหนัง เช่น สะเก็ดเงิน (Psoriasis), โรคมะเร็งที่ผิวหนัง (Sqaumous cell carcinoma) และ โรคผื่นอักเสบ (Eczema) รวม 37 ตัวอย่าง โดยใช้วิธี COBRA และ MSP ตรวจหาเมทิลเลชั่นที่โปรโมเตอร์ 1 และ 2 ตามลำดับ ผลจากการวิจัยพบว่า Cell line ชนิดเซลล์เยื่อบุผิวและเนื้อเยื่อปกติในส่วนโปรโมเตอร์2เกิดเมทิลเลชั่นเกือบทั้งหมด แต่มีการเกิดดีเมทิลเลชั่น (demethylation) ในบางตัวอย่างในกลุ่มของเนื้อเยื่อผิวหนัง การเกิดเมทิลเลชั่นควบคุมการทรานสคลิปชั่น (transcription) mRNA ซึ่งตรวจโดยวิธี RT-PCR และ real time RT-PCR โดย Cell line ชนิดเซลล์เยื่อบุผิวพบ mRNA ไอโซฟอร์ม 1 ทั้งหมด ในขณะที่ Cell line ชนิดเซลล์เม็ดเลือด พบว่า มีความหลากหลาย โดยเฉพาะ K562 ที่มี เมทิลเลชั่นทั้ง 2 โปรโมเตอร์ และไม่พบ mRNA ทั้ง 2 ไอโซฟอร์ม นอกจากนี้ ยังพบว่าโรคสะเก็ดเงิน มีระดับการเกิดเมทิลเลชั่นที่โปรโมเตอร์2 แตกต่างอย่างมีนัยสำคัญทางสถิต ($p< 0.005$) กับเนื้อเยื่อผิวหนังปกติ, โรคมะเร็งที่ผิวหนังและโรคผื่นอักเสบ รวมทั้ง พบmRNAไอโซฟอร์ม 2 จำนวน 58 copies/μg RNA ในโรคสะเก็ดเงิน และ 18 copies/μg RNA และ เนื้อเยื่อผิวหนังปกติ ซึ่งพบว่ามีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) การวิจัยครั้งนี้ได้ข้อมูลว่า *SHP-1* ในเนื้อเยื่อบุผิวสความัสเป็นลักษณะที่จำเพาะ ในเนื้อเยื่อชนิดต่างๆของทั้งร่างกาย อีกทั้งการเปลี่ยนแปลงของการเกิดดีเมทิลเลชั่น (demethylation) ที่โปรโมเตอร์ 2 มีความสัมพันธ์กับการเกิดพยาธิสภาพของโรคสะเก็ดเงิน ซึ่งความรู้จากการวิจัยนี้ จะเป็นองค์ความรู้ที่เพิ่มในกลไกที่เป็นสาเหตุหนึ่งในการเกิดโรค และเพิ่มแนวทางในการออกแบบยาที่จะช่วยในการรักษาโรคอีกด้วย

สาขาวิชา ชีววิทยาศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

478 96533 20 : MAJOR BIOMEDICAL SCIENCES

KEY WORD: *SHP-1*/DNA ETHYALTION/ PSORIASIS/PTPN6/ DEMETHYLATION

KRIANGSAK RUCHUSATSAWAT: TO STUDY THE ASSOCIATIONS BETWEEN SHP-1 METHYLATION IN NORMAL EPITHELIAL TISSUES AND DEMETHYLATION IN PSORIASIS. THESIS ADVISOR: PROF. APIWAT MUTIRANGURA, THESIS COADVISOR : ASSOC. PROF. NATTIYA HIRANKARN, 91 pp. ISBN 974-14-3534-7.

SHP-1 promoter hypermethylation has been studied in hematopoietic cells and observed only in various types of lymphoma and leukemia. This study reports a contrasting situation in normal epithelial tissues and an association with skin pathogenesis, particularly in psoriasis. We investigated several cell lines, 5 epithelial and 6 hematopoietic cell lines. White blood cells from normal healthy donors and normal micro-dissected epithelium of kidney, liver, breast, cervix, lung, prostate, bladder and skin were also included. Interestingly, promoter 2 hypermethylation was apparent in all epithelial cell lines and tissues. However, distinctive degrees of demethylation were noted in some skin samples. The methylation patterns of each cell line corresponded to their mRNA isoforms, in that isoform I and II could not be detected with either promoter 1 or 2 hypermethylation, respectively. We further explored whether an enhanced degree of demethylation could be observed in various dermatopathology lesions from Psoriasis, squamous cell cancers (SCC) and eczema. Thrity seven skin lesion samples were detected SHP-1 methylation of promoter 1 and 2 by using COBRA and MSP technique, respectively. While the promoter 2 methylation levels of SCC, eczemas and normal skins were not different, a significant degree of demethylation can be observed in psoriatic skin lesions, ($p<0.005$). In addition, psoriatic skin displays a higher level of *SHP-1* isoform II than normal skin ($p<0.05$). In conclusion, this study discovered an unprecedented role of *SHP-1* methylation in tissue specific expression and its alteration in a non-malignant human disease besides the transcription inhibition in leukemia and lymphoma. Furthermore, the promoter demethylation may play an important role in skin pathogenesis by enhancing *SHP-1* isoform II transcription in psoriatic skin lesions.

Field of study: Biomedical Sciences
Academic year: 2006

Student's signature KRIANGSAK RUCHUSATSAWAT
Advisor's signature
Co-advisor's signature

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my advisor Professor Apiwat Mutirangura, for his competent supervision, guidance, encouragement and criticism, which have inspired me to accomplish my study.

I am extremely grateful to my thesis co-advisor Associate Professor Nattiya Hirankarn, for her valuable suggestion, support and encouragement for the completeness of this thesis. I am very grateful to my supervisory committee, Professor Yong Poovorawan, Assistant Professor Virote Sriuranpong, Assistant Professor Visatree Kongcharoensuntorn, for their valuable suggestions and criticism.

I am indebted to Professor Hedeoki Ogawa for kindly providing HaCaT, Associate Professor Chintana Chirathaworn for U937 and Molt4, and Associate Professor Watchara Kasinrerk for Daudi, Jurkat, and K562, and Assistant Professor Pokrath Hansasura for both BLCLs. We also would like to express our gratitude to the department of pathology, dermatology unit, department of medicine, and Associate Professor Nopadon Noppakun for paraffin tissues.

My sincere appreciation is also expressed to my colleagues, especially Lupus Research Unit and Molecular Biology and Genetics of Cancer Development for supports since the first time I started my research and inspired me.

I am particularly indebted to the Royal Golden Jubilee Ph.D. grant, the Thailand Research Funds, Molecular Biology and Genetics of Cancer Development Research Unit, Chulalongkorn University, and National Center for Genetic Engineering and Biotechnology, Thailand for supporting equipment and other utilities.

Finally, much appreciation is special expressed to my parents, my wife who is Assistant Professor Jongkonnee Wongpiyabovorn, for their love, kindness, encouragement and moral support throughout this study.

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LIST OF ABBREVIATIONS

aa	=	amino acid
bp	=	Base pair
CpG	=	Dinucleotide conaining cytosine and guanine respectively, p represents the phosphate group
°C	=	Degree celsius
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide containing the adenine (A), thymine (T), cytosine (C) and guanine (G).
kb	=	Kilobase
ml	=	Millilitter
µl	=	Microlitter
µM	=	Micromolar
TE	=	Tris-ethylene dianine tetraacetic acid
SDS	=	Sodium dodecyl sulphate
rpm	=	Round per minute

cDNA	=	Complementary deoxyribonucleic acid
DEPC	=	Diethylpyrocarbonate
kDa	=	Kilodalton
gm	=	Gram
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
v/v	=	Volume by volume
w/v	=	Weight by volume
Taq	=	*Thermus aquaticus*
MHC	=	Major histocompatibility complex
APCs	=	Antigen presenting cells
LFA	=	Leukocyte function associated antigen
ICAM	=	Intercellular adhesion molecule
CLA	=	Cutaneous lymphocyte antigen
ELAM	=	Endothelial leucocyte adhesion molecule
VLA	=	Very late antigen

VCAM	=	Vascular cell adhesion molecule
BLCL	=	B-lymphoblastoid cell
SCC	=	Squamous cell carcinoma
MSP	=	Methylation specific PCR
COBRA	=	COmbined Bisulfite Restriction Analysis

CHAPTER I

INTRODUCTION

Background and Rationale

This proposal aims to study the association between SHP-1 gene, DNA methylation and skin diseases. SHP-1, a protein-tyrosine phosphatase with two src-homology 2 domains, is primarily expressed in hematopoietic cells. It behaves as a key regulator controlling intracellular phosphotyrosine levels in lymphocyte [1]. It is projected as the negative regulator for signaling of cell proliferation and differentiation of of hematopoietic cells such as cKit/SCF receptor, IL-3 receptor and erythropoietin receptor [2-4]. However, the functional role of SHP-1 in epithelial cell is much less understood. In previous report, the SHP-1 protein is located in the cytoplasm of hematopoietic and in the nucleus of epithelial cells [5]. SHP-1 encode the initiated amino acid sequences being MLSRG in epithelail cell lines as compared to MVR in hematopoietic cell lines. Two promoters direct the expression of *SHP-1* transcription using distinct promoters. It is little known in the usage of their promoters in different cell types. Recently, *SHP-1* promoter hypermethylation has been studied in hematopoietic cells and were observed only in various types of lymphoma and leukemia [6-9]. *SHP-1* has been reported as tumor suppressor gene in hematopoietic cell lines and epithelial cell lines such as estrogen receptor (ER) negative breast cancer cell lines and some colorectral cancer cell lines [10].

Psoriasis is a chronic inflammatory skin disease. The disease affected around 2% of the global population. It is characterized by T lymphocyte infiltration and hyperproliferation of epidermis in involved areas of the skin [11]. The pathogenesis of psoriasis remains unclear. Interestingly, transgenic mice with keratinocytes expressing a constitutively active *Stat3* (K5.Stat3C mice) develop a skin phenotype either spontaneously, or in response to wounding, that closely resembles psoriasis [12]. This information indicates that both *SHP-1* methylation and psoriasis are linked to STAT3 functions, pointing at a possible association between SHP-1 methylation and psoriasis.

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 transcriptional silencing of the gene in normal development. It has been demonstrated that the different tissue show regulate differences in 5-methylcytosine content and demethylases which define as tissue specific methylation [13-15]. This is would be expected if only a subset of all the genes in the genome are to be expressed in a single tissue type. Since identification of new tissue specific methylation control genes is crucial for a better understanding of human development and tissue differentiation processes [16-18].

This study proposed to answer whether DNA methylation play an important role for scheming the pattern of SHP-1 isoform. Moreover, we try to explore whether DNA methylation control SHP-1 expression the between hematopoetic and epithelial cell line. Finally, we plan to fine the association between SHP-1 isoform and skin diseases, especially psoriasis.

Research Questions

1. Whether *SHP-1* methylation play an important role for scheming the pattern of isoform?
2. Whether the level of *SHP-1* methylation of promoter 1 and 2 in hematopoietic cell differ from epithelial cell lines ?
3. What is the level of *SHP-1* methylation of promoter 1, 2 in normal epithelial tissue?
4. What are the variations of *SHP-1* methylation in pathologic skin from various conditions eg,, Carcinogenesis, proliferation, inflammation and injury?
5. Whether the level of SHP-1 mRNA Isoform I, II in normal epthelia differ from skin diseases?

Objectives

1. To detect *SHP-1* methylation of promoter 1,2 in hematopoietic cell lines and epithelial cell lines.

2. To detect SHP-1 mRNA isoform I, II in hematopoietic cell lines and epithelial cell lines.

3. To detect *SHP-1* methylation of promoter 1and 2 in normal epithelial tissue.

4. To detect SHP-1 mRNA isoform I and II in normal and skin diseases lesion.

Hypothesis

1. There are difference of promoter regions of SHP-1 methylation in non-hematopoietic cell and epithelial cell lines.

2. DNA methylation plays a role in controlling SHP-1 gene expression in hematipoietic cell line and epithelial cell lines.

3. Demethylation of promoter of SHP-1 associated with skin diseases including psoriasis.

6. Conceptual Framework

1. Whether *SHP-1* methylation play an important role for scheming the pattern of isoform?

2.Whether the level of *SHP-1* methylation of promoter 1, 2 in hematopoietic cell differ from epithelial cell lines ?

↓

To detect DNA methylation of SHP-1 promoter region 1 and 2 .

↓

COBRA and MSP techniques are used for detection SHP-1 methylation in promoter region 1 and 2, respectively

3. What is the level of *SHP-1* methylation of promoter 1, 2 in normal epithelial tissue?

↓

To detect DNA methylation of SHP-1 promoter region 1 and 2 in paraffin embedded epithelia .

↓

COBRA and MSP techniques are used for detection SHP-1 methylation in promoter region 1 and 2, respectively.

4. What are the variations of *SHP-1* methylation in normal epithelia especial skin diseases eg., Carcinogenesis, proliferation, inflammation and injury?

↓

To select the typical type of the skin diseases such as SCC, Psoriasis and eczema.

To detect DNA methylation of SHP-1 promoter region 1 and 2 .

5. Whether the levels of SHP-1 mRNA Isoform I, II in hematopoietic cell differ from epithelial cell lines?

↓

To detect the SHP-1 mRNA isoform by semi-quatitative RT-PCR and Real time PCR

6. Whether the level of SHP-1 mRNA Isoform I, II in normal epithelia differ from skin disease?

↓

To detect the SHP-1 mRNA isoform by semi-quantitative RT-PCR and Real time PCR

Expected Benefits and Application

The results of this study will be benefit in psoriatic molecular pathophysiology and the epigenetic modification of *SHP-1* promoter may assist in developing new and effective targeted therapies.

Research Methadology

	1-3	4-6	7-9	10-12	13-15	16-18
Collected Sample and Culture cell line	→	→	→			
DNA and RNA preparation		→	→	→		
MSP, COBRA ,Cloning and Sequencing			→	→	→	
DNA methylation Quantitation			→	→	→	
RNA Quantitation			→	→	→	
Data collection, analysis and writing thesis			→	→	→	

CHAPTER II

REVIEW OF RELATED LITERATURES

I. SHP-1

1. *SHP-1* GENE

SHP-1(PTP1C, HCP, SH-PTP1, PTPN6), a non-receptor protein tyrosine-phosphatase containing an SH2 domain and protein tyrosine phosphatase in tandem, plays an important role in regulating growth and proliferation process depending on cell types [7, 19-21]. The SHP-1 gene is located on chromosome 12p13. It comprised of 17 exons and spans approximately 17 kb of DNA with transcription size of 2.4-2.6 kb. The gene comprises of two promoters, directing the expression of two isoforms [1]. SHP-1 gene encodes two forms of SHP-1 protein due to different translation initiation codons located with exons1 and 2, respectively. Both forms of SHP-1 proteins are synthesized using identically restricted exons. The major differences between the two forma appear at N-terminal amino acid sequences, MLSRG form I and MVR from II. The difference in activity of two isoforms of SHP-1 proteins is marginal and negligible. Promoter 1 is located approximately 7 kb upstream from promoter 2 (Figure 1). Both promoters contain the regconition sequences for transcription factors such as SP-1 and/or AP2. Promoter 2 contains an inverted GATA box approximately 250 bp from the identified transcription starting site (CAP site), a second GATA sequence, a CCAAT box, and a properly-spaced TATA box region between 390 and 490 bp from the CAP site.

In contrast, promoter 1 has an important motif for its expression, located about 190 bp upstream of CAP site which contains two 12 bp repeats, each with an E-box. This region is regulated by the heterodimer or homodimer of upstream stimulatory factors USF1 and/or USF2. Promoter 1 also contains another motif containing 1 bp repeats, the NF-ΚB binding site, lacated 105 bp up stream of the E box. This motif is regulated by different complex of NF-ΚB dimmer [22]. Exon 3, 4 and exon5, 6 and exons 8-10 encode the N-terminal SH-2 domain, C-terminal SH2 domain and catalytic domain, respectively. The translation stop codon is located within exon 16, and no known protein is encoded by exon 17.

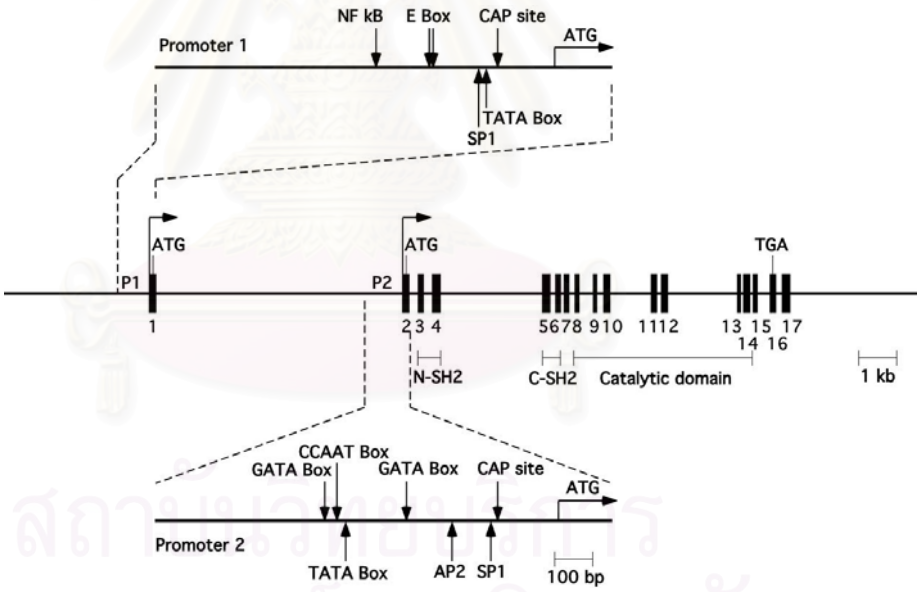

Figure 1. Schematic representation of the SHP-1 gene. The black boxes represent the exons. P1 and P2 indicated the position of promoters 1 and 2 which are active in non-hematopoietic and hematopoietic cells, respectively. The positions of the translation initiation sites (ATG) and the termination codons are indicated.

