CHAPTER II
LITERATURE REVIEWS

Cancer is a notorious disease of multiple gene defect functions. The initiation and progression of human cancer are due to multiple processes which involve the accumulation of genetic and epigenetic changes in somatic cells [15]. In order for cells to start dividing uncontrollably, genes which normally regulate cell growth must be inactivated or mutated. Proto-oncogenes are genes which promote cell growth and mitosis, and tumor suppressor genes discourage cell growth, or temporarily halt cell division from occurring in order to carry out DNA repair.

The enormous catalog of cancer cell genotypes or "hallmarks of cancer" is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes (novel capabilities acquired during tumor development) represents the successful breaking of an anticancer defense mechanism hardwired into cells and tissues [16]. Genetic defects and epigenetic alterations are the main mechanisms of cancer development. Genetic defects such as mutation and deletion can lead to the dysfunction of genes. Although epigenetic alteration does not change the DNA sequence, it can affect gene expression by chemical modifications including DNA methylation and histone deacetylation. The hypermethylation of CpG islands in promoter regions of tumor suppressor genes can cause their transcriptional inactivation [11-13].

Cancer is accepted as one of critical public health problems worldwide. It is a huge challenge for scientists and researchers to study the important molecular biology, carcinogenesis and treatment options for several types of cancer, including the fatal disease of biliary system named "cholangiocarcinoma".

1. Cholangiocarcinoma (CCA)

1.1 Definition and pathology

CCA is a malignant tumor of the biliary tree including the intrahepatic and extrahepatic portions, but not include carcinoma of the gall bladder and the ampulla of Vater. Different features of CCA, both macroscopic and microscopic, might be significantly associated with the variety of tumor transformation processes and clinical outcomes. CCA can be divided anatomically into two main types: intrahepatic (70%) and extrahepatic tumors (30%) [17]. In the case of intrahepatic CCA, growth characteristics are grossly classified into 3 representative types including mass-forming (MF), periductal-infiltrating (PI), or intraductal growing (ID) types [18] as shown in Figure 2-1. The MF type is the most common, accounting 70-80% of intrahepatic CCA [19]. The tumor shows an expansile and solid nodule or mass in the hepatic parenchyma. The tumor borders between the cancerous and non-cancerous portions, are relatively clear. The PI type exhibits diffuse infiltration along the portal pedicle and is usually associated with biliary stricture. For the ID type which is the least common, the tumors are confined within the dilated part of an intrahepatic large bile duct, with no, or mild, tumorous extension beyond the bile duct walls. The affected bile ducts usually show marked localized dilatation [20]. These tumor patterns can be evaluated by imaging and are useful to preoperative staging and managing the surgical strategies. However, advanced intrahepatic CCA usually shows mixed types.

For microscopic classification, the most common histopathology of CCA (> 95%) is adenocarcinoma showing a glandular and/or papillary structure with a variable fibrous stroma. In addition, several special types or histologic variants are also classified as detailed in Table 2-1 [21]. In the case of liver fluke infection, there is no dominant histological type of CCA when compared to affected persons from non-endemic areas. Most studies have demonstrated a relationship between histological grade and post-operative outcome, although stage is more important for the outcome in CCA patients [22]. For CCA staging, it is based on the tumor-node-metastasis (TNM) system which is the same procedure as followed in the International Hepato-Pancreato-Biliary Association [23].

In CCA, tumor cells can spread via various routes. The patterns of spreading include;

- *Direct invasion*: tumor cells invade directly into the adjacent liver parenchyma through the sinusoid.

- *Infiltration along the billiary tree*: carcinoma cells spread along the bile duct wall via lymphatic permeation with variable desmoplastic reaction. For the intrahepatic large bile duct, tumor cells also infiltrate into the paribiliary glands.

- *Vascular and lymphatic permeation*: tumor cell often infiltrate into the portal tracts and invade the portal vessels (lymphatics, portal venules). Lymphatic permeation is common in CCA while vascular invasion is lesser than in Hepatocellularcarcinoma (HCC).

- *Perineural or intraneural invasion*: nerve involvement has been reported in both intrahepatic and extrahepatic CCA, particularly tumors at the large portal tracts.

Intrahepatic metastasis usually develops in all CCA cases at relatively advanced stages. The incidence of metastasis in lymph nodes is higher than in HCC. Blood-borne spread occurs later to the lungs in particular but also the bones, adrenals, kidneys, spleen and brain [24].

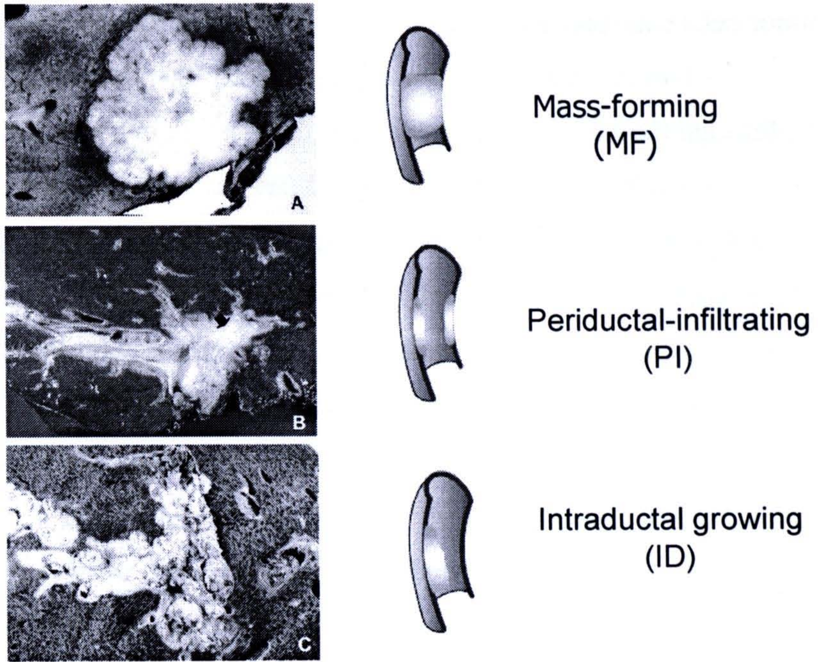


Figure 2-1 Macroscopic types of intrahepatic CCA. (A) MF type; (B) PI type; (C) ID type [modified from 6, 20].

Table 2-1 Histopathological classification of CCA [21].

Common types	**Special types and histologic variants**
Adenocarcinoma	Cholangiocellular carcinoma
Papillary (adeno) carcinoma	Mucinous carcinoma
	Signet ring cell carcinoma
	Adenosquamous and squamous cell carcinoma
	Mucoepidermoid carcinoma
	Sarcomatous CCA
	Clear cell variant CCA
	Lymphoepithelioma-like carcinoma

1.2 Epidemiology

Worldwide, CCA accounts for approximately 15% of liver cancers, but its incidence and relative frequency among all liver cancers vary in different geographic areas. In the endemic areas of HCC such as China and southern Africa, CCA accounts only a small fraction of liver cancers. In contrast to the countries where HCC is rare, the numbers of CCA trends to be exceeded [25]. Among Caucasians, the relative frequency of CCA in histologically confirmed primary liver cancers is 19-25% [26]. However, Data have shown that the incidence and mortality rate of CCA, especially intrahepatic CCA, are increasing in several different regions such as the US, Oceania and Western Europe [2]. The highest incidence of CCA has been found in Southeast Asia where the *Opisthorchis viverrini* (OV) infection is endemic. The experimental and epidemiological evidence implicate opisthorchiasis in the etiology of CCA in this endemic area [9-10]. The prevalence of liver fluke related CCA in the northeast of Thailand accounts for the highest incidence of this cancer worldwide with truncated age-standardized incidence of ages >35 years up to 317.6 per 100,000 population [4]. CCA remains an important public health problem in Thailand because the incidence and fatality rates are still very high. The disease is notoriously difficult to diagnose and is usually fatal because of the late clinical stage and the lack of effective

non-surgical therapy. Surgical resection can be potentially curative, but few patients are available for surgery [5-6].

