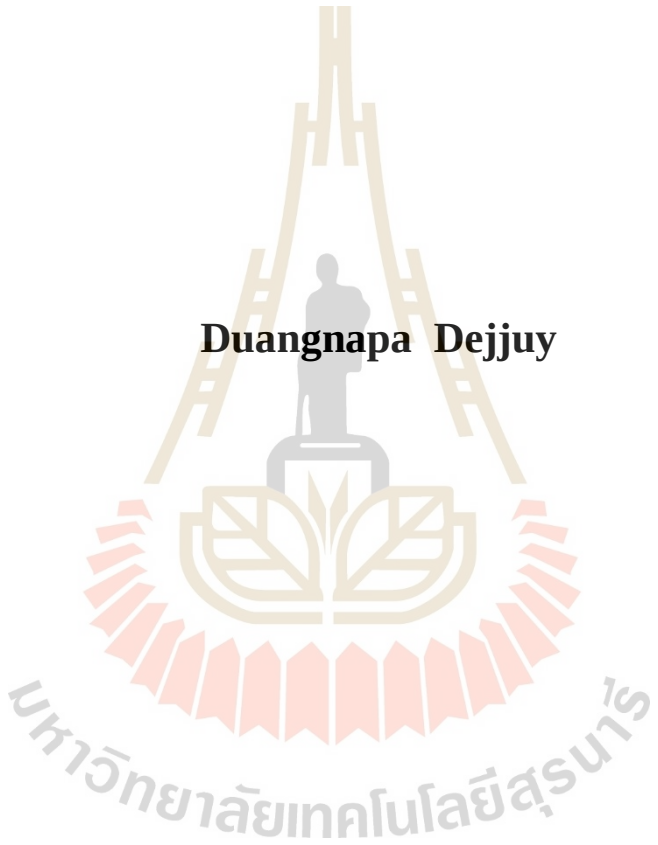
REGULATION OF WILMS' TUMOR (*WT1*) GENE EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIC CELL

Duangnapa Dejjuy



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การควบคุมการแสดงออกของยีน Wilms' tumor (*WT1*) ในเซลล์มะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์

นางดวงนภา เดชจุ้ย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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REGULATION OF WILMS' TUMOR (*WT1*) GENE EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIC CELL

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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ดวงนภา เดชจุ้ย : การควบคุมการแสดงออกของยีน Wilms' tumor (*WT1*) ในเซลล์มะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์ (REGULATION OF WILMS' TUMOR (*WT1*) GENE EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIC CELL)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ เทคนิคการแพทย์หญิง ดร.วิไลรัตน์ ลื้อนันต์ศักดิ์ศิริ
157 หน้า.

Wilms' tumor 1 (WT1) เป็น transcription factor ชนิดหนึ่ง มีบทบาทสำคัญต่อการควบคุมกลไกทางชีวภาพที่หลากหลาย การแสดงออกแบบ overexpression ของ WT1 พบได้ในเซลล์มะเร็งเม็ดเลือดขาวหลายชนิด เช่น Acute lymphoblastic leukemia (ALL) และ Chronic myeloid leukemia (CML) ในการวิจัยครั้งนี้ มีวัตถุประสงค์ในการควบคุมอัตราการเจริญเติบโต และการกระตุ้นให้เซลล์มะเร็งเม็ดเลือดขาวตายแบบ apoptosis ด้วยการใช้เทคโนโลยี RNA interference (RNAi) โดยทำการออกแบบ small interference RNA (siRNA) ที่จำเพาะต่อ WT1 mRNA ขึ้นมาใหม่ ซึ่งในการศึกษาวิจัยครั้งนี้เรียกว่า WT1-siRNA เพื่อใช้กับเซลล์มะเร็งเม็ดเลือดขาว โดย WT1-siRNA นี้ได้ถูกโคลนเข้าสู่เวคเตอร์ pPRIME-CMV-GFP-FF3 ที่มี Green fluorescent protein (GFP) เป็นยีนบ่งชี้ จากนั้นทำการผลิต Lentivirus ด้วยเทคนิคตกตะกอนด้วยแคลเซียม ลำดับต่อมา ทำการนำไวรัสที่ผลิตได้เข้าสู่เซลล์มะเร็งเม็ดเลือดขาว CML ชนิด K562 เซลล์มะเร็งที่ได้รับ WT1-siRNA และ C-siRNA จะถูกนำไปคัดเลือกเอาเฉพาะเซลล์ที่มีการแสดงออกของ GFP ด้วยเครื่อง flow cytometry ทำให้ได้ K562-WT1-siRNA-GFP$^+$ และ K562-C-siRNA-GFP$^+$ และนำไปทำการทดลองเพื่อหาอัตราการเจริญเติบโต การกระตุ้นการตายแบบ apoptosis และการเปลี่ยนแปลงของเซลล์ในระดับโมเลกุลต่อไป หลังจากทดสอบ พบว่า การแสดงออกของ WT1 mRNA ลดลงอย่างเห็นได้ชัดที่ 72 ชั่วโมง ในเซลล์ K562-WT1-siRNA-GFP$^+$ นอกจากนี้ ยังพบการลดการแสดงออกของไซโตไคน์ ที่เกี่ยวข้องกับการมีชีวิตรอดของเซลล์ ซึ่งได้แก่ Interleukin-2 (IL-2) และตัวรับของ IL-2 ซึ่งได้แก่ IL-2RB และ IL-2RG อีกด้วย ผลจากการลดการแสดงออกของ WT1 ทำให้เซลล์มะเร็งเม็ดเลือดขาว ถูกยับยั้งอัตราการเจริญเติบโตที่ระยะเวลาการทดสอบที่ 3 6 12 24 48 72 และ 96 ชั่วโมง ซึ่งมีการยับยั้งโดยประมาณ 10±0% 12±10% 16±7.5% 25±6.5% 40±7.0% 44±9.5% และ 88±9.1% ตามลำดับ ยิ่งไปกว่านั้นยังพบการกระตุ้นเซลล์ในระยะ early apoptosis เพิ่มขึ้นอีกประมาณ 70% เมื่อเวลาการทดสอบผ่านไปเพียง 12 ชั่วโมงเท่านั้น จากนั้นได้ทำการทดลองเพื่อยืนยันผลการกระตุ้นการเกิด apoptosis ด้วยการตรวจวัดหาค่ากิจกรรมของเอ็นไซม์ caspase-3/7 พบว่า มีการเพิ่ม activity ของเอ็นไซม์ caspase-3/7 จากประมาณ 507±32 Relative Fluorescent Unit (RFU) เป็น 1,487±425 RFU หรือประมาณสามเท่าในเวลาเพียง 48 ชั่วโมง อีกประการหนึ่ง จากการศึกษาพบว่า

การแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการ apoptosis อันได้แก่โปรตีน caspase-7 เพิ่มขึ้นภายใน 48 ชั่วโมง ยิ่งไปกว่านั้น จากการศึกษาผลของ WT1-siRNA ต่อการลดอัตราการเจริญเติบโตและการกระตุ้นการตายของเซลล์มะเร็งเม็ดเลือดขาวของผู้ป่วยเด็ก ที่ได้รับการวินิจฉัยว่าป่วยเป็นโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์ ALL subtype L1 (ALL-L1) พบว่า WT1-siRNA สามารถยับยั้งอัตราการเจริญเติบโตของเซลล์ L1-WT1-siRNA ได้ประมาณ 79±14% ที่ 48 ชั่วโมง เมื่อทดสอบการทำงานของ caspase-3/7 พบว่ามีค่าเพิ่มสูงขึ้นจาก 1,823±347 RFU เป็น 5,104±836 RFU และมีการกระตุ้นการแสดงออกของโปรตีน caspase-7 อย่างมีนัยสำคัญ ซึ่งสอดคล้องกับการตรวจพบว่าเซลล์ได้เข้าสู่ขั้น early apoptosis จำนวน 36.6±6.35% และขั้น late apoptosis จำนวน 33.25±9.8% เมื่อเปรียบเทียบกับ L1-C-siRNA การเพิ่มขึ้นของโปรตีน caspase-7 เป็นการยืนยันผลการตายของเซลล์มะเร็งเม็ดเลือดขาวของผู้ป่วยด้วย WT1-siRNA ขณะเดียวกันได้ทำการทดลองกับเลือดของอาสาสมัครสุขภาพดีเพื่อเป็นกลุ่มควบคุม พบว่า WT1-siRNA ไม่มีผลต่อการเปลี่ยนแปลงทางด้านการเจริญและการตายของเซลล์แต่อย่างใด WT1-siRNA สามารถลดระดับการแสดงออกของ WT1 IL-2 IL-2RB และ IL-2RG mRNA ได้ ซึ่งผลนี้สอดคล้องกับการศึกษาในระดับโปรตีน พบว่าโปรตีน WT1 ถูกลดการแสดงออกลงอย่างมีนัยสำคัญ จากผลการศึกษาครั้งนี้สามารถสรุปได้ว่า WT1-siRNA ที่ได้ทำการออกแบบขึ้นมาใหม่ สามารถนำมาใช้ในการยับยั้งการเจริญเติบโตของเซลล์ และกระตุ้น apoptosis ของเซลล์มะเร็งเม็ดเลือดขาวทั้งชนิด cell line และ L1 primary leukemic cells ได้อย่างมีประสิทธิภาพ ผลการศึกษานี้สามารถนำไปประยุกต์ใช้เพื่อการวิจัยในการรักษาโรคมะเร็งเม็ดเลือดขาว หรือการทดสอบการรักษาโรคมะเร็งในคลินิกต่อไป

สาขาวิชาจุลชีววิทยา
ปีการศึกษา 2556

ลายมือชื่อนักศึกษา _____
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DUANGNAPA DEJJUY : REGULATION OF WILMS' TUMOR (*WT1*) GENE EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIC CELL. THESIS ADVISOR : ASST. PROF. WILAIRAT LEEANANSAKSIRI, Ph.D. 157 PP.

Wilms' tumor 1 (WT1) is a zinc-finger transcription factor which plays crucial roles in various biological regulations. Overexpression of WT1 is observed in many types of hematopoietic malignancy such as acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML). This study aims to apply RNA interference technology for regulation of cell growth and apoptosis induction of leukemic cells. To this end, our new designed WT1-siRNA was cloned into pPRIME-CMV-GFP-FF3 plasmid vectors. Then, the lentiviral particles were produced by calcium precipitation method prior to transduction into CML cell line K562. The K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ control cells were then sorted by flow cytometry and cell sorter method. Both collected cell populations were subjected to cell proliferation and apoptosis determinations. The results showed significant downregulation of WT1 mRNA expression at 72 hours post-transduction. In addition, the expression of cellular survival cytokine including Interleukin-2 (IL-2) and its receptor subunits (IL-2RB and IL-2RG) were also reduced. Moreover, the proliferation rates of K562-WT1-siRNA-GFP$^+$ cells at 3, 6, 12, 24, 48, 72, and 96 hours post-transduction were inhibited for approximately 10±0%, 12±10%, 16±7.5%, 25±6.5%, 40±7.0%, 44±9.5%, and 88±9.1%, respectively. Interestingly, we found that WT1-siRNA can induce an early apoptosis for approximately 70% at 12 hours post-transduction. This result was confirmed by the study of caspase-3/7 enzymes activities. The activities of

caspase-3/7 were significantly increased from 507±32 Relative Fluorescent Units (RFU) to 1,487±425 RFU or approximately 3-fold within 48 hours, which was supported by caspase-7 protein expression using western blot analysis. Moreover, the study of the effects of WT1-siRNA on primary childhood acute lymphoblastic leukemic cells subtype L1 (ALL: L1) showed a significant inhibitory effect of WT1-siRNA on the L1-WT1-siRNA leukemic cells of about 79±14% after 48 hours post-transduction. The caspase-3/7 enzyme activities were significantly accelerated from 1,823±374 RFU to 5,104±836 RFU. Furthermore, WT1-siRNA also significantly upregulated caspase-7 protein expression and increased the amount of an early apoptosis population of L1-WT1-siRNA cells by 36.63±6.35% and late apoptosis by 33.25±9.8% when compared with L1-C-siRNA cells. These results also consisted by upregulation of caspase-7 protein expression by WT1-siRNA in the transduced cells. On the other hand, WT1-siRNA has no significant effect on proliferation and apoptosis of normal blood cells. The WT1-siRNA also downregulated WT1, IL-2, IL-2RB and IL-2RG mRNA expressions. Consistently, the level of WT1 protein expression was suppressed by WT1-siRNA. Altogether, these findings suggest that our new designed WT1-siRNA could effectively inhibit cellular growth and induce leukemic cell death by apoptosis in both leukemic cell lines and L1 primary leukemic cells. The applications of this work are future therapeutic value and clinical trials in leukemic treatment.

School of Microbiology

Academic Year 2013

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CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

AA	=	Amino acids
ABL	=	Abelson murine leukemia
ALL	=	Acute lymphoblastic leukemia
AML	=	Acute myeloid leukemia
ASR	=	Age standardize rate
ATCC	=	American type cell culture
BCL-2	=	B-cell lymphoma 2
BCR	=	Breakpoint cluster region
BF	=	Bright field
CD	=	Cluster of differentiation
CDK	=	Cyclin dependent kinase
CLL	=	Chronic lymphoblastic leukemia
CML	=	Chronic myeloid leukemia
C-siRNA	=	Control- small interfering ribonucleic acid
CSF-1	=	Colony-stimulating factor-1
CTE	=	Constitutive transport element
CTGF	=	Connective tissue growth factor
CTLs	=	Cytotoxic T lymphocytes
cDNA	=	Complementary deoxyribonucleic acid
DLS	=	Delivery with lipid solution

LIST OF ABBREVIATIONS (Continued)

DMSO	=	Dimethyl sulfoxide
dsRNA	=	Double stranded RNA
ECM	=	Extra cellular metrix
EGFR	=	Epidermal growth factor receptor
EGR	=	Early growth response
EPO	=	Erythropoietin
ERK	=	Extracellular signal-regulated kinase
FACs	=	Fluorescence-activated cell sorting
FF	=	Fluorescence field
FITC	=	Fluorescein isothiocyanate
GAPDH	=	Glyseraldehyde 3-phosphate dehydrogenase
G-CSF	=	Granulocyte colony-stimulating factor
GFP	=	Green fluorescence protein
GM-CSF	=	Granulocyte macrophage colony-stimulating factor
GTP	=	Guanosine triphosphate
hTERT	=	Human telomerase reverse transcriptase
IGF-1R	=	Insulin-like growth factor-1 receptor
IGF-II	=	Insulin-like growth factor II
IL-2	=	Interleukin-2
IL-2RA	=	Interleukin-2 receptor subunit alpha
IL-2RB	=	Interleukin-2 receptor subunit beta
IL-2RG	=	Interleukin-2 receptor subunit gamma

LIST OF ABBREVIATIONS (Continued)

JAK1	=	Janus kinase-1
JAK3	=	Janus kinase-3
JNK1	=	c-Jun N-terminal kinase
KTS	=	Leusine, threonine, and serine
LAA	=	Leukemia associated antigen
LFS	=	Luekemia free survival
MAP	=	Mitogen activated protein
MAPK	=	Mitogen activated protein kinase
MDS	=	Myelodysplastic syndrome
MEL	=	Murine erythroleukemia
miRNA	=	Micro RNA
mRNA	=	Messenger ribonucleic acid
NFATc	=	Nuclear factor of activated T cell
ODC	=	Ornithine decarboxylase
PAR-4	=	Prostate apoptosis response factor
PBMCs	=	Peripheral blood mononuclear cells
PCNA	=	Proliferating cell nuclear antigen
PDGF-A	=	Platelet-derived growth factor A
Ph	=	Philadelphia chromosome
PI	=	Propidium iodide
PI3K	=	Phosphoinositide-3 kinase
PLK	=	Pyridoxal kinase

LIST OF ABBREVIATIONS (Continued)

PRAME	=	Preferentially expressed antigen in melanoma
PS	=	Phosphatidyl serine
PVDF	=	Polyvinylidene difluoride
RAR-∝1	=	Retinoic acid receptor alpha 1
RFU	=	Relative fluorescent unit
RISC	=	RNA induced silencing complex
RNAi	=	RNA interference
RT-PCR	=	Reverse transcriptase polymerase chain reaction
SDS-PAGE	=	Sodium dodecylsulfate-polyacrylamine gel electrophoresis
SEAP	=	Secreted alkaline phosphatase
siRNA	=	Small interfering ribonucleic acid
STAT5	=	Signal transducer and activators of transcription
TGF-β1	=	Transforming growth factor-beta 1
TSP1	=	Thrombospondin 1
VEGF	=	Vascular endothelial growth factor
WT1	=	Wilms' tumor 1
ZF	=	Zinc-finger

CHAPTER I

INTRODUCTION

1.1 Introduction

Cancer is one of crucial health problems of world populations. The number of cancer patients is increased to 9 million and can maximum grow up for 16 million patients within 2020 ("Cancer Facts and Figures 2012," 2012; Weinberg, 2007). The most of world's population contacts the cancer risk factors such as radiation, atomic bomb, benzene, and electro-magnetic wave in their lifestyles. The genetic abnormalities have been induced to cancer formation such as Downs' syndrome patients have higher leukemic incident rate than the normal child. Leukemia is a disease characterized by uncontrolled proliferation and dissemination of abnormal white blood cells. Leukemia could be found in all ages depending on leukemia types; acute lymphocytic leukemia (ALL) is usually found in childhood, acute myelocytic leukemia (AML) is found in both childhood and adult, chronic lymphocytic leukemia (CLL) is found in adults more than 55 years old, and chronic myeloid leukemia (CML) is mostly found in adults. In recent years, The National Cancer Institute of Thailand reported that most leukemic patients are children not over 15 years of age. All male patients classified as 0-5 years are found 3 in 17 persons (17.6%), 5-10 years are found 6 in 17 persons (35.3%), 10-15 years are found 4 in 17 persons (23.5%). For female patients, they have been classified as 7 in 13 persons (53.8%) in 5-10 years old. However, 13 in 17 of male patients were identified having as precursor cell lymphoblastic leukemia, one case is precursor T-cell lymphoblast leukemia, one case

is classified as chronic myeloid leukemia and another one case is myeloid sarcoma. In female patients, the most of them is precursor cell lymphoblastic leukemia, other one is acute myeloid leukemia and another one is acute leukemia (Attasara and Buasom, 2011). However, observation on 2004-2008 the overall leukemia incidence rates increased slightly by 0.5% per year, a consistent trend since 1992. Among death rate is found decreased by 0.8% per year among males and by 1.4% per year among females ("Cancer Facts and Figures 2012", 2012).

Two basic theories for cancer treatment are remission in the beginning diagnosis case and protection of cancer recurrent. Several effective methods such as chemotherapy, surgery, transplantation, radiation are mostly used for cancer treatment. However, these methods are inconvenient and take time. Moreover, several side effects might occur after treatment, including anti-cancer drug resistance, normal cells are destroyed together with cancer cells in case of chemotherapy, surgery scar, and graft rejection, low immune system and ease of microbial infection in case of bone marrow transplantation. In addition, anti-cancer drug also decreases the life quality of cancer patients. Therefore, researchers have tried to discover a novel therapy for side effects reduction and drug resistance decreasing while patients have high opportunity to recover from the disease. RNA interference (RNAi) is new technology that many scientists play high attention for cancer treatment research. The principle of RNAi technology is blocking and destroying specific mRNA resulted to inhibit gene transcription. Gene therapy using RNAi technology is suitable for genetic disorder disease such as cancer treatment. Wilms' tumor 1 (WT1) is a type of transcription factor, it plays important roles in cellular proliferation and survival of various cancer cells such as breast cancer (Loeb, Evron, Patel, and Sharma, 2001),

lung cancer (Oji, Miyoshi, and Maeda, 2002), ovarian cancer (Barbolina, Adlay, and Shea, 2008), pancreatic cancer (Wolfgang, Luise, and Johannes, 2009) and leukemia (Bergmann, Miething, and Maurer, 1997), especially in human chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Although, myeloid leukemia cell line (K562) has been shown to be less than 60% of *WT1* expression by real time RT-PCR when compared with pancreatic cancer (Wolfgang et al., 2009). However, regulation of *WT1* expression in CML is still important to study. Because of the potential of cancer gene therapy has never been explored previously. Because of WT1 is variably expressed in the type and subtype of leukemia, it is used as the molecular marker for leukemia diagnosis and prognosis. WT1 overexpressed in adult AML, ALL, pediatric AML, and pediatric ALL (Rosenfeld, Cheever, and Gaiger, 2003). It shows the potential for suppression of vascular endothelial growth factor (VEGF) expression in pediatric ALL leukemia (Moazam, Eisermann, Fraizer, and Kuerbitz, 2010). Therefore, the mRNA degradation or translation arrest which is the major mechanism(s) of RNA interference (RNAi) technology. The silencing of target mRNA is included in post-transcriptional gene regulation in eukaryotic cells. Small interference RNA (siRNA) guides the RNA interference process, resulting in a base pairing dependent downregulation of gene expression (Storvold, Andersen, Perou, and Frengen, 2006).

Wilms' tumor 1 (*WT1*) gene encodes zinc-finger transcription factor, which was originally identified as a tumor suppressor gene inactivated in Wilms' tumor (Haber and Housman, 1992). WT1 protein contains of 10 exons with an activator and repressor domain located near the N-terminal and four zinc-fingers of CH_2-His_2 at C-terminal. Total thirty six protein isoforms of WT1 are generated by the alternative

splicing processes, however, four major isoforms are expressed in various types of solid cancers, such as ovarian cancer, mesothlioma of the lung, melanoma, breast cancer (D. M. Loeb et al., 2001; Miyoshi et al., 2002). The two alternative splicing are characterized by the presence or the absence of 17 amino acids in exon 5 and/or the terminal 9 nucleotides of exon 9 which encodes three amino acids (Lysine, Threonine, and Serine: KTS) (Haber et al., 1991; Hohenstein and Hastie, 2006). Each major isoform of WT1 represented by WT1(-17AA,-KTS), WT1(+17AA,-KTS), WT1(-17AA,+KTS), and WT1(+17AA,+KTS). WT1 plays critical roles in the proliferation, apoptosis, and differentiation of cells. That is the reason which making it as a suitable therapeutic target for cancer treatment including leukemia. The overexpression of WT1 was previously reported by various groups of researchers; in blast crisis of acute myeloid leukemia (80-90%), chronic myeloid leukemia, acute lymphoblastic leukemia (70-90%), and myelodysplastic syndrome are usually found both gene and protein levels (Inoue et al., 1997; Menssen et al., 1995; Miwa, Beran, and Saimders, 1992; Sugiyama, 2002). Although, the discrepancies between some reports which referred that the overexpression of WT1 were observed in B-ALL more than T-ALL. However, WT1 is a good marker for leukemia prognosis and as a target gene for treatment. In addition, in acute leukemia patients, WT1 is measured for relapse prognosis (Hu et al., 2010). In the same hand, significantly expression of WT1 specifically WT1(+17AA) isoform was observed in bone marrow of AML patients with relapse more than the patients who initially diagnosed. This observation led WT1(+17AA) to be used for AML prognosis (Gu et al., 2010). High expression of WT1 in leukemia blast crisis is also associated with poor response to chemotherapy, a higher risk of leukemic relapse, and extended free survival is significantly lower

probability (Bergmann et al., 1997). WT1 is overexpressed in proliferating-leukemia cells such as K562 and HL-60 cell lines, indicated that this gene is important for leukemia cells growth. In contrast, ones WT1 is downregulated, cells are triggered apoptosis. Moreover, cell differentiation is observed in G0/G1 accumulation (Kerst et al., 2008). Some review referred to cellular therapy for ALL at relapse treatment. One of the mechanisms is WT1 peptide which specific T-cells classified as allogenic or autogenic T- cells, consequent it was infused into the recipient to target leukemic cells (Bhojwani and Pui, 2013; O'Reilly, Dao, Koehne, Scheinberg, and Doubrovina, 2010).

This study we aim to investigate the effect of our new designed WT1-siRNA on cells proliferation and apoptosis of leukemic cell line (K562) and primary childhood acute lymphoblastic leukemic cells type L1 in order to advantage of leukemic treatment research.

1.2 Research objectives

1.2.1 To determine cell growth inhibition efficiency and apoptosis induction of our new designed WT1-siRNAs on human chronic myeloid leukemic cell line both in cellular and molecular levels.

1.2.2 To study the effect of our new designed WT1-siRNA on growth inhibition and apoptosis induction in human primary acute lymphoblastic leukemic cells from ALL patients in both cellular and molecular levels.

CHAPTER II

LITERATURE REVIEWS

2.1 Cancer

Cancer is a disease in which a normal single cell, somewhere in the body, begins to abnormally multiply, forming a tumor. Over time, the cells in the tumor all originating from a single ancestral cell which could be called monoclonal, acquire certain traits that allow them to continue proliferating and generate distinct subpopulations of cells that give no indication of a common origin, it can considered to be polyclonal. If not treated in time, the cells can leave their original tissue and invade other healthy tissue in a process called metastasis. The patient eventually may die not of the tumor itself, but from damage caused to other adjacent tissues. If detected early, the tumor can be surgically removed; very often, by the time the cancer is diagnosed, the cells have already metastasized, making it virtually impossible to remove the tumor. The tumor has been segregated into two broad categories depending on their degree of aggressive growth. First, benign tumor which is the group of cells that grew locally without invading adjacent tissues. The second category is malignant tumor which invaded nearby tissues and spawned metastasis. The epithelial tissues are the majority sources for human tumoregenesis. However, the nonepithelial tissues including sarcomas, fibroblasts, adipocytes, osteoblasts, myocytes and also contained hematopoietic tissues could be remained the malignant tumors. The causation of human cancer is identified in various events such as the exposure of carcinogenic agents for example; benzene, radium arsenic, asbestos,

diethylstilbestrol. The other risk of cancer is depending on human lifestyle which induces the cancer risk includings cigarette consumption, low in vegetables diet, alcohol use, and virus infection. Moreover, the genetic mutation has also being cause of cancer (Weinberg, 2007). The tumor has several steps of progression in termed of "Multi-step tumoregenesis" which driven by randomly occurring mutations and epigenetic alterations of DNA that affect the genes controlling cell division, survival and malignant cell phenotype. The benign tumor can develop to malignant tumor by several processes, beginning with the tumor cells grow in the epithelium layer. This cell is considered to be benign as long as the cells forming them remain on this side. Consequently, the tumor cells break through basement membrane (basal lamina) into the capillary and travel through blood stream. The mechanisms of the invading involved removes an important physical barrier to the further expansion of tumor cell populations and degrades various components of the basement membrane for harvesting growth and survival factors that have been sequestered by attachment to specialized extracellular matrix. Next step, only a few cells that survive in the capillary adhere to the blood vessel wall of another organ. Then the survival tumor cells escape from blood vessel called extravasation by the mechanisms of release the growth and survival factor involving vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF) led to induce blood vessel leakage caused some substances and tumor cells go out from the capillary and the cells proliferate to form metastasis tumor (Figure 2.1).

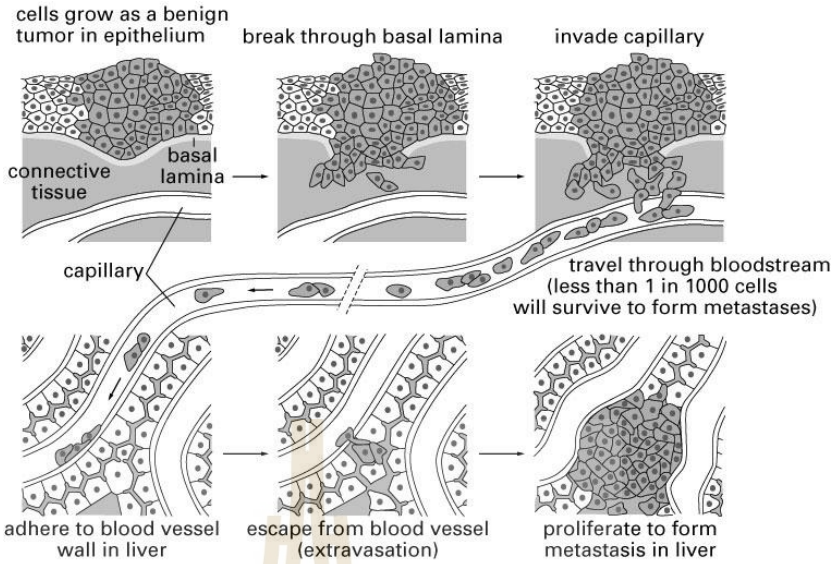

Figure 2.1 Schematic of cancer progression from benign tumor to metastasis cancer. The invasion-metastasis cascade demonstrated six distance steps. First, the benign cells grow at the epithelium. Next step, the cells break the basement membrane. Then the cells invade into the capillary blood vessel (intravasation). After that, the survival tumor cells adhere to the surface of the capillary wall. Consequently, the cells escape from the blood vessel (extravasation). The last step, tumor cells proliferate to form metastasis in another organ, this case is liver.

(Source: http://thefutureofthings.com/articles/1012/smart-bombing-cancer.html)

Another process that supports the growing of tumor is angiogenesis. The angiogenesis is the process of the construction of new capillaries by vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) stimulate endothelial cells in the vicinity to multiply the new blood vessel. The VEGF is soluble protein which secreted by various types of cells, the receptor of VEGF is expressed by endothelial cells. In addition, the hypoxia condition is the major cause for VEGF expression activation. The new generating blood vessel has the role for nutrient and

oxygen supply for the tumor survival (Weinberg, 2007). In addition, the tumor cells are virtually automatic release of angiogenic factor to attract the inflammatory and endothelial cells for capillary construction and induction to the tumor site for protection of them from the hypoxia situation (Figure 2.2). The inflammatory cells play a role for additional substance secreting, while the endothelial cells form existing blood vessel to respond an angiogenic signal and secrete protease for vessel migration. The endothelial cells proliferate to new blood vessels (Garrel, 2001).

