การศึกษาความสำคัญของระดับสัญญาณ Wnt/ß-catenin ต่อการเปลี่ยนแปลงสภาพของเซลล์มะเร็งประสาทมนุษย์ SH-SY5Y

นางสาวจันทร์จิรา สืบสุนทร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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INVESTIGATION OF THE EFFECTS OF Wnt/ß-catenin SIGNALLING MODULATION ON THE DIFFERENTIATION OF HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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จันทร์จิรา สืบสุนทร : การศึกษาความสำคัญของระดับสัญญาณ Wnt/ß-catenin ต่อการเปลี่ยนแปลงสภาพของเซลล์มะเร็งประสาทมนุษย์ SH-SY5Y (INVESTIGATION OF THE EFFECTS OF Wnt/ß-catenin SIGNALLING MODULATION ON THE DIFFERENTIATION OF HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ปริญญา น้อยสา, 105 หน้า.

มะเร็งประสาทมนุษย์ (Neuroblastoma) เป็นมะเร็งของเซลล์ต้นกำเนิดของระบบประสาทอัตโนมัติซึ่งพบได้บ่อยในทารก และเด็กอายุน้อยกว่า 10 ปี การอุบัติของมะเร็งชนิดนี้เกิดมาจากเซลล์ประสาทขั้นต้น (neuroblast) ทำให้การรักษาโรคในปัจจุบันนั้นทำได้ยาก ด้วยเหตุนี้แล้วจึงมีการวิจัยเชิงทดลองทางคลินิกโดยการรักษาด้วยวิธีอื่น เช่น การใช้โมโนโคลนอลแอนติบอดี้ หรือยาต้านมะเร็งตลอดจนการจำเพาะเจาะจงต่อสัญญาณต่าง ๆ ภายในเซลล์ที่คาดว่าจะสามารถกระตุ้นประสิทธิภาพของการรักษาให้ดีขึ้นได้ โดยมีงานวิจัยต่าง ๆ รายงานเกี่ยวกับผลของสัญญาณภายในเซลล์กับเซลล์มะเร็งและการรักษาโรคได้แก่ สัญญาณ Wnt Notch และ Hedgehog ว่ามีผลเกี่ยวกับกับการมีชีวิตรอดของเซลล์มะเร็ง การเพิ่มจำนวน รวมไปถึงเกี่ยวข้องกับการพัฒนาเปลี่ยนแปลงเซลล์ร่างกาย และเซลล์ต้นกำเนิด

สมมุติฐานของงานวิจัยนี้คือ การศึกษาเกี่ยวกับการพัฒนาเปลี่ยนแปลงของเซลล์มะเร็งกับความก้าวร้าวของเซลล์ โดยการที่เซลล์มะเร็งมีความก้าวร้าวรุนแรงขึ้นนั้นเป็นผลจากการปรับเปลี่ยนผ่านตัวกระตุ้นแบบยับยั้งผ่านสัญญาณวินซ์ (Wnt Signalling) ซึ่งเสริมฤทธิ์ และไวต่อการรักษาโรคด้วยยาต้านมะเร็งชนิด Doxorubicin โดยสัญญาณ Wnt ถูกกีดกั้นด้วยโมเลกุล XAV939 ซึ่งโมเลกุลชนิดนี้เป็นตัวกระตุ้นแบบยับยั้งที่จำเพาะเจาะจงต่อเอนไซม์ Tankyrase จากนั้นทำการศึกษาการเพิ่มจำนวนของเซลล์มะเร็ง การมีชีวิตรอดของเซลล์มะเร็ง การแสดงออกของยีนส์ และผลต่อยาต้านมะเร็ง พบว่าการยับยั้งสัญญาณ Wnt ทำให้จำนวนของเซลล์ที่มีการแสดงออกของ ß-catenin ลดลงจากร้อยละ 99.21 เป็นร้อยละ 48 ในกลุ่มของเซลล์มะเร็งที่ให้ตัวยับยั้งสัญญาณ wnt เปรียบเทียบกับเซลล์มะเร็งปกติที่ไม่ได้ถูกยับยั้งตามลำดับ ในทางตรงกันข้าม XAV939 กลับไม่ส่งผลกระทบต่อลักษณะทางสัณฐานวิทยาของเซลล์, การเพิ่มจำนวนและ/หรือการมีชีวิตรอดของเซลล์ ด้วยเหตุนี้จึงได้มีการศึกษายีนที่เกี่ยวข้องกับประสาท และยีนชั้นเนื้อเยื่อคัพภะ ซึ่งประกอบด้วย *N-Myc*, *C-Myc, TFAP2, PHOX2A, PHOX2B, PAX6, SLUG,* และ *Tuj1*. ผลการศึกษาการแสดงออกของยีนทั้งสองกลุ่มนี้เป็นสิ่งที่น่าสนใจเป็นอย่างยิ่ง เพราะยีนที่เกี่ยวกับประสาทที่เจริญเติบโตเต็มที่เช่น *Tuj1, PHOX2B, PHOX2A* มีการแสดงออกของยีนลดลงในขณะที่ TFAP2 และ PAX6 มีการแสดงออกของยีนเพิ่มขึ้น ผลของการศึกษานี้

สามารถอภิปรายได้ว่า การยับยั้งสัญญาณ Wnt/ß-catenin ทำให้มีผลกระทบต่อการพัฒนาเปลี่ยนแปลงของเซลล์มะเร็งประสาทมนุษย์ (Human neuroblastoma) ชนิด SH-SY5Y

ที่น่าสนใจยิ่งไปกว่านั้นคือ การผสานการรักษามะเร็งโดยการใช้ยาต้านมะเร็งชนิด Doxorubicin กับ XAV939 มีผลให้เกิดการเสริมฤทธิ์ของยาต้านมะเร็ง เพื่ออธิบายปรากฏการณ์นี้ จึงได้ทำการศึกษาการแสดงออกของยีนทั้งหมดในเซลล์ ด้วยการใช้ข้อมูลการแสดงออกของยีนมาวัดระดับของการลอกรหัสของยีนภายในเซลล์ รวมทั้งการค้นหาวิธีการและยารักษาโรคมะเร็งจากการใช้ DNA microarra ซึ่งปรากฏยีนเป้าหมายที่สำคัญหลายยีนในเซลล์มะเร็งที่ให้ XAV939 ตัวอย่างเช่น *TP53TG3, TAP2, DDC, CASP3, BIRC5, PSMC6*, และ *Ubiquitin C*. จากงานวิจัยนี้สามารถกล่าวได้ว่าการกระตุ้นแบบยับยั้งสัญญาณ Wnt ทำให้เกิดการเปลี่ยนแปลงของเซลล์มะเร็งประสาทมนุษย์ SH-SY5Y และทำให้เกิดการตอบสนองต่อยาต้านมะเร็งดีขึ้น ซึ่งองค์ความรู้นี้สามารถนำไปปรับปรุงประสิทธิภาพของการรักษาโรคมะเร็งประสาทมนุษย์ในปัจจุบันได้โดยการเจาะเพาะเจาะจงต่อเซลล์เสมือนเซลล์ประสาทต้นกำเนิด

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

JUNJIRA SUEBSOONTHRON : INVESTIGATION OF THE EFFECTS OF Wnt/ß-catenin SIGNALLING MODULATION ON THE DIFFERENTIATION OF HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE. THESIS ADVISOR : ASST. PROF. PARINYA NOISA, Ph.D., 105 PP.

HUMAN MALIGNANT NEUROBLASTOMA/WNT SIGNALLING/TANKYRASE INHIBITION/ANTICANCER DRUG

Neuroblastoma is an embryonic malignancy and the most common cancer in infant and childhood, arising from neuroblasts. This cancer can be hard to treat, therefore clinical trials of novel treatments, such as monoclonal antibodies or new anticancer drugs, and targeting cellular signalling might be an alternative option for effective therapy. Many studies reported that cellular signalling pathways, including Wnt, Notch, and Hedgehog, play a key role in the survival, proliferation, and differentiation of somatic cells and stem cells. In this study, we hypothesized that the differentiation of aggressive human neuroblastoma SH-SY5Y cells can be modulated by the inhibition of Wnt Signalling, and this could enhance the sensitivity of the cells to an anticancer drug, Doxorubicin. Wnt Signalling of human neuroblastoma SH-SY5Y cells were blocked by a specific Tankyrase inhibitor, XAV939, prior to characterization of cell proliferation, cell survival, gene expression, and anticancer drug sensitivity. Upon the treatment with XAV939, the number of ß-catenin positive cells was reduced from 99.21% to 48.00% in control and treated cells. In contrast, XAV939 did not alter cell morphology, cell proliferation or cell survival. Thereafter, two sets of neural and neural crest-related genes were investigated, including *N-Myc*,

C-Myc, TFAP2, PHOX2A, PHOX2B, PAX6, SLUG, and *Tuj1*. Interestingly, the mature neural genes such as *Tuj1, PHOX2B, PHOX2A* were down-regulated, while *TFAP2* and *PAX6* were up-regulated when XAV939 was supplemented into the culture. The results here suggested that the inhibition of Wnt/ß-catenin affected the differentiation of human neuroblastoma SH-SY5Y cells. Remarkably, the combination of an anticancer drug, Doxorubicin, with XAV939 was shown to enhance anticancer activity against SH-SY5Y cells. To explain this phenomenon, the global gene expression by microarray revealed the numbers of candidate target genes of XAV939, for instance, *TP53TG3, TAP2, DDC, CASP3, BIRC5, PSMC6,* and *Ubiquitin C*. Altogether, this study suggested that the inhibition of Wnt Signalling altered the differentiation of human neuroblastoma SH-SY5Y cells, and primed the cells to be more responsive to an anticancer drug. This knowledge could improve the efficacy of current treatment of human neuroblastoma by targeting stem-like cancer cells.

School of Biotechnology Student's Signature_____

Academic Year 2016 Advisor's Signature_____

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LIST OF ABBREVIATIONS

SHSY	=	SH-SY5Y human neuroblastoma
XAV939	=	3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3d]pyrimidin-4-one
FBS	=	Fetal bovine serum
DMEM	=	Dulbecco's modified Eagle's medium
P/S	=	Penicillin and streptomycin
PBS	=	Phosphate buffer saline
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
BrdU	=	Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BUdR, BrdUrd)
PFA	=	Paraformaldehyde

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Neural development is a controlled process that integrates proliferation differentiation and apoptosis of neural cells (Hagg, 2007). Abnormal neural development could lead to emotional, behavioral and learning disorders in children, as well as the growth of neural tumor. Neuroblastoma is an embryonic malignancy and the most common brain cancer found in infant, arising from neuroblasts (Zhi et al., 2012) with an incidence about six hundred and fifty cases per year in the United State (Broaddus et al., 2009) and a hundred cases per year in the United Kingdom (NHS, 2016). The neuroblast progenitors migrate out from the neural crest via the neural tube to the notochord and dorsal aorta. Then, specialized cells known as primary sympathetic ganglia (PSG) are emerged before dividing into mature sympathetic ganglia neural cells (Marshall et al., 2014). Nerve growth factor (NGF) induces normal sympathetic ganglion cells to either differentiate toward mature ganglion cells or enter program cell death (apoptosis), therefore the postnatal survival of neuroblast pre-cancer cells lead to be a malignant and resistant cells, which presents as neuroblastoma (Li and Nakagawara, 2013). The idea of an embryonal origin of human neuroblastoma is supported by the expression profile showing that human fetal adrenal neuroblasts contain gene signatures that are remarkably similar to those of neuroblastoma (Biedler et al., 1973). During *in vivo* neural differentiation, various

proteins experience changes in their expression levels as a consequence of cellular specialization (Constantinescu et al., 2007). In order to compare the alteration and differentiation of cells, the progenitor/mature neuronal markers were an option, for instance *Tuj1, NeuroD, TFAP2, PHOX2B, PHOX2A, PAX6* and *Nestin*.

Neuroblastoma has been classified into three risk categories: low, intermediate, and high (Society, 2014). The "*International Neuroblastoma Staging System*" (INSS), established in 1988, has categorized neuroblastoma according to its anatomical presence at diagnosis. The International Neuroblastoma Risk Group (INRG) risk assignment classified neuroblastoma at diagnosis based on a new INRG Staging System, INRGSS (Monclair et al., 2009a; Voss et al., 2011). Age stage and biological features encountered in tumor cells are important prognostic factors and are used for risk stratification and treatment assignment. Older children with advanced-stage disease, however, have a significantly decreased chance for cure, despite received an intensive therapy (Isaacs, 2007).

The types of treatment used for neuroblastoma, including surgery chemotherapy radiation therapy and immunotherapy. Neuroblastoma cell lines contain a side-population of cells, which express stem cell markers. These stem-like cells may represent the potential underlying mechanism for resistance and recurrence to conventional therapy (Hammerle et al., 2013). Treatment of recurrent neuroblastoma depends on many factors, including the initial risk group, localization of the recurrence, and the treatment history (Society, 2014). These cancers can be hard to treat, therefore clinical trials of novel treatments, such as monoclonal antibodies, novel anti-cancer drugs, or cancer-specific target Signalling, might be a reasonable option for an effective therapy (Institute, 2016).

It has been reported that several Signalling pathways, including Wnt, Notch, and Hedgehog (Hh), play a key role in the survival, proliferation, and differentiation of normal stem cells and somatic precursor cells (Templeton et al., 2014). These pathways are tightly regulated during embryogenesis and aberration of them could lead to tumorigenesis. Wnt/ß-catenin Signalling is correlated with numerous cancers, such as intestine, breast, prostate and lung cancers. The regulation of this pathway plays an important role in a number of adult and pediatric tumors. It has been reported that Wnt/ß-catenin Signalling could promote neural stem cell (NSC) proliferation and prevent neuronal differentiation (Zhi et al., 2012). During neural development, Wnt/ß-catenin Signalling pathway takes part in the neural crest formation, neuronal differentiation as well as neurite outgrowth during the culture of early born hippocampus neurons (Takebe et al., 2015). It was found that Wnt-1 and Wnt-2 were over-expressed in non-small cell lung cancer (NSCLC) cell lines and the inhibition of Wnt-1/-2 Signalling inhibited cell growth and induced apoptosis (Templeton et al., 2014). Moreover, small molecule; XAV939 could affect the Wnt pathway by the regulation of Axin inhibiting protein, thus this molecule presents as a new Wnt Signalling modifier for cancer therapies (Huang et al., 2009). XAV939, a Tankyrase (TNKS) inhibitor, can antagonize Wnt Signalling via stabilizing Axin protein and promoting the formation of a destruction complex, in which stimulates ß-catenin degradation. Mechanistically, XAV939 stabilized Axin protein by inhibiting the poly (ADP-ribose) polymerase (PARP) enzyme Tankyrase (TNKS). Tankyrase will then interact with the conserved domain of Axin protein and stimulate the degradation of β-Catenin through recruiting the ubiquitin proteasome activation. Interestingly, the activity of TNKS inhibitor for the alteration of differentiation of human neuroblastoma

cells has not been studied. These might present the alternative strategy to enhance the sensitivity of malignant human neuroblastoma to anticancer drug.

1.2 Research objectives

1.2.1 To investigate whether the modulation of Wnt Signalling could affect the survival, proliferation and differentiation of human neuroblastoma cells.

1.2.2 To investigate whether the alteration of Wnt Signalling in human neuroblastoma cells will show an increasing the sensitivity of anti-cancer drug in human neuroblastoma cells.

CHAPTER II

LITERATURE REVIEWS

2.1 Human Neuroblastoma

Neuroblastoma is an embryonic malignancy arising from neuroblasts (Zhi et al., 2012). Primary neuroblastoma arises in the adrenal medulla or sympathetic ganglia along the paravertebral axis. The idea of an embryonal origin for human neuroblastoma is supported by the gene expression profile, showing that human fetal adrenal neuroblasts gene signatures that are remarkably similar to those of neuroblastoma. The great majority of cases are sporadic and non-familial. About 1-2% of cases run in families and has been linked to specific gene mutations. Familial neuroblastoma in some cases is caused by rare germline mutations in the anaplastic lymphoma kinase (ALK) gene (Maris, 2010). Germline mutations in the PHOX2A or KIF1B genes (Mosse et al., 2008) have been implicated in familial neuroblastoma as well. Neuroblastoma is also a feature of neurofibromatosis type1. MYCN oncogene amplification within the tumor is a common finding in neuroblastoma. The presence of this mutation is highly correlated to advanced stages of disease (Brodeur et al., 1984).

Table 1 Stages of neuroblastoma and classification (Monclair et al., 2009)

Stages	Description
1	Localized tumor with complete gross excision, representative ipsilateral lymph nodes negative for tumor microscopically.
2A	Localized tumor with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumor microscopically.
2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor.
3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs, except as defined for stage 4S.
4S	Localized primary tumor, as defined for stage 1, 2A, or 2B, with dissemination limited to skin, liver, and/or bone marrow.

The "International Neuroblastoma Staging System" (INSS), established in 1986 and revised in 1988, stratifies neuroblastoma according to its anatomical presence at diagnosis into 4 stages as shown above in Table1 (National Cancer Institute, 2008). The new INRG risk assignment classifies neuroblastoma at diagnosis based on a new International Neuroblastoma Risk Group Staging System (INRGSS) as show in Table 2.

Table 2 International Neuroblastoma Risk Group Staging System (INRGSS)

Risk	**Description**
L1	Localized disease without image-defined risk factors.
L2	Localized disease with image-defined risk factors.
M	Metastatic disease.
MS	Metastatic disease "special" where MS is equivalent to stage 4S.

The new risk stratification is based on the new INRGSS staging system, age (dichotomized at 18 months), tumor grade, *N-myc* amplification, unbalanced *11q* aberration, and ploidy into four pre-treatment risk groups: very low low intermediate and high risk (Maris et al., 2007). In some patients with neuroblastoma, new tumors form at different times and locations early in the child's life. Considerable evidence suggests that neuroblastoma is initiated in uterus during sympathoadrenal development and it goes through an embryonal precancer phase. During normal sympathoadrenal development, expression of the proto-oncogene *MYCN* is high in the early post migratory neural crest, where it regulates the ventral migration and expansion of

neural crest cells. *MYCN* protein level gradually reduces in differentiating sympathetic neurons, suggesting that sympathoadrenal maturation requires low or absent *MYCN* expression (Marshall et al., 2014) .

The amount of ß-catenin which is accumulated in nucleus indicates risk of activation of Wnt/ß-catenin Signalling in human neuroblastoma. The result of ß-catenin accumulation provides new information for neuroblastoma initiation and progression, and suggests a potential drug target for neuroblastoma treatment (Zhi et al., 2012). The treatment of neuroblastoma depends on the stage of the cancer, the child's age, and the prognostic markers. The types of treatment used include surgery chemotherapy radiation therapy and immunotherapy. Neuroblastoma cell lines contain a side-population of cells which express stem cell markers. These stem-like cells may represent the potential underlying mechanism for resistance to conventional therapy and recurrence of neuroblastoma in patients (Hammerle et al., 2013). Recurrent of neuroblastoma after initial treatment, known as a recurrence or relapse, are commonly found. The treatment of recurrent neuroblastoma depends on many factors, including the initial risk group, sites of recurrence, and treatment history. For low and intermediate risk neuroblastoma that recurs in the same area where they started, surgery with or without chemotherapy may be an effective therapy. For high-risk cancers or those that recur in distant parts of the body, the intensive treatment is needed, and may include a combination of therapeutic approaches (such as MIBG; metaiodobenzylguanidine radiotherapy) (AmericanCancerSociety, 2014). Intensive treatment with high-dose chemotherapy/radiation therapy followed by a stem cell transplant might be an option. Because these cancers are hard to treat, clinical trials of a novel treatment, such as monoclonal antibodies or new anticancer drugs, will be a reasonable strategy.

2.1.1 Human Neuroblastoma Cell Lines

2.1.1.1 SH-SY5Y cells

SH-SY5Y is a human derived cell line used in scientific research. This cell line was isolated from a bone marrow biopsy taken from a four year-old female with neuroblastoma. SH-SY5Y cells are often used as in vitro models of neuronal function and differentiation (Biedler et al., 1973). Most differentiation SH-SY5Y cell line was considered to be differentiated based on morphology, without much additional characterization. In several studies, the differentiation SH-SY5Y cells were assessed by measuring the neurite length (Nicolini et al., 1998). During *in vivo* neural differentiation various proteins changes are considered. In order to compare undifferentiated with differentiated cells, the following neuronal markers were interested. Neurogenin is a transcription factor that induces neurogenesis and inhibits the differentiation of neural stem cells into astrocyte (Constantinescu et al., 2007). Tyrosine hydroxylase (TH) the marker of dopaminergic neurons (Gates et al., 2006). Microtubule-associated protein 2 (MAP-2) is a neuronal cytoskeletal phosphoprotein that binds to tubulin and stabilizes microtubules, essential for the development and maintenance of neuronal morphology, cytoskeleton dynamics and organelle trafficking (Binder et al., 1985). Tuj1 is a neuron-specific of tubulin. Nestin is a marker for neuronal progenitor cells, member of intermediate filaments. Nestin not expressed in mature cells and terminal neuronal cell differentiation which is associated with down-regulation of this protein (Duggal and Hammond, 2002). SH-SY5Y cells are adrenergic neurons in phenotype but also express dopaminergic markers and have been used to study Parkinson's disease and other neural disorder disease (Biedler et al., 1973).

Table 3 List of various marker proteins

genes	**Type/Function**	**References**
Beta-Actin	conserved proteins	Hanukoglu, I., Tanese, N.,
Tuj1	neuron-specific class of tubulin	Constantinescu, Reichmann,
C-MYC	oncogene protein	Constantinescu, Reichmann,
N-MYC	oncogene protein, It's expressed in the	Knoepfler PS et al., 2002
MASH1	Autonomic neurons	Casarosa et al., 1999
PHOX2A	Noradrenergic cell types that regulates the expression of tyrosine hydroxylase	Johnson KR et al., 1996
PHOX2B	a homeodomain transcription factor. It is expressed in the nervous system (cardiovascular, digestive and respiratory	ncbi.nlm.nih.gov: paired-like homeobox 2b
TH	dopaminergic neurons	Constantinescu, Reichmann,
NeuroD1	Transcription factors for growth and differentiation of neurons and expressed	Constantinescu, Reichmann, & Janetzky, 2007)
PAX3	Transcription factors	ncbi.nlm.nih.gov: PAX3
PAX6	Transcription factor present during	Jordan T et al., 1992
SLUG	β-catenin–T-cell factor (TCF)-4	Damian Medici et al., 2008
SOX10	Transcription factors: important for neural crest and peripheral nervous	ncbi.nlm.nih.gov: SOX10 SRY (sex determining region
TFAP2a	Transcription factor corresponding to	ncbi.nlm.nih.gov:TFAP2A

Table 3 (continued)

genes	**Type/Function**	**References**
Nestin	epithelial stem cells=precursors	Constantinescu, Reichmann,
Cyclin A2	Express in dividing somatic cells.	Yam CH et al., 2002
p21	regulator of cell cycle progression at G1	Gartel AL, Radhakrishnan
p53	prevents cancer formation, thus, functions	Surget S et al., 2013

2.1.1.2 IMR-32 cells

A continuous hyper-diploid human cell line, IMR-32, was derived from neuroblastoma tissue. At least two morphologically distinct cell types comprise the population of IMR-32. These two cell types have the same characteristic chromosomal constitution and hyper-diploid stem line number, indicating a common clonal origin (Tumilowicz et al., 1970). IMR-32 cells established from an abdominal mass occurring in a 13 months old Caucasian male. These cells can be made to express a differentiated phenotype by using differentiating agents like 5-bromo-deoxyuridine (BrdU). IMR-32 is more suitable for scientific research because when it is differentiated, it mimics large projection neurons of the human cerebral cortex. It is originated from human with large size, and has previously been used in studies related to the stability of the Amyloid protein precursor (APP) (Neill et al., 1994). Moreover, It has another evidence suggested that human IMR-32 neuroblastoma cells could be a useful cell line for investigating paired helical filaments formation. Louhivuori, L.M. et al. (2009) study on the differentiation of IMR-32 cell line and found that mRNA for

TRPA1 and TRPM8 are strongly upregulated in differentiating IMR-32 cells (Louhivuori et al., 2009).

