# CELLULAR ACTIVITIES OF SERINE-ARGININE PROTEIN KINASE INHIBITORS IN CUTANEOUS AND OCULAR MELANOMA CELLS

JESADAGORN SIRIWATH

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Biochemistry)
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Thesis entitled "Cellular activities of Serine-Arginine Protein Kinase inhibitors in cutaneous and ocular melanoma cells"

By JESADAGORN SIRIWATH

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** CELLULAR ACTIVITIES OF SERINE-ARGININE PROTEIN KINASE INHIBITORS IN CUTANEOUS AND OCULAR MELANOMA CELLS
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## ABSTRACT

Melanoma is a tumor resulting from the malignant transformation of melanocytes in various organ, include skin and eye. This cancer is a serious health problem in countries with high sun or UV exposure. Due to a generally late detection, high invasive and metastatic properties, and the lack of effective treatments in melanoma. That reason lead to melanoma has very poor prognostic and high mortality rate. Aberrant alternative splicing lead to the expression of aberrant mRNA transcripts has been associated in the progression of various cancers. Serine/Arginine-riched Splicing Factors (SRSFs) are responsible for an alternative splicing and their functions are regulated by phosphorylation via Serine-Arginine Protein Kinases (SRPKs) that have been reported an overexpression in melanoma cells. In this study, the effects of SRPK1/2-specific inhibitor SRPIN340 and SRPK1-specific inhibitor SPHINX31 (SRPKi) were demonstrated. The phosphorylation profile in A375 cutaneous and 92-1 ocular melanoma cells in comparison with HaCat keratinocyte, were investigated by Western blot. The result found that most of phosphorylation profile more highly expressed in A375 and 92-1 than HaCat. Then, cell viability in A375 in comparison with 92-1, were determined by MTT viability assays. The result showed that the effect of SRPKi on viability of melanoma cells were presented as dose- and time-dependent manners. Next, the molecular effects of SRPKi treatment on melanoma cells were determined, using Western blot. Suppression of phosphorylated forms (pSRSFs) was observed. Then, SRSFs translocation were

confirmed by Immunocytofluorescent (ICF) and show that SRSFs presented in cytoplasmic greater than nucleus. Proliferation ability of melanoma cells was examined by clonogenic assay, the results showed that both SRPIN340 and SPHINX31 reduced the size of cancer cell colonies. Particularly, in 92-1 cell showed more sensitive to SRPIN340 than A375 cell. Furthermore, both SRPIN340 and SPHINX31 could reduce AKT phosphorylation through dysfunction of SRPKs. The findings from this study should serve as the basis information for targeting SRPKs as an alternative therapeutic strategy and its downstream pathways.

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JESADAGORN SIRIWATH

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# ABBREVIATIONS

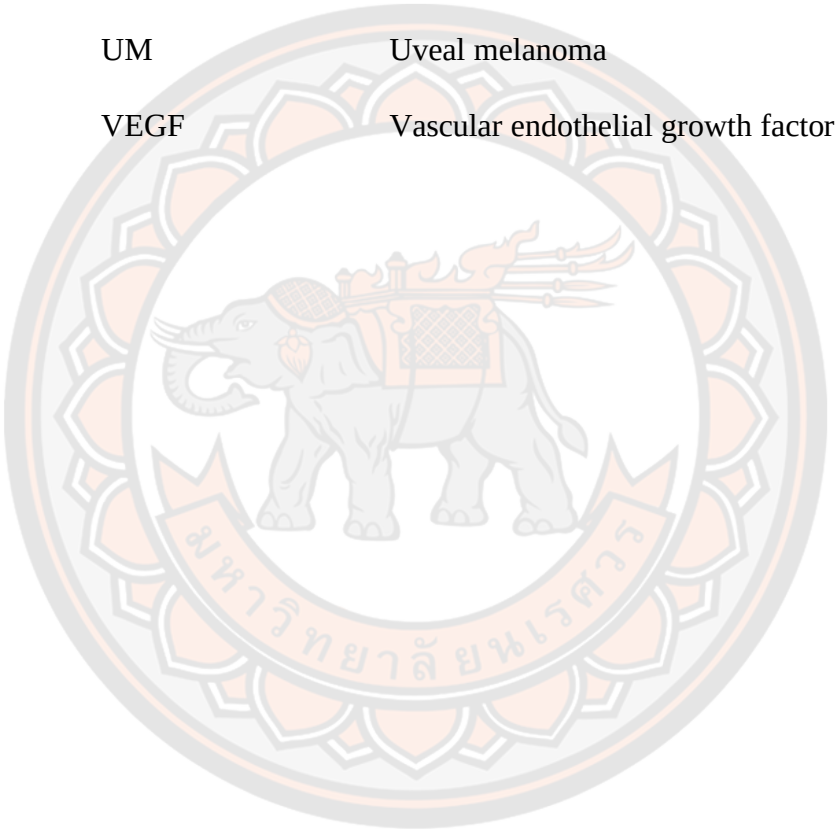
| | |
|---|---|
| AM | Acral melanoma |
| AMD | Age-related macular degeneration |
| AML | Acute myelogenous leukemia cell |
| AS | Alternative splicing |
| ASR | Age-standardized incidence rate |
| CLKs | CDC2-like kinases |
| CM | Cutaneous melanoma |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Fetal bovine serum |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GNAQ | Guanine nucleotide binding protein (G protein) |
| GNA11 | G Protein Subunit Alpha 11 |
| hnRNPs | Heterogeneous nuclear ribonucleoproteins |
| HRP | Horseradish peroxidase |
| ICF | Immunocytofluorescence |
| kDa | Kilo Dalton |
| MAPK | Mitogen-Activated Protein Kinase |
| MC1R | Melanocortin receptor 1 |

# ABBREVIATIONS (CONT.)

| | |
|---|---|
| MM | Mucosal melanoma |
| mRNA | Messenger ribonucleotide acid |
| MTT | (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) |
| MW | Molecular weight |
| NRVMs | Neonatal rat ventricular myocytes |
| p53 | Tumor protein 53 |
| PBS | Phosphate-buffered saline |
| PHLPP | PH domain and Leucine rich repeat Protein Phosphatases |
| PVDF | Polyvinylidene fluoride |
| RAC1B | Ras-related C3 botulinum toxin substrate 1B |
| RBPs | Retinol-binding proteins |
| RIPA | Radio-immunoprecipitation assay |
| RPE | Retina pigment epithelial |
| RPMI | Roswell park memorial institute |
| RNA | Ribonucleic acid |
| RRM | RNA recognition motif |
| SDS | Sodium dodecyl sulfate |
| shRNA | short hairpin RNA |
| siRNA | Small interfering RNA |
| snRNP | Small nuclear ribonucleoprotein |

# ABBREVIATIONS (CONT.)

| | |
|---|---|
| SPHINX31 | SR protein phosphorylation inhibitor X 31 |
| SRPIN340 | SR protein phosphorylation inhibitor 340 |
| SRPKs | Serine-arginine protein kinase |
| SRSFs | Serine/arginine-rich splicing factors |
| UM | Uveal melanoma |
| VEGF | Vascular endothelial growth factor |



# CHAPTER I

# INTRODUCTION

## Introduction and Research Significance

In everyday life, human is generally sensitized with mutagens and carcinogens, especially from the environmental exposure, then promote cancer development and progression. A melanoma is a tumor produced by the malignant transformation of melanocytes, which located at various anatomic locations, including skin and ocular region. The risk factors of melanoma include exposure of ultraviolet (UV) radiation with genetic defects and lead to promote melanoma development. The main etiology is sun exposure over a lifetime and remarkably, Recent evidence showed that the risk of melanoma was higher in people who expose to UVA more than UVB. The incidence of malignant melanoma has been rapidly increasing worldwide. Overall, melanoma ranks the fifth most common malignancy in men and the seventh most common malignancy in women, accounting for 5% and 4% of all new cancer cases, respectively. Although it is a rare disease, a number of death cases are caused by metastasize to secondary sites, such as brain, lymph nodes and liver. Current diagnosis and treatment of melanoma may not sufficient to cure the tumor completely. The incidence of melanoma varies greatly between countries and these different incidence patterns are ascribed to variations in racial skin phenotype including Thailand. Therefore, understanding the basic molecular pathogenesis and cellular mechanism contributes to melanoma development is essential. The information of initial study on molecular carcinogenesis will be developed for being early diagnostic biomarkers or to provide alternative therapeutic strategy for melanoma patients.

In 2020, several evidences mentioned the contribution of molecular event in melanoma, especially the alteration of alternative splicing. The researchers suggest the profiling of prognostic alternative splicing in melanoma, systemic analysis of the prognosis-related RNA alternative splicing signals in melanoma, and alternative splicing events, are indicators for the prognosis of uveal melanoma.

Alternative splicing is the post-transcriptional modification process 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, Alternative splicing is categorized to be additional hallmarks of cancer. Many important genes in various types of human cancer present the alternative splicing isoforms and the oncogenic properties of them. In melanoma, there is the number of research articles showed the aberrant alternative splicing occurred in many important genes and presents their contributions to promoting melanoma development and procession. The aberrant genes regulate alternative splicing by SR protein family.

There are 3 major classes of the regulator of alternative splicing including heterogeneous nuclear ribonucleoproteins (hnRNPs), RNA-binding proteins (RBPs), and serine and arginine-rich (SR) protein family. Especially, SR protein family is the important classes of splicing regulatory proteins that play an essential role in the alternative splicing of pre-mRNA to mature mRNA. Moreover, upstream modulators of these regulators, serine-arginine protein kinase (SRPKs) are splicing regulatory proteins that play an essential role in the phosphorylation of the serine residuals in the serine/arginine-rich repeats (RS domain) of various mRNA splicing factors such as, SC35, U2AF65, and alternative splicing factor or SRSF. The expression and activation of SRPKs may lead to aberrant alternative splicing and potentially contribute to various diseases and in particular to the development of cancer. The important alterations occur in the mRNA splicing included the overexpression or dysregulation of function in regulatory spicing factors that mentioned as SRPKs and also the abnormality of their target molecules, which are SR protein can generate multiple abnormal protein isoforms involved in the oncogenesis and progression of several cancers.

There are several studies demonstrated that SRPK can be act as an oncogenic protein, which regulate alternative splicing of various tumor suppressor genes for initiating the cancer progression. Moreover, SRPK can be a target protein for cancer treatment. For example, mice subcutaneously injected with melanoma cell after SRPIN340 (SRPK1/2 inhibitor) treatment, showed significantly reduce in tumor growth. This result may be associated with impairing SRPK1 nuclear translocation and SR protein phosphorylation in the intracellular environment by SRPK protein

inhibitor. Therefore, the effect of SRPK protein inhibitors on the cellular activity of SRPK and the cellular phenotype of cancer cell is interesting.

This study aims to determine the effect of SRPK protein inhibitors on SR protein (SRSFs) phosphorylation in cutaneous and ocular melanoma cell lines. Next, dose- and time-dependent inhibitory effects of SRPK protein inhibitors on proliferation and growth will be verified. In addition, target protein of proliferation and growth-related signaling pathway will be monitored. The expectation of this study is to demonstrate that SRPKs could be able to develop as the specific therapeutic target for melanoma.

**Research Objectives**

1. To investigate the phosphorylation profile of SRSFs in cutaneous melanoma (A375) and ocular melanoma (92-1) cells in comparison with non-cancerous cells (HaCat).

2. To investigate the half maximal inhibitory concentration (IC50) of SRPK protein inhibitors (SRPIN340 and SPHINX31) in A375, HaCat, and 92-1 cells (for selection the most efficient inhibitory activity).

3. To investigate the effect of SRPK inhibitor on SRSF phosphorylation and subcellular translocation in A375 and 92-1 cells.

4. To determine the effect of SRPK inhibitor on the growth and the activation of growth-related signaling pathway in A375 and 92-1 cells.

**Research Scope**

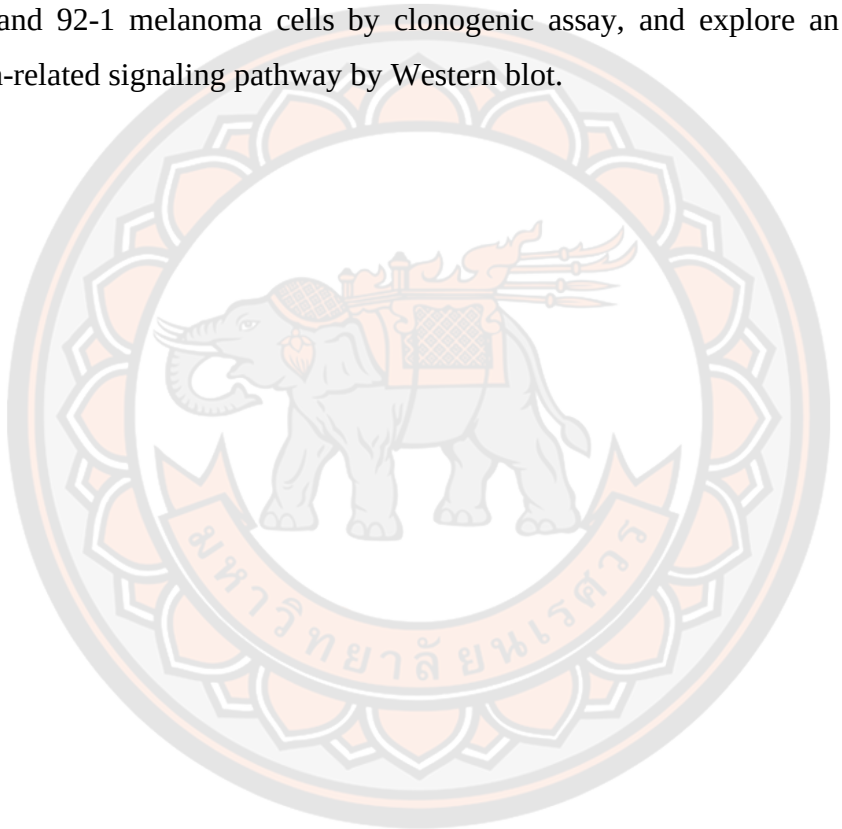
The research proposal is designed into 4 parts;

**Part 1**: Investigate the phosphorylation profile of SRSFs in cutaneous melanoma (A375), and ocular melanoma (92-1) compared with keratinocyte (HaCat) cells by Western blot.

**Part 2:** Investigate IC50 of the half maximal inhibitory concentration of SRPK protein inhibitors (SRPIN340 and SPHINX31) in A375, and 92-1 compared with HaCat cells by MTT assay.

**Part 3:** Investigate the effect of a selected SRPK inhibitor on target protein phosphorylation and translocation in A375 and 92-1 melanoma cell lines by Western blot and immunocytofluorescence.

**Part 4:** Determine the effect of a selected SRPK inhibitor on the growth of A375 and 92-1 melanoma cells by clonogenic assay, and explore an activation of growth-related signaling pathway by Western blot.



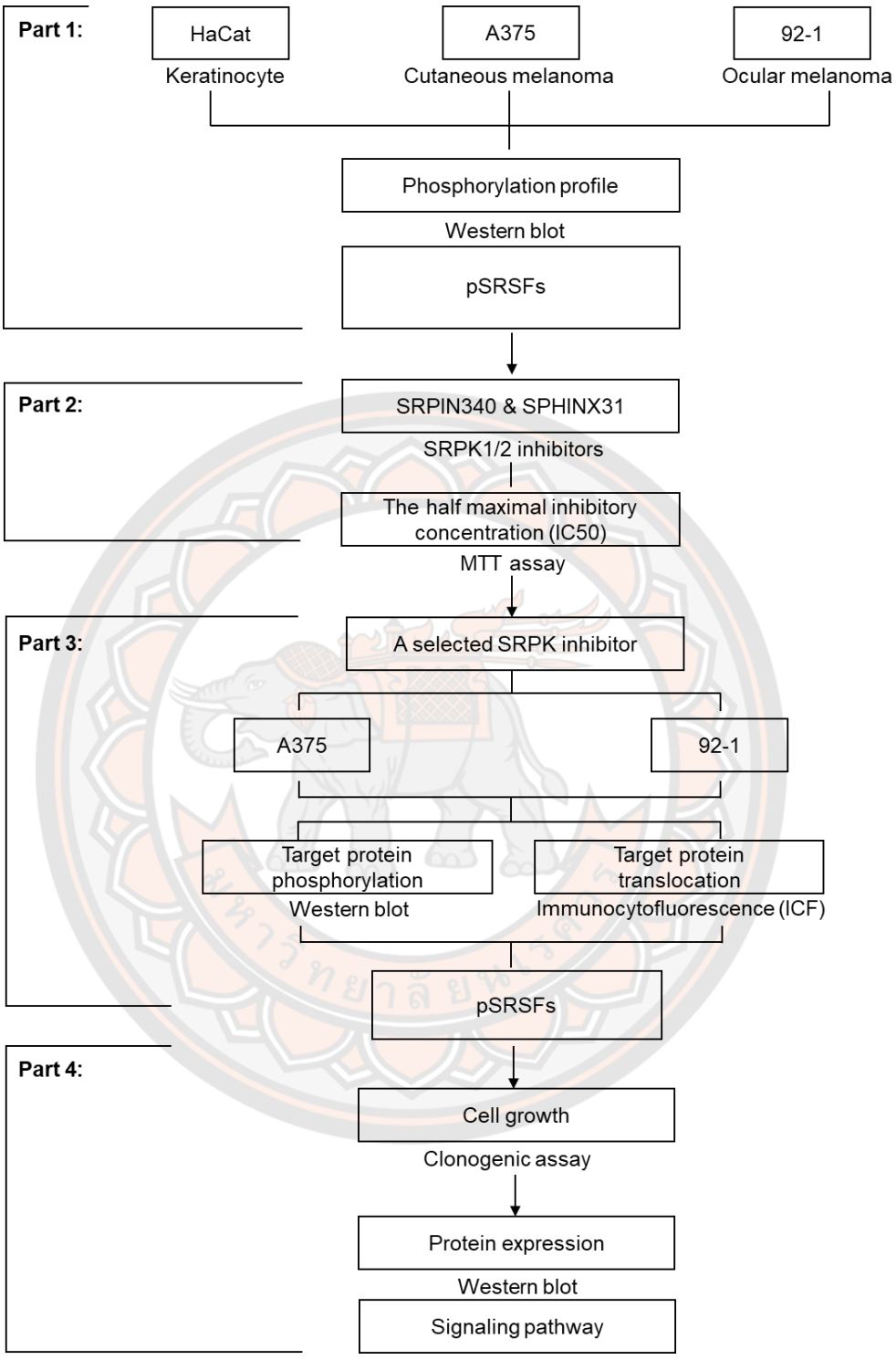



**Figure 1** Schematic diagram shown scope of this study.

**Keywords**

Alternative splicing, Melanoma, Phosphorylation, SRPK, SRSF

**Research Hypothesis**

SRPK inhibitors (SRPIN340 and SPHINX31) can reduce the growth of melanoma cells (cutaneous and ocular melanoma cells) by reduced splicing factors (SRSFs) phosphorylation and deactivation of the specific growth-related signaling pathway.

**Expected outcomes**

1. SRPK inhibitors (SRPIN340, SPHINX31) can block SRPK activity in melanoma cells, which will be observed by decrease of SRSF phosphorylation and alter subcellular translocation.

2. SRPK inhibitors (SRPIN340, SPHINX31) can reduce melanoma cell proliferation and growth through the growth-related signaling pathway.

# CHAPTER II

# LITERATURE REVIEWS

## 1.1 Melanoma

1.1.1 Morphology and classification

Melanoma, the most aggressive type of skin cancer, develops from melanocytes and accounts for approximately 4% of all cancer types diagnosed in adolescents worldwide. Even the incidence rate of melanoma is lower than other types of skin cancer, it can grow rapidly and metastasize into the secondary organs such as liver when it is not detected and treated in early stage. Therefore, late-stage melanoma can be difficult to treat and terrible. The 5-year survival rate ranges between 15% and 60% depending on the stage of the melanoma (Ma F.C. et al., 2019). Current therapeutic options for melanoma patients consist of surgical excision, chemotherapy, immunotherapy, and targeted therapy. Additionally, metastatic melanoma is one of the most highly mutated, heterogeneous and lethal types of cancer (Kozar I. et al., 2019).

The classification of melanoma is based on the tissue from which the primary tumor arises and includes four main subtypes; First, cutaneous melanoma (CM), which arises in non-glabrous skin. Second, uveal melanoma (UM) which develops from melanocytes in the uveal tract of the eye. Third, acral melanoma (AM), a distinct form that originates in glabrous skin of the palms, soles and nail beds Forth, mucosal melanoma (MM), the rarest subtype, which arises from melanocytes in the mucosal lining of inter-nal tissues (Rabbie R. et al., 2019) as shown in figure 2.

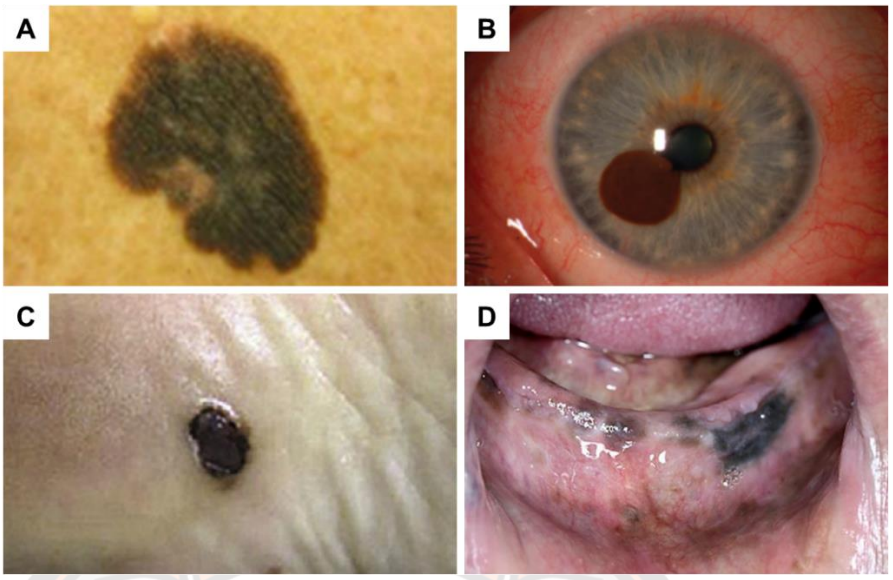



**Figure 2** Morphology of melanoma subtypes. cutaneous melanoma (A), uveal melanoma (B), acral melanoma (C), and mucosal melanoma (D).

**Source**: (A) Klebanov N. et al., 2019; (B) Kaliki S. et al., 2017; (C) Moon K.R. et al., 2018; (D) Deinlein T. et al., 2017

1.1.2 Cutaneous melanoma: epidemiology, risk factor and carcinogenesis

Cutaneous melanoma is a disease with low incidence that develops from abnormality of melanocytes located predominantly in the epidermis. However, it is highly capable of spreading and more resistant to chemotherapy than other types of skin cancers until leading to the cause of death (Laikova K.V. et al., 2019). They are unnoticeable or asymptomatic in early stages but the signs in severity with metastasis into other organs will be presented. The worldwide incidence of cutaneous melanoma has been increasing annually at a more rapid rate compared to any other type of cancer (Leonardi G. C. et al., 2018). Studies of the occurrence of melanoma across the South American continent have shown a strong correlation between white-skinned European ancestry and the risk of melanoma. The burden imposed by melanoma is borne primarily by Australasian, North American, and European populations, as well as individuals from all populations who are elderly male (Laikova K.V. et al., 2019) as shown in figure 3. In 2019, a report from the United States found that estimated

new case 96,480 case and over 7,230 case who die from this cancer (Siegel R.L. et al., 2019).