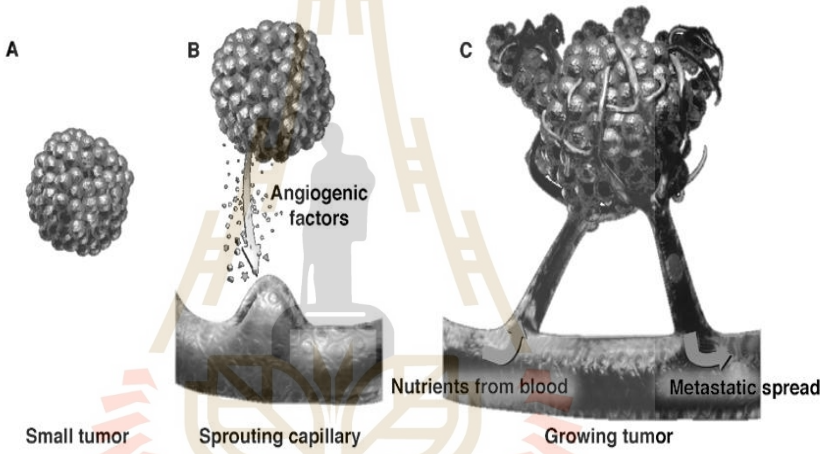

Figure 2.2 Schematic representation of new vessels development in tumor angiogenesis. The formation of tumor is generated at some organ in the body (A). A tumor induces the sprouting capillary by releasing of angiogenic factors (B). A lot of new blood vessels form covered the tumor for nutrients and oxygen supply. Tumor cells are moving on the vessels for metastatic spread to another organ, tumor growth (C) (Garrel, 2001). (Source: http://www.angioworld.com/DominiqueGarrel.html)

2.2 Leukemia

Blood cells are usually derived from bone marrow and they have the unique ability to give rise to all of the different mature blood cell types. The lineage of blood cells is classified into three series including erythrocytic cells, lymphocytic cells and myelocytic cells. The erythroid cells (red blood cells) play a role for oxygen carrying into all systems of the body. The lymphocytes derived from the common lymphoid progenitors and play a role for activation of adaptive immune system. The lymphoid lineage is developed to mature T-cells, B-cells and natural killer cells. Other myelocytes are derived from the common myeloid progenitors, they are developed for various types of blood cell involved granulocytes, megakaryocytes and macrophages. However, granulocytic cells are classified by 3 groups depended on nuclei formations and the granules containing as followed, eosinophil, basophil, and neutrophil (Figure 2.3). The myelocytes have critical roles for innate immunity, adaptive immunity and blood clotting.

(“Heamatopoiesis:http//en.wikipedia.org/wiki/Haematopoiesis, ” 2012).

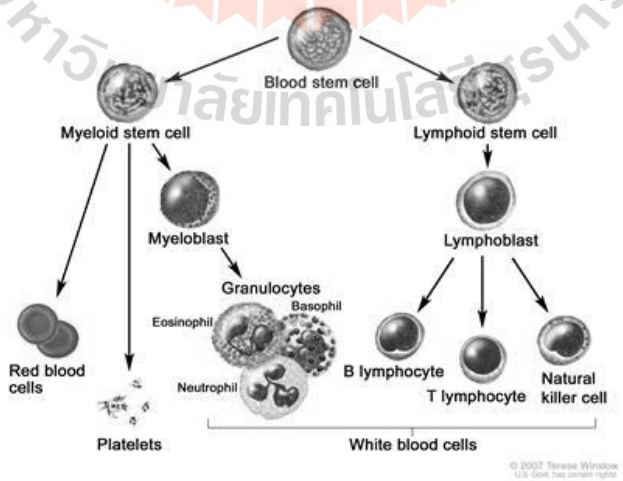

Figure 2.3 Schematic representation of blood cell lineage. (source: www.cancer.gov)

Leukemia is a type of cancer which occurs in white blood cell especially in blasts crisis, this disease is divided into 2 major groups involved acute leukemia and chronic leukemia depended on how quickly the disease development. Leukemic incidence rates of the world population are the number of new cases of leukemia as usually present as a specific number per 100,000 populations. In 1993-1997, the average incidence of these diseases were 8.5 and 5.2 in 100,000 persons for males and females, respectively (Parkin et al., 2002). However, in The United States of America, the report showed that the number of leukemic patients was found around 57% for males (http://www.leukemia.org/all_page?item_id=9346, 2009). Moreover, in 2012 the estimate new cases and death of leukemia in USA was reported by American Cancer Society. The report shows that leukemic patients are diagnosed with CML for 3,210 in males and 2,220 in females where as the death rate is reported for 370 and 240 in males and females, respectively ("Cancer Facts and Figures 2012", 2012). As notice, the incidence of leukemia in Thai people has increasing from the past (Mendessohn, Howley, Islael, and Liotta, 2001). Leukemic incidence was found in children approximately 38.7% when compared to the children patients with the same age. It should be concerned from many countries especially, undeveloped and developing countries. Moreover, in 2009 the incident new cases of leukemia at USA are observed as 44,790 cases for adults and 3,509 cases for 0-14 years of children. However, the incident of chronic leukemia was found more than acute leukemia around 11% of cases in USA. The ratio of leukemic incidence has been classified by race and ethnicity for 12.8, 7.0 and 7.3 cases per 100,000 for American of European, American Indian and Asia Pacific Islander populations, respectively. In 2009 there are around 4,390 dead cases from CLL, 1,400 cases from ALL, 9,000 cases from AML

and 450 cases from CML (http://www.leukemia.org/all_page?item_id=9346, 2009). In Thailand, the incidence of these diseases is rarely low by world standard. In 1995 to 1997, the estimated age standardized rate (ASR) for Thailand is 4.1 and 3.5 per 100,000 persons for males and females, respectively (Sriplang et al., 2003). Consistently, it was found around 4.2 and 3.7 per 100,000 populations in 1998-2000. Acute lymphoblastic leukemia was found in males more than females. In addition, this type of leukemia shows highest incident rate in childhood 0-4 years of age (Jootar, 2000). In 2001-2003 the incident rate of leukemia in Thailand was found 4.9 and 3.7 in male and female patients by ASR rate (Khuhaprema et al., 2010). In 2011, the report revealed that the incident rates of leukemia in USA population not difference from 2010 observation. By mean, 12.3 was found in 2010 and 12.5 was found in 2011. All of children cancer patients (0-19 years old) were identified as leukemia around 27.2%. The new cases of the USA leukemic patients were diagnosed for 44,600 cases in 2011. Each of cases was classified by the type of leukemia as shown in Figure 2.4. Moreover, the mortality rate of leukemic patient was observed for 21,780 people in 2011 (“Leukemia and lymphoma society: fighting blood cancers”, 2012). The increasing number of estimated new cases of leukemia is reported for 47,150 cases in 2012 whereas, the death rate are expected for 23,540 cases. Although, the number of deaths in 2012 is significantly increased from 2011 when compared with the death rates in 2004 to 2008, we found that death rates of leukemia have been decreased by 0.8% per year among males and by 1.4% per year among females (“Cancer Facts and Figures 2012”, 2012).

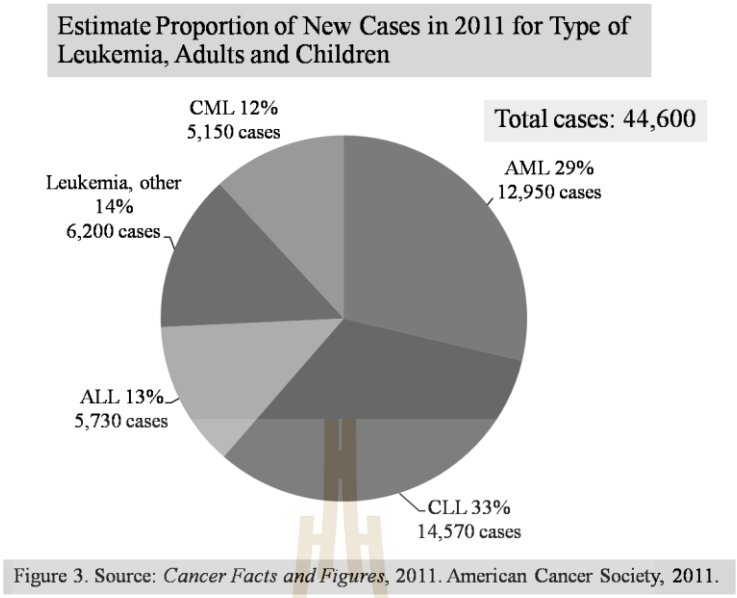

Figure 2.4 The estimate proportion of new cases of various types of USA leukemic patients both in adults and children in 2011.

Despite, CML patients are found less than other types of leukemia. However, the most cases of CML occurred in adults. Approximately 2.9% of leukemic children patients were classified as CML. Gender and age are the factor risk of leukemia, male is recorded for leukemia more than female and older more than younger. Leukemic incident rates of CML are found significant increasing in the person who is 65 years or older. However, the relative survival rate of CML from 2001 to 2007 was shown around 55.2%, which is the second level from AML (23.6%). These situations indicated that AML is the highest cause of death and the second one is CML ("Leukemia and lymphoma society: fighting blood cancers", 2012). Consistently, The American Cancer Society has reported about the 5-year relative survival rate, for CML the values are increased from 31% in 1990-1992 to 55% in 2001-2007 ("Cancer Facts and Figures 2012", 2012). Next, the estimated deaths of four majors of leukemia

are order from high to low as follow: AML (9,050), CLL (4,380), ALL (1,240), and CML (270). Among CML, the number of female patients who pass away is 170 and male patients is 100 deaths, recorded in 2011 ("Leukemia and lymphoma society: fighting blood cancers.," 2012).

2.2.1 Chronic myelogenous leukemia

Chronic myelogenous leukemia is a rare type of leukemia that tends to affect older males. More than 90% of cases are dued to a gene abnormality called “Philadelphia chromosome” (Figure 2.5) which occurred from the translocation between chromosomes 9 and 22 within blood cells. The new gene on this chromosome led to encode a protein called tyrosine kinase which caused the out of control of white blood cells growth. Moreover, these abnormal cells resist to old and die (Benjamin and Wedro, 2011). CML progresses through 3 phases: chronic, accelerate, and blast. The chronic phase of disease is the mature cell proliferation phase. In an accelerate phase, additional cytogenetic abnormalities occur and blast phase is the rapidly growth of immature cells (Sawyers, 1999). CML patients were diagnosed in chronic phase around 90-95% and then within and average 4-6 years the disease will transform through accelerate phase and finally to blast phase within 3-5 years. The histopathologic findings in the peripheral blood are standard method for CML diagnosis. Moreover the detection of the Philadelphia chromosome in bone marrow cells is the best for accuracy diagnosis (Besa and Krishnan, 2011; Druker, 2008).

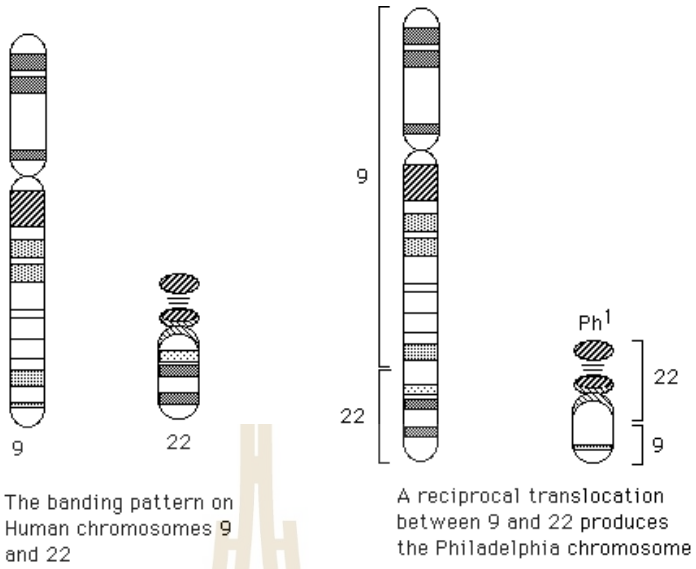

Figure 2.5 The translocation involved a piece of chromosome 9 and the mostly of chromosome 22 resulting a hybrid gene called Philadelphia chromosome "Ph^+". The ABL and BCR (breakpoint cluster region) are the normal genes located on chromosome 9 and 22, respectively. The translocation of middle of chromosome 22 to chromosome 9 (q34:q11) led to produce hybrid gene and the result of Philadelphia chromosome generates BCR-ABL fusion gene. In general the protooncogene ABL gene encodes tyrosine kinase enzyme that plays a role for growth regulation. The translation of BCR-ABL gene encodes the protein which deregulated the tyrosine kinase activity. The present of this protein in CML patients is strong evidence of its pathogenic role (Druker, 2008).

2.2.2 Acute Lymphoblastic Leukemia

Leukemia which has diagnosed in the most cases of childhood especially the first-five years old is Acute Lymphoblastic Leukemia (ALL). More than 80 cases per million per year of 2-3 years old of childhood were diagnosed for ALL. In general, white blood cell count of ALL patients is 50,000 per microlitre. The major risk factors

of ALL are depended on the exposure to some chemical such as benzene, petroleum products, pesticides, hair dyes, tobacco smoking, and ionizing radiation. In addition, human T-cell leukemia virus 1(HTLV-1) and Epstein-Barr virus infections have also been increased the risk of acute leukemia with T-cells and B-cells leukemia, respectively (Sandler and Ross, 1997). The precursor B-cell ALL was found more than precursor T-cell ALL, approximately 75% and 25%, respectively (Mittal and Meehan, 2001). The cellular surface markers that used to define a positive reaction of blast cells of B-cell lineage are CD19, CD20, CD22, CD24, and CD79a. While, T-cell lineage markers are CD1a, CD2, CD3, CD4, CD5, CD7, and CD8, CD2, CD5, and CD7 are antigens of immature T-cell markers (Abdul-Hamid, 2011). In addition, T-cell and B-cell ALL can additionally express myeloid antigen or stem cell antigen CD34 (De Waele et al., 2001). The French-American-British (FAB) criteria are classified ALL to 3 groups by cells morphology, the characteristic of chromatin, the amount of nucleoli and cytochemical staining. In addition, by the method of immunophenotypic classification based on cell surface markers is divided B-cell ALL for 4 subtypes and T-cell ALL for 2 subtypes, by the degree of maturation (Table 2.1), including L1 (homogeneous cells) which is frequently found in children, around 74% of cases occurring in 15 years of age or younger, L2 (heterogeneous cells) which is usually found in adult, approximately 66% of cases occurring in patients older than 15 years of age, and L3 usually called Burkitt's type blast cells which is least found when compared with L1 and L2 (Abdul-Hamid, 2011; Mahajan, 2007). In the present, children with ALL are cured and successful around 90% which in contrast with adult ALL, cured only 40%. These situations let the researchers to observe and characterize the genetic abnormalities of ALL in order to increase ALL understanding

(Moorman, 2012). The current WHO classification of B-cell ALL defines seven genetic subtypes including: t(9;22)(q34;q11.2)/BCR-ABL1, MLL/11q23 translocation**,** t(12;21)(p13;q22)/ETV6 RUNX1, t(1;19)(q23;p13.3)/ TCF3-PBX1, t(5;14)(q31;q32)/IGH@-IL3, hyperdiploidy and hypodiploidy (Kebriaei, Anstasi, and Larson, 2003; Moorman, 2012).

Table 2.1 Morphologic and immunophenotypic classification of acute lymphocytic leukemia. (Mittal and Meehan, 2001)

Morphologic classification		Immunophenotypic classification
FAB type	Salient features of blasts	B-cell lineage:
L1	Small cells with scant cytoplasm; nucleoli indistinct and not visible	- Early precursor B-cell ALL - Common ALL - Precursor B-cell ALL - B-cell ALL
L2	Large, heterogeneous cells with moderately abundant cytoplasm; clefting and indentation of nucleus; large and prominent nuclei	T-cell lineage: - Precursor T-cell ALL - T-cell ALL
L3	Large cells with moderately abundant cytoplasm; regular, oval-to-round nucleus; prominent nucleoli; prominent cytoplasmic basophilia and cytoplasmic vacuoles	

2.2.3 Treatment of cancer

In the present, the most common effective methods for cancer treatment are surgery, chemotherapy, and radiation (Figure 2.6). However, these conventional approaches led inducing many side effects on patients included pain, fatigue, loss of appetite, bleeding, infection, lymph edema (P. Attasara and R. Buasom, 2011). The chemotherapy treatment for CML and other leukemias usually use anti-cancer drugs with a single agent or by combination of several drugs such as Imatinib (Gleevec), nilotinib (Tasigna), and Dasatinib (Sprycel) ("Cancer Facts and Figures 2012", 2012).

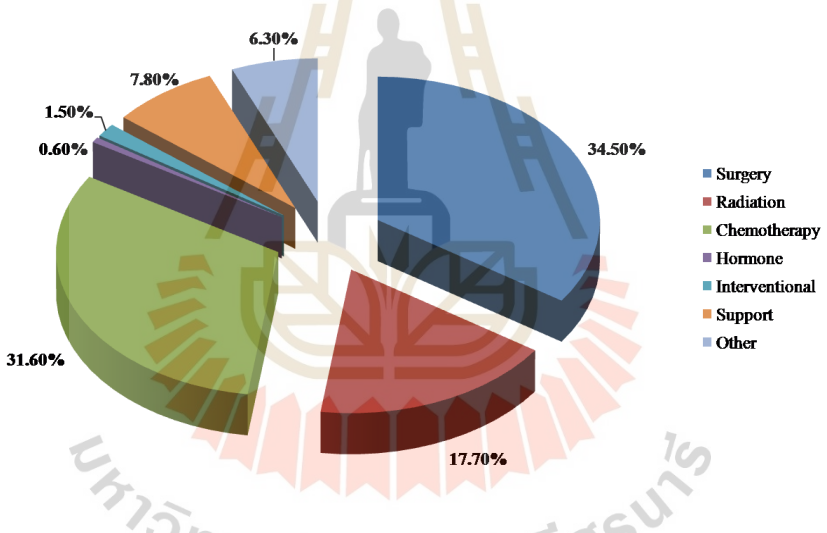

Figure 2.6 The effective methods for cancer treatment (P Attasara and R Buasom, 2011).

2.3 Wilms' tumor 1 (WT1)

Wilms' tumor 1 (WT1) gene was first identified in 1990, it locates at chromosome 11p13 (Call et al., 1990). *WT1* gene consists of 10 exons and led to produce 3 kb of mRNA. Further, it is translated to 52-54 kDa protein size. Basic structure of WT1 protein consisted of 2 major regions. First: the regulatory domain

which remains at the N-terminal and contains with proline/glutamine rich region. This domain is necessary for the transcriptional regulatory function of WT1. These transactivation domain consists of various regions which have different roles including the repression region which is located at 84-124 amino-acid residues, activation domain which is located at 181-250 amino acid residues, self-association domain which is located at 1-180 amino acid residues and among of RNA recognition in the N-terminal which is located at 11-72 amino acid residues. Second, zinc-finger domain remained at C-terminal region contains of Early Growth Response (EGR) family zinc-finger type which is 2 cysteines-histidines cross-link. Each zinc-finger locates at the position of 323-347, 353-377, 383-405, and 414-438 amino acid residues (Haber et al., 1991; Sigmaaldrich). A Krupple-like zinc-finger has a crucial role for DNA/RNA binding and involved nuclear localization. The position of each region is located at 323-438 amino acid residues for DNA binding domain, at 291-350 amino acid residues and at in zinc-fingers 2/3 for nuclear localization signal. Not only in N-terminal that contains RNA recognition but C-terminal is also remained of RNA recognition site in the first of zinc-finger domain (Figure 2.7) (Englert, 1998; Yang, Han, Suarez Saiz, and Minden, 2007). Each zinc-finger is separated from the next one by short intron (Haber et al., 1991). The present of KTS insertion more increases the flexibility of the linker between zinc-finger than the absent of KTS insertion (Figure 2.10) (Stetefeld and Ruegg, 2005).

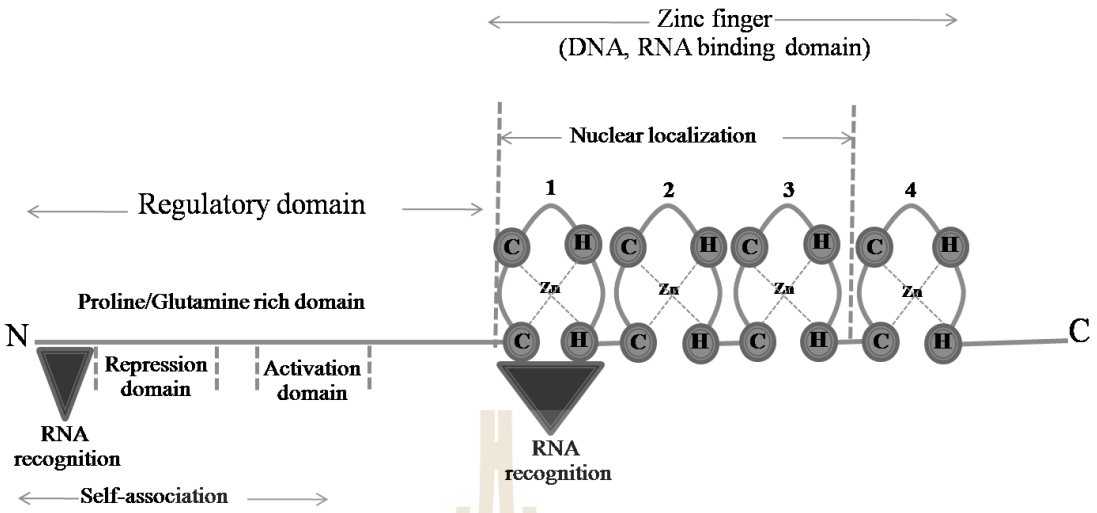

Figure 2.7 Schematic representation of basic structure of WT1 protein. The N-terminal transactivation domain contains proline/glutamine-rich region, which is almost identical to the RNA recognition, repression, activation, and self-association domains. The C-terminal is consisted of four-loops of $Cysteine_2$-$Histidine_2$ cross-link zinc-finger domains, which represent the DNA, RNA binding domains and nuclear localization region along with the two-alternative splicing event (Englert, 1998; Yang et al., 2007).

The simplified structure of each four major isoforms of WT1 divided by two alternative splicing regions. Splice I comprises exon 5, which consists of 51 nucleotides and encodes specific sequences of 17 amino acids including 5 residues of serine and 1 residue of threonine which are effective sites for protein phosphorylation (Haber et al., 1991) and interaction with PAR-4 (prostate apoptosis response factor), which is a co-activator of transcription (Richard, Schumacher, Royer-Pokora, and Roberts, 2001) (Figure 2.7). The alternative splice II results from the use of an alternative splice-donor sequence between zinc-fingers 3 and 4 at the exon 9 and 10,

respectively. The three residues insertion of lysine, threonine, and serine (the KTS sequence) (Figure 2.8) occur in the 3′ end of exon 9 and resulted to interrupt the spacing between the third and forth zinc-fingers which lead to impact with DNA binding role (Yang et al., 2007). WT1 has 4 major isoforms and 36 minor isoforms, generates by RNA splicing, RNA editing, alternative splicing and initial translation with different start codon sites such as AUG (start codon) which generates 52-54 kDa, CUG-73 at the upstream of start codon AUG which synthesize 60-62 kDa protein size, and the beginning of translation at AUG127, the protein of 36-38 kDa will be encoded. The review of Morrison and colleagues referred that four main isoforms are generated by translation initiation at AUG start codon encoding protein products for 52-54 kDa depended on present or absent of 17 amino acids insertion (A. A. Morrison, Viney, Saleem, and Ladomery, 2008). As mention above, four major isoforms of WT1 derived from two alternative splicing processes. Each isoforms is named depending on the difference between 2 alternative splice sites including WT1(-17AA/-KTS), WT1(+17AA/-KTS), WT1(-17AA/+KTS), and WT1(+17AA/+KTS). Representative variant isoforms were called variant A, variant B, variant C, and variant D, respectively (Figure 2.9). Each variant of WT1 isoforms generates different protein size involving variant A consists of 429 amino acids and encodes 47.19 kDa, variant B contains 446 amino acids and encodes 49.06 kDa, variant C comprises of 432 amino acids and encodes 47.52 kDa, and variant D consists of 449 amino acids which encodes 49.39 kDa (Graidist, 2009). The schematic representation of each WT1 isoforms was shown in Figure 2.9. Moreover, the fifth isoform was discovered by Haber and Gessler, two researcher groups that worked about genomic structure of WT1. These isoform is WT1(-17AA/-KTS), however it is

a little differ from variant A because of it has 68 amino acids insertion at the N-terminal region resulted containing of 497 amino acids which encodes 54.67 kDa (Gessler, Konig, and Bruns, 1992; Haber et al., 1991).

Figure 2.8 Sequence of the two alternative WT1 splices. The nucleotide sequence of the two inserted splices and of their derived amino acids are shown. Splice I consists 51 bp encoding 17 amino acids, whereas splice II contains 9 bp encoding 3 amino acids (Lysine, Threonine, and Serine: KTS). In both cases, the first codon is splice between the alternative splice and the upstream exon, and the normal reading frame is maintained downstream of the insertion. (Harber et al., 1991)

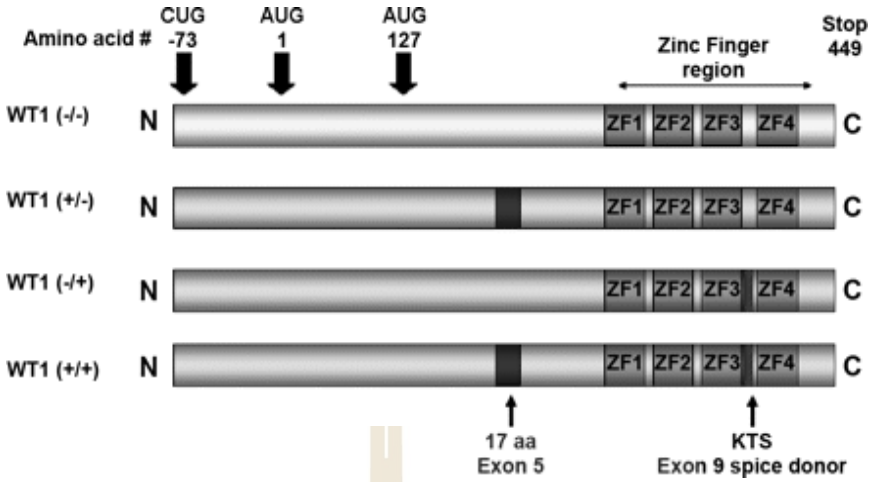

Figure 2.9 Schematic representations of WT1 isoforms. The best studied splice variants of WT1 are shown (mammalian-specific 17 amino acids encoded by exon 5, and 3 amino acids (KTS) encoded by an alternative splice donor site in exon 9). These generate 4 mainly studied WT1 isoforms, with a molecular mass of 52-54 kDa. Translation at an upstream CUG codon results in the generation of larger WT1 isoforms with a mass of 62-64 kDa. Initial translations that start at a downstream AUG codon (127AUG) give rise to smaller isoforms of 32-34 kDa (A. A. Morrison et al., 2008).

2.4 The biological roles of WT1 in mammalian cells

WT1 plays a special role as a transcription factor. It has zinc-finger domain belong to an early growth response (EGR) family. The main functional roles of WT1 can be separated as 4 functions including regulation of cellular growth, regulation of cancer spread, regulation of cell senescence, and regulation of cellular apoptosis. Each roles of WT1 has various types of gene to be regulatory target such as WT activates *IGF-II* for growth stimulation (Nichols, Re, Yan, Garvin, and Haber, 1995) and represses *IGF-II* for growth inhibition (Ward, Pooler, and Miyagawa, 1995). WT1

activates *amphiregulin, P21, Bax, Bak* where as *PDGF-A, TGF-B1, CTGF, EGFR, IGF-IR, c-Myb, c-Myc, hTERT, BCL-2,* and *ODC* are repressed and indicated the role of WT1 as tumor suppressor protein. However, WT1 activates *c-Myc*, *BCL-2* and represses *TSP1* suggested that WT1 plays a role as oncogenic function (Graidist, 2009; Lee and Haber, 2001).

2.4.1 Effect of WT1 protein on its downstream genes

2.4.1.1 Platelet-Derived Growth Factor A-Chain (PDGFA)

WT1 is a transcription factor that regulates many growth related genes including the platelet-derived growth factor A-chain (PDGFA) via functional domains in the N-terminus and C-terminus. WT1 suppresses the PDGFA expression by inhibition of PDGFA promoter using zinc-finger domain. The different isoforms of WT1 led to regulate with different values such as WT1(-KTS) isoform can bind with PDGFA promoter and gave a Kd of 100±30 nM while WT1(+KTS) isoform showed higher than WT1(–KTS) (130±40nM) (Fagerlund, Ooi, and Wilbanks, 2012).

2.4.1.2 Amphiregulin

Amphiregulin is a member of the epidermal growth factor family. It plays a role during kidney differentiation. This gene is directly regulated by WT1 which can bind to its promoter and led to activate Amphiregulin transcription (Lee et al., 1999). Amphiregulin expression was induced by WT1(-KTS) isoform.

2.4.1.3 B-cell lymphoma-2 (BCL2)

The protooncogene *BCL-2* acts as anti-apoptotic function in many cancer cells. It is a downstream target of *WT1* gene because *WT1* gene has a consensus sequence with BCL-2 and both of them are binding with high affinity. WT1 can

regulate BCL-2 by the activating of its promoter resulted to transcriptional upregulate the BCL-2 expression (Mayo et al., 1999). In AML patients, WT1 and BCL-2 are found approximately 74% (113/152) and 84% (127/152), respectively. The coexpression of both genes in the less than 60 years old AML patients indicated that they have the risk to induce the death rate and reduce rate of continuing complete remission (CRR). While, the patient who ages more than 60 years old had no prognosis impact (Karakas et al., 2002). The knockdown of WT1 and BCL-2 by siRNA inhibit cellular proliferation of WT1-expressing leukemic cells such as K562 and HL-60 cells within 48 hours post-transduction (Glienke et al., 2007).