1.3 Etiology

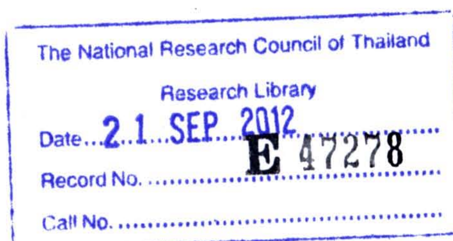
Several etiological or risk factors have been accounted for the development of CCA which seem to be associated with chronic inflammation, increased epithelial proliferation and probable cause of accumulation of DNA damage. These relevant risk factors include primary sclerosing cholangitis (PSC), liver fluke infection (*O. viverrini and Clonorchis cininesis*), hepatolithiasis (intrahepatic biliary stones), congenital biliary tract diseases (e.g. Caroli's syndrome, anomalous biliary-pancreatic malformation, and choledochal cysts), chemical carcinogen exposures, and hepatitis C virus infection. Other probable risk factors have been also described such as liver cirrhosis, hepatitis B virus infection, HIV infection (adjusted odds ratio 5.9), and diabetes (adjusted odds ratio 2.0), but pathogenesis is unclear [6, 27].

1.4 Clinical manifestations

The presentation of CCA is primarily governed by anatomic location. The most common presenting clinical features of perihilar or extrahepatic tumors are biliary obstruction, jaundice, pale stool, dark urine, and pruritus. Intrahepatic CCA presents as mass lesions while the obstructive symptoms are rare. Right upper quadrant pain, fever, night sweats, and rigors may occur due to cholangitis. CCA usually presents in the advanced stage of the disease. This is particularly true with more proximal intrahepatic and perihilar tumors obstructing one duct, which often occur with systemic manifestations of malignancy such as malaise, fatigue and weight loss. Other symptoms may coexist, related to hepatitis C infection, cirrhosis or systemic metastases. In some patients, the tumors are detected incidentally as a result of abnormal liver function tests or ultrasound scans performed for other indications [22, 28].

1.5 Treatments

Treatment options for CCA are determined by the local extent of disease, vascular involvement, the presence or absence of metastases, and available local expertise. Surgical resection can be potentially curative, but few patients are suitable for surgery. The choice to perform surgery with curative intent depends on the site of the tumor within the liver or the biliary tract [3, 6]. In addition to surgical resection, both palliative chemotherapy and radiotherapy have been used as adjuvant therapy for CCA. Chemotherapy has been used in an attempt to control disease, and improve survival and quality of life in patients with unresectable, recurrent and metastastic CCA. There is no single chemotherapy regimen which is universally used, and enrollment in clinical trials is often recommended when possible. Various chemotherapeutic agents, either alone or in combination, have been tested. Chemotherapy agents used to treat CCA include 5-fluorouracil (5-FU) with leucovorin, mitomycin C, or doxorubicin, gemcitabine as a single agent, or gemcitabine plus cisplatin, irinotecan, or oral 5-FU prodrugs uracil–tegafur (UFT) [22, 29-30]. From the clinical studies, the response rate of CCA to chemotherapy is relatively poor in which partial responses have been observed in only about 10-20% of cases [31-33]. Improvement in the survival of CCA patients will not only rely on more advanced surgical techniques but also from the improvement in chemotherapeutic treatments. The data of drug sensitivity and drug resistance in CCA are still limited when compared to other cancers. This may be due to the fact that CCA has previously been considered as a rare cancer in developed countries [34-35]. In addition, other options have been experimentally developed and used for CCA treatment such as liver transplantation, biliary decompression and stenting, and photodynamic therapy [6, 22].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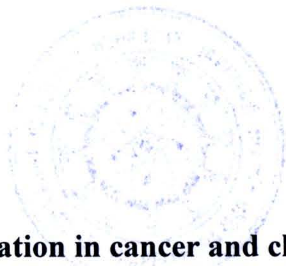

2. Genetic alteration in cancer and cholangiocarcinoma

2.1 Genetic alterations and cancer development

The genes that are defective in many types of cancer are still being discovered and characterized, but they appear to fall into two major categories: proto-oncogenes and tumor suppressor genes. Proto-oncogenes encode proteins that play an important role in normal cells in growth control, differentiation, and expression of other genes. Many of these proteins function as growth factors or play a part in formation of the receptor apparatus that allows a cell to respond to external stimuli such as growth factors. In some cases, proto-oncogenes code for enzymes called protein kinases that alter the function of target proteins by catalyzing the phosphorylation. Other proto-oncogenes act as transcription factors that regulate the amount of other proteins within the cell by altering the transcription of DNA into RNA. The defective versions of these genes, known as oncogenes, can cause a cell to divide and grow in an unregulated manner. Proto-oncogenes may be converted into oncogenes by one of three mechanisms including DNA mutations, chromosome rearrangement, or gene amplification. Tumor suppressor genes function in many important cellular processes including the regulation of transcription, DNA repair and cell-to-cell communication. The inactivation of tumor suppressor genes, usually when both copies are affected by deletion or mutation, provides a competitive growth advantage and an abnormal cellular behavior.

The mechanisms of genetic alterations leading to tumorigenesis include mutation, chromosome rearrangement, gene duplication and amplification, loss of heterozygosity (LOH) (http://www. cancerquest.org) and aneuploidy [36].

2.1.1 Mutation

Mutation is an alteration in DNA sequence. This change may or may not lead to a change in the protein coded by the gene. Different types of mutations are described as follows.

2.1.1.1 Point mutation

Point mutation is the changes that alter a single nucleotide along a DNA strand. The coding region may be changed by point mutation into three ways including;

- *Nonsense mutations*: The new codon causes the protein to prematurely terminate, producing a product that is shortened and often does not function e.g. nonsense mutation at the codon 461 (AAG to TAG) of MLH1 in hereditary nonpolyposis colorectal cancer (HNPCC) [201].

- *Missense mutations*: The new codon causes an incorrect amino acid to be inserted into the protein. The effects on the function of the protein depend on what is inserted in place of the normal amino acid e.g. BRCT missense mutation (Met-1775 → Arg-1775) affects BRCA1 function [202].

- *Silent mutation*: The codon for an amino acid is changed, but is still coded for the same amino acid e.g. silent mutation nucleotide 744 G --> A, Lys172Lys, in exon 6 of BRCA2 in breast and ovarian cancers [203].

2.1.1.2 Frameshift mutation

The deletion or insertion of any number of bases other than a multiple of three bases, such framshift mutation causes the affected codon and all of the codons that follow to be misread. This leads to a very different and often nonfunctional protein product for example frameshift mutation c.31delC (p.L11X) in the MLH1 gene [204].

2.1.2 Chromosome rearrangement

This genetic alteration is the large-scale mutation of DNA, often at the level of the chromosome, including;

2.1.2.1 Translocations

These changes involve the breakage and movement of chromosome fragments. Often, breaks in two nonhomologous chromosomes allow the transfer and rejoining of a fragment of one chromosome to another. This rearrangement would not cause much trouble, if all the genes are still normally expressed. However, it can be a harmful event if the genes are not transcribed and translated appropriately in their new location. The expression level of genes might be increased or decreased from the effect of translocation. Chromosomal translocations are common in leukemia and lymphomas e.g. t(9;22)(q34;q11) in chronic myeloid leukemia (CML) and have been less commonly identified in solid tumors.

2.1.2.2 Inversion

This type of genetic change occurs when a DNA segment is released from the chromosome and then rejoined in the opposite orientation. This rearrangement can lead to abnormal gene expression, either by activating an oncogene or inhibiting a tumor suppressor gene.

2.1.3 Gene duplication and amplification

2.1.3.1 Gene duplication

Through replication errors, a gene or group of genes may be copied more than one time within a chromosome. This is different from gene amplification in that the genes are not replicated outside the chromosome and they are only copied one extra time.

2.1.3.2 Gene amplification

A flaw of normal DNA replication can lead to the production of multiple copies of the genes those are located on the region of the chromosome. Sometimes, so many copies of the amplified sequences can actually form their own small pseudo-chromosome called double-minute chromosomes. These amplified copies of gene can be transcribed and translated, leading to an overproduction of mRNA and protein. If an oncogene is located in the amplified region, resulting overexpression of that oncogene lead to deregulated cell growth. Gene amplification occurs quite often in cancer cells, for example, the amplification of myc oncogene in a wide range of tumors and the amplification of *Erb*B-2 or HER-2/*neu* oncogene in breast and ovarian cancers [205-206].

2.1.4 Loss of heterozygosity (LOH)

LOH, also termed "allelic loss", is loss of one parental copy of a gene in an organism that previously had two different alleles. This genetic damage is caused by two mechanisms. These include the deletion of an allele and the recombination event which leaves the cell with two copies of the chromosomal region but both come from the same parent for example LOH of p53 in CCA [108] .

