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A STUDY OF TETRATRICOPEPTIDE REPEAT DOMAIN12 (*TTC12*) METHYLATION IN LEUKEMIA

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รุ่งทิวา วัฒนวราภรณ์ : การศึกษาดีเอ็นเอเมทิลเลชั่นบนยีนเตทตระไตรโคเปปไทด์ รีพีท โดเมน 12 ในผู้ป่วยลิวคีเมีย (A STUDY OF TETRATRICOPEPTIDE REPEAT DOMAIN12 (*TTC12*) METHYLATION IN LEUKEMIA) อ. ที่ปรึกษา : ศ.นพ. อภิวัฒน์ มุทิรางกูร, 59 หน้า. ISBN 974-14-2634-8

จากการศึกษาก่อนหน้านี้ เพื่อการหายีนต้านมะเร็งโพรงหลังจมูก (NPC) ในบริเวณที่เกิด loss of heterozygosity (LOH) บนโครโมโซม11q จากการศึกษาเมทิลเลชั่นของยีนเตทตระไตรโคเปปไทด์ รีพีท โดเมน 12 (tetratricopeptide repeat domain 12; *TTC12*) ซึ่งเป็นยีนซึ่งอยู่ในบริเวณที่เกิด LOH และ down regulate ในผู้ป่วยมะเร็งโพรงหลังจมูก กลับพบว่ายีนดังกล่าวมีเมททิลเลชั่นในเฉพาะ cell line ที่เป็นมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน ซึ่งได้แก่ Daudi (B-lymphoblastoid line), Jurkat (acute T cell leukemia), Molt4 (acute lymphoblastic leukemia) แต่ไม่มีเมททิลเลชั่นใน K562 (chronic myelogenous leukemia), HN12 และ Hela (epitherial cell lines) ดังนั้นสมมติฐานคือยีน *TTC12* น่าจะทำหน้าที่เป็นยีนต้านมะเร็งในผู้ป่วยมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน (acute lymphoblastic leukemia: ALL) เพื่อพิสูจน์สมมติฐานดังกล่าว จึงทำการศึกษาเมททิลเลชั่น ในไขกระดูกผู้ป่วย ALL จำนวน 29 ราย เปรียบเทียบเม็ดเลือดขาวของปกติจำนวน 10 ราย โดยใช้เทคนิค combined bisulfite restriction analysis (COBRA)พบความแตกต่างของดีเอ็นเอเมทิลเลชั่นในผู้ป่วย ALL สูงกว่าในคนปกติอย่างมีนัยสำคัญ ($p< 0.0001$) และระดับของเมททิลเลชั่นไม่ขึ้นกับชนิดของ ALL ($p>0.1$) ซึ่งผลดังกล่าวได้รับการยืนยันโดยการโคลนและศึกษาลำดับเบส นอกจากนี้จากการศึกษาการแสดงออกของยีน *TTC12* โดยใช้เทคนิค RT-PCR พบว่ากลุ่ม cell line ที่ไม่มีเมททิลเลชั่น ได้แก่ K562, HN12 และ Hela มีการแสดงออกของยีน *TTC12* สูง ในขณะที่ cell line ที่มีเมททิลเลชั่นสูง ได้แก่ Daudi, Jurkat และ Molt4 ไม่พบการแสดงออกของยีน *TTC12* ซึ่งผลดังกล่าวสอดคล้องกับตัวอย่างไขกระดูก โดยที่กลุ่มผู้ป่วยที่มีเมทิลเลชั่นสูง พบการแสดงออกของยีน *TTC12* ต่ำกว่ากว่ากลุ่มผู้ป่วยที่มีเมทิลชั่นต่ำอย่างมีนัยสำคัญ จากผลการทดลองการแสดงออกของยีน *TTC12* ระหว่างกลุ่มผู้ป่วย ALL ที่มีดีเอ็นเอเมททิลเลชั่นในระดับสูงและในระดับต่ำเปรียบเทียบกัน พบว่ากลุ่มที่มีเมททิลเลชั่นสูงส่งผลให้ mRNA ของยีน *TTC12* ที่ต่ำลงอย่างมีนัยสำคัญ ($p<0.05$) นอกจากนี้ยังศึกษาระดับเมททิลเลชั่นในตัวอย่าง remission ALL จำนวน 8 ราย ผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดทีเซลล์จำนวน 4 ราย และเซลล์เม็ดเลือดต้นกำเนิดจำนวน 5 ราย พบว่ามีระดับเมทิลเลชันแตกต่างกันในระหว่างกลุ่ม และยังพบว่าเมทิลเลชั่นบนยีน *TTC12* ได้ขยายไปจนถึงปลาย 5'UTR ของยีน ดังนั้น ระดับเมททิลเลชั่นบนยีน *TTC12* ที่สูงขึ้นอาจชักนำให้เกิดมะเร็งเม็ดเลือดขาวโดยการยับยั้งระดับการแสดงออกของ mRNA แม้ว่ายังไม่มีรายงานการศึกษาหน้าที่ของยีน *TTC12* อย่างแน่ชัด แต่การทดลองโดยใช้คอมพิวเตอร์ พบว่ายีนนี้ประกอบด้วย 2 โดเมนหลัก คือ โดเมน tetratricopeptide repeat (TPR) และ โดเมน armadillo repeat (ARM) จึงคาดว่าโปรตีน TTC12 อาจจะจับกับกับโปรตีนตัวอื่นๆ โดยผ่านทางโดเมน TPR และ โดเมน ARM ในการกระตุ้นให้เกิดกระบวนการต่างๆ ของเซลล์ รวมทั้งการควบคุมวัฏจักรเซลล์ อย่างก็ตาม สมมติฐานหน้าที่ของโปรตีน TTC12 ดังกล่าวควรจะได้รับการศึกษาเพิ่มเติม ซึ่งจะเป็นความรู้ด้านชีววิทยาของมะเร็ง และสามารถนำไปประยุกต์ใช้ในการตรวจวินิจฉัยและการรักษาโรคมะเร็งต่อไปในอนาคต

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....รุ่งทิวา วัฒนวราภรณ์.....

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา.....

467 47740 30: MAJOR MEDICAL SCIENCE

KEYWORDS: *TTC12* / LEUKEMIA / DNA METHYLATION / TUMOR SUPPRESSOR GENE

ROONGTIWA WATTANAWARAPORN: A STUDY OF TETRATRICOPEPTIDE REPEAT DOMAIN12 (*TTC12*) METHYLATION IN LEUKEMIA. THESIS ADVISOR: PROF. APIWAT MUTIRANGURA, M.D. Ph.D., 59 pp. ISBN 974-14-2634-8

We searched a candidate tumor suppressor gene of nasopharyngeal carcinoma (NPC) on the critical region of loss of heterozygosity on chromosome 11q. Unexpectedly, we accidentally discovered that tetratricopeptide repeat domain 12 (*TTC12*), an NPC down regulated gene located in the LOH region, hypermethylated in leukemic cell lines. These cells included Daudi (B-lymphoblastoid line), Jurkat (acute T cell leukemia), Molt4 (acute lymphoblastic leukemia). No methylation was observed in K562 (chronic myelogenous leukemia) and those of epitherial cell lines, HN12 and Hela, were nonmethylated. Therefore, we hypothesized that the methylation of *TTC12* may be specific in leukemogenesis *in vivo*. To prove this hypothesis, we tested 29 acute lymphoblastic leukemia (ALL) in comparison with normal white blood cells (WBC) from 10 healthy volunteers using combined bisulfite restriction analysis (COBRA) technique. In ALL group, the results significant demonstrated hypermethylated than in normal WBC ($p< 0.0001$), and methylation level do not depend on ALL subtypes ($p>0.1$). The results were confirmed by cloning and sequencing. We tested *TTC12* expression by RT-PCR, nonmethylated cells, K562, HN12 and Hela, expressed *TTC12*, whereas hypermethylated cells, Daudi, Jurkat and Molt4, did not express. The same situation has also detectable *in vivo*. We compared the proportion between TTC12 and GAPDH and found significant increase in expression of TTC12 in hypomethylated ALL when compared with hypermethylated cases ($p<0.05$). In addition, we tested *TTC12* methylation in 8 remission ALL, 4 T cell lymphomas and 5 stem cells. The result showed different level of methylation each groups. The methylation on *TTC12* expands through over 5'UTR. Therefore, hypermethylation in *TTC12* may induce leukemogenesis by inhibiting its mRNA. Although the function of *TTC12* has not been identified *in silico*, two conserved domains have been found including the tetratricopeptide repeat (TPR) domain and the armadillo repeat (ARM) domains. TTC12 might mediate protein-protein interaction with other proteins through TPR and ARM domains to trigger some cellular process including cell cycle control. Nevertheless, this hypothesis as well as the function(s) of TTC12 should be investigated and this will provide new knowledge of cancer biology that could be applied in cancer diagnosis as well as therapeutic in the future.

Field of Study : Medical Science

Academic Year : 2006

Student's Signature Roongtiwa Wattanawaraporn

Advisor's Signature Apiwat Mutirangura

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LIST OF ABBREVIATIONS

TTC12	=	Tetratricopeptide repeat domain12
NPC	=	Nasopharyngeal carcinoma
ALL	=	Acute lymphoblastic leukemia
ANLL	=	Acute non-lymphoblastic leukemia
WBC	=	White blood cell
BM	=	Bone marrow
COBRA	=	Combined bisulfite restriction analysis
PCR	=	Polymerase chain reaction
RT-PCR	=	Reverse transcriptase polymerase chain reaction
S.D	=	Standard deviation

CHAPTER I

INTRODUCTION

Background and Rationale

We searched a candidate tumor suppressor gene of nasopharyngeal carcinoma (NPC) on the critical region of loss of heterozygosity (LOH) on chromosome 11q [1] ,and the gene expression in this LOH region was down regulated in NPC by using cDNA microarrays [2]. As a result of these previous studies, we obtained *TTC12* gene and we hypothesized that this gene should be a candidate tumor suppressor gene in NPC. We evaluated *TTC12* methylation level in several cell lines, Daudi (B lymphoblast), Jurkat (acute T cell leukemia), Molt4 (acute T lymphoblastic leukemia), K562 (chronic myelogenous leukemia), HN12 (head and neck cancer), SW480 (colorectal adenocarcinoma) and HeLa (cervical cancer), by combined bisulfite restriction analysis (COBRA) technique. Unexpected result, the leukemic cell lines were nearly complete methylation including Daudi, Jurkat and Molt4. On the other hand, non-methylation in K562 and epitherial cell lines, HN12, SW480 and HeLa. Therefore, we suggested that *TTC12* should be a candidate tumor suppressor gene in acute lymphoblastic leukemia (ALL).

The main mechanism of epigenetic modification is the DNA methylation. Methylation of cytosine bases in CpG dinucleotides with in DNA aids in the organization of the mammalian genome. DNA methylation is involved in inactivating one of two X chromosome in women, establishes tissue-specific patterns of gene expression, effects on cellular development and differentiation, and represses proviral and retrotransposon sequences in the genome [27]. Patterns of DNA methylation are often altered in tumor cells. Two distinct changes have been observed, termed DNA "hypermethylation" and "hypomethylation". In tumor DNA hypermethylation usually occurs in a focal fashion affecting the CpG rich promoter regions of tumor suppressor genes, causing or supporting their transcription inactivation. In contrast, genome-wide losses of DNA methylation (global hypomethylation) have been regarded as a common epigenetic event in malignantcies [30], which contribute to carcinogenesis may possibly mediated

through facilitating chromosome instability and regulating the expression of proto-oncogenes.

Hypermethylation of CpG islands surrounding tumor suppressor gene promoter leads to transcriptional silencing and has been linked to human malignancies including acute lymphoblastic leukemia (ALL) [3-12]. In ALL, promoter hypermethylation is a common mechanism of gene regulation and silencing that participates in the regulation of genes implicated in major cellular pathways, such as cell cycle regulation (*CDKN2B, CDKN2A, TP73, PTEN, NES-1* and *LATS-1*) [11], apoptosis (*p14, PYCARD, APAF1, ASPP-1* and *DAPK1*) [3,10], or cell adhesion (*CDH13* and *CHD1*) [10]. Then, we hypothesized that similar to several candidate tumor suppressor genes, the methylation of *TTC12* may be specific in leukemogenesis *in vivo*.