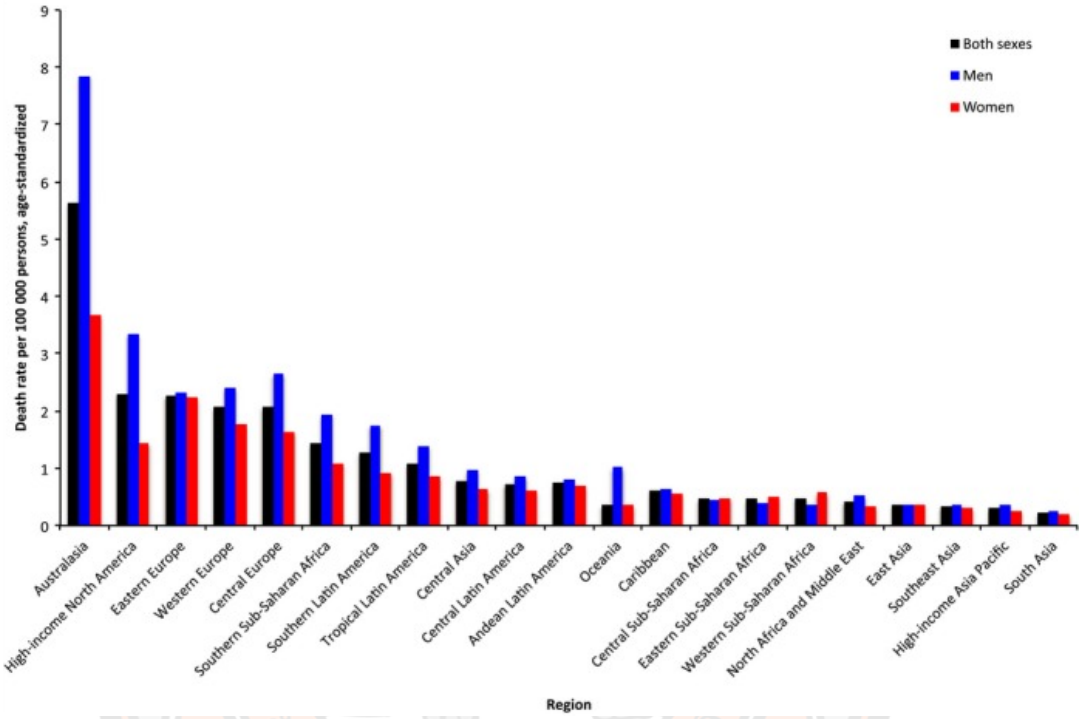



**Figure 3** Melanoma mortality rates in 21 world regions.

**Source**: Karimkhani C. et al., 2015

In Thailand, melanoma is a rare disease but possess the most common cause of mortality among skin cancers. There have been reports the age-standardized incidence rate (ASR) of melanoma in male of the northern region of Thailand was approximately 0.5 per 100,000 population. Remarkably, the ASR of melanoma in female was 0.4, 0.4, 0.5, and 0.27 per 100,000 population in Phitsanulok, Chiang Mai, Lamphun, and Lampang respectively (Suwanrungraung K. et al., 2007; Imsamran W. et al., 2015) as shown in figure 4.

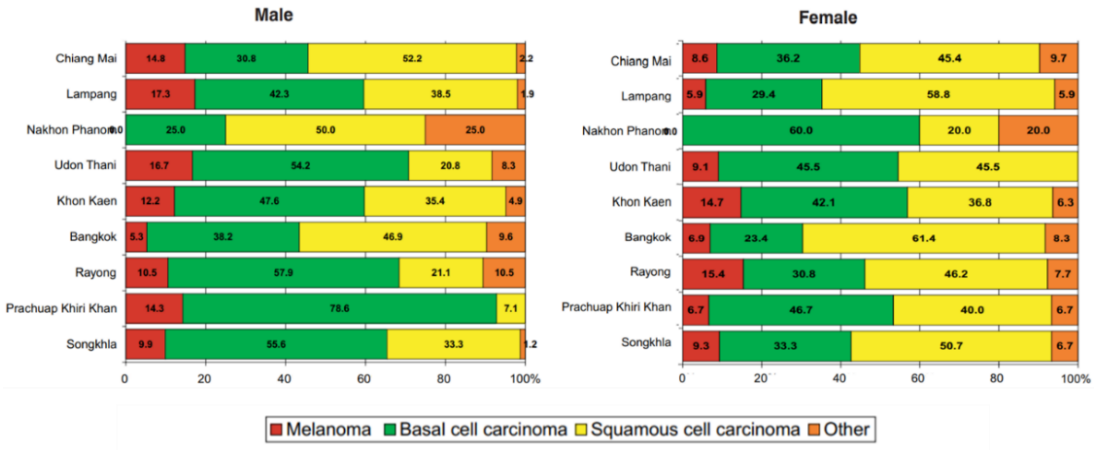



**Figure 4** Incidence report of melanoma in Thailand.

**Source:** Suwanrungraung K. et al, 2007

Ultraviolet (UV) radiation from sunlight is the main environmental risk factor for melanoma development. The increased risk of melanoma due to sun exposure is directly associated with the UV radiation level and in particular to the UV-B spectrum (Laikova K.V. et al., 2019). Generally, UV radiation is an important risk factor for all skin cancers and is considered as a complete carcinogen because it is responsible for damaging DNA and gene mutations, which lead to carcinogenesis.

Carcinogenesis is the formation of a cancer, which normal cells are transformed into cancer cells. The process is characterized by changes at the cellular, genetic, and epigenetic levels and abnormal cell division. Normally the balance between proliferation and programmed cell death, in the form of apoptosis, is maintained to ensure the integrity of tissues and organs. but cancer can survive and develop process involving initiation, growth, promotion, conversion, propagation, invasion and metastasis (Liu Y. et al., 2015) as shown in figure 5.

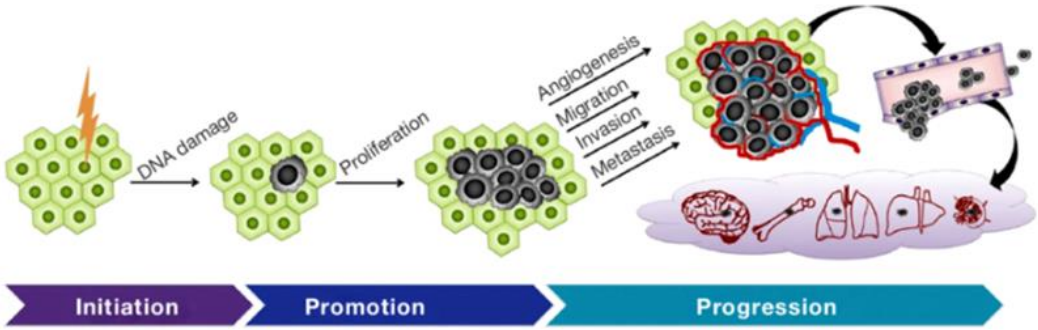



**Figure 5** Three-phase process of carcinogenesis when carcinogen administration based on animal models of primary cancers.

**Source:** Liu Y. et al., 2015

In addition, sun exposure patterns and timing have been associated in a number of studies with an increased risk of melanoma (Strickland F.M. et al., 2000; De Fabo E.C. et al., 2004; Noonan F.P. et al., 2012; Wäster P. et al., 2017). Particularly, intense and intermittent sun exposure is associated with a higher risk compared to a chronic continuous pattern of sun exposure that is more frequently associated with actinic keratosis and non-melanoma skin cancers. In response to UV-induced DNA damage, skin keratinocytes produce the melanocyte stimulating hormone (MSH) that binds the melanocortin receptor 1 (MC1R) on the melanocytes that produce and release melanin. The melanin pigment is a shield for preventing further DNA damage from UV radiation (Nasti T.H.  et al., 2015) as shown in figure 6.

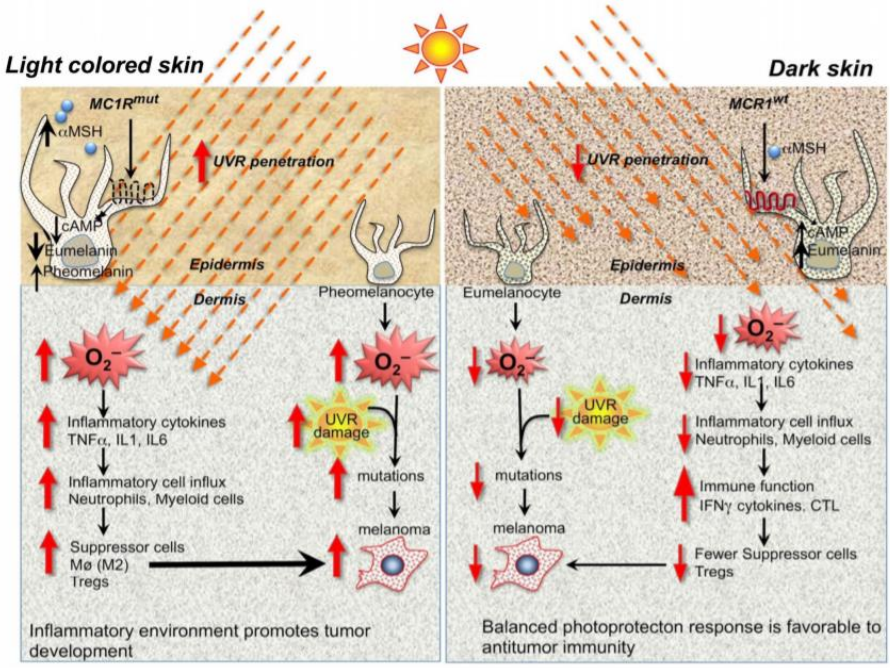



**Figure 6** Mechanism of ultraviolet radiation on DNA damage. In dark-colored individuals, there is balanced inflammation, less ROS production, higher T-cells activity that remove mutated cells and leads to microenvironment favorable to antitumor immunity. The light-colored individuals have high inflammatory response, high ROS generation, less T-cell activity and leads tumor development.

**Source:** Nasti T.H. et al., 2015

Furthermore, abnormalities at the gene level can develop cutaneous melanoma. For example, melanoma is associated with abnormalities and mutations of human Serine/threonine-protein kinase B-raf (BRAF) gene. Common mutation of BRAF is BRAF(V600E) which accounting as 50% approximately in cutaneous melanoma. BRAF proteins are associated with the transmission of the Mitogen-Activated Protein Kinase (MAPK) pathway, a pathway that deals with cell proliferation (Ascierto P.A. et al., 2012).

1.1.3 Ocular melanoma: epidemiology, risk factor and carcinogenesis

Ocular melanoma is a rare disease that develops from abnormality of melanocytes located predominantly in the eye (Amaro A., et al. 2017). The human

eye consists of 3 main layers include the outer layer composes of sclera and cornea as. In addition, it is lined by conjunctiva. The next layer is the middle layer or uveal track that is a wall in the middle of 3 layers. The blood vessels and melanocytes are most found in this position. It consists of 3 main parts which are iris, ciliary muscle, and choroid. About the final layer is the inner layer where nerves of the eye will be found. It consists of an important part is retina (Kaliki S. et al., 2017). Ocular melanoma can be classified and named according to the location of disease such as Uveal melanoma or commonly recognized as UM and another type, Conjunctival melanoma as shown in figure 7.as UM and another type, Conjunctival melanoma as shown in figure 7.

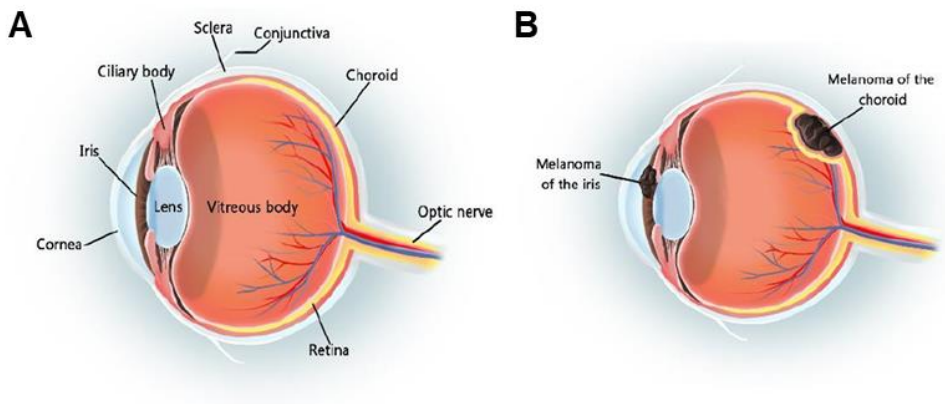



**Figure 7** Anatomy of the eye (A). Ocular melanoma (B).

Ocular melanoma incidence varies by sex, race, and country. Males are higher incidence than females and this cancer has the higher incidence in European Americans who are white skin when compared with blacks and Asians (Krantz B. A. et al., 2017). However, the evidence is unclear for ocular melanoma in Thailand because it is often reported to combine with melanoma in other areas. Even though, the initial stages of the disease will not lead to death. In the event of it is not diagnosed, cancer can develop to affect vision and spread to various organs, especially the liver until causing danger your life.

The mechanism and risk factor of occurrence of ocular melanoma is similar to the mechanism described above of cutaneous melanoma. However, abnormalities or mutations at the gene level related to cancer are different. For example, there are

many studies of ocular melanoma found that the GNAQ / GNA11 mutation is 84% (Van Raamsdonk C.D. et al., 2009; Van Raamsdonk C.D. et al., 2010). Other studies present the GNAQ / GNA11 mutation in patients with metastasis from ocular melanoma in 96 percent mutation, that results in cells proliferation and migration into cancer cells (Chattopahdyay C. et al., 2016) as shown in figure 8.

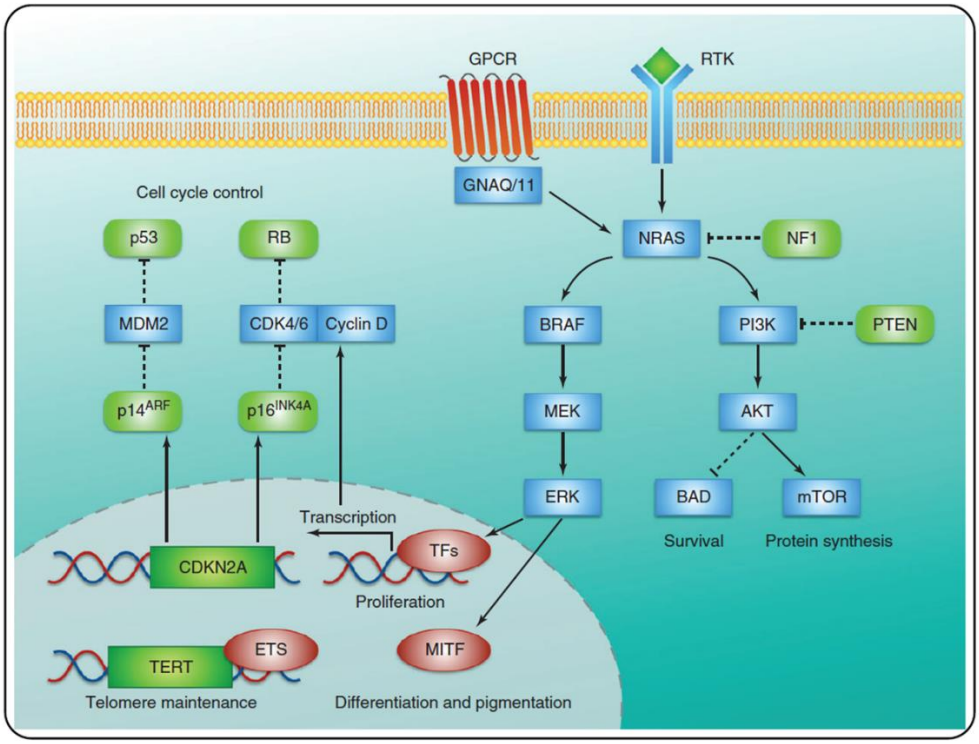



**Figure 8** Signaling pathways underlying melanoma development. Common mutation of BRAF is BRAF(V600E) in cutaneous melanoma. BRAF proteins are associated with the transmission of the Mitogen-Activated Protein Kinase (MAPK) pathway, a pathway that deals with cell proliferation. GNAQ / GNA11 mutation in ocular melanoma. GNAQ / GNA11 are associated with PI3K/AKT/mTOR pathway and MAPK pathway that results in cells proliferation and migration into cancer cells.

**Source:** Guterres A.N. et al., 2018

### 1.2 Alternative splicing and dysregulation in melanoma

1.2.1 Alternative splicing in cancer

The hallmarks of cancer consist 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 (Hanahan D. et al., 2000). In 2011, four additional hallmarks were reported such as genomic instability, inflammation, abnormal metabolism and evasion of the immune system. In addition, last alternative splicing is one of the hallmarks in cancer (Hanahan D. et al., 2011). 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 M., 2013) as shown in figure 9. One of the interesting hallmarks of cancer is alternative splicing which regulate gene expression in a post-transcriptional manner, involve in the biological aspects of cancer cells such as cell proliferation, differentiation, and respond to internal and external cells (da Silva M.R. e t al., 2015).

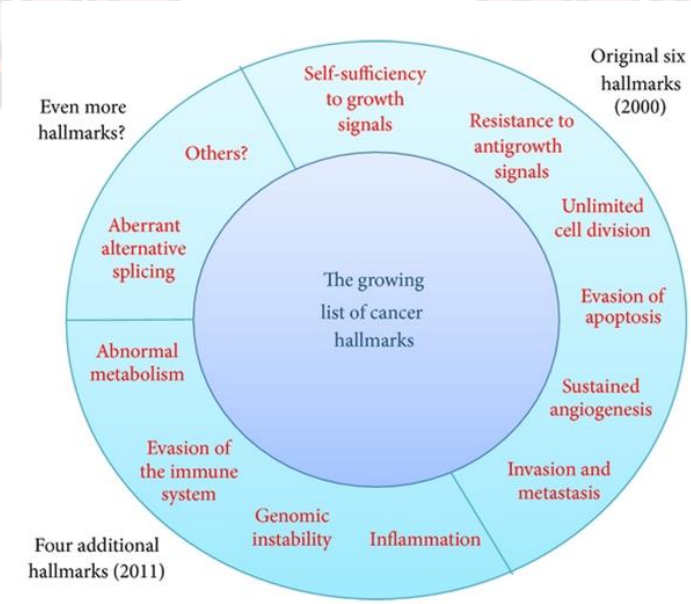



**Figure 9** The Hallmarks of Cancer.

**Source:** Ladomery M., 2013

Generally, alternative splicing 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, D. L., 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 demonstrated (Cooper, G., 2006) Figure 10. In human, more than 95% of roughly 30,000 human genes undergo alternative splicing to encode two or more splice isoforms (Pan, Q. et al., 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 Serine and arginine-rich proteins (SR proteins) or Serine/arginine-rich splicing factors (SRSFs). Alterations in the alternative splicing pattern such as overexpression or dysregulation in regulatory splicing factors, include Serine-Arginine protein kinases (SRPKs) and CDC2-like kinases (CLKs) lead to promote oncogene involved angiogenesis, invasion, metastasis, apoptosis, and survival in cancer (da Silva M.R. et al., 2015).

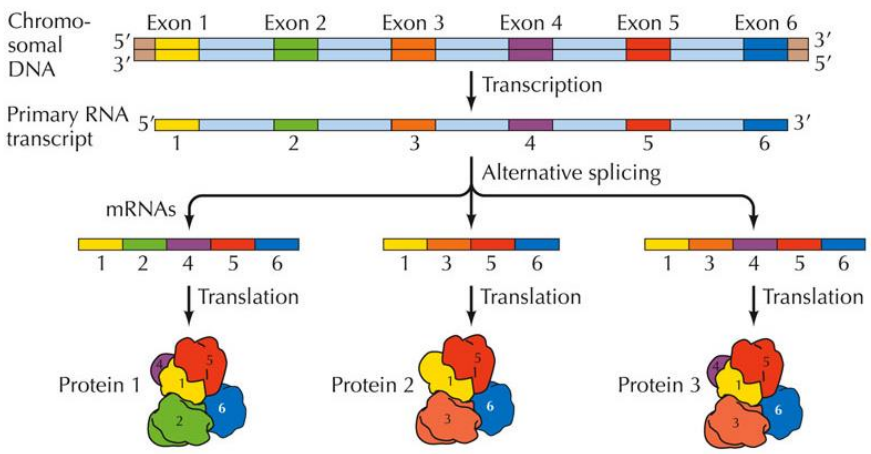



**Figure 10** Alternative splicing generates various protein isoforms.

**Source:** Cooper, G. et al, 2006

1.2.2 Alternative splicing dysregulation in melanoma

Alterations in RNA-binding proteins (RBPs) due to mutation, copy number variation or else are frequently observed in human cancers. Abnormal RBP expression also contributes to tumorigenesis via regulation on gene/protein homeostasis in metastatic melanoma. Moreover, alternative splicing dysregulation is associated with a splicing switch to pro-angiogenic vascular endothelial growth factor 165 (VEGF165) and lead to increase the tumor mass and size (Gammons M.V. et al., 2014). Furthermore, there are number of research articles summarized the upregulation of SRSFs in 9 cancer tissue by Genvestigator and presented that SRSF1 is the most upregulated as the first ranking in 8 cancer types including colorectal, lung, osteosarcoma, breast, ovarian, pancreatic, glioma cancer and melanoma (Zhou, X. et al., 2019) as shown in figure 11.

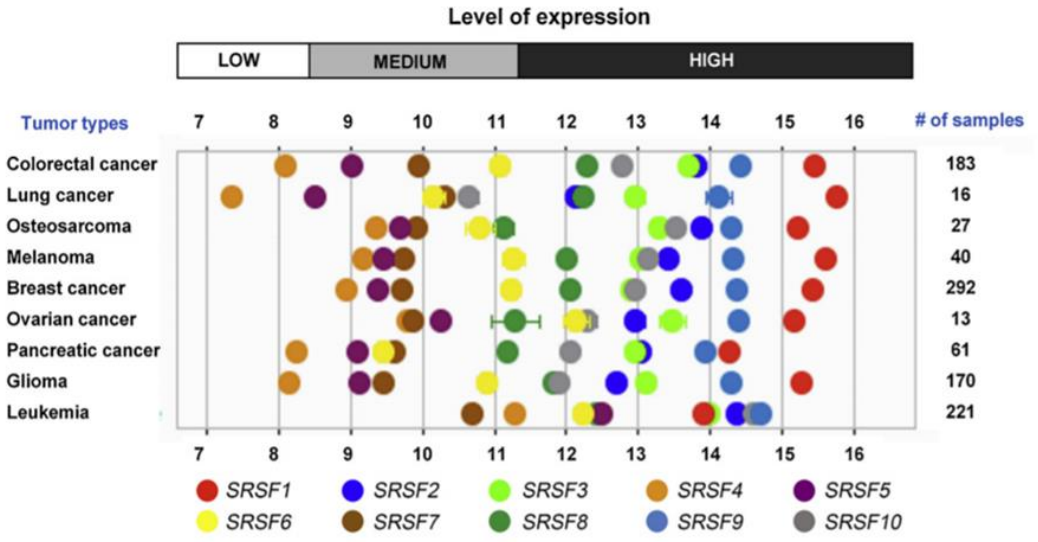



**Figure 11** Expression profile of SRSF genes in 9 human tumor types.

**Source:** Zhou X. et al., 2019

### 1.3 Serine-arginine protein kinases (SRPKs)

1.3.1 SRPKs structure

Serine-arginine protein kinases (SRPKs) are serine/threonine kinases that especially recognize and phosphorylate to SR protein family at serine/arginine-rich

repeats (RS domains) in a processive manner (Aubol B.E. et al., 2013; Ghosh G. et al., 2011). This protein family consists of four members in mammalian cells including SRPK1, SRPK1a, SRPK2, and SRPK3 whereas SRPK1 is found that it highly expressed in testicles and pancreas, SRPK2 is essentially found in the nervous system (Wang H.Y. et al., 1998). Both SRPK1 and SRPK2 are expressed in other human organs such as heart and skeletal muscle. While they slightly expressed in the lung, liver, and kidney (Wang H.Y. et al., 1998). Moreover, the expression of SRPK3 seems to be in muscle cells (da Silva M.R. et al., 2015). All SRPKs structure consists of kinase domain, a feature common among tyrosine kinases, that is bifurcated by a large insert domain. The insert domain regulates sub-cellular localization. In addition, the important regulatory domain is N-terminal and/or C-terminal extensions (Zhou Z. et al., 2013) as shown in figure 12.

