CHAPTER II

REVIEW OF RELATED LITERATURES

1.DNA methylation in mammals

Epigenetic modifications of the DNA do not alter the sequence code; however, they are heritable and involved in regulation of gene transcription . DNA methylation, the addition of a methyl group to the 5′carbon of deoxycytosine, is one such epigenetic modification found in DNA [1]. 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′) [2]. 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 [3]. 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 [4]. Most CpG islands are unmethylated in normal cells; however, there are certain conditions where these sequences become methylated and form part of gene regulation [5]. The majority of CpG islands on the inactive X-chromosome in a female cell are methylated [6], and certain CpG island-like sequences in the vicinity of imprinted genes have been found to be methylated in an allele-specific manner [7]. Furthermore, it has been found that some CpG islands become methylated with age [8]. 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 [9]. 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 [10]. Their role in mature cells is less clear, but Dnmt3b appears to be necessary for maintaining methylation of pericentromeric heterochromatin [11]. Following differentiation the patterns are replicated during mitosis by the maintenance DNA Mtase Dnmt1 [9]. 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 [9](Fig.2-1).
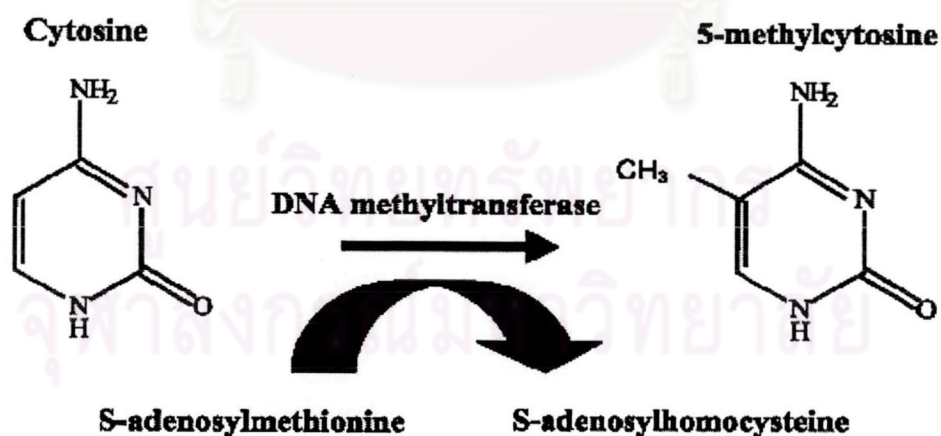

Figure 2-1. Cytosine methylation. The DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethione to cytosine, producing 5-methylcytosine and S-adenosylhomocysteine.

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 [12].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 [13]. One of the methylcytosine binding proteins, MBD4, has also been shown to act as a demethylase with similar 5-MCDG activity [14]. While another methylcytosine-binding protein, MDB2, has been reported to have demethylase activity, this has not been confirmed by others [15].