2.4.1.4 Interleukin 2 (IL-2)

Interleukin 2 (IL-2) is a crucial cytokine for induction of T-cell growth, differentiation, and functional activation. It was first identified in 1976 as a growth factor for bone-marrow-derived T-lymphocytes (Morgan, Ruscetti, and Gallo, 1976). Human T-cell acts by signals of IL-2 inserted via IL-2 receptor subunit complexes on cell surface. The IL-2 receptor contains three subunits such as subunit alpha (IL-2RA), subunit beta (IL-2RB), and subunit gamma (IL-2RG) or common cytokine subunit gamma chain. All of them play a key role for intracellular signal transduction mediated by IL-2 (Figure 2.10). Each isoform was original classified by the potential of IL-2 binding affinity; high (K_d = 10^{-11} M), intermediate (K_d = 10^{-9} M), and low (K_d = 10^{-8} M) affinity IL-2 receptor (Leonard, 1996; Smith, 1988; Taniguchi, 1995). The IL-2RA contains 8 exons which localized on chromosome 10p14-15 however, it cannot act as the signal transduction by only itself because the cytoplasmic domain of IL-2RA contains only 13 amino acid residues which is too small (Ishida et al., 1985; Leonard et al., 1985). Therefore, other receptor subunits are required. IL-2RB

contains 10 exons located on chromosome 22q11.2-12 and consists of 525 amino acid residues. It is characterized as cytokine receptor superfamily which high affinity capacity. IL-2RG located on human chromosome Xq13.1 and encodes 64 kDa protein sizes and consists of 347 amino acid residues with in 8 exons. IL-2RG was original detected by coimmunoprecipitation with Il-2RB (Kim, Imbert, and Leonard, 2006; Takeshita et al., 1992). The structure of cytoplasmic domain of IL-2RG contains with Src homology region 2 which plays a role for downstream signaling via its interaction with phosphotyrosine residues of various signal transducing effectors such as IL-2, IL-4, IL-9, and IL-15. Three subunits of IL-2 receptor will be good function depend on their association and dissociation rate constants and affinities for IL-2 binding (Matsuoka et al., 1993). IL-2 receptor heterotrimer complex shows high affinity IL-2 binding, on the other hand, heterodimer of IL-2RA and IL-2RB complex shows no signal transducing ability for cell growth. In addition, heterodimer of IL-2RB and IL-2RG complex exhibited intermediate-affinity IL-2 binding and both of them are indispensable subunit for the functional IL-2 receptor complex which supported an increasing of the IL-2 binding affinity. IL-2 receptor responded to IL-2 in terms of tyrosine phophorylation of IL-2RB and also induction of protooncogenes such as *c-Myc, c-Fos,* and *c-Jun* (Asao et al., 1993). All of these indicated that IL-2RB and IL-2RG are important and adequate for formation of functional IL-2 receptor complexes; IL-2RA only increases the IL-2 binding affinity of the IL-2RB and IL-2RG complex. Moreover, IL-2RG could respond with various cytokines such as IL-4, IL-7, IL9, and IL-15 which served as multireceptor subunits (Kim et al., 2006). The IL-2 gene expression is controlled with the transcriptional regulation of several transcription factors involving Sp1/EGR1 which are zinc-finger transcription factor

like WT1. The Sp1 is a sequence-specific zinc-finger transcription factor that recognizes GGGGCGGGGC motifs (GC boxes). Among EGR1, it contains a DNA binding domain consisted of three zinc-finger motifs and responsible for cell growth and apoptosis (Gashler and Sukhatme, 1995). EGR1 regulates IL-2 transcription via its synergistic interaction with NFATc (Nuclear factor of activated T cell) (Decker, Skerka, and Zipfel, 1998).

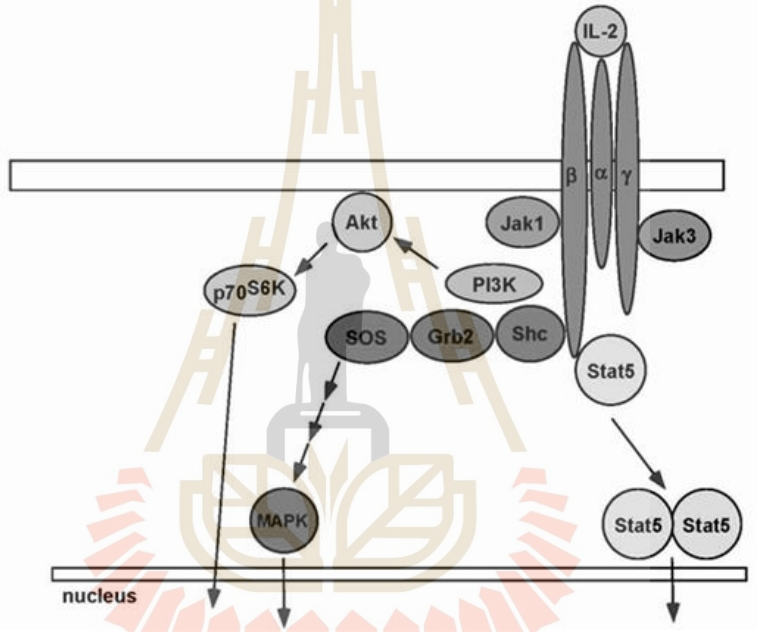

Figure 2.10 Growth and differentiation of T-cells, B-cells, and natural killer cells are regulated by T-cell-derived cytokine; Interleukin 2 (IL-2). IL-2 signaling is mediated by specific binding with IL-2 receptor subunit which consists of 3 subunits; alpha, beta, and gamma. The protein kinase Jak3 interacted with IL-2RG domain, are also activated after IL-2 binding. Then autophosphorylation is occurred with Jak3 and further activates the phosphorylation of STAT5 and induces dimerization of STAT5 complex. The complex is translocated into the nuclease and stimulating of cytokine production and expression. The IL-2 pathway is interned by the binding of IL-2 with its receptors. (http://www.sabiosciences.com/pathway.php?sn=IL-2_Pathway)

2.4.1.5 Insulin-like Growth Factor II (IGF-2)

The insulin-like growth factor II (IGF-2) locates at the short arm of chromosome 11 (11p15.5) like WT1 (11p13). IGF-2 plays a role as a growth promoting hormone and can bind with both IGF-2 receptor and IGF-1 receptor. The expression of IGF-2 is observed in many different tissues and can be found in various stages of tissue development. Upregulation of IGF-2 important for kidney development, results to enhance cellular hyperproliferation and plays a key role in tumorigenesis (Kaneda et al., 2007). The ablation of WT1 leads to inhibit mesenchyme differentiation, and upregulate IGF-2 expression detected by the increasing of ERK1/2 phosphorylation, results to induce Wilms tumor malignant in mice model (Hu et al., 2011). WT1(-KTS) isoform can bind multiple site in the promoter3 of IGF-2, whereas WT1(+KTS) isoform binds to +63/+71 position site at exon 5 with high affinity force. Upregulation of WT1(-KTS) represses IGF-2 promoter3 activity (Drummond et al., 1992). This result showed the tumor suppressor function of WT1. However, WT1 plays an oncogenic function role in various cancers, wild-type WT1 shows the positive regulation of IGF-2 by transcriptional activation of endogenous IGF-2 in Wilms' tumor (RM1) cell line (Nichols et al., 1995). It can confirm that IGF-2 is downstream target of WT1.

2.4.1.6 Insulin-like Growth Factor-I Receptor (IGF-1R)

The transcription of insulin-like growth factor-I receptor (IGF-1R) can be suppressed by WT1. Therefore, the downregulation of endogenous WT1(-17AA/+KTS) or WT1(+17AA/+KTS) isoform by siRNA specific WT1 transcript leads to induce IGF-1R overexpression, decreases cellular survive, and inhibit chemoresistance of anti-cancer drugs such as 1,3-bis(2-chloroethyl)-1

nitrosourea and cisplatin related DNA damage in human glioblastoma cell lines. The result showed growth inhibition of glioblastima cells in dose-dependent manner (Chen et al., 2011). This can confirm that WT1 is an oncogene in glioblastomas. On the other hands, WT1 plays a role as tumor suppressor gene by repression of IGF-1R in Wilms' tumor cells in dose-dependent manner (Werner et al., 1993).

2.4.1.7 Growth factor independent-IB (GFI1B)

Growth factor independent-IB (GFI1B) is transcription factor located downstream of the BCR-ABL translocation in CML cells. It plays roles for commitment and maturation of hematopoietic cell population. GFI1B overexpresses in various leukemic cells including erythropoietic and megakaryocytic malignancies and advance CML. Co-transfection of GFI1B-siRNA and BCR-ABL-siRNA showed significantly decreased of cellular proliferation of K562 cells and further downragulated the expressions of *MDR1*, *MPR1* and *c-Myc* genes (Koldehoff, Zakrzewski, Beelen, and Elmaagacli, 2013).

2.4.2 Leukemia and WT1 expression

Wilms' tumor 1 (*WT1*) gene is a member of the early growth response family (*EGR1, EGR2, EGR3* and *EGR4*). It is located in the nucleus of cells. *WT1* is expressed in the developing kidney, gonads, spleen and mesothelium and brain (Hesketh, 1997). In addition, WT1 has an important in cell signaling during heamatopoiesis development. The expression of WT1 in hematopoiesis progenitor cell is necessary for the survival of progenitor populations. That because, WT1 is co-expressed with Erythropoietin (EPO) which is a crucial factor for embryonic hematopoiesis. The mechanisms of this process are the binding of WT1(-17AA/-KTS)

and EPOR promoter, resulted trans-activation of both EPO and EPOR promoter, driving of hematopoiesis (Scholz and Kotsianti, 2005). In the same, WT1(-KTS) also binds and activates Est1 gene which is one of the transcription factor, generates tumor angiogenesis (Wagner, Michiels, Schedl, and Wagner, 2008). WT1 protein plays an important role in normal genitourinary development. Two alternative splicing have been generated four alternative products: (variant A) WT1(-17AA/-KTS), (variant B) WT1(+17AA/-KTS), (variant C) WT1(-17AA/+KTS) and (variant D) WT1(+17AA/+KTS). However, the ratio of variant A:B:C:D isoforms are 1:2.5:3.8:8.3 have been found in kidney cell (Gu et al., 2010). Furthermore, WT1(+17AA/+KTS) isoform is a predominant variant in all cells which is a strong transcriptional repressor that binding to the sequence of GC-rich of DNA that EGR1 binds as a transcriptional activator. In addition, the behavior of each of WT1 isoform has been difference for example, WT1-KTS isoform plays a role as typical transcription factor that binds to the target genes such as *BCL2*, *c-Myc*, *Wnt4*, *amphiregulin* and *podocalyxin* (Roberts, 2005) and shows high DNA binding affinity. In term of WT1+KTS isoform causes disrupt of DNA binding activity and interact with ubiquitous splice factor U2AF65 and mRNA in vivo resulted coimmunoprecipitation of endogenous WT1-U2AF65 protein. In addition, WT1 protein has also combined or interact to RNA and localized in mRNP (ribonucleoprotein) particles in leukemia cells (Davies et al., 1998). Consistent with prior refer, WT1(+KTS) also plays a role in mRNA metabolism or even splicing process (Herb, 2010).

The high expression level of *WT1* almost found in hematological malignancies such as ALL, AML (Yanada et al., 2004), CML (Na, Kreuzer, Lupberger, Dorken,

and Coultre, 2005) and MDS (Dao and Scheinberg, 2008; Tamaki et al., 2000). There are several studies had summarized that *WT1* gene expression with high level could be used as a molecular marker for prognostic an acute leukemia of childhood (Bergmann, Miething, and Maurer, 1997; Spanaki et al., 2007). With the same reason, WT1 is very sensitive marker to diagnose the present or reappearance of leukemic cells (Cilloni, 2009). The most case of AML patients could be found a high level of *WT1* gene expression but in mild cases of around 10% of them showed normal level of *WT1* expression (Cilloni, 2009). Therefore, some of researchers used *PRAME* which is human melanoma antigen recognized by cytotoxic T cell as co-molecular marker with *WT1* for detection of hematological malignancies including acute and chronic leukemia (Qin et al., 2009). Human chronic myeloid leukemia cell line (K562) was used as positive control for the study of *WT1* expression level in leukemia researchs. K562 cell has a high *WT1* expression level (7,896 *WT1* copies/10^4 ABL copies) using real time RT-PCR (Spanaki et al., 2007). Thirty five percent of childhood with leukemic patients including ALL and AML have been expressed *WT1* level at a mean of 4250±1776.41 *WT1* copies/10^4 ABL copies while very low expression was found in negative control sample (Spanaki et al., 2007). However, *WT1* gene expression has been down regulated after human bone marrow of four patients with acute myelogenous leukemia cells was induced cell differentiation toward myeloid lineage as detected at 10 days of culture. Moreover, *WT1* gene expression was significant increased all of four isoforms after human cytokines such myeloid growth factor: GM-CSF and G-CSF were used for leukemic cell differentiation especially after 20 days of culture when compared with normal hematopoietic cells (Harnandez Calballero et al., 2007). The expression of WT1(-17AA/-KTS) in WT1-expressing

cells led to generate several phenotypes of cell included the induction of small-size cell shape, reduction of cell-substratrum adhesion, and enhancement of cell-migration and invasion in several cancer cell lines. The abnormally changes of cellular morphology affected from the aberration of alpha-actinin and gelsoline gene; a cytoskeletal architecture (Jomgeow et al., 2006).

In the clinical level study, WT1 expresses in bone marrow can be observed however, peripheral blood is a major source for WT1 study. AML patients who have the risk of relapse, WT1 expression is detected to significantly increase when compared to patient who is lower risk. The overexpression of WT1 in AML peripheral blood at diagnosis is associated with poorer leukemia free survival (LFS) which independent of age and cytogenetic risk-group whereas, it is not significant correlation with LFS was observed when bone marrow aspirate was the source for measuring WT1 expression (Gray et al., 2012).

2.4.3 WT1 and apoptosis

The programmed cell death (PCD) is the general process of the cell for regulation of apoptosis. Therefore, the major gold target for destroy cancer cell is focus on the PCD process. However, PCD is classified by three types including type I-III. Type I PCD (classical apoptosis) is involved both intrinsic and extrinsic pathway of apoptosis which are occurred via the mitochondrial destroyed and via death receptors stimulation, respectively. Type II PCD is characterized by the changes of cellular morphology such as autophagic and double-membrane vacuoles. The mitochondria or endoplasmic reticulum is found in the cytoplasm of the death cell. Type I and II are called caspase-dependent apoptosis. The last one is type III PCD

which is caspase-independent apoptosis or called necrosis, this type occurred without pronounced nuclear chromatin condensation (Figure 2.11). Type III of PCD will occur in the cell which shows high level of BCL-2/Bax ratio (Barbier et al., 2009).

WT1 affects both of negative and positive on apoptosis depended on transcriptional control of cell surface receptors, such as IGF1R and EGFR, which both are correlated with BCL-2, BAK, and A1/BFL1 apoptotic gene. Moreover, WT1 can inhibit p53 by binding, stabilization and functional inactivation of p53 protein. In addition, WT1 plays a role as anti-apoptotic function by increasing the resistance of immatinib induced apoptosis in K562 cells. The using of antisense oligonucleotides of WT1 or WT1-siRNA has an ability to promote apoptosis. These indicated that WT1 as a protein with has an important roles to keep the cells alive and cellular proliferation and enhance the risk of malignant transformation (Mayo et al., 1999; Debra J. Morrison, English, and Licht, 2005; Nichols et al., 1995).

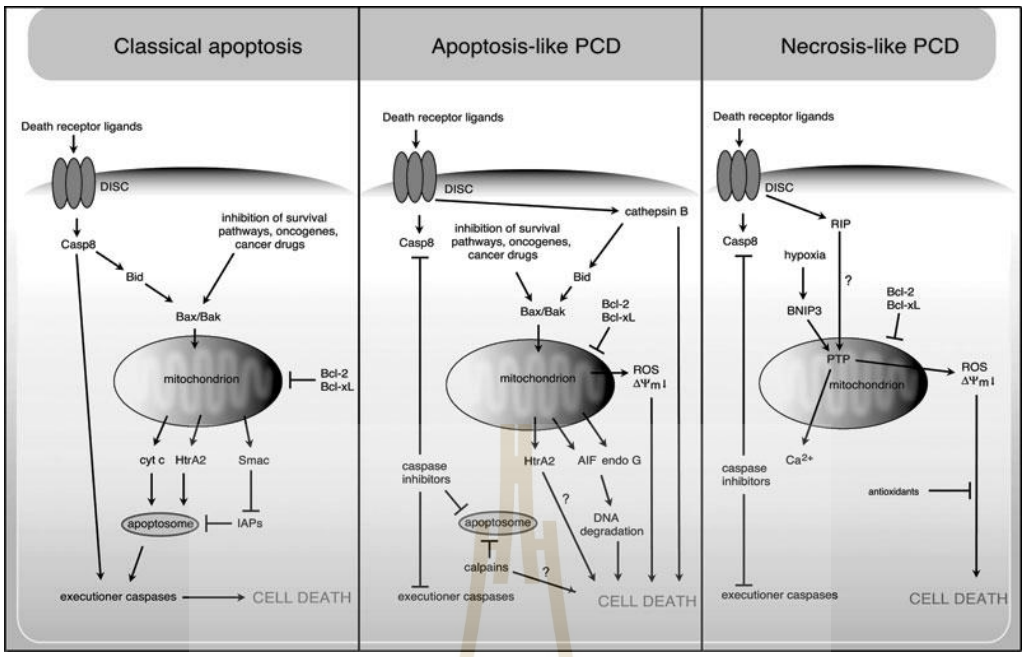

Figure 2.11 The pathways of programmed cell death which contained 3 types. Type I is classical apoptosis, involving both intrinsic and extrinsic apoptotic pathways which is depended on caspases activation. Type II is apoptosis-like programmed cell death which is caspase-dependent pathway. Type III program cell death (PCD) is necrosis or caspase-independent cell death (Barbier et al., 2009; Kogel and Prehn, 2003).

One of the several reports summarized that differentiation of cancer cell by the change of cell shape and the increasing of cellular mobility are causing the expression of WT1 isoform in WT1-expressing cells. WT1(-17AA/-KTS) isoform is the target for their studies. They found that WT1(-17AA/-KTS) led to significantly reduce cellular attachment ability approximately 35-40% when tested with the specific extracellular metrix (ECM); fibronectin, by observation of the number of unattached cells after treat with 3 types of ECM including fibronectin, collagen I, and laminin. However, the attachment of cancer cell is relating between extracellular matrix substratum and cellular adhesion molecule such as integrin subunit. The induction of

cellular attachment resulted to reduce cancer metastasis property and vice versa. WT1(-17AA/-KTS) can regulates integrin subunit led to control the motility of cells. These results can explain that WT1(-17AA/-KTS) inhibits the movement of cancer cells via integrin signaling pathway. Moreover, the integrin subunit will bind with actin binding complexs within the intracellular of cell surface such as alpha-actinin I, gelsolin, and cofilin. All of these three complexes are the major proteins target of WT1 (-17AA/-KTS) isoform (Jomgeow and Tima, 2009). Furthermore, the induction, degradation and rearragement of filamentous actin (F-actin) are the choices for type III programmed cell death stimulation of cancer cells. In addition, the ratio of F-actin and G-actin is decreased by the induction of type III programme cell death by CD47 ligation on CLL cells.

Despite WT1is known as apoptosis regulator but it is depended on cell type. The different WT1 isoforms exhibit different effects which vary by the nature of cells. BCL-2 is an early identified gene for direct target of WT1. The first role of WT1 function is tumor suppresser gene which it can suppress the BCL-2 promoter resulted to induce the apoptosis of cells (Hewitt, Hamada, McDonnell, Rauscher, and Saunders, 1995). Moreover, WT1(-17AA/-KTS) also inhibit the BCL-2 promoter activation in the transiently transfected Hela cells. Therefore, the downregulation of WT1 would lead to upregulate the expression of BCL-2. However, some report revealed that WT1(-KTS) isoform can activate the BCL-2 promoter led to demonstrate coexpression of WT1 and BCL-2 in Saos2 cells (Mayo et al., 1999). In summarized the role of WT1 on apoptosis induction or suppression is depended on cell types such as total WT1 can caused the increasing of apoptosis in the metanephric blastema cells; caused the suppression of endogenous BCL-2 in DHL-4 cells and

activation of K562 cells survival. WT1(+17AA/+KTS) has the role for induction of apoptosis in HepG2 cells and in the other hand it also suppresses of caspase-3 and -9 and Bax expression. Among WT1(+17AA/-KTS), it has the responsibility for induction of apoptosis in Saos-2, U2OS, and M1 leukemic cells. Moreover, it can activate BCL-2 promoter in Saos-2 and CV1 cells and also downregulate pro-apoptotic Bax expression. WT1(-17AA/+KTS) isoform can activate apoptosis in HepG2 cells and also suppresses executioner caspase-3 and -9 and pro-apoptotic Bax expression like WT1(+17AA/-KTS) isoform. The last WT1(-17AA/-KTS) isoform which can induce BCL-2 promoter of Saos2 and CV1 cells. Furthermore, it also inhibits the BCL-2 promoter of Hela cells and other function is upregulation of Bfl-1/A1 expression (Simpson et al., 2006). In addition, WT1 indirectly acts with BCL-2 by coexpression of interacting gene such as *Par-4*, the Par-4 binds along with WT1 binding site on the BCL-2 promoter resulted to suppress the activity of BCL-2 promoter (Cheema et al., 2003). In the molecular structure of Par-4, it has leucine-zipper domain which is crucial for cooperation with WT1 to regulate apoptosis-specific gene transcription (Sells et al., 1997). In addition, downregulation of WT1 through specific-RNAi also induces cancer cell death and promotes the sensitization of anti-cancer drugs including doxorubicin and cisplatin in B16F10 murine melanoma cell line. The result showed that viability of B16F10 is reduced from 80% in cells treated with cisplatin alone to 20% using WT1-2 RNAi/cisplatin combination, while WT1-2 RNAi/doxorubicin combination resulted in 38% cell viability compared to 60% viable rate from doxorubicin alone. Moreover, the inhibitory effect of WT1-2 RNAi/drug combination are 91% and 82% when

simultaneously treated with ciaplatin and doxorubicin, respectively (Zapata-Benavides et al., 2012).

2.4.4 WT1 and cellular proliferation

WT1 plays an essential role for cellular proliferation. There was a report which concluded that WT1 is a surrogate marker of hematopoietic cells. Researchers revealed that the stimulation of hematopoietic cell growth by cytokines combination led to increase cellular proliferation and simultaneously upregulates the expression of *WT1* by quantitative RT-PCR and CD71 antibody staining analyzed by flow cytometry. The results showed that after the samples of peripheral blood of healthy volunteer, umbilical cord blood, and peripheral blood mononuclear cells were stimulated the cellular proliferation by addition of cytokines: GM-CSF and CSF in the culture medium then cultured for 21 days. The data indicated that at day 14 the highest proliferation was observed all cell groups which are the same result of the data of K562 positive control. Moreover, their report also showed a high expression level of WT1 at day 14 of culture. This report supports that WT1 is a crucial factor for cellular proliferation. Therefore, the high expression of WT1 could be used for monitoring of acute leukemia relapse (Olszewski, Huang, Chou, Duerst, and Kletzel, 2005). In addition, WT1 is also known as a tumor cell proliferation marker. The overexpression of WT1 in leukemic cell such as K562 and HL-60 also causes the induction of proliferation, in contrast, once WT1 was downregulated cellular growth was inhibited and cell population was also accumulated in G0/G1 phase of cell cycle (Kerst et al., 2008). In the MRD patients, the high level of WT1 expression was observed as increasing in accordance with the aggressiveness of diseases subtype in

MDS such as RARS which showed the highly increase the WT1 level from 570 to 67,000 copies/μg (Tamura et al., 2010). Not only leukemic cells, WT1 also plays a role in neuroblastoma cells proliferation. However, the report indicated that the WT1 mRNA expression level was not correlates with the histologic grade, Evans stages (I, II, III, and IV), and prognosis of neuroblastoma cells. Especially, in NB69 cells which found the overexpression of WT1, these cells were significantly promoted the proliferation after endogenous WT1 was knockdowned by WT1 antisense oligonucleotides in neuroblastoma cells. This data indicated that WT1 plays a key role for suppression of neuroblastic cells proliferation, however, WT1 expression is observed in differentiation tumor cells more than primitive immature of neuroblastoma cells (Wang et al., 2011). This indicates consistent with various cell lines including, leukemic cell line (M1) studied in vivo (C.B.-17 scid/scid mice) (S. I. Smith, Down, and Boyd, 2000) and prostate tumor (Fraizer, Leahy, and Priyadarshini, 2004). The exogenous WT1(-17AA/-KTS) isoform has been transfected into leukemic cells (NB4), the data showed inhibitory effect of NB4 cells growth. Moreover, the ratio of S-phase in WT1(-17AA/-KTS) transfected-NB4 cells have also been increased and supported the expressions of p21 and cyclin A1 proteins which are essential for cellular proliferation (Shen et al., 2006). That indicates the growth regulatory role of WT1 which depended on cell types.

2.4.5 WT1 and hematopoietic differentiation

In the normal hematopoietic cells, WT1 expression is observed in $CD34^{+}$ cells. Consequently, it will be reduced during cellular differentiation and cannot detect WT1

expression in the mature cells (Baird and Simmons, 1997). Differentiation induction of leukemic cells has many possible mechanisms included;

1) Membrane-mediated event and signal transduction pathway which interaction of growth factors and cell surface receptors on the cells. Signaling was generated from the plasma membrane into the nucleus in order to support up- and/or downregulation of genes expression (Ghysdeal et al., 2000). Polar/apolar chemical inducers cause pulse shift at the level of plasma membrane, alterations in cell surface architecture, reduction in cell size, activation of $Ca2^{+}$ ion transport early in the precommitment phase, and other biochemical events including shortening of cell cycle G1 phase (Tsiftsoglou and Robinson, 1985).

2) Receptor-mediated processes which base on the differentiation induction via receptor-like cellular components. Bisacetamides, hydroxamic acid and ureidopyridine derivatives have been used for forming a complex with intracellular components to generate receptor-like cellular components (review of Tsiftsoglou et al,2003).

3) Remodeling of the superfine structure of chromatin which occurred via nuclear condensation and irreversible of DNA replication of MEL cell differentiation. This differentiation correlates with upregulation of alpha and beta-globin genes via increase in DNAse I hypersensitivity at a sequence of both genes.

4) Alterations in transmethylation of DNA and RNA. DMSO induces methylation of total cytoplasmic and $polyA^{+}$ RNA. However, increasing of RNA methylation may affect their physicochemical behavior and facilitate transport from the nucleus into cytoplasm.

5) Cell lineage restricted transcriptional factors acting as regulators of reprogramming of hematopoietic cell fate under normal and leukemia state. GATA-1 can activate erythroid lineage differentiation, GATA-2 serves as cell survival and proliferation, p53 activates along progenitors and erythroids differentiation, and c-Myc activates hemaopoietic cells differentiation.

6) The potential role of protooncogenes in differentiation and apoptosis. The c-Myc affects cell differentiation and apoptosis involved in reprogramming of hematopoietic progenitor cells by releasing of cytochrom c from mitochondria to cytosol. This event generates CD95/Fas ligand which necessary for activation of apoptosis via caspases pathways.

The role of WT1 in myeloid differentiation has been evaluated that the isoform of WT1(-17AA/-KTS) has the potential for suppression of Cyclin E expression resulted to inhibit cell cycle progression of G-CSF mediated differentiation (Loeb et al., 2003) while, the overexpression of WT1(+17AA,+KTS) inhibit G-CSF mediated differentiation (Inoue, Tamaki, Ogawa, and al., 1998). In addition, BCL-2 family member; *A1* is classified as the direct target gene of WT1. It is usually used for differentiation marker of myeloid and apoptotic resistance detection in hematopoietic cells. WT1(-17AA/-KTS) isoform can bind and upregulate to the endogenous A1 promoter resulted in chemotherapy resistance and spontaneous differentiation upon IL-3 withdrawal of murine myeloblast (32D cl3) cells (Simpson et al., 2006). This differentiation was observed by both morphological change such as nuclease condensation and circular shape of nuclei induction and cell-surface antigen changes especially it shows significantly induce of granulocytic marker Gr-1 (neutrophil

specific antigen) and CD69 (marker of activated monocytes and nuetrophils) expression.

The role of WT1 in lymphoid differentiation, the reduction of WT1 expression level was observed during hematopoietic progenitor cells differentiation, rather more in mature cells. Moreover, in $CD34^{+}HLA\text{-}DR^{-}$ and $CD34^{-}CD33^{+}$ primitive leukemic cells, WT1 expression was significantly higher than more mature progenitor differentiated $CD34^{+}HLA\text{-}DR^{+}$ and $CD34^{-}CD33^{-}$ leukemic cells, respectively. On the other hands, mature T-cell and B-cell ALL cell lines such as Molt-3, Molt-4, Tall-1, Jurkat, and Ball-1 showed very low levels of WT1 expression. This suggested that the differentiation of leukemic cells correlated WT1 expression level and downregulation of WT1 can observe during leukemic cells differentiation (Inoue et al., 1997). In addition, CCTCT-binding factor (CTCF) is a highly conserved 11 zinc-finger protein, it is the multifunctional protein that can activate or repress transcription and as the regulator of promoter-enhancer interactions. WT1 transcription factor can regulate *Wnt4* gene in mouse model by control state of chromatin in CTCF domain. Although, WT1 exhibits indirect regulation of CTCF, the correlated function between WT1 and CTCF proteins is suggested the role of WT1 control hematopoietic cell differentiation via CTCF. The overexpression of CTCF suppresses apoptotic induction and enhances proliferation of leukemic cells. Thus, inhibition of CTCF activates apoptotic cell death and decreases leukemic cell growth (Gurudatta and Corces, 2011; Zhang et al., 2014). Knock-down of CTCF using antisense oligonucleotides can regulate K562 myeloid differentiation into erythroid lineage and not affect for megakaryocytic lineage (Torrano et al., 2005).

