MICRORNA REGULATION OF HUMAN CHOLINE KINASE ALPHA GENE EXPRESSION AND FUNCTION IN CANCER CELL LINES

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by

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

>	More than
<	Less than
×	Multiply by
~	Approximately
%	Percentage
°C	Degree Celsius
α or a	Alpha
β or b	Beta
µg	Microgram
µl	Microliter
µM	Micromolar
A260	Absorbance at 260 nm
A260/280	Absorbance ratio at 260 and 280 nm
ADP	Adenosine diphosphate
AKT	Serine/threonine kinase
AMO	Anti-miRNA oligonucleotide
APS	Ammonium persulfate
AR	Androgen receptor
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
APS	Ammonium persulfate
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
CCl_4	Carbon tetrachloride
CDP	Cytidine-diphosphate
CDP-Cho	Cytidine-diphosphocholine
chk	Choline kinase (mRNA or gene)
CHKA	Choline kinase alpha (protein)
CHKB	Choline kinase beta (protein)
chka	Choline kinase alpha (mRNA or gene)
chkb	Choline kinase beta (mRNA or gene)
CK	Choline kinase (protein)
CKI	Choline kinase inhibitor
CL	Cardiolipin
cm^2	Centimetre squared
CO_2	Carbon dioxide
c-Src	Proto-oncogene tyrosine protein kinase Src
C_t	Threshold cycle
CTP	Cytidine-5'-triphosphate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

dT	Deoxy-thymine nucleotides
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* - 'for example'
EGFR	Epidermal growth factor receptor
EK	Ethanolamine kinase (protein)
ek	ethanolamine kinase (gene)
ER	Endoplasmic reticulum
ESCC	Esophageal squamous cell carcinoma
et al.	*et alii* - 'And others'
EtBr	Ethidium bromide
Ets	E26 transforming sequence
FBS	Fetal bovine serum
g	Gravity
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine cytosine
HC-3	Hemicholinium-3
HCl	Hydrochloric acid
HeLa	Human cervical adenocarcinoma cell line
HepG2	Human hepatocarcinoma cell line
HIF-1α	Hypoxia-inducible transcription factor 1α
H-MMuLV	H Minus Moloney Murine Leukemia Virus
HRE	Hypoxia response elements
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
kb	Kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
LB	Luria Bertani
MCF7	Human breast adenocarcinoma cell line
mg	Milligram
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
miRNAs	MicroRNAs
mL	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
ncRNAs	Non-coding RNAs
nM	Nanomolar
nm	Nanometer
NSCLC	Non-small-cell lung cancer

nt	nucleotide
OD	Optical density
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCho	Phosphocholine
PCR	Polymerase chain reaction
PE/PtdEtn	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEMT	Phosphatidylethanolamine methyltransferase
PG	Phosphatidylglycerol
PI/PtdIns	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
piRNAs	Piwi-interacting RNAs
PKA	Protein kinase A
PKC	Protein kinase C
pre-miRNAs	Precursor miRNAs
pri-miRNAs	Primary miRNAs
PS/PtdSer	Phosphatidylserine
psi	Pound-force per square inch
qPCR	Quantitative real-time PCR
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RhoA	Ras homolog gene family, member A
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPS18	40S Ribosomal Protein S18
rRNAs	Ribosomal RNAs
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
siRNA	Small interfering ribonucleic acid
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Tm	Melting temperature
tRNA	Transfer RNA
U	Units
UTR	Untranslated region
UV	Ultraviolet
V	Volt
v/v	Volume/volume
VLDL	Very Low-Density Lipoprotein
X	Times
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

PENGAWALATURAN EKSPRESI DAN FUNGSI GEN KOLINA KINASE ALFA MANUSIA OLEH MIKRORNA DALAM TITISAN SEL KANSER

ABSTRAK

Kolina kinase alfa (CHKA) ialah enzim pertama dalam tapak jalan Kennedy yang mempunyai fungsi bukan pemangkin dalam perkembangan tumor. Peningkatan ekspresi *chka* merupakan ciri klinikal dalam sel malignan dan tisu kanser. MikroRNA (miRNA) adalah pengawalatur postranskripsi ekspresi gen yang efisien. Sehingga kini, pengawalaturan ekspresi gen *chka* oleh miRNA tidak pernah dilaporkan. MiRNA ialah molekul RNA kecil bertetali tunggal dengan kepanjangan ~22 nukleotida yang berfungsi sebagai pengawalatur postranskripsi ekspresi gen. Objektif kajian ini adalah untuk mengeksploitasi kebolehan pengawalaturan ekspresi gen oleh miRNA yang mensasarkan *chka* untuk menurunkan ekspresi *chka* di dalam sel kanser payudara (MCF7), servik (HeLa) dan hati (HepG2) serta mengkaji kesan penurunan tahap protein CHKA terhadap proses sel. miRNA yang menunjukkan potensi tinggi untuk berinteraksi dengan bahagian 3'UTR gen *chka* telah diramal menggunakan perisian bioinformatik atas talian, seterusnya disenaraipendekkan dengan kriteria pemilihan yang ditetapkan dan calon miRNA terpilih telah diuji secara *in vitro* untuk mengkaji interaksi miRNA:mRNA dalam sel kanser. miR-32-5p, miR-367-3p dan miR-876-5p masingmasing menunjukkan interaksi kuat dengan mRNA *chka* secara *in silico* dengan tenaga bebas minimum (MFE) yang lebih rendah daripada -1.00 kcal/mol dan memenuhi kriteria-kriteria lain yang menunjukkan interaksi miRNA:mRNA yang kuat. Perlekatan ketiga-tiga miRNA tersebut pada tapak benih di 3'UTR mRNA *chka*

telah disahkan secara eksprimen dalam sel MCF7, HeLa dan HepG2 menggunakan asai lusiferase. Kesan interaksi miRNA: mRNA terhadap ekspresi *chka* pada tahap mRNA dan protein telah dikaji dengan qRT-PCR dan blot western. Fungsi sel yang terkesan oleh perubahan ekspresi *chka* yang diaruh oleh miRNA pula telah dikaji menggunakan asai apoptosis sel, asai kitar sel dan asai penyembuhan luka. Berbanding dengan kawalan negatif, tahap ekspresi *chka* dalam HeLa, HepG2 dan MCF-7 selepas transfeksi miR-32-5p, miR-367-3p dan miR-876-5p ialah 0.47, 0.62 dan 0.76 kali ganda masing-masing dan ini menunjukkan miR-32-5p menyebabkan penurunan yang paling ketara. Kesan yang hampir sama iaitu penurunan *chka* sebanyak 0.33 kali ganda dalam sel MCF7 juga diperolehi apabila miR-32-5p dan miR-876-5p digabungkan. Transfeksi miR-32-5p juga menghasilkan penurunan tahap protein CHKA yang paling ketara dalam MCF7 (0.20 kali ganda) dan HeLa (0.46 kali ganda) berbanding miRNA lain yang telah diuji. Spesifisiti mimik miRNA terhadap *chka* telah disahkan menggunakan asai pelapor lusiferase *firefly* dan rawatan bersama dengan perencat spesifik miRNA. Ketiga-tiga miRNA dan pasangan miR-32-5p+miR-367-3p masing-masing menyebabkan ~50% sel HeLa dan MCF7 mengalami apoptosis. Mir-367-3p menyebabkan kitar sel MCF7 terhenti pada fasa G_0/G_1 dengan paling ketara (77% daripada keseluruhan populasi sel). Fungsi penyembuhan luka jatuh mendadak kepada <50% kawasan luka sembuh walaupun selepas 96 jam pada sel MCF7, HeLa dan HepG2 yang dirawat dengan miR32-5p secara tunggal atau secara kombinasi dengan miR-367-3p. Data yang dikutip telah membuktikan hubungan songsang antara ketiga-tiga calon miRNA yang dikaji dengan tahap ekspresi gen *chka*. Hasil kajian juga menyokong potensi miR-32-5p, miR-367-3p dan miR-876-5p sebagai penanda biologi untuk prognosis awal penyakit kanser dan sebagai penawar bersifat anti-kanser pada jenis kanser yang menunjukkan ekspresi *chka* yang lebih tinggi.

