SILENCING OF SRSF1 TO INDUCE CHOLANGIOCARCINOMA CELL DEATH

PHICHAMON PHETCHAHWANG

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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By PHICHAMON PHETCHAHWANG
has been approved by the Graduate School as partial fulfillment of the requirements
for the Master of Science in Biochemistry of Naresuan University

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Title SILENCING OF SRSF1 TO INDUCE CHOLANGIOCARCINOMA CELL DEATH
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ABSTRACT

Cholangiocarcinoma or CCA is a malignance cancer arising from abnormal growth of bile duct epithelial. CCA present the high incidence and mortality rate in Southeast Asian countries, especially in Thailand. CCA development does not have any specific symptoms. Therefore, CCA difficult to detect or diagnosis in early state, then developed into advance stages with highly metastasis which resulting in ineffective treatment and leading to high mortality rate. Several strong evidences presented the association of CCA development and progression with aberrant alternative splicing. These processes regulated by Serine/Arginine-rich splicing factors or SRSFs. Dysregulation of SRSFs can generated the aberrant transcript variants and cancer related protein isoforms of many important genes in CCA. Some of the spliced genes mentioned in CCA related to anti-apoptosis and promoted cancer cells evading from death. Previous studies reported high expression of SRSF1 in various cancers and the result from our group also found that SRSF1 was upregulated in CCA patient tissues and in CCA cell lines. This study aimed to investigate the role of SRSF1 on CCA cell death. Particularly, to clarify the specific type of death. SRSF1 mRNA and protein were verified that predominantly upregulated in KKU-055 and KKU-213A (CCA cells) when compared with MMNK-1 (cholangiocyte). Next, to study the effect of SRSF1-silencing by 2 specific siRNAs (siSRSF1-1 and siSRSF1-2), SRSF1 expression was depleted in both CCA cell lines. Especially, the higher efficiency was observed in siSRFS1-2 transfection. For the effect of SRSF1-silencing on CCA cell death, SRSF1-silencing increased the number of death cell and the specific morphologies of death cell

including apoptosis, autophagy and necrosis were observed under transmission electron microscope (TEM). For apoptosis, SRSF1-silencing was slightly increase apoptotic cell number and caspase 3/7 enzyme activity which confirmed by upregulation of BAX and downregulation of Bcl-2. For autophagy-dependent cell death, SRSF1-silencing was downregulated p62 protein and upregulated ATG5 and Beclin-1 mRNA, and increased the LC3B-II and LC3B-I protein expression ration. From these results suggest that high expression of SRSF1 promotes CCA cell evading from death which verified by SRSF1 silencing by siRNAs can induce CCA cell to apoptosis and autophagy-dependent cell death. Therefore, these results might be used as supportive information for applying the SRSF1 targeting as an alternative treatment strategy for CCA.

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PHICHAMON PHETCHAHWANG

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ABBREVIATIONS

%	Percent
AAD	Aminoactiomycin D
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease activating factor 1
AS	Alternative splicing
ATG	Autophagy-related genes
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

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
FBS	Fetal bovine serum
FLIP	FLICE-like inhibitory protein
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
hnRNPs	Heterogeneous nuclear ribonucleoproteins
HtrA2/Omi	High temperature requirement protein A2
ICAD	Inhibitor of CAD
IkB	Inhibitor of NF-kB
kDa	Kilo Dalton
LC	Liquid Chromatograph

ABBREVIATIONS (CONT.)

LC3B	Microtubule-associated proteins 1A/1B light chain 3B
Mcl-1	Myeloid cell leukemia 1
mg	Milligram
mg	Milligram
min	Minute
mL	Milliliter
mRNA	Messenger ribonucleotide acid
NF-kB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer
p53	Tumor protein 53
p62	Nucleoporin p62/ Sequestosome-1
p73	Tumor protein 73
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PUMA	p53 upregulated modulator of apoptosis
RBPs	Retinol-binding proteins
RNA	Ribonucleic acid

ABBREVIATIONS (CONT.)

RT-PCR	Reverse transcription-polymerase chain reaction
S.D.	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Smac	Second mitochondria-derived activator of caspase
SR	Serine and arginine-rich proteins
SRPKs	Serine/Arginine protein kinases
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
ULK1	Unc-51 Like autophagy activating kinase 1
WIPI1	WD repeat domain phosphoinositide-interacting protein 1
WIPI2	WD repeat domain phosphoinositide-interacting protein 2
XIAP	X-linked inhibitor of apoptosis protein

ABBREVIATIONS (CONT.)

^{o}C	Degree Celsius
µg	Microgram
µl	Microliters
µM	Micro Molar
µm	Micrometer

CHAPTER I

INTRODUCTION

Rational of the study

Cholangiocarcinoma (CCA) is epithelial cell malignancy arising in various locations of the biliary duct. The risk factor of CCA involving freshwater fish consumption with the contaminating of the metacercaria of carcinogenic liver fluke, *Opisthorchis viverrini.* The infection by this liver fluke and intake of carcinogen during food fermentation will promote CCA development. There are no 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. Current diagnosis and treatment of CCA are not enough effective or cure the tumor completely. Also, CCA shows high incidence in Southeast Asia, particularly in Thailand where CCA cases were often reported in the North and Northern region. Therefore, understanding the molecular processes of CCA development and the various molecules that contributes to cholangiocarcinogenesis is very important. The knowledge for an initial study on molecular carcinogenesis will develop a biomarker to diagnose early or to develop options CCA patient treatment.

The molecular process that is recently mentioned is alternative splicing (AS). AS is one of the post-transcriptional modification of RNA synthesis for gene expression that results in a single gene (multi-exon gene) coding for multiple RNA transcripts and protein isoforms. In 2013, AS is categorized to be additional hallmarks in cancer. Many important genes in various types of cancer presented their aberrantly splicing and the oncogenic properties of them. In CCA, there is the number of research articles shown the aberrant AS occurred in many important genes and presents their contributions to promote CCA development and procession. The AS is regulated by the function of SR protein family.

SR protein family is the important classes of splicing regulatory proteins those play an essential role in the AS of pre-mRNA to mature mRNA. Serine/Arginine-rich Splicing Factors or SRSFs is encoded by 12 gene members (SRSF1 to SRSF12). There are the combinations of SRSFs function with spliceosome to regulate splicing

mechanism. Dysregulation of SRSFs can be generated the aberrant transcript variants and cancer related-protein isoforms. Cancer cells often display aberrant AS profiles, expressing isoforms that stimulate cell proliferation and migration or improve the ability for resisting to cell death.

There are several studies demonstrated SRSF1 can be acts as an oncogenic protein which function in regulating AS of various tumor suppressor genes for initiating the cancer progression (Anczuków et al., 2012). Remarkably, our research group investigated the expression of 12 members (SRSF1-SRSF12) of SR proteins and demonstrated that SRSF1 is highly expressed in CCA cells compare with normal cells. Interestingly, SRSF1 protein also expressed in CCA tissue (tumor area when compared with adjacent non-tumor area). In addition, suppression of SRSF1 expression by specific siRNAs can inhibits cancer cell proliferation which also increased the population of death cells.

The present study aimed to investigate the roles of SRSF1 depletion on cell death in CCA cells, particularly to clarify the specific type by cancer cell death morphologies. Apoptosis- and autophagy-specific marker genes expression will be determined. In addition, specific phenotypes of each type of cell death will be monitored. The expectation of this study is to investigate that can SRSF1 be able to develop as the specific therapeutic target for CCA.

Objectives of study

1. To confirm the mRNA and protein expression of SRSF1 in CCA cell lines.

2. To investigate the effect of SRSF1 silencing on cell viability of CCA cell lines.

3. To study the effect of SRSF1 silencing on death morphologies and types of cell death in CCA cell lines.

4. To investigate the effect of SRSF1 silencing on apoptosis of CCA cells both in apoptotic cellular phenotypes and in pro-/ anti-apoptotic protein expressions.

5. To investigate the effect of SRSF1 silencing on autophagy-dependent cell death of CCA cells by autophagic marker gene and protein expressions.

Scope of study

The research study is divided into 5 parts as shown in Figure 1;

Part 1: Investigate the mRNA and protein expression profile of SRSF1 in CCA cell lines (KKU-055, KKU-213A) by RT-PCR and western blot. (Corresponding to objective 1)

Part 2: Investigate the effect of SRSF1 silencing on cell viability of CCA cell lines by trypan blue dye exclusion assay. (Corresponding to objective 2)

Part 3: Investigate the effect of SRSF1 silencing on death morphologies and types of cell death in CCA cells (KKU-055 and KKU-213A) under transmission electron microscope (TEM). (Corresponding to objective 3)

Part 4: Investigate the effect of SRSF1 silencing on apoptosis of CCA cells (KKU-055 and KKU-213A) to (1) determine apoptotic cell population by Annexin V/7-AAD staining; Flow cytometry, (2) Apoptosis enzyme activity by Apo-ONE® Homogeneous caspase 3/7 assay, (3) determining pro-/anti-apoptotic expression in protein level, (Corresponding to objective 4)

Part 5: Investigate the effect of SRSF1 silencing on autophagy-dependent cell death of CCA cells (KKU-055 and KKU-213A) to determine autophagic marker gene expression in mRNA and protein level by RT-PCR and western blot. (Corresponding to objective 5)

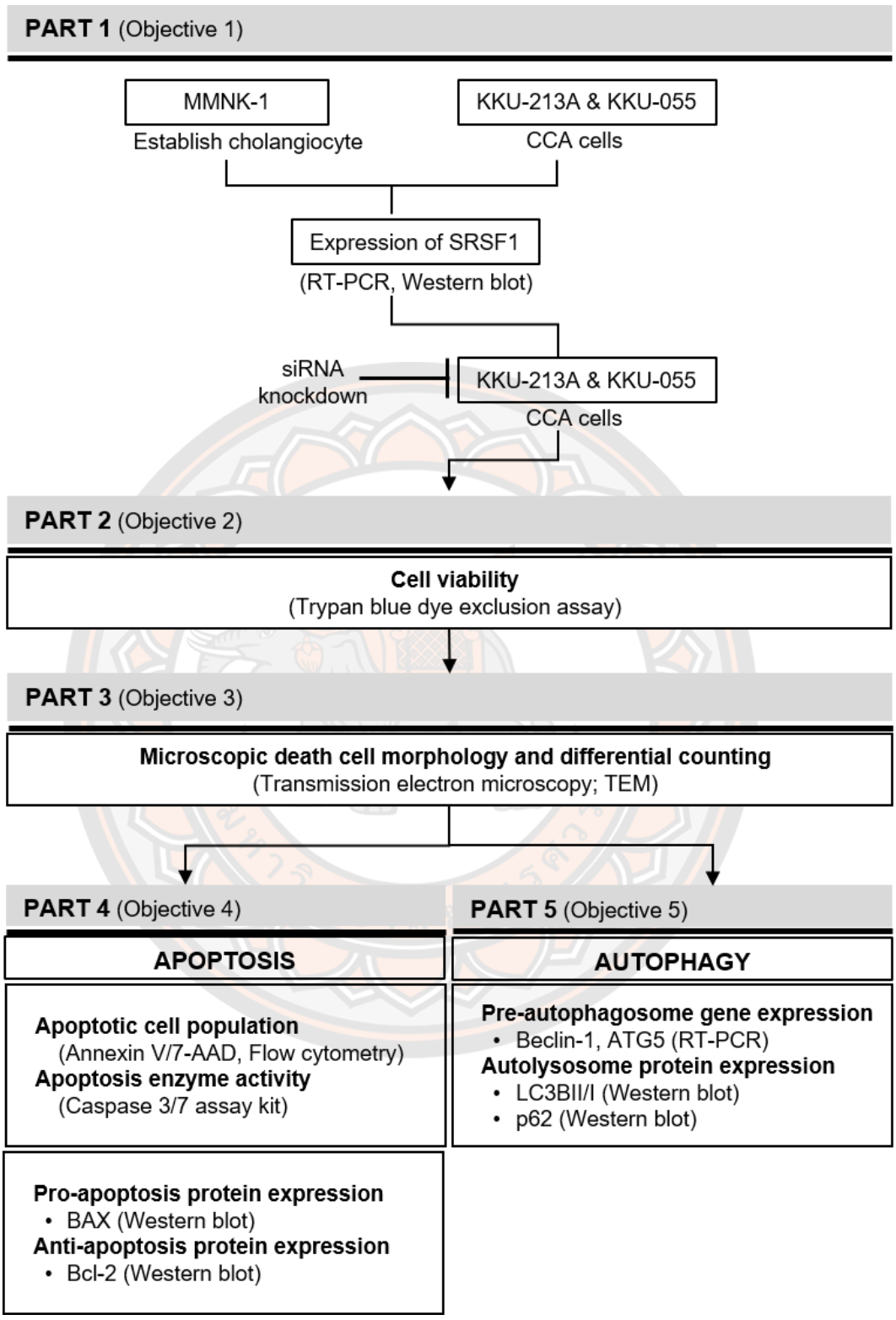


Figure 1 Schematic diagram shown scope of this study.

Hypothesis of Study

SRSF1 can induces CCA cell death through specific underlying mechanism of cell death which demonstrated by cell death morphology, specific genotypes (expression of marker genes/proteins) and specific phenotypes.

Anticipated outcome

1. SRSF1 silencing can induces cellular death of CCA cells through the specific underlying mechanism.

2. The basic information for research continuing to develop SRSF1 as the precision therapeutic target for CCA intervention.

CHAPTER II

REVIEW LITERATURE

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is epithelial cell malignancy arising from the biliary tree. There are two types including intrahepatic and extrahepatic parts (Kendall et al., 2019) as shown in Figure 2. Based on their size, interlobular and septal bile ducts are considered as small intrahepatic bile ducts while segmental and area are considered as large intrahepatic bile ducts. The rick factor of CCA is involving freshwater fish consumption with contaminating metacercaria of *Opisthorchis viverrini*. The infection by liver fluke and carcinogenic substances during food fermentation will promote CCA development. There are no specific symptoms in early stages but will present the signs in severity with metastasis into the liver, lung, lymph node or other secondary organs. Current diagnosis and treatment of CCA such as surgery, chemotherapy or radiation are not effective. Therefore, patients developed into an advance stage with metastasis and leading to death.

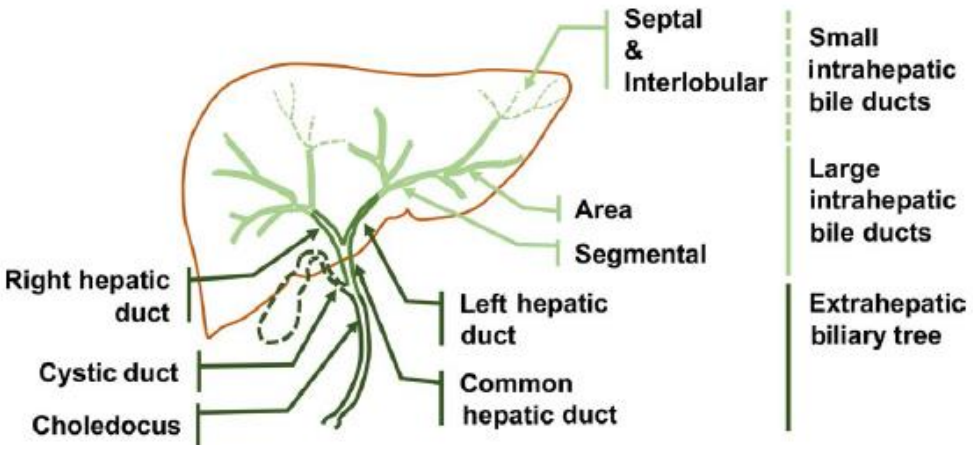


Figure 2 Extrahepatic tumors, intrahepatic tumor resulting in biliary duct dilation.

Source: Kendall et al., 2019

The incidence of CCA shows high frequency in Southeast Asia, particular in Thailand found this disease often reported in North and Northern Thailand. Data refer to the period 1971–2009, there are approximately 85 cases per 100,000 and in northern are 14.6/100,000 which associated with the cases of liver fluke (*O. viverrini*) infection (Banales et al., 2016) as shown in Figure 3.

In 2018, New cancer patient in Thailand, CCA was second and six ranks in top 10 cancer disease with high mortality in Thai population in men and woman, respectively as shown in Table 1 (National Cancer Institute Thailand, 2018).

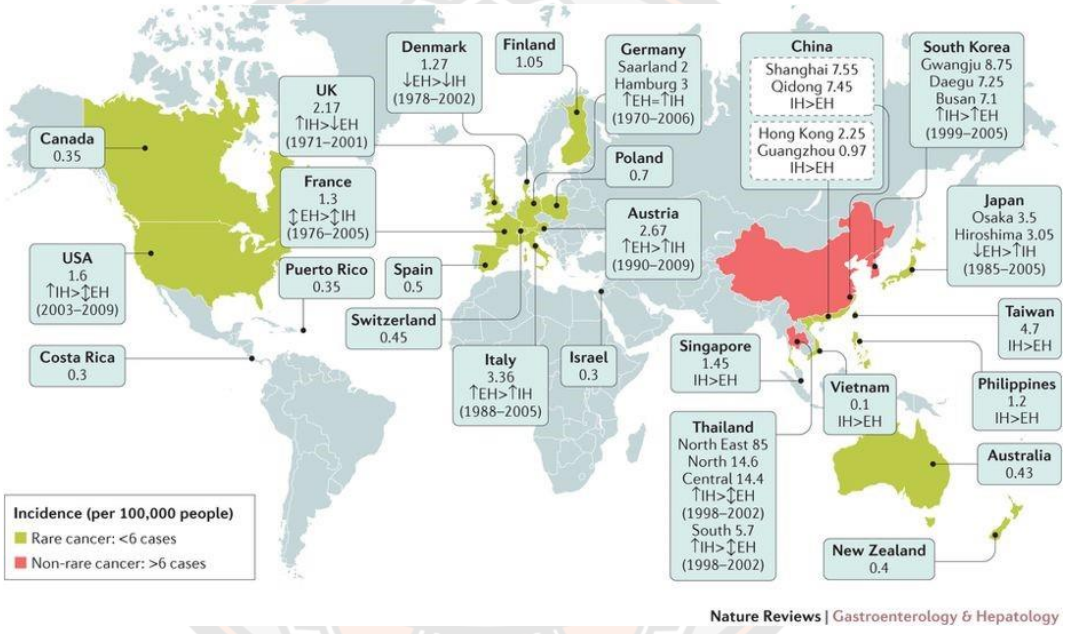


Figure 3 Incidence report of CCA worldwide.

Source: Banales et al., 2016

Table 1 Top 10 new cancer patient in Thailand: 2018.

Rank	Male		Female	
	Disease	**%**	**Disease**	**%**
1	Colon and Rectum	19.7	Breast	40.8
2	**Liver and bile duct cancer**	18.2	Cervix uteri	14.2
3	Trachea, Bronchus and Lung	13.6	Colon and Rectum	11.1
4	Prostate	7.5	Copus uteri	5.3
5	Oral cavity	7.3	Trachea, Bronchus and Lung	4.8
6	Esophagus	5.7	**Liver and bile duct cancer**	4.4
7	Non-Hodgkin lymphoma	3.1	Oral cavity	3.5
8	Nasopharynx	3.0	Ovary	3.1
9	Oropharynx	2.4	Stomach	1.9
10	Stomach	2.3	Non-Hodgkin lymphoma	1.6

Source: National Cancer Institute Thailand, 2018

Cholangiocarcinogenesis

Carcinogenesis is the differentiation or development of a cancer from normal healthy cells transform in to cancer cells. 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. The first step of carcinogenesis, initiation, the mutation of genes arising spontaneously or induced by a carcinogenic agent. Genetic alterations can result in dysregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation. Promotion is the second step that occurs on those cells already mutated by an initiator. The expression of the genome mediated through promoter-receptor interactions. The final stage of progression, where genetic and

phenotypic changes and cell proliferation occur. This involves a fast increase in the tumor size, invasive and metastatic potential as shown in Figure 4 (Liu et al., 2015).

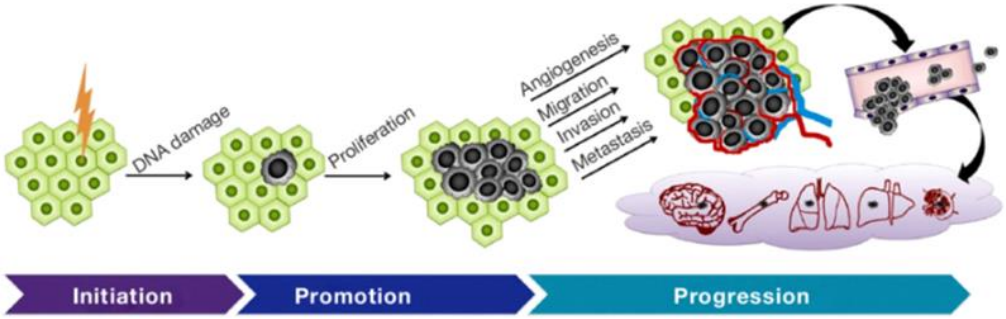


Figure 4 Three-phase process of carcinogenesis.

Source: Liu et al., 2015

For cholangiocarcinogenesis, the infection by the carcinogenic liver fluke attached the host cell epithelium in the bile duct. Then, this chronic inflammatory process effects epithelial cell by stimulating fibrosis around the bile duct. 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 unlimitless of epithelial cell proliferation as show in Figure 5 (Prueksapanich et al., 2018).

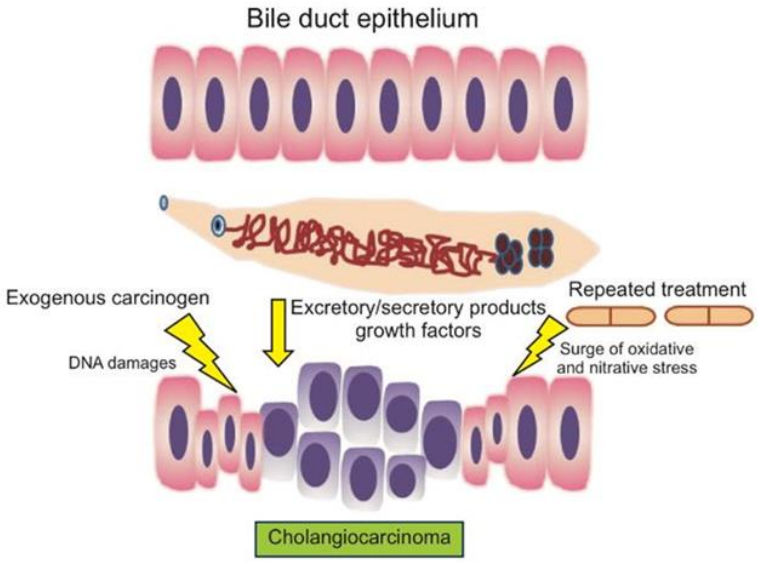


Figure 5 The mechanism of process cholangiocarcinoma.

Source: Prueksapanich et al., 2017

The hallmarks of cancer

The hallmarks of cancer comprise the biological capabilities acquired during the multistep development of human tumors. In 2000, there are original six hallmarks including self-sufficiency to growth signals, resistance to antigrowth signal, unlimited cell division, sustained angiogenesis, invasion, metastasis, and evasion of apoptosis. In 2011, there are four additional hallmarks such as inflammation, genomic instability, evasion of the immune system and abnormal metabolism and last alternative splicing is one of the hallmarks in cancer. Recently, re-visiting of the cancer hallmark regarding to a number of studies presented the contribution of mRNA splicing alteration to cancer development. Therefore, in 2013, the researchers proposed that aberrant alternative splicing may be included as a novel hallmark of cancer (Ladomery, 2013) as shown in Figure 6.

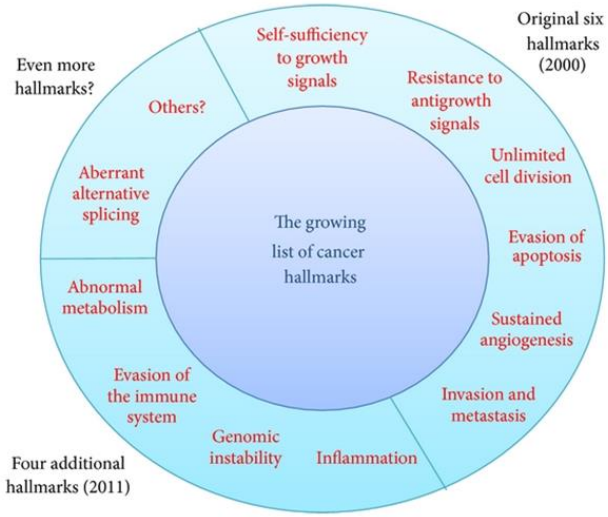


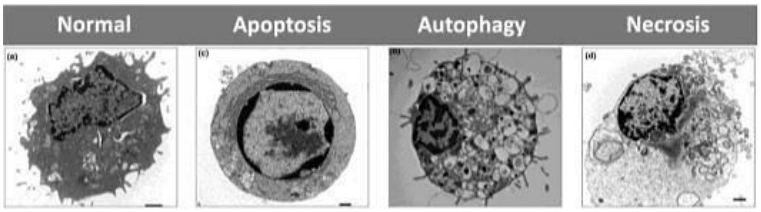
Figure 6 The hallmarks of cancer.

Source: Ladomery., 2013

Cell death characteristics, mechanisms, and specific markers

Cell death is a normal process that is essential to development and homeostasis of multicellular organisms. It is tightly regulated since either too much or too little cell death can lead to pathological defects, such as neurodegeneration or cancer. There are three major pathways of cell death including apoptosis autophagy and necrosis, which are distinguished, based on morphological appearances and molecular features (Azad & Gibson, 2010; Thornton et al., 2017) as shown in Figure 7 and Table 2.

In cancer, two main death phenotypes that predominantly occur in cancer cell death are apoptosis and autophagy (Letai, 2015). The majority of recent therapeutic regimens aim to directly kill tumor cells and often successfully reduces tumor mass. Depending on the treatment scheme. For example, Bcl-2 Homology region 3 (BH3)-mimetics (Particularly, ABT-199 and Obatoclax) mimic BH3-activity by binding and inhibiting pro-survival Bcl-2 proteins, specific compounds that induce apoptosis directly at the Bcl-2 level (Labi & Erlacher, 2015). In addition, the induction or inhibition of autophagy can provide therapeutic benefits to patients by synthesize of autophagy modulators as the new therapeutic strategies in cancer (Dalby, Tekedereli, Lopez-Berestein, & Ozpolat, 2010).