The various domains of SHP-1 encoded different exons are shown below the exons. The schematic representations of promoter regions 1 and 2 are enlarged in the figure. The CAP sites (position+1) and the initiation codons are shown for both promoters regions. The positions of a putative TATA box, an SP-1 recognition sequence, an AP2 binding site, two GATA boxes and CCAAT box are shown in promoter 1 region. The positions of a TATA box, a SP-1 recognition site, two repeat E-boxes which bind USF1 and/or USF2, and NF-κB binding site are shown in the promoter 2 region [23].

Previous studies distinguished between levels and types of *SHP-1* mRNA isoforms in various cell types. Studies in cell lines have shown that isoform I is normally expressed at lower level in epithelial and barely discovered in hematopoietic cells whereas isoform II exclusively and vigorously expressed in hematopoietic cells. Interestingly, the SHP-1 protein is located in the cytoplasm of hematopoietic and in the nucleus of epithelial cells [5]

2. SHP-1 structure and regulation

2.1 Structure

SHP-1 have two N-terminal SH2 domains (N-SH2 and C-SH2), a classic PTP domain and a C-terminal tail (C-tail) (Figure 2). It has two tyrosyl phosphorylation sites in their C-tails, which are phosphorelated differentially by receptor and non-receptor protein-tyrosine kinases (PTK). SHP-1 protein consisted of 595 aa, or 68 KDa. Its crystal structure has been determines by molecular replacement method and refined to

a final crystallographic. The protein can be divided into four parts; N-SH2 domain contains 108 aa, C-SH2 domain contains 112 aa. PTPase contains 162 and C-terminal region (C –tail) , ~ 60 aa.

2.2 Regulation mechanism of SHP-1

2.2.1. Role of the SH2 domains

SHP-1 SH2 domains (particularly the N-SH2) also regulated PTP activity [24]. Basal SHP-1 activity is low, but addition of a phosphotyrosyl peptide that binds the N-SH2 (Tyr-*P* peptide ligand) markedly simulates catalysis. Bisphosphorylated ligands (ligands containing two phosphotyrosine residues) that engage both SH2 domains provide even more effective stimulation. In its crystal structure, the 'backside' of the N-SH2 domain (the surface opposite the Tyr-*P* peptide-binding pocket) is wedged into the PTP domain. The simultaneously obstructs the catalytic cleft and contorts the Tyr-*P* peptide-binding pocket. Thus, in the basal state, the PTP domain is inhibited by the N-SH2 and the PTP domain impairs Tyr-*P* peptide binding to N-SH2. The C-SH2 has minimal interaction with the PTP domain; its Tyr-*P* binding pocket is unperturbed. These data suggest that C-SH2 surveys that the cell for appropriated Try-*P* proteins. If it binds to a bisphosphorylated ligand, the effective increase in local concentration of the N-SH2 ligand reverses inhibition by the PTP domain, enabling release of the N-SH2 and enzyme activation.

Resent studies support such a model for SHP-1 interactions with glycoprotein 49B (gb49B) [25]. Alternatively, high-affinity ligands for the N-SH2 might cause activation in absence of C-SH2 binding. The similarity of the SHP-1 PTP domain structure to the SHP-2 strong suggest that the molecular details of SHP-1 regulation are analogous [26].

2.2.2. Role of C-tail

The C-tail has also been proposed to regulate SHP-1 activity, but this remains controversial. Early work suggested that tyrosyl phosphorylation increased catalytic activity of SHP-1. However, this study did not distinguish the effects of phosphorylation from SH-2 domain association, and other reports found no effect of phosphosrylation on activity. Recently, protein-ligation techniques were used to replace Tyr542 or Try580 of SHP-2 with a non-hydrolyzable phosphotyrosine mimetic, and phosphorylation at either position was found to stimulate catalysis by two or three fold [27]. Mutagenesis and protease-resistance studies suggest that stimulation by Tyr542-P involves intra-molecular engagement of N-SH2 domain, whereas, Tyr580-P stimulates by binding to C-SH2 domain. These studies are challenging, although the fold-activation observed was low compared with previous reports. It also remain unclear how C-SH2 engagement stimulates SHP activity, which showed no effect of C-SH2 engagement [28].

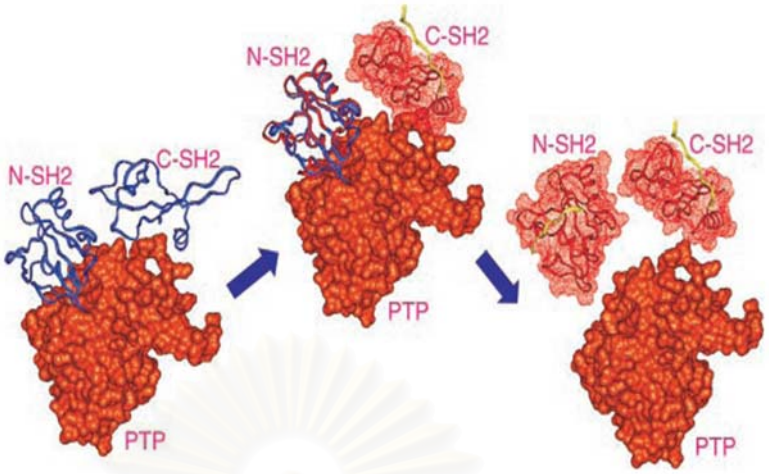

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Figure 2. A proposed mechanism for the activation of SHP-1. From *left* to *right*, the tree structural models represent the auto-inhibited, C-SH2 domain-stimulated, and activated conformations. The molecular surface of PTP domains is shown in *orange*. The SH2 domains modeled from SHP-1 structure is depicted by *blue* and *red* coils, respectively [29].

3. SHP-1 signaling

SHP-1 is an important negative regulator, as illustrated by the 'motheaten mice' phenotype [30]. The motheathen mice die prematurely as a consequence of hemorrhagic interstitial pneumonitis associated with intra alveolar accumulation of macrophages and neutrophiles in the distal lung units [31] and with enhanced production of TNF-α by alveolar macrophages [32]. Together these observations support the contention that unregulated leukocytes activation plays a fundamental etiological role in the pathogenesis of mice disease and underscore the importance of signaling effectors and pathways of inflammatory process.

The association of impaired SHP-1 function with a diminution in cell death rate that has important role for the inflammatory process. Involvement of SHP-1 in regulation of apoptosis cell raises the possibility that reduction in spontaneous apoptosis contributes in motheaten mice by regulating signaling through the Ag receptors and various Ag receptor comodulators [33]. Also, the function of SHP-1 to down-regulate mitogenic signaling cascades has been demonstrated in many cellular systems and in a diversity of receptors. For example, SHP-1 attenuated IL-3 induced tyrosine phosphorylation in DA-3 cell by SHP-1 binding with IL-3R β –chain and β –chain dephosphorytion [34]. Similarly, impaired SHP-1 activity is associated with hyperproliferative response to IL-3 and erythropoitetin in DA-3 cells [35] and with IL-2 independent growth of transformed T cells [36]. The latter effect again appears to reflect the capacity of SHP-1 to associated with cytokine receptor (IL-2R) and to dephosphorelate the β –chain of the activated receptor, leading to diminished phosphorylation of associated Janus family tyrosine kinases (JAK1 and JAK3) [36].

In other pathway, SHP-1 may also exert a positive function. For example, SHP-1 participates in activation of MEK by binding with MEKK-X (unidentified activators) with connecting the activator MEK. resulting in MAPK activation in CSF-1 treatment of macrophage [37]. Moreover, the observation that catalytically inactive SHP-1 overexpressed in in human embryonic kidney cells suppress MEK and MAPK activation by the epidermal growth factors receptor [38].

However, SHP-1 signaling in higher eukaryote, in which the same or similar molecules are activated by different mechanisms and function in different pathways depending on the cellular background [39,37,40].

4. Human Cancer

SHP-1 or *PTPN6* has been considered a tumor suppressor gene in hematopoietic cells due to its promoter hypermethylation in various types of lymphoma and leukemia [6-9]. *SHP-1* methylation would have been detectable in other malignant genomes [7]. *SHP-1* promoter hypermethylation, discernible in various lymphomas/leukemias, increases lymphoma developmental potential by silencing *SHP-1* transcription. The epigenetic regulation has been attributed to promoter 2 [8, 9, 41]. It is important to note that the mechanism inducing the methylation has been described. A recent study conducted on T cell lymphoma indicates that STAT3, in part, transform cells by inducing the epigenetic silencing in cooperation with DNMT1 and, apparently, histone deacetylase 1 [42].

II. DNA METHYLATION

DNA methylation is a major epigenetic mechanism of gene silencing. It profound effects on x chromosome inactivation, genomic imprinting, immobilization of mammalian transposons and suppression of transcriptional noise [43-46]. DNA methylation of promoter region in CpG island, involves the addition of the methyl group to carbon 5 position of cytosine ring [47]. It has shown to be associated with

transcriptional silencing of the genes in normal development [48]. CpG methylation with in gene promoter can act directly by affecting the binding of certain transcription factors to DNA or by recruiting methyl binding proteins (eg., MeCP2, MBD2) that in turn attach to chromatin modifier complexs including histones deacetylases [49]. The cause occurs deacetylation of adjacent histones and subsequent chromatin condensation and gene silencing [50].

1. Methylated cytosine and CpG islands

CpG dinucleotide is a connecting base-pairs between cytosine and guanine in the same strand. Groups of the CpG dinucleotide are often clustered into CpG islands, the area of hight CpG density, 0.5-5 kb and found every 100 b. The presence of CpG island is determined base on these criterias; (1) At least 200 bases length, (2) GC content more than 50%, (3) CpG/GpC ratio more than 0.6 [51, 52] . In vertebrate DNA, the CpG sequence is a signal for methylation by specific cytosine DNA methyltransferase. In higher animals, only as little as 3% to 4% of the total cytosine were methylated. Nevertheless, if there are cytosines on both sides of double stranded DNA, as much as 80% to 100% of nucleotide, there will be two cytosines possible to methylation. On the contrary, if only one is methylated, the doublet is call hemimethylated.

CpG island are frequently associated with location of genes. In the case of genes showing widespread expression, CpG islands are always found at the 5′ ends of genes. It occurs near transcription start sites of approximately 50% of all mammalian

genes [53]. Frequently CpG dinucleotides occur in the center of *cis* control elements of genes, a position in which their interference with recognition and binding site is expected, and of the extending into the first exon. However, the associated CpG island are quite often found some distance downstream of the transcription-initiation site for gene which shows restricted expression pattern. Many studies suggest that the methylated CpG on CpG islands involved gene expression control. It is estimated that there are 45,000 CpG island in human genome and approximately 50% of 70,000 genes in human are associated with the islands .

2. DNA methyltransferase and level of DNA methylation

The enzyme that transfers methyl group s-adenosyl methionine to cytosine ring, cytosine 5-methyltransferases that have been characterized in the number of eukaryotes. The reaction is catalyzed by a family of enzymes call DNA methylatransferase (DNMT) such as DNMT1, DNMT2, DNMT3a, DNMT3b. The active mammalian DNA methylatransferase, encoded by *Dnmt1* gene, is capable of methylation both unmethylated DNA (by *de novo*) methylation and hemimethylated DNA (by maintaince methylation). These gene is highly conserved among eukaryotes and itsorthologs have been identified in various species, including human (*DNMT1*). The DNA methyltransferase recognizied hemi-methylated DNA while replicating and adding methyl group o the newly synthesized strand in the position on the template strand.

This process is maintenance of methylation, the newly synthesized strand will receive the same methylation pattern as the parental DNA. Furthermore, DNMT3a and DNMT3b act as *de novo* methyltransferase and they are report to aid in maintenance methylation.

3. Abnormal DNA methylation and cancer

DNA methylation processes associated with implantation of embryo, organogenesis, fetus growth and disease [54, 55]. The DNA methylation changes dramtically in the case of tumorginesis. These are many epigenetic rearrangement characterstis for cancer; (1) general demethylation [56], (2) hypermethylation individual genes [57] , (3) hypermethylation of CpG island of a number of housekeepting genes [58] and tumor suppressor genes. Hypermethylated DNA also leads to transcriptional repression of these genes and to abnormalities in intracellular processes. Methylation of the promoters of *P16* gene (or *INK4A*) in several cancers which showed an example of abnormal methylation. The protein is inhibitor of cyclin-dependent protein kinase. Moreover, defective expression of *P16* leads to abnormalities in the cell cycles that involved in tumor growth [59]. There are different types of cancers which are associated with methylation of different combination of tumor suppressor genes. For example, in the case of adenocarcinoma methylation of promoter [5] of *K-RAS, P53 and DPC4* can be seen in 80%, 60% and 50 % of patient respectively. Whereas, in case of ovarian carcinoma methylation of promoter of *DOC2/DAB2, BTAK/AURORA* and *K-RAS* 36%, 30% and 22% respectively.

These differences can reflect different genesis of various cancer. Several reports showed that decrease in expression of tumor suppressor genes correlates with decrease in acetylation and increase in methylation of histones in promoter regions of these genes. However, application of inhibitors of histone deacetylases alone did not lead to reactivation of transcription, the combination of these inhibitors with inhibitors of DNA-methyltransferases restored the normal level of expression. Furthermore, application of only inhibitors of DNA-methyltransferases leads to significant decrease in methylation of histones. Also, methyl-DNA-binding proteins MBD2 and MeCP2 specifically interact with abonormally methylated promoters of tumor suppressor genes such as promoter *P14* and *P16*.

III. PSORIASIS

1. Epidemiology

Psoriasis is a common chronic skin disease affecting up to 2.0% in many populations. There are differences described for various countries, geographical regions [60]. For instance, the percent frequency rates range from 0% (Samao) to 11.8% (Arctic-Kasach'ye). In USA the prevalence of psoriasis was estimated to be around 4.6% while in Canada it was 4.7%. For Europe including UK, Norway and Croatia, it showed a variation with range form 1.6, 1.4 and 1.55%, respectively. In East Africa, it was 0.7%. Also, In Asia including China and Japan, there are 0.4 and 0.5 %. However, in Thailand, the prevalence of this disease still has not been clear.

It has reported about gender in the disease. The point prevalence was 4.2% for males and 3.3% for female in Denmark. However, the ratio of this disease was not significantly difference.

2. Spectrum of clinical disease

The clinical morphology of psoriatic lesions shows features that are characteristized and can be classified. The hall mark of this disease which is sharply demarcated erythematous plaque covered with silvery scale. The characterization of psoriasis is restricted to lesional morphology (nummular, inverse, geographica, anularis, circinata, etc.) in addition to important distinctions such as pustular and nonpustular psoriasis (Table 1). However, equally important seems to be described the actual clinical status as the momentary stage of disease activity.

Table 1. Clinical forms of non-pustular and pustular psoriasis

Non-pustular psoriasis	Pustular psoriasis
Psoriasis vulgaris: Type I (early onset) Type II(late onset)	A) Generalized: Von Zumbusch type Impetigo herpetiformis
Guttate psoriasis	B) Localized: Palmo-plantar pustular
Erythodermic psoriasis	psoriasis
Drug-induce psoraisis	Acrodermatitis continua
	Annular pustular psoriasis

In addition to the great variability of the psoriatic disease with different degrees of activity, extent of body involvement, frequency of relapses, variable age onset, a distinct role of trigger factors seems likely. For example, the trigger factors have been described such as physical trauma (Koebner phenomenon), streptococcal infection, psychological stress and Drug (lithium beta adrenergic antagonism, angiotensin-converting enzyme inhibitors, nonsteroidal anti-inflammatory drugs, iodine, digoxin). The impact of environmental factors could be small. However, there are many factors involved the processing of this disease still have evaluated.

3. Genetic studies of Psoriasis

There are several reports showed that psoriasis correlated with genetic basis. Gunnar Lomholt who explored the relative roles of the environment and heredity in residents of the Faroe Island, and Farber and Nall who studied concordance rates in monozygotic twins (~70%) and documented kindreds with multiafflicted family members [61]. HLA class I allele was one of earliest candidate genes for predisposition of psoriasis specifically HLA-Cw6. A dosage effect of HLA-Cw*0602 has been reported, where heterozygotes have a relative risk of developing psoriasis of 8.9 compared with 23.1 in homozygous individuals. Also, homozygous individuals experience an earlier onset but not necessarily a more severe disease course [62]. Several other psoriasis–susceptibility loci have been mapped by using linkage analysis, haplotypes harboring disease-susceptibility alleles with single-nucleotide polymorphism (SNP) analysis, genome wide scans.

