MECHANISTIC INSIGHTS OF SRPK INHIBITORS IN CHOLANGIOCARCINOMA CELL APOPTOSIS

PREENAPAN CHANGPHASUK

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Biochemistry
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Thesis entitled "Mechanistic insights of SRPK inhibitors in cholangiocarcinoma cell apoptosis"

By Preenapan Changphasuk

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for the Master of Science in Biochemistry of Naresuan University

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ABSTRACT

Cholangiocarcinoma (CCA) is a cancer that arising from abnormal growth of bile duct epithelium. It is a common cancer among Thai population derived from unhygienic food consumption. Regarding no early-diagnosis and inefficient treatment, therefore, CCA represents very poor prognosis and low survival rate. The molecular mechanisms underlying CCA development are still unclear, however, dysregulation of mRNA splicing is suspected to play a major role as a number of oncogenic aberrant spliced-transcripts in CCA have been reported. Aberrant splicing is caused by the increased activity of Serine/Arginine rich-splicing factors (SRSFs) that translocate into the nucleus as phospho-SRSFs after activated by Serine-arginine protein kinases (SRPKs). In this study, the effects of two SRPK inhibitors (SRPIN340 and SPHINX31) in two CCA cell lines (KKU-213A and TFK-1) were investigated. SRPIN340 and SPHINX31 increased the number of dead cells once stained with calcein-AM/PI as dose-dependent manner. The type of dead cell induction was defined as apoptosis by increasing of apoptotic cell population, diffused cytoplasmic cytochrome c and upregulation of cleaved caspase-3 by

Annexin-V/7AAD staining (flow cytometry), immunocytofluorescence and Western blot analysis, respectively. Moreover, Western blot analysis using anti-phospho-epitope of SRSF protein family members revealed lower phospho-SRSFs band intensities for representing the reduction of SRSFs phosphorylation. Particularly, inhibition of nuclear-cytoplasmic translocation of predominant SRSF1 was demonstrated by immunocytofluorescence and Western blotting of subcellular protein fractions. To link these phenotypes with aberrant gene splicing, Bridging Integrator 1 (BIN1) gene was selected. Both SRPIN340 and SPHINX31 can decrease BIN1+12A (oncogenic/anti-apoptotic isoform) once observed by RT-PCR. These results provide the strong evidences that suggests the targeting SRPKs could be an alternative strategy for developing the precise CCA treatment.

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ABBREVIATIONS

%	Percent
AAD	Aminoactiomycin D
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease activating factor 1
AS	Alternative splicing
Bad	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-B	Bcl-2-like protein 10
Bcl-W	Bcl-2-like protein 2
Bcl-xL	B-cell lymphoma large
BECLIN	Beclin-1
Bid	A Bax-like BH3 protein
BIM	Bcl-2-like protein 11
BIN1	Bridging intergrator 1
bp	Base pair
CAD	Caspase-activated DNase
Caspases	Cysteine aspartyl-specific protease
CCA	Cholangiocarcinoma
cDNA	Complementary DNA

ABBREVIATIONS (CONT.)

cDNA	Complementary DNA
c-IAP 1	Cellular inhibitor of apoptosis protein 1
c-IAP 2	Cellular inhibitor of apoptosis protein 2
CLKs	CDC2-like kinases
CO_2	Carbon dioxide
DAMPs	Damage-associated molecular patterns
dNTP	Deoxynucleoside triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FAS	FAS Cell Surface Death Receptor
FBS	Fetal bovine serum
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hnRNPs	Heterogeneous nuclear ribonucleoproteins
ICAD	Inhibitor of CAD
IkB	Inhibitor of NF-kB
kDa	Kilo Dalton
LC	Liquid Chromatograph
MCL-1	Myeloid cell leukemia 1
mg	Milligram
mg	Milligram

ABBREVIATIONS (CONT.)

min	Minute
mL	Milliliter
mRNA	Messenger ribonucleotide acid
NF-kB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer
p53	Tumor protein 53
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PUMA	p53 upregulated modulator of apoptosis
RBPs	Retinol-binding proteins
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
S.D.	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SR	Serine and arginine-rich proteins
SRPKs	Serine-Arginine protein kinases

ABBREVIATIONS (CONT.)

SRSF1	Serine/Arginine-rich splicing factor 1
SRSFs	Serine/Arginine-rich splicing factors
siRNA	small interfering RNA
siSRSF1	small interfering RNA specific to Serine/Arginine-rich splicing factor 1
TAE	Tris Acetate-Ethylenediaminetetraacetic acid
T_m	Melting temperature
XIAP	X-linked inhibitor of apoptosis protein
^oC	Degree Celsius
µg	Microgram
µl	Microliters
µM	Micro Molar
µm	Micrometer

CHAPTER I

INTRODUCTION

Introduction and Research Significance

Cholangiocarcinoma (CCA) is a severe type of cancer that occurs in the epithelium of the bile duct. Thailand has a high incidence of CCA, which is found in the north and northeast. The main reason was freshwater fish consumption with contaminating metacercaria of *Opisthorchis viverrini* (OV) together with other factors such as exogenous carcinogen which generate the cellular DNA damage, excretory/secretory products as an important growth factors or growth stimuli, and repeated treatment with parasitic drugs generate. Treatment for CCA is determined by the stage at which the cancer is detected. Since there are no specific symptoms in the early stages, most of the patients treated for CCA are more advanced or invasive and spread to other organs. As a result, the mortality rate of CCA patients has increased every year.

In CCA, aberrant alternative splicing has been reported to result in the production oncogenic isoforms of various important genes in many cancers and recognized as an another hallmark of cancers. For example, splicing of CD44 into CD44v6 encourages the growth ability of colon cancer cells, splicing of VEGF into VEGF165 promotes the angiogenesis capacity in breast and melanoma cancers, and splicing of BIN1 into BIN1+12A plays a crucial role in the evasion of apoptosis and facilitates cancer cell survival. Serine/arginine-rich splicing factor (SRSFs) is the SR protein family that serves as a key molecule in regulation, there are consists of 12 members of SRSFs. Previous studies from our group found that SRSF1 predominantly upregulated in CCA cell lines and CCA patients' derived tissues. The main activity of SRSFs is regulated by Serine-arginine protein kinase (SRPKs). SRPKs are responsible for phosphorylation to SRSFs at arginine-serine rich (RS) domain into active SRSFs. Dysregulations of SRSFs and SRPKs might be leading to abnormal protein synthesis with overexpression, functional alteration or reveals the oncogenic properties of the protein isoforms. The subsequence activation initiated by SRSFs were phosphorylated

by SRPKs, then SRSFs will be translocated from the cytoplasm to nucleus for targeting synthesized mRNA.

Therefore, this study aims to determine the inhibition of SRSFs phosphorylation using two SRPK inhibitors (SRPIN340 and SPHINX31) in 2 CCA cell lines (KKU-213A, an intrahepatic CCA with OV-associated and TFK-1, an extrahepatic CCA with non-OV associated). Regarding to the preliminary result of viability, the effect of SRPK inhibitors on apoptosis will be designed, inhibitory effect on SRSF1 activation, decreasing of cytoplasmic-to-nuclear translocation of SRSF1 protein. Remarkably, the downstream splicing which direct to cellular apoptosis will be investigate and the splicing of BIN1 into BIN1+12A is selected. The researcher expects those the results and knowledge from this study will be supported as the basis for development or as a new target molecule in the treatment of CCA.

Research Objectives

1. To study the effect of SRPK inhibitors on CCA cell apoptosis (death phenotype examination and specific apoptotic protein expression).
2. To investigate the effect of SRPK inhibitors on SRSFs phosphorylation, especially for SRSF1.
3. To investigate the effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1 in CCA cells.
4. To investigate the effect of SRPK inhibitors on an alternative splicing of BIN1 in CCA cells.

Research Scope

The research proposal is designed into 4 parts as shown in Figure 1;

Part 1: Study the effect of SRPK inhibitors (SRPIN340, SPHINX31) on CCA cell apoptosis in KKU-213A and TFK-1: (1) differential count of live and dead cell by live/dead dual staining, (2) determine apoptotic cell population by AnnexinV/7-AAD staining using Flow cytometry, (3) determine pro-apoptotic protein expression by immunocytofluorescence and western blotting (corresponding to objective 1)

Part 2: Investigate the effect of SRPK inhibitors (SRPIN340, SPHINX31) on SRSFs and SRSF1 phosphorylation in KKU-213A and TFK-1 by western blotting (corresponding to objective 2)

Part 3: Investigate the effect of SRPK inhibitors (SRPIN340, SPHINX31) on cytoplasmic-to-nuclear translocation of SRSF1 protein in KKU-213A and TFK-1: (1) determine the SRSF1 subcellular localization by Immunocytofluorescence, (2) determine SRSF1 protein expression in subcellular protein fractionation (cytoplasmic protein, nuclear protein) by western blotting (corresponding to objective 3)

Part 4: Investigate the effect of SRPK inhibitors (SRPIN340, SPHINX31) on an alternative splicing of BIN1 gene KKU-213A and TFK-1 by RT-PCR using BIN1 and BIN1+12A specific primers (corresponding to objective 4)

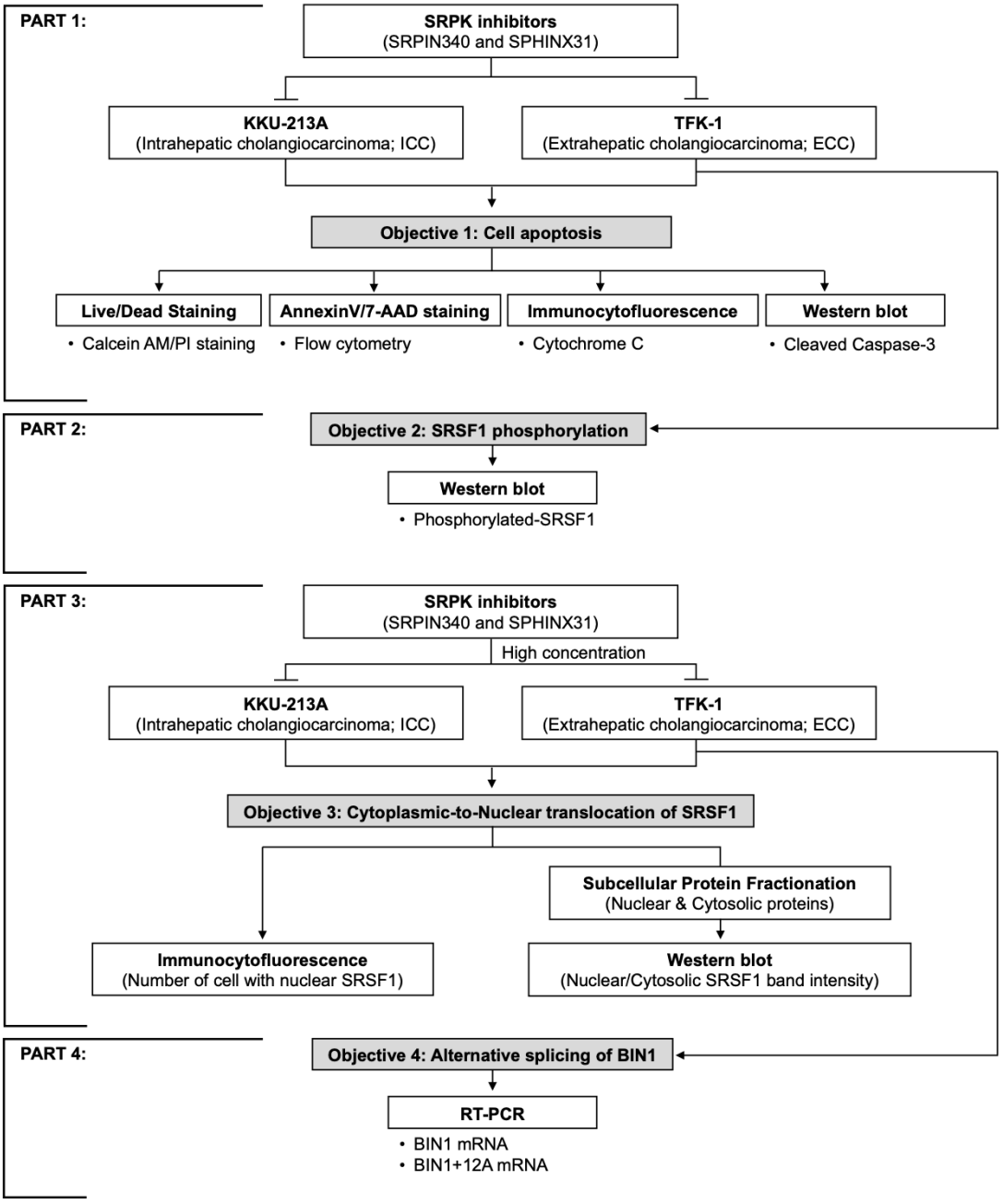

Figure 1 Schematic diagram shown scope of this study.

Keywords

Apoptosis, Bridging Integrator 1 (BIN1), Cholangiocarcinoma (CCA), Serine/arginine-rich splicing factor (SRSF1), Serine-arginine protein kinase (SRPKs), SRPK inhibitors

Research Hypothesis

SRPK inhibitors can induce CCA cell to apoptosis by inhibiting the activation of SRPKs. Therefore, phosphorylation of SRSF1 protein is decreasing and then, cannot translocating into nucleus. The reduce the aberrant alternative splicing of BIN1 gene via correcting anti-apoptotic BIN1+12A and switching into pro-apoptotic BIN1 (wild-type) for reducing CCA cell survivability.

Anticipated outcomes

1. Attenuation of SRSF1 activation (by SRPK inhibitors) can reduce CCA cell survivability through the correction of BIN1 splicing.

2. The basic information for research continuing to develop splicing machinery (SRPKs/SRSFs) as the precision therapeutic target for CCA.

CHAPTER II

REVIEW LITERATURE

1. Cholangiocarcinoma (CCA)

1.1 Anatomical classification

Cholangiocarcinoma (CCA) is a bile duct cancer that originating in the epithelium of the biliary tract. CCA can be divided into 2 groups according to anatomical positions includes extrahepatic cholangiocarcinoma (eCCA) and intrahepatic cholangiocarcinoma (iCCA). eCCA can be divided into distal CCA and hilar. For in part iCCA includes can be further classified into three subtypes: (i) mass-forming tumors, (ii) periductal-infiltrating type, and (iii) intraductal based on their growth pattern (Kodali et al., 2021) as shown in Figure 2.

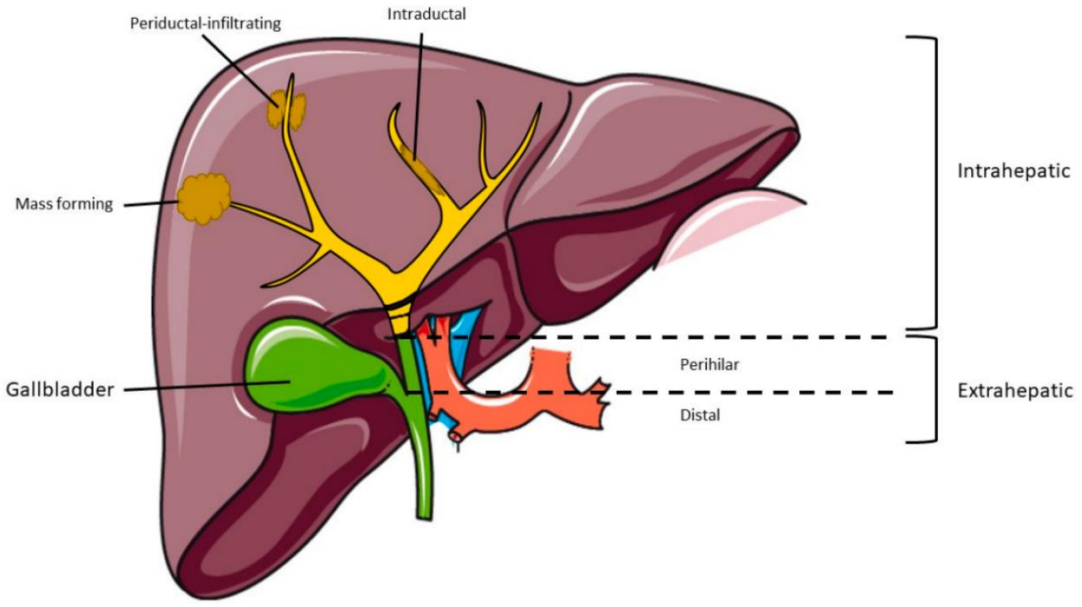

Figure 2 Classification of cholangiocarcinoma by anatomic location.

Source: Kodali et al., 2021

1.2 Incidence

CCA has been increasing in incidence globally, as shown in many studies. In addition, CCA has not specific symptoms in the early stage but there are present the sign in severity or advanced stages with cancer cell metastasize to other secondary organs. The patients usually present with advanced disease and hence it is associated with a high mortality rate. Global age-standardized annual mortality rates for CCA. Data refer to the periods 2000–2004 (2002), 2005–2009 (2007) and 2010–2014 (2012). Yellow indicates countries/regions with low mortality (<2 deaths per 100,000 people), orange indicates countries/regions with mortality between 2 and 4 deaths per 100,000 people, and red indicates countries/regions with high mortality (>4 deaths per 100,000 people). Mortality in eastern countries/regions in which CCA is highly prevalent (that is, Thailand, China, Taiwan, and South Korea) (Banales et al., 2020) as shown in Figure 3.

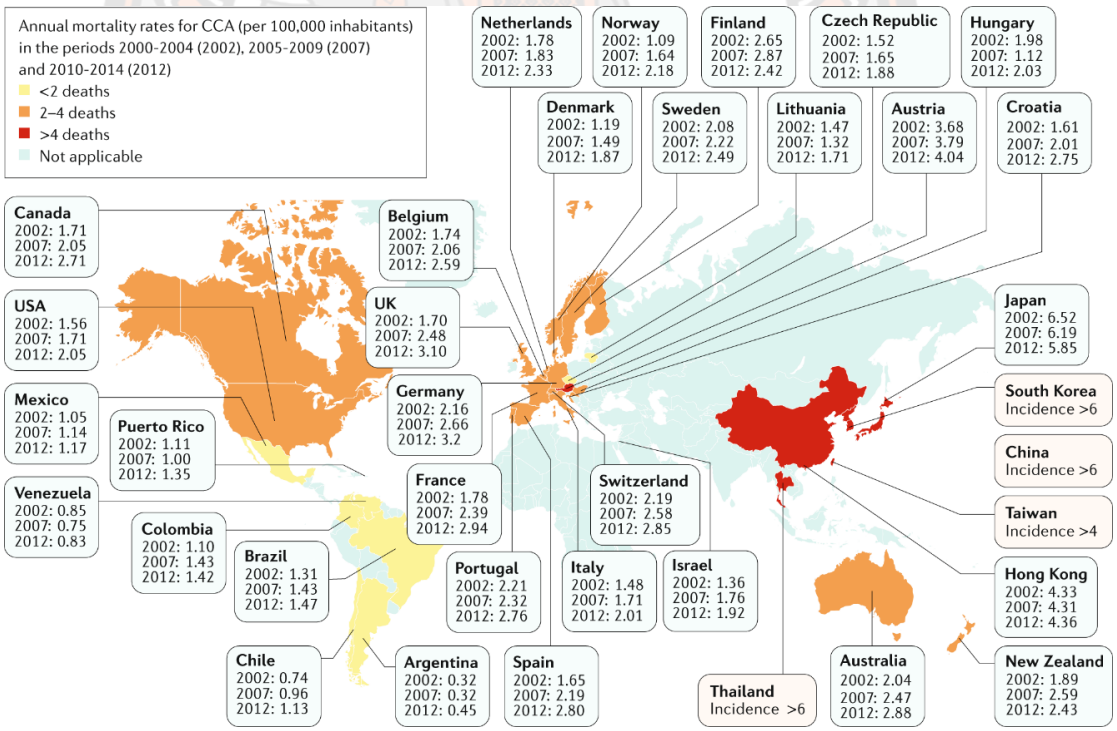

Figure 3 The worldwide incidence of cholangiocarcinoma.

Source: Banales et al., 2020

1.3 Risk factors

A common characteristic amongst many of these risk factors is that they are associated with chronic inflammation of the biliary epithelium and bile stasis. Several recognized risk factors have increased globally over recent decades and could be contributing to increasing CCA rates. For instance, high alcohol consumption, tobacco smoking, Hepatolithiasis, Cirrhosis, Caroli's disease and viral infections (hepatitis B virus (HBV) and hepatitis C virus) (Banales et al., 2020) In Thailand, risk factors of CCA are often caused by freshwater fish consumption with contaminating metacercaria of *Opisthorchis viverrini* (Prueksapanich et al., 2018). The higher *Opisthorchis viverrini* prevalence correlates with a higher CCA as show in Figure 4.

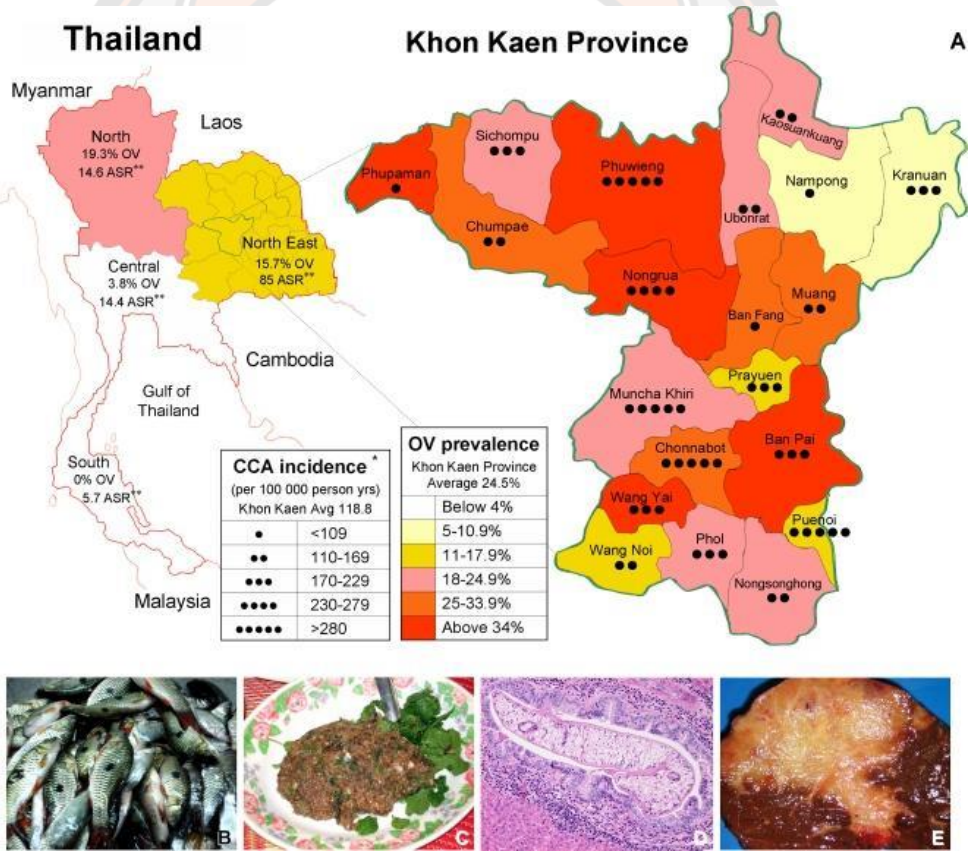

Figure 4 Incidence of CCA and the important risk factor of CCA in Thailand.

Source: Sripa., 2008

1.4 Treatment

Treatment options for iCCA include surgical resection, locoregional therapies, chemotherapy, radiation, and liver transplantation. Overall, surgical not an option for patients with cirrhosis and portal hypertension or for larger tumors with vascular invasion. Liver transplantation may be a better option in patients with cirrhosis. Chemotherapy in combination with radiation have shown improvement in survival in patients with cholangiocarcinoma and a combination of Cisplatin plus Gemcitabine is currently the first-line treatment and standard of care for these tumors (Kodali et al., 2021).

1.5 Cholangiocarcinogenesis

The process of carcinogenesis beginning with a single cell that has acquired malignant properties through cellular DNA damage. There are divided into at least three stages including initiation, promotion, and progression (Liu et al., 2015) as shown in Figure 5.

Initiation, mutations of DNA or genes arising spontaneously or induced by a carcinogenic agent. Genetic alterations can result in deregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation.

Promotion, the expression of the genome mediated through promoter-receptor interactions.

Progression, where genetic and phenotypic changes and cell proliferation occur. This involves a fast increase in the tumor size, invasive and metastatic potential.

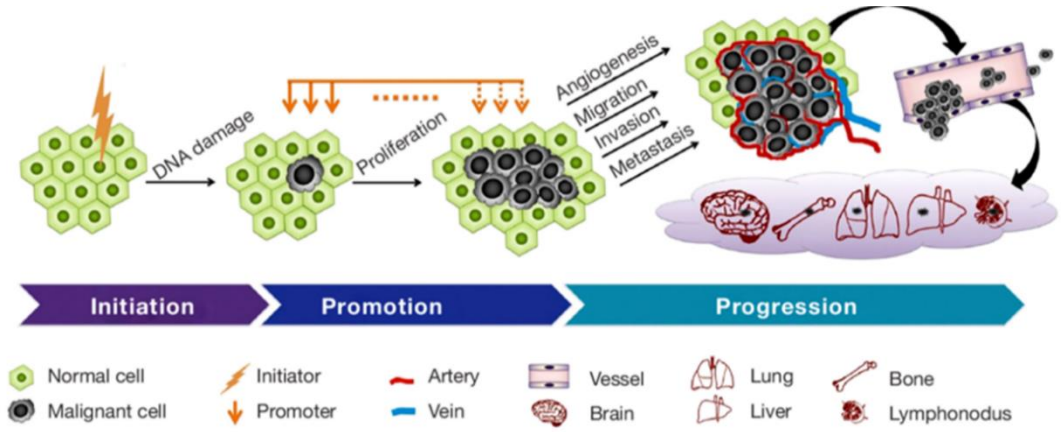

Figure 5 Three-phase process of carcinogenesis when carcinogen administration based on animal models of primary cancers.

Source: Liu et al., 2015

For CCA, the infection by *Opisthorchis viverrini* attached the host cell epithelium in the bile duct, stimulating fibrosis and stimulating chronic inflammatory process. In addition, may be triggered by external factors including of exogenous carcinogen which generate the cellular DNA damage, excretory/secretory products as an important growth factors or growth stimuli, and repeated treatment with parasitic drugs generate the surge of oxidative and nitrative stress which finally resulting in limitless of epithelial cell proliferation (Prueksapanich et al., 2018) as show in Figure 6.

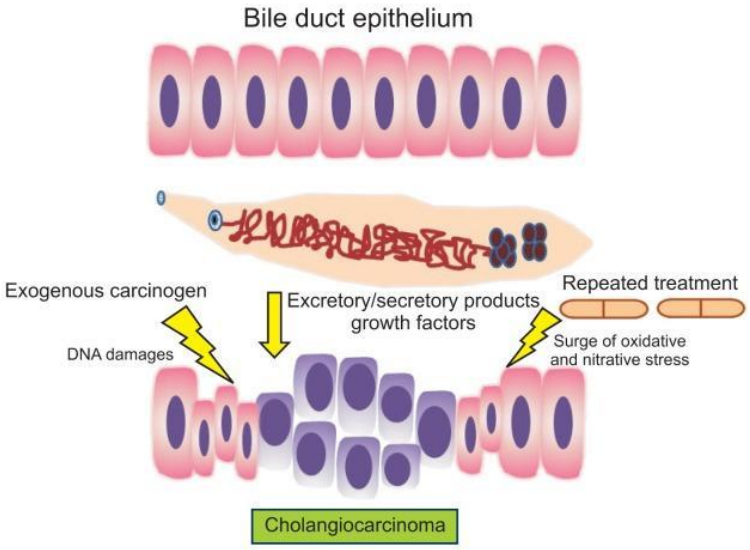

Figure 6 The mechanism of process cholangiocarcinoma.

Source: Prueksapanich et al., 2018

2. Cancer cell death

2.1 Resisting of cell death is a cancer hallmark

In 2000, Doug Hanahan and Bob Weinberg published a paper in which they suggested that all cancers share six common features, or hallmarks (Hanahan et al., 2000). They were self-sufficiency in relation to growth signals; insensitivity to growth inhibitory signals; limitless replicative potential; the ability to evade apoptosis; the ability to sustain angiogenesis; and lastly, the ability to invade tissues and metastasize. In 2011, they extended the cancer hallmarks to a list of ten. The four new hallmarks were the ability to evade the immune system, the presence of inflammation, the tendency towards genomic instability, and dysregulated metabolism (Hanahan et al., 2011). And finally in 2013, A systematic dysregulation of alternative splicing should therefore be considered yet another hallmark of cancer (Ladomery, 2013) as shown in Figure 7.

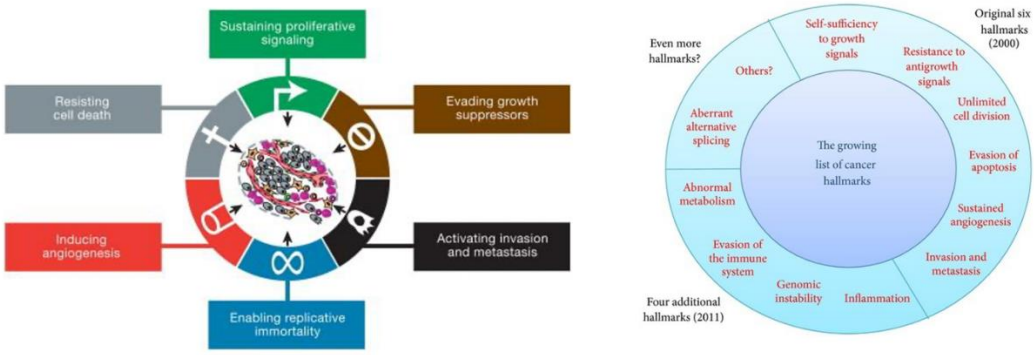

Figure 7 Six hallmarks of cancer in 2000 (left) and additional hallmarks of cancer (right).

Source: Hanahan et al., 2000 and Ladomery., 2013

Normally, program cell death helps keep an organism healthy through growth and development, maintaining body tissue by removing infected or damaged cells. It is tightly regulated since either too much or too little cell death can lead to pathological defects. But cancer cells do not follow this process, they evade cell death. The cancer cells may alter the mechanisms that detect the damage or irregularities, preventing proper signaling and cell death activation.

2.2 Common types of cell death

Three major types of cell death have been described depending on the developmental context:

Type I or apoptosis, featuring with cytoplasmic shrinkage, nuclear pyknosis, karyorrhexis, DNA fragmentation, and plasma membrane blebbing, and eventually apoptotic body formation.

Type II, or autophagy, showing extensive cytoplasmic vacuolization to form autophagosome, phagocytosis and subsequent lysosomal degradation.

Type III or necrosis, manifesting with distinctive morphology, is different from type I and type II cell death. It is morphological changes contain organelle and plasma membrane rupture, finalizing with disposal of cell corpses absent of evident phagocytic and lysosomal involvement. Which are distinguished, based on

morphological appearances and molecular features (Azad and Gibson, 2010; Kroemer et al., 2009) as shown in Figure 8 and Table 1.

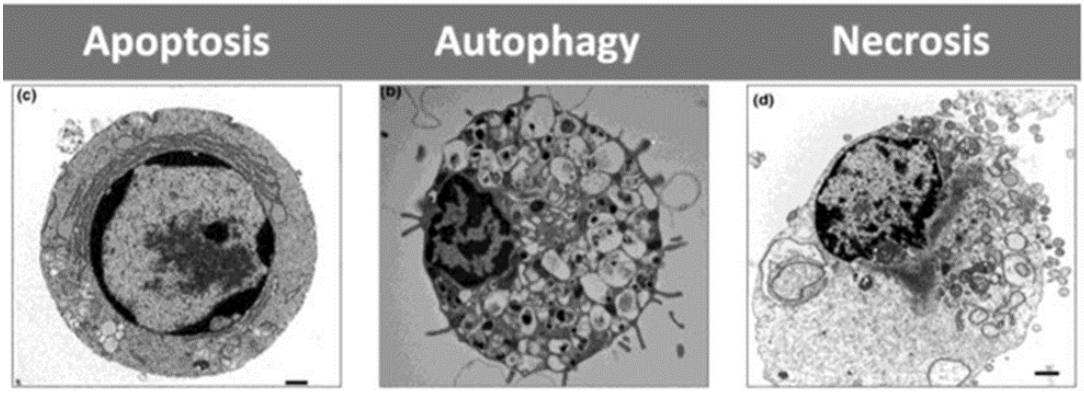

Figure 8 Morphological features of the major types of cell death.

Source: Azad and Gibson, 2010

Table 1 Summary features of the major types of cell death (Azad et al., 2009)

Cell Death Features	**Type I (Apoptosis)**	**Type II (Autophagy)**	**Type III (Necrosis)**
General morphology	Shrink	-	Swell
Chromatin condensation	++	+	-
Autophagosomes	-	++	+
Caspase activation	++	-	-

Other research has revealed apoptosis is attenuated in those tumors that succeed in progressing to states of high-grade malignancy and resistance to therapy (Adams and Cory, 2007; Lowe et al., 2004).

3. Apoptosis

The term “apoptosis” was derived from a Greek word that means “falling off” and often referred to as "Programmed cell death". Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations

in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001).

3.1 Apoptotic mechanisms (Extrinsic pathway, Intrinsic pathway, Caspase-independent pathway)

The mechanism of apoptosis is very complex. Currently, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Wani et al., 2023 as shown in Figure 9. There is now evidence that the two pathways are linked and molecules in one way can influence another. There is an additional pathway is perforin/granzyme pathway (Elmore et al., 2007) as shown in Figure 10.