The aberrant methylation is linked to transcriptional gene silencing, novel tumor suppressor genes can be identified using methylated CpG [13]. Therefore, we COBRA PCR [14] to evaluate the *TTC12* methylation status and expression level in normal white blood cells (WBC), bone marrows of ALL patients, remission ALL and T-cell lymphoma, and cord blood stem cells. The benefit of this study will be to understand the function of *TTC12* in normal WBC, ALL and other samples, and may be apply as tumor marker in the future.

Objective

To study methylation status of *TTC12* gene in normal WBC and ALL .

Question

Primary question

Does *TTC12* may function as tumor suppressor gene in ALL ?

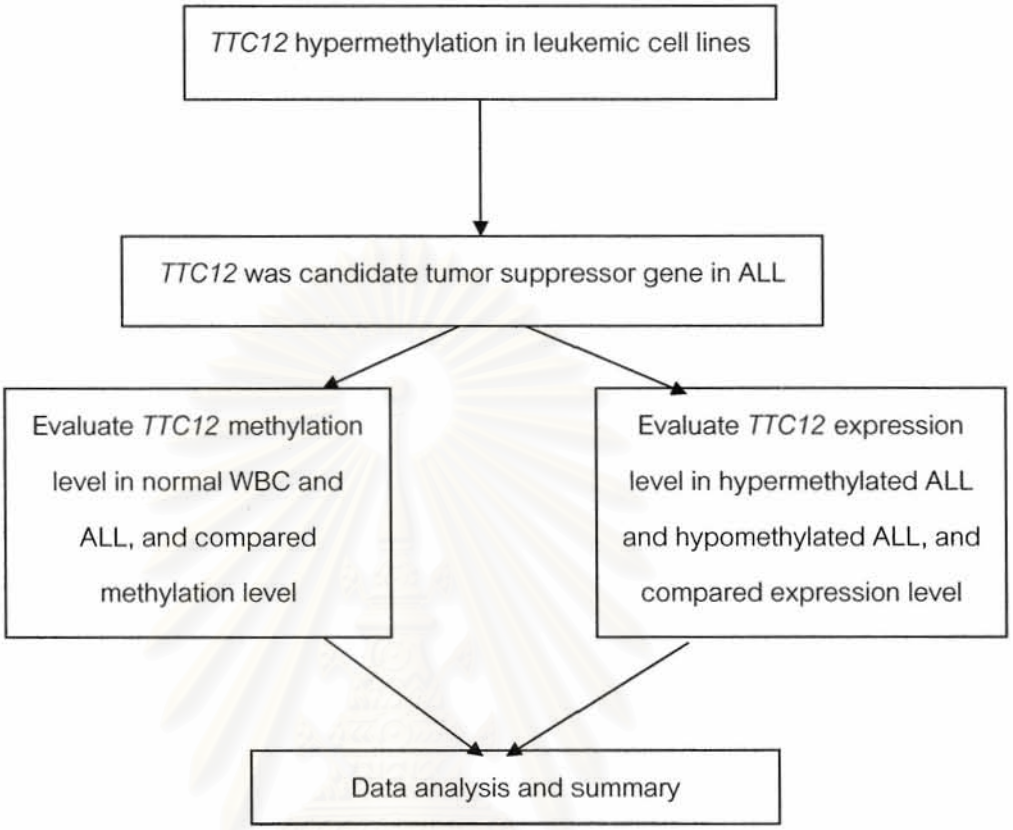
Secondary question

- How about the level of *TTC12* methylation between normal WBC and ALL?
- How about the level of *TTC12* methylation in other samples?
- How about *TTC12* expression in ALL?
- Does the level of *TTC12* methylation depend on ALL subtypes?
- Does methylation on *TTC12* expand through over 5'UTR?

Hypothesis

- *TTC12* may function as tumor suppressor gene in ALL
- The level of *TTC12* methylation in ALL should be higher than in normal WBC.
- The level of *TTC12* methylation in different samples should be different methylation level.
- The expession of *TTC12* correlate inversely with the methylation level.
- The level of *TTC12* methylation does not depend on ALL types.
- Methylation on *TTC12* expands through over 5'UTR.

Conceptual Framework



Operation definition

Hypermethylation: more methylation than in normal WBC

Hypomethylation: methylation level in normal WBC

Non-methylation: no methylation

Expected benefit

The benefit of this study will be to understand the function of *TTC12* in normal WBC, ALL and other samples, and may be apply as tumor marker in the future.

Research Methodology

1. Bone marrow, peripheral blood and stem cell collection.
2. DNA extraction.
3. Sodium bisulfite treatment and desalted with DNA clean-up system.
4. Primer design for CORBA *TTC12*.
5. Polymerase chain reaction (PCR).
6. Restriction enzymes digestion.
7. Gel eletropholesis and quantitation with Molecular Dynamics phosphoimager.
8. Cloned and sequenced
9. RT-PCR

CHAPTER II

REVIEW OF RELATED LITERATURES

1. DNA methylation in mammals

Epigenetic modifications of the DNA do not change the coding sequence; however, they are heritable and involved in regulation of gene transcription. DNA methylation, one such epigenetic modifications found in DNA, is the mechanism that add a methyl group to the 5′carbon of deoxycytosine [15]. In mammals, the major target for DNA methylation is a found in CpG –dinucleotide. CpG dinucleotide is cytosine located next to a guanine(5′-CpG-3′) [16]. These targets of methylation are not equally distributed in the genome, but found in long CG-rich sequences present in satellite repeat sequences, middle repetitive rDNA sequences, centromeric repeat sequences and CpG islands. CpG islands are sequences longer than 200 bp with a GC content of over 50% (in contrast to a genome-wide average of about 40%) and an observed over expected ratio of CpG of 0.6 or greater [17]. Interestingly, CpG islands are found mainly in the 5′ -regions of house-keeping genes as well as some other specifically tissue expressed genes and usually extend from the promoter region into the first exon and sometimes into intron 1 [18]. Most CpG islands are unmethylated in normal cells; however, there are certain conditions where these sequences become methylated and form part of gene regulation [19]. The majority of CpG islands on the inactive X-chromosome in a female cell are methylated [20], and certain CpG island-like sequences in the vicinity of imprinted genes have been found to be methylated in an allele-specific manner [21]. While CpG islands are usually unmethylated, other GC-rich sequences, e.g. the centromeric repeat sequences and satellite sequences, are highly methylated in normal cells.

1.1 Mechanisms of DNA methylation

DNA methylation is mediated by a family of DNA methyltransferase(Mtases) that includes Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 is a maintenance Mtase that primarily replicates methylation patterns, while Dnmt3a and Dnmt3b are capable of methylating previously unmethylated DNA, referred to as de novo methylation [22]. DNA methylation patterns are established during differentiation, and serve to suppress genes unnecessary for the function of the mature cell. This involves de novo methylation of DNA, and requires Dnmt3a and Dnmt3b [23]. Their role in mature cells is less clear, but Dnmt3b appears to be necessary for maintaining methylation of pericentromeric heterochromatin [24]. Following differentiation the patterns are replicated during mitosis by the maintenance DNA Mtase Dnmt1 [22]. During mitosis, Dnmt1 recognizes hemimethylated CG dinucleotides in the parent DNA strand, and catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM) to the cytosine residues in the unmethylated daughter DNA strand, producing symmetrically methylated sites and maintaining methylation patterns [22](Fig.2-1).

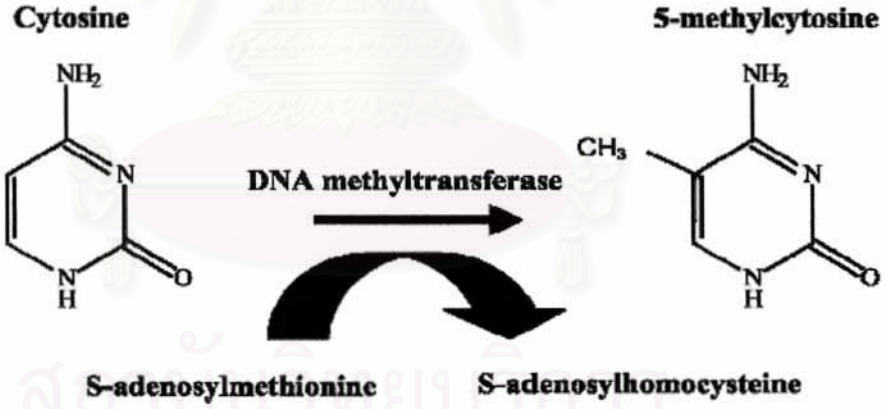


Figure 2-1 Cytosine methylation. The DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethione to cytosine, producing 5-methylcytosine and S-adenosylhomocysteine.

1.2 DNA demethylation

Demethylation of DNA also occurs, and involves at least two mechanisms. Passive demethylation occurs when Dnmt1 is unable to methylate newly synthesized DNA during replication. This is the mechanism by which 5-azacytidine (5-azaC), an irreversible DNA Mtase inhibitor, hypomethylates DNA, and certain DNA binding factors may also block cytosine methylation during S phase [25].The second mechanism may involve DNA demethylases. One protein demonstrating this activity is 5-methylcytosine DNA glycosylase (5-MCDG) and requires RNA for its demethylating function. Both enzyme and RNA exist in a larger complex that also contains an RNA helicase and GT mismatch DNA glycosylase activity [26]. One of the methylcytosine binding proteins, MBD4, has also been shown to act as a demethylase with similar 5-MCDG activity [27]. While another methylcytosine-binding protein, MDB2, has been reported to have demethylase activity, this has not been confirmed by others [28].

1.3 Mechanisms of gene suppression

The methylation of CG sequences can affect nearby gene expression. Hypomethylation of regulatory sequences usually correlates with gene expression, while methylation results in transcriptional suppression. In contrast, methylation of coding sequences generally has little effect on gene expression [29]. Methylation suppresses transcription by at least three mechanisms. Methylation of recognition sequences prevents the binding of some transcription factors such as AP-2 [30]. A family of methylcytosine binding proteins has been described, which inhibit binding of transcription factors to promoters [31] in Fig 2-2. Interestingly, all three DNA Mtases can also suppress gene expression directly independent of their methylation activity [32]. Finally, some methylcytosine binding proteins such as MeCP2 and MBD2 can promote chromatin condensation into an inactive configuration through interactions with chromatin inactivation complexes containing histone deacetylases [31]. This can affect gene expression at a distance from the methylated region, and is described in detail later.

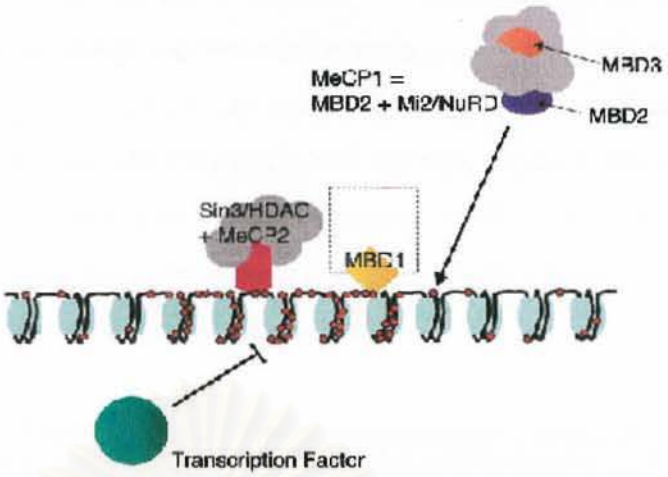


Figure 2-2 Mechanisms of transcriptional repression by DNA methylation. A stretch of nucleosomal DNA is shown with all CpGs methylated (red circles). Below the diagram is a transcription factor that is unable to bind its recognition site when a methylated CpG is within it. Many transcription factors are repelled by methylation. Above the line are protein complexes that can be attracted by methylation, including the methyl-CpG-binding protein MeCP2 (plus the Sin3A histone deacetylase complex) and the MeCP1 complex comprising MBD2 plus the NuRD corepressor complex. MeCP2 and MBD1 are chromosome bound proteins, whereas MeCP1 may be less tightly bound.