MICRORNA REGULATION OF HUMAN CHOLINE KINASE ALPHA GENE EXPRESSION AND FUNCTION IN CANCER CELL LINES

ABSTRACT

Choline kinase alpha (CHKA), the first enzyme of Kennedy pathway, has noncatalytic function in tumour onset and progression. Overexpression of *chka* is a clinical feature in malignant cells and cancerous tissues. microRNAs (miRNAs) are efficient posttranscriptional regulators of gene expression. To date, the regulation of *chka* gene expression by miRNAs has never been reported. The objective of this work was to exploit the gene regulatory capabilities of *chka*-targeting miRNAs to downregulate *chka* expression in breast (MCF7), cervical (HeLa) and liver (HepG2) cancer cells and study the effects of the downregulated CHKA protein levels on cellular processes. miRNAs exhibiting increased potential of interacting with the 3'UTR of *chka* gene were predicted using online available bioinformatic tools, shortlisted based on predefined selection criteria and the shortlisted miRNA candidates were tested *in-vitro* to study the miRNA:mRNA interaction in cancer cells. miR-32-5p, miR-367-3p and miR-876-5p each exhibited strong *in silico* interaction with *chka* mRNA, releasing free energy (MFE) lower than -1.00 kcal/mol and fulfilling other predefined criteria for determining strong miRNA:mRNA interactions. Binding of the selected three miRNAs with respective seed sites on 3'UTR of *chka* mRNA was experimentally validated in MCF7, HeLa and HepG2 cells by luciferase assay. Effects of miRNA:mRNA interaction on *chka* expression at mRNA and protein levels were examined by qRTPCR and western blot. Cellular functions impacted by miRNA mediated differential *chka* expression were studied by cell apoptosis assay, cell cycle assay and wound healing assay. Compared to negative control, *chka* expression levels, in HeLa, HepG2 and MCF7, as a result of transfection with miR-32-5p, miR-367-3p

and miR-876-5p were 0.47-fold, 0.62-fold, and 0.76-fold, respectively in MCF7; 0.32-fold, 0.38-fold, and 0.63-fold, respectively in HeLa; 0.44-fold, 0.49-fold and 0.51-fold, respectively in HepG2 indicating maximum reduction was inflicted by miR-32-5p. Similar effect was observed when miR-32-5p was combined with miR-876-5p, which resulted in 0.33-fold *chka* downregulation in MCF7 cells. Correspondingly, transfection of miR-32-5p also produced the strongest suppression of CHKA protein levels in MCF7 (0.20-fold) and HeLa (0.46-fold) compared to other miRNAs tested. The specificity of miRNA mimics towards *chka* was verified by the firefly luciferase reporter assay and co-treatment with miRNA specific inhibitors, which led to reversal of the decreased *chka* levels produced by the miRNAs. The three miRNAss and the pair of miR-32-5p+miR-367-3p each initiated ~50% cells to undergo apoptosis in HeLa and MCF7 cells. Maximum (77% of cell population) cell cycle arrest at the G_0/G_1 phase was prompted by miR-367-3p in MCF7. Wound repair function plummeted with <50% wound area healed even after 96 hrs in MCF7, HeLa and HepG2 treated with miR-32-5p alone and in combination with miR-367-3p. The data collected in this study showed an inverse relationship between the three miRNA candidates and *chka* expression level, thus, proving the potential of miR-32-5p, miR-367-3p and miR-876-5p as biomarkers for early cancer prognosis and anti-cancer remedies in cancer types with over expressed *chka* as a clinical observation.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Choline Kinase (CK) is the first biological catalyst that phosphorylates extracellular choline metabolite to generate phosphorylcholine (PCho), illustrating the first step of Kennedy pathway that bio-fabricates phosphatidylcholine (PC), a component critical for eukaryotic cell membrane structure (McMaster, 2018). Human choline kinase enzyme is encoded by two different genes choline kinase alpha (*chka)* and choline kinase beta (*chkb)*, both these subunits are 60% alike. However, *chka* further undergoes alternative splicing to produce two variants, *chka1* and *chka2*. The splice variants code for the proteins CHKA1 and CHKA2 respectively. CHKA2 subunit consists of 18 amino acids more than the CHKA1 protein subunit (Malito et al., 2006). Although all the CK isozymes share the same catalytic activity, physiologically, CHKA plays a critical role in regulation of apoptosis and cell cycle, cell proliferation and transformation (Ramírez de Molina et al., 2008) whereas the CHKB counterpart is involved in bone homeostasis and muscle development (Suzuki et al., 2020). Increased expression and activation of CHKA triggered an altered choline metabolism profile and increased phosphocholine (PCho) content, distinctly observed in most cancers (Anna Granata et al., 2015). Overexpression of choline kinase-alpha is a predominant feature of varied cancer types, namely breast, lung, colorectal, prostate (Arlauckas et al., 2016). Ravaging implications of increased CHKA levels on cell growth, proliferation, initiation, and progression of cancer demonstrated the enzymes' oncogenic nature, characterising overexpression of CHKA as the prognosis marker for cancer detection and indicator of tumour response to anti-cancer therapies (Z. Chen et al., 2016). Inhibition of CHKA, has since been considered a promising therapeutic

approach attracting extensive scientific attention. Choline kinase inhibitors (CKI) MN58b (Mazarico et al., 2016), RSM-932A (Lacal and Campos, 2015) have entered phase I clinical trials and newly designed CHKA inhinitors: EB-3D and EB-3P under extensive research due to positive results on HepG2 cells (Sola-Leyva et al., 2019). Downregulation of CHKA expression with shRNA (Krishnamachary et al., 2009) and CHKA gene silencing RNA interference (RNAi) triggered apoptosis and selectively inhibits cancer cell growth whilst safeguarding normal cells (Glunde et al., 2005). However, miRNA effected regulation of CHKA expression is yet to be thoroughly explored.

Functionally mature miRNAs belong to a family of small (18-22 nucleotide), noncoding single stranded RNA molecules that function as posttranscriptional regulators of gene expression and are reported to control the activity of nearly 30% of protein coding genes. miRNAs operate primarily by binding to the complementary sequences of the 3' untranslated region (3'UTR) of target mRNA thereby causing either degradation of target mRNAs or inhibition of protein translation (Kuhn et al., 2008). Since their discovery in the early 1990s, miRNAs have observed significant roles in cellular defence mechanisms, signalling pathways, tumorigenesis, and cell death (Schickel et al., 2008). Importantly, individual miRNAs can act upon numerous target mRNAs and every mRNA can be targeted by multiple miRNAs, allowing for
enormous combinatorial complexity and regulatory potential (Elton & Yalowich, 2015). Research to employ miRNAs as potential prognostic biomarkers for cancer detection has been initiated following extensive data suggesting dysregulation of miRNA expression in the diseased state clinical samples (Hwang & Mendell, 2006). It is, therefore, vital to determine miRNAs targeting a specific gene and experimentally validate the effect of its miRNA-mRNA interactions on the biological functions

modulated by the target gene to verify its therapeutic potential (Elton & Yalowich, 2015).

