THE EFFECT OF BUDESONIDE AND 5-AZACYTIDINE ON THE LEVELS OF METHYLATION AT THE CpG ISLANDS OF HUMAN CHOLINE KINASE ALPHA (*ckα*) AND BETA (*ckβ*) PROMOTER

MUHAMMAD AL BUNYAMIN BIN ABDUL RAHMAN

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by

MUHAMMAD AL BUNYAMIN BIN ABDUL RAHMAN

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
bp	Base pair
BSA	Bovine serum albumin
cm^2	Centimeter square
cm^3	Centimeter cube
C	Cytosine
CpG	Cytosine phosphate Guanine
CK	Choline kinase
ckα	Choline kinase alpha
ckβ	Choline kinase beta
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy nucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	Double stranded deoxy nucleic acid
EDTA	Ethylenediamine tetraacetic acid
et al.	et alii – 'And others'
EtBr	Ethidium bromide
FBS	Fetal bovine serum
g	Gram

g/mol	Gram per mole
g/L	Gram per litre
GC	Guanine sytosine
IP	Immunoprecipitation
MCF-7	Human breast adenocarcinoma cell line
$MgCl_2$	Magnesium chloride
min/kb	Minute per kilobase pair
mL	Milliliter
mM	Millimolar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NcoI	Nocardia corallina
ng	Nanogram
^{o}C	Degree celcius
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCho	Phosphocoline
PCR	Polymerase chain reaction
pH	Potential hydrogen
sec	Second
TAE	Tris acetate-EDTA buffer
Taq	Thermus aquaticus
TF	Transcription factor

T_m	Melting temperature
V	Volt
uL	Microliter
µM	Micromolar
U/ml	units per millilitre
ug/ml	Microgram per milliliter
xg	Fold gravity
%	Percentage
UV	Ultraviolet
v/v	Volume per volume
~	Approximately

KESAN BUDESONIDE DAN 5-AZASITIDINA TERHADAP PARAS METILASI DNA PADA KEPULAUAN CpG DI PROMOTER KOLINA KINASE MANUSIA ALPHA (*ckα*) DAN BETA (*ckβ*)

ABSTRAK

Kolina kinase (CK) merupakan enzim yang pertama di dalam tapak jalan CDP-kolina untuk sintesis fosfatidilkolina, komponen utama fosfolipid membran. Dalam manusia, CK dikodkan oleh gen *ckα* dan *ckβ* yang menghasilkan tiga isoform protein yang dikenali sebagai CKα1, CKα2 dan CKβ. CKα terlibat dalam pembentukan tumor manakala CKβ telah dikaitkan dengan distrofi otot. Metilasi DNA ialah tanda epigenetik penting dalam pengawalaturan ekspresi gen. Kajian ini melaporkan status metilasi *ckα* dan *ckβ* manusia pada kepulauan CpG selepas rawatan agen epigenetic (Budesonide dan 5-azasitidina). Dalam analisis silico terungkap kepulauan CpG pada setiap promotor ckα dan ckβ melalui alat prediksi MethPrimer dan EMBOSS CpGPlot dan lima puluh sembilan dan enam puluh dua pengikat laman faktor transkripsi pada pulau CpG ckα dan ckβ, masing-masing melalui alat prediksi Mat Inspektor dan TFBIND. Status metilasi yang diramalkan pada kepulauan CpG telah dianalisa melalui rawatan epigenetik pada sel MCF-7. Tujuh kawasan daripada empat belas kawasan promoter kepulauan CpG telah berjaya diamplifikasikan dengan menghasilkan produk PCR pada saiz yang diharapkan. Tujuh kawasan ini disasarkan untuk analisis selanjutnya. MCF-7 telah dikultur dan dibahagikan kepada empat kumpulan, terdiri daripada dua kumpulan rawatan iaitu kumpulan rawatan budesonide (70 μM, 24 jam) dan kumpulan rawatan 5-azasitidina (1 μM; 96 jam) dan dua kumpulan kawalan, iaitu kumpulan kawalan budesonide (1% DMSO; 24 jam) dan kumpulan kawalan 5-azacytidine (1% DMSO; 96

jam). DNA genom daripada semua kumpulan telah diekstrak selepas pengeraman mereka dengan ubat epigenetik. Fragmentasi DNA genom untuk semua kumpulan mendedahkan DNA telah difragmentasi dari 200 bp hingga 3000 bp. DNA terfragmentasi ini kemudiannya tertakluk kepada prosedur IP. Proses memperbanyak oleh IP telah berjaya dibuktikan melalui amplifikasi kawalan DNA dan pencernaan oleh *NcoI* dengan membandingkan intensiti band sebelum dan selepas IP. Amplifikasi tujuh kawasan pulau CpG selepas IP promotor *ck* mendedahkan kebanyakan produk PCR pada saiz yang dijangkakan, tetapi tidak konsisten dalam intensiti band dalam kawasan CpG yang berbeza oleh kumpulan yang sama dan dalam kalangan kumpulan. Daripada tujuh kawasan ini, kawasan ckα-2ndCpG-2, ckβ-3rdCpG-4 dan ckα-4thCpG-7 menunjukkan paras tinggi status metilasi selepas rawatan ubat budesonide, manakala kawasan ckβ-1stCpG-1 dan ckβ-3rdCpG-4 menunjukkan paras rendah status metilasi selepas rawatan ubat 5-azasitidina. Walau bagaimanapun, analisis ini memerlukan penyiasatan lanjut, kerana hanya menganalisis tujuh dan bukannya empat belas kawasan CpG promoter *ckα* dan *ckβ*. Analisis semua empat belas kawasan pulau CpG promotor *ckα* dan *ckβ* boleh memberikan maklumat yang jelas mengenai status metilasi kepulauan CpG kedua-dua promoter *ckα* dan *ckβ*.

THE EFFECT OF BUDESONIDE AND 5-AZACYTIDINE ON LEVELS OF DNA METHYLATION AT THE CpG ISLANDS OF HUMAN CHOLINE KINASE ALPHA (*ckα*) AND BETA (*ckβ*) PROMOTER

ABSTRACT

Choline kinase (CK) is the first enzyme in the CDP-choline pathway for the synthesis of phosphotidylcholine, a major component of membrane phospholipid. In human, CK is encoded by *ckα* and *ckβ* genes which produce three protein isoforms known as CKα1, CKα2 and CKβ. CKα is involved in tumorigenesis while CKβ is associated with muscular dystrophy. DNA methylation is an important epigenetic mark in gene expression regulation. This study reports on the methylation status of human *ckα* and *ckβ* promoter CpG islands after treatment with epigenetic drugs (budesonide and 5-azacytidine). *In silico* analyses revealed multiple putative CpG islands on each *ckα* and *ckβ* promoter through MethPrimer and EMBOSS CpGPlot prediction tools and fifty-nine and sixty-two putative transcription factor binding sites on *ckα* and *ckβ* promoter CpG islands respectively through Mat Inspector and TFBIND prediction tools. The methylation status of predicted putative CpG islands were analysed through epigenetic drugs treatment on MCF-7 cell line. Seven regions of out fourteen regions of the promoter CpG islands were successfully amplified by producing PCR products at their expected sizes. These seven regions were targeted for further analysis. MCF-7 were cultured and divided into four groups, consisting of two treatment groups which were budesonide (methylating agent) treated group (70 µM; 24 hours) and 5-azacytidine (demethylating agent)group (1 µM; 96 hours) and two control groups, which were budesonide control group (1% DMSO; 24 hours) and 5-azacytidine control group (1%