2.1.5 Aneuploidy

Aneuploidy is a genetic change that involves the loss or gain of entire chromosomes. Due to problems in the cell division process, the replicated chromosomes may not separate into the daughter cells accurately. This can result in cells that have too many chromosomes or too few chromosomes. This genetic defect is also described in cancer cells. Normally, humans have 46 chromosomes in their cells, but cancer cells often have many more. However, there is currently an ongoing debate as to whether or not all cancers are aneuploid.

2.2 Genetic alterations and cholangiocarcinoma

The development of CCA, as with most tumors, is probably a complex multistage carcinogenesis dependent on an interaction between environment factors and host genetics (Figure 2-2) [3]. Most of the putative environment risk factors for CCA cause chronic inflammation of biliary epithelium and may result in the accumulation of mutigenetic alterations leading to initiation, promotion, progression and aggressive behaviors, such as metastasis, of the tumor. The combination of several evidences suggests a putative mechanism of CCA pathogenesis as a four stage cascade [10, 24, 37]. These include; **(1)** ***the exposure to endogenous and exogenous risk factors***, as mentioned above, causes chronic biliary irritation and inflammation and/or cholestasis through the biochemical and/or mechanical processes. **(2)** ***the genotoxic events*** are caused from nitric oxide (NO) overproduction by inflammatory cells. NO and related oxygen radicals can inactivate and injure the biomolecules within the cells, especially DNA via direct oxidative damage. The overproduced NO has also been reported as having an immunosuppressive effect particularly in lymphocyte proliferation [38-40]. In addition, inflammatory cytokines can induce several processes such as the modulation of gene expression, the inactivation of detoxification gene expression, and the activation of carcinogen metabolism. These effects enhance the ultimate carcinogens adducting and damage to DNA [41-42]. **(3)** ***the dysregulation of DNA repair and apoptosis***, damaged DNA usually leads to DNA mismatch repair mechanism, or if the damage is unable to repair, cell is stimulated to apoptosis process. However, cell may be survived by its apoptosis-resistant characteristic, oncogene activation and tumor suppressor gene inactivation. These events increase the rate of cell proliferation and can lead to histomorphological changes of the cell. And **(4)** ***histomorphological change***, it is suggested that CCA arises from a precancerous lesion which follows the hyperplasia-dysplasia-carcinoma process. The most studied molecular alterations for CCA which are related to dysregulation of cell growth and survival pathways, aberrant gene expression, invasion and metastasis, tumor microenvironment, and evasion from immune surveillance are detailed in Table 2-2.

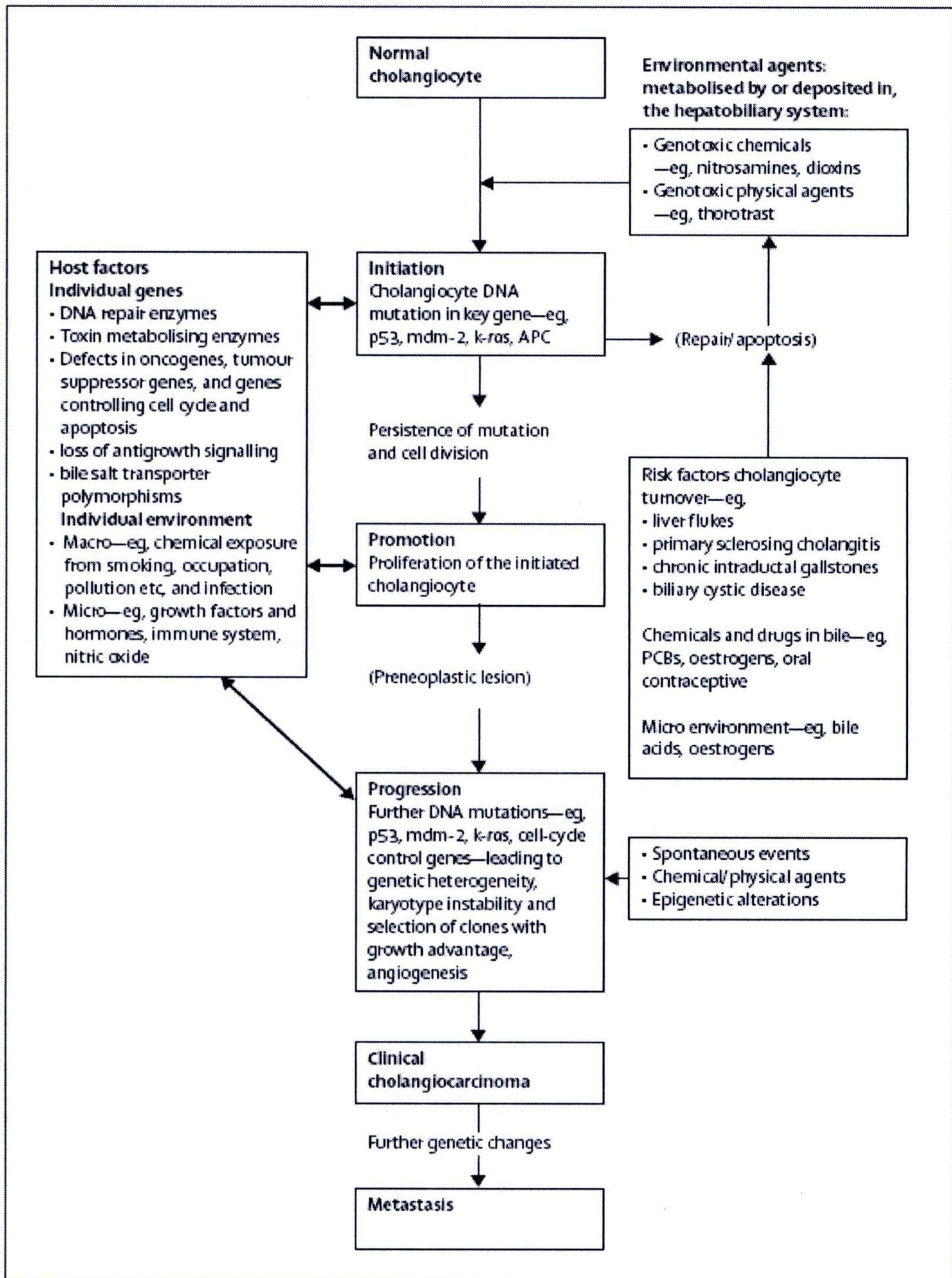

Figure 2-2 The proposed mechanism for pathogenesis of cholangiocarcinoma [3].

Table 2-2 Molecular aberrations of cholangiocarcinoma [43].

Role in cholangiocarcinogenesis	Molecular alteration
Autonomous proliferation signaling	IL-6, gp130/ gp80 upregulation
	HGF/c-met upregulation
	EGF/c-erbB2 overexpression
	COX-2 upregulation
	K-ras mutation
Loss of antigrowth signaling	*p53* mutation
	p21 mutation
	Mdm-2 overexpression
	p16 mutation
	TGF-β deletion
Evasion of apoptosis	FLIP upregulation
	NO inhibiton casepases
	Bcl-2 upregulation
	Mcl-1 upregulation
	COX-2 upregulation
	TRAIL and its receptor alterations
Unlimited replicative potential	Telomerase expressed
Angiogenesis	VEGF expressed
Metastasis and invasiveness	E-cadherin and α-catenin decreased
	MMP overexpression
	Aspartyl β-hydroxylase expression
	WISP1v expression
	Galectin-3 upregulation
	CD44 aberrant expression
	TIMP3 downregulation

3. Epigenetic alteration in cancer and cholangiocarcinoma

3.1 Epigenetics, DNA methylation and gene silencing

The modern definition of "epigenetic" refers to a heritable change in the pattern of gene expression that is mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene [44-45]. Several epigenetic mechanisms which regulate gene expression including DNA methylation, histone modifications and RNA interference, have been described [46-47].

DNA methylation can inactivate gene expression by hypermethylation of promoter CpG islands in tumors [14, 49, 190], while remain unmethylated in normal cells. However, there are several clear exceptions to this rule. DNA methylation is also believed to play important role in normal cells including X-chromosome inactivation in female, control of imprinted genes, regulation of cell-type specific expression and suppression of testis specific genes [48-49].