Cell Death Features	Apoptosis (PCD I)	Autophagy (PCD II)	Necrosis
General morphology	Shrink	–	Swell
Chromatin condensation	++	+	–
Vacuolization (Autophagosomes)	–	++	+
Exteriorization of phosphatidylserine	++	+	–
Caspase activation	++	–	–
Processing of LC3	–	++	–

Figure 7 Features of apoptosis, autophagy and necrosis cell death.

Source: Azad & Gibson, 2010

Table 2 Summary of death morphological features.

Feature	**Apoptosis**	**Autophagy**	**Necrosis**
Membrane	Blebbing	Intact	Lysed
Nucleus	Shrinkage Chromatin condensation DNA/Nuclear fragmentation	Nucleus and chromatin minor changes	Swollen and late fragmentation of nucleus
Organelles	Apoptotic bodies	Autophagosomes and autolysosomes	Swelling and released

Source: Thornton et al. 2017

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 & Mak, 2009) as shown in Figure 8.

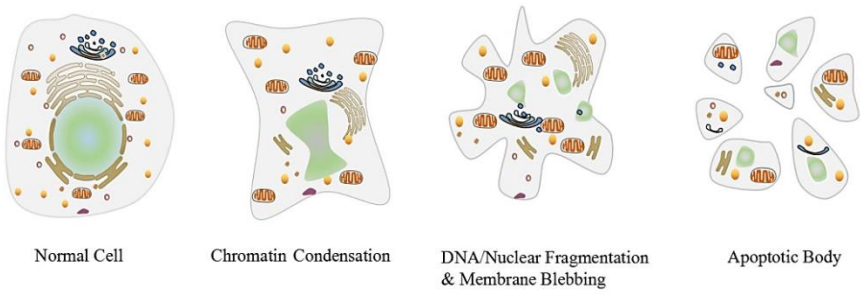


Figure 8 The process of apoptosis and characteristic cell changes.

Source: Brenner & Mak, 2009

Apoptosis pathway is controlled by many genes, such as tumor suppressor genes (p53) and oncogenes (AKT and Bcl-2). In addition, there are many molecules associated in the process which act as anti-apoptotic and pro-apoptotic functions. For apoptosis, mitochondria play an important role in the secretion of apoptotic agents such as cytochrome C and apoptosis-inducing factor (AIF). Apoptosis occurs when linked Cascade of cell signaling using caspase enzymes (caspase-mediated events) and pro-apoptotic proteins induced death but also co-modulated with anti-apoptotic proteins as inhibitors of death. Induction of apoptosis occurs in 2 pathways including extrinsic pathway and intrinsic pathway (Brenner & Mak, 2009) as shown in Figure 9.

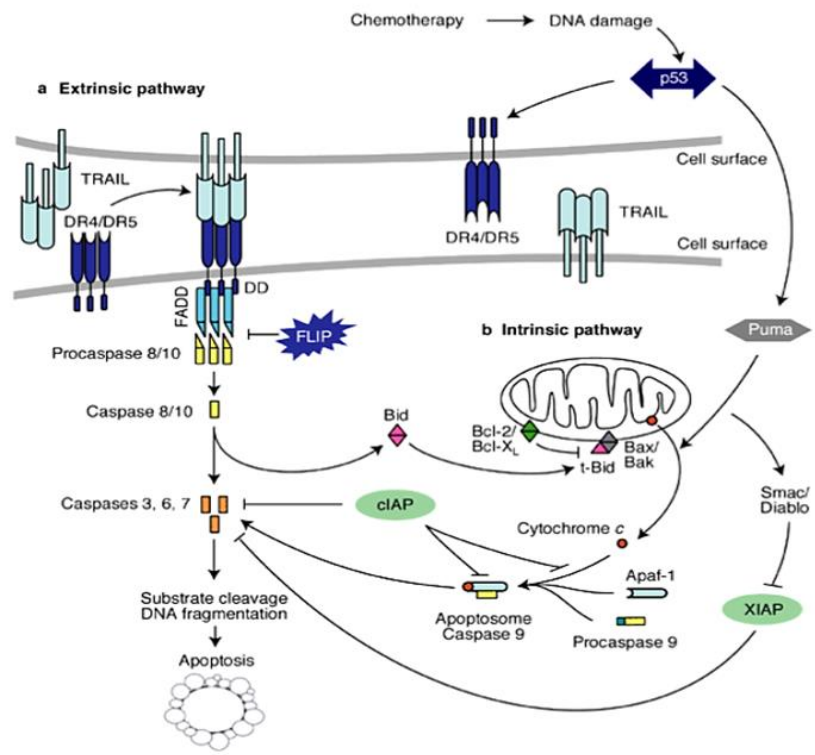


Figure 9 Apoptosis pathway and mechanism.

Source: Brenner & Mak, 2009

First, Death-receptor-induced extrinsic pathway: External pathways occurs when death receptors (DRs), such as tumor necrosis factor receptor (TNFR), Fas receptor (FasR), or TNF-related apoptosis-inducing ligand (TRAILR1 or DR4 and TRAILR2 or DR5). recognized by their specific ligands. Then, formed a structure that consists of the protein adapter and initiator caspase (procaspase 8 or 10), called "death-inducing signaling complex" (DISC), encouraging pro-caspase to be initiator caspase, which will induce the effector caspases (caspase 3 and 7) to work and lead to apoptosis.

Second, Mitochondrial-apoptosome-mediated apoptotic intrinsic pathway: The path from the interior with Mitochondria. The apoptosis is mediated that occurs when stimulated by various stimuli such as ultraviolet light (UV), gamma radiation, oxidative stress or oxidative stress that causes DNA damage. Apoptosis has many other regulators which can be divided into 2 groups of control including pro-apoptosis members (e.g.

PUMA, BAX, BAK) and anti-apoptotic members (e.g. Bcl-2 and Bcl-xL). When pro-apoptosis BAX and BAK promote MOMP and the release of cytochrome c from the mitochondria. Cytochrome c associates with APAF1 and caspase-9 to form the apoptosome, which activates caspase-3 lead to apoptosis.

Therefore, if the activation of anti-apoptosis is high up-regulated will be one of the causes of the cancer process. Consequently, the study of apoptosis can use various controls as a marker or as an active target of drugs on the way of death as shown in Table 3.

Table 3 Pro-apoptosis and anti-apoptotic members.

Proapoptotic regulators	**Anti-apoptotic regulators**
Bid	Bcl-xL
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)

Table 3 (Cont.)

Proapoptotic regulators	Anti-apoptotic regulators
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)	

Source: Brenner & Mak, 2009

Autophagy-associated cell death

Autophagy or self-eating is an important process of cells to eliminate or destroy the components of damaged cells or proteins dysfunction for working as a source of energy for cells either to reuse degraded cellular components. The process of autophagy can activate in many conditions, for example, starvation, hypoxia, and oxidative stress. If autophagy dysregulation, it may lead to various diseases such as degeneration of neurons or the emergence of various types of cancers. Importantly, the autophagy process can be both acted as a form of tumor-promoting and tumor-suppressive (Kimmelman & White, 2017). Autophagy process in cancer starts from various conditions such as hypoxia, starvation and oxidative stress.

These conditions will stimulate the initiation of autophagosome that consists of Autophagy related proteins (ATG) and Microtubule-associated protein 1A/1B-light chain 3 (LC3), which are Ubiquitin-like proteins in binding to the 3 destructive proteins, including LC3A (found in peri-nuclear), LC3B (found in cytoplasm and nuclear) and LC3C (found in nucleuses). The formation of autophagy is most often found in LC3B by cytosolic LC3B-I. It is stimulated to be combined with phosphatidylethanolamine (PE) as LC3B-II which is reacted with ATG7, ATG3 and ATG5-ATG12, respectively.

Subsequently, the protein or organelle that needs to be destroyed caught with p62/sequestosome-1, p62 is captured with LC3B-II in Autophagosome, Autophagosome-lysosome fusion, resulting in autolysosome formation, is essential for autophagy flux. The molecular players facilitating this step are largely unknown. Degradation of the autophagosome content is catalyzed by lysosomal proteases resulting in the recycling of nutrients and energy back to the cytoplasm as shown in Figure 10. Therefore, dysfunction in the autophagy process can encourage cancer to continue to survive and develop into aggressive (Fulda, 2017; Maycotte & Thorburn, 2011).

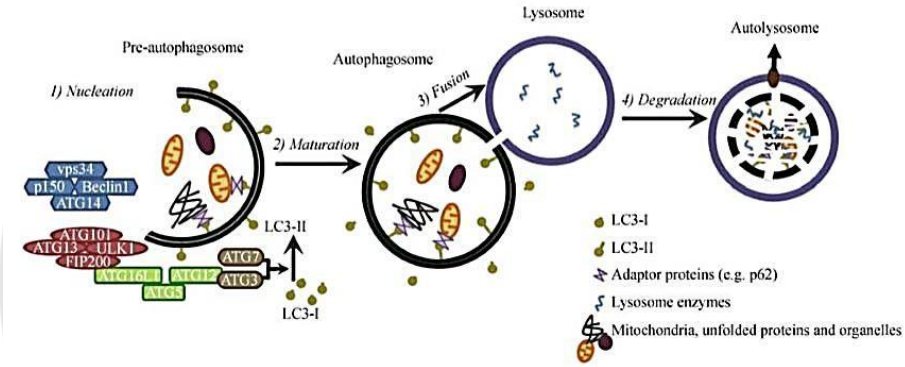


Figure 10 Molecular mechanism of autophagy.

Source: Maycotte & Thorburn, 2011

The role of autophagy in cell death, autophagy-dependent cell death was defined by Nomenclature Committee on Cell Death (Galluzzi et al., 2018), is morphologically defined (especially by transmission electron microscopy (TEM)) as a type of cell death that accumulate of autophagosomes and autolysosomes in the cytoplasm.

Autophagy-dependent cell death has three types, their distinguishing characteristics and modes of lethality. (1) Excessive bulk autophagy, resulting from a massive induction of autophagosomes and autolysosomes. Although membrane integrity is maintained, there is ballooning of the perinuclear space (PNS). (2) Excessive mitophagy, autophagy-dependent selective elimination of mitochondria. (3) Autosis, involving changes in cell morphology, including PNS swelling and focal

rupture of the plasma membrane (Bialik, Dasari, & Kimchi, 2018) as shown in Figure 11.

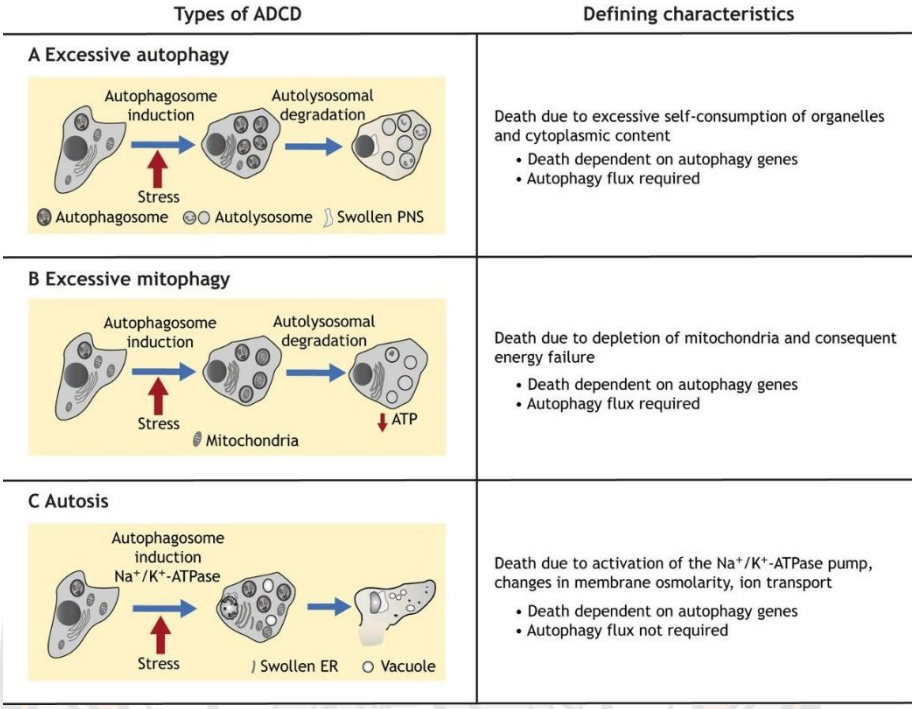


Figure 11 Summary of the three types of autophagy-dependent cell death (ADCD), their distinguishing characteristics and modes of lethality.

Source: Bialik, Dasari, & Kimchi, 2018

For example, autophagy can death from over-eating elimination of intracellular organelles and cytosol through excessive bulk autophagy. A recent work characterized the morphology of A549 cells in which autophagy-dependent cell death was induced by Resveratrol. At long-time treatment, as autophagy flux continued, the researchers observed the cytoplasm is overwhelmed by the presence of autophagic and empty vacuoles. Notably, these cells are almost devoid of any intracellular organelles (Dasari et al., 2017).

These observations, conducted in a systematic manner in a classic autophagy-dependent cell death, lethality may result from extreme levels of autophagy flux that

lead to overconsumption of cellular organelles and rerouting of cellular membrane sources to support autophagosome generation.

In general, the studying of autophagy process commonly used autophagic markers includes Beclin1, ULK1, WIPI1 (which is not necessary for autophagy but can be used as a marker), WIPI2, ATG5, LC3, and p62/SQSTM1 (Yoshii & Mizushima, 2017).

Alternately, monitoring autophagy by electron microscopy in mammalian cells. Transmission electron microscopy (TEM) was used to monitor the ultrastructure of autophagic structures and identify the cargo during this process due to its high resolution (Kim, Sohn, Kim, & Kim, 2018) as shown in Figure 12.

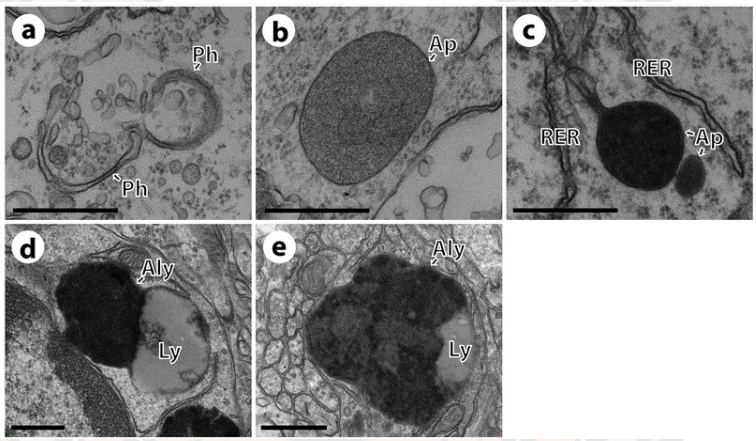


Figure 12 The cell morphology of autophagy observed under Transmission electron microscopy (TEM), (a) Phagophore containing fragments of cytoplasmic organelles; (b) Early autophagosome; (c) Late autophagosome; (d) Early autolysosome; and (e) Late autolysosome. Abbreviations are: Ap, autophagosome; Aly, autolysosome; G, Golgi apparatus; M, mitochondria; N, nucleus; Ph, phagophore; RER, rough endoplasmic reticulum. Size bars: 500 nm.

Source: Kim, Sohn, Kim, & Kim, 2018

Necrosis

Necrosis has been characterized as accidental, uncontrolled, passive and energy-independent cell death. This necrotic process has long been described because of extreme physicochemical stress (for example, osmotic shock, heat and high concentration of hydrogen peroxide). The observed morphological changes comprise rapid cytoplasmic swelling with the organelle breakdown and the rupture of the plasma membrane releasing damage-associated molecular pattern molecules (DAMPs) that alert the innate immune system within the stroma (Proskuryakov, Konoplyannikov, & Gabai, 2003) as shown Figure 13.

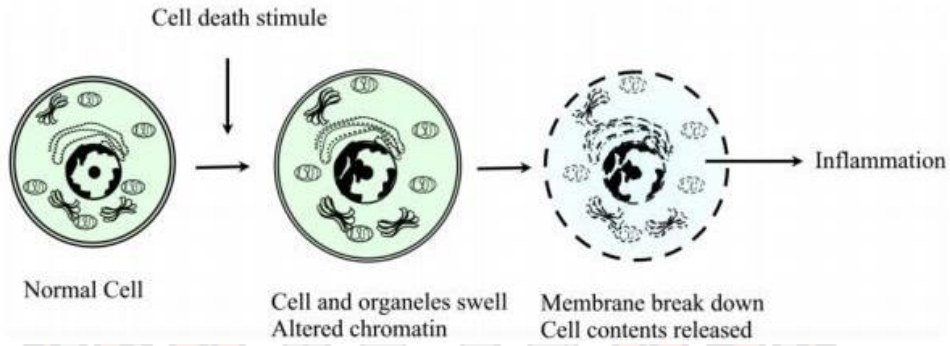


Figure 13 Morphological characteristics of necrosis involve the membranous swelling of the organelles, DNA degradation, and finally the release of the cytoplasmic content that affects the neighbor cells, provoking an inflammatory response.

Source: Proskuryakov, Konoplyannikov, & Gabai, 2003

The molecular hallmark of necrosis is extremely depletion of ATP level, which is believed to be the underlying cause of cell death. There is a metabolic disruption accompanied by energy depletion and loss of ATP that leads to cellular edema. Therefore, the mitochondria become round and swollen, the endoplasmic reticulum dilates, the lysosomes are disrupted, and the formation of plasma membrane protrusions.

Alternative splicing

Alternative splicing (AS) is one of post-transcriptional modification processes which 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, 2003). In this process, 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 & Hausman, 2006) as shown in Figure 14. In human, more than 95% of roughly 30,000 human genes undergo alternative splicing to encode two or more splice isoforms (Pan, Shai, Lee, Frey, & Blencowe, 2008). 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).

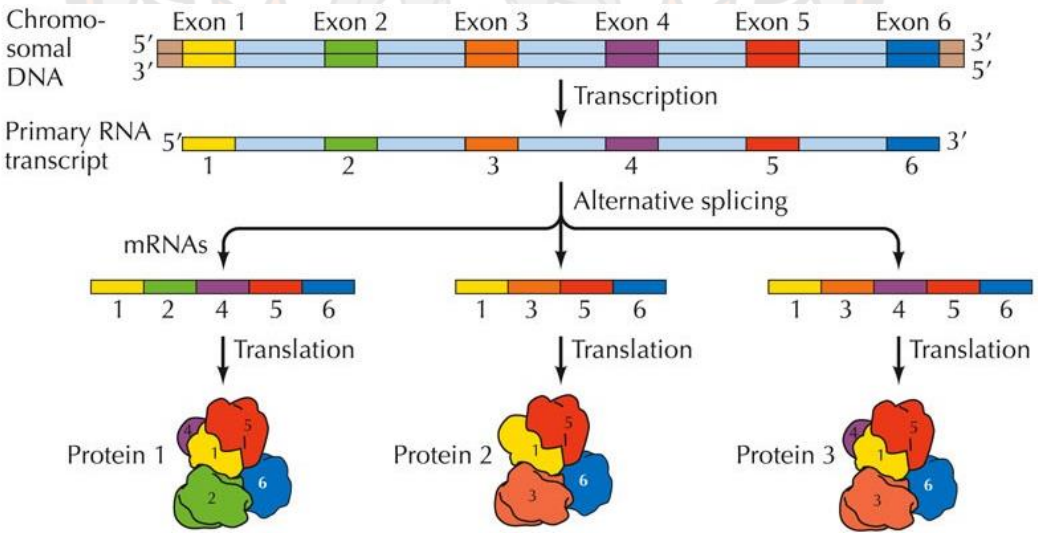


Figure 14 Alternative mRNA splicing generates various protein isoforms.

Source: Cooper & Hausman, 2006

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 (Sanford et al., 2009; Sapra et al., 2009).

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 (Anko et al., 2012) as shown in Figure 15.

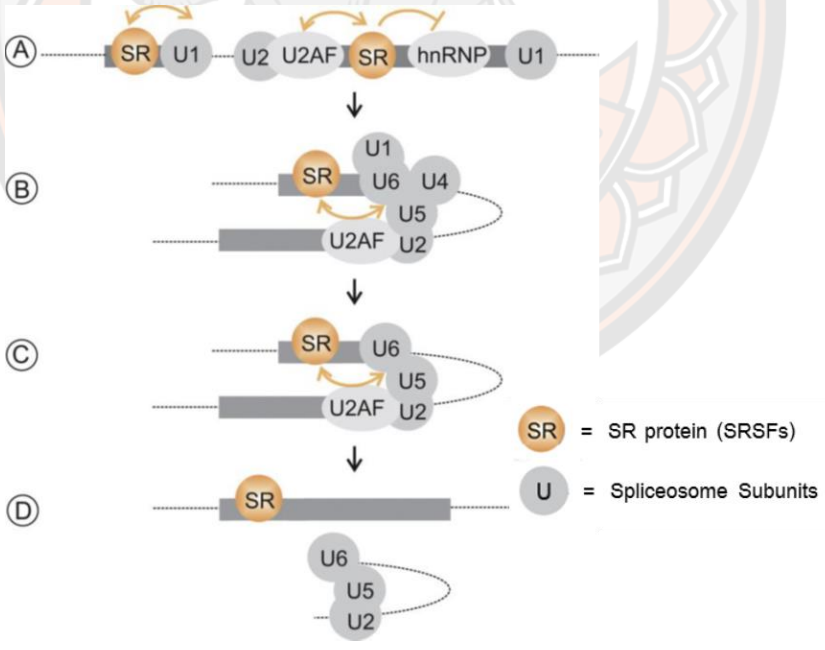


Figure 15 Integration of SRSFs with spliceosome units.

Source: Anko et al., 2012

There are consists of 12 members of SRSFs including SRSF1 to SRSF12, actually, each SRSF presented their previous nomenclature as listed in Table 4, such as AS2/AFS is SRSF1. Each member containing 2 important domains (Anko, 2014). First, different number of RNA recognition motif (RRM) at N-terminal which are the mRNA binding site by specific consensus binding sequences of their targeted mRNA. Second, 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 as show in Figure 16.

Table 4 Nomenclature of SRSFs and GenBank accession numbers.

SR protein family	Alternative names	mRNA (GenBank Accession No.)	Nt (bases)
SRSF1	SF2/ASF, SRp30a	NM_006924.5	5,343
SRSF2	PR264, SC35, SRp30b	NM_003016.4	3,003
SRSF3	SRp20	NM_003017.5	4,228
SRSF4	SRP75	NM_005626.5	2,300
SRSF5	HRS, SRP40	NM_001039465.2	1,621
SRSF6	B52, HEL-S91, SRP55	NM_006275.6	4,353
SRSF7	9G8, AAG3	NM_001031684.3	2,356
SRSF8	DSM-1, SRP46	NM_032102.3	4,349
SRSF9	SRp30c	NM_003769.3	1,152
SRSF10	SRp38, SRrp40, TASR	NM_006625.6	3,940
SRSF11	NET2, p54	NM_004768.5	3,812
SRSF12	SRrp35	NM_080743.5	3,589

Source: Prakasit Khamsuwan, Unpublished data

Figure 16 The protein structures of 12 SRSFs.

Source: Anko, 2014

SRSFs in cancer

The expression of SRSFs may leads to aberrant alternative splicing and potentially contributes to various diseases and in particular to the development of cancer. The important alterations occur in the mRNA splicing are including the overexpression or dysregulation of regulatory splicing factors that mentioned as SRSFs and also the abnormality of their specific kinase enzyme such as Serine-Arginine protein kinases (SRPKs), CDC2-like kinases (CLKs). There are a number of research articles reported that SRSFs can promote the tumorigenesis, angiogenesis, tissue invasion, metastasis, apoptosis evasion, or survival in cancer. These aspects of cancer biology are supported by mRNA or protein isoforms that predominate in tumor cells (da Silva et al., 2015) as shown in Figure 17. For example, SRSF1 regulated BIN splicing to BIN1+12A isoforms that play a role in the evasion of apoptosis and cancer cell survival (Anczuków et al., 2012).

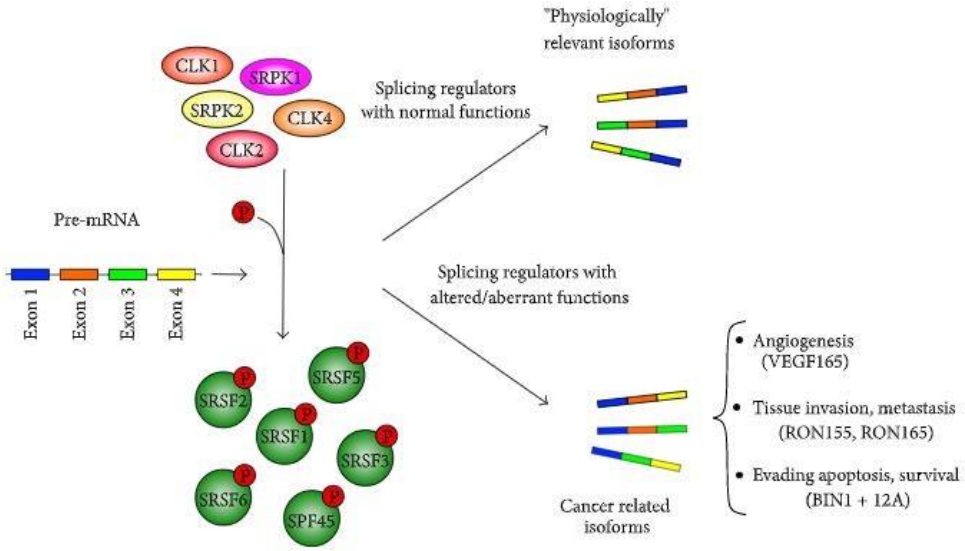


Figure 17 Dysregulation of splicing factors activities and the outcomes in cancer cells

Source: da Silva et al., 2015

SRSF1 structure and function

In various types of cancer, SRSF1 is the member of SR protein family that predominantly expressed among the other members, which also demonstrated the strongly evidences of its functional involves in cancer development and progression in colorectal cancer (L. Chen et al., 2017), breast cancer (Anczukow et al., 2015) or lung cancer (Jiang et al., 2016). There is a study that summarized the upregulation of SRSFs in 9 cancer tissue by Genvestigator and presented that SRSF1 is the most upregulated as the first ranking in 8 cancer types including colorectal, lung, osteosarcoma, breast, ovarian, pancreatic, glioma cancer and in the second rank for melanoma (Zhou et al., 2019) as shown in Figure 18.

