CHAPTER II

REVIEW OF RELATED LITERATURE

Endogenous DNA Double-strand Breaks (EDSBs)

DNA damage can occur by intrinsic insults including base pair mismatches during DNA replication, collapse of replication forks, and attack by reactive oxygen species produced during normal cellular metabolism. Moreover, DNA damage can also be induced by extrinsic insults such as exposure to ultraviolet light, ionizing radiation, or environmental mutagens. One particularly harmful form of DNA damage is DSBs. In normal cells, DSBs occur spontaneously at background levels, which are termed EDSBs. These breaks are induced by a number of mechanisms including intermediates of different DNA recombination processes such as decatenation of intertwined DNA molecules by topoisomerase II and LINE-1 retrotransposition, programmed cleavage by specific endonuclease during immunoglobulin gene rearrangement, and products converted from single-strand lesions (SSLs) (1-5). Previously, Knudonson *et al.* estimated that in normal human cells about 1% SSLs were converted to about 50 EDSBs per cell in each cell cycle. This number is calculated as if EDSBs are produced by 1.5-2.0 Gy of sparsely ionizing radiation, which is a physiologic dose of normal cellular environment (1). The production of such breaks is a dynamic process that occurs in two steps. The first step is the abundant production of SSLs of different types during normal cell cycle. These lesions cause collapse of replication forks from single-strand breaks (SSBs). The second step is the conversion of some SSLs into EDSBs during the S phase of cell cycle (1).

EDSBs are particularly dangerous lesions if they occur during the replication of the genome and during the segregation of duplicated chromosomes into daughter cells. Proper genome duplication is hampered by EDSBs. If broken chromosomes are carried through mitosis, the acentric chromosome fragments will not partition evenly between daughter cells. Therefore, eukaryotes have developed several checkpoints to prevent

cells from starting DNA replication (the G1/S checkpoint), from progressing with replication (the intra S checkpoint), or from going into mitosis (the G2/M checkpoint), if they contain damaged DNA (6-10). Furthermore, all eukaryotes have evolved several mechanisms to deal with DSBs, which indicates the importance and difficulty of repairing this type of DNA injury. The fidelity of EDSB repair is importance to the fate of the cell. The failure to repair EDSBs or their inaccurate repair can lead to chromosomal instability (CIN) that contributes to carcinogenesis. CIN phenotype is characterized by the gross rearrangement of chromosomes. Common chromosomal aberrations include the loss or gain of whole chromosomes or chromosomal fragments, and the amplification of chromosomal segments (9). Numerous studies employing mouse models and cellular models have demonstrated the correlation between the formation of DSBs and the generation of chromosomal aberrations. The chicken B-cell line DT40 cells without functional Ku70 and/or Rad54 were generated as a cellular model for NHEJ and HR, respectively (10). Disruption of the *RAD54* gene, which encoded a component of the HR pathway, caused radiosensitivity, whereas inactivation of *Ku70*, which encoded a component of the NHEJ pathway, was not detectable effect on survival after γ-irradiation. Disruption of *RAD54* also increased the rates of chromosomal aberration, mainly in the form of chromatid type breaks. A low dose of γ-irradiation markedly increased the number of breaks in this mutant. The *Ku70* mutation did not significantly affect chromosomal instability in DT40 cells, which suggested that HR is the main pathway to repair DSBs in these cells. Other evidence for the involvement of DSBs in chromosomal aberrations came from studies in mouse model. The first know DSB-repair-defective mouse mutant was the SCID (severe combined immunodeficiency) mouse, which carried a spontaneous mutation that prevents the production of mature B and T cells, owing to a defect in joining the DSB intermediate in V(D)J recombination. These mice not only had a defect in the development of their immune system, but also were hypersensitive to ionizing radiation (12).