2.1.1.3 SK-N-SH cells

SK-N-SH is a neuroblastoma cell line that displays and grows in adherent culture. Treatment with all-trans-retinoic acid causes these cells to differentiate and adapt a neuronal phenotype, characterized by extensive neurite outgrowth. This makes them particularly useful for delineating Signalling pathways involved in neuronal differentiation. In addition, the SK-N-SH cells are known to form tumors in immunocompromised mice. This cell line was established in 1970 from metastatic cells found in the bone marrow aspirate of a four year old female of unknown ethnicity (Biedler et al., 1973).

2.2 Cellular Signalling pathways in Human Neuroblastoma

Several pathways including Wnt, Notch, Hedgehog (Hh) and PTEN pathways, play a key role in the survival, proliferation, and differentiation of normal stem cells and somatic precursor cells (Reya et al., 2001). Interestingly, these pathways are aberrantly regulated in cancer and even to a greater degree in a defined subset of cancer precursor cells resulting in abnormal transformation and possibly inciting tumorigenesis (Pardal et al., 2003). Figure1 depicts the regulation and interplay of these Signalling pathways (Templeton et al., 2014).

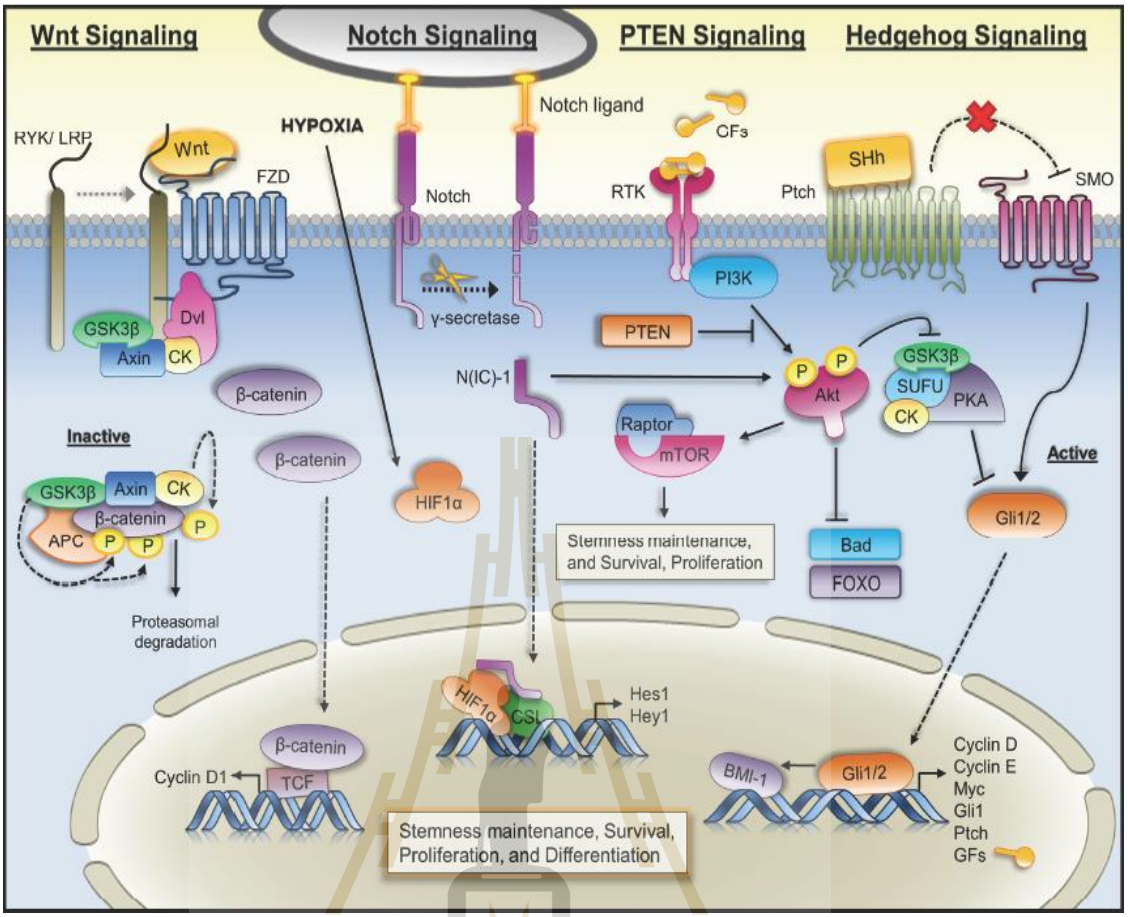


Figure 1 An Overview of selected signalling pathways related to cancer stem cell maintenance, survival, proliferation, and differentiation. The Wnt, Notch, PTEN, and Hedgehog Signalling pathways have been implicated in the development and maintenance of cancer stem cells. These novel regulatory mechanisms may promote self-renewal and differentiation and thereby provide avenues for therapeutic interventions. Akt, protein kinase B; APC, adenomatous polyposis coli; BMI-1, BMI 1 polycomb ring finger oncogene; CK, casein kinase; CSL, suppressor of hairless; Dvl, dishellved; FZD, frizzled; FOXO, forkhead box O transcription factor family; GFs, growth factors; GSK3B, glycogen synthase 3 beta; HIF-α, hypoxia inducible factor-alpha; LRP, low denisity lipoprotein receptor-related protein; mTOR,

mammalian target of rapamycin; N(IC)1, notch intracellular domain-1; PI3K, phosphatidylinositol-3-kinase; PKA, protein kinase A; PTEN, phosphatase and tensin homolog; Ptch, patched; RTK, receptor tyrosine kinase; RYK, related to receptor tyrosine kinase; SHh, sonic Hedgehog protein; SMO, smoothened; SUFU, suppressor of fused homolog; TCF, T cell factor transcription factor; Wnt, Wingless (Templeton et al., 2014).

2.2.1 Notch Signalling pathway

Notch pathway plays critical roles in cell differentiation, proliferation and survival, and has oncogenic or tumor suppressor effects in a variety of malignancies (Zage et al., 2012). Notch pathway is an intercellular communication system between a transmembrane ligand and receptor that transduces cell fate regulatory signals (Pannuti et al., 2010). These contextual antagonist roles of Notch Signalling in embryonic development can either maintain progenitor/stem cell characteristics or induce tissue-specific differentiation. These diametric roles of Notch pathway activation can possibly explain its tumor suppressive and oncogenic effects. For example, Notch oncogenic activity has most commonly been reported in the context of Notch3 upregulation in Non-Small Cell Lung Cancer (NSCLC) (Westhoff et al., 2009). Active Notch1/2 in Small Cell Lung Cancer (SCLC) lines resulted in tumor suppressive effects, while a NSCLC ALDH subpopulation has been shown to be dependent on Notch activity for proliferation and clonogenicity (Sriuranpong et al., 2001). Specifically, hypoxia increases the expression of genes downstream of Notch and induces hypoxia inducible factor-1 alpha (HIF-1α), a global regulator of oxygen homeostasis, to interact with the intracellular domain of Notch. Notch Signalling, as measured by γ-secretase cleavage product N(IC)-1, is active in human lung tumor

tissue samples and hypoxic lung tumor cell lines, but not normoxic tumor cell lines (Eliasz et al., 2010). NOTCH Signalling is an evolutionarily conserved Signalling pathway that regulates cell fate during development and postnatal life. It has been increasingly linked to carcinogenesis, although its role in cancer seems to be highly context and tissue specific. Although NOTCH Signalling is required for lung development, little is known about its role in lung cancer (Chen et al., 2007). Notch pathway also inhibits neuronal differentiation and maintains sympathetic precursors in a proliferative state. Furthermore, Notch pathway is activated by the homeobox transcription factor PHOX2B, an important regulator of peripheral sympathetic nervous system differentiation that was found to be mutated in some cases of familial and sporadic neuroblastoma. In neuroblastoma cell lines, several studies indicated that Notch Signalling prevents neuronal differentiation; in fact, neuroblastoma cell differentiation is inhibited by Notch1, and Notch overexpressing neuroblastoma cells are resistant to RA differentiation (Ferrari-Toninelli et al., 2010).

2.2.2 Shh Signalling pathway

Recently, many studies suggested the essential role of Sonic hedgehog (Shh) Signalling in the regulation of neural crest development (Zhou et al., 2010). The Hh proteins, Sonic hedgehog (Shh), Desert hedgehog, and Indian hedgehog, act as ligands for the receptor Patched 1 protein (Ptch1) that is located on the cell membrane (Taipale et al., 2002). Hedgehog signal transduction is initiated by the binding Hh proteins to Ptch1. Ptch1 inhibits the activity of a transmembrane protein that activates factors downstream of Hh Signalling pathway when those ligands are not bound to Ptch1. SMO stimulates a Signalling cascade that results in the activation of the transcription factors Gli proteins (GLI1, GLI2, and GLI3) when ligands are bound to Ptch1. GLI1 is amplified in glioma and is a strong positive activator of downstream

target genes in the nuclear, and GLI1 is a transcriptional activator of Hh Signalling itself. Therefore, GLI1 staining in the nucleus by immunohistochemistry is a marker of activation for Hh Signalling (Jeong and McMahon, 2005).

2.2.3 PTEN Signalling pathway

PTEN, a protein tyrosine phosphatase, is a known tumor suppressor gene PTEN/MMAC1 (phosphatase, tensin homologue/mutated in multiple advanced cancers) is a tumor suppressor protein that has sequence homology with dual-specificity phosphatases, which are capable of dephosphorylating both tyrosine phosphate and serine/threonine phosphate residues on proteins. The mutational of PTEN/MMAC1 gene in various tissues, especially endometrium, brain, prostrate, and ovary can form tumor (Ali et al., 1999). The observation that loss of PTEN causes or contributes to tumorigenesis in multiple tissues suggests that PTEN may play a role in self-renewal and differentiation of TICs. Addition, some studies have shown that PTEN plays a potential role in lung stem cell homeostasis and cell differentiation (Yanagi et al., 2007). Furthermore, Huang, H. C., et al. (2014) found that Aβ results in over-expression of Phosphatase and tensin homolog (PTEN), a negative regulator of PIP3. Curcumin depresses Aβ-induced up-regulation of PTEN induced by Aβ. These results imply that curcumin inhibits Aβ-induced tau hyperphosphorylation involving PTEN/Akt/GSK-3β pathway (Huang et al., 2014).

2.2.4 Wnt/β-Catenin Signalling pathway

The Wnt Signalling pathway is one of signal transduction pathways (Nusse, 2005). Three Wnt Signalling pathways have been characterized; (i) the canonical Wnt pathway: leads to regulation of gene transcription; (ii) the non-canonical planar cell polarity pathway: regulates the cytoskeleton that is responsible for the shape of the cell, and (iii) the noncanonical Wnt pathway: regulates calcium inside the cell (Nusse

and Varmus, 1992). Wnt Signalling was first identified for its role in carcinogenesis, but has since been recognized for its function in embryonic development. Its role in embryonic development was discovered when genetic mutations in Wnt pathway proteins produced abnormal fruit fly embryos (Goessling et al., 2009). Wnt Signalling also controls tissue regeneration in adult bone marrow, skin and intestine. Activation of Wnt signal transduction pathways is depend on the binding of Wnt ligand protein that regulate many processes including cell proliferation, migration and differentiation. The binding of Wnt ligand can stabilize the transcription co-activator β-catenin, which enters the nucleus to regulate Wnt pathway target genes. Multiple genes encodes for Wnt ligands and Wnt receptors:frizzleds and Ryk/Derailed (Eisenmann, 2005).

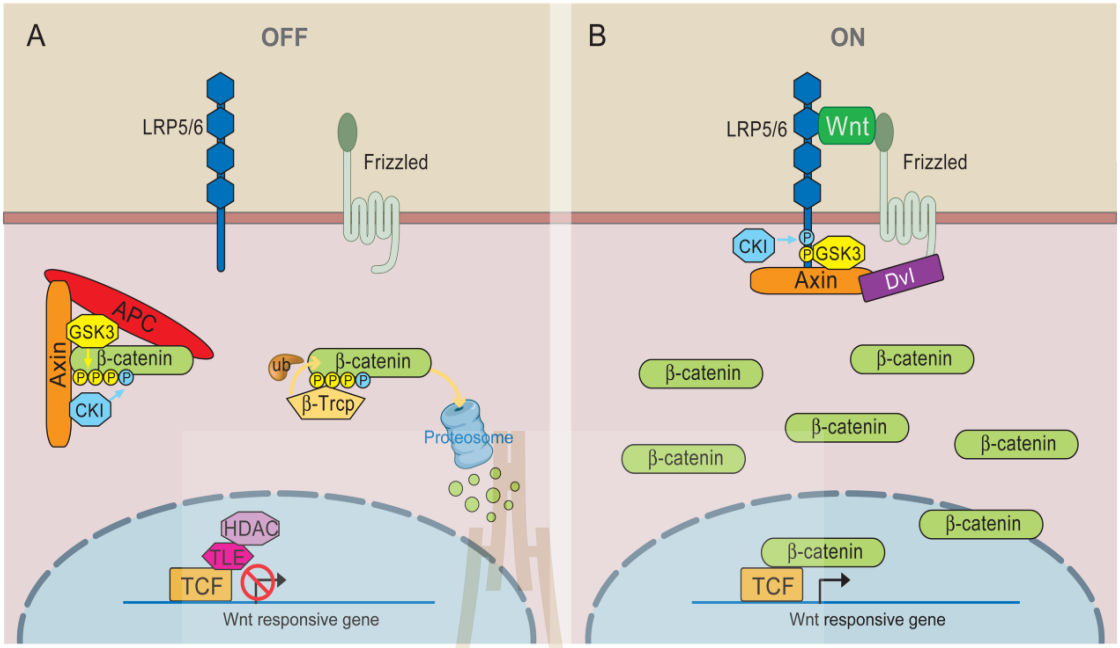


Figure 2 Overview of Wnt/ß-catenin Signalling: A) In the absence of Wnt ligand, ß-catenin forms a destruction complex with Axin, APC, GSK3 and CK1, and phosphorylated by CK1 subsequently by GSK3. Phosphorylated ß-catenin is recognized by E3 ubiquitin ligase ß-Trcp, which targets ß-catenin for proteosomal degradation (He et al., 2004). B) In the presence of Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation, and Axin recruitment. This disrupt Axin-mediated phosphorylation/degradation of ß-catenin, allowing ß-catenin to accumulate in the nucleus where it serves as a co-activator for TCF to activate Wnt responsive genes. This elimination of ß-catenin is prevented by ß-catenin go through the nucleus, and Wnt target genes are repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) (MacDonald et al., 2009).

The activated of Wnt/ß-catenin pathway occur when a Wnt ligand binds to a seven-pass transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close relative LRP5. The formation of a Wnt-Fz-LRP6 complex together with the recruitment of the scaffolding protein Dishevelled (Dvl) results in LRP6 phosphorylation and activation and the recruitment of the Axin complex to the receptors. The inhibition of Axin-mediated β-catenin phosphorylation proceed the stabilization of β-catenin. β-catenin will accumulates and transfer to the nucleus to form complexes with TCF/LEF and activates Wnt target gene expression (MacDonald et al., 2009).

A critical and most studied Wnt pathway is canonical Wnt Signalling, which functions by regulating the amount of the transcriptional co-activator β-catenin that controls key developmental gene expression programs (Nusse, 1997). WNTs and their effectors are important for cancer progression including tumor initiation, tumor growth, cell death, differentiation and metastasis. In addition, over activation of Wnt Signalling by mutation is a major factor in oncogenesis in the human colon and other tissues (Polakis, 2000). Mutations in the Wnt pathway are often related to human birth defects, cancer and other diseases (Clevers, 2006). A role for WNTs in cancer was first described in mouse models of mammary cancer and in human and mouse colon cancer. Aberrant overexpression of WNT1 induced by a proviral insertion at the Wnt1 locus induces spontaneous mammary hyperplasia and tumors in mice. The studies found that WNT1 and other WNTs promoted the stabilization of β-catenin (CTNNB1) and the activation of CTNNB1-dependent transcription (Anastas and Moon, 2013).

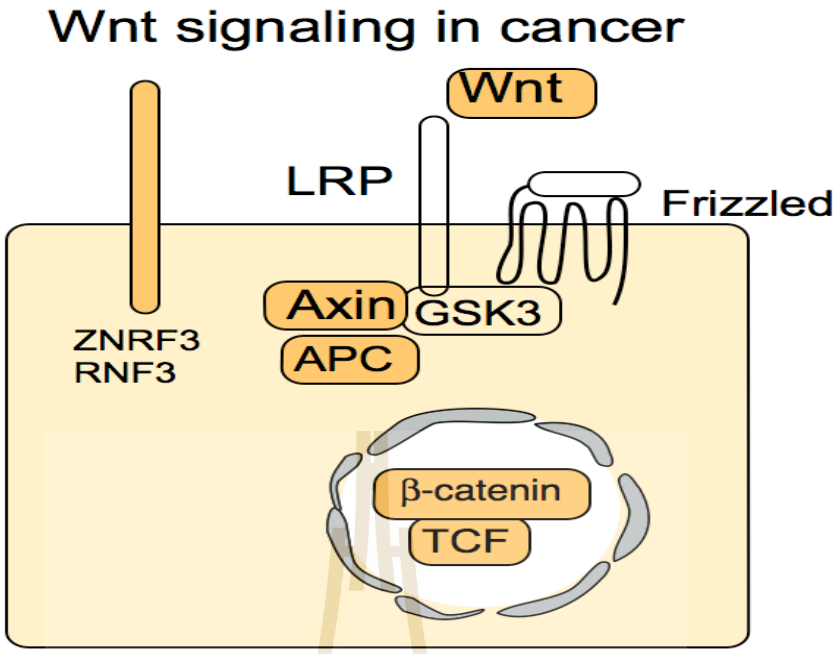


Figure 3 Wnt related genes in Cancer: Components of Wnt Signalling pathway have been implicated in human tumors or experimental cancer models including: Wnt-1 was found as oncogene in murine breast cancer (Nusse and Varmus, 1992), APC was isolated as a tumor suppressor gene in human colon cancer (Polakis, 2000), AXIN1 gene which mutate was reported in human hepatocellular carcinomas (Satoh et al., 2000). A repressor can be a tumor suppressor gene and an activator a dominant oncogene, TCF1 act as a tumor suppressor gene (Roose and Clevers, 1999). Mutant Tcf1 in mice was developing adenomas in the gut and mammary glands, and ZNRF3 and RNF3 are mutated in some tumors (Koo et al., 2012) and (Jiang et al., 2013).

Thus, Wnt signalling pathways contribute to both the maintenance and differentiation of a variety of multipotent progenitor cells in developing embryos and adults. Subpopulations (tumor-initiating cell) of leukemia stem cells that are capable

of forming tumors with short latency in mice (Anastas and Moon, 2013) as well as myeloid progenitors isolated from patients with CML have increased levels of nuclear CTNNB1 and increased WNT–CTNNB1 reporter activity. This suggests that WNT–CTNNB1 signalling is up-regulated in leukemia initiating cells (Jamieson et al., 2004). Barker, N. et al. (2009) found that the activating of WNT signalling by deleting APC in a subset of colon progenitor cells induces the rapid development of adenocarcinoma. In contrast, deleting APC in other cells does not induce tumors. This indicates that the activation of CTNNB1 signalling in progenitor cells drives tumorigenesis (Barker et al., 2009).

Inhibition of the WNT–CTNNB1 pathway in a variety of cancer cells increases cell sensitivity to chemotherapeutic agents. For example, WIF1 increases PC3 prostate cancer cell sensitivity to paclitaxel and etoposide, but has no effect on DU145 cell death (Ohigashi et al., 2005). Increasing the activity of WNTs lead to cancer cells sensitize to chemotherapy (Anastas and Moon, 2013). Although WNT signalling pathways have been difficult to target, improved drug-discovery platforms and new technologies have facilitated the discovery of agents that can alter WNT signalling in preclinical models in humans (MacDonald et al., 2009).

Table 4 Wnt related genes and human diseases

genes	**Function**	**Human Disease**	**References**
ß-catenin (CTNNB1)	+ Primary Wnt effector, Oncogene	Cancer	(Korinek et al., 1997; Morin et al., 1997)
Axin1-2	− Facilitates β-catenin degradation, Tumor suppressor	LOF Caudal duplication, Cancer	(Oates et al., 2006; Satoh et al., 2000)
APC	− Facilitates β-catenin degradation, Tumor suppressor	LOF Familial adenomatous polyposis, Cancer	(Kinzler et al., 1991; Nishisho et al., 1991)
WTX	− Facilitates β-catenin degradation, Tumor suppressor	LO Wilms tumor	(Major et al., 2007; Rivera et al., 2007)
TCF4 (TCF7L2)	- β-catenin transcriptional partner	Cancer, Type2 diabetes	(Florez et al., 2006; Grant et al., 2006)
Tankyrase (TNKS)	− Facilitates β-catenin degradation	Cancer	Anasta et al.,2013
GSK3-β	− Facilitates β-catenin degradation, inhibition of tumor necrosis factor	Alzheimer disease, Cancer	Hoeflich et al., 2000; Anasta et al.,2013

Flahaut, M. et al. (2009) reported the way to elucidate genes and pathways involved in chemoresistance in neuroblastoma cells including neuroblastoma cell lines exhibited overexpression of the MDR1 and the Wnt receptor FZD1 gene (Flahaut et al., 2009). These genes are shown to mediate doxorubicin (DoxR) chemoresistance through the activation of Wnt/β-catenin pathway. The mechanisms underlying the chemoresistant phenotype in neuroblastoma were addressed by gene expression profiling. Wnt/β-catenin mediated of FZD1 in neuroblastoma resistant to chemotheraphy (Flahaut et al., 2009). Gene was included in the identified upregulated genes, although the highest overexpressed transcription in cell lines was the frizzled-1 Wnt receptor (FZD1) gene, an essential component of the Wnt/β-catenin pathway. FZD1 upregulation in resistant variants was shown to mediate sustained activation of the Wnt/β-catenin pathway as revealed by nuclear b-catenin translocation and target genes transactivation. The significant restoration of drug sensitivity in FZD1-silenced cells confirmed the FZD1-associated chemoresistance. From this result, it represents the implication of the Wnt/β-catenin pathway in neuroblastoma chemoresistance and identifies potential new targets to treat aggressive and resistant neuroblastoma.

2.3 Stem Cells and Differentiation

Stem cells are un-differentiated cells that can differentiate into specialized cells and can divide to produce more cells. In mammals, there are two broad types of stem cells: embryonic stem cells; isolated from the inner cell mass of blastocysts, and adult stem cells; found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system in the body. In a developing embryo, stem cells can differentiate into all the specialized cells such as ectoderm, endoderm and mesoderm

but also maintain the normal regenerative organs, such as blood, skin, or intestinal tissues (Tuch, 2006). The major properties that classify stem cell are self-renewal: are ability to go through numerous cycles of cell division while maintaining the undifferentiated state, and potency to differentiate into specialized cell types.