DMSO; 96 hours). Genomic DNA from all groups was extracted following their respective incubation time with the epigenetic drugs. Fragmentation of the genomic DNA for all groups revealed fragmented DNA ranging from 200 bp to 3000 bp. These fragmented DNAs were then subjected to IP procedure. Enrichment process of the IP was successfully proven through control DNA amplification and digestion by *NcoI* by comparing the band intensity before and after IP. Amplification of seven *ck* promoter CpG island regions after IP revealed most PCR products at expected sizes, but there were inconsistency in band intensity within CpG regions of similar group and among the groups. Out of these seven regions, ckα-2ndCpG-2, ckβ-3rdCpG-4 and ckα-4thCpG-7 regions showed higher level of methylation status after budesonide drug treatment, while ckβ-1stCpG-1 and ckβ-3rdCpG-4 regions showed lower level of methylation status after 5-azacytidine drug treatment. However, this analysis warrants further investigation, since only seven instead of fourteen CpG regions of *ckα* and *ckβ* promoter were analysed. Analysis of all fourteen *ckα* and *ckβ* promoter CpG island regions could give clearer information on both *ckα* and *ckβ* promoter CpG island methylation status after the treatment of epigenetic drugs.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Phospholipids

Cell membranes are structures which form boundary layers between two cellular compartments and play an extraordinarily vital part in the metabolism of cells (O'Brien, 1967). Most lipids on the membrane contain a phosphate group and they are known as phospholipids (Karp, 2004). Phospholipids are the major component of all cell membranes (Figure 1.1) and due to their amphiphilic structures (possess both hydrophilic and hydrophobic properties), they can form lipid bilayers.

Most membrane phospholipids have a glycerol backbone, which is called phosphoglycerides (Karp, 2004). Membrane phosphoglycerides have an additional group attached to the phosphate, either choline, forming phosphatidylcholine (PC) or ethanolamine, forming phosphatidylethanolamine (PE) (Karp, 2004). PC is the most abundant phospholipid in mammalian cells, comprising 40–50% of total phospholipids (Vance, 2015).

1.1.1 Phosphatidylcholine (PC)

PC is known as lecithin (Kuan, *et al*., 2014) (Figure 1.2) and it is the most abundant membrane phospholipid (40–60%) in eukaryotic cells (Kent, 2005). PC accounts for a major portion of phospholipids compare to phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin and sphingomyelin (SM) and thus plays important roles in structural maintenance of the phospholipid bilayer (Cui and Houweling, 2002; Wu *et al*., 2008; Gibellini and Smith, 2010). Phosphatidylcholine

and lysophosphatidylcholine are also major structural features of serum lipoproteins (Kent, 2005). Phosphatidylcholine is also involved in signal transduction, serving as a substrate or activator for phospholipases in signal transduction pathways. Implication of the phospholipid metabolites and derivatives in different mitogenic signaling pathways was extensively described as well (Kent, 2005; Cui and Houweling, 2002; Gallego-Ortega *et al*., 2011). PC biosynthesis is also needed for normal to very low-density lipoprotein secretion from hepatocytes (Gibellini & Smith, 2010). It is well known that together with polyphosphoinositides (PI) and PE, PC is the second messenger and major source of the arachidonic acid (AA) and its eicosanoid metabolites released by agonist activation of phospholipase A (PLA) in a variety of cells (Exton, 1994). PC hydrolysis in response to agonists and phorbol ester mainly resulted from activation of a phospholipase D (PLD) yielding phosphatidic acid (PA), and that much of the DAG arises due to the action of phosphatidate phosphohydrolase (PAP) on PA (Exton, 1990; Shukla and Halenda, 1991; Bilk *et al*., 1993). Besides, PC might act as an autocoid to modulate cellular functions as it causes Ca^{2+} influx across the plasma membrane in intact cells (Putney, 1980; Billah and Anthes, 1990).

1.2 Biosynthesis of Phosphatidylcholine

PC can be synthesized de novo in all mammalian cells by two pathways, (i) the CDP-choline pathway and (ii) the PE methylation pathway (Aoyama *et al*., 2004). Diacylglycerol is the precursor for PC (Kent, 1995). In yeast, diacylglycerol is less important in rapidly growing cells, where the CDP-diacylglycerol branch is the principal pathway for the biosynthesis of PC (Kent, 1995). In most eukaryotic cells, PC and PE

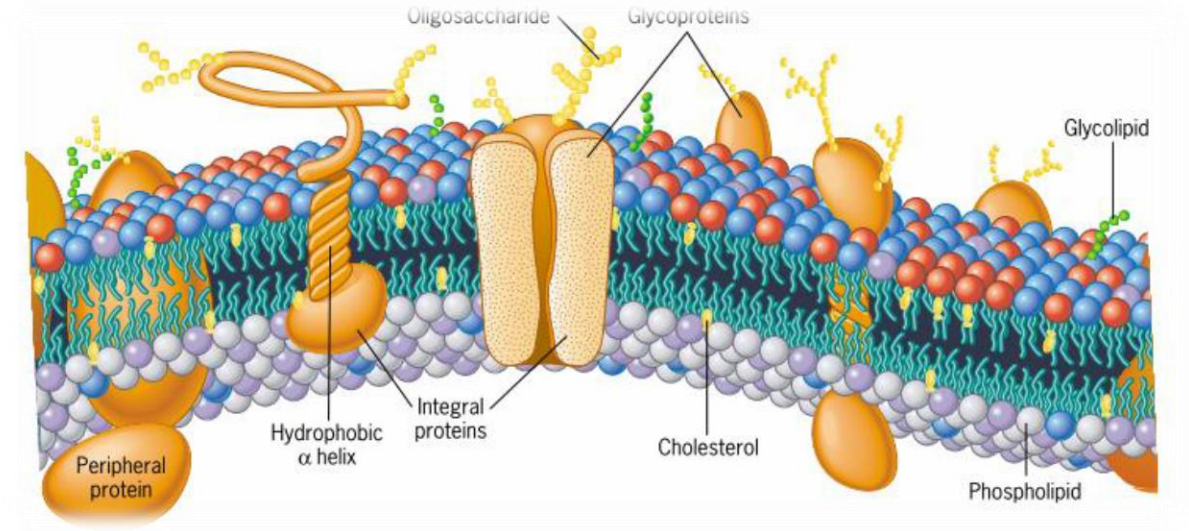

Figure 1.1: The structure of a cell membrane. The cell membrane is composed of lipid bilayers and membrane proteins. The external surface of most membrane proteins, as well as a small number of the phospholipids contain short chains of sugars, making them glycoproteins and glycolipids (Karp, 2004)