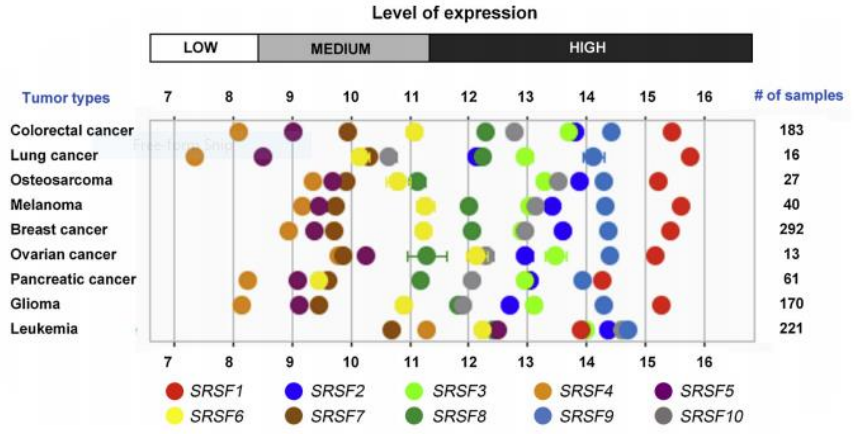


Figure 18 The expression profile of SRSFs in 9 human tumor types plotted by Genevestigator.

Source: Zhou et al., 2019

Serine/Arginine Splicing Factor 1 (SRSF1) or SF2/ASF is the archetype member of the SR protein family of splicing regulators. The SRSF1 gene located on the 17q22 chromosome and transcript in to 5,343 bp mRNA which arranged in 4 exons. The modular domains of 248 amino acids of SRSF1 consist of two RRMs: (i) a canonical RRM at the N-terminus, followed by a pseudo-RRM and (ii) and a C-terminal RS domain that is shorter than that of most other SR proteins (Das & Krainer, 2014) as show in Figure 19.

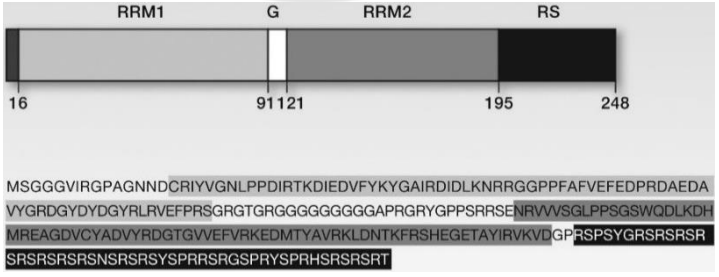


Figure 19 Modular domain structure of SRSF1.

Source: Das & Krainer, 2014

SRSF1 and cell death in cancer

There is one important previous report studied the role of SRSF1 overexpression affects alternative splicing in MCF-10A (breast cancer cell lines). Them investigated AS of BIM (gene name: BCL2L11), a pro-apoptotic Bcl-2 family member with a critical role in luminal apoptosis during acinar morphogenesis. The results show that SRSF1 overexpression promotes the inclusion of a novel alternative 3' exon, generating two new BIM isoforms including BIMγ1 and BIMγ2. Resulting in cannot binding to Bcl-2 (anti-apoptosis) protein then decreased the activated/cleaved caspase 3 and allowed cancer cell evasion of apoptosis (Anczuków et al., 2012) as show in Figure 20.

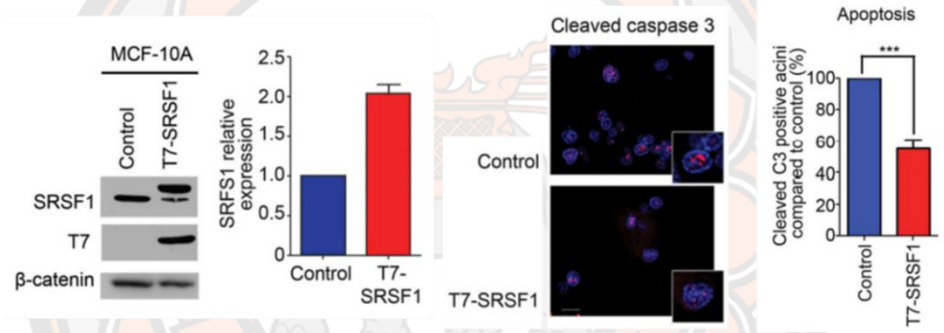


Figure 20 Overexpression of SRSF1 decreased breast cancer apoptosis.

Source: Anczuków et al., 2012

Remarkably the alternative splicing in CCA.

Summary of oncogenic spliced genes in CCA was reported by Yosudjai, J. 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), Anterior gradient2 (AGR2vH) (Yosudjai, Wongkham, Jirawatnotai, & Kaewkong, 2019) as shown in Figure 21. Of note, some of them related to anti-apoptosis and chemo-resistant including CD44v8-10 and

Δ133p53. Therefore, abnormal genes were associated to promote CCA to evading cell death.

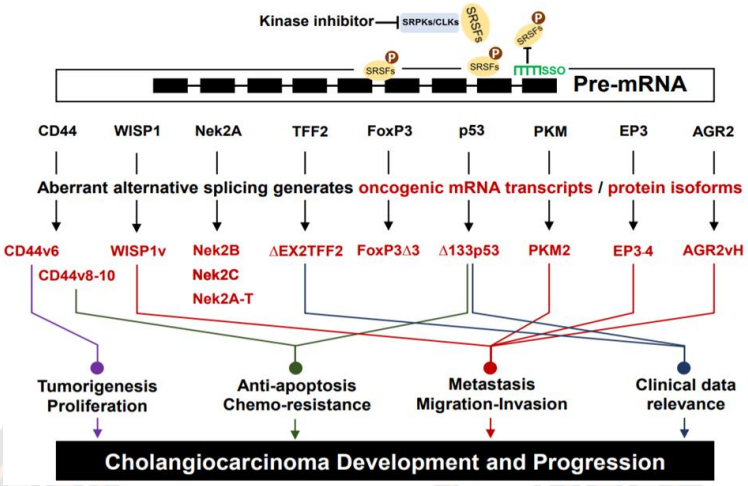


Figure 21 The spliced mRNA transcript and their functional involves in CCA.

Source: Yosudjai, Wongkham, Jirawatnotai, & Kaewkong, 2019

SRSF1 in CCA: preliminary information

Previous results from Pawee Tangwiriyarotkul studied the expression profile of SR protein family members (SRSF1-SRSF12) and found that SRSF1 was predominantly upregulated in CCA cells (KKU-055, KKU-100, KKU-213, and KKU-214) when compared with established cholangiocyte (MMNK-1) as show Figure 22.

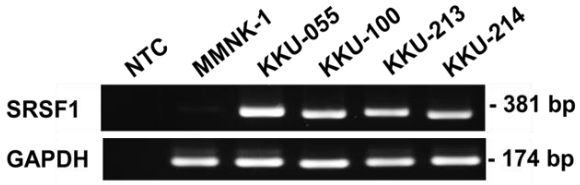


Figure 22 The gene expression of SRSF1 in CCA cell lines compared with established cholangiocyte.

Source: Pawee Tangwiriyarotkul, Unpublished data.

The expression of SRSF1 mRNA resulted from Pawee Tangwiriyarotkul correlated with the study from Sirintip Srisuksai that determined the expression of SRSF1 protein in CCA tissues derived from operative CCA patients. The result demonstrated that SRSF1 was up-regulated significantly in 9 CCA cases from the tumor tissues (T) compare with adjacent non-tumor (N) tissues as show Figure 23.

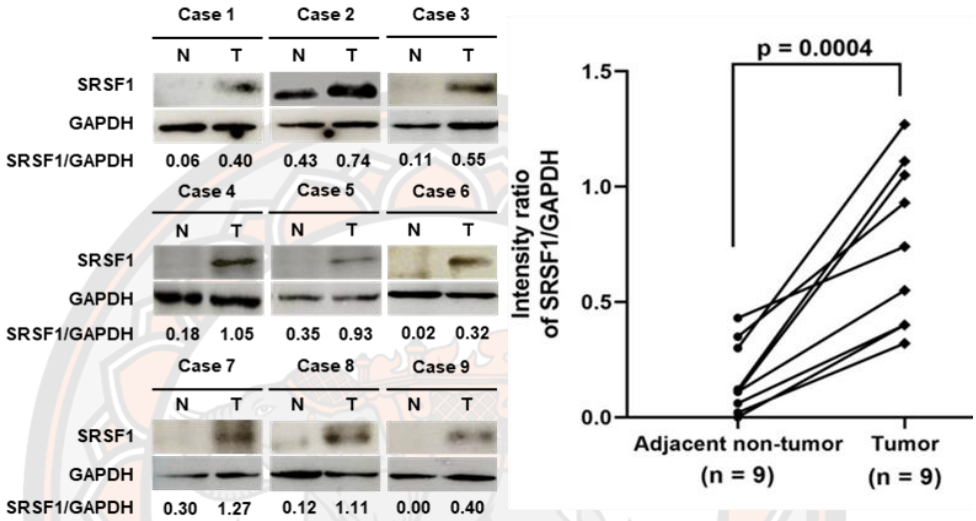


Figure 23 The expression of SRSF1 in 9 matched-pair tissues from CCA patients and statistically analysis.

Source: Sirinthip Srisuksai, Unpublished data.

Role of SRSF1 on CCA survivability

From studies in our group, to investigate the role of SRSF1 on CCA cell proliferation and colony formation. Pawee Tangwiriyarotkul demonstrated the successful silencing of SRSF1 expression using specific siRNA in CCA cells and presented the depletion of SRSF1 expression correlated with significantly reduction of cell proliferation in CCA (KKU-055 and KKU-213) as show Figure 24.

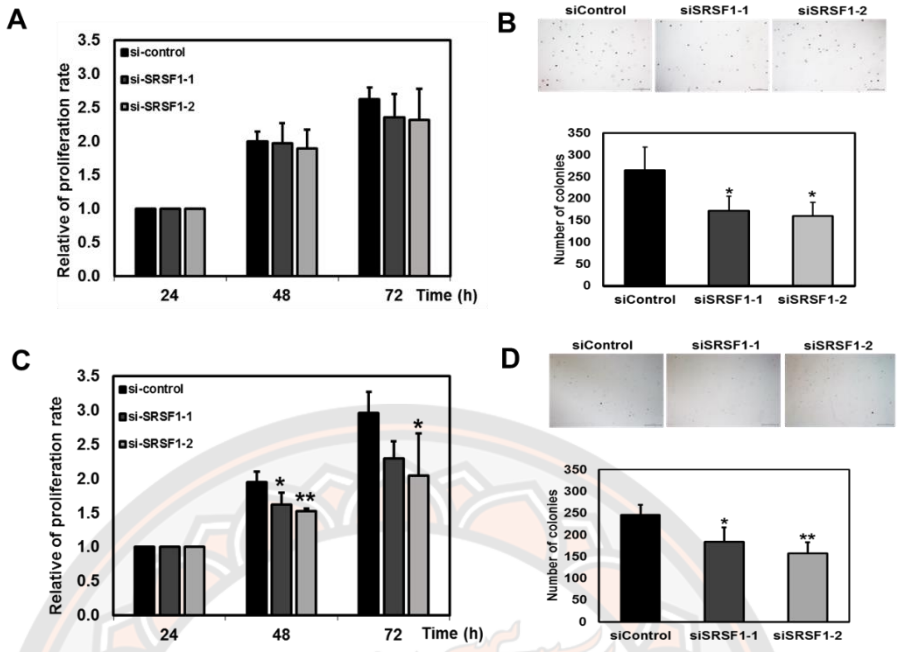


Figure 24 The SRSF1-silencing decrease cell proliferation and colony formation in CCA cell lines. (A) and (C) SRSF1 knockdown on cell proliferation at 24, 48 and 72 h in KKU-055 and KKU-213. (B) and (D) SRSF1 knockdown on colony formation in KKU-055 and KKU-213.

Source: Pawee Tangwiriyarotkul, Unpublished data

Therefore, this study aimed to investigate the silencing of SRSF1 to induce CCA cell death. First, the mRNA and protein expression of SRSF1 in CCA cell lines were confirmed, and silencing efficiency of SRSF1 both in mRNA and protein levels were validated. Next, the effect of SRSF1 silencing on cell death phenotypes of CCA cells was observed. Particularly to clarify the specific type of cancer cell death by monitor death morphological change of cell death phenotypes under transmission electron microscopy (TEM). Subsequently, to investigate Apoptosis- and autophagy-specific marker genes expression. The expectation of this study is to investigate that can SRSF1 be able to develop as the specific therapeutic target for CCA by targeting SRSF1 leading to CCA cell death.

CHAPTER III

RESEARCH METHODOLOGY

Laboratory materials

Chemicals and instruments used in this study are listed below:

1. Instruments

1.1 0.5 ml PCR tube (LC premium, Thailand)

1.2 1.5 ml microcentrifuge tube (LC premium, Thailand)

1.3 5% CO2 Incubator (Shel lab, USA)

1.4 6-well plate (SPL life science, Korea)

1.5 Agarose gel electrophoresis apparatus (ATTO, Japan)

1.6 Glass pipette (Precicolor HBG, Germany)

1.7 Glove-powder free (Sri Trang, Thailand)

1.8 ImageQuant™ LAS 500 (GE Healthcare Life Science, UK)

1.9 Inverted microscope (Olympus, USA)

1.10 Laminar Flow hood (NUAIRE, USA)

1.11 Micropipette (Proline ®Plus, USA)

1.12 Microplate reader (Biochrom Ltd, UK)

1.13 Mini Trans-Blot® Cell (Bio-Rad laboratories, USA)

1.14 Neubauer hemocytometer (Fisher Sciencitific, Germany)

1.15 Pipette aid (TOPSCIEN ®, China)

1.16 Polyvinylidene fluoride (PVDF) membrane (Bio-Rad laboratories, USA)

1.17 Refrigerated centrifuge (Hettich, Germany)

1.18 T100™ Thermal Cycler (Bio-Rad laboratories, USA)

1.19 Vortex (SPSLAB, USA)

1.20 Water bath (Memmert, USA)

1.21 Muse® Cell Analyzer (Millipore sigma, USA)

1.22 JEM-1010 Transmission Electron Microscope (JEOL, Japan)

1.23 Cubee™ Mini-Centrifuge (BIOGENOMED, Taiwan)

2. Chemicals

2.1 100 bp DNA Ladder (Smobio, Hsinchu City, Taiwan)

2.2 1×MyTagTM HS Red Mix (Bioline, Taunton, Massachusetts)

2.3 30% Acrylamide: Bis (19:1) (National diagnostics, USA)

2.4 Agarose (Invitrogen, NY)

2.5 Albumin from bovine serum (Fluka, USA)

2.6 Apo-ONE® Homogeneous caspase 3/7 kit (Promega, USA)

2.7 Beta-mercaptoethanol (Gibco, Grand Island, NY)

2.8 Clarity™ Western ECL Substrate (Bio-Rad laboratories, USA)

2.9 Coomassie Brilliant Blue G-250 (Bio-Rad laboratories, USA)

2.10 Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltman, MA)

2.11 Dimethyl sulfoxide (DMSO) (Sigma-aldrich.St. Louis, NY)

2.12 Fetal bovine serum (FBS) (Gibco, Waltman, MA)

2.13 Glutaraldehyde, 25% Solution (Bio basic, USA)

2.14 HisenScriptTM RH [-] RT-PreMix Kit (Intron Biotech, Seoul, South Korea)

2.15 Lipofectamine 2000 (Thermo fisher, USA)

2.16 jetOPTIMUS® DNA Transfection Reagent (Polyplus, USA)

2.17 jetOPTIMUS® Buffer (Polyplus, USA)

2.18 Methanol (RCI labscan, Thailand)

2.19 Muse™ AnnexinV & Death Cell Reagent (Merck Millipore, USA)

2.20 Novel Juice (Gene DireX, Hsinchu City, Taiwan)

2.21 Opti-MEM I reduced serum medium (Gibco, Waltman, MA)

2.22 Sequencing grade trypsin (Promega, Germany)

2.23 Penicillin 100 U/mL (Gibco, Waltman, MA)

2.24 Antibiotic-Antimycotic,100X (Fisher Scienctific, Germany)

2.25 Phosphate buffered saline (PBS) Tablets (Amresco, E.U.)

2.26 Ribozol® reagent (Life science, USA)

2.27 Streptomycin 100 µg/mL (Gibco, Waltman, MA)

2.28 TAE buffer (Bio basic, CA)

2.29 TEMED (Bio basic, CA)

2.30 Trypsin/EDTA (Gibco, Waltman, MA)

2.31 Tween 20 (Life science, TH)

2.32 Primary Antibody

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

2) Anti-LC3B: antibody produced in rabbit (Cell signaling, USA)

3) Anti-P62/QSTM1: antibody produced in rabbit (Elabscience, USA)

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

5) Anti-CASP3: antibody produced in rabbit (Elabscience, USA)

6) Anti-CASP8: antibody produced in rabbit (Elabscience, USA)

7) Anti-CASP9: antibody produced in rabbit (Elabscience, USA)

8) Anti-BAX: antibody produced in rabbit (Elabscience, USA)

9) Anti-Bcl-2: antibody produced in rabbit (Elabscience, USA)

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

2.33 Secondary Antibody

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

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

Biological materials

1. Cell lines

MMNK-1 (immortalized cholangiocyte), KKU-055 and KKU-213A (CCA cells) were provided by Cholangiocarcinoma Research Institute, Khon Kaen University. MMNK-1 is a highly differentiated immortalized cholangiocyte linage by SV40T and hTERT transfections (Maruyama et al., 2004). Particularly, KKU-055 was established from a poorly differentiated CCA patient whereas KKU-213A was a high-invasive cell line originated from adenosquamous CCA tumor with well differentiation (Sripa et al., 2020) as show in Figure 25.

Cells were cultured in DMEM, supplemented with 10% v/v FBS with of 100 Unit/ml of penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, MA) and maintained at 37°C and 5% CO2. Cells were sub-cultured twice a week. At 70-80% confluence, cells were detached form culture flask using 0.025% w/v trypsin/EDTA.

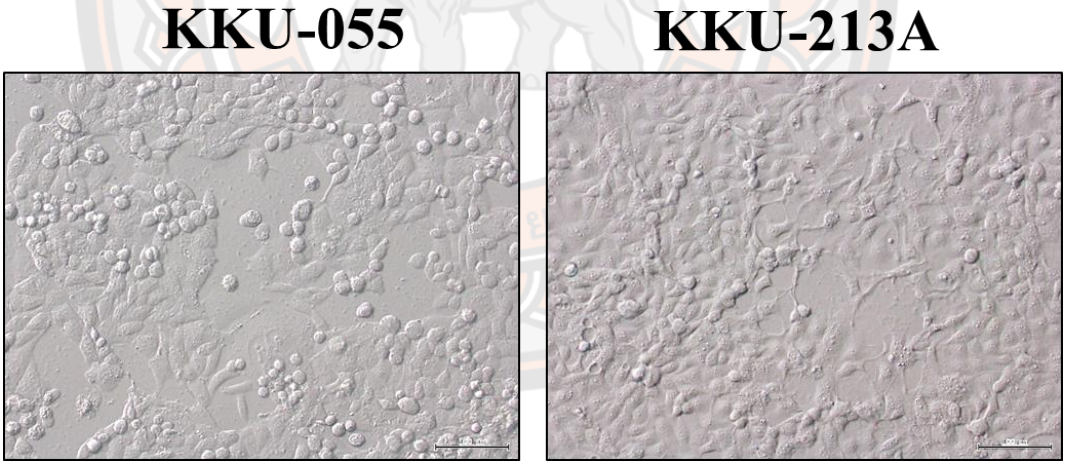


Figure 25 The morphology of cultured KKU-055 and KKU-213A.

2. Silencing of SRSF1

Two siRNAs targeting SRSF1 were followed Jiang et al., 2016 (5'-CCAACAAGATAGAGTATAA-3') and Fu et al., 2013 (5'-GGAAAGAAGATATGACCTA-3') (Fu et al., 2013) as show in Figure 26. Remarkably, siRNA targeting nucleotide 1,778-1,796 of SRSF1 mRNA from Jiang et al., 2016 was named as siSRSF1-1 whereas siRNA targeting nucleotide 700-718 of SRSF1 mRNA from Fu et al., 2013 was named as siSRSF1-2. In addition, the negative control siRNA (Ambion, Thermo Fisher Scientific, Waltman, MA) were used as experimental control of transfection.

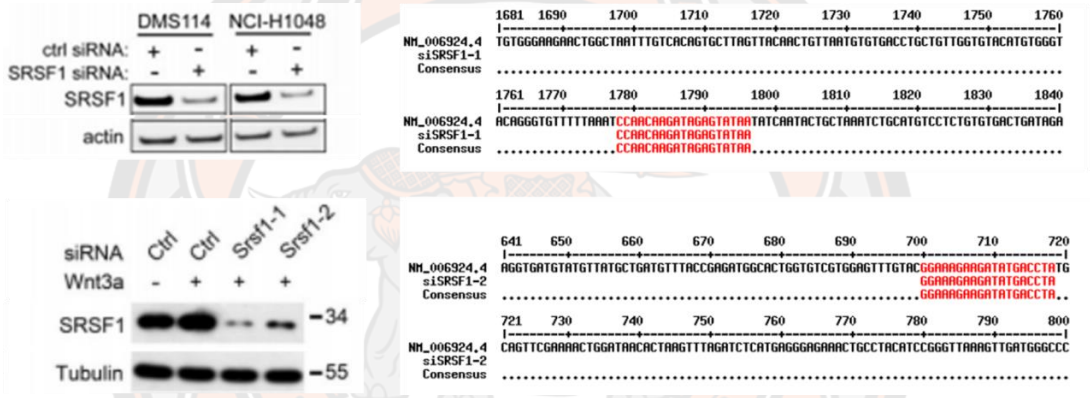


Figure 26 Efficiency and binding sequence of siSRSF1 from Jiang et al., 2016 in lung cancer cells (upper panel) and from Fu et al., 2013 in lung cancer cells (lower panel).

3. SRSF1 specific primer

SRSF1 was amplified by specific primers that previously designed by Pawee Tangwiriyarotkul for his M.Sc. Thesis (2019) for specific amplification of SRSF1. Amplification of Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) is used as an internal control. The sequences of primers used in this study were listed in Table 5.

Table 5 Sequences of SRSF1 specific primers and internal control.

Name	Forward primer 5'→3'	Reverse primer 5'→3'	Product (bp)
SRSF1	TTAGATCTCATGAGGGA GAAACT	CAAAGACACGAAGGG AATGT	381
GAPDH	TTGCCATCAATGACCCC TTCA	CGCCCCACTTGATTTT GGA	174

4. Autophagy genes in CCA specific primer set

ATG5 were amplified by specific primers that newly design for specific amplification of ATG5 gene. Beclin-1 genes primers were ordered which following the publication of (W. Wang et al., 2013). The sequences of primers used for autophagy genes were listed in Table 6.

Table 6 Sequences of primers for Autophagy genes in CCA.

Name	Forward primer 5'→3'	Reverse prime 5'→3'	Product (bp)
ATG5	GTGAACATCTGAGCTAC CCG	GCAGTGGAGGAAAGC AGAGG	266
Beclin-1	GGCTGAGAGACTGGATC AGG	CTGCGTCTGGGCATA ACG	127

Methods

1. RNA extraction and cDNA synthesis

Total RNAs were extracted from CCA cell lines KKU-055, KKU-213A, and also MMNK-1 cell using Ribozol RNA extraction reagent (Life science, USA) and use as a template for cDNA synthesis using HisenScript^{TM} 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.

2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression levels of SRSF1 were explored by PCR amphification 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 95°C for 5 min, followed by 38 amplification cycles with 30 s at 95°C, 30s at 53°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, as follows: 51.1°C for ATG5 and 60°C for Beclin-1. The PCR product were analyzed by 2% agarose gel electrophoresis after mixed with staining dye, Novel Juice (Gene DireX, Hsinchu City, Taiwan) and detected by Image Quant ^{TM} LAS 500 (GE Healthcare Life Science, UK). The intensity of each band was quantitated using Image J software as a semi-quantitative expression.

3. Western blotting analysis

Total protein was isolated from cell lines using 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). The protein concentrations were determined by Bradford assay. First step is to separate the protein in a sample using SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad laboratories, USA). The membranes were blocked using 5% skim milk and then probed with specific primary antibody for overnights at temperature 4°C and with specific secondary antibody for 1 h. The protein bands were detected with enhanced chemiluminescence (ECL) detecting system (Bio-Rad laboratories, USA), imaged by Image Quant TM LAS 500 (GE Healthcare Life Science, UK). The signal intensity of each band was quantitated using Image J software as a semi-quantitative expression.

4. Silencing of SRSF1 by small interfering RNA (siRNA)

Specific siRNAs transfections were carried out using Lipofectamine 2000 (Thermo Fisher, USA). siRNAs targeting SRSF1 (described above) and negative control siRNA (Ambion, Thermo Fisher Scientific, Waltman, MA) were used. Briefly, cell lines were seeded into 6-well plate for 2×10^5 cells/well and cultured in complete media at 37°C in a 5% CO_2 until the monolayer cell is approximately 80% confluency. siRNAs were transfected at a final concentration of 20 nM in Opti-MEM I reduced serum medium (Gibco, Waltman, MA) and incubate at 37°C in a 5% CO_2 for 6 h. Before replaced by complete media for 48 h. RNA was extracted for evaluating the knockdown efficiency using RT-PCR and western blot.

5. Trypan blue dye exclusion assay

Suppression of SRSF1 in KKU-055 and KKU-213A cell viability was evaluated with Trypan blue dye exclusion assay. Cells were seeded in a 6-well plate at $2x10^5$ cells/ well. After knockdown SRSF1 for 48 h, cells were trypsinized and then, 20 µl of harvested cell was mixed with an equal volume of 0.4% trypan blue solution (1:1) (Sigma, USA). Then, the living cells (Cells that can remove color) and dead cells (dyed cells) were counted under a microscope using hemocytometer.

6. Flow cytometry

Cells were plated in a 6-well plate at $2x10^5$ cells per well after knockdown by siSRSF1 for 48 h, 75 µl of Muse™ AnnexinV & Death Cell Reagent (Merck Millipore, USA) and equal volume with $4x10^5$ cells of each groups were mixed. After 20 min incubation at room temperature, the numbers of live, death and apoptosis cells (early and late stages) were analyzed using Muse® Cell Analyzer and the attached analytical software (Millipore sigma, USA).