Extrinsic or death receptor pathway: occurs when death receptors (DRs) such as tumor necrosis factor receptor (TNFR), Fas receptor (FasR), DR3, TNF-related apoptosis-inducing ligand (TRAILR1 or DR4 and TRAILR2 or DR5) binding to specific death ligands to form an adaptor protein and initiator caspase called a "death-inducing signaling complex" (DISC), which activates the enzyme caspase-8, caspase-3, caspase-6 and caspase-7 leading to apoptosis.

Intrinsic or mitochondrial pathway: occurs when triggered by stimuli such as oxidative stress, ultraviolet light, DNA damage. Mitochondria are damaged and cytochrome c is released into the cytoplasm which is controlled by 2 groups of control including pro-apoptosis members (e.g. PUMA, BAX, BAK) and anti-apoptotic members (e.g. Bcl-2 and Bcl-xL). Then, Cytochrome c associates with APAF1 and caspase-9 to form the apoptosome to activate the enzyme caspase-9, caspase-3, caspase-6 and caspase-7 leading to apoptosis.

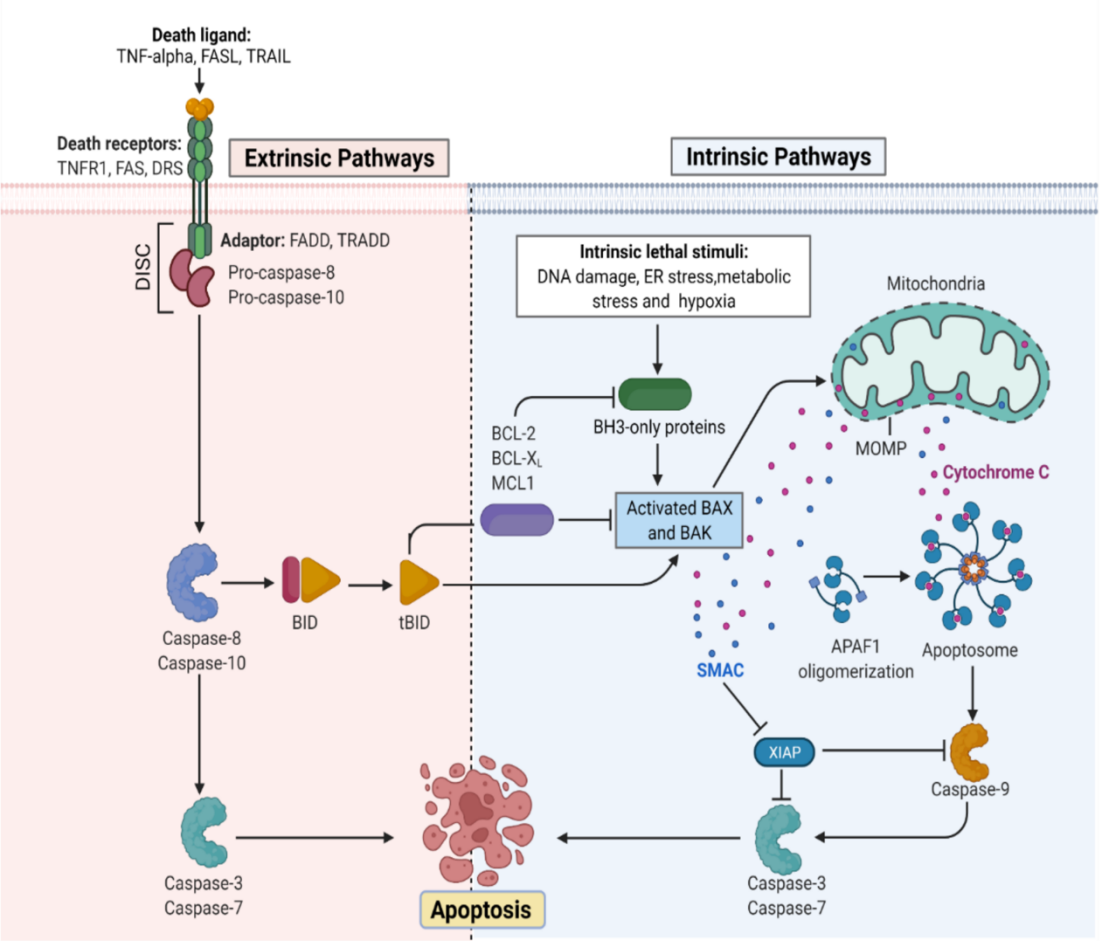

Figure 9 Schematic representation of Extrinsic pathway and Intrinsic pathway.

Source: Wani et al., 2023

Perforin/granzyme Pathway: perforin is mediated, with granzyme A stimulating a caspase-independent apoptosis pathway through single-stranded DNA damage. granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome c release activate caspase-3 either directly or via caspase-10, leading to apoptosis.

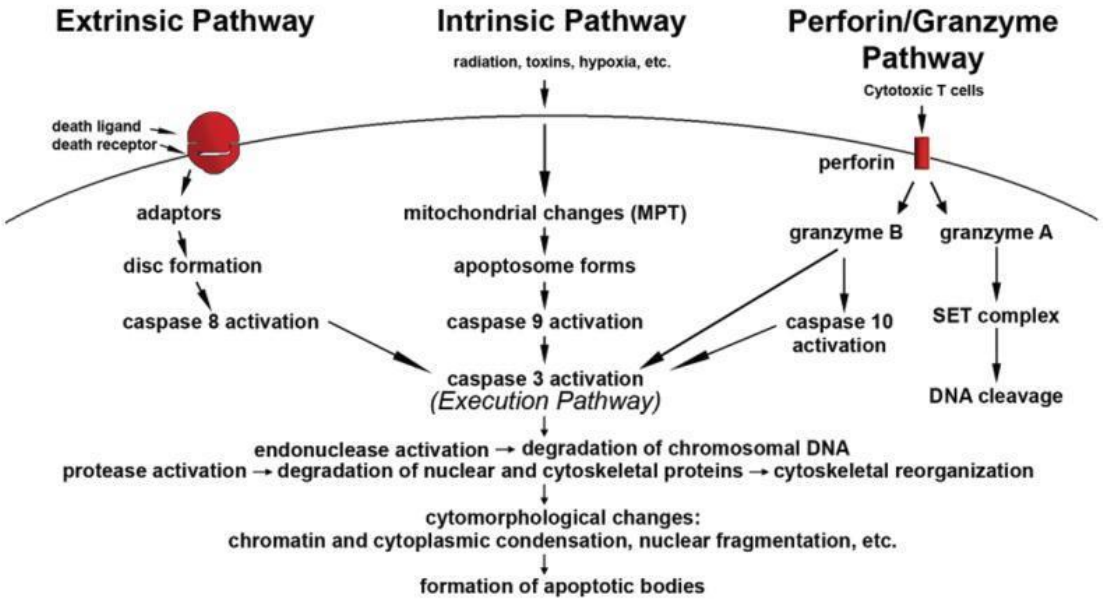

Figure 10 Schematic representation of Perforin/granzyme Pathway.

Source: Elmore et al., 2007

Therefore, if the activation of anti-apoptosis is high up-regulated will be one of the causes of the cancer process. Pro-apoptosis and anti-apoptotic members as shown in Table 2.

Table 2 Pro-apoptosis and anti-apoptotic members (Brenner et al., 2009)

Proapoptotic regulators	**Anti-apoptotic regulators**
Bid	Bcl-X_L
Bad	Bcl-2
Bax	Bcl-w
BIM	Bcl-B
BAK	Mcl-1
Puma (p53 upregulate mediator of apoptosis)	FLIP (FLICE-inhibitory protein)
AIF (apoptosis inducing factor)	XIAP (X-linked inhibitor of apoptosis protein)
Cytochrome C	c-IAP1 (cellular inhibitor of apoptosis 1)
Apaf-1 (apoptosis protease-activating factor 1)	c-IAP2 (cellular inhibitor of apoptosis 2)
p53	Survivin
p73	NF-kB
Smac/DIABLO (second mitochondria-derived activator)	ICAD
Caspases (Cysteine aspartyl-specific proteases)	
HtrA2/Omi	
IkB (inhibitor of NF-KB)	
CAD (caspase activated DNase)	

3.2 Pattern of apoptosis activation

3.2.1 Physiologic apoptosis

Apoptosis, when the body is Physiological, Can be found from the embryo stage to the adulthood is the result of (1) Loss of growth factor signaling during Embryogenesis, turnover of lymphocytes in bone marrow and Thymus gland, (2) A decrease in the levels and function of hormones in the Mammary glands, Endometrium, Ovary's corpus luteum, and Prostate gland, (3) Loss of white blood cell

viability signaling after the activation of white blood cells has been eliminated, resulting in a decrease in the number of white blood cells, and (4) Strong recognition of self-antigens induces activation of two apoptosis pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptors pathway. (Maślińska., 2018)

3.2.2 Pathologic apoptosis

Apoptosis, when the body is pathological, is the result of (1) The destruction of genetic material within cells. The BH3-only sensors within the cytoplasm of cells activate BH1-3 pro-apoptotic proteins, (2) Accumulation of misfolded proteins within cells by BH3-only sensors that activate pro-apoptotic proteins or may be caused by direct activation of the enzyme caspase, and (3) When cells undergo viral infection, viral proteins induce apoptosis pathways by stimulating the intrinsic or mitochondria pathway in these cells. In addition, cytotoxic T lymphocytes also assist in the destruction of viral-infected cells through apoptosis by stimulating the extrinsic or death receptors pathway through the action of the caspase enzyme (Blomgren et al., 2007).

Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome (ALS), acquired immunodeficiency syndrome (AIDS) and neurode-generative diseases such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, and Amyotrophic Lateral Sclerosis.

3.3 Morphological characteristic of cell apoptosis

Apoptosis is a particular form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes are including chromatin condensation, DNA and Nuclear fragmentation, Membrane blebbing, and present of apoptotic bodies (Brenner et al., 2009; Belizário et al., 2015) as shown in Figure 11 and Figure 12.

Cell apoptosis does not induce inflammatory reactions because: (1) apoptotic cells do not release intracellular elements to the surrounding interstitial tissue, (2)

apoptotic cells are rapidly phagocytosis, and (3) phagocytosis does not produce anti-inflammatory cytokines. (Savill et al., 2000)

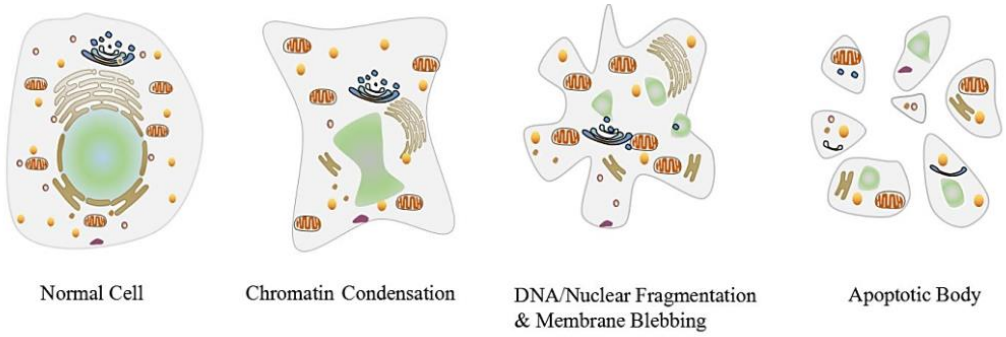

Figure 11 The process of apoptosis and characteristic cell changes.

Source: Brenner et al., 2009

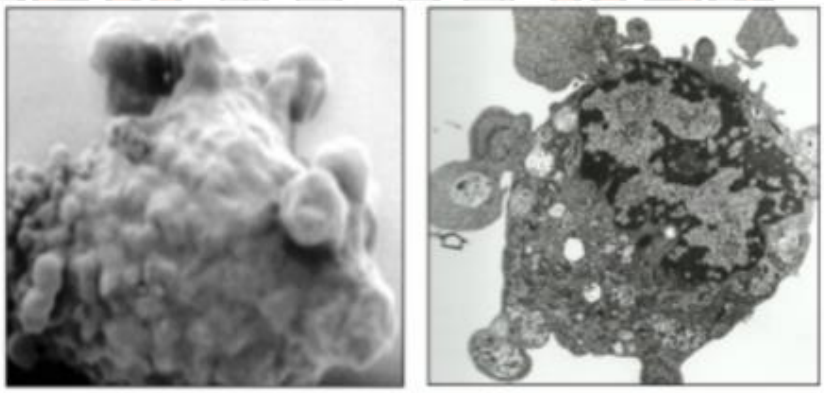

Figure 12 The morphological changes at the microscopy level under scanning electron microscope; SEM (left) and transmission electron microscopy; TEM (right).

Source: Belizário et al., 2015

4. Alternative splicing and regulation

Alternative splicing (AS) is an essential mechanism to increase the complexity of gene expression, and it plays an important role in cellular differentiation and organism development (Wang et al., 2015). AS regulated the mechanism during gene expression that results in a single gene transcribed into several mRNA transcript variants and subsequently translated into multiple protein isoforms (Black et al., 2003). The particular exons of a gene may be included within or excluded from the final, processed mRNA produced from that gene. Consequently, the proteins translated from alternatively spliced mRNAs will contain differences in amino acid sequence and structure as demonstrate (Cooper et al., 2006) as shown in Figure 13. Abnormal of the process may lead to disruption of normal cellular function and the eventuality of disease, Cancer is one of those diseases.

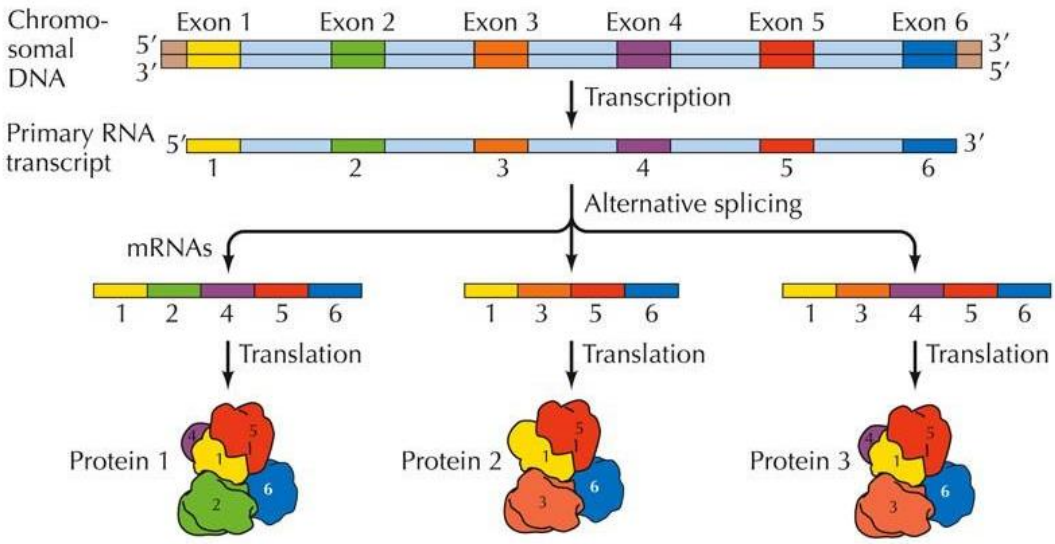

Figure 13 Alternative mRNA splicing generates various protein isoforms.

Source: Cooper et al., 2006

4.1 Regulation by splicing regulatory protein families

For this regulatory machinery, not only the function of spliceosome, there are several groups of protein families contribute in this process including Heterogeneous nuclear ribonucleoproteins (hnRNPs), RNA-binding proteins (RBPs), and Serin and

arginine-rich proteins (SR proteins) or Serine/arginine-rich splicing factors (SRSFs). During post-transcriptional modification, Serine/Arginine-rich splicing factors or SRSFs are the important classes of splicing regulatory protein that plays an essential role in the AS of pre-mRNA to mature mRNA. Particularly in the mRNA splicing process, spliceosome is a protein that regulates AS and role play splice site selection, intron removal and exon ligation. SRSFs are the RNA binding protein which joining to plays important role in the spliceosome assembly. The functional involves of SRSF together with spliceosome presents more efficiency.

Modulation of spliceosome in AS process is divided into 4 main steps. First step, SRSFs bind to snRNP U1 and U2 recognize the 5' splice site and bind to the branch point. second, U4/U6–U5 tri-snRNP complex associates with the forming spliceosome and third, removing U1 and U4 then finally, the U2, U6 and U5 has intron excision and exon ligation (Änkö et al., 2012) as shown in Figure 14.

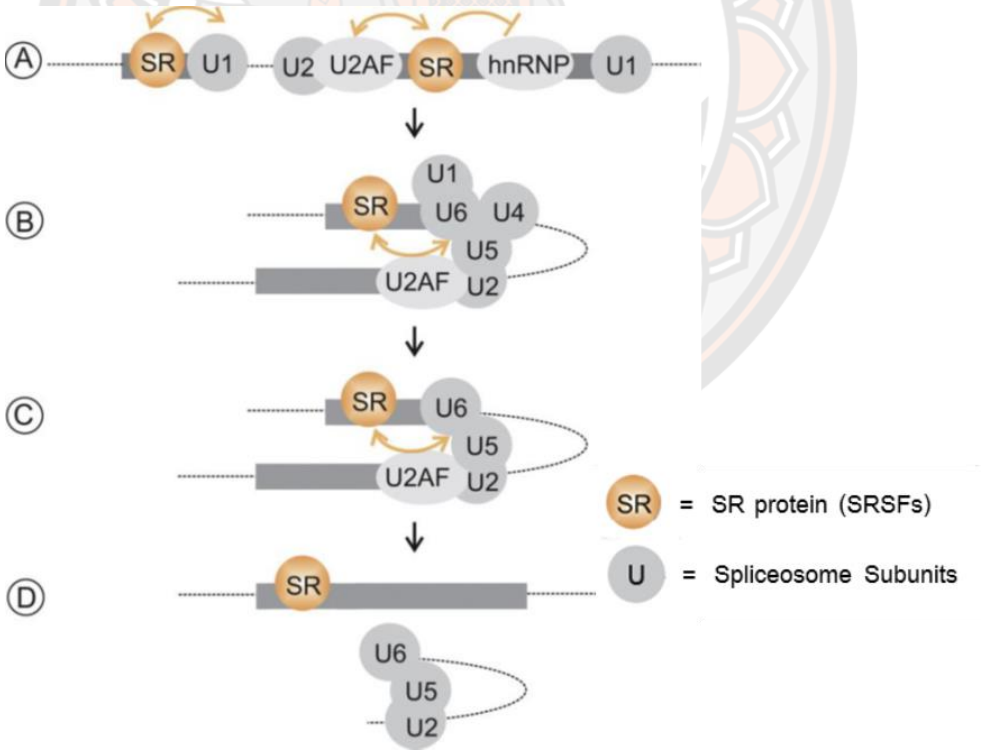

Figure 14 Integration of SRSFs with spliceosome units.

Source: Änkö et al., 2012

4.2 Serine/arginine-rich splicing factors (SRSFs)

SRSFs function as trans-acting factors to facilitate pre-mRNA splicing. There are consists of 12 members of SRSFs including SRSF1 to SRSF12. Each member containing 2 important domains: (1) RNA recognition motif (RRM) at N-terminal which are the mRNA binding site by specific consensus binding sequences of their targeted mRNA, and (2) Arginine-serine rich (RS) domain at C-terminal which are presented the ordering of enriched serine and arginine residues that plays important role the protein-protein interactions, nuclear localization signal and targeted residue of phosphorylation (Änkö., 2014) as show in Figure 15.

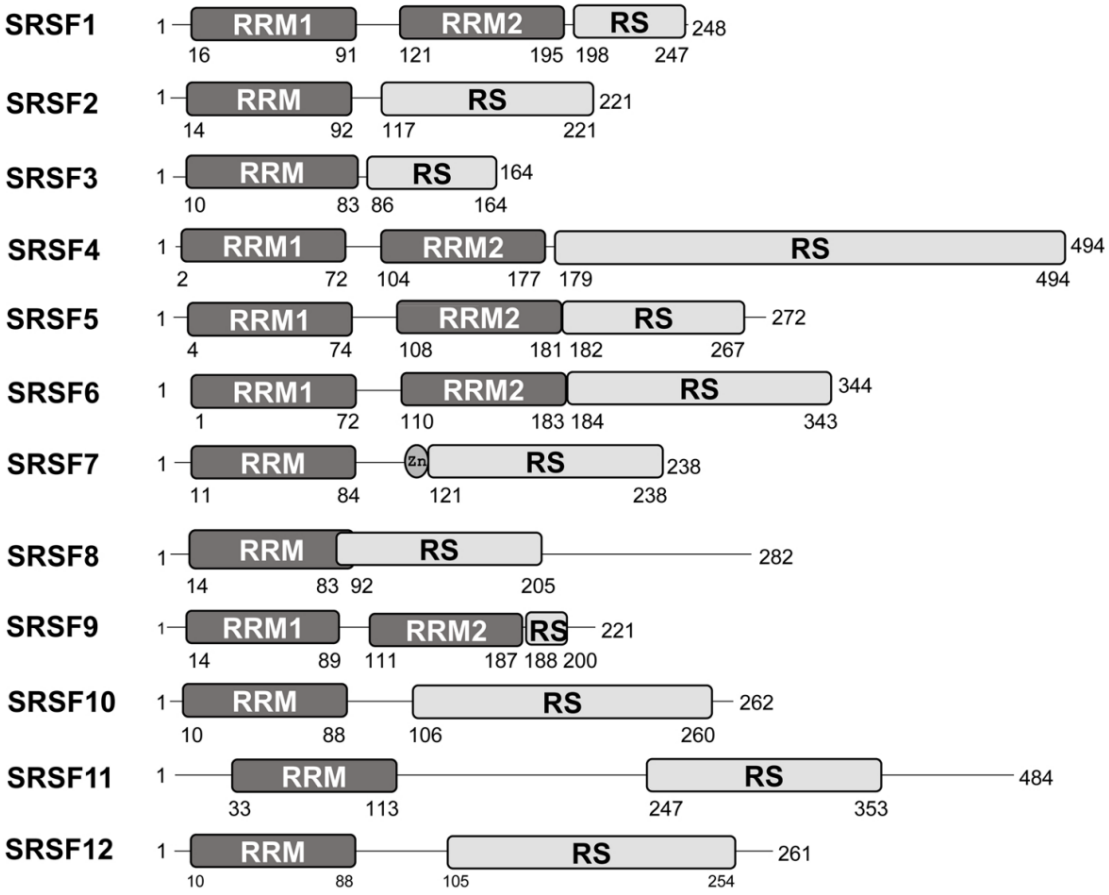

Figure 15 Schematic representation of domain structure of SR proteins and the numbers refer to amino acid positions.

Source: Änkö., 2014

Their previous nomenclature of 12 members SR protein during the course of their discovery was rename such as AS2/AFS is rename as SRSF1, the number of amino acid, protein sequence and functional information of reference number by UniProt and the molecular weight of commercial detectable as shown in Table 3

Table 3 Nomenclature, amino acid, UniProt identifiers and the molecular weight of 12 member of SRSFs (Summary by Preenapan Changphasuk)

SR protein family	Synonym	Amino acid	UniProt identifiers	Detectable MW (kDa)
SRSF1	SF2/ASF, SRp30a	248	Q07955	32 (Invitrogen)
SRSF2	PR264, SC35, SRp30b	221	Q01130	35 (Invitrogen)
SRSF3	SRp20	164	P84103	19 (Invitrogen)
SRSF4	SRP75	494	Q08170	57 (Invitrogen)
SRSF5	HRS, SRP40	273	Q13243	39 (acbam)
SRSF6	B52, HEL-S91, SRP55	334	Q13247	40 (acbam)
SRSF7	9G8, AAG3	238	Q16629	27 (acbam)
SRSF8	DSM-1, SRP46	282	Q9BRL6	32 (Labome)
SRSF9	SRp30c	221	Q13242	24 (Invitrogen)
SRSF10	SRp38, SRrp40, TASR	262	075494	37 (mybiosource)
SRSF11	NET2, p54	484	Q05519	54 (Invitrogen)
SRSF12	SRrp35	261	Q8WXF0	30 (biocompare)

4.3 Regulation of SRSFs by SRPKs

During post-transcriptional modification, serine-arginine protein kinases or SRPKs are splicing regulatory protein that plays an essential role in the phosphorylation of the serine residuals in the serine/arginine-rich repeats (RS domain) of various mRNA splicing factors and alternative splicing factor/splicing factors or SRSFs (Chan et al., 2013).

The majority of SRPKs reside in the cytoplasm, where they phosphorylate SRSFs (Giannakouros et al., 2011) and migrate into the nucleus via interaction with transportin-SRs. In the nucleus, SRSFs can be phosphorylated by Cdc-like kinases (CLKs) (Li et al., 2023) as shown in Figure 16.

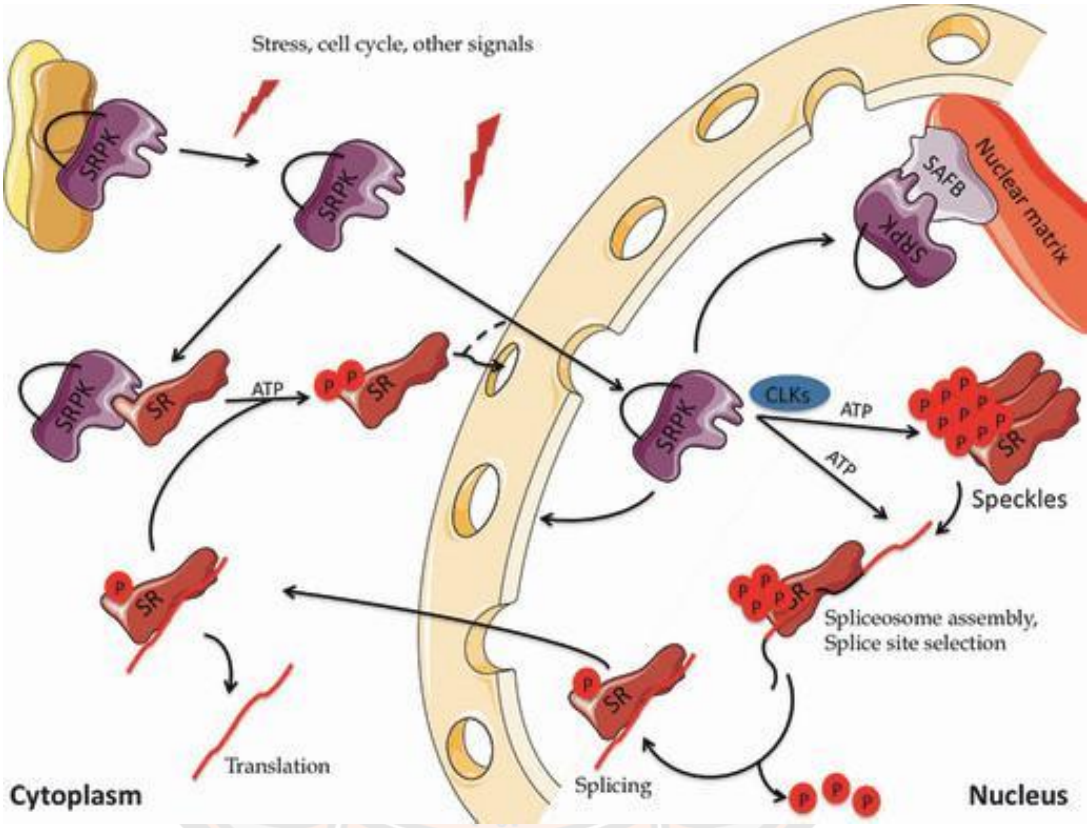

Figure 16 Regulation of SRSRs phosphorylation by SRPKs and CLKs. In the cytoplasm, SRSFs are phosphorylated by SRPKs at arginine-serine rich (RS) domain and transport their target into the nucleus. Whereas CLKs may encourage the phosphorylation of SR proteins in the nucleus leading to regulate mRNA splicing.

Source: Giannakouros et al., 2011

4.4 Serine-arginine protein kinases (SRPKs)

This protein family consists of three members in mammalian cells including SRPK1, SRPK2, and SRPK3. They are serine/threonine kinases that especially recognize and phosphorylate to SR protein family at serine/arginine-rich (RS) domain.

Accumulated evidence has indicated that SRPK1 and SRPK2 have been found overexpressed in various types of cancer, for example, breast, colon, pancreatic carcinomas, leukemia, gliomas, and ovary, which is correlated with advanced tumor staging and poor prognosis (Nikas et al., 2019)

5. Aberrant alternative splicing in cancers

Aberrant alternative splicing as an alternative hallmark of cancer (Ladomery., 2013). The aberrant mRNAs and their encoded proteins have unique properties that confer new properties of growth, differentiation, and other cellular characteristics to the cancer cell (Fackenthal et al., 2008).

5.1 Reports of aberrant alternative splicing in cancers

There are several studies have reported the important role of the abnormal overexpression of SRSFs can promote the tumorigenesis, angiogenesis, tissue invasion, metastasis, apoptosis evasion, or survival in cancer (da Silva et al., 2015) as shown in Figure 17.

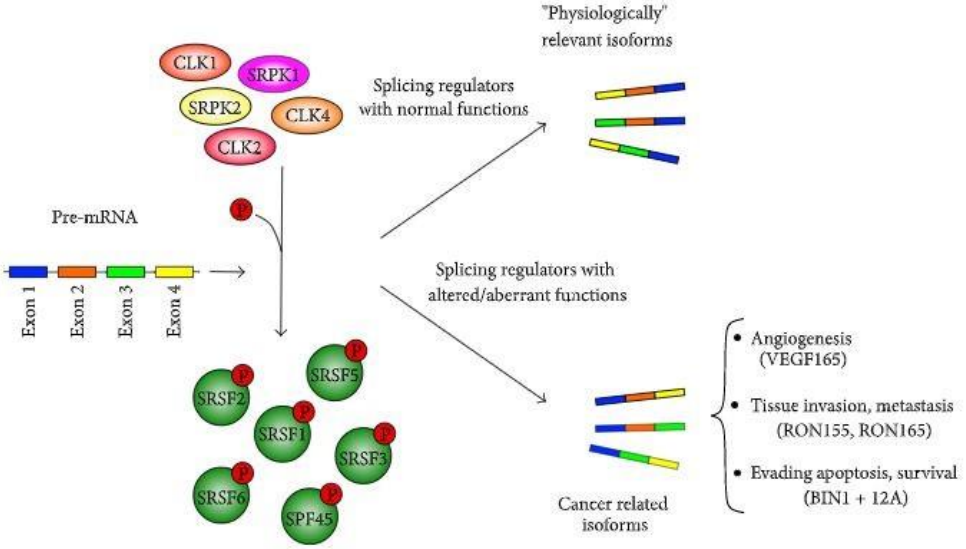

Figure 17 Dysregulation of splicing factors activities and the outcomes in cancer cells

Source: da Silva et al., 2015

5.2 Relationship between aberrant splicing and apoptosis related genes in cancers

As mentioned previously, Gene splicing also known as alternative splicing is one of the hallmarks of cancer (Refer to Figure 7). Cancer cells subvert this process to produce isoforms that benefit cell proliferation or migration and evade apoptosis. Many genes encode for splice variants having opposite roles in cell apoptosis. Isoforms involved in preventing cell death such as (1) BCL2L1, a member of the Bcl-2 family which generates two isoforms, BCL-xL prevents apoptosis and BCL-xS promotes apoptosis, (2) FAS, a member of the TNF-receptor superfamily which promoting the extrinsic pathway of apoptosis. An alternatively spliced isoform, soluble Fas (sFAS) is produced by the skipping of exon 6 resulting in evade apoptosis, (3) CASP-2 also known as Caspase 2 is a pro-apoptotic protein and tumor suppressor. Splicing of CASP-2 into Casp-2S isoforms will leading to the anti-apoptotic protein and (4) Bridging Integrator 1 (BIN1) is tumor suppressor that functions by interacting with and inhibiting c-MYC. Inclusion of exon 12A of BIN1 generates a protein isoform that no longer binds Myc, leading to eliminates BIN1 tumor-suppressive function.

5.3 Bridging Integrator 1 (BIN1)

Bridging Integrator 1 (BIN1) is tumor suppressor that functions by interacting with and inhibiting c-MYC (Esmailzadeh et al., 2011) as shown in Figure 18.

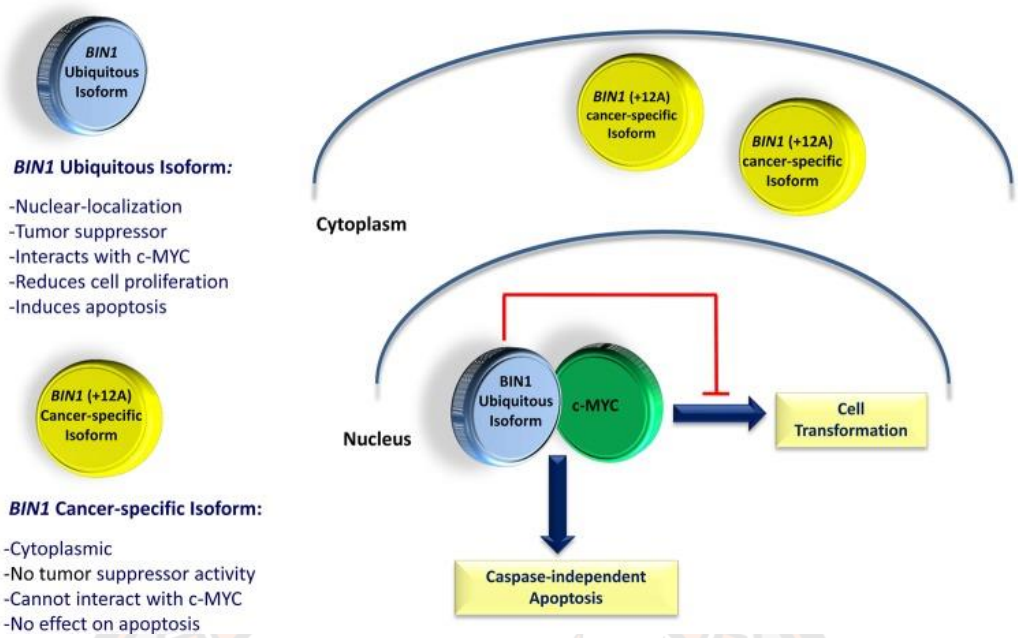

Figure 18 The function of BIN1 ubiquitous isoform and BIN1 cancer-specific isoform.