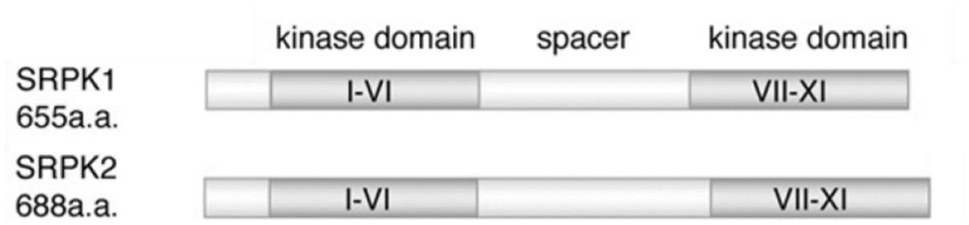



**Figure 12** Serine-arginine protein kinases (SRPKs) structure.

**Source:** Liang N.et al., 2014

1.3.2 SRPKs function

During post-transcriptional modification, serine-arginine protein kinases or SRPKs are splicing regulatory protein that plays an essential role in the phosphorylation of the serine residuals in the serine/arginine-rich repeats (RS domain) of various mRNA splicing factors such as, SC35, U2AF65, and alternative splicing factor/splicing factors or SRSFs (Chan C.B. et al. 2013). Moreover, the majority of SRPKs reside in the cytoplasm. Under some condition, like osmotic shock, SRPK1 is dissociated from the cytoplasmic co-chaperone complex and translocated into the nucleus to phosphorylate SR proteins to regulate the RNA splicing in response to the stress condition (Chan C.B. et al. 2013) as shown in figure 13.

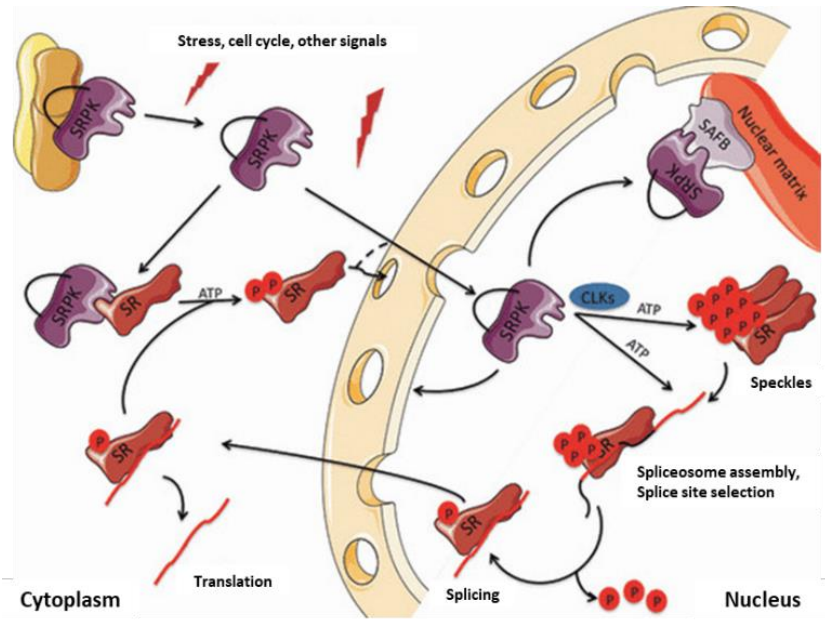



**Figure 13** Serine-arginine protein kinases (SRPKs) function. SRPKs are cumulative in the cytoplasm, where they phosphorylate SR proteins and encourage their nuclear import to regulate the RNA splicing.

**Source:** Giannakouros T. et al., 2011

1.3.3 SRPKs and SR protein family

SR proteins are RNA binding proteins which share common RNA binding motifs that plays an essential role in the mRNA splicing process. The structure of SR protein consists of 2 major domains (Änkö, M. L., 2014). First, 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 is 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. SR proteins consist 12 members including SRSF1 to SRSF12 multiple names have been given to SR proteins during the course of their discovery for example, ASF/SF2 becomes SRSF1; C35 is renamed as SRSF2; and SRp20, SRp75, SRp40, SRp55, and 9G8 are known as SRSF3 to SRSF7, respectively. In addition, the molecular weight of predicted (by Molecular Weight Calculator - .NET DLL Version) and commercial detectable was shown in Table 1.

**Table 1** Nomenclature and molecular weight of SR protein family (Summary by Jesadagorn Siriwath)

| SR protein | Alternative names | Amino acids | Calculated MW (kDa) | Detectable MW (kDa) |
|---|---|---|---|---|
| SRSF1 | SF2/ASF, SRp30a | 248 | 27.3 | 32 (Invitrogen) |
| SRSF2 | PR264, SC35, SRp30b | 221 | 24.3 | 35 (Invitrogen) |
| SRSF3 | SRp20 | 164 | 18.0 | 19 (Invitrogen) |
| SRSF4 | SRp75 | 494 | 54.3 | 57 (Invitrogen) |
| SRSF5 | HRS, SRP40 | 273 | 30.0 | 39 (abcam) |
| SRSF6 | B52, HEL-S91, SRP55 | 334 | 36.7 | 40 (abcam) |
| SRSF7 | 9G8, AAG3 | 238 | 26.2 | 27 (abcam) |
| SRSF8 | DSM-1, SRP46 | 282 | 31.0 | 32 (Labome) |
| SRSF9 | SRp30c | 221 | 24.3 | 24 (Invitrogen) |
| SRSF10 | SRp38, SRrp40, TASR | 262 | 28.8 | 37 (mybiosource) |
| SRSF11 | NET2, p54 | 484 | 53.2 | 54 (Invitrogen) |
| SRSF12 | SRrp35 | 261 | 28.7 | 30 (biocompare) |

These SR proteins encourage U1 snRNP binding to the 5’ splice site and U2 snRNP binding to the 3’ splice site. they also bridge the communication between these initial splice site recognition events in the pre-spliceosome and the mature spliceosome which is a protein that regulates alternative splicing and role play splice site selection, intron removal and exon ligation (Zhou Z. et al., 2013 and Änkö, M. L.

et al., 2012). The above process is regulated by Cytoplasmic SRPKs which are responsible for initial phosphorylation of RS domain of SR proteins to transport their target protein to nuclear, whereas nuclear CDC2-like kinases (CLKs) may encourage the phosphorylation of SR proteins in the nucleus. Therefore, the two families of kinases may be responsible in a synergistic to regulate splicing, RNA export, and other processes of RNA metabolism in cells (da Silva M.R. et al., 2015) as shown in figure 14.

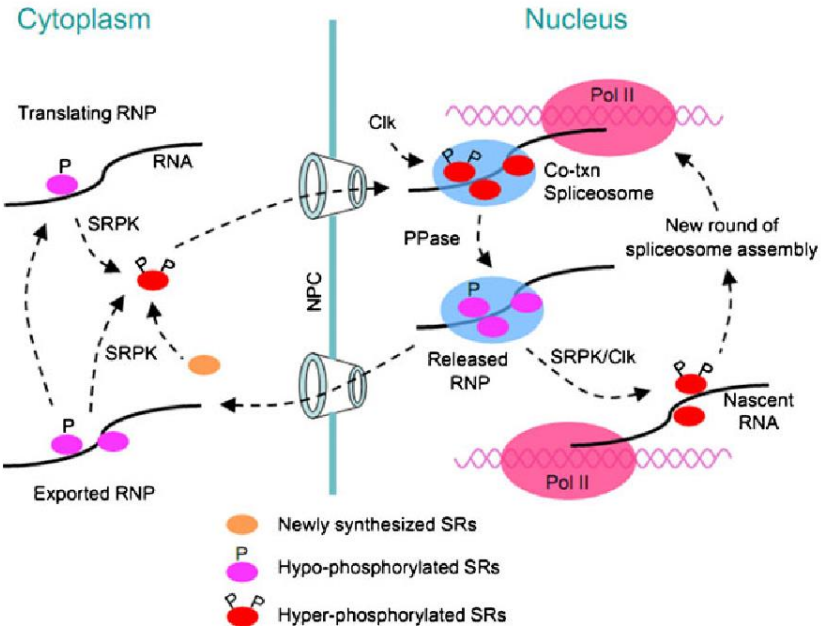



**Figure 14** Regulation of SR protein phosphorylation by SRPKs. SRPKs phosphorylate arginine-serine rich (RS) domain of SR proteins in cytoplasm and transport their target into the nucleus. Whereas CLKs may encourage the phosphorylation of SR proteins in the nucleus. Therefore, the two families of kinases are be responsible in a synergistic to regulate splicing, RNA export, and other processes of RNA metabolism in cells.

**Source:** Zhou Z. et al., 2013

In humans, there is reported of the location of the protein kinases in Human osteosarcoma (U2OS cells). The class of splicing kinases include SRPKs and CLKs have a distinct cellular localization, may depend on the different functional roles of protein kinases in splicing regulation as shown in figure 15 (Corkery D.P. et al., 2015).

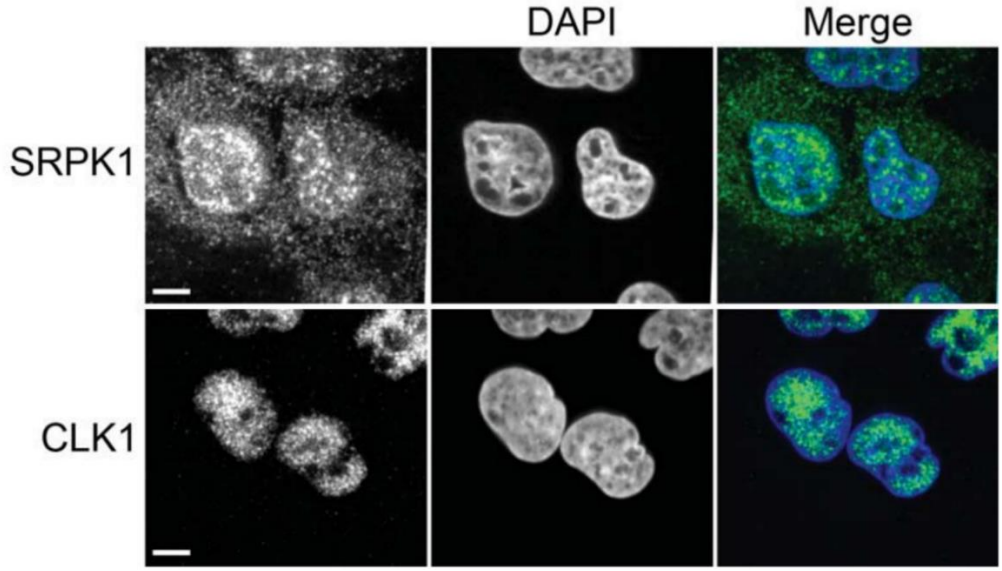



**Figure 15** Cellular localization of splicing kinases (SRPK1 and CLK1).

**Source:** Corkery D.P. et al., 2015

1.3.4 SRPKs in cancer

The expression of SRPKs may lead to aberrant alternative splicing and potentially contribute 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 function in regulatory spicing factors that mentioned as SRPKs and also the abnormality of their target molecules which are SR protein can generate multiple abnormal protein isoforms involved in the biogenesis and progression of several cancers (da Silva M.R. et al., 2015) as shown in figure 16.

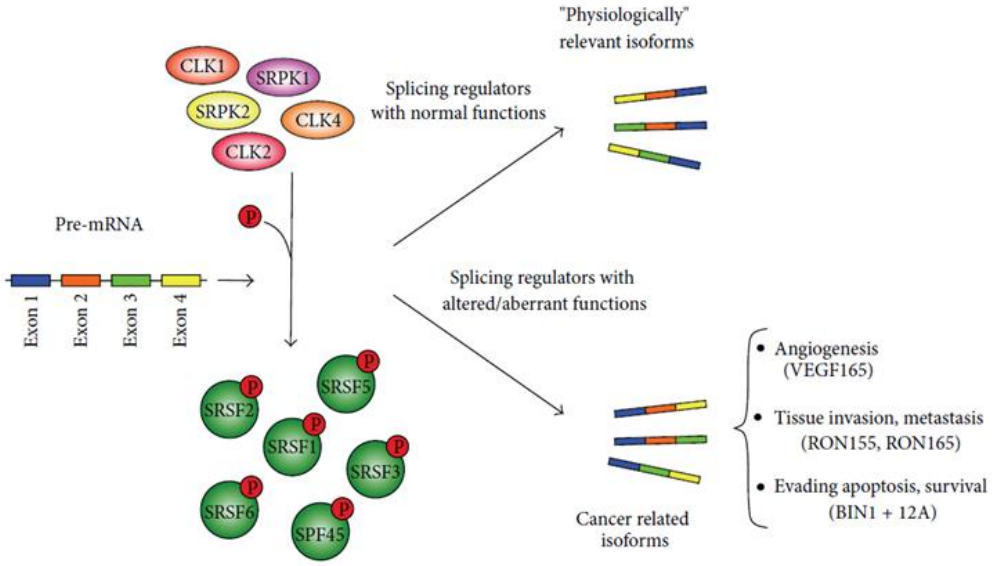



**Figure 16** Dysregulation of splicing factors activity in cancer cells.

**Source:** da Silva M.R. et al., 2015

There are a number of research articles reported overexpression of SRPK1 and SRPK2 in different types of cancer including breast (Hayes G.M. et al., 2007), colon (Hayes G.M. et al., 2007), pancreatic carcinomas (Hayes G.M. et al., 2006), leukemia (Jang S.W. et al., 2008), non-small cell lung carcinoma (Gout S. et al., 2012), squamous cell lung carcinoma (Gout S. et al., 2012), gliomas (Wu Q. et al., 2013), ovary (Odunsi K. et al., 2012), and hepatocellular carcinoma (Zhou B. et al., 2013). The increasing of SRPKs expression can promote the tumorigenesis, angiogenesis, tissue invasion, metastasis, apoptosis evasion, or survival in cancer (da Silva M.R. et al., 2015). Furthermore, targeting SRPK1 using short hairpin RNA (shRNA) in tumor of melanoma resulted in significantly reduced tumor growth (Gammons M.V. et al., 2014). as shown in figure 17.

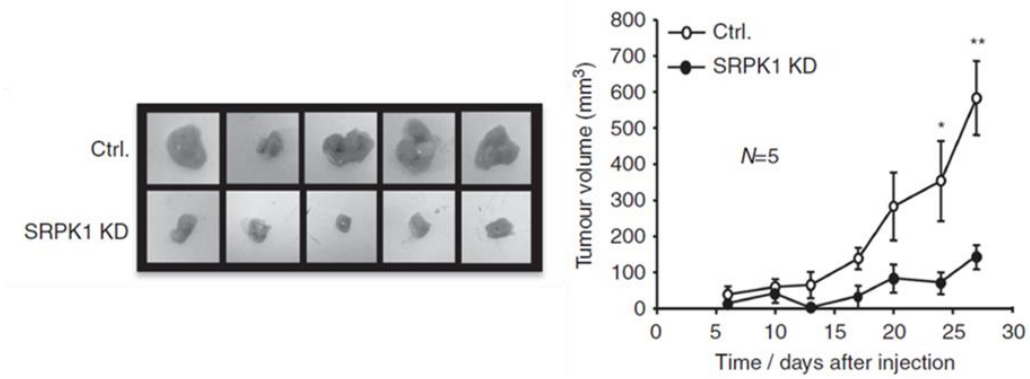



**Figure 17** Tumor volume of melanoma after SRPK1 Knockdown.

**Source:** Gammons M.V. et al., 2014

1.3.5 SRPK targeting and activation of molecular crosstalk

Alternative spliced variants of several oncogenes and tumor suppressors have been shown to be important for their tumorigenicity. Serine-arginine protein kinase 1 (SRPK1), a major regulator of splicing factors, is involved in other cancer progression and plays a role in chemo-sensitivity (Odunsi K. et al., 2012).

The PI3K/AKT/mTOR pathway plays an important role in the regulation of signal transduction and biological processes such as cell proliferation, apoptosis, metabolism and angiogenesis. Compared with those of other pathways, the components of the PI3K/AKT/mTOR pathway are complicated. The regulatory mechanisms and biological functions of the PI3K/AKT/mTOR pathway are important in many human diseases, including ischaemic brain injury, neurodegenerative diseases, and cancer. (Xu F. et al., 2020).

Interestingly, SRPKs are involved in the phospho-regulation of the SR protein family of splicing factors via the EGF-AKT-SRPK signal transduction axis (Zhou et al., 2012). Previously reported SRPK1 can act as either oncogene or tumor suppressor. SRPK1 presented tumor suppressor activity since its inactivation in mouse embryonic fibroblasts could induce cell transformation. This phenotype has been related to the impairing of phosphatase PHLPP recruitment to AKT which leads to hyperactivation of AKT. Moreover, SRPK1 overexpression is also tumorigenic because excess SRPK1 squelches PHLPP1 and leads to AKT phosphorylation (Zhou et al., 2012; Wang P. et al., 2014) as shown in figure 18.

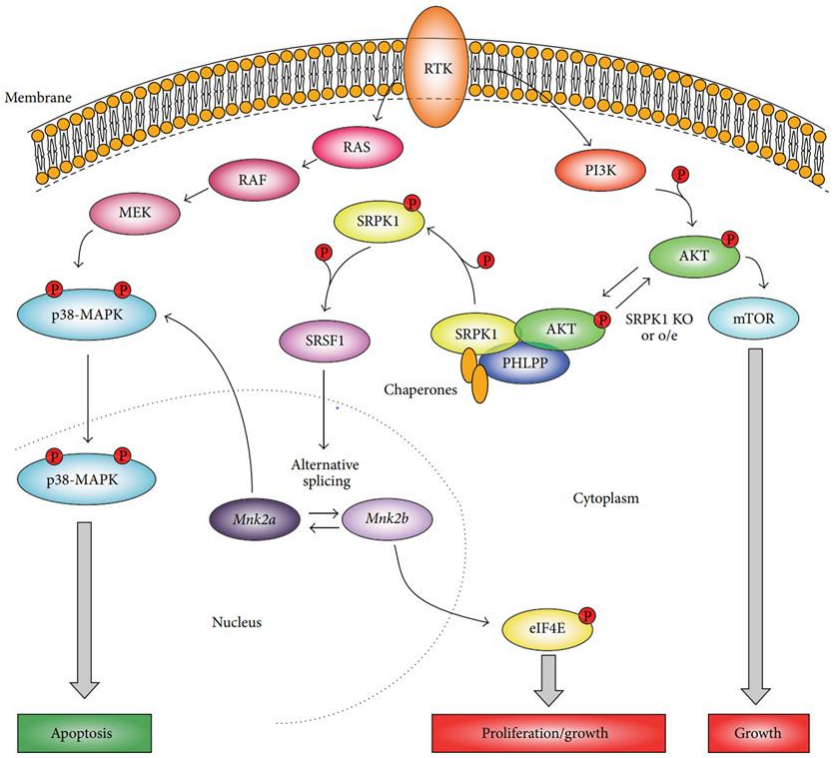



**Figure 18** Cellular pathways related to SRPK activity.

**Source:** da Silva M.R. et al., 2015

There is an article reported siRNA-mediated SRPK1 can decrease cell proliferation rate, cell migration, tumorigenic potential, and slower cell cycle progression in ovarian cancer (Odunsi K. et al., 2012). SRPK1-mediated alterations of MAPK/AKT signaling pathways were associated with these phenotypes, because the levels of phosphorylated (activated) MAPK/AKT protein were decreased in the SRPK1 knockdown cells by specific shRNA knockdown in SKOV3 ovarian cancer cell (Odunsi K. et al., 2012) as shown in figure 19.

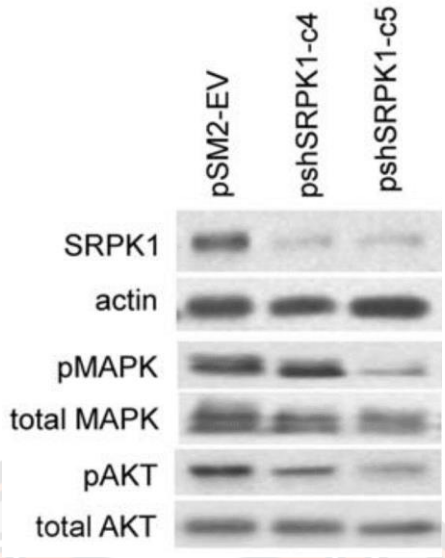



**Figure 19** SRPK1-mediated alterations of MAPK/AKT signaling pathways in ovarian cancer cell (SKOV3 cell).

**Source:** Odunsi K. et al., 2012

### 1.4 SRPK inhibitors

There are several studies have reported the important role of the abnormal activity of SRPK, the kinase enzymes, for tumorigenesis and poor patient prognosis of human cancers. In the occurrence of metastasis, high SRPK1 expression, for example, correlates with poor breast cancer disease outcome and preferential metastasis to the lungs and brain (Van Roosmalen W. et al., 2015). The role of SRPKs in tumor metastasis has been credited to their benefits to the expression of pro-metastatic spliced isoforms, such as Rac1b (Gonçalves V. et al., 2014), RONΔ165 (Moon H. et al., 2014) and the pro-angiogenic VEGF165, which have been related to metastatic melanoma (Gammons M.V. et al., 2014). Therefore, these data collectively suggest that SRPK are potential targets for the development of antimetastatic drugs.

The role of SRPK in cancers as well as in other human diseases, such as viral infections and dysregulated vascular proliferation, has led to the search of small molecules capable of inhibiting their catalytic activity (da Silva M.R. et al., 2015). These compounds – which include SRPIN340 (Fukuhara T. et al., 2006), MVRL09 (Gammons M.V. et al., 2013), SPHINX (Gammons M.V. et al., 2013), SRPIN803

(Morooka S. et al., 2015), SPHINX31 (Batson J. et al., 2017), and SRPKIN-1 (Hatcher J. et al., 2018) have presented promising experimental results in in vitro and *in vivo* studies as shown in figure 20.