The fundamental goal of this dissertation was to take advantage of the gene regulatory capabilities of *chka* targeting miRNAs to deregulate CHKA expression in HeLa, HepG2 and MCF7 cancer cells and study the advantageous effects of the downregulated CHKA levels on cellular processes modulated by CHKA in the oncogenic state. miRNAs harbouring the potential to significantly downregulate *chka* expression by binding onto the 3'UTR of *chka* mRNA were predicted using online software and shortlisted based on the predefined criteria to obtain a few of the most qualified miRNAs which were tested for their *chka* deregulating capabilities *in vitro* in HeLa, HepG2 and MCF7 cancer cell lines each representative of a distinct cancer type. Following the successful miRNA mediated downregulation of *chka* expression, its impact on the cellular functions governed by *chka* namely, cell apoptosis, cell cycle progression and wound repair were scrutinised.

1.2 Problem Statement and Research Objective

Overexpression of *chka* is a clinical feature observed in several cancer types. Dysregulated miRNAs have been reported to be responsible for varied disease conditions. Yet, regulation of *chka* by miRNAs in cancer has not been thoroughly explored.

Objective: This study was designed to identify the potential miRNAs that efficiently regulate the *chka* expression, and the effect of the miRNA mediated differential *chka* expression on CHKA participating cell signalling pathways – apoptosis pathway, cell cycle regulation and wound repair activity.

1.2.1 Specific Research Objectives

1. To predict (*in silico*) the potential miRNAs that regulate the expression of human *chka.*
2. To test the effect of bioinformatically predicted miRNA mimics on the expression of *chka* at mRNA level and protein level.
3. To validate the potential *chka*-targeting miRNAs by transfection of miRNA specific inhibitors.
4. To verify the binding of potential miRNAs to the 3'-UTR of *chka* mRNAs by luciferase assay.
5. To investigate the combinatorial effect of multiple miRNAs on the expression of *chka.*
6. To study the effect of downregulated CHKA protein expression on the biological functions in cancer cell lines.

1.2.2 Research Plan Outline

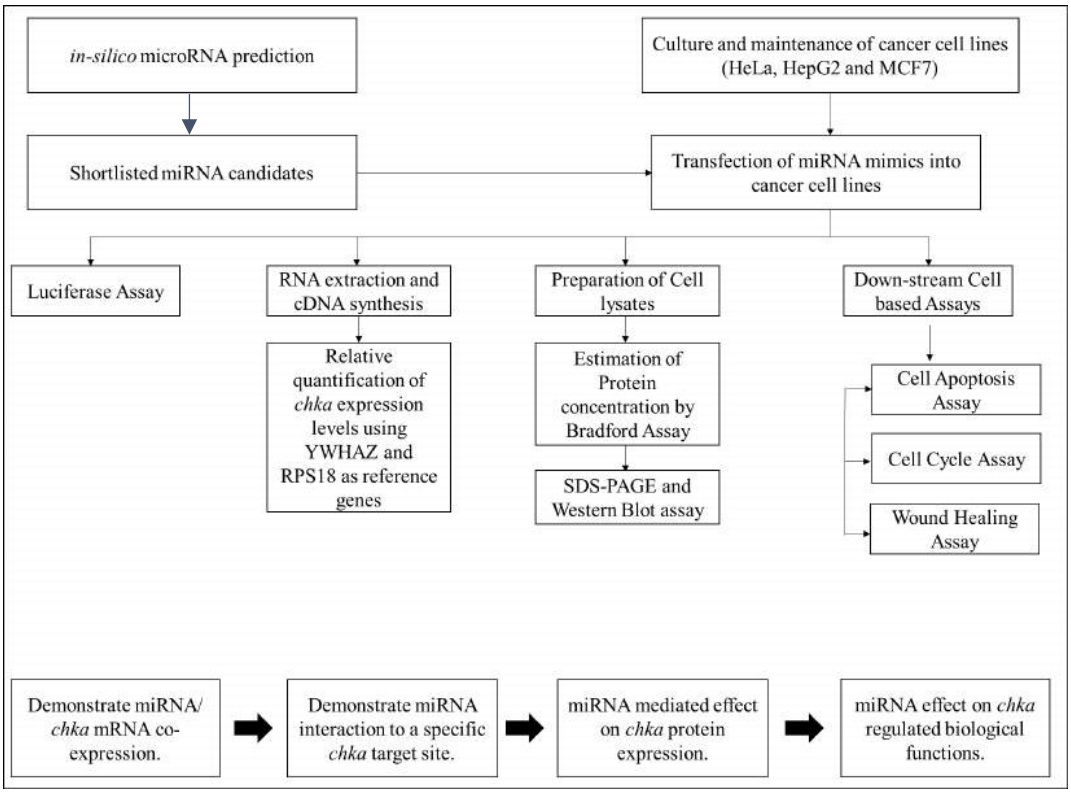

Figure 1.1 Experimental workflow for the study

1.3 Literature Review

1.3.1 Phosphatidylcholine (PC)

Phosphatidylcholine (PC), also known as lecithin, is the most abundant lipid (>50%) in a eukaryotic cell membrane but only 10% bacteria have PC comprised membranes (Sohlenkamp et al., 2003). Along with a predominant function in the framework of cell membranes and lipoproteins via formation of a bilayer matrix (Vance, 2008), PC is also involved in signal transduction as lipid secondary messengers (Exton, 1994) and is vital for cell viability (Esko et al., 1982). Within major phospholipids, PC has a special status because of its (1) bilayer – promoting properties and (2) zwitterionic state at physiological pH (Sohlenkamp et al., 2003). PC is specifically required for very low-density lipoprotein (VLDL) which cannot be substituted for by any other phospholipid moiety (Robinson et al., 1989).

1.3.2 Biosynthesis of PC

The cellular laboratory fabricating most membrane lipid components including phosphatidylcholine is centralized in the endoplasmic reticulum (Ballas & Bell, 1981). PC synthesis occurs via three distinct pathways: one is the Kennedy pathway for *de novo* biosynthesis of PC and phosphatidylethanolamine (PE) (Figure 1.2), the enzymes for which are ubiquitously present in all cells (Kanno et al., 2007). Formation of high energy intermediates CDP-choline for the end-product PC and CDP-ethanolamine for PE led to the two branches of the Kennedy pathway (Gibellini & Smith, 2010). The second is the Lands cycle, in which phospholipase A2 removes fatty acids at the sn-2 position of PC, which results in the formation of lysophosphatidylcholine. This can be used in a reverse reaction, the addition of a fatty acid at the sn-2 position, to yield PC. This re-acylation is catalyzed by lysophosphatidylcholine acyltransferases (Moessinger et al., 2014).