1.4 DNA methylation and chromatin structure

The relationship between DNA methylation and chromatin structure is currently under active investigation. The binding of some deoxy methylcytosine binding proteins to methylated sequences attracts complexes containing co-repressors and histone deacetylases, leading to a change in the chromatin structure from an open, transcriptionally active form to a more compact, inactive form, inaccessible to the transcription machinery. This is illustrated in Fig. 2-3. Perhaps the best-characterized methylcytosine binding protein mediating this effect is MeCP2. MeCP2 contains a deoxy methylcytosine binding region as well as a transcriptional repression domain.MeCP2 associates with the Sin3A histone deacetylase complex, consisting of at least seven proteins including the transcriptional repressor Sin3A and histone deacetylases HDAC1 and HDAC2. These enzymes remove acetyl groups from histones, which in turn leads to

a transcriptionally inactive chromatin structure [32]. Recent evidence suggests that changes in histone acetylation are important in the aging process, and that promoting histone deacetylation in some organisms increases longevity [33]. Since DNA methylation and histone acetylation are intimately linked,both are likely to play a role in aging . The relationship of DNA methylation to other histone modifications like methylation, phosphorylation, ubiquitination and others, referred to as the histone code [34], is less clear at present, but this area is developing rapidly.

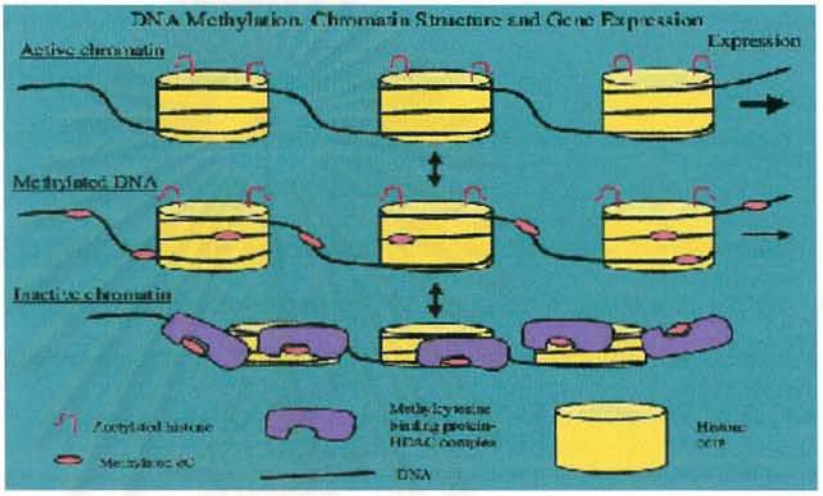


Figure 2-3 Chromatin inactivation by DNA methylation and histone deacetylation. The methylation of DNA sequences permits binding of the chromatin inactivation complex, which deacetylates histones and promotes chromatin condensation.

1.5 Important of DNA methylation in normal cells

The importance of DNA methylation in the function of normal cells is evidenced by its role in differentiation of development, X chromosome inactivation, genomic imprinting, maintenance of chromatin structure, and suppression of "parasitic" DNA [35].

1.6 DNA methylation in cancer

Tumorigenesis is known to be a multistep process in which defects in various cancer genes accumulate. Virtually every tumor type has revealed an enormous complexity of altered gene functions, including activation of growth-promoting genes as

well as silencing of genes with tumor growth-suppressing functions, all contributing to uncontrolled growth. Hanahan and Weinberg proposed that cancer gene functions can be classified into six essential alterations in cell physiology, including self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. It is now clear that the genetic abnormalities found in cancers will not provide the complete picture of genomic alterations. Epigenetic changes, mainly DNA methylation and, more recently, modification of histones, are now recognized as additional mechanisms contributing to the malignant phenotype. The study of these epigenetic changes on a genome-wide scale is referred to as epigenomics [36].

Hypomethylation and hypermethylation are most often used to describe relative states, best understood in relative to an expected, "normal" setting or degree of methylation (i.e. the level of methylation that in seen in nonmalignant cells). Hypomethylation and hypermethylation of DNA are relative terms and denote less or more methylation than in some standard DNA. When applied to cancer epigenetic, that standard is normal tissue. However, there are considerable differences in the amounts and distribution of DNA methylation among different vertebrate tissues because DNA methylation is not only species specific but also tissue-specifc [37].

The discovery of extensive cancer associated DNA hypomethylation in the human genome [38] preceded that of cancer-linked DNA hypermethylation. DNA hypomethylation in cancer often affects more of the genome than does hypermethylation so that net losses of genomic 5-methylcytosine are seen in many human cancers (fig.2-4). The role in carcinogenesis of cancer linked hypermethylation of transcription control regions is clear because of the consequent transcriptional silencing of genes important for prevention of cancer (tumor suppressor gene) .The biological significance of DNA hypomethylation in cancer is less understood.

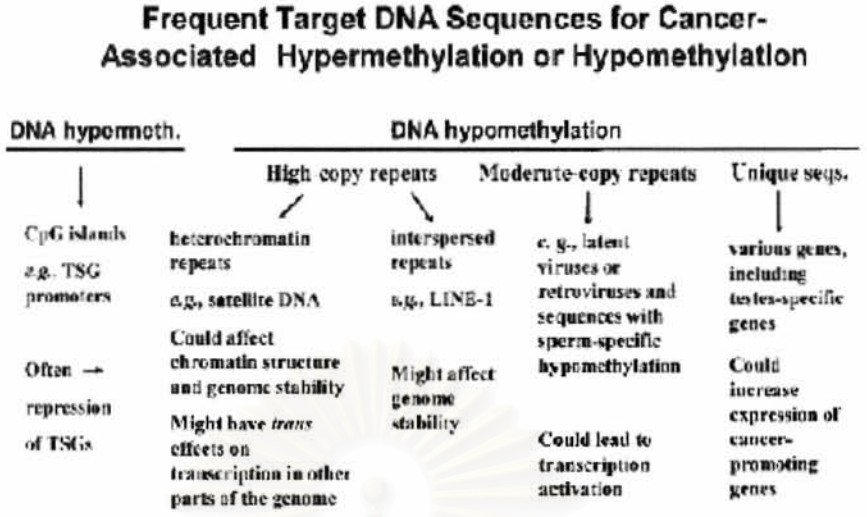


Figure 2-4 A summary of the most frequent types of sequences affected by cancer specific DNA hypermethylation or hypomethylation. TSG, tumor suppressor gene

1.7 Methylation and tumor suppressor gene

Epigenetic changes play a significant role in tumor formation and progression. Overall, the genome of malignant cells is characterized by global DNA hypomethylation and reductions of specific histone modifications. On the other hand, promoter hypermethylation and deacetylation of CpG islands results in aberrant transcriptional silencing of tumor suppressor genes [39]. According to Knudson's two-hit model [40], complete loss of function of a tumor suppressor gene requires loss of function of both gene copies. Epigenetic silencing of the wild-type allele of a tumor suppressor gene by aberrant promoter hypermethylation and histone deacetylation can be considered as the second hit in this model, resulting in complete loss of function of the gene. Aberrant tumor suppressor gene DNA methylation in human cancer cells is cooperatively maintained by DNMT1 and DNMT3b [41].

2. Tetratricopeptide repeat domain 12 (*TTC12*) gene

TTC12, a novel human gene, was identified and characterized by using bioinformatics. It, consists of 22 exons, has two isoforms of cDNA.FLJ20535

corresponded to nucleotide position 55-2255 of FLJ13859, and nucleotide position 52-2169 of FLJ13859 was the coding region (figure 2-5). TTC12 is comprised of tetratricopeptide repeat domain (TPR) domain and three armadillo (ARM) domains. TPR domain of TTC12 was most homologous to that of SMAP1, while ARM1-ARM3 domains of TPARM were most homologous to ARM7-ARM9 domains of CTNNB1 (also known as beta-catenin). TTC12 might be implicated in the WNT-beta-catenin signaling pathway, and its mRNA was expressed in testis, prostate, lung, germinal center B-cells, and also in neuroblastoma, teratocarcinoma, colon cancer, and gastric cancer [42] (figure 2-6).

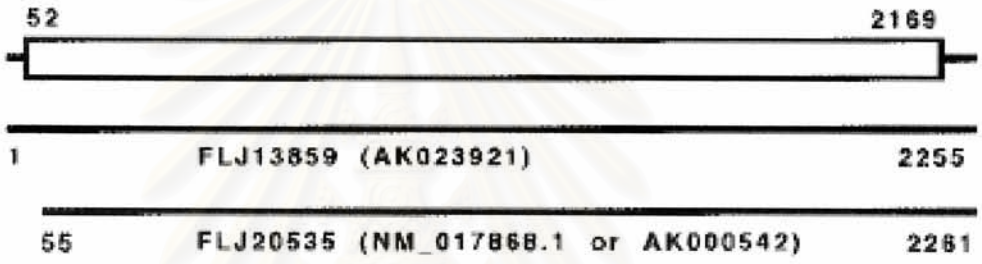


Figure 2-5 Schematic presentation of TTC12 cDNAs. The cDNA is shown by a bold bar (UTR) with an open box (coding region). Nucleotide position 52-2169 is the coding region of TTC12 cDNA. FLJ20535 (NM_017868.1 or AK000542) is a 5'-truncated TTC12 cDNA, while FLJ13589 (AK023921) is the representative TTC12 cDNA spanning the complete coding sequence.

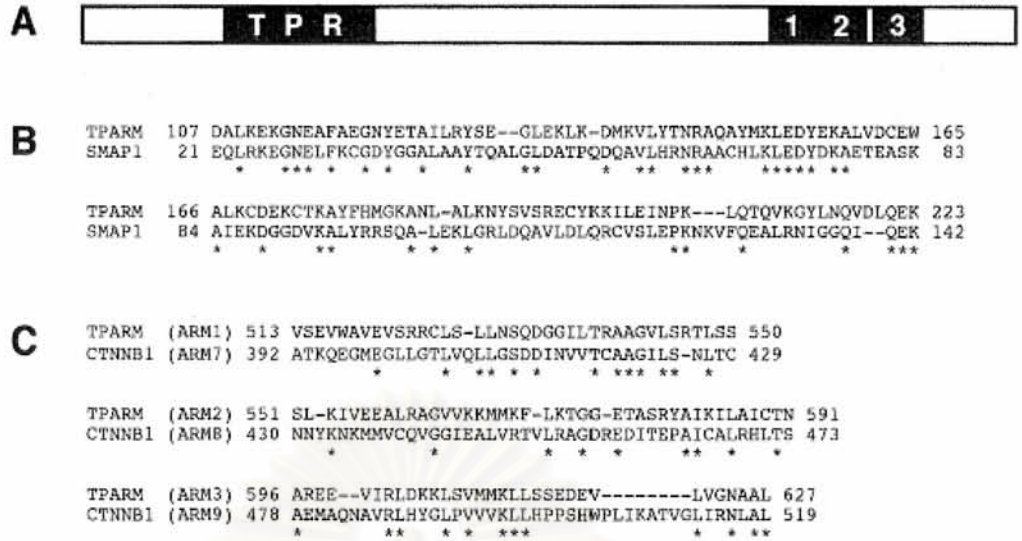


Figure 2-6 Domain structure of TTC12 protein. A. Schematic presentation of TTC12 protein. Tetratricopeptide repeat domain (TPR) and armadillo repeat domains (ARM1-ARM3) are shown by black boxes. B, Alignment of TTC12 or TPARM and SMAP1 proteins in the TPR domain. Amino-acid sequence of SMAP1 (smooth muscle cell associated protein-1) is derived from NP_061141.2. Amino-acid residues are numbered on both sides. Conserved amino-acid residues are shown by asterisks below the alignment. C, Alignment of TTC12 or TPARM and CTNNB1 proteins in the ARM domains. Amino-acid sequence of CTNNB1 (β-catenin) is derived from NP_001895.1. Amino-acid residues are numbered on both sides. Conserved amino-acid residues are shown by asterisks below the alignment. ARM1-ARM3 of TTC12 are homologous to ARM7-ARM9 of CTNNB1, respectively.

SMAP1, stromal membrane-associated protein 1, is a novel species of GTPase-activating protein (GAP) that preferentially acts on ADP-ribosylation factor 6 (Arf6). Arf6 is a small-GTPase that regulates the membrane trafficking between the plasma membrane and endosome [43].