DNA methylation, the enzymatic addition of a methyl (-CH_3) group from a methyl donor, S-adenosyl-methionine, to the carbon-5 position of cytosine residues, occurs at cytosines that are followed by a guanine (so-called CpG dinucleotides) (Figure 2-3). Non CpG sequences such as CpNpG or non-symmetrical CpA and CpT may also exhibit methylation, but generally at a much lower frequency [50-51]. This modification is catalyzed by enzymes known as DNA methyltransferases (DNMTs), of which three are known in humans: DNMT1, DNMT3a, and DNMT3b. DNMT1 are primarily involved in maintenance of methylation patterns, whereas DNMTs 3a and 3b affect de novo DNA methylation [52]. As DNMT1 acts primarily on hemimethylated DNA, it is often associated with DNA replication complexes [53], whereas DNMT3a and 3b are primarily expressed during development [54].

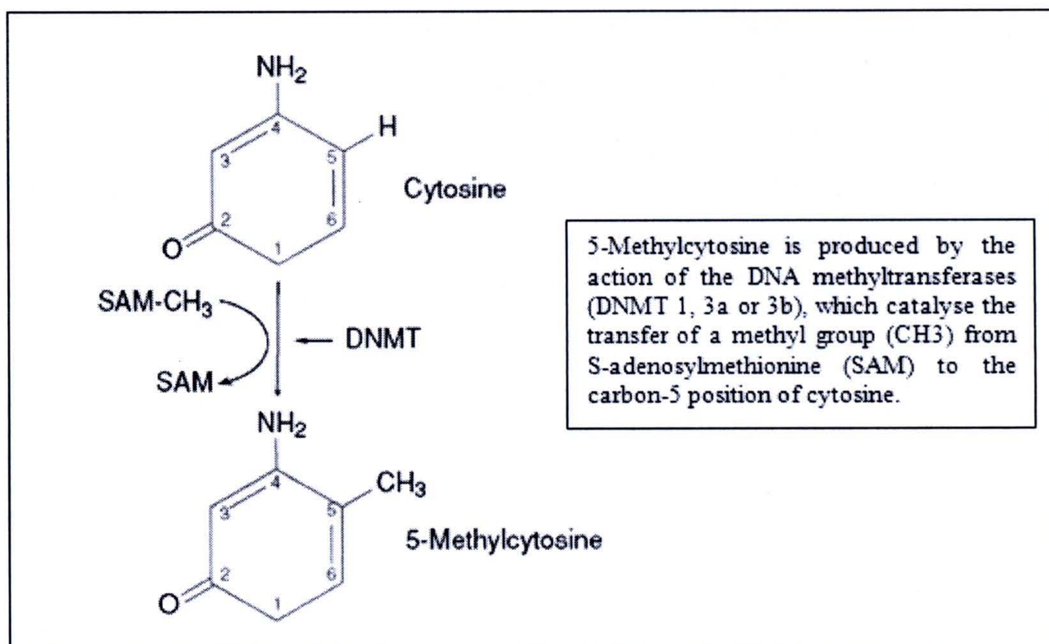

Figure 2-3 Mechanism of DNA methylation [14].

The bulk of the genome displays a clear depletion of CpG dinucleotides, presumably due to the high rate of deamination of 5-methylcytosine to thymine. CpG sites in the genome are mainly methylated (~70%). By contrast, small stretches of DNA, called CpG islands, ranging from 0.5-5 kb, are comparatively rich in CpG dinucleotides and are normally unmethylated in adult tissue. This pattern of DNA methylation is stably inherited from one cell generation to the next. The genome consists of an estimated 30,000 CpG islands and 50–60% of these are associated with genes, usually within the promoter region. The methylation of CpG islands within gene promoters has been shown to be associated with transcriptional repression of the corresponding genes [55-57].DNA methylation plays a role in transcriptional silencing through a direct binding of specific transcriptional repressor to methylated DNA or inhibition of transcription factor binding. The DNA methylation imprints are recognised by the methyl-CpG-binding proteins, the target being the 5′ methylted CpG sequence [58-60]. There are 6 members of methyl-binding proteins which have been described. MBD1, MBD2, MBD3, MeCP2, and KAISO are all involved in transcriptional repression, whereas MBD4 functions in DNA mismatch repair since it

has a glycosylase domain that removes thymidine from T:G mismatches [44]. Methyl-CpG-binding proteins have been shown to recruit and associate with histone deacetylase (HDAC), histone methyltransferase (HMT), and large protein complexes such as NuRD complex and Sin3a complex which contain chromatin remodeling activities for creation and maintenance of transcriptional repressive status (Figure 2-4) [14].

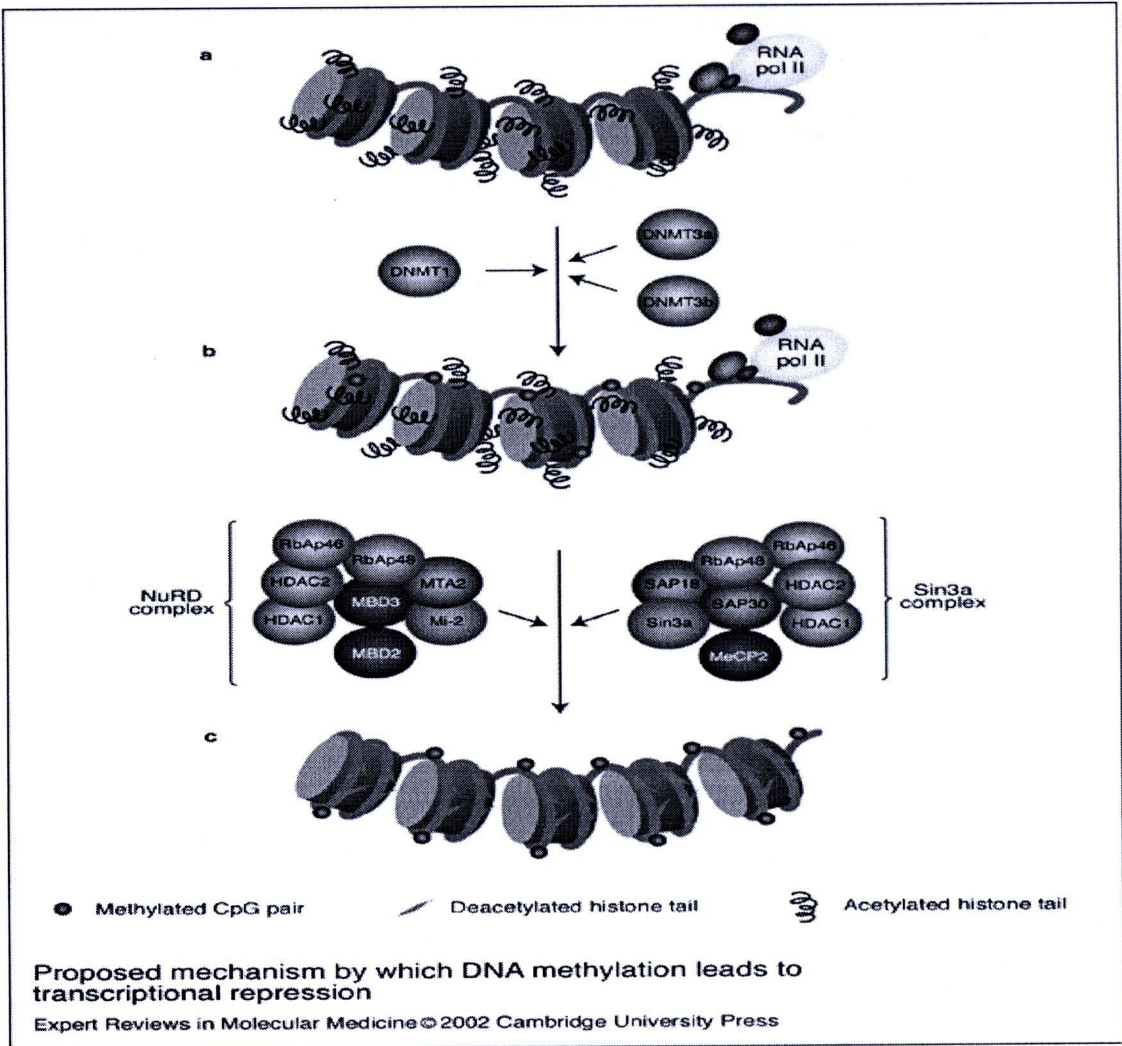

Figure 2-4 Proposed mechanism by which DNA methylation leads to transcriptional repression [14].