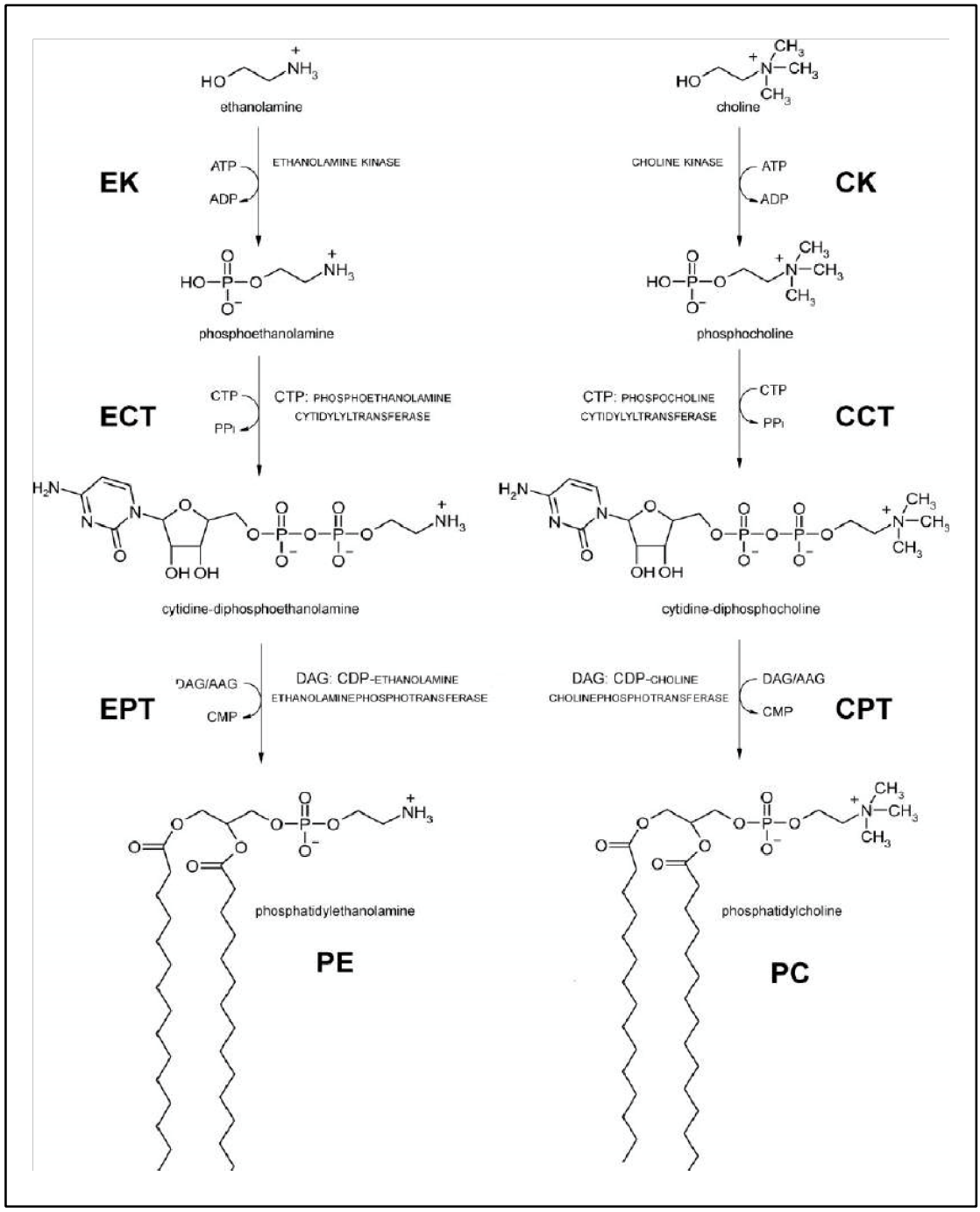

Figure 1.2 Kennedy Pathway. Biochemical reactions and the enzymes involved in the two branches of the Kennedy pathway for biosynthesis of PC and PE (Gibellini & Smith, 2010).

The third pathway for PC biosynthesis is via sequential methylation of phosphatidylethanolamine by phosphatidylethanolamine-N-methyltransferase (PEMT) (Kanno et al., 2007). PC biosynthesis via the PEMT pathway is restricted to the liver cells (Moessinger et al., 2014). Activated PEMT is localised in the endoplasmic reticulum where the enzyme synthesizes PC resultant of three repeated methylation reactions of PE with the methyl groups donated by S-adenosylmethionine for every reaction. A molecule of S-adenosylhomocysteine is formed for each PC molecule generated (Kelly & Jacobs, 2011). ~30% hepatocytic PC is contributed by the PEMT pathway, the remaining 70% being supplied by CDP-Choline pathway (Moessinger et al., 2014). However, in choline limiting diets, PEMT pathway is crucial for maintaining the PC supply in liver (Vance, 2014).

CDP-Choline Pathway: The uptake of exogeneous 'choline' by the cell (Figure 1.3) marks the commencement of the Kennedy pathway and its phosphorylation to phosphocholine by the cytosolic enzyme 'choline kinase' is the first step of the CDP-Choline Pathway. This step utilizes ATP (Adenosine Tri Phosphate) as the source of the phosphate group leaving ADP (Adenosine Di Phosphate) as a by-product of the reaction (Brophy et al., 1977). The next step, the rate limiting step, is a reaction between Phosphocholine and CTP (cytidine Tri Phosphate) catalysed by the nuclear/cytosolic CTP: Phosphocholine cytidylyltransferase (CCT) to yield a high energy donor CDP-Choline (Cytidine Diphosphate-Choline) along with the release of a pyrophosphate (Gibellini & Smith, 2010; Kennedy & Weisst, 1956).

The substitution of cytidine monophosphate in CDP-Choline by a 1,2-diacylglycerol moiety (product of phosphatidate phosphatase activity on phosphatidic acid) catalysed by CPD-choline:1,2-diacylglycerol choline phosphotransferase (located in Golgi bodies and the endoplasmic reticulum) leads to the production of the final product of