β-catenin is the protien in canonical Wnt signaling pathway or transcriptional control and interact with E-cadherin [44]. Wnt signalling that involved cell development [45, 46] (figure 2-7).

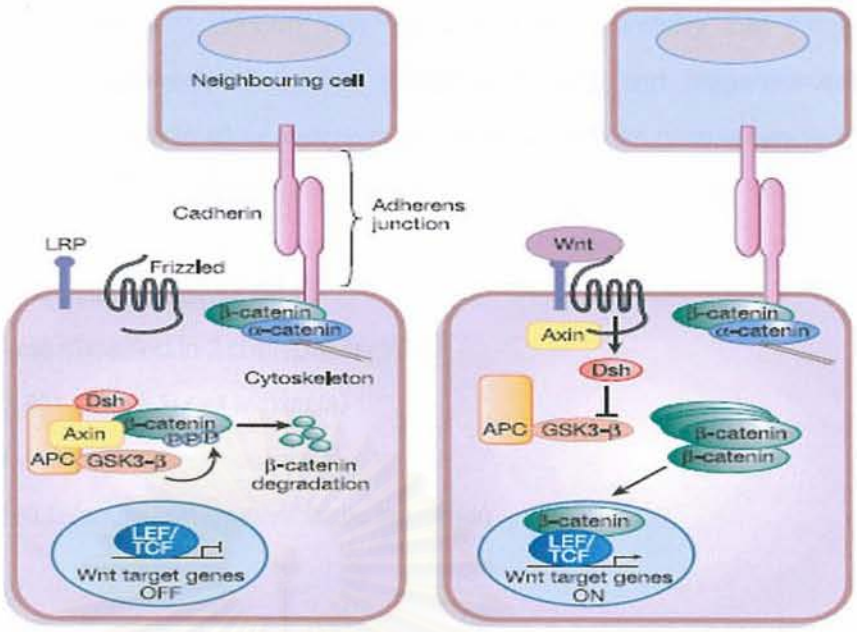


Figure 2-7 In the absence of Wnt signalling, β-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation. β-catenin also exists in a cadherin-bound form and regulates cell-cell adhesion. In the presence of Wnt signaling, β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where it binds Lef/Tcf transcription factors, thus activating target genes.

2.1 SMAP1 and cancer

Erythropoiesis is regulated by erythropoietin and microenvironment of erythropoietic organs. SMAP1 may play an important role in the erythropoietic stimulatory activity of the stromal cells [47]. In addition, an ALL patient was found chromosome translocation of *MLL* gene fusion to SMAP1 [48].

2.2 β-catenin and cancer

The E-cadherin-catenin complex plays a crucial role in epithelial cell-cell adhesion. Disturbance in protein-protein interaction in the E-cadherin-catenin adhesion complex is one of the main events in the early and late steps of cancer development. An inverse correlation is found between expression of the E-cadherin-catenin complex and the invasive behaviour of tumour cells [49]. In many tissues, activation of Wnt signalling has

also been associated with cancer. This has raised the possibility that the tightly regulated self-renewal mediated by Wnt signalling in stem and progenitor cells is subverted in cancer cells to allow malignant proliferation such as gastric cancer, colon cancer, leukemia etc [46].

3. Acute lymphoblastic leukemia (ALL)

ALL was classified in 3 subtypes by WHO.

1. B-ALL (acute B cell leukemia)
2. T-ALL (acute T cell leukemia)
3. ANLL (acute non lymphoblastic leukemia)

ALL is the most common malignancy in children. It accounts for one-fourth of all childhood cancers and approximately 75% of all cases of childhood leukemia [50]. ALL is a malignant neoplasm of lymphocytes characterized by the accumulation of immature blood cells in the bone marrow. These abnormal cells are arrested in the lymphoblast stage of the normal maturation pathway. Aberrations in the proliferation and differentiation of these cells are common, and normal hematopoiesis is suppressed. Patients have failed to produce normal blood cells, leading to anemia, infection, and bleeding [51]. Moreover , leukemic cells can infiltrate many organs, thus causing enlargement and dysfunction. Symptoms result from varying degree of anemia, neutropenia and thrombocytopenia [52].

ALL is a heterogenous disease with distinct biological and prognostic groups. Diagnosis relies on traditional cytomorphological and immunohistochemical evaluationof the leukemic blasts [53]. ALL developes as a consequence of malignant transformation of a single abnormal progenitor cell that has the capability to expand by indefinite self-renewal. In pediatric ALL, there is evidence that these events occur in committed lymphoid precursors. It appears that they may occur earlier because there is evidence of mutation in multiple cell lineages [54]. The events that lead to the process of malignant transformation are complex and multifactorial. It has been proposed that ALL results from spontaneous mutations that may occur in lymphoid cell of B or T-cell lineage or in their precursor cells [55]. During normal lymphoid development, lymphocyte precursor

may be at a higher risk for spontaneous mutation because of the intrinsically regulated mutagenic activity occuring during the process of gene rearrangement and the high rate of proliferation in these cells. Many of the described molecular mutations bear evidence of immunoglobulin VDJ and T-cell receptor (TCR) recombinase activity. The rearrangement of immunoglobulin and TCR genes has also been studied as a marker of clonality in ALL of pre-B lineage [56].

Leukemia transformation is unlikely to be the result of a single event but rather the culmulation of multiple processes involving complex interactions. The causes of leukemia include genetic [57], physical [58], chemical [59] and infectious causes [60]. Like all human cancer, ALL occurs as a result of specific genetic changes [54, 61]. Cytogenetic abnormalities have been detected in more than 60% of ALL, including hyperdiploidy, translocations, inversion and deletions [62-64]. Although many of these abnormalities are detectable by routine cytogenetic analysis, others require molecular assay [65]. Molecular identification of the gene located at the sites of these aberrations has led to the isolation and characterization of numerous oncogenes and tumor suppressor genes providing valuable clues to the mechanisms of leukemogenesis [54, 62, 66]. These leukemogenic genes are normal genes that have become altered by mutations, fusion to other genes, rearrangement, or loss. The genes that are potentially leukemogenic in hematopoietic cells can be conveniently grouped into five families. The first consists of genes that convey growth-stimulating signals from the cell membrane to the nucleus. The second is composed of genes that activate transcription; the protein products of these genes bind to specific DNA sequence near target genes and enhancer the synthesis of mRNA. The third family comprises genes involved in tissue differentiation. The fourth consists of genes involved in programmed cell death. The fifth comprises anti-oncogenes that may normally function to suppress tumor development. The products of a gene may have more than one function, depending on the assay used to detect its biological activity [54].

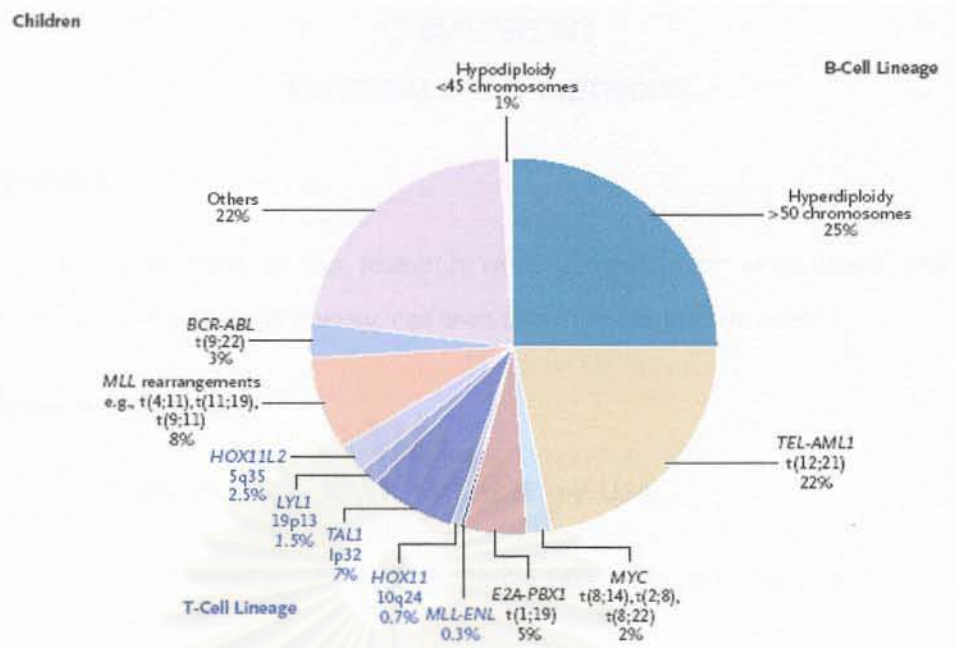


Figure 2-8 Estimated frequency of specific genotypes of ALL in children

The cause of genetic alterations in ALL occurs from mutations [67] (figure 2-8) including epigenetic mechanisms. The hypermethylation of CpG islands surrounding tumor suppressor gene promoter leads to transcriptional silencing and has been linked to human malignancies including acute lymphoblastic leukemia (ALL) [3-12]. In ALL, promoter hypermethylation is a common mechanism of gene regulation and silencing that participates in the regulation of genes implicated in major cellular pathways, such as cell cycle regulation (*CDKN2B, CDKN2A, TP73, PTEN, NES-1* and *LATS-1*) [11], apoptosis (*p14, PYCARD, APAF1, ASPP-1* and *DAPK1*) [3,10], or cell adhesion (*CDH13* and *CHD1*) [10].

CHAPTER III

MATERIALS AND METHODS

Specimens

The specimens in this research were comprised of white blood cell from peripheral blood and bone marrow, cell lines and cord blood stem cells.

Research Instruments

1. Pipette tip : 10 µl, 100 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-rad, Elkay, USA)
3. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
4. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
5. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
6. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
7. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
8. Microcentrifuge tube rack (USA/ Scientific plastics)
9. Thermometer (Precision, Germany)
10. Parafilm (American National Can, USA)
11. Plastic wrap
12. Stirring-magnetic bar
13. Combs
14. Electrophoresis chamber set
15. Pipette boy (Tecnomara, Switzerland)

16. Vortex (Scientific Industry, USA)

17. pH meter (Eutech Cybernatics)

18. Stirring hot plate (Bamstead/Thermolyne, USA)

19. Balance (Precisa, Switzerland)

20. Microcentrifuge (Fotodyne, USA)

21. DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)

22. Thermal cycler (Touch Down, Hybraid USA)

23. Power supply model 250 (Gibco BRL, Scothland)

24. Power poc 3000 (Bio-Rad)

25. Horizon 11-14 (Gibco BRL, Scothland)

26. Beta shield (C.B.S scientific. Co.)

27. Heat block (Bockel)

28. Incubator (Memmert)

29. CO_2 incubator (Shel lab)

30. Thermostat shaking-water bath (Heto, Denmark)

31. Spectronic spectrophotometers (Genesys5, Milon Roy USA)

32. UV Transilluminator (Fotodyne USA)

33. UV-absorbing face shield (Spectronic, USA)

34. Gel doc 1000 (Bio-RAD)

35. Refrigerator 4 ^{0}C (Misubishi, Japan)

36. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

37. Water purification equipment (Water pro Ps, Labconco USA)

38. Water bath

39. Storm 840 and ImageQuaNT solfware (Molecular dynamics)

40. Gel star nucleic acid gel stain (Cambrex Bio Science)

Reagents

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular glade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Hydrochloric acid (Merck)

1.9 Hydroquinone (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Isopropanol (Merck)

1.15 Sodium chloride (Merck)

1.16 Sodium dodecyl sulfate (Sigma)

1.17 Sodium hydroxide (Merck)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40%acrylamide/bis solution 19:1 (Bio-Rad)

1.22 Wizard DNA Clean-up System (Promega)

1.23 QIAamp DNA blood mini kit (QIAGEN)

1.24 Trizol (Invitrogen)

1.25 DEPC water

1.26 cDNA synthesis kit (Fermentas)

1.27 pGEM-T easy vector kit (Promega)

2. Cell culture reagents

2.1 Dulbecco's modified Eagle's medium; DMEM (Gibco)

2.2 RPMI 1640 (Gibco)

2.3 10% fetal bovine serum; FBS (Sigma)

2.4 penicillin and streptomycin

2.5 phosphobuffer saline; PBS

3. Reagents of PCR

3.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL, Perkin Elmer)

3.2 Magnesium chloride (GibcoBRL, Perkin Elmer)

3.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

3.4 Oligonucleotide primers (BSU) in appendix B

3.5 HotStar*Taq* polymerase (Qiagen)

3.6 Genomic DNA sample

4. Restriction enzyme

3.1 *Taq*I and buffer *Taq*I (Fermentus)

3.2 *Bst*UI and 10X NEBuffer 2 (Biolabs)

Methods

Subjects and Sample collection

After clinical diagnosis and informed consents were obtained, bone marrow (BM) of acute lymphoblastic leukemia (ALL) patients, remission BM and BM of T-cell lymphoma patients for genetic analysis were collected from the different groups. All patients were diagnosed by experienced hematologist.