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 [16]. Methylation suppresses transcription by at least three mechanisms. Methylation of recognition sequences prevents the binding of some transcription factors such as AP-2 [17]. A family of methylcytosine binding proteins has been described, which inhibit binding of transcription factors to promoters [18] in Fig 2-2. Interestingly, all three DNA Mtases can also suppress gene expression directly independent of their methylation activity [19]. 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 [18]. This can affect gene expression at a distance from the methylated region, and is described in detail later.
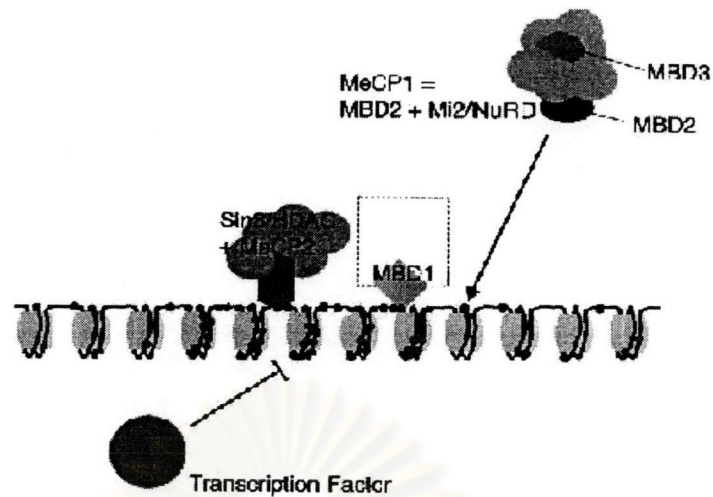
Figure 2-2. Mechanisms of transcriptional repression by DNA methylation. A stretch of nucleosomal DNA is shown with all CpGs methylated (red circles). Below the diagram is a transcription factor that is unable to bind its recognition site when a methylated CpG is within it. Many transcription factors 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 MeCP1complex 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 [19]. Recent evidence suggests that changes in histone acetylation are important in the aging process, and that promoting histone deacetylation in some organisms increases longevity [20]. 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 [21], is less clear at present, but this area is developing rapidly.
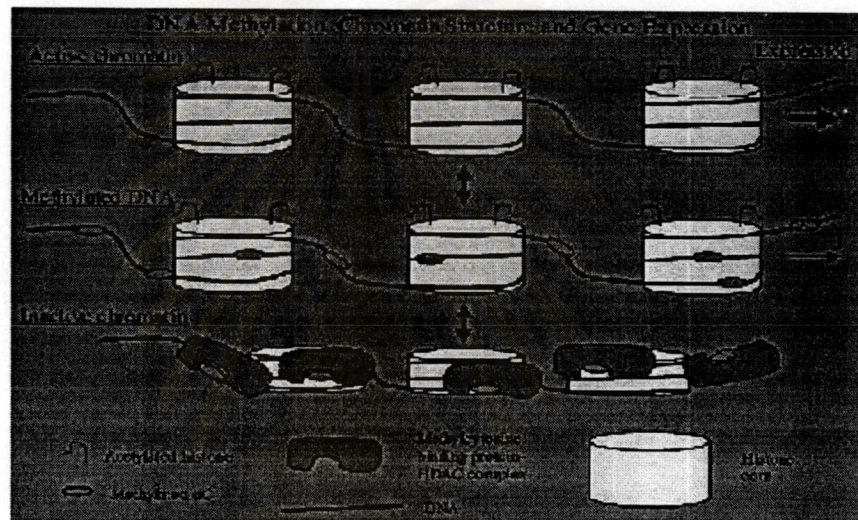
Figure 2-3. Chromatin inactivation by DNA methylation and histone deacetylation. The methylation of DNA sequences permits binding of the chromatin inactivation complex, which deacetylates histones and promotes chromatin condensation.

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 [22].

1.5.1 X-chromosome inactivation

X-chromosome inactivation is a process that occurs in all mammals, resulting in selective inactivation of alleles on one of the two X-chromosome in females. It provides a mechanism of dosage compensation, which overcomes sex differences in the expected ratio of autosomal gene dosage to X-chromosome gene dosage (which is 2:1 in male but 1:1 in females). Males with a single X-chromosome are constitutionally hemizygous for X-chromosome genes, but female become functionally hemizygous by inactivating one of the parental X-chromosome alleles. Not all gene on the X-chromosome are subject to inactivation; gene which escape X-inactivation include ones where there is a function homolog on the Y-chromosome, and some genes where gene dosage dose not seem to be important. In mammals, X-chromosome inactivation appears to be initially controlled by a single gene, *Xist* gene, at the early stage in development (blastula stage). *Xist* gene in human encodes a mature 15 kb RNA product which located on Xic (X-chromosome inactivation center) located at Xq 13 in human and uniquely encoded only by the inactive X. The inactivation occurred randomly, either maternal or paternal X-chromosome may be inactivated. Methylation contributes to inactivation of the suppressed X-chromosome in female, resulting in equal male and female dosing for X-linked genes [23].

1.5.2 Silencing of parasitic DNA sequences

Transposable elements are DNA sequences that capable of moving from one site to another (so-called "jumping genes") and that are present in numerous copies throughout the human genome. These elements resemble viral DNA and are commonly referred to as "parasitic DNA". Repression of transposons is required to prevent DNA damage due to unconstrained transposition [24] or that transcription of a large excess of irrelevant promoters would constitute an unacceptable level of transcription noise that would interfere with gene expression programs [25]. Increased transcription of elements in human and mouse cells has not so far been found to lead to increased transposition. elements that are incapable of transposition, must be silenced to suppress transcription noise. DNA methylation deficiency is the activation of transposable element derived

promoters. Like much of the mammalian genome, transposable element related sequences are heavily methylated and transcriptionally silent in somatic cells.