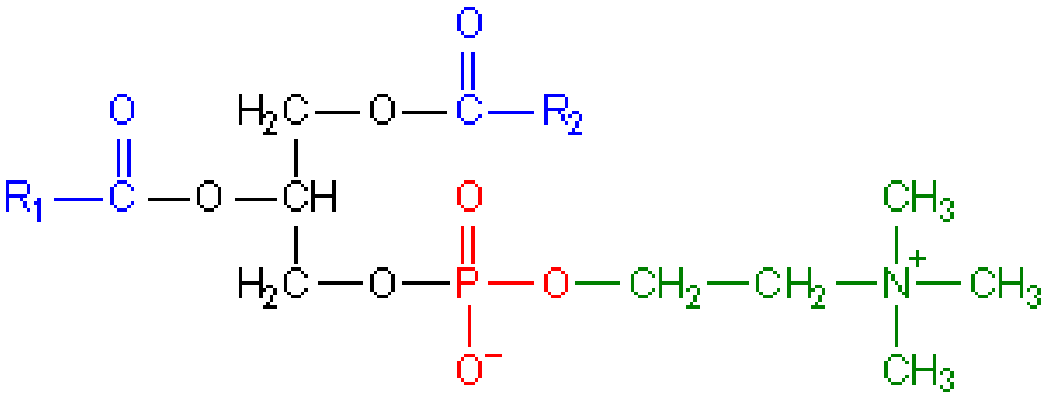

Figure 1.2: Chemical structure of phosphatidylcholine. It incorporates choline as polar head group and is a glycerophospholipid which acts as common membrane lipid. It is the most abundant phospholipid required for the synthesis of membranes in eukaryotic cells (Lacal, 2005).

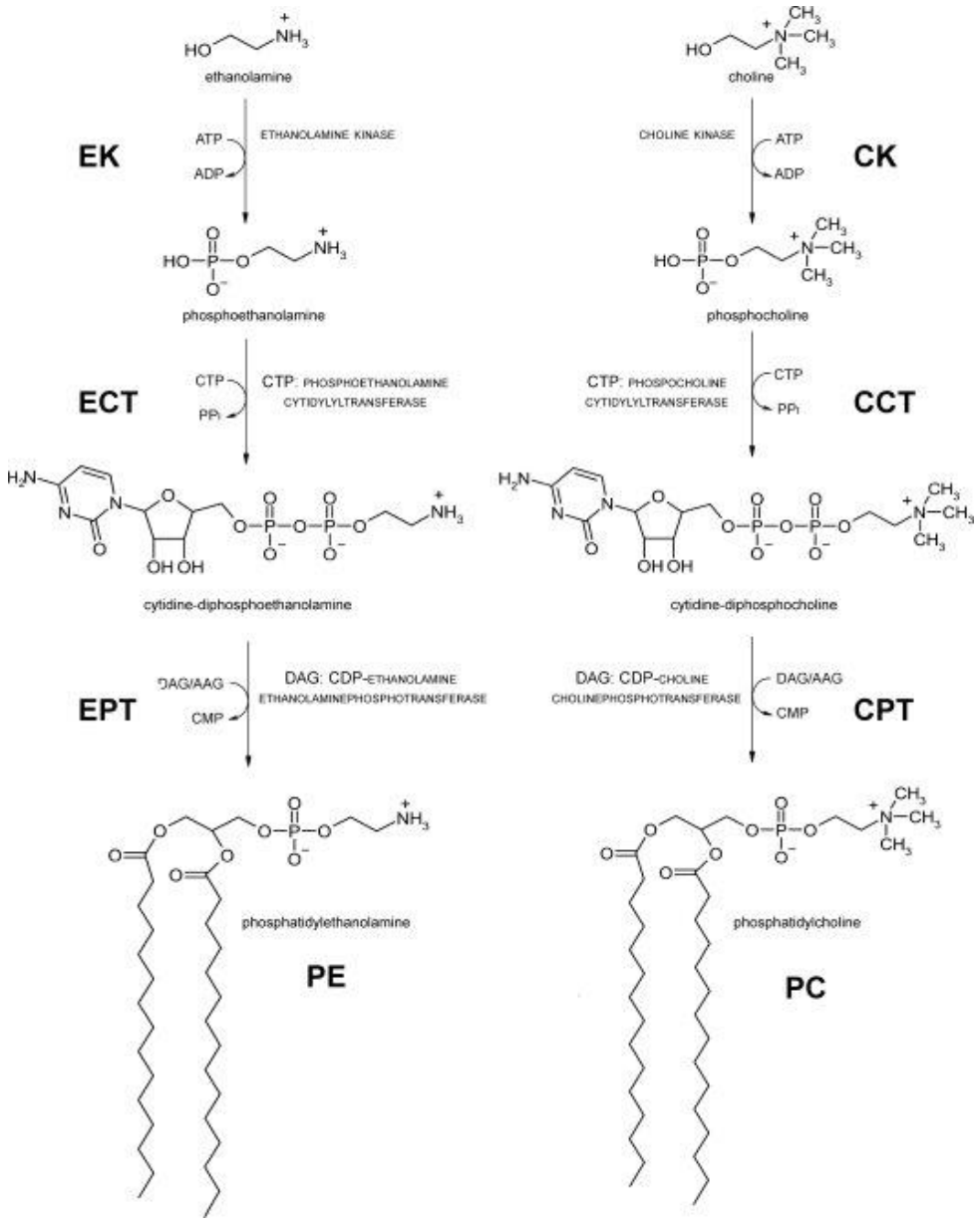

Figure 1.3: Two branches of Kennedy pathway: (i) CDP-ethanolamine pathway and (ii) CDP-choline pathway. First enzyme: ethanolamine kinase (EK), choline kinase (CK). Second enzyme: phosphoethanolamine cytidylyltransferase (ECT), phosphocholine cytidylyltransferase (CCT). Third enzyme: ethanolaminephosphotransferase (EPT), cholinephosphotransferase (CPT) (Gibellini and Smith, 2010).

are synthesized through aminoalcoholphosphotransferase reaction, which uses sn-1,2-diacylglycerol and either novel high-energy intermediates CDP-choline (Kennedy pathway) or CDP-ethanolamine, respectively (Gibellini and Smith, 2010; Fagone and Jackowski, 2013). Hence, the two branches of the Kennedy pathway are often referred to as CDP-choline and CDP-ethanolamine pathway, respectively (Figure 1.3) (Gibellini and Smith, 2010). CDP-choline pathway also exists in some pathogenic bacterial systems (Sohlenkamp *et al*., 2003). However, this pathway is not utilized for PC biosynthesis in those bacteria such as the Gram-negative *Haemophilus influenzae*, *Pseudomonas aeruginosa* and the Gram-positive *Streptococcus pneumoniae*, because they do not have significant PC in their membrane components. Instead, they use this pathway for the synthesis of P-Cho. The P-Cho moiety is transferred most likely from CDP-choline to their cell surface polysaccharides. This P-Cho modification of cell surface constituents seems to be very vital for their survival in the host animals (Aoyama *et al*., 2004).