2.5 RNA interference technology

RNA interference (RNAi) is the mechanism that supports gene regulation of the cell. The short stretches of 23-25 nucleotide double strand RNAs are designed as siRNA (small interfering RNAs). The complementary of mRNA can be able to degrade by siRNAs which have a specific sequence of mRNA target. The double stranded RNAs is cleaved by the RNase III family member; dicer and generates small interfering RNA (siRNA). The mRNA of interest is degraded by one strand of siRNA which associated with a protein assembly called the RNA induced silencing complex (RISC). Napoli and co-workers (Napoli, Lemieux, and Jorgensen, 1990) reported about gene silencing mechanism in plants at the first time in 1990. Cejka and co-workers (Cejka, Losert, and Wacheck, 2006) referred that the use of antisense oligonucleotide and short single stranded stretches of DNA or RNA with complementary sequence to their target mRNA, was the state of the art for gene silencing experiment. The suppression of interested gene by injected double stranded RNA into in *Caenorhabditis elegens* resulted in potent gene silencing, which was confirmed as a mechanism by Fire and co-workers (Fire et al., 1998)

RNAi is based on posttranscriptional gene silencing. There are three steps for general model describing RNAi mechanisms. First, when double strand RNA was introduced into cells. It was recognized by dicer, as enzyme of RNase III family of dsRNA- specific ribonucleases. Double strand RNA was cleaved to 19-23 bp with 2-nucleotides overhangs in 3’ end. Second, four different subunits of RNA induced silencing complex (RISC) contained with a helicase, exonuclease, endonuclease and homology searching domains. This complex has a specific catalytic activity (slicer) that has been suggested to selectively degrade on of the strands. Double strand RNA

was incorporated into a multiprotein of RISC complex, this process required 5' phosphorylation (Banan and Puri, 2004; Nykanen, Haley, and Zanore, 2001; Tomari and Zamore, 2005). Third, siRNA is unwound to be a single strand. Antisense strand which bind to the RISC complex plays a role for complementary with target mRNA and then cleaved at a single site in a specific region between the duplex of siRNA and target mRNA. By mean, mRNA degradation by endonuclease and exonuclease at 10 nucleotide from the 5' end of the siRNA (Elbashir, Lendeckel, and Tuschl, 2001). However, siRNA has been protected from the recognition of nuclease in cell when the single strand RNA was loaded by RISC and it can cleavage the target mRNA. Masiero and co-workers refer to the successful to introduce siRNA into the cytoplasm without the cleavage by Dicer. They found that introduction of chemically synthesized siRNA into the cytoplasm bypassing the first step (Dicer cleavage) (Figure 2.12). Another, used of plasmids or viral vectors for carried siRNA or shRNA. The last requirement, start with the nuclear cleavage by the Drosha RNase III endonuclease to generate miRNAs with 60-70 nt stem loop.

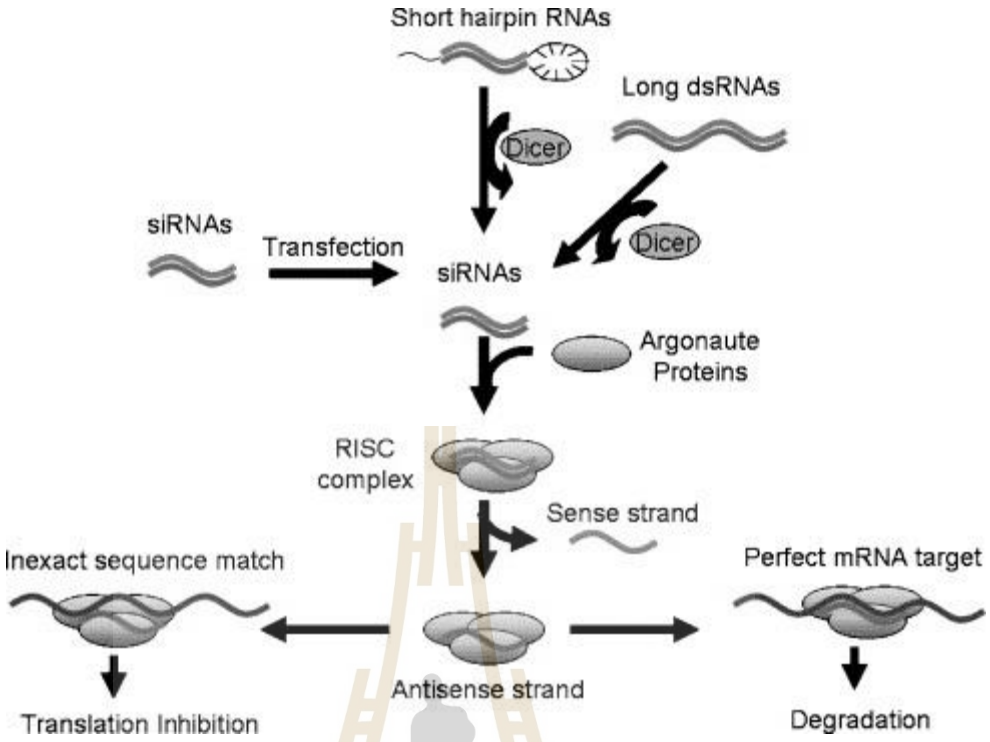

Figure 2.12 siRNA interference mechanism: short hairpin RNAs and long dsRNA are processed by Dicer to form siRNA. Small interfering RNAs can also be directly delivered into cells through transfection. The double-stranded siRNA complexes bind with argonaute proteins to form the RISC complex. The sense strand is removed from the double stranded siRNA. This leaves the antisense strand to anneal to the target mRNA. When the sequence has a perfect match, the target mRNA is degraded, but if the sequence has an inexact sequence match, the RISC complex blocks mRNA translation (Tebes and Kruk, 2005).

2.6 Biology of lentivirus and lentiviral transduction efficiency

Recently, lentivirus is an important tool for gene transfer both *in vitro* and *in vivo*. Lentivirus is another class of retrovirus that be able transduced into both dividing and non-dividing cells which made them especially attractive for gene

transfer into differentiated and non-dividing cells (Blesch, 2004). The original studies about viral-mediated gene transfer has been used the retroviruses as vectors. Since it has strong advantage, retroviral vector genomic sequences can integrate into the host genome which led it able for stable transduction. The retrovirus contains of a double strand RNA genome which consisting of 5' long terminal repeats (LTR) for viral integration and viral gene transcription regulation. Other region nearly the 5' LTR is the region for tRNA primer binding and reverse transcription start site. Followed by, the region of *gag*, *pol*, and *env* genes that encode for the viral structure proteins. The *gag* gene encodes the protease and reverse transcriptase/RNaseH, whereas, *pol* gene encodes integrase enzymes, another, *env* gene encodes envelope glycoproteins. Prior of 3' LTR, it has a region of purine-rich which supports for the initiation of plus strand cDNA synthesis during reverse transcription. The 3' LTR serves for polyadenylation signals (Coffin, 1996; B. E Huber and I. Magrath, 2007). A little bit different to retroviruses, lentiviruses are identified as HIV-base vectors that are subclass of retroviruses. HIV-vector or lentiviral vector consists of six additional genes including *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*. In addition, HIV-base vector uses VSV -G protein pseudotyping for viral construction (B. E Huber and I Magrath, 2007). Lentivirus virions are produced by the packaging cell line (293T cells), the packaging vectors are encoded for construction of lentiviral then the virus particles have been released out of the 293T cells by membrane fusion. Lentivirus particles entry into cells is begun by the binding of the env-encoded glycoprotein to its cellular receptor such as phosphatidyl serine. After binding, the virus envelope fuses with the plasma membrane and genome of virus is trigger to host nuclease then integration with host genome (Coffin, 1996) (Figure 2.13).

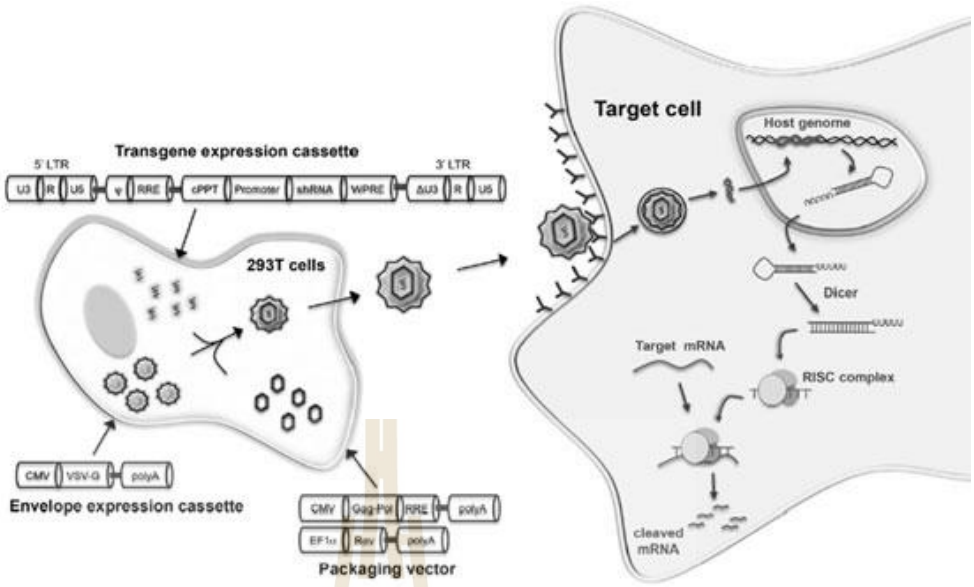

Figure 2.13 Schematic of non-replicating lentiviral vector for stable shRNA expression (Available from WWW.creative-biogene.com by (Heiser)).

Lentivirus was transduced into target cell as difficult as depended on cell type. The enhancement of lentiviral transduction efficiency was improved by several research groups. For example, the uses of condition medium derived from dyeing monocytes (MCM) in the initial stage of lentiviral transduction and supported 2-10 folds of transduction efficiency into Monocyte dendritic cells (Masurier et al., 2007).

CHAPTER III

MATHERIALS AND METHODS

3.1 Materials and Methods (Part 1: Study the effect of WT1-siRNA on leukemic cell line)

3.1.1 Cell culture

Human chronic myeloid leukemic cell line (K562) was cultured in complete RPMI (cRPMI) including Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, USA), penicillin (100U/ml), streptomycin (100 µg/ml). K562 cells were maintained in a humidified incubator at 37°C and 5% CO_2. Human embryonic kidney cells (293T/17) were used as packaging cells. These cells were cultured in complete DMEM (cDMEM) contained with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) supplemented with 10% FBS (Hyclone, USA), penicillin (100U/ml), streptomycin (100 µg/ml). Cells were maintained in a humidified incubator at 37°C and 5% CO_2. cDMEM medium with 4.5 g/L glucose and without antibiotic was used for lentiviral production processes.

3.1.2 Plasmids preparation, lentiviral production, titration, transduction, and GFP$^+$ cell sorting.

3.1.2.1 Plasmids preparation

Five plasmids including pPRIME-CMV-GFP-FF-WT1-siRNA (WT1-siRNA), pPRIME-CMV-GFP-FF-control-siRNA (C-siRNA), and packaging plasmids including pLP1 (encodes icosahedral capsid (gag) and reverse transcriptase polymerase enzyme (pol) genes), pLP2 (encodes Rev protein), and pPLv (encodes psuedotype of vasicular stomatitis virus glycoprotein (VSV-G))**.** Each plasmid was amplified by transformation competent *E.coli* (XL-10 Gold) according to transformation protocol (Stratagene, USA). The best clone of each plasmid was selected and cultured in 10 ml starter of NZY$^+$ broth for 8-12 hours by shaking at 250 rpm in 37°C shaking incubator. The mixture was then transferred into 400 ml of NZY$^+$ broth and further incubated at 37°C shaking incubator for 16-20 hours for bacterial expanding. Total of bacteria was packed by centrifugation at 5,000 rpm for 10 minutes. Plasmid extraction was performed using Purelink Hipure Plasmid Mexiprep kit (Invitrogen, USA). Briefly, packed cells were completely suspended with 10 ml resuspension buffer, then cells were lysed with 10 ml lysis buffer and mixed gently. Lysed cells were precipitated by 10 ml precipitation buffer and centrifuged at 12,000 x g for 10 minutes. Supernatant was loaded onto the equilibrated column and allowed the solution to drain by gravity flow. The column was washed by 60 ml of wash buffer and the flow-through was discarded. The plasmid was eluted from the column by 15 ml elution buffer. The eluted plasmid was precipitated by 10.5 ml isopropanol and centrifuged at 12,000 x g, 4°C for 30 minutes and supernatant was discarded. Next, DNA pellet was washed by 5 ml of 70% ethanol and centrifuged

at 12,000 x g, 4°C for 5 minutes prior to remove the supernatant. The purified plasmid DNA was resuspended with 200 µl sterilized nuclease free water. Plasmids concentration was measured (Nanodrop spectrometer). Plasmids were stored at -20°C until use.

3.1.2.2 Lentiviral production

Lentiviral supernatant was produced by calcium chloride precipitation method. The mixture of lentiviral vectors containing 4 plasmids including 6.5 µg of pLP1, 2.5 µg of pLP2, 3.5 µg of pLPv and either 10 µg of WT1-siRNA or 10 µg of C-siRNA were prepared in 0.5 ml of 0.25 mM CaCl2 and BBS buffer. The mixture was then incubated for 15 minutes at room temperature and transfected into 293T/17 packaging cells (80-90% confluence). Cultured dish was incubated at humidified, 37°C, 5% CO_2 for 18 hour. The medium was removed from the dish after incubation. The fresh high-glucose DMEM medium without antibiotic was then added into the culture. The dish was re-incubated for 24 hours for lentiviral supernatant harvesting.

3.1.2.3 Lentiviral titration

Lentiviral supernatant was harvested and centrifuged at 1,200 rpm, 4°C, 5 minutes for removing cell debris. The supernatant was then filtrated through 0.45 µm low-protein attach syringe filter. Serial dilution of viral supernatant was prepared for viral titer determination. Briefly, the $4x10^5$ cells of 293T/17 cells were overnight cultured in 6-well plate before titration process. The serial dilution of filtrated lentiviral supernatant with 16 µg/ml final concentration of polybrene was transduced into 293T/17 cells, and incubated at 37°C, 5% CO_2 for 3 days. After incubation, 293T/17 was trypsinized and washed with PBS. One milliliter of cell

suspension was prepared for GFP checking by flow cytometry (FACSCalibur, Becton Dickinson, USA) using CellQuest Pro Software vision 5.2.1. The percentage of GFP positive cells were represented as population of transduced cells. Lentiviral titer was calculated by the formula as followed:

$$\text{Lentiviral titer} = \frac{\%\ GFP^{+}\ \text{cells} \times \text{seeding number of 293T/17} \times \text{dilution factor}}{100}$$

Lentiviral titer unit represents in TU/ml (Transduction Unit/ml)

3.1.2.4 Lentiviral transduction

K562 cells were cultured in cRPMI medium overnight prior to transduction. At the time of transduction, lentiviral supernatant was concentrated by ultracentrifugation at 28,000 rpm, 4ºC for 2.06 hours. After that, lentiviral pellet was suspended with high glucose DMEM without antibiotic at 10X final concentration. Total $2x10^6$ cells of K562 were added into 10X viral supernatant containing 8 μg/ml of polybrene. In order to increase viral transduction efficiency, spin transduction at 1,800 rpm, 25ºC, for 45 minutes was performed. After that, cells were incubated for 12 hours at 37°C, 5% CO_2 in 90% humidity. Transduction approach was performed triplication using fresh concentrated lentiviral supernatant. After finishing of the third transduction, cells were incubated in cRPMI medium for 48 hours at 37°C, 5% CO_2 in 90% humidity. Forty eight hours after transduction, K562 cells with GFP expression was sorted by flow cytometry and cell sorter (FACs). K562 cells which were transduced with WT1-siRNA and exhibit GFP expression referred to

K562-WT1-siRNA-GFP$^+$ cells while K562 cells which were transduced with C-siRNA and showed GFP expression referred to K562-C-siRNA-GFP$^+$ cells.

3.1.2.5 Cell sorting

K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were elementary checked by fluorescent inverted microscope. These cells were sorted based on GFP expression by FACSVantage cell sorter (Becton-Dickinson). For this step, K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were harvested and centrifuged at 1,200 rpm, 4°C, 5 minutes. Cells were washed twice with PBS and resuspended with 5 ml of PBS. The concentration of cell suspension should be around 500,000-800,000 cells/ml to avoid cell clumping in the FACs nuzzle. Prior to sorting process, the workflow system of the machine was cleaned for protection of contamination in the sorted cell population. The K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were then separately collected and centrifuged at 1,500 rpm, 4°C, 5 minutes and washed with PBS. Cells were cultured in cRPMI medium for the further experiments.

3.1.3 Cell proliferation assay

The K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were separately plated at density of 10,000 cells/well in 100 µl of cRPMI medium in 96-well plate and incubated at 0, 3, 6, 12, 24, 48, 72, and 96 hours. At the end of each time point, 20 µl of CellTiter 96 Aqueous One solution reagent (Promega: USA) were added into each well and incubated for 2 hours at 37°C with 5% CO_2. In order to stop the reaction, 25 µl of 10% SDS was added into each well. The absorbance of each well was measured at 490/620 nm using ELISA plate reader.

3.1.4 Apoptosis assay using Apo-One Homogeneous Caspase-3/7 reagent

The K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were separately plated at a density of 10,000 cells/well in 100 µl of cRPMI medium in 96-wells plate. At specific time points: 0, 3, 6, 12, 24, 48, 72, and 96 hours 100 µl of Apo-One Homogeneous Caspase-3/7 reagent (Promega, USA) were added into each well. The plate was incubated for 4 hours at room temperature and protected from the light. The absorbance at $499_{Ex}/521_{Em}$ nm was recorded using a fluorescence spectrometer. The absorbance value was represented by caspase-3 and -7 enzymes activity which correlated with apoptosis induction.

3.1.5 Apoptosis assay

Apoptosis induction was confirmed using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, USA). The K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were separately incubated at 0, 3, 6, 12, 24, 48, 72, and 96 hours. Total of $1x10^5$ cells of K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were packed and washed twice with PBS, then cells were resuspended with 100 µl of 1X buffer solution, 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI). The reactions were incubated for 15 minutes at room temperature in dark. After that, 400 µl of 1X buffer solution was added into the tube and apoptotic cells were analyzed by flow cytometry (FACSCalibur) (Becton-Dickinson) using Cellquest Pro software.

3.1.6 mRNA expression by RT-PCR

3.1.6.1 Total RNA extraction

Total RNA of treated cells: K562-WT1-siRNA-GFP$^+$ cells and control cells: K562-C-siRNA-GFP$^+$ was extracted by Total RNA purification Mini Kit (Geneaid, Taiwan) according to manufacturer's protocol. Total RNA concentration was measured by Nanodrop ND-1000 spectrometer (Thermo Fisher Scientific Inc., USA.). For protection of RNA degradation, 1 μl of RNase Out (Invitrogen, USA) was added into 25 μl of total RNA. RNA was stored at -70°C until used.

3.1.6.2 Primers

Various primer sets were used to amplify the specific genes. The sequences of primers, annealing temperature, PCR product size, and the amplified position are demonstrated in the Table 3.1

Table 3.1 Primer sequences and PCR conditions involved in the experiment.

Genes	Primer sequences	Annealing temp. (°C)	Product Size (bp)	References
WT1	R:5'-TCAAAGCGCCAGCTGGAGTTT-3' F: 5'-AGACATACAGGTGTGAAACC-3'	51	225	(Damon et al., 2000)
IL2	R:5'- TGGGAAGCACTTAATTATCAAGTC-3' F:5'-CGTAATAGTTCTGGAACTAAAGGG -3'	60	150	(Kim et al., 2006)
IL-2RB	R:5'-CGGTGTTCCTGCAGTTG-3' F:5'-CAGTATGAGTTTCAGGTGCG-3'	50	205	(Kim et al., 2006)
IL-2RG	R:5'-CCAACAGAGATAACCACGG-3' F:5'-CGCTACACGTTTCGTGTTC-3'	60	152	(Kim et al., 2006)
GAPDH	R: 5'GTACTCAGCGGCCAGCATCG-3' F: 5'-AGCCACATCGCTCAGACACC-3'	60	302	(Yao et al., 2006)

3.1.6.3 Complementary DNA (cDNA) synthesis

Total RNA was converted into cDNA using Superscript First-Strand Synthesis System (Invitrogen, USA). The reverses transcription was performed in a final volume of 24 µl reaction mixed which containing of 1X RT buffer, 0.83 mM of deoxynucleotide triphophate solution (dNTPs mixed), 2.08 pmole of specific reverse primer, 100 units of reverse transcriptase, 700 ng of RNA template and adjust to final volume by diethylpyrocarbonate (DEPC) treated water. After incubation at 42ºC for 60 minutes, the reverse transcription reaction was terminated by heating at 95ºC for 5 minutes. The newly synthesized specific cDNA was amplified by PCR or kelp at -20°C until used.

3.1.6.4 Polymerase Chain Reaction (PCR)

The PCR mixture containing 1X PCR buffer, 0.2 mM of dNTPs mixed, 2 mM of $MgCl_2$, 0.2 pmole of primers (Table 3.1), 1 unit of Taq DNA polymerase, 10.0 µl cDNA was prepared and adjusted to 25 µl/reaction by DEPC treated distill water. The PCR reaction was started by heating the PCR mixture at 95°C for 5 minutes for denature initiation, then 40 cycles of amplification step were done at 95°C for 1 minute, appropriated annealing temperature (Table 3.1) was set for 30 seconds, 72°C for 45 seconds in extension step then followed by 72°C for 5 minutes in final extension and held the reaction at 4ºC until use. PCR product was loaded on 1.5% agarose gel and electrophoresis was performed. One hundred base pair DNA ladder was used for DNA marker. PCR products were stained with ethidium bromide and UV-visualized by Gel Documentation system.

3.1.7 Western blot analysis

3.1.7.1 Protein lysate preparation

K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were harvested at specific time and washed with PBS. Cells were packed by centrifugation at 1,200 rpm, 4°C, 5 minutes and lysed by CellLytic M reagent with the ratio of 10^6 cells/100 µl of CellLytic M reagent (Sigma, USA) for 15 minutes on shaker. Protein lysate was cleared by centrifugation at 12,000 rpm for 15 minutes for packing the cellular debris. Collection of protein-containing-supernatant was performed and replaced to microcentrifuge tube.

3.1.7.2 Protein concentration determination (Bradford)

Protein concentrations were determined using Bradford protein assay (Sigma, USA). The first step is BSA standard preparation; BSA was prepared for 200, 400, 800, 1,000, 2,000, and 3,000 µg/ml. The standard 3.1 ml Bradford assay consists of mixing 1 part of the protein sample (or BSA standard) with 30 parts of the Bradford reagent. The mixture was incubated for 5-45 minutes at room temperature and light protection. The color development of mixed-BSA standard was detected at 595 nm absorbance by nanodrop ND-1000 using Bradford protocol. Protein concentration determination is performed with the same method of BSA standard. Then, the absorbance of protein sample was compared to the standard curve and the protein concentration was presented in the unit of µg/ml.

3.1.7.3 Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared by 10% separating gel and 3.5% stacking gel. Twenty micrograms of protein was mixed with loading dye and boiled for 5 minutes for protein denature before loading into the SDS-PAGE. The Benchmark prestained protein ladder (Invitrogen, USA) was used for determination of protein markers. Protein was separated on SDS-PAGE by 120 Volt, 70 mAmp of constant current for 1-2 hours in the running buffer. The separated protein was transfered to Polyvinylidene difluoride (PVDF) membrane (Whatman, USA) using blotting buffer for 1 hour with 100 Volt, 400 mAmp. The membrane was blocked by blocking buffer (5% skim milk) overnight. The membrane was washed twice with 1% skim milk and probed with specific primary antibodies (1:100 of polyclonal anti-WT1 antibody (Santacruz, C19) or 1:1000 of polyclonal anti-actin antibody (Santacruz, H196) or 1:1000 of anti-caspase-7 antibody (Sigma, C7724) in 1% skim milk at room temperature by rocking for 2 hours or overnight for caspase-7 antibody. The membrane was washed three times with 1% skim milk for 10 minutes. Consequently, the membrane was probed with secondary antibody which is horseradish peroxidase conjugated either with anti-mouse or anti-rabbit secondary antibodies (1:5000 of horseradish peroxidase conjugated polyclonal goat anti-rabbit IgG antibody (Santacruz, SC-2004). The reaction was incubated by gentle shaking for 1 hour. The secondary antibody was removed and the membrane was washed three times with 0.1% blocking buffer. The complex was detected with Super Signal West Pico chemiluminescent mixing reagents (Pierce) for 5 minutes and exposed to the film.

3.2 Materials and Methods (Part 2: Study the effect of WT1-siRNA on primary leukemic cells)

3.2.1 Primary blood sample collection and cell preparation

Peripheral blood (PB) was collected from sixteen new diagnosis childhood patients with acute lymphoblastic leukemia with informed consents and the approval of Suranaree University of Technology and Maharaj Nakhonratchasima hospital's Ethics Committees. Diagnosis for each patient was made according to the FAB criteria (Bennett et al., 1976). PB was also obtained from healthy volunteers and used as normal controls. Mononuclear cells (MCs) were isolated using Ficoll-paque Plus (GE Healthcare:Sweden) density gradient and centrifugation with 400 x g at 25°C for 30 minutes. Peripheral blood mononuclear cells (PBMCs) were collected from the interface of the density gradient separation for WT1-siRNA transduction.

3.2.2 Lentiviral production and transduction

Lentivirus was produced by using the same protocol above. Viral supernatant of WT1-siRNA and C-siRNA were concentrated and resuspended with cDMEM without antibiotic for 30X final concentration. Total $2x10^6$ cells of PBMCs were added into 30X viral supernatant presented of 32 μg/ml polybrene and 10 mM HEPES (Sigma: H9268). For transduction efficiency enhancement, lentiviral supernatant was spin at 1,800 rpm, 25ºC, for 1 hour. After that, cells were incubated for 6 hours at 37°C, 5% CO_2 in 90% humidity. Transduction approach was performed for three times. Cells were incubated in cDMEM medium for 48 hours at 37°C, 5% CO_2 in 90% humidity. Acute lymphoblastic leukemic cells (ALL) subtype L1 which express

GFP after WT1-siRNA and C-siRNA transduction referred for L1-WT1-siRNA cells and L1-C-siRNA cells, respectively. In the same hand, PBMC from normal volunteer which was transduced with WT1-siRNA or C-siRNA referred to PBMC-WT1-siRNA cells and PBMC-C-siRNA cells, respectively.

3.2.3 Cell proliferation assay

The L1-WT1-siRNA cells, L1-C-siRNA cells, PBMC-WT1-siRNA cells, and PBMC-C-siRNA cells were separately plated at density of 10,000 cells/well in 100 µl of cRPMI medium in 96-well plate and incubated at 0 and 48 hours. At the end of each time point, 20 µl of CellTiter 96 Aqueous One solution reagent (Promega: USA) were added into each well and incubated for 2 hours at 37°C, 5% CO_2. In order to stop the reaction, 25 µl of 10% SDS was added into each well. The absorbance of each well was measured at 490/620 nm using ELISA plate reader.

3.2.4 Apoptosis assay (Caspase-3/7 activity)

Forty-eight hours after transduction, L1-WT1-siRNA cells and L1-C-siRNA cells or PBMC-WT1-siRNA cells and PBMC-C-siRNA cells were plated at a density of 10,000 cells/well in 100 µl of cDMEM in 96-well plate. At 0 and 48 hours post-transduction, cells were evaluated apoptotic enzyme activity using Apo-One Homogeneous Caspase 3/7 reagent according to manufacturer's protocol. The absorbance at $499_{Ex}/521_{Em}$ nm was recorded using a fluorescence spectrometer. The absorbance value was represented by Caspase-3 and -7 enzymes activity which correlated with apoptosis induction.

3.2.5 Apoptosis assay (Annexin V-FITC/PI)

Apoptosis induction was confirmed using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen: Cat.No. 556547). At 0 and 48 post-transduction, L1-WT1-siRNA cells, L1-C-siRNA cells, PBMC-WT1-siRNA cells and PBMC-C-siRNA cells were washed twice with PBS. The pellet was suspended with 100 µl of 1X buffer solution, 5 µl of Annexin V-FITC and 5 µl of PI were added into cells suspension. The reaction was incubated for 15 minutes at room temperature in dark. After that, 400 µl of 1X buffer solution was added into the tube and apoptotic cells were analyzed by flow cytometry using Cellquest Pro software.

3.2.6 mRNA expression (RT-PCR)

L1-WT1-siRNA cells, L1-C-siRNA cells, PBMC-WT1-siRNA cells, and PBMC-C-siRNA cells were harvested at 48 hours post-transduction. The PCR mixture containing 1X PCR buffer, 0.2 mM of dNTPs mixed, 2 mM of $MgCl_2$, 0.2 pmole of primers (Table 3.1), 1 unit of Taq DNA polymerase, 10.0 µl cDNA was prepared and adjusted to 25 µl/reaction by DEPC treated distill water. The PCR reaction was started by heating the PCR mixture at 95°C for 5 minutes for denature initiation, then 40 cycles of amplification step was done at 95°C for 1 minute, appropriated annealing temperature (Table 3.1) was set for 30 seconds, 72°C for 45 seconds in extension step then followed by 72°C for 5 minutes in final extension and held the reaction at 4ºC until use. PCR product was loaded on 1.5% agarose gel and electrophoresis was performed. One hundred base pair DNA ladder was used for DNA marker. PCR

products were stained with ethidium bromide and UV-visualized by Gel Documentation system.

3.2.7 Western blot analysis

L1-WT1-siRNA cells, L1-C-siRNA cells, PBMC-WT1-siRNA cells and PBMC-C-siRNA cells were harvested at 48 hours post-transduction and lysed using CellLytic M reagent (Sigma, C2978) for 15 minutes. The concentration of protein lysate was determined using Bradford protein assay (Sigma, B6916) according to manufacturer protocol. Western blot analysis was performed using the same protocol as in the part of leukemic cell line.

3.2.8 Statistics

All data were expressed as the mean values ± the standard deviation (SD). Differences between the treated and control were analyzed by paired samples t-test. A probability of $P<0.05$ was considered statistically significant. All statistic tests were calculated with SPSS software (version 17.0, SPSS Inc, USA.)