International Psoriasis Genetics Consortium provided this in Table 1. For example, the major genetic determinant for psoriasis is within the *PSORS1* region of the MHC on chromosome 6p21, as reported by several groups [63-66] accounting for 30-50% genetic susceptibility. The second most well characterized disease-susceptibility locus *(PSORS2)* resides within 17q24-q25. This locus has been identified by independent family sets [67, 68]. There are two candidate genes (*SLC9A3R1 and NAT9*) in this region. In psoriatic patients, the rSNPs of interest may regulate expression of two this genes. One is SLC9A3R1(solute carrier family 9, isoform 3 regulating factor 1) contains phosphoprotein that associates with members of the ezrin-radixin-moesin family. It can negatively regulate immune synapse function, influencing negative selective during development (central tolerance) and thus leading to emergence of autoreactive T cells [69]. The other, NAT9 (*N*-acetyltransferase family), modifies glucosylation patterns of various immuno-regulatory proteins including MHC class I, and components of immunological synapse.

Although the linkage of genotyping data and psoriatic disease have be proved in many reported as previous. However, the studies of genetic of this disease should be validated in further.

Table 2. The correlation chromosome and Psoriasis

Locus symbol	Chromosome region	Candidate Gene	Reference
PSORS1	6p21	Corneodesmosin (CDSNgene), HCR, HLA class I	Capon F,et al., 1999 [63], Trembath RC,et al.,1997 [64], Nair RP,et al.,1997 [65], Enlund F,et al., 1999 [66]
PSORS2	17q24-25	Immunoglobulin super family gene cluster,SLC9A3R1, NAT9	Thomfohrde J,et al., 1994 [67], Nair RP,et al.,1997 [65] , Enlund F,et al., 1999 [66], Speckman RA,et al.,2003 [68],Helm C,et al., 2003 [70]
PSORS3	4p		Matthews D,et al., 1996 [71]

PSORS4	1q21-q23	Epidermal differentiation complex gene cluster	Capon F,et al., 1999 [63], Bhalerao J,et al.,1998 [72] , Capon F,et al.,1999 [73]
PSORS5	3q21	SLC12A8	Enlund F,et al.,1999 [74]
PSORS6	19p13-q13		Lee YA,et al.,2000 [75]
PSORS7	1p35-p34		Veal CD,et al.,2001 [76]
-	16q12-13		Nair RP,et al.,1997 [65]
-	20p		, Trembath RC,et al.,1997 [64]

Note:SLC9A3R1, solute carrier family 9isoform3 regulatory factor 1; SLC12A8, solute carrier family 12 isoform 8; NAT9, N-acetyl-transferase super family

4. Pathogenetic mechanism for psoriasis

T Lymphocyte

Psoriasis is believed to be a multifactorial genetic disorder with the phenotype depending on the patient's genetic background and environmental factors. The disease is characterized by keratinocyte hyperproliferation and early differentiation as a consequence of autoimmune reaction [77]. The precise pathogenetic mechanism of epidermal hyperproliferation in psoriasis remains unclear. Keratinocytes, fibroblasts, antigen-presenting cells, T-cell and endothelial cells have been proposed as candidate for primary defect. However, the abnormal regulation of T cell-keratinocyte interaction with a complex cytokine network is involved [78]. The antigen –independent activation of T lymphocytes results in release cytokine stimulating inflammation and proliferation of keratinocytes and T lymphocytes. Chang et al (1992) have demonstrated that cytokines secreted by the psoriatic epithelial cell potentiated T cell activation to greater extent than cytokines secreted by the normal epidermal cells [79]. It is also postulated that only psoriasis keratinocytes response to activated T cells messages with hyperproliferation, because of the specific recepters or signal-transducting mechanism [80]. The exact mechanism of hyperproliferation of keratinocytes is still unknown. It may involved in the cell cycle time for example psoriatic epidermis takes in 4 days while normal epidermis takes 26 days [78].

However, there is some controversy whether the cell types undergoing this increased the number of cell cycles are stem cells [81]. or transiently amplifying cells [82]. Currently psoriasis is considered to an autoimmune disease by candidate skin autoantigens that have cross-reactivity with bacterial antigens include keratins. However, the trigger with in psoriasis plaques remains unclear. The importance of T cell activation has been reported such as Krueger et al show that T cell dependent inflammatory process in skin that accelerates the growth of epidermal and vascular cells in psoriasis lesions [83]. There are three phases of activation of T cells. First, T cells bind to antigen presenting cells (APCs) by LFA-1 and CD2 of T cells and ICAM-1 and LFA-3 of APC. Next, antigenic peptide is bound to either Major histocompatibility complex class I or II (MHC-I, MHC-II) on APC, which is recognized by T cells receptor as a specific interaction. Finally, a non-antigen specific cell-cell interaction takes place involving CD 28, CD86, CD2 and IL-2R of T cell and CD80, CD28, and LFA3 of the APC and IL-2. T cells migrate to the skin in a process call rolling. This involved CLA (cutaneous lymphocyte antigen) on T cells and E-selectin (ELAM, endothelial leucocyte adhesion molecule-1) on endothelial cells. In order to T cells to bind to the endothelium, the T cell surface proteins must be activated by chemokines, and LFA-1/ICAM-1 and VLA/VCAM interact (LFA-1= leukocyte function associated antigen, ICAM intercellular adhesion molecule, VLA= very late antigen, VCAM= vascular cell adhesion molecule). Diapediesis, migration through the vessel wall, occur after binding.

As a final step, activated T cells and secretion of other inflammatory cells (local macrophage, dendritic cells, and vascular endothelial cells), and keratinocytes induce the keratinocytes change (hyperproliferation) and expression of adhesion molecules by endothelial cells. Activated keratinocytes produce growth factors stimulating neutrophil influx, vascular alterations, and keratinocytes hyperplasia [81-83].

Langerhans cells

Langerhans cells are the dendritic APC cells of epidermis. After antigen exposure, their ability to stimulate T cell activation increases with up regulated synthesis of cell surface receptors (CD80, CD86, CD40, ICAM1) in a process. Activated Langerhans cell migrate from skin to lymph nodes presenting the antigen to nodal native T cells. These T cells recognize antigens bound to class I or class II MHC molecules, which leads to T cell activation, CLA expression (making the access to skin possible), and differentiation into type 1 or 2 effector lymphocytes.

Keratinocytes

There is hypothesizing that the primary defect resides in keratinocytes, the defective epidermal keratinocytes could be activated by physical or chemical injury increasing the synthesis and release of cytokines. This results in antigen-independent activation of T lymphocytes releases additional cytokines stimulating inflammation and proliferation of keratinocytes and T lymphocytes. Moreover, the evidence for a keratinocytes defect have shown that cytokines secreted by psoriatic epidermal cells potentiated T lymphocyte activation to a greater extent than cytokines secreted from

normal epidermal cells. It is also postulated that only psoriatic keratinocytes respond to activated T lymphocytes messages with hyperproliferation, it means that the specific receptors or signal-transducing mechanism. Furthermore, Bata-Csorgo *et al* who reported that normal keratinocytes did not respond to psoriatic T cell supernatant. However, hyperproliferation of keratinocytes in psoriasis remains unsolved. There are supports of this phenomenal such as the cell cycle time and the fraction of cells dividing. The cell cycle times of hyperproliferation of keratinocytes in psoriatic epidermal occurs in 4 days whereas normal keratinocytes occurs in 26 days. The other phenomenal, in psoriatic hyperplastic epidermis, an increase in the fraction of cells dividing before the dividing cells enter terminal apoptosis is plausible [84].

Management of psoriasis

Management of psoriasis ranges from topical therapies for limited disease to systemic therapies for more widespread disease [85, 86]. Despite various available treatments, the adverse effect or inadequate efficacy of the therapies involved have invoked a necessity for safer and more effective treatment. The advent of therapies based on mechanisms that target critical molecular pathways has evoked considerably interest [87].

CHAPTER III

MATERIALS AND METHODS

Samples

Cell lines and Patient collected samples

Hematopoietic cell lines, Daudi, Jurkat, Molt4, U937, K562, and epithelial, Hela, HEp2, SW480, HepG2 and HaCaT, were used. Additionally, two EBV transformed B-lymphoblastoid cells (BLCLs) were generated from two donors. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Paisly, UK) or RPMI1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Sigma, St Louis, MO) and antibiotics (50U/ml penicillin, 50 μg/ml streptomycin). Cells were incubated at 37 °C in 5% CO_2.

Several archival paraffin embedded tissues derived from normal and adjacent normal tissues of several tumor patients of various locations of kidney, liver, breast, cervix, lung, bladder and OTC-embedded frozen normal cervical sections were obtained [88]. Nine to 10 each of normal, collected from normal skin removed during plastic surgery, and various pathologic skins, including chronic stable plaque type psoriasis, guttate psoriasis, squamous cell carcinoma (SCC) and chronic eczema were chosen for the study. All patients with dermatological condition were diagnosed clinically by an experienced dermatologist and free form any skin therapies 4 weeks prior to taking biopsy samples.

The diagnosis were confirmed by skin biopsy and revealed under experienced dermato-pathologist as typical pathology for each skin condition. The tissues were microdissected as previously described [89]. Additional cases of chronic plaque type were microdissected to separate between upper and lower section of their psoriatic epidermis. Several samples of white blood cells (WBC) from normal healthy individuals were also included. An additional seven cases each of healthy donor and chronic stable plaque type psoriasis pathologic skin conditions were obtained by 6 millimeter wide punch biopsies under local anesthesia for RNA analysis. All psoriasis patients were of the chronic stable plaque type and did not receive any treatment other than 2 cases who sporadically used low potency topical corticosteroids at least 4 weeks prior to taking biopsy samples.

Materials

1. Cell lines: Hematopoietic cell lines such as Daudi, Jurkat, Molt4, U937, K562 and BLCL

   : Epithelial cell lines such as Hela, HEp2, SW480, HepG2 and HaCaT
2. E.coli (DH5α)
3. pGEM-T easy vector
4. DNA purification kit (Promaga, USA)
5. DNA clean up kit (Promaga, USA)
6. MicroAmp PCR tube
7. Microcentrifuge tube: 0.5 and 1.5 ml. (AxyGen® Scientfic, USA)

8. Polypropylene conical tube 15 and 50 ml. (AxyGen® Scientfic, USA)
9. Pipette tip: 10, 100, 200, and 1000 µl
10. Cryotube (Nunc, USA)
11. Glassware: Beaker, Flask, Cylinder and reagent bottles (Pyrex,USA)
12. Tissue Culture Flask, Culture plate, Sterile serological pipette 1,5 and 10 ml (Costar, USA)
13. Counting chambers

Equipments

1. Centrifuge
2. Refrigerated microcentrifuge (Universal 16R Hettich, USA)
3. -70 °C freezer (Forma Scientific, USA)
4. -20 °C freezer (Forma Scientific, USA)
5. Light microscopy
6. DNA thermal cycler (GeneAmp PCR system,Pekin Elmer, USA)
7. Stereo microscope
8. Spectrophotometery (Shimadzu UV-160A, Japan)
9. CO_2 humidified incubator
10. Autoclave
11. Hot air oven (Memmert, West Germany)
12. Multi-block heater
13. Microwave oven

14. Water bath (Memmert, West Germany)

15. Phosphor-imager Storm 840 and Image Quant solfware (Molecular dynamics)

Reagents

1. Phenol (Sigma, USA)

2. Chloroform (Merck, German)

3. Isoamyl alcohol (Merck, German)

4. Sodium acetate (Sigma, USA)

5. Absolute ethanol (Merck, German)

6. Isophpanol (Merck, German)

7. Trizol reagent (Gibco, USA)

8. Reagents for PCR analysis

    a. 10x PCR buffer

    b. Deoxynucleotide triphosphase (dNTPs) Promaga, USA

    c. Taq DNA polymerase (Quigen, USA)

9. Reagents for cDNA synthesis

    a. Improm II (Promaga, USA)

    b. RNasin ribonuclease inhibiter (Promaga, USA)

10. Restriction enzyme: T*aq* I, *Tas I*

11. Agarose gel (FMC Bioproducts, USA)

12. NuSieve agarose (FMC Bioproducts, USA)

13. Ethidium bromide (Sigma, USA)

14. Guanidium thiocyanate (GTG) (USB, USA)
15. 2-Mercaptoethanol (2-ME) (Sigma, USA)
16. Glycogen (Sigma, USA)
17. Diethylpyrocarbonate (DEPC)
18. X-gal (Promaga, USA)
19. IPTG (Promaga, USA)
20. RPMI-1640 medium (Gibco BRL, USA)
21. Fetal bovine serum (Gibco BRL, USA)
22. Penicilin/Streptomycin (Gibco BRL, USA)
23. Dnase I (Gibco BRL, USA)
24. Trysin (Gibco BRL, USA)
25. Trypan blue (Gibco BRL, USA)
26. Hepes (Merck, USA)
27. NaCl (Sigma, USA)
28. HCl (Merck, German)
29. NaOH (Merck, German)
30. KCl (Sigma, USA)
31. Na_2CO_3 (Sigma, USA)
32. $NaHCO_3$ (Sigma, USA)
33. Na_2HPO_4 (Sigma, USA)
34. KH_2PO_4 (Sigma, USA)

35. NaN_3 (Sigma, USA)

36. $MgCl_2$(Sigma, USA)

37. Sodium-N-Lauroyl-sarcosinate ($C_{15}H_{28}NNaO_3$) (Sigma, USA)

38. Dimethyl sulfoxide (DMSO) (Sigma, USA)

39. LB medium (Gibco BRL, USA)

40. 40% acrylamide/bis solution 19:1 (Bio-RAD, USA)

41. Sodium bisulfite (Sigma, USA)

42. Hydroquinone (USB, USA)

Methods

DNA extraction

DNA sample was extracted by a standard technique with a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction.

Cell lines and Tissue samples

Briefly, Five to ten x 10^6 cells or 100 mg of tissue sample was wash with 1.0 ml of PBS buffer twice time for remove the culture mediun or residual blood. The sample was lysed with 1.0 ml of digestion buffer containing lysis buffer2 and 1/10 volume of proteinase K solution; 20 mg Proteinase K in 1.0 ml of 1% SDS-2mM EDTA, should be prepare 30 min before use.

The sample was incubated in water bath at 37 °C for overnight (16-24 h) for complete digestion. Adding 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) shake vigorously for 15 seconds and then centrifuge at 10,000 g for 5 min. The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 0.5 volume 7.5 M Amonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then mix by inversion. The DNA should immediately from a stringy precipitate and then the DNA was recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and then the pellet was washed with 1 volume of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 100 µl of the double distilled water at 65°C until dissolved. The final preparation had a ratio of absorbance at 260 nm to absorbance at 280 nm of > 1.8. The purified DNA was stored at -70 °C until the DNA methylation assay.

Paraffin embedded tissue

The paraffin embedded tissue sample used micro-dissection techniques as previously described Mutirangura, 1999 #10}. Briefly, the DNA was extracted from paraffin wax embedded blocks. 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 a disposable needle no.21 mounted on a 5 ml syringe under microscope.

The microdisseced tissue fragments was transferred from slide to 1.5 ml microcentrifuge tube which containing 200 µl of Lysis buffer2 and 1/10 volume of proteinase K solution; 20 mg Proteinase K in 1.0 ml of 1% SDS-2mM EDTA. The sample was incubated in water bath at 37 °C for overnight (16-24 h) for complete digestion. After adding 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) , the sample was shaken vigorously for 15 seconds and then centrifuge at 10,000 g for 5 min. The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 0.5 volume 7.5 M Amonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then mix by inversion. The DNA should immediately from a stringy precipitate and then the DNA was recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and then the pellet was washed with 1 volume of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 50 µl of the double distilled water at 65°C until dissolved. The DNA was stored at -70 °C until the DNA methylation assay.

White blood cells (WBC)

Five to ten ml of whole blood was centrifuged for 10 min at 1,500 g. The supernatant was removed and the buffy coat was collected to a new polypropylene tube. The buffy coat was added with 10 volumes of cold lysis buffer 1 (or 10 ml), then was mixed thoroughly and incubated at -20 °C for 5 min. The tube was centrifuged for 10 min at 1000 g, then remove supernatant.

The pellet was added with 3 ml of cold lysis buffer 1, then was mixed thoroughly and was centrifuged at 1000 g for 5 min. The supernatant was discard and added 900 µl of lysis buffer 2, 10 µl of Proteinase K solution (20 mg of Proteinase K in 1.0 ml of 1% SDS-2mM EDTA, should be prepare 30 min before use), and 50 of 10% SDS, then mix vigorously for 15 seconds. The sample was incubated in water bath at 37 °C for overnight (16-24 h) for complete digestion. Adding 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) shake vigorously for 15 seconds and then centrifuge at 10,000 g for 5 min. The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 0.5 volume 7.5 M Amonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then mix by inversion.

The DNA should be immediately removed from the stringy precipitation and then the DNA was recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and then the pellet was washed with 1 volume of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 50 µl of the double distilled water at 65°C until dissolved. The DNA was stored at -70 °C until the DNA methylation assay.

Bisulfite treatment of DNA

After extraction, all DNA samples were treated with sodium bisulfite as previously described [90]. Briefly, 1µg /50 µl of genomic DNA was denatured in 0.22 M NaOH (5.5 µl of 2M NaOH) at 37°C for 10 min. Thirty microliters of 10mM hydroquinone and 520µl of 3M sodium bisulfite were added for 16-20 hrs at 50°C. The DNA was desalted with the the DNA Clean-up kit (Promaga, Medison, WI), and then was desulfonated by incubation in 0.33 M NaOH (5.5 µl of 3M NaOH) at 25°C for 3 min. The DNA was ethanol precipitated by adding 1µl of 20ng/ml glycogen, 23 µl of 7.5 M Amonium acetate (CH_3COONH_4), 240 µl of cold (100%) absolute ethanol and then mix by inversion. The DNA should precipitate by incubation at -20 °C for 2 hrs, and then the DNA was recovered by centrifugation at 23,000 g (14,000 rpm) for 15 min. The supernatant was removed and then the pellet was washed with 1.0 ml of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 50 µl of the double distilled water at 65°C until dissolved. The DNA was stored at -70°C until the DNA methylation assay.