Source: Esmailzadeh et al., 2011

Under normal physiology, pre-mRNA of BIN1 will splice into BIN1 wild-type for action as Pro-apoptotic protein. Whereas, when aberrant splicing, BIN1 will splice into BIN1+12A isoform (BIN1+12A or BIN1_V4, exon 14-15-16 skipping) for action as Anti-apoptotic protein (Esmailzadeh et al., 2011) as shown in Figure 19.

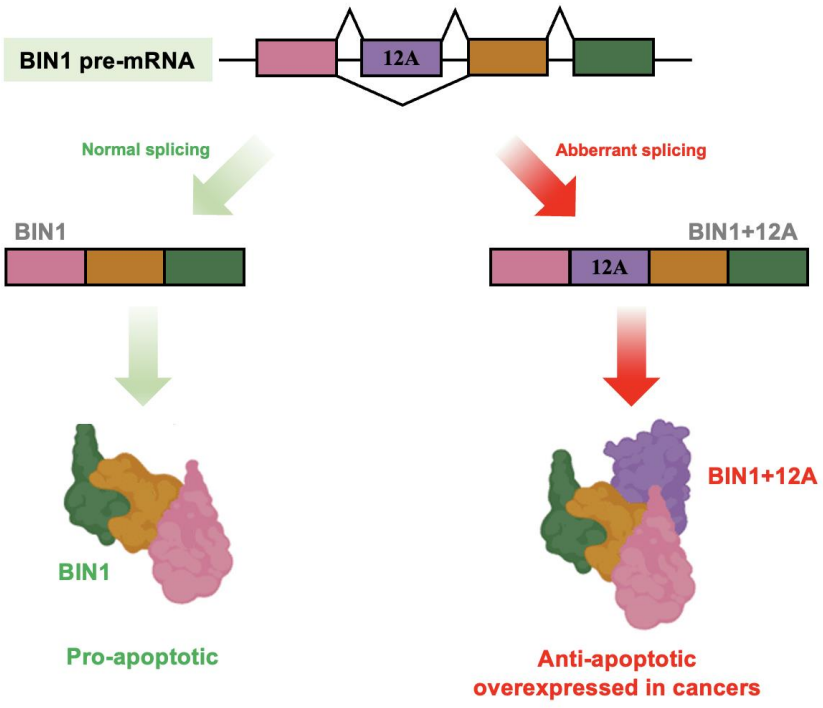

Figure 19 Aberrant alternative splicing of BIN1 promoted cancer progression.

Source: Modified from Pio R. et al., 2009

5.4 Relationship between BIN1 and SRSF1 protein

In non–small cell lung cancer (NSCLC) showed that the aberrant splicing of BIN1 was under the control of serine/arginine-rich splicing factor 1 (SRSF1). RT-PCR indicated that the expression levels of BIN1 were decreased in SRSF1 overexpressed cells compared with that of control cells, on the contrary, BIN1+12A was increased in SRSF1 overexpressed cells compared with that of control cells in H460 cells (Wang et al., 2019) as shown in Figure 20.

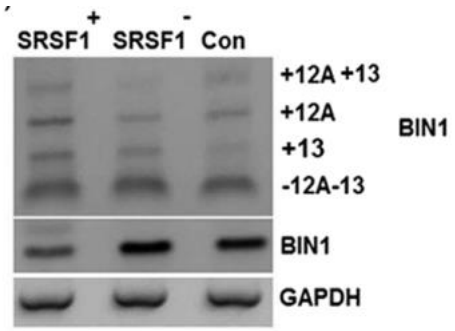

Figure 20 The expression levels of BIN1 and BIN1+12A under the control of SRSF1 in H460 cells.

Source: Wang et al., 2009

6. Aberrant alternative splicing in CCA

Summary of oncogenic spliced genes in CCA was reported by Yosudjai et al in 2019. The aberrant alternative splicing occurred in many important genes in CCA such as CD44 (CD44v6/CD44v8-10), Wnt-Inducible Secreted Protein 1 (WISP1v), NIMA-related kinase 2 (Nek2A, Nek2B, Nek2C), Trefoil Factor 2 (ΔEX2TFF2), Forkhead box protein 3 (Foxp3Δ3), Tumor Protein 53 (Δ133p53), Pyruvate kinase (PKM2), E Protanoid receptor 3 (EP3-4) and Anterior gradient2 (AGR2vH). For example, CD44v8-10 and Δ133p53 related to anti-apoptosis and chemo-resistant. The abnormal genes were associated to promote CCA to evading cell death (Yosudjai et al., 2019) as shown in Figure 21 and Table 4.

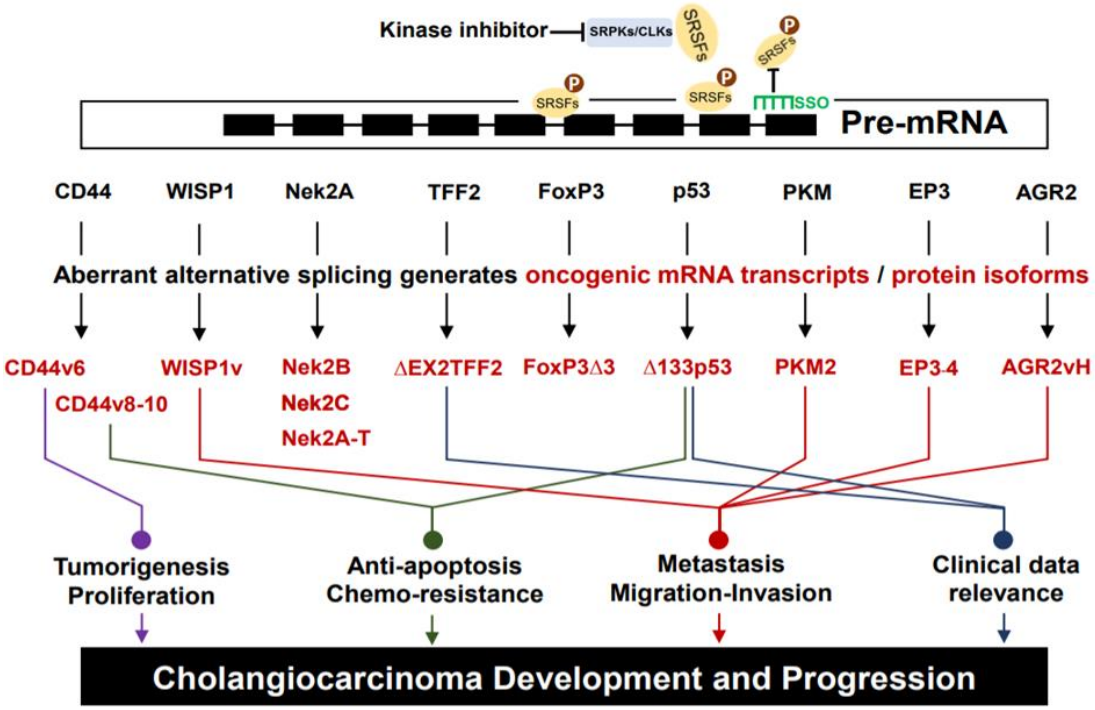

Figure 21 The spliced mRNA transcript and their functional involves in CCA.

Source: Yosudjai et al., 2019

Table 4 The spliced mRNA transcript and their function involves in CCA (Yosudjai et al., 2019)

Gene	Spliced transcripts	Function	Reference
CD44	CD44v6 CD44v8-10	Proliferation Anti-apoptosis	Yun KJ et al., 2022 Thanee M et al., 2016
Wnt-inducible secreted Protein 1	WISP1v	Neural and lymphatic invasion	Tanaka S et al., 2003
Serine/threonine-protein kinase Nek2	Nek2B Nek2C	Function unknow	Kokuryo T et al., 2007
Trefoil factor 2	ΔEX2TFF2	Independent prognostic marker	Kamlua S et al., 2012
Forkhead box protein 3	Foxp3Δ3	Function unknow	Harada K et al., 2012
Tumor protein 53	Δ133p53	prognostic marker 5-Fluorouracil resistant	Nuttasirikul N et al., 2013 Nuttasirikul N et al., 2015
Pyruvate kinase	PKM2	Neural invasion	Yu G et al., 2015
E prostanoid receptor 3	EP3-4R	Proliferation, migration, and invasion	Du M et al., 2015

6.1 SRSF profiling and SRSF1 targeting in CCA

There are number of research articles summarized the upregulation of SRSFS in 9 cancer tissue by Genvestigator and presented that SRSF1 is the most upregulated as the first ranking in many types of cancer, such as colorectal, lung, osteosarcoma, breast, ovarian, pancreatic, glioma cancer and melanoma (Zhou et al., 2019) as shown in Figure 22.

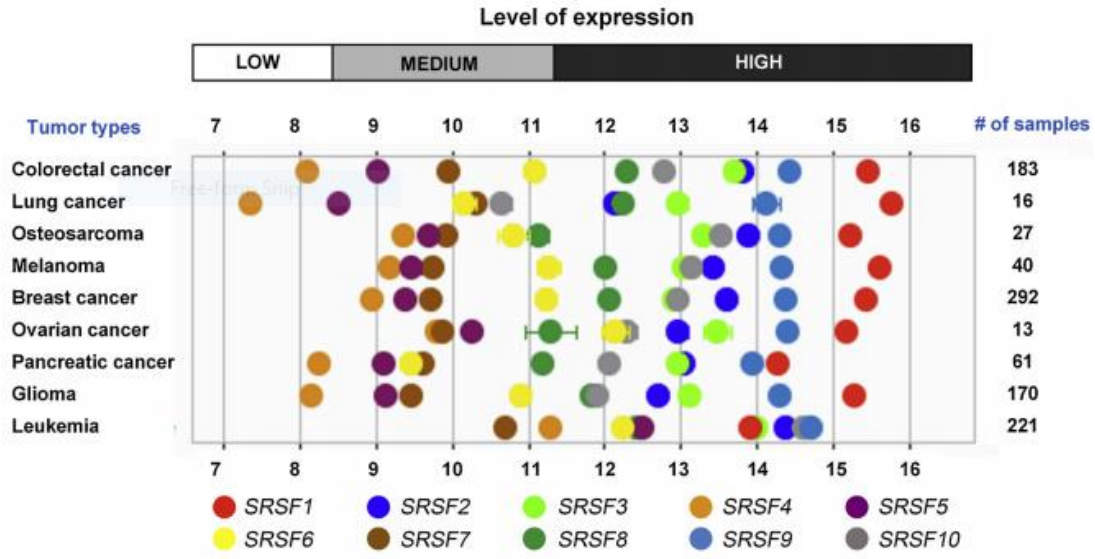

Figure 22 Expression profile of SRSF genes in 9 human tumor types.

Source: Zhou et al., 2019

Furthermore, previous result from Pawee Tangwiriyarotkul studied the expression profile of SR protein family members using silencing strategy. The result shown that SRSFs was upregulated in CCA cells (KKU-055, KKU-100, KKU-213A, and KKU-213B) and SRSF1 was high expression in CCA cells when compared with established cholangiocyte (MMNK-1) as show in Figure 23.

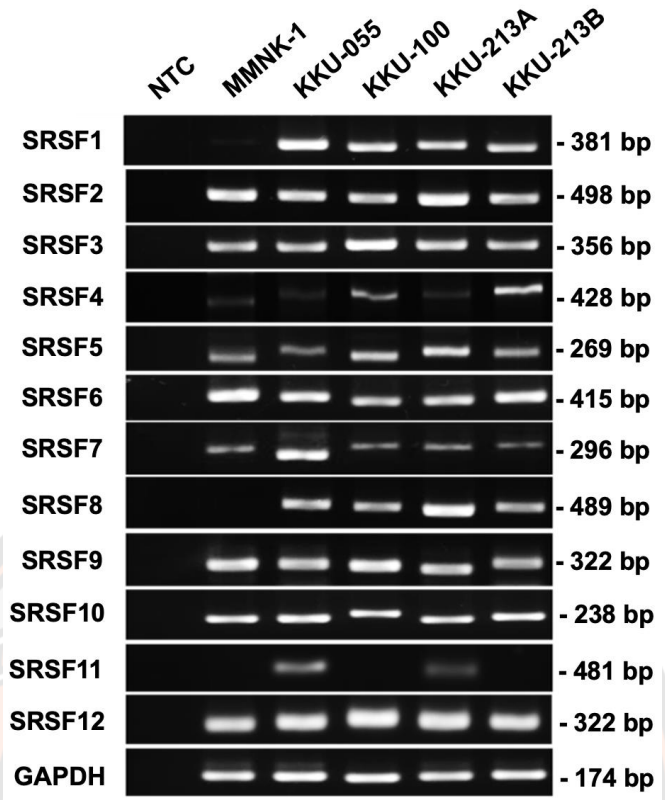

Figure 23 The gene expression profile of SRSFs in CCA cell lines compared with established cholangiocyte.

Source: Pawee Tangwiriyarotkul, Unpublished data.

And then, the previous study from Sirintip Srisuksai studied that determined the expression of SRSF1 protein in CCA tissues derived from operative CCA patients. The result shown that SRSF1 was up-regulated significantly in 9 CCA tissues (T) compare with adjacent non-tumor (N) as show in Figure 24.

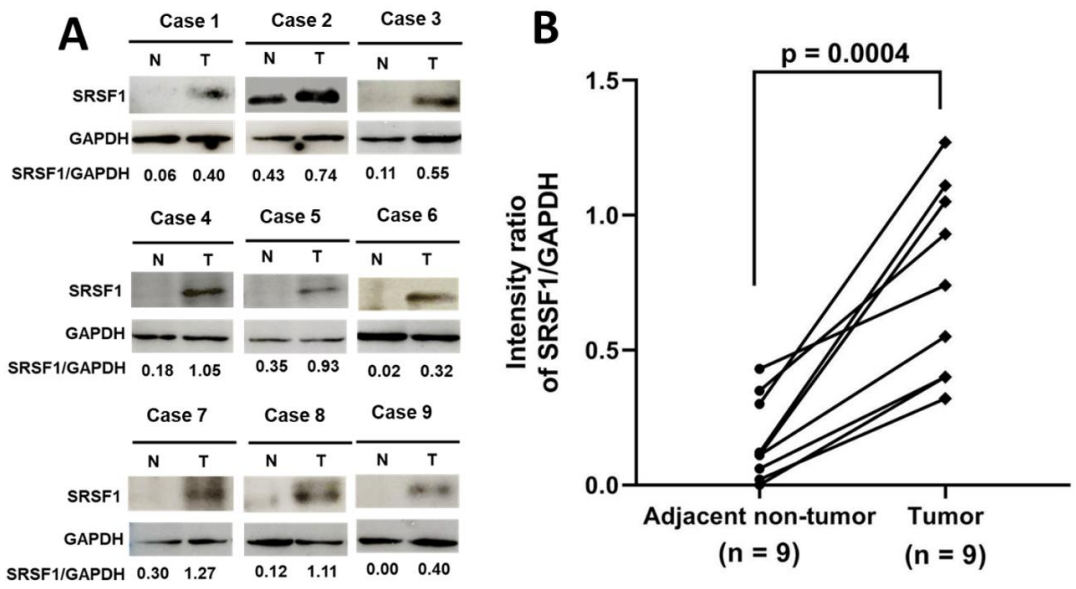

Figure 24 The expression of SRSF1 in 9 matched-pair tissues from CCA patients (A) and statistically analysis (B).

Source: Sirinthip Srisuksai, Unpublished data.

In addition, the previous study from Chaturong Inpad studied that determined the suppress SRSF1 expression by 2 specific siRNA on the dead induction in KKU-213A CCA cell line using live/dead dual staining and observed under a fluorescence microscope. After SRSF1 suppression, we found the increased number of dead cell staining (which analyzed from red fluorescent). When comparison with control group as show in Figure 25.

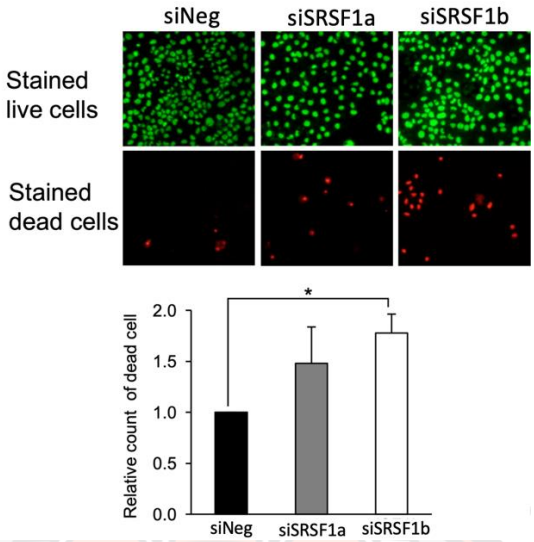

Figure 25 The effect of siSRSF1 on the dead induction in KKU-213A cell.

Source: Chaturong Inpad, Unpublished data.

The previous study from Phattarin Pothipan studied that determined the suppress SRSF1 expression by 2 specific siRNA on cytochrome c pattern in KKU-213A CCA cell line and observed under a fluorescence microscope. The results form control cell shows cytoplasmic spot or granular pattern of cytochrome c staining. Whereas siSRSF1 group, we can observe the diffuse pattern which represent the leak of cytochrome c from mitochondria as show in Figure 26.

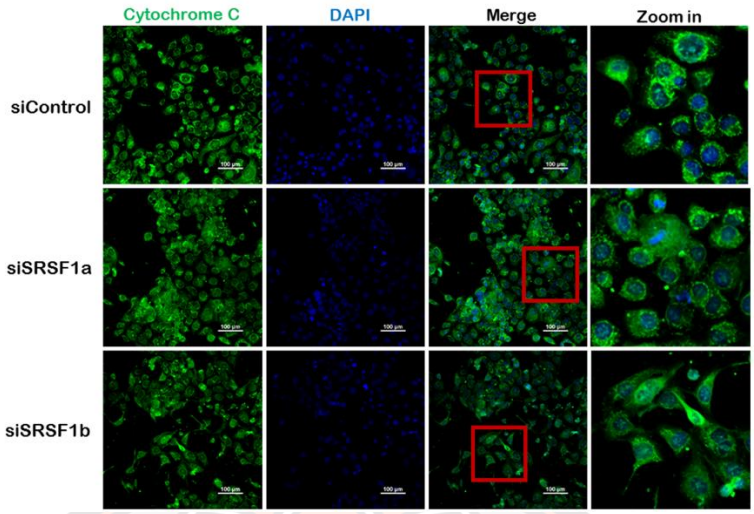

Figure 26 The effect of siSRSF1 on cytochrome c pattern in KKU-213A cell.

Source: Phattarin Pothipan, Unpublished data.

6.2 Studies of SRPK inhibiting in cancers

As mentioned above, SRPKs are proteins that regulate pre-mRNA splicing via phosphorylate to SR protein family at RS domain and resulting in SRSF activation. Accumulated evidence has indicated that SRPK1 and SRPK2 have been found overexpressed in different types of cancer including breast, colon, pancreatic carcinomas, leukemia, non-small cell lung carcinoma, squamous cell lung carcinoma, gliomas, ovary, and hepatocellular carcinoma which is correlated with advanced tumor staging and poor prognosis (Nikas et al., 2019). So, SRPKs inhibition is currently being studied in cancer.

Previous reporting in non-small cell lung cancer (NSCLC). The result shown that overexpression of SRPK1 promoted the growth and migration, while knocking down the expression of SRPK1 inhibited the growth, migration, and tumorigenicity (Liu et al., 2016) as shown in Figure 27.

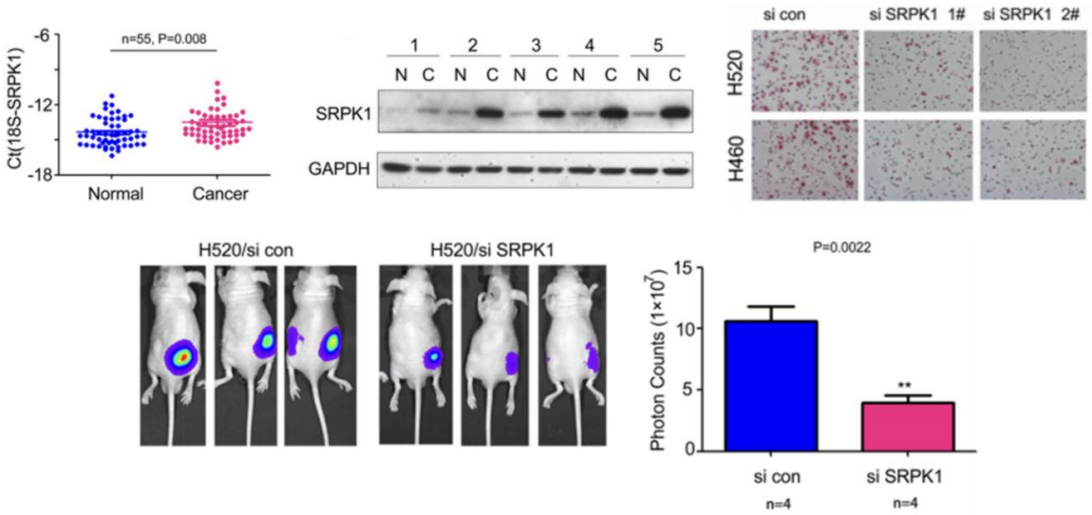

Figure 27 Knocking down the expression of SRPK1 inhibited NSCLC cell.

Source: Liu et al., 2016

Previous reported that reduced SRPK1 expression suppressed the migration of BLBC cells in vitro and breast cancer metastasis to the lungs in vivo through interacting with the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway. All in all, SRPK1 downregulation enhances apoptosis and sensitivity to chemotherapy in hormone positive (van Roosmalen et al., 2015)

Gammons et al. reported that targeting SRPK1 using short hairpin RNA (shRNA) in tumor of melanoma resulted in significantly reduced tumor growth (Gammons et al., 2014) as shown in Figure 28.

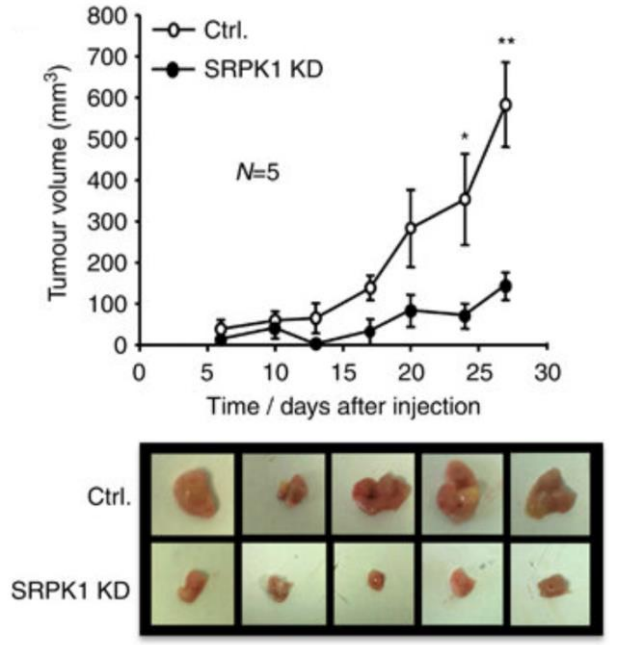

Figure 28 Tumor volume of melanoma after SRPK1 knockdown.

Source: Gammons et al., 2014

In 2018, SPHINX31 (SRPK1-specific inhibitor) was reported in leukemia. In the study of growth, SPHINX31 can decrease the cell growth rate as dose dependent manner in many leukemia cell line (Tzelepis et al., 2018) as shown in Figure 29.

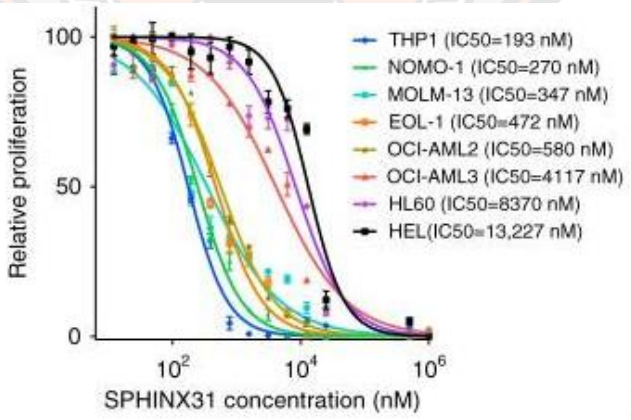

Figure 29 The effect of SPHINX31 on leukemia cell viability.

Source: Tzelepis et al., 2018

In addition, previous study in Human Umbilical Vein Endothelial Cells (HUVEC) showed that SPHINX31 was significantly reduced compared with negative controls in a dose-dependent manner. Treatment with SPHINX31 in the HUVECs led to a significant reduction of the percentage and fluorescence intensity of Ki-67. Overall, SPHINX31 can reduce the proliferation of HUVECs (Gu et al., 2022) as shown in Figure 30.

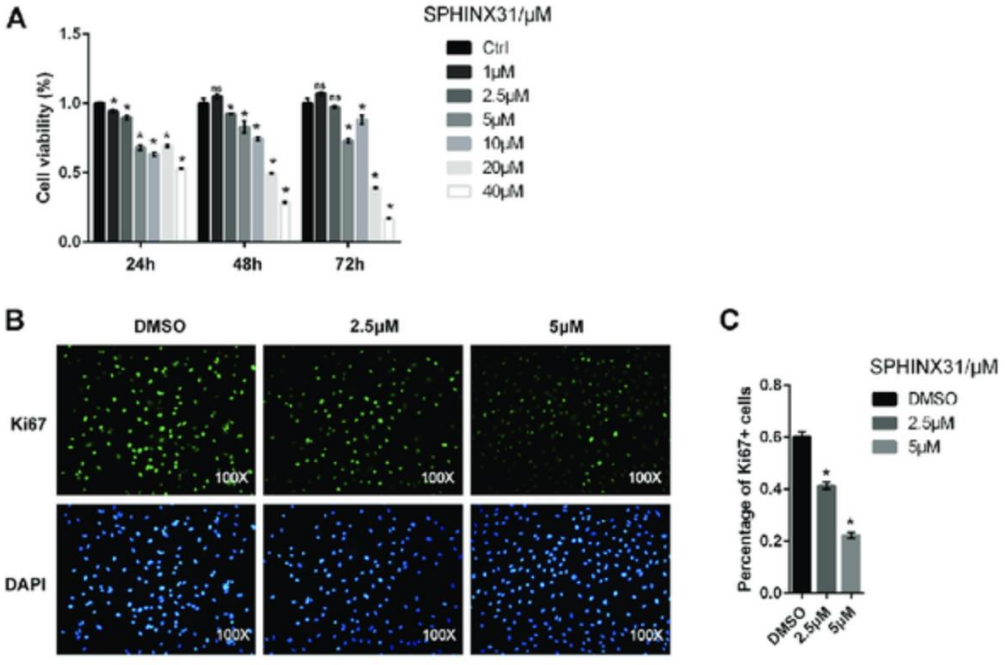

Figure 30 Effects of SPHINX31 (SRPK1-specific inhibitor) on cell viability in HUVECs

Source: Gu et al., 2022

Previous study in endometrial cancer showed SPHINX31 can decrease the kinase activity of SRPK1 by reducing the phosphorylation form of SRSFs, then reduced Survivin and increase cleaved PARP for apoptotic induction in SPEC-2 cells (Kurimchak et al., 2020) as shown in Figure 31.

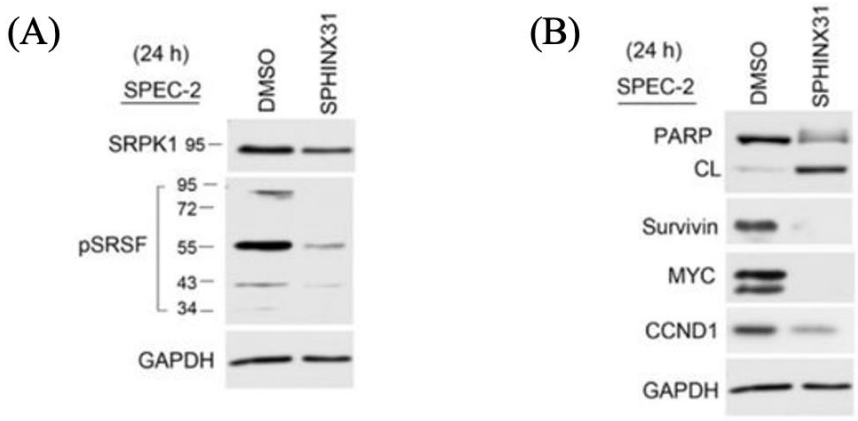

Figure 31 The effect of SPHINX31on SRPK1 activity and SRSF phosphorylation (pSRSFs) (A), and the effect of SPHINX31 on apoptotic protein marker (B) in SPEC-2 cell.

Source: Kurimchak et al., 2020

In 2022, He et al demonstrated that both SRPK1 inhibitors (SPHINX31 and SRPIN340) and siRNA can increase the apoptotic poppulation. The result of protein expression showed the increasing of cleaved PARP and cleaved caspase-3 in ENKTL (extranidal NK/T-cell lymphoma) cells (He et al., 2022) as shown in Figure 32.

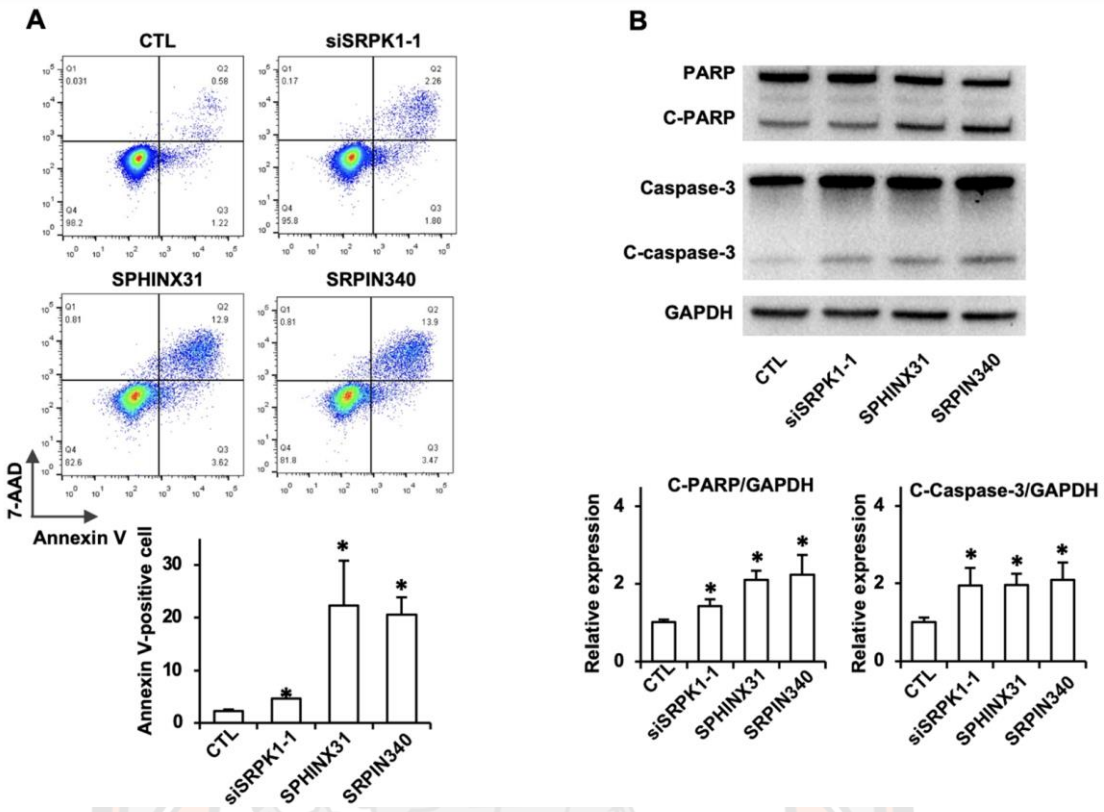

Figure 32 SRPK1 inhibition promoted apoptosis in ENKTL cells. The apoptotic poppulation (A) The level of apoptotic protein (B).

Source: He et al., 2022

Previous study in leukemia cell line, the result showed that SRPIN340 can inhibit the survival of all leukemia cell line. Moreover, SRPIN340 can trigger early and later events of apoptosis in Jurkat cells (Siqueira et al., 2015) as shown in Figure 33.