3.2 Epigenetics and application for cancer therapy

The potential reversibility of epigenetic states offers exciting opportunities for novel cancer drugs that can reactivate epigenetically silenced tumor-suppressor genes [61-63]. Blocking either DNA methyltransferase or histone deacetylase activity could potentially inhibit or reverse the process of epigenetic silencing. Inhibitors of DNA methyltransferases and histone deacetylases are the two major drug widely used for epigenetic inhibition to date, although others are expected to be added in the near future. DNA methyltransferase inhibitors are clinically more advanced than histone deacetylase inhibitors. They have been extensively tested in phase I-III clinical trials. In addition, the prototypical DNMT inhibitor 5-azacytidine (i.e., Vidaza) has recently been approved by the Food and Drug Administration (FDA) as an antitumor agent for the treatment of myelodysplastic syndrome. There are two major types of DNMT inhibitors, nucleoside and nonnucleoside compounds. Nucleoside derivatives, such as 5-Azacytidine, 5-aza-2′-deoxycytidine (decitabine) and zebularine can inhibit DNMTs by trapping the enzymes in the form of covalent protein-DNA adduct (Figure 2-5A). As a result, cellular DNMT is rapidly depleted and genomic DNA is concomitantly demethylated as a result of continued DNA replication. In nonnucleoside derivatives, they can also inhibit DNMT activity. These substances directly block DNMT activity without the inherent toxicity caused by the covalent trapping of the enzyme. The widely used nonnucleoside compounds include EGCG, RG108, procaine (Figure 2-5B) [64].

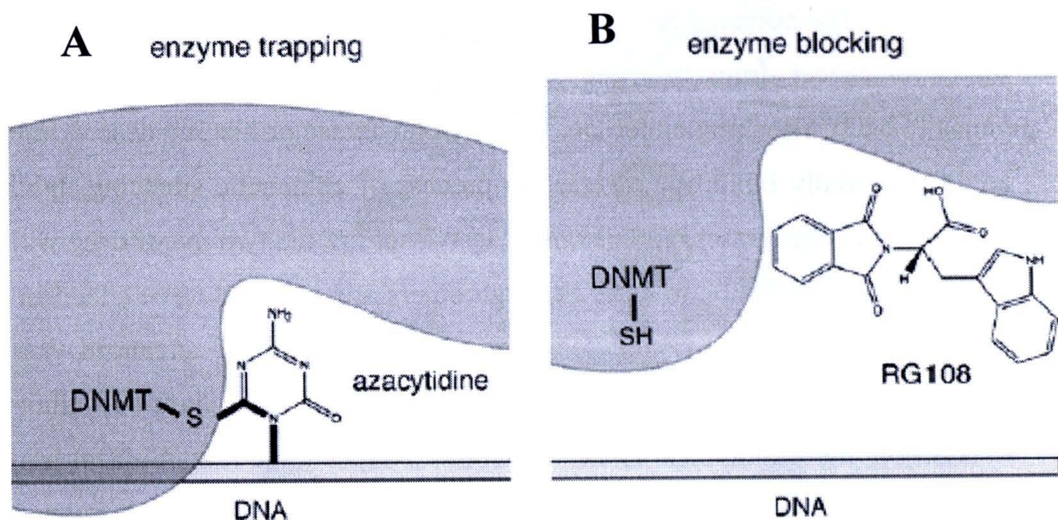

Figure 2-5 DNA methyltransferase (DNMT) inhibition by enzyme trapping (A) or enzyme blocking (B) [64].

For HDAC inhibitors, they are subdivided into several subcategories on the basis of their chemical structures. Some of HDAC inhibitors are wildly used in experimental studies or the clinical trials. These include phenylbutyrate, vaproic acid (VPA) and trichostatin (TSA) [65]. HDAC inhibitors can inhibit HDAC activity through binding the active site pocket of deacetylase. However, in some cases, the treatment with a HDAC inhibitor alone is not sufficient to induce gene re-expression [66-67], probably due to the role of other epigenetic modifications besides histone acetylation, such as CpG island methylation, in maintaining the silencing of such genes. A strategy to overcome this problem is the combination of HDAC inhibitors with the DNMT inhibitors for increasing of higher activation than either compound alone [66, 68-69]. The combined strategies of anticancer therapy may ultimately offer the most successful approach. The combined approach may lead to reduction in the dose of each drug used and reduce the side effects of each individual agent. How to combine the demethylating agents and histone deacetylase inhibitors with conventional chemotherapy to achieve the best outcome remains to be determined. If the exact methylation profiles of tumors are available and drugs targeting the specific

genes are obtainable, then the treatment of cancer could be more focused and rational [70].

3.3 Hypermethylation of tumor suppressor genes and cancer

Hypermethylation of CpG islands, often associated with the promoters of genes, can occur throughout tumor development. It is estimated that in tumors there are average 600 CpG islands aberrantly methylated compared to normal tissues, although this can vary widely between tumors types and within particular histological subtypes [71]. Moreover, methylation does not occur randomly, as there are CpG islands that are methylated in multiple tumors types, while other CpG islands are methylated only in certain tumor types [71-72]. It has been suggested that during carcinogenesis most cancers need to develop certain characteristics such as evasion of apoptosis, insensitivity to antigrowth signals, limitless replicative potential, self-sufficiency in growth signals, sustained angiogenesis, and tissue invasion and metastasis [16]. Hypermethylation and silencing of many tumor suppressor genes can affect these features of cancers. In addition, the epigenetic silencing of DNA repair genes and drug detoxification-mediated genes can also affect acquired drug resistance of tumors (Table 2-3) [14, 49].

Table 2-3 Examples of genes that can become epigenetically inactivated in tumors [49].

Function	Epigenetically inactivated genes
Evasion of apoptosis	APAF-1, Caspase-8, DAPK, DLC-1, Fas, p14ARF, p53, p73, SHP1, TMS1, TRAIL-R1, XAF1
Insensitivity to anti-growth signals	CyclinD2, ERα, LOT1, p15INK4b, p16INK4a, p21WAF1, p27KIP1, p57KIP2, Pax5, PTEN, RARα, RASSF1A, TGFbRI, TGFbRII, 14-3-3σ
Limitless replicative potential	pRb, CDX1, GATA-4, GATA-5, Myf-3, SOCS-3
Angiogenesis	THBS1, THBS2, VHL
Intercellular adhesion and tissue invasion	ADAM23, E-Cadherin, H-Cadherin, Cav-1, CD44, CLCA2, CLDN-7, gelsolin, laminin-5, Maspin, OPCML, TIMP3, SLIT2
DNA repair	MLH1, MGMT, BRCA1, FancF
Drug metabolism, detoxification	CytP4501A1, GSTp1, P-gp, RFC

3.4 Hypermethylation of tumor suppressor genes and cholangiocarcinoma

Unlike genetic investigations, the study of epigenetic changes in CCA is still limited. Previous studies have reported high frequency of methylation in 78-85% of CCA in at least one locus [73-74]. Hypermethylation in CCA have been reported in tumor suppressor genes (Table 2-4) involved to cell cycle regulation (*p16*, *14-3-3σ*, *RASSF1A*, *p73*, *p14*, *p15*), apoptosis (*DAPK*, *TMS1/ASC*, *SEMA3B*), and cell adhesion (*APC*, *E-cadherin*, *TIMP3*, *THBS1*) [73-80]. Furthermore, several reports demonstrated hypermethylation of DNA repair genes and genes involved in carcinogen metabolism as for example *hMLH1*, *MGMT* and *GSTP1* [73-74, 81].

Table 2-4 Epigenetic aberration of cholangiocarcinoma [73-82].