Methylation analysis of the promoter regions by the COBRA and MSP method

All SHP-1 bisulfite oligonucleotide sequences were derived from Genbank (accession number U47924.1). DNA methylation in the promoter 2 region of SHP-1 was detected by duplex methylation specific PCR (MSP) using methylation specific primers and non-methylation specific primers.

The methylation specific primers sequences were identical to those previously published [91]. The methylation specific forward primer sequences (FM) were 5′-TGT-GAA–CGT-TAT-TAT-AGT-ATA-GCG-3′ and reverse primer (RM) 5′-CCA-AAT–AAT-ACT-TCA-CGC-ATA-CG-3′ (162952-162975, 163103-163125) and the non-methylation forward specific ones (FU) were 5′-GTG-AAT-GTT–ATT-ATA-GTA–TAG-TGT-TTG-G-3′ and non-methylation reverse primer (RU) 5′-TTC–ACA-CAT-ACA-AAC-CCA-AAC-AAT-3′ (162953-162980, 163091-163114). A duplex MSP reaction was carried out in 20 µl mixture containing 1xPCR buffer (Tris-Cl, KCl, $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, pH 8.3), 1 unit HotStarTaq DNA Polymerase, 200 nM of each dNTPs (Qiagen), 0.25 µM of each forward and reverse primers and 2 µl of the bisulfite treated DNA. The mixture was subjected to 1 cycle at 95°C for 10 min, 40 PCR cycles, each consisted of 1 min at 95°C for denaturation, 1min at 58°C for annealing, and 1 min at 72°C for extention. The final extension step was at 72°C for 10 min in a thermalcycler (GeneAmp PCR system 2400, Perkin Elmer, USA). The PCR products, 174 and 162 bp for methylated and non-methylated sequences, respectively, were separated by electrophoresis on 6% non-denaturing polyacrylamide gel. The amplicons were analyzed by staining with cyber green and calculating the percent methylation upon visualization on a phosphor-imager using the Image Quant software (Molecular Dynamics, Pharmacia Amersham).

SHP-1 promoter 2 methylation level was calculated as a percentage of intensity of the methylated sequence divided by the sum of methylated and non-methylated amplicons.

On the micro-dissected paraffin-embedded tissues, semi-nested PCR was performed. Two µl of the duplex MSP product were added to a solution containing a nested forward primer (NF), 5'-AGY-GTG-GGT-TAG-GGA-GGG-3' (bisulfited nucleotides 163031-163048) and both of the reverse duplex PCR (RM and RU) oligonucleotides. The amplification reaction was carried out as described above. The duplex amplicons, 94 and 83 bp for methylated and non-methylated sequences, respectively, were analyzed by the phosphor-imager as previously described.

COBRA (COmbined Bisulfite Restriction Analysis) [92] by semi-nested PCR was applied for detection of methylation in the promoter 1 region. The forward (5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′) and reverse (5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-A 3′) primers (bisulfited nucleotide number 158572-158592, 158743-158722, respectively) were used to generate a 172 bp amplicon. The primary PCR amplification was carried out in 20 µl mixture containing 1xPCR buffer (Tris-Cl, KCl, $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, pH 8.3), 1 unit HotStarTaq DNA Polymerase(Qiagen), 200 nM of each deoxynucleotide triphosphates (dNTPs), 0.5 µM of each forward and reverse primers and 2 µl of the bisulfite treated DNA.

The mixture was subjected to 1 cycle at 95°C for 10 min, 40 PCR cycles, each consisted of 1 min at 95°C for denaturation, 1min at 44 °C for annealing, and 1 min at 72°C for extention. The final extension step was at 72°C for 10 min in a thermalcycler (GeneAmp PCR system 2400, Perkin Elmer, USA). One µl of primary PCR was transferred for semi-nested PCR with 5′-AAC-CCA-AAC-CAA-ATA-AAA-3′ (bisulfited nucleotide number 158657-158640) as the reverse primer. Amplification conditions were identical to those in the primary PCR and generated an 86 bp amplicon. The PCR product was digested by *Taq I* restriction enzyme (MBI fermentas, Flamborough, Ontario, Canada) at 65 °C for 16 hrs and separated by 6% acrylamide gel electrophoresis.

Bisulfite DNA sequencing

For promoter 1, bisulfite treated genomic DNA was amplified by using primer 5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′ and 5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-3′ (bisulfited nucleotides 158572-158592 and 158743-158722, respectively). For promoter 2, bisulfite treated genomic DNA was amplified using primers 5′-GTT-TTA-TAG-GGT-TGT-GGT-GAG-AAA-TT-3′ and 5′-ACA-CAT-ATA-TAC-CTT-ACA-CAC-TCC-AAA-3′ (bisulfited nucleotides 162912-162937, 163153-163127, respectively). The PCR products were cloned into the pGEM-T easy vector (Promega, Southam, UK) according to the manufacturer's protocol. The ligated products were transformed into *E. coli* DH5α.

The clones were selected by X-gal/IPTG and amplicilin resistance. Recombinant plasmid was purified by DNA purified PCR kit (Promaga, WI) according to the manufacturer's protocol.

For sequencing Analysis, the purified recombinant plasmid I (pGEM-T easy containing 86 bp PCR product of promoter 1 region) and recombinant plasmid II (pGEM-T easy containing 242 bp PCR product of promoter 2 region) 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 (sense) and distilled water was added to bring the final volume to 20µl 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 2400)

The sequencing reaction was purified using simplified ethanol precipitation. Ten microliters of sequencing reaction was added with 2 µl of 3M Sodium acetate (NaOAC), 50 µl 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 for 20 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 of 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.

RNA extraction

Total RNA was extracted from cell lines as well as skin biopsies using the Trizol reagent (Life technologies, Inc.) according to the manufacturer's instructions. Ten to six x 10^6cells from each cell line sample or 50-100 mg from fresh skin which is cut off into small pieces and then is homogenized using homogenizer, was mixed with 1 ml Trizol reagent. After vortexing, RNA was extracted by mix of 200 µl chroloform. The sample was shaked vigorously for 15 seconds and incubated at room temperature for 10 minutes. The sample was centrifuged at 12,000 g for 15 minutes at 4 ^{o}C. The aqueous phase containing RNA was transferred to new microcentrifuge tube, adding 0.5 Isopropyl alcohol and incubation at room temperature for 10 min. RNA was precipitated by centrifugation at 12000 g for 10 min at 4^{o}C. RNA pellet was washed twice time with 1 ml of cooled 75% ethanol. RNA pellet was dry at room temperature for 5 min and resuspend the RNA pellet in 50 µl DEPC treated sterile water. RNA was keep at – 70^{o}C for used as template for RT-PCR .

RT-PCR and Real-time RT-PCR

The RNA preparation was dissolved in 20 µl of RNase free distilled water containing 40 U of ribonuclease inhibiter (Promega), 50 ng of the RNA. The RNA was incubated at 70°C for 5 min with 15 pmol of oligo dT primer followed by at 25°C for 5 min, 42°C for 1 hr, 70°C for 15 min and then keep at 4°C. To each sample we added 19 µl of a reaction mixture containing 1x Improm II buffer (10mM Tris-HCl, pH 8.3, 1.5 mM MgSO4, 50mM KCl and 0.001% gelatin), 5U of RT reverse transcriptase (Promega, Santhan, UK) and 0.2 mM deoxynucleotide triphosphates(dNTPs). cDNA was amplified using the exon3-4 primer 5′-CCC-ACC-CTG-ACG-GAG-AGC-3′ (163729-163438), and either the exon 1 primers 5′-CTC-CCT-ACA-GAG-AGA-TGC-TGT-CC-3′ (158721-158743) or the exon 2 primers 5′-ACT-GGG-AGC-TGC-ATC-TGA-GG-3′ (163438-163419). All SHP-1 RT-PCR oligo sequences were obtained from Genbank (accession number NM 080549.2). GAPDH was used as a control as previously published [93]. The PCR reactions carried out in 20 µl mixture containing 1xPCR buffer (Tris-Cl, KCl, $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, pH 8.3), 1 unit Taq DNA Polymerase, 200 nM of each dNTPs (Qiagen), 0.1875 µM of each exon1or exon2 and exon3-4 and 1 µl of the cDNA, for isoform I and II respectively. The mixture was subjected to 1 cycle at 95°C for 5 min, 40 PCR cycles, each consisted of 30 second at 94°C for denaturation, 1min at 60°C for annealing, and 1 min at 72°C for extention. The final extension step was at 72°C for 10 min in a thermalcycler (GeneAmp PCR system 2400, Perkin Elmer, USA).

The amplicons are 156 and 239 bp for Isoform I and II, respectively. The products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining to ensure and proportionate specific amplicons.

Real-time PCR was performed in a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA) using QuantiTect SYBR Green I (Qiagen, Hilden, Germany), according to the manufacturer's instructions in a total volume of 20 µl. Cycling conditions were as follows: 95°C for 15 min; 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, for 40 cycles, followed by melt analysis from 65 to 95°C. The SHP-1 (II) primers were same as above. PCR reactions were conducted in parallel to normalize for differences in cDNA synthesis. To correlate the threshold (Ct) values from the amplification plots to copy number, a standard curve was generated using the pGEM-T SHP-1(II) plasmid. PCR product melt curves were analyzed for a specific peak. The products were visualized by agarose gel electrophoresis and then ethidium bromide staining to ensure and proportionate specific amplicons. A non template control was run with every assay, and all determinations were performed to achieve assay.

Data Analysis

Levels of *SHP-1* promoter 2 methylation and isoform II among skin lesions were compared and significant differences were determined by unmatched two-tail T-test using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL).

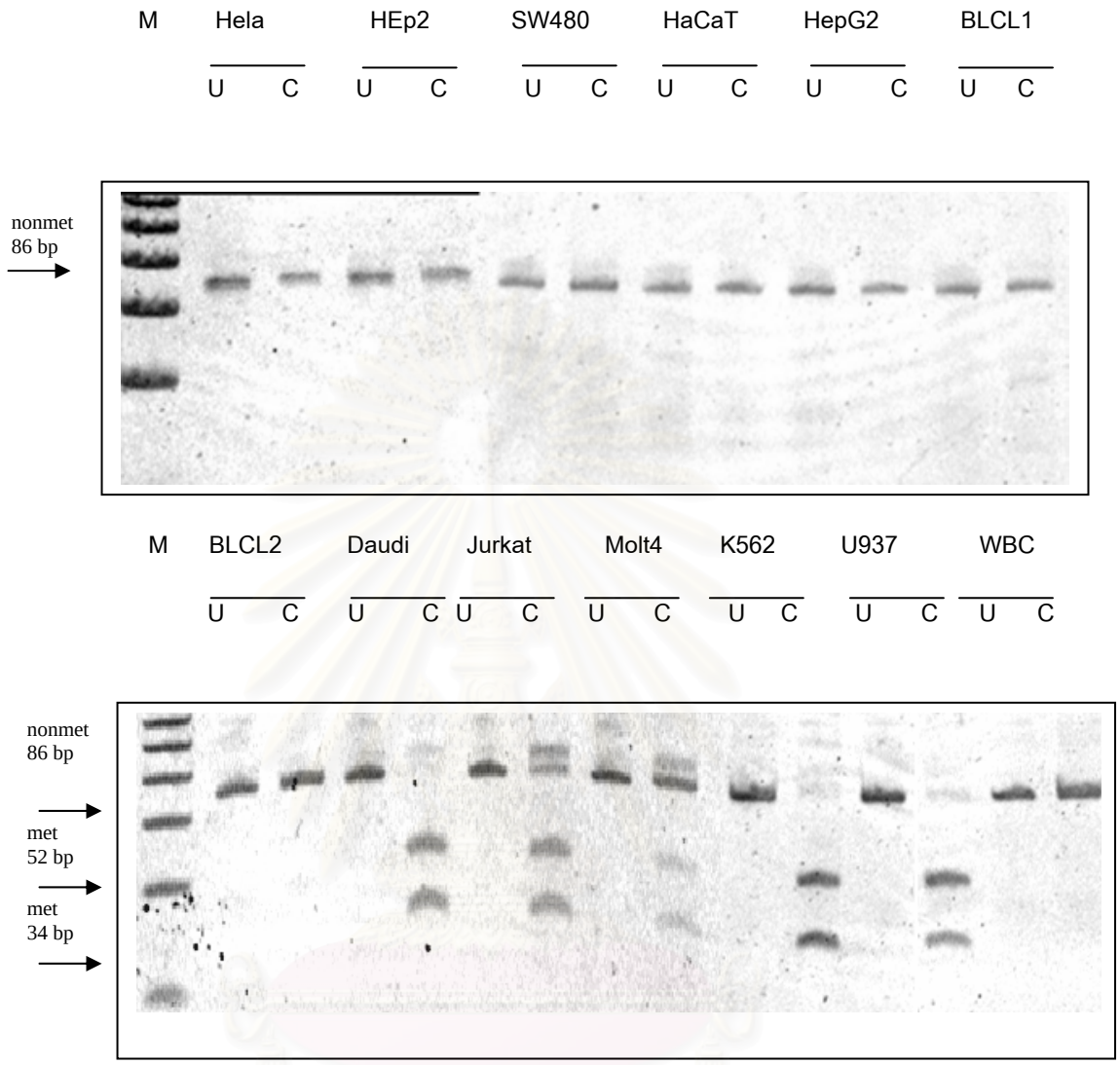
CHAPTER IV

RESULTS

1. *SHP-1* methylation and isoforms in human cell lines

Cell type specific SHP-1 isoforms, distinguishable by promoter transcriptional activity have been reported [5]. Whereas most hematopoietic cells express high levels of isoform II, the majority of epithelial cell line transcripts display lower levels of isoform I. Hence, our first experiment was aimed at determining if this tissue specific transcription process is regulated by promoter hypermethylation. A new COBRA approach was designed to analyze promoter 1 and the published MSP [91] was modified to study promoter 2 (Figure 3). The results demonstrate distinctive methylation patterns for hematopoietic and epithelial cell lines. While promoter 2 hypermethylation was constantly detected in all epithelial cell lines (Hela, HEp2, SW480, HaCaT and HepG2), promoter 1 without exception was non-methylated. In hematopoietic cells, a variety of methylation patterns could be observed. Whereas methylation was not detected in WBC, it was discernible in some hematopoietic cell lines. Promoter 1 was non-methylated in both BLCLs, and methylated in Daudi, K562 and U937. Both promoters were incompletely methylated in Jurkat and Molt4. Limited or absence amounts of promoter 2 methylation were demonstrated in both BLCLs, Daudi and U937. Finally, promoter 2 of K562 was hypermethylated.

(A) Promoter 1



(B) Promoter 2

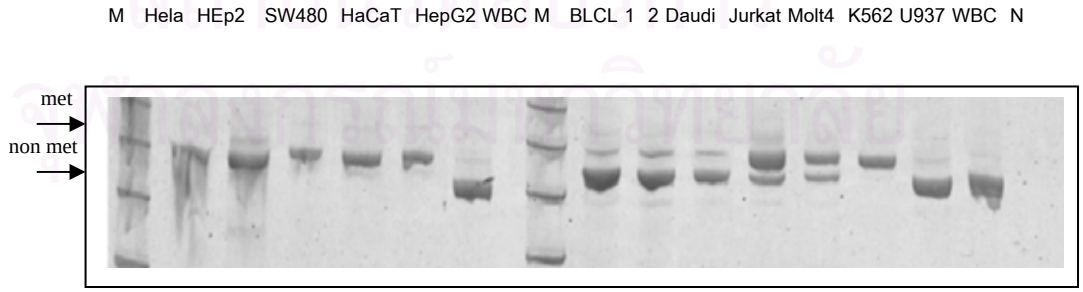


Figure 3. (A) SHP-1 promoter methylation in various epithelial and hematopoietic cell line. Promoter 1 methylation is detectable by COBRA.

The PCR products, indicated by non-met arrow, are 86 bp. Merthylated amplicons, indicated nt met arrow, are digestible to 52 and 34 bp. M is standard size DNA marker, U is uncut, and C is digested amplicon. Cell sorces of genomic DNA are listed above of each lane. (B) For promoter2 , duplex MSP yield 2 DNA fragments. Methylated amplicons, indicated by met arrow, are 174 bp, and nonmethylated products, indicated by non-met arrow, are 162 bp, respectively. N is negative control.

We confirm the new COBRA method by bisulfite, PCR, cloning and sequencing. Promoter 1 region of WBC and Daudi cell line displayed the non-methylated and methylated sequences, respectively.