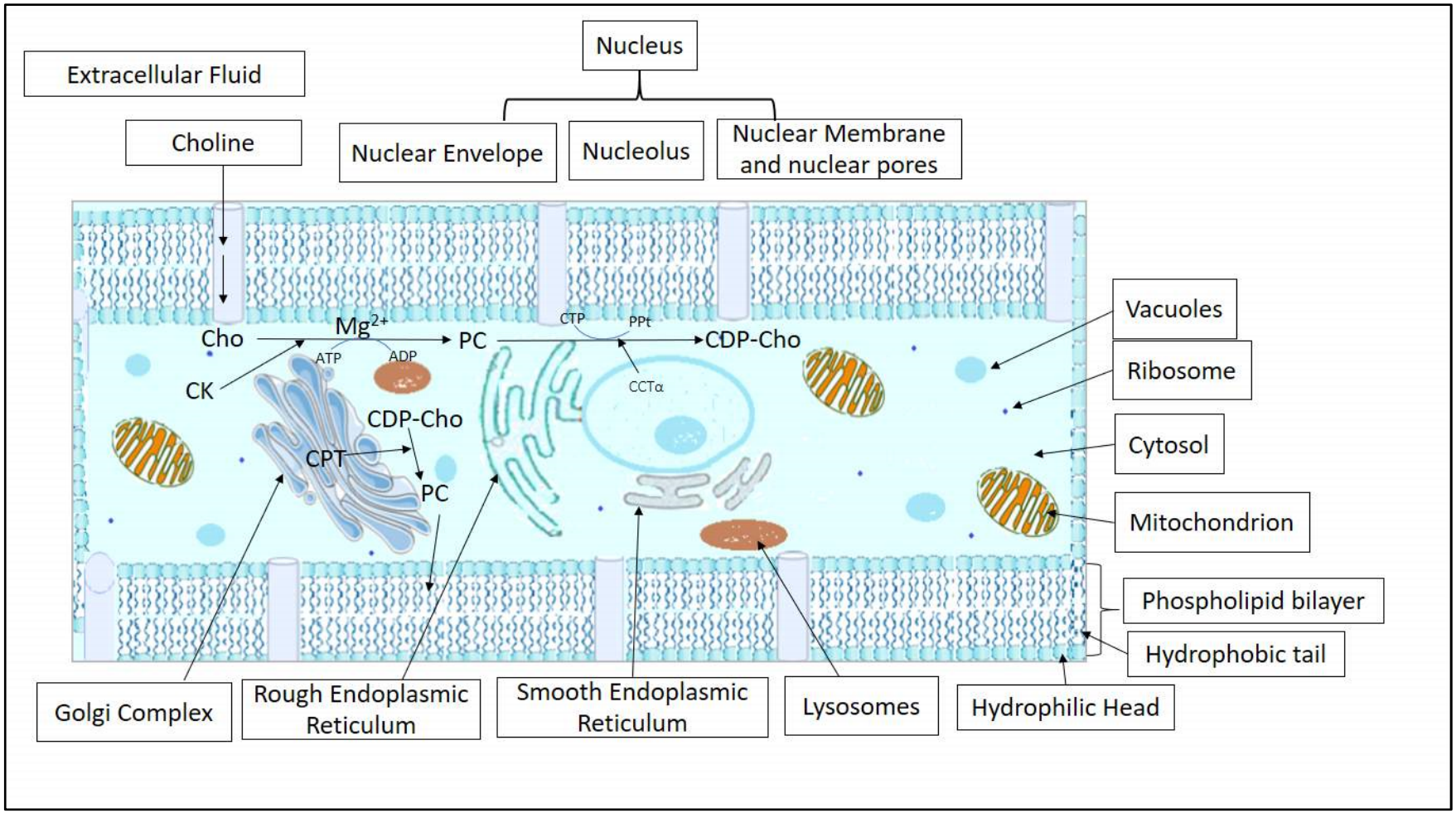

Figure 1.3: Localization of the biochemical reactions involved in the CDP-Choline pathway in the cell leading to the formation of the phospholipid: Phosphatidylcholine (PC)

the CDP-Choline pathway i.e., Phosphatidylcholine (PC) (Gibellini & Smith, 2010). The analogous pathway for PE involves the CDP-ethanolamine branch of the Kennedy pathway (Figure 1.2), which uses a series of similar reactions, except for the involvement of ethanolamine instead of choline to form PE (Gibellini & Smith, 2010).

1.3.3 Choline Kinase (CK)

Enzymatic potential of CK was first described in Brewer's yeast in 1953 (Wittenberg, 1953). Thorough mapping of CK catalytic activity and implications of its non-catalytic function via research was only initiated in the 2000s following CK purification from rat kidney in 1984 by Ishidate and his co-workers (Ishidate et al., 1984). CK gene was then successfully cloned from *Saccharomyces cerevisiae* and subsequently expressed in *E. coli* in 1989 (Hosaka & Yamashita, 1989). Following this, cDNA encoding mammalian CK from human glioblastoma was cloned, characterised, and then expressed in *E. coli* in 1992 by two separate research groups (Hosaka & Yamashita, 1989). Furthermore, cloning and characterisation of different genes and cDNAs of CK extracted from yeast (Gibellini and Smith, 2010), rodents (Aoyama et al., 2002) and plants (Monks et al., 1996) and its subsequent expression in *E. coli* has been established. Triggered by the cellular uptake of dietary (free) choline, CK catalyses the phosphorylation of choline with phosphoryl group donated by ATP to produce the very first intermediate of the Kennedy pathway in the presence of Mg^{2+} as co-factor (Moessinger et al., 2014). Apart from having higher catalytic efficiency for choline (Malito et al., 2006), CK also partakes in the enzyme assisted phosphorylation of ethanolamine as a substrate to produce phosphoethanolamine (PEtn) suggesting a close structural similarity between CK and ethanolamine kinase (EK), the enzyme that catalyses the phosphorylation of ethanolamine to PE (Sung & Johnstone, 1967). Unlike

CK, EK is highly specific for ethanolamine as a substrate for phosphorylation concluded from the research on Drosophila melanogaster (Lykidis et al., 2001).

1.3.4 Variants of the enzyme CK

In eukaryotes, alternative splicing of the mRNAs is a crucial mechanism to increase the complexity of gene regulation thereby governing the protein diversity pool (Figure 1.4) (Wang et al., 2015). > 95% of human genes have been reported to undergo splicing in a developmental, tissue-specific or signal transduction-dependant manner (Wang et al., 2015). CK enzyme, in humans, is encoded by two separate genes choline kinase alpha (*chka*) (refer Appendix A for *chka* gene map) and choline kinase beta (*chkb*) located on chromosomes 11q13.2 and 23q13.33 (Ensembl Genome Browser v48, Gene view: http://www.ensembl.org/) (Aoyama et al., 2004). Operative on the principle of alternative splicing, the *chka* transcript generates two CK mRNA splice variants known as *chka1* (GenBank Reference number: NM_212469.1) and *chka2* (GenBank Reference number: NM_001277.2) (Wu & Vance, 2010). These two *chka* variants are further translated into CHKA proteins CHKA1 and CHKA2 containing 435 (50kDa), 453 (52kDa) amino acids respectively and the *chkb* isoform is translated into CHKB protein containing 394 (45kDa) amino acids (Aoyama et al., 2004; Gruber et al., 2012). The translated protein products of the two splice variants of *chka* gene are identical in sequence except for an additional 18 amino acid stretch added to the CHKA2 protein isoform (Aoyama et al., 2004). Amino acid sequence identity between the CHKA (CHKA1 and CHKA2) and CHKB, which originate from two separate genes, is ~56% (Hong et al., 2010). In humans, each isoform of active CK enzyme exists either as dimeric (homo- or hetero-) or tetrameric complexes and remains inactive in monomeric form (Aoyama et al., 2004). Different combination of the CK