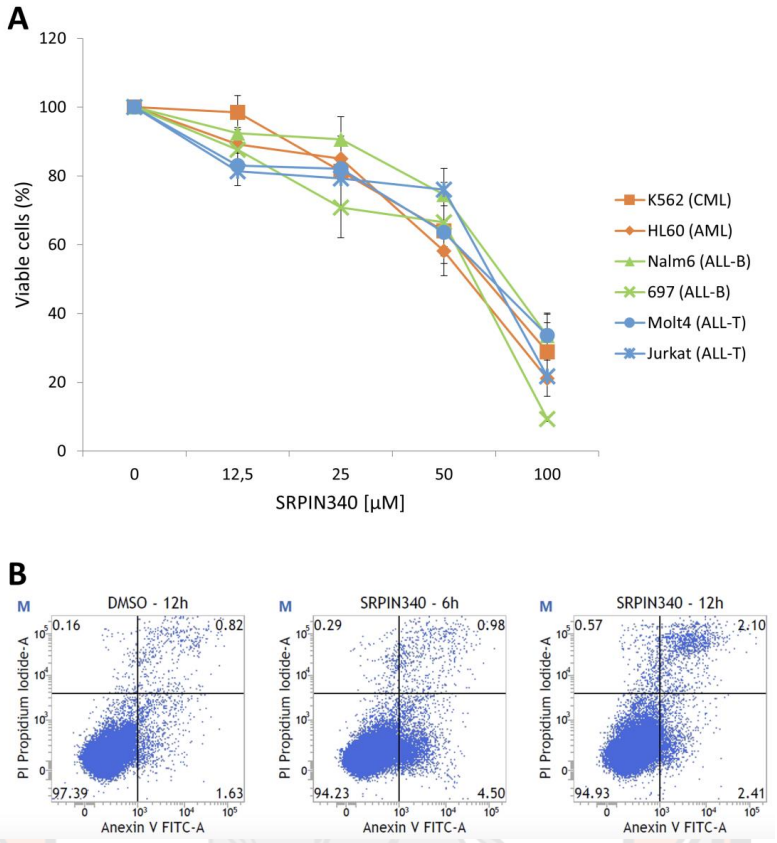

Figure 33 The effect of SRPIN340 on leukemia cell viability (A) and cell apoptosis (B).

Source: Siqueira et al., 2015

Moreover, the effect of SRPIN340 demonstrated that phosphorylation of SRSF4, SRSF6, SRSF5, and SRSF2 can decreased during treatments of both the HL60 and Jurkat cell line (Siqueira et al., 2015) as shown in Figure 34.

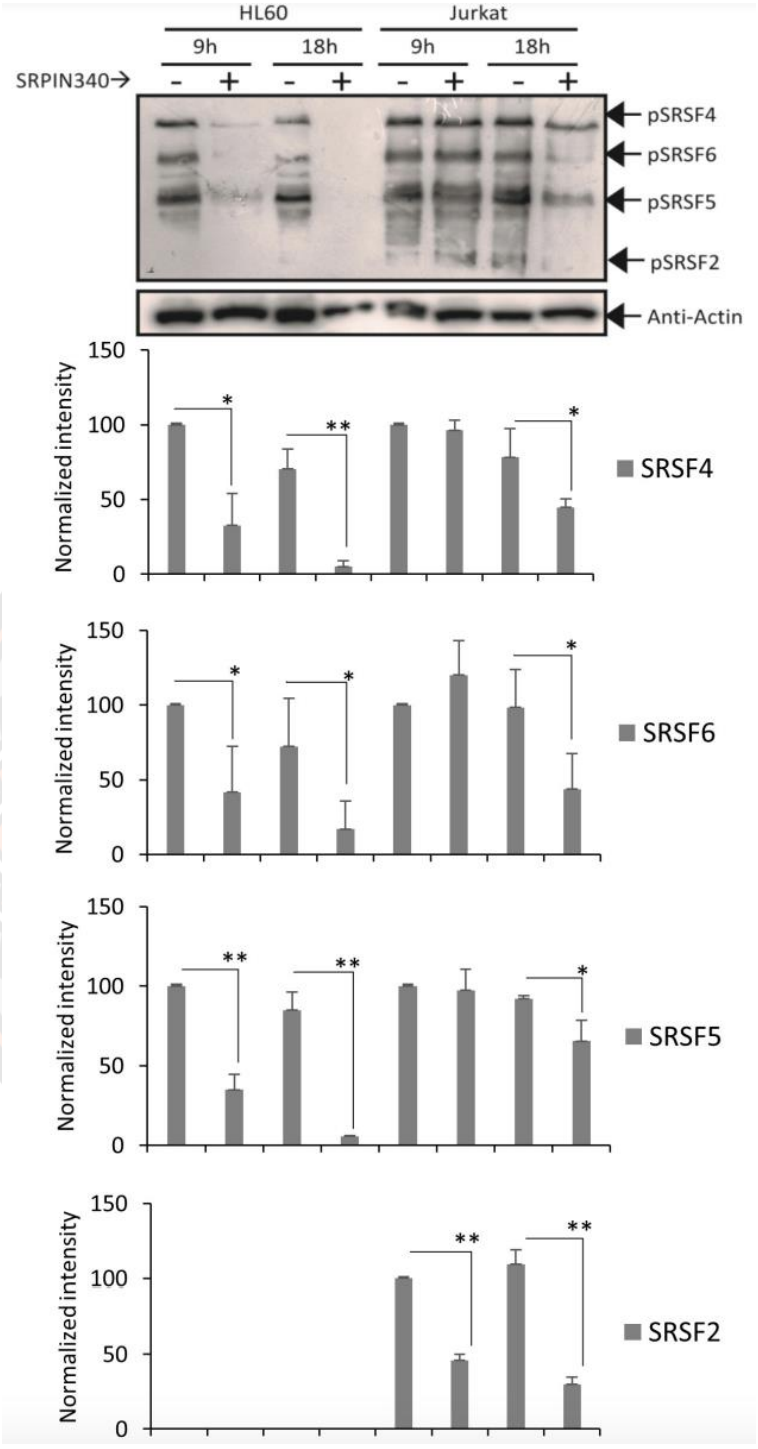

Figure 34 The effect of SRPIN340 on SR protein phosphorylation (pSRSF) in leukemia cell.

Source: Siqueira et al., 2015

Previous study in RPE cell line, the result showed that SPHINX31 can reduce the phosphorylation profile of SRSF1 as dose dependent manner (Batson et al., 2017) as shown in Figure 35.

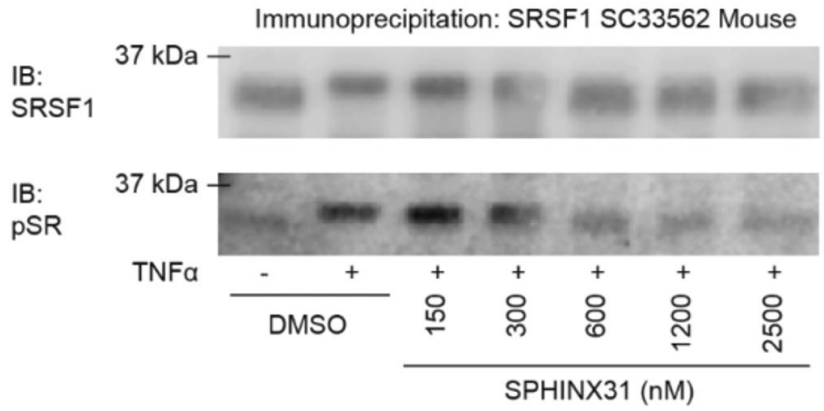

Figure 35 The effect of SPHINX31 on SRSF1 phosphorylation in RPE cell.

Source: Batson et al., 2017

Previous study in A375 cell line, to determine the phosphorylation profile of SRSFs by using docking blocker of SRPK1 (DBS1) which is inhibitor blocked protein-protein interaction (PPI) between SRPK1 and SRSF1. The results showed DBS1 can decreased phosphorylation of SRSF, especially in pSRSF1/2 when compared with control group (Li et al., 2021) as shown in Figure 36.

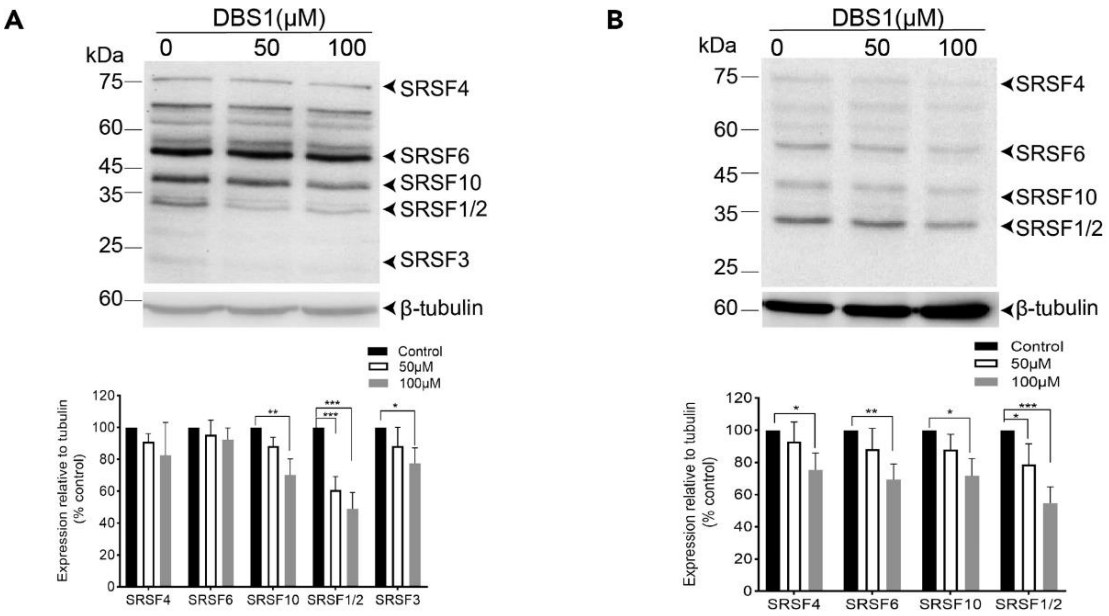

Figure 36 The effect of DBS1on SRSF phosphorylation (pSRSFs) in A375 (A) and Hela cell line (B).

Source: Li et al., 2021

Previous reported that suppression of SRSF phosphorylation using SPHINX31 resulted in significantly reduced pSRSF1, pSRSF2 and pSRSF9. In addition, SPHINX31 can also reduce migration activity of melanoma cells (Siriwath et al., 2021) as shown in Figure 37.

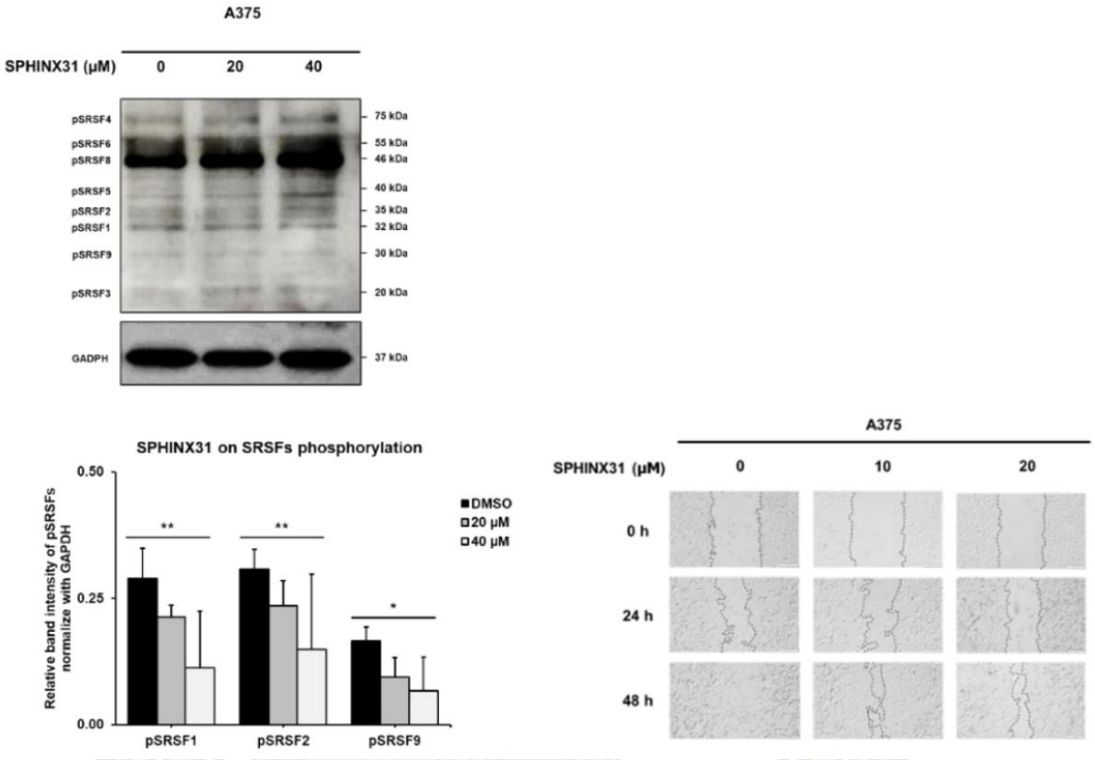

Figure 37 The effect of SPHINX31 treatment on SRSF phosphorylation (pSRSFs) (left) and effect on migration abilities of the melanoma cell (right).

Source: Siriwath et al., 2021

Previous reported that reduced SRPK1 expression suppressed the migration of BLBC cells in vitro and breast cancer metastasis to the lungs in vivo through interacting with the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway. All in all, SRPK1 downregulation enhances apoptosis and sensitivity to chemotherapy in hormone positive (van Roosmalen et al., 2015)

The effect of SRPK inhibitors was reported by Moreira et al in 2018. SRPK inhibitors were effective reduced SRPK1 nuclear translocation and phosphorylation of SR proteins at both EGF-treated and -no treated in the intracellular environment in B16F10 melanoma cell (Moreira et al., 2018) as shown in Figure 38.

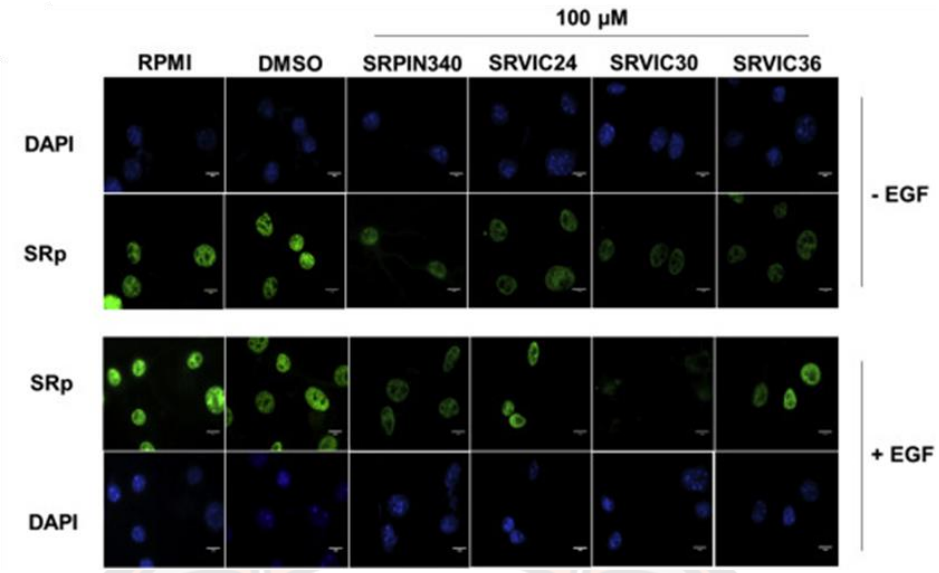

Figure 38 Effect of compounds on subcellular localization of SRPK1 and cellular activity of SRSF phosphorylation.

Source: Moreira et al., 2018

Previous study in T24 cell showed that SRPIN340 showed significantly prevent the nuclear translocation of the protein kinase; SRPK1 and SRPK2 as show in immunofluorescent image and western blotting (Sigala et al., 2021) as shown in Figure 39.

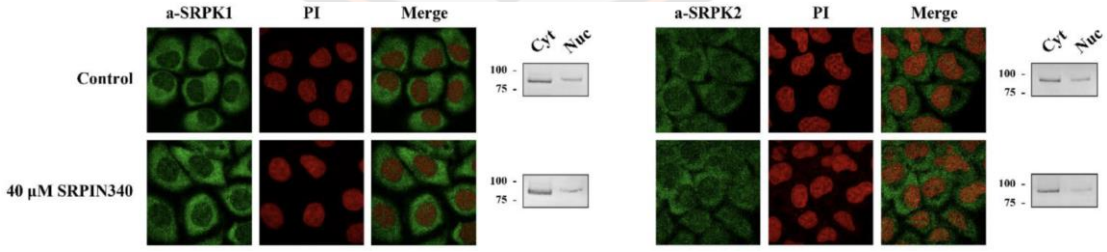

Figure 39 Effect of SRPIN340 on nuclear translocation of SRPK1 and SRPK2 in T24 cell.

Source: Sigala et al., 2021

Moreover, previous study demonstrated that SRPIN340 can reduced nuclear protein levels of SRSF1 in HEK293T cell (Gu et al., 2022) as shown in Figure 40

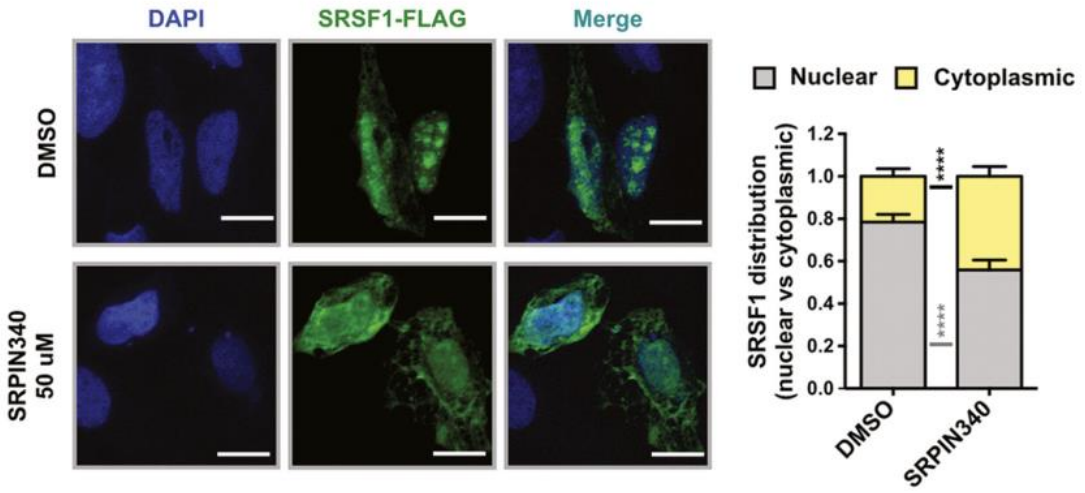

Figure 40 The effect of SRPIN340 on SRSF1 accumulation and the ratio of nuclear and cytoplasmic intensity of SRSF1 signal in HEK293T cell.

Source: Gu et al., 2022

In HuCCA-1 cells, the result showed HuCCA-1 cells treated with the indicated concentrations of SPHINX31 for 24 h showed decreased expression of SRSF1 phosphorylation and decreased SRSF1 nuclear localization in HuCCA-1 cells treated with SPHINX31 (Supradit et al., 2022) as shown in Figure 41.

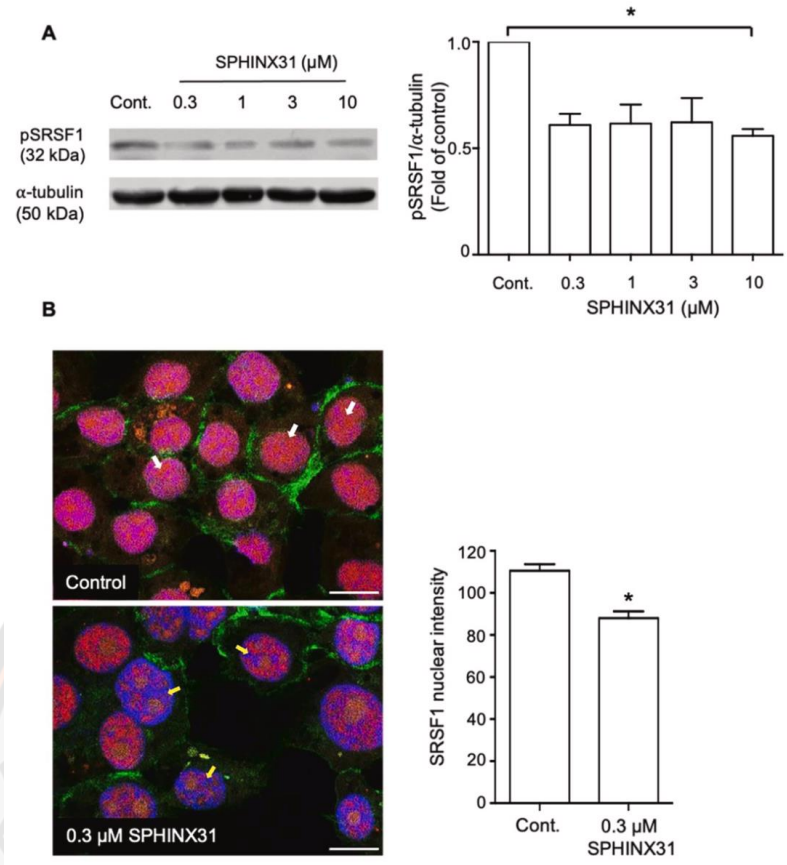

Figure 41 Effect of SPHINX31 on SRSF1 phosphorylation and nuclear localization in HuCCA-1 cells (A). SRSF1 nuclear localization in HuCCA-1 cells treated with SPHINX31 (B).

Source: Supradit et al., 2022

7. SRPK inhibitor

7.1 Development of SRPK inhibitors and their structure

Accumulated evidence has indicated that SRPKs have been found overexpressed in different types of cancer. Therefore, these data suggest that SRPKs are potential targets for the development of anti-metastatic drugs.

The role of SRPKs in cancers has leading to the search of small molecules capable of inhibiting their catalytic activity (da Silva et al., 2015). These compounds which include SRPIN340 (Fukuhara et al., 2006), MVRL09 (Gammons et al., 2014), SPHINX (Gammons et al., 2014), SRPIN803 (Morooka S. et al., 2015), SPHINX31

(Batson et al., 2017), and SRPKIN-1 (Hatcher et al., 2018) have presented promising experimental results in in vitro and in vivo studies as shown in Figure 42.

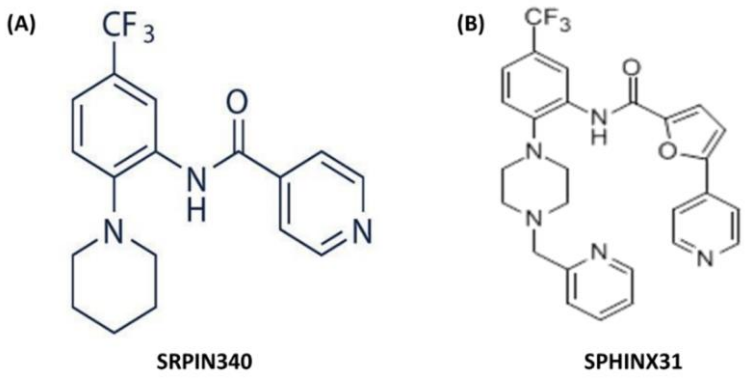

Figure 42 The structure of two main important SRPK inhibitors; SRPIN340 (A). SPHINX31 (B).

Source: Cayman Chemical, USA

For the specificity of these SRPK inhibitor reported by Batson et al in 2017, A radiolabeled ATP competition assay was investigated for 50 key kinases, which representative of the human kinome. The result shown that 1 μM of SPHINX31 was significantly inhibitory SRPK1 activity (Batson et al., 2017) as shown in Figure 43.

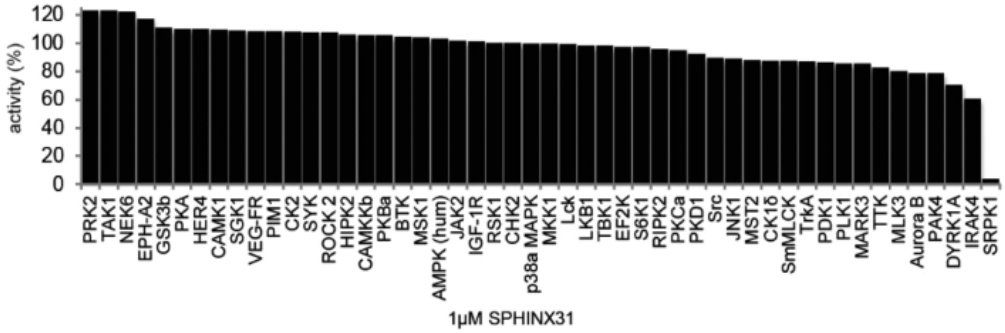

Figure 43 The effect of SPHINX31 against a series of key 50 enzymes that represent the inhibition of SRPK1.

Source: Batson et al., 2017

7.2 Mechanism of action of SRPK inhibitors

The main SRPK1/2 inhibitor was SRPIN340 (SR Protein Inhibitor 340), and subsequently modified the molecular structure to be specific to SRPK1 to be SPHINX31 (SR Protein Inhibitor X 31). SRPKs are ATP-competitive inhibitors that compete for ATP binding at the ATP binding site, causing SRPKs to not phosphorylation to SRSFs, keeping SRSFs in an inactive form and unable to control the alternative splicing process (Batson et al., 2017; Siqueira et al., 2015) as shown in Figure 44.

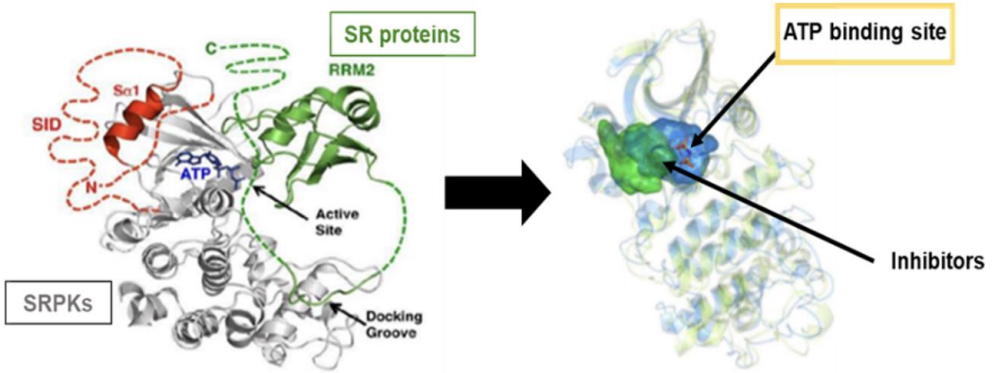

Figure 44 Mechanism of action of SRPK inhibitors.

Source: Batson et al., 2017; Siqueira et al., 2015

SRPIN340 (SR protein phosphorylation inhibitor 340) has a structure of N-[2-(1-piperidinyl)-5(trifluoromethyl)phenyl] isonicotinamide, which potently inhibits both SRPK1 and SRPK2 activity but not inhibit other kinases, for example Clk1 and Clk4. Therefore, SRPIN340 is a highly selective inhibitory for SRPK1 and SRPK2, that resulted in the reduction of phosphorylation of SR proteins (Fukuhara et al., 2006) as shown in Figure 45.

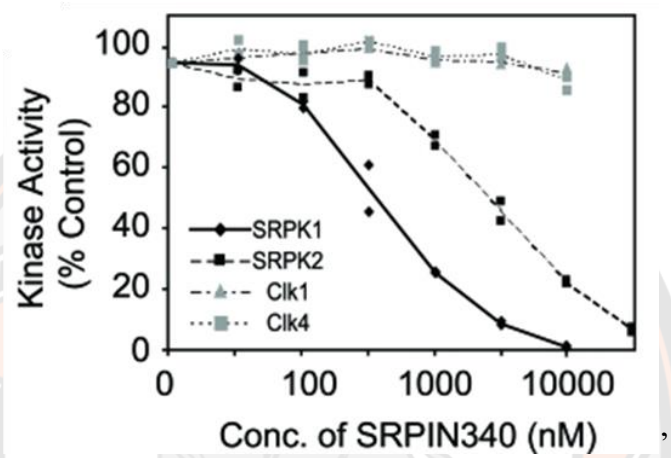

Figure 45 The effect of SRPIN340 for various protein kinases.

Source: Fukuhara et al., 2006

SPHINX31 (SR protein phosphorylation inhibitor X 31) has a structure of S-(4-pyridinyl)-N-[2-[4-(2-pyridinylmethyl)-1-piperazinyl]-5-(trifluoromethyl)phenyl]-2-furancarboxamide, which potently inhibits SRPK1 activity (Batson et al., 2017) as shown in Figure 46.

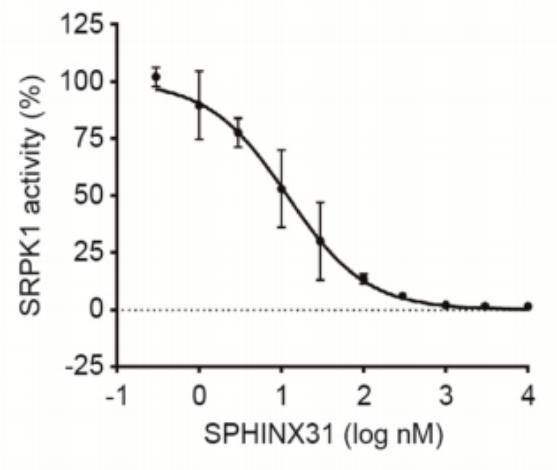

Figure 46 The effect of SPHINX31 for SRPK1 activity.

Source: Batson et al., 2017

Therefore, these information lead to the experimental design and objective in this recent study. To study the effect of SRPK inhibitors (SRPIN340 and SPHINX31) in CCA cell lines. First, to study the effect of SRPK inhibitors on apoptosis, activity of SRSF1 protein, cytoplasmic-to-nuclear translocation properties of SRSF1 protein and determined alternative splicing of BIN1 gene in CCA cell lines.

CHAPTER III

RESEARCH METHODOLOGY

Laboratory materials

Materials and instruments

- 0.5 ml PCR tube (LC premium, Thailand)
- 1.5 ml microcentrifuge tube (LC premium, Thailand)
- 15 ml centrifuge tube (LC premium, Thailand)
- 50 ml centrifuge tube (LC premium, Thailand)
- 6-well plate (SPL life science, Korea)
- 12-well plate (SPL life science, Korea)
- 24-well plate (SPL life science, Korea)
- 96-well plate (SPL life science, Korea)
- 96-well black plate (SPL life science, Korea)
- 5% CO_2 Incubator (Shel lab, USA)
- Agarose gel electrophoresis apparatus (ATTO, Japan)
- Amersham ImageQuant™ 800 (Cytiva, UK)
- Cell Culture Slide 8 well (SPL life science, Korea)
- Confocal microscope (Nikon, Tokyo)
- Cubee™ Mini-Centrifuge (BIOGENOMED, Taiwan)
- BLooK LED transilluminator (Gene Direx, Taiwan)
- Fluorescence microscope (Zeiss, Germany)
- Glass pipette (Precicolor HBG, Germany)
- Glove-powder free (Sri Trang, Thailand)
- ImageQuant™ LAS 500 (GE Healthcare Life Science, UK)
- Inverted microscope (Olympus, USA)
- Laminar Flow hood (NUAIRE, USA)
- Micropipette (Proline ®Plus, USA)
- Microplate reader (Biochrom Ltd, UK)
- Microwave

- Mini Trans-Blot® Cell (Bio-Rad laboratories, USA)
- Muse® Cell Analyzer (Millipore sigma, USA).
- Neubauer hemocytometer (Fisher Sciencitific, Germany)
- Pipette aid (TOPSCIEN ®, China)
- Polyvinylidene fluoride (PVDF) membrane (Bio-Rad laboratories, USA)
- Refrigerated centrifuge (Hettich, Germany)
- T100™ Thermal Cycler (Bio-Rad laboratories, USA)
- Sonicator ultrasonic probe (Merck Millipore, USA)
- Vortex (SPSLAB, USA)
- Water bath (Memmert, USA)

Chemicals

- 30% Acrylamide: Bis (29:1) (National diagnostics, USA)
- 100 bp DNA Ladder (Smobio, Hsinchu City, Taiwan)
- 1×MyTagTM HS Red Mix (Bioline, Taunton, Massachusetts)
- Agarose (Invitrogen, NY)
- Albumin from bovine serum (Fluka, USA)
- Antibiotic-Antimycotic,100X (Fisher Scienctific, Germany)
- Ammonium persulphate (APS) (HIMEDIA, India)
- Beta-mercaptoethanol (Gibco, Grand Island, NY)
- Clarity™ Western ECL Substrate (Bio-Rad laboratories, USA)
- Coomassie Brilliant Blue G-250 (Bio-Rad laboratories, USA)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltman, MA)
- Dimethyl sulfoxide (DMSO) (Sigma-aldrich.St. Louis, NY)
- Enhanced chemiluminescence (ECL) (Bio-Rad laboratories, USA)
- E.Z.N.A.® Total RNA Kit 1 (Omega BIO-TEK, USA)
- Fetal bovine serum (FBS) (Gibco, Waltman, MA)
- Glycine (Merck Millipore, USA)
- Guava® ICF Instrument Cleaning Fluid (Luminex Corporation, USA)
- Halt™ Protease inhibitor Cocktail, 100X (Thermo scientific, USA)
- HisenScriptTM RH [-] RT-PreMix Kit (Intron Biotech, Seoul, South Korea)
- Immobilon® ECL ultra western HRP substrate (Merck Millipore, USA)

- Live/Dead Double staining kit (Merck Millipore, USA)
- Methanol (RCI labscan, Thailand)
- Muse™ AnnexinV & Death Cell Reagent (Merck Millipore, USA)
- Novel Juice (Gene DireX, Hsinchu City, Taiwan)
- Penicillin 100 U/mL (Gibco, Waltman, MA)
- Paraformaldehyde (SIGMA-ALDRICH, USA)
- Phosphatase Inhibitor Cocktail Set V, 50X (Merck Millipore, USA)
- Phosphate buffered saline (PBS) Tablets (Amresco, E.U.)
- Radioimmunoprecipitation (RIPA) buffer (SIGMA-ALDRICH, USA)
- Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, MA)
- SDS (Bio basic, CA)
- Streptomycin 100 µg/mL (Gibco, Waltman, MA)
- SRPIN340 (Cayman Chemical, USA)
- SPHINX31 (Cayman Chemical, USA)
- Tris-acetate-EDTA (TAE buffer) (Bio basic, CA)
- Tris (powder) (Bio-Rad laboratories, USA)
- Tetramethylethylanediamine (TEMED) (Bio basic, CA)
- Trypsin/EDTA (Gibco, Waltman, MA)
- Tween 20 (Life science, TH)

Biological materials

Cell lines

KKU-213A was provided by Cholangiocarcinoma Research Institute, Khon Kaen University. It is a high-invasive cell line originated from intrahepatic cholangiocarcinoma with well differentiation (represented OV-associated CCA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) FBS with of 100 Unit/ml of penicillin and 100 µg/ml streptomycin and maintained at 37°C and 5% CO_2. Cells were sub-cultured twice a week. At 70-80% confluence, cells were detached form culture flask using 0.025% w/v trypsin/EDTA.