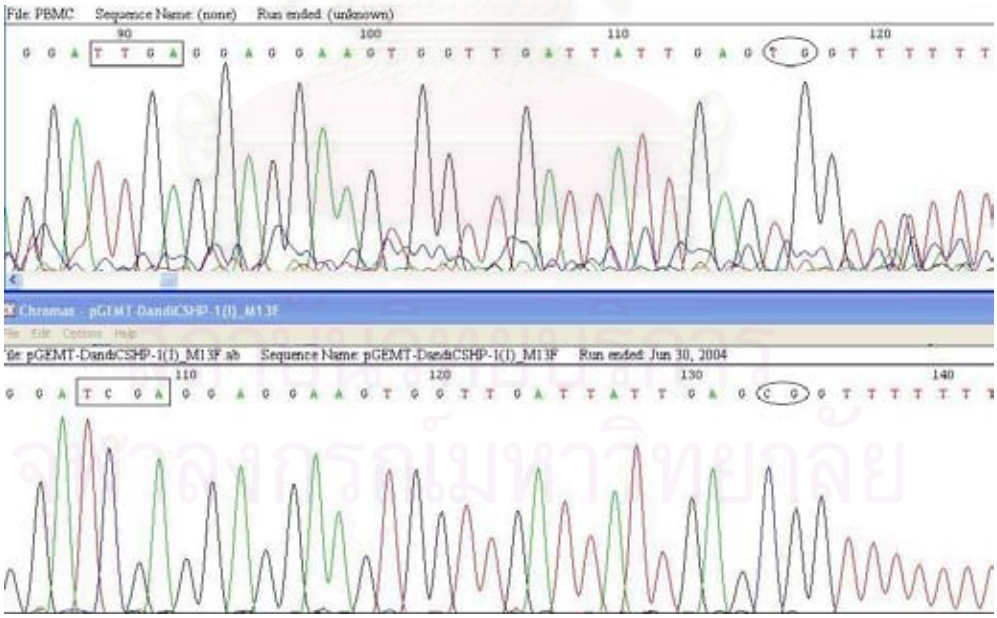


Figure 4. shown the electrophoregram of nucleotide sequence of WBC and Daudi. Taq 1 restriction enzyme cutting site indicated by rectangular and complete bisulfite treated DNA indicated by ellipse circle.

For promoter 2, there are three patterns such as methylated, non-methylated and partial band. We also used Hep2, Daudi and Molt4 cell lines for confirming complete, absent and partial methylation, respectively. We designed the two set of primer (FT, RT primer) that to cover the SHP-1 promoter 2 with in eleven CpG sites. Bisulfite treated DNA, PCR, cloning and sequencing were done. Almost of eleven CpG site of five colonies of recombinant pGEM-T HEp2 displayed methylated sequence for instant the recombinant clone 1 showed methylated CpG (Figure 5). While, the recombinant pGEM-T Daudi displayed all of non-methylated CpG site (Figure 6).

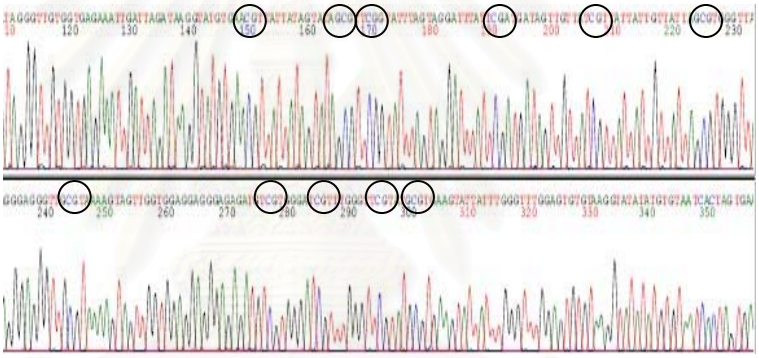


Figure 5. Example of the methylated CpG site of recombinant pGEM-T Hep2 clone 1 is shown. Black and red circles are methylated and non-methylated CpG dinucleotide, respectively.

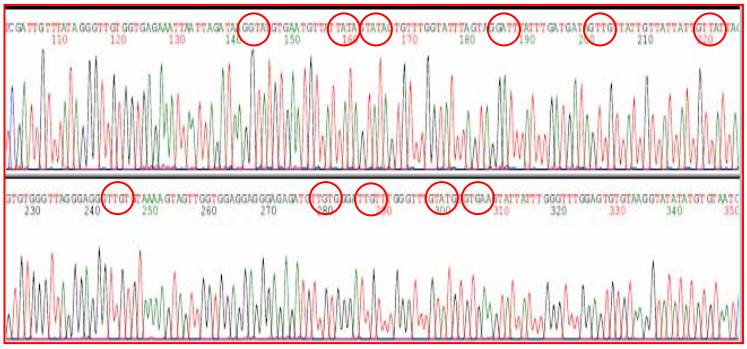


Figure 6. Example of the non-methylated CpG site of recombinant pGEM-T Daudi clone 1 is shown. Black and red circles are methylated and non-methylated CpG dinucleotide, respectively.

Interestingly, the recombinant pGEM-T Molt4 displayed a variety pattern of methylaed CpG site. For instant, clone 1 showed methylated site at position 2, 3, 4, 5, 7, 9 and 10 (Figure7). Whereas, clone 9 only showed methylated site at position 2 (Figure 8).

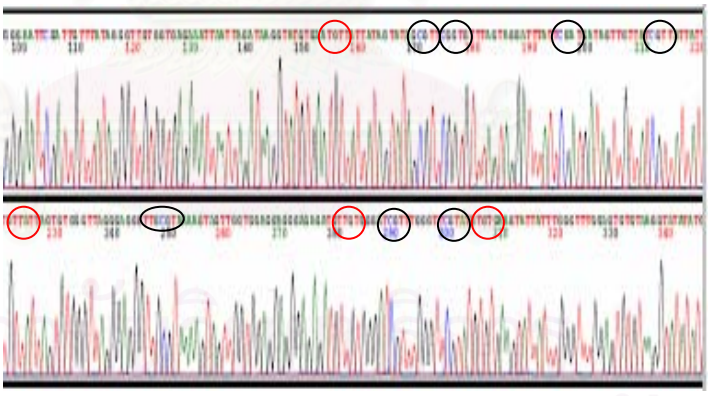


Figure 7. Example of the methylated CpG site of recombinant pGEM-T Molt4 clone 1 is shown. Black and red circles are methylated and non-methylated CpG dinucleotide, respectively.

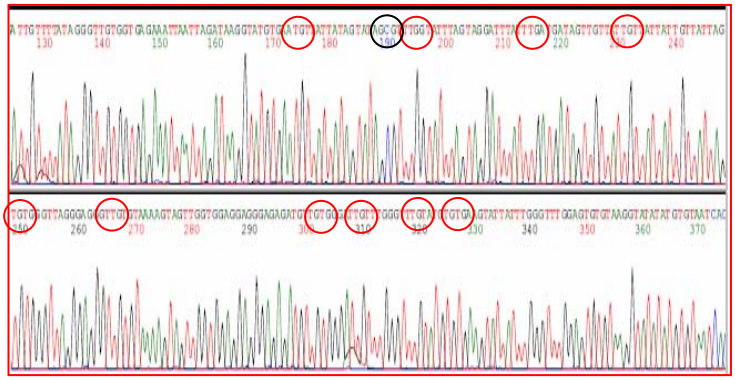


Figure 8. Example of the methylated CpG site of recombinant pGEM-T Molt4 clone 9 is shown. Black and red circles are methylated and non-methylated CpG dinucleotide, respectively.

Therefore, these results confirmed the promoter 2 region of HEp2 were methylated, Daudi were non-methylated and Molt4 present mosaic form (Figure 9).

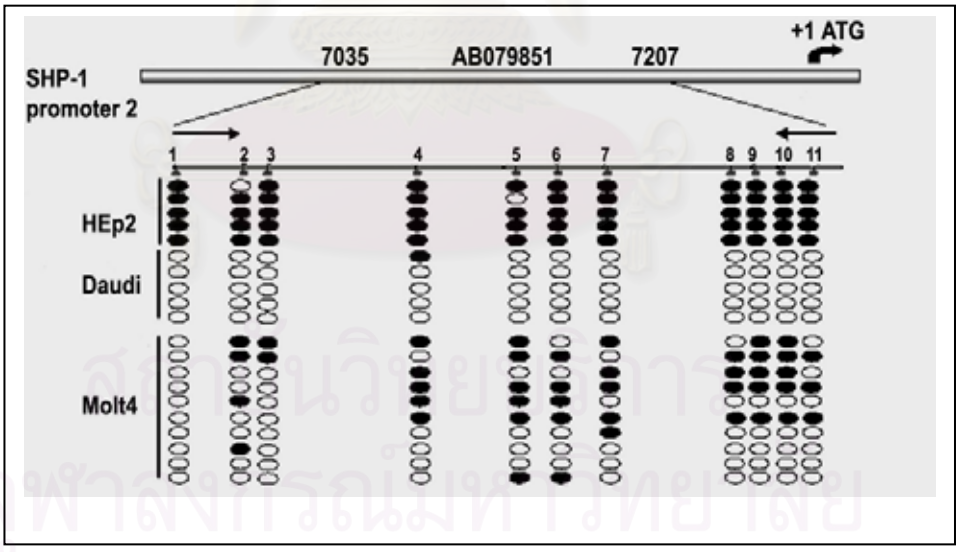


Figure 9. Methylation status of CpG nucleotide at promoter 2of HEp2, Daudi, and Molt4. The upper-most vertical bar locates the relative nucleotide sequence in

Genbank (accession number AB079851). The numbers and arrows indicate the start and end of sequenced nucleotide in relation to the transcriptional start codon, ATG (nucleotide 7604). The numbers of the line below indicate CpG nucleotides. Each circle exemplifies the methylation status of each selected clone. Black and while circles are methylated and non-methylated CpG dinucleotide, respectively.

We observed a striking inverse correlation between promoter methylation and transcriptional activity. As for promoter 1, DNA methylation was discovered in Daudi, Jurkat, Molt4, K562 and U937 while isoform I cDNA was not detectable. In contrast, promoter 1 of all epithelial cells and both BLCLs was non-methylated and transcribed. WBCs, however, were devoid of both methylation and isoform I, suggesting another transcriptional silencing mechanism. cDNAs, 239 bp, derived from isoform II were observed when amplifying *SHP-1* RNA between exon 2 and 3-4 (Figure 10). All human epithelial cell lines including HeLa, HEp2, SW480, HaCaT and HepG2 were promoter 2 hypermethylated and *SHP-1* mRNA isoform II deficient. In contrast, *SHP-1* isoform II was strongly expressed in all promoter 2 non-methylated human hematopoietic cells, BLCLs, Daudi and U937 (Figure 10). Both promoter 1 and 2 were methylated in K562 and consequently, we found limited amounts of both *SHP-1* mRNA isoforms.

The high isoform II levels from promoter 2 of Jurkat and Molt4 are noteworthy since they displayed incomplete promoter 2 methylation (Figure 10). Our findings confirmed and summarized the strong association between methylation of both promoters and their activities (Table 3).

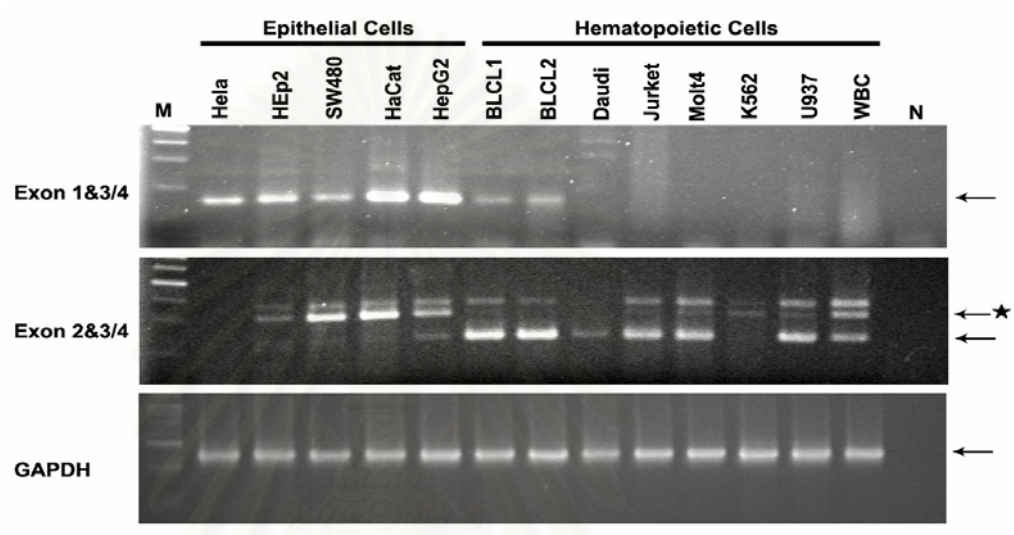


Figure 10. SHP-1 RT-PCR result of epithelial and hematopoietic cell lines. Cells are listed on the top of each lane. M is standard DNA marker, and N is negative control. Arrows indicated the locations of expected amplicons. Exon1&3-4 is RT-PCR between exon 1 and exons 3-4, and the aplicon size is 156 bp. Exon 2&3-4 amplifies 239 bp of isoform II. GAPDH was used in the RT-PCR as internal control.

Table 3. DNA methylation of Promoter 1 and 2 of SHP-1 of hematopoietic (A) and non-hematopoietic cell lines(B).

(A) Hematopoietic cell lines

Name	Cell type	Promoter 1 (COBRA)	Promoter 2 (MSP)	SHP-1 mRNA
BLCL donor I	Infected EBV B Lymh.	Unmet	Unmet	I+II
BLCL donor II	Infected EBV B Lymh.	Unmet	Unmet	I+II
Daudi	B Lymphoblast	Met	Unmet	II
Jurkat	T Lymphoblast	Unmet (30.63%) Met (69.37%)	Unmet (43.43%) Met (56.57%)	II
Molt4	T Lymphoblast	Unmet (61.57 %) Met (38.42%)	Unmet (47.31%) Met (52.69%)	II
K562	Myelogenous Leukemia	Met	Met	Not found
U937	Histocytic cell (monocyte)	Met	Unmet	II
WBC	Whilte Blood cell	Unmet	Unmet	II

(B) Epithelial cell lines

Name	Cell type	Promoter I (COBRA)	Promoter II (MSP)	(I)SHP-1 mRNA
HELA	Cervical carcinoma	Unmet	Met	I
HEp2	Cacinotumor of Lynx	Unmet	Met	I
SW480	Colorectal adenocarcinoma	Unmet	Met	I
HaCaT	Immortalized human keratinocyte	Unmet	Met	I
HepG2	Hepatocyte	Unmet	Met	I

Met = methylation

Unmet = unmethylation

2. *SHP-1* methylation in normal epithelia *in vivo*.

The complete promoter 2 methylation in epithelial cell lines prompted us to hypothesize that the epigenetic control was tissue specific rather than indicative for carcinogenesis. Accordingly, we screened the methylation in normal epithelia of several organs. Several stratified epithelial including skin, bladder and cervix, large exocrine gland, breast, and large solid organs mainly comprising epithelial tissues such as lung, kidney and liver were investigated (Table 4). Whereas no promoter 2 methylation was found in WBCs from healthy donors, complete methylation was demonstrated in almost all epithelial cells (Figure 11). We also summarized this result in figure 12.

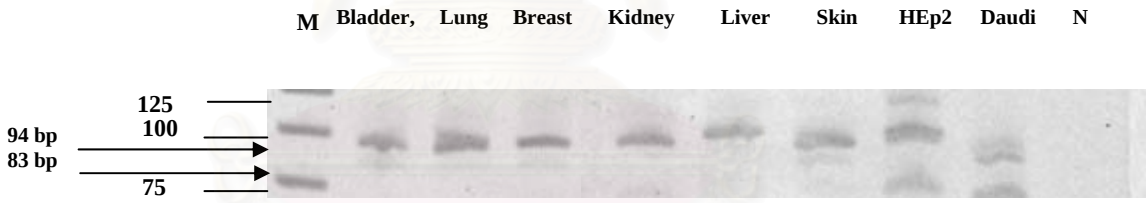


Figure 11. SHP-1 promoter 2 methylation in epithelial, Representative results of seminested duplex MSP of epithelial tissue obtained from microdissected, paraffin-embedded tissue of bladder, Lung, Breast, Kidney, Liver and skin. M is standard DNA marker. HEp2 is methylated control, indicated by arrow, are 94 bp. Daudi is non methylated control, indicated by arrow, are 83 bp. N is negative control.

Table 4. shown promoter 2 methylation of 27 microdisection paraffin-embedded tissues from normal of epithelial organ .

Source of organ	Slide no.	non-met	met
Breast (n=5)	B469AN	0	100
	B766AN	0	100
	B1068DN	0	100
	B1340CN	0	100
	NB1	0	100
LIVER (n=3)	H4706DN2	0	100
	H4269AN1	0	100
	H6450PN5	0	100
KIDNEY(n=3)	4166BN KID	0	100
	2458BN KID	0	100
	21158ANKID	0	100
LUNG (n=2)	5895AN LUNG	0	100
	7717BN LUNG	0	100
BLADDER (n=2)	BC4	0	100
	BC5	0	100
PAP SMEAR n=3	CXF34N	0	100
	CXF42N	0	100
	CC44	0	100
SKIN (n=9)	J1	0	100
	J2	46.8	53.2
	J3	0	100
	J4	0	100
	J5	0	100
	ฝ่ามือ3 (H1)	0	100
	ฝ่ามือ4 (H2)	0	100
	ฝ่าเท้า (H3)	0	100
	S46-5123	0	100

Normal tissue	Met range(%)	mean, met	Standard division (SD)
Breast	100	100	0
Liver	100	100	0
Kidney	100	100	0
Lung	100	100	0
Bladder	100	100	0
Pap smear	100	100	0
skin	53.2-100	94.8	15.6

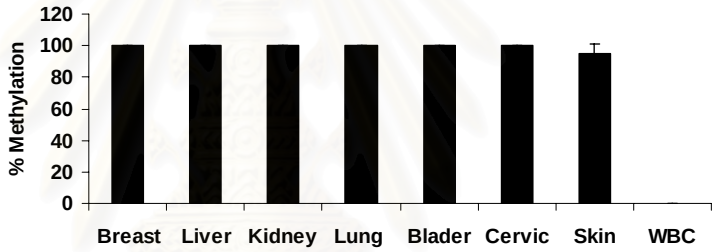


Figure 12. Average level and stand deviation (SD) of promoter methylation in epithelial tissues of several organs and WBC, as list below. T indicated the methylation SD within individual cell types. Note that 0 SD can be observed in most cases.