The control cases were composed of ten peripheral bloods of healthy volunteers randomly. Twenty nine BM of ALL, eight samples of remission BM whom were free from ALL and four BM of T-cell lymphoma were studied. A criterion to choose BM ALL samples was having percentage of cancer cells more than 65% by flow cytometry. Additionally, five samples of cord blood stem cells were cultered with varied conditions for a different number of cell growth, and flow cytometry. All of them were hematopoietic

stem cells, but in one case, some of stem cells were shifted to myeloid cell more than the others.

In addition, several cell lines that are epithelial cell lines, HN12, HeLa and SW480, and hematopoietic cell lines, K562, Molt4, Duadi and Jurkat, were cultured and harvested.

Cell culture

HN12, K562, HeLa and SW480 were maintained in DMEM. Molt4, Duadi and Jurkat were cultured in RPMI 1640. All of these cell lines were supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin-streptomycin and were incubated at 37°C with 5% CO_2.

DNA extraction

Cord blood stem cells were extracted DNA by DNA mini kit, and other samples were followed conventional method below.

The extraction of DNA from cell lines, peripheral blood and bone marrow leukocyte was performed as follow:

1. 5-10 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.
2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.
3. Centrifuge for 8 minutes at 1,000 g, then remove supernatant.
4. Add 3 ml. Cold lysis buffer1, mix thoroughly and centrifuge for 8 minutes at 1,000g.

(Cell line samples were centrifuged for 8 minutes at 400g, remove media and wash with PBS. Centrifuge for 8 minutes at 1,000 g, then remove supernatant and start the 5th step.)

5. Discard supernatant afterward add 900µl lysis buffer2, 10µl proteinase K solution (20 mg Proteinase K in 1.0 ml. Of 1% SDS-2mM EDTA, should be prepare 30 min before use.), and 10% SDS 50µl. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in 37°C shaking waterbath overnight for complete digestion.

7. Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.

9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dried the pellet. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the digested DNA in 20-300µl of the double distilled water at 37°C until dissolved.

RNA extraction

The extraction of RNA from cell lines and bone marrow leukocyte was performed as follow:

1. 5-10 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.

2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3. Centrifuge for 8 minutes at 1,000 g, then remove supernatant.

4. Add 3 ml. Cold lysis buffer1, mix thoroughly and centrifuge for 8 minutes at 1,000g.

(Cell line samples were centrifuged for 8 minutes at 400g, remove media and wash with PBS. Centrifuge for 8 minutes at 1,000 g, then remove supernatant and start the 5th step.)

5. Discard supernatant afterward add 1 ml trizol and mix rapidly by pipette until homogeneity.

6. Incubate 5 minutes at roomtemp., add 200µl cold chloroform and shake vigorously 15 seconds by hand.

7. Incubate 3 minutes at roomtemp. and cold centrifuge at 12,000g for 15 minutes

8. Transfer the aqueous phase to a mew tube, add 500µl cold isopropanol.

9. Incubate 10 minutes at roomtemp. and cold centrifuge at 12,000g for 10 minutes

10. Discard supernatant afterward wash RNA pellet by add 1ml cold 75% ethanol.

11. Vortex and cold centrifuge at 7,500g for 5 minutes

12. Remove supernatant, air dry RNA pellet not completely dry.

13. Resuspend RNA pellet in 10-50µl of DEPC water at 55°C until dissolved.

Primer design for COBRA

Primers were designed to amplify the methylated and unmethylated allele equally. The primer design mention about the difference between methylated allele and unmethylated allele after standard sodium bisulfite treatment and PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated

cytosine residues to cytosine. This sequence conversion lead to the methylation-dependent creation of new restriction enzyme site. This is also dictated by the availability of restriction enzyme sites. Here general strategy:

1. Identify the region 5′UTR of *TTC12* / CpG islands. Make a restriction map of the area (all enzymes). This is the unconverted map.
2. Copy sequence and paste in a text editor.
3. Convert all C to T except for CG. First convert all CG to XG. Then convert all C to T. Then convert all X to C. Make a restriction map of this converted sequence (Methylated map)
4. Convert all remaining C to T. Make a restriction map of this converted sequence (Unmethylated map)
5. Find restriction enzyme sites that are unique to the methylated map (not in the unconverted or unmethylated map). These are the best touse. If none is available, find restriction sites that are present in the methylated map but absent in the unmethylated map.

In this study use *Taq*I cutting TCGA sequence at COBRA1 and *Bst*UI cutting CGCG sequence at COBRA2 to digest amplicon from methylated DNA. (see primer sequence in appendix).

<u>COBRA *TTC12* PCR</u>

1. Preparing of DNA template
   - 1.1 Dilute DNA of each various samples in 50 μl dH_2O
   - 1.2 Add 5.5 μl 2M NaOH (from fresh stock) and mix well
   - 1.3 Incubate at 37 °C for 10 minutes.
   - 1.4 Add 30 μl of the dilute hydroquinone (dilution1:10 of 55 mg hydroquinone in 5 ml ddH_2O), then vortex.
   - 1.5 Add 520 μl bisulfite (bisulfite 1.88 g in 5 ml ddH_2O, bring pH to 5.0 with 5 drops of 19.5 M NaOH), then vortex.
   - 1.6 Incubate at 55 °C for 16-18 hours.
   - 1.7 Desalt samples with the Wizard DNA Clean-up System,Promega by adding 1ml Wizard ™ resin to each tube and mix.

1.8 Add to syringe attached to column anchored on the vacuum manifold and apply vacuum.

1.9 Once drained, wash with 2 ml 80% isopropanol and apply vacuum.

1.10 Once drained, elute DNA from column by adding 50 µl heated (50-70 °C) ddH_2O and centrifuge 1 minute at maximum speed.

1.11 Denature the sample with freshly prepared 5.5 µl 3mM NaOH and incubate at 37 °C for minute.

1.12 Neutralize by adding 5M-ammonium acetate and 2.3 volume of absolute ethanol.

1.13 Precipitate overnight at −20 °C centrifuge 15 minutes at 14,000 rpm wash with 70% ethanol and dry pellet.

1.14 Elute DNA with 10 µl TE buffer, then ready for PCR.

2. Reaction and condition

The PCR reaction was preformed in a total volume of 20 µl. Bisulfite treated DNA in 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphates (dNTPs) and 0.2 U HotStar*Taq* polymerase. Each of primer pair was performed in optimal concentration 0.2 µM. In PCR reaction, the initial denaturation step was 95°C for 10 minutes then followed by 35 cycles of denaturation at 95 °C for 1 minute , annealing at 54 °C (for COBRA1) or 60 °C (for COBRA2) for 1 minute ,extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes. Then, digested 5 µl of PCR product with 0.2 µl *Taq*I and 1 µl buffer *Taq*I or 0.2µl *Bst*UI and 1 µl NEBuffer2 for COBRA1 and COBRA2, respectively in total volume 10 µl . Incubate at 65°C (*Taq*I) or 60°C (*Bst*UI) overnight. Afterward, separate PCR products by 8% acrylamide gel, electrophorase acrylamide gel in 1XTBE at 100 v until dye front reaches the end of gel. Estimate size of digesting product by compare with 25 bp marker.

RT-PCR

1. Synthesis of cDNA template using cDNA synthesis kit (Fermentas). Total volume 20 μl per reaction.

   1.1 50 ng of RNA was incubated for 5 minutes at 70°C with 15 pmol of oligo dT primer.

   1.2 Add the mixture of 5X reaction buffer 4 μl, ribonuclease inhibitor 1 μl, 10 mM dNTP 2 μl and MuL V-reverse transcriptase 1 μl

   1.3 Incubate at 42°C for 60 minutes and 70°C for 10 minutes, then ready for PCR.

2. Reaction and condition

   The PCR reaction was preformed in a total volume of 10 μl. cDNA template 1 μl in 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphates (dNTPs) and 0.2 U HotStar*Taq* polymerase . Each of primer pair, GAPDH and FLJ13859 primers, was performed in optimal concentration 0.2 μM. In PCR reaction, the initial denaturation step was 95 °C for 10 minutes then followed by 35 cycles of denaturation at 95 °C for 1 minute , annealing at 53 °C for 1 minute ,extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes. Then, separate PCR products by 2% agarose gel in 1XTBE at 100 v until dye front reaches the end of gel. Estimate size of PCR products by compare with 100 bp marker (see primer sequence in appendix).

Cloning and sequencing

For COBRA1 and COBRA2, bisulfite DNA was amplified by using COBRA1 and COBRA2 primers, respectively. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced.

Data analysis

Quantitation is performed with a Molecular Dynamics Phosphorimager.

$$\text{The percentage of methylation in } \textit{TTC12} = \frac{\text{Intensity of cut band}}{\text{Intensity of (cut +uncut) band}} \times 100$$

$$\text{The percentage of expression in } \textit{TTC12} = \frac{\text{Intensity of TTC12 product}}{\text{Intensity of (GAPDH+TTC12) band}} \times 100$$

We used the SPSS software for windows 10.0 for statistical analysis. The difference of methylation and expression was determined by compared mean of percent methylation and percent expression, respectively. To compare mean of percent methylation and percent expression between two groups of indepedent samples, we used nonparametric test ; Mann-Whitney U test, and we used ANOVA to compare mean of methylation level among three or more groups of samples. In addition, the correlation of methylation level at COBRA1 and COBRA2 were analyzed by Pearson correlation.

CHAPTER IV

RESULTS

We searched a candidate tumor suppressor gene of nasopharyngeal carcinoma (NPC) on the critical region of loss of heterozygosity on chromosome 11q [1], and the previous study found that this region was down regulated in NPC [2]. Therefore, we hypothesized that *TTC12* gene should be a candidate tumor suppressor gene in NPC.

TTC12 methylation in leukemic cell lines

To proved our hypothesis, we examined the methylation status in several cell lines by COBRA technique. COBRA1 was designed according to the region and sequence in figure 4-1 and 4-2. DNA was bisulfited and converted C to U then to T after amplification but not methyl C. The bisulfite DNA was amplified by PCR and cut with *Taq*I to recognize prior sequence of CCGA. This enzyme cut TCGA sequence, so only methylated DNA was cut. The PCR product of 200 bp was digested to 123 bp and 77 bp if methylated. Band intensity was measured and calculated percentage of methylation.

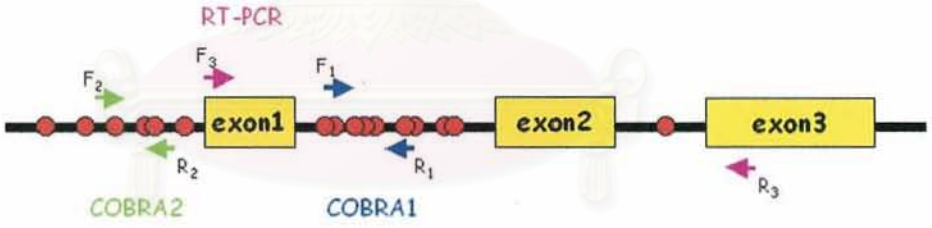


Figure 4-1 *TTC12* gene map for design primers. The CpG islands were shown by red circles. At COBRA1 region, F_1 and R_1 primers are created between exon1 and exon2 sequence. COBRA2 region, F_2 and R_2 primers are designed on 5'UTR; untranslated region. F_3 and R_3 are primers for RT-PCR.