Gene	Incidence of methylation (%)
RASSF1A	65-85
DAPK	3-6
p14	25-30
p15	54
p73	27
14-3-3 σ	59.5
p16	17-83
APC	27-47
hMLH1	25-45
E-cadherin	21-48
MGMT	11.4-33
TIMP3	9
GSTP1	34
RARβ2	16
TSBS1	11
PTEN	0
BLU	20
SEMA3B	100
SFRP1	63.4

3.5 Techniques for detection of DNA methylation

Nowadays, several existing and emerging techniques are used to investigate epigenetic events including DNA methylation and histone modification. Each method has advantages, disadvantages and areas of applicability. The decision to choose the method(s) for epigenitic analysis depends on the researcher's needs and suitability of the method(s). For DNA methylation study, several techniques are used for evaluation of methylated cytosines including digestion of DNA with methylation-sensitive restriction enzymes followed by Southern blotting or polymerase chain reaction (PCR). However, large amounts of DNA are required for Southern blotting, which limits the use of this method. This limitation is counteracted by performing PCR, but still both methods depend on a complete enzymatic digestion of the DNA in order to avoid false-positive results. At present, the most frequently used DNA methylation analysis methods employ a combination of bisulfite treatment and PCR. These include methylation-specific PCR (MSP), methylation-specific single strand conformation polymorphism (MS-SSCP), combined bisulfite restriction analysis (COBRA), bisulfite sequencing (BS), pyrosequencing and MethyLight. In addition, high-throughput methods such as methylation-specific oligonucleotide (MSO) array, chromatin immunoprecipitation on DNA microarrays (ChIP-chip), promoter microarray and HumanMethylation27 BeadChip are also used for methylation study, but these technologies are relatively high expenditure and specific software is required for data analysis [83-84]. Histone modification is another key player in epigenetic silencing as well as DNA methylation. A few well-known assays are used to study this modification including chromatin immunoprecipitation (ChIP) and ChIP-on-chip assay [85-86].

3.5.1 Methylation specific PCR (MSP)

The most popular method for methylation study is methylation-specific PCR (MSP) which developed by Herman and colleagues [87]. This method can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes. MSP entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for the DNA sequence now associated with methylated versus unmethylated DNA. It is very sensitive, permitting the analysis of small and heterogeneous samples, including paraffin-embedded material. In addition to the primer sets for unmethylated and methylated DNA which have undergone a chemical modification, a third primer set can be used that anneals to any DNA sequence (unmethylated or methylated) that has not undergone chemical modification. This reaction is optional but serves as a control for the success of the bisulfite treatment.

3.5.2 Methylation-specific single strand conformation polymorphism (MS-SSCP)

A more widespread procedure combines a bisulfite treatment and PCR-single-strand conformation polymorphism analysis [88]. In a first step, the converted DNA is amplified with primers that have no CpG sites in the corresponding region of the original DNA, and as such amplify both unmethylated and methylated DNA. Sequence differences between amplified products from unmethylated and methylated DNA are visualized on a SSCP gel.

However, MS-SSCP analysis is not always straightforward because more than two bands or a smear can be observed. These unfavorable patterns hinder accurate quantification, and therefore PCR primer design and the electrophoretic conditions should be thoroughly optimized.

3.5.3 Combined bisulfite restriction analysis (COBRA)

COBRA is a PCR-based method that uses sodium bisulfite treated DNA as a template for amplification followed by restriction digestion and band quantitation of PCR products [89]. The combination of bisulfite treatment and PCR results in the conversion of unmethylated cytosine residues to thymine while methylated cytosine residues remain as cytosine. This sequence conversion can lead to the methylation-dependent creation of new [90] or retention of pre-existing restriction enzyme recognition sites. The primers used in the PCR do not span CpG dinucleotides so that the amplification step does not discriminate between templates according to their original methylation status. The fraction that has a newly created or retained restriction site containing a CpG(s) is a direct reflection of the percentage of DNA methylation at that site in the original genomic DNA (Figure 2-6).

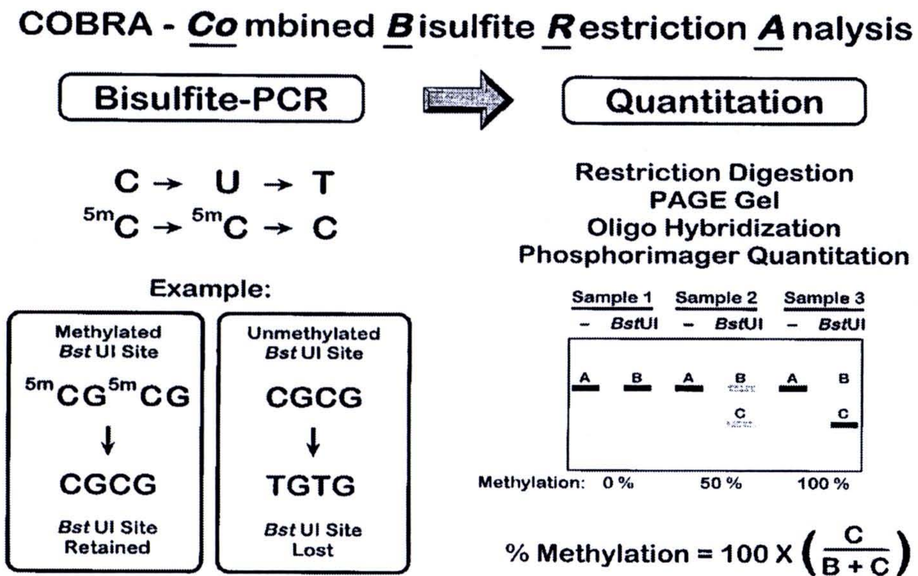

Figure 2-6 Outline of the COBRA procedure [89].

3.5.4 Bisulfite sequencing (BS)

Bisulfite sequencing is a "gold-standard" method used to determine the methylation status of each cytosine over an amplified region of a given gene, a method now used routinely for studies of CpG-islands (CGI) [91]. The underlying principle is based on the ability of sodium bisulfite to deaminate cytosine (C) residues into uracil (U) in genomic DNA, whereas the methylation cytosine residues are resistant to this modification. After PCR amplication, the U is amplified as thymine (T). Cloning and subsequent sequencing of the DNA fragments containing the CGIs then provide information on the methylation status of each C within the island (Figure 2-7) [92]. However, bisulfite sequencing requires DNA sequencing, cloning, and PCR product, which taken together make it a very time-consuming and relatively costly technique. The new sequencing technology has been developed to resolve these limitations, such as pyrosequencing method.