7. Caspase 3/7 activity assay

Cell were plated in a 96-well black plate at $2x10^4$ cells per well for 24 h after knockdown by siSRSF1 for 48 h. After 24 h, cell was seeded in a 96-well black plate, activity of caspase 3/7 were analyzed using Apo-ONE® Homogeneous caspase 3/7 assay according to the manufacturer's instruction (Promega, Madison, USA). The fluorescence signals of each well were measured by a fluorescence microplate reader, EnSpire Multimode Plate reader (Parkin Elmer, Waltham, MA). Regarding to the measurement of fluorescent intensities, the assay suggested setting the excitation wavelength at 499 nm and emission wavelength at 512 nm.

8. Transmission electron microscopy (TEM)

KKU-055 and KKU-213A cells with knockdown of SRSF1 by siRNAs were grown on 6-well at 72 h. After experiments, total cells both floated and adhered cells were harvested and washed with PBS. Then, immediately fixed in 2.5% glutaraldehyde in 0.1 M PBS buffer followed by post-fixation with 1% osmium tetroxide for 1 h at room temperature. The specimens were then dehydrated with ascending concentrations of ethanol (50–100%) and embedded in Spurr's epoxy resin. Then, thin sections (60–80 nm) were cut with an ultramicrotome and mounted on copper mesh grids. The sections were then stained with 1% uranyl acetate and lead citrate and examined by TEM, JEM-1010 (JEOL, Japan), which using the facility and services from Department of Anatomy, Faculty of Medicine, Khon Kaen University.

9. Statistical analysis

The data were presented as mean±SD of independent experiments with biological triplicates. Statistical analyses were performed using Unpaired Student's t-test (two tail) for comparison between each group by GraphPad Prism 8. Significance level of $p < 0.05$ was considered to be significant *$=p<0.05$, **$=p<0.01$, ***$=p<0.001$ and *Ns* = non-statistically significant.

CHAPTER IV

RESULTS

Confirmation of SRSF1 mRNA expression in CCA cell lines compared with established cholangiocyte

Determination of the mRNA expression of SRSF1 in CCA cells (KKU-055 and KKU-213A) and cholangiocyte (MMNK-1) was comparative analyzed by RT-PCR. The result showed that SRSF1 were predominantly upregulated in CCA cells which can be observable that KKU-055 presented the higher expression level of SRSF1 mRNA when compared with KKU-213A (Figure 27).

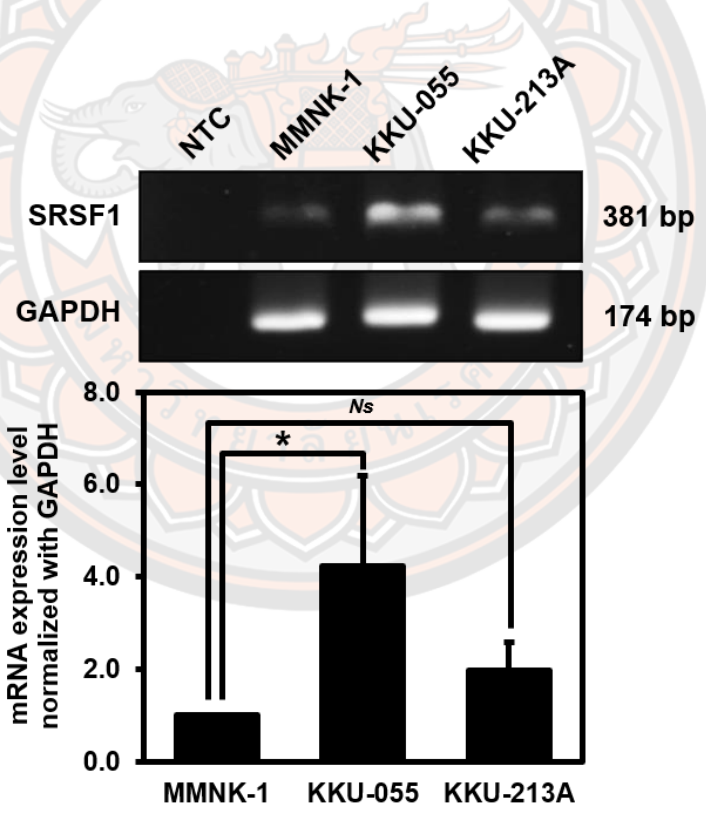


Figure 27 The mRNA expression of SRSF1 in CCA cells (KKU-055 and KKU-213A) compared with cholangiocyte (MMNK-1), The data represent mean ± SD from triplicate experiments, *Ns* = non-statistically significant and **p<0.05.*

Confirmation of SRSF1 protein expression in CCA cell lines compared with established cholangiocyte

Determination of the protein expression of SRSF1 in CCA cells (KKU-055 and KKU-213A) and cholangiocyte (MMNK1) was comparative analyzed by western blotting. The results showed that SRSF1 protein was obviously upregulated in KKU-055 and KKU-213A when compared with MMNK-1 (Figure 28). Remarkably, the protein band intensity of SRSF1 in KKU-055 is higher intensity than in KKU-213A which corresponded to the result of SRSF1 mRNA expression among these cells.

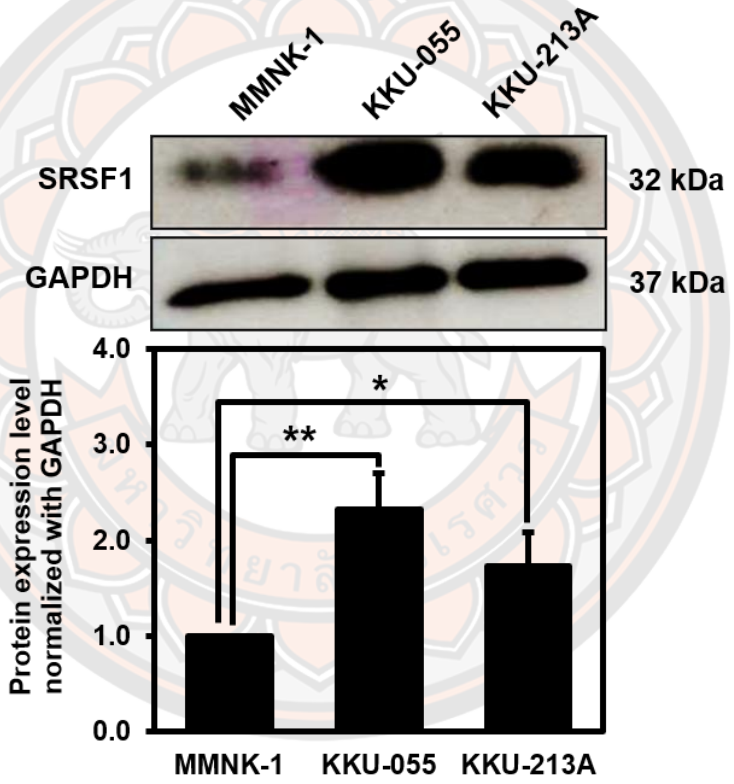


Figure 28 The protein expression of SRSF1 in CCA cells (KKU-055 and KKU-213A) compared with cholangiocyte (MMNK1), The data represent mean ± SD from triplicate experiments, *$p<0.05$ and **$p<0.01$.

Suppression of SRSF1 in CCA cells by specific siRNAs

Two siRNA targeting SRSF1 and control siRNA were separately transfected into KKU-055 and KKU-213A cells. Then, RNA and protein from these transfected group and control siRNA transfected was extracted, RT-PCR and western blotting was performed for evaluating the knockdown efficiency. The knockdown efficiency of two siRNA was observed at 48 h. The result confirmed that the mRNA expression and protein expression of SRSF1 was decreased after transfection by both siSRSF1-1 and siSRSF1-2 in KKU-055 and KKU-213A cells. Especially, siSRSF1-2 was significantly decreased mRNA and protein expression level of SRSF1 in KKU-055 and KKU-213A cells when compared with control siRNA transfection (Figure 29 and 30).

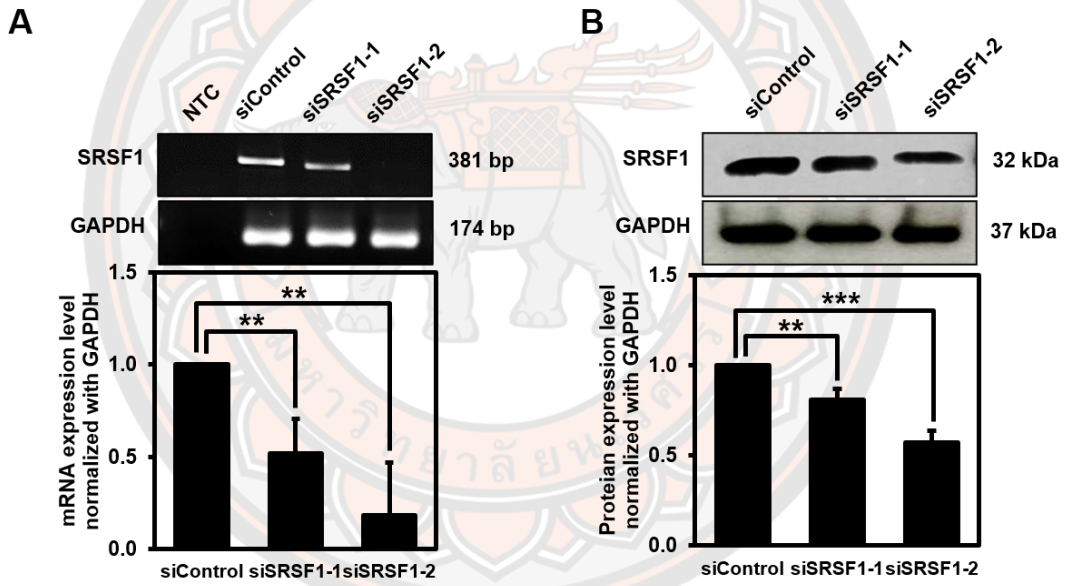


Figure 29 Suppression of SRSF1 using specific siRNAs. The mRNA (A) and protein (B) expression of SRSF1 were significantly decreased after 48 h transfection of two siRNAs (siSRSF1-1 and siSRSF1-2) in KKU-055 cells when compared with control siRNA transfected cell and determined by RT-PCR and western blot. The data represent mean ± SD from triplicate experiments, **$p<0.01$ and ***$p<0.001$.

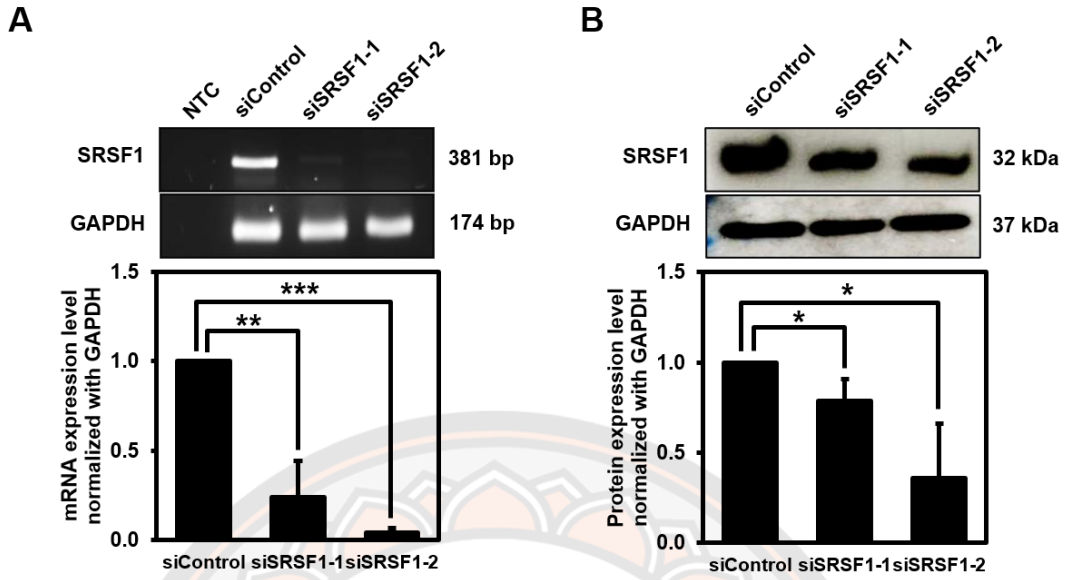


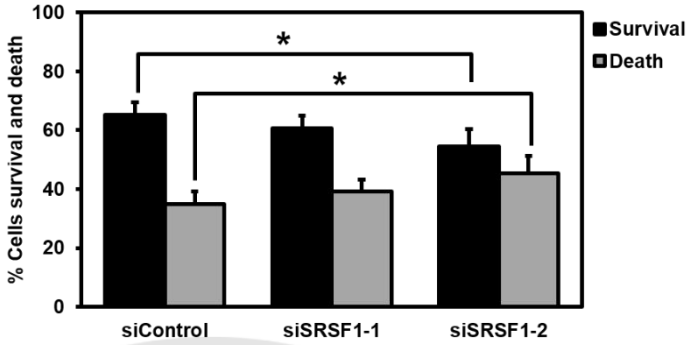
Figure 30 Suppression of SRSF1 using specific siRNA. The mRNA (A) and protein (B) expression of SRSF1 were significantly decreased after 48 h transfection of two siRNAs (siSRSF1-1 and siSRSF1-2) in KKU-213A cells when compared with control siRNA transfected cell and determined by RT-PCR and western blot. The data represent mean ± SD from triplicate experiments, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Silencing of SRSF1 decreased CCA cell viability

Investigation of the effect of SRSF1-silencing on the cell viability in KKU-055 and KKU-213A for 48 h, using trypan blue exclusion dye assay and counting viable cells (Cells that can remove trypan blue staining dye) and dead cells (trypan blue stained cells) under the light microscope. The result showed that silencing of SRSF1 expression decreased the number of viable cells and increased the number of dead cells in both CCA cells. Analysis of separated the number of viable cells (Survival) and dead cells (Death) that shows clearly that the suppression of SRSF1 expression can induces CCA cell death (Figure 31A and 32A). Of note, after transfection by siSRSF1-2, the cell confluency was obviously reduced in KKU-055 and KKU-213A cells when compared with negative control siRNA transfection (Figure 31B and 32B).

However, the death cell was observed and counted in Negative siRNA transfection (siControl) as approximately 35% and 20% for KKU-055 and KKU-213A, respectively (Figure 31A and 32A). Therefore, the effect of Negative siRNA transfection (Transfection control) was validated by compared with untransfected CCA cells (Untransfected control). The result confirmed with triplicate experiment demonstrated that siControl transfection did not affect CCA cell viability as recorded in appendix B experimental results; Raw data (Table B7 and B8).

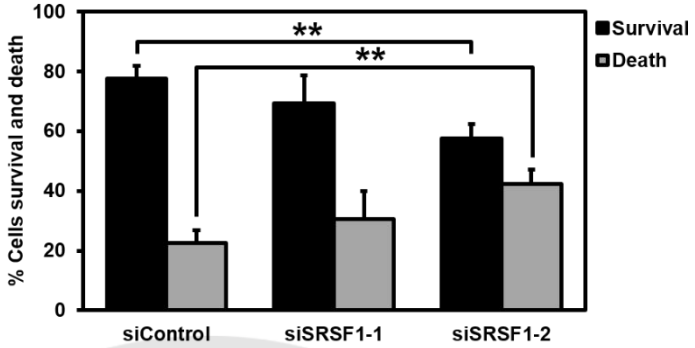
A



B

Figure 31 Cell viability at 48 h of siRNAs transfection in KKU-055 (A). Cell confluency (B) at 48 h of siRNAs transfection in KKU-055 using 20x magnification. The data represent mean ± SD from triplicate experiments, **p<0.05.*

A



B

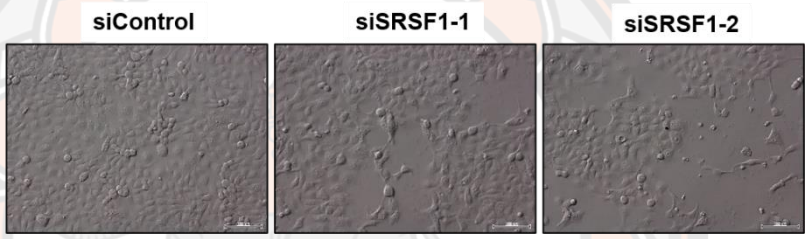


Figure 32 Cell viability at 48 h of siRNAs transfection in KKU-213A (A). Cell confluency (B) at 48 h of siRNAs transfection in KKU-213A using 20x magnification. The data represent mean ± SD from triplicate experiments, **$p<0.01$.

Morphological observation of death phenotypes of SRSF1-silencing CCA cells under transmission electron microscope (TEM).

To investigate the effect of SRSF1-silencing on death morphological changes in KKU-055 and KKU-213A at 72 h, the observation was performed by transmission electron microscope (TEM) regarding to that marked cellular changes were apparently observed in nucleus, cytoplasmic organelles and membrane features. The typical findings, such as apoptosis, autophagy, and necrosis, were observed in KKU-055 and KKU-213A treated with the siSRSF1-2 compared with Negative siRNA (Figures 33 and 34). Of note, siSRSF1-2 was selected based on the higher capacity on silencing efficiency and the cellular effects the obviously presented in the experiments above.

Firstly, the cells with viable and normal cellular morphology were marked as normal reference. Almost cell from the Negative siRNA-treated cells (Figures 33A and 34A) showed normal morphology and nuclear shape. In siSRSF1-treated cells showed apoptosis morphology; chromatin condensation, nuclear fragmentation, and membrane blebbing (Figures 33B and 34B). Autophagy morphology; Autophagosome accumulation (Figures 33C and 34C). Necrotic morphology; membrane lysed and nucleus swollen (Figures 33D and 34D).

The result showed that suppression of SRSF1 expression in KKU-055 by siSRSF1-2 was increased a number of cells with apoptosis and autophagy-dependent cell death when compared with Negative siRNA transfection. A similar result was also observed in KKU-213A, increased a number of cells with apoptosis and autophagy-dependent cell death when compared with Negative siRNA transfection.

Remarkably, KKU-213A predominantly demonstrated the higher autophagosome accumulation in the cell than KKU-055. These results might be suggested that suppression of SRSF1 expression can induce CCA cell enters to apoptosis and autophagy-dependent cell death.

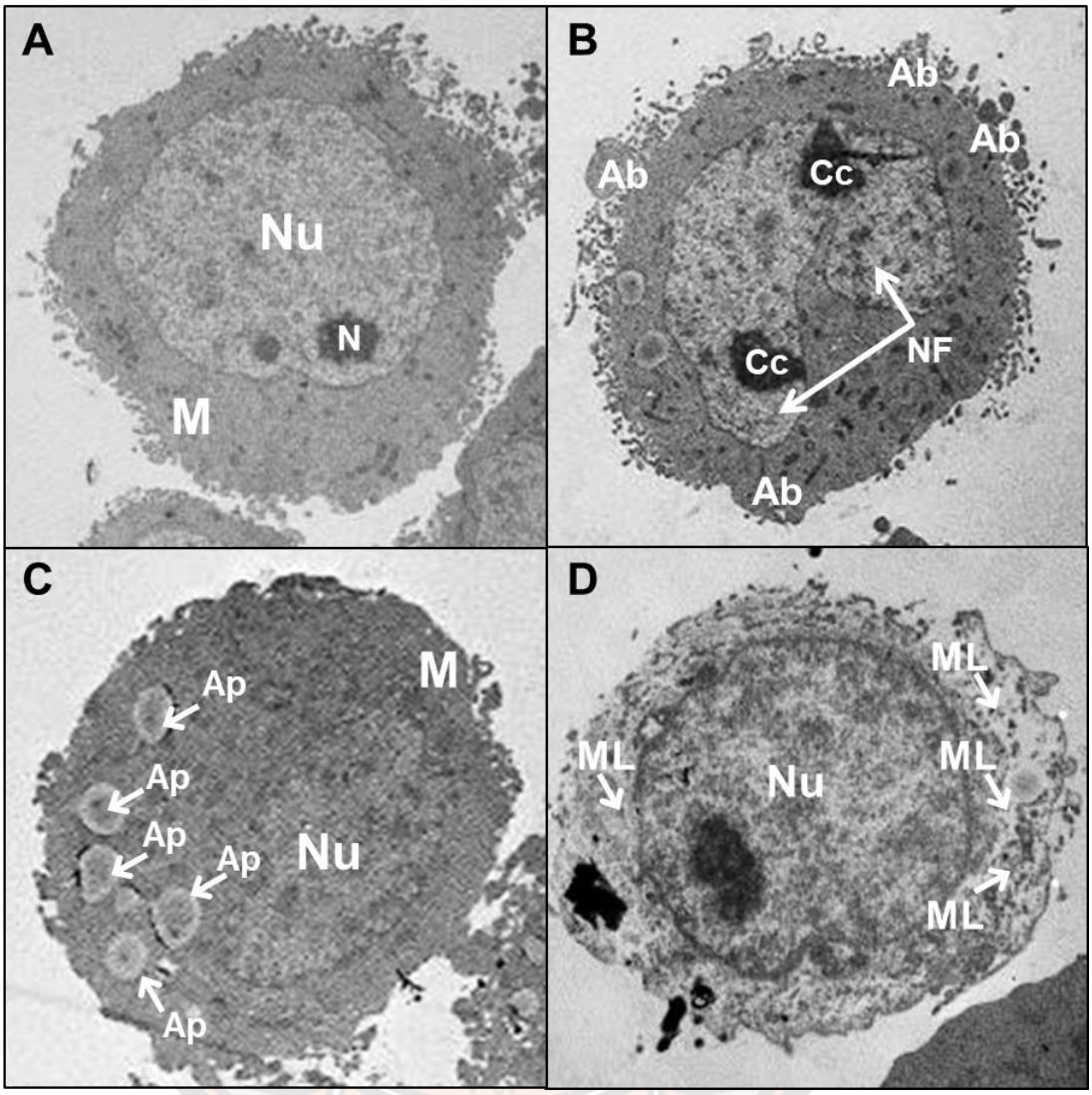


Figure 33 Representative cellular images of death cell morphologies. Normal cell which captured from Negative siRNA transfected KKU-055 cell (A). Apoptotic cell which captured from siSRSF1 transfected KKU-055 cell (B). Autophagic cell which captured from siSRSF1 transfected KKU-055 cell (C). Necrotic cell which captured from siSRSF1 transfected KKU-055 cell (D). The alphabets and the arrows pointed to the specific intracellular phenotypes including: Nu: nucleus, N: Nucleolus, M: membrane, Ab: apoptotic bodies, Cc: chromatin condensation, NF: nuclear fragmentation, Ap: autophagosome, ML; membrane lysed.

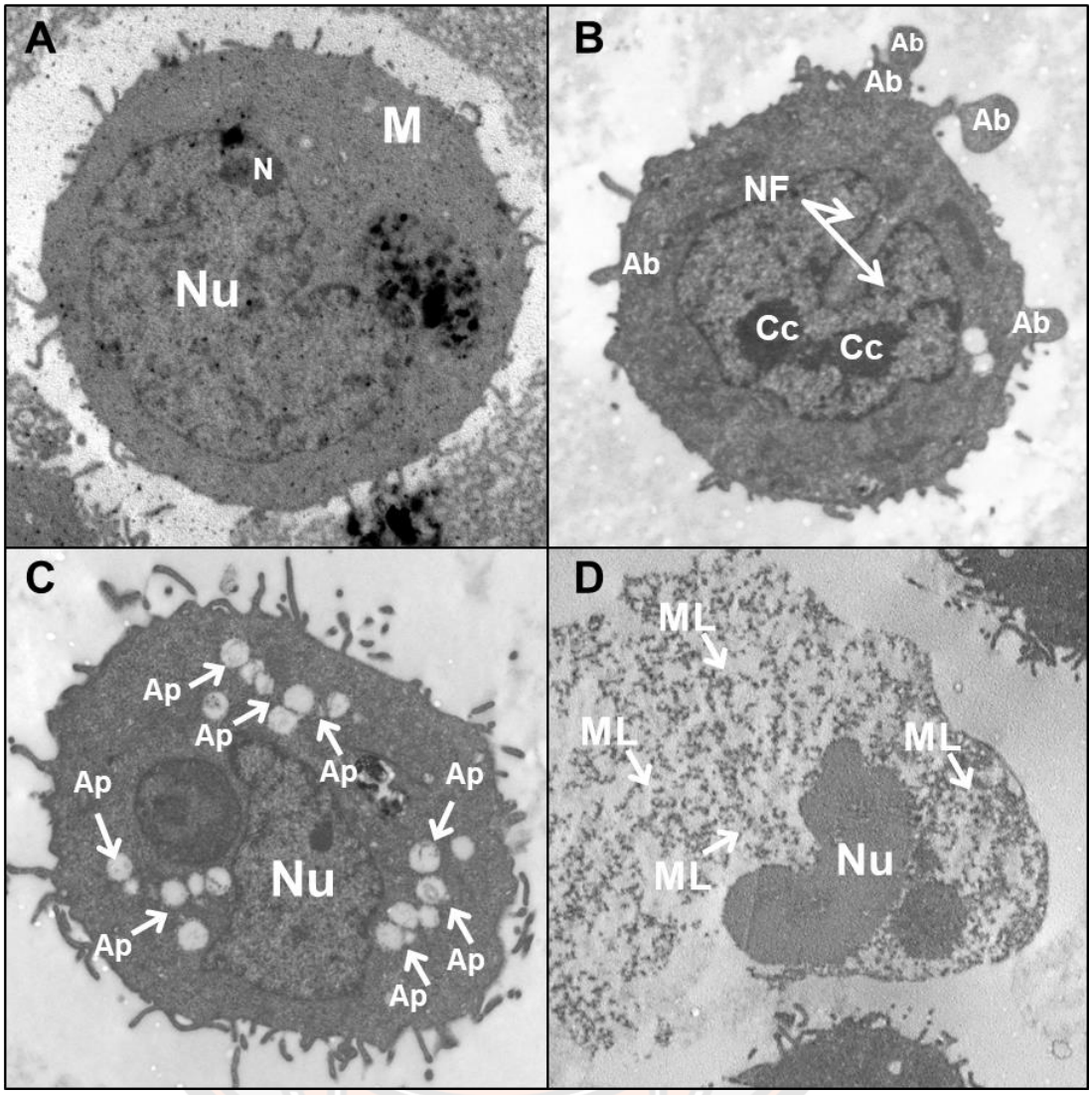


Figure 34 Representative cellular images of death cell morphologies. Normal cell which captured from Negative siRNA transfected KKU-213A cell (A). Apoptotic cell which captured from siSRSF1 transfected KKU-213A cell (B). Autophagic cell which captured from siSRSF1 transfected KKU-213A cell (C). Necrotic cell which captured from siSRSF1 transfected KKU-213A cell (B). The alphabets and the arrows pointed to the specific intracellular phenotypes including: Nu: nucleus, N: Nucleolus, M: membrane, Ab: apoptotic bodies, Cc: chromatin condensation, NF: nuclear fragmentation, Ap: autophagosome, ML; membrane lysed.