To clarify, the differences between the different types of samples analysed. Both paraffin embedded tissues and epithelial cell lines are different from fresh tissue in many instances, including expression patterns. Therefore, we compared OTC-embedded frozen normal cervical sections with paraffin embedded tissues.

The methylation status is not depended on tissue preservation (Figure 13). It is noteworthy that incomplete methylation was sporadically identifiable in 1 out of 9 skin cases (Figure 12).

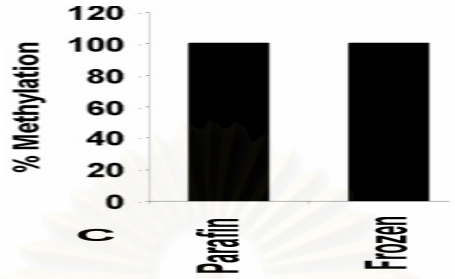


Figure 13. Epithelial DNA from paraffin-embedded tissues and frozen section. The heights of each bar represent percentage of promoter 2 methylation of each tissue types.

3. *SHP-1* promoter 2 met/demethylation in skin diseases

To explore the significance of promoter 2 demethylation in skin, we investigated if and which types of pathologic conditions possessing larger degrees of loss of methylation could be observed (Table 5).

Keratinocytes from psoriasis, SCC, and eczema lesions were collected using micro-dissected paraffin embedded tissues and their methylation levels were compared with those of normal skins (Figure 14). In addition to the 8 out of 9 normal skins, hypermethylation was discernible in the majority of eczema and SCC cases. In contrast, demethylation was found in almost all, 8 out of 9, cases of psoriasis. The average methylation level of normal skin is 94.8% whereas in psoriasis it is 68.1%.

From this it can be concluded that the levels of promoter 2 methylation in psoriasis are distinct from those in healthy skin lesions ($p<0.005$). (Figure 14). Since promoter activity and expression of many proteins change with course of keratinocyte terminal differentiation and pattern of epidermal differentiation is altered in psoriatic skin, we investigated the demethylaton in distinctive compartments. Several psoriatic skins were microdissected and divided into upper and lower half of the epidemis and the promoter methylation levels were compared. No significant demethylation level between psoriasis with different clinical sign, chronic stable plaque type and guttate psoriasis, were not distinguishable .

Table 5. shown promoter 2 methylation of 37 microdisection paraffin embedded skin diseases including Psoriasis, SCC and Eczema.

Skin disease	Slide no.	nonmet	met
Psoriasis (n=9)	827	28	72
	681	36.83	63.17
	680	0	100
	611	40.27	59.73
	609	15.27	84.73
	583	47.74	52.26
	497	60.27	39.73
	465	27.32	72.68
	361	31.62	68.38
SCC (n=10)	003/44	100	0
	65/44	0	100
	200/44	0	100
	373/44	0	100
	791/44	0	100
	816/44	43.85	56.15
	1829/44	0	100
	1303/45	0	100
	465/46	0	100
	989/46	0	100
Eczema (n=8)	44-46	0	100
	286-46	0	100
	544-46	0	100
	611-46	20	80
	658-46	0	100
	779-46	0	100
	997-46	100	0
	1223-46	0	100

Skin disease	Met range(%)	mean, met	Standard division (SD)
skin normal	53.2-100	94.8	15.6
Psoriasis	39.73-100	68.075	17.608
SCC	0-100	85.615	14.616
Eczema	0-100	85	35.05

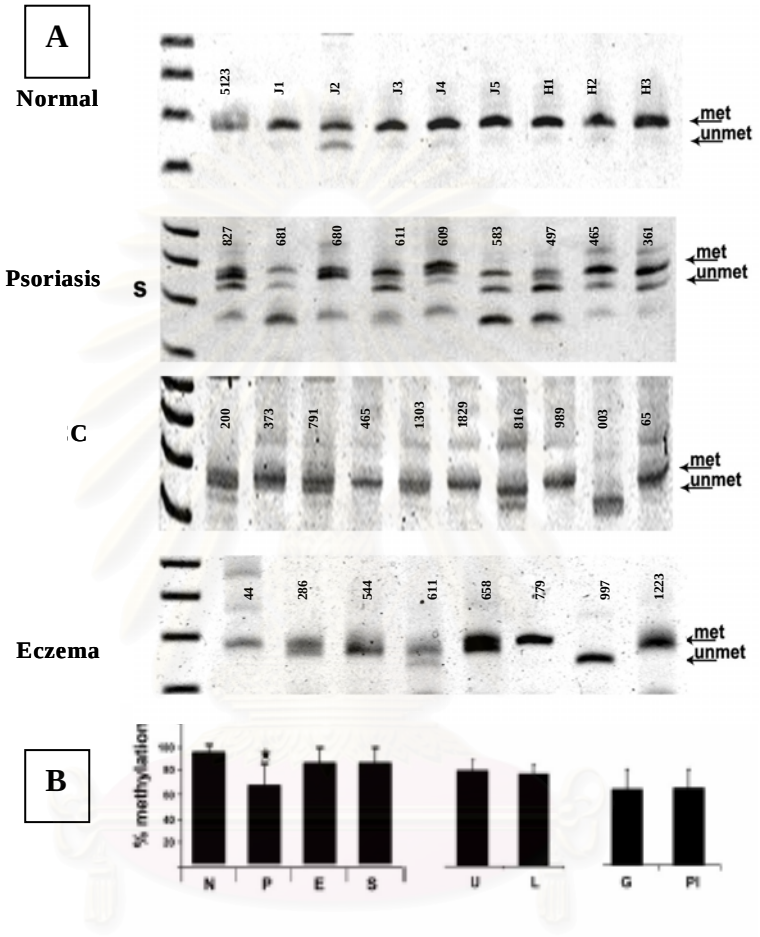


Figure 14. SHP-1 promoter 2 methylation in various skin lesions, normal, psorisis, eczema, and SCC. (A) Examples of seminested duplex PCR shows result. Met and non-met arrows are expected 94 bp methylated and 83 bp nonmethylated amplicons, respectively. (B) Methylation percentages and SD; N, P, E, S, U, L, G, Pl are the levels of normal skins, psoriasis, eczema, SCC, upper and lower part of microdissected psoriatic skin epidermis, and guttate and chronic

plaque type psoriasis, respectively. The star indicates significant differences of $p<0.005$ between psoriasis and normal skins.

We also compared between the mRNA expression of *SHP-1* isoforms in fresh biopsies of psoriatic lesions to that in normal skin by semi-quantitative RT-PCR. Interestingly, the association between promoter 2 methylation and the amount of isoform II mRNA in psoriatic skins was similar to that observed in the previously analyzed promoter 2 incompletely methylated cell lines, Jurkat and Molt4. Psoriatic skin lesions predominantly expressed isoform II, identifiable from all cases, whereas promoter 2 activities were absent or decreased in the majority of normal skin samples (Figure 15A). The isoform II mRNA level of psoriatic skin lesions as measured by real time PCR of exon 2 and 3-4 RT-PCR were average 58 copies whereas in normal skin they amounted to 18 copies per 1µg of total RNA (Figure 15B). This indicated that *SHP-1* promoter 2 activity of psoriatic lesions was significantly higher than in normal skins ($p<0.05$). This finding confirms the increment of promoter 2 activity in psoriasis by a demethylation mechanism compared to normal skin.

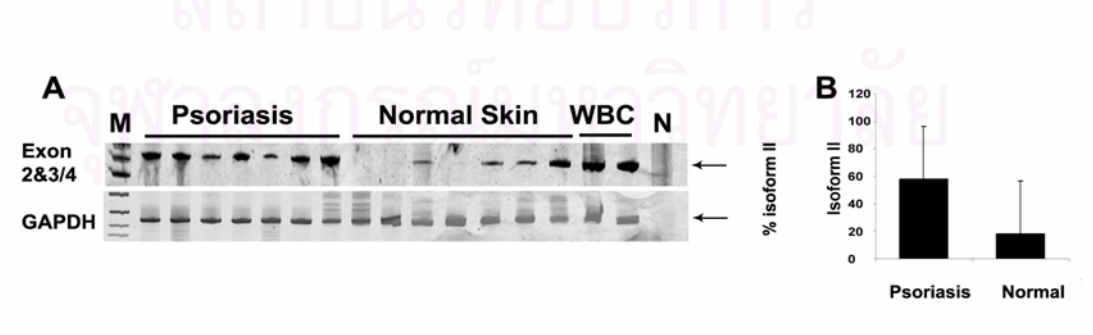


Figure 15. SHP-1 RT-PCR of psoriatic skin lesions and normal skins. (A) cell types are listed on the top of each lane. M is standard DNA marker, and N is negative

control. Arrows indicated the locations of expected amplicons. Exon 1 and 3-4 is RT-PCR between exon 1 and exon 3-4, and the amplicon is 156 bp. Exon 2 and 3-4 amplifies 239 bp of isoform II. GAPDH is RT-PCR results located at the bottom of (A). (B) The mean and SD of isoform II level of psoriasis and normal skin proportion to 1 μg total RNA are height of each bar and T, respectively. The real time RT-PCR showed significant difference at $p<0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

Alteration of gene expression is at the center of cellular pathogenesis. Accordingly, DNA methylation should be associated with human diseases since epigenetic modification usually silences gene transcription. Normally, DNA methylation is important in several biological processes, such as x chromosome inactivation and genomic imprinting [43-46, 94, 95]. As for abnormal conditions, promoter hypermethylation of several tumor suppressor genes and generalized hypomethylation have been reported to promote the potential for malignant transformation [91, 94, 96-98]. To the best of our knowledge, this study has been the first to prove that the tissue specific promoter methylation status can be altered in a non-malignant human disease condition. In contrast to previously described cases of lymphomas/leukemias, the *SHP-1* promoter 2 is hypermethylated in normal epithelia and its activity is consequently limited. Finally, we selectively analyzed several pathologic skin lesions and demonstrated that promoter 2 demethylation usually arose in psoriatic skin lesions and consequently triggered up-regulation of SHP-1 isoform II. According to previous data, mRNA derived from promoter 2 is distinctive from promoter 1 transcripts in both structure and quantity [5, 99]. The sequence of isoform II differs from isoform I by embracing few different amino acids at the N-terminal. Additionally, a strikingly large amount of mRNA is usually generated from promoter 2 [5].

Moreover, the molecular physiologic responses to up-regulate cell growth of SHP-1 in hematopoietic and epithelial cells are distinct. SHP-1 isoform II has been projected as a negative regulator for signaling of cell proliferation and differentiation of hematopoietic cells via cytokine and non-cytokine receptors such as the cKit/SCF receptor, the IL-3 receptor, the CSF-1 receptor, and the erythropoietin receptor [2-4, 100, 101]. Conversely, it has been reported to be a positive regulator of cell signaling via the MAP kinase pathway in non-hematopoietic cells [5]. In the course of this study, we found demethylation of the promoter 2 region of *SHP-1* correlated with up-regulation of *SHP-1* isoform II mRNA in psoriatic skin lesions. Hence, up-regulation of the promoter 2 in psoriatic skin lesions should result in a down stream SHP-1 signal transduction pathway different from that in normal skin. Interestingly, psoriasis is a T-cell mediated disease [102] involving many defects in the regulation of the transcription factors: signal transducer ad activator of transcription (STAT-1 alpha), interferon regulated factor-1 (IRF-1) and NF kappa B. Moreover, the pathogenesis leads to alteration in intracellular signal transduction pathways including MAP kinase and JAK-STAT pathways which lead to loss of growth and differentiation control when the cells are subjected to physico-chemical and immunological stress (37). Consequently, it is crucial to further elucidated if and which particular role SHP-1 isoform II plays in the pathologic psoriasis signal transduction network.

This association-study does not indicate that the *SHP-1* demethylation is specific to psoriasis. In contrast, some other skin lesions possessed the epigenetic loss, therefore we presume that promoter 2 demethylation is a dynamic physiologic process and that its prolongation or sensitisation implicates psoriatic pathogenesis [38]. In this study, we could observe a variety of methylated sequences in some hematopoietic malignant cell lines such as Jurkat and Molt4. This indicates that epigenetic modification is mosaic even if the cancer cells are subject to selective clonal expansion. Accordingly, the variation should result from a gradual loss of methylation *in vitro*. Furthermore, in addition to most psoriasis samples, some cases of skin lesions also sporadically disclosed demethylation [103-105]. Finally, a recent study on lymphoma has proven that down regulation of *STAT3* mRNA results in *SHP-1* demethylation [42]. Since several cytokines and growth factors are responsible for STAT3 activity, *SHP-1* methylation being controlled by STAT3 supports our hypothesis [106]. However, STAT3 may act differently on the *SHP-1* promoter in epithelial cell. A study reported by Sano S et al, has demonstrated that STAT3 up-regulation is an important mechanism in psoriatic pathogenesis [12, 107]. Hence, if on a molecular level STAT3 activity is related to *SHP-1* methylation in epithelial cells, there must be additional tissue specific factors to trigger the opposite outcome, demethylation instead of hypermethylation.

Psoriasis is a common chronic skin disease affecting up to 2.0% in many populations. It is believed to be a multifactorial genetic disorder with the phenotype depending on the patient's genetic background and environmental factors. The disease is characterized by keratinocyte hyperproliferation and early differentiation as a consequence of autoimmune reaction [77]. Management of psoriasis ranges from topical therapies for limited disease to systemic therapies for more widespread disease. Despite various available treatments, the adverse effect or inadequate efficacy of the therapies involved have invoked a necessity for safer and more effective treatment. The advent of therapies based on mechanisms that target critical molecular pathways has evoked considerable interest [87]. For example in cancer, when there is a clear cut "molecular driver," as in Philadelphia chromosome–positive chronic myelogenous leukemia, Imatinib therapy induces dramatic and often durable clinical responses in most patients. The therapeutic index is high, and optimally effective treatment can be achieved at a dose below the maximum tolerance dose [87]. Thus, our novel finding in psoriatic molecular pathophysiology, the epigenetic modification of *SHP-1* promoter 2, may assist in developing new and effective targeted therapies

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APPENDIX

APPENDIX

BUFFERS AND REAGENTS

1. Lysis buffer I

Sucrose	109.54	g
Tris base	1.21	g
$MgCl_2$	1.02	g
Triton X-100	10	ml

Adjust volume to 1.0 litre with dH_2O

2. Lysis buffer II

NaCl	292.20	g
0.5 M EDTA	48	ml

Adjust volume to 1.0 litre with dH_2O

3. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
dH_2O	80	ml

Adjust volume to 100 ml with dH_2O, and sterlize by autoclaving

4. 3 M Sodium acetate (CH3COONa)(pH 5.3)

Sodium acetate	40.82	g
dH2O	80	ml

Adjust the pH to 5.3 by adding conc.HCl

Adjust volume to 100 ml with dH_2O, and sterile by autoclaving

5.1 M Tris (pH)

Tris base	121	g
dH_2O	700	ml

Adjust the pH to 7.0 by adding conc.HCl

Adjust volume to 1.0 litre with dH_2O, and sterile by autoclaving

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate. 2 dH_2O	186.6	g
dH_2O	700	ml

Adjust the pH to 8.0 by adding conc.NaOH

Adjust volume to 1.0 litre with dH_2O, and sterile by autoclaving

7. 20 mg/ml Proteinase K (Stock solution)

Proteinase K	20	g
dH_2O	1	ml

store at -20 ^{o}C

8. 10% Sodium dodecyl sulfate (SDS)

SDS	100	g
dH_2O	870	ml

Adjust the pH to 7.2 by adding conc. HCl (a few drop)

Adjust volume to 1.0 litre with dH_2O

9. 10X TBE buffer (pH 8.3)

Tris base	121.1	g
EDTA.2H_2O	3.7	g
dH_2O	800	ml
Slowly add the boric acid, anhydrous	55.6	g

Adjust the pH to 8.3 by adding conc. HCl

Adjust volume to 1.0 litre with dH_2O

10. LB broth

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Adjust the pH to 7.0 by adding 5N NaOH

Adjust volume to 1.0 litre with dH_2O with sterile water, and sterlize by autoclaving

11. LB agar

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Agar	20	g

Adjust the pH to 7.0 by adding 5N NaOH

Adjust volume to 1.0 litre with dH_2O with sterile water, and sterlize by autoclaving

Pour into Petri dishes (25 ml/100 mm plate)

12. Sodium Bisulfite

Sodium Bisulfite	3.76	g
dH_2O	10	ml

Adjust the pH 5.0 by adding 10 M NaOH (5 drop)

13. Hydroquinone

Hydroquinone	55.4	mg
dH_2O	50	ml

Protected from light by cover with foil

J Mol Med (2006) 84: 175–182
DOI 10.1007/s00109-005-0020-6

ORIGINAL ARTICLE

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SHP-1 promoter 2 methylation in normal epithelial tissues and demethylation in psoriasis

Received: 19 June 2005 / Accepted: 21 October 2005 / Published online: 31 December 2005

Abstract *SHP-1* promoter hypermethylation has been studied in hematopoietic cells and observed only in various types of lymphoma and leukemia. This study reports a contrasting situation in normal epithelial tissues and an association with skin pathogenesis, particularly in psoriasis. We investigated several cell lines, five of them were epithelial and six were hematopoietic, white blood cells from normal, healthy donors, and normal microdissected epithelium of kidney, liver, breast, cervix, lung, prostate, bladder, and skin. Interestingly, promoter 2 hypermethylation was apparent in all epithelial cell lines and tissues. However, distinctive degrees of demethylation were noted in some skin samples. The methylation patterns of each cell line corresponded to their mRNA isoforms, in that isoforms I and II could not be detected with either promoter 1 or 2 hypermethylation, respectively. We further explored whether an enhanced degree of demethylation could be observed in various dermatopathology lesions. While the promoter 2 methylation levels of squamous cell cancers, eczemas, and normal skins were not different, a significant degree of demethylation can be observed in psoriasis ($p<0.005$). In addition, psoriasis displays a higher level of *SHP-1* isoform II than normal skin ($p<0.05$). In conclusion, this study discovered an unprecedented role of *SHP-1* methylation in tissue-specific expression and its alteration in a nonmalignant human disease besides the transcription inhibition in leukemia and lymphoma. Furthermore, the promoter demethylation may play an important role in skin pathogenesis by enhancing *SHP-1* isoform II transcription in psoriatic skin lesions.