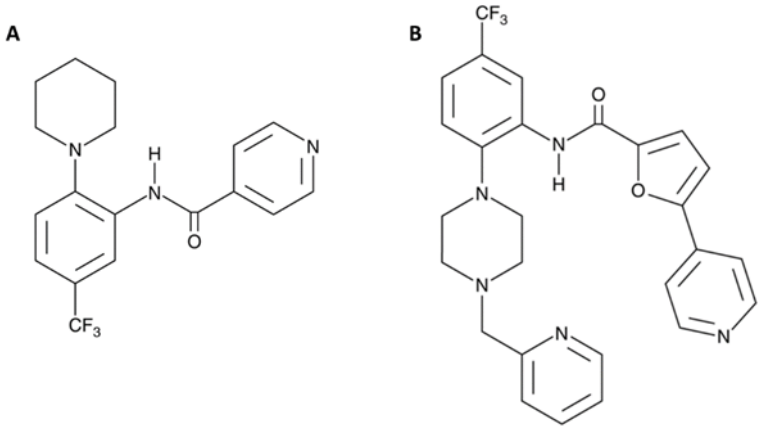



**Figure 20** The structure of two main important SRPK inhibitors; SRPIN340 (A). SPHINX31 (B).

**Source:** Cayman Chemical, USA

1.4.1 SRPK inhibitors as pharmacological substance

Serine/arginine (SR) proteins such as serine arginine-rich splicing factor 1 (SRSF1) regulate mRNA splicing by binding to the mRNA in a phosphorylated form. Specific splice factor kinases are responsible for the phosphorylation of the SRSF proteins and can be used to modulate the splicing events. For example, serine-arginine protein kinases 1 (SRPK1) regulates splicing of pro-angiogenic VEGF-A165a through phosphorylation of SRSF1, supporting SRSF1 nuclear translocation and binding to the proximal splice site in VEGF-A pre-mRNA (Batson J. et al., 2017). There are a number of research studies reported the regulation of this kinase can restore the balance of mRNA isoforms to normal, without affecting cell function such as knockdown or inhibition of SRPK1 in melanoma model can lead to distal splice site selection, generation of antiangiogenic VEGF-A165b, and reduced disease progression (Gammons M.V. et al., 2014).

Remarkably, SRPK inhibitors have been proposed as potential therapeutics in angiogenic-related diseases such as choroidal neovascularization in Age-Related Macular Degeneration (AMD) (Gammons M.V. et al., 2013), viral replication such as Hepatitis C Virus (Karakama Y. et al. 2010), and especially, as the potential anticancer agents. The example for utilizing SRPK inhibitor as the anticancer agents is metastatic melanoma (Gammons M.V. et al., 2014) However, the existing class of SRPK inhibitor, such as tricyclic quinoxaline derivatives is neither specific nor potent. Therefore, the development of highly selective kinase inhibitors is challenging due to the high similarity of the ATP binding site, the position of phosphorylation of SRPKs, is usually targeted (Batson J. et al., 2017) as show in figure 21.

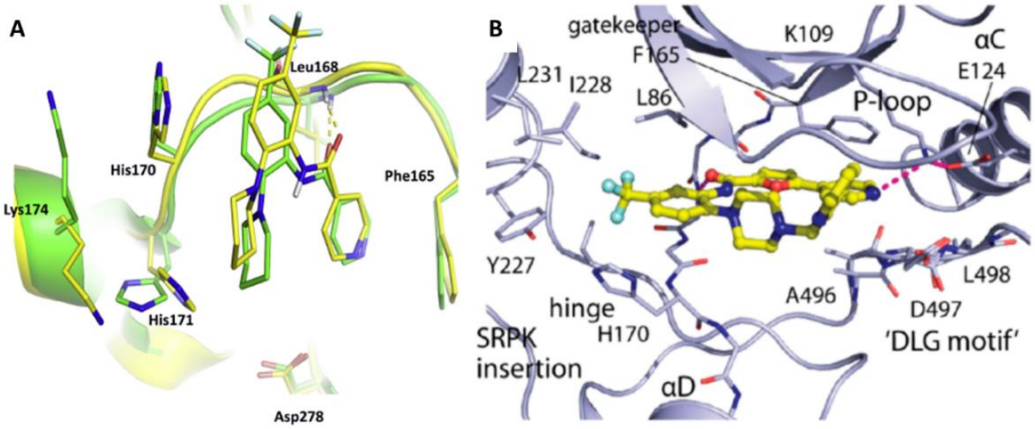



**Figure 21** Cocrystal structure of SRPIN340 bound to SRPK1 (A). Cocrystal structure of SPHINX31 bound to SRPK1 (B).

**Source:** Moreira G.A. et al., 2018; Batson J. et al., 2017

1.4.2 SRPIN340

SR protein phosphorylation inhibitor 340 or SRPIN340 has a structures of N-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide, which potently inhibits SRPK1 activity with a Ki value of 0.89 µM and SRPK2 activity with a Ki value of 1 µM. Moreover, this compound is a selective inhibitor of SRPK1/2 because it can inhibit both SRPK1 and SRPK2 but not inhibit other kinases, for example Clk1 and Clk4. In fact, concentrations up to 10 µM of SRPIN340, it fails to inhibit 140 protein

kinases. Therefore, SRPIN340 is a highly selective inhibitory for SRPK1 and SRPK2, that resulted in the reduction of phosphorylation of SR proteins (Fukuhara T. et al., 2006) as shown in figure 22.

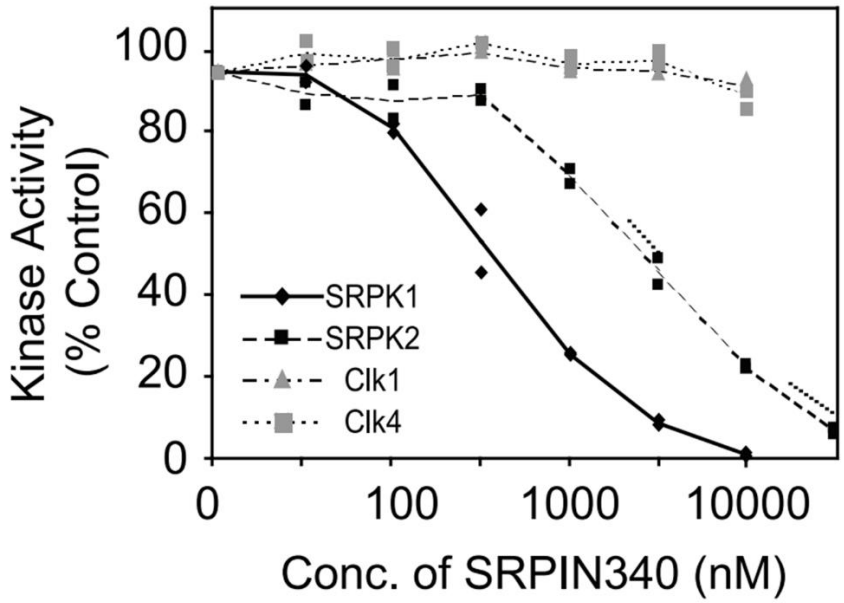



**Figure 22** The effect of SRPIN340 for various protein kinases. SRPIN340 is a highly selective inhibitor of SRPK1/2 because it can inhibit both SRPK1 and SRPK2 but not inhibit other kinases, for example Clk1 and Clk4.

**Source:** Fukuhara T. et al., 2006

In additionally, SRPIN340 inhibit SRPKs function, phosphorylate SRSFs, and combat viral infections that require SRSFs-dependent RNA processing (Fukuhara T. et al., 2006). Furthermore, it can exert anti-angiogenic effects in retinal neovascularization and in some tumors that suppress SRSFs-dependent proangiogenic VEGF165 isoform (Tsang J. et al., 2019). Recently, SRPIN340 treatment can reduce the infarct size in myocardial infarction via suppression cell apoptosis. (Huang J. et al., 2019) as shown in figure 23.

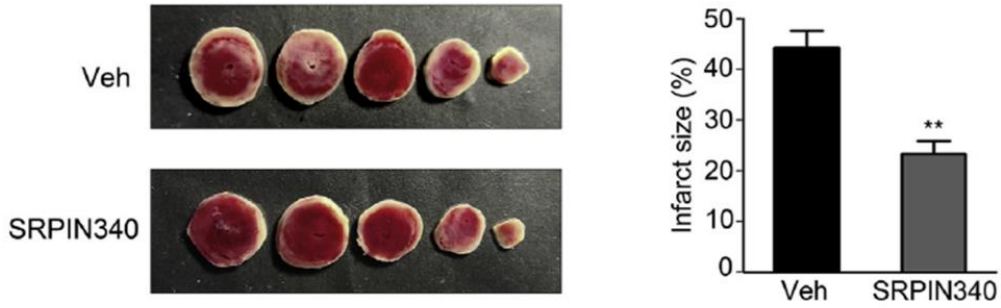



**Figure 23** The effect of SRPIN340 protects heart muscle.

**Source:** Huang J. et al., 2019

In cancer cell, it seems that SRPIN340 treatment can decrease leukemia cell viability and increase cell apoptosis in acute myelogenous leukemia cell (AML). However, lower cytotoxicity was observed in peripheral blood mononuclear cell (PBMC), due to SRPK1 and SRPK2 overexpression in AML but not express in PBMC (Siqueira R.P. et al., 2015) as shown in figure 24.

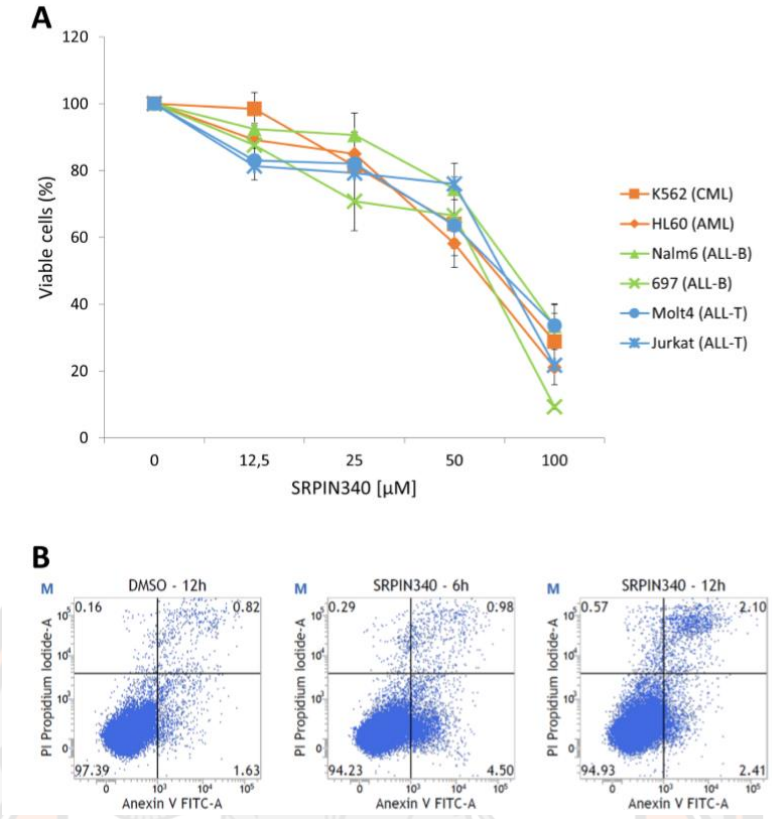



**Figure 24** The effect of SRPIN340 treatment on leukemia cell viability (A) and cell death (B).

**Source:** Siqueira R.P. et al., 2015

Furthermore, there is article study reported SR protein phosphorylation decreased during SRPIN340 treatments in HL60 and Jurkat leukemia cells (Siqueira R.P. et al., 2015) as shown in figure 25. This result relates to an effect on SRPK dysfunction.

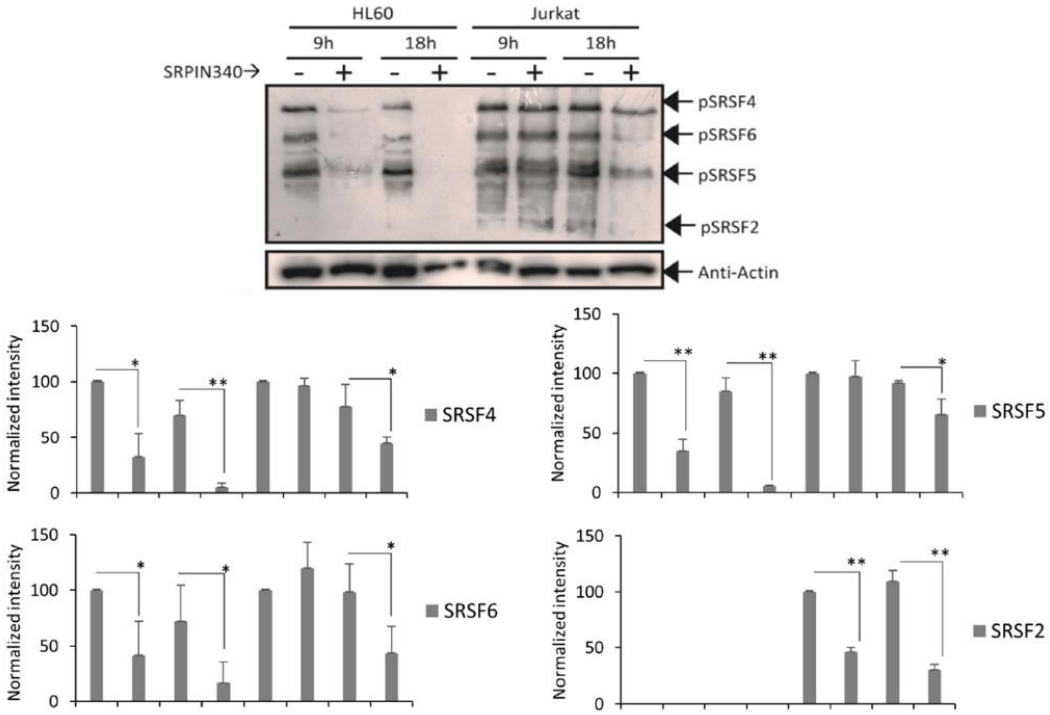



**Figure 25** The effect of SRPIN340 on SR protein phosphorylation.

**Source:** Siqueira R.P. et al., 2015

Moreover, there is a study determined the combined SRPIN340 and GSK690693 (AKT inhibition) also impaired SR proteins phosphorylation in accordance with an improved suppression of SRPK activity in human leukemic cell lines (Siqueira R.P. et al., 2020) as shown in figure 26.

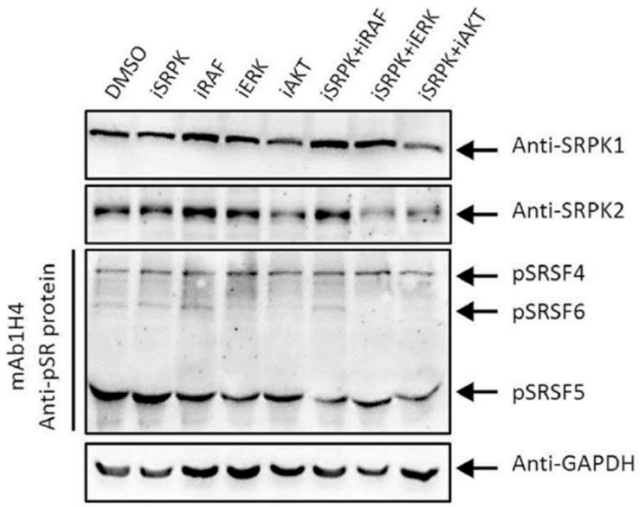



**Figure 26** Effect of SRPK and AKT inhibitor impaired SR proteins phosphorylation.

**Source:** Siqueira R.P. et al., 2020

Additionally, previous study performed that concentration of SRPIN340 can control the activation or inactivation of AKT phosphorylation (Ser473 and Thr308) compare with DMSO treated cell in neonatal rat ventricular myocytes (NRVMs) (Huang J. et al., 2019). Cells were treated with vehicle (DMSO) or SRPIN340 (10 mM) at 0, 0.5, 3, 12 and 24 h. Pan-AKT and phosphorylation at Ser473 and Thr308 were examined under these conditions using Western blot as shown in figure 27.

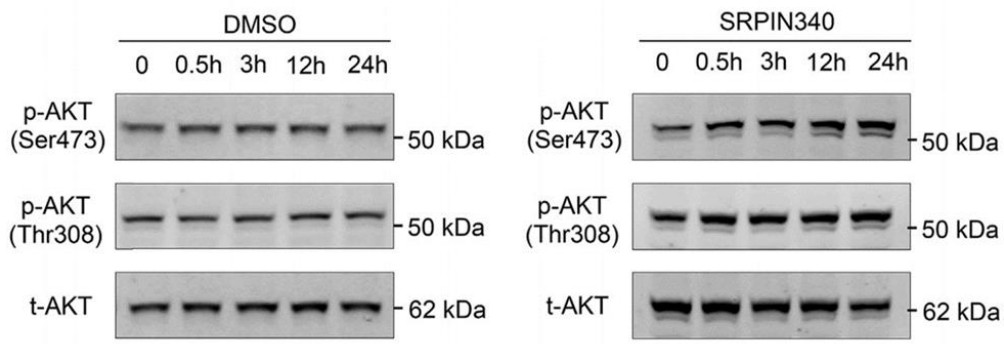



**Figure 27** Effects of SRPIN340 on AKT phosphorylation in neonatal rat ventricular myocytes (NRVMs) cell.

**Source:** Huang J. et al., 2019

1.4.3 SPHINX31

SR protein phosphorylation inhibitor X 31 or SPHINX31 has a structure of 5-(4-pyridinyl)-N-[2-[4-(2-pyridinylmethyl)-1-piperazinyl]-5-(trifluoromethyl)phenyl]-2-furancarboxamide, which potently inhibits SRPK1 activity with a IC50 of 5.9 nM, that resulted in the reduction of phosphorylation of SR protein like SRPIN340 (Batson J. et al., 2017) as shown in figure 28.

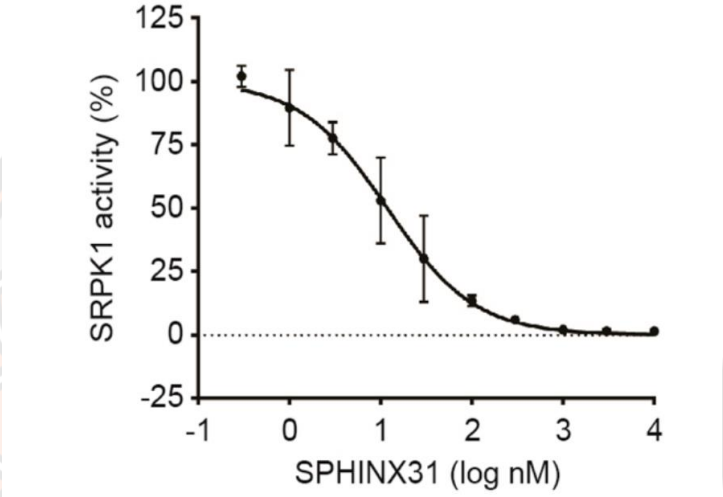



**Figure 28** The effect of SPHINX31 for SRPK1 activity. SRPIN340 is a highly selective inhibitor of SRPK1 by kinase assay**.**

**Source:** Batson J. et al., 2017

A radiolabeled ATP competition assay was investigated for 50 key kinases. This compound is a selective inhibitor of SRPK1 because the 96% inhibition of SRPK1 activity by 1 μM of SPHINX31 but it fails to inhibit other kinases. Thus, SPHINX31 is a highly selective inhibitory for only SRPK1 (Batson J. et al., 2017) as shown in figure 29.

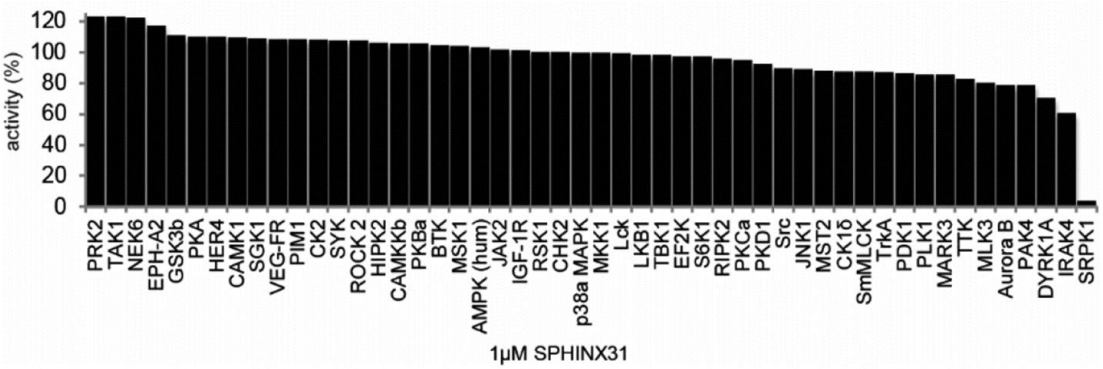



**Figure 29** The effect of SPHINX31 against a series of key 50 enzymes that represent the inhibition of SRPK1.

**Source:** Batson J. et al., 2017

In additionally, the same study reported it can increase anti-angiogenic VEGF165b expression after SPHINX31 treatments in Retina Pigment Epithelial (RPE) cell. Therefore, it is a potential topical therapeutic for neovascular eye disease (Batson J. et al., 2017) as shown in figure 30.

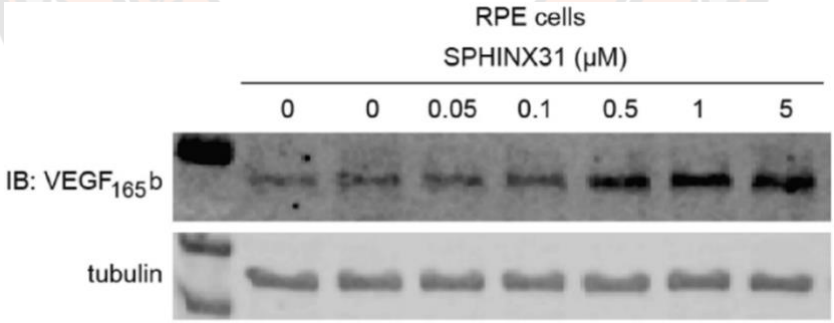



**Figure 30** The effect of SPHINX31 on VEGF165b expression in RPE cell.

**Source:** Batson J. et al., 2017

Moreover, the effect of SPHINX31 treatment on cell viability was investigated (Tzelepis K. et al., 2018). It showed that SPHINX31 can suppress cell viability in acute myelogenous leukemia cell as shown in figure 31.

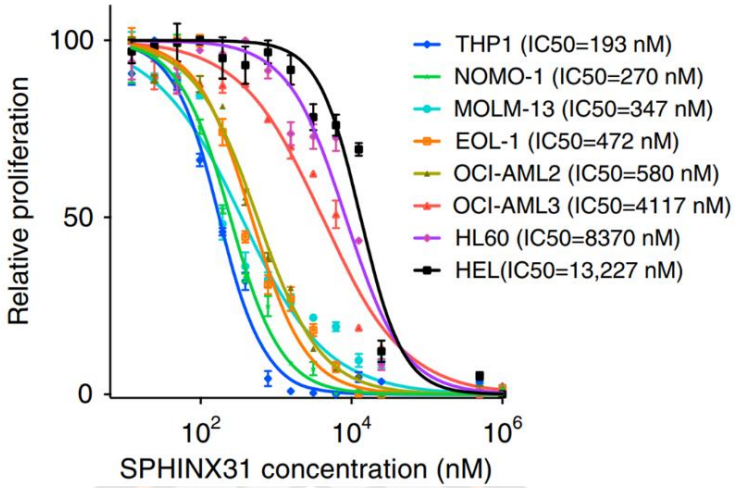



**Figure 31** The effect of SPHINX31 treatment on leukemia cell viability.

**Source:** Tzelepis K. et al., 2018

Previous study performed that effect of SPHINX31 treatment on SRSF1 phosphorylation. It showed that SPHINX31 can decrease SRSF1 phosphorylation in PC3 prostate cancer cell (Batson J. et al., 2017) as shown in figure 32.