EDSB Repair

DSBs can easily lead to gross chromosomal aberrations if not rejoined quickly. Even if repaired quickly, the repair process may be error-prone, and may eventually be detrimental to the organism. Therefore, mammalian cells have mechanisms for rapidly transmitting the damage signal to the cell cycle arrest or apoptotic machineries and DNA repair mechanisms (13). Cell cycle arrest is necessary in order to give the cell enough time for repair. In some cases, it may be more prudent for the cell to undergo apoptosis when faced with excessive or unrepairable DNA damage. Both these processes are as effective barriers to carcinogenesis (8, 14). Another important barrier to genomic instability and carcinogenesis is DSB repair. DNA damage response pathways involve conserved proteins that act together to translate the signal of damaged DNA into responses of cell cycle arrest and DNA repair. These groups comprise sensor proteins that recognize damaged DNA directly or indirectly, transducer proteins typically PIKKs such as ATM and DNA-PKcs that relay and amplify the damage signal, and effector proteins that control cell cycle progression, chromatin restructuring and DNA repair. Recruitment of DNA damage-associated PIKKs to DNA lesions is thought to be a principal step in their activation and in their function in checkpoint signalling and DNA repair. Although these PIKKs have an affinity for DNA, recruitment to DNA lesions is facilitated by specific partner proteins. For example, DNA-PKcs recruitment to DSBs is mediated by the Ku70–Ku80 heterodimer and ATM recruitment to DSBs is mediated by Nbs1, which are subunit of Mre11–Rad50–Nbs1 (MRN) protein complex (15, 16). Mammalian cells employ at least three mechanisms for DSB repair, homologous recombination (HR), DNA-PK dependent non-homologous end joining (NHEJ) and ATM dependent NHEJ. HR operates only in the S/G2 phases of the cell cycle when a sister chromatid is available. NHEJ, which simply pieces together the broken DNA ends, can function in all phases of the cell cycle and is the predominant repair pathway in mammalian cells (7, 8, 17). DSB pathways depend on different DSB sensor proteins to recognize the presence of DNA DSB, then trigger the activity of different protein kinases, further activating a series of different downstream effectors, seems to favor this "competition" possibility. However, the possibility that the two

pathways are act in concert to repair DSBs cannot be totally excluded. In animal model studies, mice with defects in both HR and NHEJ display synthetic phenotypes of viability and tumorigenic potential and a synergistic effect of these genes on genomic stability, suggesting that these two repair pathways cooperate in DSB repair (17).

I. Homologous recombination

In contrast to NHEJ, HR can repair DSBs by using undamaged sister chromatid as a template. Therefore, HR generally results in the accurate repair of the DSB. HR appears to involve a large number of proteins, including RAD51, RAD52, RAD54, BRCA1, BRCA2, the RAD51 paralogs (RAD51B, C, D), XRCC2 and XRCC3, and the MRN complex. A schematic outline of HR with its variations is shown in Figure 2. The first event is DNA recognition and resection to yield single-strand overhangs. This step could be controlled by ATM (ataxia telangiectasia mutated protein) since NBS1 is part of the MRN complex and is a direct substrate for ATM phosphorylation. The NBS1 subunit appears to be important for nuclear transport and for transmitting signals from DNA damage sensors to MRN. The RAD50 subunit of MRN has ATPase activity that is believed to facilitate DNA unwinding, whereas MRE-11 subunit has 3'-5' exonuclease activity. Next event, the RAD51 forms nucleoprotein complexes on single-strand DNA (ss DNA) tails coated by RPA (replication protein A) to initiate strand exchange. The RAD51 paralogs act as accessory proteins and are believed to facilitate the action of RAD51. In this step, the proteins mutated in inherited forms of breast cancer, BRCA1 and BRCA2, are also involved at an early point of DSB repair. Recently, it was demonstrated that BRCA2 interacts with RAD51 and RPA at the DSB and helps load RAD51 onto the DNA or to organize RAD51 filaments. Immunoprecipitation experiments suggested that BRCA1 and BRCA2 associated with RAD51 *in vivo*. BRCA1 and BRCA2 are physically associated with DSB repair foci, together with RAD51 and PCNA. Altogether, BRCA1 and BRCA2 are believed to be important at early points of HR. Then, the RAD52 helps RAD51 form DNA exchange intermediates. *In vitro* studies demonstrated that RAD52 forms ring structures consisting of seven RAD52 monomers and a hole that interacts with both ssDNA and dsDNA. The RAD54 protein has ATPase

activity and is related to DNA helicases. Thus, it is possible that RAD54 helps RAD51 and RAD52 unwind the DNA at the DSB to facilitate access of other repair factors. Following sister-chromatid pairing, the RAD51 nucleoprotein filament searches for the homologous duplex DNA. After the search has been successfully completed, DNA strand exchange generates a joint molecule between the homologous damaged and undamaged duplex DNAs. Finally, the gaps are filled by DNA polymerase and the breaks are sealed by ligase. However, the DNA polymerase and ligase necessary for this step have not been identified (5, 18-20).