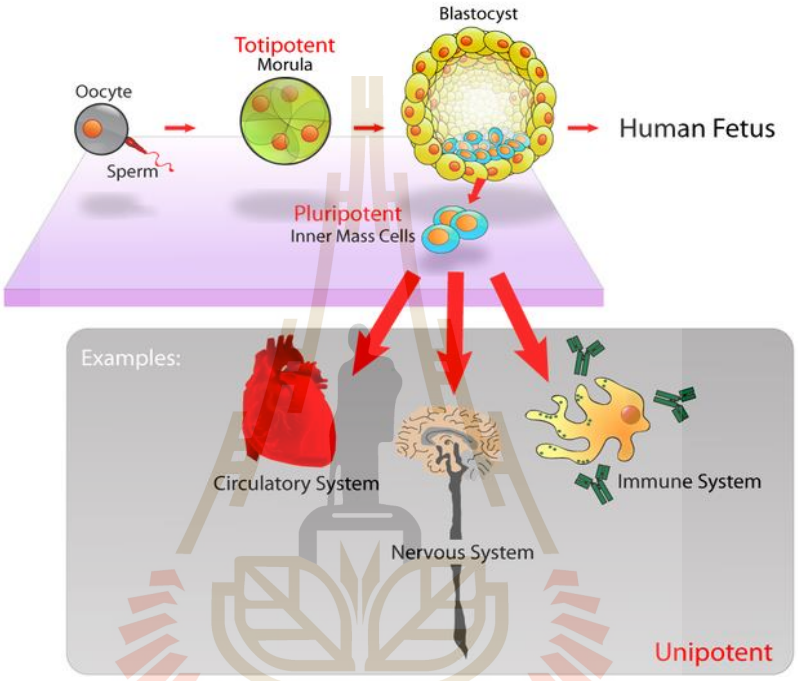


Figure 4 Stem cells and differentiation. Pluripotent embryonic stem cells originate from inner cell mass (ICM) cells within a blastocyst. These stem cells can become any tissue in the body, excluding a placenta. Only cells from an earlier stage of the embryo, known as the morula, are totipotent, able to become all tissues in the body and the extra embryonic placenta (Thomson et al., 1998).

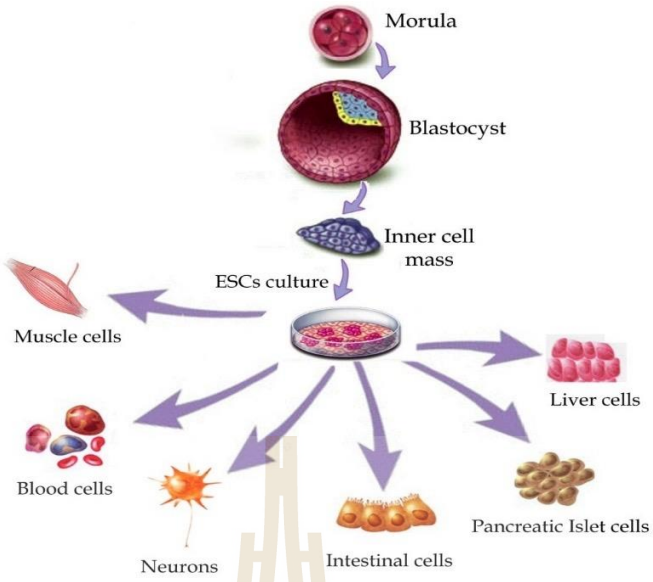


Figure 5 Cellular differentiation is the process, which cell becomes specialized cell types. Differentiation occurs during the development of a multicellular organism as the organism changes from a simple zygote to a complex system of tissues and cell types (Mirella Meregalli, 2011).

Differentiation continues in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. These changes are largely due to highly controlled modifications in gene expression. The differentiated cells can have very different physical characteristics despite having the same genome. In cytopathology, the level of cellular differentiation (Figure5) is used as a measure of cancer progression. "Grade" is a marker of how a cell in a tumor is differentiated (AmericanCancerSociety, 2014). Differentiation therapy aims to force the cancer cell to resume the process of maturation. Although differentiation therapy does not destroy the cancer cells, it restrains their growth and allows the application of more conventional therapies such as chemotherapy to eradicate the malignant cells (medicinenet, 2016). The goals of differentiation therapy may include the

simultaneous differentiation of all phenotypes or the selection of mature cell type such as neuronal. Several substances have been reported to induce differentiation in neuroblastoma cell lines. Using either retinoic acid (RA) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA), growth inhibition was shown. When both drugs were combined, cytostasis in addition to cell differentiation was observed. Since phorbol esters such as TPA act as tumor promoters, the cytostatic effects of the combination of RA plus TPA may have little therapeutic potential. RA was shown to decrease the expression of the N-Myc protooncogene, which is prevalent in neuroblastomas, before induction of neuronal differentiation (Preis et al., 1988).

2.4 Differentiation of Human Neuroblastoma Cells

Neuronal differentiation can be induced in vitro by exposure to different agents, such as tetradecanoylphorbol acetate, brain derived neurotrophic factor (BDNF), norepinephrine, RA (Constantinescu et al., 2007). The goals of differentiation therapy may include the simultaneous differentiation of all phenotypes or the selection of only one mature cell type, for instance neuronal cells. Several substances have been reported to induce differentiation in neuroblastoma cell lines. Among these are RA4 and TPA. Treatment with these drugs in vitro resulted in the formation of a spread out, polar phenotype with neuritic processes and a reduction of cell growth (Preis et al., 1988).

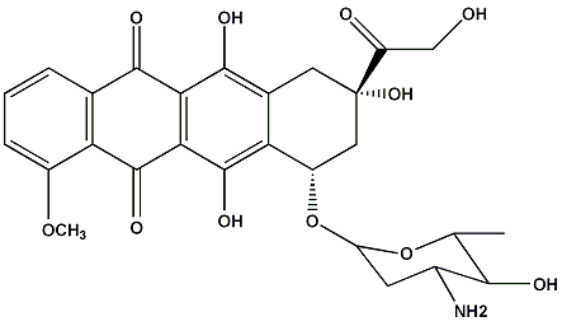
The effects of RA on SH-SY5Y cells are well characterized by RA exerts its effects by binding to two classes of receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs) (Al Tanoury et al., 2013). RA induces differentiation through regulation of the transcription of neurotrophin receptor genes, the Wnt

Signalling pathway (involving type II protein kinase A (PKA). Some differentiated SH-SY5Y cells are a suitable model for Parkinson diseases (PD) study, because SH-SY5Y stops proliferation, contain dopaminergic neuron properties, and show similar susceptibility to neurotoxins and neuroprotective agents as primary neurons. A variety method to differentiate SH-SY5Y cells has been evaluated; however, the development of an optimal differentiated SH-SY5Y dopaminergic cell model for PD requires further evaluation. Alternatively, treatment with RA resulted in partial differentiation towards the neuronal phenotype, and the proportion of neuroblast-neuronal cells was increased from Herb-A treatment induced some differentiation of the neuroblast like cells; however, the differentiation process required a longer time period than differentiation induced by RA (Preis et al., 1988). Herbimycin A and RA affected the growth and morphology of the neuroblastoma lines SK-N-SH, SH-SY5Y, and Kelly. Kelly cell line was selected for its high N-Myc gene amplification, which correlates with malignant progression in neuroblastoma (Preis et al., 1988). Kelly cell line is also responsive, albeit somewhat less sensitive than the SK-N-SH lines, emphasizes the potential of the RA and Herbimycin A. Differentiated SH-SY5Y cells expresses a variety of neuronal-specific markers, including NA, growth-associated protein (GAP-43), receptors for neurotrophic factors, neuropeptides, neurosecretory granular, neuron-specific enolase (NSE), neuronal nuclei (NeuN), vesicle proteins such as synaptophysin, and neuronal-specific cytoskeletal proteins, including microtubule associated protein (MAP), Tau, and neurofilament proteins. MAP, GAP-43, NeuN, and synaptophysin are classical markers of mature neurons (Israel Society for Neuroscience Eilat, 2006). Whereas pp60vsrc for SK-N-SH, which expresses both neuroblasts and non-neuronal cell types (Preis et al., 1988).

2.5 Anticancer Drug

Anticancer, or antineoplastic, drugs are used to treat malignancies, or cancerous growths. Drug therapy may be used alone, or in combination with other treatments, such as surgery, and radiation therapy (Cancer Weekly April 15, 2003). Anticancer or Chemotherapy, which are usually given drugs into a vein. The drugs enter the bloodstream and travel throughout the body to reach and destroy cancer cells. This makes chemo useful for treating neuroblastoma that has spread to the lymph nodes, bone marrow, liver, lungs, or other organ (AmericanCancerSociety, 2014). Some cancers are primarily treated by anti-cancer drugs and many cancers are managed by a combination of surgery, radiotherapy and drug treatments. Anticancer drugs are used to control the growth of cancerous cells. Cancer is commonly defined as the uncontrolled growth of cells, with loss of differentiation and commonly, with metastasis, spread of the cancer to other tissues and organs. Cancers are malignant growths. Although benign tumors may be fatal if untreated, due to pressure on essential organs, as in the case of a benign brain tumor, surgery or radiation is the preferred methods to treat. Drug therapy is used when the tumor has spread, or may spread, to all areas of the body. Several classes of drugs may be used in cancer treatment, depending on the nature of the organ involved. For example, breast cancers are commonly stimulated by estrogens, and may be treated with drugs that inactivate the sex hormones. Some children with neuroblastoma are treated with chemo either before surgery (neoadjuvant chemotherapy) or after surgery (adjuvant chemotherapy), the main chemo drugs used include: Doxorubicin (Adriamycin), Cyclophosphamide or ifosfamide, Cisplatin or carboplatin, and Vincristine (Cancer Weekly April 15, 2003). The most common combination of drugs includes carboplatin (or cisplatin)

cyclophosphamide doxorubicin and etoposid. The possible side effects of chemotherapy include hair loss, mouth sores, and loss of appetite. Along with the effects listed above, some drugs can have specific side effects. For example: Cyclophosphamide and ifosfamide can damage the bladder, which can cause blood in the urine. Doxorubicin work by damaging the RNA or DNA that tells the cell how to copy itself in division. If the cells are unable to divide, they die. The faster the cells are dividing, the more likely it is that chemotherapy will kill the cells, causing the tumor to shrink. They also induce self-death or apoptosis. Chemotherapy; will kill all cells that are rapidly dividing. The "normal" cells will grow back and be healthy but in the meantime, side effects occur. Doxorubicin can cause heart damage. Doctors try to reduce this risk as much as possible by limiting the doses of doxorubicin and by checking the heart with a test called an echocardiogram (an ultrasound of the heart) during treatment. The researcher reported the most commonly used dosage of doxorubicin is 40 to 60 mg/ml every 21 to 28 days. Alternatively, 60 to 75 mg/ml every 21 days. The lower doses are recommended for patients with inadequate marrow reserves due to old age, prior therapy, or neoplastic marrow infiltration (Doxorubicin, Gibco® and www.drug.com).



Doxorubicin

Chemical Formula: $C_{27}H_{29}NO_{11}$
Exact Mass: 543.17
Molecular Weight: 543.52
Elemental Analysis: C, 59.66; H, 5.38; N, 2.58; O, 32.38

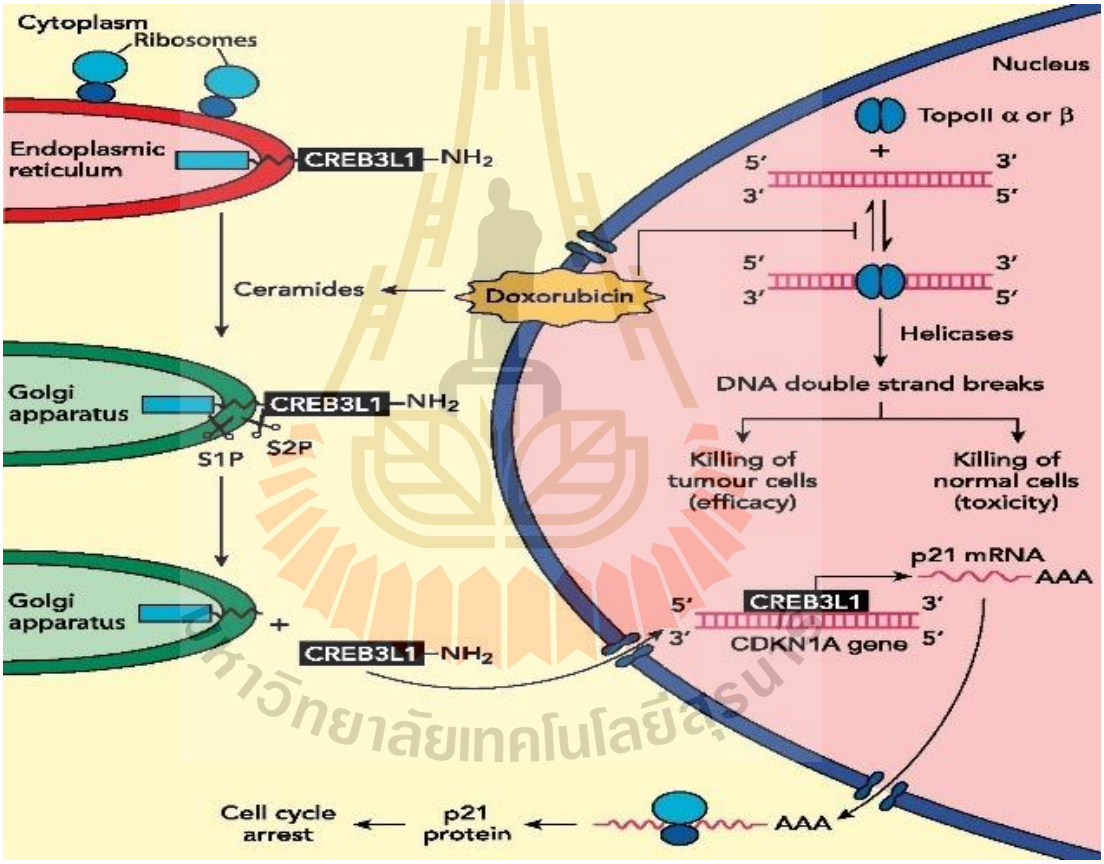


Figure 6 Doxorubicin and its mechanism in cells: Doxorubicin treatment known as Chemotherapy. Doxorubicin work by damaging the RNA or DNA that tells the cell how to copy itself in division. If the cells are unable to divide, they die. The faster the cells are dividing, the more likely it is that chemotherapy will kill the cells, causing the tumor to shrink. They also induce self-death

or apoptosis. Chemotherapy; will kill all cells that are rapidly dividing. The "normal" cells will grow back and be healthy but in the meantime, side effects occur.

2.6 XAV939: Tankyrase inhibitor

XAV939:3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3d] pyrimidin-4-one, a kind of small molecule tankyrase (TNKS) inhibitor and synthetized using a chemical genetics approach (Tian et al., 2014b). XAV939 was able to block Wnt signaling through stabilizing Axin protein and increasing β-catenin destruction in colon cancer cell lines(Wu et al., 2016). XAV939 stabilizes axin by inhibiting the poly-ADP-ribosylating enzymes tankyrase (TNKS) 1 and tankyrase2. Both tankyrase isoforms interact with a highly conserved domain of axin and stimulate its degradation through the ubiquitin-proteasome pathway (Figure 7) (Huang et al., 2009). Moreover, It inhibits proliferation of the β-catenin dependent colon carcinoma cell line DLD-1. Promotes cardiomyogenic development in mesoderm progenitor cells (Huang et al., 2009). Treatment of XAV939 promoted apoptosis and reduced the invasiveness of SH-SY5Y cells dependent on telomere shortening (Tian et al., 2014a). TNKS1 played a role in tumor progression. The XAV939 could induce apoptosis of NB cells partly by inhibiting Wnt/β-catenin signaling through TNKS1. However, it has not been reported whether XAV939 also has effect on stemness of NB CSCs, and the involved mechanism that would contribute to targeted therapy (Tian et al., 2014b).

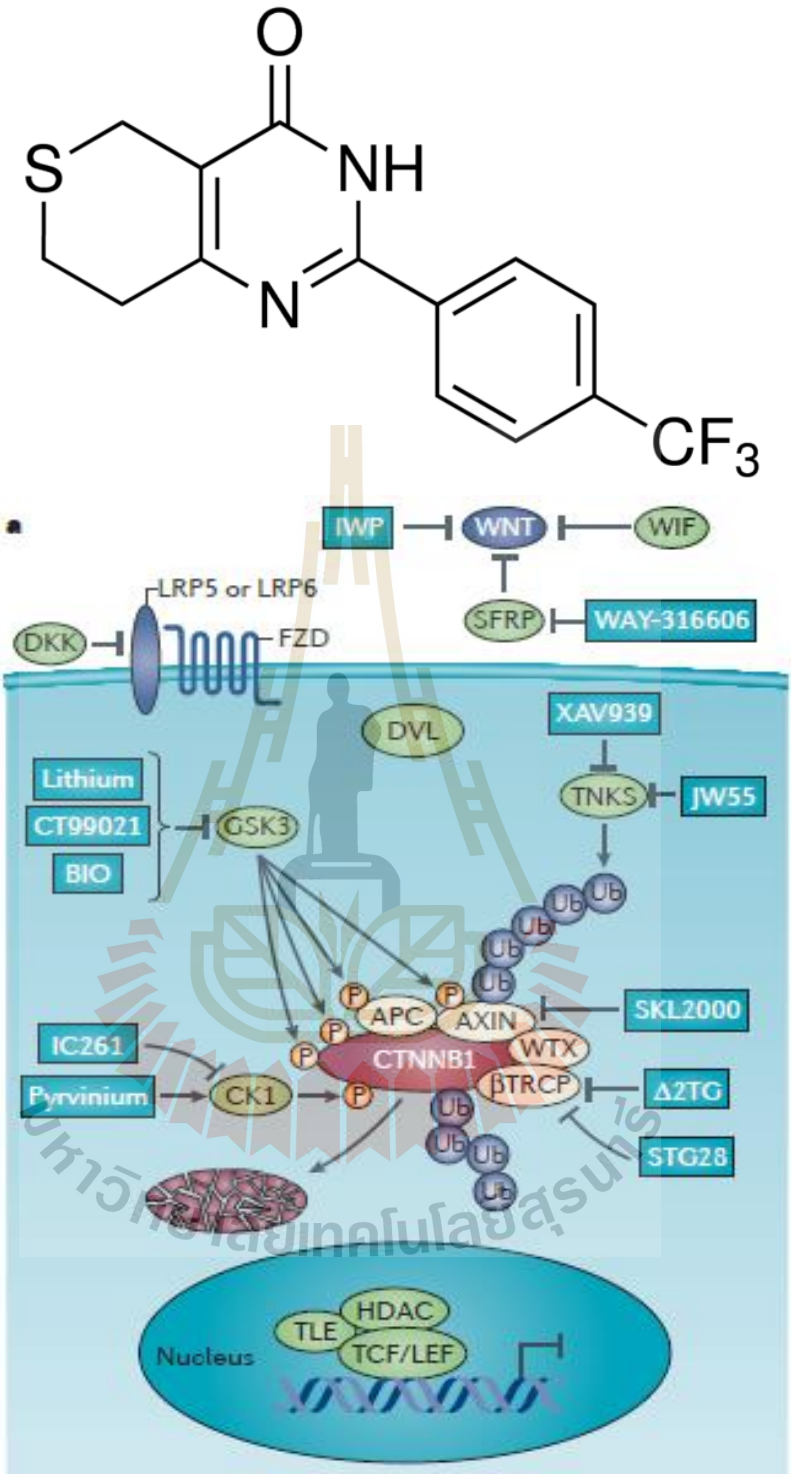


Figure 7 XAV939 and its mechanism in cells: XAV939 treatment known as Wnt inhibitor (Anastas and Moon, 2013).

CHAPTER III

MATERIAL AND METHODS

3.1 Materials

3.1.1 Cell Line

The human SH-SY5Y neuroblastoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 20% fetal bovine serum: FBS (Invitrogen, Carlsbad, CA), 10,000 units penicillin and 10 mg streptomycin/ml. The cell lines were grown at 37°C in a 95% air and 5% CO2 humidified incubator (Ferrari-Toninelli et al., 2010).

3.1.2 Primers

genes	Size (bp)	Forward	Reverse
ß-actin	351	TCACCACCACGGCCGAGCG	TCTCCTTCTGCATCCTGTCG
Tuj1	148	GCTCAGGGGCCTTTGGACATCTCTT	TTTTCACACTCCTTCCGCACCACATC
C-MYC	478	TACCCTCTCAACGACAGCAG	TCTTGACATTCTCCTCGGTG
MASH1	279	TCGCACAACCTGCATCTTTA	CTTTTGCACACAAGCTGCAT
Nestin	209	CAGCTGGCGCACCTCAAGATG	AGGGAAGTTGGGCTCAGGACTGG
NeuroD1	219	AGCCCTCTGACTGATTGCAC	GTCTATGGGGATCTCGCAGC
N-MYC	200	CTTCGGTCCAGCTTTCTCAC	GTCCGAGCGTGTTCAATTTT

genes	Size (bp)	Forward	Reverse
PAX6	275	AACAGACACAGCCCTCACAAACA	CGGGAACTTGAACTGGAACTGAC
PHOX2A	293	CTTGGCCTTCTTTGGATGCG	AGCCCCTCCACTCCTCTAAC
PHOX2B	206	CGAGCAAGGAAAAGGCACAC	ACGGTCACGTAGAGGAGACA
SLUG	221	AACAGTATGTGCCTTGGGGG	AAAAGGCACTTGGAAGGGGT
SOX10	228	TCCAGGCCCACTACAAGAGC	CAATGTCCACGTTGCCGAAG
TFAP2a	447	TCAAGTACGAGGACTGCGAG	CCTCGATGGCGTGAGGTAAG
CyclinA2	451	TCCATGTCAGTGCTGAGAGG	GAAGGTCCATGAGACAAGGC
p21	221	GAGGCCGGGATGAGTTGGGAGGAG	CAGCCGGCGTTTGGAGTGGTAGAA
p53	265	CCCCTCCTGGCCCCTGTCATCTTC	GCAGCGCCTCACAACCTCCGTCAT

3.1.3 Antibodies

Antibody	Product name	Description	Dilution
	Primary Antibody		
Tuj1	Anti-Tuj1	Mouse monoclonal to Tuj1	1:500
AP-2 alpha	Anti-human AP-2	Mouse Anti-human AP-2	1:250
BrdU	5-bromo-2'-	Mouse	1:500
Anti-BrdU	Anti-BrdU	Rat monoclonal to BrdU	1:1000
ß-catenin	Anti-beta Catenin	Rabbit polyclonal to beta	1:500
	Secondary Antibody		

Donkey anti-Goat IgG	Donkey anti-Goat IgG (H+L) Secondary	Goat IgG (H+L) Polyclonal Secondary Antibody	1:1000
Goat anti-Mouse IgG	Goat anti-Mouse IgG (H+L) Secondary	Mouse IgG (H+L) Polyclonal Secondary Antibody	

3.1.4 Instruments

Autoclave	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Sorvall RC5C plus, Kendro laboratory Products, USA Eppendrof centrifuge 5810 R, Eppendrof, USA
-70 OC Deep freezer	Heto, Ultra Freeze, Denmark.
-20 OC Freezer:	Heto, HLLF 370, Denmark. MyBio LFT420, DAIREI, Denmark
Gel Document set	White/Ultraviolet Transilluminator GDS7500, UVP, USA
Gel electrophoresis apparatus	Mini Protean® 3 cell, BioRad, USA
Heat Box	HB1, Wealtee Corp., USA
Microarray (Transcriptome Analysis Console3.0)	Gene Level Differentiation Expression Analysis, Affymetrix Ltd, Santa Clara, CA, USA
CO2 Incubator	Thermo Fisher Scientific, Thermo Electron LED GmbH, FORMA STERI-CYCLE i160, Robert-Bosch-Strasse,

	Langenselbold, Germany
Laminar hood:	Holten LaminAir HBB 2448, Denmark. BH2000 Series ClassII Biological Safety Cabinets, BHA120 & BHA180, Clyde-Apac
Laser Scanning Confocal Microscope (LSCM)	Confocal Microscope Nikon A1Rsi, NIKON INSTRUMENTS INC., Melville, NY, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA Eppendorf 54154, Eppendorf, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
Spectrophotometer	Nanodrop 2000, Thermo Scientific, USA
Microplate reader	SPECTROstar Nano, BMG Labtech, UK
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA

3.2 Methods

3.2.1 RNA Extraction

This procedure was performed according to RNeasy Mini Kit (QIAGEN Company, Ltd). Briefly, the appropriate volume of buffer RLT was added to the cells. One time volume of 70% ethanol was added to the lysate, and mixed well by pipetting. Then, 700 μl of the sample, including any precipitate was transferred to RNeasy Mini spin column, placed in a 2 ml collection tube, and centrifuged for 15s at 15,000 rpm. RNA concentration was verified by Nano Drop micro-volume spectrophotometer (Thermo Scientific, Wilmington, USA). RNA quality was observed by agarose gel

electrophoresis. The RNA samples were kept at -80°C or proceeded immediately to reverse transcription experiment.