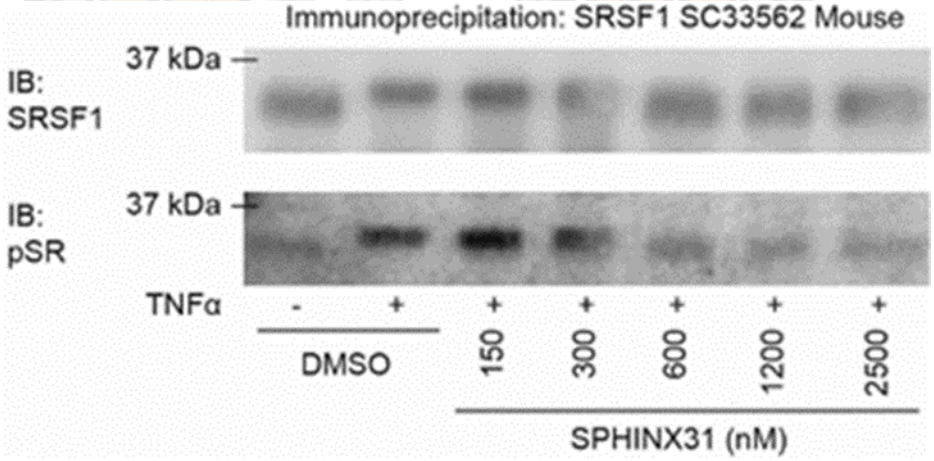



**Figure 32** The effect of SPHINX31 treatment on SRSF1 phosphorylation in PC3 prostate cancer cell**.**

**Source:** Batson J. et al., 2017

1.4.4 SRPK inhibitors in melanoma

Even though, melanoma is a rare disease, but the disease is found to have a relatively high mortality rate. The essential cause of death in melanoma is metastasis of the primary tumor. Angiogenesis, one of the hallmarks of cancer is considered for the metastasis and growth tumor cancer. There are reported that study the effects of SRPK inhibitors on cancer cells. For example, mice were injected subcutaneously by A375 cell, which is melanoma cell after SRPIN340 treatments found that SRPIN340 significantly reduced tumor growth (Gammons M.V. et al., 2014) as shown in figure 33.

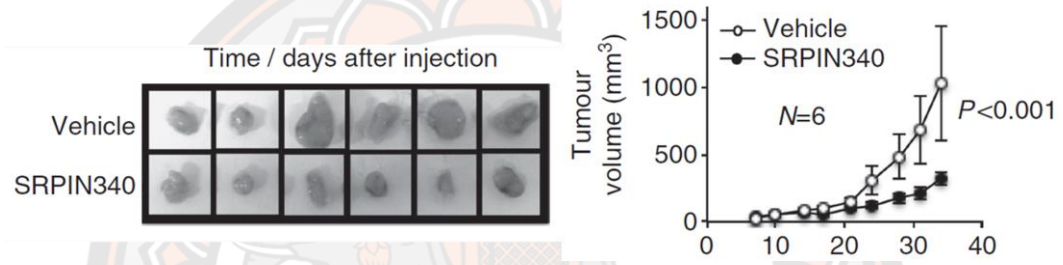



**Figure 33** The effect of SRPIN340 on tumor growth of melanoma *in vivo.*

**Source:** Gammons M.V. et al., 2014

Previous study performed that SRPIN340 significantly decrease colony formation in B16F10 murine melanoma model as dose-dependent manner compared with DMSO treated cell (Moreira G.A. et al., 2018) as shown in figure 34.

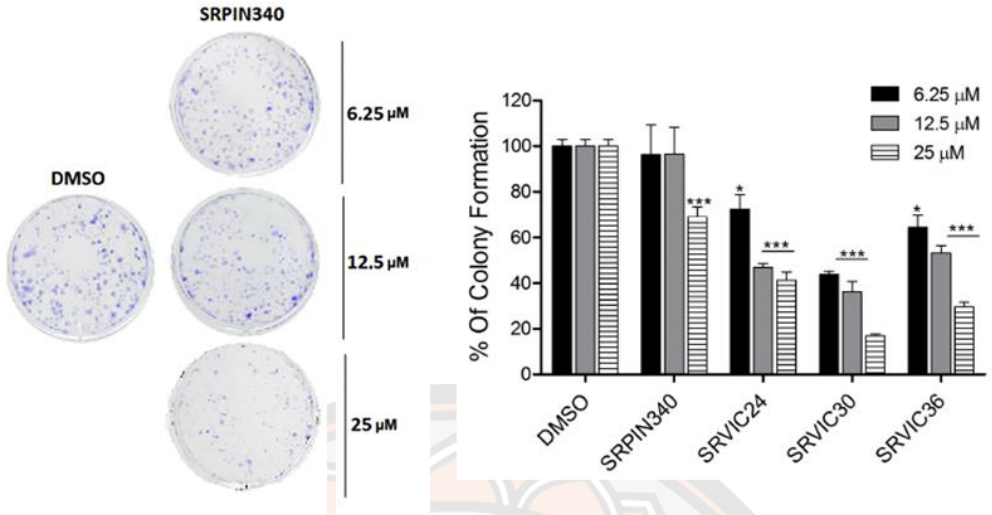



**Figure 34** Effect of SRPKs specific inhibitors on colony formation in B16F10 murine melanoma model.

**Source:** Moreira G.A. et al., 2018

Moreover, migration ability of melanoma cell was decrease when treated SRPIN340 as dose dependent manner in B16F10 cell (Moreira G.A. et al., 2018) as shown in figure 35.

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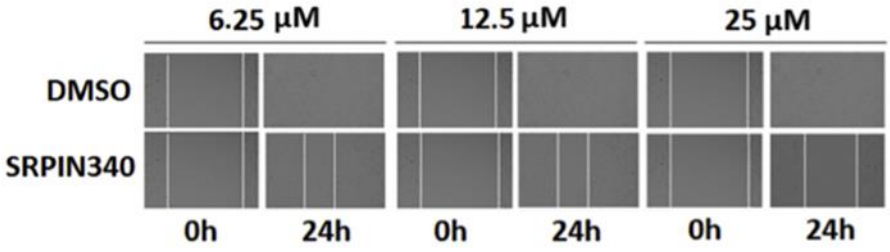


**Figure 35** Effect of SRPIN340 on migration ability in B16F10 murine melanoma model.

**Source:** Moreira G.A. et al., 2018

Remarkably, the role of SRPK inhibitors were effective in impairing SRPK1 nuclear translocation and SR protein phosphorylation in the intracellular environment in murine melanoma model (B16F10 cell) (Moreira G.A. et al., 2018) as shown in figure 36.

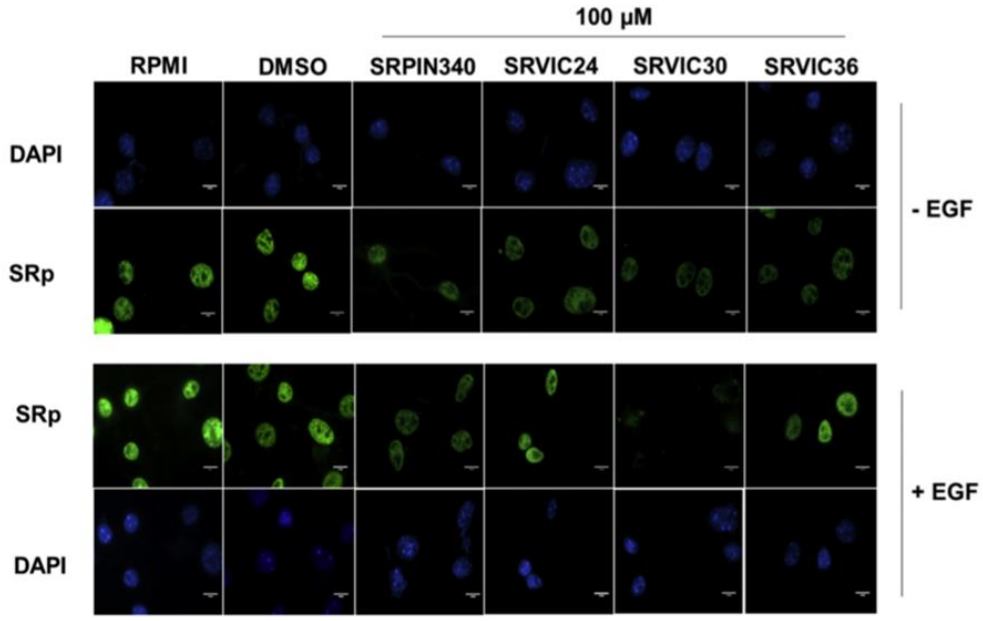


**Figure 36** The effect of SRPK inhibitor subcellular localization and cellular activity of SRSF phosphorylation.

**Source:** Moreira G.A. et al., 2018

Therefore, these information lead to the experimental design and objective in this recent study. Priorly, to investigate the phosphorylation profile of SRSFs in melanoma model demonstrated by cutaneous melanoma (A375) and ocular melanoma (92-1) cells with comparable non-cancerous cell (HaCat). Next, the half maximal inhibitory concentration of SRPK protein inhibitors (SRPIN340 and SPHINX31) in A375, and HaCat, 92-1 cells will be determined for further study on SRSF phosphorylation and subcellular translocation. Moreover, the effect of a selected SRPK inhibitor on the growth and activation of growth-related signaling pathway will be considered.

# CHAPTER III

# RESEARCH METHODOLOGY

**Laboratory materials**

Instruments

- 1.5 ml microcentrifuge tube (LC premium, Thailand)
- 5% $CO_2$ Incubator (Shel lab, USA)
- 6-well plate (SPL life science, Korea)
- Agarose gel electrophoresis apparatus (ATTO, Japan)
- Glass pipette (Precicolor HBG, Germany)
- Glove-powder free (Sri Trang, Thailand)
- Fluorescence microscope (Zeiss, Germany)
- ImageQuantTM LAS 500 (GE Healthcare Life Science, UK)
- Inverted microscope (Olympus, USA)
- Laminar Flow hood (NUAIRE, USA)
- Micropipette (Proline ®Plus, USA)
- Microplate reader (Biochrom Ltd, UK)
- Mini Trans-Blot® Cell (Bio-Rad laboratories, USA)
- Neubauer hemocytometer (Fisher Sciencitific, Germany)
- Pipette aid (TOPSCIEN ®, China)
- Polyvinylidene fluoride (PVDF) membrane (Bio-Rad laboratories, USA)
- Refrigerated centrifuge (Hettich, Germany)
- T100™ Thermal Cycler (Bio-Rad laboratories, USA)
- Vortex (SPSLAB, USA)
- Water bath (Memmert, USA)
- Cubee™ Mini-Centrifuge (BIOGENOMED, Taiwan)

Chemicals

- 30% Acrylamide: Bis (29:1) (National diagnostics, USA)
- Albumin from bovine serum (Fluka, USA)

- Beta-mercaptoethanol (Gibco, NY)
- Clarity™ Western ECL Substrate (Bio-Rad laboratories, USA)
- Coomassie Brilliant Blue G-250 (Bio-Rad laboratories, USA)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, MA)
- Dimethyl sulfoxide (DMSO) (Sigma-aldrich.St. Louis, NY)
- Fetal bovine serum (FBS) (Gibco, Waltman, MA)
- F12 Nutrient Mixture (Ham's F12) (Merck Millipore, USA)
- Glutaraldehyde, 25% Solution (Bio basic, USA)
- Methanol (RCI labscan, Thailand)
- Phosphatase Inhibitor Cocktail Set V, 50X (Merck Millipore, USA)
- Sequencing grade trypsin (Promega, Germany)
- Penicillin 100 U/mL (Gibco, MA)
- Antibiotic-Antimycotic,100X (Fisher Scienctific, Germany)
- Phosphate buffered saline (PBS) Tablets (Amresco, E.U.)
- Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, MA)
- Streptomycin 100 µg/mL (Gibco, Waltman, MA)
- SRPIN340 (Cayman Chemical, USA)
- SPHINX31 (Cayman Chemical, USA)
- Tris-acetate-EDTA (TAE) buffer (Bio basic, CA)
- Tetramethylethylenediamine (TEMED) (Bio basic, CA)
- Trypsin/EDTA (Gibco, MA)
- Tween 20 (Life science, TH)
- Anti-p-SRSFs: antibody produced in mouse (Merck Millipore, USA)
- Anti-GAPDH: antibody produced in rabbit (Merck Millipore, Germany)
- Anti-AKT: antibody produced in rabbit (Cell Signaling Technology, Germany)
- Anti-p-AKT: antibody produced in rabbit (Cell Signaling Technology, Germany)
- Anti-mouse IgG: antibody produced in goat (Fisher Scienctific, Germany)
- Anti-rabbit IgG: antibody produced in goat (Fisher Scienctific, Germany)
- Anti-rabbit IgG-FITC: antibody produced in goat (Merck Millipore, Germany)

**Biological materials**

Cell lines

The HaCat (Keratinocyte cell) and A375 (Cutaneous melanoma cell) were provided from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The 92-1 (Ocular melanoma cell) were purchased from Merck Millipore, USA. Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) were used to subpassage and culture HaCat, A375 and 92-1, respectively. The culture media were supplemented with 10% (v/v) Fetal Bovine Serum (FBS) with of 100 Unit/ml of penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, USA) and maintained at 37°C and 5% $CO_2$. Cells were sub-cultured twice a week using 0.025% (w/v) trypsin/EDTA.

**Methods**

SRPK inhibitors usage

SRPIN340 and SPHINX31 were purchased from Cayman chemical, USA. Cells at ~70% confluence was serum starved for at least 12 hours and treated with different concentration of SPHINX31 (depended on experimental designs), with 0.5% (v/v) DMSO was added to vehicle control.

Protein extraction and concentration measurement

Total cell proteins were isolated from cell lines using Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1% sodium dodecyl sulphate, 50 mM Sodium fluoride) with phosphatase inhibitor (Merck Millipore, USA). The protein concentrations were determined by Bradford assay. Briefly, BSA 0.5 mg/ml was dilute to 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/μl in 96 well plates as standard proteins. Then, 1 μl of extracted proteins (10-folds dilution) was used as unknown protein. They were mixed with Bradford solution 200 μl/well. After 4 hours incubation, the absorbance was measured at 595 nm. The protein concentration in the sample was calculated.

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

The equal number of proteins from each treatment (different concentration of SRPKi) was loaded and separated using SDS-PAGE gel (5% stacking gel and 12-15% separating gel) and then, transferred to PVDF membrane (Bio-Rad Laboratories, USA). The membranes were blocked using 5% chemiluminescent blocker (Merck Millipore, USA), then probed with primary antibody: mouse antihuman phosphoepitopes of SRSFs 1:1,000 (Merck Millipore, USA), Rabbit antihuman Akt 1:1,000, Rabbit antihuman phosphor-Akt 1:1,000 (Cell Signaling Technology, USA) at 4°C for overnight and probed with secondary antibody: HRP-conjugated goat-anti-mouse IgG 1:5,000 (Thermo Fisher Scientific, Bremen, Germany) for 1 hours. The protein bands were detected with Immobilon® ECL Ultra Western HRP Substrate (Merck Millipore, USA), imaged by Image Quant TM LAS 500 (GE Healthcare Life Sciences, UK). The signal intensity of each band was quantitated using ImageJ software as a semiquantitative expression normalized by the expression of GAPDH protein.

Immunocytofluorescence (ICF)

The cells (50,000 cells/well) were cultured in in 6 well plate for 24 hours. Cells were treated with SRPK inhibitors at 20 and 40 µM for 24 hours. The cells were washed PBS at 2 times. Next, fixed 4% (v/v) paraformaldehyde-containing PBS for 30 min at room temperature and then washed PBS 2 times 5 min. After washing, permeabilization with 0.2% (v/v) Triton-X containing PBS for 5 min at room temperature and then washed PBS at 1 time for 2 min. For blocking and non-specific binding was blocked by 1:20 FBS in PBS for 20 min. Cells were incubated with primary antibody: mouse antihuman phosphoepitopes of SRSFs 1:250 (Merck Millipore, MA, USA) at 4°C for overnight followed by secondary antibody goat anti-mouse IgG-FITC (Merck Millipore, MA, USA) and then washed PBS at 2 times. Furthermore, add 4′,6-diamidino-2-phenylindole or DAPI 1:10,000 (Merck Millipore, MA, USA) and then washed PBS at 2 times. The stained cells were examined under a fluorescence microscope.

Cell proliferation assay

The effect of SRPKi on cell viability was determined by measuring cell viability using 3-[4,5dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) assay. A375 and 92-1 cells was seeded in 96 well plates at $2\times10^3$ cells/well and then treated with vary concentration of SRPKi (0, 20, 40, 60 and 80 µM), incubated for 24, 48, and 72 hours. Subsequently, 0.5 ug/ml of MTT was added and incubated at 37°C for 4 hours. Then, removed the medium-containing MTT and dissolved the formazan crystals by DMSO (Sigma-Aldrich, MO, USA). The absorbances at 540 nm is observed by Microplate Spectrophotometer, the OD was used for calculating.

Clonogenic assay

A375 and 92-1 cells were plated at 500 and 2,000 cells/well, respectively, in 6 well plate. After 24 hours, cells were treated with SRPKi at 20 and 40 µM for 72 hours. The medium was changed every 3 days for a period of 10 days for A375 cell and 20 days for 92-1 cell. Cancer cell colonies were fixed in 4% (v/v) paraformaldehyde, stained by crystal violet and counted under the microscope. Number of colonies was counted by using ImageJ software and the results were expressed as a percentage of the untreated control cultures.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) from biological triplicate experiments. The statistical significance of the differences more than three different groups was determined with ANOVA. $P<0.05$ was considered to indicate a statistically significant difference. The significant indicator is; * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

# CHAPTER IV

# RESULTS

**The phosphorylation profile of SR protein family in melanoma cells compared with non-melanoma cell.**

Western blot was performed for estimating the phosphorylated form of SRSFs protein in A375 and 92-1 melanoma cells in comparison with HaCat non-melanoma cells. SRSFs phosphorylation was evaluated by anti-phosphoepitope SR protein antibody and represented in various molecular weight (MW) of SRSFs phosphorylation. The result showed the upregulation of pSRSFs in both A375 and 92-1 cells in comparison with HaCat cell (figure 37A and figure 38A). Additionally, band intensities of predominant pSRSFs were measured by ImageJ software and represented an increasing in pSRSF1/pSRSF12, pSRSF2/pSRSF7, and pSRSF3/pSRSF9 band intensities, while a decreasing in pSRSF4/pSRSF11, pSRSF5/pSRSF10, pSRSF6, and pSRSF8 band intensities in A375 cell in comparison with HaCat cell (figure 37B).

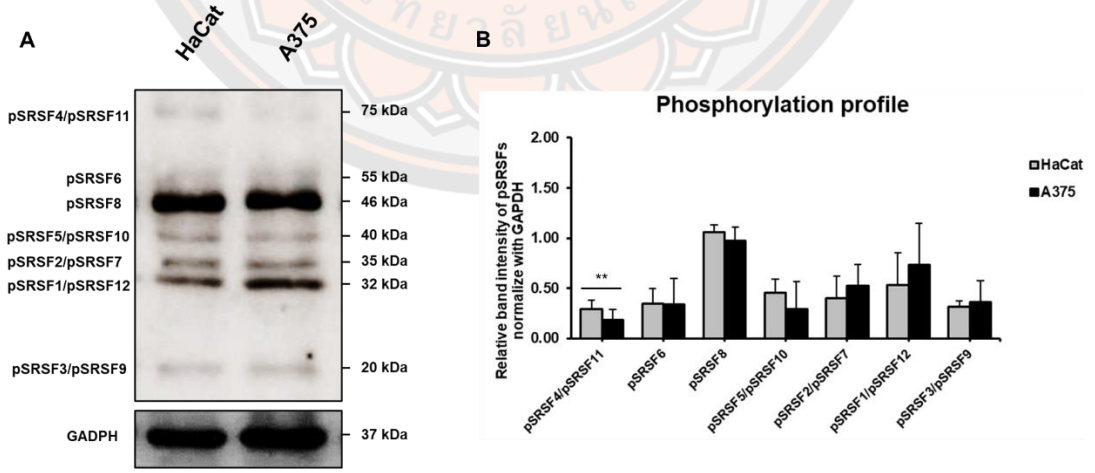


**Figure 37** The phosphorylation profile of SR protein family in A375 melanoma cell compared with HaCat non-melanoma cell (A). Band intensity analysis of the

phosphorylation profile by ImageJ software (B) ** P<0.01 (the represented figure was selected from 3 independent experiments).

Furthermore, 92-1 cell showed the significantly increase in pSRSF1/pSRSF12, pSRSF3/pSRSF9, and pSRSF4/pSRSF11 band intensities. However, pSRSF2/pSRSF7, pSRSF5/pSRSF10, and pSRSF6 represented the upregulation of band intensities but not significant, which may be because these pSRSFs may have an important play role on both normal and cancer cells (figure 38B). Remarkably, both A375 and 92-1 cells showed an increasing in pSRSF1/pSRSF12, pSRSF2/pSRSF7, and pSRSF3/pSRSF9 band intensities. These pSRSFs may be important molecules, which play a role in melanoma development.

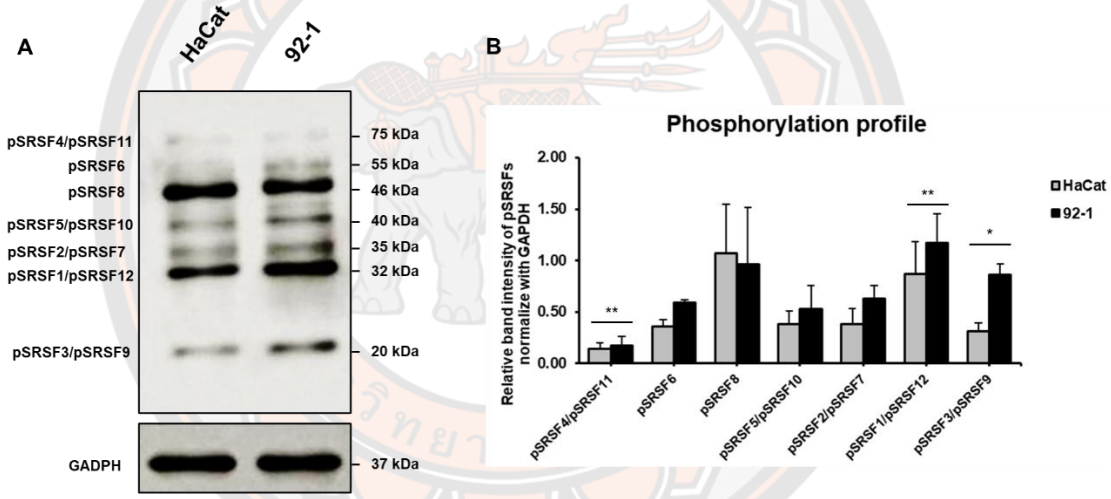


**Figure 38** The phosphorylation profile of SR protein family in 92-1 melanoma cell compared with HaCat non-melanoma cell (A). Band intensity analysis of the phosphorylation profile by ImageJ software (B) * P<0.05, and ** P<0.01 (the represented figure was selected from 3 independent experiments).