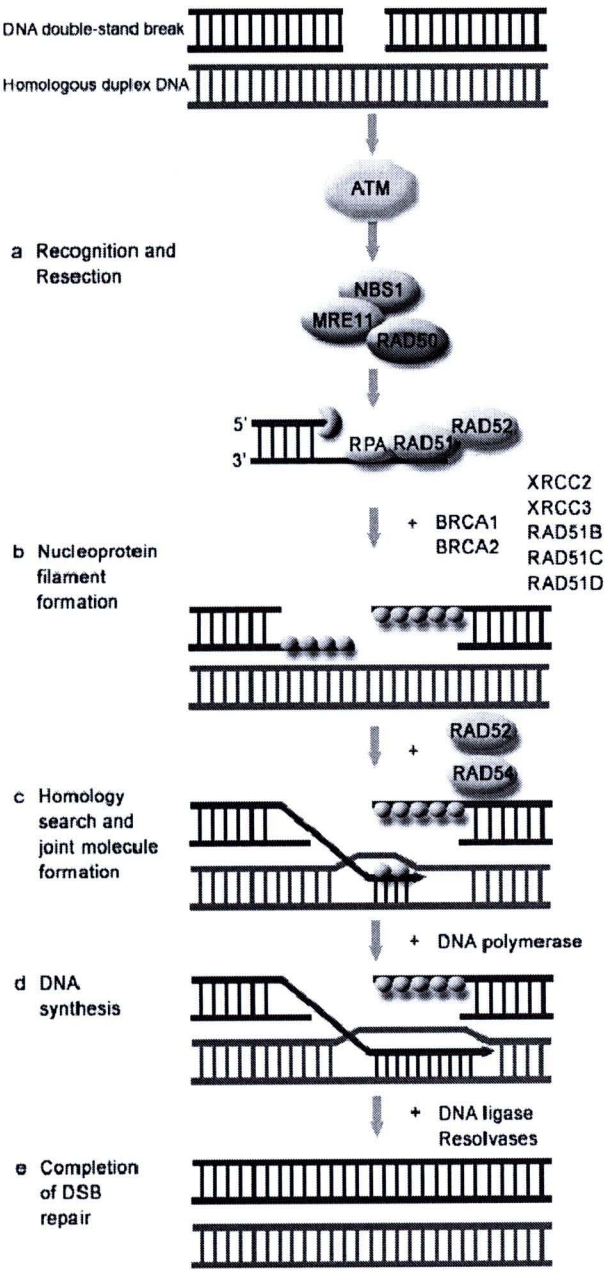

Figure 1 DSB repair via homologous recombination. (a) DSB formation triggers ATM sensor and NBS1 in MRN complex is phosphorylated by ATM. Processing of the ends occurs by the MRX complex and results in the formation of 3′ single stranded (ss) DNA overhangs. (b) The ssDNA-binding protein replication protein A (RPA) binds to the ssDNA overhangs, and RAD51 and RAD52 are recruited to the DSB. Both RPA and RAD2 help to load Rad51 onto ssDNA to form ssDNA–RAD51 nucleoprotein filaments. Other proteins implicated in the orchestration of a proper RAD51 response include BRCA1, BRCA2 and the Rad51 paralogues, XRCC2, XRCC3, RAD51B, RAD51C and RAD51D. (c) This nucleoprotein filament searches for the homologous duplex DNA in the undamaged sister chromatid. A successful search results in strand invasion, strand exchange and joint molecule formation. A reaction is stimulated by the RAD52 and RAD54 proteins. (d) DNA synthesis by DNA polymerases generates the genetic information that is required to seal the break. (e) Ligation and the resolution of the two double helices joined by strand exchange complete this error-free repair event.

II. DNA-PK dependent Non-homologous end joining

NHEJ uses little or no homology to couple DNA ends. This pathway is not only used to repair DSBs generated by exogenous DNA-damaging agents, such as IR, but also required to process the DSB intermediates that are generated during V(D)J recombination. NHEJ plays the predominant role under most conditions in mammalian cells. This is mainly because, in mammalian cells with a large and complex genome, searching for homologous sequences for precise DSB repair by HR does not seem to be efficient. Furthermore, because a significant proportion of the genome in mammalian cells consists of extragenic sequences that do not code for amino acids, the trade-off between the use of NHEJ to repair DSBs, with the relatively minor cost of imprecise repair, and efficient DSB repair to avoid lethality caused by unrepaired damage, becomes acceptable (7). The possible three steps have been suggested for the repair of DSBs *via* NHEJ: (i) end binding, (ii) end processing, and (iii) ligation (Fig. 3). In the initial step, the Ku heterodimer, which consists of Ku70 (70 kDa) and Ku86 (86 kDa), binds the DNA ends. The end-binding activity of Ku indicates that it has an early role in the NHEJ process. Ku-bound DNA end attracts the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), a 470-kDa polypeptide with a protein kinase domain near its carboxyl terminus. DNA-PKcs can subsequently phosphorylate several cellular target proteins as well as itself. At present, it is unclear which phosphorylation targets of DNA-PKcs are relevant *in vivo*. The second step, end processing is particularly important for repairing of more complex non-complementary ends. They require their terminal processing *via* nucleolysis and polymerization before the final ligation step. Several enzymes have been implicated to function in this step of NHEJ including RAD50–MRE11–NBS1 (MRN) complex, Artemis and WRN (Werner syndrome helicase). The MRE11 subunit of MRN complex is 3'-5' exonuclease. Thus, the role of the MRN complex involves in the unwinding and/or nucleolytic processing of the ends. Artemis, a 5'-3' exonuclease, has also been identified to interact with DNA-PKcs and is also phosphorylated by this kinase. Upon complex formation and DNA-PKcs-mediated phosphorylation, Artemis acquires endonucleolytic activity capable of opening the hairpin loop during V(D)J recombination as well as removing both 5' and 3' protruded