3.2.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA of SH-SY5Y cell lines was converted into cDNA by using GeneAmp PCR system 9700 and iScript cDNA synthesis Kit from Bio-Rad Laboratories, Inc., according to the manufacturer's protocol.

3.2.3. Gene Expression

The DNA was amplified by M0267S ThermoPol enzyme from New England Biolabs, Inc., PCR was performed by using cDNA templates and 1 μM final concentration of primer (Macrogen, Korea). The appropriate anneal temperature are 55-57 °C. Primer sequences and the expected lengths of amplify products are listed in Materials 3.1.2. Semi-quantitative of gene expression was calculated by target gene density divide by β-actin density (lnt*mm^2).

Semi-quantitative=Target Gene density/β-actin density

3.2.4 Cell Viability Assay

Cell viability was determined by MTT assay. MTT 5 μl (25 mg/ml) was added to each well of 96 well plates, including one set of wells with MTT but no cells (negative control). All should be done aseptically. Cells were incubated for 2 hours at 37°C in culture incubator. Then lysis buffer was added and incubated overnight. The spectrophotometer was required for measurement the absorbance at 570 nm with a reference filter of 630 nm. The percentage of viable cells was determined by comparison with negative control. For cell treatment, cells were treated for 5 days with 0, 1, 2, 5 and 10 µg/ml of 3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one (XAV939).

For cell number optimization and standard curve, cells were seed in the various number starting from 1000 cells/well to 13,000 cell/wells to determine optimize cell number then standard curve of cell viability versus cell number was plotted. The standard curve of cell viability was performed by using the various concentration of doxorubicin drug, i.e., 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, and 50 μg/ml.

3.2.5 Immunofluorescence

SH-SY5Y treated and non-treated cells (control) were grown separately on a glass coverslip coated with Geltrex®, Gibco for 5 days. Cells were washed twice with phosphate-buffered saline (PBS) and fix in 4% paraformaldehyde (PFA) for 10 min then washed three times with PBS. Then, permeabilization step was performed by incubating the cells with PBS containing 0.1% Triton X-100 for 1 hr (0.5ml/well). Cells were then incubated with primary antibody at appropriate dilution in PBS containing 1% FBS at 4ºC, overnight. Each well was washed three times by PBS for 5 minutes and incubated with secondary antibody dilute at 1:1000 in PBS for 30 min in dark and washed three times by PBS. For morphological evaluation, slides was mounted and examined by confocal laser scanning microscope (Nikon A1Rsi, Nikon Company, Ltd). The images were taken by two color channels including DAPI and TRITC. The interpreted images were shown in Merge photos of both DAPI and TRITC. The percentage of morphologically differentiated cells was determined by counting of each treatment, eight randomly chosen fields were photographed at each time point.

3.2.6 Cell Proliferation Assay

Cell proliferation was observed by BrdU incorporation: alter differentiation SH-SY5Y cells were incubated at 37 °C for 2 hr in media supplement with 10 μM BrdU. Cells were washed three times with 1X PBS. Cells were fixed by 4% PFA (freshly

made) for 10 min. For immunofluorescence, cells was treated as above, with the exception that the permeabilization step was followed by a DNA denaturation using 2M HCl at 37°C for 1 hour in order to make the DNA accessible to the antibody. Each well was rinsed with PBS for six times and incubated in PBS buffer contain 10% FBS, 0.5% Triton x-100 for 1 hour (0.5ml/well). Anti BrdU monoclonal antibody 200 μl (DSHB; 1:1000 in PBS containing 1% serum) was added to each well and incubated at 4°C, overnight. Cells were washed three times with PBS and incubated with secondary antibody diluted in PBS for 30 min in dark. Finally cells were washed with PBS six times in dark before mounting slide step and proceed to observe under confocal laser scanning microscope (Nikon A1Rsi, Nikon Company, Ltd).

3.2.7 Microarray Assay

This procedure was performed according to GeneChip® WT PLUS Reagent Kit (Affymetrix Company, Ltd). Briefly, First and double strand cDNA were synthesized followed by cRNA. T7 RNA polymerase was added to each sample to perform cRNA purification and quantitation. Then, RNA was remove from the template by added RNaseH (44 μl/sample) and incubated for 50 min. Gene fragmentation, labeling was perform followed by Hybridization on WT ship (See more: Step by Step method in Appendices)

3.2.8 Anticancer Drug Sensitivity Test

The cytotoxicity of drug to cell was assessed by MTT assay. The experiments were set into two groups; (a): Cell treated with various concentrations of doxorubicin alone and (b) Cell treated with same concentration of doxorubicin combined with 10 μg/ml of XAV939. The concentrations of doxorubicin are 0.001, 0.005, 0.01, 0.05, 0.5, 1 and 5 mM with 5 days treatment time. The optical density was determined by microplate reader at 570 nm.

3.2.9 Statistical Analysis

The data was present by mean ± SD. Two-Way ANOVA was used to compare between treatment groups and non-treated group. The differences were considered statistically significant at $p<0.05$.

CHAPTER IV

RESULTS

4.1 Inhibition of Tankyrase Reduces the Accumulation of β-catenin in Human Neuroblastoma Cells

To evaluate the effects of Wnt inhibition on human neuroblastoma cells, XAV939 was supplemented into NB cell culture. XAV939 is a compound that inhibits Wnt pathway through the inhibition of Tankyrase (TNKS) and the up-regulation of Axin1. Immunofluorescence was performed to determine whether TNKS inhibition can cause the accumulation of β-catenin and others protein in SH-SY5Y cells. As shown in Figure 7, upon the treatment of XAV939, the levels of β-catenin, was reduced, suggesting the inhibition of Wnt Signalling. The levels of total β-catenin inside nucleus were decreased in 10 μM XAV939 treated cells compared with the control groups (Figure8). Moreover, total number of β-catenin positive cells was highly decreased in treated cells about 99.21 to 48.00. This suggested that TNKS inhibition can reduce the expression of β-catenin in NB cell line by reducing β-catenin nuclear translocation.

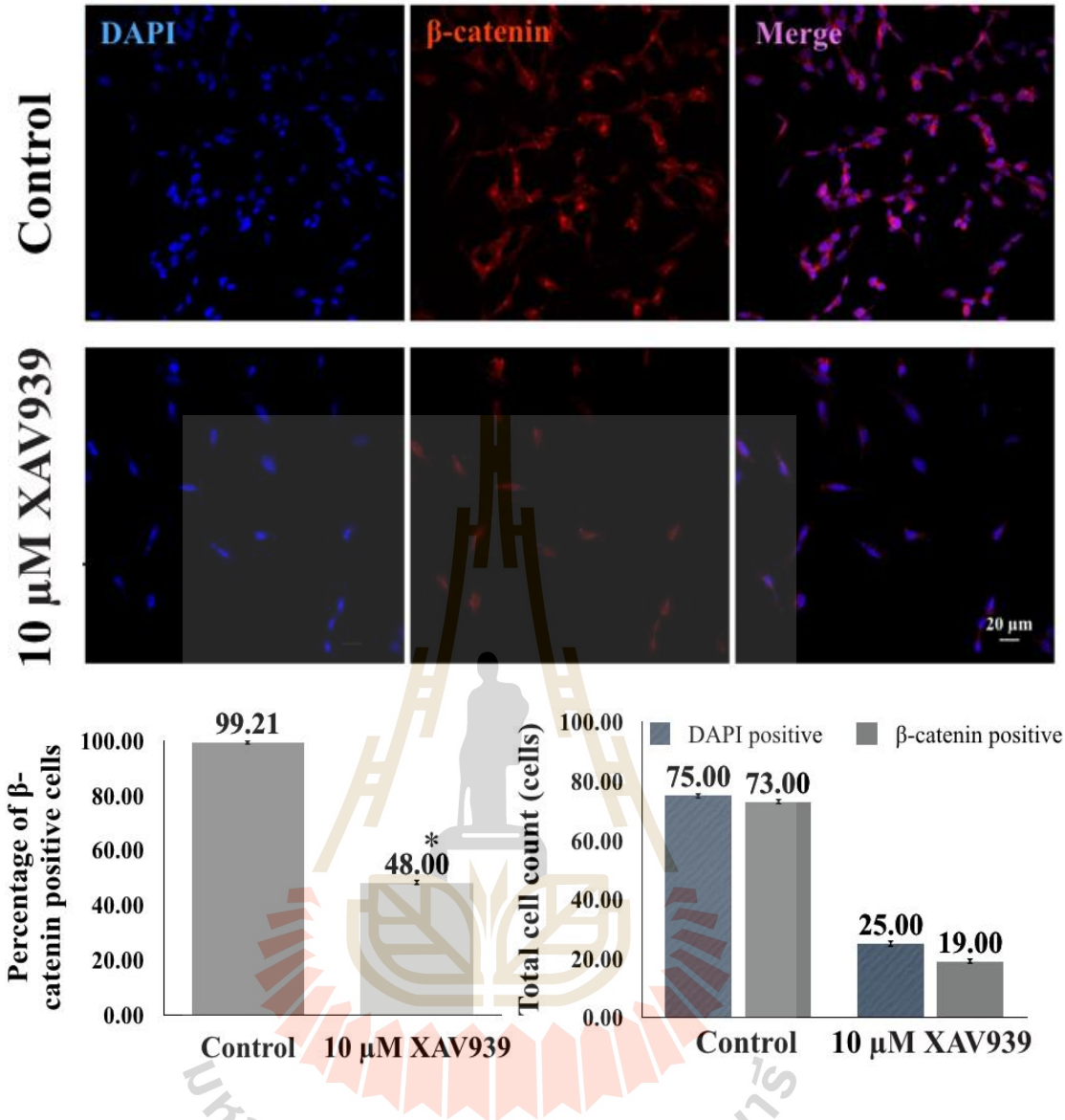


Figure 8 β-catenin translocation staining of SH-SY5Y cells, compared between control and 10 µM XAV939 treatment. Graph of percentage of β-catenin in cell was shown. The images were taken by two color channels including DAPI and TRITC. The interpreted images were shown in Merge photos of both DAPI and TRITC. Duration of XAV939 treatment is 5 days, 100x magnification.

According to Huang et al. (2009), they found that XAV939 could affect the mechanism of Wnt pathway by inhibition the regulation of Axin protein and presents a new target of Wnt pathway for cancer therapies. XAV939, a Tankyrase inhibitor, antagonizes Wnt Signalling by Axin stabilization. Thereafter, Axin can form a destruction complex and promote β-catenin degradation. Thus, it was shown that the expression and nuclear localization of β-catenin in SH-SY5Y cells was evidently decreased after treated cell with 10 µM XAV939 (Figure 8).

4.2 Inhibition of Wnt Signalling Did Not Affect Cell Morphology of SH-SY5Y Cells

To evaluate the effect of Wnt inhibition on cell morphology of human neuroblastoma cells, cell morphology was observed on a daily basis (Figure 9). After 5 days of treatment of 1 5 and 10 µM XAV939, SH-SY5Y cells showed no morphological changes, as compared with the control cells (Figure 9 A-D). These results indicated that the inhibition of Wnt by XAV939 did not affect SH-SY5Y cell shape and morphology.

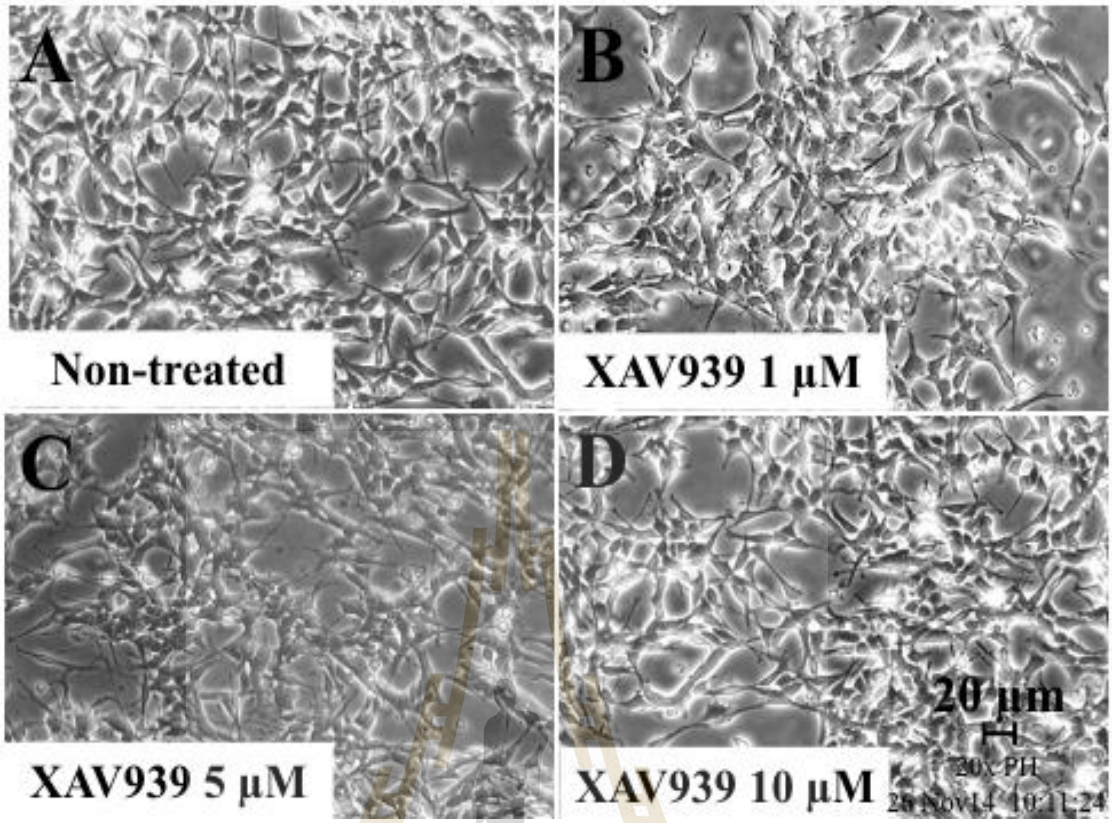


Figure 9 SH-SY5Y cells were treated with tankyrase inhibitor (XAV939) at various concentrations, compared the control; (A) non-treated. (B) Cell line was treated at 1 µM, (C) 5 µM, and (D) 10 µM of XAV939.

4.3 Inhibition of Wnt Signalling Did Not Affect Cell Viability and Proliferation of Human Neuroblastoma Cells

It has been reported that XAV939 could inhibit the proliferation of cells growth by attenuating the expression of Wnt Signalling (Tian et al., 2013). The effect of Wnt inhibition on proliferation of SH-SY5Y cells was performed by supplemented XAV939 into SH-SY5Y culture at concentrations 0, 1, 5, and 10 µM. Cell viability of SH-SY5Y were assessed by MTT cell viability assay. Treatment cells were compared

to non-treated cell as a control. At all these concentrations, XAV939 did not affect the cell viability of human NB cell line (Figure 10).

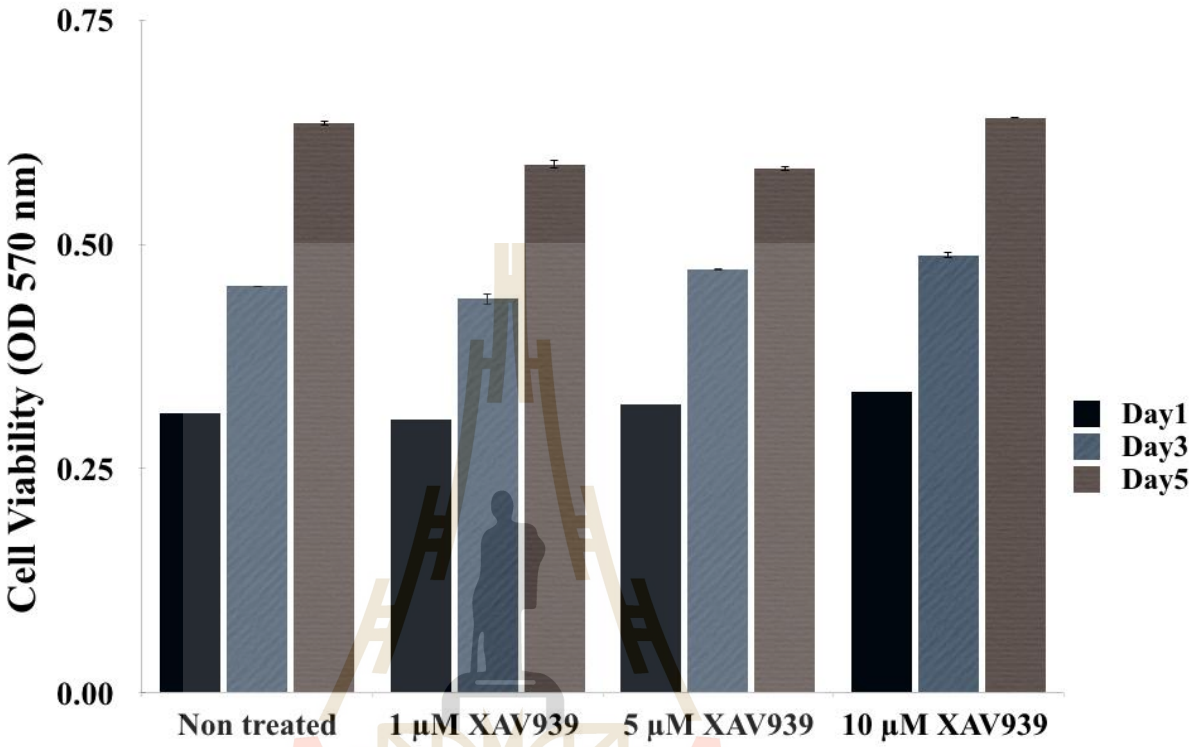


Figure 10 Bar graph showed the relative cell viability of treated cells compared to control. In both treated and untreated cell line. The influence of XAV939 on the cell viability was evaluated by MTT assays at day 1 3 and day 5, following the treatment at various concentrations. The data represented the means and standard deviation from three experiments in triplicate. The statistical analyses were conducted using Two-Way ANOVA (*$p \leq 0.05$).

In addition, cell proliferation was confirmed by BrdU cell proliferating assay after 5 days of XAV939 treatment. The newly synthesized DNA strands will incorporated with BrdU and detected by antibody against BrdU. The secondary antibody was conjugated with Alexa flour 488 (green color), and whole nuclei was

labeled by DAPI (blue), where they could be visualized under fluorescence microscope (Figure 11). The mean fluorescence intensity of BrdU protein in control and 10 µM XAV939 treatments was 98.00%±0.50%, and 97.4%±0.50%, respectively. Moreover, gene expression by RT-PCR was perform (Figure 12, Cyclin A2). Expression level of CyclinA2 was not different, compared between the control and XAV939 treated cell.

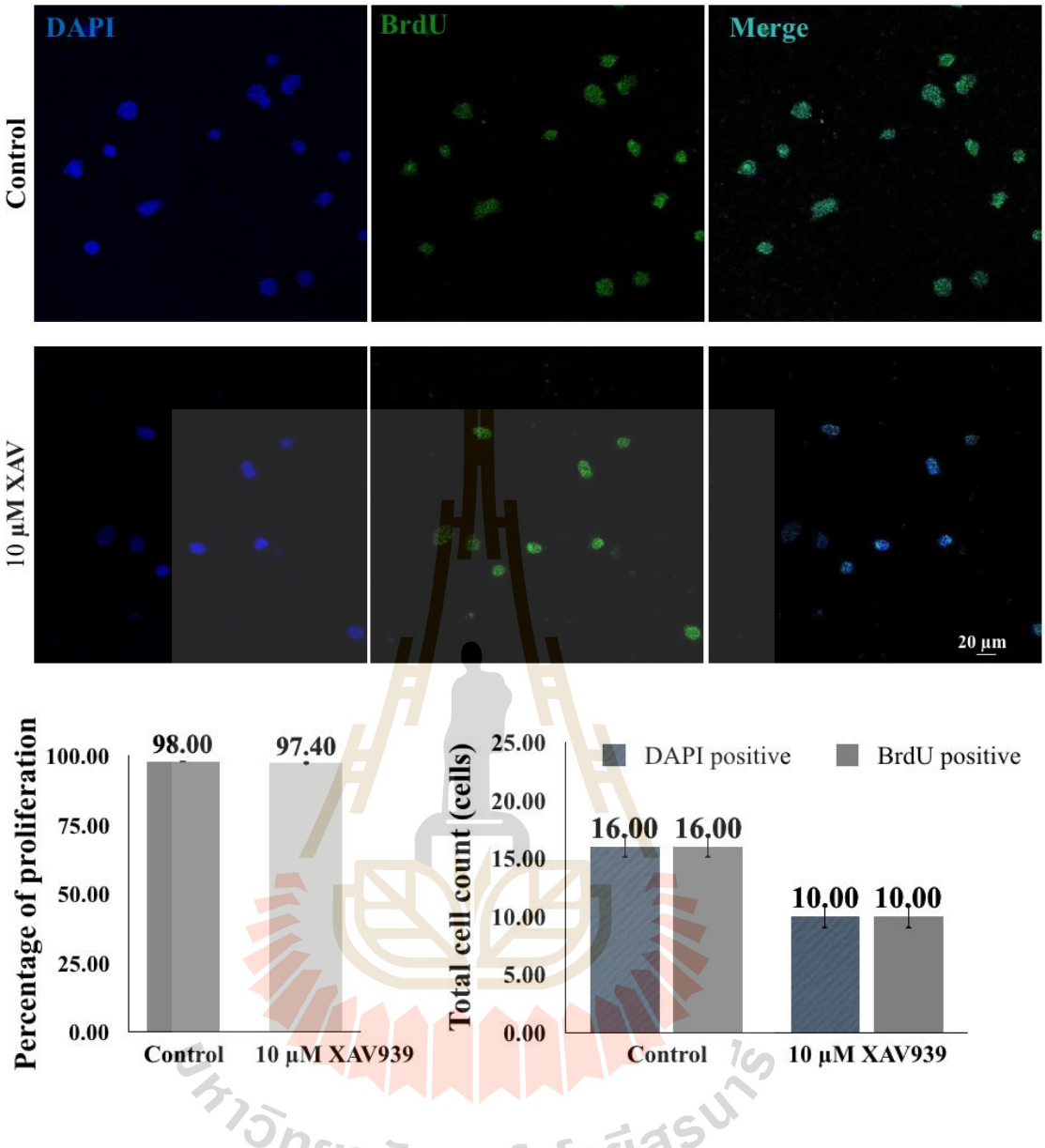


Figure 11 TNKS1 inhibitor did not affect the proliferation of SH-SY5Y cells. The representative diagrams of stained SH-SY5Y cells in control group (non-treated) and 10 µM XAV939 group. The bar graph is the average percent of cells in control group and XAV939-treated groups. The statistical analyses were conducted using One-Way ANOVA (*$p \leq 0.05$).