1.2.1 CDP-Choline Pathway

In most mammalian cells, PC is synthesized mainly via the CDP-choline Pathway (Cui and Vance, 1996). The Kennedy pathway enzymes are found ubiquitously in eukaryotes (Gibellini and Smith, 2010). Although Kennedy pathway is not found in bacteria, but some of its enzymatic components are found and used for the modification of phosphocholine in the components of cell surface (Gibellini and Smith, 2010).

At first, choline kinase (CK) catalyzes the choline phosphorylation to phosphocholine using a molecule of ATP and Mg2+ as cofactor (Figure 1.3) (Aoyama *et al*., 2004; Gibellini and Smith, 2010). Then, in the rate-limiting step of the Kennedy pathway, the phosphocholine cytidylyl transferase (CCT) uses phosphocholine and cytidine-5'-triphosphate to form the high-energy donor CDP-choline with the release of pyrophosphate (Figure 1.3) (Gibellini and Smith, 2010). Cholinephosphotransferase (CPT) catalyzes the final reaction of the pathway, using CDP-choline and a lipid anchor such as DAG or alkyl-acylglycerol (AAG) to form PC and cytidine-5'-monophosphate as byproducts (Figure 1.3) (Aoyama *et al*., 2004; Gibellini and Smith, 2010).

1.3 Choline Kinase (CK)

CK is an enzyme that was discovered in 1953 (Wu and Vance, 2010) which catalyzes the phosphorylation of choline to phosphocholine with the consumption of one molecule of ATP in the presence of Mg2+ as cofactor. This enzyme is important for the biosynthesis of PC, a major phospholipid in eukaryotic membranes (Aoyama *et al*., 2004; Gibellini and Smith, 201; Gallego-Ortega *et al*., 2011; Fagone and Jackowski, 2013). In 1953, Wittenberg and Kornberg first reported the isolation and biochemical characterization of CK and they showed that the enzymatic activity is commonly distributed in different tissues, including liver, brain, kidney and internal mucosa (Fagone and Jackowski, 2013). Besides, generation of phosphocholine from CK activity has been mentioned as an important event in the growth factor-induced mitogenesis in fibroblasts (Ramirez de Molina *et al*., 2002). CK has also been found to cooperate with several mitogens (Ramirez de Molina *et al*., 2002).

1.3.1 Isoforms of Choline Kinase

Two isoforms of *ck* gene, *ckα1 and ckα2* were previously reported from rat liver cDNA library (Uchida, 1994). ckα 1 (50kDa, 435 amino acids ; NCBI accession number NP_997634) and ckα2 (52kDa, 453 amino acids ; NCBI accession number NP_001268) were shown to be derived from the identical gene (*ckα*) by an alternative splicing as amino acid sequence showed about 60% similarity between these two isoforms. These two isoforms are differing by the presence of an 18-residue insert in ckα2 (Aoyama *et al*., 2004). ckß was clearly shown to be the separate gene (*ckß*) product as reported by Aoyama *et al*., 1998 through the cloning of cDNA from rat kidney cDNA library. ckß could encode 45kDa protein of 394 amino acids (NCBI accession number NP_005189) (Aoyama *et al*., 1998; Gruber *et al*., 2012).

Northern and western blots showed that mammalian ckα and ckß isoforms are ubiquitously expressed in different tissues (Gruber *et al*., 2012). There are proofs for different metabolic and biological functions between the two isoforms that has been reported before (Lacal, 2015). One of the differences between the isoforms is that ckα is essential for mouse embryo development while ckβ is non-essential for mouse embryo development (Lacal, 2015). However, there is no evidence that suggests a significant differential role between ckα1 and ckα2 (Lacal, 2015). A significantly high expression of ckα was detected in both testis and liver, whereas a high expression of ckβ was found in heart and liver (Aoyama *et al*., 2004). ckα is mainly involved in cell growth and proliferation (Gallego-Ortega *et al*., 2009). On the other hand, other than having a role in phospholipid biosynthesis, ckß is also involved in muscle development (Kuan *et al*., 2014).

1.4 Choline Kinase and Disease Development

Over the years, CK has been discovered to be involved in many cases of uncontrolled cell proliferation and tumor growth (Lacal, 2015). The elevated PCho and tCho levels that have been detected in human cancers are caused by interplay between severals enzymes, which are at the core of choline metabolism (Glunde *et al*., 2011).

Dual choline and ethanolamine kinase activity are display by both ckα 1 and ckα 2 homodimers with a lower K_m for choline, whereas the ckß homodimer essentially has ethanolamine kinase activity, and the ckα – ckß heterodimer has intermediate substrate specificity (Aoyama *et al*., 2002; Gallego-Ortega *et al*., 2009). The proportions of different homodimer and heterodimer populations are tissue-specific (Aoyama *et al*., 2002), and research with knockout mice showed that ckα loss, but not ckß loss, is embryonically lethal (Sher *et al*., 2006; Wu *et al*., 2008). Although the activity of yeast choline kinase (CKI) can be upregulated by protein kinase A (PKA)-dependent phosphorylation at both Ser30 and Ser85, there has been only one report indicating that PKA-mediated phosphorylation partially regulates human CK (Wieprecht *et al*., 1994). Thus, the upregulation of CK activity in cancer seemingly results from an increase in CKα expression, which would lead to a higher proportion of CKα – CKα dimers in cancer cells and in turn a higher CK activity level than CKα – CKß heterodimers or CKß – CKß homodimers (Glunde *et al*., 2008). Besides, in another research paper, Kuan and his colleagues demonstrate the participation of the protein kinase C (PKC) signaling pathway in the regulation of *ckβ* gene transcription by Ets and GATA transcription factors. They postulate that phorbol-12-myristate-13-acetate (PMA) represses *ckβ* promoter activity through binding of Ets and GATA transcription factors via the

activation of PKC signaling pathway (Kuan *et al*., 2014). Overexpression of CKα has been reported in several human tumour-derived cell lines of several origins, as well as in biopsy samples of lung, colon and prostate carcinomas, among others, which were compared with matched normal tissue from the same patient (Gallego-Ortega *et al*., 2009; de Molina *et al*., 2002; de Molina *et al*., 2007).