Oliginal

actggccttcagagccgagcagaggacgctgctttgggccccagagcatgggaaggtcttggctgcggtctgggacggctgcggtctgggacctgcatgg
agggcccgggacttgcggacgcctgggagcggttgctgggcaacagtgcgtgcggcctggcctctttgcgcccggcgggcagcgcgcgtctggggcag
gctccgcgcggggctcgggcacaggtccccgctgccgcagccctaccgagtgaagcactgagcgcggcgccgcttcgccatcacctggctctggccct
ctcaccctagcgtaggggggcactggccaggcagtgaggaccctgagatccatgccctgggagacagaggttgtctcctgtccatgcacccgcgcgcctg
ccttctaaccttcataccgttggg

After treat bisulfite and methylation all CG

aTtggTTttTagagTCGagTagaggaCGTtgTtttgggTTTTagagTatgggaaggtTttggTtgCGgtTtgggaCGgTtgCGgtTtgggaTTtg
Tatggagg gTTCGggaTttgCGgaTgTTTgggagCGgttgTtgggTaaTagtgCGtgCGgTTtggTTtTtttgCGTTCGgCGggTagCGCG
CGtTtggggTaggTttTCGCGCGgggTtCGggTaTaggtTTTCGTtgTCGTagTTTtaTCGagtgaagTaTtgagCGCGgCGTCGTtt
CGTTatTaTTtggTtTtggTTTtTtTaTTTtagCGtagggggTaTtggTTaggTagtgaggaTTTtgagatTTatgTTTtgggagaTagaggttgtT
tTTtgtTTatgTaTTCGCGCGTTtgTTttTtaaTTttTataTCGttggg

Figure 4-2 A diagram sequence at COBRA1 region. The upper and lower sequence are before and after treated bisulfite sequence, respectively. Primers (underlined) and *Taq*I site (squared) are covering. The non-methylated cytosines are changed to thymine after treat bisulfite, but the methylated cytosines are still unchanged.

These cells lines were Daudi (B lymphoblast), Jurkat (acute T cell leukemia), Molt4 (acute T lymphoblastic leukemia), K562 (chronic myelogenous leukemia), HN12 (head and neck cancer), SW480 (colorectal adenocarcinoma) and HeLa (cervical cancer). The result showed hypermethylation in leukemic cell lines, Daudi, Jurkat and Molt4, except K562. In contrast, epithelial cell lines, HN12, SW480 and HeLa were non-methylated (figure 4-3).

Next, we evaluated the *TTC12* expression in cell lines. This gene transcribed into two isoforms, FLJ13859 and FLJ20535. FLJ 20535 is a 5'-truncated of TTC12, while FLJ 13859 is the complete coding sequence of TTC12. Therefore, FLJ13859 mRNA rather than FLJ20535 mRNA was the representative in *TTC12* transcription [42]. To test *TTC12* expression, we created primers in exon1 and exon3 of *TTC12* (figure 4-1). The result showed inverse correlation between the methylation and expression of *TTC12*. The hypermethylated cells, Daudi, Jurkat and Molt4, were not expressed TTC12, but the non-methylated, K562, HN12, SW480 and HeLa, were expressed (figure 4-3). These

result indicated novel epigenetic control of *TTC12* in hematopoietic cells. It is interesting if this methylation possess carcinogenic potential or tissue specific.

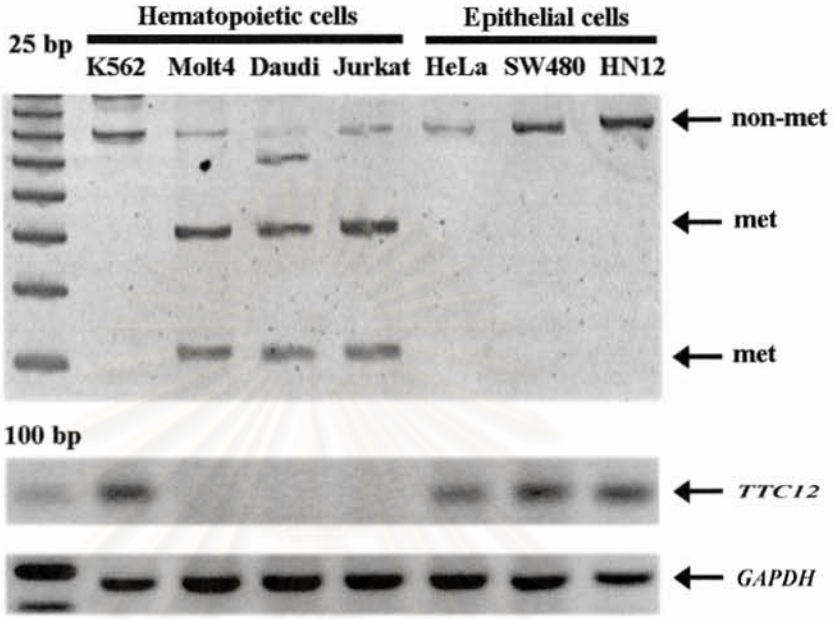


Figure 4-3 *TTC12* methylation correlated inversely with its expression in cell lines. (A) COBRA analysis in several cell lines. The arrows show non-methylated and methylated amplicons. (B) RT-PCR of TTC12 isoform and GAPDH.

To prove the reliability of this COBRA analysis, we calibrated our experiment by mixing K562 and Jurkat which non-methylated and nearly complete hypermethylated cell lines, respectively (figure 4-4 A). We did twice repeats for each experiment. The result showed no variation between intra- and inter- assays. The percentage of measured *TTC12* methylation correlated linearly with the percentage of actual methylation (figure 4-4 B).

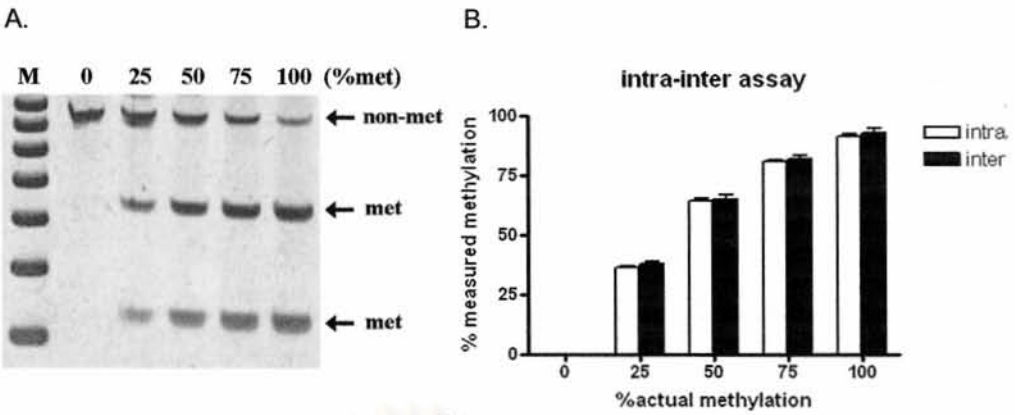


Figure 4-4 Intra- and inter- assay variation of COBRA analysis. (A) COBRA1 of a mixture of K562 and Jurkat, non-methylated and nearly complete hypermethylated cells, respectively. The arrows show non-methylated and methylated bands. (B) The average percentage of *TTC12* methylation between measured methylation (y axis), the methylated amplicons, and actual methylation (x axis), the proportion of K562 and Jurkat DNA. The bar value is mean and error bars represent ranges across experiments .

TTC12 methylation and expression in white blood cells

Next, we evaluated if TTC12 methylation status in leukemic and normal white blood cells. This included twenty-nine bone marrows from acute lymphoblastic leukemia (ALL) patients and white blood cell (WBC) from peripheral blood of ten healthy volunteers. We examined *TTC12* methylation status at COBRA1 and TTC12 expression. The result indicated the association between this epigenetic regulation and leukemogenesis. While in normal WBC, *TTC12* was hypomethylated and the mRNA level was high, hypermethylated *TTC12* was commonly detectable in ALL ($p < 0.0001$) (figure 4-5 A and figure 4-6). To confirm and characterize *TTC12* methylation status, we cloned and sequenced hypomethylated and hypermethylated *TTC12* amplicons from ALL (figure 4-5 B). Majority of CpG dinucleotides of hypermethylated cells were methylated but there were few methylated CpG from hypomethylated cells. Methylation also inhibit expression in ALL, the mRNA level of in hypermethylated ALL was less significantly than in hypomethylated ALL ($p<.05$) (figure 4-5 A and figure 4-7).

However, there was no difference in the level of *TTC12* methylation among ALL types ($p > 0.1$) (figure 4-8 c).

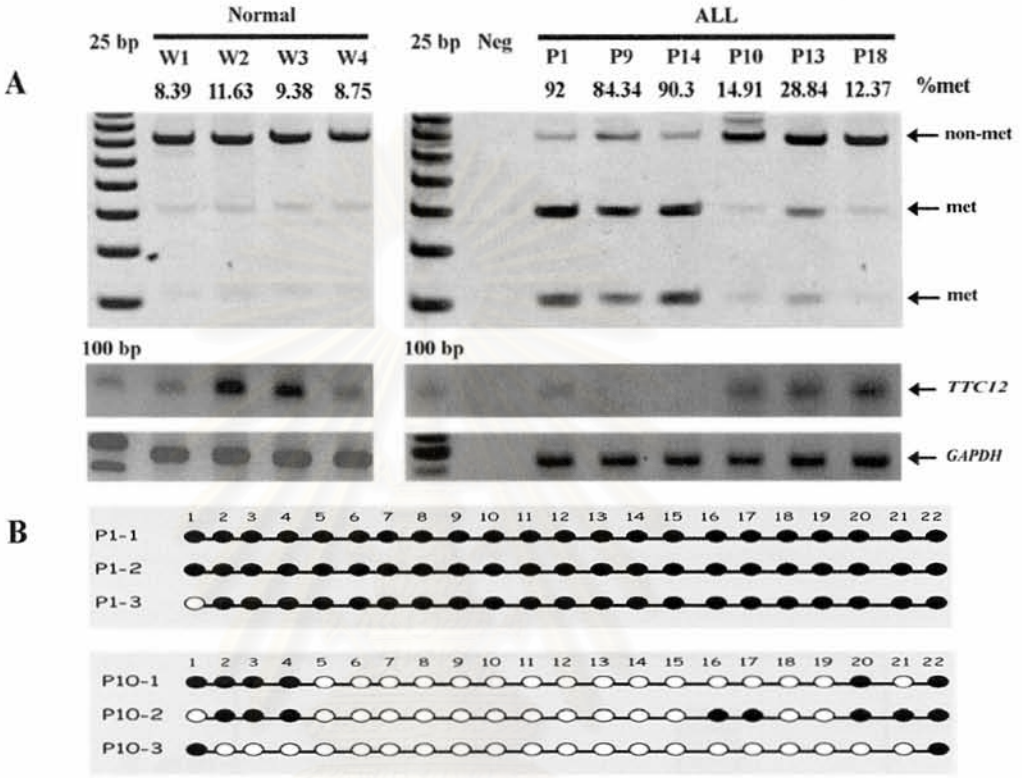


Figure 4-5 *TTC12* methylation and expression normal WBC and ALL. (A) COBRA analysis and RT-PCR. W and P are normal WBC and ALL, respectively. The percent *TTC12* methylation was shown in each case. The arrows indicate non-methylated amplicons, methylated amplicons, *TTC12 cDNA* and *GAPDH cDNA*, respectively. (B) Bisulfite sequencing at COBRA1 Methylated and unmethylated cytosines of each clone are shown by closed and open circles, respectively.