From the result of morphological observation of death phenotypes of SRSF1-silencing CCA cells those observed under transmission electron microscope (TEM). The data was analyzed and interpreted into the number and graph to suggest the comparative trend of death induction by targeting SRSF1.

The relative frequencies of normal morphology, apoptosis, autophagy, and necrosis in the cell population were shown by counting at more 50 cells in each group. Apoptosis and autophagy predominantly occur both in KKU-055 and KKU-213A when suppressed SRSF1 compare with siControl as shown in Figure 35. Next, we declined the number of normal cells from the analysis, the result showed that apoptosis, autophagy, and necrosis in KKU-055 and KKU-213A not difference between siControl and siSRSF1-2 as shown in Figure 36. Finally, we declined the number of necrosis from the analysis, the result shown that apoptosis was occurred frequently between 45.1% (KKU-055) and 38.5% (KKU-213A). Similar autophagy occurred frequently between 17.7% (KKU-055) and 24.6% (KKU-213A) as shown in Figure 37. These results might be suggested that trend of death induction both in CCA cells has two death morphology predominantly including apoptosis and autophagy-dependent cell death.

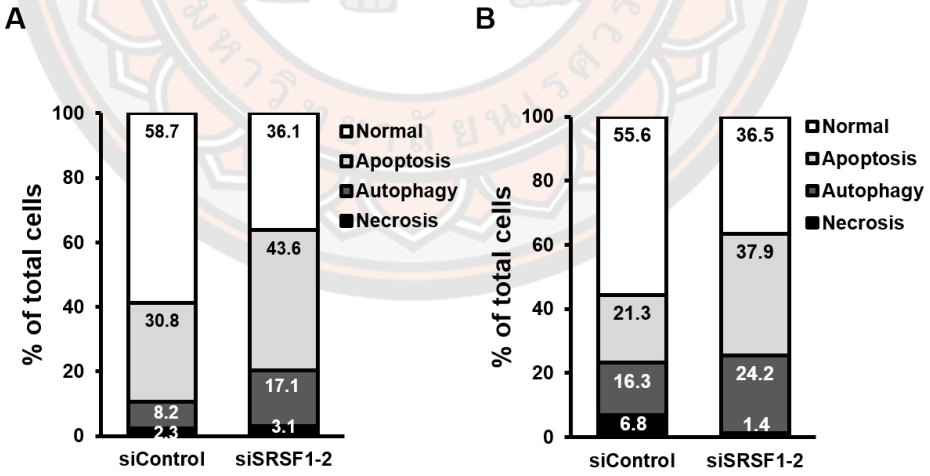


Figure 35 The relative frequencies of normal morphology, apoptosis, autophagy, and necrosis in the cell population were shown by counting at more 50 cells in each group by three different examiners. KKU-055 (A) and KKU-213A (B).

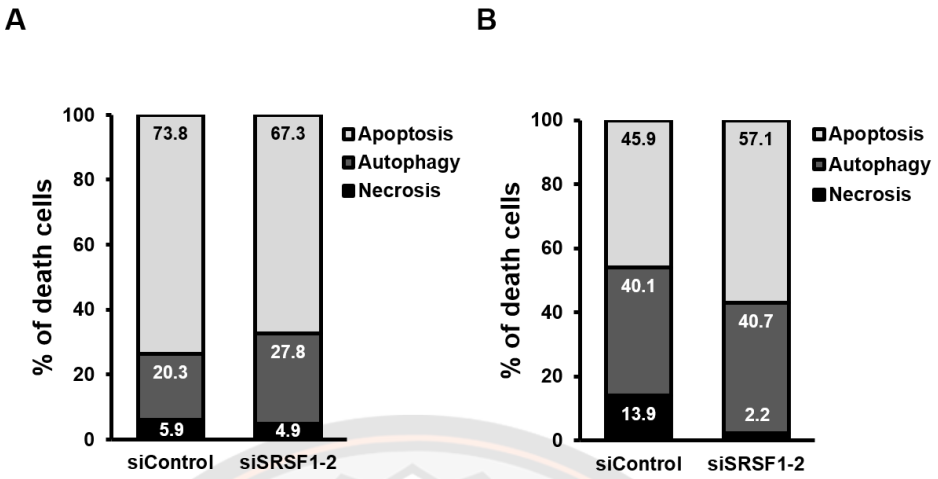


Figure 36 The relative frequencies of death cell in the cell population including apoptosis, autophagy, and necrosis were shown by counting at more 50 cells in each group by three different examiners. KKU-055 (A) and KKU-213A (B).

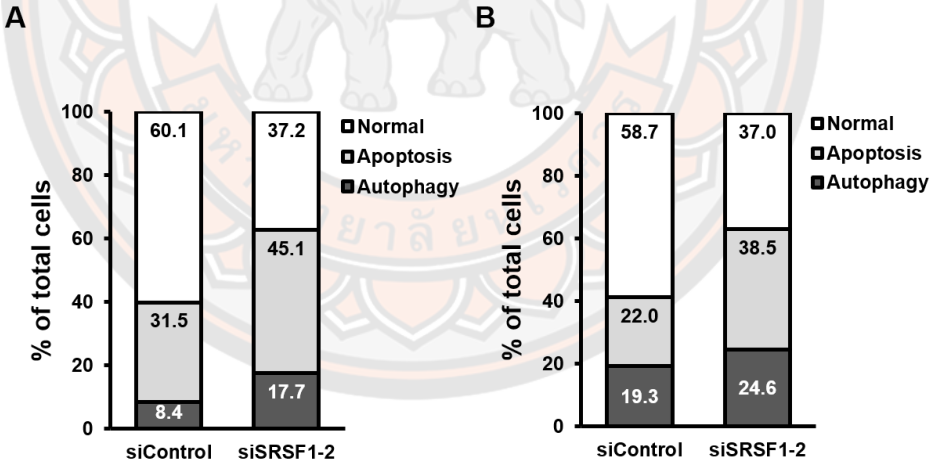


Figure 37 The relative frequencies of normal morphology, apoptosis, and autophagy in the cell population were shown by counting at more 50 cells in each group by three different examiners. KKU-055 (A) and KKU-213A (B).

The effect of SRSF1-silencing on CCA cells apoptosis

Determination of the effect of SRSF1-silencing on apoptosis, siSRSF1 was transfected to KKU-055 and KKU-213A cells compared with control siRNA transfection, then the observed apoptotic cellular phenotypes by staining with AnnexinV/7-AAD and analysis by flow cytometry. The result showed that silencing of SRSF1 expression was slightly increased the apoptotic cell population both in early and late stages of apoptosis in KKU-055 and KKU-213A (Figure 38 and 39). Especially, siSRSF1 in KKU-055 was slightly increased late stage apoptosis, while siSRSF1 in KKU-213A was slightly increased early stage apoptosis.

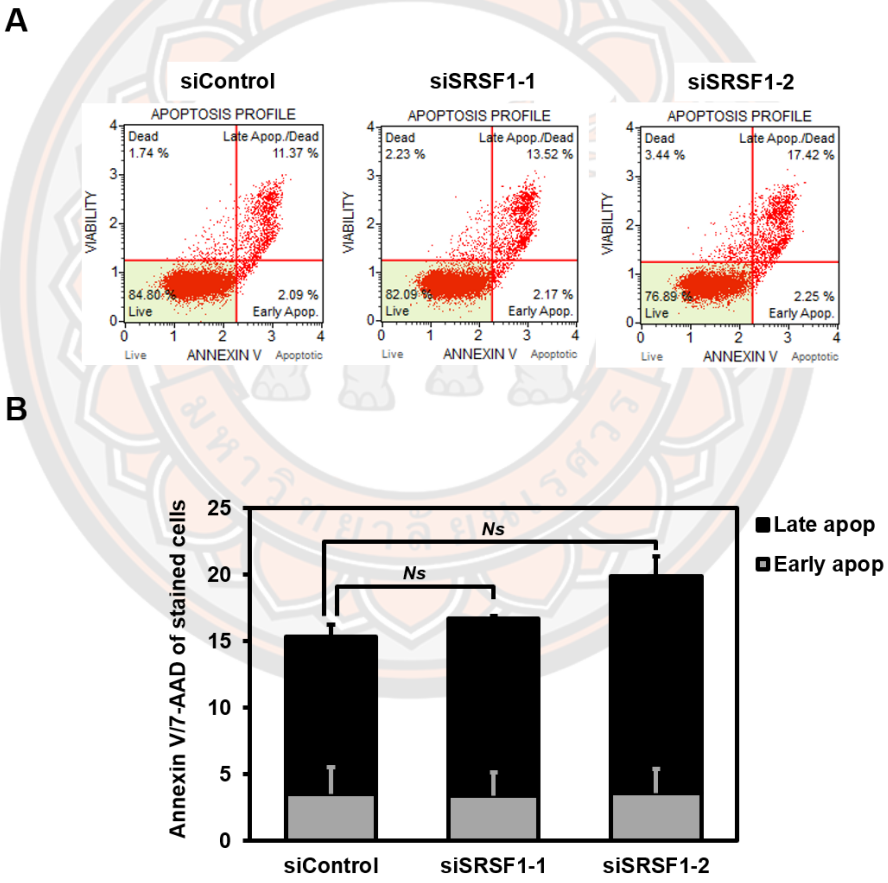


Figure 38 Effect of SRSF1 silencing on apoptotic cellular phenotypes of KKU-055 (the represented figure was selected from 3 independent experiments) (A) and analysis in percentages of apoptotic cell population (B). The data represent mean ± SD from triplicate experiments, *Ns* = non-statistically significant.

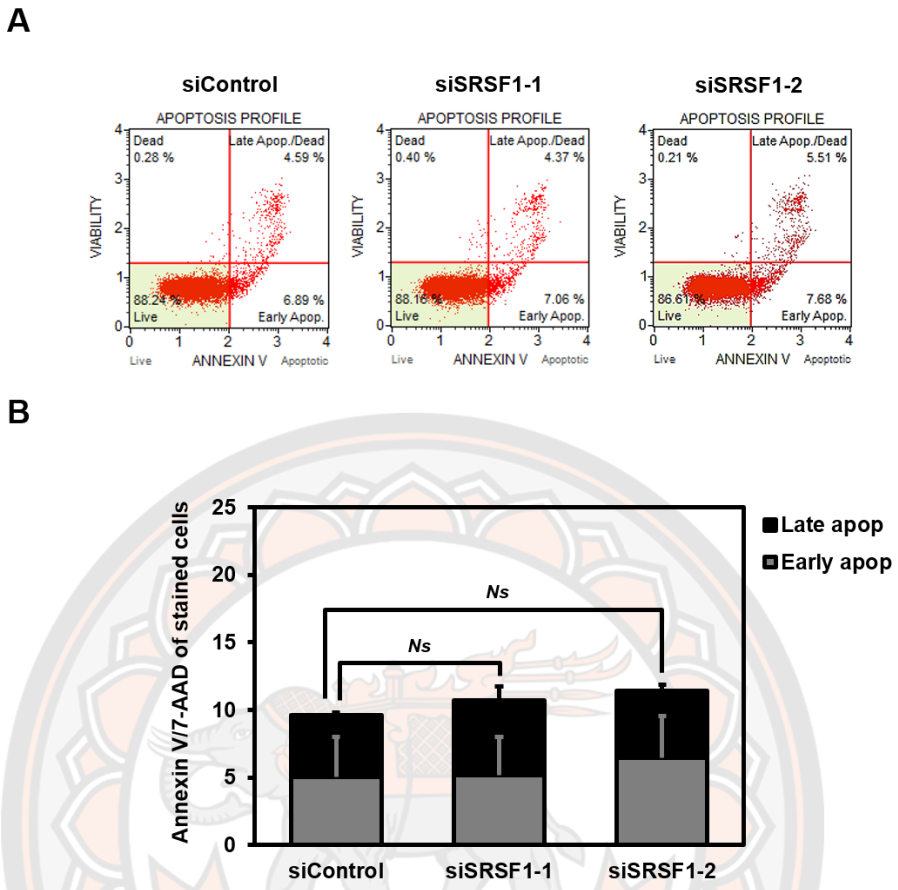


Figure 39 Effect of SRSF1 silencing on apoptotic cellular phenotypes of KKU-213A (the represented figure was selected from 3 independent experiments) (A) and analysis in percentages of apoptotic cell population (B). The data represent mean ± SD from triplicate experiments, *Ns* = non-statistically significant.

In addition, to determine the effect of SRSF1- silencing on apoptosis, the measurement of caspase 3/ 7 activity assay was also performed using Apo-ONE® Homogeneous caspase 3/7 assay. The result demonstrated that silencing of SRSF1 expression can activated caspase 3/7 activity in KKU-055 and KKU-213A (Figure 40).

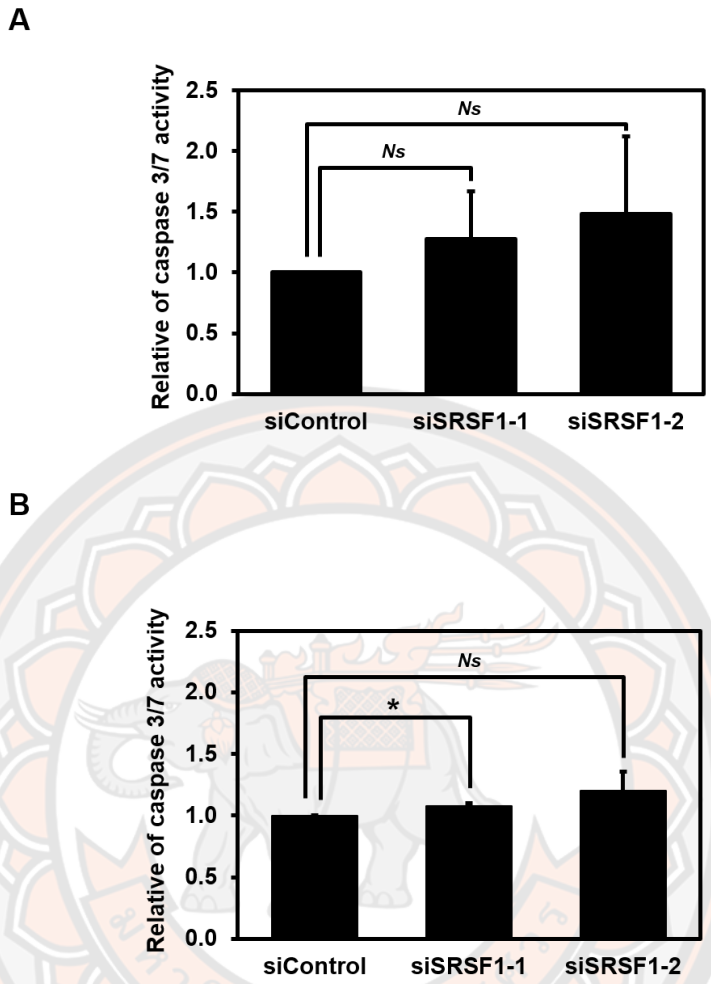


Figure 40 Effect of SRSF1 silencing on an activation of Caspase 3/7 activity in KKU-055 (A) and KKU-213A (B). The data represent mean ± SD from duplicate experiments, *Ns* = non-statistically significant and **p<0.05.*

To confirm the effect of SRSF1-silencing on apoptosis using apoptotic markers. The determination of them by western blotting was performed, the result showed that the expression levels of BAX (pro- apoptotic proteins) were increased while the expression levels of Bcl- 2 (anti- apoptotic protein) was decreased after transfecting siSRSF1 for 48 h (Figures 41 and 42). Therefore, the silencing of SRSF1 can increased CCA cell apoptosis.

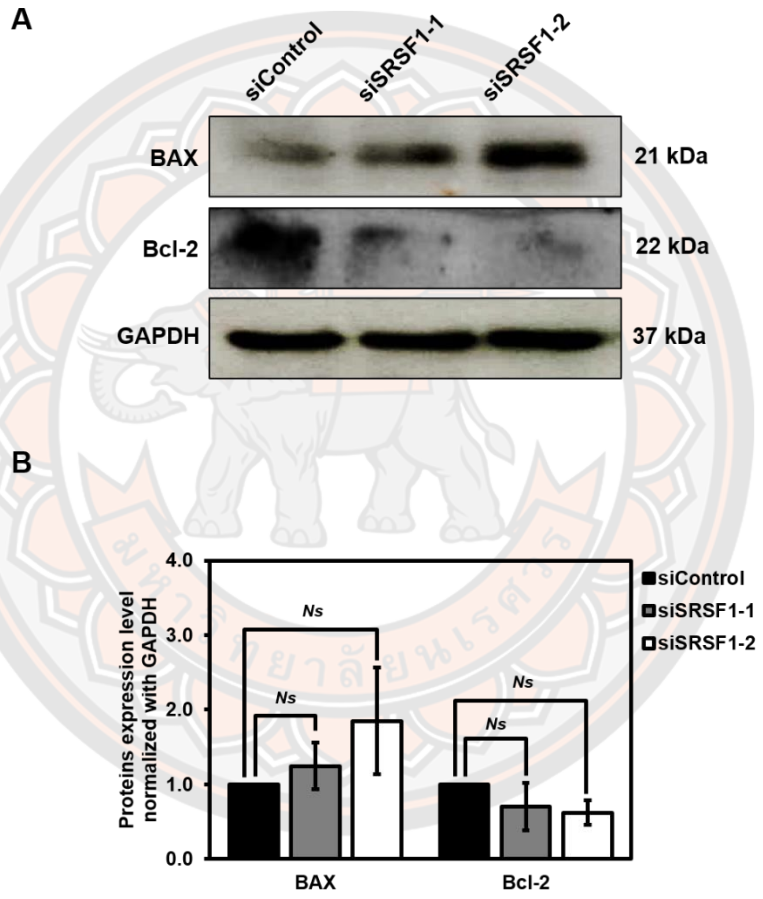


Figure 41 The expression of apoptotic marker in KKU-055. The expression levels of BAX and Bcl-2 (A). Data analysis as shown in graph (B). The data represent mean ± SD from duplicate experiments, *Ns* = non-statistically significant.

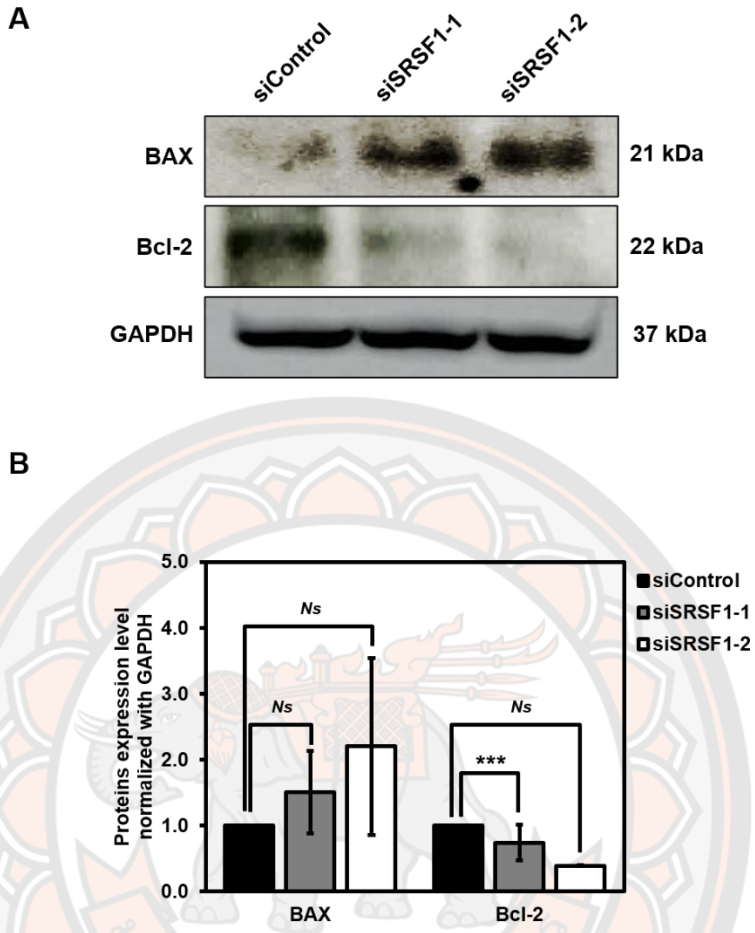


Figure 42 The expression of apoptotic marker in KKU-055. The expression levels of BAX and Bcl-2 (A). Data analysis as shown in graph (B). The data represent mean ± SD from duplicate experiments, *Ns* = non-statistically significant and ****p>0.001.*

The effect of SRSF1-silencing on CCA cell autophagy-dependent cell death

Determination of the effect of SRSF1-silencing on autophagy-dependent cell death, siSRSF1 was transfected to KKU-055 and KKU-213A cells compared with control siRNA transfection for 48 h, then observed the expression of autophagic gene and protein markers using RT-PCR and western blot. The result showed that silencing of SRSF1 expression increased Beclin-1 and ATG5 genes expression (gene-related in the formation of autophagosome) in KKU-055 and KKU-213A (Figures 43 and 44).

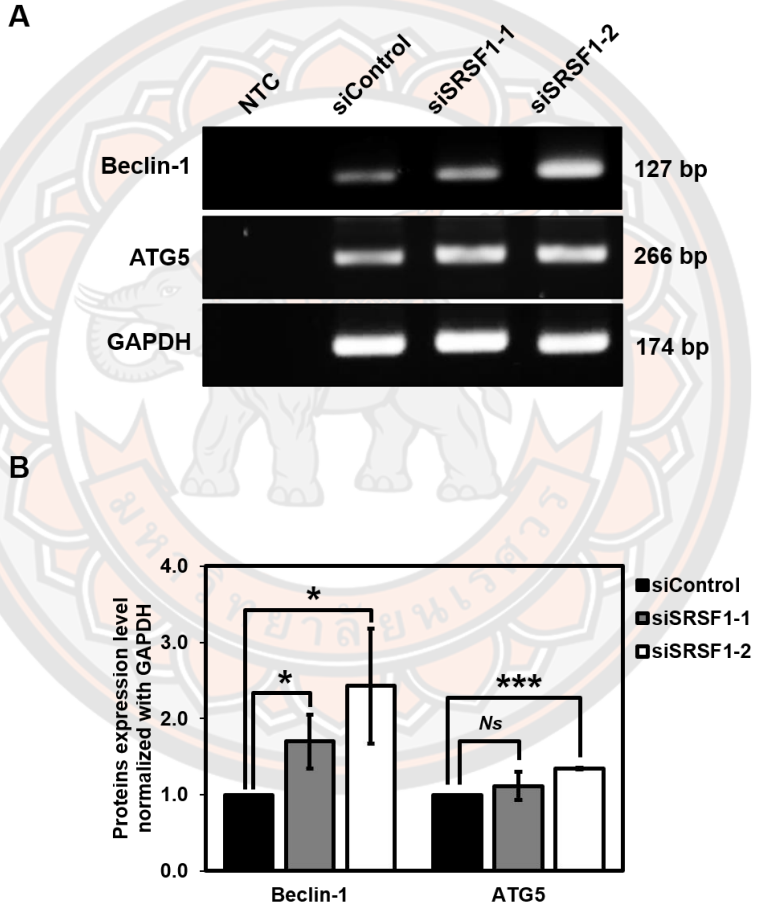


Figure 43 Effect of SRSF1 suppression on autophagic marker expression of KKU-055. mRNA expression of Beclin-1, and ATG5 determined by RT-PCR (A). Data analysis as shown in graph (B). The data represent mean ± SD from triplicate experiments, *Ns* = non-statistically significant, **p<0.05* and ****p>0.001.*

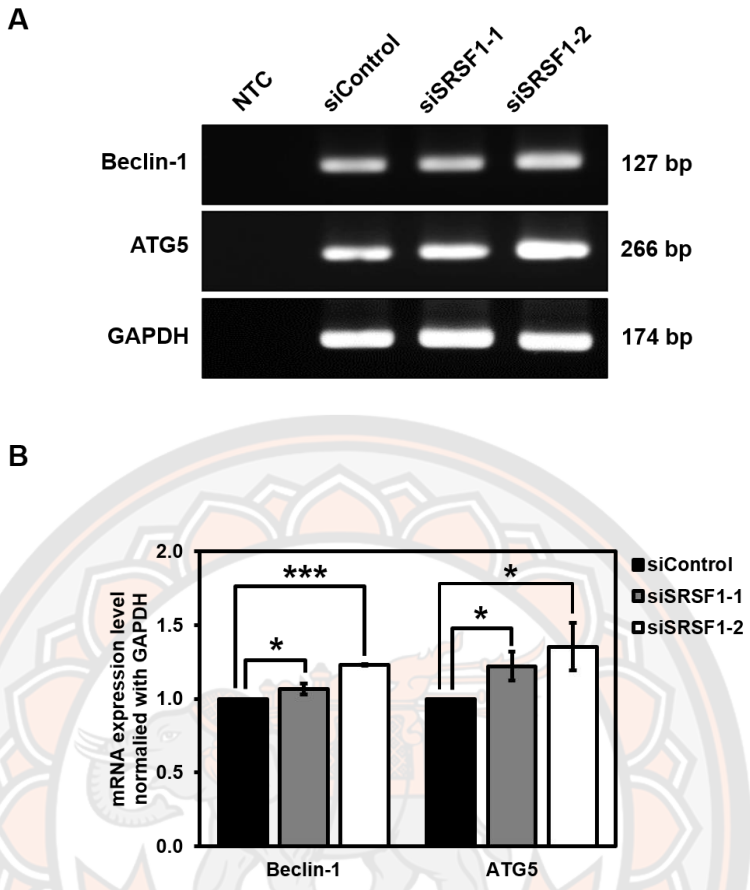


Figure 44 Effect of SRSF1 suppression on autophagic marker expression of KKU-213A. mRNA expression of Beclin-1, and ATG5 determined by RT-PCR (A). Data analysis as shown in graph (B). The data represent mean ± SD from triplicate experiments, *$p<0.05$ and ***$p>0.001$.

In addition, also increased the ratio between LC3B-II and LC3B-I protein (protein-related in the formation of autophagosome) and decreased p62 protein (protein-related in the degradation of autophagosome and lysosome) in KKU-055 and KKU-213A cells (Figures 45 and 46).