ends for NHEJ repair. WRN is 3'-5' exonuclease with a preference for recessed 3' ends, and stimulated by Ku but inhibited by DNA-PKcs. WRN can displace DNA-PKcs from DNA-PK holoenzyme bound to a DNA end. In the final step, the coordinated assembly of Ku and DNA-PKcs on DNA ends is followed by recruitment of the DNA Ligase IV–XRCC4 complex that is responsible for the rejoining step. This complex lies at the center of the NHEJ pathway. It is present in all eukaryotes including yeast, which lacks DNA-PKcs but not Ku. Ligase IV is stabilized by forming a tight complex with XRCC4 and then stimulates its DNA ligation activity (5, 17, 20).

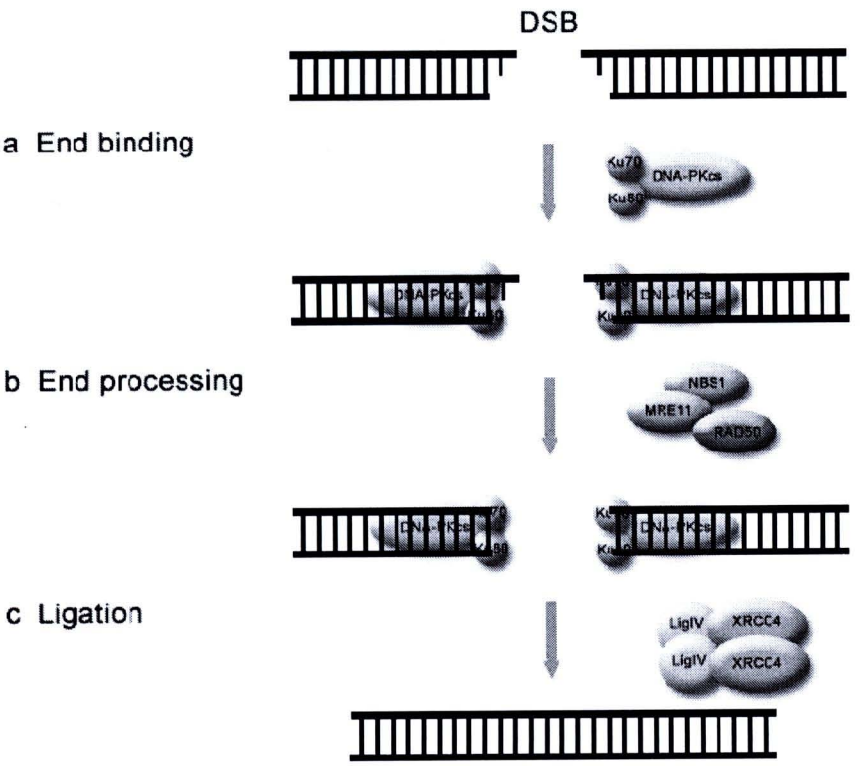

Figure 2 DSB repair via non homologous end-joining. (a) After induction of a DSB, the Ku proteins bind DNA end of DSB and recruit DNA-PKcs to this end leading to activation of DNA-PKcs. (b) The RAD50–MRE11–NBS1 (MRN) complex might be required to process broken end making it available for ligation. (c) This is followed by end-to-end ligation by the Ligase IV–XRCC4 complex. (MRE11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1; XRCC4, X-ray-repair-crosscomplementing defective repair in Chinese hamster mutant 4)

III. ATM dependent Non-homologous end joining

ATM dependent is a sub pathway of NHEJ exists that can repair DSB with higher fidelity and that there are additional factors that can improve the fidelity of NHEJ. BRCA1, one of the key members of HR, has been suggested to play this accessory role in NHEJ, both in vitro and in vivo. In particular, ATM-activated, Chk2-phosphorylated BRCA1 was shown to be important in promoting precise end-joining activity. MRN complex is recently shown to play a crucial role in DNA DSB repair by recruiting ATM to DSB sites, leading to activation of this DNA repair network (15, 21). In addition, the MRN complex is involved in the initial processing of DSBs due to its nuclease activity and DNA binding capability (22). These activities reside in the Mre11 protein, which is required to trim the ends of DSBs, thus facilitating DSB repair, especially HR, by producing the overhanging DSB ends required for efficient HR (23). However, as BRCA1 has been shown to inhibit the nuclease activity of Mre11 (24), and Chk2 phosphorylates BRCA1, leading to inhibition of MRN foci formation, which is thought to play a role in both HR and NHEJ complex (25), it is possible that this inhibition is required for precise end-joining, because exonuclease digestion would result in the loss of nucleotides, leading to imprecise NHEJ if these digested ends were rejoined directly showing that error-prone rejoining is characterized by an increase in the formation of >2 kb deletions in DSB repair by NHEJ in BRCA1-deficient cells and that this error-prone repair phenotype is linked to Chk2 phosphorylation, lends critical support to this model (7).