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Keywords *SHP-1* · DNA Methylation · Psoriasis · *PTPN6* · Demethylation

Abbreviations SCC: Squamous cell carcinoma · COBRA: combined bisulfite restriction analysis · MSP: methylation-specific PCR

Introduction

SHP-1 or *PTPN6* has been considered a tumor suppressor gene in hematopoietic cells due to its promoter hypermethylation in various types of lymphoma and leukemia [1–4]. Surprisingly, during our study, while *SHP-1* methylation would have been detectable in other malignant genomes, instead, the epigenetic modification was discovered in normal epithelial tissues. This information is very interesting not only because it presents a paradox situation but also because identification of new tissue-specific methylation control genes is crucial for a better understanding of human development and tissue differentiation processes [5, 6]. The aim of this study was to explore *SHP-1* methylation and transcription profile in various cell and tissue types and to elucidate if this epigenetic modification could be altered in any epithelial pathological conditions.

SHP-1, a nonreceptor protein tyrosine phosphatase containing an SH2 domain in tandem and protein tyrosine phosphatase, plays an important role in regulating the growth and proliferation process depending on cell types [2, 7–9]. The gene comprises two promoters, directing the expression of two isoforms [10]. Promoter 1 is located approximately 7 kb upstream from promoter 2. Previous studies distinguished levels and types of *SHP-1* mRNA isoforms in various cell types. Studies in cell lines have shown that isoform I is normally expressed at lower level in epithelial and barely discovered in hematopoietic cells, whereas isoform II is exclusively and vigorously expressed in hematopoietic cells. Interestingly, the SHP-1 protein is located in the cytoplasm of hematopoietic and in the nucleus of epithelial cells [11]. *SHP-1* promoter hypermethylation, discernible in various lymphomas/leukemias, increases the lymphoma development potential by silencing *SHP-1* transcription. The epigenetic regulation has been attributed to promoter 2 [3, 4]. It is important to note that the mechanism inducing the methylation has been described. A recent study conducted on T-cell lymphoma indicates that STAT3, in part, transforms cells by inducing the epigenetic silencing in cooperation with DNMT1 and, apparently, histone deacetylase 1 [12].

Interestingly, transgenic mice with keratinocytes expressing a constitutively active *Stat3* (K5.Stat3C mice) develop a skin phenotype either spontaneously or in response to wounding, which closely resembles psoriasis [13]. Psoriasis is a benign chronic inflammatory skin disease. The skin lesions are characterized by sharply demarcated erythematous plaques of various sizes covered with silvery scaling. These changes are caused by intense skin inflammation with infiltration of inflammatory cells into the dermis and epidermis and strongly enhanced keratinocyte proliferation [14]. This information indicates that both *SHP-1* methylation and psoriasis are linked to STAT3 functions, pointing at a possible association between *SHP-1* methylation and psoriasis.

Psoriasis is a common chronic skin disease affecting up to 2.0% in many populations. It is believed to be a multifactorial genetic disorder with the phenotype depending on the patient's genetic background and environmental factors. The disease is characterized by keratinocyte hyperproliferation and early differentiation as a consequence of autoimmune reaction [15]. Management of psoriasis ranges from topical therapies for limited disease to systemic therapies for more widespread disease. Despite various available treatments, the adverse effect or inadequate efficacy of the therapies involved has invoked a necessity for safer and more effective treatment. The advent of therapies based on mechanisms that target critical molecular pathways has evoked considerable interest [16]. Our novel finding in psoriatic molecular pathophysiology, the epigenetic modification of *SHP-1* promoter 2, may assist in developing new and effective targeted therapies.

Materials and methods

Sample

Cell lines and patient samples

Hematopoietic cell lines, Daudi, Jurkat, Molt4, U937, K562, and epithelial cell lines, Hela, HEp2, SW480, HepG2, and HaCaT, were used. Additionally, two Epstein–Barr virus transformed B-lymphoblastoid cell lines (BLCLs) were generated from two donors. All cells were cultured in Dulbecco's modified Eagle's medium 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, 50 μg/ml streptomycin). Cells were incubated at 37°C in 5% CO_2.

Several archival paraffin-embedded tissues derived from normal and adjacent normal tissues of several tumor patients of various locations of kidney, liver, breast, prostate, cervix, lung, bladder, and OTC-embedded frozen normal cervical sections were obtained [17]. Nine to ten each of normal skins, collected from normal skin removed during plastic surgery, and various pathological skins, including chronic stable plaque type psoriasis, guttate psoriasis, squamous cell carcinoma (SCC), and chronic eczema, were chosen for the study. All patients with dermatologic condition and who were free from any skin therapies 4 weeks prior to taking biopsy samples were diagnosed clinically by an experienced dermatologist. The diagnosis was confirmed by skin biopsy and was revealed by an experienced dermatopathologist as typical pathology for each skin condition. The tissues were microdissected as previously described [18]. Additional cases of chronic plaque type psoriasis were microdissected to separate between upper and lower section of their psoriatic epidermis. Several samples of white blood cells (WBCs) from normal healthy individuals were also included. An additional seven cases each of healthy donor and chronic stable plaque type psoriasis pathological skin conditions were obtained by 6-ml-wide punch biopsies under local anesthesia for RNA analysis. All of the psoriasis patients were of the chronic stable plaque type and did not receive any treatment except the two cases who sporadically used low-potency topical

corticosteroids at least 4 weeks prior to taking biopsy samples.

Bisulfite treatment for DNA

After extraction, all DNA samples were treated with sodium bisulfite as previously described [19]. Briefly, 1 µg of genomic DNA was denatured in 0.22 M NaOH at 37°C for 10 min. Thirty microliters of 10 mM hydroquinone and 520 µl of 3 M sodium bisulfite were added for 16–20 h at 50°C. The DNA was purified and incubated in 0.33 M NaOH at 25°C for 3 min, ethanol precipitated, then washed with 70% ethanol, and resuspended in 20 µl of H_2O.

Methylation analysis of the promoter regions by the COBRA and MSP method

All *SHP-1* bisulfite oligonucleotide sequences were derived from GenBank (accession number U47924.1). DNA methylation in the promoter 2 region of *SHP-1* was detected by duplex methylation-specific polymerase chain reaction (PCR) (MSP) using methylation-specific primers and nonmethylation-specific primers. The methylation-specific primer sequences were identical to those previously published [20]. The methylation-specific primer sequences were 5′-TGT-GAA-CGT-TAT-TAT-AGT-ATA-GCG-3′ and 5′-CCA-AAT-AAT-ACT-TCA-CGC-ATA-CG-3′, and the nonmethylation-specific ones were 5′-GTG-AAT-GTT-ATT-ATA-GTA-TAG-TGT-TTG-G-3′ and 5′-TTC-ACA-CAT-ACA-AAC-CCA-AAC-AAT-3′. A duplex MSP reaction, containing bisulfite-treated DNA and 375 nM each of all oligonucleotides, was performed for 35 cycles with the annealing temperature set at 58°C. The PCR products, 174 and 162 bp for methylated and nonmethylated sequences, respectively, were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. The amplicons were analyzed by staining with cyber green and calculating the percent methylation upon visualization on a phosphor imager using the Image Quant software (Molecular Dynamics, Pharmacia Amersham). The *SHP-1* promoter 2 methylation level was calculated as a percentage of intensity of the methylated sequence divided by the sum of methylated and nonmethylated amplicons.

On the microdissected, paraffin-embedded tissues, seminested PCR was performed. Two microliters of the duplex MSP product was added to a solution containing a nested forward primer, 5′-AGY-GTG-GGT-TAG-GGA-GGG-3′ (bisulfited nucleotides 163031-163048), and both of the reverse duplex PCR oligonucleotides. The amplification reaction was carried out as described above. The duplex amplicons, 94 and 83 bp for methylated and nonmethylated sequences, respectively, were analyzed by the phosphor imager as previously described.

Combined bisulfite restriction analysis (COBRA) [21] by seminested PCR was applied for detection of methylation in the promoter 1 region. The forward (5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′) and reverse (5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-A 3′) primers (bisulfited nucleotide number 158572-158592, 158743-158722, respectively) were used to generate a 172-bp amplicon during 35 cycles of PCR with the annealing temperature set at 44°C. One microliter was transferred for seminested PCR with 5′-AAC-CCA-AAC-CAA-ATA-AAA-3′ (bisulfited nucleotide number 158657-158640) as the reverse primer. Amplification conditions were identical to those in the primary PCR and generated an 86-bp amplicon. The PCR products were digested by *Taq I* (MBI Fermentas, Flamborough, Ontario, Canada) at 65°C for 16 h and separated by 6% acrylamide gel electrophoresis.

Bisulfite DNA sequencing

For promoter 1, bisulfite-treated genomic DNA was amplified by using primers 5′-GTT-TTT-GTA-GTG-TTA-TTG-GTT-3′ and 5′-AAA-CAA-CAT-CTC-TCT-ATA-AAA-3′ (bisulfited nucleotides 158572-158592 and 158743-158722, respectively). For promoter 2, bisulfite-treated genomic DNA was amplified using primers 5′-GTT-TTA-TAG-GGT-TGT-GGT-GAG-AAA-TT-3′ and 5′-ACA-CAT-ATA-TAC-CTT-ACA-CAC-TCC-AAA-3′ (bisulfited nucleotides 162912-162937, 163153-163127, respectively). The PCR products were cloned into the pGEM-T easy vector (Promega, Santhan, UK) and sequenced.

RT-PCR and real-time RT-PCR

Total RNA was extracted from cell lines as well as skin biopsies using the Trizol reagent (Life technologies, Inc.) according to the manufacturer's instructions. The RNA preparation was dissolved in 20 µl of RNase-free distilled water containing 40 U of ribonuclease inhibitor (Promega), and 50 ng of the RNA was incubated for 5 min at 70°C followed by 45 min at 42°C with 15 pmol of oligo deoxythymidine primer. To each sample, we added 19 µl of a reaction mixture containing 1× ImProm-II buffer (10 mM Tris–HCl, pH 8.3, 1.5 mM MgSO4, 50 mM KCl, and 0.001% gelatin), 5 U of reverse transcriptase (RT) (Promega), and 0.2 mM deoxynucleotide triphosphates. cDNA was amplified using the exons 3–4 primer 5′-CCC-ACC-CTG-ACG-GAG-AGC-3′ and either the exon 1 primer 5′-CTC-CCT-ACA-GAG-AGA-TGC-TGT-CC-3′ or the exon 2 primer 5′-ACT-GGG-AGC-TGC-ATC-TGA-GG-3′. All *SHP-1* RT-PCR oligo sequences were obtained from GenBank (accession number NM080549.2). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control as previously published [22]. Both PCRs were performed for 35 cycles with the annealing temperature set at 60°C.

Real-time RT-PCR was performed in a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA) using QuantiTect SYBR Green I (Qiagen, Hilden, Germany), according to the manufacturer's instructions in a total volume of 20 µl. Cycling conditions

were 95°C for 15 min, 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles, followed by melt analysis from 65 to 95°C. The *SHP-1* (II) primers were same as above. PCRs were conducted in parallel to normalize for differences in cDNA synthesis. To correlate the threshold (Ct) values from the amplification plots to copy number, a standard curve was generated using the pGEM-T *SHP-1*(II) plasmid. PCR product melt curves were analyzed for a specific peak, and the products were visualized by the agarose gel electrophoresis and ethidium bromide staining to ensure and proportionate specific amplicons. A non-template control was run with every assay, and all determinations were performed to achieve assay.

Statistical analysis

Levels of *SHP-1* promoter 2 methylation and isoform II among skin lesions were compared, and significant differences were determined by unmatched two-tail *t* test using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL, USA).

Results

SHP-1 methylation and isoforms in human cell lines

Cell-type-specific *SHP-1* isoforms, distinguishable by promoter transcriptional activity, have been reported [11]. Whereas most hematopoietic cells express high levels of isoform II, the majority of epithelial cell line transcripts display lower levels of isoform I. Hence, the aim of our first experiment was to determine if this tissue-specific transcription process is regulated by promoter hypermethylation. A new COBRA approach was designed to analyze promoter 1, and the published MSP [20] was modified to study promoter 2 (Fig. 1a). The results demonstrate distinctive methylation patterns for hematopoietic and epithelial cell lines. While promoter 2 hypermethylation was constantly detected in all epithelial cell lines (Hela, HEp2, SW480, HaCaT, and HepG2), promoter 1 without exception was nonmethylated. In hematopoietic cells, a variety of methylation patterns could be observed. Whereas methylation was not detected in WBC, it was discernible in some hematopoietic cell lines. Promoter 1 was nonmethylated in both BLCLs and methylated in Daudi, K562, and U937. Both promoters were incompletely methylated in Jurkat and Molt4. Limited or absent amounts of promoter 2 methylation were demonstrated in both BLCLs, Daudi, and U937. Finally, promoter 2 of K562 was hypermethylated. Bisulfite, PCR, cloning, and sequencing were applied to confirm both MSP and COBRA results. Figure 1b shows examples of bisulfite sequencing results of HEp2, Daudi, and Molt4, confirming complete, absent, and partial methylation, respectively.

We observed a striking inverse correlation between promoter methylation and transcriptional activity. As for promoter 1, DNA methylation was discovered in Daudi, Jurkat, Molt4, K562, and U937, while isoform I cDNA was not detectable. In contrast, promoter 1 of all epithelial cells and both BLCLs was nonmethylated and transcribed. WBCs, however, were devoid of both methylation and isoform I, suggesting another transcriptional silencing mechanism (Fig. 1a,c). cDNAs, 239 bp, derived from isoform II were observed when amplifying *SHP-1* RNA between exons 2 and 3–4 (Fig. 1c). All human epithelial cell lines, including HeLa, HEp2, SW480, HaCaT, and HepG2, were promoter 2 hypermethylated and *SHP-1* mRNA isoform II deficient. In contrast, *SHP-1* isoform II was strongly expressed in all promoter-2-nonmethylated human hematopoietic cells, BLCLs, Daudi, and U937 (Fig. 1a,c). Both promoters 1 and 2 were methylated in K562, and consequently, we found limited amounts of both *SHP-1* mRNA isoforms. The high isoform II levels from promoter 2 of Jurkat and Molt4 were noteworthy since they displayed incomplete promoter 2 methylation (Fig. 1a,b). These findings confirmed the strong association between methylation of both promoters and their activities.

SHP-1 methylation in normal epithelia in vivo

The complete promoter 2 methylation in epithelial cell lines prompted us to hypothesize that the epigenetic control was tissue specific rather than indicative for carcinogenesis. Accordingly, we screened the methylation in normal epithelial tissues of several organs. Several stratified epithelial tissues, including skin, bladder, and cervix, and large solid organs mainly comprising epithelial tissues, such as prostate gland, breast, lung, kidney, and liver, were investigated. Whereas no promoter 2 methylation was found in WBCs from healthy donors, complete methylation was demonstrated in almost all epithelial cells (Fig. 2a,b). The methylation status is not depended on tissue preservation (Fig. 2c). It is noteworthy that incomplete methylation was sporadically identifiable in one out of nine skin cases (Figs. 2 and 3).