Advanced histological tumour grade and negative estrogen receptor status in breast carcinoma correlated with the increased enzymatic activity and overexpression of CKα (de Molina *et al*., 2002), which is consistent with the elevated levels of PCho and tCho that are seen in breast cancers (Gribbestad *et al*., 1999). Conversely, no significant correlation was found with age, tumour size, progesterone receptor status, vascular invasion and histological tumour type, or with expression of p53, ERBB2 or Ki-67, in these breast tumours (de Molina *et al*., 2002). These clinical findings provide CKα as potential prognostic marker of some cancers, such as non-small-cell lung cancer suggesting that CKα expression and activity is directly associated with elevated cancer cell proliferation and malignancy (de Molina *et al*., 2007). CKα expression and activity levels have not yet been studied in metastases.

A part from that, transcriptional control of the regulators of choline metabolism by factors related with cancer has been most clearly demonstrated for CK. For instance, in the liver, the binding of JUN to a distal activator protein 1 (AP1) element in the promoter region of CKα mediates its expression, suggesting a correlation between the function of AP1 in cell proliferation and transformation of CK (Aoyama *et al*., 2007). Besides, there is a close correlation that was observed between region of high total

choline-containing compounds (tCho) levels and hypoxia in vivo (Glunde *et al*., 2008), which was determined to be due to the regulation of CKα expression by the transcription factor hypoxia-inducible factor 1 (HIF1) (Glunde *et al*., 2008). A potential role for the proto-oncogene MYC was suggested in studies that showed increase levels of CK and PCho in Myc+/+ compared with Myc−/− rat fibroblasts (Morrish *et al*., 2009).

Although there is significant sequence identity between ckα and ckß, evidence for different metabolic and biological functions has been reported. The most difference has been found in KO mice studies. CKβ is expressed at a higher level in hindlimb muscle, suggesting that this isoform has a specialized role in this tissue (Fagone and Jackowski, 2013). Removal of CKα results in embryonic lethality as it is necessary for mouse embryo development (Wu *et al*., 2008). Conversely, CKß is dispensable for mouse embryo development and CKß KO mice are viable but acquire rostrocaudal muscular dystrophy (Sher *et al*., 2006) due to reduced PC biosynthesis and increased catabolism of PC (Wu *et al*., 2010). A similar disease has been found in humans correlate to mutations of the *ckß* gene (Mitsuhashi and Nishino., 2013). In humans, genetic mutations of the *ckβ* gene cause congenital muscular dystrophy (CMD), a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy (Mitsuhashi *et al*., 2011).

1.5 Epigenetics

The way of genes being expressed are controls by the human genome that is composed of billions of sequence arrangements containing a bioinformatics code. This code is further dependent upon heritable non-static epigenetic organization of histone

scaffolding that surrounds the DNA and comprises the "epigenome" (Mazzio and Soliman, 2012). Epigenetic processes are vital for development and differentiation processes (Jaenisch and Bird, 2003). Epigenetic mechanisms also protect a DNA sequence against viral genomes that would hijack and change cellular functions (Jaenisch and Bird, 2003). DNA methylation, RNA-associated silencing and histone modification are three systems used to initiate and sustain epigenetic silencing. Unravelling the correlation between these components has led to surprising and rapidly evolving new concepts, showing how they interact and stabilize each other (Egger *et al*., 2004) (Figure 1.4).

1.5.1 DNA Methylation

DNA methylation is found exclusively at cytosine residues in eukaryotes ranging from plants to humans. It involves the addition of a methyl group to the carbon at position 5 of the cytosine ring by DNA methyltransferases (DNMT), generating 5-methyl cytosine (5mC) (Figure 1.5) (Santos *et al*., 2005). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3b with its isoforms, and DNMT3L (Robertson, 2002). Methylation can be de novo (when CpG dinucleotides on both DNA strands are unmethylated) or maintenance (when CpG dinucleotides on one strand are methylated). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful *de novo* methyltransferases (Costello and Plass, 2001). The important roles of this post-synthetic modification through DNMTs have been shown using several mouse experiments. For instance, it is vital for mammalian embryonic development as shown by early lethality in mice that lack DNA methyltransferases (Dnmts) (Li *et al*., 1992; Okano *et al*., 1999). Dnmt-null mice have

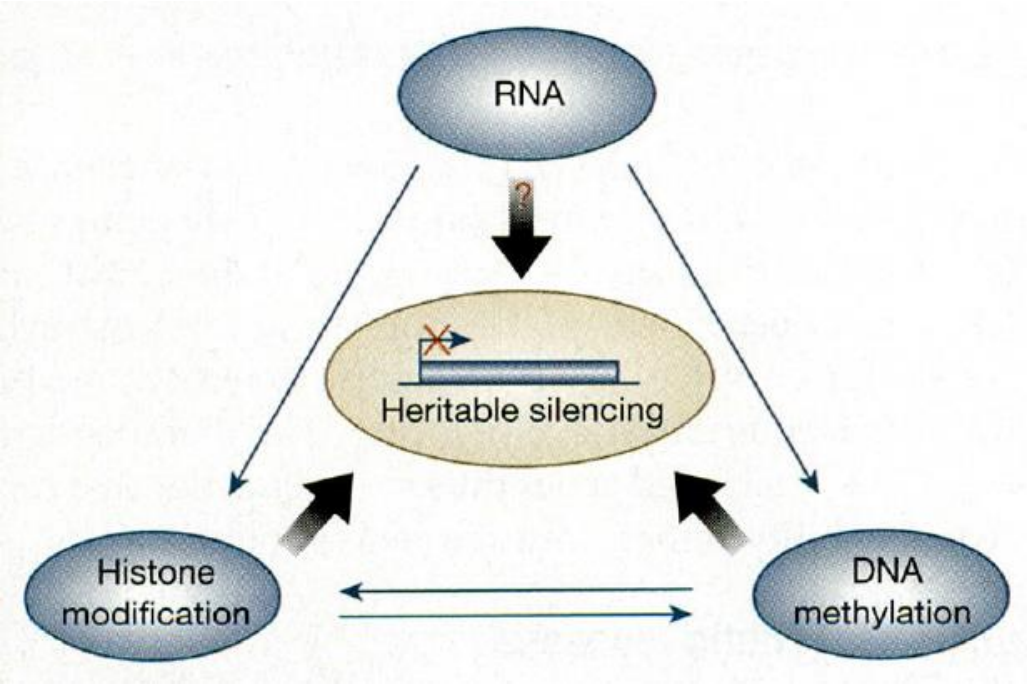

Figure 1.4: Interaction between DNA methylation, RNA and histone modification in heritable silencing. Histone deacetylation and other modifications, especially the methylation of lysine 9 within histone H3 (H3-K9) residues located in the histone tails, cause chromatin condensation and block transcriptional initiation. Histone modification can also attract DNA methyltransferases to begin cytosine methylation, which in turn can reinforce histone modification patterns conducive to silencing. Study with yeast and plants clearly showed the involvement of RNA interference in the establishment of heterochromatin states and silencing. RNA triggering of heritable quiescence might therefore also be involved in higher animals. (Mazzio and Soliman., 2012).