1.5.3 DNA methylation and development

Dramatic changes in overall methylation of DNA occur at different periods of embryogenesis, development, and differentiation to adult cells [26]. The genome of the primodial germ cells of the embryo are not methylated to any extend. After glonadal differentiation and as the germ cell begin to develop, de novo methylation occurs leading to substantial methylation of the DNA of mammalian sperm and egg cells in Fig.2-4.The sperm genome is more heavily methylated than the egg genome. The genome of the fertilized oocyte is an aggregate of the sperm and egg genome and so it and the very early embryo are substantially methylated with methylation differences at paternal and maternal alleles of many genes. Later on, at the morula and early blastula stages in the preimplantation embryo, genome-wide demethylation occurs. A wave of demethylation initially erases presetmethylation patterns in the first days of embryogenesis. This is followed by several waves of de novo methylation that eventually establish adult patterns of gene methylation. In differentiated cells, methylation patterns change relatively little and are perpetuated after DNA replication through the high affinity of DNA metnyltransferase for hemimethylated DNA

Evidence that DNA methylation is important in development comes from the observation that disrupting both *DNMT1* alleles in embryonic stem cells results in embryonic death [27]. Dnmt3a and Dnmt3b are also essential for mammalian development; homozygous Dnmt3a deficiency causes running and death at 4 weeks of age, while Dnmt3b deficiency is embryonic lethal [28].
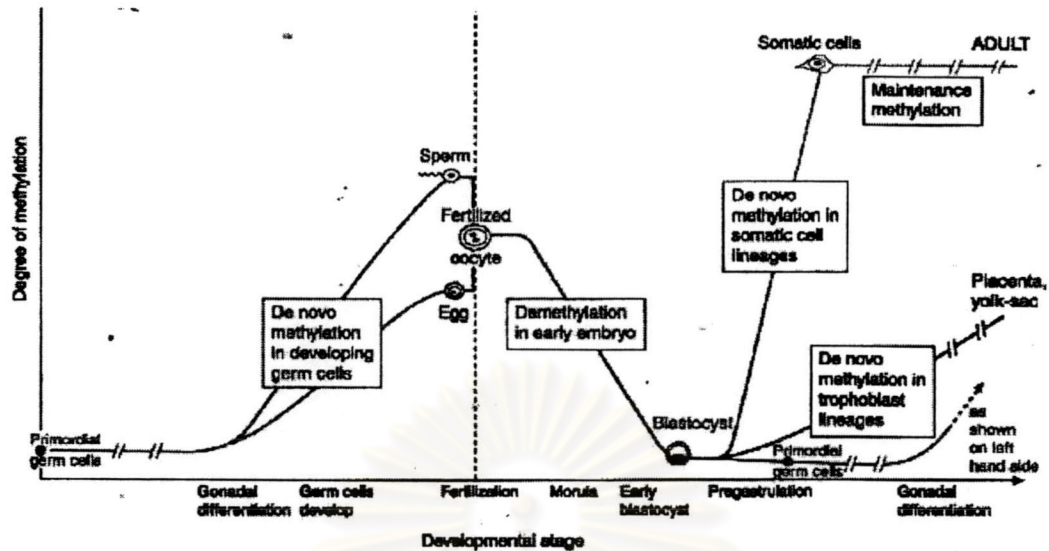
Figure 2-4. Changes in DNA methylation during mammalian development

1.6 DNA methylation in aging

Early studies examined changes in total genome d^mC content. Overall, total d^mC levels tend to decrease (hypomethylation)with aging in most vertebrate tissues. Demethylation has been reported in salmon, mice, rats, cows and humans, and occurs in the brain, liver, small intestine mucosa, heart, and spleen [29]. In contrast, rat lung genome DNA does not demethylate as a whole, and total d^mC content in rat kidneys increases [30]. However, these studies, relying on techniques measuring total d^mC content, did not define whether the methylation changes occurred in transcriptionally irrelevant sequences, or whether the changes occurred in the vicinity of genes with effects on gene expression. Thus the significance of the changes determined by this approach is uncertain. Changes in DNA methylation also occur outside of coding regions. For example, repetitive DNA sequences in brains from aged rats contain greater amounts of than d^mC identical sequences from younger rats, again measured using methylation sensitive enzymatic digestion [31]. Demethylation of repetitive DNA sequences also occurs in other tissues including the liver, thymus and heart [32]. This may promote chromosomal translocations with aging [33]. Mobile DNA elements known as retrotransposons are normally repressed by DNAmethylation. These sequences can demethylate with age, correlating with activation [34]. Similarly, sequences encoding endogenous retroviruses, also normally repressed by extensive methylation, have been reported to demethylate

with age, and this process may be responsible for the increase in expression of these parasites that occurs with aging [35].Again, cause and effect in most instances need to be established.