Genomic Instability and Carcinogenesis

Genomic instability is believed to play a critical role in the malignant process. It has been proposed that genomic instability can be divided into two types including microsatellite instability (MIN), and chromosomal instability (CIN) (26). Cancers develop instability either at the sequence level, termed MIN or at the chromosomal level, termed CIN, but not generally at both levels. Since these instabilities are rarely found to coexist in tumors, it seems that one form of instability is sufficient to drive tumorigenesis (26).

I. Microsatellite Instability and Carcinogenesis

Microsatellites are repetitive DNA sequences dispersed throughout the human genome. The most common microsatellite in humans is a dinucleotide repeat of cytosine and adenine $(CA)_n$. Somatic alteration in microsatellite sequences due the loss or gain of one or more repeat units is termed MIN (9, 27). MIN is a direct consequence of defects in nucleotide mismatch repair (MMR). The MMR machinery removes misincorporated nucleotides from the DNA molecule and has been shown to be involved in genomic instability. The MIN is found in subsets of several cancer types, and germ-line defects in MMR underlie a cancer syndrome (28). The first clue to the role of MMR cancer came with the discovery of a group of sporadic (non-familial) colorectal cancers that exhibited widespread alterations of poly (A) tracts in their genomes. MIN occurs in most cancer in patients with hereditary nonpolyposis colon cancer (HNPCC) (29). Studies of HNPCCs revealed mutation in MMR genes such as *MSH1* and *MLH2*, which encode proteins that repair nucleotide mismatches (30, 31). Moreover, targeted mutations in mouse homologues cause a spectrum of MIN and cancer susceptibility phenotypes. *Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$ mice have high levels of MIN in all somatic tissues tested and highly penetrant colon cancer susceptibility (32). MIN has been observed in bacteria with defects in the MMR genes *mutS* or *mutL*, and showed that *Saccharomyces cerevisiae* with defects in the yeast homologues of either *mutS* or *mutL* exhibited a similar MIN phenotype. Six human *mutS* or *mutL* homologues genes are known that, when recessively inactivated lead to a MIN phenotype in cancer patients (28, 33).

II. Chromosomal Instability and Carcinogenesis

CIN is the dominant phenotype of cancer cells and is recognized as numerical and structural aberrations of the genome. CIN has been related to genetic repair and mitotic control pathways, but the underlying mechanisms appear complicated and remain to be clarified (32). More than 100 genes are expected to cause CIN when mutated in eukaryotic cells, including genes that are involved in telomere metabolism, chromatid cohesion, spindle assembly and dynamics, cell-cycle regulation, DNA repair and checkpoint controls (34). For examples, mutations in the kinetochore binding proteins MAD, BUB, and the securins, key components of the large multi-protein cascade known as the anaphase-promoting complex (APC) in eukaryotes, increased chromosomal instability by different mechanisms (34, 35). *MAD2*$^{+/-}$ cells showed premature entry into anaphase and increased percentage of chromosome loss. Whereas, *hSecurin*$^{-/-}$ cells showed defect of sister chromatid separation at anaphase and increased nondisjunction (35). Targeted mutagenesis against the MAD2 gene in karyotypically stable, continuous cell lines led to CIN. The RecQ family of DNA helicases plays important roles in DNA repair, replication and recombination pathways (36). Defects of the RecQ family helicases encoded by the *BLM*, *WRN* and *RECQ4* genes in humans, give rise to Bloom's (BS), Werner's (WS) and Rothmund–Thomson (RTS) syndromes, respectively. These disorders are associated with cancer predisposition and premature aging. They also exhibited various types of chromosomal instability including elevated frequencies of sister chromatid exchanges (SCEs) (37, 38). Furthermore, the BRCA1 and BRCA2 proteins have been considered as caretakers of CIN because these proteins take part in DNA repair, cell-cycle checkpoint control, protein ubiquitination and chromatin remodeling. For example, the previous study demonstrated that murine lymphocytes carrying homozygous mutations of *Brca2*, frequently developed multiple cytogenetic lesions including chromosomal fragmentation, multi-radial formations, and random translocations (39, 40).