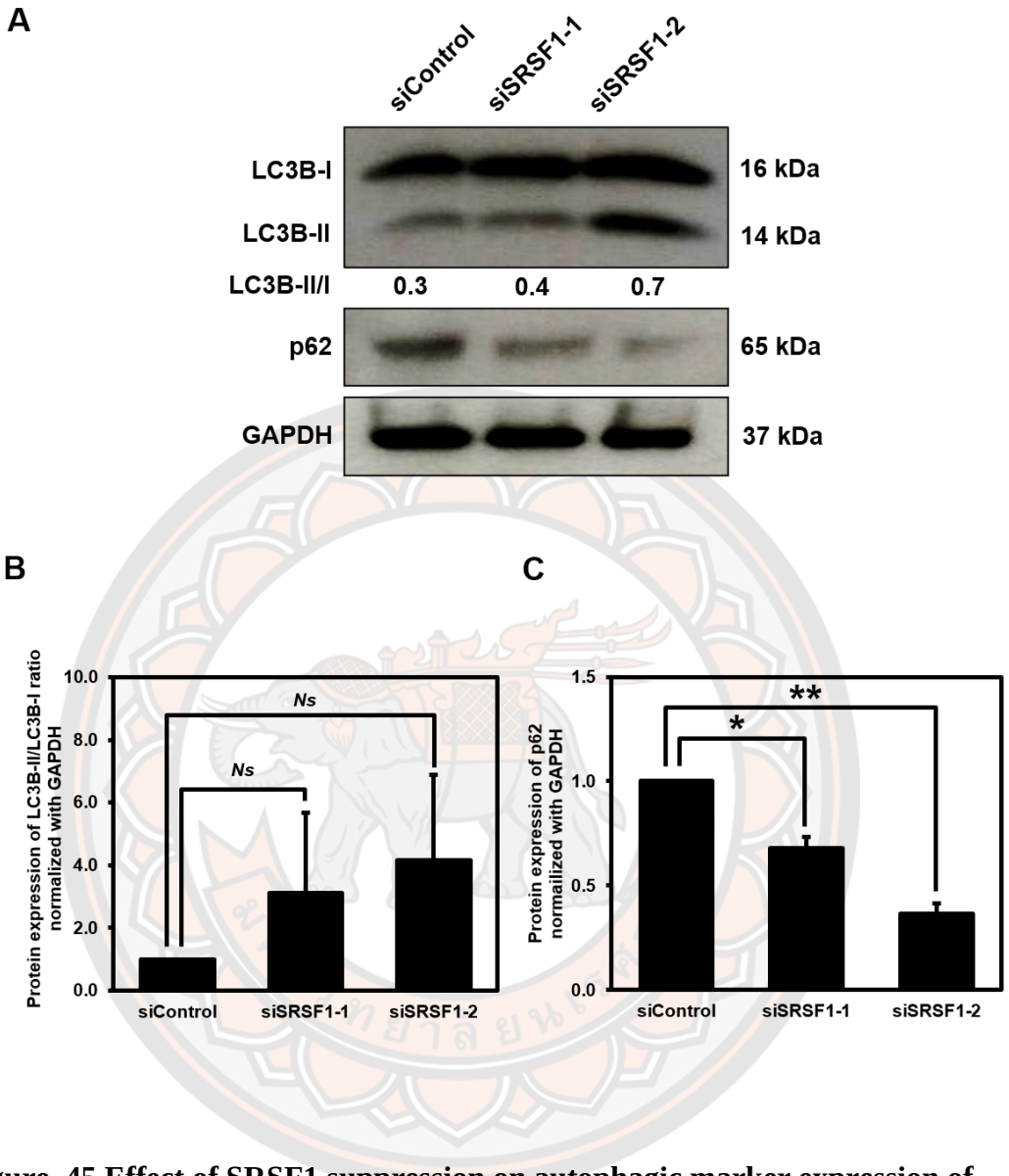


Figure 45 Effect of SRSF1 suppression on autophagic marker expression of KKU-055 (A). LC3B-II/LC3B-I ratio and p62 data analysis as shown in graph (B and C). The data represent mean ± SD from triplicate experiments, *Ns* = non-statistically significant, **p<0.05* and ***p>0.01*.

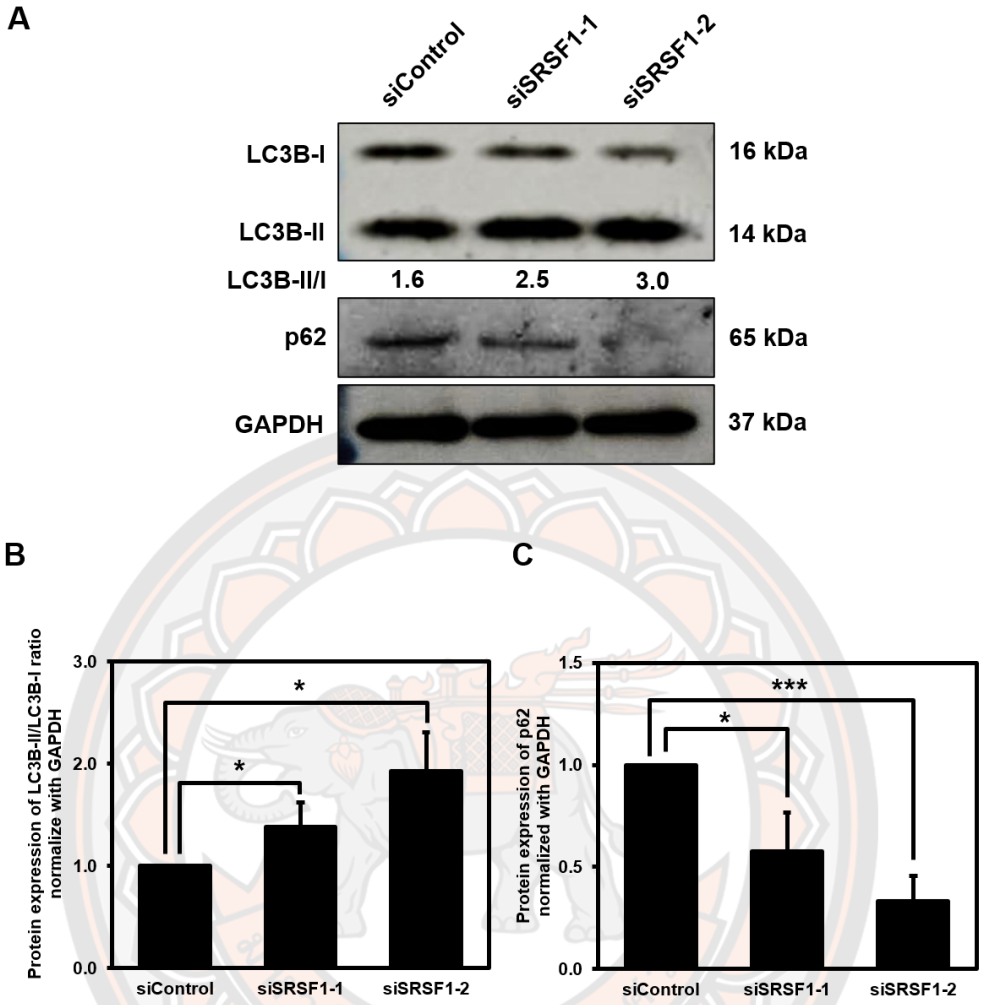


Figure 46 Effect of SRSF1 suppression on autophagic marker expression of KKU-213A (A). LC3B-II/LC3B-I ratio and p62 data analysis as shown in graph (B and C). The data represent mean ± SD from triplicate experiments, *p<0.05 and ***p>0.001.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Cholangiocarcinoma (CCA) is a malignant cancer arising from epithelial cell of bile duct. CCA shows high incidence in Northeast and North of Thailand. CCA is normally diagnosis in advanced stage and with high metastatic, resulting in ineffective treatment and high mortality rate. Our recent review article summarized the aberrant alternative splicing occurred in important genes in CCA. Remarkably, they present their contributions with cancer development and drive cancer to more aggressiveness (Yosudjai, Wongkham, Jirawatnotai, & Kaewkong, 2019). We focused SRSFs or "Serine/Arginine-rich splicing factors, important classes of splicing regulator regarding that SRSFs plays essential role to enhance the alternative splicing processes of pre-mRNA and importantly, dysregulation of SRSFs can be generated aberrant transcript variants or oncogenic-protein isoforms.

Previously, another member in our research group (Pawee tangwiriyarotkul) attempted to determine the expression of SRSFs in CCA cells compared established cholangiocyte (MMNK-1). Our result showed that SRSF1 was upregulated in CCA cells when compared with MMNK-1. For protein expression SRSF1 were predominantly upregulated in CCA cells parallel with another member in our research group (Sirinthip Seesuksai) attempted to determine SRSF1 in clinical specimen. She detected SRSF1 protein expression in CCA tissues by compared between the protein extract from tissue section of tumor area and adjacent non-tumor of 9 matched-pair tissues. All cases presented higher SRSF1 intensity in tumor tissues. Statistically analysis revealed the upregulation of SRSF1 in tumor area was significantly higher than non-tumor area. In addition, a previous study from Pawee tangwiriyarotkul was observed its biological effect in carcinogenesis CCA cells. He determines the cell proliferation and colony formation in KKU-055 and KKU-213A by interrupted the SRSF1 using specific siRNA. The results show that SRSF silencing decreased cell proliferation and colony formation in CCA cells.

In this study, we focused on SRSF1 by determination the effect of SRSF1 silencing on cell death of CCA cells. We started to confirm the expression of SRSF1 in KKU-055 and KKU-213A when compared with MMNK-1, our result showed that SRSF1 was upregulated in KKU-055 and KKU-213A when compared with MMNK-1 both in mRNA and protein expression level (Figures 27 and 28). Especially, in KKU-055 which was poorly differentiated, tend to be more aggressive and have a worse prognosis than KKU-213A with a well-differentiated cancer cell. Poorly differentiated cancers are high grade while well-differentiated cancers are low grades. (Telloni, 2017). These results indicated that SRSF1 expression correlated with the cancer differentiation grades.

For the efficiency of SRSF1 silencing at 48 h in KKU-055 and KKU-213A, we used 2 sequences of siRNA (si-SRSF1-1 and siSRSF1-2) references from 2 research articles in lung cancer that different site of targeting SRSF1 mRNA structure (Fu et al., 2013; Jiang et al., 2016). The result showed that SRSF1 silencing significantly decreased the mRNA and protein expression level in these two CCA cell lines. Especially, higher efficiency observed by the second sequence of siRNA (siSRSF1-2) (Figures 29 and 30).

Experimentally cell viability of SRSF1 silencing in KKU-055 and KKU-213A were determined by trypan blue dye exclusion assay and separately counting viable cells and dead cells under the light microscope. The result showed that silencing of SRSF1 expression decreased the number of viable cells and increased the number of dead cells. In addition, the cell confluence was reduced in both CCA cells (Figures 31 and 32).

The result from this recent study associated with the previous studies from Pawee tangwiriyarotkul, when he performed the SRSF1 silencing in KKU-055 and KKU-213A, the results show that SRSF1 silencing decreased cell proliferation and colony formation in both CCA cells. Our result in CCA cells correlated with the previous reports, the knockdown of SRSF1 caused a decrease of cell viability and colony formation in colon cancer (Chen et al., 2017) and recent studies, knockdown of SRSF1 inhibit cell proliferation and cell cycle progression via regulated splicing of NEAT1 in glioma cancer (Zhou et al., 2019).

For the classification of death cell morphology in to the particulat types. In 2012, Phimsen and colleagues used transmission electron microscopy (TEM), to study apoptosis and necroptosis morphological change in CCA (M213) and HeLa cell by induction of DNA damage in p53-insufficient cells by siGanp (Phimsen et al., 2012) as shown in Figure 47. That led the way for using TEM to study CCA cell death morphological change in this study. Investigation of the effect of SRSF1-silencing on death morphological change in KKU-055 and KKU-213A at 72 h observed under TEM. CCA cells treated with the siSRSF1-2 when compared with Negative siRNA, the typical findings; such as apoptosis, autophagy, and necrosis (Figures 33 and 34).

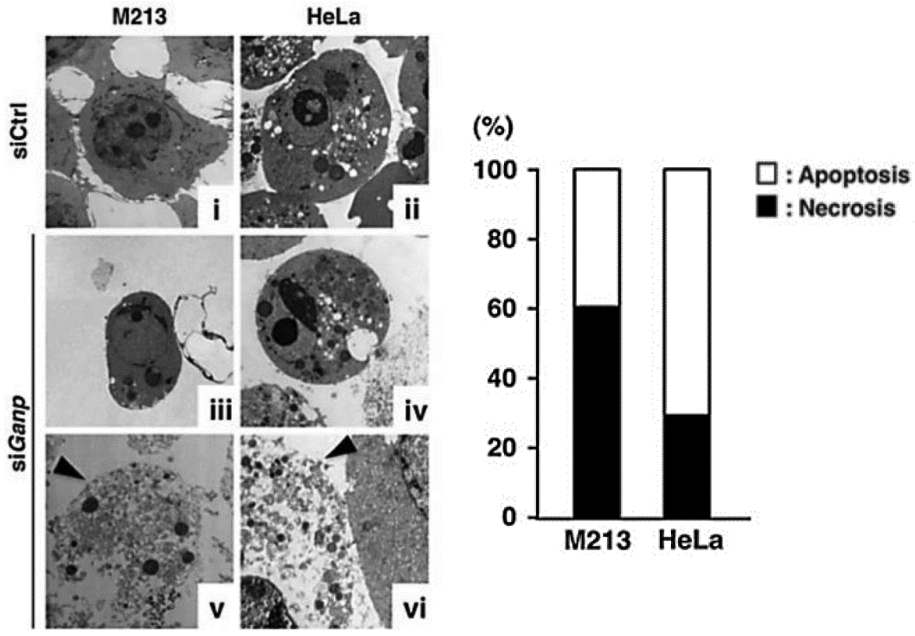


Figure 47 Cellular images of death cell morphologies in M213 and HeLa cells treated with siControl and siGanp for 48 h were analyzed by electron microscopy and the relative frequencies of cell apoptosis and necrosis in the dead cell population.

Source: Phimsen et al., 2012

Moreover, we analyzed the relative frequencies of normal cell, apoptosis, autophagy, and necrosis both in CCA cells population were shown by counting a number of cells in each group. Apoptosis and autophagy predominantly occur both in KKU-055 and in KKU-213A when suppressed SRSF1 compare with siControl (Figure 35). Next, we declined the number of normal cells from the analysis, the result shown that apoptosis, autophagy, and necrosis in KKU-055 and KKU-213A not difference between siControl and siSRSF1-2 (Figure 36). Finally, we declined a number of necrosis from the analysis, the result shown that apoptosis was occurred frequently between 45.1% (KKU-055) and 38.5% (KKU-213A). Similar autophagy occurred frequently between 17.7% (KKU-055) and 24.6 % (KKU-213A) as shown in Figure 37.

In addition, we declined the number of normal cells and necrosis from the analysis, the result shown that apoptosis occurred predominantly in KKU-055 (70.7%) and KKU-213A (58.4%) while autophagy occurred as minority count in KKU-213A (41.6%) and KKU-055 (29.3%) as shown in Figure 48. This result suggested that death morphology in both CCA cells has two main types including apoptosis and autophagy-dependent cell death.

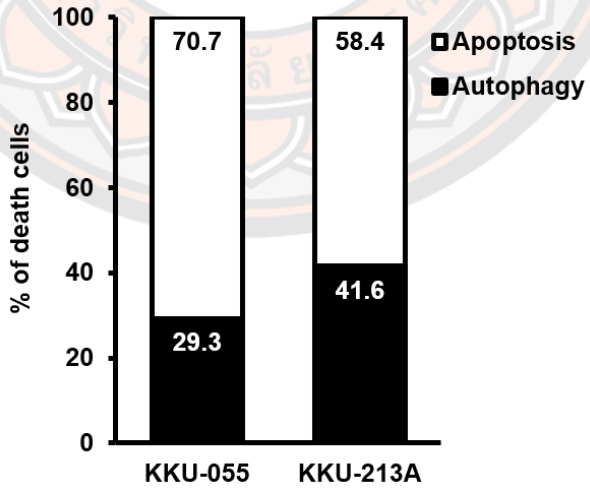


Figure 48 The relative frequencies of apoptosis, and autophagy in the KKU-055 and KKU-213A cell population were shown by counting at more 50 cells in each group by the three different examiners.

The survival of the cell, which subsequently determined the effect of SRSF1-silencing on apoptosis in KKU-055 and KKU-213A at 48 h by Annexin V and Death cell assay and caspase 3/7 activity assay. The population of the apoptotic cells was slightly increased apoptotic cell number (Figures 38 and 39) and activated caspase 3/7 activity (Figure 40) both in CCA cells. We confirmed the genotypic of apoptosis by western blot to determined apoptotic marker expression, the result showed that the expression levels of BAX (pro-apoptotic) was increased while the expression levels of Bcl-2 (anti-apoptotic) was decreased after transfecting siSRSF1 for 48 h (Figures 41 and 42). In addition, we also determined the protein expression of cleaved caspase-3, the result showed that the expression level of cleaved caspase-3 slightly increased both in CCA cells but the below figures were obtained from a single experiment as shown in Figure 49.

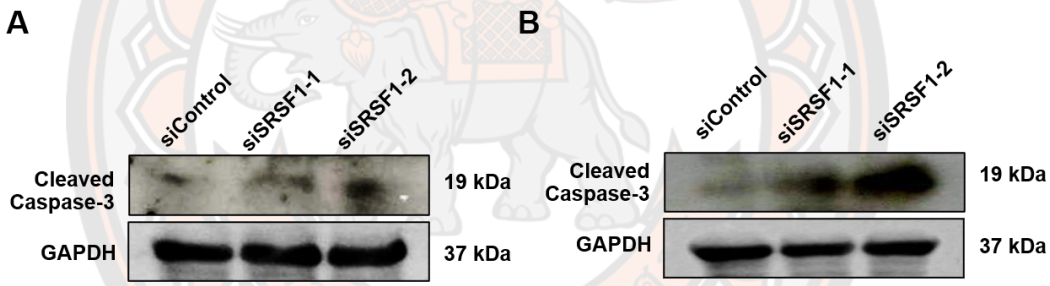


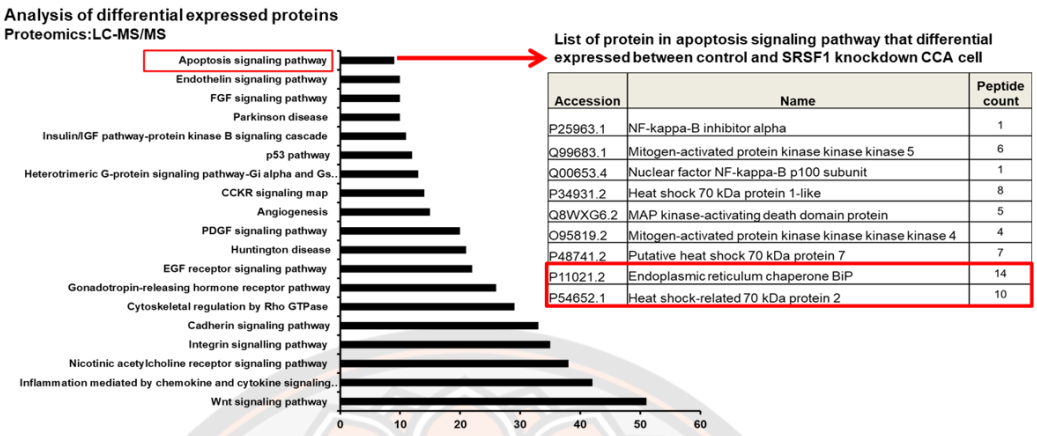
Figure 49 The expression of apoptotic marker in KKU-055. The expression levels of cleaved caspase-3 in KKU-055 (A) and KKU-213A (B).

There are several points found in the results those need to mention. The significant upregulation of BAX and significant downregulation of Bcl-2, slightly increase of early and late apoptosis cell population and higher activity of caspase 3/7 but not statistically significants. These results can be discussed by the timing of biochemical events of apoptosis activation cascades and sequentially reveals the apoptosis cell phenotypes.

As in the mechanism of apoptosis activation, the initial phase involves with an activation of Bcl-2 proteins and depolarization of the mitochondria is a relatively fast process, which happens within a several hours after stimuli triggering (Sundquist, Moravec, Niles, O'Brien, & Riss, 2006). Therefore, knockdown of SRSF1 at 48 h can activated BAX and suppressed Bcl-2 expression (Figures 41 and 42) as in the initial phase of apoptosis, but still not yet activated the last activation of caspase 3/7 in common pathway (final step of apoptosis) (Figure 40). In addition, the apoptosis cellular phenotype still not yet presented which cannot stained by Annexin V (Figures 38 and 39).

In addition, another important result from Pawee Tangwiriyarotkul on proteomic and PANTHER analysis of SRSF1 silencing CCA cells to identified differentially expressed proteins, his results demonstrated that suppression of SRSF1 differential expressed 9 proteins belonging to apoptosis pathway. Especially, we found BiP/GRP78 and HSP70 most differential expressed between control and SRSF1 silencing CCA cell as show in Figure 50A. Furthermore, we also proved that SRSF1 silencing can decreased BiP/GRP78 and HSP70 expression resulting CCA cell enter to death as show in Figure 50B. In addition, this study corresponded with the previous studies in lung cancer, knockdown of SF2/ASF increase apoptotic cells number and upregulated cleaved caspase-3 in A549 and NCI-H157 cells (Ezponda et al., 2010). Therefore, the suppression of SRSF1 can increase CCA cell apoptosis.

A



List of protein in apoptosis signaling pathway that differential expressed between control and SRSF1 knockdown CCA cell

Accession	Name	Peptide count
P25963.1	NF-kappa-B inhibitor alpha	1
Q99683.1	Mitogen-activated protein kinase kinase kinase 5	6
Q00653.4	Nuclear factor NF-kappa-B p100 subunit	1
P34931.2	Heat shock 70 kDa protein 1-like	8
Q8WXG6.2	MAP kinase-activating death domain protein	5
O95819.2	Mitogen-activated protein kinase kinase kinase kinase 4	4
P48741.2	Putative heat shock 70 kDa protein 7	7
P11021.2	Endoplasmic reticulum chaperone BiP	14
P54652.1	Heat shock-related 70 kDa protein 2	10

B

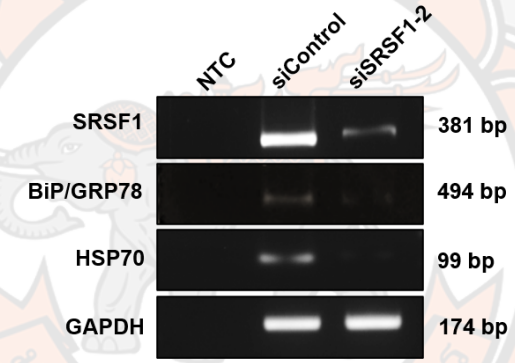


Figure 50 The suppression of SRSF1 in KKU-213 cell on apoptosis. Proteomic and PANTHER analysis classified identifying-proteins based on Biological process and ranked apoptotic signaling pathway in one of major class (A). Suppression of SRSF1-2 can decrease mRNA expression of BIP/GRP78 and HSP70 in KKU-213 when compared with siControl (B).

Next, we determined the effect of SRSF1-silencing on autophagy-dependent cell death in KKU-055 and KKU-213A at 48 h. there were determined autophagic marker expression by RT-PCR and western blot. The results showed that the mRNA expression of Beclin-1 and ATG5 was increased (Figures 43 and 44). In addition, the suppression of SRSF1 expression was increased the ratio between LC3B-II and LC3B-I protein and decreased p62 protein in KKU-055 and KKU-213 cells (Figures 45 and 46). These results suggested that SRSF1 not only relate to apoptosis of CCA cell. But we also proved the contribution of SRSF1 in autophagy of CCA cell.

Although, autophagy was initially defined as a survival mechanism of cell due to its role maintaining homeostasis under stressful conditions, several reports have revealed its dual role in cancer (Ávalos et al., 2014) and the therapeutic potential of its modulation (Chude & Amaravadi, 2017; Marinković, Šprung, Buljubašić, & Novak, 2018). In recent studies have described that autophagy has a therapeutic potential in CCA (Perez-Montoyo, 2020). Due to the dual role of autophagy in cancer cells, its modulation either by activation or by inhibition has emerged as a promising therapeutic strategy for cancer treatment, as shown summarized in Table 7.

Table 7 Preclinical studies with autophagy modulators in CCA.

Autophagy inhibitors					
Compound	**Mechanism of action**	**Preclinical models**	**Effect on CCA**	**Level of inhibition**	**Reference**
Capsaicin (major pungent component of chili peppers)	Interferes with NF-kB and AP-1 signaling	In vitro: QBC939, SK-ChA-1 and MZ-ChA-1. In vivo: QBC939 xenograft	Inhibition of 5-FU induced autophagy in vitro and in vivo via activation of PI3K/Akt/mTOR pathway, increasing sensitivity to 5-FU	Initiation: activatesmTOR	(Hong et al., 2015)
Chloroquine (antimalaria agent)	Alters acidic environment of lysosomes, induces sustained ER stress and CHOP-mediated apoptosis	In vitro: QBC939 cells	Induces apoptosis through activation of multiple death pathways and increases sensitivity to cisplatin	Fusion: Inhibits autophagosome fusion with lysosomes	(Jia et al., 2018)
Resveratrol (natural phenol, phytoalexin, produced by plants against infections)	Sirt1 agonist. Promotes deacetylation of FOXO1, blocking FOXO1 binding to Atg7	In vitro: QBC939 cells	Induces apoptosis by increasing oxidative stress and mitochondrial dysfunction.	Initiation: inhibits Foxo1-Atg7 activation	(He et al., 2018)

Table 7 (Cont.)