1.7 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, nonaging 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.2-5).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 (tumour suppressor gene) .The biological significance of DNA hypomethylation in cancer is less understood.

Frequent Target DNA Sequences for Cancer-Associated Hypermethylation or Hypomethylation
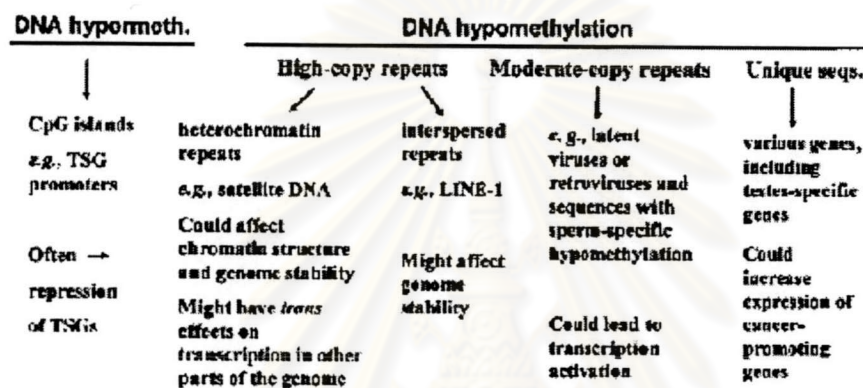

Figure 2-5. A summary of the most frequent types of sequences affected by cancer specific DNA hypermethylation or hypomethylation. TSG, tumor suppressor gene

1.8 Global genomic hypomethylation

The malignant cell can have 20-60% less genomic 5-methylcytosine than its normal counterpart. The loss of methyl groups is accomplished from various sources, but mainly by hypomethylation of the "body" (coding region and introns) of gene, demonstrated, for example, in the case of gramma-globin, *H-Ras* and *c-myc* genes; and through demethylation of repetitive DNA sequence that account for 20-30% of the human genome. Hypomethylation potentially promotes cancer via four mechanism: chromosome instability , reactivation of transposon element, activation of protooncogene and loss of imprinting. Undermethylation of DNA might favor mitotic recombination leading to loss of heterlozygosity, as well as promoting karyotypically detectable rearrangements. Additionally, extensive demethylation in centromeric sequences is common in human tumors and may play a role in aneuploidy. Supporting this postulate,

it has been shown that murine embryonic stem cell nullizygous for the *DNMT1*gene exhibit significantly elevated rates of genetic deletions [39]and that patients with germline mutations in the order *DNMT3b* have numerous chromosome aberrations [40]. Hypomethylation of the malignant cell DNA can also reactivate intragenomic parasitic DNA: loss of methylaton has been observed in LINE-1 (long interspersed nuclear elements, *LINES*) and Alu repeats in cancer cells [41].

1.8.1 Hypomethylation of highly repeated, interspersed DNAsequences

Hypomethylation has been observed very often in DNA repeats in diverse cancers (Figure 4). The phenomenon of repeat induced gene silencing, which has been seen in mammals as well as plants and fungi [42], is probably related to the finding that mammalian DNA repeats tend to be highly methylated in postnatal somatic tissues. The repeats that display tumor-associated hypomethylation include endogenous retrotransposons. Retrotransposons or retroviral-derived elements can have their transcription upregulated in vivo by DNA demethylation. This was concluded from studies of Dnmt1 knockout mouse embryos, interspecies mammalian hybrids, and mice with an inherited epigenetically controlled phenotype whose expression is regulated by a genetically linked retrotransposon (IAP)[43]. Also, there is evidence for frequent activation of expression of full-length transcripts from retrotransposons in certain types of murine cancer [44].