Genomic instability appears early in tumorigenesis and is believed to play a critical role in the malignant process (41). Oncogene activations or increasing number of copies of oncogenes and loss of tumor-suppressor genes are found in cancer cells with CIN. CIN can result in oncogene activations by translocation or gene amplification (9). Translocations can directly activate oncogenes by different mechanisms. First, the elevated expression of some oncogenes can result from proximity to strong transcriptional elements brought close to the target oncogene by translocation. Burkitt's lymphoma (BL) cells contain a t(8;14) reciprocal translocation that juxtaposes *c-myc* and strong transcriptional regulatory elements within the IgH locus on chromosome 14. This translocation results in potent transcriptional activation of *c-myc*, leading to overexpression and cellular transformation (42, 43). Second, Translocation can result in the creation of a fusion gene that encodes a novel oncogenic protein. The Philadelphia (Ph) chromosome, associated with chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL), represents this mode of oncogene activation through the generation of a BCR–ABL fusion protein with non-receptor tyrosine kinase hyperactivity that functions in the RAS-signaling pathway which drives cellular proliferation. The Ph chromosome is the product of a reciprocal translocation between the long arms chromosomes 9 and 22 (44, 45). Besides translocation, Amplification of proto-oncogenes is another common means of oncogene activation in numerous tumor types. Gene amplification is generally defined as an increase in the cellular copy number of a gene or genomic region relative to the rest of the genome. However, the mechanisms leading to amplification within a tumor have remained obscure (9). Gene amplification is an important process in human cancers. It is clearly associated with tumor progression, has prognostic significance and even provided a target for therapy in the case of breast cancers carrying HER2/neu amplification. In contrast, loss of large regions of a chromosome might promote tumorigenesis by the inactivation of tumor suppressor genes. Massive chromosomal deletion is frequently seen in myelodysplastic syndrome (MDS), a precursor of acute myeloid leukemia (AML). This deletion occurs frequently in chromosomes 5, 7 and 20. One candidate tumor suppressor is the ETF1 translation

factor on chromosome 5, while candidate genes on chromosomes 7 and 20 remain to be elucidated (46, 47).

DNA methylation

DNA methylation is a change in DNA by chemical modification, involving the addition of a methyl group to the carbon-5 position of cytosine in a CpG dinucleotide in human which disperse throughout the whole genomes both in noncoding repetitive sequences and genes (Figure 3). DNA methylation does not change in DNA coding sequence because both cytosine and 5-methylcytosine base pair with guanine. DNA methylation is associated with condensation of chromatin, stabilization of chromosomes, transcriptional silencing of X chromosome, genomic imprinting and tissue-specific silencing of gene expression (48, 49). The Enzymes responsible for the process of DNA methylation are DNA methyltransferases (DNMTs) that can be divided in to maintenance and *de novo* DNMTs. DNMT1 is the maintenance methyltransferase responsible for reproducing the parental DNA methylation pattern into daughter cells during DNA replication. The newly synthesized DNA contains hemimethylated sites that provide the signal for DNMT1 to transfer a methyl group to a cytosine residue from its cofactor, S-adenosylmethionine (SAM). If maintenance methylation does not occur either by a decrease in capacity or fidelity of DNMT1 activity and/or decreased levels of SAM, the daughter DNA will lose a pattern of DNA methylation. DNMT3A and DNMT3B are *de novo* methyltransferases responsible for adding methyl groups to CpG dinucleotide of unmethylated DNA (49). Both enzymes in *de novo* methylation are necessary for proper development of mammalian embryos by establishing new methylation patterns, especially DNMT3B for methylation of specific genomic regions such as pericentromeric repetitive sequences and CpG islands on the inactive X chromosome (50, 51).

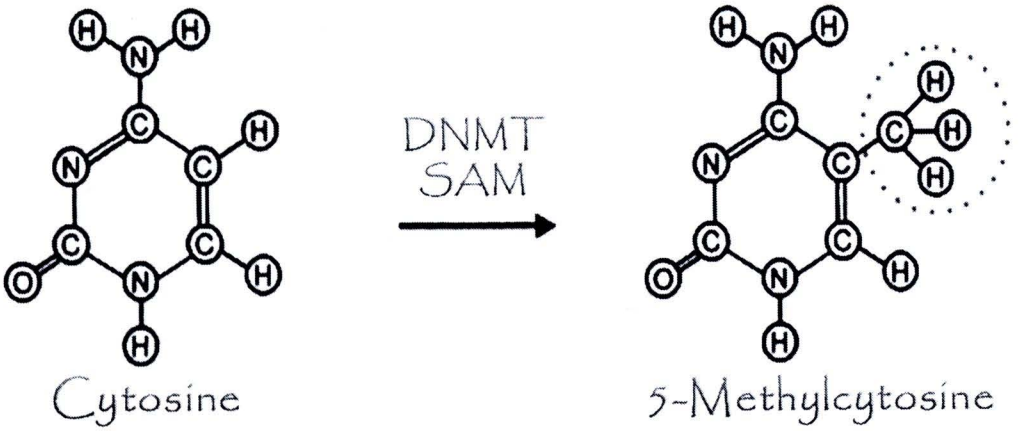

Figure 3 Cytosine methylation. The DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethione to cytosine, producing 5-methylcytosine and S-adenosylhomocysteine.