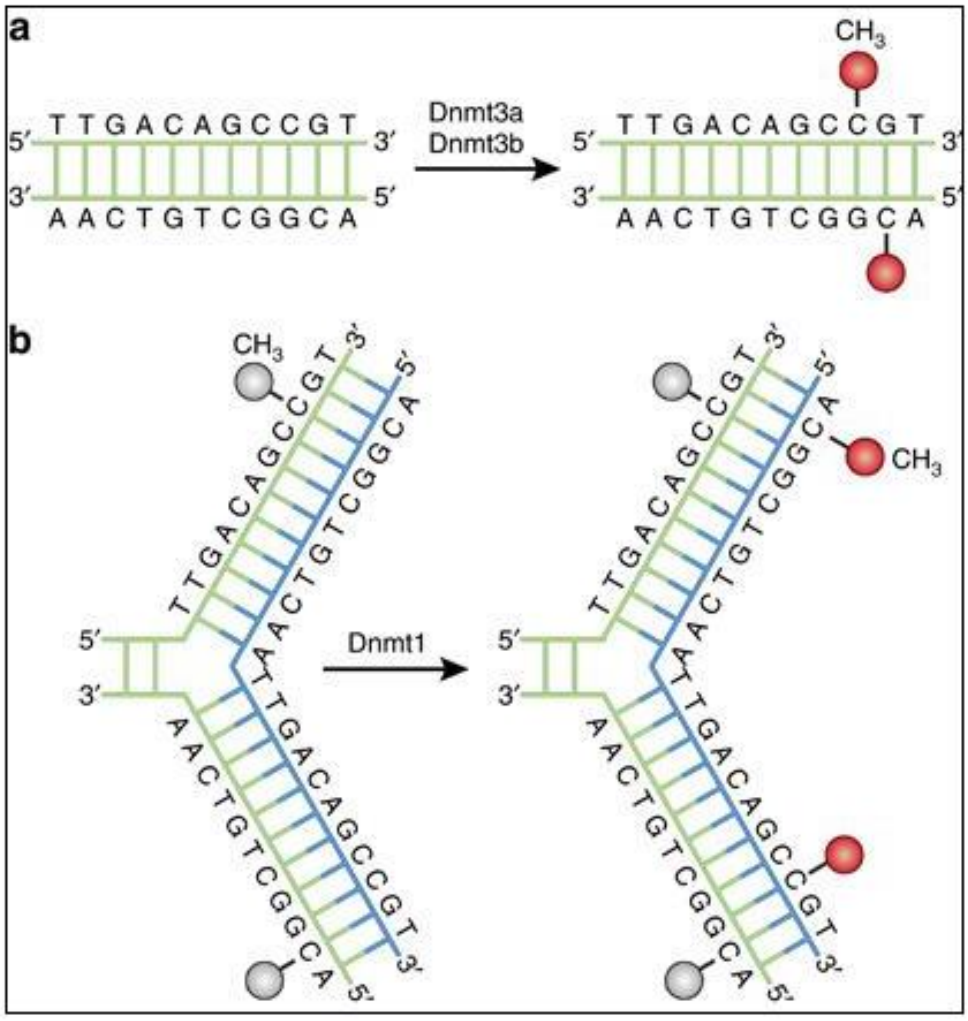

Figure 1.5: DNA methylation. A family of DNA methyltransferases (DNMTs) catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (mC). (a) DNMT3A and DNMT3B are the de novo DNMTs and transfer methyl groups (red) onto newly synthesized DNA. (b) DNMT1 is the maintenance DNMT which preserves DNA methylation pattern during replication. During semiconservative replication, the parental DNA strand retains the original DNA methylation pattern (gray). Subsequently, DNMT1 associates at the replication focus and precisely replicates the original DNA methylation pattern by adding methyl groups (red) onto the newly formed daughter strand (blue) (Moore *et al*., 2013).

reduced DNA methylation levels, but the exact reasons for death during development are unclear. It has been suggested in two research papers that methylation of cytosine residues in the context of CpG dinucleotides could serve as an epigenetic mark in vertebrates (Holliday and Pugh, 1975; Riggs, 1975). Both papers proposed that sequences could be methylated *de novo*, that methylation can be inherited through somatic cell divisions by a mechanism involving an enzyme that recognizes hemimethylated CpG palindromes, that the presence of methyl groups could be interpreted by DNA-binding proteins and that DNA methylation directly silences genes. Although several of these key tenets turned out to be correct, the correlations between DNA methylation and gene silencing have proved to be challenging to resolve (Jones, 2012).

Understanding the roles of DNA methylation requires consideration of the distribution of methylation across the genome. More than half of the genes in vertebrate genomes have short CpG-rich regions known as CpG islands and the rest of the genome is depleted for CpGs (Jones, 2012).

DNA methylation mostly occurs on cytosines that precede a guanine nucleotide or CpG sites. There is confirmation of non-CpG methylation in undifferentiated mouse and human embryonic, however these methylations are lost in develop tissues (Ramsahoye *et al*., 2000). Evidence suggests that DNA methylation of the gene body is correlated with a higher level of gene expression in dividing cells (Hellman and Chess, 2007; Ball *et al*., 2009; Aran *et al.,* 2011), but in slowly dividing and nondividing cells such as the brain cells, gene body methylation is not correlated with increased gene expression (Aran *et al.*, 2011; Guo *et al.*, 2011, b; Xie *et al.*, 2012). More thorough examination of

the murine frontal cortex has recently revealed that although the bulk of methylation occurs within CpG sites, there is a significant percentage of methylated non-CpG sites (Xie *et al.*, 2012). However, the role of non-CpG methylation is still unclear.

1.5.2 DNA Methylation and CpG Promoter

In mammals, methylation is confined to CpG dinucleotides, which are hugely depleted from the genome except at short genomic regions called CpG islands, which are usually located at gene promoters (Ioshikhes and Zhang, 2000). CpG islands are stretches of DNA roughly 1000 base pairs long that have a higher CpG density than the rest of the genome but often are not methylated (Bird *et al*, 1985). Roughly about 70% of CpG islands reside within gene promoters (Saxonov *et al*, 2006). In particular, the CpG islands are often imbedded in promoter of housekeeping genes (Gardiner-Garden and Frommer, 1987). CpG islands within the promoter regions are highly conserved between mice and humans (Illingworth *et al*., 2010).

The location and preservation of CpG islands throughout evolution define that these particular regions possess a functional importance. Impairment of transcription factor binding, recruitment of repressive methyl-binding proteins, and stabilizing the silence gene expression are the effect of methylation within that CpG islands (Klose and Bird, 2006).Few mechanisms have been suggested to account for transcriptional repression by DNA methylation. One of the mechanisms involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters (Das and Singal, 2004). Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NFkB, recognize sequences that

contain CpG residues and binding of each of these TF has been shown to be inhibited by methylation (Singal and Ginder, 1999; Tate and Bird, 1993). The local concentration of CpGs within the promoter could affect the strength of gene repression. Indeed, it is established that methylation of CpG-rich promoters is incompatible with gene activity, yet no conclusive picture has emerged for promoters containing low amounts of CpGs (Boyes and Bird, 1992; Hsieh, 1994). Most CpG island promoters remain unmethylated even in cell types that do not express the gene (Bird, 2002). Nevertheless, changes in DNA methylation linked to tissue-specific gene expression have been seen sporadically on CpG-rich promoters (Song *et al*., 2005).