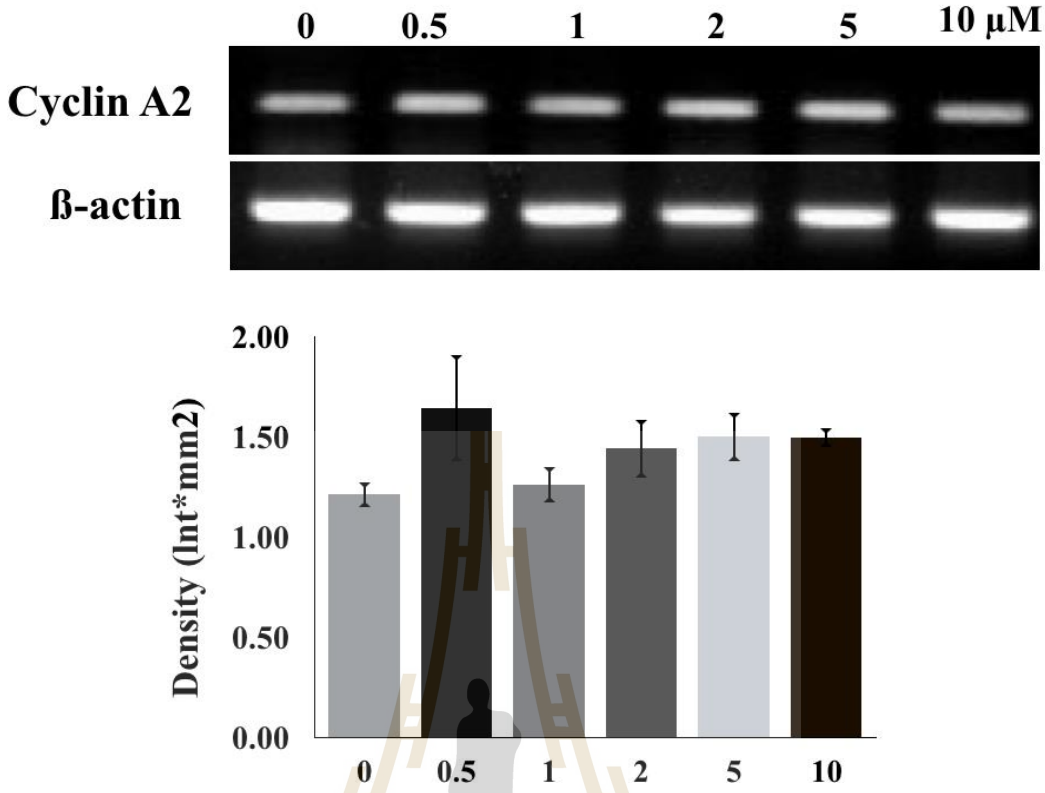


Figure 12 Cyclin A2 expression. RT-PCR analysis in the control and XAV939 treated SHSY-5Y at 0.5, 1, 2, 5 and 10 µM was assessed after 5 days. β-actin was used as an internal control gene expression of SH-SY5Y cells.

4.4 Inhibition of Wnt signalling by tankyrase inhibitor alters differentiation of SH-SY5Y cells

From those results of cell viability and proliferation experiments, showing that the inhibition of Wnt Signalling did not affect these two parameters of SH-SY5Y cells. The alteration of SH-SY5Y cells by Wnt Signalling inhibition was next determined by changing of their gene expression (Figure 13). Noteworthy, it was found that XAV939 treatment led to the downregulation of mature neuronal genes, including *Tuj1, PHOX2B,* and *PHOX2A* while the relative expression level of neural

progenitor markers, which are TFAP2a and PAX6 were significantly higher than control cells (Figure 13). The results indicated that the inhibition of Wnt by XAV939 repressed the maturation of SH-SY5Y cells to some extent.

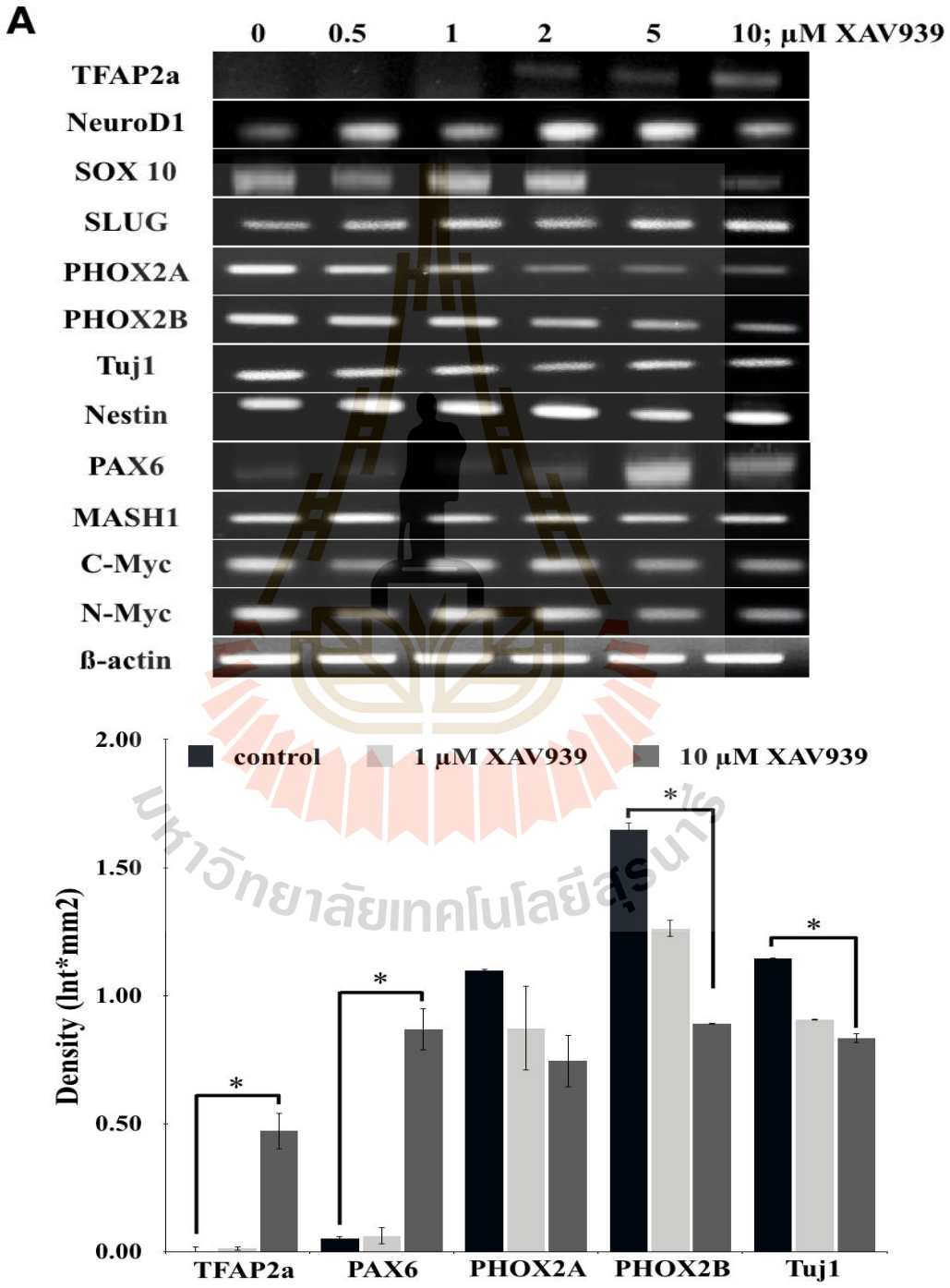


Figure 13 Tankyrase inhibition altered the expression levels of different lineage specific genes in SH-SY5Y cells. RT-PCR analysis in the control and XAV939 treated SHSY-5Y at 0.5, 1, 2, 5 and 10 µM was assessed after 5 days. β-actin was used as an internal control gene (A). The bar graph showed mean ± S.D. of the ratio interest proteins/β-actin band intensity obtained by pooling the results from 3 independent experiments in SH-SY5Y cells.

In addition, Tuj1 and TFAP2a which are markers of mature neurons and neural progenitors, respectively, were monitored after RT-PCR experiments. The immunofluorescence results showed that β-tubulinIII (Tuj1) protein was highly expressed in the control SH-SY5Y cells (Figure 14a). The treatment of XAV939 resulted in the clear reduction of Tuj1 protein (Figure 14b). Interestingly, the treated cells were increased the expression of TFAP2a protein (Figure 14c). These results indicated that inhibition of Wnt Signalling by XAV939 reduced the expression of mature neuronal proteins and primed the expression of neural progenitor markers of human NB cells.

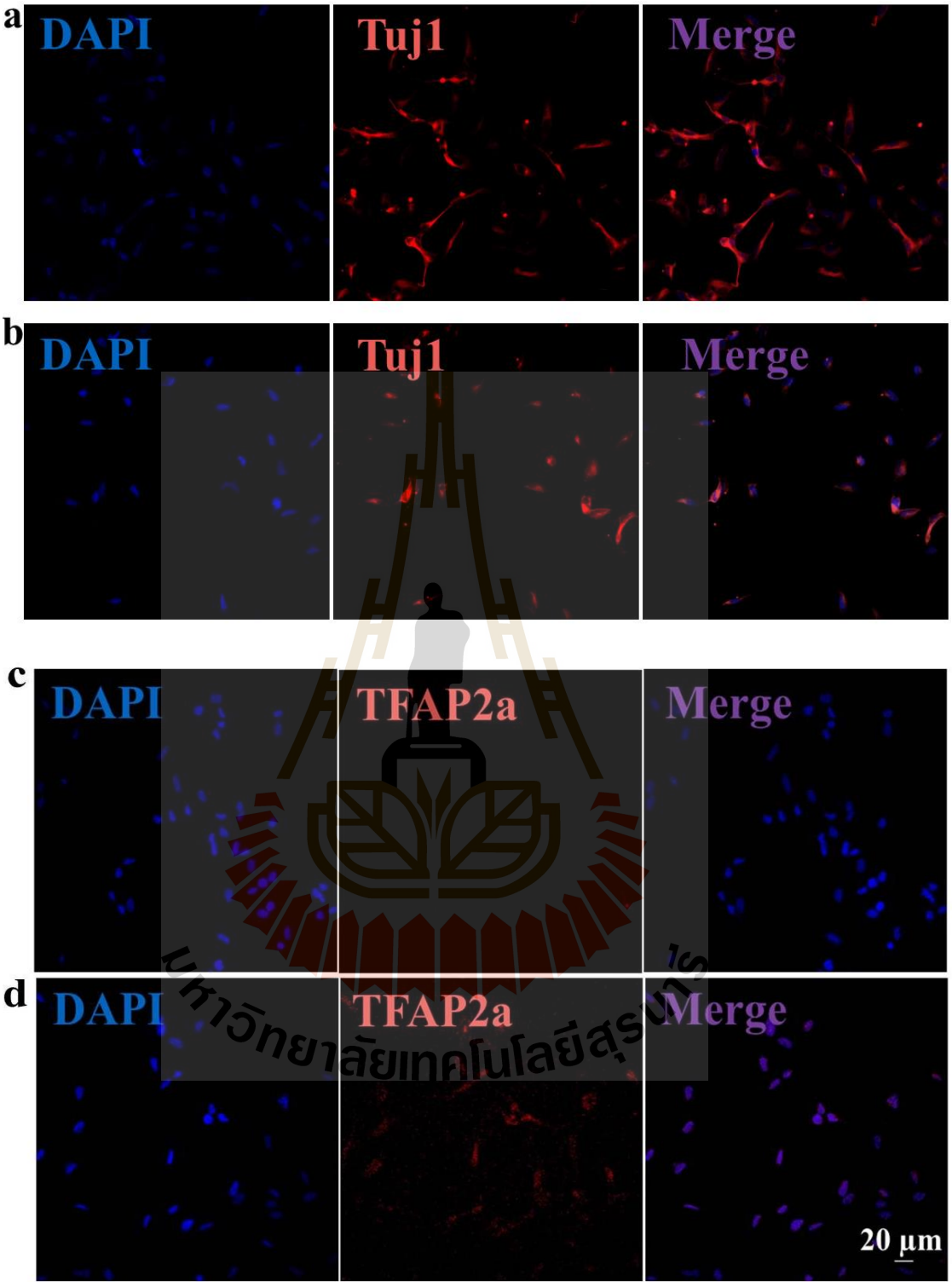


Figure 14 Tuj1 (a-b) and TFAP2a (c-d) marker staining of SH-SY5Y. Control (a&c), 10 µM XAV939 cell treatment (b&d). Beta- tubulin and TFAP2a stain with Alexa Fluor® 594 (red); while nucleus stained with DAPI (blue). The

images were taken by two color channels including DAPI and TRITC. The interpreted images were shown in Merge photos of both DAPI and TRITC. XAV939 treatment for 5 days, 100x magnification

4.5 Inhibition of Wnt Signalling by tankyrase inhibitor showed synergistic effect with anticancer drug against the survival of neuroblastoma cells

To determine whether XAV939 reduces cell viability and survival of SH-SY5Y cells, we performed an anti-cancer drug activity by MTT in vitro. The formazan is converted by mitochondria reductase enzyme of survives cells line was measure at OD 570 nm and OD 630 as negative reference absorbance. The absorbance of negative control (DMEM+ 10% FBS coated well with Geltrex) was used as subtraction value. The positive control (non-treated cell without doxorubicin), control of anti-cancer drug (Doxorubicin) group were use the same concentration with doxorubicin plus 10 µM XAV939 group. From these results it is evident that the XAV939 enhanced the sensitivity of anti-cancer drug activity when compare with the result of merely doxorubicin. This result was correlate with the expression level of apoptosis genes which are p21 and p53 which is increasing in treated cells (Figure 16).

As shown in Figure 15, specific activity of XAV939 in SH-SY5Y cells resulted in a significant decrease cell survival rate in co-treatment of anti-cancer drug with 10 µM XAV939, it was show lower cell survival rate than control group or neither doxorubicin alone group ($p < 0.05$). These results indicate that the growth inhibitory effects of XAV939 on SH-SY5Y cells are due to TNKS1-dependent inhibition.

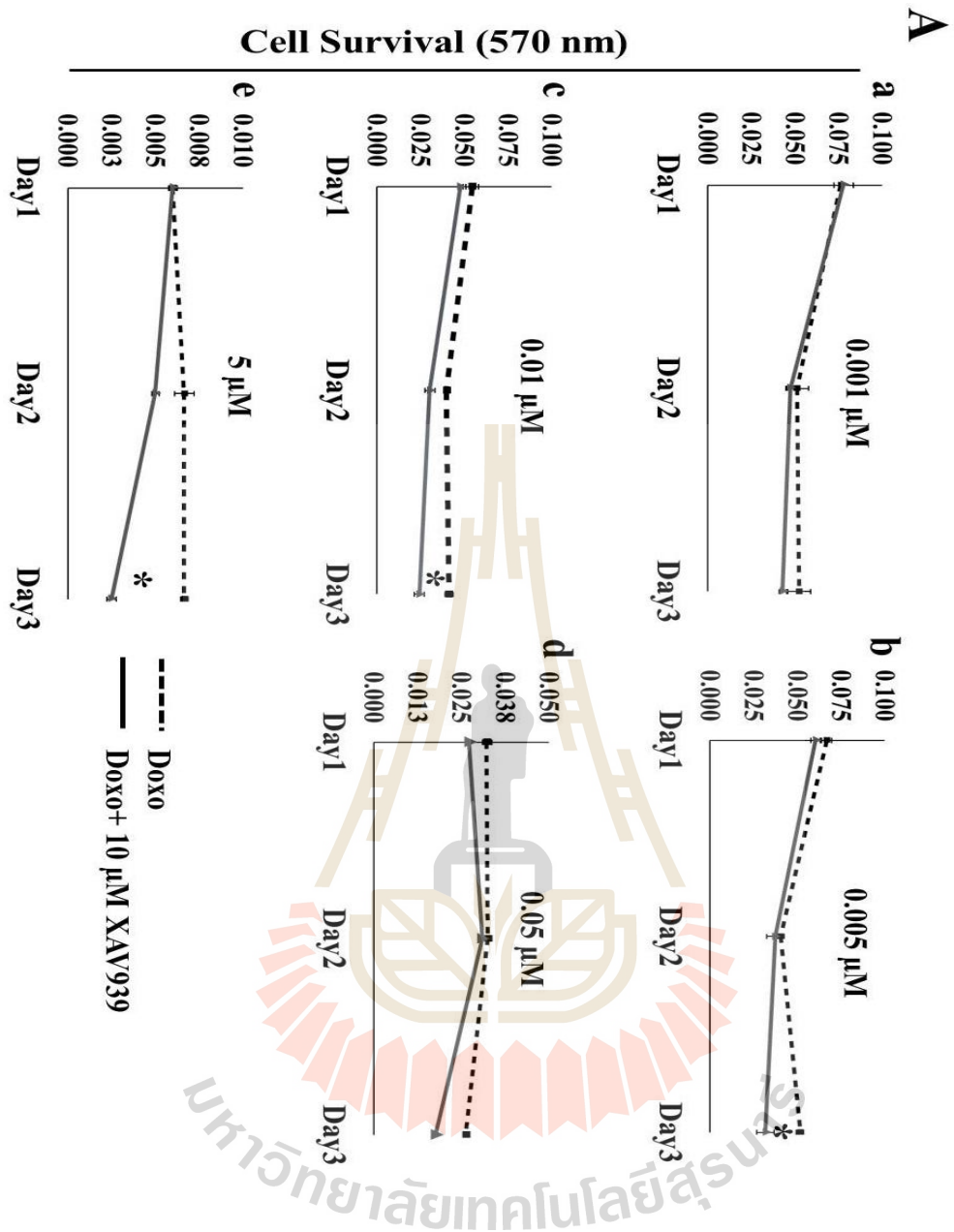


Figure 15 Anti-cancer activity affects human neuroblastoma (SH-SY5Y) cells in co-treatment with XAV939 and doxorubicin. A; Cytotoxicity by MTT assay (a) Neuroblastoma cells were treated with 0.001 µg/ml Doxorubicin or both of Doxorubicin and 10 µg XAV939 for 3 days. (b) 0.005 µg/ml, (c) 0.01, (d) 0.05 and (e) 5 µg/ml, respectively.

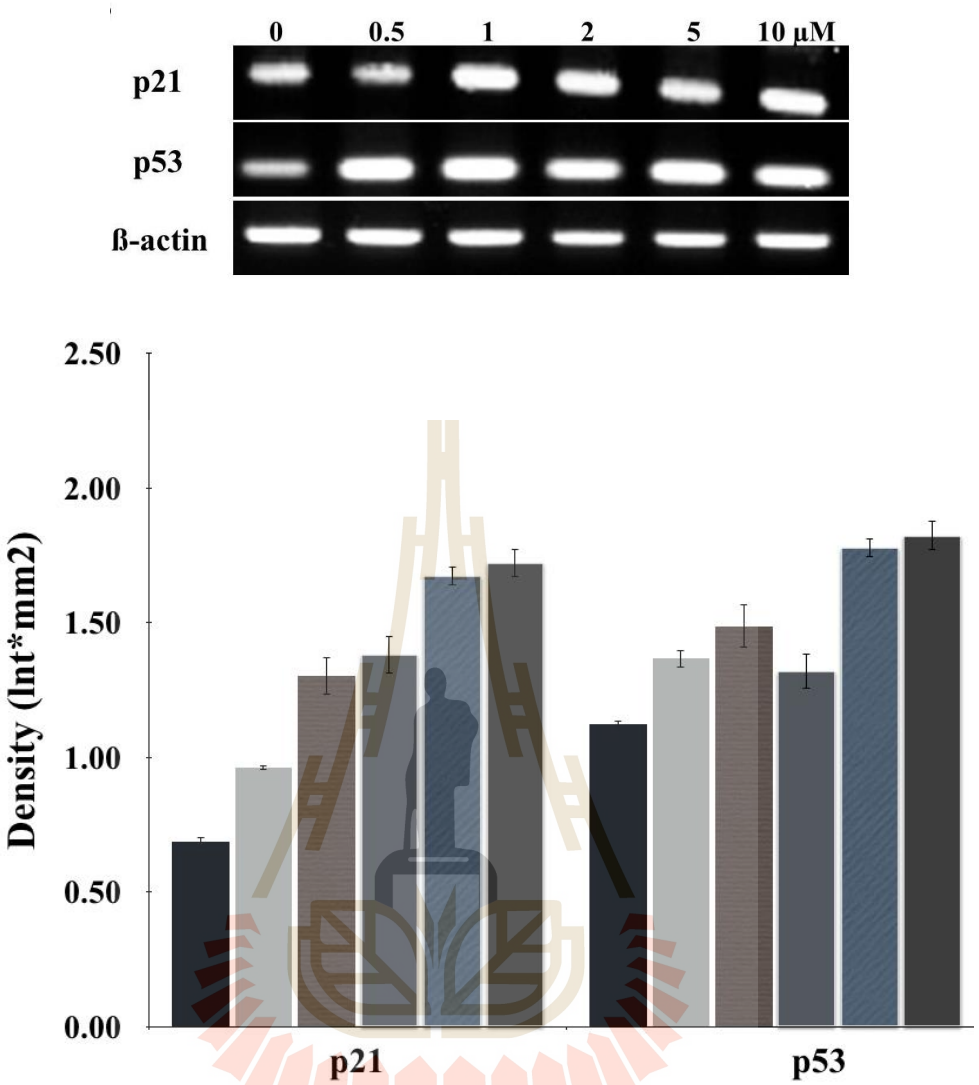


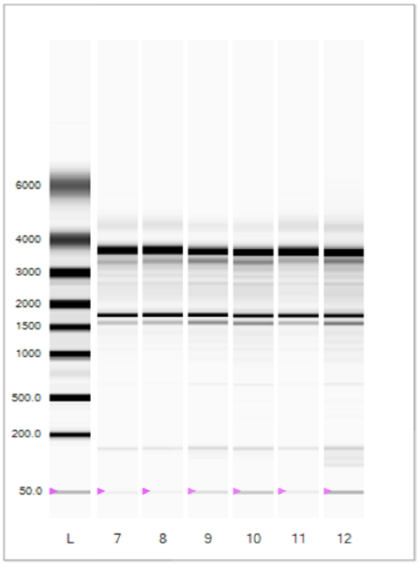
Figure 16 p21 and p 53 expression. RT-PCR analysis in the control and XAV939 treated SHSY-5Y at 0.5, 1, 2, 5 and 10 µM was assessed after 5 days. β-actin was used as an internal control gene in SH-SY5Y cells.

In our study, the sensitivity of doxorubicin was preferable in XAV939 treated cells as figured out in Figure 15, specific activity of XAV939 resulted in a significant decrease survival rate in combination of anti-cancer drug and 10 μM XAV939 group.

Thus, global gene expression of SH-SY5Y cells by microarray (Transciptome Analysis Console 3.0) was conducted, allowing the description of wide genome expression changes in the treatment and control groups (Table 5, Figure 17-18).

4.6 Global Gene Expression of SH-SY5Y Cells Changed Upon the Treatment of XAV939

Global genome expression by microarray preprocessing from RNA extraction in both of the treated and control cells. Then, the quality of RNA was validated by RNA analyzer (figure 16), and prepared the dataset for the application of data analysis methods. The method used to identify differentially expressed genes was the fold change. A change of at least one fold (up or down) was considered meaningful. However, the two-fold threshold was arbitrarily chosen. The result was shown as Hierarchical graph (Figure 18), Chromosome summary (Figure19), Volcano plot (Figure20), of 1 and 10 μM XAV939, respectively.