Global Hypomethylation

Genomic hypomethylation has been recognized as a common epigenetic change during cancer development (52-55). Global losses of methylation in cancer may lead to alterations in the expression of proto-oncogenes critical to carcinogenesis and may facilitate chromosomal instability (54, 56). Genome-wide hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the whole genomes both in noncoding repetitive sequences and genes (57, 58). Previous studies have described the hypomethylation of genomic repetitive sequences, a marker of the global genomic hypomethylation, in several malignancies including carcinoma of urinary bladder, liver, prostate, and colon (59-64). In those reports, Southern blotting and/or distinguishing PCR amplicons by methylation sensitive restriction enzymes were used to qualitatively evaluate the methylation status of LINE-1,

a highly repeated and widely interspersed human retrotransposon, in isolated DNA from whole tissue samples. Additional studies described a quantitative method to analyze LINE-1 methylation by using COBRA (65). Chalitchagorn *et al.* (66) applied COBRA to evaluate the methylation status of LINE-1 repetitive sequences in genomic DNA derived from microdissected samples from several human normal and neoplastic tissues. They found that methylation of LINE-1 in leukocytes was independent of age and gender. In addition, normal tissues from different organs showed tissue-specific levels of methylated LINE-1. Globally, most carcinomas including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, revealed a greater percentage of hypomethylation than their normal tissue counterparts. Furthermore, DNA derived from sera of patients with carcinoma displayed more LINE-1 hypomethylation than those of noncarcinoma individuals. Finally, in a colonic carcinogenesis model, they detected significantly greater hypomethylation in carcinoma than those of dysplastic polyp and histological normal colonic epithelium. Thus, the methylation status is a unique feature of a specific tissue type and the global hypomethylation is a common epigenetic process in cancer, which may progressively evolve during multistage carcinogenesis.

Global Hypomethylation and Carcinogenesis

Global hypomethylation may play crucial roles in carcinogenesis. Since genome-wide losses of DNA methylation has been found in many types of human cancer and associated with the degree of malignancy. Global hypomethylation potentially promotes cancer development through three possible mechanisms: oncogene activation, transposable element reactivation and chromosomal instability (56, 67).

I. Oncogene Activation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells. It might be a mechanism of activation of oncogenes in addition to the mutation activation of oncogenes. The evidences supporting this notion came from the studies of hypomethylation of *CMYC* and *HRAS*

oncogenes in bladder cancer and non-small cell lung cancer, respectively (68, 69). As mentioned above, one possible mechanism of global hypomethylation causing cancer may be the activation of oncogenes, which involve in tumor progression. A good example is the protease urokinase plasminogen activator (uPA), which is required for tumor invasion and metastasis. *uPA* gene is expressed in highly metastatic but not expressed in non-metastatic breast cancer cell lines. In non-metastatic cancer cell line, MCF-7 cells were treated with 5-aza-2-deoxycytidine, a DNA methylation inhibitor. The result showed activation of *uPA* and induction of metastasis in treated MCF-7 cells (70).

II. Reactivation of Transposable Elements

Hypomethylation in cancer cells may also lead to transcriptional activation of mobile genetic elements called retrotransposons. In normal cells, retrotransposons are highly methylated leading to inhibit of both gene expression and mobility of the inserted DNA. The function of methylation is to defense the genome from the deleterious effects of transposable elements. However, decreased methylation and consequent reactivation of expression of retrotransposons has been detected in a large number of human cancers. The most abundant retrotransposons in human genome are Long Interspersed Nuclear Element type 1 (LINE-1 or L1) which intersperse 17% throughout the genome. Full length LINE-1s are 6 kb and have two open reading frames, ORF1 and ORF2. ORF1 encodes p40, an RNA-binding protein. ORF2 encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate (71). Loss of LINE-1 methylation can potentially lead to transcriptional activation, retrotransposition and integration at new site in the genome. Dangers resulting from LINE-1 reactivation in cancer cells comprise insertional mutagenesis and disturbance of transcriptional activity and gene regulation. Active LINE-1 transcripts have been detected in human testicular cancers (72), which intriguingly have the appearance of embryonal carcinoma or yolk sac tumor cells (73). Metastatic cells originating from these cancers also express LINE-1 transcripts. This study suggested that LINE-1 encoded proteins may function as oncoproteins in some tumors. The p40 product of LINE-1 ORF1 has also been found to be abundantly