TFK-1 was purchased from Cell Resource Center for Biomedical Research, Tohoku University. Cells originated from extrahepatic cholangiocarcinoma (represented non-OV associated CCA). Cells were cultured in Roswell Park Memorial

Institute (RPMI) 1640 Medium, supplemented with 10% (v/v) FBS with of 100 Unit/ml of penicillin and 100 µg/ml streptomycin and maintained at 37°C and 5% CO_2. Cells were sub-cultured once a week. At 70-80% confluence, cells were detached form culture flask using 0.025% w/v trypsin/EDTA.

The morphology of KKU-213A and TFK-1 cells were shown in Figure 47.

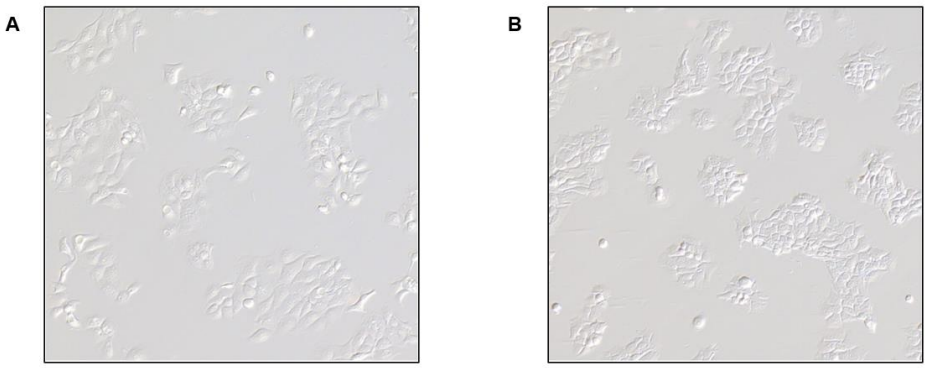

Figure 47 The morphology of KKU-213A (A) and TFK-1 (B) cells, under 10X microscope.

SRPK inhibitors

SRPIN340 and SPHINX31 were purchased from Cayman chemical, USA. SRPK inhibitors dissolved in DMSO and stored at 4°C. Cells at 70-80% confluence were treated with different concentration of SRPK inhibitors (0, 10 and 20 µM) for 18 hours with 0.5% (v/v) DMSO was added to vehicle control.

Primers

Primer for BIN1_All and BIN1+12A isoform was amplified by specific primers that previously designed by Preenapan Changphasuk in figure 48 and 49. Beta-actin is used as an internal control. The sequences of primers used in this study were listed in Table 5.

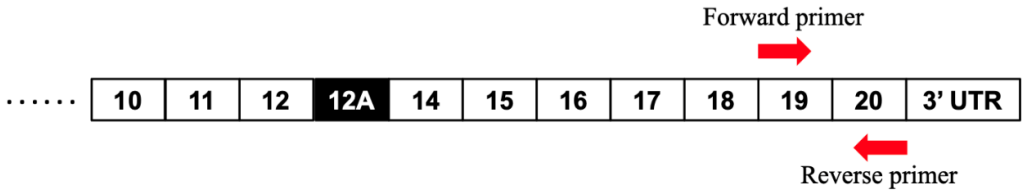

Figure 48 Binding sequence of BIN1 specific primer for splicing all isoforms used in RT-PCR

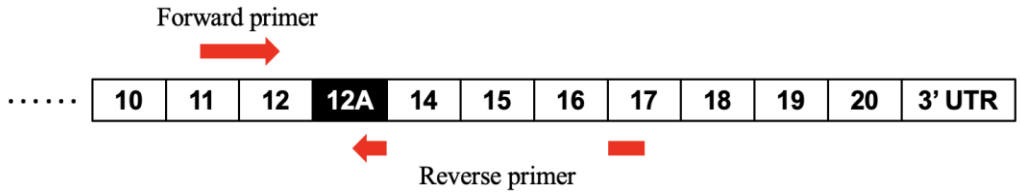

Figure 49 Binding sequence of BIN1 specific primer for splicing 12A isoform (BIN1+12A or BIN1_V4, exon 14-15-16 skipping) used in RT-PCR

Table 5 Sequences of specific primers for BIN1_All, BIN1+12A isoform and internal control gene.

Name	Forward primer 5'→3'	Reverse primer 5'→3'	Product (bp)
BIN1_All	TGATGTGGTGCTGGTGATCC	GACCTAATCTTTGGGAGAACGCC	311
BIN1+12A	GGCCCAGCCCAGAAAGAAAAGTA	GCCTTTCCGGAGCTGAGATGGG	214
BETA-ACTIN	AGTCGGTTGGAGCGAGCATC	GGGCACGAAGGCTCATCATT	295

Antibodies

Primary Antibody

1) Anti-SRSF1: antibody produced in mouse (Invitrogen, NY)

2) Anti-pSRSFs (anti-Phosphoepitope SR Protein): antibody produced in mouse (Merck Millipore, USA)

3) Anti-Cleaved Caspase-3 (Asp175) (5A1E): antibody produced in rabbit (Cell signaling Technology, USA)

4) Anti-Cytochrome C: antibody produced in rabbit (Cell signaling Technology, USA)

5) Anti-GAPDH: antibody produced in rabbit (Merck Millipore, Germany)

6) Anti-Beta-Actin: antibody produced in rabbit (Cell signaling Technology, USA)

7) Anti-LaminB1: antibody produced in rabbit (Cell signaling Technology, USA)

Secondary Antibody

1) HRP conjugated Anti-mouse IgG: antibody produced in goat (Fisher Scienctific, Germany)

2) HRP conjugated Anti-rabbit IgG: antibody produced in goat (Fisher Scienctific, Germany)

3) FITC conjugated Anti-rabbit IgG: antibody produced in goat (Merck Millipore, Germany)

4) Cy3 conjugated Anti-mouse IgG: antibody produced in goat (Merck Millipore, Germany)

Methods

Preparation of SRPK inhibitors

Cells were plated in a disk at $8x10^5$ cells per disk. After 24 hours, cells at 70-80% confluence will be treated with SRPK inhibitors at 0, 10 and 20 µM for 18 hours, with 0.5% (v/v) DMSO was added to vehicle control.

Live/dead dual staining

Cells were plated in a 96-well black plate at $1.6x10^4$ cells per well. After 24 hours, cells were treated with SRPK inhibitors at 0, 10 and 20 µM for 18 hours. Next, centrifuged at 1,500 rpm, 5 min, 25°C and then added 50 µl (1 µl of 1 µM Cyto-dye, 1 µl of 1 mg/ml PI and 1,000 of Staining buffer) and incubated 37°C, 15 min. The cells were examined under a fluorescence microscope (Zeiss, Germany).

Flow cytometry with AnnexinV/7-AAD staining

Cells were plated in a 24-well plate at $1x10^5$ cells per well. After 24 hours, cells were treated with SRPK inhibitors at 0, 10 and 20 µM for 18 hours. 75 µl of MUSE™ AnnexinV & Death Cell Reagent (Merck Millipore, USA) and equal volume with $5x10^4$ cells of each group were mixed. After 20 min incubation at room temperature in dark, the numbers of live cells, death cells and apoptosis cells (early and late stages) were analyzed using Muse® Cell Analyzer and the attached analytical software (Millipore sigma, USA).

Immunocytofluorescence (ICF)

The cells were culture in 6 well plate at $5x10^4$ cells per well for 24 hours. Cells were treated with SRPK inhibitors at 0, 10 and 20 µM for 18 hours. The cells were washed PBS at 2 times. Next, fixed 4% (v/v) paraformaldehyde-containing PBS for 30 min at room temperature and then washed PBS 2 times, 5 min. After washing, permeabilization with 0.2% (v/v) Triton-X containing PBS for 5 min at room temperature and then washed PBS at 1 time for 2 min. For blocking and non-specific binding was blocked by 1:20 FBS in PBS for 20 min. Cells were incubated with primary antibody: Rabbit anti-human Cytochrome C 1:1,000 (Cell signaling Technology, USA), mouse anti-human SRSF1 1:250 (Invitrogen, NY) at 4°C for

overnight followed by secondary antibody goat anti-rabbit IgG-FITC 1:100 (Merck Millipore, MA, USA), goat anti-mouse IgG-Cy3 1:100 (Merck Millipore, Germany) and then washed PBS at 2 times. Furthermore, add 4',6-diamidino-2-phenylindole or DAPI 1:10,000 (Merck Millipore, MA, USA) and then washed PBS at 2 times. The stained cells were examined under a confocal microscope (Nikon, Tokyo).

Protein extraction, Protein fractionation and concentration measurement

Total cell proteins were isolated from cell lines using Radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1% sodium dodecyl sulphate, 50 mM Sodium fluoride) with phosphatase inhibitor (Merck Millipore, USA) and protease inhibitor (Thermo scientific, USA).

For protein fractionation, Total cell proteins were isolated from cell lines using Buffer A (10mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.1% NP-40, 0.5 mM DTT) and centrifuged at 5000 rpm, 1 min, 4°C (supernatant is cytoplasmic protein). Next, the pellets were extracted by Buffer B (50mM HEPES KOH pH 7.9, 10% glycerol, 420 mM NaCl, 5 mM $MgCl_2$, 10 mM KCL, 0.1 mM EDTA, 1 mM DTT), which contained a high concentration of detergent. Then, sonicated and centrifuged at 15,000 rpm, 15 min, 4°C (supernatant is nuclear protein).

The protein concentrations were determined by Bradford assay. Briefly, BSA 0.5 mg/ml was dilute to 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/µl in 96 well plate as standard protein. Then, 1 µl of extracted protein (10-flods protein) was used unknown protein. They were mixed with Bradford solution 200 µl/well. The absorbance was measured at 595 nm. The protein concentration in the sample was calculated.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot

The equal number of proteins from each treatment (difference concentration of SRPK inhibitors) was loaded and separated using SDS-PAGE (5% stacking gel and 12-15% separating gel) and then, transferred to PVDF membrane (Bio-Rad laboratories, USA). The membranes were blocked using 5% bovine serum albumin (BSA) (Capricorn scientific, USA), then probed with specific primary antibody:

Rabbit anti-human cleaved Caspase 3 1:1,000 (Cell signaling Technology, USA), Rabbit Anti-human GAPDH 1:10,000 (Merck Millipore, Germany), Mouse anti-Phosphoepitope SR Protein 1:1,000 (Merck Millipore, USA), Rabbit anti-human Beta-actin 1:1,000 (Cell signaling Technology, USA), Mouse anti-human SRSF1 1:10,000 (Invitrogen, NY), Rabbit anti-human Lamin B1 1:1,000 (Cell signaling Technology, USA), for overnights at temperature 4°C and with specific secondary antibody: HRP conjugated-Goat anti-mouse IgG and HRP conjugated-Goat anti-rabbit IgG for 1 hours in dark. The protein bands were detected with enhanced chemiluminescence (ECL) detecting system (Bio-Rad laboratories, USA), immobilon® ECL ultra western HRP substrate (Merck Millipore, USA) and imaged by ImageQuant™ LAS 500 (GE Healthcare Life Science, UK). The signal intensity of each band was quantitated using Image J software as a semi-quantitative expression.

RNA extraction, cDNA synthesis and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

At 70-80% confluent culture cells were harvested for RNA extraction. Total RNAs was extracted from CCA cell lines KKU-213A and TFK-1 cell using E.Z.N.A.® Total RNA Kit 1 (Omega BIO-TEK, USA) and use as a template for cDNA synthesis using HisenScript™ RH [-] RT-PreMix Kit (Intron Biotech, Seoul, South Korea) according to the manufacturer's instructions. All cDNA samples were stored in -20°C until use.

The expression levels of BIN1 was explored by PCR amplification using cDNA as a template and using 1×MyTagTM HS Red Mix (Bioline, Taunton, Massachusetts) in a total volume of 20 µl. PCR condition were performed by initial denaturation 94°C for 5 min, followed by 35 amplification cycles with 30 s at 94°C, 30s at 62°C annealing temperature and 30 s at 72°C final step with 5 min at 72°C. The annealing step consisted of 30 s at one of various annealing temperatures regarding to the optimization.

The PCR product was analyzed by 2% agarose gel electrophoresis after mixed with staining dye, Novel Juice (Gene DireX, Hsinchu City, Taiwan) and detected by ImageQuant™ LAS 500 (GE Healthcare Life Science, UK). The intensity of each

band was quantitated using Image J software as a semi-quantitative expression. Analysis normalized by the intensity of Beta-Actin bands.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) from biological triplicate experiments. The statistical significance of the differences between two different groups was determined with Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. The significant indicators are; * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

CHAPTER IV

RESULTS

Effect of SRPK inhibitors on dead induction of CCA cell.

To study the effect of SRPK inhibitors (SRPIN340, SPHINX31) on dead induction of CCA cell. 10 and 20 μM concentration of both inhibitors were treated in KKU-213A and TFK-1 cells for 18 hours, differential count of live and dead cell was monitored by live/dead dual staining. Esterase enzyme from viable cell catalyze calcein into calcein-am, then emits green fluorescence. On the other hand, PI can pass through nucleus and binds to the DNA of death cell, staining of nucleic acid and emit red fluorescence. Finally, the cells will be examined under a fluorescence microscope.

For KKU-213A cell line, the result demonstrated that after SRPK inhibitors treated, the number of stained dead cell was obviously much higher when in comparison with DMSO (control group) (Figure 50A). We analyzed from red fluorescent count, effect show as dose-dependent manner from both SRPK inhibitors, especially in 10 μM concentration of SPHINX31 and 20 μM concentration of both SRPK inhibitors. The result showed that there was a significantly increased the number of stained dead cell after treated SRPK inhibitors. (Figure 50B).

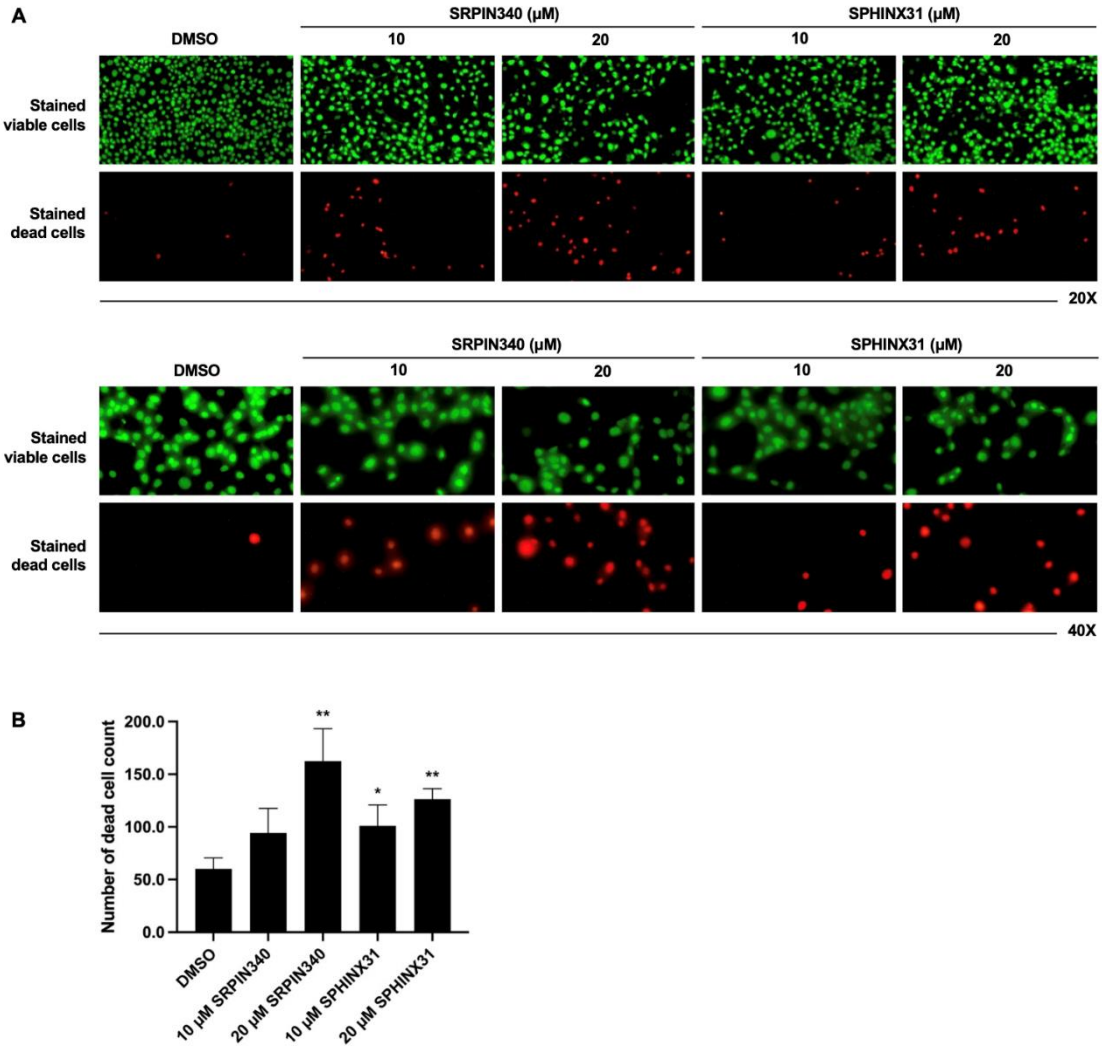

Figure 50 Effect of SRPIN340 and SPHINX31 on the dead induction in KKU-213A cell. Fluorescence images of live/dead dual staining was observed under a fluorescence microscope using 20X and 40X; live cells were stained green while dead cells were stained red (A). The data were analyzed from red fluorescent count (B). *p<0.05, **p<0.01, and ***p<0.001 (The represented figure was selected from 3 independent experiments)

For TFK-1 cell line, the result showed that SRPK inhibitors treated cell can increase the number of stained dead cell when in comparison with DMSO (control group) (Figure 51A). In addition, the analyzed from red fluorescent count, effect show as dose-dependent manner. SRPIN340 and SPHINX31 treated was a significantly increased the number of stained dead cell (Figure 51B). Therefore, these results can summary that both inhibitors could increase number of dead cells.

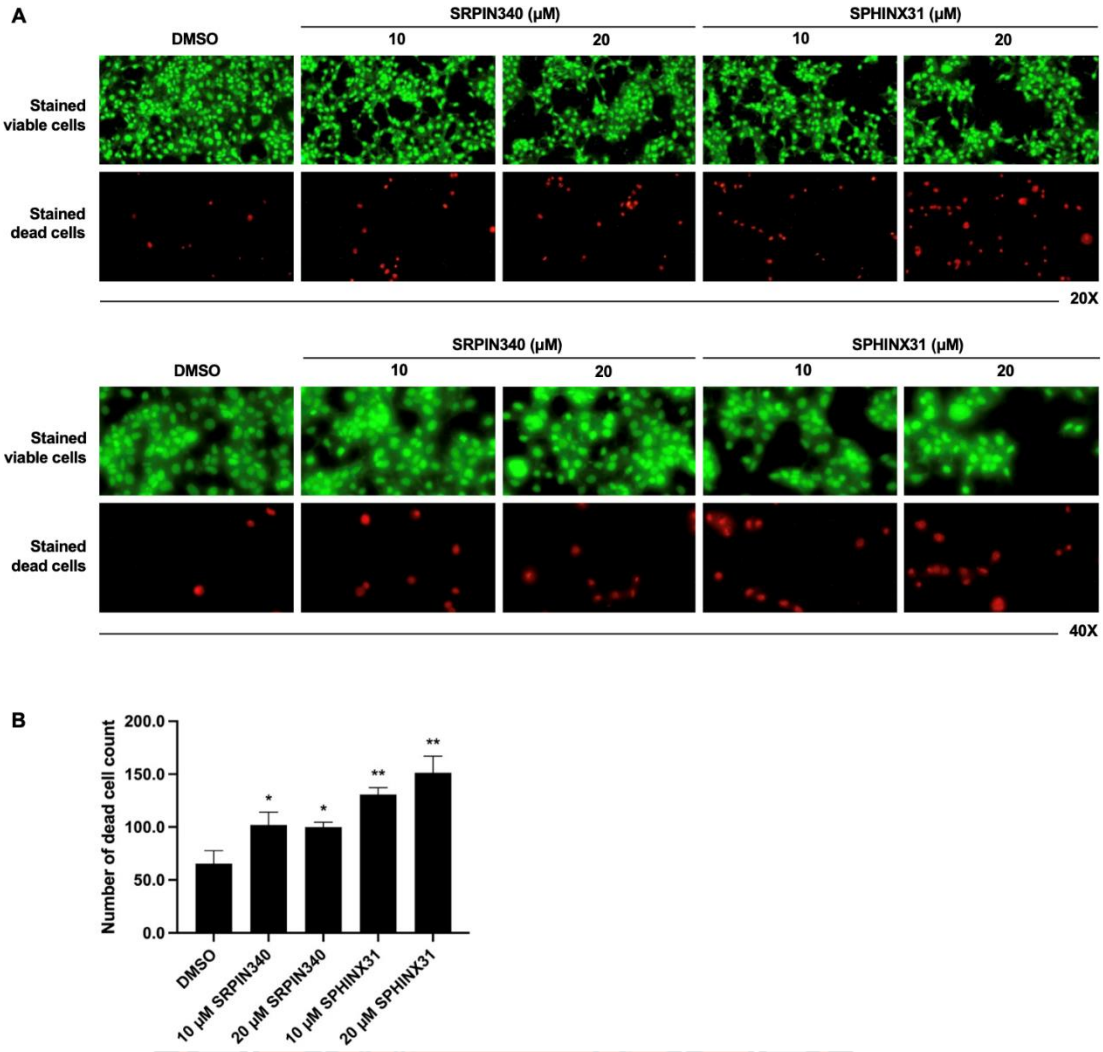

Figure 51 Effect of SRPIN340 and SPHINX31 on the dead induction in TFK-1 cell. Fluorescence images of live/dead dual staining was observed under a fluorescence microscope using 20X and 40X; live cells were stained green while dead cells were stained red (A). The data were analyzed from red fluorescent count (B). *$p<0.05$, **$p<0.01$, and ***$p<0.001$ (The represented figure was selected from 3 independent experiments)

Effect of SRPK inhibitors on apoptotic cell population of CCA cell.

As the previous result showed that SRPK inhibitors could increase death cells in CCA cell. We next examined the effect of SRPK inhibitors (SRPIN340 and SPHINX31) on apoptotic cell population of CCA cell. 10 and 20 μM concentration of both SRPK inhibitors were treated in KKU-213A and TFK-1 cells for 18 hours, determine apoptotic cell population by AnnexinV/7-AAD staining using Flow cytometry. Therefrom, the numbers of live, death and apoptosis cells (early and late stages) were analyzed.

The results showed that both SRPIN340 and SPHINX31 were increased the apoptotic cell population in KKU-213A cell (Figure 52A). The effect shows as dose-dependent manner, especially in SPHINX31 treated cells presented the higher apoptotic cell population when compared with DMSO (control group) (Figure 52B).

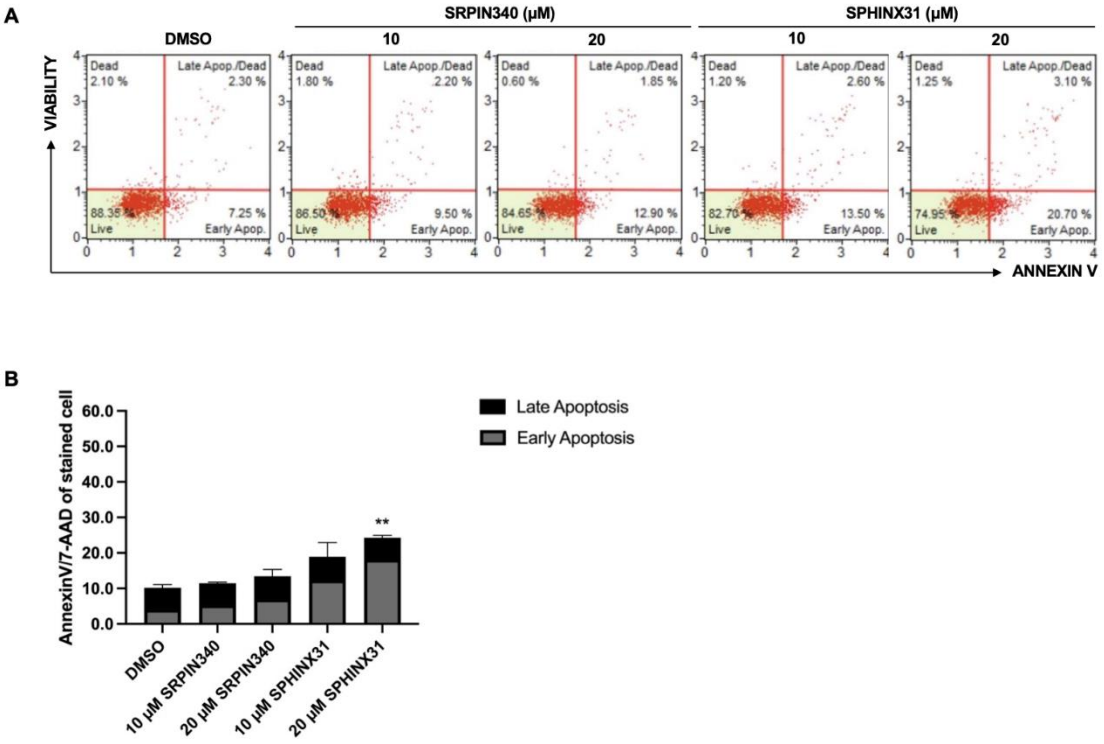

Figure 52 Effect of SRPIN340 and SPHINX31 on the apoptotic cell population in KKU-213A cell. The represented figure was selected from 3 independent experiments and analysis in percentages of apoptotic cell population (A). The data represent mean ± SD from duplicate experiments (B)

For TFK-1 cell line, the result showed that SRPK inhibitors treated cell can increase apoptotic cell population when in comparison with DMSO (control group) (Figure 53A). Additionally, the analyzed in percentages of apoptotic cell population, effect show as dose-dependent manner from both SRPK inhibitors, especially in SPHINX31 treated cell was significantly when in comparison with DMSO (control group) (Figure 53B).

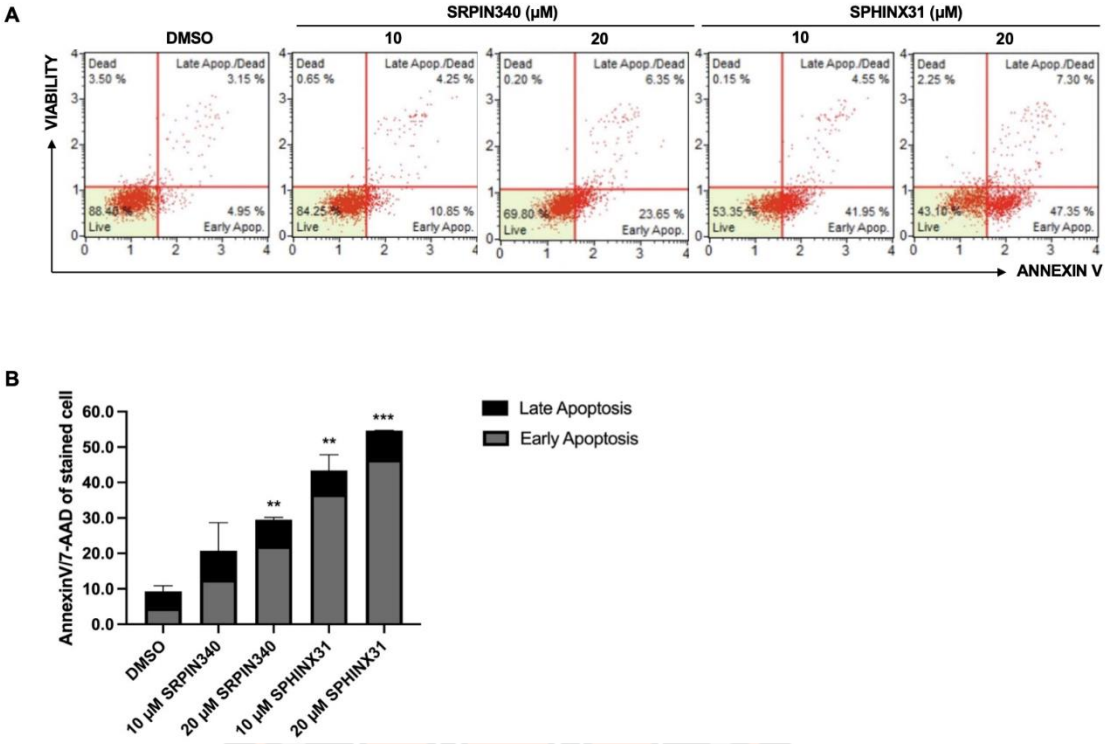

Figure 53 Effect of SRPIN340 and SPHINX31 on the apoptotic cell population in TFK-1 cell. The represented figure was selected from 3 independent experiments and analysis in percentages of apoptotic cell population (A). The data represent mean ± SD from duplicate experiments, *p<0.05 (B).

Effect of SRPK inhibitors on cytochrome c pattern of CCA cell.

Regarding to the dead mechanism, cytochrome c is a one of molecule in apoptotic pathway which released from mitochondria into the cytoplasm to activate the enzyme caspase-3 leading to apoptosis. Therefore, to determine the effect of SRPK inhibitors (SRPIN340, SPHINX31) on cytochrome c pattern of CCA cell. 10 and 20 μM concentration of SRPK inhibitors were treated in KKU-213A and TFK-1 cell for 18 hours, Immunocytofluorescence (ICF) was used to observe cytochrome c pattern. The cells were examined under a confocal microscope. Green fluorescents represent as cytochrome c localization and merge with 4',6-diamidino-2-phenylindole (DAPI) as nuclei mapping, analyzed in staining pattern.

For KKU-213A cell line, the results demonstrated that after SRPK inhibitors treated, DMSO (control cell) show cytoplasmic spot or granular pattern of cytochrome c staining. Whereas SRPIN340 and SPHINX31 treated cell could observe the diffuse pattern which represent the leak of cytochrome c from mitochondria, especially in high concentration of SRPK inhibitors (Figure 54).

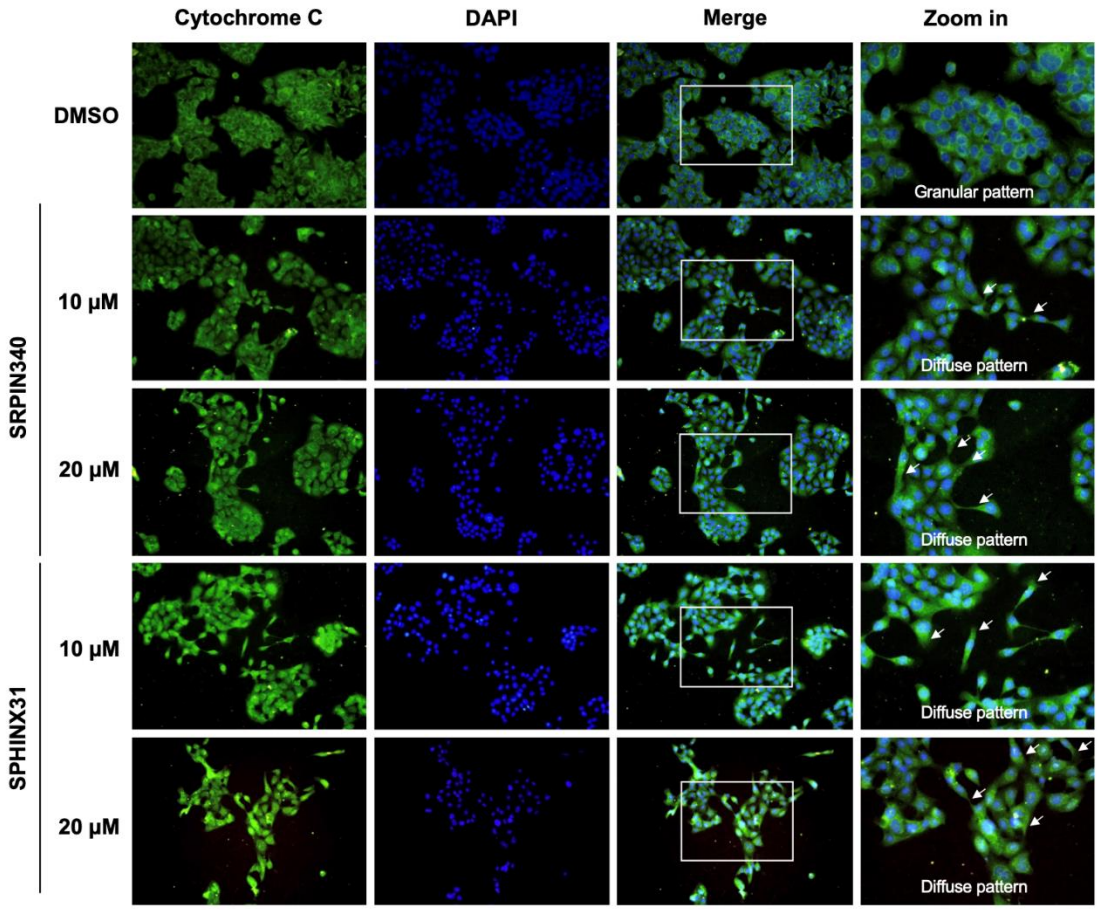

Figure 54 Effect of SRPIN340 and SPHINX31 on the cytochrome c pattern in KKU-213A cell. The accumulation of fluorescent antibody stain specific cytochrome c (green fluorescent), DAPI marks the nucleus and monitor by immunocytofluorescent (ICF) Scale bars are 100 μm.