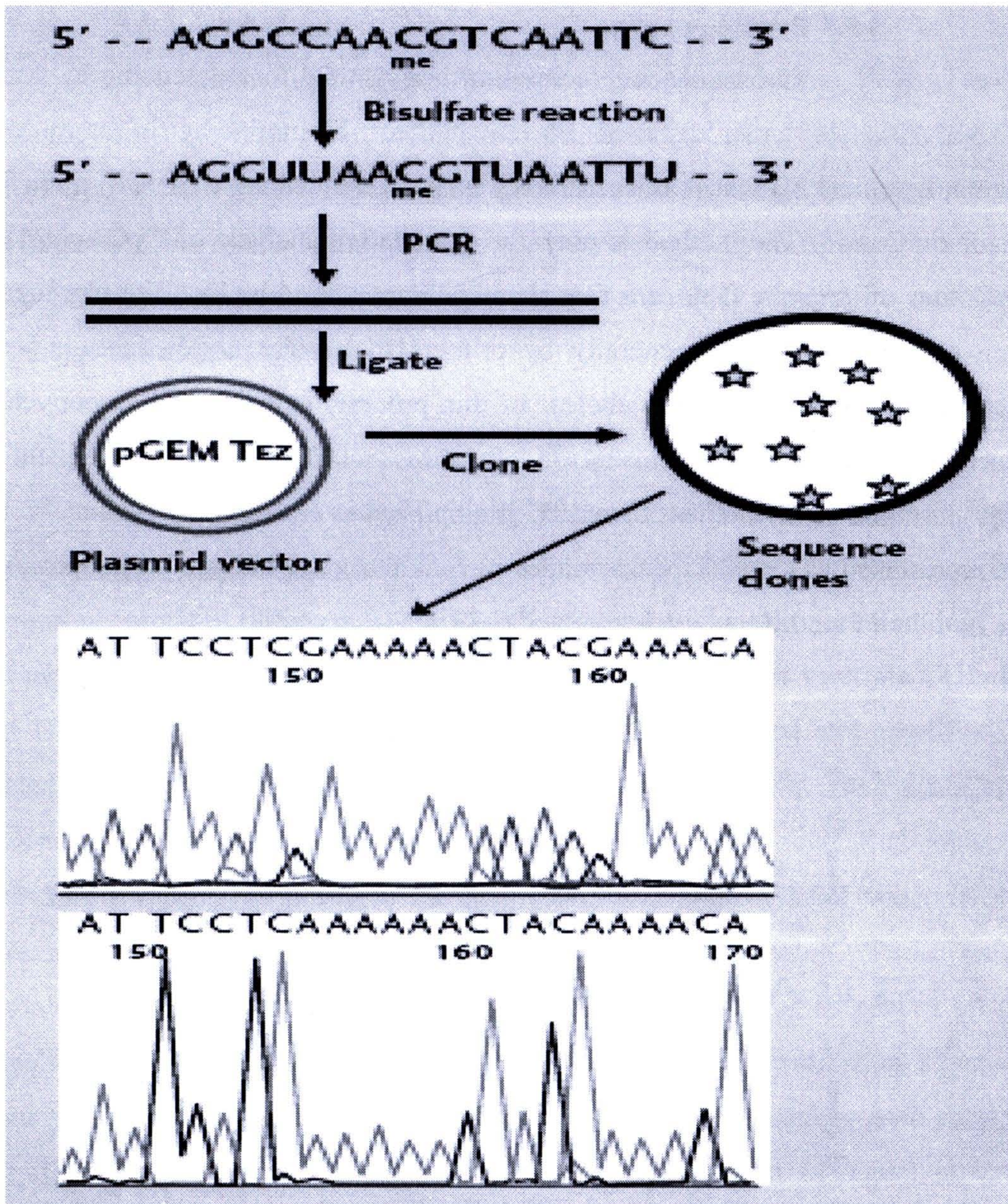

Figure 2-7 The workflow of bisulfite sequencing (BS) [92].

3.5.5 Pyrosequencing

Pyrosequencing technology is a method for sequencing by synthesis in real time [93]. It is based on an indirect bioluminometric assay of the pyrophosphate (PPi) that is released from each deoxynucleotide (dNTP) upon DNA-chain elongation. The method is used for quantitative analysis of CpG methylation, CpG sites of genomic DNA are first chemically converted by bisulfite treatment and then amplified by PCR, generally by primers that make no distinction between methylated and unmethylated alleles. In this process, cytosine (C) is converted to uracil (U), whereas methylcytosine (^{m}C) remains unchanged. In the subsequent PCR, U is amplified as thymidine (T) and ^{m}C is amplified as C. In the Pyrogram, ^{m}C and C are represented as C and T peaks, respectively. These peak heights are proportional to the number of methylated alleles at each CpG site (Figure 2-8) [94].

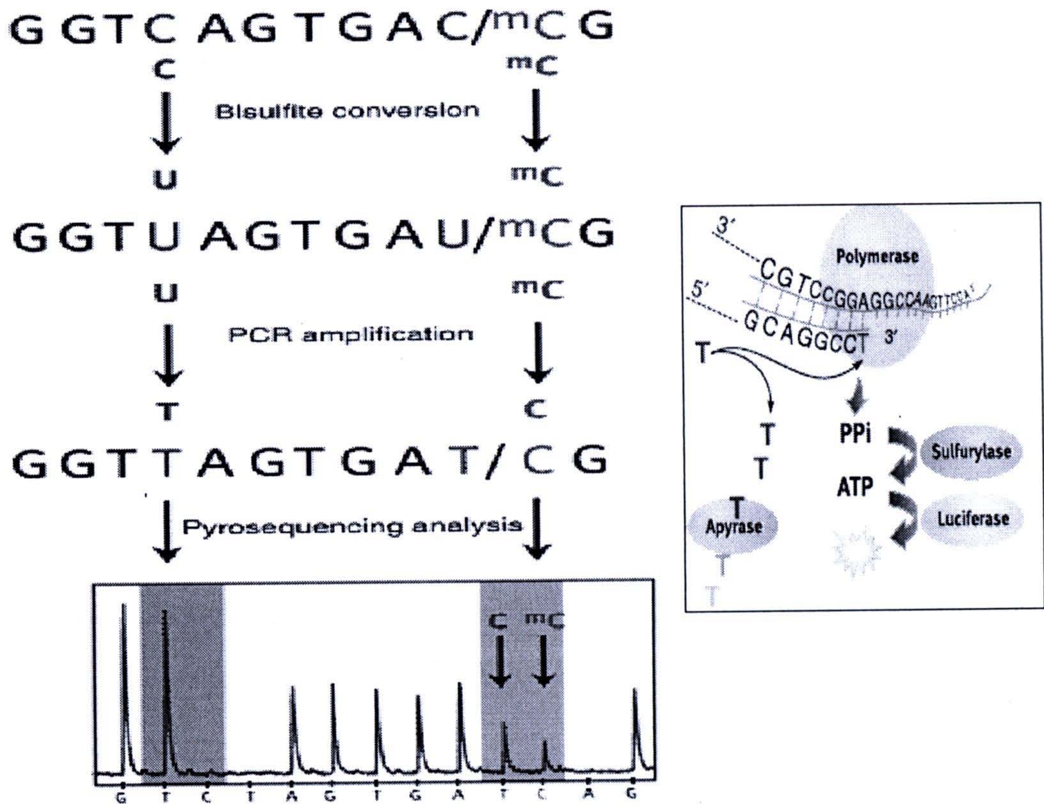

Figure 2-8 The workflow of pyrosequencing [94].

3.5.6 MethyLight

MethyLight is a fluorescence-based real-time PCR Taq-Man technology for high-throughput methylation detection and quantification using DNA methylation specific probes. It is highly quantitative assay which has good precision and linearity. This method can be used for methylation analysis of small amounts of DNA [95-96]. However this technique cannot accurately determine methylation percentage at a single CpG site [92].

3.5.7 Methylation-specific oligonucleotide (MSO) array

MSO array was initially designed to provide a high-throughput method for fine mapping of CpG sites in a known CGI using a hybridization based microarray protocol (Figure 2-9) [97-98]. Subsequently, the methodology was adapted to interrogate simultaneously the methylation status of multiple CGIs [99]. Test DNA is restricted with *Kpn*I and *Nde*I, bisulfite modified and amplified, and labeled with Cy5, resulting in a pool of labeled targets with altered nucleotide sequences due to their differential methylation status. Sets of short oligonucleotides (~21–25-mers), corresponding to the methylated and unmethylated versions of the CGI, are designed to provide coverage of the entire region. These pair of oligonucleotides are synthesized and immobilized in triplicates as probes on glass slides. After hybridization of the targets to the probes, hybridization signals are captured, quantified, and analyzed. The percentage of methylation for each short CG-rich fragment (2–4 CpG sites) is determined by comparison of signal intensities between the paired "unmethylated" and "methylated" oligonucleotide probes.

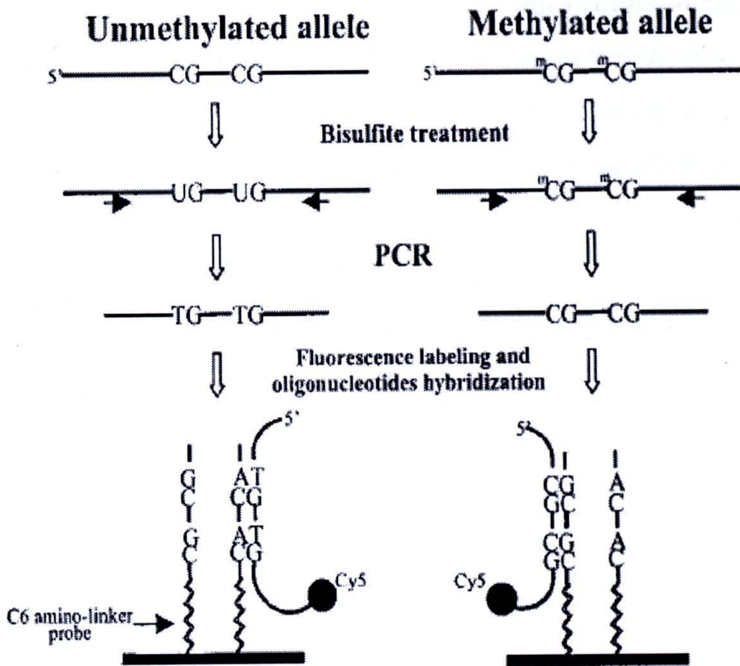

Figure 2-9 Diagram illustrating how to analyze DNA methylation by methylation specific oligonucleotide (MSO) array [97].