Well# 7 Sample 7 control1K

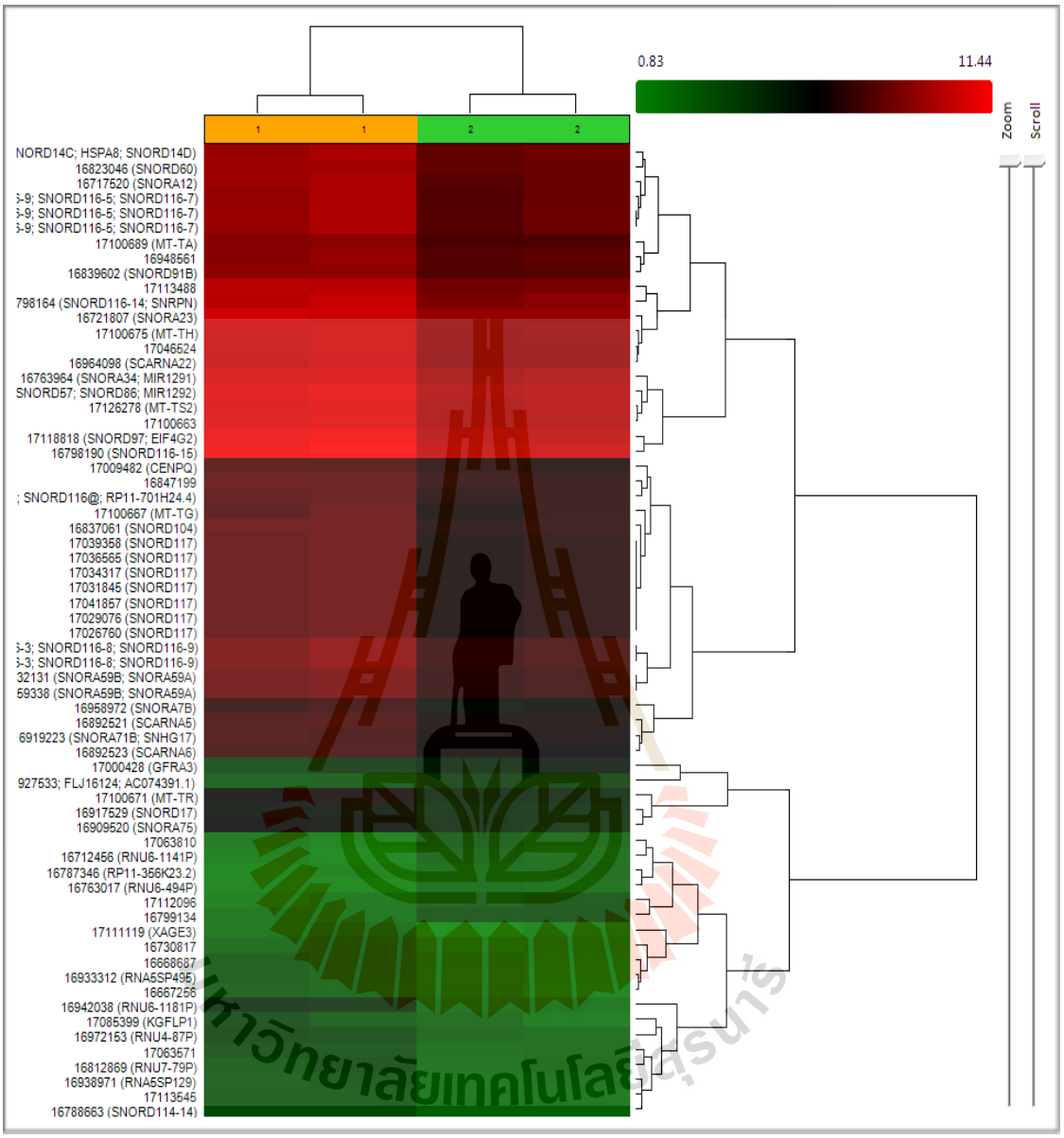
Fragment Number		Start Time	End Time	Area	% of Total Area
1	18S	40.20	41.75	79.09	16.78
2	28S	47.00	48.85	165.50	35.12

RNA Area: 471.27
RNA Concentration: 188.47 ng/µl
Ratio[28S/18S]: 2.09
RQI: 10.0

Sample	RNA Area	RNA Concentration (ng/µl)	Ratio (28s/18s)	RQI
L: Ladder	400.08	160.00	-	-
7: Control1	471.27	188.47	2.09	10
8: Control2	608.70	243.43	2.05	10
9: 1 XAV1	251.97	100.77	2.23	9.9
10: 1XAV2	154.92	61.95	2.01	9.8
11: 10 XAV1	428.94	171.54	2.21	10
12: 10 XAV2	148.93	59.56	1.92	9.7

Figure 17 RNA analysis by Experion™ Automated Electrophoresis System of control, 1 and 10 μM XAV939 treated cells including ladder (L). Bands of 18s and 28s ribosomal RNA were detected and total RNA was calculated. RQI 9-10 were interpreted as a good RNA purity and acceptable for further downstream process such as Microarray.

A


0.83
11.44
Zoom
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1
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NORD14C; HSPA8; SNORD14D)
16823046 (SNORD60)
16717520 (SNORA12)
ɜ-9; SNORD116-5; SNORD116-7)
ɜ-9; SNORD116-5; SNORD116-7)
ɜ-9; SNORD116-5; SNORD116-7)
17100689 (MT-TA)
16948561
16839602 (SNORD91B)
17113488
798164 (SNORD116-14; SNRPN)
16721807 (SNORA23)
17100675 (MT-TH)
17046524
16964098 (SCARNA22)
16763964 (SNORA34; MIR1291)
SNORD57; SNORD86; MIR1292)
17126278 (MT-TS2)
17100663
17118818 (SNORD97; EIF4G2)
16798190 (SNORD116-15)
17009482 (CENPQ)
16847199
; SNORD116@; RP11-701H24.4)
17100667 (MT-TG)
16837061 (SNORD104)
17039358 (SNORD117)
17036565 (SNORD117)
17034317 (SNORD117)
17031845 (SNORD117)
17041857 (SNORD117)
17029076 (SNORD117)
17026760 (SNORD117)
ɜ-3; SNORD116-8; SNORD116-9)
ɜ-3; SNORD116-8; SNORD116-9)
32131 (SNORA59B; SNORA59A)
59338 (SNORA59B; SNORA59A)
16958972 (SNORA7B)
16892521 (SCARNA5)
6919223 (SNORA71B; SNHG17)
16892523 (SCARNA6)
17000428 (GFRA3)
927533; FLJ16124; AC074391.1)
17100671 (MT-TR)
16917529 (SNORD17)
16909520 (SNORA75)
17063810
16712456 (RNU6-1141P)
16787346 (RP11-356K23.2)
16763017 (RNU6-494P)
17112096
16799134
17111119 (XAGE3)
16730817
16668687
16933312 (RNA5SP495)
16667256
16942038 (RNU6-1181P)
17085399 (KGFLP1)
16972153 (RNU4-87P)
17063571
16812869 (RNU7-79P)
16938971 (RNA5SP129)
17113545
16788663 (SNORD114-14)

B

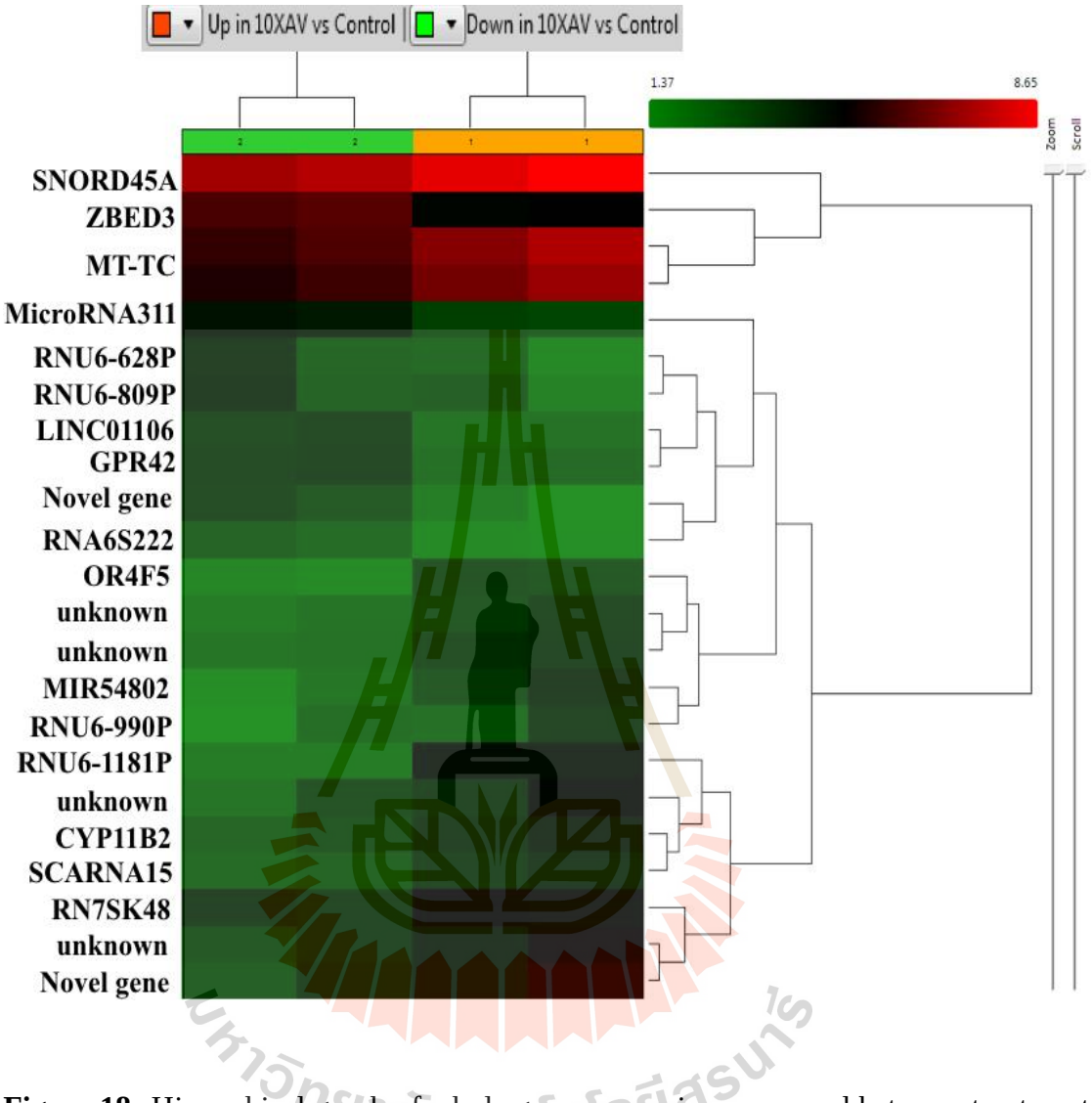


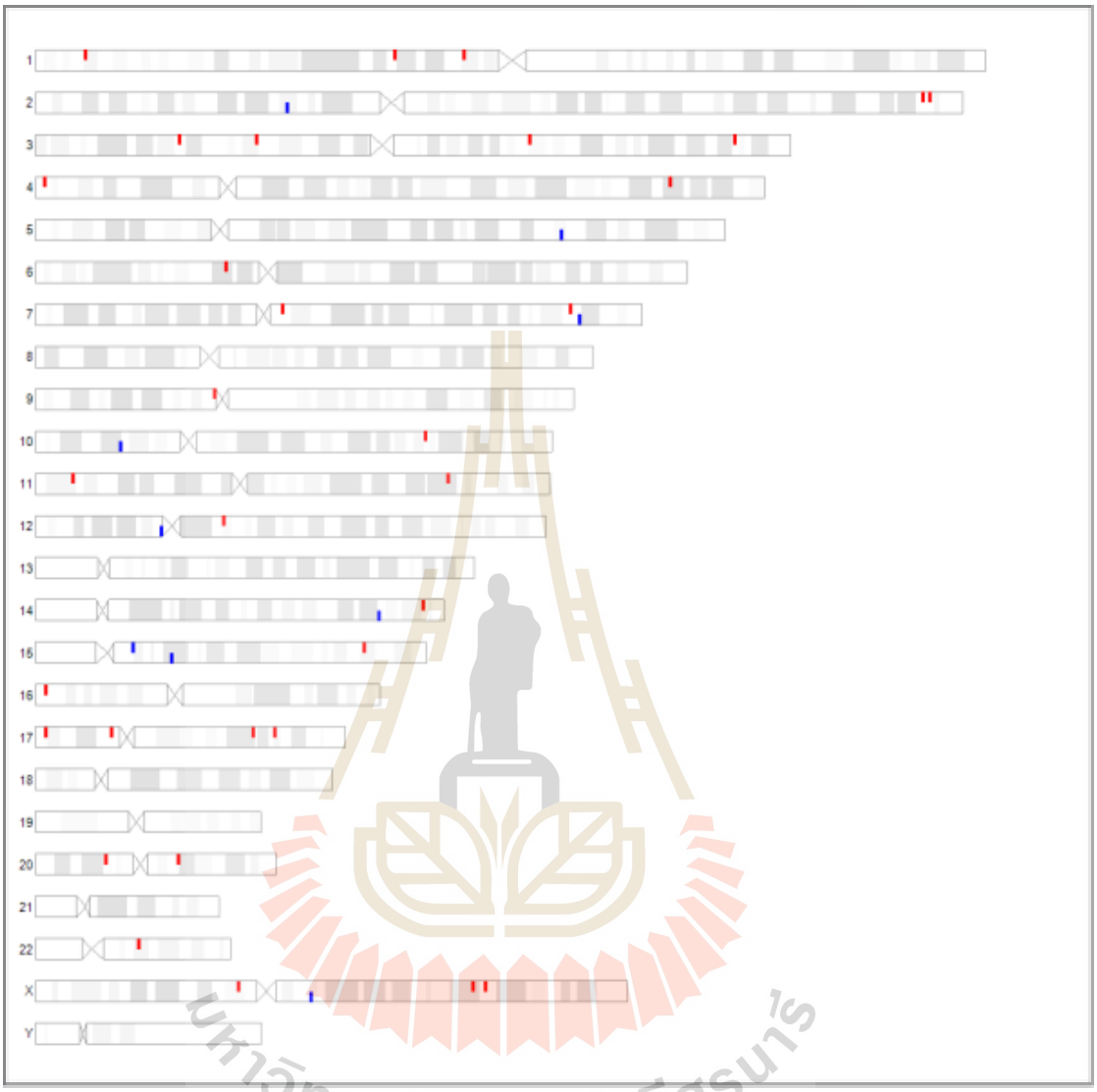
Figure 18 Hierarchical graph of whole gene expression compared between treatment 1 µM (A) and 10 µM XAV939 (B) with non-treat by Gene Level differential Expression Analysis, HuGene-2_0-st Microarray, (**p<0.05). Red was gene up-regulation in XAV939 treated calls while Green was down- regulation. Black is uncharacteristic and/or non-express.

The result of whole genome expression are total 48286 genes in treatment group with 10 µM XAV939 (Figure 18B) and 48226 genes in 1 µM XAV939 (Figure 18A) respectively.

Table 5 The result of genes up and down regulations between 10 µM XAV939 and control group (non-treated).

Differentiation Genes Expression Analysis: Microarray (Transciptome Analysis Console 3.0)	
Genes	**Fold Change**
Up- regulation	
TFAP2a	1.24
Neurotrophin	1.24
MYC	1.07
PAX3	1.01
Down regulation	
Tuj1	-1.25
NeuroD1	-1.22
TH	-1.14
GSK3β	-1.09
SOX10	-1.05
β-catenin	-1.02
PHOX2B	-1.02

A



B

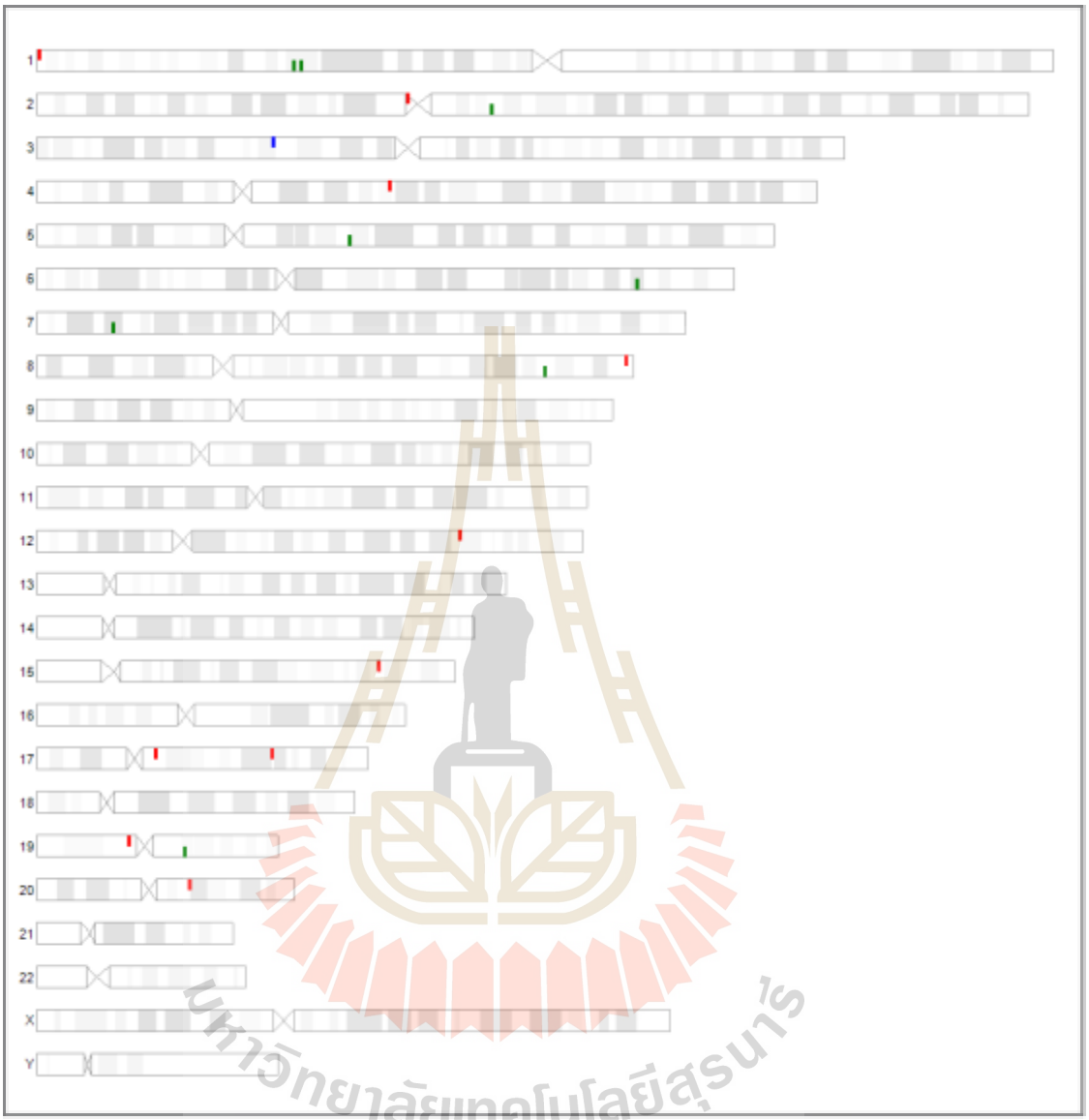


Figure 19 Chromosome summary of 1 (A) and 10 (B) µM XAV939 treated cell by Gene Level differential Expression Analysis by HuGene-2_0-st (**p<0.05).

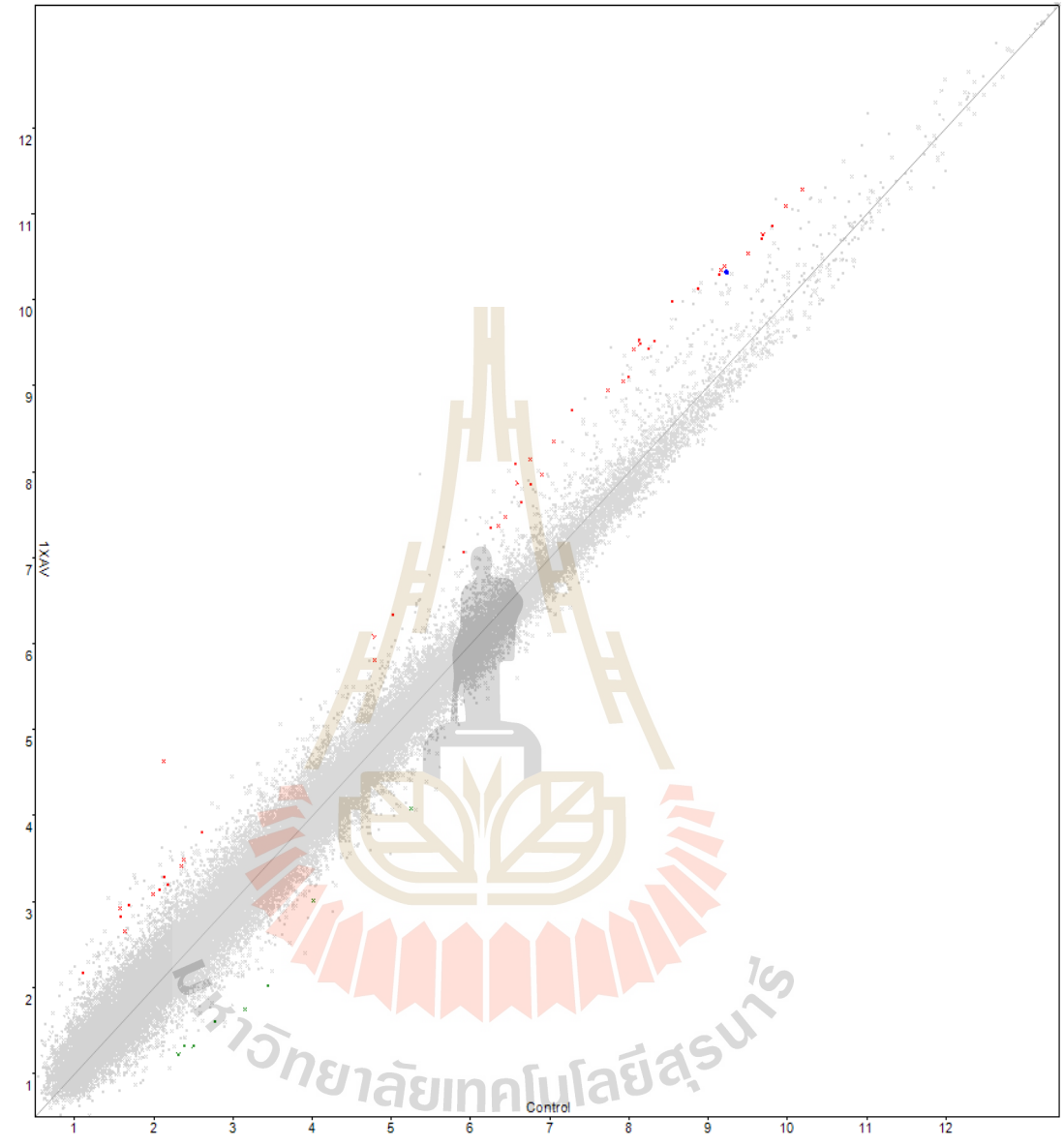
TAC analysis results were interpreted in Hierarchical graph (Figure 18), Chromosome summary (Figure 19), Volcano plot (Figure 20) and the result show that 23 genes are differentially expressed 15 genes up-regulates and 8 down-regulate in 10 µM XAV939 (Panel B, Figure 18-19) while 65 genes different in 1 µM XAV939 (Panel A, Figure 18-19) with 57 genes up-regulate and 8 genes down-regulate in treatment group compare with non-treated. The results were repeated measure by ANOVA (paired), -2 < fold change> 2, ANOVA p-value <0.05 was interpret in both experiments (1 µM XAV939 vs Control and 10 µM XAV939 vs Control).

These TAC result may supported to anti-cancer drug sensitivity that the differentiated stages of human neuroblastoma was found to support doxorubicin activity significantly more effectively than parental cell (control cell), which was far more effective than doxorubicin alone when added to XAV939 treated neuroblastoma cells because genes related Apoptosis/Necrosis such as CASP3, CASP7, CASP 9, JUN, and BIRC5 was increase to 1.22, 1.11, 1.09, 1.35, 1.47, respectively (Figure 20 and Table6). In addition, NPHP3-AS1 and UBC which are ubiquitin C genes; play important role to ubiquitinal proteasome degradation was increase in neuroblastoma treated XAV939 similarity with other representative genes in cell damage and cell stress as shown as Table 6. Whereas, XXbac; TAP2; gene resistant to drug activity was decrease. Wnt molecule and Wnt ligand including FZD, DVL, LRP was decreased too.

Table 6 The result of genes related to various degradation and apoptosis pathway compared between 10 μM XAV939 and control group (non-treated).