2. Retrotransposon : long interspersed nuclear elements, *LINES*

2.1 Overview of Retroelements

Although once thought of as "junk" DNA, the importance of interspersed elements in the genome has become increasingly appreciated in recent years. It has been estimated that at least one third of the mammalian genome consists of these elements in various forms [45]. In a broad sense they are collectively referred to as transposable elements, which encompass both transposons and retrotransposons . Transposons have inverted terminal repeats, encode a transposase activity, and move

from one site to another through a "cut and paste" mechanism [46]. Retrotransposons, which move by a "copy and paste" mechanism, proceed through an RNA intermediate largely dependent on their encoded reverse transcriptase activity. However, they may utilize the host's reverse transcriptase [47]. In this manner a copy of the original can be integrated into a new genomic location. Therefore, stability of the genome depends upon keeping these movable and amplifiable elements transcriptionally repressed. DNA methylation plays a key role in the regulation of gene expression overall, including keeping transposable elements transcriptionally silent [48]. Altering the methylation status of repetitive elements may foster a surge of events leading to toxicity, and a consideration of this is the focal point of this review.

2.2 Retrotransposable Elements: LINE-1

Retrotransposable elements are categorized as either autonomous or nonautonomous elements, where autonomous refers to the property of self-sufficiency for mobility. There are two classes of autonomous elements: long terminal repeat (LTR) and non-LTR retrotransposons. Similar in structure to retroviruses, although lacking a functional env gene, LTR retrotransposons encode proteins necessary for retrotransposition. Likewise, non-LTR retrotransposons also encode a reverse transcriptase and endonuclease that play a role in their ability to mobilize themselves and other non-autonomous elements [47]. A basic difference between the LTR and non-LTR retrotransposons is their method of recombination. LTR retrotransposons move by first being transcribed into RNA, followed by reverse transcription leading to a DNA copy that recombines with genomic DNA. Non-LTR retrotransposons move through a somewhat different RNA-mediated event, discussed below [49]. Up to several kb in length, the non-LTR retrotransposons are commonly referred to as long interspersed nuclear elements (LINEs). LINE-1 repeats constitute about 15% of the human genome, but of the about 4×10^5 copies of LINE-1 elements in the human genome, only about 30-60 are estimated to be competent for transposition Structurally, they contain an internal promoter for RNA polymerase II, a 5' untranslated region (UTR), two open reading frames (ORFs), and a 3' terminal polyadenylation site [50]. The ORF1 protein is an RNA

binding protein [51], while ORF2 encodes both a reverse transcriptase and a DNA endonuclease (Fig 2-6.)[52].
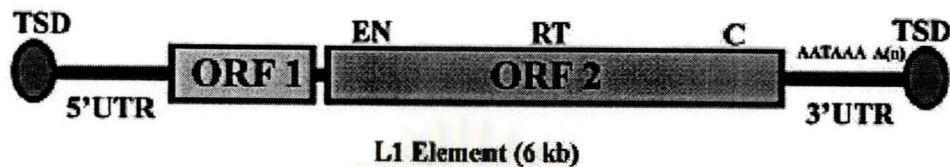

Figure 2-6. Non-LTR elements contain an internal promoter for RNA polymerase II, a 5' untranslated region (UTR) and a 3' deoxyadenosine (A)-rich tract.

2.3 Genomic Consequences of Long Interspersed Nuclear Element-1 (LINE-1)

At the forefront of genomic consequences due to retrotransposon expression and movement is insertional mutagenesis. Insertion of these elements, whether random or targeted, represents a mutation, and therefore, retrotransposition poses a clear risk to the stability of the genome. Not only is movement of these elements critical but also their capability to transduce surrounding DNA sequences. At times this may promote genomic diversification(exon shuffling) [53], but more apparent is the possible contribution to mutagenicity. On a larger scale, fully LINE-1 and their transduced sequences can result in chromosomal rearrangements [54]. Medically, muscular dystrophy, characterized by a progressive loss of muscle strength in humans, has been associated with a LINE-1 insertion within exon 48 of the dystrophin gene [55]. These findings supported recent LINE-1 retrotransposition activity and directly demonstrated the consequential toxicity associated with the aberrant regulation of these elements. Additionally, altered regulation of gene expression by insertion of LINE-1 elements as a direct mutation has been documented numerous times. cited twenty-one examples of sequence element inclusion from Drosophila, sea urchin, human, and mouse genomes that serve a function in terms of transcriptional competency. Counterpart to insertions are deletions and duplications, which can arise from unequal crossing-over and mispairing of homologous