Autophagy activators					
Compound	**Mechanism of action**	**Preclinical models**	**Effect on CCA**	**Level of Activation**	**Reference**
Dihydroartemisinin (active compound fromArtemisiaannua)	ROS-mediated ER stress through DAPK activation promoting the disruption Beclin1-Bcl2	In vitro: KKU-452, KKU-023 and KKU-100, KKU-223 and MMNK-1	Induces apoptosis-dependent and autophagy-mediated apoptosis-independent cell death	Initiation: disruption of Beclin1-Bcl2	(Thongchot et al., 2018)
MiR-124 (associated with STAT3 regulation)	Targets EZH2 and STAT3 signaling pathway inducing ER stress	In vitro: HuCCT1, KMBC and MZChA1. In vivo MZChA1 transfected to stably express low levels of miR-124 or shEZH2	Induces autophagy-related cell death via EZH2-STAT3 signaling axis in vitro and tumor-suppressive function in vivo	Initiation: disruption of Beclin1-Bcl2	(Ma et al., 2018)
Piperlongumine (small molecule extracted from plants)	Inhibits the antioxidant enzyme glutathione S-transferase P, leading to elevated ROS via pathways (p38/JNK, MAPK and NN-KB)	In vitro: HuCCT-1	Induces apoptosis and autophagy through ROS-activated Erk signaling	Initiation: disruption of Beclin1-Bcl2	(H. T. Chen et al., 2019)

This recent results in this thesis suggest that the high expression of SRSF1 can be promoted cancer cells evading cell death. The effect of SRSF1-silencing can induce apoptosis and autophagy-dependent cell death in CCA. Therefore, SRSF1 can be used target for the precision therapeutic strategy of CCA. for example, switching oligonucleotides (SSO) as a strategy to interfere with aberrant splicing, can prevent SRSF proteins from interacting with exon splicing enhancers (ESEs) located on the pre-mRNA (B. D. Wang & Lee, 2018), or to design the specific monoclonal antibody, protein inhibitor, or compounds extraction for suppression molecules about regulate SRSF1 function in CCA. Therefore, these results might be used as supportive information for applying the SRSF1 targeting as an alternative treatment strategy for CCA.

Conclusion

Serine/Arginine-rich Splicing Factors 1 or SRSF1 is the splicing regulatory protein that plays an essential role in the alternative splicing of pre-mRNA to mature mRNA. Dysregulation of SRSF1 can be generated the aberrant transcript variants and cancer related-protein isoforms to promote cancer evading cell death. We determined the mRNA and protein expression of SRSF1 in CCA cells (KKU-055and KKU-213A) compared with cholangiocyte (MMNK-1). The result presented SRSF1 mRNA and protein was predominantly up-regulated in CCA cells. Next, we performed the silencing of SRSF1 by 2 specific siRNAs in KKU-055 and KKU-213A. SRSF1-silencing CCA cells decreased cell survival and increase cell death, which can be confirmed by death morphological change in KKU-055 and KKU-213A at 72 h observed under transmission electron microscope (TEM) by presented type of death cell including the apoptosis and autophagy predominantly occurs in CCA cells. Molecular effect of SRSF1 silencing on apoptosis was verified by slightly increase early and late apoptotic cell population and activated caspase 3/7 activity, and also confirmed with apoptotic marker expression such as increased the expression of BAX and decreased expression of Bcl-2. In addition, we also proved the contribution of SRSF1 in autophagy of CCA cell. SRSF1-silencing CCA cells increased Beclin-1 and ATG5 mRNA and also increased the ratio between LC3B-II and LC3B-I protein and decreased p62 protein. This study revealed the high expression of SRSF1 in CCA cells promotes cancer cell evading cell death. When silencing of SRSF1 can induces CCA cells enter to apoptosis and autophagy. These are the informative data suggest that SRSF1 may involve promoting cancer cells evading cell death. Therefore, SRSF1 maybe support the possibility of applying the SRSF1 targeting to serve as an alternative target for the treatment strategy of CCA.

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APPENDIX

APPENDIX A LIST OF SOLUTIONS AND BUFFERS

1. Agarose gel electrophoresis

1.1. 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.

1.2. 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.

2. cDNA synthesis master mix

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

3. PCR master mix

Component	1x Reaction	
Distilled water 18 Ω (autoclaved)	8.7	µl
PCR mastermix (Bioline)	10	µl
Forward primer	0.4	µl
Reverse primer	0.4	µl
cDNA template	0.5	µl
Total	20	µl

4. SDS-PAGE and Western Blot

4.1 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.

4.2 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.

4.3 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	

4.4 10% Ammonium persulfate

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

4.5 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	

4.6 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.

4.7 10X Bolt & Mohoney transferring buffer

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

4.8 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.

4.9 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	

4.10 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.

5. Culture Medium

5.1 Complete media

Dulbecco's Modified Eagle Medium (DMEM) 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^{o}C and warmed in water bath at 37^{o}C before use.

5.2 Antibiotic free media

Dulbecco's Modified Eagle Medium (DMEM) was supplement with 10% v/v fetal bovine serum (FBS). Media was stored at 4^{o}C and warmed in water bath at 37^{o}C before use.

6. Transfection mixture

Control siRNA or siSRSF1 transfection complexes were prepared separately according to the manufacturer's protocol. The 6 µl Lipofectamine^{TM} 2000 reagent (Invitrogen) was dilute in 125 µl OptiMEM and incubated at room temperature for 10 min. For each siRNA to be transfected 75 ng were dilute in OptiMEM (GIBCO) up to 125 µl. The µl Lipofectamine/ OptiMEM suspension was combined with the siRNA/ OptiMEM one. Following 10 min incubation at room temperature, 250 µl of siRNA/ Lipofectamine were added to each well. Protocol was adjusted to smaller or larger cell volumes and numbers according to the protocol.

7. Fixative reagent

7.1 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.

APPENDIX B Experimental results; Raw data

Table B1 Expression of SRSF1 in CCA cells using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
MMNK-1	122.35	20.10	0.03	1.00
KKU-055	134.91	87.24	1.34	3.94
KKU-213A	140.48	45.07	0.92	1.95

Table B2 Expression of SRSF1 in CCA cells using western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
MMNK-1	117.12	68.55	0.59	1.00
KKU-055	126.81	164.97	1.30	2.22
KKU-213A	139.39	135.07	0.97	1.66

Table B3 Expression of SRSF1 knockdown efficiency in KKU-055 cells using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
Control siRNA	135.03	98.96	0.73	1.00
siSRSF1-1	133.14	50.34	0.38	0.52
siSRSF1-2	147.08	1.84	0.01	0.02

Table B4 Expression of SRSF1 knockdown efficiency in KKU-055 cells using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
Control siRNA	139.28	136.22	0.98	1.00
siSRSF1-1	135.00	102.13	0.76	0.77
siSRSF1-2	132.28	77.82	0.59	0.60

Table B5 Expression of SRSF1 knockdown efficiency in KKU-213A cells using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
Control siRNA	136.28	137.36	1.01	1.00
siSRSF1-1	131.49	30.90	0.23	0.23
siSRSF1-2	124.12	4.42	0.04	0.04

Table B6 Expression of SRSF1 knockdown efficiency in KKU-213A cells using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	SRSF1	SRSF1/GAPDH	
Control siRNA	118.27	102.71	0.87	1.00
siSRSF1-1	117.22	76.97	0.66	0.76
siSRSF1-2	114.04	45.54	0.40	0.46

Table B7 Effect of SRSF1 suppression on cell viability in KKU-055 cells using Trypan blue dye exclusion assay

KKU-055				
Sample	**% Cell survival and cell death**			
	Survival	**Death**	**Total**	**S.D.**
Untransfected control	80.12	18.88	100.00	0.39
Transfection control	80.68	19.32	100.00	0.89
Control siRNA	65.16	34.84	100.00	4.27
siSRSF1-1	60.80	39.20	100.00	4.07
siSRSF1-2	54.52	45.48	100.00	5.78

Table B8 Effect of SRSF1 suppression on cell viability in KKU-213A cells using Trypan blue dye exclusion assay

KKU-213A				
Sample	**% Cell survival and cell death**			
	Survival	**Death**	**Total**	**S.D.**
Untransfected control	91.95	8.05	100.00	0.83
Transfection control	91.89	8.11	100.00	1.51
Control siRNA	77.47	22.53	100.00	4.39
siSRSF1-1	69.37	30.63	100.00	9.15
siSRSF1-2	87.63	42.37	100.00	4.64

Table B9 Effect of SRSF1 suppression on cell apoptotic population in KKU-055 using Flow cytometry

KKU-055				
Sample	**Apoptotic cell number**			
	Early apoptosis	**Late apoptosis**	**Death**	**S.D.**
Control siRNA	3.49	11.85	15.34	2.85
siSRSF1-1	3.39	13.30	16.69	1.53
siSRSF1-2	3.53	16.32	19.85	0.40

Table B10 Effect of SRSF1 suppression on cell apoptotic population in KKU-213A using Flow cytometry

KKU-213A				
Sample	**Apoptotic cell number**			
	Early apoptosis	**Late apoptosis**	**Death**	**S.D.**
Control siRNA	5.06	4.58	9.64	3.12
siSRSF1-1	5.14	5.54	10.68	2.75
siSRSF1-2	6.43	4.99	11.42	3.19

Table B11 Effect of SRSF1 suppression on apoptotic enzyme in KKU-055 using Caspase 3/7 activity assay

KKU-055				
Sample	**Relative apoptotic enzyme**			
	1	**2**	**Mean**	**S.D.**
Control siRNA	1.00	1.00	1.00	0.000
siSRSF1-1	1.00	1.56	1.28	0.394
siSRSF1-2	1.03	1.94	1.48	0.641

Table B12 Effect of SRSF1 suppression on apoptotic enzyme in KKU-213A using Caspase 3/7 activity assay

KKU-213A				
Sample	**Relative apoptotic enzyme**		**Mean**	**S.D.**
	1	**2**		
Control siRNA	1.00	1.00	1.00	0.000
siSRSF1-1	1.09	1.07	1.08	0.019
siSRSF1-2	1.09	1.31	1.20	0.153

Table B13 Effect of SRSF1 suppression on pro-apoptosis (Cleaved caspase-3) in KKU-055 using Western blot

Sample	**Band Intensity**			**Normalized with GAPDH**
	GAPDH	**Cleaved caspase-3**	**Cleaved caspase-3 /GAPDH**	
Control siRNA	145.01	28.85	0.20	1.00
siSRSF1-1	147.14	42.79	0.29	1.46
siSRSF1-2	138.41	60.46	0.44	2.20

Table B14 Effect of SRSF1 suppression on pro-apoptosis (Cleaed caspase-3) in KKU-213A using Western blot

Sample	**Band Intensity**			**Normalized with GAPDH**
	GAPDH	**Cleaved caspase-3**	**Cleaved caspase-3 /GAPDH**	
Control siRNA	136.78	32.49	0.24	1.00
siSRSF1-1	129.26	91.32	0.71	2.97
siSRSF1-2	129.91	127.05	0.98	4.12

Table B15 Effect of SRSF1 suppression on pro-apoptosis (BAX) in KKU-055 using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	BAX	BAX/GAPDH	
Control siRNA	100.53	66.59	0.66	1.00
siSRSF1-1	102.38	80.52	0.79	1.19
siSRSF1-2	90.16	85.73	0.95	1.44

Table B16 Effect of SRSF1 suppression on pro-apoptosis (BAX) in KKU-213A using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	BAX	BAX/GAPDH	
Control siRNA	108.15	63.93	0.59	1.00
siSRSF1-1	107.30	74.23	0.69	1.17
siSRSF1-2	105.75	99.53	0.94	1.59

Table B17 Effect of SRSF1 suppression on anti-apoptosis (Bcl-2) in KKU-055 using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	Bcl-2	Bcl-2/GAPDH	
Control siRNA	100.53	66.83	0.66	1.00
siSRSF1-1	102.38	55.86	0.55	0.82
siSRSF1-2	90.16	35.77	0.40	0.60

Table B18 Effect of SRSF1 suppression on anti-apoptosis (Bcl-2) in KKU-213A using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	Bcl-2	Bcl-2/GAPDH	
Control siRNA	108.15	98.52	0.91	1.00
siSRSF1-1	107.30	75.85	0.71	0.78
siSRSF1-2	105.75	37.69	0.36	0.39

Table B19 Effect of SRSF1 suppression on autophagic marker (LC3B) in KKU-055 using Western blot

Sample	Band Intensity			Ratio
	LC3B-I	LC3B-II	LC3B-II/ LC3B-I	
Control siRNA	30.29	6.05	0.20	1.00
siSRSF1-1	45.27	19.97	0.44	2.21
siSRSF1-2	48.28	23.83	0.49	2.47

Table B20 Effect of SRSF1 suppression on autophagic marker (LC3B) in KKU-213A using Western blot

Sample	Band Intensity			Ratio
	LC3B-I	LC3B-II	LC3B-II/ LC3B-I	
Control siRNA	65.65	103.10	1.57	1.00
siSRSF1-1	54.77	114.65	2.09	1.33
siSRSF1-2	42.79	114.40	2.67	1.70

Table B21 Effect of SRSF1 suppression on autophagic marker (p62) in KKU-055 using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	p62	p62/GAPDH	
Control siRNA	110.69	66.10	0.60	1.00
siSRSF1-1	105.34	45.18	0.43	0.72
siSRSF1-2	102.89	20.29	0.20	0.33

Table B22 Effect of SRSF1 suppression on autophagic marker (p62) in KKU-213A using Western blot

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	p62	p62/GAPDH	
Control siRNA	108.15	40.07	0.37	1.00
siSRSF1-1	107.30	19.84	0.18	0.50
siSRSF1-2	105.75	11.17	0.11	0.29

Table B23 Effect of SRSF1 suppression on autophagic marker (Beclin-1) in KKU-055 using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	Beclin-1	Beclin-1/GAPDH	
Control siRNA	151.80	74.90	0.49	1.00
siSRSF1-1	141.45	97.47	0.69	1.41
siSRSF1-2	157.90	146.85	0.93	1.91

Table B24 Effect of SRSF1 suppression on autophagic marker (Beclin-1) in KKU-213A using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	Beclin-1	Beclin-1/GAPDH	
Control siRNA	159.68	120.40	0.75	1.00
siSRSF1-1	152.83	122.87	0.80	1.07
siSRSF1-2	159.66	148.23	0.93	1.23

Table B25 Effect of SRSF1 suppression on autophagic marker (ATG5) in KKU-055 using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	ATG5	ATG5/GAPDH	
Control siRNA	162.00	127.02	0.78	1.00
siSRSF1-1	163.57	148.13	0.91	1.16
siSRSF1-2	158.94	168.56	1.06	1.35

Table B26 Effect of SRSF1 suppression on autophagic marker (ATG5) in KKU-213A using RT-PCR

Sample	Band Intensity			Normalized with GAPDH
	GAPDH	ATG5	ATG5/GAPDH	
Control siRNA	140.47	95.62	0.68	1.00
siSRSF1-1	136.94	101.75	0.74	1.09
siSRSF1-2	140.47	158.47	1.13	1.66

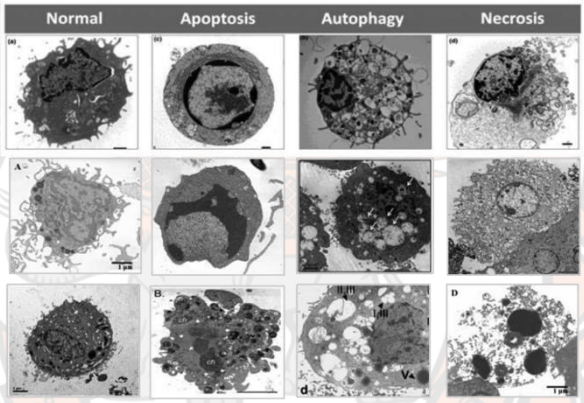
Death cell-TEM counting sheet

Name: …….. **Date:** .....
Sample: ……..
Sample code: **1**…… **2**……..
3…… **4**…….

Marked phenotypes

Features	Apoptosis	Autophagy	Necrosis
Membrane	Blebbing	Intact	Lysed
Nucleus	Shrinkage Chromatin condensation DNA fragmentation	Nucleus and chromatin minor changes	Swollen and late fragmentation of nucleus
Organelles	Apoptotic body	Autophagosome and autolysosome	Swelling and released

Figure morphology



Sample		Normal	Apoptosis	Autophagy	Necrosis	Total cells
……	1					
	2					
	3					
	4					
……	1					
	2					
	3					
	4					

Remarks:……
……..
……..
……

Reference:

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Figure B27 Death cell-TEM counting sheet (for at least 2-independent examiners)

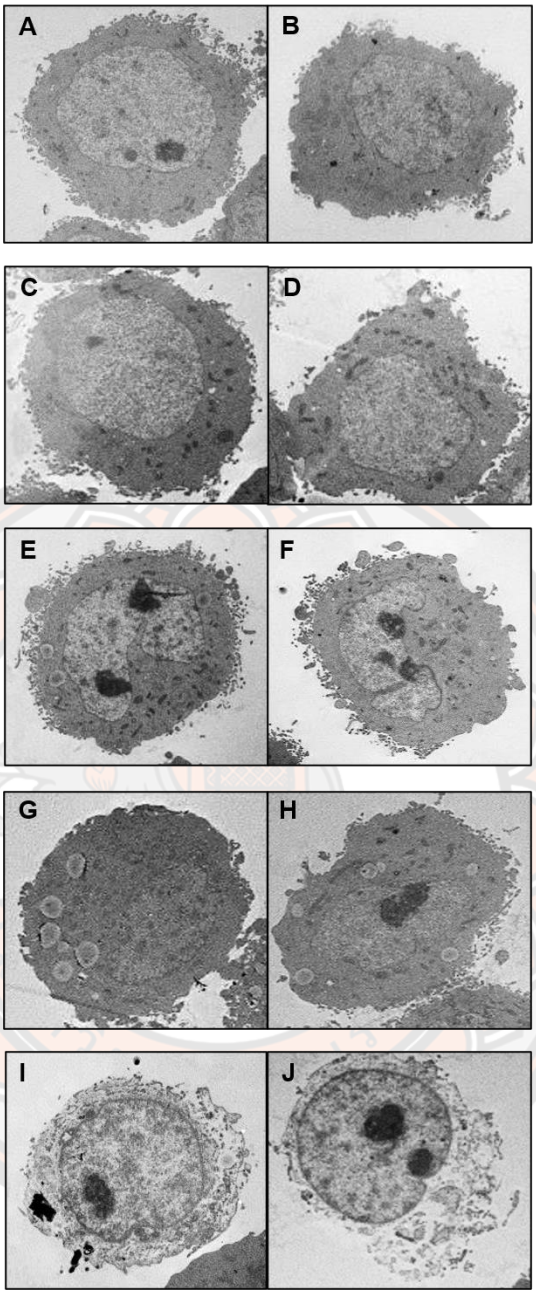


Figure B28 Representative cellular images of death cell morphologies in KKU-055 transfected cells under TEM observation. Negative siRNA transfected KKU-055 cells with normal morphology (A-B). siSRSF1 transfected KKU-055 cells with normal morphology (C, D), apoptotic cells (E, F), autophagic cells (G, H) and necrotic cells (I, J).

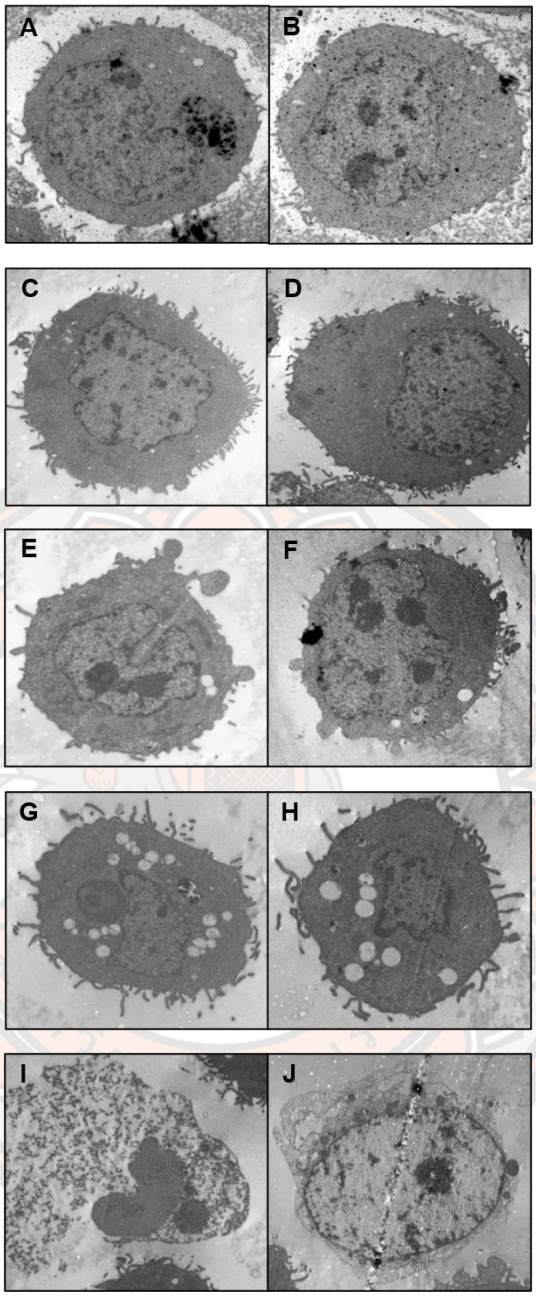


Figure B29 Representative cellular images of death cell morphologies in KKU-213A transfected cells under TEM observation. Negative siRNA transfected KKU-213A cells with normal morphology (A-B). siSRSF1 transfected KKU-213A cells with normal morphology (C, D), apoptotic cells (E, F), autophagic cells (G, H) and necrotic cells (I, J).

APPENDIX C Research presentation and publication

1. Research presentations

Poster presentations

Phichamon Phetchahwang, Pawee Tangwiriyarotkul, Sirithip Seesuksai, Sopit Wongkhan and Worasak Kaewkong. Targeting SRSF1 to induce apoptosis and autophagy-dependent cell death in cholangiocarcinoma. **The 45th annual meeting of Korean Cancer Association and 5th International Cancer Conference (KCA2019).** Seoul Dragon City Hotel, Seoul, Korea. 20-21 June 2019

2. Research publication

Proceeding (Oral presentation)

Phichamon Phetchahwang, Pawee Tangwiriyarotkul, Sirithip Seesuksai, Nuttanan Hongsrichan and Worasak Kaewkong. Silencing of SRSF1 expression induces apoptosis and autophagy-dependent cell death in Cholangiocarcinoma Cell. **The 21st National Graduate Research Conference March 27, 2020 at Khon Kaen University (NGRC2020)** (Full paper published in the proceeding book).

Review article (in Thai)

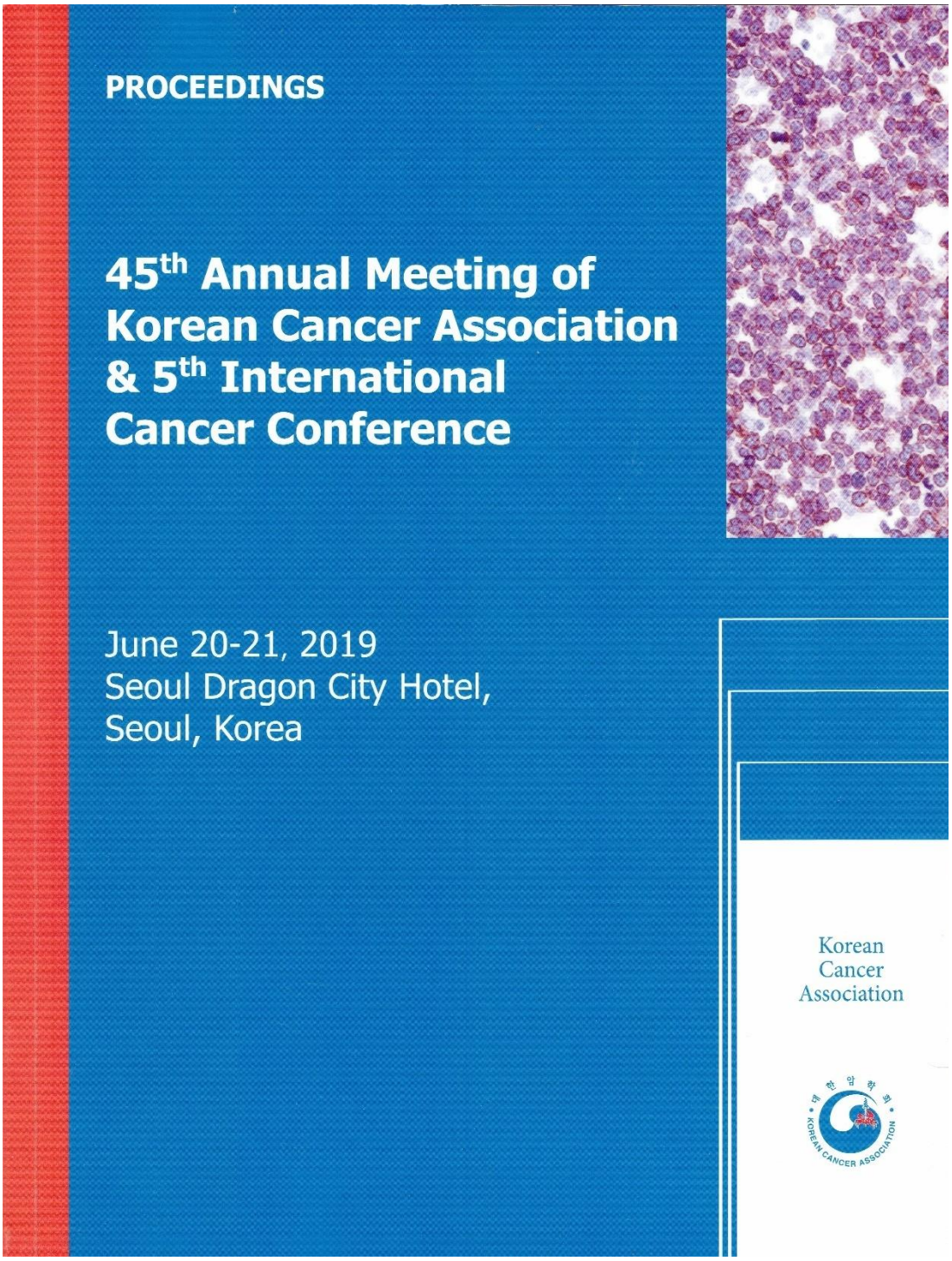
Phichamon Phetchahwang and Worasak Kaewkong. Elevating the autophagic process by balancing the biological clock (review article). Srinagarind Medical Journal (SMJ). 2020: 35(3); May-June 2020.

Manuscript preparation (for submission to Gene-to-Cell journal)

Phichamon Phetchahwang, Pawee Tangwiriyarotkul, Sirithip Seesuksai, Nuttanan Hongsrichan, Sopit Wongkham, Worasak Kaewkong. Targeting SRSF1 induces apoptosis and autophagy-dependent cell death of cholangiocarcinoma cells.