Gene Level Differentiation Expression Analysis of 10 XAV939 & Control			
Genes Symbol	Genes	Fold Change	Annotation
NPHP3-AS1, UBC	ubiquitin C	1.35, 1.47	Ubiquitinal-Proteasome
ZBED8	zinc finger, BED type 8	1.45	
BIRC5	baculoviral IAP repeat 5	1.28	Apoptosis pathway
CASP3, 7, 9	caspase	1.22, 1.11, 1.09	Apoptosis/Necrosis
MAPK12, 14, 7, 4	mitogen-activated protein kinase 12	1.29, 1.07, 1.02, 1.04	Stress Activation
PSMC2, 3, 4, 5, 6	proteasome 26S subunit, ATPase	1.19, 1.05, 1.04, 1.04, 1.58	Proteasome Degradation
TP53TG3; 3C; 3B	TP53 target 3; 3C; 3B	1.17	DNA-damage and DOX-apoptosis.
FADD	Fas (TNFRSF6)-associated via death domain	1.14	
TP63	p53 related gene	1.14	
UBE2D1	ubiquitin-conjugating enzyme	1.07	
NFKB1	NF-kappa-B	1.07	
JUN	jun proto-oncogene	1.03	Apoptosis pathway
FZD1, 2, 8, 10	Frizzled receptor	-1.08, -1.26, -1.06, -1.38	
LRP 5, LRP6	LDL receptor related protein	-1.10, -1.06	
DVL1, 2, 3	dishevelled segment polarity	-1.09, -1.11, -1.11	
APC	adenomatous polyposis coli	-1.20	
MDM2	MDM2 proto-oncogene, E3 ubiquitin protein ligase	-1.46	
HLA-A	major histocompatibility class I, A	-1.38	
DDC	Dopamine gene	-1.23	ROS oxidation to reduce stress and cell death
XXbac; TAP2	novel protein, TAP2-HLA	-1.23	Resistance gene

A



B

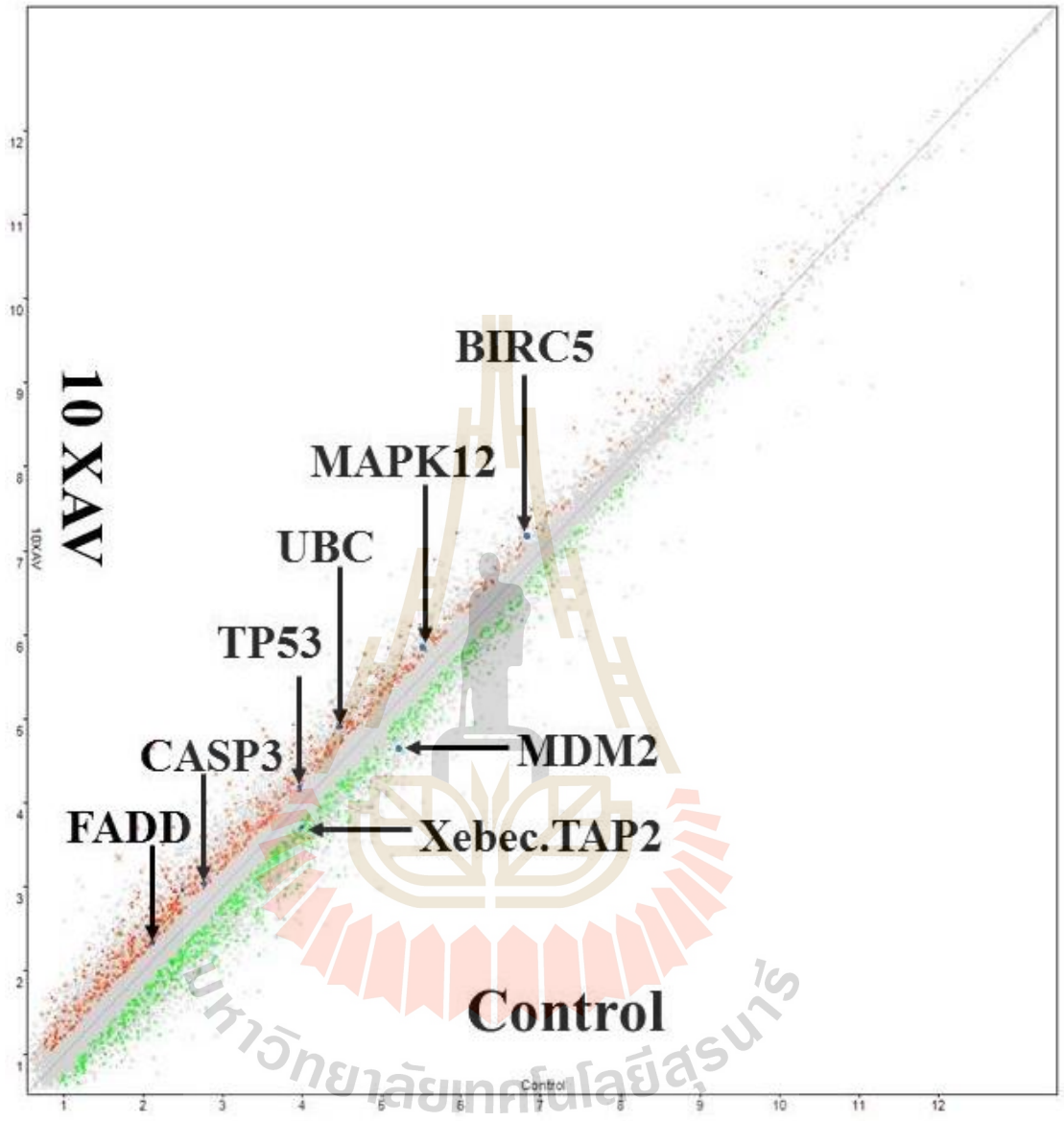


Figure 20 Volcano plot of 1 µM XAV939 (A) and 10 µM XAV939 (B) treated cells compared to control group (non-treated) by Gene Level differential Expression Analysis HuGene-2_0-st (**p<0.05).

Moreover, pathways summary in the 10 µM XAV939 experimented groups versus control group were shown as Figure 21-22. Some genes, such as Wnt related genes and apoptosis genes could have important biological effects even though their changes in expression are less than two-fold. The fold changes of each gene measured in the treated and control SH-SY5Y cells were calculated by measured intensities and referred as a ratio.

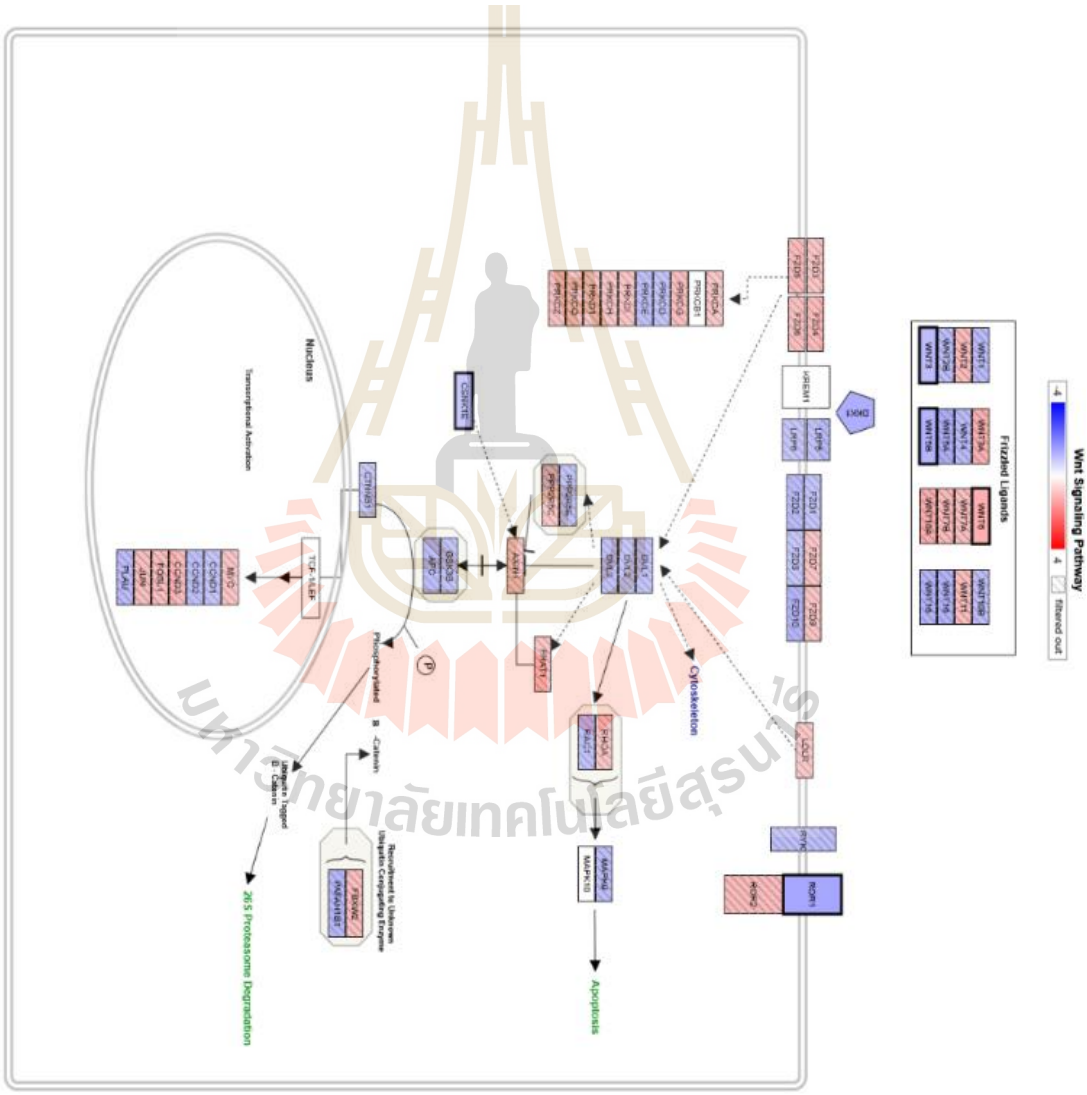


Figure 21 Wnt Pathway summary in 10 µM XAV939 compared to control derived from Gene Level differential Expression Analysis by HuGene-2_0-st (**p<0.05).

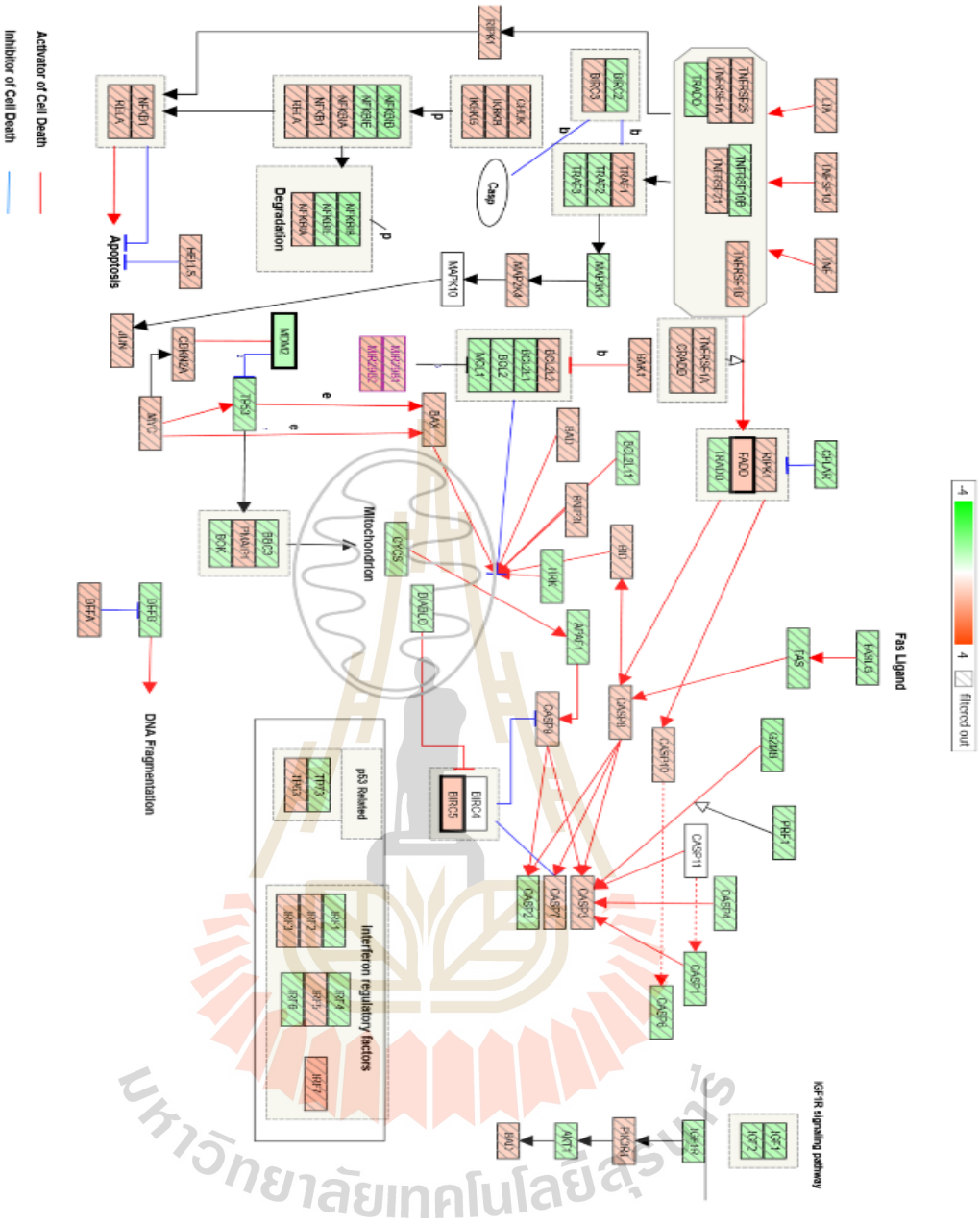


Figure 22 Apoptosis pathway of 10 µM XAV939 compared to control derived from Gene Level differential Expression Analysis by HuGene-2_0-st (**p<0.05).

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

WNT signalling pathway has been indicated as therapeutic targets in cancer because WNT and their downstream effectors regulate various processes that are important for cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis (Anastas and Moon, 2013). In addition, it has been reported that Wnt Signalling pathways play a key role in the survival, proliferation, and differentiation of normal stem cells and somatic precursor cells (Reya et al., 2001). Wnt/β-catenin Signalling could promote neural stem cell (NSC) proliferation (Hirsch et al., 2007) in order to prevent neuronal differentiation (Muroyama et al., 2004).

Huang et al., (2009) indicated that XAV939 could affect the mechanism of Wnt pathway by inhibition the regulation of Axin protein and present a new target Wnt pathway for cancer therapies (Huang et al., 2009). XAV939, Tankyrase inhibitor, is an antagonist of Wnt Signalling via stimulates β-catenin degradation by stabilizing Axin and can form a destruction complex. Thus, our results shown that the expression of β-catenin translocated in NB nucleus was highly decreased (Figure 8) in 10 μM XAV939 treated cell. β-catenin does not influence cell proliferation directly but plays the significant role on NB stages and the alteration of the cell. Cell proliferation was shown in Figure 9-12 XAV939 treated SH-SY5Y cells does not alter cell morphology,

significantly promote and/or neither inhibited cell proliferation upon cell viability and newly DNA stranded synthesize. These results contrast to Hirsch et al., (2007) and Muroyama et al., (2004) they reported that Wnt/β-catenin Signalling could promote neural stem cell proliferation.

According to (Zhi et al., 2012) they found that Id genes may mediate the function of Wnt/β-catenin Signalling in neuroblastoma initiation, C-Myc and CyclinD1 plays crucial role in neuroblastoma proliferation; however, our RT-PCR results show that the expression of C-MYC was not significantly different in treated and non-treated cell. This result was related to cell a proliferation result which is XAV939 does not affected cell proliferation. Moreover, other direct target genes of Wnt/β-catenin pathway such as Tuj1, PHOX, and TFAP2a were examined.

Genes expression study was comparable between RT-PCR and global gene expression by microarray, those genes are altered upon Wnt Signalling inhibition. Interestingly, Tuj1 and TFAP2a, markers of mature neurons and neural progenitors, respectively were altered in treatment cells. Tuj1 protein was highly expressed in the control SH-SY5Y cells while TFAP2a expressing in treated cells (Figure 14). Importantly, the immunofluorescence results showed the clear reduction of Tuj1 protein in 10 μM XAV939 treatment (Figure 14b) and increased the expression of TFAP2a protein (Figure 14d). These results indicated that inhibition of Wnt Signalling by XAV939 reduced the expression of mature neuronal proteins and primed the expression of neural progenitor markers of human NB cells.

Neuroblastoma recurrence or known as relapse after treatment depends on many factors, including the initial risk group, where the cancer recurs, and what treatments have been used (Oncology, 2015). Intensive treatment with small molecule which is inhibiting cell signalling pathway, high-dose chemotherapy/radiation therapy followed

by a stem cell transplant might be another option. Flahaut M et al., 2009 reported that to elucidate genes and pathways involved in chemo-resistance in neuroblastoma cells including neuroblastoma cell lines exhibited over expression of some gene which are mediate chemo-resistance in doxorubicin (DoxR) and patients, through Wnt/β-catenin pathway activation such as FZD1 gene (Flahaut et al., 2009). Pathway activity showed that DoxR treatment significantly suppressed the wnt pathway activity in cells. Expression of β-catenin and GSK3β (S-9) were significantly increased in DoxR treated and untreated (Vangipuram et al., 2012). In our study, the sensitivity of human neuroblastoma cells to doxorubicin was increased in XAV939 treated cells, compared to the control (Figure 15). Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis (Tacar et al., 2013). It can also induce histone eviction from transcriptionally active chromatin, leading to DNA damage response, epigenome and transcriptome are deregulated in doxorubicin-exposed cells (Pang et al., 2013).

XAV939 promote cell apoptosis and reduce cell invasion, accompanied by telomere shortening and unaltered telomerase activity in SH-SY5Y cells (Tian et al., 2014b). XAV939 treatment induced some signs of apoptosis appeared, including the condensation and bright stained of nuclear chromatin. The results indicated that XAV939 treatment and RNAi-TNKS1 reduced the expression of stemness of human neuroblastoma cells (Tian et al., 2013). The XAV939 could induce apoptosis of NB cells partly by inhibiting Wnt/β-catenin signaling through TNKS1. However, it has not been reported whether XAV939 also has effect on stemness of NB CSCs, and the involved mechanism that would contribute to targeted therapy (Tian et al., 2014b). Noteworthy, our study represented that the specific activity of XAV939 highly decrease human neuroblastoma survival rate in co treated with doxorubicin or neither

doxorubicin (p<0.05). These results indicated that the growth inhibitory effects of XAV939 on SH-SY5Y cells are due to TNKS1-dependent inhibition. According to Kim et al., (2007) found that the increased expression of Wnt inhibitory factor promoted the apoptosis of tumor cells and significantly inhibited tumor growth (Kim et al., 2007). XAV939 inhibit the stemness of human neuroblastoma by attenuating Wnt/β-catenin signalling via repression of TNKS1. The treatment of human neuroblastoma with XAV939 was correlated with increased apoptosis, increased chemosensitivity and reduced invasiveness in human neuroblastoma cells (Tian et al., 2014b). Therefore, our study substantiated that the modulation of Wnt inhibitory molecule could lead to improve cancer therapeutics treatment and their recurrence issue.

5.2 CONCLUSION

1. The inhibition of Wnt/β-catenin by Tankyrase inhibitor (XAV939) affected the accumulation of β-catenin in SH-SY5Y cells. β-catenin was highly decrease in XAV939 treated cells compared to control cells from 99.21% to 48.00% in control and treated cells, respectively.

2. The inhibition of Wnt/β-catenin might not alter either cell morphology, cell proliferation or cell survival. Percentage of cell proliferation in XAV939 treated cells and control are 98.00 and 97.40, respectively.

3. The treatment of XAV939 resulted in differentiated SH-SY5Y stages and affect gene expression by RT-PCR and neural marker staining.

4. The combination of 10 μM XAV939 significantly enhanced anticancer activity of doxorubicin against SH-SY5Y cells.

5. Results of Microarray could explain sensitivity enhancement to Doxorubicin of SH-SY5Y cells, target genes of XAV939, for instance p21, p53, Ubiquitin C, CASP3 which are apoptosis, cell death related genes were increase.

6. Human malignant neuroblastoma cells could be sensitized to anticancer drug by alteration key cellular Signalling pathways, for instance Wnt Signalling, and this approach serves as an effective option for aggressive malignancy treatment.

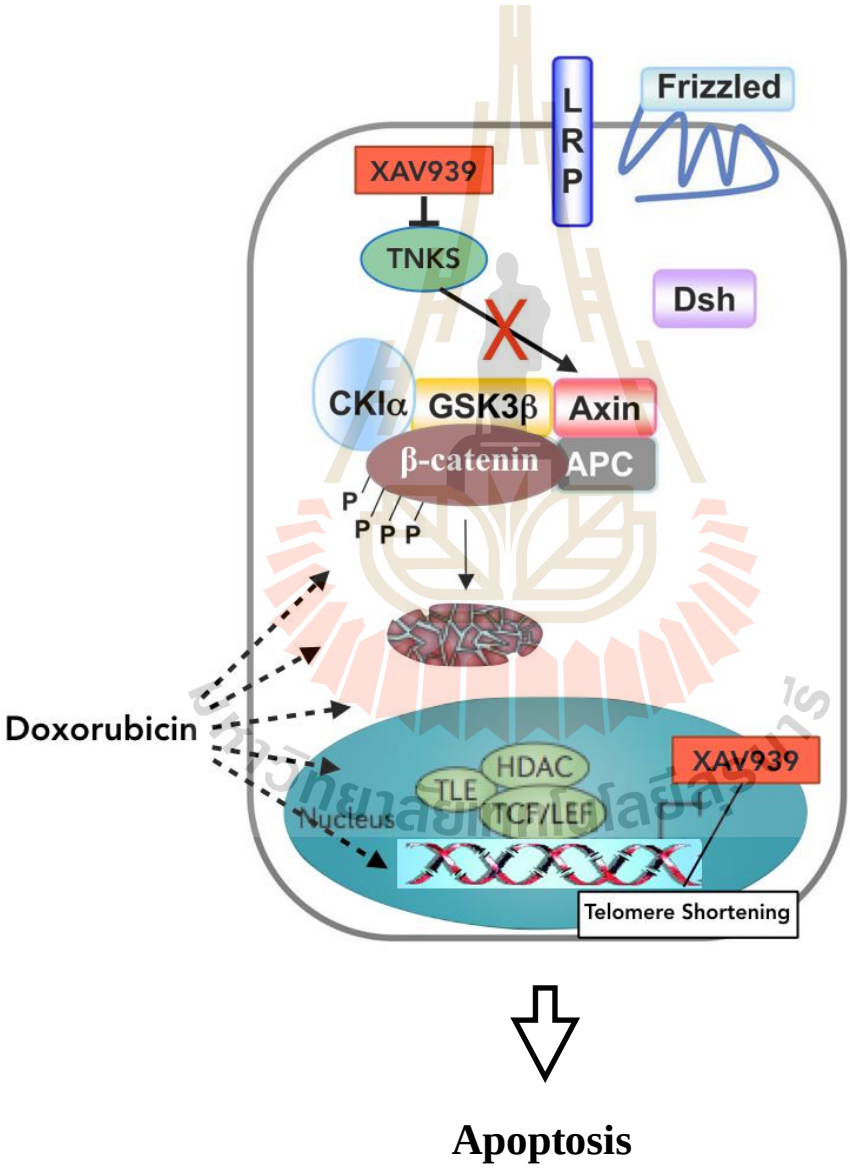


Figure 23 Synergistic action of Doxorubicin and XAV939 via Wnt/β-catenin signalling

5.3 Suggestion further work

The understanding of cellular mechanisms occur in XAV939 treated neuroblastoma cells should be conducted to clarify the exact mechanisms. These could improve cancer resistance therapy and lead to a novel approach for life-threatening disease by targeting the key genes conferring stemness and drug resistance to human neuroblastoma.