LINE-1 sequences . As much as a 3% frequency of DNA deletions due to LINE-1 retrotransposition has been proposed [56]. Gilbert et al. (2002) observed a large deletion of the genomic DNA following the retrotranspositional event. A common mechanism preceding this deletion, among other alterations, was shown to involve cleavage of the genomic top strand. Variations of this model also suggest that chimaeric LINE-1s, large deletions, and long duplications are also possible [57]. Clinically, inactivating mutations arising from LINE-1-mediated recombination can lead to the accumulation of mutations in specific target genes during cancer and development [58]. This highlights the fact that LINE-1 elements are capable of reshaping the genome through direct mutation.

2.4 Relationship of DNA methylation status in LINE-1 to carcinogenesis

LINE-1 sequences in the human has been characterized structurally as having a promoter region which controls the expression of the two open reading frames, ORF1 and ORF2 . Expression of these regions, encoding an RNA binding protein, a reverse transcriptase, and an endonuclease, is required for integration of a new copy of the original element into the genome. Given the distribution of these elements, both their movement and expression can lead to unstable conditions within the genome. Therefore, an important aspect of DNA methylation is its connection to the host-defense system, which acts to offset the threats from these largely parasitic sequences by maintaining them in a methylated, transcriptionally silent state [24]. Genome instability is a common feature of tumorigenesis [59]. There have been occasional reports of cancer-associated retrotransposition-like insertions involving LINE-1 sequences [60], and they may mobilize cellular RNAs at low frequencies [61]. Their activation can also lead to transcriptional interference involving neighboring genes [62]. However, retrotransposition of endogenous elements is implicated in disease much less frequently for humans than for mice [63]. Extensive hypomethylation results in genome instability reflected by an increase in mutation frequency [64]. Hypomethylation-induced transcriptional activation of LINEs contributes to this instability [65] . Furthermore, hypomethylation of LINE-1 sequences has been observed in various cancers. LINE-1 hypomethylation was observed in chronic lymphocytic leukemia vs normal mononuclear blood cells [66], urinary

bladder carcinomas compared to normal bladder [67], hepatocellular carcinomas vs non-tumorous `normal' or cirrhotic tissue [65], and prostate carcinomas vs normal prostate and other normal tissues [68]. In a chemically induced mouse hepatoma, LINE-1 hypomethylation was also seen [69]. That study involved methylation-sensitive representational difference analysis (MS-RDA) to survey HpaII-sensitive fragments in the tumor vs normal liver, with conformation of the results by Southern blotting [69]. LINE-1 hypomethylation was the only alteration seen repeatedly in the MS-RDA banding patterns from different tumors. Hypomethylation, including of single-copy DNA sequences, was observed more frequently than hypermethylation. In these studies of murine and human tumors, it was not demonstrated that hypomethylation of LINE-1 sequences increased the use of these repeats as transcription units in cancer, but it has been hypothesized that this hypomethylation might sometimes lead to the retrotransposition of the very small percentage of retrotransposition-competent copies of LINE-1 [68]. This led to the speculation that demethylation of LINE-1 sequences may promote genomic instability and facilitate tumor progression .

3. Background of the experiment approach

This study has designed a new method to be able to study LINE-1 methylation from paraffin embedded tissues, normal white blood cell and serum (view in 3.2). Thus all tumor tissue types can be selected and studied. This PCR uses principle of COBRA (combined bisulfite restriction analysis) method [70]. Previously, reports of LINE-1 genomic DNA methylation status were performed by either Southern blotting or distinguish PCR amplicons by methylation sensitive enzyme (MS-PCR)

Disadvantages of Southern blot analysis and MS-PCR

- Southern blot analysis required a large amount of DNA.

- Southern blot analysis required a high quality of DNA.

- Mix type of tissue from fresh specimen.

- Incompatible with DNA isolated from paraffin sections.

- Methylation-sensitive restriction enzyme digestion followed by PCR is prone to false-positive results since even low levels of spurious incomplete digestion can result in a

PCR product. This problem is exacerbated in samples derived from paraffin sections.

Advantages COBRA method

- compatibility with paraffin sections.
- quantitative accuracy.
- applicability to large numbers of samples.
- COBRA circumvents incomplete digestion by restriction digestion of a purified PCR product, rather than of the original genomic DNA.