CHAPTER IV

RESULTS

4.1 Results (Part 1: Leukemic cell line)

4.1.1 Transduction efficiency of lentivirus in K562 cells

In this study, we used a new designed WT1-siRNA sequences which were specific for human WT1 coding region. Lentiviral production was done by using calcium chloride precipitation method. The human embryonic kidney (293T/17) cells were used as packaging cells. Forty-eight hours after transfection, 293T/17 cells could produce lentiviral particles which were indicated by high density of GFP expression. This finding showed that our lentiviral vectors system successfully transfected into packaging 293T/17 cells. High viral titer was varied depending on the number of 293T/17-GFP$^+$ cells and the density of GFP expression. The 293T/17-C-siRNA-GFP$^+$ cells and 293T/17-WT1-siRNA-GFP$^+$ cells were illustrated by fluorescent inverted microscope as shown in Figure 4.1A and 4.1B, respectively. These figures showed high density of GFP expression and the most of packaging cells were completely transfected with our lentiviral production protocol. In order to determine of lentivirus transduction efficiency, viral titer was performed based on GFP expression of transduced-293T/17 cells. After lentiviral supernatant collection, 293T/17 cells were transduced with various dilutions of C-siRNA viral supernatant. The amount of 293T/17-C-siRNA-GFP$^+$ was demonstrated with various numbers and densities as shown in Figure 4.2A. Based on lentiviral titer determination, we found that 10^6-10^7 Transduction Unit (TU/ml) of lentiviral supernatant were the best titer for

transduction into the target cells. However, our preliminary study exhibited low yield of lentiviral transduction into human hematopoietic cells. Therefore, we applied ultracentrifugation process for enhancement of lentiviral transduction efficiency (Figure 4.2). To this end, both viral supernatant of C-siRNA and WT1-siRNA were separately concentrated by ultracentrifugation at 28,000 rpm, 4°C for 2.06 hours. The K562 cells were transduced with concentrated C-siRNA or WT1-siRNA viral supernatant by using spinning procedure. Forty-eight hours post transduction, transduced-K562 cells exhibited GFP expression. Both of K562-C-siRNA-GFP$^+$ cells and K562-WT1-siRNA-GFP$^+$ cells were sorted for GFP positive cells collection. The percentage of K562-C-siRNA-GFP$^+$ population was obtained approximately 87.90% after sorting and 85.40% was found in K562-WT1-siRNA-GFP$^+$ cells (Figure 4.3). These results indicated that sorting method is appropriated for cellular collection based on fluorescent expression. After sorting, both of K562-C-siRNA-GFP$^+$ cells and K562-WT1-siRNA-GFP$^+$ cells were captured using fluorescent inverted microscope to compare together at different time points. Death cells induction was clearly observed in K562-WT1-siRNA-GFP$^+$ cells, whereas K562-C-siRNA-GFP$^+$ cells could not be detected at 48 hours of the experiment. There are many signs that can be classified death cells or viable cells by using fluorescent microscope. For example, cellular morphology changes during cell death, the smaller size of death cells than normal viable cells, cell shrinking, a lot of cell debris, and the reduction of fluorescent intensity on the cells as shown in Figure 4.4. These results suggest that lentivirus is a useful vehicle for siRNA delivery into the target cells including chronic myeloid leukemia. Moreover, lentivirus based on GFP reporter gene is more comfortable and easier ways to follow up and measure the efficiency of viral infection. Furthermore, a

new designed of WT1-siRNA sequence affects the induced cell death in these leukemic cells after 24 hours of experiment.

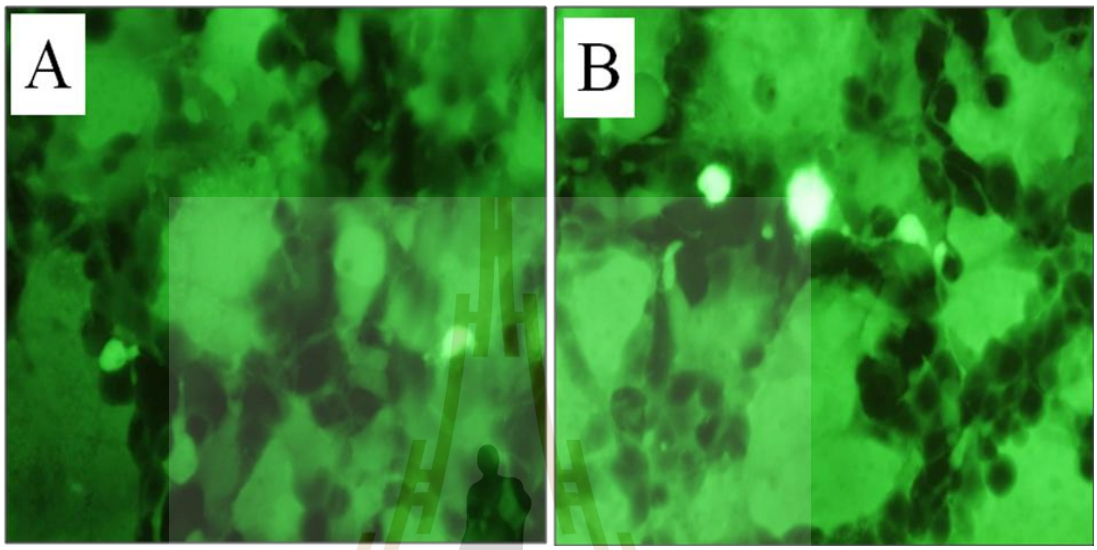

Figure 4.1 Demonstration of GFP-expressing 293T/17 packaging cells after transfection processes. The packaging cells were transfected with lentivirus vectors for lentiviral production. Forty-eight hours after transfection, 293T/17 cells were captured by fluorescent microscope with 20X magnification power. The 293T/17-C-siRNA-GFP^{+} cells are shown in panel A and 293T/17-WT1-siRNA-GFP^{+} cells are shown in panel B.

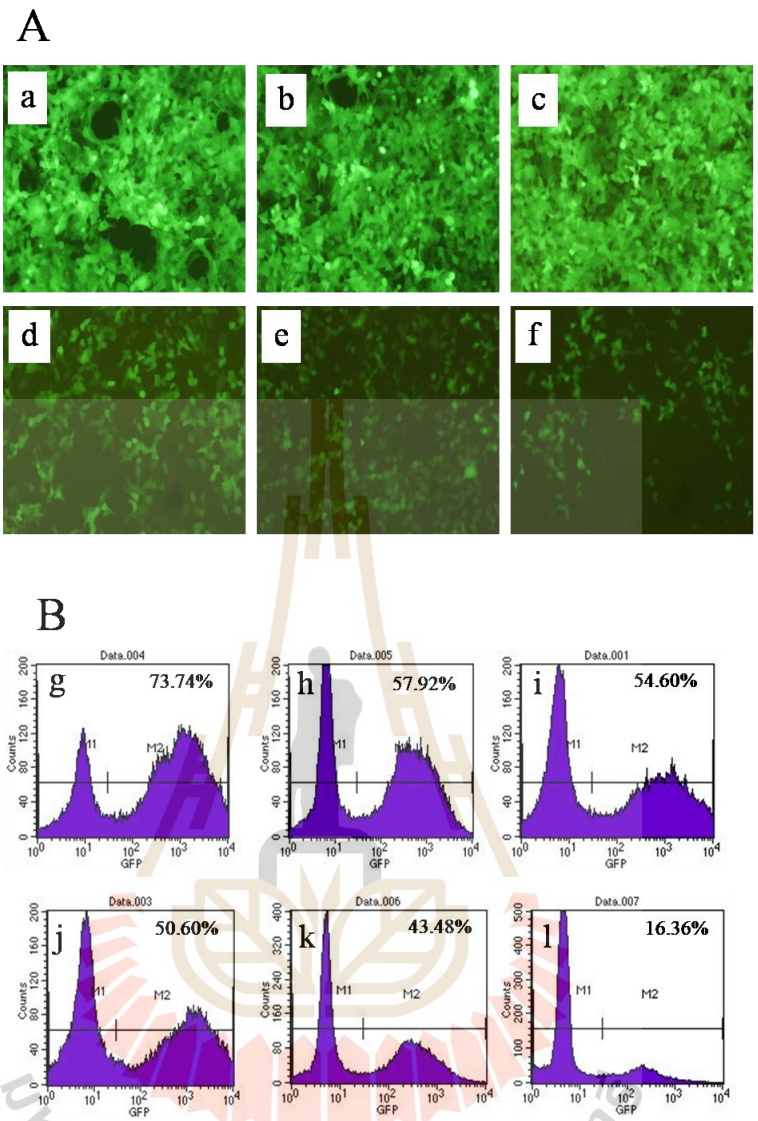

Figure 4.2 Lentiviral titer determination based on GFP expression of 293T/17 cells. The 293T/17 cells were transduced with various concentrations of lentivirus supernatant. The GFP expressing-293T/17 cells are shown in (A). Serial dilution of lentiviral transduction shows differentiation of GFP expression: dilution factors of 1, 1.5, 3, 15, 30, and 150 are demonstrated in a, b, c, d, e, and f, respectively. The amount of 293T/17-GFP^{+} cells was analyzed by flow cytometry (B) which presented in percentage of GFP expression. The histograms of each dilution factors are showed in g, h, i, j, k, and l, respectively.

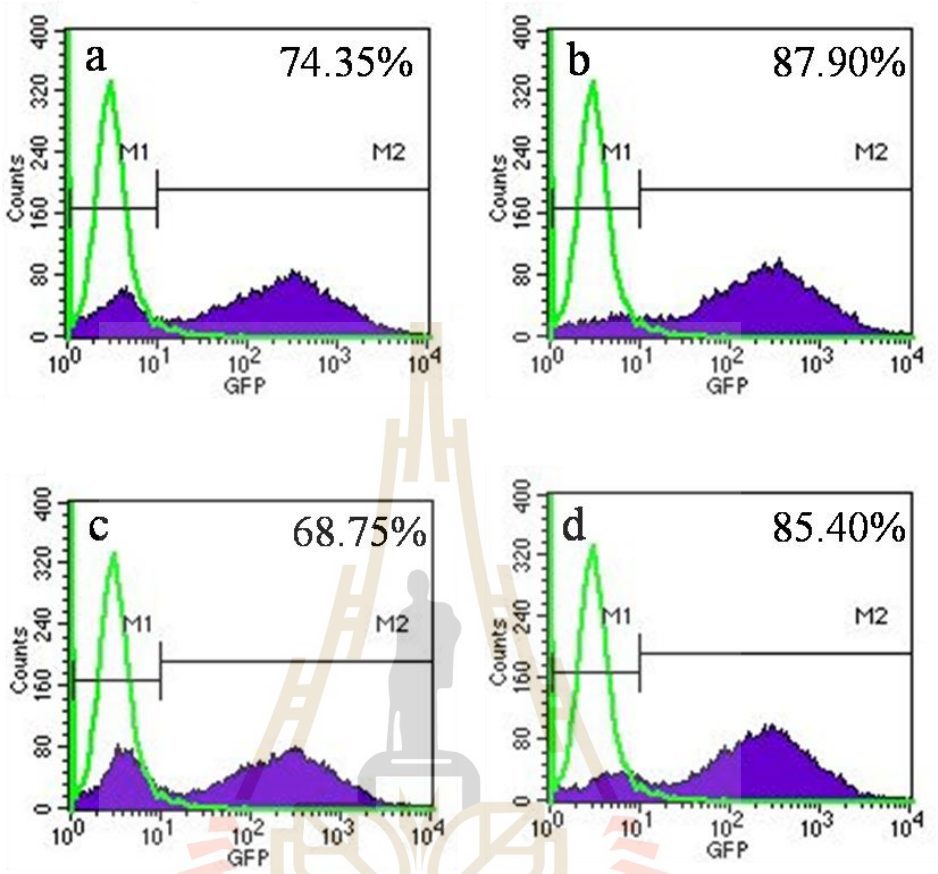

Figure 4.3 The comparison of K562-GFP^{+} cells populations between previous and post sorting processes. Histogram represented the population of K562-C-siRNA-GFP^{+} cells before cell sorting (a) and after cell sorting (b). Histogram represented the population of K562-WT1-siRNA-GFP^{+} at previous sorting (c) and post sorting (d).

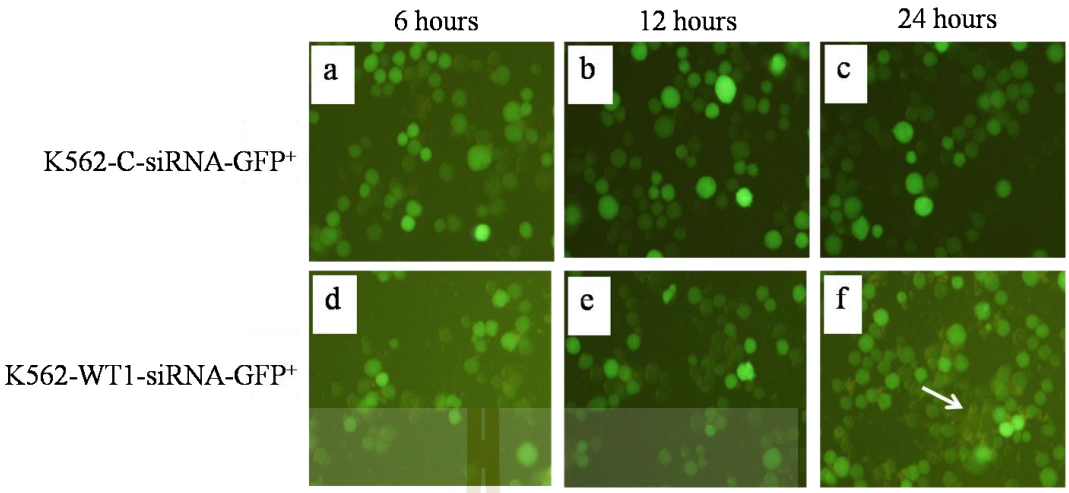

Figure 4.4 Demonstration of GFP expression of K562 cells after C-siRNA and WT1-siRNA transductions. Panels a, b, and c represented the morphology of K562-C-siRNA-GFP$^+$ cells at 6, 12, and 24 hours, respectively. The K562-WT1-siRNA-GFP$^+$ cells are shown in panel d, e, and f represented at 6, 12, and 24 hours post-transduction, respectively. The white arrow indicates dead cells of K562-WT1-siRNA-GFP$^+$ cells.

4.1.2 WT1-siRNA inhibits cell growth of K562 cells

After transduction, total viable cells of K562-C-siRNA-GFP$^+$ cells and K562-WT1-siRNA-GFP$^+$ cells were counted by Trypan blue exclusion method. The amount of viable cells was calculated at specific time points and plotted as a growth inhibition curve. The number of viable cells of K562-WT1-siRNA-GFP$^+$ cells was significantly decreased at time dependent manner while normal cells growth was observed in K562-C-siRNA-GFP$^+$ cells (Figure 4.5A, Figure 4.5B). The result showed that WT1-siRNA led to inhibit leukemic cells growth nearly 90% at 96 hours post-transduction (Figure 4.6). The inhibitory effects of WT1-siRNA on K562 cells were confirmed by MTT assay which demonstrated a gradual increase of percentage of inhibition from 10±1.6%, 12±8.2%,16±10.6%, 25±13.2%, 40±3.5%, 44±19.8%, and 88±4.6% at 3, 6, 12, 24, 48, 72, and 96 hours, respectively (Figure 4.7). The result indicated the significant inhibitory effect of WT1-siRNA on the growth of K562 cells at 48 hours forward after transductions whereas no inhibitory effect was observed in C-siRNA treated cells. These data suggest that WT1 expression is an important factor for cell survival and proliferation of K562 leukemic cells. Therefore, the silencing of *WT1* using siRNA led to reduce cell viability.

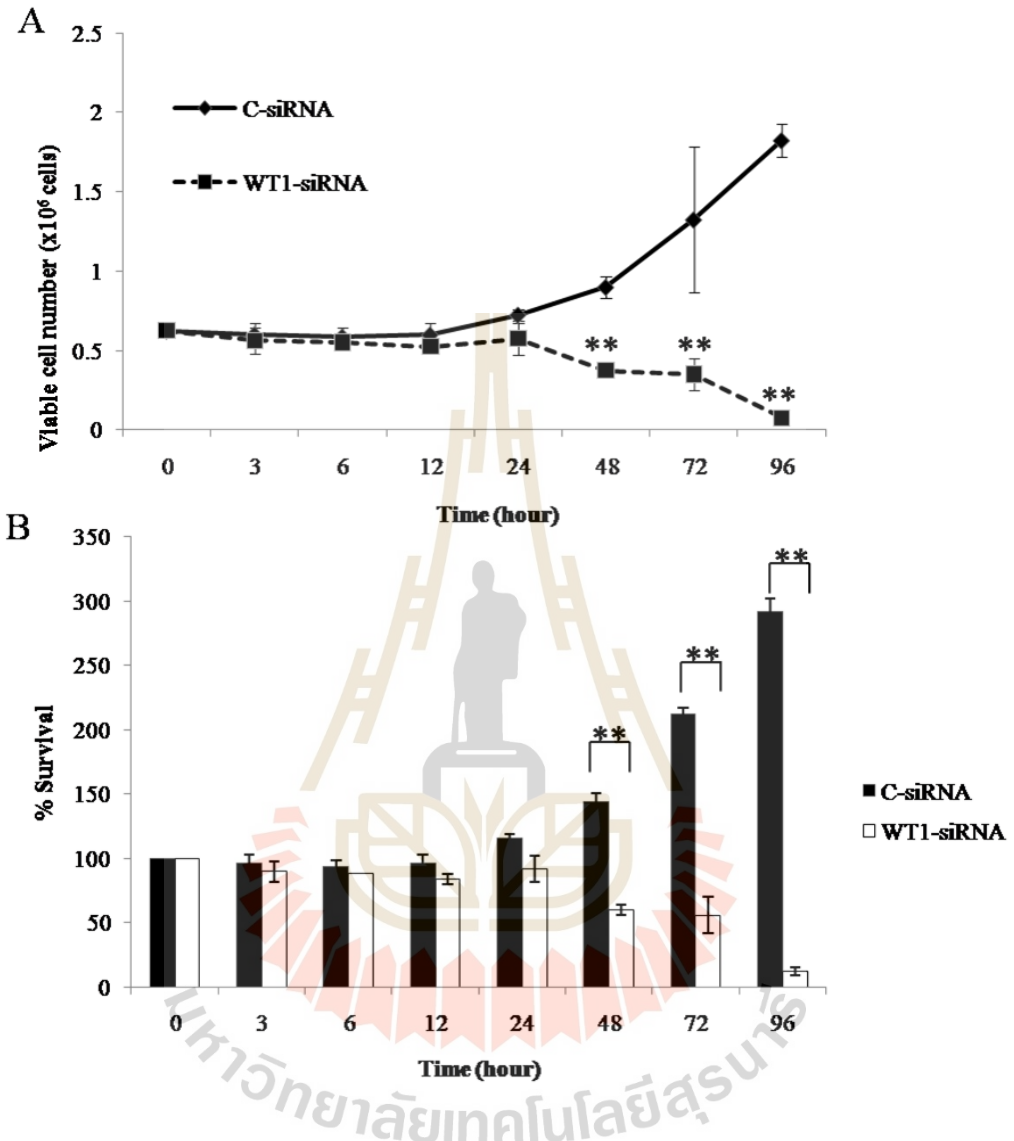

Figure 4.5 Evaluation of WT1-siRNA inhibits K562 cells growth. The inhibitory effect was determined at specific time points: 0, 3, 6, 12, 24, 48, 72, and 96 hours post-transduction. K562-WT1-siRNA-GFP^{+} cells and K562-C-siRNA-GFP^{+} cells were harvested for analysis of cell growth by using trypan blue exclusion method. The number of viable cells is represented in panel A. The percentage survival of transduced-K562 cells is represented in panel B. Assays were performed in triplicate reactions. (**$p<0.01$ by paired samples t-test)

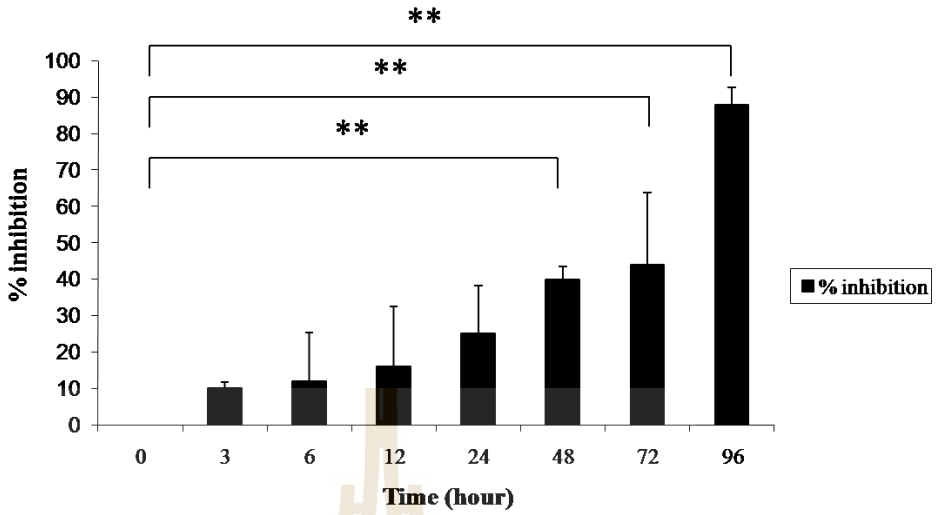

Figure 4.6 Inhibitory effect of WT1-siRNA against K562 cells growth. The percentage of inhibition was calculated from viable number of K562-WT1-siRNA-GFP^{+} subtracted with number of K562-C-siRNA-GFP^{+} cells. Assays were performed in triplicate reactions. (**$p<0.01$ by paired samples t-test)

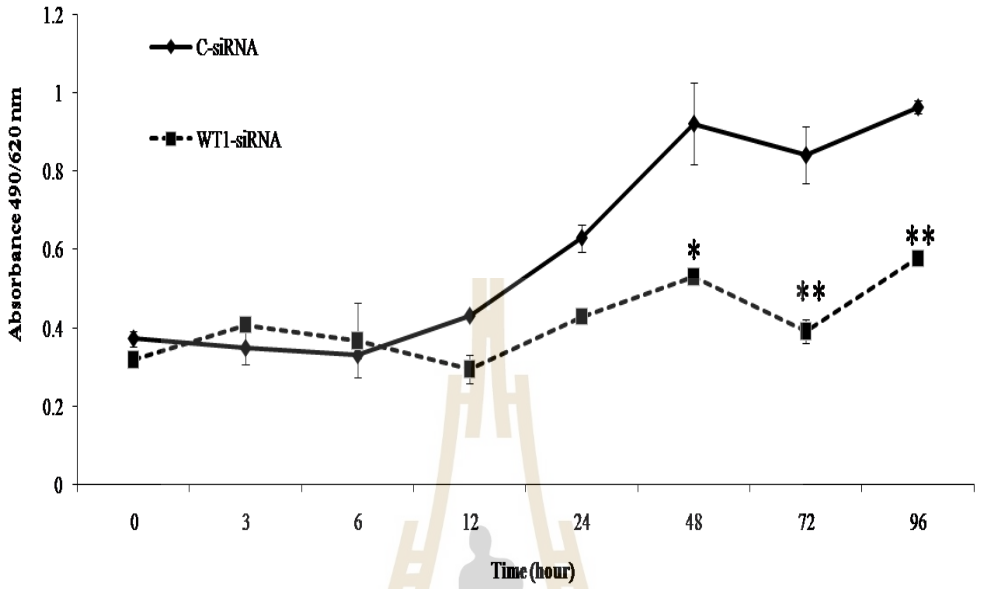

Figure 4.7 The inhibitory effect of WT1-siRNA on cellular proliferation of K562 cells by MTT assay. K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were harvested at 0, 3, 6, 12, 24, 48, 72, and 96 hours post-transduction. Relative proliferation rates are represented in absorbance value which was measured at 490 nm and subtracted with 620 nm. Assay was performed in triplicate reactions. (*$p<0.05$, **$p<0.01$ by paired samples t-test)

4.1.3 Apoptosis induction by WT1-siRNA

Previous results indicated that the silencing of WT1 expression clearly inhibited cellular proliferation and growth of leukemic cells. Therefore, we further determined the mechanism of cell death whether it was induced by apoptosis pathway. To this end we investigated the caspases-3 and -7 enzyme activities which are important in apoptosis pathway. Furthermore, detection of cell membrane change during apoptosis was also performed by Annexin V-FITC/PI staining. The reactions were performed as descripbed in Materials and Methods. In principle, caspase-3 and caspase-7 cleaved the non-fluorescent substrate (Z-DEVE-R110; Rhodamine-110-bis (CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)) in order to remove the DEVD peptide. Next the Rhodamine 110 was excited and emitted at 499 and 521 nm, respectively to determine fluorescent intensity of apoptotic cells via caspase-3 and -7 activities. After experiments were done, the results showed that caspase-3 and -7 were significantly activated at 48 hours for 1,487±425 RFU or around 3 folds and the highest activities were observed at 96 hours for 2,151±189 RFU or around 5 folds in K562-WT1-siRNA-GFP^{+} cells when compared with the pararel control. In contrast, the lower activities of caspase-3 and -7 were found in K562-C-siRNA-GFP^{+} cells at 48 and 72 hours for 507±32 RFU and 399±65 RFU, respectively (Figure 4.8). These results suggested that WT1-siRNA could induce apoptosis via the activation of apoptotic enzymes including executioner caspase-3 and caspase-7 (caspase-dependent apoptosis pathway). Furthermore, detection of cell membrane change during apoptosis had also carried on by Annexin-V FITC/PI staining. This method gives us more detail of early and late apoptosis stages. To accomplish this, the transduced cells were stained with Annexin V-FITC and PI prior to analyze by flow cytometry method. The

early stage of apoptosis was detected by Annexin V-FITC positive and PI negative cells. The population of early apoptotic cells was shown as the value of lower right position in a dot plot diagram (Figure 4.9A). On the other hand, late stage of apoptosis was detected by double positive of Annexin V-FITC and PI (Figure 4.9A). These results showed that the highest percentage of early apoptotic cells were observed in WT1-siRNA treated cells at 12 and 24 hours for 70±1% and 76±0.49%, respectively (Figure 4.9B). The lower percentage of these cell populations decreased to 50±0.98% and 38±1.2% at 48 and 72 hours, respectively. However, late apoptosis stage was demonstrated for 48±0.93% and 60±0.59% at 48 and 72 hours, respectively (Figure 4.9C). These results supported the apoptosis induction of WT1-siRNA on leukemic cells. Moreover, intrinsic apoptotic pathway was clarified by its activated executioner-caspases activities and complete effect was observed at 48 hours of experiment forward. Interestingly, WT1-siRNA could not induce significant necrosis of treated cells.

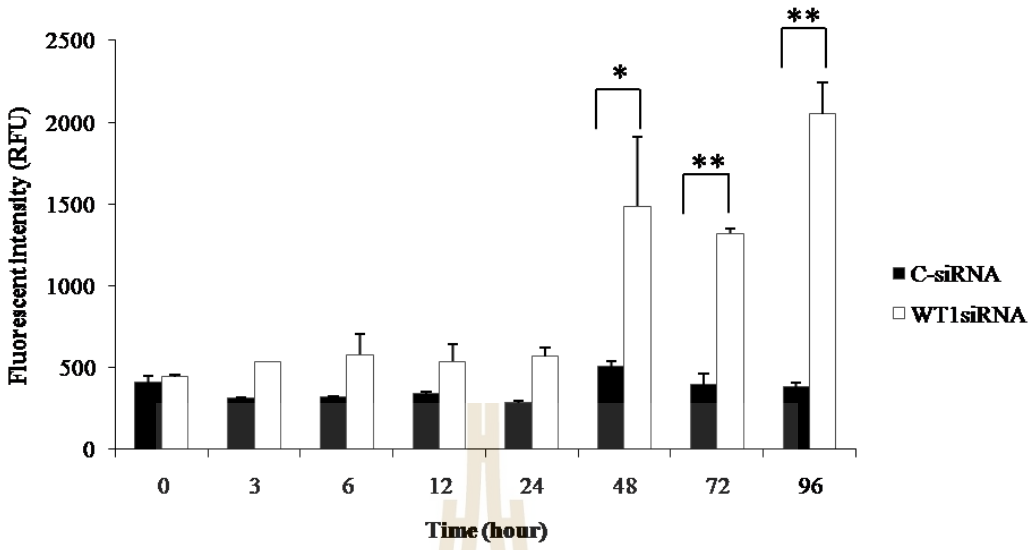

Figure 4.8 WT1-siRNA activates apoptotic enzymes; caspase-3/7 activities on K562 leukemic cells. K562-WT1-siRNA-GFP^{+} cells and K562-C-siRNA-GFP^{+} cells were collected at 0, 3, 6, 12, 24, 48, 72, and 96 hours post-transduction for analysis of apoptotic rates by using Apo-One Homogenouse Caspase 3/7 apoptosis kit (Promega). Fluorescent intensity was directly represented an apoptotic enzymes activity. The increasing of caspase enzyme activities represented as apoptotic induction by WT1-siRNA. Assay was performed in triplicate (*$p<0.05$, **$p<0.01$ by paired samples t-test).