**Effect of SRPK inhibitors on cell viability in melanoma cells.**

The cytotoxic potential of SRPKs-specific inhibitors was evaluated against A375 and 92-1 cells before tested its activity next experiments. Cell viabilities were determined by MTT assay in 24 hours interval for 3 days. The result performed 80 µM concentration of SRPIN340 could significantly decrease of A375 cell viability

(67.00±2.65) in 24 hours (figure 39A). In 48 and 72 hours, a decreasing in SRPIN340 treated-A375 cell viability showed 41.00±1.00, and 24.00±7.64 respectively (figure 39B and 39C).

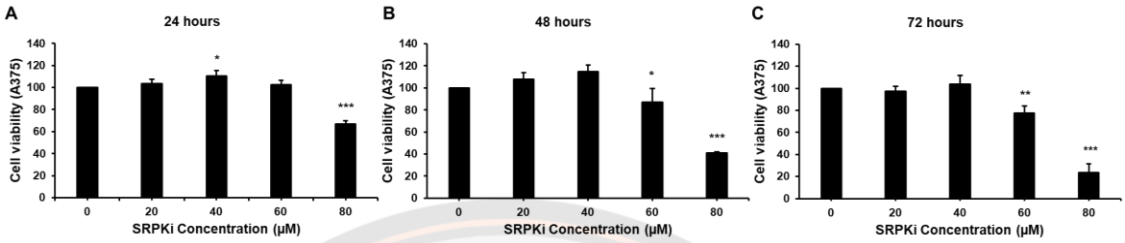


**Figure 39** The effect of SRPIN340 treatment (in vary concentration) on the viability of A375 cells in 24 hours interval for 3 days (A-C) * P<0.05, ** P<0.01, and ***P<0.001.

Remarkably, the treatment with 48 and 72 hours showed that SRPIN340 can significantly suppress more the viability of 92-1 than A375 cell, especially in 80 μM concentration. Due to a decreasing in SRPIN340-treated 92-1 cell viability showed 13.00±4.36, and 8.00±2.08 cell viability for 48 and 72 hours respectively (figure 40B and 40C). Thus, it seems that SRPIN340 is more sensitive 92-1 than A375 cell viability for long time durations.

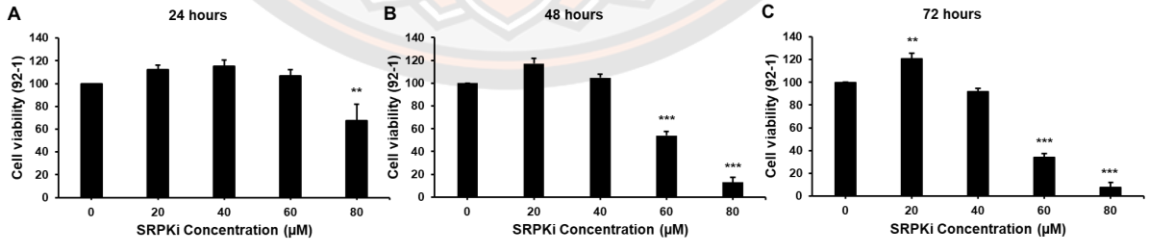


**Figure 40** The effect of SRPIN340 treatment (in vary concentration) on the viability of 92-1 cells in 24 hours interval for 3 days (A-C) ** P<0.01, and ***P<0.001.

Additionally, SPHINX31 could significantly suppress the viability of 92-1 cell greater than A375 in 24 hours. Because 40, and 60 µM SPHINX31 treated 92-1 cell showed 76.00±2.96, and 74.00±4.77 cell viability respectively (figure 42A). However, it seems that SPHINX31 more sensitive A375 than 92-1 cell in 48 and 72 hours after treatment.

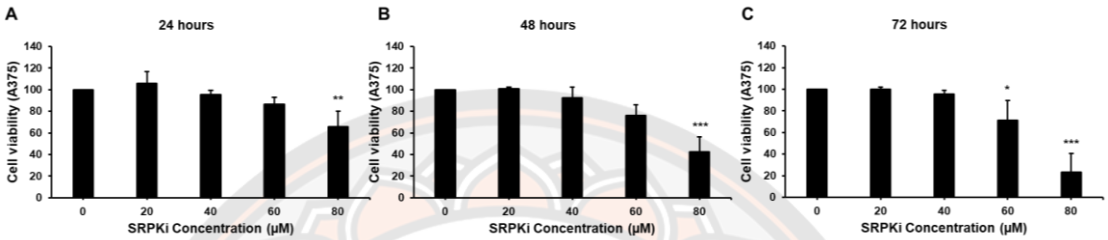


**Figure 41** The effect of SPHINX31 treatment (in vary concentration) on the viability of A375 cells in 24 hours interval for 3 days (A-C) * P<0.05, ** P<0.01, and ***P<0.001.

Especially, at 80 µM concentration of SPHINX31 showed a decreasing in A375 cell viability for 48 hours (42.00±14.01) and 72 hours (23.00±17.21) respectively (figure 41B and 41C). Thus, it seems that SPHINX31 is more sensitive A375 than 92-1 cell viability for long time durations.

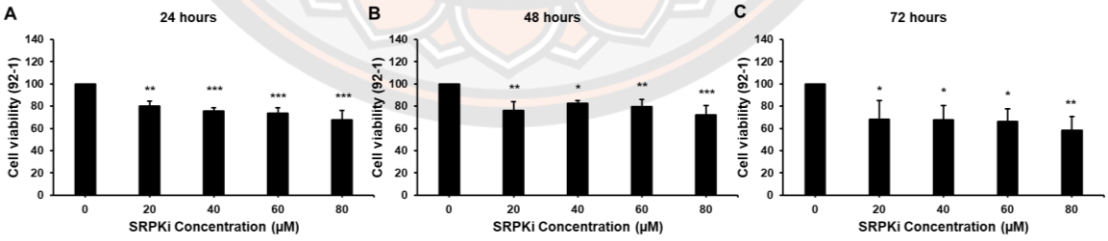


**Figure 42** The effect of SPHINX31 treatment (in vary concentration) on the viability of 92-1 cells in 24 hours interval for 3 days (A-C) * P<0.05, ** P<0.01, and ***P<0.001.

Furthermore, the half maximal inhibitory concentration (IC50) of SRPIN340 showed time dependent manner in melanoma cells but SPHINX31 showed time dependent manner in only A375 cell (table 2). Therefore, we can summary that the

effect of SRPIN340 on cell viability as in dose- and time- dependent manners both A375 and 92-1 cells. However, the effect of SPHINX31 on cell viability as in dose- and time- dependent manners only A375 cell.

**Table 2** The half maximal inhibitory concentration (IC50) of SRPIN340 and SPHINX31 in melanoma cells.

| SRPK inhibitors | Cell lines | The half maximal inhibitory concentration (IC50) (μM) | | |
|---|---|---|---|---|
| | | 24 hours | 48 hours | 72 hours |
| SRPIN340 | A375 | > 80.00 | 76.41±1.54 | 70.08±2.16 |
| | 92-1 | > 80.00 | 61.76±1.30 | 54.87±1.29 |
| SPHINX31 | A375 | > 80.00 | 67.37±5.60 | 58.35±9.47 |
| | 92-1 | > 80.00 | > 80.00 | > 80.00 |

**Effect of SRPK inhibitors on SRSFs phosphorylation in melanoma cells.**

Regarding to the result of phosphorylation profile of SRSFs upregulated in A375 and 92-1 cells. Moreover, the result of cell viability, 20 and 40 μM of SRPKs-specific inhibitors were selected for further study on the molecular function of SRPKs-specific inhibitors in the melanoma cells.

The result showed that SRPIN340 could reduce the phosphorylated form of SRSFs, especially at 40 μM concentration (figure 43A). Additionally, band intensities of predominant pSRSFs were mentioned in phosphorylation profile experiment. There was a significantly decrease in pSRSF1/pSRSF12 (0.17±0.04), and pSRSF2/pSRSF7 (0.16±0.06) band intensities. Moreover, other pSRSFs including pSRSF3/pSRSF9, pSRSF4/pSRSF11, pSRSF5/pSRSF10, pSRSF6, and pSRSF8 band intensities were reduced but not significant in SRPIN340-treated A375 cell (figure 43B).

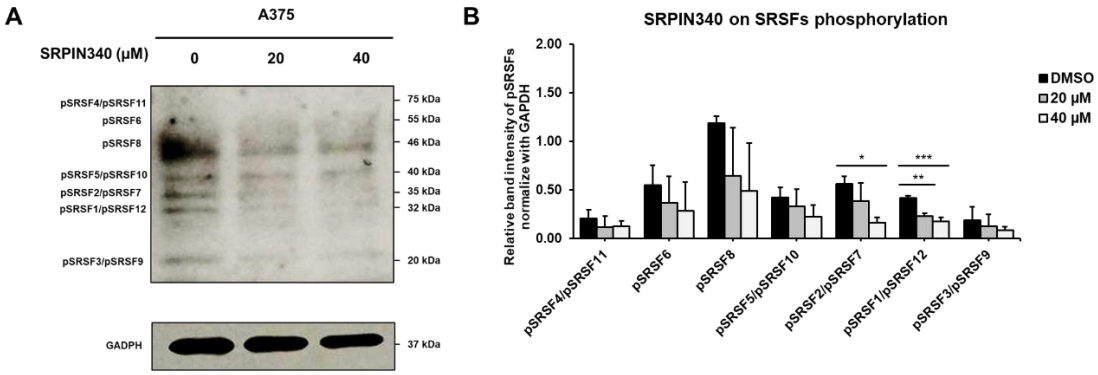


**Figure 43** Effect of SRPIN340 on SRSFs phosphorylation in A375 cell (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B) * P<0.05, ** P<0.01, and ***P<0.001 (the represented figure was selected from 3 independent experiments).

Additionally, 40 μM concentration of SRPIN340 could significantly reduce the phosphorylation of SRSFs (figure 44A) such as pSRSF1/pSRSF12 (0.31±0.09), pSRSF2/pSRSF7 (0.37±0.08), and pSRSF3/pSRSF9 (0.14±0.015) in 92-1 cell. However, we found that there was a reducing of pSRSF4/pSRSF11, pSRSF5/pSRSF10, pSRSF6, and pSRSF8 band intensities but not significant in SRPIN340-treated 92-1 cell (figure 44B).

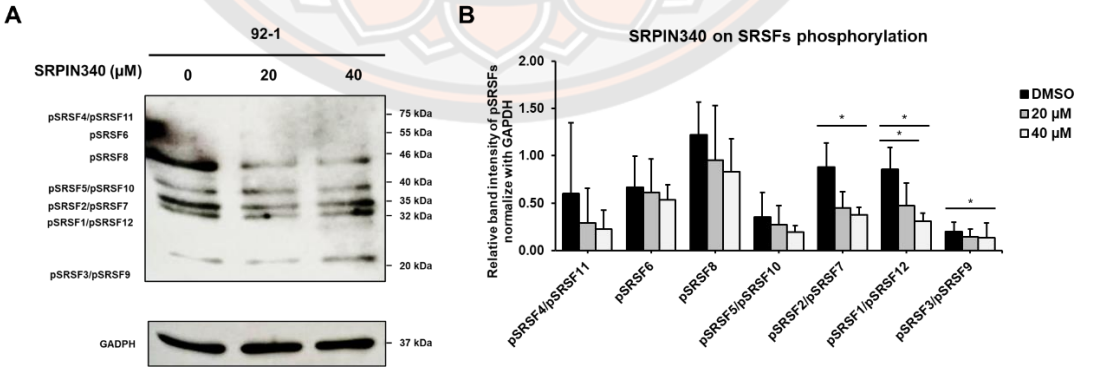


**Figure 44** Effect of SRPIN340 on SRSFs phosphorylation in 92-1 cell (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B) * P<0.05 (the represented figure was selected from 3 independent experiments).

Moreover, the effect of SPHINX31 on melanoma cells was observed same. We found that SPHINX31 could reduce the phosphorylation of SRSFs in A375 cell (figure 45A). Band intensities of predominant pSRSFs were measured. The results showed that there was a significantly decrease in pSRSF1/pSRSF12 (0.11±0.03), and pSRSF2/pSRSF7 (0.15±0.02) band intensities. Moreover, the effect of SPHINX31 showed a reducing of pSRSF3/pSRSF9, pSRSF4/pSRSF11, pSRSF6, and pSRSF8 band intensities, especially at 40 µM concentration of SPHINX31 but not significant (figure 45B).

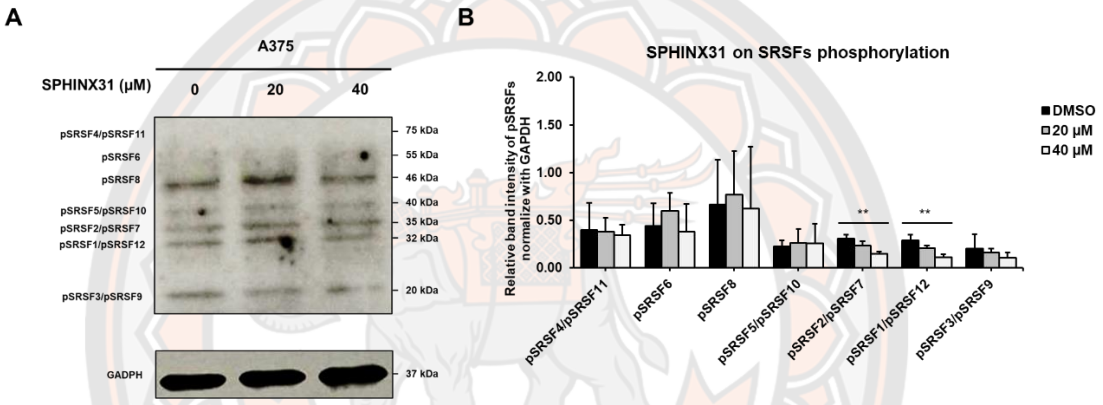


**Figure 45** Effect of SPHINX31 on SRSFs phosphorylation in A375 cell (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B) ** P<0.01 (the represented figure was selected from 3 independent experiments).

Furthermore, SPHINX31 could significantly reduce the phosphorylation of SRSFs (figure 46A) including pSRSF1/pSRSF12 (0.21±0.12), and pSRSF2/pSRSF7 (0.29±0.01) band intensities. Additionally, we found that there was a decreasing in pSRSF3/pSRSF9, pSRSF4/pSRSF11, pSRSF5/pSRSF10, pSRSF6, and pSRSF8 band intensities but not significant in SPHINX31-treated 92-1 cell (figure 46B). Therefore, we can summary that both SRPIN340 and SPHINX31 can suppress the SRSFs phosphorylation in melanoma cells as dose dependent manner.

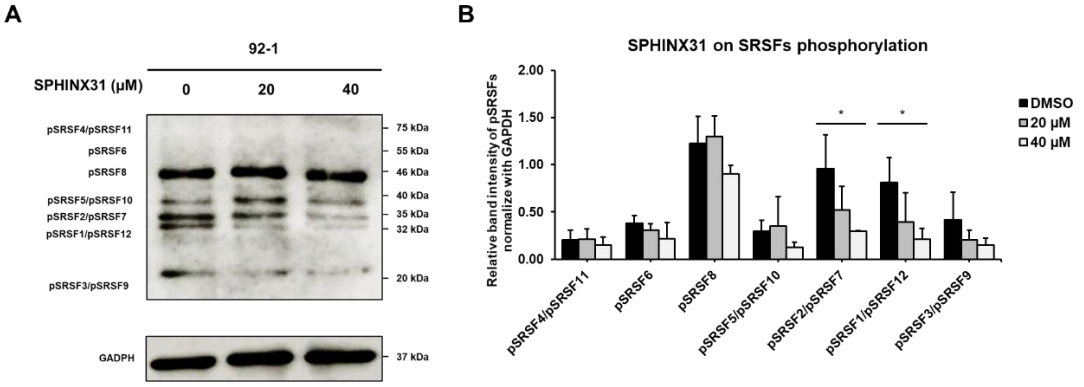


**Figure 46** Effect of SPHINX31 on SRSFs phosphorylation in 92-1 cell (A). Band intensity analysis of SRSFs phosphorylation by ImageJ software (B) * P<0.05 (the represented figure was selected from 3 independent experiments).

**Effect of SRPK inhibitors on SRSFs translocation in melanoma cells.**

Regarding to SRPKs function that phosphorylated SRSFs, it can translocate into the nucleus for regulating the aberrant alternative splicing. Although, effect of SRPKs-specific inhibitors on SRSFs translocation in melanoma cells was investigated. 20, and 40 μM concentration of SRPK-specific inhibitors were selected to treat in A375 and 92-1 cells for 24 hours, pSRSFs translocation was monitored by immunocytofluorescent (ICF). For A375 cell line, the result demonstrated that SRPIN340 increased a number of fluorescent antibody stain specific pSRSFs (green fluorescent) strong intensity (figure 47A). Moreover, SPHINX31 could increase the accumulation of pSRSFs in cytoplasm of A375 cell, especially in 40 μM concentration of SRPKs specific inhibitors at pointed by white arrow (figure 47B).

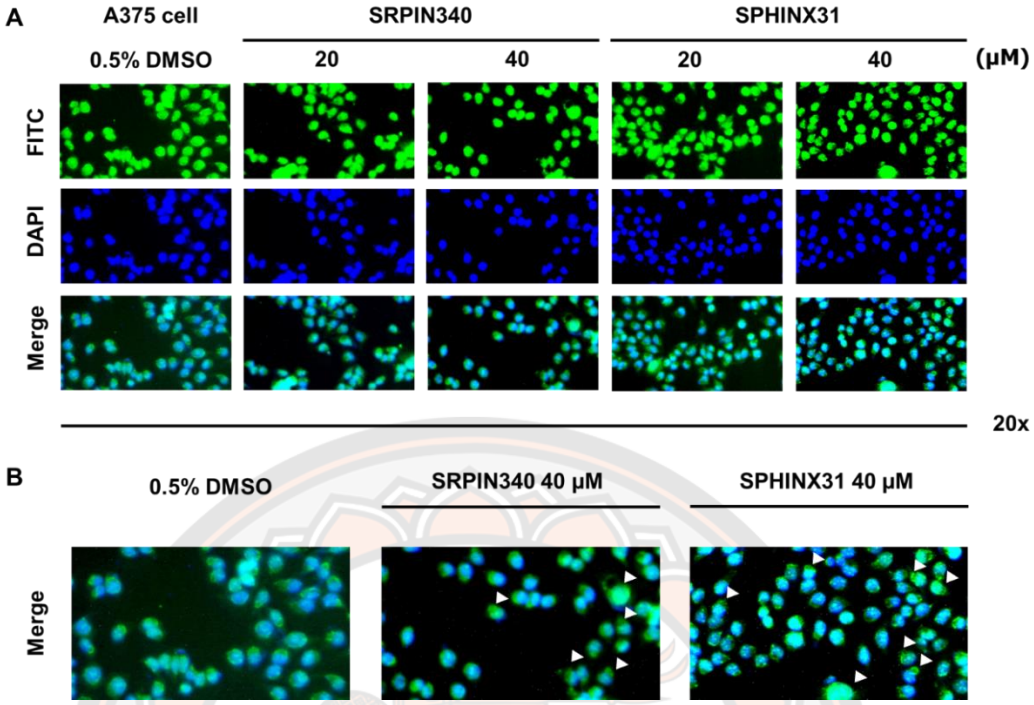


**Figure 47** Effect of SRPIN340 and SPHINX31 on the phosphorylated form of SRSFs localization in A375 cell (A). The accumulation of fluorescent antibody stain specific pSRSFs (green fluorescent) strong intensity in cytoplasm was observed by immunocytofluorescent (ICF) (B).

For 92-1 cell line, treatment with SRPIN340 could increase pSRSFs positive cells (figure 48A). Furthermore, SPHINX31 could increase the accumulation of pSRSFs in cytoplasm of 92-1 cell, especially in 40 μM concentration of SRPKs specific inhibitors at pointed by white arrow (figure 48B). Therefore, we can summary that both SRPIN340 and SPHINX31 could increase the accumulation of pSRSFs in cytoplasm of melanoma cells.

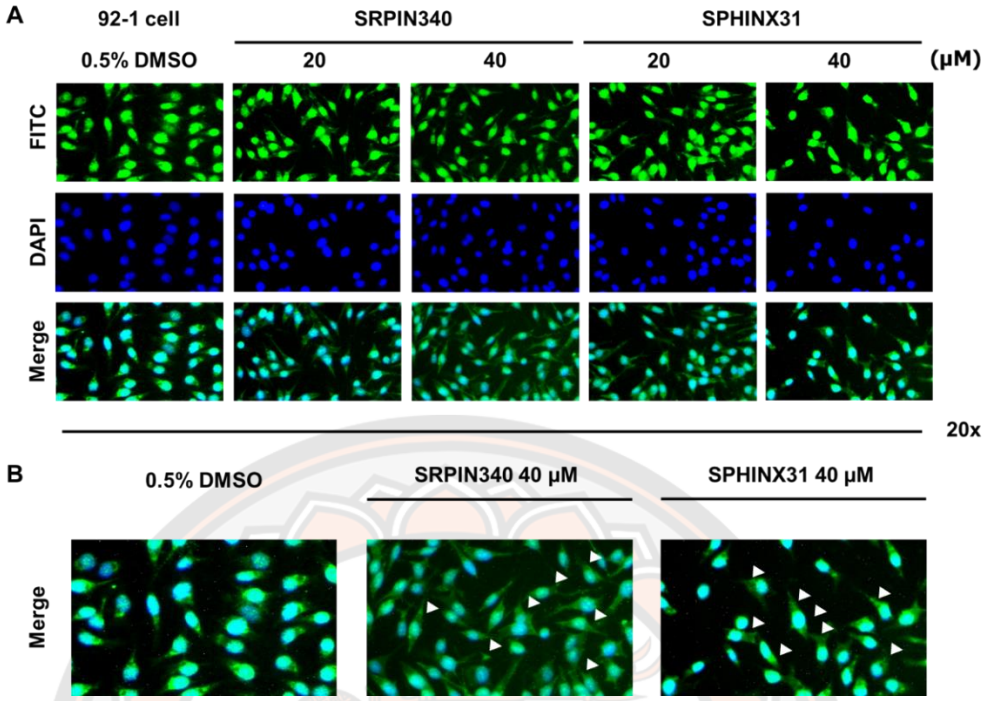


**Figure 48** Effect of SRPIN340 and SPHINX31 on the phosphorylated form of SRSFs localization in 92-1 cell (A). The accumulation of fluorescent antibody stain specific pSRSFs (green fluorescent) strong intensity in cytoplasm was observed by immunocytofluorescent (ICF) (B).

**Effect of SRPK inhibitors on colony formation ability of melanoma cells.**

After the cells had been treated by 20, and 40 μM concentration of SRPKs specific inhibitors, colonies formation was determined by clonogenic assay and monitored for 10 days and 20 days in A375 and 92-1 cells respectively. The results showed that A375 cell was more effective SPHINX31 than SRPIN340 at 20 μM concentration (figure 49A). For the quantitative analysis of A375 cell colonies, we used ImageJ to detect and count. SPHINX31 can significantly decrease the size and number of A375 cell colonies (199.00±46.12) at 40 μM concentration while SRPIN340 can rarely suppress A375 cell colonies (243.00±44.19) (figure 49B).