3.1 COBRA LINE-1

Methylation-dependent sequence differences are introduced into the genomic DNA by the standard sodium bisulfite treatment and then PCR amplified . This combination of bisulfite treatment and PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine. This sequence conversion can lead to the methylation-dependent creation of new restriction enzyme sites or it can lead to the methylation-dependent retention of pre-existing sites such as BstUI (CGCG). The general outline of the method is depicted in Fig 2-7.The COBRA LINE-1 PCR technique is designed to evaluate CpG nucleotide at 270 and 285 of LINE 1.2, an active LINE-1 locus. The sequence at position 267-270 will be AAT (T/C) and 284-287 will be T (T/C) GA. Thus TasI will digest hypomethylation sequence at 270 and TagI cut de novo methylation at 285 in figure 2-8. The summation of proportion of TagI and TasI digested fragments is always close to 100%. From preliminary study interestingly, this COBRA LINE-1 technique has no failure rate of PCR from paraffin embedded tissue in which the little amount of DNA usually degrades. The underlining reasons of this improvement should be due to the shorter of the amplicon size and the significantly larger in number of LINE-1approximately 3-4,000 full-length copies per cell, as the DNA template.
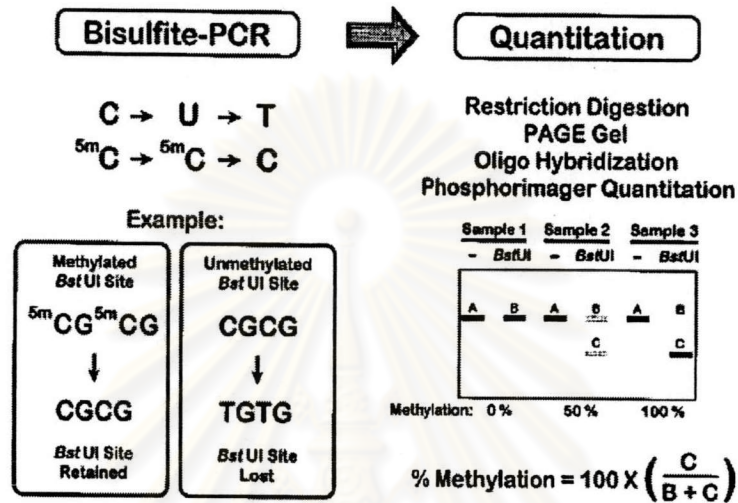
Figure 2-7. Outline of the COBRA procedure. COBRA consists of a standard sodium bisulfite PCR treatment followed by restriction digestion and quantitation
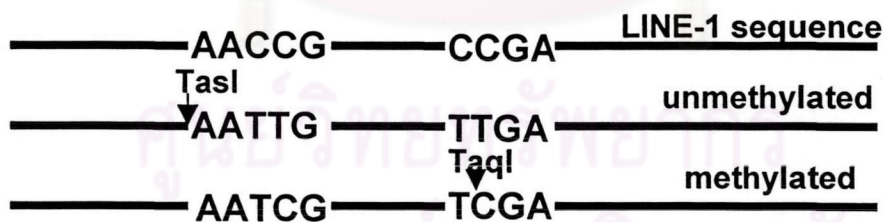

Figure 2-8. Combined bisulfite restriction analysis (COBRA LINE-1). The technique was designed from DNA sequence in 5ꞌUTR of LINE-1.2 from NCBI accession number M80343.

3.2 Plasma nucleic acids as new tools for molecular diagnostics

Free circulating nucleic acids found in blood plasma and sera have been of special interest to investigate over recent years in the development of noninvasive methods for disease diagnosis and monitoring. In 1989, following the observation of decreased strand stability in the plasma DNA of cancer patient, Stroun and coworkers suggested that the circulating DNA from cancer patients might carry certain characteristic of tumor DNA[71]. Cell-free DNA in the plasma of cancer patients shows characteristics identical to those present in the tumor, including oncogene mutations, microsatellite alteration, epigenetic changes, mitochondrial DNA mutations and viral genomic sequences. The discovery of plasma tumor DNA has important implications for early cancer detection, monitoring and prognostication. Aberrant methylation of the p16 gene is common in both lung and liver cancer tissues, and two recent reports describe its detection in the sera of several of the same patients[72]. Similar results have also been reported in prostate cancer. In this study detects methylation status of LINE-1 in stomach cancer by using sera.