1.5.3 DNA Methylation and Cancer Development

There was one study postulated that epigenetic changes could influence cancer progenitor cell formation, cancer progression, and formation of stage-specific metastatic cancer (Byler *et al*., 2014). As compared with normal cells, the malignant cells represent major disruptions in their DNA methylation patterns (Baylin and Herman, 2000). It involves both histone modifications and DNA methylation at specific CpG residues. Many oncogenes including *ras* and *src*, become activated by mutations during carcinogenesis (hypermethylation) (Frew *et al*., 2008; Sarkar and Faller, 2011; Mataga *et al*., 2012; Sarkar *et al*., 2013). Conversely, many tumor suppressor genes including both cell-cycle inhibitors and pro-apoptotic genes are silenced by methylation of CpG islands in their promoter sites (hypomethylation) (Heerboth *et al*., 2014). There are several examples of silenced tumor suppressor genes in cancers, for instance, p21, p16, p27, differentiation marker RARβ2, and imprinted pro-apoptotic gene ARHI (in breast

and ovarian cancer) (Monier, 1990). Up to date, there are numerous genes have been found to undergo hypermethylation in cancer (Table 1.1).

Many tumors show some kind of hypermethylation of one or more genes (Table 1). Lung cancer is the cancer type that has been mostly studied and more than 40 genes were found to have several degrees of alteration in DNA methylation patterns. Among the various genes studied, the usually hypermethylated genes include *RAR_*, *RASSF1A*, *CDNK2A*, *CHD13*, and *APC* (Tsou *et al*., 2002). Hypermethylation lead to loss of expression of a variety of important genes for the development of breast cancer such as steroid receptor genes, cell adhesion genes, and inhibitors of matrix metalloproteinases (Yang *et al*., 2001). Genes that commonly associated with hypermethylation in breast cancer are the *p16NK4A*, estrogen receptor (*ER*) alpha, the progesterone receptor (*PR*), *BRCA1*, *GSTP1*, *TIMP-3*, and E-cadherin.

Besides, many leukemia and other hematologic diseases are associated with hypermethylation. Several genes, such as the calcitonin gene, *p15INK4B*, *p21Cip1/Waf1*, the *ER* gene, *SDC4*, *MDR*, and others were seen to be hypermethylated in a variety of hematologic cancers (Leone *et al*., 2002). 65% of myelodysplastic syndromes were found to have hypermethylation in calcitonin

Table 1.1: Several genes commonly methylated in human cancer and their role in tumor development. Abbreviations: *APC,* adenomatous polyposis coli; *BRCA1,* breast cancer 1; *CDKN2A/p16,* cyclin-dependent kinase 2A; *DAPK1,* death-associated protein kinase 1; *ER,* estrogen receptor; *GSTP1,* glutathione S-transferase Pi 1(Das and Singal, 2004).

Gene	Role In Tumour Development	Site of Tumour	References
APC	Deranged regulation of cell proliferation, cell migration, cell adhesion, cytoskeletal reorganization and chromosomal stability	Breast Lung Esophageal	Virmani *et al*., 2001 Kawakami *et al*., 2000
BRCA1	Implicated in DNA repair and transcription activation	Breast Ovarian	Kawakami *et al*., 2005 Chan *et al*., 2002
CDKN2A/p16	Cyclin-dependent kinase inhibitor	Gastrointestinal Head and neck	Herman *et al*., 1995 Sanchez-Cespedes *et al*., 2000
DAPK1	Calcium/calmodulin-dependent enzyme that phosphorylates serine / threonine residues on protein; Suppression of apoptosis	Lung	Harden *et al*., 2003
E-cadherin	Increasing proliferation, invasion, and/or metastasis	Breast Thyroid Gastric	Graff *et al*., 1995 Graff *et al*., 1998 Waki *et al*., 2002
ER	Hormone resistance	Breast Prostate	Yang *et al*., 2001 Li *et al*., 2000
GSTP1	Loss of detoxification of active metabolites of several carcinogens	Prostate Breast Renal	Lee *et al*., 1994 Esteller *et al*., 1998 Esteller *et al*., 1998

gene and *p15*. Additionally, detection of *p15* methylation at diagnosis was associated with lower survival rate (Leone *et al*., 2002). Hypomethylation is the second type of methylation irregularity that is seen in a wide variety of malignancies (Feinberg and Vogelstein, 1983; Kim *et al*., 1994). Progressive increase in the grade of malignancy can be seen in a number of cancers such as breast, cervical, and brain and all of these cancer types are closely related to global hypomethylation (Ehrlich., 2002). Long interspersed nuclear elements are the most plentiful mobile DNAs or retrotransposons in the human genome. Hypomethylation of these mobile DNAs causes transcriptional activation as has been found in many types of cancer including urinary bladder cancer (Jürgens *et al*., 1996) and it also causes disruption of expression of the adjacent genes as well (Das and Singal., 2004).

1.6 Methylating and demethylating agents

DNA methylation, histone modification, nucleosome remodeling, and RNA-mediated targeting has been deeply studied over the last few years as these events are involved in the regulation of biological processes that are essential to the genesis of cancer (Dawson and Kouzarides, 2012). Epigenetic modifications are appealing to be as therapeutic targets in cancer due to their dynamic nature and potential reversibility (Einav Nili *et al*., 2008). Various compounds that alter DNA methylation patterns are recently being examined as single agents or in combinations with other drugs in clinical settings, such as budesonide, a DNA hypermethylating agent and 5-Azacytidine, a DNA hypomethylating agent (Einav Nili *et al*., 2008).

1.6.1 Budesonide

Budesonide (Figure 1.6) is a hypermethylating agent and known synthetic glucocorticoid with clinically significant anti-inflammatory effects (Tao *et al*., 2002; Orta *et al*., 2010). It is a glucocorticoid used to control mild-to-moderate persistent asthma (Pereira *et al*., 2007). Budesonide has been shown to prevent the formation of lung adenomas and adenocarcinomas in mice when administered either by inhalation or in the diet (Wattenberg *et al*., 1997; Estensen *et al*., 2004). As a chemopreventive agent, budesonide appears to reduce both the growth rate of tumors and the progression of adenomas to adenocarcinomas through hypermethylation of genes (Pereira *et al*., 2007). When budesonide was administered into mice with lung tumors, very rapidly, within days, the methylation of tumor DNA was increased and resulting in DNA that was no longer hypomethylated (Pereira *et al*., 2007).