expressed in invasive malignant breast carcinoma compared with nonmalignant carcinoma or normal tissues (74). This observation proposed that LINE-1 expression was up-regulated during tumor progression. Additionally, LINE-1 insertional mutageneses have been found in sporadic cancers. LINE-1 insertions disrupt the *APC* gene and *CMYC* gene in a sporadic tumor of the colon and breast, respectively (67). In the disrupted *APC* gene, the nucleotide sequences in and around the insertion site exhibited the signature of retrotransposon integration (67). A role of genome hypomethylation in permitting transposition in cancer cells is not resolved, but there is substantial evidence for the unleashing of transcription of large numbers of retrotransposon sequences in a methylation dependent manner.

The association between Global Hypomethylation and Chromosal Instability

Chromosome aberrations are common finding in human cancers. It has been suggested that DNA methylation could be involved in the control of chromosome stability. In support of this notion, there is a study in ICF (Immunodeficiency, Centromeric region instability and Facial anomalies) syndrome, a rare genetic disorder in humans. This syndrome is caused by inherited mutations in the DNA methyltransferase *DNMT3B* (75). In all somatic cells of ICF patients, the pericentromeric heterochromatin of chromosomes 1 and 16 is abnormally hypomethylated. Mitogen stimulation of lymphocytes from ICF patients resulted in a high frequency of abnormalities involving chromosomes 1 and 16, and a lesser degree of chromosome 9 (64). Similarly, when Pro B cells are treated with the DNA methylation inhibitors 5-azadeoxycytidine and 5-azacytidine, the hypomethylated Pro B cells exhibited chromosomal anomalies in the pericentromeric region of chromosome 1 (76). From both studies, it inferred that global hypomethylation could induce CIN. Furthermore, DNA hypomethylation and CIN of the pericentromeric heterochromatin regions on chromosomes 1 and 16 had also been observed in ovarian epithelial tumors, breast adenocarcinomas and Wilms tumors (77). It implied that genomic hypomethylaion had a causal role in carcinogenesis by promoting CIN. Experimental studies from colorectal cancers by Vogelstein's group also supported this idea. They showed that colorectal

tumors without MIN exhibited a striking defect in chromosome segregation, resulting in gains or losses of chromosomes. Furthermore, this group investigated the correlation between DNA methylation and genomic instability in colorectal cancer cell lines. They differentiated the methylation and consequently expression inhibition of exogenous retroviral genes in MMR-proficient (MMR^+) versus MMR-deficient (MMR^-) cell lines. MMR^- tumor cells inadequately expressed the foreign gene but MMR^+ tumor cells efficiently expressed. Therefore, MMR^- cell lines had higher potential of retroviral vector methylation than MMR^+ cell lines. Finally, they proposed that hypomethylation was positively associated with CIN, but not MIN (26). To further explore the link between DNA hypomethylation and CIN, Jaenish and colleagues showed that murine embryonic stem cells carrying defect of *DNMT1* gene ($DNMT1^{-/-}$) exhibited significantly elevated mutation rates at both the endogenous hypoxanthine phosphoribosyltransferase (*Hprt*) gene and an integrated viral thymidine kinase (*tk*) transgene. Gene deletions were the predominant mutations at both loci (78). Moreover, this group also generated mice carrying mutant *DNMT1* allele, which reduced DNMT1 expression. They found that mutant mice developed aggressive T cell lymphomas at 4 to 8 months of age and cancer cells displayed chromosomal aberrations (54). In the final experiment, they studied the effect of DNA hypomethylation on tumor-prone mice carrying mutations in both the Neurofibromatosis 1 (*Nf1*) and *p53* tumor suppressor genes. They found that $Nf1^{+/-}/p53^{+/-}$ (NPcis) mice showed a significant increase in loss of heterozygosity (LOH) rate when a hypomorphic *Dnmt1* allele was introduced (54). All studies of Jaenish's group indicated that mammalian DNA methylation played an important role in maintaining genome stability and a causal role in tumor formation by promoting CIN. *Another potential connection between global hypomethylation and CIN, the evidence* demonstrates indirect mechanisms by which retrotransposons could promote CIN in human cancer. Hypomethylation of the satellite sequences was thought to be the cause of decondensation of pericentromeric chromatin and an increased propensity for chromosomal breaks and rearrangements in this region. In a similar fashion, hypomethylation of transposable elements dispersed in the genome could facilitate illegitimate recombination. In favor of this idea, LINE-1 sequences are enriched at the

ends of 3p14.1 and 9p21 deletions in carcinomas and homozygous deletions arise preferentially in chromosomal regions with high LINE-1 content. It has also been suggested that LINE-1 sequences were involved in the formation of double-minute circular chromosomes in cancer cells.