For TFK-1 cell line, treatment with SRPK inhibitors could observe the diffuse pattern, especially in high concentration more than DMSO (control group) which could observe the granular pattern of cytochrome c staining. Furthermore, the result showed that after SRPK inhibitors treated cell confluence less than DMSO, this indicates that cell death increased after treatment (Figure 55). There results demonstrated that after SRPK inhibitors treated could increase the leak of cytochrome c from mitochondria into cytosol content and represent cell apoptosis.

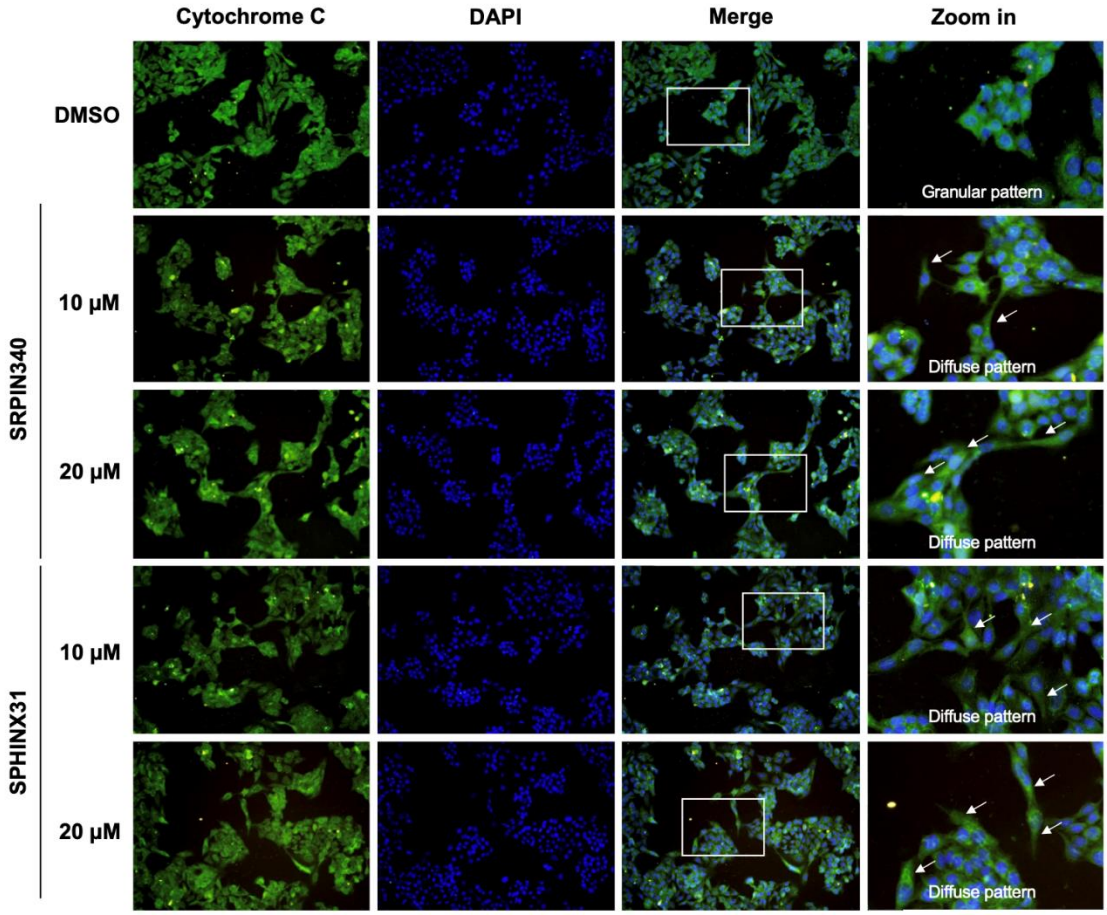

Figure 55 Effect of SRPIN340 and SPHINX31 on the cytochrome c pattern in TFK-1 cell. The accumulation of fluorescent antibody stain specific cytochrome c (green fluorescent), DAPI marks the nucleus and monitor by immunocytofluorescent (ICF) Scale bars are 100 µm.

Effect of SRPK inhibitors on apoptotic protein expression of CCA cell.

In addition, to determine the effect of SRPK inhibitors (SRPIN340, SPHINX31) on CCA apoptotic protein expression of CCA cell. 10 and 20 µM concentration of both SRPK inhibitors were treated in KKU-213A and TFK-1 cell for 18 hours, determine protein expression of cleaved caspase-3 (pro-apoptotic protein) by western blot.

The result showed that after SRPK inhibitors treated in KKU-213A cell was increased the expression levels of cleaved caspase-3 when in comparison with DMSO (control group) (Figure 56A). Additionally, band intensities of cleaved caspase-3 was measured by ImageJ software and represented an increasing as dose-dependent manner from both SRPK inhibitor, especially in 20 µM concentration of SPHINX31 but not significant (Figure 56B).

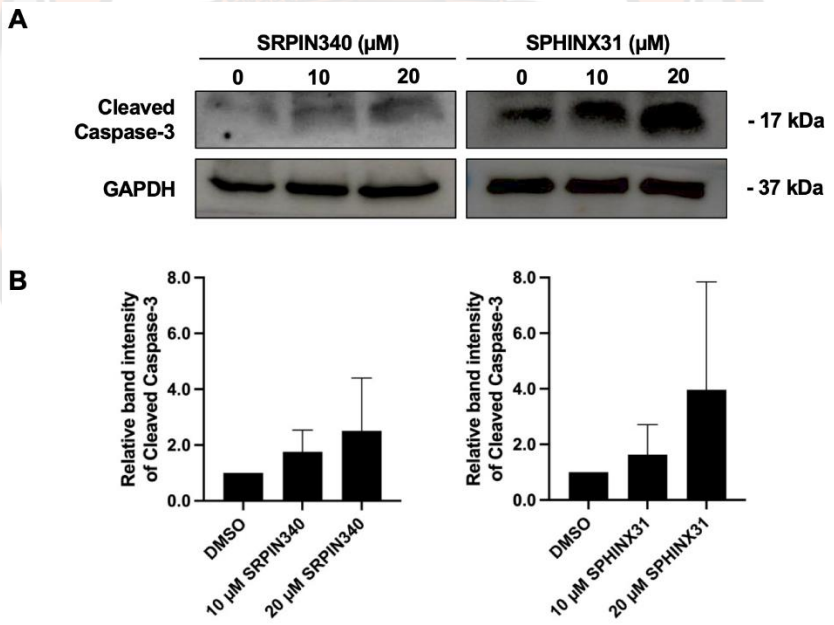

Figure 56 Effect of SRPIN340 and SPHINX31 on the expression of apoptotic marker in KKU-213A cell. The expression levels of cleaved caspase-3 (A). Band intensity analysis of cleaved caspase-3 by ImageJ software (B). (The represented figure was selected from 3 independent experiments).

Furthermore, SRPK inhibitors treated TFK-1 cell demonstrated that the expression levels of cleaved caspase-3 were increasing as dose-dependent manner from both SRPK inhibitor when in comparison with DMSO (control group) (Figure 57A). Band intensities was measured by ImageJ software and the result showed that 20 μM concentration of SPHINX31 was a significantly increasing in cleaved caspase-3 band intensity (Figure 57B).

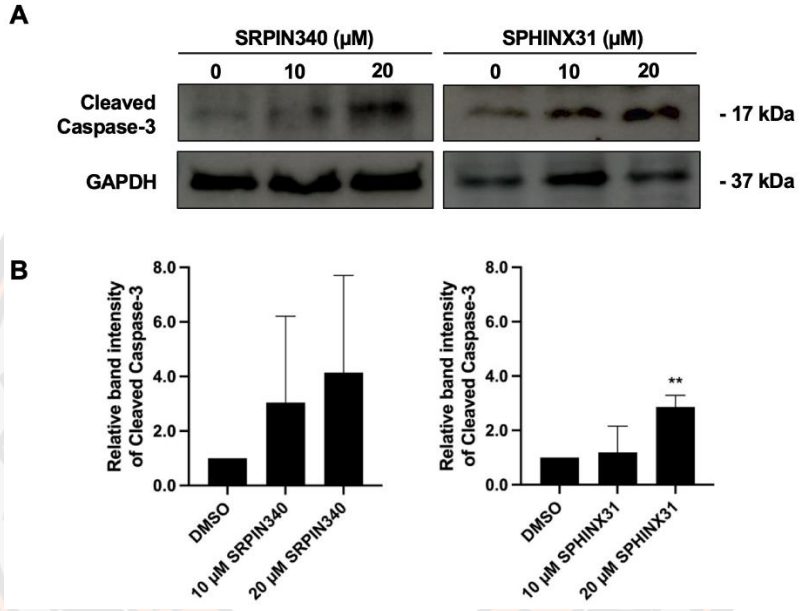

Figure 57 Effect of SRPIN340 and SPHINX31 on the expression of apoptotic marker in TFK-1 cell. The expression levels of cleaved caspase-3 (A). Band intensity analysis of cleaved caspase-3 by ImageJ software (B). *$p<0.05$ (The represented figure was selected from 3 independent experiments).

Effect of SRPK inhibitors on phosphorylation profile of SRSFs protein in CCA cell.

As mentioned above, SRPKs are responsible for phosphorylation to SRSFs into active SRSFs. Therefore, the molecular function of SRPKs-specific inhibitors were studies. To determine the effect of SRPK inhibitors (SRPIN340, SPHINX31) on phosphorylation profile of SRSFs protein in CCA cell. 10 and 20 μM concentration of SRPK inhibitors were treated in KKU-213A and TFK-1 cell for 18 hours, Western blotting was performed for the phosphorylated form of SRSFs protein. SRSFs phosphorylation (pSRSFs) was evaluated by mouse anti-phosphoepitope SR protein antibody and represented in various molecular weight (MW) of pSRSFs. Additionally, band intensities of pSRSFs were measured by ImageJ software.

For KKU-213A cell, the results showed the downregulation of pSRSFs from both SRPK inhibitors when in comparison with DMSO (control group) (Figure 58A). The band intensities of pSRSFs were measured by ImageJ software and represented an decreasing of pSRSF5/pSRSF10 and pSRSF2/pSRSF7 band intensities as dose-dependent manner from SRPIN340 treated cell, especially in 20 μM concentration of SRPIN340 was significant, while a decreasing in pSRSF5/pSRSF10 and pSRSF2/pSRSF7 band intensities in 10 μM concentration of SPHINX31 treated cell and increasing in pSRSF5/pSRSF10 and pSRSF2/pSRSF7 band intensities in 20 μM concentration but not significant. Additionally, the result showed the significantly decrease in pSRSF1/pSRSF12 band intensity in 20 μM concentration of both SRPK inhibitors (Figure 58B).

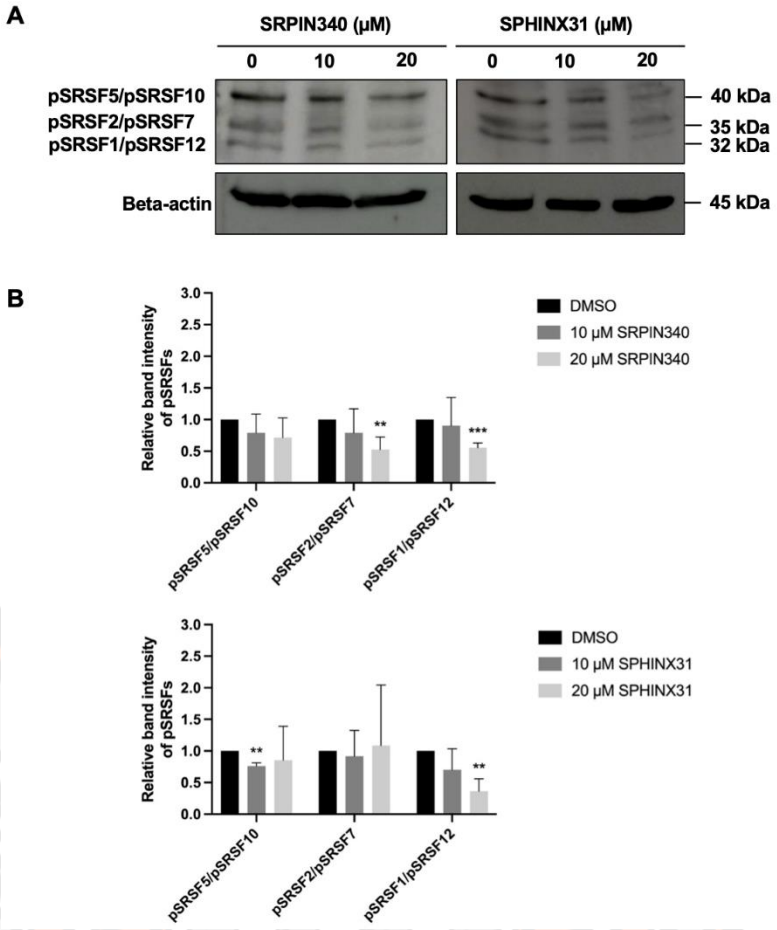

Figure 58 Effect of SRPIN340 and SPHINX31 on SRSFs phosphorylation in KKU-213A cell. The expression levels of SRSFs phosphorylation (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B). **p<0.01, and ***p<0.001 (The represented figure was selected from 3 independent experiments).

Furthermore, TFK-1 cell showed the downregulation of pSRSFs as dose-dependent manner from both SRPK inhibitors when in comparison with DMSO (control group) (Figure 59A). SRPIN340 could significantly decrease in pSRSF5/pSRSF10, pSRSF2/pSRSF7 and pSRSF1/pSRSF12 band intensities. In addition, the effect of 10 μM concentration of SPHINX31 was significantly decrease band intensities of pSRSF5/pSRSF10, pSRSF2/pSRSF7 and pSRSF1/pSRSF12, while an increasing in 20 μM concentration of SPHINX31 but not significant (Figure 59B). Remarkably, there results demonstrated that SRPK inhibitors could inhibit function of SRPKs by suppress the SRSFs phosphorylation, especially in pSRSF1/pSRSF12.

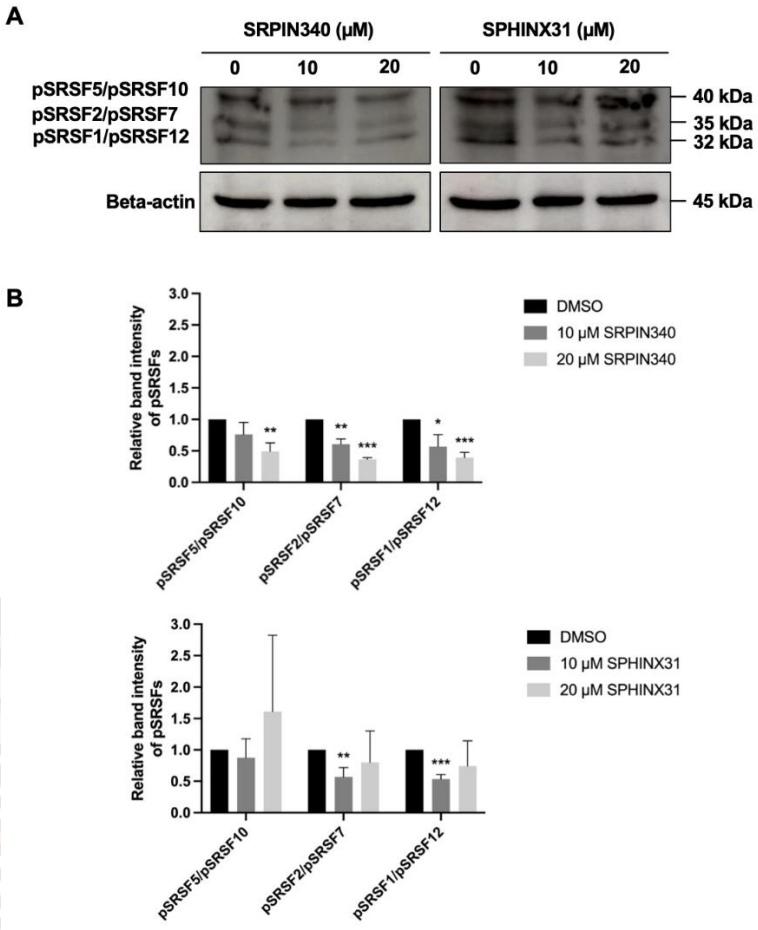

Figure 59 Effect of SRPIN340 and SPHINX31 on SRSFs phosphorylation in TFK-1 cell. The expression levels of SRSFs phosphorylation (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B). **p<0.01, and ***p<0.001 (The represented figure was selected from 3 independent experiments).

Effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1 protein.

Previous result showed that SRSFs phosphorylation downregulated after SRPK inhibitors treated CCA cell, especially in pSRSF1 protein from high concentration of SRPIN340 and SPHINX31. Therefore, 20 μM of SRPK inhibitors were selected for further experiment. Regarding to SRSF1 that translocate from cytoplasm into nucleus as phospho-SRSF1 (pSRSF1) after their activation by SRPKs. Therefore, the downregulation of pSRSF1 is suspected to control a major role in cytoplasmic-to-nuclear translocation of SRSF1 protein. To determine the effect of SRPK inhibitors (SRPIN340, SPHINX31) on cytoplasmic-to-nuclear translocation of SRSF1 protein of CCA cell. 20 μM concentration of SRPK inhibitors were treated in KKU-213A and TFK-1 cell for 18 hours, Immunocytofluorescence (ICF) was used to observe SRSF1 protein localization. The cells were examined under a fluorescence microscope. Red fluorescents represent as SRSF1 protein localization and merge with 4’,6-diamidino-2-phenylindole (DAPI) as nuclei mapping.

The result demonstrated that after SRPIN340 and SPHINX31 treated KKU-213A cell, DMSO (control cell) showed SRSF1 protein could accumulated in nuclear more than cytoplasm area. Whereas SRPIN340 and SPHINX31 treated cells could increased the accumulated of SRSF1 in cytoplasm of KKU-213A cell (Figure 60).

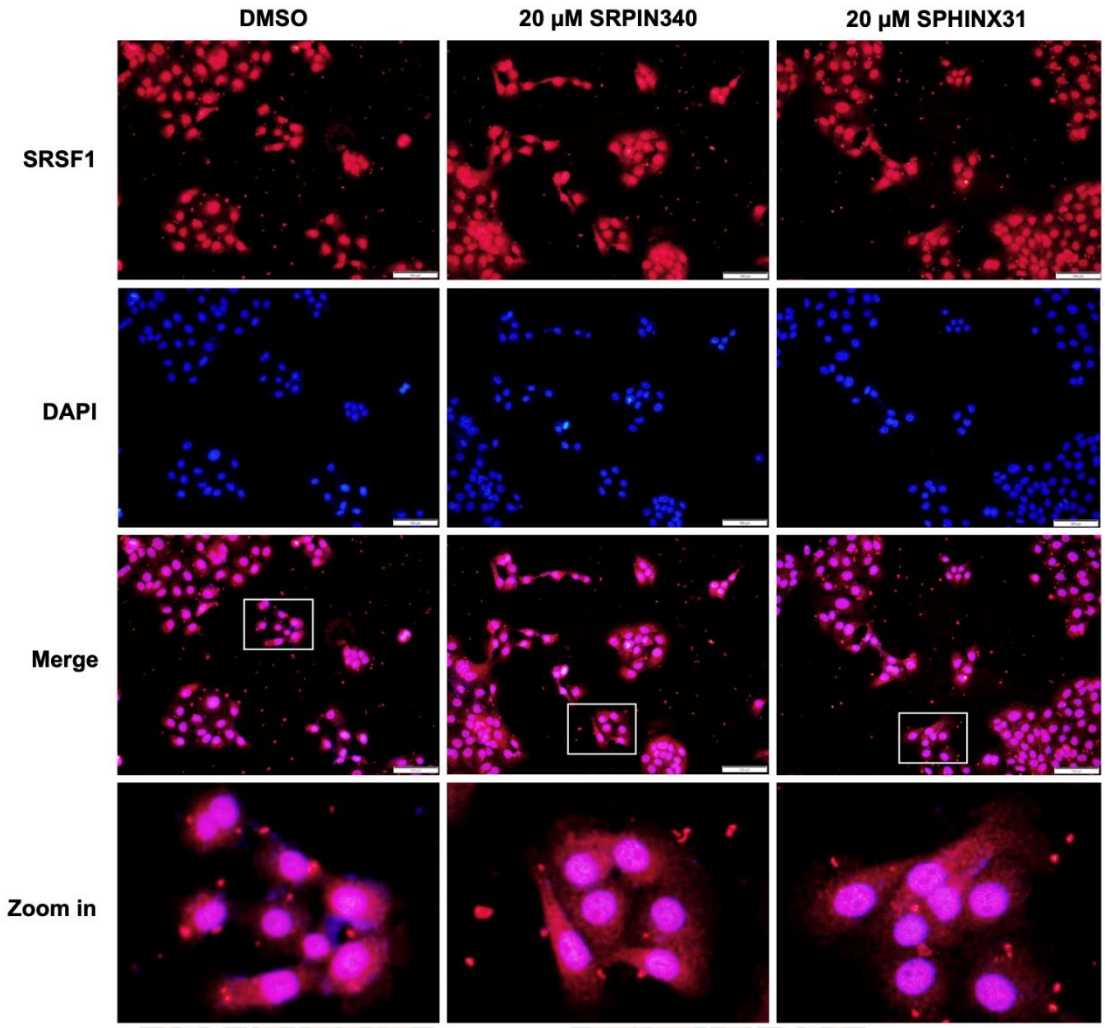

Figure 60 Effect of SRPIN340 and SPHINX31 on cytoplasmic-to-nuclear translocation of SRSF1 protein in KKU-213A cell. The accumulation of fluorescent antibody stain specific SRSF1 (red fluorescent), DAPI marks the nucleus and monitor by immunocytofluorescent (ICF) Scale bars are 100 μm.

In addition, SRPK inhibitors also increased the cytoplasmic accumulation of SRSF1 protein in TFK-1 cell when in comparison with DMSO (control group). Therefore, SRPIN340 and SPHINX31 could decrease the cytoplasmic-to-nuclear translocation of SRSF1 (Figure 61).

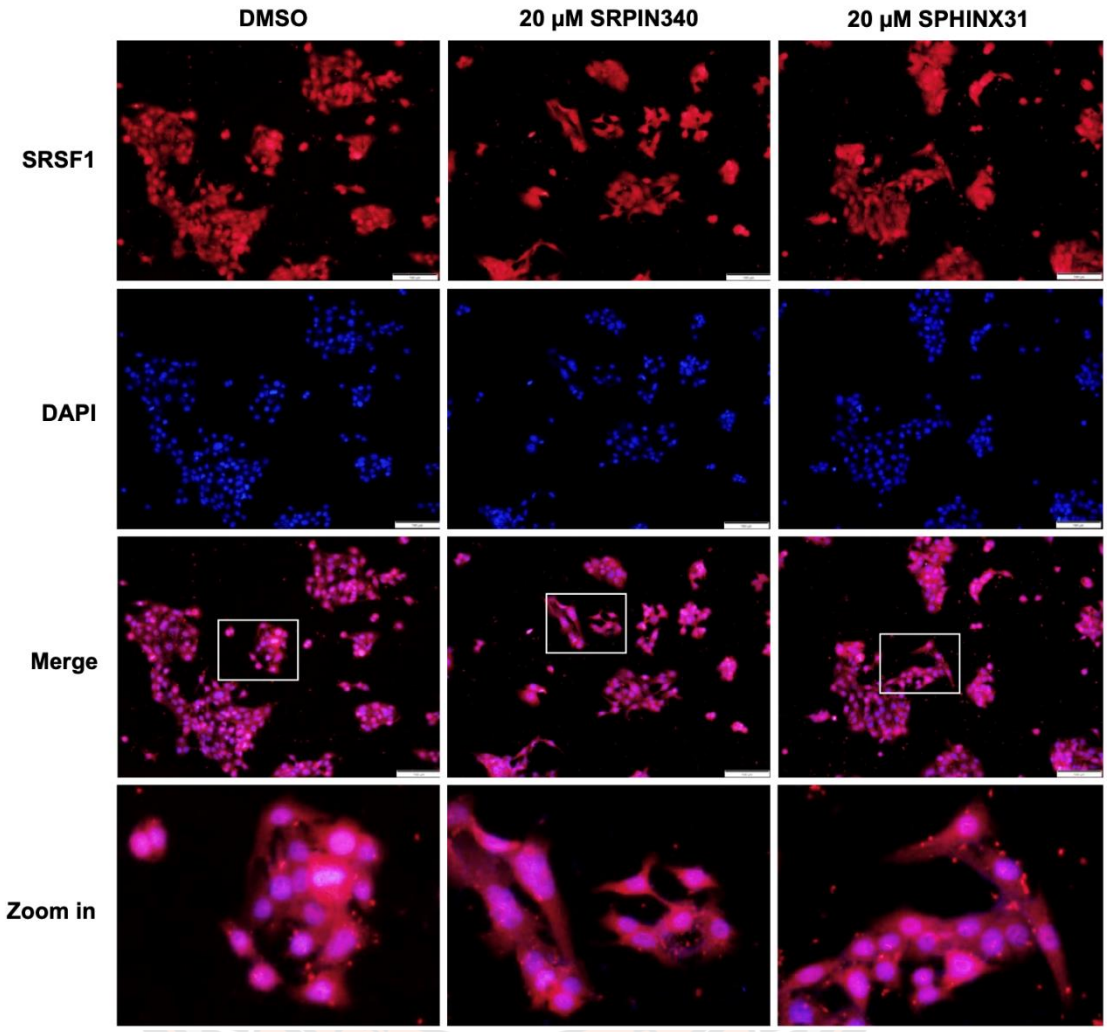

Figure 61 Effect of SRPIN340 and SPHINX31 on cytoplasmic-to-nuclear translocation of SRSF1 protein in TFK-1 cell. The accumulation of fluorescent antibody stain specific SRSF1 (red fluorescent), DAPI marks the nucleus and monitor by immunocytofluorescent (ICF) Scale bars are 100 μm.

To confirm the effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1. The determination of them by subcellular protein fractionation (cytoplasmic protein and nuclear protein) and western blotting was performed, 20 μM concentration of SRPIN340 and SPHINX31 was treated in CCA cell for 18 hours, cytoplasmic and nuclear protein of SRPK inhibitors or untreated cells (DMSO) were extracted.

For KKU-213A cell, western blot analysis of the subcellular protein fractions revealed a cytoplasmic accumulation of SRSF1. The result demonstrated that after SRPK inhibitors treated, the nuclear protein expression of SRSF1 decreased when in comparison with DMSO (control group). Whereas the cytoplasmic protein expression of SRSF1 increased when in comparison with DMSO (control group) (Figure 62A and B).

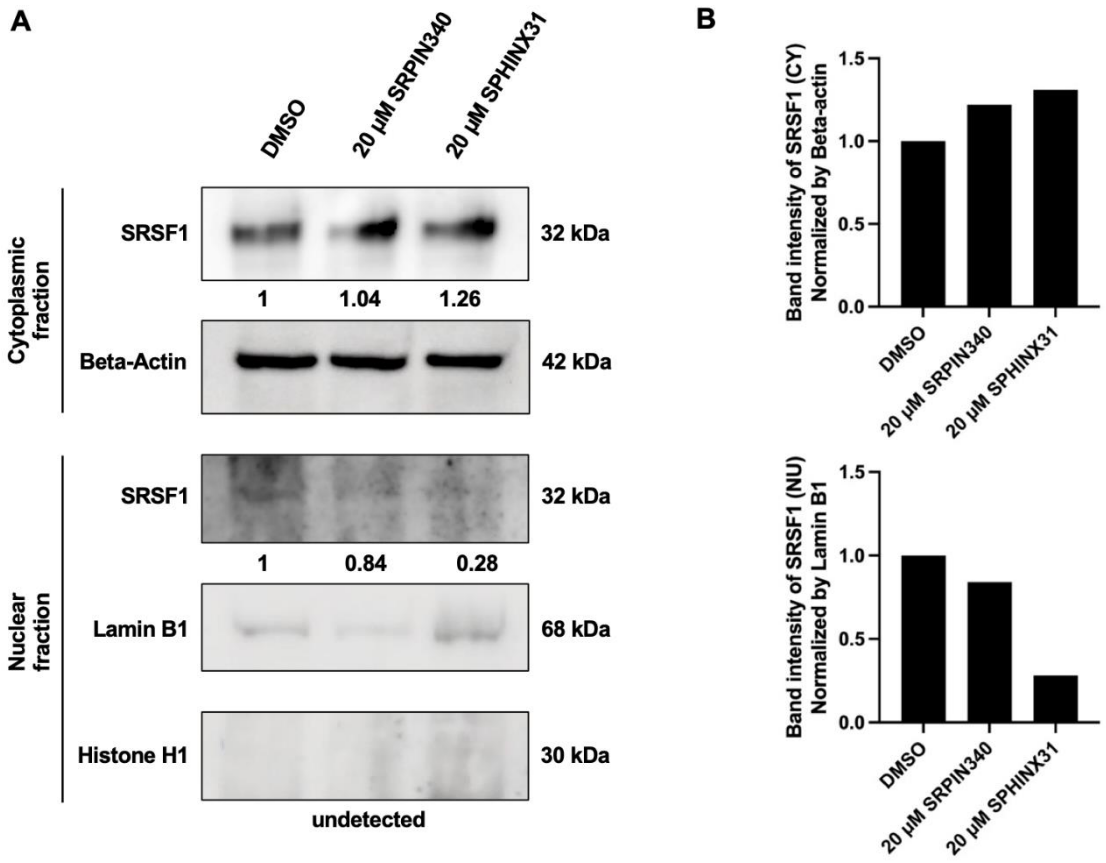

Figure 62 Effect of SRPIN340 and SPHINX31 on cytoplasmic-to-nuclear translocation of SRSF1 protein in KKU-213A cell. The nuclear expression of SRSF1 and cytoplasmic expression of SRSF1 detected by western blot (A). Band intensity analysis by ImageJ software (B). Figures were obtained from a single experiment.

For TFK-1 cell, the result showed that after SRPK inhibitors treated, the nuclear protein expression of SRSF1 decreased when in comparison with DMSO (control group). Whereas the cytoplasmic protein expression of SRSF1 increased when in comparison with DMSO (control group) (Figure 63A and B). There results confirm that SRSF1 phosphorylation was blocked, then they accumulated in the cytoplasm.

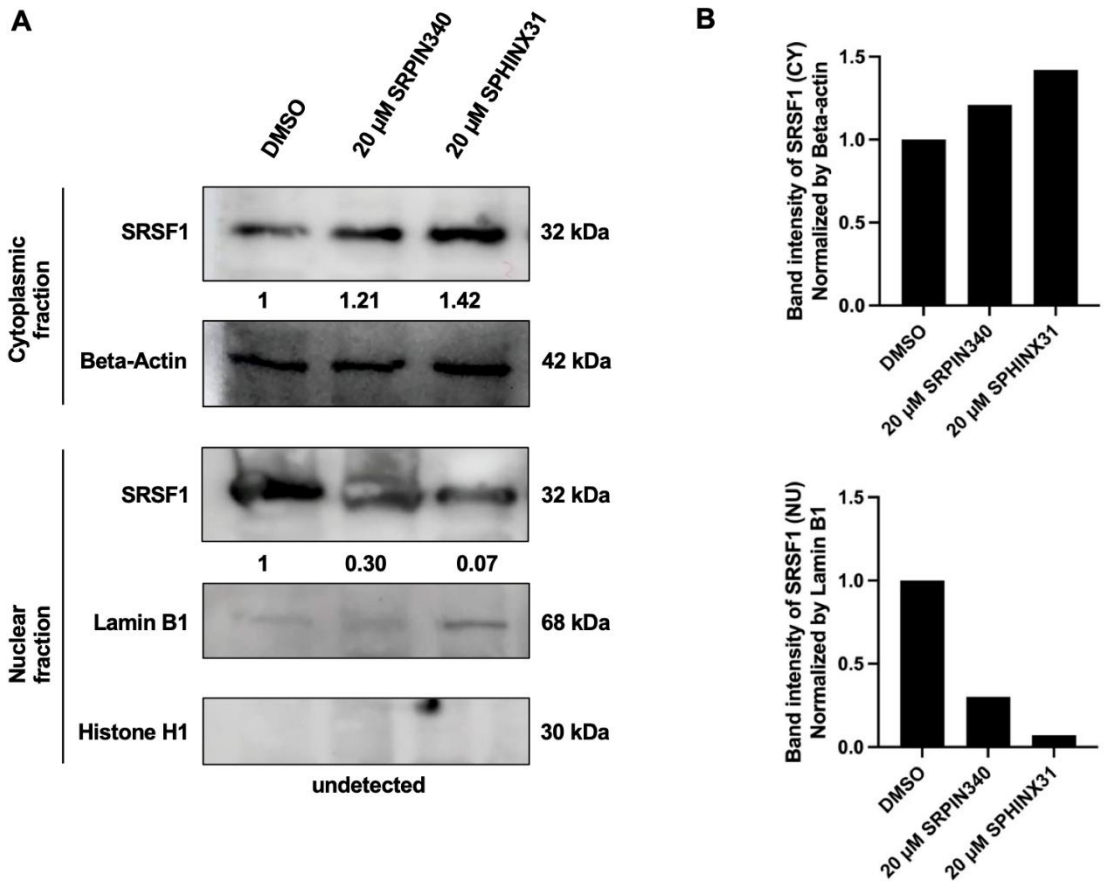

Figure 63 Effect of SRPIN340 and SPHINX31 on cytoplasmic-to-nuclear translocation of SRSF1 protein in TFK-1 cell. The nuclear expression of SRSF1 and cytoplasmic expression of SRSF1 detected by western blot (A). Band intensity analysis by ImageJ software (B). Figures were obtained from a single experiment.