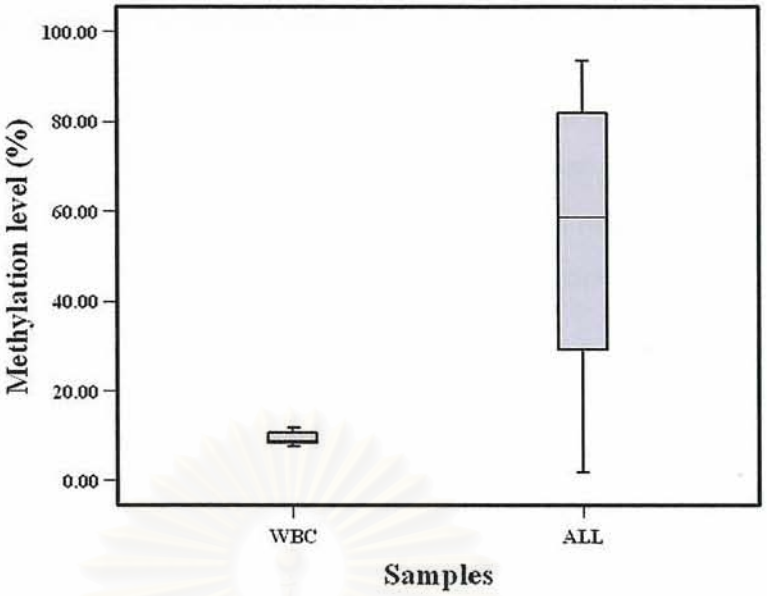


Figure 4-6 The average of *TTC12* methylation percentage between normal WBC and ALL. The box plots show mean and error bars of each groups.

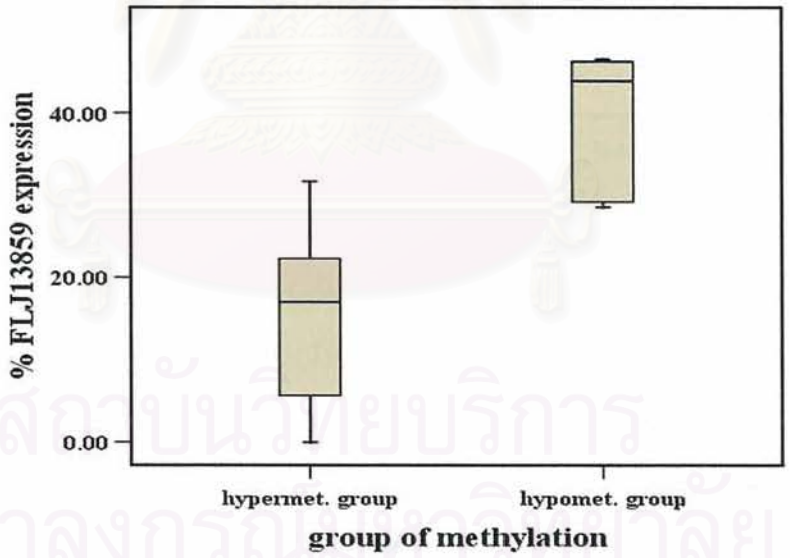


Figure 4-7 The average level of FLJ13859 between hypermethylated and hypomethylated ALL. The box plots show mean and error bars of each groups.

TTC12 methylation is leukemogenesis

We tested methylation level of *TTC12* methylation in other cell types. Eight samples of remission bone marrows (BM) were derived from ALL patients whom were free from ALL. Five samples of cord blood stem cells were harvested from cell culture, including four samples of BM T-cell lymphoma. The methylation levels in each type were shown in table4-1 and figure 4-8.

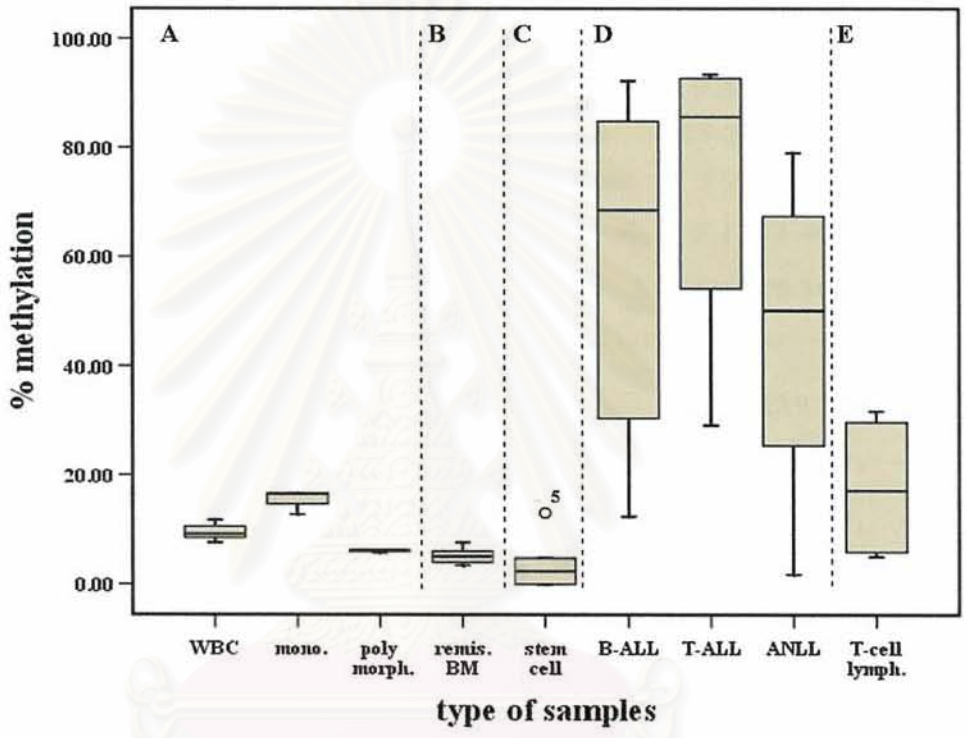


Figure 4-8 *TTC12* methylation levels in different sample types. Y axis displays the percentage of *TTC12* methylation. Types of samples (A-E) are the methylation levels of A; normal WBC, mononuclear cells and polymorphonuclear cells, B; remission BM, C; cord blood stem cells, D; B-ALL, T-ALL and ANLL, E; T-cell lymphoma, respectively. A circle indicate the number of case that significant difference of methylation level in stem cell group. WBC, BM, ALL and ANLL are white blood cell, bone marrow, acute lymphoblastic leukemia and acute non-lymphoblastic leukemia, respectively. Hypermethylation is specific to leukemic cells and no methylation is detectable in normal cells including stem cells. Norworthily, intermediate level of *TTC12* methylation are

discoverable in lymphoma. Therefore, we compared mean of methylation level among ALL types and T cell lymphoma. There were significant difference in the level of *TTC12* methylation among B-ALL and T cell lymphoma, and T-ALL and T cell lymphoma ALL types ,($p < 05$) and ($p < 01$), respectively (figure 4-8 d and e).

Table 4-1 *TTC12* methylation level of different sample types

	% methylation [mean (S.D)]
(A) normal WBC	9.42 (1.34)
mononuclear cells	15.28 (2.23)
polymorph. cells	5.94 (0.24)
(B) remission BM	5.12 (1.40)
(C) cord blood stem cell	4.01 (5.39)
(D) ALL	
B-ALL	59.41 (29.70)
T-ALL	73.45 (30.27)
ANLL	44.87 (28.28)
(E) T cell lymphoma	17.74 (13.90)

(A), (B), (C), (D) and (E) were type of samples. WBC, white blood cell; polymorph. cells, polymorphonuclear cells; BM, bone marrow; ALL, acute lymphoblastic leukemia; % methylation, the percentage of *TTC12* methylation; S.D, standard deviation.

TTC12 methylation in stem cell

We interested in *TTC12* methylation level in stem cell. Five stem cells were collected from two umbilical cord blood stem cells, CB1 and CB2, that were cultured, treated with the different conditions, harvested at day 8, and flow cytometry with $CD7^+$ and $CD34^+$. The data of each case showed in table 4-2. *TTC12* methylation level of each

case and mean of stem cell methylation were shown in table4-10, table 4-1 and figure 4-8, respectively.

Table 4-2 The percentage of $CD7^+$ and $CD34^+$ antigen

condition	$CD7^+$	$CD34^+$
(1)		
MSC +IL - 1 α	32.90 (4.80)	54.91 (5.36)
(2)		
MSC + FL + TPO	26.73 (3.70)	52.73 (4.56)
(3)		
FL + TPO	4.55	56.12

Umbilical cord blood was cultured with different conditions. MSC, mesenchymal stem cell; IL-1α, recombinant human interleukin-1alpha; FL, recombinant human Flt3-L; TPO, recombinant human thrombopoietin. Flow cytometry with $CD7^+$ and $CD34^+$ antigen, and the percentage indicate mean and standard diviation for each condition.

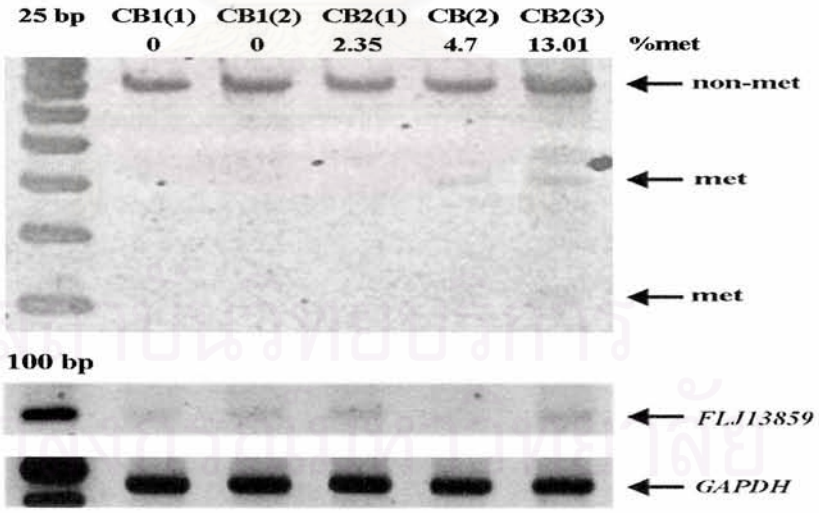


Figure 4-9 *TTC12* methylation and expression in stem cells. Upper picture is COBRA analysis, and the lower is RT-PCR. CB, cord blood stem cell; the numbers in parenthesis are conditions to culture stem cells. The percent *TTC12* methylation was shown in each

case. The arrows indicate non-methylated amplicons, methylated amplicons, *FLJ13859 cDNA* and *GAPDH cDNA*, respectively

TTC12 methylation on 5'UTR

After test methylation on COBRA1 region, we suggested that the *TTC12* methylation status should be expanded to 5'UTR. We designed primer on COBRA2 region for detect methylated expansion. COBRA2 was designed according to the region and sequence in figure 4-1 and 4-10. The bisulfite DNA was amplified by PCR and cut with *Bst*UI. This enzyme cut CGCG sequence, so only methylated DNA was cut by *Bst*UI. We evaluated COBRA2 methylation in some cases of ALL and correlated with methylation in COBRA1. The methylation at COBRA1 correlated directly with COBRA2 (r = .797) (figure 4-11). We summed up that the methylation of *TTC12* gene expand through 5'UTR.

Oliginal

gcagcaggcgctgctttagttaccagcactttgcttcctccgtctccccgcgccaggcatcgggcatcccaacctcgactttggccttcaacttggcgcgctccg
gagctagagctctgcggtagcgaatctgatcgcgtgtccatgcccgccccctgtcccgcctcctggcttttgaccccgtccttggtcccgccccgggccctgg
ctcctggccccgcccttggtcccgccccgggccccctggctcctggccccgccccagcccaggcaggtcactgcgccatttcctgtccaaagctgggcgaa
tcaggtcggtgccgcggggtaccccggaatccccccctgggagctggtcctgc

After treat bisulfite and methylation all CG

GTagTaggCGTtgTtttagttaTTagTaTtttgTttTTtTCGtTtTTTCGCGTTaggTatCGggTatTTTaaTTtCGaTtttggTTttTaaTttgg
CGCGTtTCGgagTtagagTtTtgTggtagCGaatTtgatCGCGtgtTTatgTTTgTTTTTtgtTTCGTTtTTtggTttttgaTTTCGtTTttggt
TTCGTTTCGggTTTtggTtTTtggTT TCGTTTttggtTTCGTTTCGggTTTTtggTtTTtggTTTCGTTTTagTTTaggTaggtTaT
tgCGTTatttTTtgtTTaaagTtgggCGaatTaggtCGgtgTCGCGgggtaTTTCGgaatTTTTTTtgggagTtggtTTtgT

Figure 4-10 A diagram sequence at COBRA2 region. The upper and lower sequence are before and after treated bisulfite sequence, respectively. Primers (underlined) and *Bst*UI site (squared) are covering. The non-methylated cytosines are changed to thymine after treat bisulfite, but the methylated cytosines are still unchanged.