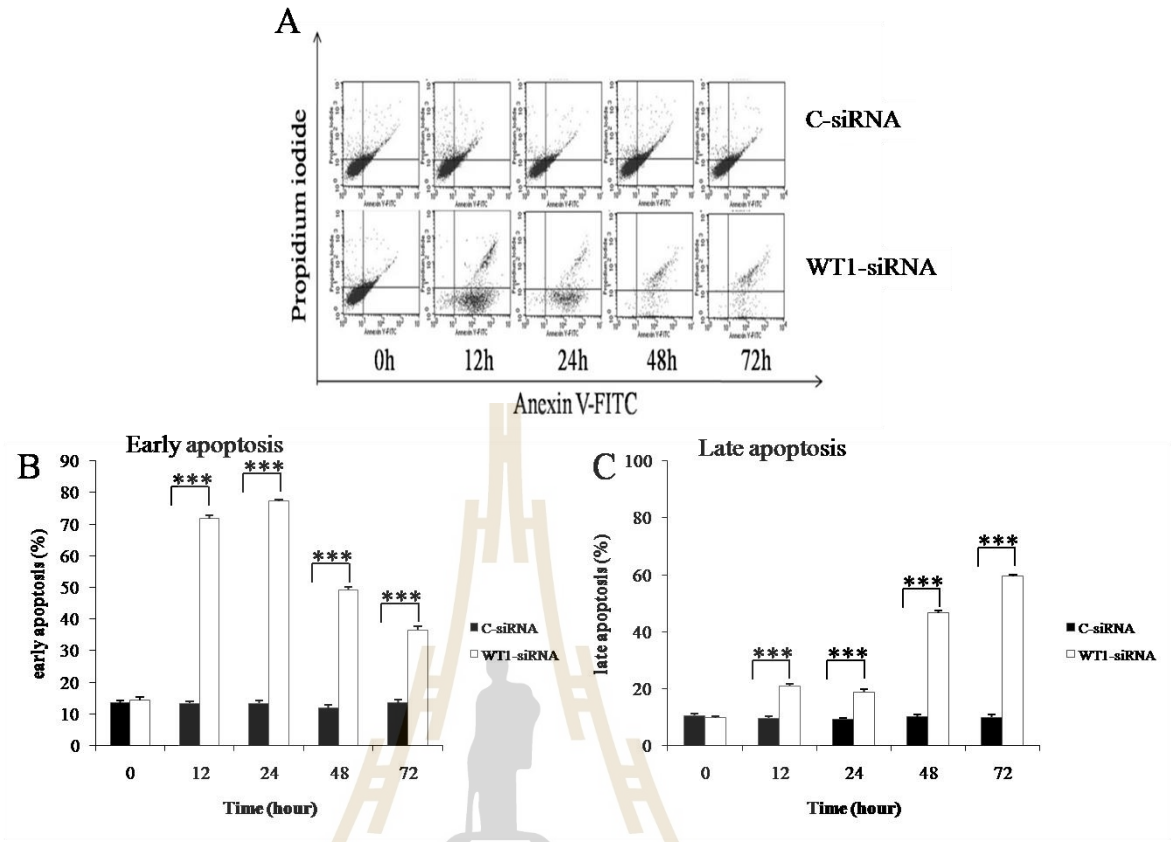

Figure 4.9 The effect of WT1-siRNA on apoptotic stages of treated K562 cells was detected by flow cytometry based on cellular membrane change during apoptotic induction. K562-WT1-siRAN-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells were subjected to determine the apoptotic induction. Total 1x10^5 cells were collected and stained with Annexin V-FITC/PI. Flow cytometry analysis was then performed. The early and late apoptosis were represented on lower right and upper right in a quadrant diagram, respectively (A). Data analysis of early apoptosis and late apoptosis are shown in panel B and panel C, respectively. Assay was performed in triplicate reactions (***p<0.001 by paired samples t-test).

4.1.4 Effects of WT1-siRNA in molecular levels of transduced K562 cells

4.1.4.1 mRNA expressions

To examine WT1 mRNA silencing effects induce by WT1-siRNA. WT1-expressing leukemic cells (K562) were transduced with WT1-siRNA based on lentiviral system. Total RNA of treated cells: K562-WT1-siRNA-GFP$^+$ cells and control cells: K562-C-siRNA-GFP$^+$ cells was extracted and mRNA expression was analyzed by using RT-PCR. Results showed that WT1-siRNA could significantly downregulate the expression of WT1 mRNA at 72 and 96 hours post-transduction (Figure 4.10). Two major isoforms of WT1 including WT1(-17AA) and WT1(+17AA) were shown in 2 separate bands on agarose gel. The downregulation of WT1 mRNA was occurred with both WT1(-17AA) and WT1(+17AA) expressions. These results indicate that WT1-siRNA could inhibit major isoforms of WT1 mRNA. Moreover, WT1-siRNA also extremely inhibited the expression of survival cytokine including IL-2 and its receptors; IL-2RB and IL-2RG (Figure 4.10A). These results suggested that our new designed WT1-siRNA directly inhibited WT1 mRNA expressions. Typically WT1 can be found in various types of leukemia that represent as a marker for leukemia prognosis. The downregulation of both genes by WT1-siRNA led K562-WT1-siRNA-GFP$^+$ cells death. In contrast, C-siRNA could not inhibit WT1 mRNA expression resulted in observing normal growth of control K562-C-siRNA-GFP$^+$ cells. Furthermore, human cytokine such as interleukin-2 (IL-2) which is important for leukemic cells survive was demonstrated its expression after WT1-siRNA treatment. The result exhibited the inhibitory effect of IL-2 expression at 96 hours post-transduction. However, IL-2 is a cytokine that activates cell growth via binding with IL-2 receptors. IL-2 receptor contains with 3 receptor subunits including

subunit alpha (IL-2RA), subunit beta (IL-2RB), and subunit gamma (IL-2RG). Each subunit can identify by IL-2 binding affinity. Heterotrimerization of all subunits (ABG) is classified as a high affinity of IL-2 binding and this complex exhibits high signal into the cell. However, heterodimer of IL-2RA and IL-2RB show pseudo-high affinity of IL-2 binding while heterodimer of IL-2RB and IL-2RG are identified as intermediate affinity. Thus, IL-2RB and IL-2RG expressions were demonstrated in this study. This result showed that IL-2RG expression could not be detected after 48 hours while IL-2RB was downregulated at 72 hours post-transduction in WT1-siRNA treatment. The results suggest that WT1-siRNA suppresses the function of IL-2 receptor subunits beta and gamma that caused to downregulate IL-2 mRNA expression.

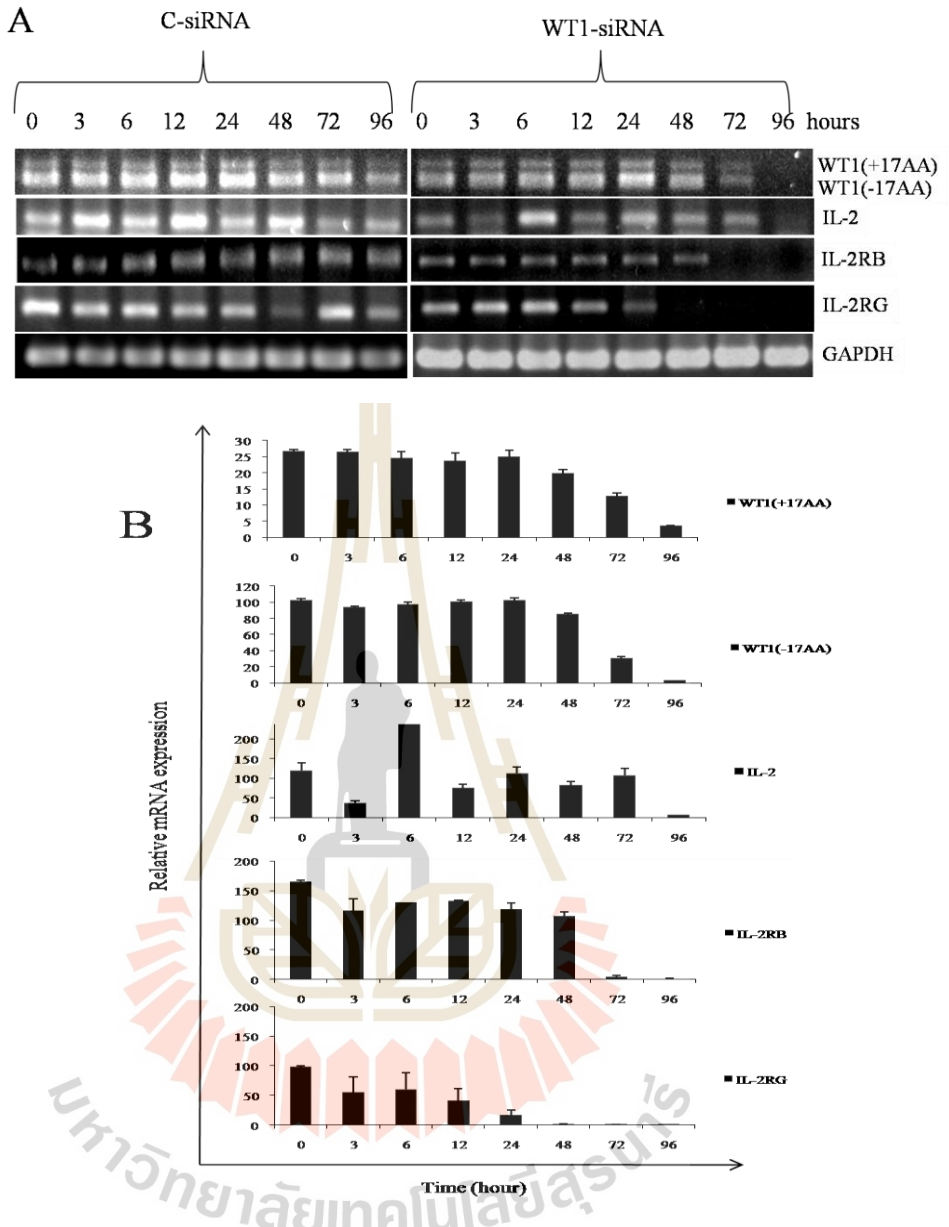

Figure 4.10 Demonstration of mRNA expressions on K562-WT1-siRNA-GFP$^+$ cells and K562-C-siRNA-GFP$^+$ cells. Cells were collected at 0, 3, 6, 12, 24, 48, 72, and 96 hours post-transduction. Total RNA was converted to cDNA. WT1, IL-2, IL-2RB and IL-2RG mRNA expressions were analyzed by RT-PCR analysis. GAPDH was used as an internal control. PCR products were demonstrated (A). The relative expressions of interested mRNA of K562-WT1-siRNA-GFP$^+$ cells were determined based on the expression of GAPDH (B).

4.1.4.2 Protein expressions

Wilms' tumor 1 (WT1) encodes transcriptional factor that plays an important role for cell growth regulation in leukemic cells. The silencing of WT1 results in cell growth suppression and cellular apoptosis induction. In this study, WT1-siRNA system led to downregulate WT1 mRNA expression. Moreover, it can inhibit K562-WT1-siRNA-GFP$^+$ cells growth. Furthermore, western blot analysis was performed in order to study the effect of WT1-siRNA on protein expression level. The result showed that WT1-siRNA induced WT1 protein suppression at 72 and 96 hours post-transduction according with the results of mRNA expression study. Moreover, WT1-siRNA also clearly inhibited both of WT1(+17AA) which represented as 54 kDa protein band and WT1(-17AA) which represented as 52 kDa protein band. The inhibition of WT1 both gene and protein levels were consistent with cell growth suppression by WT1-siRNA treatment. Here, we also determined the expression level of apoptotic enzyme protein in caspase-7 by using western blot analysis. The result showed three sizes of caspase-7 isoforms including; 35 kDa of precursor caspase-7, 30 kDa of cleaved caspase-7 (active form), and 15 kDa of cleaved caspase-7 (inactive form) (Figure 4.11A). The decreasing expression of each isoform of caspase-7 was observed at 72 and 96 hours. These results suggest that WT1-siRNA activated K562 cell death via apoptosis pathway by activated caspase-7 expression. However, the expression of each caspase-7 isoform was decreased at 72 and 96 hours. These findings indicated that K562-WT1-siRNA-GFP$^+$ cells were completely death and led to stop translation process. Caspase is the member of cysteine proteases mediated with high substrate specificity (Cohen, 1997). Normally, caspase-7 is expressed as a dimeric zymogen with 23-residues N-terminal prodomain and remains as dimer after

activation (Chai et al., 2001). During the activation process, initiator caspase such as caspase-8, proteolyses after the Asp198 residue of pro-caspase-7 located between the large P20 and small P11 subunits (Chai et al., 2001; Cohen, 1997). Therefore, the prodomain of caspase-7 is cleaved after the Asp23 residue. The crucial cleavage in caspase-7 activation is at Asp198 because the cleavage at Asp23 alone does not produce an active caspase-7 (Shi, 2002). Caspase-7 is cleavable by caspase-3, -9,-10 and granzyme B to get the products of active (long form) and inactive (short form) enzymes. An inactive form of caspase-7 could inhibit the activity of its active form. The data analysis of protein expression level was performed by measurement of protein band density by using Image J software. The density of each protein band was calculated by comparing with control of untransduced K562 cells. The data represented in folds of expression (Figure 4.11B).

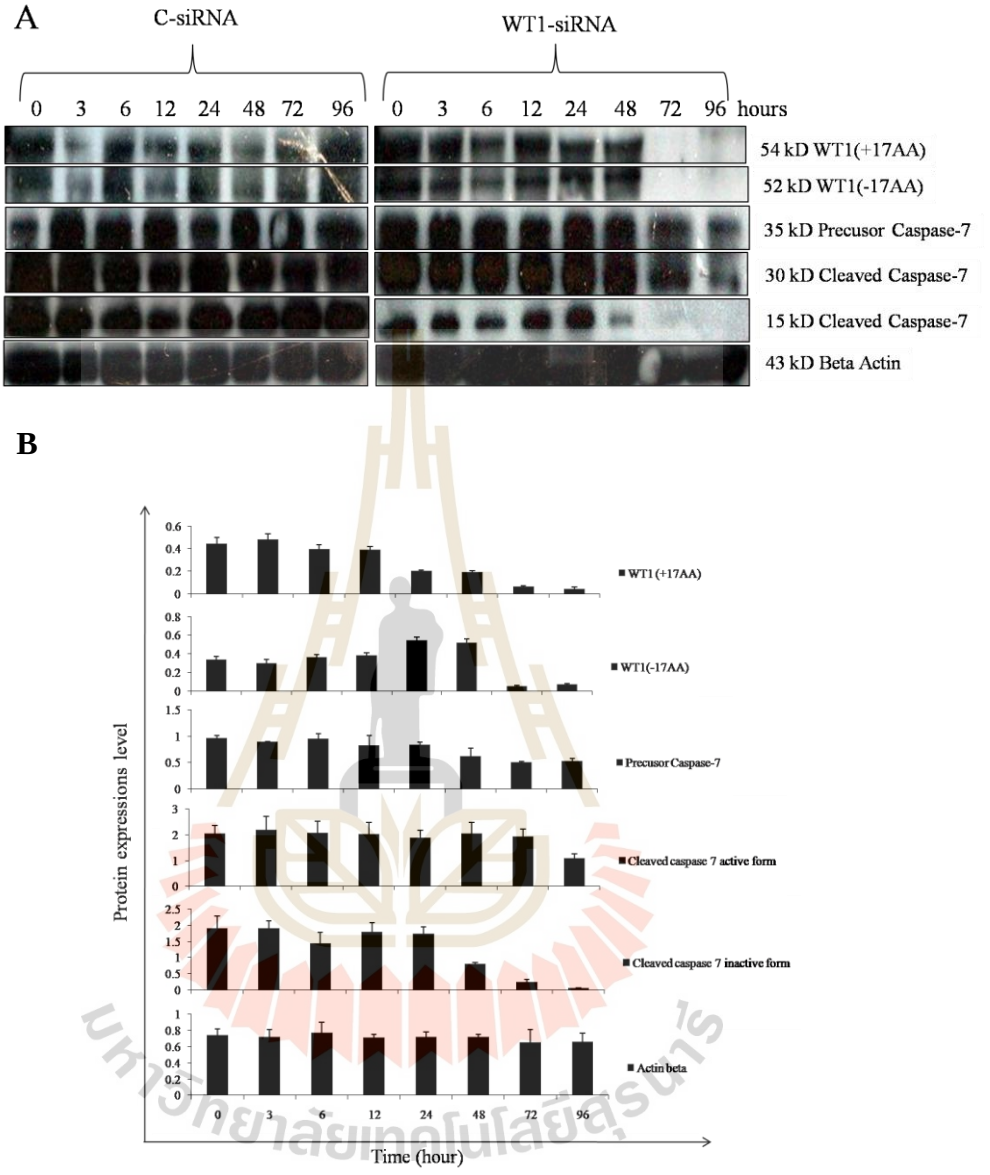

Figure 4.11 Protein expressions analysis of K562 cells after WT1-siRNA treatment. At specific time points, transduced K562 cells were collected and protein extraction was performed. Protein expression was analyzed by Western blot analysis. The expression of each protein of K562-C-siRNA-GFP$^+$ and K562-WT1-siRNA-GFP$^+$ cells are shown in panel A. The density of each band of protein was measured by Image J software and shown in panel B.

4.2 Results (Part 2: primary leukemic cells)

4.2.1 Effects of WT1-siRNA on normal blood cells

In order to evaluate the effects of WT1-siRNA on normal blood cells, peripheral blood cells were collected from healthy volunteers after gave written informed consent. WT1-siRNA and C-siRNA were transduced into peripheral blood mononuclear cells (PBMCs) for control groups of the next primary leukemic cells study. The results showed that WT1-siRNA had no affect on normal whole white blood cells. The number of dead cells was not differently change as compared together between C-siRNA and WT1-siRNA (Figure 4.12A). As the same manner, the proliferation rate of these cells was not decreased at 48 hours of the experiment (Figure 4.12B). Although the increasing of caspase-3/7 activities was observed at 48 hours as compared to 0 hour, there were no differentiation of apoptotic enzymes activities between C-siRNA transduced cells and WT-siRNA transduced cells at 48 hours (Figure 4.12C). These results suggest that WT1-siRNA has no effects for the normal white blood cells in terms of cells survival, death induction and apoptosis mechanisms.

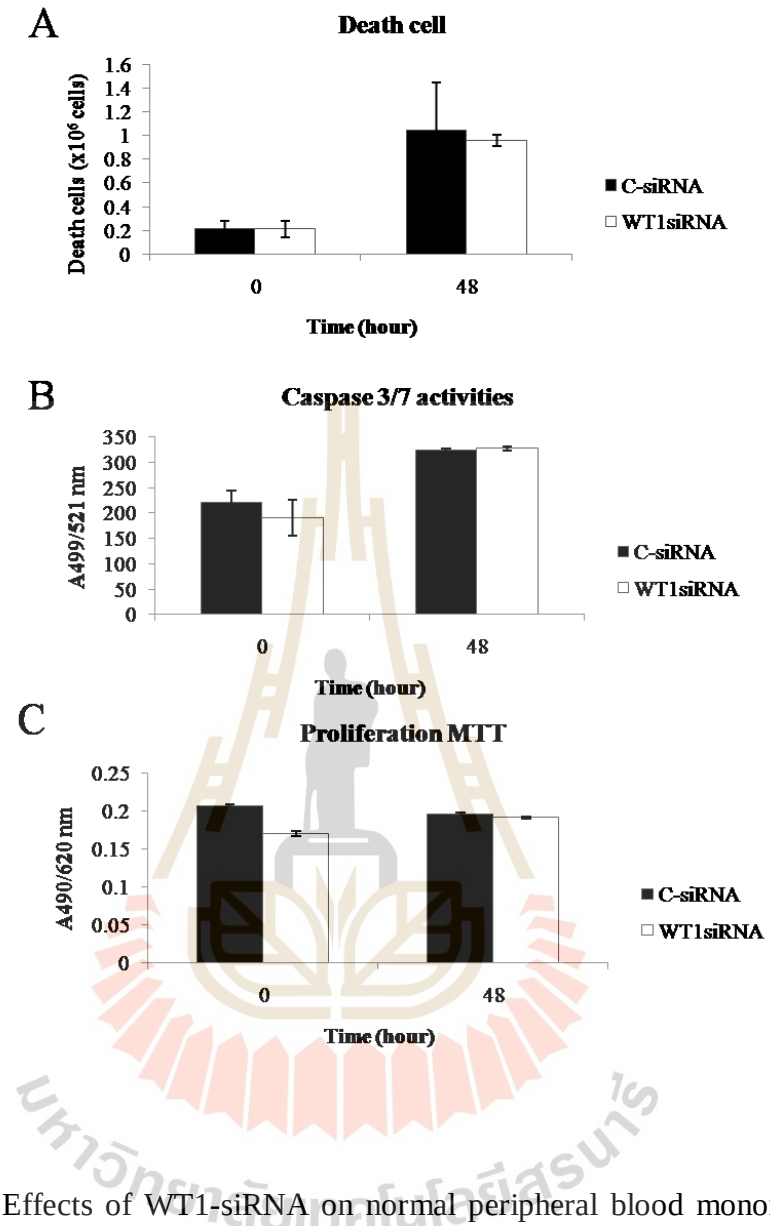

Figure 4.12 Effects of WT1-siRNA on normal peripheral blood mononuclear cells. PBMCs from healthy volunteers were collected and separated by ficoll paque by using gradient centrifugation method. PBMCs were transduced with WT1-siRNA and C-siRNA. At 0 and 48 hours post-transduction, PBMCs were collected for determination of dead cell count (A). The proliferation of the transduced cells was measured by MTT assay (B). Apoptosis induction as evaluated by determination of apoptotic enzymes activities in caspase-3/7 (C) (n=3).

4.2.2 Proteins expression of transduced normal PBMCs cells

After the process of WT1-siRNA transduction, PBMCs-WT1-siRNA cells and PBMCs-C-siRNA cells were collected for Western blot analysis. The transduced cells were subjected to compare the expression level of WT1. We found that WT1 expression level of PBMCs-WT1-siRNA cells was not change from the control cells. However, the level of caspase-7 seems to increase in WT1-siRNA treatment (Figure 4.13A). The expression density of each protein bands was measured by using Image J software. Both of C-siRNA and WT1-siRNA transduced cells showed the same ratio of WT1 expression as 51%±0.1 and 49%±0.1, respectively (Figure 4.13B). The increasing of caspase-7 expression was observed in PBMCs-WT1-siRNA group around 55±0.6% while PBMCs-C-siRNA group showed the expression of 45±0.6% (Figure 4.13C). Results suggest that WT1-siRNA has no affect on WT1 expression level of normal PBMCs cells. However, WT1-siRNA had slight effect on caspase-7 expression of the transduced cells as compared to the control. These data indicate that our new designed WT1-siRNA is not impact on endogenous WT1 protein in normal blood cells and can not induce apoptosis in these cells.

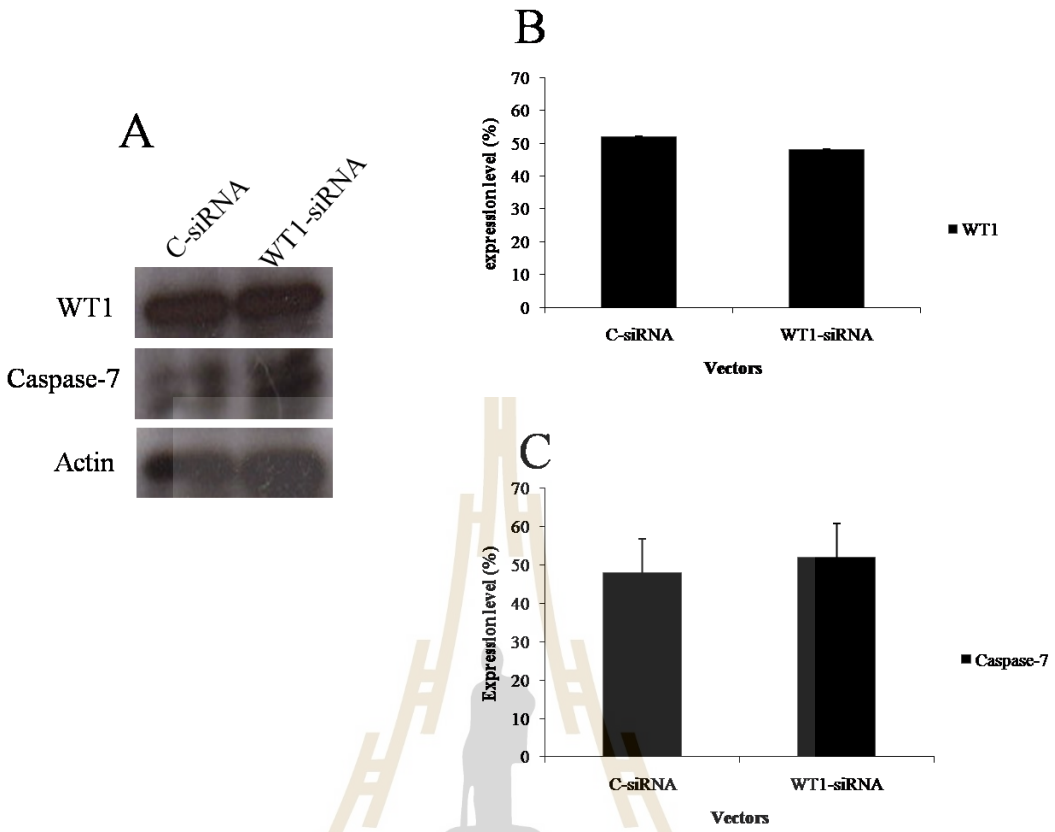

Figure 4.13 Western blot analysis of WT1 and caspase-7 in normal peripheral blood mononuclear cell after WT1-siRNA transduction (A). Immage J software was used for protein bands density determination of WT1 (B) and caspase-7 (C). Twenty micrograms of protein derived from PBMCs-WT1-siRNA cells and PBMCs-C-siRNA cells were used. Protein bands were analysis by horse-radish peroxidase reaction with exposure to the film (A).

4.2.3 WT1-siRNA could not induce apoptosis in normal PBMCs cells

We confirmed the effect of WT1-siRNA on apoptosis induction in normal PBMCs cells. PBMCs-WT1-siRNA cells and PBMCs-C-siRNA cells were collected for Annexin V-FITC/PI stainning in order to evaluate apoptosis induction. Apoptotic cells population was shown as dot plot diagram (Figure 4.14A). The number of an early apoptosis was evaluated as shown in lower left of quadrant (Figure 4.14B) and late apoptosis was demonstrated from upper left of quadrant (Figure 4.14C). However, the upper right quadrant which represented necrosis cells population was not differently observed in both at 0 hour and 48 hours of the experiment. Based on our results, we hypothesized that WT1-siRNA could not activate apoptosis in normal PBMCs cells. Our findings were consistent with the results from caspase-3/7 activities assay.

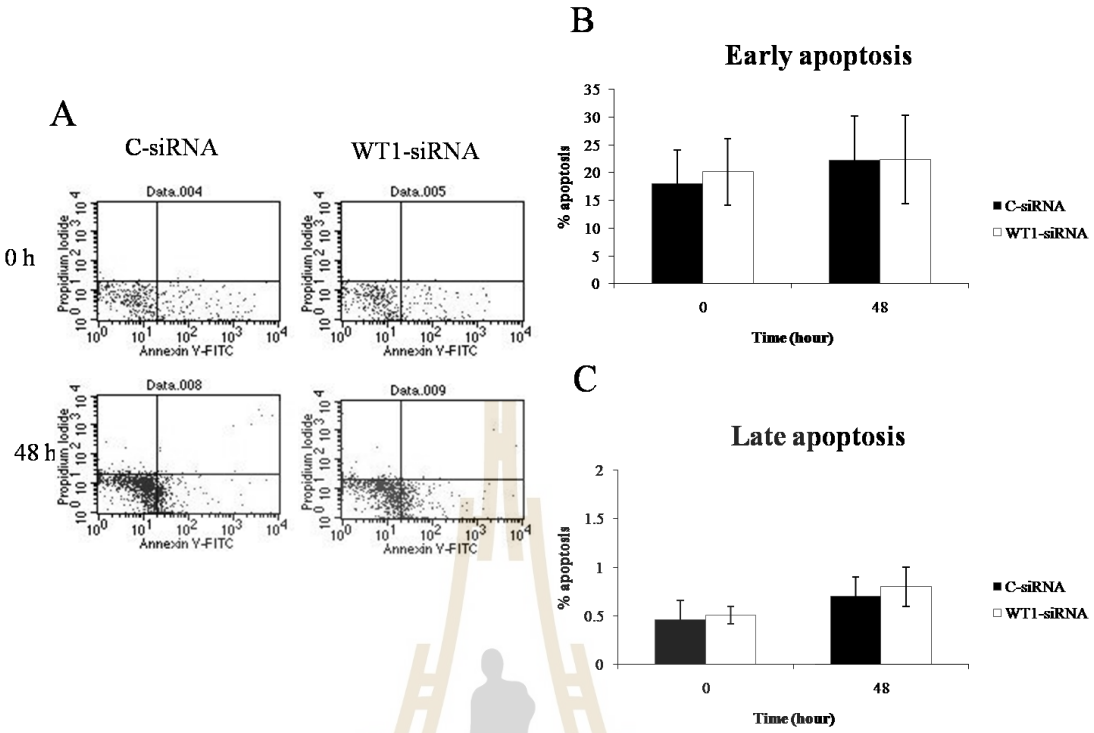

Figure 4.14 Apoptosis of normal PBMCs cells induced by WT1-siRNA treatment. Flow cytometry analysis based on Annexin V-FITC/PI was performed at 0 and 48 hours of experiment. Dot plot diagrams demonstrated apoptotic population of PBMCs-WT1-siRNA cells and PBMCs-C-siRNA cells (A). The average percentage of early (B) and late (C) apoptosis cells were determined in the 3 cells populations by using Annexin V-FITC/PI staining (n=3).

4.2.4 Sample collection from childhood ALL patients

The peripheral blood mononuclear cells of sixteen cases of childhood acute lymphoblastic leukemic patients (ALL) subtype L1 were collected by sterile technique. All of volunteers are the patients at Maharaj Nakhonratchasima hospital. In order to determine the effects of WT1-siRNA on primary childhood ALL hematopoietic cells, PBMCs were collected from ALL (L1) patients who are 3-15 years of age with informed consent. Ten milliliters of blood were collected by sterile technique and added into vacuum blood collection tube contained anticoagulant reagent. The blood sample was kept in ice boxes for transferring to Suranaree University of Technology's laboratory.

4.2.5 Lentiviral transduction efficiency in primary ALL cells

It is difficult for siRNA transferring into primary blood cells. In this study, we tried to find out the appropriated strategies for lentiviral transduction into primary ALL leukemia cells. The concentration of lentivirus supernatant was performed as 30X by ultracentrifugation and used for ALL transduction. Moreover, we also tested the enhancement of transduction efficiency. Concentrated lentiviral supernatant was supplemented with 32 μg/ml of polybrene and 10mM HEPES for increasing of transduction efficiency. Polybrene is cationic polymer which acts by neutralizing the charge repulsion between viral particles and salicylic acid on cell surface (Davis, Rosinski, Morgan, and Yarmush, 2004). It means that the polybrene is the first step of viral binding with target cells which called as initial adsorption of transduction process (Davis, Morgan, and Yarmush, 2002). Moreover, the spinoculation was done by centrifugation of target cells and viral supernatant at 1,800 rpm at 30°C for 1 hour

in order to enhance the efficacy of lentivirus transduction. The transduction efficiency was measured by flow cytometry based on GFP expression on target cells. Approximately 20% of infected cells were analyzed. The PBMCs from primary childhood ALL (L1) which were transduced by WT1-siRNA or C-siRNA are coded as L1-WT1-siRNA cells and L1-C-siRNA cells respectively. Both of L1-WT1-siRNA cells and L1-C-siRNA cells were subjected to further measure for proliferation inhibition, apoptosis induction, cellular differentiation, and molecular analysis.