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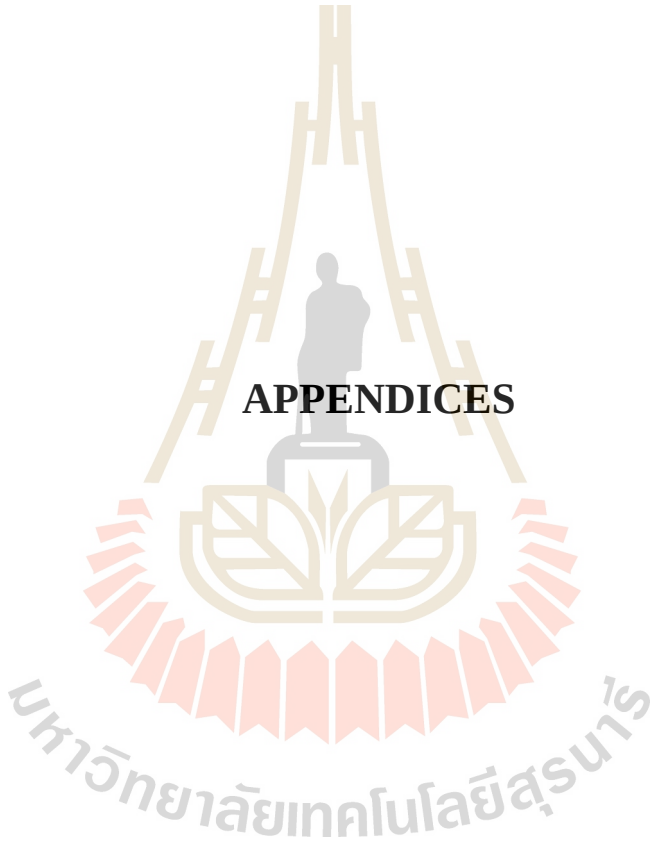
APPENDICES



APPENDIX A

ADDITIONAL MATERIALS

A1. SH-SY5Y (ATCC® CRL-2266™)

Organism	Homo sapiens, human
Tissue	bone marrow
Morphology	epithelial
Culture Properties	mixed, adherent and suspension
Biosafety Level	1
Disease	neuroblastoma
Age	4 years
Gender	female

Applications

This cell line is a suitable transfection host.

A2. Additional antibody and information

Antigen Name	Type	Company	Cat #	Dilution
PAX6		DSHB		1:500
PAX7		DSHB		1:500
PAX3		DSHB		1:500
AP-2 alpha		DSHB		1:200
BrdU		DSHB		1:500
Nestin		DSHB		1:200
Neurogenin2	Rabbit	Santacruz	sc50402	1:200
beta-catenin	goat poly	Santacruz	sc1496	1:250
Oct-04		Santacruz	sc8628	1:500
Peripherin		Santacruz	sc7604	1:500
NGN3	rabbit	Santacruz	sc25654	1:500
Brn3a		Santacruz	sc8429	1:500

A3. Additional markers

Gene	Forward	Tm	Reverse	Tm	Ta	cycles	Size (bp)
beta-Actin	TCACCACCACGGCCGAGCG	67	TCTCCTTCTGCATCCTGTCG	59		25	351
Tuj1	GCTCAGGGGCCTTTGGACATCTCTT	66	TTTTCACACTCCTTCCGCACCACATC	65	63	25	148
Brn3A	TGCCTATACTCTGGTGGCCT	60	TGTTTTCGCCCAACATGCAG	59		35	543
ChAT	ACTGGGTGTCTGAGTACTGG	58	TTGGAAGCCATTTTGACTAT	52		35	451
C-MYC	TACCCTCTCAACGACAGCAG	59	TCTTGACATTCTCCTCGGTG	56		30	478
Cyclin A	TCCATGTCAGTGCTGAGAGG		GAAGGTCCATGAGACAAGGC		56	25	451
Cyclin D2	TGCAGAAGGACATCCAACC		AGGAACATGCAGACAGCACC		58	25	184
MAP2	GGTGCCTTTGGAGAGCATGG	61	GGAACTCCATCTTCGAGGCT	59		-	565
MASH1	TCGCACAACCTGCATCTTTA	57	CTTTTGCACACAAGCTGCAT	58		30	279
Nestin	CAGCTGGCGCACCTCAAGATG	64	AGGGAAGTTGGGCTCAGGACTGG	65		30	209
NeuroD1	AGCCCTCTGACTGATTGCAC	60	GTCTATGGGGATCTCGCAGC	60		30	219
N-MYC	CTTCGGTCCAGCTTTCTCAC	58	GTCCGAGCGTGTTCAATTTT	57		30	200
p21	GAGGCCGGGATGAGTTGGGAGGAG	68	CAGCCGGCGTTTGGAGTGGTAGAA	67		25	221
p53	CCCCTCCTGGCCCCTGTCATCTTC	68	GCAGCGCCTCACAACCTCCGTCAT	69		30	265
PAX3	AGCCGCATCCTGAGAAGTAA	58	CTTCATCTGATTGGGGTGCT	57		30	148

A3. continues

Gene	Forward	Tm	Reverse	Tm	Ta	cycles	Size (bp)
PAX6	AACAGACACAGCCCTCACAAACA	62	CGGGAACTTGAACTGGAACTGAC	61		30	275
Peripherin	AGAGTCTAACGTGCGAGGTG	59.4	TAGGGTTTGGGCTTTGAGCA	59.5		30	514
PHOX2A	CTTGGCCTTCTTTGGATGCG	59	AGCCCCTCCACTCCTCTAAC	60		30	293
PHOX2B	CGAGCAAGGAAAAGGCACAC	60	ACGGTCACGTAGAGGAGACA	59		30	206
SLUG	AACAGTATGTGCCTTGGGGG	59	AAAAGGCACTTGGAAGGGGT	59		30	221
SOX1	CAATGCGGGGAGGAGAAGTC	60	CTCTGGACCAAACTGTGGCG	61		30	464
SOX10	TCCAGGCCCACTACAAGAGC	61	CAATGTCCACGTTGCCGAAG	60		35	228
SOX2	CCCCCGGCGGCAATAGCA	65	TCGGCGCCGGGGAGATACAT	65		35	448
TFAP2a	TCAAGTACGAGGACTGCGAG	59	CCTCGATGGCGTGAGGTAAG	60		30	447
TH	GTACTTCGTGCGCCTCGAGGTG	65	GCGTGGACAGCTTCTCAATTTC	60		35	719
VAChT	GACGTGAAGATCGGGGTGCTGTT	65	CGAGGAAGGCGAGGGGAATGTTAC	65		35	565

APPENDIX B

STEP-BY-STEP METHODS

B1. MTT ASSAY for cell proliferation

1. To set a standard curve, seed cells at different cell number in 100 ul medium overnight.
2. Treated cells with experimental conditions and 25 ul MTT (5 mg/ml) was added to each well and the cell incubated for 2 hours at 37 C.
3. 100 ul lysis buffer, a 10% SDS solution, was added to each well, and the plates incubated overnight, 37 C.
4. MTT conversion in each well was determined the absorbance/ optical density (OD) at 562 nm.

$$\% \text{ Cell Proliferation/Survival} = \frac{(\text{OD of treated cells} - \text{OD of lysis control}) * 100}{(\text{OD of healthy cells} - \text{OD of lysis control})}$$

N.B. Lysis control = Cells were plated at the same densities as the experiment wells, but were lysed and incubated overnight, prior to the addition of MTT.

B2. BrdU Labeling & Immunocytochemistry Protocol

1. Plate cells in 24 well plate, and grow cells overnight.
2. BrdU* is added to each well to a final concentration of 10uM and incubated at 37 C for 2 hours.
3. Medium is aspirated from the wells and washed 3X in PBS.
4. Fix for 10 min in 3.5-4% paraformaldehyde (freshly made).
5. Aspirate PFAP and wash with PBS 3 times store at 4ºC.
6. **DNA Denaturation**: 0.2ml of 2M HCL in water is added to each well and

incubated at 37 C for 1 hour.

7. Rinse 6X by PBS. (5 min each)
8. Incubate in PBS buffer containing 10% goat serum, 0.5% Triton x-100 for 1 hour (0.5ml/well for 4-well chamber slide).
9. Aspirate solution and add 200 ul of anti BrdU monoclonal antibody (DSHB; 1:1000 in in PBS containing 1% serum) for 1 hr at RT.
10. Wash with PBS 3 x 10'.
11. Incubate with secondary antibody diluted in PBS for 30 min in dark.
12. Repeat same washes as step 6 in dark.
13. Mount slides and stored in fridge.

B3. SOP-PR/1: Immunocytochemistry on Fixed Cells

1. Grow cells in chamber slides or cover slips and wash cells twice with PBS.
2. Fix for 10 min in 3.5-4% paraformaldehyde (freshly made).
3. Wash with PBS 3 times (store at 4ºC for later ICC).
4. Incubate in PBS buffer containing 10% appropriate serum (FBS), 0.1% Triton x-100 for 1 hour at RT (0.5ml/well for 4-well chamber slide).
5. Incubate with primary antibody at appropriate dilution in PBS containing 1% serum (FBS) for 1 hour at RT or O/N at 4ºC (200ml/well for 4-well chamber slide).
6. Wash with PBS 3 times.
7. Incubate with secondary antibody diluted in PBS for 30 min in dark.
8. Repeat same washes as step 6 in dark.
9. Mount slides with DAPI.

B4. Microarray experiments: GeneChip® WT PLUS Reagent Kit

Additional Reagent Required

Absolute Ethanol, Molecular Biology Grade

Input RNA Quantity

IMPORTANT: The RNA volume must be ≤ 5 µL (≤ 3 µL if poly-A RNA controls are used).

Table A: Total Input RNA Limits

RNA Input	Total RNA
Recommended	100 ng
Minimum	50 ng
Maximum	500 ng

Table B: Thermal Cycler Programs

Program	Heated Lid Temp	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C or 105°C	25°C for 60 min	42°C for 60 min	4°C for 2 min		10 µL
Second-Strand cDNA Synthesis	RT or disable	16°C for 60 min	65°C for 10 min	4°C for 2 min		30 µL
In Vitro Transcription cRNA Synthesis	40°C or 50°C	40°C for 16 hr	4°C hold			60 µL
2nd-Cycle Primers-cRNA Annealing	70°C or 105°C	70°C for 5 min	25°C for 5 min	4°C for 2 min		28 µL
2nd-Cycle ss-cDNA Synthesis	70°C or 105°C	25°C for 10 min	42°C for 90 min	70°C for 10 min	4°C hold	40 µL
RNA Hydrolysis	70°C or 105°C	37°C for 45 min	95°C for 5 min	4°C hold		44 µL
Fragmentation	93°C or 105°C	37°C for 60 min	93°C for 2 min	4°C hold		48 µL
Labeling	70°C or 105°C	37°C for 60 min	70°C for 10 min	4°C hold		60 µL
Hybridization Control	65°C or 105°C	65°C, 5 min				Variable
Hybridization Cocktail	99°C or 105°C	95°C or 99°C, 5 min	45°C, 5 min			Variable

Section 1: Prepare RNA with Poly-A RNA Controls

Table 1: Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th Dilution to Add to Total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Table 1 provides a guideline when 50, 100, 250 or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

TIP: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

Table 2: Total RNA/Poly-A RNA Control Mixture

Component	Volume for One Reaction (µL)
Total RNA Sample (50-500 ng)	variable
Diluted Poly-A RNA Controls (Fourth Dilution)	2
Nuclease-free Water	variable
Total Volume	**5**

TIPS for Reagent Preparation

- Mix Enzymes by gently vortexing and mix Buffers by thoroughly vortexing to dissolve precipitates.
- Include ~5% overage to correct for pipetting losses when preparing Master Mixes.
- Master Mixes and samples should be mixed thoroughly by gently vortexing followed by a quick spin to collect contents of tube or well.
- Immediately after incubation, spin the sample tubes briefl y, then place on ice before proceeding to the next step.
- Enzyme should be added last and just before adding the Master Mix to the reaction.

Section 2: Synthesize First-Strand cDNA

1. On ice, prepare First-Strand Master Mix.

Table 3: First-Strand Master Mix

Component	Volume for One Reaction (µL)
First-Strand Buffer	4
First-Strand Enzyme	1
Total Volume	**5**

2. On ice, transfer 5 µL of the First-Strand Master Mix to individual tube or well. Add 5 µL of the Total RNA/Poly-A Control Mixture (Table 2).

3. Incubate for 60 min at 25°C, then for 60 min at 42°C in a thermal cycler using the "First-Strand cDNA Synthesis" program (Table B).

4. Proceed immediately to Second-Strand cDNA Synthesis.

Section 3: Synthesize Second-Strand cDNA

1. On ice, prepare Second-Strand Master Mix.

NOTE: Pre-cool the thermal cycler block to 16°C while you are preparing the Second-Strand Master Mix.

Table 4: Second-Strand Master Mix

Component	Volume for One Reaction (µL)
Second-Strand Buffer	18
Second-Strand Enzyme	2
Total Volume	**20**

2. On ice, transfer 20 µL of the Second-Strand Master Mix to each (10 µL) fi rst-strand cDNA sample.

3. Incubate for 60 min at 16°C, then for 10 min at 65°C in a thermal cycler using the "Second-Strand cDNA Synthesis" program (Table B).

NOTE: Disable the heated lid of the thermal cycler or keep the lid off during the Second-Strand cDNA Synthesis.

4. Proceed immediately to In Vitro Transcription.

Section 4: Synthesize cRNA by In Vitro Transcription

1. Transfer the second-strand cDNA sample to room temperature while preparing IVT Master Mix.

2. At room temperature, prepare the IVT Master Mix.

NOTE: Transfer the IVT Buffer to room temperature for ≥ 10 min before preparing the IVT Master Mix.

Table 5: IVT Master Mix

Component	Volume for One Reaction (µL)
IVT Buffer	24
IVT Enzyme	6
Total Volume	**30**

3. At room temperature, transfer 30 µL of the IVT Master Mix to each (30 µL) second-strand cDNA sample.

4. Incubate the IVT reaction for 16 hr at 40°C in a thermal cycler using the "In Vitro Transcription cRNA Synthesis" program (Table B).

5. Proceed to cRNA Purification, or freeze immediately.

TIP: STOPPING POINT. The cRNA sample can be stored at –20°C.

Section 5: Purify cRNA

Before beginning the cRNA purification:

- Preheat the Nuclease-free Water to 65°C for at least 10 min.
- Mix the Purification Beads thoroughly before use. Aliquot the appropriate amount, and keep at room temperature. For each reaction, 100 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage will be needed.

NOTE: This entire procedure is performed at room temperature.

1. Bind cRNA to Purification Beads.

A. Mix the Purification Beads container to re-suspend the magnetic particles. Add 100 µL of the magnetic beads to each (60 µL) cRNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.

B. Mix well by pipetting up and down 10 times. Incubate for 10 min.

C. Move the plate to a magnetic stand for ~5 min to capture the magnetic beads.

D. Carefully aspirate and discard the supernatant without disturbing the magnetic beads. Keep the plate on the magnetic stand.

2. Wash the Purification Beads.

A. While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well andincubate for 30 sec.

B. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the magnetic beads.

C. Repeat Step A and Step B twice for a total of 3 washes. Completely remove the final wash solution.

D. Air-dry on the magnetic stand for 5 min until no liquid is visible. Additional time may be

required. Do not over-dry the beads.

3. Elute cRNA.

A. Remove the plate from the magnetic stand. Add 27 µL of the preheated (65°C) Nuclease-

free Water to each sample and incubate for 1 min.

B. Mix well by pipetting up and down 10 times.

C. Move the plate to the magnetic stand for ~5 min to capture the magnetic beads.

D. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.

E. Place the purifi ed cRNA on ice, and proceed to quantitation.

TIP: STOPPING POINT. The purifi ed cRNA sample can be stored at –20°C.

Section 6: Assess cRNA Yield

Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm using a NanoDrop® Spectrophotometer or equivalent quantitation instrument.

Section 7: Synthesize 2nd-Cycle ss-cDNA

1. On ice, prepare 15 µg of cRNA in a volume of 24 µL with Nuclease-free Water.

2. Add 4 µL of 2nd-Cycle Primers to each (24 µL) cRNA sample.

3. Incubate for 5 min at 70°C, 5 min at 25°C, then for 2 min at 4°C in a thermal cycler using the "2nd-Cycle Primers-cRNA Annealing" program (Table B).

4. Place the cRNA/2nd-Cycle Primers sample on ice.

5. On ice, prepare 2nd-Cycle ss-cDNA Master Mix.

Table 6: 2nd-Cycle ss-cDNA Master Mix

Component	Volume for One Reaction (µL)
2nd-Cycle ss-cDNA Buffer	8
2nd-Cycle ss-cDNA Enzyme	4
Total Volume	**12**

6. Transfer 12 µL of the 2nd-Cycle ss-cDNA Master Mix to each (28 µL) cRNA/2nd-Cycle Primers sample.

7. Incubate for 10 min at 25°C, 90 min at 42°C, then for 10 min at 70°C in a thermal cycler using the "2nd-Cycle ss-cDNA Synthesis" program (Table B).

8. Proceed immediately to RNA Hydrolysis.

Section 8: Hydrolyze RNA using RNase H

1. On ice, add 4 µL of RNase H to each (40 µL) 2nd-Cycle ss-cDNA sample.

2. Mix thoroughly, followed by a quick spin.

3. Incubate for 45 min at 37°C in a thermal cycler using the "RNA Hydrolysis" program (Table B).

4. Place the hydrolyzed 2nd-Cycle ss-cDNA sample on ice.

5. On ice, add 11 µL of Nuclease-free Water to each (44 µL) hydrolyzed 2nd-Cycle ss-cDNA sample.

6. Mix thoroughly and proceed to ss-cDNA Purifi cation, or freeze immediately.

TIP: STOPPING POINT. The hydrolyzed ss-cDNA samples can be stored at –20°C.

Section 9: Purify 2nd-Cycle ss-cDNA

Before beginning the ss-cDNA purification:

- Preheat the Nuclease-free Water to 65°C for at least 10 min.
- Mix the Purifi cation Beads thoroughly before use. Aliquot the appropriate amount, and keep at room temperature. For each reaction, 100 µl plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage will be needed.

NOTE: This entire procedure is performed at room temperature.

1. Bind ss-cDNA to Purifi cation Beads.

A. Mix the Purifi cation Beads container to resuspend the magnetic particles. Add 100 µL of the magnetic beads to each (55 µL) hydrolyzed ss-cDNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.

B. Add 150 µL of 100% ethanol to each sample. Mix well by pipetting up and down 10 times. Incubate for 20 min.

C. Move the plate to a magnetic stand for ~5 min to capture the magnetic beads.

D. Carefully aspirate and discard the supernatant without disturbing the magnetic beads. Keep the plate on the magnetic stand.

2. Wash the Purifi cation Beads.

A. While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 sec.

B. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the magnetic beads.

C. Repeat Step A and Step B twice for a total of 3 washes. Completely remove the fi nal wash solution.

D. Air-dry on the magnetic stand for 5 min until no liquid is visible. Additional time may be required. Do not over-dry the beads.

3. Elute ss-cDNA.

A. Remove the plate from the magnetic stand. Add 30 µL of the preheated (65°C) Nuclease-free Water to each sample and incubate for 1 min.

B. Mix well by pipetting up and down 10 times.

C. Move the plate to the magnetic stand for ~5 min to capture the magnetic beads.

D. Transfer the supernatant, which contains the eluted ss-cDNA, to a nuclease-free tube.

E. Place the purifi ed ss-cDNA on ice, and proceed to quantitation.

TIP: STOPPING POINT. The purifi ed cDNA sample can be stored at –20°C.

Section 10: Assess ss-cDNA Yield

Determine the concentration of a ss-cDNA solution by measuring its absorbance at 260 nm using a NanoDrop® Spectrophotometer or equivalent quantitation instrument.

Section 11: Fragment and Label ss-cDNA

1. On ice, prepare 5.5 µg of ss-cDNA in a volume of 31.2 µL with Nuclease-free Water.

2. On ice, prepare Fragmentation and Labeling Master Mix.

Table 7: Fragmentation Master Mix

Component	Volume for one reaction (µL)
Nuclease-free Water	10
10X cDNA Fragmentation Buffer	4.8
UDG, 10 U/µL	1
APE 1, 1,000 U/µL	1
Total Volume	**16.8**

3. Transfer 16.8 µL of the Fragmentation Master Mix to each (31.2 µL) 5.5 µg ss-cDNA sample.

4. Incubate for 60 min at 37°C, then for 2 min at 93°C in a thermal cycler using the "Fragmentation" program (Table B).

5. On ice, transfer 45 µL of the fragmented ss-cDNA sample to individual tube or well.

6. On ice, prepare Labeling Master Mix.

Table 8: Labeling Master Mix

Component	Volume for one reaction (µL)
5X TdT Buffer	12
DNA Labeling Reagent, 5 mM	1
TdT, 30 U/µL	2
Total Volume	**15**

7. Transfer 15 µL of the Labeling Master Mix to each (45 µL) fragmented ss-cDNA sample.

8. Incubate for 60 min at 37°C, then for 10 min at 70°C in a thermal cycler using the "Labeling" program (Table B).

9. Proceed immediately to WT Cartridge Array Hybridization.

Section 12: WT Cartridge Array Hybridization

Please refer to Affymetrix® WT PLUS Reagent Kit User Manual for Array Strip and Array Plate Hybridization.

NOTE:

- Ensure that the reagent is completely thawed before use. Store DMSO at room temperature after the first use. DMSO will solidify when stored at 2-8°C.
- This procedure requires the use of the GeneChip® Hybridization, Wash and Stain Kit (not supplied).

1. Heat the 20X Hybridization Controls for 5 min at 65°C in a thermal cycler using the "Hybridization Control" program (Table B).

2. At room temperature, prepare Hybridization Master Mix.

Table 9: Hybridization Master Mix for a Single Reaction

Component	49 or 64-Format	100 or 81/4-Format	169-Format	Final Concentration
Fragmented and Labeled ss-cDNA	**5.2 µg**	**3.5 µg**	**2.3 µg**	**23 ng/µL**
Control Oligonucleotide B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	11 µL	7.5 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X
DMSO	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	19.9 µL	13.5 µL	9.3 µL	
Total Volume	**160 µL**	**109 µL**	**73 µL**	

Please refer to specific probe array package insert for information on array format.

3. Add the Hybridization Master Mix to individual tube containing the biotin-labeled ss-cDNA sample to prepare Hybridization Cocktail.

Table 10: Hybridization Cocktail for a Single Array

Component	49 or 64-Format	100 or 81/4-Format	169-Format
Hybridization Master Mix	160 µL	109 µL	73 µL
Fragmented and Labeled ss-cDNA	~60 µL (5.2 µg)	41 µL (3.5 µg)	27 µL (2.3 µg)
Total Volume	**220 µL**	**150 µL**	**100 µL**

4. Incubate the Hybridization Cocktail for 5 min at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the "Hybridization Cocktail" program (Table B).

5. Inject the appropriate amount (Table 11) of the specifi c sample into the array.

Table 11: Probe Array Cartridge Volumes for Hybridization Cocktail

	49 or 64-Format	100 or 81/4-Format	169-Format
Volume to Load on Array	200 µL	130 µL	80 µL

6. Hybridize with rotation at 60 rpm for 16 hr at 45ºC.

Table 12: Fluidics Protocol

Component	49 or 64-Format	100 or 81/4-Format	169-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0007

BIOGRAPHY

Miss Junjira Suebsoonthron was born on March 26th, 1990 in Udonthani, Thailand. She graduated with the bachelor degree of Biotechnology, Mae Fah Luang University, Thailand in 2013. During her master degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2013-2016), she presented research work in the SUT 4th International Colloquium, September 3-6, 2015, Suranaree University of Technology, Nakhorn Ratchasima, Thailand (Oral presentation; in "Investigation of the effects of Wnt/ß-Catenin signalling modulation on the differentiation stage of human neuroblastoma cell line"; recipient certificated of high Scientific Impact). The 41st Congress on Science and Technology of Thailand (STT41), November 6-8, 2015, Suranaree University of Technology, Nakhonratchasima, Thailand (Poster presentation). The International Conference on Stem Cells: Development and Management, Nov. 30 - Dec. 2, 2015, Naresuan University, Thailand (Oral presentation; in "Inhibition of Wnt/B-catenin signalling alters the differentiation and sensitivity of human neuroblastoma SH-SY5Y cells to an anti-cancer drug"). The 4th SUT International Agricultural Colloquium, June 29, 2016, Suranaree University of Technology, Nakhonratchasima, Thailand (Poster presentation; recipient of most poster popular vote Award).