Effect of SRPK inhibitors on an alternative splicing of BIN1 gene.

Regarding to SRPKs function that phosphorylated SRSFs, it can translocate into the nucleus for regulating the aberrant alternative splicing to result in the production oncogenic isoforms, BIN1 into BIN1+12A plays a crucial role in the evasion of apoptosis and facilitates cancer cell survival. To investigate the effect of SRPK inhibitors (SRPIN340, SPHINX31) on an alternative splicing of BIN1 gene. 20 μM concentration of SRPK inhibitors were treated in CCA cell for 18 hours, then observed the expression of BIN1 gene (BIN1 and BIN1+12A specific primers) using RT-PCR

The result showed that after SRPK inhibitors treated KKU-213A cell decreased BIN1+12A which promote anti-apoptotic function. whereas, BIN1 was recovered, by switching back into BIN1 wild-type that action as pro-apoptotic protein (Figure 64A). The band intensity of BIN1 wild-type and BIN1+12A were measured. SPHINX31 could significantly decrease BIN1+12A isoform which could explain the restoration of apoptosis (Figure 64B).

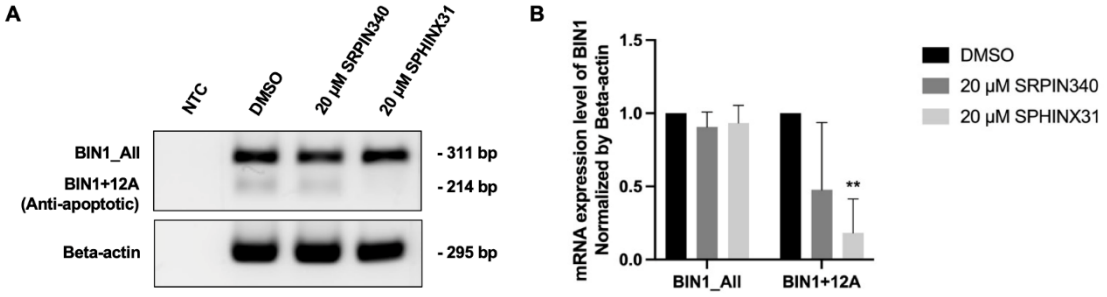

Figure 64 Effect of SRPIN340 and SPHINX31 on an alternative splicing of BIN1 gene in KKU-213A cell. mRNA expression of BIN1wild-type (BIN1_All), and BIN1+12A isoform determined by RT-PCR (A). The data represent mean ± SD from triplicate experiments, **p<0.01 (B).

For TFK-1 cell, SRPIN340 and SPHINX31 could significantly decrease BIN1+12A isoform when in comparison with DMSO (control group) (Figure 65A and B).

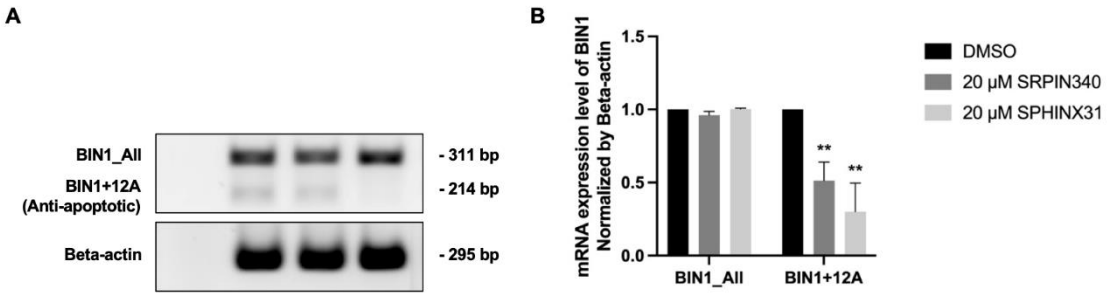

Figure 65 Effect of SRPIN340 and SPHINX31 on an alternative splicing of BIN1 gene in TFK-1 cell. mRNA expression of BIN1wild-type (BIN1_All), and BIN1+12A isoform determined by RT-PCR (A). The data represent mean ± SD from triplicate experiments, **p<0.01 (B).

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Cholangiocarcinoma (CCA) is a severe type of cancer that occurs in the epithelium of the bile duct. Thailand has a high incidence of CCA, which is commonly reported from the north and northeastern regions (Banales et al., 2020). The main reasons were freshwater fish consumption with contaminating metacercaria of *Opisthorchis viverrini* (OV) together with other factors such as exogenous carcinogens, excretory/secretory products from OV acting as important growth stimuli, and repeated treatment with parasitic drugs (Prueksapanich et al., 2018). Treatment for CCA is determined by the stage at which the cancer is detected. Since there are no specific symptoms in the early stages, most of the patients treated for CCA are more advanced or have metastasis, leading to the mortality rate of CCA patients having increased every year (Kodali et al., 2021). Aberrant alternative splicing has been reported to result in oncogenic isoform production from various important genes in many cancers and is recognized as another hallmark of cancers (Ladomery et al., 2013). The Serine/arginine-rich splicing factor (SRSFs) is the SR protein family that serves as a key molecule in regulation. It consists of 12 members of SRSFs named as SRSF1 to SRSF12 (Änkö et al., 2012). The main activity of SRSFs is regulated by Serine-arginine protein kinase (SRPKs), which are responsible for phosphorylating SRSFs at their arginine-serine rich (RS) domain into active SRSFs. Then, phosphorylated-SRSFs translocate to the nucleus for targeting multi-exon pre-mRNA (Giannakouros et al., 2011). Dysregulation of SRSFs and SRPKs leads to abnormal protein synthesis with overexpression and functional alteration to gain oncogenic properties (Yodsudjai et al., 2019). Accumulated evidence has indicated that SRPK1 and SRPK2 have been found overexpressed in various types of cancer, for example, breast, colon, pancreatic carcinomas, leukemia, gliomas, and ovary, which is correlated with advanced tumor staging and poor prognosis (Nikas et al., 2019). The effect of SRPK protein inhibitors on the cellular activity of cancer cells is interesting.

SRPIN340 was demonstrated the inhibitory effect on both SRPK1 and SRPK2 activities (Fukuhara et al., 2006) whereas SPHINX31 was verified as only SRPK1-specific inhibitor (Batson et al., 2017).

In this study, we focus on SRPK function by determination the effects of SRPK inhibitors on CCA cell lines, especially in the role of dead induction. We started to investigate differential count of live and dead cell by live/dead dual staining and analyzed from red fluorescent count. The result showed SRPIN340 and SPHINX31 can significantly increase the number of stained dead cell in both KKU-213A and TFK-1 cell as dose-dependent manner, especially in high concentration of SRPK inhibitors (Figure 50 and Figure 51). Interestingly, an increasing in these number of stained dead cell similar to previous study, which showed that SPHINX31 can decrease the cell growth rate as dose dependent manner in many leukemia cell line (Tzelepis et al., 2018) (Figure 29). Moreover, SPHINX31 was significantly reduced the percentage and fluorescence intensity of Ki-67 which indicates that SPHINX31 can reduce the proliferation of HUVECs (Gu et al., 2022) (Figure 30).

Next, we examine apoptotic cell population to confirm CCA cell apoptosis by AnnexinV/7-AAD staining using Flow cytometry. The numbers of live, death and apoptosis cells were analyzed, and we found that both SRPIN340 and SPHINX31 were increased the apoptotic cell population both in early and late stages in CCA cell as dose-dependent manner, especially in SPHINX31 treated TFK-1 cell (Figure 52 and Figure 53). Our results corresponded to the previous report that SRPK1 inhibitors (SPHINX31 and SRPIN340) can increase the apoptotic population in ENKTL (extranidal NK/T-cell lymphoma) cells (He et al., 2022) (Figure 32). Moreover, the result from leukemia cells shown that SRPIN340 can trigger early and late apoptosis in leukemia cells (Siqueira et al., 2015) (Figure 33).

Furthermore, we determine the effect of SRPK inhibitors on cytochrome c pattern of CCA cell by Immunocytofluorescence under a fluorescent microscope. The results demonstrated that DMSO showed the cytoplasmic spot or granular pattern of cytochrome c staining. Whereas SRPIN340 and SPHINX31 treated cell could observe the diffuse pattern which represent the leak of cytochrome c from mitochondria into cytosol content and represent CCA cell apoptosis, especially in high concentration of SRPK inhibitors (Figure 54 and Figure 55). Furthermore, this result associated with

the previous studies from Sujitra Madee and Donnapha Boontang, they study cytochrome c pattern in KKU-055 CCA cell line, the result show that both SRPIN340 and SPHINX31 treated cell could observe the diffuse pattern which represent the leak of cytochrome c from mitochondria (Figure 66)

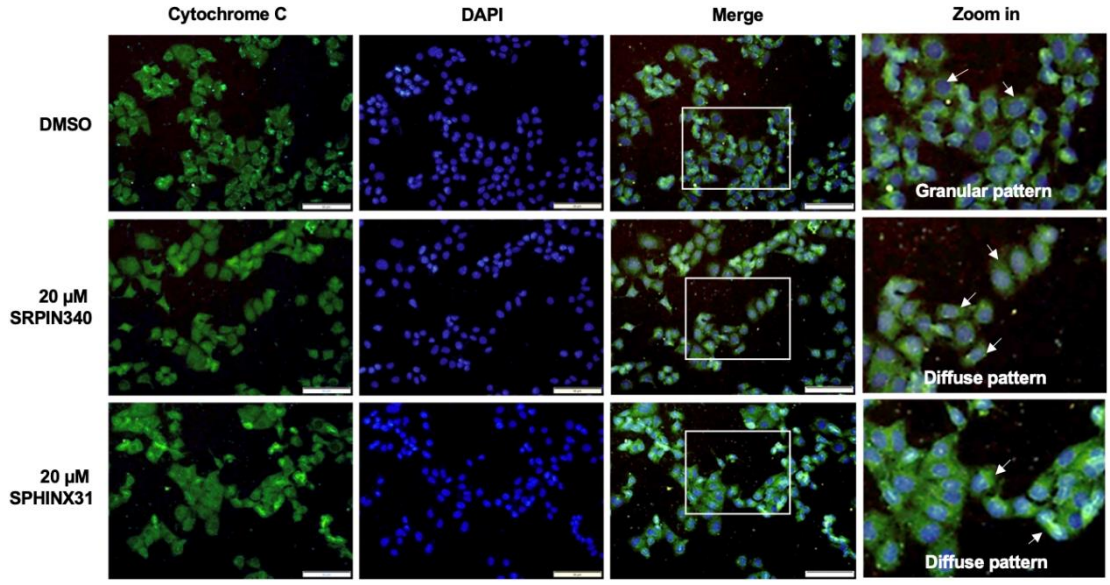

Figure 66 Effect of SRPIN340 and SPHINX31 on the cytochrome c pattern in KKU-055 cell. The accumulation of fluorescent antibody stain specific cytochrome c (green fluorescent), DAPI marks the nucleus and monitor by immunocytofluorescent (ICF) Scale bars are 50 µm.

Source: Sujitra Madee and Donnapha Boontang, 2022, Unpublished data.

In addition, western blot was used to detecting the protein expression of cleaved caspase-3 which is a pro-apoptotic protein. The result showed that after SRPK inhibitors treated in CCA cell was increased the expression levels of cleaved caspase-3, especially in 20 µM concentration of SPHINX31 in TFK-1 cell (Figure 56 and Figure 57). The result from this recent study associated with the previous studies in endometrial cancer showed SPHINX31 can reduced Survivin and increase cleaved PARP for apoptotic induction in SPEC-2 cells (Kurimchak et al., 2020) (Figure 31). Moreover, SRPIN3401 and SPHINX31 can increase the protein expression of cleaved

PARP and cleaved caspase-3 in ENKTL (extranidal NK/T-cell lymphoma) cells (He et al., 2022) (Figure 32). Furthermore, we also determined the expression of pro-apoptotic gene; Caspase-3 and BAX in KKU-213A cell by qRT–PCR. the result showed that the expression level of Caspase-3 and BAX slightly increased but the below figure were obtained from a single experiment (Figure 67).

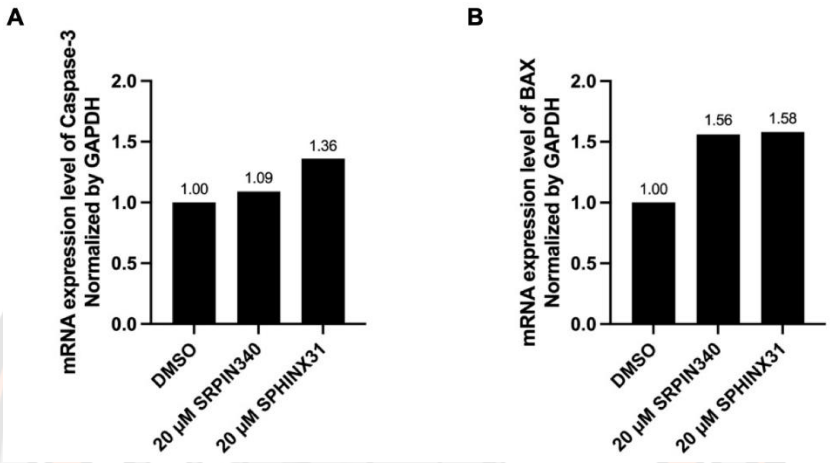

Figure 67 Effect of SRPIN340 and SPHINX31 on mRNA expression of Caspase-3 (A). and BAX in KKU-213A cell (B).

In addition, previous studies from Thirawut Miprawat and Siriwan Noieam, they observe the morphology of apoptosis (Nuclear condensation) in KKU-055 cell by DAPI staining assay, and we found that both SRPIN340 and SPHINX31 can induced DNA condensation and these effects were dose-dependent (Figure 68).

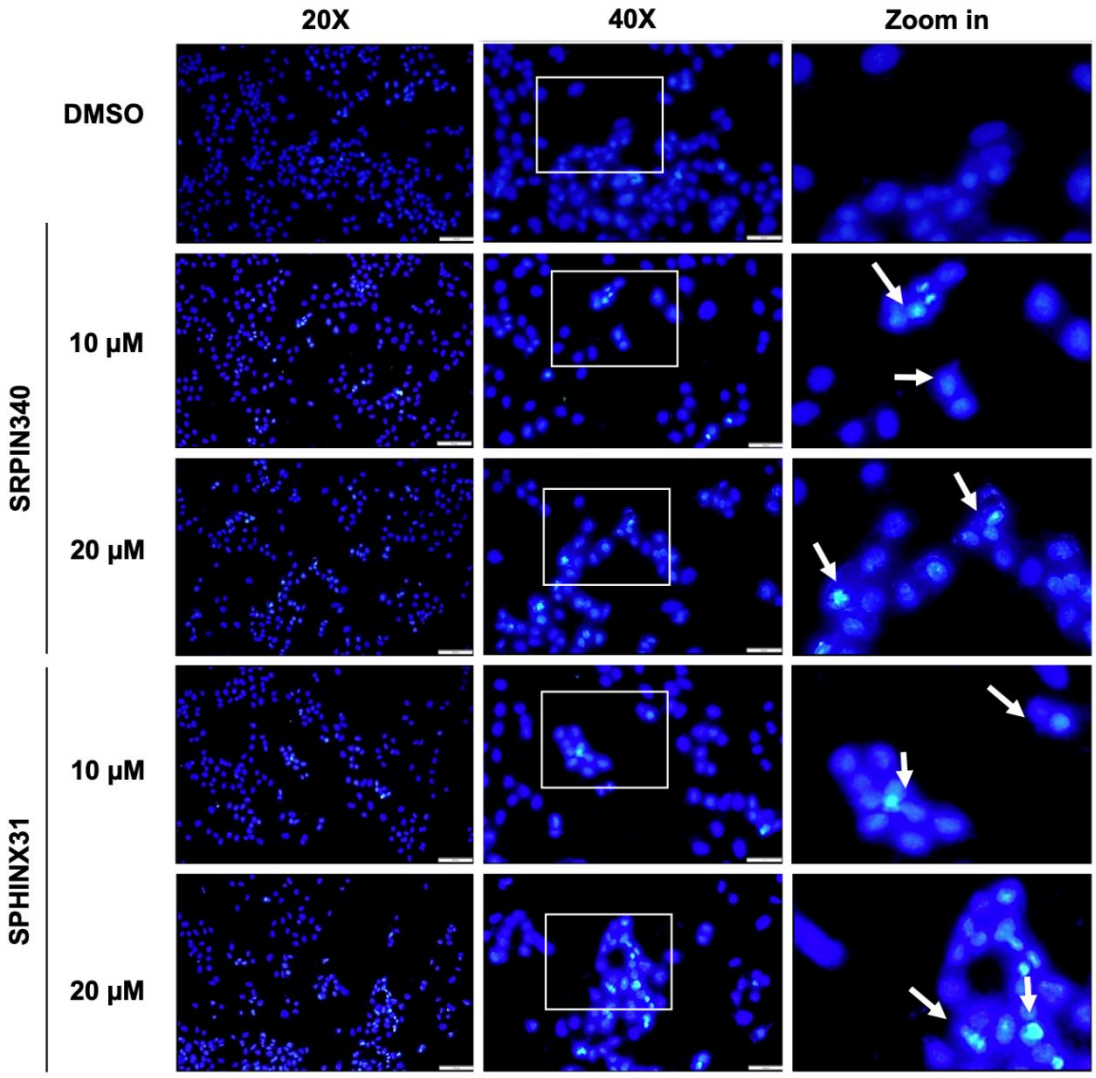

Figure 68 Effect of SRPIN340 and SPHINX31 on DNA damage and condensation in KKU-055 cell.

Source: Thirawut Miprawat and Siriwan Noieam, 2022, Unpublished data.

Regarding to SRPKs are responsible for phosphorylation to SRSFs into active SRSFs or SRSFs phosphorelation (pSRSFs). Therefore, the molecular function of SRPK inhibitors were studies in CCA cell line. Western blotting was performed for the phosphorylated form of SRSFs protein and followed by mouse anti-phosphoepitope SR protein antibody and represented in various molecular weight (MW) of pSRSFs. The result showed the downregulation of pSRSFs from both SRPK inhibitors.

SRPIN340 and SPHINX31 could decreas the band intensity of pSRSF5/pSRSF10, pSRSF2/pSRSF7 and pSRSF1/pSRSF12, especially in high concentration from both SRPK inhibitors in CCA cell. There result demonstrated that SRPK inhibitors could inhibit function of SRPKs by suppress the SRSFs phosphorylation, especially in pSRSF1/pSRSF12 (Figure 58 and figure 59). Interestingly, a decreasing in pSRSF similar to previous study, which showed that SPHINX31 can decrease the kinase activity of SRPK1 by reducing the phosphorylation form of SRSFs in SPEC-2 cells (Kurimchak et al., 2020) (Figure 31). SRPIN340 can decreased the phosphorylation of SRSF4, SRSF6, SRSF5, and SRSF2 both the HL60 and Jurkat cell line (Siqueira et al., 2015) (Figure 34). In RPE cell line, the result showed that SPHINX31 can reduce the phosphorylation profile of SRSF1 as dose dependent manner (Batson et al., 2017) (Figure 35). In A375 cell line, docking blocker of SRPK1 (DBS1) can decreased phosphorylation of SRSF, especially in pSRSF1/pSRSF2 (Li et al., 2021) (Figure 36). Moreover, this study corresponded with the previous studies in CCA cell. SPHINX31 could suppress SRSF1 phosphorylation (Supradit et al., 2022) (Figure 41). In addition, this result associated with the previous studies from Jesadagorn Siriwath, SRPIN340 and SPHINX31 can suppress many pSRSF including pSRSF2/pSRSF7 and pSRSF1/pSRSF12 in 92-1 and A375 cell line. Therefore, we can summary that SRPK inhibitor could significantly downregulated pSRSF1 in CCA cell (Siriwath et al., 2021).

Regarding to SRSF1 that translocate from cytoplasm into nucleus as phospho-SRSF1 (pSRSF1) after their activation by SRPKs. Therefore, we determine the effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1 protein of CCA cell. Immunocytofluorescence (ICF) was used to observe SRSF1 protein localization, and we found that SRPIN340 and SPHINX31 could decrease the cytoplasmic-to-nuclear translocation of SRSF1 (Figure 60 and Figure 61). Next, to confirm the effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1. The determination of them by subcellular protein fractionation (cytoplasmic protein and nuclear protein) and western blotting was performed. The result demonstrated that after SRPK inhibitors treated, the nuclear protein expression of SRSF1 decreased when in comparison with DMSO. Whereas the cytoplasmic protein expression of SRSF1 increased when in comparison with DMSO (Figure 62 and Figure 63). There

results confirm that SRSF1 phosphorylation was blocked, then they accumulated in the cytoplasm. In addition, this study corresponded with the previous studies in B16F10 melanoma cell. SRPIN340 were effective reduced SRPK1 nuclear translocation and phosphorylation of SR proteins at both EGF-treated and -no treated (Moreira et al., 2018) (Figure 38). Moreover, SRPIN340 showed significantly prevent the nuclear translocation of the protein kinase; SRPK1 and SRPK2 in T24 cell (Sigala et al., 2021) (Figure 39). SRPIN340 can reduced nuclear protein levels of SRSF1 in HEK293T cell (Gu et al., 2022) (Figure 40). In addition, this study corresponded with the previous studies in CCA cell. Immunofluorescence image showed that SPHINX31 could suppress SRSF1 nuclear translocation (Supradit et al., 2022) (Figure 41).

Regarding to inhibition of SRSFs phosphorylation by SRPK inhibitors were verified and provided the limitation of SRSF1 translocation to target gene splicing in the nucleus for further neutralizing of gene-splicing errors. Therefore, finally experiment we observed the expression of BIN1 gene (BIN1 and BIN1+12A specific primers) using RT-PCR. The result showed that after SRPK inhibitors treated cell decreased BIN1+12A which promote anti-apoptotic function. whereas, BIN1 was recovered, by switching back into BIN1 wild-type that action as pro-apoptotic protein (Figure 64 and Figure 65). Interestingly, we also determined the gene expression of MCL-1, the result showed that after SRPK inhibitors treated cell increase MCL-1S that action as pro-apoptotic protein in KKU-213A (Figure 69) and TFK-1 cell line (Figure 70).

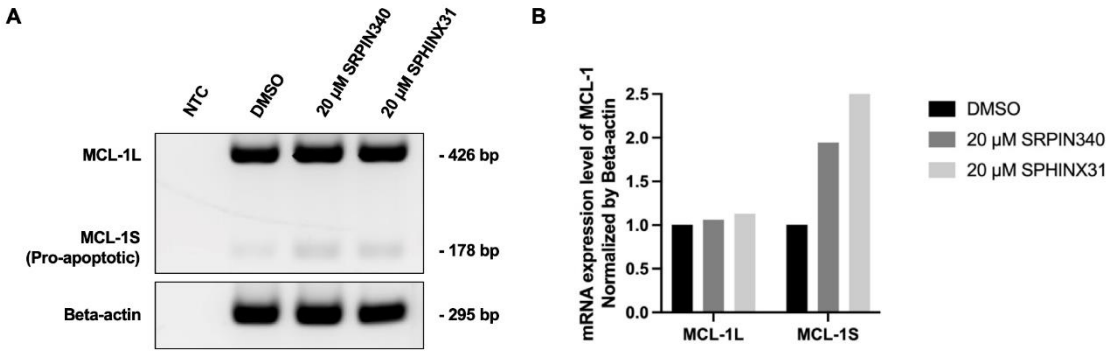

Figure 69 Effect of SRPIN340 and SPHINX31 on an alternative splicing of MCL-1 gene in KKU-213A cell. mRNA expression of MCL-1L, and MCL-1S isoform determined by RT-PCR (A). The data represent from single experiment (B).

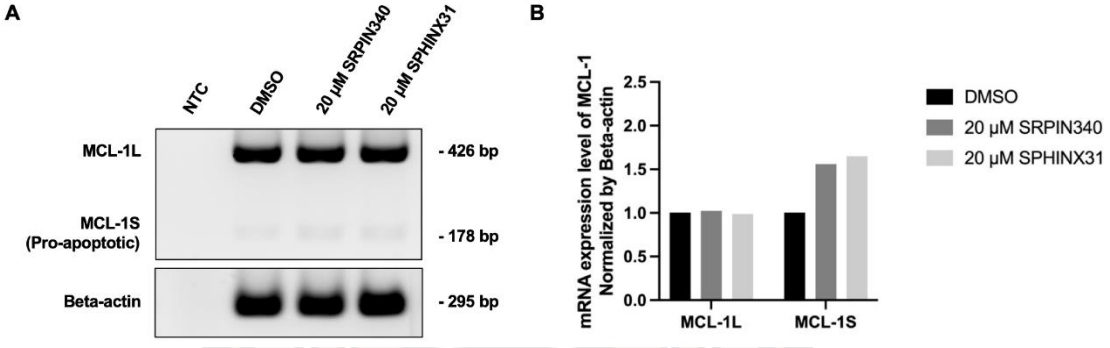

Figure 70 Effect of SRPIN340 and SPHINX31 on an alternative splicing of MCL-1 gene in TFK-1 cell. mRNA expression of MCL-1L, and MCL-1S isoform determined by RT-PCR (A). The data represent from single experiment (B).

This result associated with the previous studies from Kumlai munsuk, she studies the splicing of Fas Cell Surface Death Receptor (Fas) gene in KKU-213A cell by RT–PCR. The results showed that SRPK inhibitors can slightly increase Fas-fl (Full-length; pro-apoptotic isoform) and significantly decrease Fas-tr (Truncated-exon 6 skipping, anti-apoptotic isoform). Figures were obtained from duplicate experiment (Figure 71).

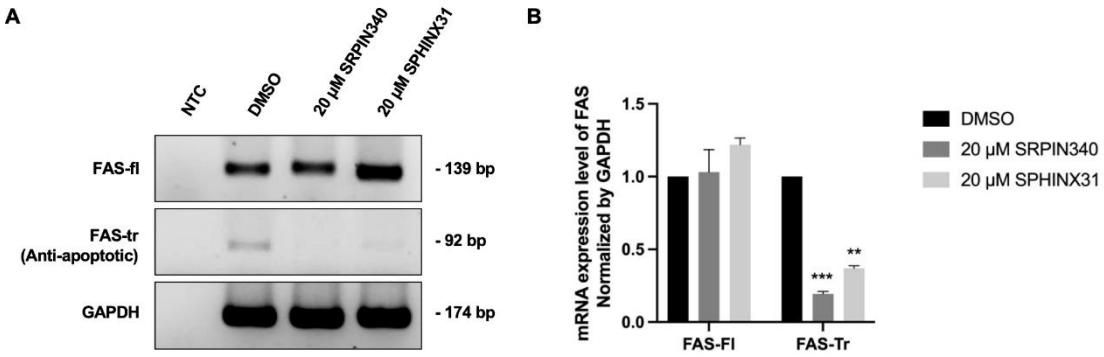

Figure 71 Effect of SRPIN340 and SPHINX31 on an alternative splicing of FAS gene in KKU-213A cell. mRNA expression of FAS-fl, and FAS-tr isoform determined by RT-PCR (A). The data represent mean ± SD from duplicate experiments, **p<0.01 (B).

Source: Kumlai munsuk, 2023, Unpublished data.

Conclusion

Serine-Arginine Protein Kinases (SRPKs) are the enzymes that have an essential role in regulating numerous aspects of mRNA splicing through the phosphorylation of splicing factors, Serine/Arginine-rich splicing factors (SRSFs). After that SRSFs can translocate from cytoplasm into the nucleus as phospho-SRSFs and splices target mRNA. Of note, dysregulations of SRSFs and SRPKs expression or mutations elicit fallacies in gene splicing, leading to the generation of oncogenes. Therefore, we aimed to explore the cellular effects of SRPK inhibitors (SRPIN340 and SPHINX31) in CCA cell lines (KKU-213A and TFK-1). SRPIN340 and SPHINX31 treatments in CCA cells increased dead cell number as in dose dependent. Higher apoptotic population, diffused cytochrome C localization and cleaved caspase-3 upregulation were approved that SRPKs inhibitors promoted CCA cell apoptosis. Next, SRPK inhibitors can be inactivate function of SRPK through suppression of SRSFs phosphorylation, especially in SRSF1. In addition, the result presented accumulated of SRSF1 in cytoplasm after SRPK inhibitors treated CCA cell and we also found that SRPK inhibitors can decrease the cytoplasmic-to-nuclear translocation of SRSF1 which demonstrated the blockage of acting mode of SRSF1 as splicing factor. Finally, recovering of wild-type BIN1 from BIN1+12A, an anti-apoptotic spliced isoform, could explain the restoration of cell apoptosis. This study will be serving as the basis information for targeting SRPKs or SRSFs and contribute to future studies of functions and drug development targeting.

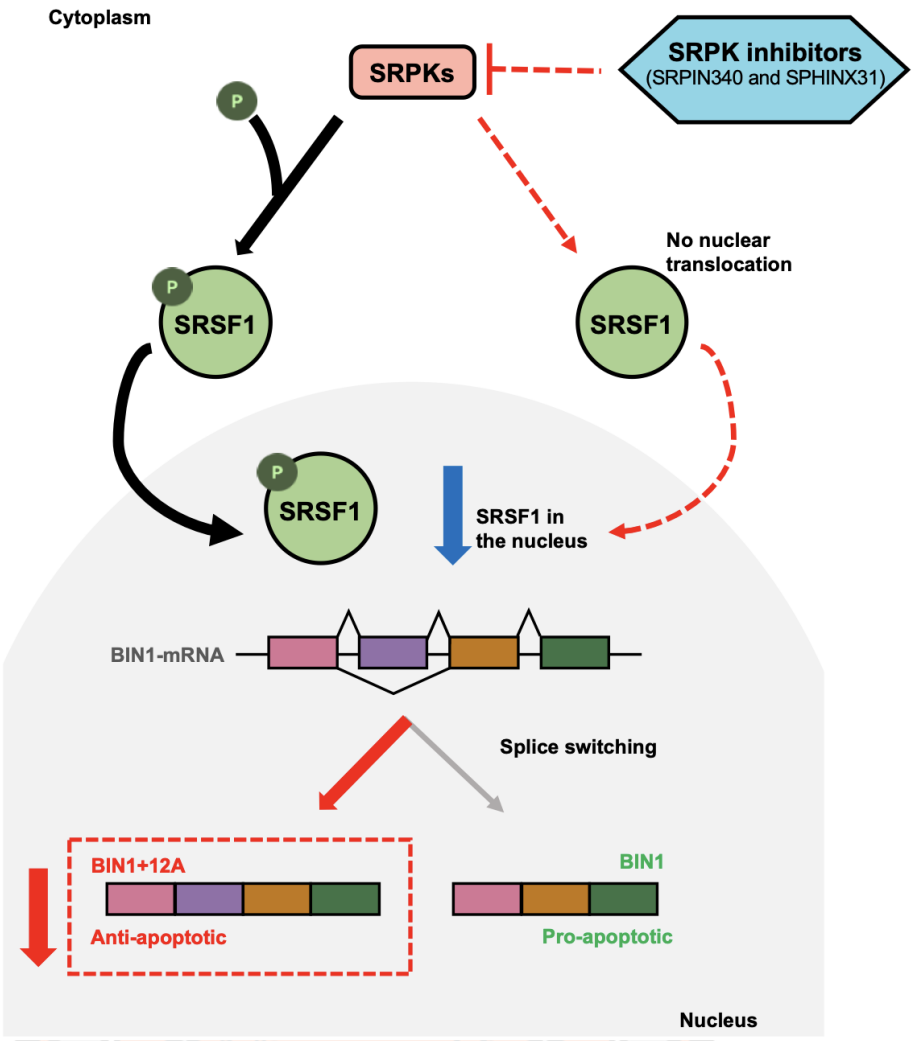

Figure 72 Graphical abstract of this study

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APPENDIX

APPENDIX A DOCUMENTATION OF ETHICS IN HUMAN RESEARCH

AF 04-09/5.0

COE No. 021/2022

IRB No. P1-0018/2565

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร

99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596-8752

หนังสือรับรองการยกเว้นพิจารณาจริยธรรมโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : กลไกการทำงานของสารยับยั้งโปรตีนเอสอาร์พีเคที่ส่งเสริมการตายแบบอะพอพโทซิสของเซลล์มะเร็งท่อน้ำดี

ผู้วิจัยหลัก : ผู้ช่วยศาสตราจารย์ ดร. วรศักดิ์ แก้วก่อง

สังกัดหน่วยงาน : คณะวิทยาศาสตร์การแพทย์

ผู้ร่วมวิจัย : นางสาวปรีณาพรรณ ช่างผาสุข

วิธีทบทวน : แบบยกเว้น (Exemption Review)

รายงานความก้าวหน้า : ไม่ต้องส่ง รายงานความก้าวหน้า (Progress Report)

ลงนาม:.....

(นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล)

ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์

มหาวิทยาลัยนเรศวร

วันที่รับรองการยกเว้นพิจารณาจริยธรรม : 11 มีนาคม 2565

หมายเหตุ

1. ไม่ต้องส่ง รายงานความก้าวหน้า (Progress Report) และรายงานสรุปผลการวิจัย (Final Report)
2. หากมีการแก้ไขโครงการวิจัยภายหลังการรับรอง ให้ผู้วิจัยดำเนินการส่งส่วนแก้ไขเพิ่มเติมโครงการวิจัย (Amendment) หรือจัดทำเป็นโครงการวิจัยใหม่

APPENDIX B LIST OF SOLUTIONS AND BUFFERS

1. Culture Medium

1.1 Complete media

Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 was supplement with 10% w/v fetal bovine serum (FBS), 100 Unit/ml of penicillin and 100 µg/ml streptomycin. Media was stored at 4°C and warmed in water bath at 37°C before use.