3.5.8 Chromatin immunoprecipitation on DNA microarrays (ChIP-chip)

ChIP was developed to identify and characterize the interactions of specific genomic DNA sequences associated with a target protein such as transcription factor, histone, or histone deacetylase, etc. in the context of intact cells [100]. In this method, living cells are first fixed with formaldehyde to crosslink protein-protein and protein-DNA complexes in situ. Next, the crosslinked genomic DNA with specific target protein is isolated and immunoprecipitated using specific antibody to the target protein. To study the effects of DNA methylation/chromatin structure on gene expression by ChIP-chip methodology (Figure 2-10), specific antibodies to 5-methylcytosine as well as MDB proteins (e.g. MeCP2) are often used to pull down methylated DNA [207-208]. After reverse crosslinking, DNA is extracted and purified before amplify with PCR to generate chromatin amplicons. Amplicons from experimental immunoprecipitation are labeled with Cy5, and the input reference amplicons are labeled with Cy3. Labeled probes are applied on the

CGI array for hybridization. Data can be utilized to study the interaction between particular target protein and specific CpG sites of genes [83]. A human promoter array has commercially developed for ChIP experiments (Affymetrix). This is a single array with more than 4.6 million probes titled through more than 25,500 human promoter regions proximal to transcriptional start sites and probes for approximately 59% CpG islands annotated by UCSC in the NCBI human genome assembly (Build 34) [83]. Some study uses the ChIP-chip technology for methylation studies called "methylome" which refers to the complete set of methylated DNA sequences in a cell [101].

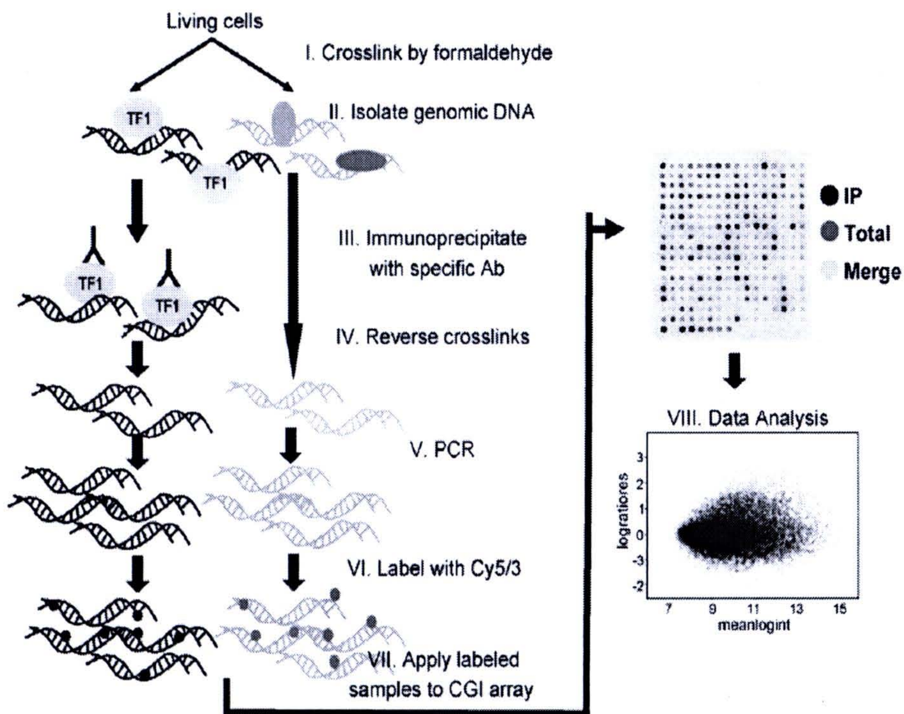

Figure 2-10 Workflow of chromatin immunoprecipitation (ChIP) on DNA microarray (ChIP-chip) analysis [83].

3.5.9 Genome-wide methylation array/promoter arrays

Promoter arrays have been developed to study a large set of CGIs found in the human and mouse genomes (the University Health Network Microarray Center (UHNMC): www.microarray.ca). A number of 20K CG-rich sequence clones can be assessed and used to distinguish promoter methylation status of human (12,192 CGIs) and mouse (7296 CGIs) genome. These arrays clearly provide a high-throughput, genome-wide screen of promoters with CGIs undergoing methylation changes due to experimental or pathological conditions. For this technology, the expenditure for setting-up routine hybridization is relatively high since it requires hybridization stations, equipment for quality controls of the targets (e.g. Nanodrop), and a scanner for capturing signals. In addition, because of the large volume of data generated in one experiment, pre- and post-experimental consultations on experimental design and data mining are critical elements for this kind of study. Specific software packages provided by the vendors and others developed in-house are required to generate meaningful results. Custom designed or small-scale promoter methylation arrays have recently become commercial available that simultaneously profile the methylation status of the gene of interests from one sample (NimbleGen System; Panomics). This is a high-throughput analysis of promoter methylation that costs less than the genome-wide CGI array [83].

3.5.10 HumanMethylation27 BeadChip (Illumina assay)

The HumanMethylation27 BeadChip uses Infinium technology to perform genome-wide screening of DNA methylation patterns. The high throughput quantitative measurements of DNA methylation are determined for 27,578 CpG dinucleotides spanning 14,495 genes. In this assay, only 1 μg of genomic DNA is required for bisulfite modification prior to performing the automatic Infinium assay. After bisulfite conversion, whole-genome amplification (WGA) and enzymatic fragmentation are performed for each sample. The bisulfite-modified WGA-DNA sample is purified and applied to the BeadChip. In hybridization step, WGA-DNA strand anneals to locus specific DNA oligomers linked to individual bead types. Two bead types correspond to each CpG locus, one to methylated (C) and the other to the unmethylated (T) state. Allele-specific primer annealing is followed by single-base

extension using DNP- and Biotin-labeled ddNTPs. Both bead types for the same CpG locus will incorporate the same type of labeled nucleotide, determined by the base preceding the interrogated "C" in the CpG locus, and will be detected in the same color channel (Figure 2-11). After extension, the array is fluorescently stained, scanned, and measured the intensities of the unmethylated and methylated bead types. DNA methylation values are recorded for each locus in each sample. DNA methylation values are continuous variables between 0 and 1, representing the ratio of the intensity of the methylated bead type to the combined locus intensity [102].

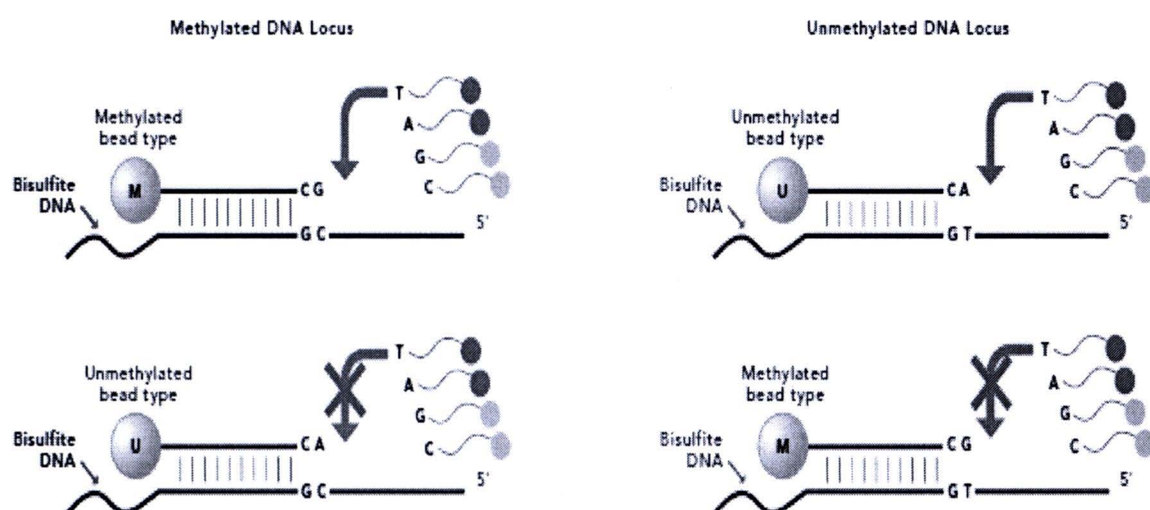

Figure 2-11 The Infinium assay for methylation study. The assay detects methylation status at individual CpG by typing bisulfite-converted DNA. Methylation protects C from conversion (left), whereas unmethylated C is converted to T (right). A pair of bead-bound probes is used to detect the presence of T or C by hybridization followed by single-base extension with a labeled nucleotide [102].