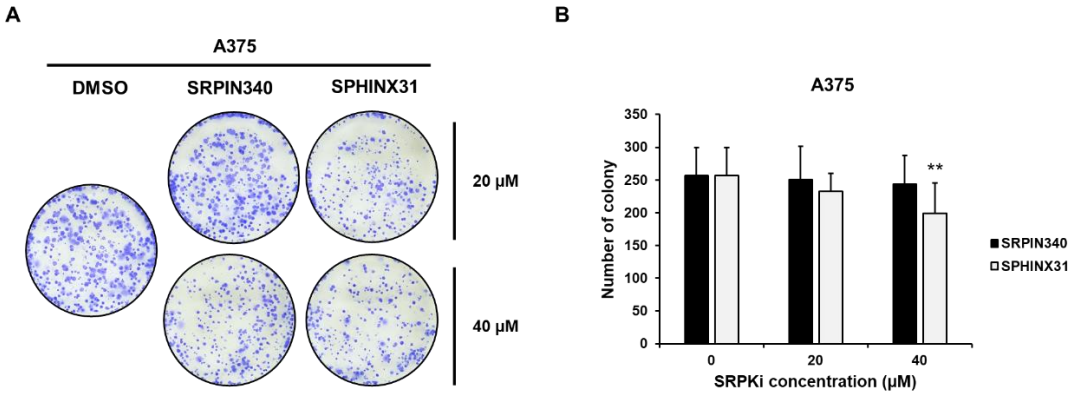


**Figure 49** Suppression of SRSF phosphorylation by SRPKs specific inhibitors had affected on colony formation ability of A375 cell (A). A375 Colonies were counted by ImageJ software with trended as in dose-dependent manner (B) ** P<0.01 (the represented figure was selected from 3 independent experiments).

For 92-1 cell colonies, the result showed that SRPIN340 (88.00±10.58) was more effective than SPHINX31 (174.00±72.00) at 40 µM concentration (figure 50A). For the quantitative analysis of 92-1 cell colonies, we found that SRPIN340 can significantly reduce the size and number of 92-1 cell colonies at 20, and 40 concentration while SPHINX31 can insignificantly decrease (figure 50B). Therefore, we can summary A375 cell is more sensitive SPHINX31 while SRPIN340 can be more effective 92-1 cell.

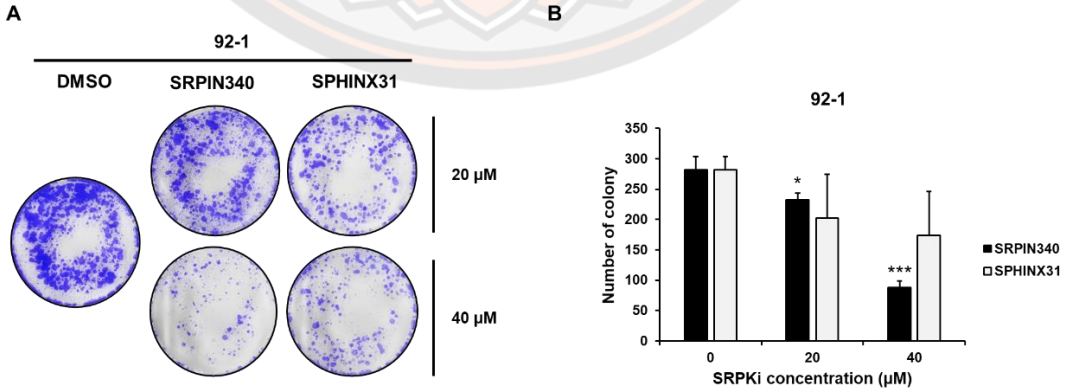


**Figure 50** Suppression of SRSF phosphorylation by SRPKs specific inhibitors had affected on colony formation ability of 92-1 cell (A). 92-1 Colonies were counted by

ImageJ software with trended as in dose-dependent manner (B) * P<0.05, and *** P<0.001 (the represented figure was selected from 3 independent experiments).

**Effect of SRPK inhibitors on the activation of growth-related signaling pathway.**

Regarding to SRPKs are involved in the phospho-regulation of the SRSFs via the EGF-AKT-SRPK signal transduction axis. Although, effect of SRPKs specific inhibitors on the activation of growth-related signaling pathway in melanoma cells was monitored. After 20 and 40 µM of SRPKs specific inhibitors treatment in A375 and 92-1 cells for 24 hours, target proteins were detected by western blot. For A375 cell, the result showed that treatment with SRPIN340 could reduce p-AKT (Ser473), which related cell survival signaling (figure 51A). Band intensities of p-AKT (Ser473) were measured. The result showed that there was a significantly decreasing in p-AKT at Ser473 (0.55±0.14) band intensity (figure 51B).

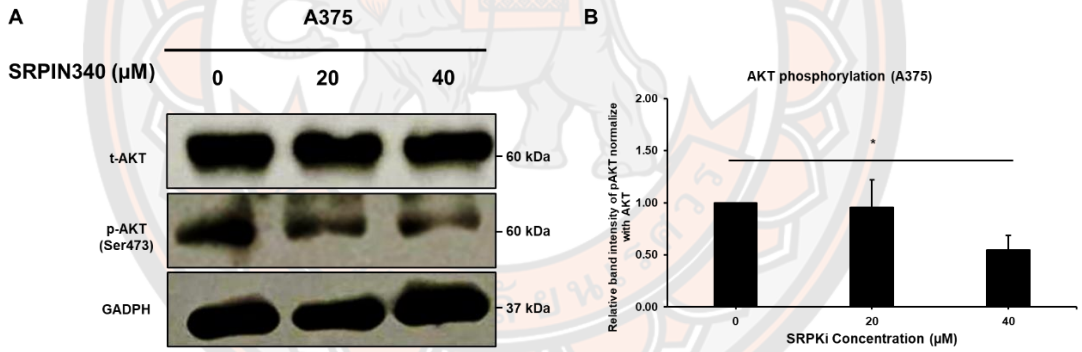


**Figure 51** Effect of SRPIN340 on AKT phosphorylation (Ser473) in A375 cell (A). Band intensity analysis of AKT phosphorylation (Ser473) by ImageJ software (B) * P<0.05 (the represented figure was selected from 3 independent experiments).

For 92-1 cell, we found that treatment with SRPIN340 could decrease p-AKT (Ser473) (figure 52A). However, band intensities of p-AKT were measured. The results showed that SRPIN340 could not reduce p-AKT at Ser473 (1.00±0.19) band intensity (figure 52B).

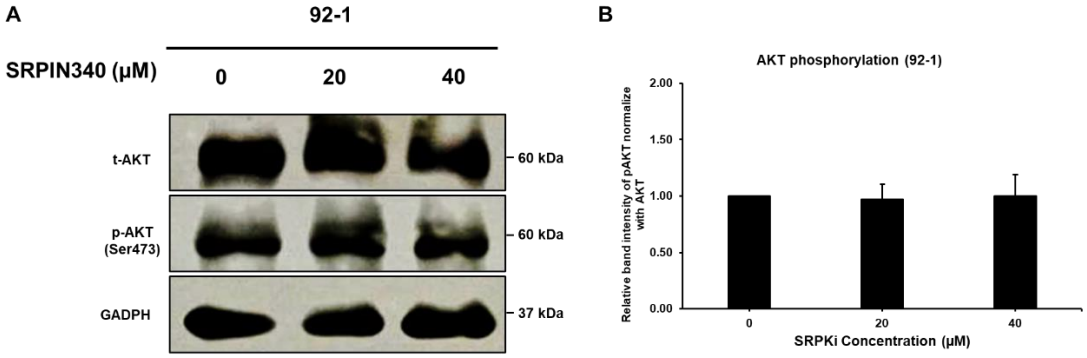


**Figure 52** Effect of SRPIN340 on AKT phosphorylation (Ser473) in 92-1 cell (A). Band intensity analysis of AKT phosphorylation (Ser473) by ImageJ software (B). (the represented figure was selected from 3 independent experiments).

Furthermore, treatment with SPHINX31 was demonstrated in melanoma cells. Treatment with SPHINX31 could reduce p-AKT (Ser473) (figure 53A). Band intensities of p-AKT (Ser473) were measured. The result showed that treatment 40 μM of SPHINX31 could significantly decrease p-AKT at Ser473 (0.51±0.13) band intensity while 20 μM of SPHINX31 could reduce p-AKT at Ser473 but not significant in A375 cell (figure53B).

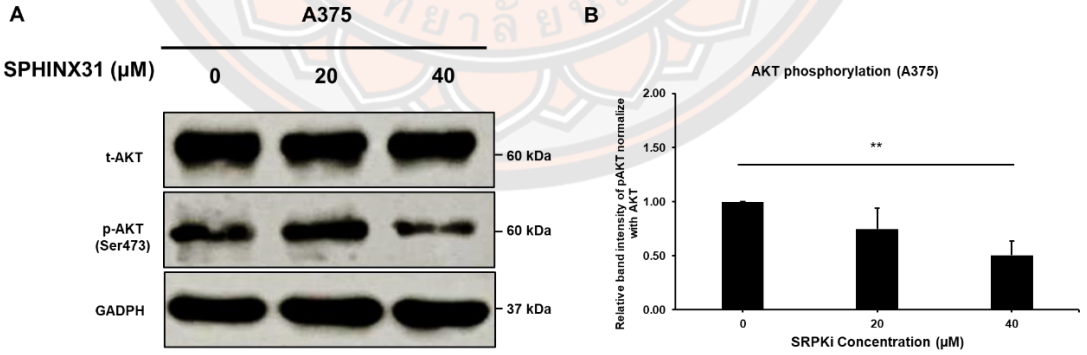


**Figure 53** Effect of SPHINX31 on AKT phosphorylation (Ser473) in A375 cell (A). Band intensity analysis of AKT phosphorylation (Ser473) by ImageJ software (B) ** $P<0.01$ (the represented figure was selected from 3 independent experiments.

For 92-1 cell, the result showed that SPHINX31 could decrease phosphorylation of AKT (Ser473) (figure 54A). However, band intensities of p-AKT (Ser473) were measured. We found that treatment with 40 µM of SPHINX31 could reduce p-AKT at Ser473 (0.67± 0.52) but not significant in 92-1 cell (figure 54B). Therefore, we can summary that SRPIN340 and SPHINX31 tend to reduce the AKT phosphorylation via dysfunction of SRPKs in melanoma cells.

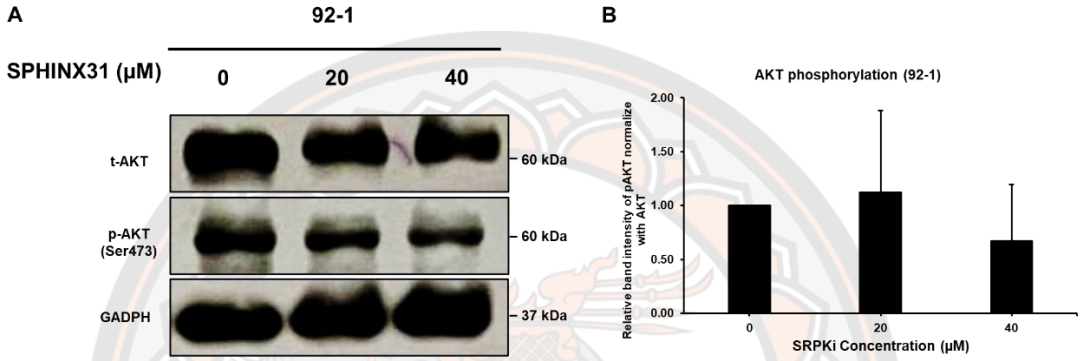


**Figure 54** Effect of SPHINX31 on AKT phosphorylation (Ser473) in 92-1 cell (A). Band intensity analysis of AKT phosphorylation (Ser473) by ImageJ software (B). (the represented figure was selected from 3 independent experiments).

# CHAPTER V

# DISCUSSION AND CONCLUSION

## Discussion

Melanoma is, the malignant transformation of melanocyte, an aggressive cancer which is a serious health problem in countries with high sun or UV exposure, including Thailand. Even though, it is a rare incidence, but an amount of mortality cases is caused by metastasize to secondary sites, including the lymph nodes, lung, liver, bone and brain (Braeuer R.R. et al., 2013) lead to very poor prognosis and ineffective treatment. Our research group review article summarized the contribution of aberrant gene splicing in cancer development and progression. Alternative splicing function was regulation of the mechanism of gene expression, which results in a single gene transcribed into several mRNA transcript and subsequently translated into multiple protein isoforms within cells (Black, D. L., 2003). In this process, specific exons of a gene may be included within or excluded from pre-mRNA. Consequently, the proteins translated from alternatively spliced mRNAs could contain different amino acid sequence and structure (Cooper, G., 2006). In human, more than 95% of roughly 30,000 human genes undergo alternative splicing to encode two or more splice isoforms (Pan, Q. et al., 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 Serine and arginine-rich proteins (SR proteins) or Serine/arginine-rich splicing factors (SRSFs). Remarkably, the upregulation and activation of Serine-Arginine Protein Kinases or SRPKs, splicing factor kinases, play essential role to phosphorylation of Serine-Arginine Rich Splicing Factors or SRSFs for regulating the aberrant alternative splicing and potentially encourage to various diseases and in particular especially to the development of various cancer (da Silva M.R. et al., 2015). That phenomenon is related to the cancer cell aggressiveness (David and Manley, 2010; Kim and Kim, 2012).

Regarding to SRPKs function that control cancer progression via the aberrant alternative splicing, it led to develop substance that can inhibit SRPKs function, SRPKs specific inhibitors. However, previous study showed both SRPIN340 and SPHINX31 are the most common use in research fields. In this study, we focus on the association of specific SRPKs function, which phosphorylated SRSFs, and effect to the aggressive phenotype and related signaling pathway of melanoma cells. We evaluated the phosphorylation profile of SRSFs in A375 cutaneous melanoma cell and 92-1 ocular melanoma cell in comparison with HaCat keratinocyte cell by western blot analysis and followed by anti-phosphoepitope SR protein antibody and represented in various molecular weight (MW) of SRSFs phosphorylation. Our funding showed an increasing in pSRSF1/pSRSF12, pSRSF2/pSRSF7, and pSRSF3/pSRSF9 band intensities, which is possible that these pSRSFs may be an important molecule that play a role in A375 and 92-1 melanoma cells (figure 37 and figure 38). Similarly, previous study found that the upregulation of SRSFs in 9 cancer tissue by Genvestigator and showed an increasing in SRSF1, SRSF2, SRSF3, SRSF9, and SRSF10 expression in melanoma tissue (Zhou, X. et al., 2019) (figure 11). For pSRSFs overexpression in melanoma cells might to regulate the aberrant alternative splicing for development of various cancer or cancer cell aggressiveness (da Silva M.R. et al., 2015; David and Manley, 2010).

In 2018, the research article showed the effect of SRPK1/2-specific inhibitor, SRPIN340 treatment on cell viability of acute myeloid leukemia cells. They found that SRPIN340 treatment could reduce the leukemia cell viability, which directly associated with dysfunction of SRSK1/2 (Siqueira R.P. et al., 2015) (figure 24). In addition, another previous study performed that SRPK1-specific inhibitor, SPHINX31 treatment could decrease the leukemia cell viability via dysfunction of SRPK1 (Tzelepis K. et al., 2018) (figure 31). These evidences represented SRPK1/2 function associated aggressive cancer progression. In our result, we treated with both SRPIN340 and SPHINX31 on melanoma cell. The results showed that SRPIN340 can significantly reduce cell viability in both A375 and 92-1 cell as in dose- and time-dependent manners (figure 39 and figure 40). However, the effect of SPHINX31 on cell viability as in dose- and time- dependent manners, appeared only A375 cell (figure 41 and figure 42). Furthermore, our preliminary experiment showed that both

SRPIN340 and SPHINX31 have a highly toxicity in HaCat cell. Because HaCat cell is a Keratinocyte cell but both A375 and 92-1 are abnormal melanoma, which are different types of cells. Although, treatment with SRPK-specific inhibitors in different types of cells will have different effects.

In order to investigate the molecular mechanism, previous study presented that SRPIN340 treatment in leukemia cells could decrease SRSFs phosphorylation as dose-dependent manner (Siqueira R.P. et al., 2015) (figure 25). Moreover, there were a report showed that SPHINX31 treatment could inhibit SRSF1 phosphorylation in PC3 prostate cancer cell (Batson J. et al., 2017) (figure 30). Therefore, we designed the experiment to investigate phosphorylated inhibitory efficiency. Our result showed the both SRPIN340 and SPHINX31 can significantly decrease some of SRSFs phosphorylation including pSRSF1/pSRSF12, and pSRSF2/pSRSF7 in melanoma cells as dose-dependent manner, especially in high concentration of SRPKs specific inhibitors (figure 43-46). Remarkably, a decreasing in these pSRSFs similar to previous studies, which showed that SRPIN340 treatment could reduce pSRSF2 leukemia cells (Siqueira R.P. et al., 2015) and SPHINX31 treatment could reduce pSRSF1 in prostate cancer cell (Batson J. et al., 2017). Next, we confirmed the dysfunction of pSRSFs by SRPKs specific inhibitors was monitored by ICF. We found that both SRPIN340 and SPHINX31 can increase the accumulation of pSRSFs in cytoplasm of SRPKs specific inhibitors-treated melanoma cells, especially in 92-1 cell (figure 48). Therefore, SRPKs-specific inhibitors tend to decrease the proliferation ability of melanoma cell via interruption SRSFs nuclear translocation. Interestingly, previous article performed that SRPIN340 could be effective in impairing SRPK1 nuclear translocation and SRSFs phosphorylation in the intracellular environment in B16F10 murine melanoma model (Moreira G.A. et al., 2018) (figure 36).

Previously in study 2014, the researchers found that the essential role of SRPK1 associated pro-angiogenic VEGF splicing for tumor angiogenesis in metastatic melanoma and they use SRPIN340 for proof its activity. They found that tumor growth in SRPIN340 treated group was decreased in comparison with control group (Gammons M.V. et al., 2014) (figure 33). Recently, there is article study reported SRPK inhibitor’s activity could reduce colony formation of B16F10 murine

melanoma cell as dose dose-dependent manner (Moreira G.A. et al., 2018) (figure 34). Although, our study presented the effect of SRPKs specific inhibitors on growth ability in A375 and 92-1 melanoma cells and demonstrated reducing of the size and number of melanoma colonies after both SRPIN340 and SPHINX31 treatment. However, it seems A375 cell is more effective SPHINX31 (figure 49) while SRPIN340 can be more effective 92-1 cell (figure 50). Moreover, extended experiments from a member in our research group selected A375 cell for treating SPHINX31's activity on dead evasion and migration abilities. Cancer cell behaviors were followed by live and dead staining and wound healing assays for mimicking the dead-evading and metastasis associated phenotypes, the dead evasion of SPHINX31 treated A375 cell was decreased when in comparison with control group (figure 55).

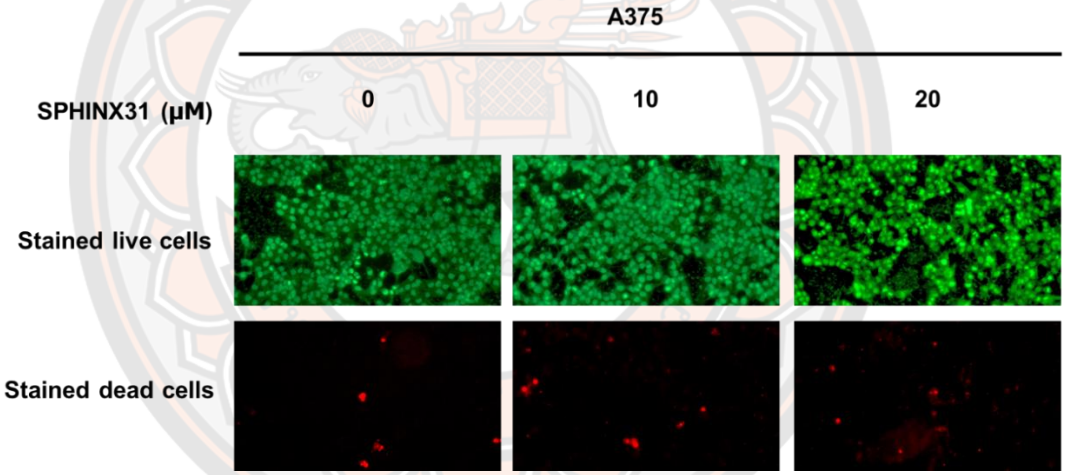


**Figure 55** Specific suppression of SRPK1 activity by SPHINX31 had affected on dead evasion of A375 cell by live and dead staining assay.

While the migration ability was also slower as well, especially in 48 hours (figure 56). Although, the effect of SPHINX31 could decrease the dead evasion and migration ability of A375 cell.

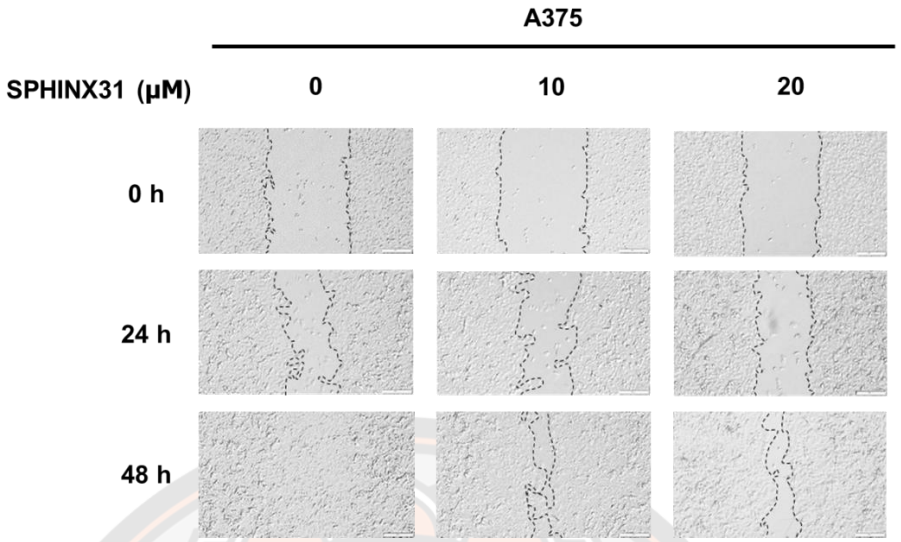


**Figure 56** Specific suppression of SRPK1 by SPHINX31 had affected on migration abilities of A375 cell by wound healing assay.

Regarding to SRPKs were involved in the regulation of the SRSFs phosphorylation through the EGF-AKT-SRPK signal transduction axis (da Silva M.R. et al., 2015). Moreover, previous study showed SRPIN340 administration could increase AKT phosphorylation in neonatal rat ventricular myocytes (NRVMs). They assumed that SRPIN340 could impair SRPK1-dependent recruitment of protein phosphatase, PHLPP1 to AKT at certain concentrations (Huang J. et al., 2019) (figure 27). Therefore, our group confirmed the effect of SRPKs-specific inhibitors on the activation of growth-related signaling pathway. Our result showed both SRPIN340 and SPHINX31 could decrease p-AKT (Ser473) in SRPIN340- and SRPHINX31-treated melanoma cells in comparison with control group as dose-dependent manner (figure 51 and figure 53). Therefore, both SRPIN340 and SPHINX31 tend to inactivate the AKT phosphorylation via dysfunction of SRPKs in melanoma cells.