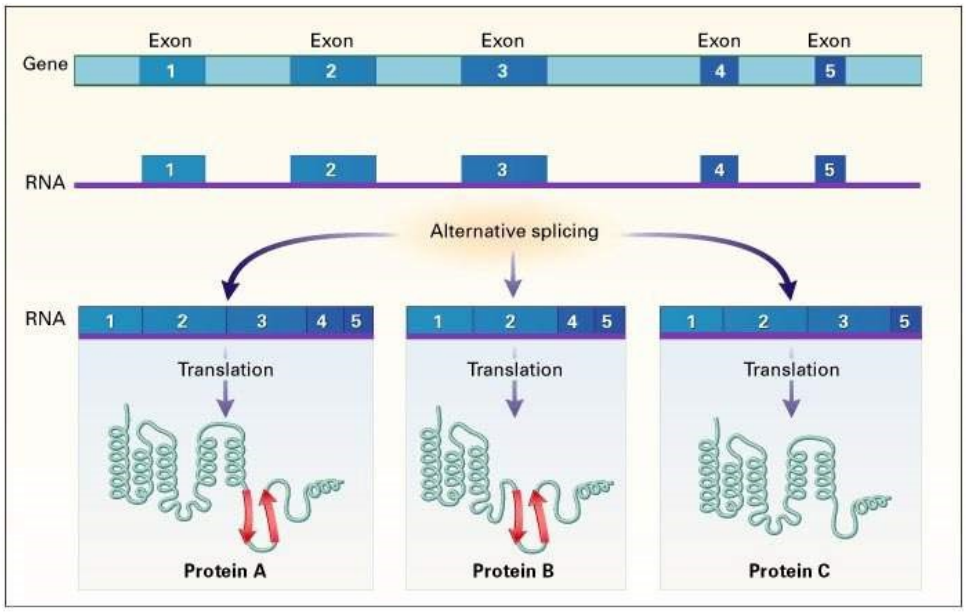

Figure 1.4: Pre-mRNA splicing and alternative splicing of eukaryotic genes. Pre-mRNA splicing - In primary transcript, the intronic sequences (light blue boxes) are removed, leaving behind only exonic sequences (dark blue boxes), which are later linked together to produce mature mRNAs. Alternative splicing - The alternative splicing of the exonic sequences generates different mRNAs that encode proteins with different properties (Wang et al., 2015).

isoforms (a/a, b/b or a/b) have significant inference on enzyme activity with a/a homodimer being the most active form and b/b homodimer categorised as the least active while the a/b heterodimer exhibited an intermediary activity. Activity of CK, in each cell type, is regulated not only by the level of each isoform in the cell but also by the combination of each isoform subunit (a1, a2 and b) to generate active dimer complexes (a1/a1, a1/a2, a2/a2, a1/b, a2/b, and b/b), strongly proposing that the molecular mechanism of regulation of CK activity in a cell type is extremely complicated (Aoyama et al., 2004; Aoyama et al., 2002). Enzymatically, CHKA and CHKB are known to perform the same catalytic activity. Gallego-Ortega and his coworkers found that under whole cell conditions CHKA1 can function as both CK and EK, but CHKB only affects the production of phosphatidylethanolamine (PEtn), indicating that besides their similarity in their primary sequences, the two isoforms are implicated in different metabolic pathways (Gallego-Ortega et al., 2009).

1.3.5 Physiological Significance of CK isoforms

According northern and western blot data, in mammals, both *chka* and *chkb* mRNAs, as well as their encoded protein isoforms, are ubiquitously expressed, although CHKA is highest in the testes and CHKB is relatively high in heart and liver (Gruber et al., 2012). Differential distribution of the two CK isoforms among tissues suggests that CHKA and CHKB isoforms have specific functions in the cellular pathways governing physiology and development of eukaryotes (Aoyama et al., 2002) Balance between CHKA and CHKB might, however, be important for cell cycle regulation (Gruber et al., 2012). There is no evidence pointing towards the specific roles of CHKA1 and CHKA2 (Lacal, 2015).

Distinctive *in-vivo* functions of CHKA and CHKB could only be clearly understood after the successful generation of knockout mice. Homozygous *chka*$^{-/-}$ mutant mice were recovered at the blastocyst stage but could not survive past the 7.5 days embryonic stage highlighting the indispensable role of CHKA in early embryogenesis (Wu & Vance, 2010). Also, the presence of homozygous *chka*$^{-/-}$ embryonically lethal (Wu et al., 2010). Spontaneously occurring genomic deletion in murine *chkb*, on the other hand, is not lethal, but results in neonatal forelimb bone deformity and hindlimb muscular dystrophy (Wu et al., 2009; Wu & Vance, 2010). The mitochondria of *chkb*$^{-/-}$mice were abnormally large with compromised mitochondrial function (Wu & Vance, 2010). In humans, mutations in the *chkb* gene incurs congenital muscular dystrophy, a heterogeneous group of inherited diseases clinically characterised by muscle weakness and hypotonia in early infancy (Mitsuhashi et al., 2011). Structural abnormalities in mitochondria are a recurring feature also observed in rostrocaudal muscular dystrophy in mice (Mitsuhashi & Nishino, 2013). Kuan and his colleagues have demonstrated the regulation of CHKB transcription by Ets and GATA transcription factors via protein kinase C (PKC) signalling pathway (Kuan et al., 2014) . Ral-GDP dissociation stimulator (Ral-GDS) and phosphatidylinositol-3-kinase (PI3K), the two Ras effectors, selectively activated CHKA (Ramírez de Molina et al., 2002) and RhoA induced CHKA activation was achieved by the contribution from the ROCK kinases (Ramírez De Molina et al., 2005). *Chka/a* dimer is the most active and effective in the biosynthesis of PC as well as EK and the *chkb/b* dimer activity is affected towards EK production *in vivo,* however, its activity for phosphocholine fabrication remains nugatory (Gallego-Ortega et al., 2009). Overexpression of CHKA has been

scientifically evidenced in laboratory processed human tumor derived cell lines from varied origins and in biopsy samples of colorectal, lung, and prostrate carcinomas.

Conclusions were drawn after the data obtained from tumor samples with that of the CHKA levels in normal tissues from the same patient (de Molina et al., 2007).

1.3.6 Regulation of CK

Despite comprehensive testament to the contribution of CK in carcinogenesis by regulating related signalling pathways, only a limited literature is available on the regulation of CK expression in mammals (Wu & Vance, 2010). Regulation of CK expression is overseen by several elements. The very first documented factor being polycyclic aromatic hydrocarbons that drastically induced CK activity in rat liver cytosol (Wu & Vance, 2010). The activation of CK was prevented by detoxicating CCl_4 treated hepatic cells with either cycloheximide or actinomycin D implicating RNA and protein synthesis in the increased CK activity (Wu & Vance, 2010). Consequent research in mouse liver indicated that CHKA and not CHKB was induced by the treatment with CCl_4 (Aoyama et al., 2002). The adapted mechanism for increasing CHKA was by increased binding of the transcription factor c-jun, enhanced by treatment with CCl_4, to a distal Activator Protein-1 element (–887/–867 from the transcription start site) on *chka* promoter (Aoyama et al., 2007). In 3T3 fibroblasts, mitogenic growth factors activate CK activity (Warden & Friedkin, 1984). Very first report of phosphorylation and therefore the regulation of CK in the yeast, *Saccharomyces cerevisiae,* was by activation of Protein kinase A activation (Kim & Carman, 1999). It was later revealed that CK phosphorylation is stimulated by Protein kinase A on multiple serine residues, at positions, serine-30, and serine-85 (Yu et al., 2002) and by PKC, at positions, serine25 and serine-30 (Choi et al., 2005). Induction

of CK expression in zinc-depleted yeast cells resulted in increased CK activity, both *in vitro* and *in vivo* and a subsequent increase in the synthesis of phosphatidylcholine via Kennedy pathway (Soto & Carman, 2008). Treatment with myo-inositol resulted in a significant decrease in CK enzyme amount and its mRNA abundance in *S cerevisiae* (Hosaka et al., 1990). In humans, CHKA protein levels are regulated by forming a complex with EGFR in a c-Src dependent manner (Miyake & Parsons, 2012). Replicating the effect in yeast, Protein kinase A catalyses the phosphorylation of the human CK isoform, CHKB, at serine positions- serine-39 and serine-40 (C. C. Chang et al., 2016). Protein kinase A assisted CHKB phosphorylation increased CHKB enzyme's catalytic efficiencies for choline and increased sensitivity of CHKB to hemicholinium-3 (HC-3) led inhibition (C. C. Chang et al., 2016). Glunde et al., (2008) and Bansal et al., (2012) showed that CHKA expression could be regulated by the binding of hypoxia-inducible factor-1α (HIF-1α) to hypoxia response elements (HREs) in the chkα promoter region. Kuan et al., (2014) proved that Ets and GATA transcription factors repressed *chkb* promoter activity via PKC signalling pathway following treatment with phorbol-12-myristate-13-acetate. The effect of histone acetylase inhibitor, trichostatin A (TSA) treatment on *chka* and *chkb* mRNA levels recommend contribution of epigenetic mechanisms in modulating the expression of these genes (C. S. Ling et al., 2015). Regulation of CHKA in neuronal differentiation of neuroblastoma cells is modulated by KBM2B binding to the Box2 by forming a complex with other unidentified proteins, the model for which is described in detail in (Domizi et al., 2019).