4.2.6 WT1-siRNA induces cellular proliferation inhibition and apoptotic induction of primary ALL (L1) cells

The inhibitory effect of WT1-siRNA on primary ALL cells growth was observed at 48 hours after transduction. The result showed that WT1-siRNA could inhibit the growth of L1-WT1-siRNA cells for 79±14%, while L1-C-siRNA cells were inhibited for 49±9% (Figure 4.15A). Significant reduction of cellular proliferation was demonstrated on L1-WT1-siRNA cells when compared to L1-C-siRNA cells as 0.27±0.005 and 0.21±0.02, respectively (Figure 4.15B). According to inhibitory effect, WT1-siRNA induces L-WT1-siRNA cells death for 2.6±0.3 folds when compared to L1-C-siRNA cells (Figure 4.15C). Moreover, the mechanisms of WT1-siRNA induce ALL subtype L1 leukemia cell death is occurred via the activation of apoptosis enzyme activities. Caspase-3 and caspase-7 are executioner caspases that play a key role at the last cascade of apoptosis process. The increasing of caspase-3/7 activities are directly represented apoptosis induction. In this study, we used our new designed WT1-siRNA for ALL subtype L1 dead induction. The result showed that caspase-3/7 activities showed 5,104±836 RFUs in

L1-WT1-siRNA cells while L1-C-siRNA cells were detected enzymes activities as 1,823±374 RFUs (Figure 4.15D). These results suggest that WT1-siRNA is not only inhibits of ALL subtype L1 cells growth but also significantly induces cells death via apoptosis process when compared to C-siRNA.

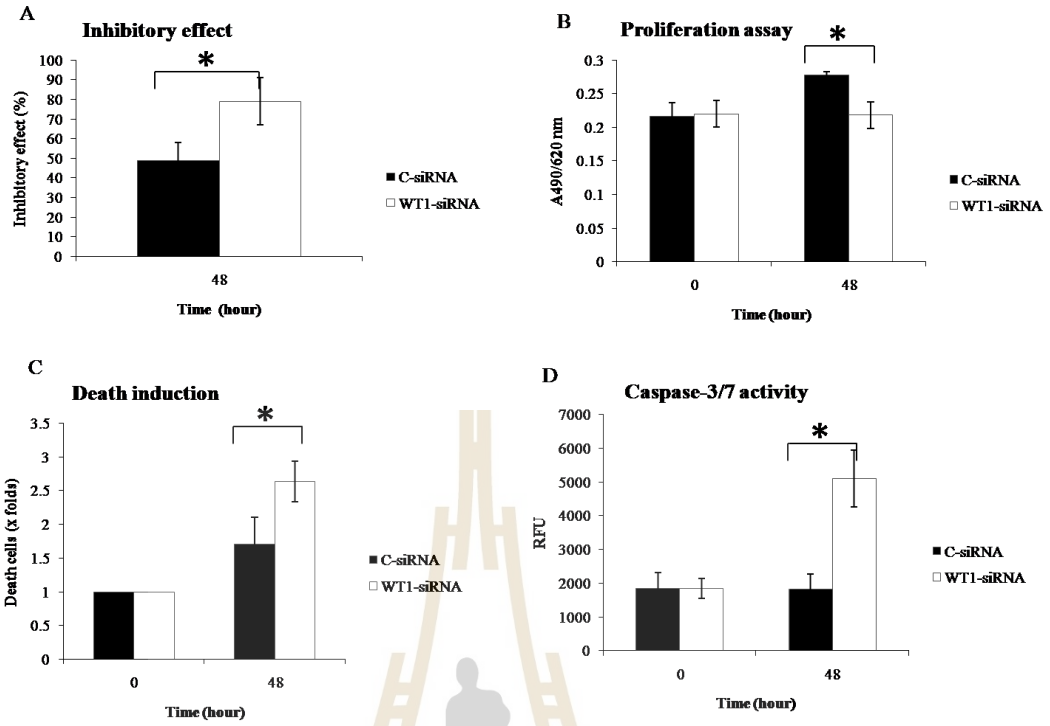

Figure 4.15 WT1-siRNA shows inhibitory effect and dead induction on primary ALL subtype L1 cells. Forty eight hours after transduction, L1-WT1-siRNA cells and L1-C-siRNA cells were collected for viable and dead cells count by using trypan blue exclusion method. Inhibitory effect was calculated by subtraction of the initial viable cell number and converted to percent inhibition (A). Proliferation assay by using MTT analysis was performed on transduced cells (B). Fold of dead induction was calculated from dead cell count and subtraction with initial dead cell number (C). Apoptotic induction was analyzed by determination of caspase-3/7 activities (D). Data represented on average cases (n=5).

4.2.7 Apoptosis induction

WT1 is an important gene for growth regulation in various cells types of leukemia. Our new designed WT1-siRNA could downregulate WT1 of childhood ALL subtype L1 in both gene and protein levels at 48 hours post-transduction. In this study, we demonstrated the effect of WT1-siRNA to induce apoptosis in ALL subtype L1 cells. Five childhood ALL patiant samples were determined apoptosis induction based on anexin V/PI procedure after WT1-siRNA treatment. All five samples showed the same trend of early and late apoptosis populations as shown in the lower right quadrant and upper right quadrant, respectively. The diagram clearly exhibits population of early and late apoptotic cells in WT1-siRNA treatment at 48 hours post-transduction (Figure 4.16A). WT1-siRNA significantly enhances both early and late apoptosis for 36.63±6.35% and 33.25±9.8% at 48 hours of experiment, respectively (Figure 4.16B and 4.16C). WT1-siRNA significantly induced apoptosis in L1-WT1-siRNA cells when compared with L1-C-siRNA cells. This result suggests that our new designed WT1-siRNA exhibits as a good factor for ALL subtype L1 apoptosis induction which has potential for treatment in leukemia in the future.

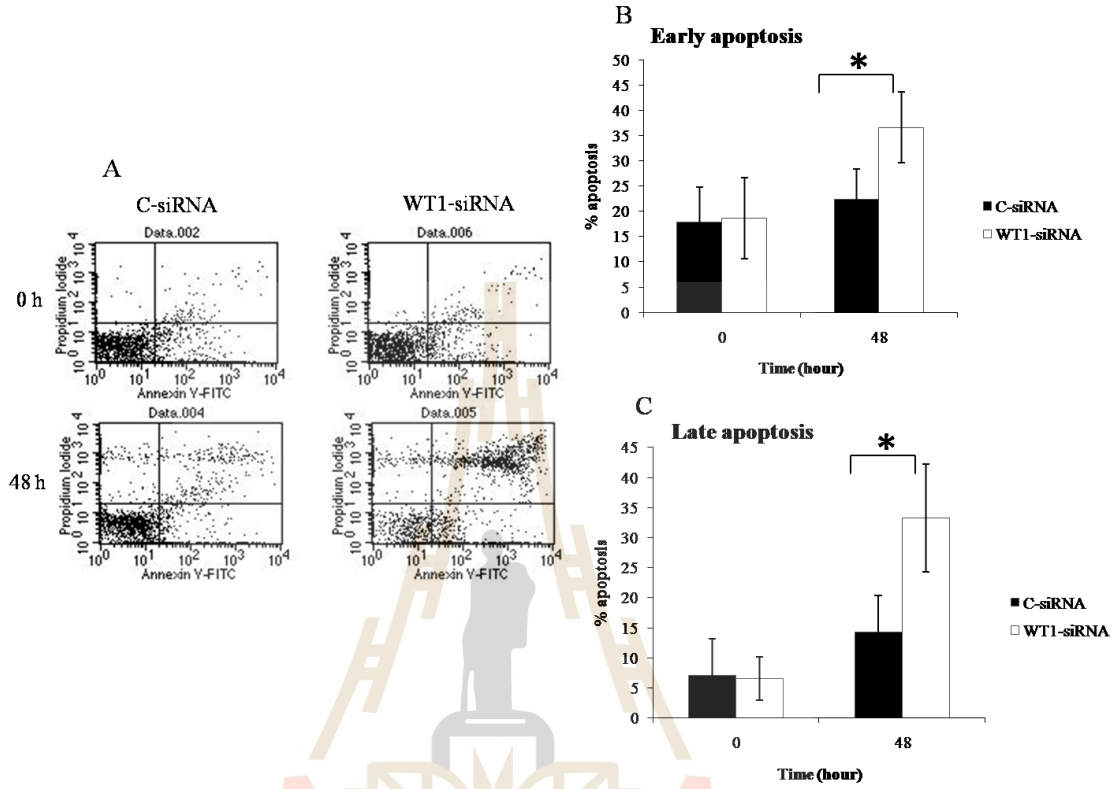

Figure 4.16 Apoptosis of primary childhood ALL subtype L1 samples induced by WT1-siRNA treatment. Flow cytometry analysis based on anexin V/PI staining was performed at 0 and 48 hours of experiment. Dot plot diagrams showed apoptotic population of L1-WT1-siRNA cells and L1-C-siRNA cells (A). The average percentage of early apoptosis (B) and late apoptosis (C) were determined in the 5 cells population by using annexin V/PI staning. The data showed significant statistically (n=5) (paired samples t-test $p \leq 0.05$).

4.2.8 Protein expression level

WT1 is a transcription factor which plays a critical role to regulate cellular proliferation and differentiation. The expression of WT1 on hematopoetic cells is biomarker for leukemia diagnosis that involved as a target for MRD detection (Kerst et al., 2008). In this study, two cases from sixteen cases were collected for Western blot analysis. We downregulated WT1 by specific new designed WT1-siRNA based on lentivirus transduction. Western blot analysis was performed, protein bands were showed in Figure 4.17A. The result showed inhibition of WT1 protein expression at 48 hours post-transduction for 38±0.6% in L1-WT1-siRNA sample where as L1-C-siRNA sample showed 62±0.6% of WT1 expression level (Figure 4.17B). WT1 suppression led to induce apoptosis of L1-WT1-siRNA-GFP+ cells via the increasing of caspase-7 enzyme expression which is well known protien of apoptosis cascade. Caspase-7 is an executioner enzyme that further activiats apoptosis process. This result demonstrated that caspase-7 was detected for 60±1.1% in L1-WT1-siRNA sample while 40±1.1% was found in L1-C-siRNA sample (Figure 4.17C). However, WT1-siRNA could not affect to downregulate WT1 protein expression in PBMCs-WT1-siRNA and PBMCs-C-siRNA when compared altogether. Moreover, the expressing of caspase-7 was observed as the same level between PBMCs-WT1-siRNA and PBMCs-C-siRNA samples, (data not shown). These results suggest that our new designed WT1-siRNA shows significant donwregulation efficacy on WT1 protein expression as well as it could activated caspase-7 apoptotic enzyme in primary childhood ALL subtype L1 cells.

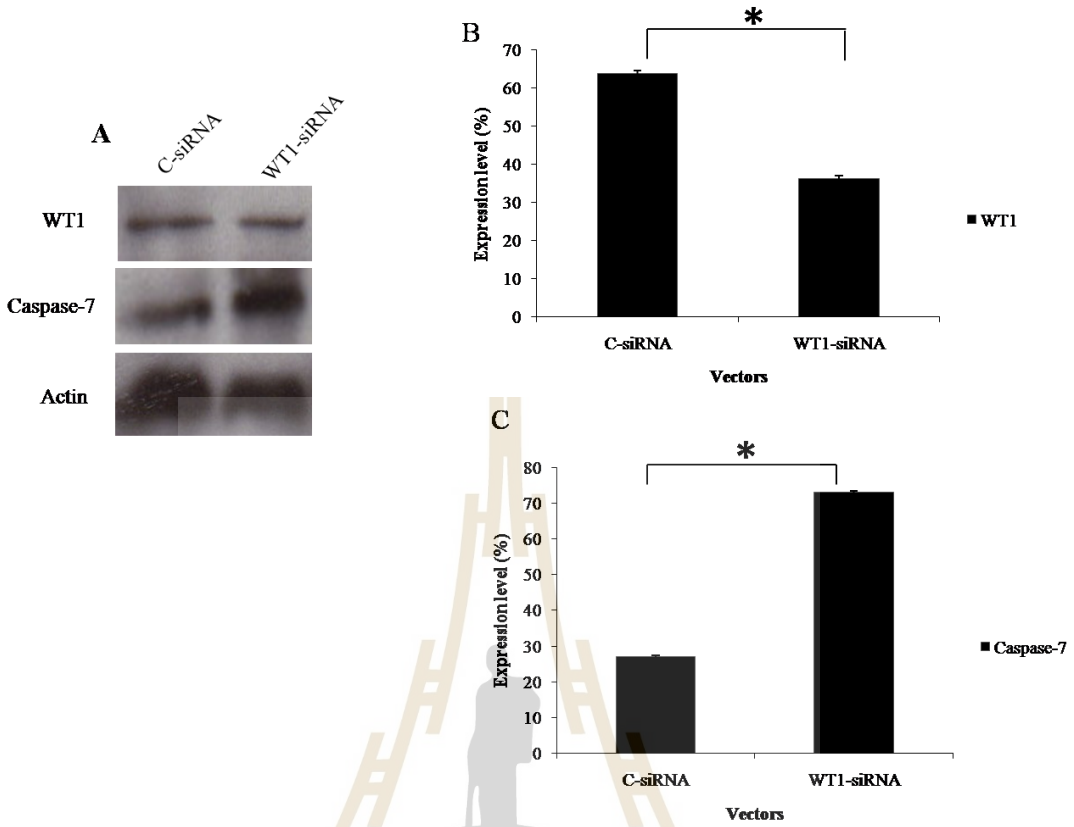

Figure 4.17 Western blot analysis of WT1 and caspase-7 in childhood primary ALL subtype L1 cells after WT1-siRNA transduction. At 48 hours after transduction, L1-WT1-siRNA cells and L1-C-siRNA cells were collected and protein extraction was performed by using CelLytic M reagent. Twenty micrograms of proteins were collected for Western blot analysis. Each protein bands were analysis by horse radish peroxidase reaction and exposed to the film (A). Image J sorfware was used for protein bands density measurement. The analysis of WT1 (B) and caspase-7 (C) was evaluated. Actin was used as a control.

4.2.9 mRNA expression level

The effect of WT1-siRNA on gene expression level was performed by reverse transcriptase PCR. This study, Two patients samples were collected based on the sufficient amount of isolated PBMC. WT1 mRNA expression level is the first target for the expression measurement. WT1-siRNA has effect to destroy WT1 mRNA of primary ALL subtype L1 cells. This indicates that WT1 is a crucial molecule for growth regulation of acute lymphoblastic leukemia (ALL). In consistency, WT1-siRNA was not only controlling cells growth but also inducing cell apoptosis which had evidences by the decreasing of IL-2, IL-2RB, and IL-2RG expression level (Figure 4.18A). The WT1 mRNA level was extreamly inhibited in L1-WT1-siRNA when compared together with L1-C-siRNA sample. Moreover, the suppression effect was also detected on the mRNA of IL-2 and its' receptors (beta and gamma subunits). WT1-siRNA could suppress the expression levels of IL-2, IL-2RB, and IL-2RG as 25±0.3%, 39±0.3%, and 45±0.6%, respectively, after transduction. These findings indicate that our new designed WT1-siRNA significantly downregulates IL-2 and it's receptors expression (Figure 4.18B). However, the expression of truncated WT1 isoform was observed as the same level in both of L1-WT1-siRNA and L1-C-siRNA samples. In this study, our new designed WT1-siRNA acts with a specific position of 1528 of WT1 mRNA sequences. Therefore, WT1-siRNA also significantly affected with WT1 mRNA expression.

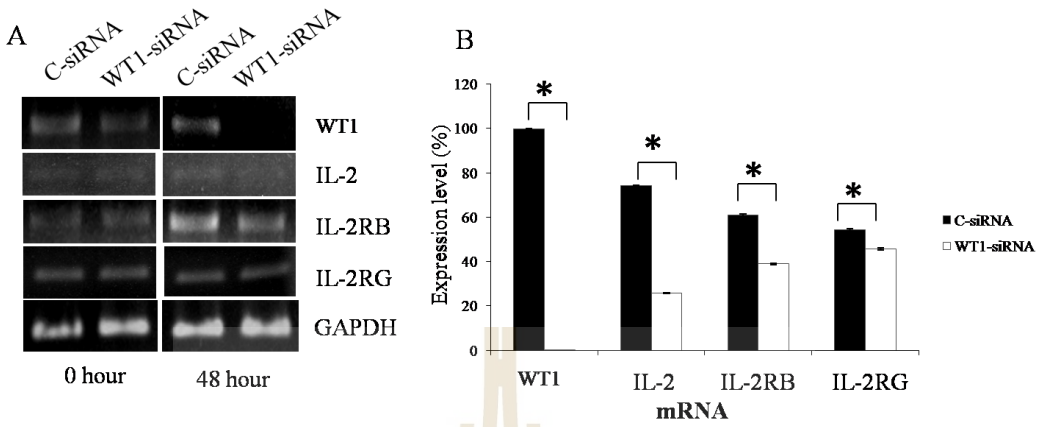

Figure 4.18 mRNA expression of primary childhood ALL subtype L1 cell was performed after WT1-siRNA transduction. At 0 and 48 hours after transduction, L1-WT1-siRNA cells and L1-C-siRNA cells were collected for total RNA extraction. Reverse transcriptase PCR was performed for gene expression study. The expression levels of various PCR products were shown (A). Each PCR products band of L1-WT1-siRNA was analyzed by Image J software which represented as percent expression level (B).

CHAPTER V

DISCUSSIONS

5.1 Leukemic cell line

Small interference RNA (siRNA) is an effective technique to silence a complementary mRNA of target cells as resulted in downregulation of specific mRNA expression. Wilms' tumor 1 (WT1) is a transcription factor that plays a key role as an oncogene in several types of leukemic cells including acute lymphoblastic leukemic cells and chronic myeloid leukemic cells. WT1 was first isolated from human embryonic kidney cells which were subset of hematopoietic cells. It is located on human chromosome 11 (band p13) and its amino acid sequence is homology with some human growth factors including EGR1 and EGR2 (Call et al., 1990). *WT1* was first identified as a tumor suppressor gene. However, the overexpression of this gene was found in various types of cancer such as epithelial ovarian cancer (Hylander et al., 2006), breast cancer (Loeb et al., 2001), and hepatocellular carcinoma (Perugorria et al., 2009). High levels of WT1 in many cancers indicate an oncogenic function in leukemogenesis and tumorigenesis. The majority of WT1 are identified in 4 isoforms depending on two alternative splicing regions. The first alternative splicing event affects the entire exon 5 and leads to presence or absence of 17 amino acids (17AA). The second alternative splicing event generates an insertion or no insertion of 3 amino acids, lysine, threonine, and serine (KTS) in the very end of exon 9 between the third and fourth zinc-fingers, affecting the conformation of zinc-finger in WT1 protein

(Haber et al., 1991; A. A. Morrison, Viney, Saleem, and Ladomery, 2008). Each major isoform generate different characters including, isoform A contains with 429 amino acids and encodes 47.19 kDa protein size which named WT1(-17AA/-KTS), isoform B contains with 446 amino acids and encodes 49.06 kDa named WT1(-17AA/+KTS), isoform C contains 432 amino acids and encodes 47.52 kDa named WT1(+17AA/-KTS), and isoform D contains 449 amino acids and encodes 49.39 kDa named WT1(+17AA/+KTS). Different isoforms of WT1 play different roles depended on cell types and its partner proteins such as Par-4 which interacts with the 17AA of WT1(+KTS) resulting to activate transcription of WT1, while the inhibition of the transcriptional activation is generated by interaction between Par-4 and zinc-fingers of WT1(+KTS) (Sells et al., 1997). The WT1(+17AA/+KTS) is a dominant isoform in AML and CML in blast crisis patients. This isoform represented nearly 50% of total WT1 expression which is analyzed by real time RT-PCR. Interestingly, expressions of WT1(+17AA/-KTS) and WT1(+17AA/+KTS) isoform were predominated in AML patients whereas WT1(-17AA/+KTS) isoform was found in CML patients but less than WT1(+17AA/+KTS) isoform. The WT1(-17AA/+KTS) isoform shows the potential to serve as an early marker of relapse onset of CML patients (Lopotova, Polak, Schwarz, Klamova, and Moravcova, 2012). Our study used new designed WT1-siRNA cloned into pPRIME-CMV-GFP-FF3 to silence WT1 expression in K562 cells both at mRNA and protein levels resulted to inhibit cellular proliferation and apoptosis induction of K562 cells.

In this study, we used lentiviral particle as a vehicle for delivery of WT1-siRNA into the K562 cells. Lentivirus is an excellent vector for gene therapy approaches because it can be transduced into dividing and non-dividing target cells. In

addition, lentivirus contains with psuedotype of VSV-G glycoprotein which encodes viral envelope for reduction of HIV-1 tolerant effects. Moreover, this system is produced only replication-incompetent lentivirus which is more safety to use. In this work, cell sorting approach was performed for accurate collection of total transduced cells prior to subject to any further experiments. The inhibition of cellular proliferation of K562-WT1-siRNA-GFP^{+} cells was observed at a time dependent manner while no effect was found in K562-C-siRNA-GFP^{+} cells. In addition, a highest value (more than 85%) of inhibitory rate was found at 96 hours post-transduction. These results suggest that WT1 is an important for the survival of K562 cells, therefore, the downregulation of WT1 by WT1-siRNA is a gold-target for inhibition of K562 cells proliferation. There are some reports indicate that the proliferation of K562 cells and fresh leukemic cells from AML and CML patients were inhibited by downregulation of WT1 via antisense oligonucleotides (Yamagami et al., 1996). In addition, the inhibition of WT1 expression caused G2/M arrest of cell division (Yamagamia et al., 1998). Our findings demonstrated that WT1-siRNA can induce apoptosis of K562 cells. Specifically, the early apoptosis detected by cell membrane permeability was increased at 12 and 24 hours post-transduction, consequently, late apoptotic population demonstrated by double positive of cell membrane permeability and DNA fragmentation was observed at 48 and 72 hours post-transduction. Results suggest that our new designed WT1-siRNA can induce apoptotic phenotypes which have evidences by cell membrane change and extremely DNA damage on K562-WT1-siRNA-GFP^{+} cells. Our findings confirm that the endogenous WT1 regulates apoptosis of leukemic cells. In addition, the high level of WT1 expression induced by BCR/ABL1 fusion protein enhances apoptotic resistance

on ABL-1 tyrosine kinase inhibitor imatinib treated K562 cells (Svensson et al., 2007). Therefore, downregulation of WT1 expression by WT1-siRNA combines with BCR/ABL-siRNA increased sensitivity of K562 cells to imatinib treatment and induce apoptosis of leukemic cells (A. H. Elmaagacli et al., 2005; Koldehoff, Steckel, Beelen, and Elmaagacli, 2007). Moreover, our new designed WT1-siRNA suppressed the expression of survival cytokine Interleukin-2 (IL-2) and its receptor subunits beta and gamma (IL-2RB and IL-2RG) transcripts. IL-2 is a T cell-derived cytokine which plays a key role to induce cellular proliferation, differentiation and functional activation. Although IL-2RB was downregulated at 72 hours and IL-2RG was suppressed at 48 hours post-transduction, IL-2 mRNA still expressed until 72 hours before it was inhibited at 96 hours post-transduction. These results suggest that IL-2 still binds to other subunits of receptor such as subunit alpha (IL-2RA) and beta (IL-2RB). In addition, heterotrimer of each subunit has an essential role for IL-2 binding affinity. Although, heterodimer of alpha-beta (AB) or beta-gamma (BG) subunits can induce IL-2 signaling pathway, however, signaling activation is depended on binding affinity property of IL-2. In addition, the heterodimerization of IL-2RB and IL-2RG exhibits intracellular signals for T-cell proliferation (Kawahara, Minami, and Taniguchi, 1994; Nakamura et al., 1994). In our study, the downregulation of IL-2RB and IL-2RG transcripts led to inhibit IL-2 mRNA expression account for one possible mechanism of cell death induction of K562-WT1-siRNA-GFP^{+} cells. This point of view has been supported by the downregulation of IL-2RG which not only effect IL-2 but also impact IL-4, IL-7, IL-9, IL-15, and IL-21 signaling pathways (Kim, Imbert, and Leonard, 2006). These results indicate that our new designed WT1-siRNA activates cell death via the

inhibition of IL-2 signaling pathway. In additional, the cytoplasmic domain of IL-2RG generates Jak/Stat stimulation via the phosphorylation of Jak3 molecule and further activates Stat phosphorylation. The phosphorylated-Stat molecule is homodimerized and moves into nucleus then activates target gene transcription and generates cytokine production (Stahl et al., 1995). Sciesielski and colleagues showed that WT1 not only inhibit IL-2 expression but IL-10 also. In addition, WT1(+17AA/-KTS) isoform stimulates IL-10 promoter as five-folds while WT1(+17AA/+KTS) can induce eight-folds of the IL-10 promoter stimulation. On the other hands, IL-10 level was reduced approximately 90% by the silencing of WT1 using RNA interference. Their results indicated that WT1 contain immune regulatory function by IL-10 signaling regulation (Sciesielski, Kirschner, Scholz, and Persson, 2010).

Our data revealed that WT1-siRNA suppressed WT1 protein expression at 72 hours and 96 hours post-transduction. This result suggests that WT1-siRNA has the inhibitory effect on WT1 expression both mRNA and protein expression levels. Our results showed the 2 specific bands of WT1 protein as 52 kDa and 54 kDa which indicate that WT1 has several isoforms based on the presence or absence of 17 amino acids encodes at exon 5 and KTS insertion or deletion at exon 9 (Avril and Michael, 2006). Our new designed WT1-siRNA can silence both of +17AA and -17AA isoforms of WT1 proteins. However, the expression of WT1 protein depends on the proliferation of cells. Kerst and coworkers showed inhibition of acute and chronic myeloid leukemic cells proliferation by reducing some growth factors in fetal craft serum and decreasing pH of cell culture medium. Moreover, they also observed inhibition of WT1 protein expression when reduction of cellular proliferation (Gunter Kerst and Marketa Kalinova, 2008). Their experiment can support our results for

using WT1-siRNA to inhibit the proliferation of K562 cells leading downregulation of WT1 expression both WT1 mRNA transcripts and WT1 protein at time dependent manner. Moreover, downregulation of WT1 mRNA expression level induced apoptosis of K562 cells which was determined the expression of procaspase-7 protein by using Western blot analysis. Caspase-7 is a member of interleukin-1 beta converting enzyme (ICE)/ced-3 subfamily. It is classified as an effector/executioner caspase and cleaved via proteolysis mechanism by initiator caspases including caspase-8, caspase-9 and caspase-10. The overexpression of caspase-7 long form without pro-domain (active form) can induce apoptosis. Our result demonstrated the expression of caspase-7 precursor (35 kDa), large subunit cleaved caspase-7 (30 kDa), and small subunit cleaved caspase-7 (15 kDa) indicated apoptotic induction of K562 cells by our new design WT1-siRNA. Consequently, the complete apoptotic induction by WT1-siRNA was found at 72 hours and 96 hours post-transduction observed by caspase-7 proteins suppression. The result suggested that our new designed WT1-siRNA activates cell death of K562 until complete apoptotic mechanism at 72 hours post-transduction. The inactive caspase-7 (short form) can inhibit the activity of active form leading repression of active form expression level. However, WT1 plays a role for positive and negative regulations of apoptosis. The positive regulation of apoptosis by mean tumor suppressor function, Wide-type WT1(-17AA/-KTS) can activate both intrinsic and extrinsic pathway of apoptosis via transcriptional regulation of endogenous caspase-8 (death-receptor mediated apoptotic enzyme), caspase-9 and proapoptotic protein Bak led to induce apoptosis in Saos-2 cells. This indicates that caspase-8, caspase-9 and Bak are the direct targets of wide-type WT1 (Debra J. Morrison, English, and Licht, 2005). The negative regulation of apoptosis represented

as oncogenic function of WT1, WT1(-17AA/+KTS) and WT1(+17AA/+KTS) inhibit the expression of caspase-3, caspase-9 and Bax apoptotic proteins (Loeb, 2006).

Downregulation of WT1(+17AA/+KTS) and WT1(-17AA/-KTS) isoforms inhibited K562 cell differentiation to megakaryopoiesis which was induced by 12-o-tetradecanoylphorbol 13-acetate (TPA). These effects were determined by Nitroblue tetrazolium (NBT) reduction and anti-CD61 marker which was reduced after inhibition of WT1 gene expression (Carrington and Algar, 2000). In addition, the report mentions that WT1-overexpressing K562 cells loss the capacity of detachment between cell and surface of culture flask. Moreover, floating cells were observed in the culture medium more than control cells which were not induced to WT1 overexpressing cells. However, result is not significant difference when compared with control cells. These findings suggest that *WT1* gene is able to increase the differentiation signal and inhibits the differentiation program. However, cell growth suppression is not promoted in this experiment. Therefore, the direct role of WT1 is regulation of cellular differentiation in megakaryopoiesis. Consistent with other reports, we found that WT1 acts as oncogenic gene. Moreover, it is related with the increasing of differentiation signal. In general, K562 cells are floating cells in liquid medium. However, in this study, we observed many adherent cells in the culture of K562-WT1-siRNA-GFP$^+$ cells while did not in K562-C-siRNA-GFP$^+$ cells culture. This finding indicated that our new designed WT1-siRNA may increase adhesion molecule on cell surface. These results showed the potential of WT1-siRNA for reduction of leukemic cells spread in blood circulation for effective leukemic treatment. To support this finding, the increasing of CD34$^+$ molecule, on the cell surface of hematopoietic cells, enhance cell-cell adhesion. In addition, actin is the

partner protein of WT1 which plays a role as cofactor of WT1 in both of nuclease and cytoplasm of the cells. The polymerization of monomeric-actin led to produce filamentous-actin and further facilitate RNA binding and promoting WT1 cytoplasmic function (Dudnakova, Spraggon, Slight, and Hastie, 2010). The WT1(-17AA/-KTS) represses the combination between integrin subunit and actin binding complex (alpha actinin1, gelsolin, and cofilin binding with actin) enhancing cancer metastasis (Jomgeow and Tima, 2009). Therefore, downregulation of WT1(-17AA/-KTS) has a high possibility to control cancer cell motility resulting to decrease cancer metastasis.