1.6.2 5-Azacytidine

Even though 5-azacytidine (Figure 1.7) has been known to have cytotoxic an effect on cancer cells since 1968, its mechanism of action was discovered more recently (Kaminskas *et al*., 2005). 5-Azacytidine is a cytidine analog, with a nitrogen atom in the place of carbon 5. In molecular level, it is phosphorylated, incorporated into DNA during replication and recognized by DNMT1. Thus, the normal reaction involving the transfer of a methyl group starts to take place (Heerboth *et al*., 2014). There will be a formation of an irreversible DNMT1–aza linkage due to the nitrogen group in the fifth position which triggers the degradation of the enzyme and leads to widespread reductions in methylation (hypomethylation) (Santi *et al*., 1984; Momparler *et al*., 2005). Rapidly dividing cancer cells are more susceptible to its effects as aza are more

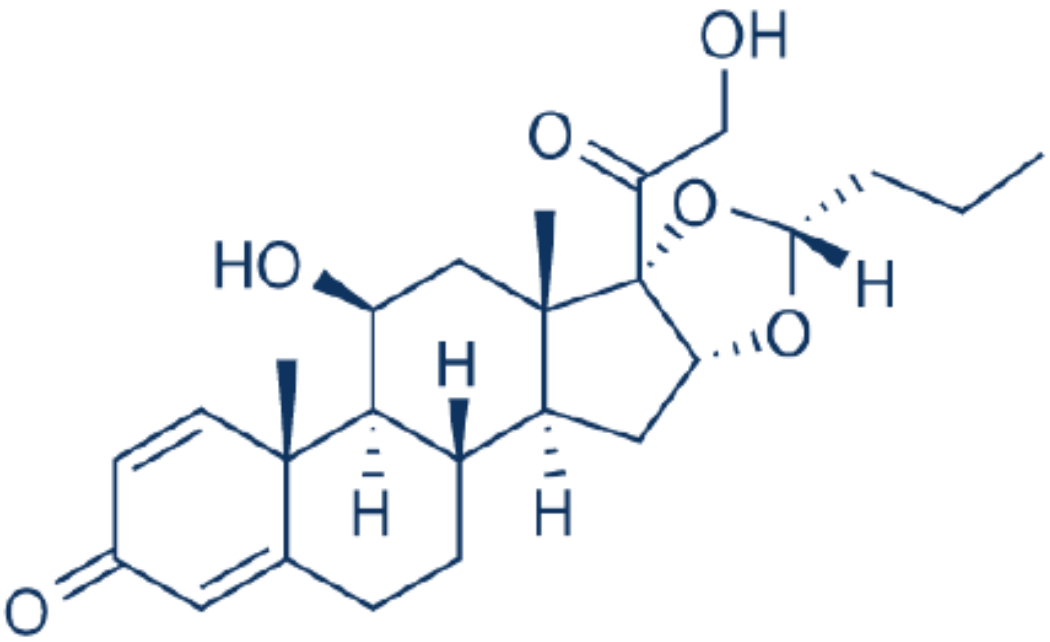

Figure 1.6: The chemical structure of budesonide. Budesonide is a synthetic anti-inflammatory glucocorticoid which acts as a hypermethylating agent. (http://www.drugfuture.com/pharmacopoeia/usp32/pub/data/v32270/usp32nf27s0_m10458.html)

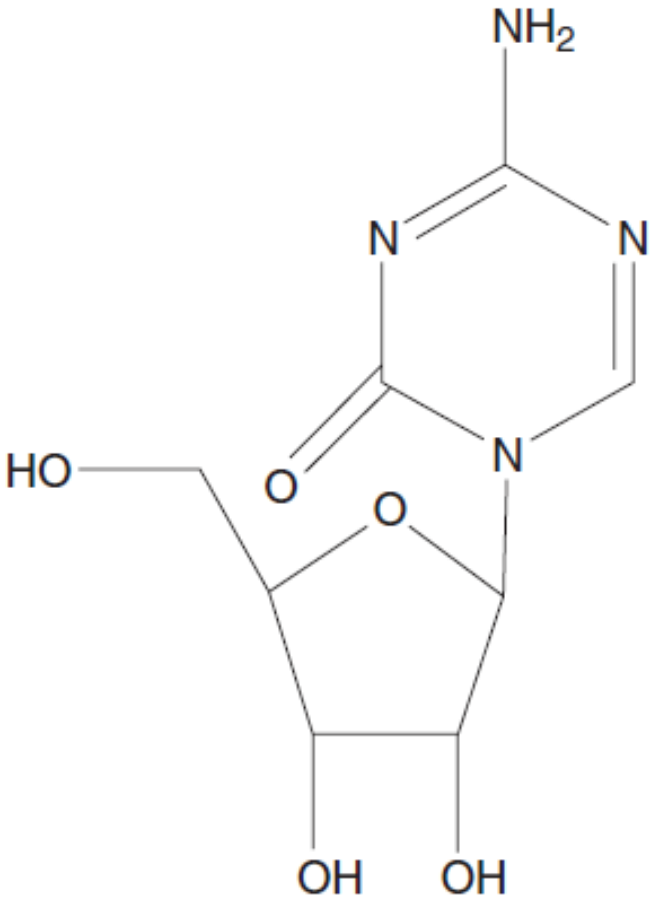

Figure 1.7: Molecular structure of 5-Azacytidine. 5-Azacytidine is a pyrimidine ring analogue in which the ring carbon 5 is replaced by nitrogen (Kaminskas *et al*., 2005).

1.7 Rationale of the study

The function of CK was shown to be essential for the biosynthesis of phosphatidylcholine. ckα and ckβ enzymes have been implicated in pathogenesis of cancer, muscular dystrophy and bone deformities, thus they become a potential target for therapy (Wu and Vance, 2010). However, the exact mechanisms or involvement of CK in disease development are still remaining unclear. The ckα has been reported to be overexpressed in 70% of human tumors. The pharmacological inhibition of ckα has been proposed as a novel anti-tumoral strategy (Gallego-Ortega *et al*., 2009).

A known epigenetics mechanism, DNA methylation can control gene expression by modulating the activity of a promoter either by increasing or reducing the promoter activity. Even though there are many previous studies that demonstrated the association between CK and cancer pathogenesis, up to date, the effects of hypomethylation and hypermethylation on the CK promoters are not yet explored. Budesonide (hypermethylating agent) and 5 Azacytidine (demethylating agent) are known to alter the pattern of gene expression (Santi *et al*., 1984; Momparler *et al*., 2005; Orta *et al*., 2010). Hypomethylation is demonstrated to stimulate gene expression, while hypermethylation causes gene repression.

Cis-acting transcriptional regulatory elements of a promoter contain recognition sites for trans-acting DNA-binding transcription factors, which function either to enhance or repress transcription (Cooper, 2000; Maston *et al.*, 2006). The presences of multiple regulatory elements within promoters are essential to control the gene regulation, which exponentially increases the potential number of unique expression patterns (Maston *et*