1.2 Antibiotic free media

Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 was supplement with 10% v/v fetal bovine serum (FBS). Media was stored at 4°C and warmed in water bath at 37°C before use.

2. SDS-PAGE and Western Blot

2.1 4X stacking gel (0.5 M Tris-HCl pH 6.8)

Tris base 60.7 g add distilled water to 800 ml for adjust the pH to 6.8 with conc. HCL and then add distilled water to 1,000 ml.

2.2 4X separating gel (1.5 M Tris-HCl pH 8.8)

Tris base 182.1 g add distilled water to 800 mL for adjust the pH to 8.8 with conc. HCl and then add distilled water to 1,000 ml.

2.3 4X sample solubilizing buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.2% bromophenol blue)

Component		
Glycerol	4	ml
β-mercaptoethanol	2	ml
1 M Tris-HCl pH 6.8	2	ml
SDS	0.8	g
Bromophenaol blue	0.02	g
Distilled water	Add to 10 ml	

2.4 10% Ammonium persulfate

1 g ammonium persulfate was dissolved in 10 ml distilled water.

2.5 10X SDS-PAGE running buffer (Tris-glycine buffer)

Component		
Tris base	30.29	g
glycine	144.13	g
20% SDS	50	ml
Distilled water	Add to 1,000 ml	

2.6 Working SDS-PAGE running buffer (Tris-glycine buffer)

100 ml of 10X SDS-PAGE running buffer and then add distilled water to 1,000 ml.

2.7 10X Bolt & Mohoney transferring buffer

Tris base 30.29 g and glycine 150.14 g were dissolved in 1,000 ml distilled water.

2.8 Working Bolt & Mohoney transferring buffer

100 ml of 10X Bolt & Mohoney transferring buffer and 200 ml of absolute methanol and then add distilled water to 1,000 ml.

2.9 TBST washing buffer (0.1% Tween-20 in TBS)

Component		
1 M Tris-HCl pH 7.4	10	ml
5 M NaCl	30	ml
Tween-20	1	ml
Distilled water	Add to 1,000 ml	

2.10 Blocking buffer (5% skim milk in 0.1% Tween-20 in TBS)

5 g of skim milk powder was dissolved in 100 ml TBST and dissolved for at least 30 minutes on a magnetic stirrer to remove all clumps.

3. Fixative reagent

3.1 2.5% Glutaraldehyde

2.5% Glutaraldehyde was dissolved in 0.1 M Phosphat buffer (PBS) pH 7.4 should be prepared before use and stored at 4 degrees, not born for 14 days.

4. Agarose gel electrophoresis

4.1. 1X Tris Acetate-EDTA (TAE) buffer

20 ml 50X TAE buffer was mixed with distilled water (15 mΩ), adjust the volume to 1000 ml. The solution was stored at room temperature.

4.2. 2 % Agarose gel

2 g agarose was dissolved in 100 ml 1X TAE Buffer and boiled until becoming homogeneous. After that, hot liquid agarose was poured to the agarose-gel-setting tray and stored at room temperature for at least 30 min for gel formation.

5. cDNA synthesis master mix

Component	1x Reaction
Total RNA	1-5 µl (1,000 ng)
Distilled water	Add to 20 µl

6. PCR master mix

Component	1x Reaction	
Distilled water 18 Ω (autoclaved)	8.2	µl
PCR mastermix (Bioline)	10	µl
Forward primer	0.4	µl
Reverse primer	0.4	µl
cDNA template	1	µl
Total	20	µl

APPENDIX C Experimental results; Raw data

Table B1 Effect of SRPK inhibitors on dead induction in KKU-213A cells using Live/Dead dual staining

KKU-213A					
Sample	**Number of dead cell count**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	72	56	52	60	10.58
10 µM SRPIN340	80	81	121	94	23.39
20 µM SRPIN340	135	156	196	162	30.99
10 µM SPHINX31	111	114	78	101	19.97
20 µM SPHINX31	121	138	120	126	10.12

Table B2 Effect of SRPK inhibitors on dead induction in TFK-1 cells using Live/Dead dual staining

TFK-1					
Sample	**Number of dead cell count**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	55	62	79	65	12.34
10 µM SRPIN340	90	114	102	102	12.00
20 µM SRPIN340	105	96	98	100	4.73
10 µM SPHINX31	137	131	124	131	6.50
20 µM SPHINX31	146	169	139	139	15.70

Table B3 Effect of SRPK inhibitors on apoptotic cell population in KKU-213A cells using Flow cytometry

KKU-213A				
Sample	**Total apoptotic cell number**		**Mean**	**S.D.**
	1	**2**		
DMSO (Control)	10.80	9.55	10.18	0.88
10 µM SRPIN340	11.25	11.70	11.48	0.32
20 µM SRPIN340	12.05	14.75	13.40	1.91
10 µM SPHINX31	21.75	16.10	18.93	4.00
20 µM SPHINX31	24.75	23.80	24.28	0.67

Table B4 Effect of SRPK inhibitors on apoptotic cell population in TFK-1 cells using Flow cytometry

TFK-1				
Sample	**Relative apoptotic cell number**		**Mean**	**S.D.**
	1	**2**		
DMSO (Control)	10.40	8.10	9.25	1.63
10 µM SRPIN340	26.35	15.10	20.73	7.95
20 µM SRPIN340	29.05	30.00	29.53	0.67
10 µM SPHINX31	40.20	46.50	43.35	4.45
20 µM SPHINX31	54.70	54.65	54.68	0.04

Table B5 Effect of SRPIN340 on apoptotic protein expression in KKU-213A cells using Western blot

KKU-213A					
Sample	**Band intensity (Cleaved Caspase-3)**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	1.00	1.00	1.00	1.00	0.00
10 µM SRPIN340	2.51	0.95	1.81	1.75	0.78
20 µM SRPIN340	4.65	1.04	1.84	2.51	1.90

Table B6 Effect of SPHINX31 on apoptotic protein expression in KKU-213A cells using Western blot

KKU-213A					
Sample	**Band intensity (Cleaved Caspase-3)**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	1.00	1.00	1.00	1.00	0.00
10 µM SPHINX31	2.86	1.20	0.84	1.63	1.08
20 µM SPHINX31	8.42	2.16	1.30	3.96	3.88

Table B7 Effect of SRPIN340 on apoptotic protein expression in TFK-1 cells using Western blot

TFK-1					
Sample	**Band intensity (Cleaved Caspase-3)**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	1.00	1.00	1.00	1.00	0.00
10 µM SRPIN340	1.56	0.89	6.68	3.04	3.16
20 µM SRPIN340	2.38	1.81	8.24	4.14	3.56

Table B8 Effect of SPHINX31 on apoptotic protein expression in TFK-1 cells using Western blot

TFK-1					
Sample	**Band intensity (Cleaved Caspase-3)**			**Mean**	**S.D.**
	1	**2**	**3**		
DMSO (Control)	1.00	1.00	1.00	1.00	0.00
10 µM SPHINX31	2.21	0.28	1.08	1.19	0.97
20 µM SPHINX31	3.09	3.13	2.37	2.87	0.43

Table B9 Effect of SRPIN340 on phosphorylation profile of SRSFs protein in KKU-213A cells using Western blot

KKU-213A						
pSRSFs	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
pSRSF5/pSRSF10	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.45	1.00	0.92	0.79	0.30
	20 µM SRPIN340	1.05	0.66	0.44	0.72	0.31
pSRSF2/pSRSF7	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.42	1.18	0.77	0.79	0.38
	20 µM SRPIN340	0.67	0.61	0.30	0.53	0.20
pSRSF1/pSRSF2	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.42	1.30	0.99	0.90	0.45
	20 µM SRPIN340	0.63	0.56	0.48	0.56	0.40

Table B10 Effect of SPHINX31 on phosphorylation profile of SRSFs protein in KKU-213A cells using Western blot

KKU-213A						
pSRSFs	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
pSRSF5/pSRSF10	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	0.80	0.78	0.70	0.76	0.06
	20 µM SPHINX31	1.43	0.76	0.37	0.85	0.54
pSRSF2/pSRSF7	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	1.38	0.75	0.63	0.92	0.41
	20 µM SPHINX31	2.17	0.73	0.36	1.09	0.96
pSRSF1/pSRSF2	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	0.40	1.06	0.65	0.70	0.33
	20 µM SPHINX31	0.24	0.59	0.25	0.61	0.20

Table B11 Effect of SRPIN340 on phosphorylation profile of SRSFs protein in TFK-1 cells using Western blot

TFK-1						
pSRSFs	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
pSRSF5/pSRSF10	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.78	0.56	0.94	0.76	0.19
	20 µM SRPIN340	0.48	0.36	0.63	0.49	0.13
pSRSF2/pSRSF7	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.58	0.54	0.70	0.61	0.08
	20 µM SRPIN340	0.37	0.33	0.39	0.36	0.03
pSRSF1/pSRSF2	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SRPIN340	0.37	0.74	0.60	0.57	0.19
	20 µM SRPIN340	0.35	0.49	0.34	0.39	0.08

Table B12 Effect of SPHINX31 on phosphorylation profile of SRSFs protein in TFK-1 cells using Western blot

TFK-1						
pSRSFs	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
pSRSF5/pSRSF10	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	0.73	0.68	1.22	0.88	0.30
	20 µM SPHINX31	1.07	0.76	3.00	1.61	1.22
pSRSF2/pSRSF7	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	0.51	0.46	0.74	0.57	0.15
	20 µM SPHINX31	0.66	0.38	1.36	0.80	0.51
pSRSF1/pSRSF2	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	10 µM SPHINX31	0.45	0.59	0.56	0.53	0.08
	20 µM SPHINX31	0.62	0.42	1.19	0.74	0.40

Table B13 Effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1 protein in KKU- 213A cells using Subcellular protein fractionation (cytoplasmic protein and nuclear protein) and Western blot

KKU-213A		
Sample	**Band intensity (SRSF1)**	
	Cytoplasmic protein	**Nuclear protein**
DMSO (Control)	1.00	1.00
20 µM SRPIN340	1.22	0.84
20 µM SPHINX31	1.31	0.28

Table B14 Effect of SRPK inhibitors on cytoplasmic-to-nuclear translocation of SRSF1 protein in TFK-1 cells using Subcellular protein fractionation (cytoplasmic protein and nuclear protein) and Western blot

TFK-1		
Sample	**Band intensity (SRSF1)**	
	Cytoplasmic protein	**Nuclear protein**
DMSO (Control)	1.00	1.00
20 µM SRPIN340	1.21	0.30
20 µM SPHINX31	1.42	0.07

Table B15 Effect of SRPK inhibitors on an alternative splicing of BIN1 gene in KKU-213A cells using RT-PCR

KKU-213A						
BIN1 isoform	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
BIN1_All	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	20 µM SRPIN340	0.97	0.96	0.79	0.91	0.10
	20 µM SPHINX31	1.07	0.89	0.84	0.94	0.12
BIN1+12A	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	20 µM SRPIN340	0.34	0.99	0.10	0.48	0.12
	20 µM SPHINX31	0.04	0.45	0.06	0.18	0.00

Table B16 Effect of SRPK inhibitors on an alternative splicing of BIN1 gene in TFK-1 cells using RT-PCR

KKU-213A						
BIN1 isoform	**Sample**	**Band intensity**			**Mean**	**S.D.**
		1	**2**	**3**		
BIN1_All	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	20 µM SRPIN340	0.93	0.98	0.97	0.96	0.03
	20 µM SPHINX31	1.01	1.00	1.00	1.00	0.01
BIN1+12A	DMSO (Control)	1.00	1.00	1.00	1.00	0.00
	20 µM SRPIN340	0.66	0.44	0.44	0.51	0.00
	20 µM SPHINX31	0.33	0.48	0.09	0.30	0.00

APPENDIX D Research presentation and publication

1. Research presentations

1.1. Poster presentations

1.1.1. Preenapan Changphasuk, Chaturong Inpad, Phattarin Pothipan and Worasak Kaewkong. Splicing kinase inhibitors induce cholangiocarcinoma cell apoptosis via the activation of the unfolded protein response (UPR) pathway. **The 48th Annual Meeting of Korean Cancer Association (KCA2022) and 2nd International Congress of Asian Oncology Society (AOS2022).** June 16-18, 2022 at Lotte Hotel Seoul, Seoul, Korea

1.1.2. Preenapan Changphasuk, Chaturong Inpad and Worasak Kaewkong. SRPK inhibitors Promotes Cholangiocarcinoma Cell Apoptosis by the Deactivation of Splicing Factor SRSF1 and Correction of BIN1 Gene Splicing. **The 16th The Conference in Medical Biochemistry: Medical Biochemistry for Precision Medicine.** August 26, 2023 at Khon Kaen University, Khon Kaen, Thailand

2. Research publication

2.1. Proceeding (Oral presentation)

2.1.1. Preenapan Changphasuk, Chaturong Inpad, Phattarin Pothipan, Suchada Phimsen and Worasak Kaewkong. Inhibition of SR-protein kinase induces cholangiocarcinoma cell apoptosis through the alteration of SR-rich splicing factor phosphorylation. **The 17th International Symposium of The Protein Society of Thailand (PST2022).** November 9-11, 2020 at Khum Kham International Convention Centre, Chiang Mai, Thailand (Full paper published in the proceeding book).

2.2. Manuscript preparation (for submission to Journal of Molecular Medicine Reports)

2.2.1. Preenapan Changphasuk, Chaturong Inpad, Kumrai Munsuk, Sukanya Horpaopan, Sasiprapa Khunchai, Suchada Phimsen, Atit Silsirivanit, and Worasak Kaewkong. SRPK inhibitors lessening the phosphorylation and nuclear-translocation of SR protein splicing factors to correct the dysregulated BIN1, MCL-1 and FAS gene splicing for enabling cholangiocarcinoma cell apoptosis.

Abstract page

The 48th Annual Meeting of Korean Cancer Association (KCA2022) and 2nd International Congress of Asian Oncology Society (AOS2022)

* Abstract No.	ABST-0139
* Abstract Category	Poster
* Topic	Molecular Biology of Cancer
* Abstract Title	Splicing kinase inhibitors induce cholangiocarcinoma cell apoptosis via the activation of the unfolded protein response (UPR) pathway
* Key Words	apoptosis, cholangiocarcinoma, SRPK
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* Text	Propose: Dysregulation of mRNA splicing is suspected to play a major role as oncogenic aberrant spliced genes have been reported in cholangiocarcinoma (CCA). Aberrant splicing is caused by the increased activity of Serine/Arginine rich splicing factor (SRSF) that translocate into the nucleus as phospho-SRSF after their activation by Serine-arginine protein kinase (SRPK), and splices target mRNA. Therefore, we aimed to explore the cellular effects of SRPK inhibitors in CCA cell lines. Methods: Dead induction of SRPK inhibitors (SRPIN340 and SPHINX31) was evaluated by calcein-AM/PI staining. Annexin-V/7AAD in flow cytometry, cleaved caspase-3 expression by western blotting, and cytochrome c cyto-staining were performed for verification of apoptotic death. Nuclear translocation of key SRSF was validated by western blot of fractionated protein. Aberrant splicing status of pro-apoptotic BIN1 gene was assessed by RT-PCR, and the activation of unfolded protein response (UPR) pathway was determined by western blot analysis. Results: SRPIN340 and SPHINX31 treatments in CCA cells increased dead cell number as in dose-dependent. Higher apoptotic population, cleaved caspase-3 upregulation and diffused cytochrome C localization were approved that SRPKs inhibitors promoted CCA cell apoptosis. Cytoplasmic accumulation was demonstrated the blockage of acting mode of SRSF1 as splicing factor. Remarkably, recovering of wild-type BIN1 from BIN1+12A, an anti-apoptotic spliced isoform, could explain the restoration of cell apoptosis. Finally, upregulation of phosphor-EIF2a and CHOP can verify the apoptotic induction was stimulated by an activation of UPR pathway. Conclusions: SRPK inhibitors eradicate SRSF1 dysregulation for correcting aberrant BIN1 splicing to regain the driver of CCA cell apoptosis.
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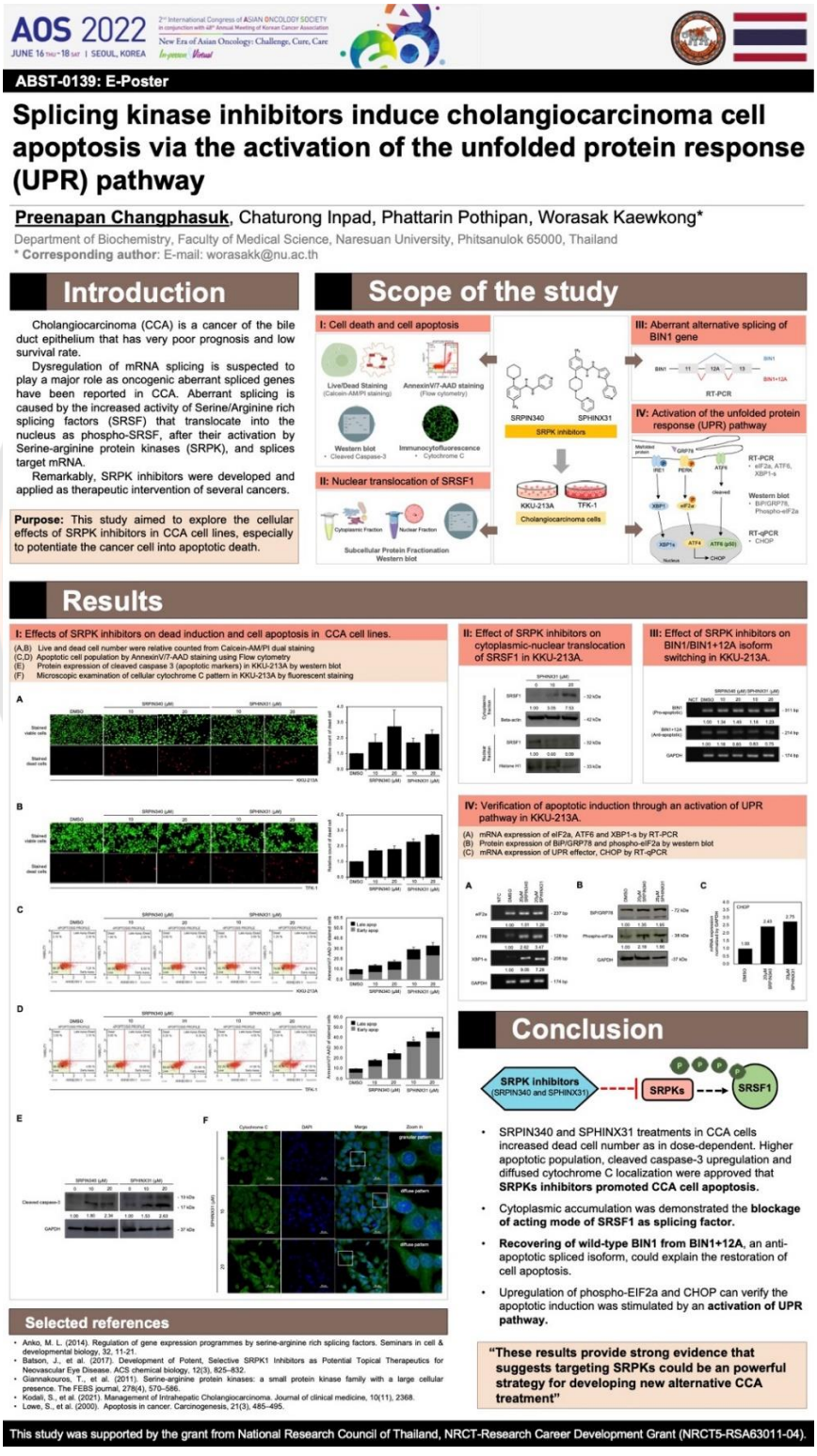
The 48th Annual Meeting of Korean Cancer Association (KCA2022) and 2nd International Congress of Asian Oncology Society (AOS2022)



Cover page

The 16th The Conference in Medical Biochemistry: Medical Biochemistry for Precision Medicine

Abstract page

The 16th The Conference in Medical Biochemistry: Medical Biochemistry for Precision Medicine

16th Conference of Medical Biochemistry 2023

SRPK Inhibitors Promotes Cholangiocarcinoma Cell Apoptosis by the Deactivation of Splicing Factor SRSF1 and Correction of BIN1 Gene Splicing

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Introduction and Objective: A number of oncogenic aberrant spliced genes have been reported in cholangiocarcinoma (CCA), therefore, dysregulation of mRNA splicing is suspected to play a major role in CCA development. This study, the splicing regulators name serine-arginine protein kinases (SRPK) are targeted by specific inhibitors to explore the cellular effects in CCA cells.

Methods: KKU-213A and TFK-1 were treated with SRPIN340 or SPHINX31. Dead induction, apoptosis cell, apoptotic activations were evaluated by calcein-AM/PI staining, Annexin-V/7AAD staining and western blotting or immunofluorescent staining of apoptotic proteins, respectively. Inhibiting the nuclear-to-cytoplasmic translocation of splicing factor SRSF1 was monitored by western blotting. In addition, splicing status of BIN1 genes was verified by RT-PCR.

Results: Increasing of dead cell numbers and apoptotic cell populations was found in SRPK inhibitors treated in KKU-213A and TFK-1 as dose dependent manner. Upregulation of cleaved caspase-3 and diffused cytoplasmic pattern of cytochrome C were verified. Remarkably, accumulation of cytoplasmic SRSF1 was confirmed that the action mode of SRPK was inhibited. Moreover, correcting of BIN1 genes splicing from oncogenic isoforms was demonstrated.

Conclusion: SRPK inhibitors deactivate splicing factor SRSF1 to correct aberrant BIN1 gene splicing, to promote CCA cell apoptosis.

Keyword: apoptosis, cholangiocarcinoma, SRPK, SRSF

References:

1.Banales, J. M.; Marin, J. J. G.; Lamarca, A.; Rodrigues, P. M.; Khan, S. A.; Roberts, L. R.; et al. *Nat Rev Gastroenterol Hepatol. Cholangiocarcinoma 2020: the next horizon in mechanisms and management*, **2020**, *17(9)*, 557-588.

2.Das, S.; Krainer, A. R. *Mol Cancer Res. Emerging function of SRSF1, splicing factor and oncoprotein, in RNA metabolism and cancer*, **2014**, *12(9)*, 1195-1204.

Preenapan Changpasuk, M.Sc. student
Naresuan University, Thailand, Biochemistry and Molecular Biology, 2021
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Poster

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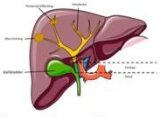
Introduction

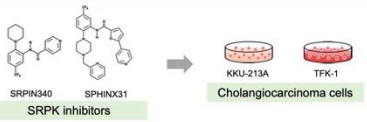


Cholangiocarcinoma (CCA) is a cancer of the bile duct epithelium that has very poor prognosis and low survival rate.

A number of oncogenic aberrant spliced genes have been reported in CCA, therefore, dysregulation of mRNA splicing is suspected to play a major role in CCA development.

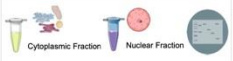
Purpose: To study the splicing regulators name serine-arginine protein kinases (SRPK) which were targeted by specific inhibitors for exploring the cellular effects in CCA cells.

Methods



I: Cell death and cell apoptosis

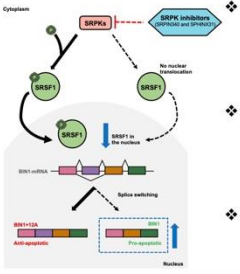
II: Nuclear translocation of SRSF1



III: Aberrant alternative splicing of BIN1 gene

Conclusion

❖ SRPIN340 and SPHINX31 treatments in CCA cells **increased dead cell numbers and apoptotic cell populations** as dose dependent manner.



❖ **Upregulation of cleaved caspase-3** and **diffused cytoplasmic pattern of cytochrome C** were verified.

❖ **Accumulation of cytoplasmic SRSF1** was confirmed that the action mode of SRPK was inhibited.

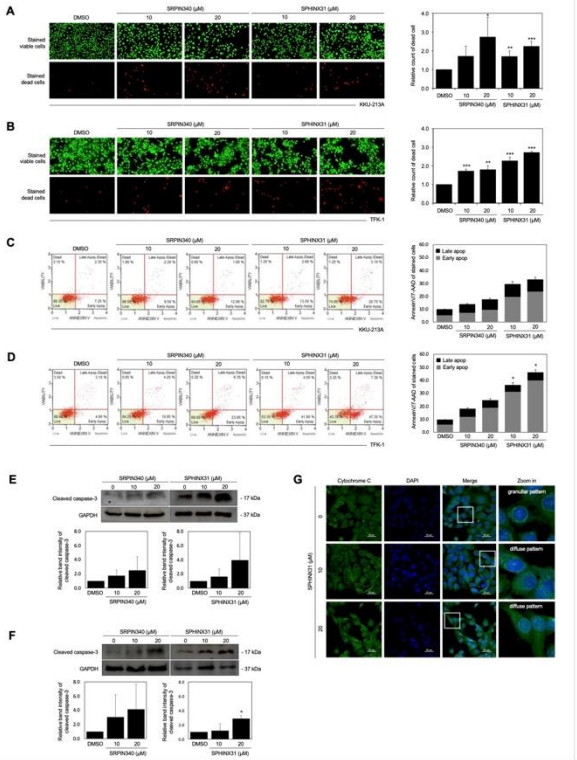
❖ **Correcting of BIN1 genes splicing** from oncogenic isoforms was demonstrated.

"SRPK inhibitors deactivate splicing factor SRSF1 to correct aberrant BIN1 gene splicing, to promote CCA cell apoptosis"

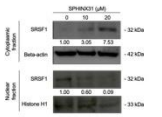
Results

I: Effects of SRPK inhibitors on dead induction and cell apoptosis in CCA cell lines.

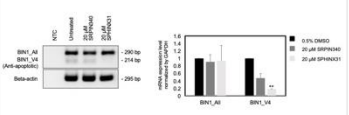
(A,B) Live and dead cell number were relative counted from Calcein-AM/PI dual staining
(C,D) Apoptotic cell population by AnnexinV/7-AAD staining using Flow cytometry
(E,F) Protein expression of cleaved caspase 3 (apoptotic markers) by western blot
(G) Microscopic examination of cellular cytochrome C pattern in KKU-213A by fluorescent staining



II: Effect of SRPK inhibitors on cytoplasmic-nuclear translocation of SRSF1 in KKU-213A.



III: Effect of SRPK inhibitors on BIN1/BIN1+12A isoform switching in KKU-213A.



Selected references

Banales, J. M.; Marin, J. J. G.; Lamarca, A.; Rodrigues, P. M.; Khan, S. A.; Roberts, L. R.; et al. *Nat Rev Gastroenterol Hepatol. Cholangiocarcinoma 2020: the next horizon in mechanisms and management*, **2020**, *17(9)*, 557-588.

Das, S.; Krainer, A. R. *Mol Cancer Res. Emerging function of SRSF1, splicing factor and oncoprotein, in RNA metabolism and cancer*, **2014**, *12(9)*, 1195-1204.

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Cover page

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Abstract page

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PROCEEDING-20220080

Oral Presentation

Inhibition of SR-protein kinase induces cholangiocarcinoma cell apoptosis through the alteration of SR-rich splicing factor phosphorylation

Preenapan Changpasuk[1], Chaturong Inpad[1], Phattarin Pothipan[1], Suchada Phimsen[1], and Worasak Kaewkong[1,*]

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ABSTRACT

Cholangiocarcinoma (CCA) is a cancer arising from abnormal growth of bile duct epithelium with a low survival rate due to inefficient treatments. The molecular mechanisms underlying CCA development are still unclear. However, aberrant mRNA splicing is suspected to play a major role since many oncogenic spliced transcripts have been reported in CCA. Aberrant splicing is caused by the dysregulation of Serine-Arginine rich splicing factors (SRSFs) that translocate into the nucleus after phosphorylation by Serine-arginine protein kinase (SRPKs) and splice targeted mRNAs. Therefore, the effects of SRPK inhibitors (SRPIN340 and SPHINX31) treatments on CCA cell lines were investigated in this study. SRPIN340 and SPHINX31 treatments of KKU-213A and TFK-1 cells increased the number of dead cells stained with calcein-AM/PI in a dose-dependent manner. Additionally, the apoptotic verification, including annexin-V/7AAD in flow-cytometry, cytochrome c staining in confocal microscopy, and cleaved Caspase-3 in western blot, all confirmed that SRPK inhibitor treatments promoted apoptosis in KKU-213A cells. Moreover, western blot analysis of the SRSF phosphorylation profile revealed a decrease in phosphorylated-SRSFs (pSRSFs). These results provide strong evidence that suggests targeting SRPKs could be a powerful strategy for developing new alternative CCA treatments.

Keywords: Cell apoptosis, Cholangiocarcinoma, SRPK, SRSF, SRPK inhibitor

1 INTRODUCTION

Cholangiocarcinoma (CCA) is a severe type of cancer that occurs in the epithelium of the bile duct. Thailand has a high incidence of CCA, which is commonly reported from the north and northeastern regions [1]. The main reasons were freshwater fish consumption with contaminating metacercaria of *Opisthorchis viverrini* (OV) together with other factors such as exogenous carcinogens, excretory/secretory products from OV acting as important growth stimuli, and repeated treatment with parasitic drugs [2]. Treatment for CCA is determined by the stage at which the cancer is detected. Since there are no specific symptoms in the early stages, most of the patients treated for CCA are more advanced or have metastasis, leading to the mortality rate of CCA patients having increased every year [3].

Aberrant alternative splicing has been reported to result in oncogenic isoform production from various important genes in many cancers and is recognized as another hallmark of cancers [4]. The Serine-arginine rich splicing factor (SRSFs) is the SR protein family that serves as a key molecule in regulation. It consists of 12 members of SRSFs named as SRSF1 to SRSF12 [5]. The main activity of SRSFs is regulated by Serine-arginine protein kinase (SRPKs), which are responsible for phosphorylating SRSFs at their arginine-serine rich (RS) domain into active SRSFs. Then, phosphorylated-SRSFs translocate to the nucleus for targeting multi-exon pre-mRNA [6]. Dysregulation of SRSFs and SRPKs leads to abnormal protein synthesis with overexpression and functional alteration to gain oncogenic properties [7]. Accumulated evidence has indicated that SRPK1 and SRPK2 have been found overexpressed in various types of cancer, for example, breast, colon, pancreatic carcinomas, leukemia, gliomas, and ovary, which is correlated with advanced tumor staging and

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Symposium Program

10:40 - 11:10	**Coffee Break & Exhibition (Chiang Thong, Chiang Sean and Viang Kham Room)**	
	Selected Oral Presentation 1 Chair: Assoc. Prof. Atit Silsirivanit	**Selected Oral Presentation 2** Chair: Dr. Sittiruk Roytrakul
11:10 - 11:25	**Selected Oral Presentation 5** Dr. Suyanee Thongchot Mahidol University Title: The Induction of Nucleolin-Specific T cells Against Triple Negative Breast Cancer by Immunogenic Nucleolin Short Peptides	**Selected Oral Presentation 8** Dr. Jittiporn Chaisaingmongkol Chulabhorn Research Institute Title: Landscape of DNA Methylation in Intrahepatic Cholangiocarcinoma Patients in Thailand
11:25 - 11:40	**Selected Oral Presentation 6** Dr. Charupong Saengboonmee Khon Kaen University Title: GABBR2 is a Potential Therapeutic Target for Cholangiocarcinoma with Diabetes Mellitus	**Selected Oral Presentation 9** Prakasit Khamsuwan Naresuan University Title: Targeting Upregulated-LAMB1 Derived from SRPK Inhibitors Treated-Cholangiocarcinoma Cell Proteome for Enhancing In Vitro Growth Inhibition Through pERK/ERK/c-Jun Axis
11:40 - 11:55	**Selected Oral Presentation 7** Preenapan Changpasuk Naresuan University Title: Inhibition of SR-protein Kinase Induces Cholangiocarcinoma Cell Apoptosis Through the Alteration of SR-rich Splicing Factor Phosphorylation	**Selected Oral Presentation 10** Orasa Panawan Khon Kaen University Title: Global- and Phospho-Proteomics Identify the Cholesterol and Fatty Acid Metabolic Pathway as a Specific Key Target of Glioma Stem-like Cells
11:55 - 12:55	**Luncheon Lecture** by **World Tech Enterprise Ltd.** Dr. Jaran Jainhuknan Title: Enhancing the Workflow Capabilities of Translational and Life Science Mass Spectrometry: New Developments in Mass Spectrometry Imaging, and Proteomics	**Luncheon Lecture** By **SCIEX** Prof. Qingsong Lin Title: Drug Target Profiling by Chemical Proteomics
	Session 5: Immunology and Inflammation Chairs: Assist.Prof. Hathairat Thananchai Assist.Prof. Aussara Panya	**Session 6: Bioinformatics and Statistics** Chair: Assoc. Prof. Varodom Charoensawan
13:00 - 13:35	**Keynote Lecture** Prof. Watchara Kasinrerk Chiang Mai University Title: Cannabinoid Receptors and T cell Regulation	**Keynote Lecture** Dr. Pimlapas Leekitcharoenphon Technical University of Denmark Title: Bioinformatics for Fighting with Infectious Diseases
13:35 - 14.00	**Invited Lecture** Dr. Suangsuda Supasai Mahidol University Title: Persistent Neuropathology and Behavioral Defects in Rats After Status Epilepticus	**Invited Lecture** Dr. Kwanjeera Wanichthanarak Mahidol University Title: Bioinformatics Meets Omics
14:00 - 14:25	**Invited Lecture** Prof. Chanitra Thuwajit Mahidol University Title: The Immunotherapy of Triple Negative Breast Cancer	**Keynote Lecture (14.00-14.35)** Assist.Prof. Balachandran Manavalan Sungkyunkwan University Title: Computational Identification of Therapeutic Peptides using Several Machine Learning Frameworks

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