Most of the publications cited above illustrate the functional effects of CHKA overexpression or inhibition, and — apart from c-Src — could not identify interaction

partners of CHKA, which could clarify the mechanism through which CHKA deregulation feeds into oncogenic signalling pathways.

1.3.7 CHKA role in oncogenesis

The increased phosphocholine and total choline levels that have been detected in human cancers, are consequential of the interaction among multiple enzymes including choline kinase. These enzymes are partakers of choline metabolism and constitute the biosynthetic and catabolic pathways of PC (Glunde et al., 2011). Choline kinase, being a candidate regulated by Rho-GTPases, mirrors the ramifications inflicted by these active Rho proteins in multifarious key cellular processes that include migration, cell adhesion, membrane transport, regulation of transcription factors, control of the cell cycle, cell proliferation, and cell apoptosis under normal conditions, and these active Rho proteins initiate malignant transformation and human tumorigenesis by enhancing metastatic properties of cancerous cells (Ramírez De Molina et al., 2005).

Upregulation of CK activity in cancer is resultant of the increased expression of CHKA isoform (Glunde et al., 2011). Exponential CHKA expression was reported as a clinical finding, for the very first time, in breast carcinomas, with elevated CHKA levels in 39% patient derived tumor specimens and is reported to be inversely proportional to estrogen receptor positivity (Arlauckas et al., 2016). Since then, dysregulated CHKA expression has also been reportedly observed in tumor samples from 47% of colon cancer, 56% of lung cancer, and 48% of prostate cancer (Arlauckas et al., 2016). Furthermore, CHKA overexpression has been a consistent characteristic of ovarian (Iorio et al., 2010), endometrial (Trousil, 2014), and pancreatic cancers (Penet et al., 2015). Molecular characterisation of the pancreatic ductal adenocarcinoma tumors by ^{1}H Magentic Resonance Spectroscopic imaging (MRSI)

identified CHKA, CHT1 and CTL1 as the primary causes of increased total choline and PC levels (Lee & Ambros, 2001). Overexpression of CHKA, reportedly, induced an aggressive phenotype in MCF7 breast cancer cells with exponentiated capacity to invade extracellular matrix and harbour increased drug resistance to 5-fluorouracil (Shah et al., 2010). A retrospective study of patients with non-small-cell lung cancer (NSCLC) with a history of surgical tumor resection defined a correlation between overexpression of CHKA and severity of the clinical outcome in patients with NSCLC. This sanctioned for the recognition of CHKA as a prognostic factor aiding in the prediction of the probability of recurrence and determine the best suited treatments (Molina et al., 2007). Elevated CHKA levels in glioma xenografts with accumulated JAS239 have been imaged *in vivo* using near infrared fluorescence OI (Weissleder & Ntziachristos, 2003). Dysregulated lipid metabolism represented by increased CHKA and decreased expression of total PC were characteristic traits in esophageal squamous cell carcinoma (ESCC) patient sera (Ma et al., 2018). In human hepatocellular carcinoma cells (HCC), overexpressed CHKA heightened their invasiveness, prompted resistance to EGFR inhibitors, and increased ability to form metastatic tumors in mice by promoting interaction of EGFR with mechanistic target of rapamycin complex 2 (Lin et al., 2017). Aberrated CHKA levels were found to be linked with tumor aggressiveness and reduced survival times of patients with HCC (Lin et al., 2017).

Ras oncogenes modulated delineation of CHKA function in phosphocholine synthesis occurs via point mutations that replace glycine with valine or aspartate with cysteine causing the target proteins to be locked in the active state, continuously releasing signals that trigger overactivation of cell cycle regulation (Janardhan et al., 2006). The

Ras proteins responsible for mediating carcinogenesis by increasing CHKA activity are modulated by two important Ras effectors, namely, Ral-GDP dissociation stimulator (Ral-GDS) and phosphatidylinositol-3-kinase (PI3K) (Janardhan et al., 2006).

Akt is a serine/threonine kinase engaged in cell growth, proliferation, and survival. CHKA has been shown to control the activation of Akt by regulating the phosphorylation of serine 473. Mutations in the Akt pathway can produce human cancers, implying that CHKA could cause tumor development and metastasis through the activation of Akt pathway (Chua et al., 2009). In T-cell lymphoma, aberrant choline metabolism, directed by overexpressed CHKA, was related to Ras-AKT/ERKMYC signaling pathway activation (Xiong et al., 2015).

In prostate cancers, CHKA acts as a molecular chaperone for androgen receptor (AR), which is a transcription factor playing a critical role in prostate cancer development, by physically interacting with the ligand binding domain of AR to increase its stability in maintaining AR signaling thereby controlling AR transcriptome (Asim et al., 2016). Non-catalytic role of CHKA protein has therefore, been evidently presented in cancer onset and progression which is independent of its prominent catalytic function in the biosynthesis of phosphatidylcholine. Hence, a potent anticancer strategy would not only inhibit CHKA activity but also subvert its proteinic form.

The promising potential of CHKA in use as biomarkers and design of the said protein targeted anticancer therapeutics required a thorough understanding of the CHKA protein behaviour under diseased and normal conditions in different species. The design of both monoclonal (Gallego-Ortega et al., 2006) and polyclonal (Too et al., 2010) antibodies facilitated the study of fluctuations in CHKA protein levels in cancer

cells allowing to quantify and compare protein expression in varied cancer types. Noninvasive imaging techniques such as magnetic resonance imaging (Gabellieri et al., 2009) and positron emission tomography (Contractor et al., 2011) detected changes in choline metabolism that were orchestrated by CHKA in tumors.

1.3.8 CHKA as a promising target for antitumor strategies

As described in section 1.1.8, the aftermath of CHKA upregulation, is altering the normal functioning of cell signalling pathways to promote carcinogenesis via both its catalytic activity in the biosynthesis of PC and non-catalytic activity in regulating cell stress. CK inhibitors specifically target and kill only cancer cells, sparing normal cells by causing metabolic wobbles that form high levels of ceramides that ultimately encourage cancer cell apoptosis (Rodríguez-González et al., 2004).

Hence, design of strategies specifically targeting CHKA as anticancer therapeutics is the scientific direction of capable potential being largely investigated. Two therapeutically effective research directions in the works are pharmacological inhibition of CHKA (Arlauckas et al., 2016) and RNA interference (RNAi) by-theway-of small interfering RNA (siRNA) or short hairpin RNA (shRNA) (Krishnamachary et al., 2009).