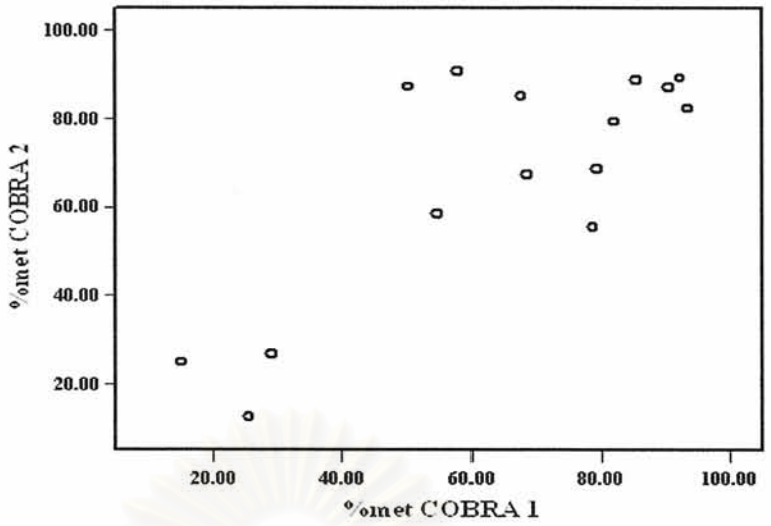


Figure 4-11 This graph represented the correlation of *TTC12* methylation level between COBRA1 and COBRA2.

CHAPTER V

DISCUSSION

From the study of Lo KW *et al.*[1], and Sriuranpong V *et al.*[2], we combined the critical regions of LOH with the reported down regulated genes in NPC for a new candidate tumor suppressor genes. From those results, we found 12 genes that fell into tumor suppressor gene criteria. Of these, since there has been no report about its' methylation status in tumor progression, *TTC12* gene, a novel gene harboring TPR and ARM domains, was subjected to be studied the epigenetic effect on the gene expression.

Surprisingly, the preliminary study demonstrated that the *TTC12* gene promoter in the head and neck cancer cell lines was nonmethylated; while, the hypermethylated was found in the leukemic cell lines. Therefore, we investigated the methylation level of *TTC12* gene promoter in ALL bone marrow compared with normal WBC samples. We found that *TTC12* gene promoter in ALL bone marrow samples was significantly hypermethylated than in normal WBC samples ($p<0.0001$). Within ALL group, we could classify samples as *TTC12* gene promoter hypermethylation and hypomethylation groups by comparing to the cut off value (mean of ALL methylation level $\pm$ S.D; 56.33 $\pm$ 29.84). From these results, we asked whether the methylation status was inversely correlated with the expression of the gene or not. As expected, TTC12 was higher expressed in the hypomethylated group and lower expressed in the hypermethylated group. ALL comprises of three subtypes; B-ALL and T-ALL, which are mononuclear cells, and ANLL, which is polymorphonuclear cells. We, therefore, investigated whether the *TTC12* gene promoter methylation level was correlated with ALL subtypes. We found that all three subtypes were hypermethylated. However, there was no significant correlation among subtypes ($p>0.05$; ANOVA). From these results, we concluded that the *TTC12* promoter hypermethylation was independent of ALL subtypes. From the evident that the location of *TTC12* is commonly deleted in malignant melanoma[68] and NPC[1] , *TTC12* can be suggested as a tumor suppressor gene and may be involved in the multistep process of ALL in most of the patients.

Although the function of *TTC12* has not been identified, *in silico*, two conserved domains have been found, including the tetratricopeptide repeat (TPR) domain and the armadillo repeat (ARM) domains. The TPR domain is located at codon 107-223, whereas the ARM domains are located at codon 513-550 (ARM1), codon 551-591 (ARM2) and codon 596-627 (ARM3)[42].

ARM, an approximately 40 amino acid long tandemly repeated sequence motif, was first identified in the Drosophila segment polarity gene armadillo; this domain was also found in the mammalian armadillo homolog beta-catenin, the junctional plaque protein plakoglobin, the adenomatous polyposis coli (APC) tumor suppressor protein, and a number of other proteins. ARM has been implicated in mediating protein-protein interactions, but no common features among the target proteins recognized by the ARM repeats have been identified. ARM-containing proteins have been involved in many cellular processes including nuclear localization, signal transduction and interaction with microtubules. Interestingly, the ARM1-ARM3 domains of TTC12 protein are most homologous to ARM7-ARM9 domains of CTNNB1 (β-catenin). These domains are the binding site of T-cell factor (TCF), the transcription factor involved in the T-cell development through the WNT-β-catenin signaling pathway[42].

TPR is a new class of interacting domain that **is found in a variety of organisms including bacteria, cyanobacteria, yeast, fungi, plants, and humans.** This protein-protein interaction domain is present in a number of proteins with diverse functions and subcellular locations, but the **common features in the interaction partners have not been defined.** By Interacting with TPR and non TPR proteins, TPR-containing proteins are implicated in a variety of cellular processes, ranging from cell cycle control to signal transduction, kinase regulation and tumorigenesis[69]. In addition, mouse TPR motifs in Ah receptor-interacting protein (AIP) is present in proteins required for cell cycle control and RNA synthesis and in steroid-binding immunopholins [70].

From these data, TTC12 might mediate protein-protein interaction with other proteins through ARM and TPR domains to trigger some cellular process including cell cycle control. According to high homology with ARM7-ARM9 domains of β-catenin, the ARMs domain of TTC12 may be involved in WNT-β-catenin signaling pathway that control the T-cell development and, in ALL, down regulation of *TTC12* that we found may

contribute to the promotion of multistep process of ALL, and perhaps of other cancers. Nevertheless, this hypothesis as well as the function(s) of TTC12 should be investigated and this will provide new knowledge of cancer biology that could be applied in cancer diagnosis as well as therapeutic in the future.

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APPENDICES

APPENDIX A

BUFFERS AND REAGENTS

1. Lysis Buffer 1

Sucrose	109.54	g
1.0 M Tris — HCl (pH 7.5)	10	ml
1.0 M $MgCl_2$	5	ml
Triton X — 100 (pure)	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator (at 4^0C).

2. Lysis Buffer 2

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml

Mix the solution and store in a refrigerator (at -20^{0}C).

5. 1.0 M Tris — HCl

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H_2O	186.6	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

Magnesium chloride.6H_2O	20.33	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride	29.25	g
Distilled water to	100	ml

Dispense the solution into aliquot and sterilize by autoclaving.

9. 10X Tris borate buffer (10X TBE buffer)

Tris — base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and store at room temperature.

10. 6X loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mixed and stored at 4^0C

11. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

12. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 8% Non-denature acrylamide gel (w/v)

40%acrylamide: Bis (19:1)	1	ml
10X TBE	0.5	ml
10% ammoniumpersulfate	100	µl
TEMED	5	µl
H_2O	3.5	ml

Dissolve by heating in microwave oven and occasional mix.

14. TE buffer

Tris base	1.21	g
5M EDTA	200	µl

Adjust pH to 7.5 with conc.HCL and adjust volume to 1.0 litre with H_2O.

APPENDIX B

Sequence of *TTC12* and primer

Human *TTC12* ACCESSION

COBRA1

We designed primers for examined the *TTC12* methylation status on intron sequence between exon 1 and exon 2 of *TTC12* gene

BEFORE BISULFITE

actggccttcagagccgagcagaggacgctgctttgggccccagagcatgggaaggtcttggctgcggtctggga
cggctgcggtctgggacctgcatggagggcccgggacttgcggacgcctgggagcggttgctgggcaacagtgc
gtgcggcctggcctctttgcgcccggcgggcagcgcgcgtctggggcaggctccgcgcggggctcgggcacag
gtccccgctgccgcagccctaccgagtgaagcactgagcgcggcgccgcttcgccatcacctggctctggccctc
tcaccctagcgtaggggggcactggccaggcagtgaggaccctgagatccatgccctgggagacagaggttgtct
cctgtccatgcacccgcgcgcctgccttctaaccttcataccgttggg

AFTER BISULFITE+METHYLATION ALL CG

aTtggTTttTagagTCGagTagaggaCGTtgTtttgggTTTTagagTatgggaaggtTttggTtgCGgtTtg
ggaCGgTtgCGgtTtgggaTTtgTatggagggTTCGggaTttgCGgaTg

F_1

TTtgggagCGgttgTtgggTaaTagtgCGtgCGgTTtggTTtTtttgCGTTCGgCGggTagCGCGCGt

TaqI site

TtggggTaggTtTCGCGCGgggTtCGggTaTaggtTTTCGTtgTCGTagTTTtaTCGagtgaagTa

R_1

TtgagCGCGgCGTCGTttCGTTatTaTTtggTtTtggTTTtTtTaTTTtagCGtaggggggTaTtg

GTTaggTagtgaggaTTTtgagatTTatgTTTtgggagaTagaggttgtTtTTtgtTTatgTaTTCGCGC
GTTtgTTttTtaaTTttTataTCGttggg

F_1 = TTTGGGAG(C/T)GGTTGTTGGG

R_1 = CAATACCCCCCTAC(G/A)CTAAA

Total amplicon = 200 bp and *Taq*I = 123+ 77

COBRA2

We designed primers for detected the expansion of *TTC12* methylation on 5′UTR of *TTC12* gene.

BEFORE BISULFITE

gcagcaggcgctgctttagttaccagcactttgcttcctccgtctccccgcgccaggcatcgggcatcccaacctcg

actttggccttcaacttggcgcgctccggagctagagctctgcggtagcgaatctgatcgcgtgtccatgcccgcccc

ctgtcccgcctcctggcttttgaccccgtccttggtcccgccccgggccctggctcctggccccgcccttggtcccgc

cccgggcccctggctcctggccccgccccagcccaggcaggtcactgcgccatttcctgtccaaagctgggcga

atcaggtcggtgccgcggggtaccccggaatccccccctgggagctggtcctgc

AFTER BISULFITE+METHYLATION ALL CG

gTagTaggCGTtgTtttagttaTTagTaTtttgTttTTtTCGtTtTTTCGCGTTaggTatCGggTatTTT

aaT

F_2 *Bst*UI site

TtCGaTttggTTttTaaTttggCGCGTtTCGgagTtagagTtTtgTggtagCGaatTtgatCGCGtgtTTat

gTTTgTTTTTtgtTTCGTTtTTtggTttttgaTTTCGtTTttggtTTCGTTTCGggTTTtggTtTTtggTT

R_2

TCGTTTttggtTTCGTTTCGggTTTTtggTtTTtggTTTGGTT

TTagTTTaggTaggtTaTtgCGTTatttTTtgtTTaaagTtgggCGaatTaggtCGgtgTCGCGgggtaT

TTCGgaatTTTTTTtgggagTtggtTTtgT

F_2 = TT(C/T)GATTTTGGTTTTTAATTTGG

R_2 = AAC(G/A)AAACCAAAAACCAAAAACC

Total amplicon = 187 bp and *Bst*UI = uncertain pattern (because more than one site)

RT-PCR

We created primers for tested *TTC12* expression, FLJ13859 isoform, status on exon 1 and exon 3 sequence of *TTC12* gene.

FLJ13859 sequence and primer

F_3

tgggcgaatcagggattccggttcacaatggatgctgataaagagaaagatttgcagaaatttcttaaaaatgtggatga
aatctccaatttaattcaggagatgaattctgatgacccagttgtgcaacagaaagctgtcctggagacagaaaagag

R_3

actactgcttatggaggaagaccaggaggaggatgaatgc

F_3 = TGGGCGAATCAGGGATTCC

R_3 = TCATTCATCCTCCTCCTGGT

PCR product = 198 bp

GAPDH primer

F = GTGGGCAAGGTATCCCTG

R = GATTCAGTGTGGTGGGGGAC

PCR product = 460 bp

BIOGRAPHY

Miss Roongtiwa Wattanawaraporn was born in Bangkok in 1981. In 2002, she graduated from faculty of Science, Chulalongkorn University in Genetics program and then attended to particulate in Medical Science program in Faculty of Medicine for her master degree. She plan to study in Ph.D. program.