Cover page

The 45th annual meeting of Korean Cancer Association and 5th International Cancer Conference (KCA2019)



Abstract page

The 45th annual meeting of Korean Cancer Association and 5th International Cancer Conference (KCA2019)

this study, we investigated the mechanisms by which Hsp70 prevents apoptotic and autophagic programmed cell death of cancer cells. Under the stress condition, Hsp70 acetylation prevented apoptosis by association with apoptotic protease-activating factor (Apaf)-1 and apoptosis-inducing factor (AIF), key modulators of caspase-dependent and -independent apoptotic pathways. Hsp70 acetylation also inhibited autophagic cell death by upregulation of autophagy-related genes and autophagosome induction. Taken together, these results suggest that Hsp70 acetylation plays as a key regulator in cell death pathways.

P1-009

Naa10 promotes cancer cell survival via Hsp70 acetylation

Yu Ran Na[1], So Jin Shin[1], Seungmee Lee[1], Hyewon Chung[1], Tae-Kyu Jang[1], Eunsom Choi[1], Chi-Heum Cho[1], Eunyoung Ha[2], Ji Hae Seo[2,*]

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The acetyltransferase Naa10 was first identified in *Saccharomyces cerevisiae*. Mammalian Naa10 might have an important role in cell survival; however, studies of the function of Naa10 in tumorigenesis have yielded controversial results. For example, the depletion of Naa10 prevented cancer cell proliferation and induced cell death, suggesting the contribution of Naa10 to cancer cell survival. In contrast, Naa10 was required for caspase activation during apoptosis and reduced cancer cell survival. Because of these conflicting reports, it is uncertain whether the role of Naa10 in cancer cell is anti-apoptotic or pro-apoptotic. Hsp70s is the one of chaperone proteins that are required for the maintenance of protein homeostasis. Hsp70 is highly induced in response to cellular stressors and contributes to cellular resistance to stress-induced cell death. To combat stressful conditions, most tumor cells maintains elevated levels of Hsp70. In this study, we found that Naa10 enhances cancer cell survival through the regulation of chaperone function of Hsp70. Upon stress induction, Naa10 promptly acetylates Hsp70 to promote the repair of denatured proteins. Subsequently, Hsp70 was deacetylated again by HDAC4. These acetylation and deacetylation states of Hsp70 were essential to maintain protein homeostasis, thus enhanced cancer cell survival under stress conditions. Therefore, Naa10-expressing cancer cells were more resistant to anti-cancer drug. Collectively, our results suggest that Naa10 promotes cancer cell survival via Hsp70 acetylation and modulation of Naa10-mediated Hsp70 acetylation might be helpful to cancer treatment.

P1-010

Targeting SRSF1 to induce apoptosis and autophagy-dependent cell death in cholangiocarcinoma

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Purpose Aberrant splicing of many important genes has been reported in cholangiocarcinoma (CCA). However, the function of splicing factors in CCA is still unclear. In this study, the roles of splicing factor SRSF1, especially on the ability to resist programmed cell death was investigated. **Methods** Expression of SRSF1 mRNA and protein were analyzed in both CCA cells and patient tissues by RT-PCR and western blot. Specific siRNAs were used to inhibit SRSF1 expression in CCA cells. The effects of SRSF1 silencing on apoptosis were studied by AnnexinV/7ADD-flow cytometry and caspases 3/7 activity assay, while effects on autophagy were determined by autophagic markers expression. Moreover, LC-MS/MS was used to explore the proteomics changes of SRSF1 silenced CCA cells. **Results** SRSF1 was predominantly upregulated in CCA cell lines and patient tissues. SRSF1-silenced CCA cells exhibited slightly increase apoptotic cell number and caspase 3/7 activity. Remarkably, autophagy was stimulated as represented by significantly increased LC3B-II/I ratio and ATG5, whereas decreased p62 expression. In addition, proteomic analysis identified differentially expressed proteins, especially 9 proteins belonging to cell death process. **Conclusions** This study shows that SRSF1 plays important role in CCA by helping cancer cells to evade from cell death with potential intracellular mediators of cell death evading abilities have been identified. SRSF1 has a lot of potential, but it will still require further characterization before it could be used as an alternative therapeutic target for CCA.

P1-011

Withaferin A induces apoptosis in neuroblastoma cells via mitochondrial dysfunction

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Poster

The 45th annual meeting of Korean Cancer Association and 5th International Cancer Conference (KCA2019)

Presentation code: **P1-010**

Cell Death

Targeting SRSF1 to induce apoptosis and autophagy-dependent cell death in cholangiocarcinoma

Phichamon Phetchahwang[1], **Pawee Tangwiriyarotkul**[1], **Sirinthip Seesuksai**[1], **Sopit Wongkham**[2], **Sittiruk Roytrakul**[3], **Worasak Kaewkong**[1,*]

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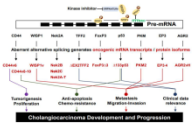
KCA Korean Cancer Association
45th Annual Meeting & 5th International Cancer Conference
제45차 대한암학회 학술대회 및 제5차 국제암컨퍼런스

BIOTEC a member of NSTDA

Introduction

Cholangiocarcinoma (CCA) is malignant cancer arising from bile duct epithelium which shows the highest incidence in Thailand.



Our group published a review article to summarize the aberrant mRNA splicing of important genes in CCA. We demonstrated a number of splicing genes and their contributions with cancer development and also driving cancer to evade cell death.

Therefore, we focused the splicing factor protein, **SRSF1** or **Serine/Arginine-rich Splicing Factor 1**. SRSF1 plays an essential role to enhance alternative splicing of pre-mRNA.

Many article reported dysregulation of SRSF1 can generated aberrant transcript variants or oncogenic-protein isoforms and relates with resisting ability to programmed-cell death in various cancer model including breast, lung and colon cancers.

Objectives: To investigate the roles of SRSF1 on the resisting cell death capacity of CCA cells, particularly to clarify the specific type of cancer cell death.

Conceptual framework & Experimental Design

Expression of SRSF1

Cell lines: MMNK1 (Established cholangiocyte) KKU-055, KKU-213 (CCA cells)
RT-PCR, Western Blot

CCA Tissue: Tumor area vs. Adjacent mom-tumor area
Western Blot

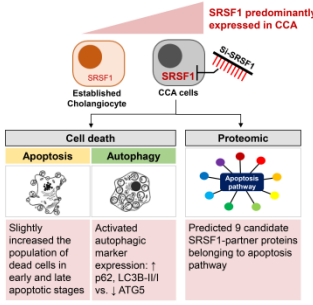
Suppression of SRSF1 expression
SRSF1 siRNAs (Fu, Y., et al, 2013 , Jiang L., et al, 2016)
RT-PCR, Western Blot

Determination of cell death
Apoptosis
AnnexinV/7-AAD staining
Caspase3/7 activity assay
Autophagy
RT-PCR (p62, ATG5)
Western blot (LC3BII/LC3BI)

Death underlying mechanism
Proteomic analysis
LC-MS/MS
Bioinformatics analysis
(PANTHER: Biological process)

Conclusion

SRSF1 is highly expressed in CCA cells and tissue. Suppression of SRSF1 can increased cell death and activated partner proteins belonging to apoptosis pathway.



"This study shows that SRSF1 plays important role in CCA by helping the cancer cells to evade from cell death. SRSF1 has a lot of potentials, but it still required further characterization before developing to be an alternative therapeutic target for CCA"

Results

Expression of SRSF1 in CCA

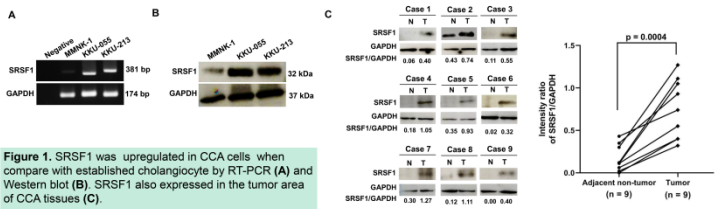


Figure 1. SRSF1 was upregulated in CCA cells when compare with established cholangiocyte by RT-PCR **(A)** and Western blot **(B)**. SRSF1 also expressed in the tumor area of CCA tissues **(C)**.

The effect of knockdown SRSF1 on cell death of CCA cell

Expression of SRSF1 knockdown

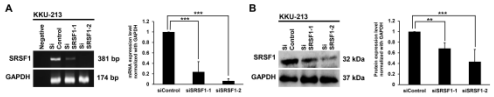


Figure 2. siRNA knockdown of SRSF1 in KKU-213 demonstrated significantly depletion of SRSF1 both in mRNA **(A)** and protein **(B)** level.

Apoptosis

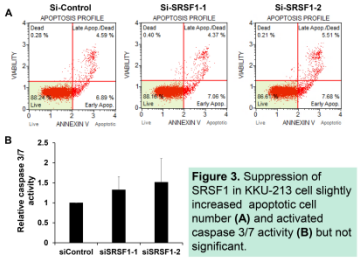


Figure 3. Suppression of SRSF1 in KKU-213 cell slightly increased apoptotic cell number **(A)** and activated caspase 3/7 activity **(B)** but not significant.

Autophagy

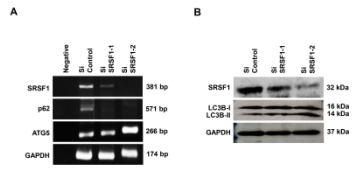
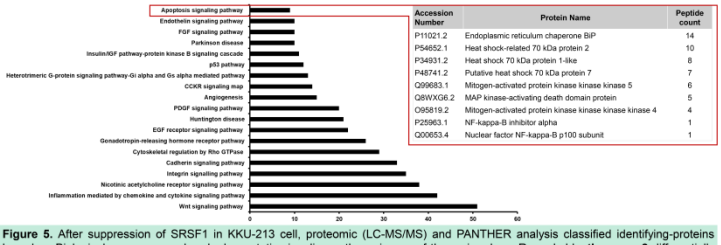


Figure 4. Suppression of SRSF1 in KKU-213 cell presented higher ATG5 and lower p62 mRNA expression **(A)** and increased LC3B-II/I ratio **(B)** which are the markers of autophagic death.

The proteomic profiles of SRSF1-silenced CCA cell



Accession Number	Protein Name	Peptide count
P11021.2	Endoplasmic reticulum chaperone BiP	14
P54652.1	Heat shock-related 70 kDa protein 2	10
P34931.2	Heat shock 70 kDa protein 1-like	8
P48741.2	Putative heat shock 70 kDa protein 7	7
Q99683.1	Mitogen-activated protein kinase kinase kinase 5	6
Q8WXG6.2	MAP kinase-activating death domain protein	5
O95819.2	Mitogen-activated protein kinase kinase kinase 4	4
P25963.1	NF-kappa-B inhibitor alpha	1
Q00653.4	Nuclear factor NF-kappa-B p100 subunit	1

Figure 5. After suppression of SRSF1 in KKU-213 cell, proteomic (LC-MS/MS) and PANTHER analysis classified identifying-proteins based on Biological processes and ranked apoptotic signaling pathway in one of the major class. Remarkably, **there are 9** differentially expressed proteins belonging to apoptosis pathway.

Discussion

This study suggested that targeting SRSF1 can induced CCA cells death. Regarding to activation of cell death marker expression, autophagy-dependent cell death was apparently approved whereas apoptosis was still unclear for SRSF1.

Next, we planned to perform Transmission electron microscopy (TEM) for investigating specific types of CCA cell death that resulting from SRSF1 suppression.

Furthermore, we would like to explore the upstream activation or molecular mechanism of SRSF1 induced cell death in CCA.

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Acknowledgements: Thailand Research Fund and Office of the Higher Education Commission (MRG6080014). Thailand Research Fund and Medical Research Council-UK (Newton Fund) (DBG5980004).

Cover page

The 21st National Graduate Research Conference March 27, 2020 at Khon Kaen University (NGRC2020)

Abstract page

The 21st National Graduate Research Conference March 27, 2020 at Khon Kaen University (NGRC2020)

21st NGRC March 27, 2020 การประชุมวิชาการเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 21 วันที่ 27 มีนาคม 2563 ณ มหาวิทยาลัยขอนแก่น

MMO1

การยับยั้งการแสดงออกของโปรตีน SRSF1 เพื่อเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสและออโตฟาจีในเซลล์มะเร็งท่อน้ำดี

Silencing of SRSF1 Expression Induces Apoptosis and Autophagy-dependent Cell Death in Cholangiocarcinoma Cell

พิชามนชุ์ เพ็ชรฉวาง (Phichamon Phetchahwang)* ปวีร์ ตั้งวิริยโรจน์กุล (Pawee Tangwiriyarotkul)*
ศิรินทิพย์ สีสุกไส (Sirinthip Seesuksai)** ดร.ณัฐนันท์ หงษ์ศรีจันทร์ (Dr.Nuttanan Hongsrichan)***
ดร.วรศักดิ์ แก้วก่อง (Dr.Worasak Kaewkong)****

บทคัดย่อ

โรคมะเร็งท่อน้ำดีเป็นโรคมะเร็งชนิดที่มีอุบัติการณ์สูงในประเทศไทย มีอัตราการตายสูงเนื่องจากวิธีการตรวจวินิจฉัยที่ยังไม่มีประสิทธิภาพเนื่องจากยังไม่มีตัวบ่งชี้สำหรับการวินิจฉัยในระยะแรก ข้อมูลล่าสุดได้มีการรวบรวมไอโซฟอร์มของโปรตีนที่เกิดจากการตัดแต่งยีนที่ผิดปกติและสัมพันธ์กับการพัฒนาของโรคมะเร็งท่อน้ำดี กระบวนการนี้ถูกควบคุมโดย Serine/Arginine-rich Splicing Factors (SRSFs) การศึกษาครั้งนี้ ผู้วิจัยจึงทำการศึกษาโปรตีน SRSF1 และผลการศึกษายืนยันว่ามีการแสดงออกสูงขึ้นในเนื้อเยื่อผู้ป่วยและในเซลล์มะเร็งท่อน้ำดี เมื่อศึกษาผลการยับยั้งการแสดงออกของ SRSF1 ด้วย small interfering RNA (siRNA) พบว่ามีการตายของเซลล์มะเร็งท่อน้ำดีเพิ่มขึ้น โดยพบการตายของเซลล์ทั้งแบบอะพอพโทซิสและออโตฟาจี ดังนั้น โปรตีน SRSF1 อาจสามารถใช้เป็นโมเลกุลเป้าหมายใหม่เพื่อพัฒนาการรักษาแบบการแพทย์แม่นยำสำหรับโรคมะเร็งท่อน้ำดี

ABSTRACT

Cholangiocarcinoma (CCA) is a high incidence cancer in Thailand which presents high mortality rate resulting from low sensitivity of diagnosis. Recent publication summarized the contribution of aberrant alternative splicing with CCA development, this process is regulated by Serine/ Arginine-rich splicing factors or SRSFs. In this study, we focused on SRSF1 and demonstrated that SRSF1 was upregulated in both CCA patients' tissues and CCA cells. After SRSF1 expression was suppressed by small interfering RNA (siRNA). Increasing of death cell number was demonstrated and categorized into apoptosis and autophagy-dependent cell death. Therefore, SRSF1 might be serving as a new alternative target for precision therapeutic strategy of CCA.

คำสำคัญ: มะเร็งท่อน้ำดี SRSF1 การตายของเซลล์

Keywords: Cholangiocarcinoma, SRSF1, Cell death

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21st NGRC March 27, 2020 การประชุมวิชาการเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 21
วันที่ 27 มีนาคม 2563 ณ มหาวิทยาลัยขอนแก่น

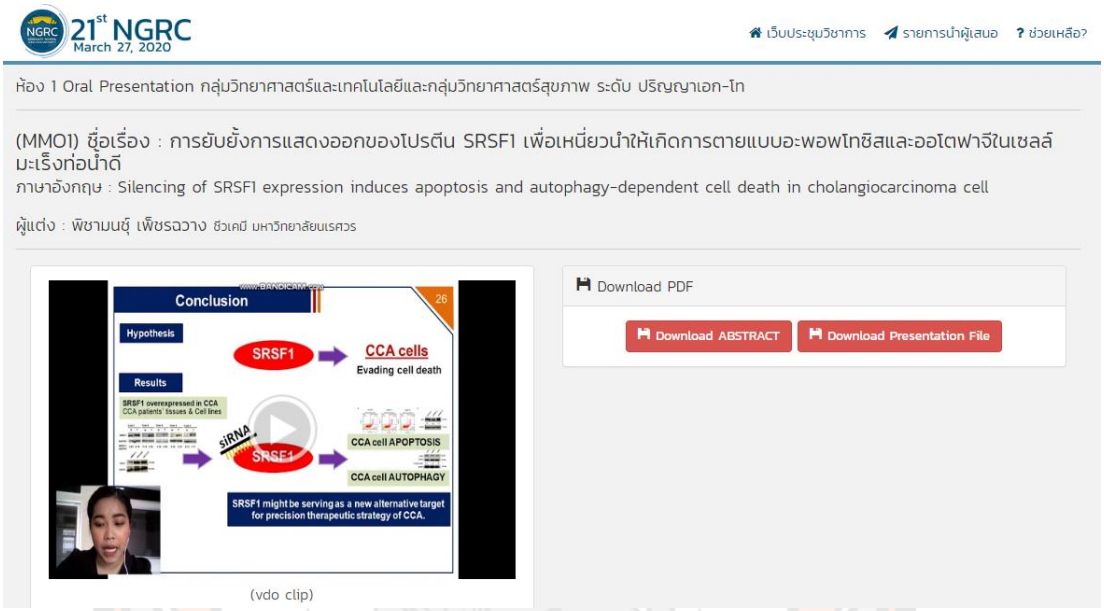
รายชื่อผลงานวิจัยที่ได้รับการพิจารณาให้นำเสนอแบบบรรยาย (Oral Presentation)
กลุ่มวิทยาศาสตร์และเทคโนโลยี ระดับปริญญาเอก และกลุ่มวิทยาศาสตร์สุขภาพ ระดับปริญญาโท (ห้อง 1)
ตามห้องนำเสนอ ในเว็บไซต์ https://conference.kku.ac.th/grc/

ที่	รหัส	เวลา	ชื่อ - สกุล	สถาบัน	สาขา	ชื่อเรื่องภาษาไทย	หน้า
1	SDO1	09.00-11.00 น.	นายบัณฑิต พรหมรักษา	มหาวิทยาลัยขอนแก่น	ชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล	การศึกษาฤทธิ์ของสารประกอบในการยับยั้งเซลล์มะเร็งท่อน้ำดี	1 [1],*,***
2	SDO4	09.00-11.00 น.	นางสาวนารีลักษณ์ ตั้งศรีศักดา	มหาวิทยาลัยขอนแก่น	กายวิภาคศาสตร์	ผลของแอลกอฮอล์ต่อการเปลี่ยนแปลงการแสดงออกของโปรตีนในอัณฑะของหนูเพศผู้เต็มวัย	2 [1],*,***
3	SDO5	09.00-11.00 น.	นางสาวจีรติ พรหมพิภักดิ์	มหาวิทยาลัยขอนแก่น	ชีวเคมี	ฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีของสารสกัดเอทานอลจากผลิตภัณฑ์น้ำลูกยอเมื่อทดสอบร่วมกับยาฟลูออโรยูราซิลในระดับหลอดทดลองและสัตว์ทดลอง	3 [1],*,***
4	MMO1	09.00-11.00 น.	นางสาวพิชามนชุ์ เพ็ชรฉวาง	มหาวิทยาลัยนเรศวร	ชีวเคมี	การยับยั้งการแสดงออกของโปรตีน SRSF1 เพื่อเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสและออโตฟาจีในเซลล์มะเร็งท่อน้ำดี	4-13 ***
5	MMO2	09.00-11.00 น.	นายอนุชา พรมกันยา	มหาวิทยาลัยขอนแก่น	การพยาบาลเด็ก	ผลของโปรแกรมเตรียมความพร้อมผู้ดูแลต่อพฤติกรรมการดูแลเด็กวัยก่อนเรียนโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันลิมโฟลาสต์ที่ได้รับยาเคมีบำบัดครั้งแรกในระยะชักนำให้โรคสงบ	14-26 ***
6	MMO3	09.00-11.00 น.	นางอรพินิต ภูวงษ์ไกร	มหาวิทยาลัยขอนแก่น	การผดุงครรภ์	การพัฒนาแบบคัดกรองปัจจัยเสี่ยงต่อการคลอดก่อนกำหนด	27-38 ***

หมายเหตุ *[1]ป.เอก *ไม่ประสงค์ตีพิมพ์ ***บทความดูในเว็บไซต์ https://conference.kku.ac.th/grc/*

Oral presentation online page

The 21st National Graduate Research Conference March 27, 2020 at Khon Kaen University (NGRC2020)



Certificate page

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21st NGRC
March 27, 2020

บัณฑิตวิทยาลัย มหาวิทยาลัยขอนแก่น

มอบเกียรติบัตรนี้ไว้เพื่อแสดงว่า

นางสาวพิชามนชุ์ เพ็ชรฉวาง

ได้นำเสนอผลงานวิจัยระดับบัณฑิตศึกษาออนไลน์ แบบบรรยาย

ระดับปริญญาโท กลุ่มวิทยาศาสตร์สุขภาพ

ในการประชุมวิชาการเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ ๒๑

ณ มหาวิทยาลัยขอนแก่น

วันที่ ๒๗ มีนาคม ๒๕๖๓

(ศาสตราจารย์สุรศักดิ์ วงศ์รัตนชีวิน)

คณบดีบัณฑิตวิทยาลัย มหาวิทยาลัยขอนแก่น

Review article (in Thai) page

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การส่งเสริมกระบวนการออโตฟาจีด้วยสมดุลของนาฬิกาชีวิต

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Elevating the Autophagic Process by Balancing the Biological Clock

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ออโตฟาจี (autophagy) หรือการกินตัวเองของเซลล์ เป็นกระบวนการที่เซลล์ใช้กำจัดองค์ประกอบของเซลล์ที่เสื่อมสภาพเพื่อป้องกันการทำงานที่ผิดปกติของเซลล์ ซึ่งจะก่อให้เกิดโรค โดยกระบวนการนี้มีส่วนเกี่ยวข้องกับนาฬิกาชีวิต ซึ่งเป็นวงจรที่ควบคุมระบบการทำงานต่าง ๆ ภายในร่างกายในขณะนอนหลับและตื่น นาฬิกาชีวิตจะกระตุ้นให้มีการหลั่งฮอร์โมนที่จำเป็นต่อการซ่อมแซมเนื้อเยื่อส่วนต่าง ๆ ในขณะเดียวกันกระบวนการออโตฟาจีร่วมทำหน้าที่กำจัดของเสียที่สะสมไว้ เมื่อร่างกายอยู่ในสภาวะพักผ่อนน้อยหรือไม่เหมาะสมจนส่งผลกระทบต่อนาฬิกาชีวิต อาจเพิ่มความเสี่ยงต่อการเป็นโรคต่าง ๆ เช่น โรคของระบบประสาทและความจำ โรคเบาหวาน โรคไขมันพอกตับ โรคหัวใจและหลอดเลือด และโรคมะเร็ง เป็นต้น ดังนั้นการพักผ่อนที่เพียงพอและเหมาะสม ตลอดจนการเลือกรับประทานอาหารที่มีประโยชน์ร่วมกับการออกกำลังกายจะช่วยปรับสมดุลนาฬิกาชีวิตและช่วยกระตุ้นกระบวนการออโตฟาจีเพื่อกำจัดโปรตีนหรือเซลล์เสื่อมสภาพ นำไปสู่การมีสุขภาพที่แข็งแรง ลดความเสี่ยงต่อการเป็นโรคและการมีชีวิตที่ยืนยาว

คำสำคัญ: การกินตัวเองของเซลล์, นาฬิกาชีวิต, ออโตฟาจี

Autophagy or self-eating is a cellular process that facilitates the elimination of deteriorated cellular components to prevent cellular dysfunction and disease development. This mechanism interrelates with the biological clock, the controlling system in the living organism through sleep-wake cycle. During sleep, biological clock stimulates tissue repairing hormone secretion, while the autophagy acts mainly on the clearance of metabolic wests. In the sleep deprivation state, the biological clock will be interfered and this could lead to several diseases such as neurodegenerative diseases, diabetes, fatty liver, cardiovascular diseases and cancers. Therefore, the healthy sleep habits combined with healthy diet and exercise will improve the biological clock and the activation of autophagy mechanism for better life and longevity.

Keywords: self-eating, biological clock, autophagy

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บทนำ

ระบบร่างกายของมนุษย์มีกระบวนการทำงานระดับเซลล์ที่ทำหน้าที่ย่อยสลายองค์ประกอบที่เสื่อมสภาพของเซลล์ เพื่อนำกลับมาใช้ใหม่เรียกว่า Autophagy หรือ ออโตฟาจี โดยศัพท์คำนี้เกิดจากการรวมคำในภาษากรีกสองคำได้แก่ คำว่า Auto- หมายถึง ตัวเอง และคำว่า Phagein หมายถึง การกิน จึงหมายถึง กระบวนการที่เซลล์กินชิ้นส่วนของตัวเอง จึงเปรียบเสมือนกระบวนการกำจัดขยะและรีไซเคิลภายในเซลล์ โดยเฉพาะองค์ประกอบที่เป็นโปรตีนเพื่อนำกรดอะมิโนกลับมาใช้ซ้ำ หากกระบวนการนี้ทำงานราบรื่น การทำงานระดับเซลล์ต่าง ๆ ก็จะเป็นปกติ ในทางตรงกันข้าม หากกระบวนการนี้ทำงานบกพร่อง การทำงานระดับเซลล์ก็อาจเกิดความผิดปกติ มีการสะสมของเสียและองค์ประกอบที่เสื่อมสภาพภายในเซลล์ ซึ่งอาจจะก่อให้เกิดโรคต่าง ๆ ยกตัวอย่างเช่น โรคสมองเสื่อม โรคอัลไซเมอร์ โรคพาร์กินสัน โรคเบาหวาน โรคไขมันพอกตับ โรคหัวใจและหลอดเลือดและโรคมะเร็ง เป็นต้น[1]

เมื่ออายุมากขึ้น การสร้างโปรตีนหรือองค์ประกอบของเซลล์ที่ผิดปกติจะเพิ่มมากขึ้น รวมทั้งกระบวนการออโตฟาจีทำงานลดลง ร่างกายจึงจำเป็นต้องมีกลไกกระตุ้นกระบวนการออโตฟาจีให้กลับมาทำงานอย่างมีประสิทธิภาพ ซึ่งหนึ่งในกลไก

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