1.3.9 Pharmacological inhibitors targeting CK to selectively inhibit cancer cells

Discovery of hemicholinium-3 (HC-3) was key in initiating the design of potent inhibitors (Hernández-Alcoceba et al., 1997). Eventual structural resolution of several human CHKAs by X-ray crystallography techniques allowed for a more specific, tailor-made design of inhibitors targeting the most effective sites of CHKA (Malito et al., 2006). Most of the CHKA inhibitors designed over the last 15 years, therefore,

utilized the information of the active site architecture of CHKA, amassed from CK Xray crystal structures (Rubio-Ruiz et al., 2021).

Small molecule CK inhibitors typically comprising of two cationic moieties in form of quaternary pyridinium salts connected by a lipophilic linker have been developed. Very first member of the small molecule inhibitors of CK discovered is HC-3 which inhibits the activity of both choline transporters and CHKA due to its structural similarity with choline (Gómez-Pérez et al., 2012). However, the inhibition of high affinity choline transporters, by treatment with HC-3, halts the cellular uptake of choline for acetylcholine synthesis causing neurotoxicity and apnoea (Ferguson, 2004). A conscious need for effective yet non-toxic CHKA targeting inhibitors inspired from the structure of HC-3 led to the development of MN58B (second generation compound of HC-3) and TCD-717, also known as RSM-932A (third generation compound of HC-3), both of which induce ER stress response by increasing the expression of inositol-requiring protein 1 (IRE1a) and glucose-regulated protein 78 (GRP78), forcing the cells to enter apoptosis, stimulation of growth inhibition, apoptosis, differentiation, and senescence (Hannun & Obeid, 2008; Sanchez-Lopez et al., 2013). These effects are restricted to cancer cells and the normal cell counterparts remain unaffected (Sanchez-Lopez et al., 2013). TCD-717 (RSM-932A) is the first CHKA specific inhibitor to complete phase I clinical trials conducted by TCD Pharma (Valladolid, Spain) at two U.S. clinical centers (NCT01215864), to be tested in patients with advanced solid tumors (Hua et al., 2020; Kall et al., 2018).

The treatment of HepG2 cells with EB-3D and EB-3P CHKA inhibitors, which are structured from bipyridinium and bisquinolinium derivatives, showed inhibition of CKHA enzyme activity, downregulation of CHKA expression, apoptosis dependent

on ER stress, inhibition of basal autophagy and deregulation of lipid metabolism (SolaLeyva et al., 2019). EB-3D, the novel inhibitor targeting CHKA, is reported to induce deregulation of the AMPK-mTOR pathway and commence apoptosis in leukemia Tcells (Mariotto et al., 2018a). CK37, which is structurally distinct from pyridinium analogues, is an uncharged CHKA inhibitor that was initially identified by *in silico* methods (Clem et al., 2011). It is only capable of affecting Akt and Erk phosphorylation at high concentrations and weakly inhibit CHKA (Clem et al., 2011). JAS239 is a novel carbocyanide dye that possesses CHKA inhibitory properties thereby inducing cell death in breast cancer cells along with conferring fluorescence in the near-infrared window that permitted real-time detection of JAS239 accumulation in the target tumors (Arlauckas et al., 2014). Compound 67, a product of fragment-based drug design approaches, showed successful cellular PCho inhibition, growth inhibition of CHKA driven cancer cell lines resulting in cell death and induction of apoptosis in MDA-MB-415 and MDA-MB-468. Normal cell lines such as MCF-10A and MCF-12A did not undergo apoptosis when they were treated with this compound suggesting the selective inhibitory effects on oncogenic cells (Zech et al., 2016).

V-11-0711, which is an ATP competitive CHKA inhibitor (Gnocchi et al., 2019), showed the highest inhibitory activity against CHKA when it was tested in the HeLa cell systems (Hudson et al., 2013). Inhibition by V-11-0711 caused reversible growth arrest (Falcon et al., 2013). The inhibitor treatment could not diminish the tumorigenic attribute of CHKA implying that oncogenic activity of the enzyme may be due to its non-catalytic protein scaffolding function not only its catalytic activity (Mori et al., 2007).

1.3.10 RNA interference helps slower CK initiated cancer cell proliferation

Beneficial effect of transcriptional silencing of CHKA reduces intracellular PCho levels (Mori et al., 2013) translating into lowered proliferation of MDA-MB-231, MDA-MB-468 and HeLa (Glunde et al., 2005) and initiate apoptosis (Gadiya et al., 2014). CHKA silencing downregulated MAPK and PI3K/AKT phosphorylation and differential expression of genes participating in cell cycle regulation, DNA repair and proliferation (Yalcin et al., 2010). Consequence being, increased differentiation, prevention of anchorage dependant growth in HeLa cells and annihilate their ability to form xenografts in athymic mice (Yalcin et al., 2010). CHKA downregulation reduced migration and cell invasiveness whilst increasing sensitivity to drugs in ovarian cancer cells (Granata et al., 2015). CHKA inhibition activates endoplasmic reticulum stress response triggering apoptosis in cancer cells (Sanchez-Lopez et al., 2013). CHKA depletion sensitises cancer cells to chemotherapy, like treatment with 5-fluorouracil (5-FU) enhancing the effect of siRNA against CHKA in MDA-MB-231 (Mori et al., 2007). siRNA against CHKA not only downregulated its expression in two triple negative human breast cancer cell lines (MDA-MB-231 and SUM-149) and two human pancreatic ductal adenocarcinoma cell lines (Pa09C and Pa20C) but also helped identify the inverse relationship between CHKA and PD-L1 at the genomic, proteomic and metabolomic levels which is governed by prostaglandin-endoperoxide synthase 2 (COX-2) and transforming growth factor beta (TGF-β). These outcomes opened doors to the design of combinatorial therapies targeting immune checkpoints and cancer metabolism by tweaking the endogenous expression of CHKA, PD-L1, COX-2 and/or TGF-β (Pacheco-Torres et al., 2021).

A gene therapy mouse model targeting CK is developed by Krishnamachary and his colleagues by intravenously injecting shRNA targeting CK, delivered through a lentiviral vector, into athymic mice bearing MDA-MB-231 xenografts (Krishnamachary et al., 2009). Their results showed that the CK mRNA levels were reduced by 35%, diminishing CK activity and tumour volume.

A gene therapy approach targeting solid tumours although, challenging to translate into humans due to ethical and technical considerations, this experiment substantiates the evidence for CK to be a potential drug target (Trousil, 2014). While the implementation of CK targeting RNAi as an anti-cancer strategy is more preferred due to its specificity, lack of side effects (as imposed by drug inhibitors), and its effectivity in targeting CK activity independent cancer cell survival (Mori et al., 2015), the delivery of siRNA or shRNA to the CHKA target site in cancer patients is quite challenging.

1.4 RNAs

Ribonucleic Acid (RNA) is a major biological macromolecule that carries the genetic information, received from the DNA in the form of nucleotides, for protein synthesis. Apart from being the transient intermediaries in the central dogma directing the translation of genetic commands from DNA into functional proteins, RNAs have a significant and broader role (Higgs & Lehman, 2015). Protein coding capacity, thus, discriminates RNAs into protein coding RNAs (mRNAs) and non-coding RNAs (ncRNAs) (Eddy, 2001; Lander et al., 2001). ncRNAs are involved in multiple biological processes, regulate physiological and developmental processes or even disease, hence, being recognised as tumor suppressors and oncogenic drivers in varied cancer types (Zhang et al., 2019). Eukaryotic ncRNA species have been classified into