The essential role of SRPKs in basic behaviors base on their benefits for slicing gene associated cancer isoforms, such as RONΔ165 (Moon H. et al., 2014), the pro-angiogenic VEGF165, and Rac1b (Gonçalves V. et al., 2014), which have been related to metastatic melanoma (Gammons M. V. et al., 2014). Therefore, these data collectively suggest that SPPKs can be used as the target for the precision therapeutic strategy of melanoma cells. Moreover, we attempted to demonstrate the

effect of SRPIN340 and SPHINX31 on the growth ability of melanoma cells through the inhibiting of SRSFs phosphorylation and inactivating of AKT phosphorylation by SRPKs specific inhibitors. These results will be serving as the basis information for targeting SRPKs as an alternative therapeutic strategy for melanoma treatment.

Further study, treatment of combined SRPKs specific inhibitors with AKT inhibitor in A375 and 92-1 cells is proposed for testing their activity on SRSFs phosphorylation and aggressive phenotypes including dead evasion, cell migration, cell proliferation, cell growth, and cell apoptosis. Moreover, we expect to design the experiment for study the molecular insight or exploration of SRPK co-partner proteins by subcellular proteomics or phosphoproteomics.

**Conclusion**

Serine-Arginine Protein Kinases or SRPKs are serine/threonine kinases regulate the alternative splicing by phosphorylation of target protein, splicing factor SRSFs. Upregulation or dysregulation of SRPKs involving the aberrant alternative splicing of many oncogenic properties, include growth ability. To study the SRPKs function and association with melanoma cells (A375 and 92-1), we performed SRPKs specific inhibitors (SRPIN340 and SPHINX31) can decrease melanoma cell viability through dysfunction of SRSFs phosphorylation and increase the accumulation of pSRSFs in cytoplasm of melanoma cell. Next, suppression of SRSFs phosphorylation had affected on the size and number of colony formation of melanoma cells by both SRPIN340- and SPHINX31- treated melanoma cells, especially in 92-1 ocular melanoma cell. In addition, both SRPIN340 and SPHINX31 can be inactivate AKT phosphorylation through dysfunction of SRPKs. This study will be serving as the basis information for targeting SRPKs as an alternative therapeutic strategy for melanoma treatment.

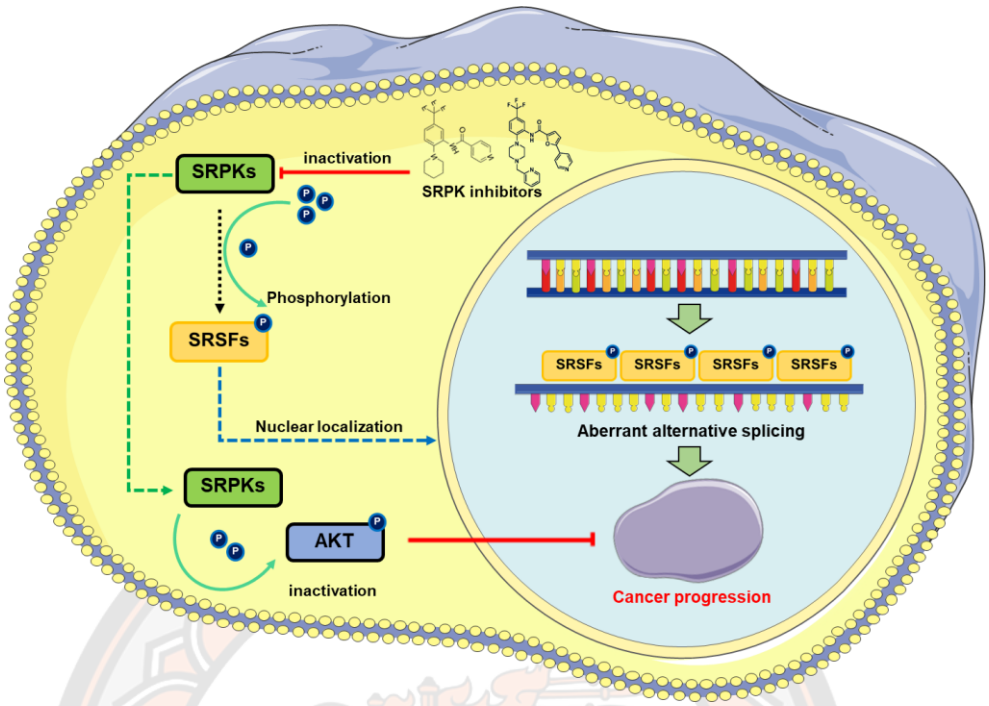


**Figure 57** Schematic representation of SRPKs specific inhibitors treatment. After treatment SRPKs cannot be phosphorylate SRSFs, it will be not translocated into the nucleus for regulating the aberrant alternative splicing. Subsequently, SRPKs specific inhibitors can be inactivated the AKT phosphorylation and lead to decrease cancer progression in melanoma cells.

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# APPENDIX

## APPENDIX A LIST OF SOLUTIONS AND BUFFERS

### 1. SDS-PAGE and Western Blot

**1.1 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.

**1.2 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.

**1.3 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 | |

**1.4 10% Ammonium persulfate**

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

**1.5 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 | |

**1.6 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.

**1.7 10X Bolt & Mohoney transferring buffer**

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

**1.8 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.

**1.9 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 | |

**1.10 Blocking buffer** Immobilon® Block - CH (Chemiluminescent Blocker)

Blocking solution volume in 30 ml to ensure efficient membrane blocking.

**2. Culture Medium**

**2.1 Complete media**

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

**2.2 Antibiotic free media**

Dulbecco's Modified Eagle Medium ( DMEM) and Roswell Park Memorial Institute (RPMI) 1640 Medium were 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.

**3. Fixative reagent**

3.1 2.5% Glutaraldehyde

2.5% Glutaraldehyde was dissolved in 0.1 M Phosphate 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 Effect of SRPIN340 on cell viability in A375 and 92-1 cell at 24 hours using MTT assay**

| Sample | SRPIN340 (24 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 104±4.04 | 110±4.93 | 103±3.79 | 67±2.65 |
| 92-1 | 100±0.00 | 112±4.36 | 115±5.57 | 107±5.29 | 68±14.15 |

**Table B2 Effect of SRPIN340 on cell viability in A375 and 92-1 cell at 48 hours using MTT assay**

| Sample | SRPIN340 (48 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 108±6.35 | 115±6.11 | 87±12.29 | 41±1.00 |
| 92-1 | 100±0.00 | 117±4.73 | 105±3.06 | 76±3.64 | 42±4.36 |

**Table B3 Effect of SRPIN340 on cell viability in A375 and 92-1 cell at 72 hours using MTT assay**

| Sample | SRPIN340 (72 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 97±4.51 | 104±7.81 | 78±6.43 | 24±7.64 |
| 92-1 | 100±0.00 | 121±7.64 | 92±10.79 | 34±2.65 | 8±2.08 |

**Table B4 Effect of SPHINX31 on cell viability in A375 and 92-1 cell at 24 hours using MTT assay**

| Sample | SPHINX31 (24 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 106±10.82 | 96±3.51 | 87±6.11 | 66±14.47 |
| 92-1 | 100±0.00 | 80±4.52 | 76±2.96 | 74±4.77 | 68±8.37 |

**Table B5 Effect of SPHINX31 on cell viability in A375 and 92-1 cell at 48 hours using MTT assay**

| Sample | SPHINX31 (48 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 101±1.53 | 93±9.87 | 76±9.85 | 42±14.01 |
| 92-1 | 100±0.00 | 76±8.10 | 83±2.50 | 79±6.45 | 72±8.61 |

**Table B6 Effect of SPHINX31 on cell viability in A375 and 92-1 cell at 72 hours using MTT assay**

| Sample | SPHINX31 (72 hours) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** | **60 µM** | **80 µM** |
| A375 | 100±0.00 | 100±2.00 | 96±3.51 | 34±18.04 | 23±17.21 |
| 92-1 | 100±0.00 | 68±16.69 | 68±13.17 | 66±11.58 | 58±12.54 |

**Table B7 Expression of phosphorylation profile of SRSFs in HaCat compare with A375 cells using Western blot**

| Sample | Band intensity | |
|---|---|---|
| | **HaCat** | **A375** |
| pSRSF4/pSRSF11 | 0.30±0.08 | 0.19±0.11 |
| pSRSF6 | 0.34±0.15 | 0.34±0.26 |
| pSRSF8 | 1.06±0.08 | 0.97±0.14 |
| pSRSF5/pSRSF10 | 0.46±0.13 | 0.29±0.28 |
| pSRSF2/pSRSF7 | 0.41±0.22 | 0.53±0.21 |
| pSRSF1/pSRSF12 | 0.53±0.32 | 0.74±0.41 |
| pSRSF3/pSRSF9 | 0.32±0.06 | 0.36±0.21 |
| GAPDH | 1.00±0.00 | 1.00±0.00 |

**Table B8 Expression of phosphorylation profile of SRSFs in HaCat compare with 92-1 cells using Western blot**

| Sample | Band intensity | |
|---|---|---|
| | **HaCat** | **92-1** |
| pSRSF4/pSRSF11 | 0.14±0.06 | 0.18±0.09 |
| pSRSF6 | 0.36±0.07 | 0.59±0.02 |
| pSRSF8 | 1.07±0.48 | 0.96±0.56 |
| pSRSF5/pSRSF10 | 0.38±0.13 | 0.53±0.23 |
| pSRSF2/pSRSF7 | 0.39±0.15 | 0.63±0.13 |
| pSRSF1/pSRSF12 | 0.87±0.31 | 1.17±0.29 |
| pSRSF3/pSRSF9 | 0.31±0.08 | 0.86±0.10 |
| GAPDH | 1.00±0.00 | 1.00±0.00 |

**Table B9 Expression of pSRSFs after SRPIN340 treatment in A375 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| pSRSF4/pSRSF11 | 0.21±0.09 | 0.11±0.11 | 0.13±0.05 |
| pSRSF6 | 0.55±0.20 | 0.37±0.27 | 0.28±0.29 |
| pSRSF8 | 1.19±0.07 | 0.64±0.50 | 0.49±0.49 |
| pSRSF5/pSRSF10 | 0.42±0.10 | 0.33±0.18 | 0.22±0.12 |
| pSRSF2/pSRSF7 | 0.56±0.08 | 0.39±0.18 | 0.16±0.06 |
| pSRSF1/pSRSF12 | 0.42±0.02 | 0.23±0.04 | 0.17±0.04 |
| pSRSF3/pSRSF9 | 0.19±0.13 | 0.12±0.12 | 0.08±0.04 |
| GAPDH | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 |

**Table B10 Expression of pSRSFs after SPHINX31 treatment in A375 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| pSRSF4/pSRSF11 | 0.40±0.29 | 0.38±0.14 | 0.35±0.11 |
| pSRSF6 | 0.44±0.24 | 0.60±0.19 | 0.38±0.29 |
| pSRSF8 | 0.67±0.47 | 0.77±0.46 | 0.62±0.65 |
| pSRSF5/pSRSF10 | 0.23±0.06 | 0.27±0.14 | 0.26±0.20 |
| pSRSF2/pSRSF7 | 0.31±0.04 | 0.23±0.05 | 0.15±0.02 |
| pSRSF1/pSRSF12 | 0.29±0.06 | 0.21±0.02 | 0.11±0.03 |
| pSRSF3/pSRSF9 | 0.21±0.15 | 0.16±0.04 | 0.10±0.05 |
| GAPDH | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 |

**Table B11 Expression of pSRSFs after SRPIN340 treatment in 92-1 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| pSRSF4/pSRSF11 | 0.60±0.75 | 0.29±0.36 | 0.23±0.20 |
| pSRSF6 | 0.67±0.33 | 0.61±0.35 | 0.53±0.16 |
| pSRSF8 | 1.22±0.35 | 0.96±0.57 | 0.83±0.35 |
| pSRSF5/pSRSF10 | 0.35±0.26 | 0.27±0.20 | 0.20±0.07 |
| pSRSF2/pSRSF7 | 0.88±0.26 | 0.45±0.17 | 0.37±0.08 |
| pSRSF1/pSRSF12 | 0.85±0.23 | 0.47±0.24 | 0.31±0.09 |
| pSRSF3/pSRSF9 | 0.20±0.10 | 0.14±0.08 | 0.14±0.15 |
| GAPDH | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 |

**Table B12 Expression of pSRSFs after SPHINX31 treatment in 92-1 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| pSRSF4/pSRSF11 | 0.20±0.10 | 0.21±0.11 | 0.15±0.09 |
| pSRSF6 | 0.38±0.07 | 0.31±0.07 | 0.21±0.17 |
| pSRSF8 | 1.23±0.29 | 1.30±0.22 | 0.90±0.09 |
| pSRSF5/pSRSF10 | 0.30±0.12 | 0.35±0.31 | 0.13±0.05 |
| pSRSF2/pSRSF7 | 0.96±0.36 | 0.52±0.24 | 0.29±0.01 |
| pSRSF1/pSRSF12 | 0.81±0.26 | 0.39±0.31 | 0.21±0.12 |
| pSRSF3/pSRSF9 | 0.42±0.29 | 0.20±0.10 | 0.15±0.07 |
| GAPDH | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 |

**Table B13 Effect of SRPIN340 on colony formation in A375 and 92-1 cells using Clonogenic assay**

| Sample | SRPIN340 | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| A375 | 257±42.79 | 251±50.52 | 243±44.19 |
| 92-1 | 281±22.05 | 232±11.14 | 88±10.58 |

**Table B14 Effect of SPHINX31 on colony formation in A375 and 92-1 cells using Clonogenic assay**

| Sample | SPHINX31 | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| A375 | 257±42.79 | 232±27.50 | 199±46.12 |
| 92-1 | 281±22.05 | 202±72.29 | 174±72.00 |

**Table B15 Expression of pSRSFs after SRPIN340 treatment in A375 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| t-AKT | 56.38±5.29 | 55.82±11.02 | 59.23±15.21 |
| p-AKT | 51.71±10.94 | 47.15±11.55 | 28.02±1.53 |
| p-AKT/t-AKT | 1.00±0.00 | 0.96±0.27 | 0.55±0.14 |

**Table B16 Expression of pSRSFs after SPHINX31 treatment in A375 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| t-AKT | 59.03±18.21 | 54.31±19.46 | 55.41±19.42 |
| p-AKT | 53.02±13.74 | 36.61±16.20 | 25.39±10.93 |
| p-AKT/ t-AKT | 1.00±0.00 | 0.75±0.19 | 0.51±0.13 |

**Table B17 Expression of pSRSFs after SRPIN340 treatment in 92-1 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| t-AKT | 71.17±11.52 | 74.45±6.51 | 54.20±10.59 |
| p-AKT | 45.27±17.54 | 44.82±11.85 | 35.38±17.60 |
| p-AKT/ t-AKT | 1.00±0.00 | 0.97±0.13 | 1.00±0.19 |

**Table B18 Expression of pSRSFs after SPHINX31 treatment in 92-1 cell using Western blot**

| Sample | Band Intensity | | |
|---|---|---|---|
| | **0 µM** | **20 µM** | **40 µM** |
| t-AKT | 72.67±9.72 | 81.57±13.26 | 56.51±14.72 |
| p-AKT | 65.23±10.95 | 46.87±19.96 | 31.43±21.60 |
| p-AKT/ t-AKT | 1.00±0.00 | 1.12±0.76 | 0.67±0.52 |

## APPENDIX C Research presentation and publication

### Research publication

**Proceeding (Oral presentation)**

Jesadagorn Siriwath, Natsupa Wiriyakulsit, Patcharee Klomkleang, Chaturong Inpad, Sittiruk Roytrakul, and Worasak Kaewkong. SPHNIX31 Suppresses Splicing Factor Phosphorylation and Inhibits Melanoma Cell growth and Aggressiveness. **The 6th RSU International Conference on Sciences and Technology April 30, 2021 at Rangsit University** (RSU conference 2021) (773).

**Manuscript preparation (for submission to Journal of Current Science and Technology: JCST)**

Jesadagorn Siriwath, Natsupa Wiriyakulsit, Patcharee Klomkleang, Chaturong Inpad, Sittiruk Roytrakul, and Worasak Kaewkong. SPHNIX31 suppresses splicing factor phosphorylation and inhibits melanoma cell growth and aggressiveness (In press).

**Cover page**

**THE 6th RSU INTERNATIONAL CONFERENCE ON SCIENCES AND TECHNOLOGY (RSUSCI-2021)**

**Abstract page**

**THE 6th RSU INTERNATIONAL CONFERENCE ON SCIENCES AND TECHNOLOGY (RSUSCI-2021)**

RSU International Research Conference 2021
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30 APRIL 2021

## SPHNIX31 Suppresses Splicing Factor Phosphorylation and Inhibits Melanoma Cell growth and Aggressiveness

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**Abstract**

Melanoma is a tumor resulting from the malignant transformation of skin or ocular melanocytes, which is a serious health problem in countries with high UV exposure. Due to the late detection, high invasive and metastatic potentials of melanoma cells, and lack of effective treatments, therefore, poor prognostic in melanoma cases led to high mortality rate among melanoma patients. The aberrant mRNA transcripts derived from alternative splicing has been contributed to the progression of various typed of cancer. Serine/Arginine-riched Splicing Factors (SRSFs) are the proteins responsible for the mRNA splicing under the specific regulation by Serine-Arginine Protein Kinases (SRPKs). This study, the effects of SRPK1-specific inhibitor SPHINX31 was investigated. Cell viability were determined in A375 (cutaneous melanoma cell) compare with 92-1 (ocular melanoma cell) by MTT viability assays. Inhibitory effect of SPHINX31 on melanoma cell viability were presented as dose- and time-dependent manners. Then, western blot analysis was performed to observe the suppression of kinase activity by SPHINX31. Decreasing of phosphorylated SRSFs (pSRSFs) was demonstrated in both cells. Growth inhibition of SPHINX31 was examined by clonogenic assay, the size and number of both A375 and 92-1 cell colonies were decreased. Remarkably, the result of SPHINX31 in other cancer phenotypes were studies in A375 cell regarding to the significant effect in growth inhibition. We found SPHINX31 had reduced the dead-evasion and migration abilities of A375 cell. The collected data should serve as a strong foundation to develop new alternative therapeutic strategies for melanoma treatment by targeting SRPK1 activation.

***Keywords:*** *Alternative splicing, Melanoma, Phosphorylation, SRPK1*

**List of oral presentation: International Conference page**

**THE 6th RSU INTERNATIONAL CONFERENCE ON SCIENCES AND TECHNOLOGY (RSUSCI-2021)**

**RSU CONFERENCE ON SCIENCE AND TECHNOLOGY 2021**

| G5 | | | | | Zoom link: https://rsucon.rsu.ac.th/zoom/SCI-G5-1Biochem |
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| G5-1 BIOSCIENCE/BIOCHEMISTRY | | | | | Room SCI-G5-1 |
| PLENARY SESSION | OPENING REMARKS 9.00-9.20 am | WELCOME SPEECH | Asst. Prof. Dr. Nares Pantaratorn | Vice President for Research Rangsit University, Thailand | Click here |
| PLENARY SESSION | PLENARY SESSION 9.20-10.00 am | New Normal for Ganja Research after COVID-19 | Asst. Prof. Dr. Surang Leelawat | College of Pharmacy Rangsit University, Thailand | |
| KEYNOTE | KEYNOTE 10.00-10.30 am | Human intestinal microbiota: The hidden gems in the gut? | Dr.Pipat Piewngam | National Institute of Allergy and Infectious Diseases, National Institutes of Health National Institutes of Health, USA | https://rsucon.rsu.ac.th/zoom/SCI-G5-1Biochem |
| KEYNOTE | KEYNOTE 10.30-11.00 am | Characterization of miRNA Profiles in The Mammary Tissue of Dairy Cattle in Response to Heat Stress | Prof. Dr. Li Qiuling | Lecturer, College of Life Sciences Langfang Normal University (LNU), China | |
| G5-1 | IN21-019 11.00-11.20 am | Development of the in-house genetically modified wheat MON71800 reference plasmid for qualitative detection by Tetraplex Real-Time PCR. | Weerasak Pitaksaringkarn; Thitirut Assawamongkholsiri; Piyanuch Sornchai; Piyarat Thammakijjawat | Biotechnology Research and Development Office Laboratory Building, Department of Agriculture Department of Agriculture, Thailand | |
| G5-1 | IN21-024//JCSAT-D-21-00008 11.20-11.40 am | Cloning and expression of Baeyer-Villiger monooxygenase from Microbacterium esteraromaticum SBS1-7 | Wirakorn Pimpasida; Akanit Wongbunmak; Thunyarat Pongtharangkul | Graduate Program in Biotechnology, Faculty of Science Mahidol University, Thailand | |
| G5-1 | IN21-053//JCSAT-D-21-00012 11.40-12.00 pm | Inhibitory effects of SPHINX31 on cutaneous and ocular melanoma cell viability with the reducing of cancer-aggressiveness phenotypes via SR protein phosphorylation blockage | Jesadagorn Siriwath; Natsupa Wiriyakulsit; Patcharee Klomkleang; Chaturong Inpad; Sittiruk Roytrakul; Worasak Kaewkong | Department of Biochemistry,Faculty of Medical Science Naresuan University, Thailand | |
| G5-1 | IN21-102 12.00-12.20 pm | Population genetic structure of Tylototriton panhai Nishikawa, Khonsue, Pomchote and Matsui, 2013 (Urodela: Salamandridae) from Phu Hin Rong Kla National Park, Thailand | Parada Peerachidacho; Porrawee Pomchote; Nontivich Tandavanitj | Faculty of Science Chulalongkorn University, Thailand | |

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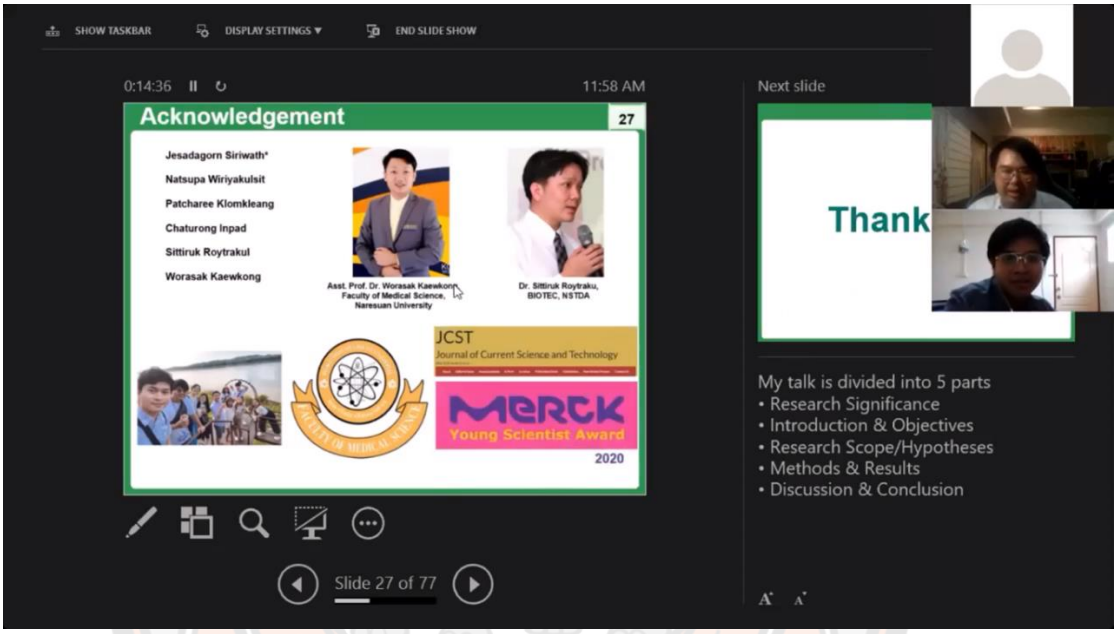

**Certificate page**

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