SHP-1 promoter 2 demethylation in psoriasis

To explore the significance of promoter 2 demethylation in skin, we investigated if and which types of pathological conditions possessing larger degrees of loss of methylation could be observed. Keratinocytes from psoriasis, SCC, and eczema lesions were collected using microdissected, paraffin-embedded tissues, and their methylation levels were compared with those of normal skins (Fig. 3a). In addition to eight out of nine normal skins, hypermethylation was discernible in the majority of eczema and SCC cases. In contrast, demethylation was found in almost all, eight out of nine, cases of psoriasis. The average methylation level of normal skin is 94.8%, whereas in psoriasis, it is 68.1%. From this, it can be concluded that the levels of promoter 2 methylation in psoriasis are distinct from those in healthy skin lesions ($p<0.005$) (Fig. 3b). Since promoter activity and expression of many proteins change with the

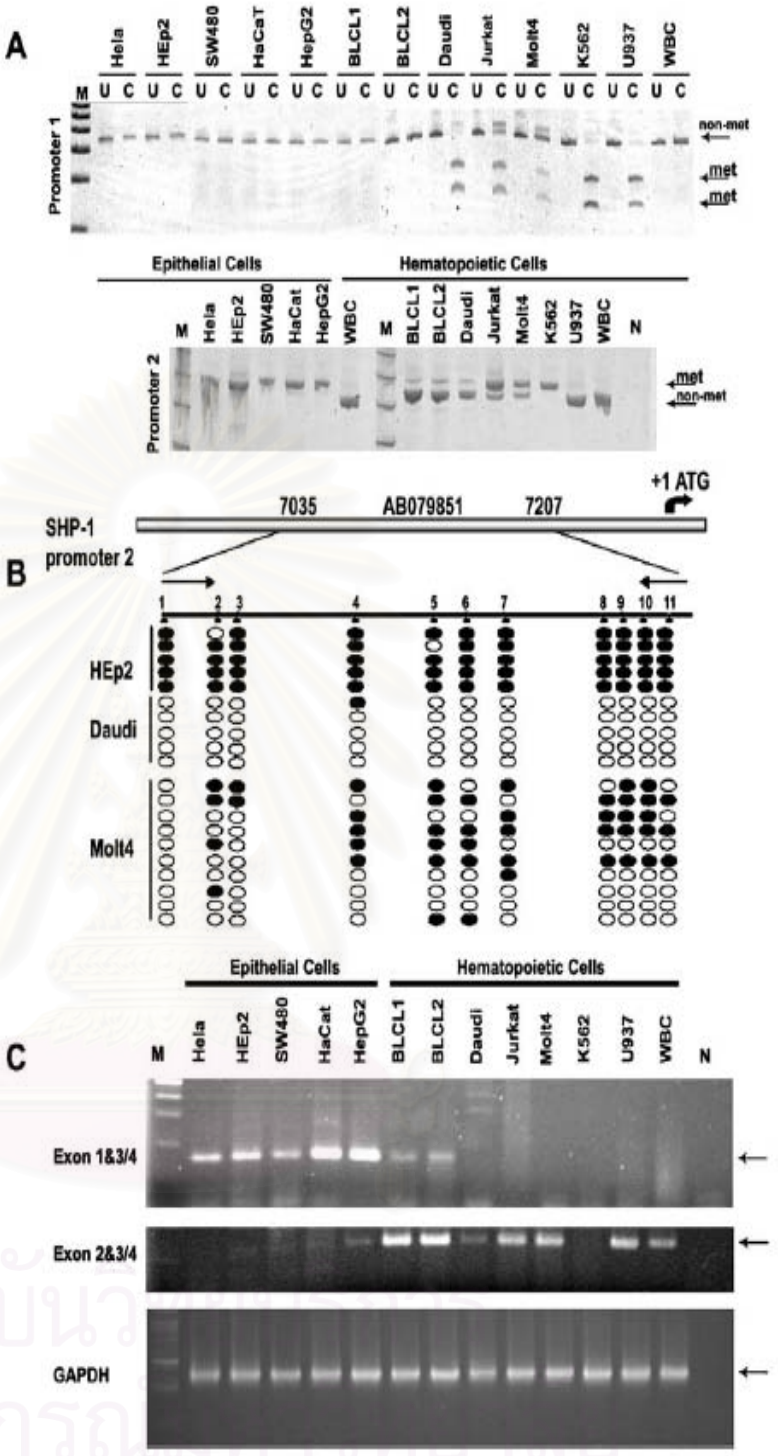

Fig. 1 *SHP-1* promoter methylation and RNA isofom in various epithelial and hematopoietic cell lines. a Promoter 1 and 2 methylation patterns. Promoter 1 methylation is detectable by COBRA. The PCR products, indicated by *non-met arrow*, are 86 bp. Methylated amplicons, indicated by *met arrow*, are digestible to 52 and 34 bp. *M* is standard size DNA marker, *U* is uncut, and *C* is digested amplicons. Cell sources of genomic DNA are listed above each lane. For promoter 2, duplex MSP yield 2 DNA fragments. Methylated amplicons, indicated by *met arrow*, are 174 bp, and nonmethylated products, indicated by *non-met arrow*, are 162 bp, respectively. *N* is negative control. b Methylation status of CpG nucleotides at promoter 2 of HEp2, Daudi, and Molt4. The uppermost *vertical bar* locates the relative nucleotide sequence in GenBank (accession number AB079851). The *numbers* and *arrows* indicate the start and end of sequenced nucleotide in relation to the trancriptional start codon, ATG (nucleotide 7604). The *numbers* of the line below indicate CpG nucleotides. *Each circle* exemplifies the methylation status of each selected clone. *Black* and *white circles* are methylated and nonmethylated CpG dinucleotides, respectively. c *SHP-1* RT-PCR result of epithelial and hematopoietic cell lines. Cells are listed on the top of each lane. *M* is standard DNA marker, and *N* is negative control. *Arrows* indicate the locations of expected amplicons. *Exon1&3/4* is RT-PCR between exon 1 and exons 3–4, and the amplicon size is 156 bp. *Exon2&3/4* amplifies 239 bp of isoform II. *GAPDH* was used in the RT-PCR as the control

course of keratinocyte terminal differentiation and the pattern of epidermal differentiation is altered in psoriatic skin, we investigated the demethylation in distinctive compartments. Several psoriatic skins were microdissected and divided into upper and lower half of the epidermis, and the promoter methylation levels were compared. No significant demethylation differences between the compartments were identified (Fig. 3b). In addition, the demethylation level between psoriasis with different clinical sign, chronic stable plaque type, and guttate psoriasis were not distinguishable (Fig. 3b).

We also compared mRNA expression *SHP-1* isoforms in fresh biopsies of psoriatic lesion and normal skin by Real Time RT-PCR. Interestingly, the association between promoter 2 methylation and the amount of isoform II mRNA in psoriatic skins was similar to that observed in the previously analyzed promoter 2 incompletely methylated cell lines, Jurkat and Molt4. Psoriatic skin lesions predominantly expressed isoform II, identifiable from all cases, whereas promoter 2 activities were absent or decreased in the majority of normal skin samples (Fig. 4a). The isoform II mRNA level of psoriatic skin

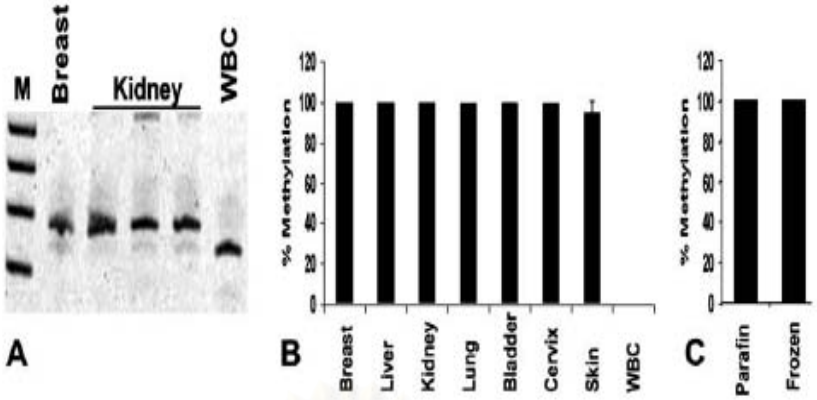

Fig. 2 *SHP-1* promoter 2 methylation in epithelia. a Representative result of seminested duplex MSP of epithelial tissue obtained from microdissected, paraffin-embedded tissue of breast, kidney, and WBC. *M* is standard DNA size marker. b Average level and standard deviation (SD) of promoter methylation in epithelial tissues of several organs and WBC, as listed below. c Epithelial DNA from paraffin-embedded tissues and frozen section. The *heights* of each bar represent percentage of promoter 2 methylation of each tissue types. *T* indicates the methylation SD within individual cell types. Note that 0 SD can be observed in most cases

lesions as measured by real-time RT-PCR of exons 2 and 3–4. The RT-PCR was average at 58 copies, whereas in normal skin, they amounted to 18 copies per 1 μg of total RNA (Fig. 4b). This indicated that *SHP-1* promoter 2 activity of psoriatic lesions was significantly higher than in normal skins ($p<0.05$). This finding confirms the increment of promoter 2 activity in psoriasis by a demethylation mechanism compared to normal skin.

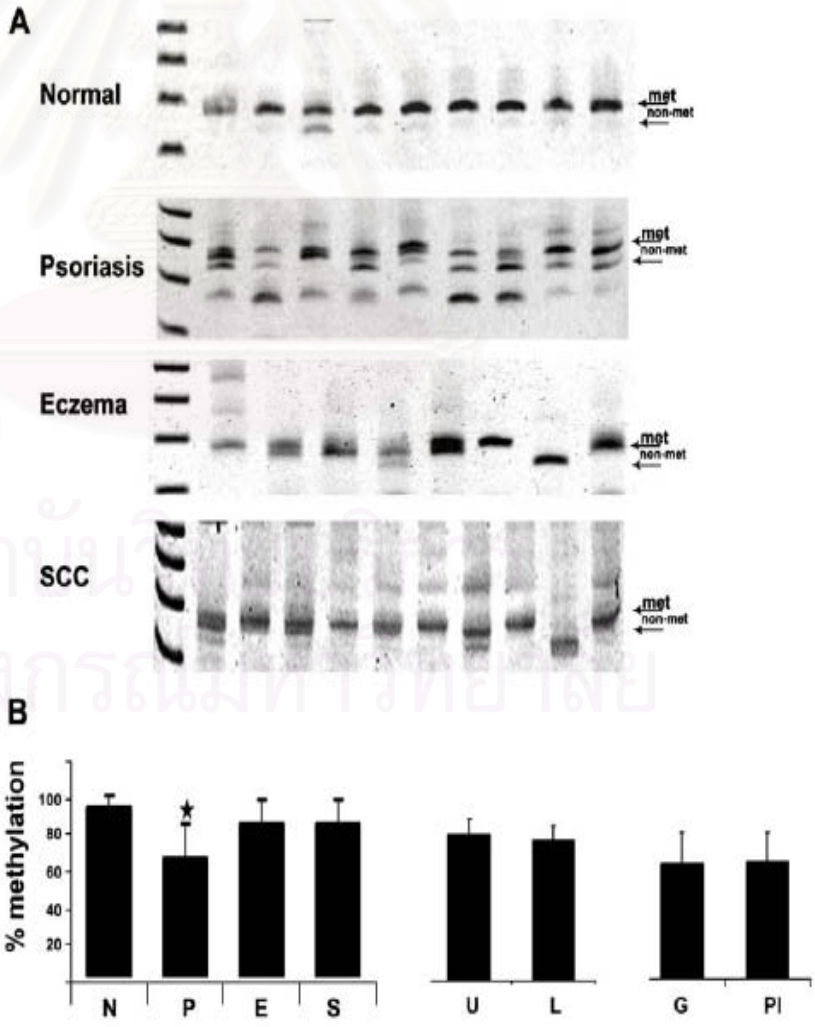

Fig. 3 *SHP-1* promoter 2 methylation in various skin lesions, normal, psoriasis, eczema, and SCC. a Examples of seminested duplex PCR results. *Met* and *non-met arrows* are expected 94-bp methylated and 83-bp nonmethylated amplicons, respectively. b Methylation percentages and SD; *N*, *P*, *E*, *S*, *U*, *L*, *G*, *Pl* are the levels of normal skins, psoriasis, eczema, SCC, upper and lower part of microdissected psoriatic skin epidermis, and guttate and chronic plaque type psoriasis, respectively. The *star* indicates significant differences of $p<0.005$ between psoriasis and normal skins

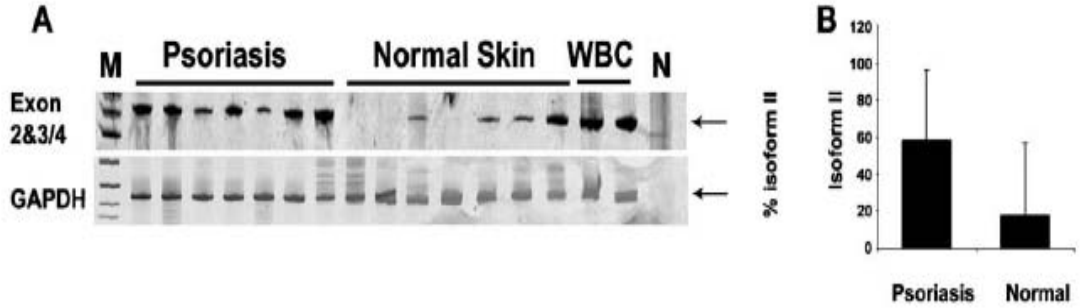

Fig. 4 *SHP-1* RT-PCR of psoriatic skin lesions and normal skins. a Cell types are listed on the top of each lane. *M* is standard DNA marker, and *N* is negative control. *Arrows* indicate the locations of expected amplicons. Exon 1 and 3/4 is RT-PCR between exons 1 and exon 3–4, and the amplicon is 156 bp. *Exon2&3/4* amplifies 239 bp of isoform II. *GAPDH* is RT-PCR results located at the bottom of a. b The mean and SD of isoform II level of psoriasis and normal skin proportion to 1 μg total RNA are the height of each bar and T, respectively. The real-time RT-PCR showed significant difference at $p<0.05$

Discussion

Alteration of gene expression is at the center of cellular pathogenesis. Accordingly, DNA methylation should be associated with human diseases since epigenetic modification usually silences gene transcription. Normally, DNA methylation is important in several biological processes, such as X chromosome inactivation and genomic imprinting [23–27]. As for abnormal conditions, promoter hypermethylation of several tumor suppressor genes and generalized hypomethylation have been reported to promote the potential for malignant transformation [20, 27–30]. To the best of our knowledge, this study has been the first to prove that the tissue-specific promoter methylation status can be altered in a nonmalignant human disease condition. In contrast to previously described cases of lymphomas/leukemias, the *SHP-1* promoter 2 is hypermethylated in normal epithelia, and its activity is consequently limited. Finally, we selectively analyzed several pathological skin lesions and demonstrated that promoter 2 demethylation usually arose in psoriatic skin lesions and consequently triggered up-regulation of *SHP-1* isoform II.

According to previous data, mRNA derived from promoter 2 is distinctive from promoter 1 transcripts in both structure and quantity [11]. The sequence of isoform II differs from isoform I by embracing few different amino acids at the N-terminal. Additionally, a strikingly large amount of mRNA is usually generated from promoter 2 [11]. Moreover, the molecular physiological responses to up-regulate cell growth of *SHP-1* in hematopoietic and epithelial cells are distinct. *SHP-1* isoform II has been projected as a negative regulator for signaling of cell proliferation and differentiation of hematopoietic cells via cytokine and non-cytokine receptors such as the cKit/stem cell factor receptor, the interleukin-3 receptor, the colony-stimulating factor–1 receptor, and the erythropoietin receptor [31–35]. Conversely, it has been reported to be a positive regulator of cell signaling via the mitogen-activated pathway (MAP) kinase pathway in nonhematopoietic cells [11]. In the course of this study, we found demethylation of the promoter 2 region of *SHP-1* correlated with up-regulation of *SHP-1* isoform II mRNA in psoriatic skin lesions. Hence, up-regulation of the promoter 2 in psoriatic skin lesions should result in a downstream *SHP-1* signal transduction pathway different from that in normal skin. Interestingly, psoriasis is a T-cell-mediated disease [36], involving many defects in the regulation of the transcription factors: signal transducer and activator of transcription (STAT-1 alpha), interferon-regulated factor 1, and nuclear factor-kappaB. Moreover, the pathogenesis leads to alteration in intracellular signal transduction pathways, including MAP kinase and JAK-STAT pathways, which lead to loss of growth and differentiation control when the cells are subjected to physicochemical and immunologic stress [37]. Consequently, it is crucial to further elucidate if and which particular role *SHP-1* isoform II plays in the pathological psoriasis signal transduction network.

This association study does not indicate that *SHP-1* demethylation is specific to psoriasis. In contrast, some other skin lesions possessed the epigenetic loss; therefore, we presume that promoter 2 demethylation is a dynamic physiological process and that its prolongation or sensitization implicates psoriatic pathogenesis. In this study, we could observe a variety of methylated sequences in some hematopoietic malignant cell lines such as Jurkat and Molt4. This indicates that epigenetic modification is mosaic even if the cancer cells are subject to selective clonal expansion. Accordingly, the variation should result from a gradual loss of methylation in vitro. Furthermore, in addition to most psoriasis samples, some cases of skin lesions also sporadically disclosed demethylation. Finally, a recent study on lymphoma has proven that down-regulation of *STAT3* mRNA results in *SHP-1* demethylation [12]. Since several cytokines and growth factors are responsible for STAT3 activity, *SHP-1* methylation being controlled by STAT3 supports our hypothesis. However, STAT3 may act differently on the *SHP-1* promoter in epithelial cell. A study reported by Sano et al. [13] has demonstrated that STAT3 up-regulation is an important mechanism in psoriatic pathogenesis. Hence, if on a molecular level, STAT3 activity is related to *SHP-1* methylation in epithelial cells, there must be additional tissue-specific factors to trigger the opposite outcome, demethylation instead of hypermethylation.

Acknowledgements This work was supported in part by grants from the Royal Golden Jubilee Ph.D. grant, the Thailand Research Funds, Molecular Biology and Genetics of Cancer Development Research Unit, Chulalongkorn University, and National Center for Genetic Engineering and Biotechnology, Thailand. For cell lines, we thank Professor Hedeoki Ogawa for kindly providing HaCaT, Dr. Chintana Chirathaworn for U937 and Molt4, Dr. Virote Sriuranpong for Hela and SW480, Dr. Watchara Kasinrerk for Daudi, Jurkat, and K562, and Dr. Pokrath Hansasura for both BLCLs. We also would like to express our gratitude to the Department of Pathology, Dermatology Unit, Department of Medicine, and our professor, Dr. Nopadon Noppakun, for paraffin tissues and clinical information. Finally, we would like to thank Ms. Petra Hirsch for critically reviewing the manuscript.

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