The blocking of WT1 directly affected in all myeloid lineage differentiation. Therefore, WT1 may play the roles for normal hematopoietic cells in the early stage of them and earlier stage of lineage commitment. In addition, WT1 mRNA was rapidly downregulated in the early phase of erythroid, megakaryocyte, and myelomonocytic cells differentiation pathway (Svedberg, Chylicki, and Gullberg, 1999). However, WT1(+KTS) isoform showed both effects for promotion and inhibition of monocytic differentiation in murine promyolocytic leukemic cells M1 (Smith, Weil, Johnson, Boyd, and Li, 1998). Additionally, regulation of WT1 has been undergo differentiation of K562 cells belong to neuronal like cells (Goodfellow et al., 2011). However, the effects of WT1 expression in cancer are depended on the ratio of four major isoforms, protein partners of WT1 and cancer types (Simpson et al., 2006). WT1 differently regulates cell cycle progression depending on its isoform. The WT1(-17AA/-KTS) induced cyclin E suppression resulting the blocked cell cycle progression, whereas WT1(+17AA/+KTS) inhibited cyclin E suppression inducing cellular proliferation (Loeb et al., 2002). In the last few years, it has been trying to search the effect of WT1 downregulation by using medicinal plant extract. Pure

curcumin could reduce K562 cell proliferation by suppression of WT1 in dose- and time-dependent manners. The suppression effect of K562 was occurred via the inhibition of protein kinase-C (PKC) pathway. In the other words, pure curcumin could interfere WT1 auto regulatory activity that caused repression of WT1 expression (Semsri, Krig, Kotelawala, Sweeney, and Anuchapreeda, 2011).

5.2 Primary leukemic cell

Wilms' tumor 1 is a zinc-finger transcription factor that locates in the chromosome 11p13. The important roles of WT1 are activation or suppression of transcription process which lead to control cellular proliferation, differentiation, and apoptosis (Loeb et al., 2002; Sugiyama, 2002). WT1 is mostly overexpressed in various types of cancers including hematopoietic malignancy like leukemia. Acute lymphoblastic leukemic cells (ALL) showed majority expression of WT1 around 70-90% by real time quantitative PCR analysis while WT1 expression in leukemic cells causes the loose of their ability to control cellular proliferation. These findings suggest that WT1 is the critical target gene for leukemic treatment (Boublikova et al., 2006). Moreover, the most cases of ALL patients (92%) were detected WT1 overexpression. However, once comparison between T-cell and B-cell ALL, significant high expression level was shown in immature B-lineage more than mature T-lineage and mature B-lineage in adult ALL patients, respectively. It has been reported that WT1 was more expressed in clinical ALL leukemic cells with myeloid marker than without myeloid marker coexpressions (Busse et al., 2009). In the same way, the higher level of WT1 expression was found in ALL with relapse, AML, and ALL, respectively (Hu et al., 2010).

Previously, we studied about the effects of our new designed WT1-siRNA on leukemic cell line (K562). The appropriated results in terms of growth inhibition and apoptosis induction were shown at 48, 72, and 96 hours post-transduction. Based on our preliminary study, we determined WT1-siRNA effect on primary ALL leukemic cells at 48 hours post-transduction. Moreover, the primary cells could not suitable for long term of culture. Therefore, 48 hours post-transduction is the optimizing time for this experiment. We used our new designed WT1-siRNA, which specific the position of 1528 of WT1 transcript for regulation of endogenous WT1 of primary ALL hematopoietic cells. Sixteen childhood patients (3-18 years of age) with acute lymphoblastic leukemia (ALL) subtype L1 were participated in this study with informed consents which have been approved from Suranaree University of Technology and Maharaj Nakhonratchasima hospital's Ethics Committees. The thirteen cases were new cases and three cases were relapse. Consequently, ten milliliter of peripheral blood was taken from each patient. Peripheral blood mononuclear cells (PBMC) were separated by using Ficoll paque protocol. The proliferation of ALL cells was determined by MTT assay after 48 hours post-transduction. WT1-siRNA inhibited cellular proliferation of primary ALL cells by decreasing of absorbance value at 0.20±0.001 when compared with 0.27±0.005 of C-siRNA or around 22% cell growth suppression. This result is consistency with previous data which reported the effects of WT1-siRNA on primary CML patients in blast crisis (UPN3). WT1-siRNA affected to decrease percent proliferation of CML from 27.5±2.7% to 18.7±2.1% and it shows significant decreasing to 8.6±1.1 % when cotreatment together with BCR-ABL-siRNA (A. H. Elmaagacli et al., 2005). Our results suggest that the silencing of endogenous WT1 leads interfere primary ALL cell

proliferation and showed significant inhibitory effect on ALL subtype L1 patient samples. Moreover, the impacts of cell death increasing and apoptosis induction of primary ALL cells were evaluated after WT1-siRNA treatment. Dead induction of primary ALL cells was measure approximately 2.6±0.3 folds in WT1-siRNA treated cells whereas only 1.7±0.4 folds were found in C-siRNA treated cells. In CML patients, WT1-siRNA induces cell death for 19.6±1.9% and increasing to 24.8±2.1% when cotransfected with BCR-ABL siRNA (A. H. Elmaagacli et al., 2005). This data indicates that CML cells have two important oncogenes (*WT1* and *BCR-ABL*) as the targets for treatment. To confirm dead induction of ALL cells by our new designed WT1-siRNA, we evaluated the activities of caspase-3 and/or caspase-7 which are the executioner caspases in the last step of apoptotic cascade. Our result exhibited the high level of caspase-3/7 enzymes activities in WT1-siRNA for 5,104±836 RFU while lower level of activity was observed in C-siRNA (1,823±374 RFU). This result indicated that the apoptotic induction mechanisms on ALL cells by WT1-siRNA occur via the activation of caspase enzyme and enhance apoptotic signs such as cell shrinking, apoptotic body induction, DNA fragmentation, chromosome condensation and cell membrane permeability. In addition, we identified apoptosis population by Annexin V-FITC/PI staining property using FACs analysis. The principal of apoptotic cell is characterized by cytoplasmic membrane permeability determination and DNA fragmentation. The membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane of apoptotic cells, thereby exposing PS to the external cellular environment. Annexin V-FITC is $Ca2^{+}$ dependent phospholipid-binding protein that has a high affinity for PS binding. Annexin V-FITC binds to cells with exposed PS indicated cellular apoptosis. Since externalization of

PS occurs in the earlier stages of apoptosis. In this study, our results exhibited 36.63±6.35% and 33.25±9.8% of early and late apoptosis populations of L1-WT1-siRNA cells at 48 hours post-transduction. The result indicated that our new designed WT1-siRNA activates both early and late apoptosis in L1-WT1-siRNA cells. In the other hand, these effects were not observed in normal blood cells as shown 22.2±8% and 22.3±8% on PBMC-C-siRNA cells and PBMC-WT1-siRNA cells at 48 hours post-transduction, respectively. Therefore, it has potential for the using of WT1-siRNA in order to further leukemic treatment and research.

The mechanisms of WT1-siRNA are specific binding and silencing of complementary WT1 mRNA which leads to inhibit WT1 protein translation. This study used new designed siRNA specific WT1 transcript in order to downregulation of WT1 both mRNA and protein levels. The primary hematopoietic samples from ALL patients were evaluated mRNA expression level. Results showed significant downregulation of various mRNAs including WT1, IL2, IL-2RB, and IL-2RG. The data suggested that, our new designed WT1-siRNA is specific for WT1 transcript and impact with essential cytokine like IL-2 which has potential to support leukemic cell growth. The function of IL-2 is depended on it receptor subunits: alpha (IL-2RA), beta (IL-2RB), and gamma (IL-2RG). The best affinity between IL-2 and receptors was occurred in trimeric complex of all receptors. However, only 2 in 3 of subunits are sufficient for IL-2 binding. Our results showed inhibition of IL-2RB and IL-2RG expression after WT1-siRNA treatment in ALL clinical samples. These results confirm the effects of our new designed WT1-siRNA to increase apoptotic induction via caspase-3/7 activation. Moreover, WT1-siRNA could significantly induce ALL cell death via downregulation of IL-2 pathway. WT1 has many isoforms generated by

RNA splicing, RNA editing, alternative splicing, and translation with different start codon which produce different protein sizes of WT1 and play different roles depended on cell types (Hohenstein and Hastie, 2006). The aberrant of truncated WT1 is contained at the 5' end of WT1 transcript which located in intron 5 and at the 3' end which located at exon 6-10 of WT1. Novel truncated-WT1 was detected by RT-PCR in many types of cancer such as human breast cancer cell line; MCF-7, CML cell line; K562, blood sample from AML patients but not in normal peripheral-blood samples. Results suggested that the novel truncated-WT1 plays a key role as tumor marker in various types of cancer (Dechsukhum et al., 2000). In addition, there is some report studied the expression level of WT1 in childhood ALL. The results showed the comparison of WT1 expression level on various subtypes of ALL. The data indicated that childhood B-cell precursor (BCP-ALL) showed significant less of WT1 expression level than childhood T-ALL cases (Boublikova et al., 2006).

Finally, we could summarize that our new designed WT1-siRNA has the potential effects on primary childhood ALL cells for inhibition of cellular proliferation, induction of apoptosis via caspases enzyme activation, enhancement of early and late apoptosis. All of data caused by downregulation of WT1 both mRNA and protein expressions which also impacted to the survival cytokine such as IL-2 and its receptors and apoptotic protein, caspase-7. Our new designed WT1-siRNA represents as a new tool for acute lymphoblastic leukemic treatment.

5.3 The use of siRNA technology for cancers treatment

The technology of siRNA silencing still have an efficiency in several types of cancer including chronic myelogenous leukemia (CML) (Koldehoff et al., 2007),

breast cancer (Navakanit, 2007), lung cancer and ovarian cancer (Numnum et al., 2007). The investigation of *Hec I* silencing by siRNA against gene causing ovarian carcinoma has been studied by Numnum and co-workers in 2007. Their study used adenoviruses vector carried siRNA against *Hec I* (AdHecI) and enhance-adenoviruses, which were modified viral capsid. Domains of Ad5 knob and Ad3 knob were changed chimeric fiber composed, as represented by F5/3. The F5/3 capsid modification could induce the infection of adenovirus into target cells. AdHecI and infectivity-enhance adenovirus (AdHecIF5/3) has been transfected into two types of ovarian carcinoma cell lines, HEY and SKOV3ip1. After 72 hours post-transfection, the *Hec I* gene knockdown was observed. However, downregulation of *Hec I* gene has been shown significant highest in HEY cell with 78% efficiency by AdHecIF5/3. Moreover, translational inhibition was demonstrated after 96 hours of AdHecIF5/3 transfection process. In the last decade, CML patients were treated by chemotherapy process. The anticancer drug; imatinib was used for CML treatment. However, imatinib-resistance will occur in some CML patients after treatment for the long time. Therefore, several scientists interested to discover a new therapeutic method for CML treatment. Koldehoff and co-worker try to use siRNA against BCR-ABL for downregulation *BCR-ABL* gene expression in immatinib-resistance CML patients. They found that after injected 10 μg/kg body weight of BCR-ABL-siRNA delivery with lipid solution (DLS with anionic lipoplex) into 47 years old philadelphia (+) CML female patient, there were a little bit side effects with dizziness to the patient. After that, they increased and decreased siRNA doses to 30 μg/kg body weight in 60 ml DLS and 10 μg/kg in 20 ml DLS, respectively. They found 70% of blast cells in blood stream after injected siRNA against *BCR-ABL* gene. In the end of this experiment, the death of

patient was an outcome. Researcher suggested that it may be caused by siRNA resistant or transfection failure because presence of serum RNase activity in blood stream of patient. In their experiment, K562 cell line and the peripheral blood cells from CML patient were treated by BCR-ABL-siRNA, both cells showed the reduction of apoptosis of 16.1% and 18.5% and increase the proliferation rate of 14.6% and 28.3%, respectively. These data showed approximately 2 folds as compared with non silencing siRNA. Moreover, *BCR-ABL* expression was decreased one day after transfection and still decreased more than log scale within 7 days (Koldehoff et al., 2007). To support these results, Wilda and co-workers used *BCR-ABL* hybrid genes as a target mRNA for treatment of leukemic cells by using BCR-ABL-siRNA. They found that the specific siRNA could destroy K562 cells and induced apoptotic cell death. Normally, WT1 overexpression is found in several leukemic cell lines such as K562, Kasumi-1; acute myeloid leukemia FAB M2 with t(8;21), MV-4-11; acute monocytic leukemia FAB M5 with t(4;11) (q21,q23) and NB-4; acute promyelocytic leukemia (FAB M3) with t(15;17). However, WT1 gene was more downregulated in K562 than another cell lines after WT1-siRNA treatment, observed by 43% real time RT-PCR and normalized to *GAPDH* expression. Moreover, co-transfection of BCR-ABL-siRNA and WT1-siRNA resulting high efficiency for inhibition of leukemic cells proliferation and induction of apoptotic rate in K562 approximately 15.3% and 26.4%, respectively. Consistency with K562 cell lines, the peripheral blood from CML patients in blast crisis showed proliferation reduction and apoptosis induction as 8.6% and 24.8%, respectively, after both siRNA treatment (Wilda, Fuchs, Wossemann, and Borkhardt, 2002). In contrast, there were a little bit effects on proliferation rate by slight decreasing after transfection with WT1-siRNA in normal

$CD34^+$ cells. Where as, the apoptotic rate was not change after WT1-siRNA transfection (AH Elmaagacli et al., 2005). Recently, CML treatment with use of siRNA silencing against only one target gene may be an insufficient for effective therapeutic. Therefore, Merkerova and co-workers (2006), interested to study downregulation of proliferating cell nuclear antigen (*PCNA*) gene, which represents as one of target gene caused CML, by using siRNA. The important role of *PCNA* gene is coordinated synthesis of leading and lagging strands of SV40 DNA, as a cofactor of polymerase δ (Bravo, Frank, Blundell, and MacDonald-Bravo, 1987). Silencing of *PCNA* gene expression leads to inhibit BCR-ABL positive CML cell proliferation such as K562, KU-812 (b3a2 BCR/ABL fusion type) and MOLM-7 (b2a2). Moreover, the function of target genes including cell cycle factors (cyclin dependent kinase: CDK1, CDK4, PLK1, ERK3 and JNK1), replication transcription and repair factors (RFC, transcription factor gene, STAT5), cell signaling gene (MAP kinase and GTPases), apoptosis relate gene and adhesion gene were disrupted after knockdown the *PCNA* gene. Their studied found that the expressions of target genes were changed after siRNA nucleofection to CML cells as observed 35 of 588 upregulated genes by gene expression array analysis. Moreover, WT1-siRNA technology was used for breast cancer cell line (MCF-7) treatment. The concentration of siRNA was varied as 25-800 nM and tested 120 hours after transfection. They found that the small level of WT1-siRNA (25 nM) can be enough for suppression of MCF-7 proliferation. Furthermore, 100 nM of WT1-siRNA resulted to decrease total number of cells approximately 2 folds after 12 hours of transfection (Navakanit, 2007).

Many researches found that human leukemia derived from the abnormal of oncogene and tumor suppressor genes. Certainly, one of them is the overexpression of *WT1*

gene. *WT1* gene is expressed in many types of solid cancers and leukemias. WT1 locates on the short arm of chromosome 11 and contains 10 exons. Moreover, it contains four zing fingers which derive from exon 7-10 at the carboxy-terminal. The zinc-fingers play a role for binding to promoter regions of DNA (Rosenfeld, Cheever, and Gaiger, 2003). Four isoforms of WT1 are derived from the process of alternatively spliced which are insertion or omission of 17 amino acids at the exon 5 in the central path of the protein and absence or presence of 3 amino acids (lysine, threonine and serine: KTS) between the position at 3 and 4 of zinc-finger domains (M Gessler, Konig, and Bruns, 1992; Harber et al., 1991). The overexpression of WT1(+17AA) isoform leads to antiapoptotic function in leukemia. This action occurs through the protection of mitochondrial membrane damages (Ito et al., 2006). However, WT1(-17AA) isoform is not effect for induction of apoptosis in leukemic cells. WT1-siRNA specific WT1(+17AA) isoform was used as a crucial tool for induction of apoptosis in leukemic cells, the result of these approach induced the activation of caspase-3 and caspase-9 of the intrinsic apoptosis pathway. This WT1-siRNA could not affect on caspase-8 of the extrinsic apoptosis pathway (Ito et al., 2006). The alternative splice II, which is insertion of KTS amino acids leads to bind with early growth factor 1 consensus sequence (Rauscher, Morris, Tournay, Cook, and Curran, 1990) and resulted to disrupt zinc-finger domains in the DNA binding affinity with a speckled pattern of expression within the nucleus (Englert et al., 1995). *WT1* gene encodes 52-54 kDa of nuclear proteins contained with a glutamine-proline rich transregulatory domain and four zinc-fingers DNA binding carboxy-terminal region (Call et al., 1990; M. Gessler et al., 1990). WT1 play a role for regulation of downstream target genes such *EGR-1, IGF-2, IGF-1-R, C-MYC* and

C-MYB with repression or coactivation and activation of a variety of growth associated genes (Rauscher et al., 1990; Scharnhorst, Van der Eb, and Jochemsen, 2001). The interaction of WT1 and target genes sush as WT1(-KTS) isoform has a zinc-finger domain which closely resembling of early growth response-1 (EGR-1) consensus sequence (CGCCCCCGC) since the EGR-1 protein also containing Cys2-His2 zinc-fingers at the C-terminal and 60% of amino acids similar to zinc-fingers 2, 3 and 4 of WT1 protein (Rauscher, 1993). However, the EGR-1 protein does not display significant binding to RNA which unlike with WT1. Whereas contrast effect was found in WT1(+KTS) isoform by mean it can not interact with EGR-1 protein (Rauscher et al., 1990). The ratio of WT1 expression was evaluated during human kidney development as 8.3:3.8:2.5:1.0 of WT1(+17AA/+KTS), WT1(-17AA/+KTS), WT1(+17AA/-KTS) and WT1(-17AA/-KTS) isoforms, respectively (Harber et al., 1991). It has been reported that WT1(-KTS) can repress the effects of WT1(+KTS) in dose-dependent manner by measuring the expression of secreted alkaline phosphatase (SEAP) after pCMVgagpol transfection into cells for CTE evaluation (Bor, Swartz, and Aorrison, 2006). Each WT1 isoform has been shown to repress and activate a variety of cellular promoters via binding to cognate DNA elements. Only WT1(+17AA/-KTS) displays transactivation effect which is required for exon 5 to regulate the other cellular factors (Moorwood et al., 1999). Several studies suggested that the mainly function of WT1(+KTS) isoform related with the post-transcriptional level with RNA processing. So that, they showed that WT1(+KTS) isoform specifically associated with a key splicing factor (U2AF65) and a putative splicing factor (WTAP) (Davies et al., 1998; Larsson et al., 1995; Ortiga et al., 2003). Moreover, WT1(+KTS) can work in conjunction with *cis*-acting of

constitutive transport element (CTE) to export unspliced RNA with retained of intron from the nuclease to cytoplasm and enhance translation processing of the target mRNA of *WT1* gene (Bor et al., 2006). In addition, the expression of WT1 is very rare in the normal blood cells and CD34+ hematopoietic progenitors (Gaiger, Reese, Disis, and Cheever, 2000) but it overexpresses in several leukemic cell lines such as K562 and HL60. However, downregulation of WT1(+17AA) leads to upregulate CD11b antigen expression and induces block NB4 cell differentiation (Gu et al., 2005). The ratio of four major WT1 isoforms is required for characterization between of normal cells and disease. Liu and colleagues tried to identify Wilms' tumors cases out of the normal kidney cells. They studied the ratio of WT1(+17AA) and WT1(-17AA) in cell sample cases which found different ratio between normal kidney and Wilms' tumor cells as 2:1 and 1:1 or 1:5, respectively. This suggestes that the difference in the relative levels of two isoform of WT1 expression could be characterized of subsets of sporadic unilateral Wilms' tumor (Liu, Wang, Deuel, and Xu, 1999). Several reports confirmed that the overexpression of WT1 was found to relate with the cases of new diagnosed and relapsed acute leukemia used RT-PCR analysis. Approximately 60-100% was found in the cases of AML and ALL patients (Rosenfeld et al., 2003). However, the expression level of WT1 was found in ALL lesser than AML cases (Inoue et al., 1994).

CHAPTER VI

CONCLUSION

This study demonstrated the downregulation of WT1 mRNA by using our new designed WT1-siRNA on human chronic myeloid leukemic cell line (K562) and primary childhood acute lymphoblastic leukemic cells subtype L1. WT1 is known as a high immunogenic antigen which overexpresses in various leukemic cells. The overexpression of WT1 leads to hyper-proliferation induction. Thus, WT1 is attractively interested as a target for immunotherapy of leukemia. Our findings revealed that WT1-siRNA contained potent inhibitory effect on endogenous WT1 expression and led to induce apoptosis of transduced leukemic cells. In addition, the proliferation of cells was significantly inhibited as 10±0.01% at 3 hours to 44±9.5% at 96 hours after WT1-siRNA transduction. These results indicated that our new designed WT1-siRNA is an effective tool for leukemic cells growth inhibition. Apoptosis induction is a gold target for this study. The K562-WT1-siRNA-GFP^{+} cells were triggered apoptosis as 70±1% and 76±0.49% within 12 and 48 hours post-transduction, respectively. Moreover, our newly designed WT1-siRNA leads to inhibit cellular proliferation as 79±14% after 48 hours of WT1-siRNA transduction. Furthermore, WT1-siRNA induces both of an early and late apoptosis of primary ALL subtype L1 as 36.63±6.35% and 33.25±9.8%, respectively. However, there is no effect was observed in normal blood cells which used as control. Furthermore, the mechanisms of WT1-siRNA which enhance ALL cells death were occurred via the

activation of caspase-3/7 activities as 1,823±347 RFU to 5,104±836 RFUs at 0 hour and 48 hours after transduction, respectively. Similarly, caspase-7 protein expression was activated up to 60±1.1% while WT1 protein was downregulated to 38±0.6% by WT1-siRNA transduction. However, WT1-siRNA showed high potential of WT1 silencing when compared to C-siRNA tranduced cells which showed 62±0.6% of WT1 expression level.

Therefore, the application of this work is therapeutic value in further research approaches and clinical trials for leukemic treatment.

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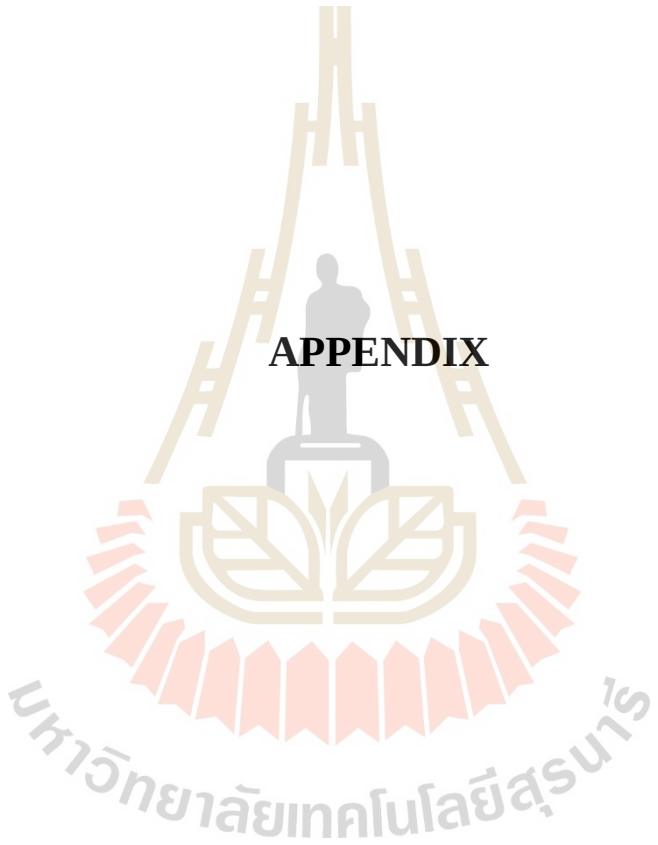
APPENDIX



APPENDIX

1. Reagent for cell culture

1.1 Complete Dulbecco's Modified Eagle Medium (cDMEM)

Mix the reagent as follow:

- DMEM	10.0	g
- FBS	10.0	ml
- $NaHCO_3$	3.7	g
- 1000 U/ml Penicillin/1000 ug/ml Streptomycin	10.0	ml
- 2 mg/ml Amphotericin B	0.4	ml

Add sterile ultra-pure water to bring a volume up to 1,000 ml and adjust pH to 7.25 then sterilize by 0.2 μm filter sterile.

1.2 cDMEM for viral production

Mix the reagent as follow:

- DMEM	10.0	g
- FBS	10.0	ml
- $NaHCO_3$	3.7	g
- D-Glucose	3.5	g

Add sterile ultra-pure water to bring a volume up to 1,000 ml and adjust pH to 7.25 then sterilize by 0.2 μm filter sterile.

1.3 Complete Roswell Park Memorial Institute 1640 (cRPMI 1640)

Mix the reagent as follow:

- RPMI 1640	10.4	g
- FBS	10.0	ml
- $NaHCO_3$	2.0	g
- 10x HEPES	10.0	ml
- 1000 U/ml Penicillin/1000 ug/ml Streptomycin	10.0	ml
- 2 mg/ml Amphotericin B	0.4	ml

Add sterile ultra-pure water to bring a volume up to 1,000 ml and adjust pH to 7.25 then sterilize by 0.2 μm filter sterile.

1.4 Trypsin/EDTA

Mix the reagent as follow:

- Trypsin	0.25	g
- EDTA	0.04	g

Add sterile PBS to bring a volume up to 100 ml.

1.5 Phosphate Buffer Saline (PBS)

Mix the reagent as follow:

- NaCl	8.00	g
- Na_2HPO_4	1.44	g
- KCl	0.20	g
- KH_2PO_4	0.24	g

Add sterile Ultra-pure water to bring a volume up to 1,000 ml. Adjust the pH of the solution to 7.4 then sterilize by autoclaved at 121°C for 15 minutes and store at room temperature.

2. Reagent for viral production

2.1 2.5 M $CaCl_2$

Mix the reagent as follow:

- $CaCl_2$ 18.375 g

Add sterile Ultra-pure water to bring a volume up to 50 ml then sterilize by 0.2 μm filter sterile. Aliquot 10 ml and freeze, store working solution at 4°C for approximately 2 months, indefinite at -20°C

2.2 2x BBS

Mix the reagent as follow:

- NaCl 4.09 g
- BES 2.66 g
- Na_2HPO_4 52.5 mg

Add sterile Ultra-pure water to bring a volume up close to 250 ml then adjust pH to 6.95 with 1 M NaOH. Add water to 250 ml then filter sterilize through a 0.2 μm filter. Aliquot 10 ml and freeze, store working solution at 4°C for approximately 2 months, indefinite at -20°C

2.3 100x Polybrene

Mix the reagent as follow:

- Hexadimethrin Bromide	16.0	mg

Add the cDMEM without antibiotic to bring a volume up to 10 ml. Sterilize by 0.2 μm filter sterile, store at 4°C and make fresh every 2 weeks.

3. Reagent for Western blot

3.1 1.5 M Tris-HCl pH 8.8 (For 4x Running gel buffer)

Mix the reagent as follow:

- Tris-Base (Mw=121.1)	9.08	g
- ddH_2O	20	ml
Adjust pH to 8.8 with HCl		
Adjust volume with ddH_2O to	50	ml

3.2 0.5 M Tris-HCl pH 6.8 (For 4x Stacking gel buffer)

Mix the reagent as follow:

- Tris-Base (Mw-121.1)	3	g
- ddH_2O	25	ml
Adjust pH to 6.8 with HCl		
Adjust volume with ddH_2O to	50	ml

3.3 30% Monomer (30.8%T, 2.7%C bis): (50 ml)

Mix the reagent as follow:

-	Acrylamide (Mw=71.08)	15	g
-	Bis-acrylamide (Mw=154.2)	0.4	g
-	ddH2O to	50	ml

Filtrate with 0.45 um filter paper and degas before use

Store in dark at 4°C (up to 3 months)

3.4 10X Running buffer: 1 L

Mix the reagent as follow:

-	Trizma base (= 0.25 M)	30.3	g
-	glycine (=1.92 M)	144	g
-	SDS (= 1%) add last	10	g
-	ddH_2O to	1000	ml

Do not adjust the pH

3.5 10% Ammonium persulfate (APS) $(NH_4)_2S_2O_8$

Mix the reagent as follow:

-	Ammonium persulfate (Mw=228.2)	0.05	g
-	ddH_2O	0.5	ml

Mix well (Freshly prepare)

3.6 10% SDS (Sodium dodecyl sulfate)

Mix the reagent as follow:

- SDS (Mw= 288.38)	0.1	g
- ddH_2O	1	ml

Mix well, aliquot 150 µl/tube and store at -20ºC

3.7 2X sample buffer

Mix the reagent as follow:

- 1.5 M Tris-HCl, pH 8.8 (130 mM)	1.73	ml
- Glucerol (20% v/v)	4	ml
- SDS (4.6% w/v)	0.92	g
- Bromophenol blue (0.02%)	0.004	g
- ddH_2O to	20	ml

3.8 Coomassie Brilliant Blue R250 (40% methanol;7% Acetic Acid)

Mix the reagent as follow:

- Coomassie brilliant blue R250 (Biorad)	0.0625	g
- Methanol	100	ml
- Acetic acid	17.5	ml
- ddH_2O to	250	ml

Mix well until dissolve and store at room temperature

3.9 Destaining gel solution I (40% Methanol;7% Acetic acid)

Mix the reagent as follow:

-	Methanol	200	ml
-	Acetic acid	35	ml
-	ddH_2O to	500	ml

Mix well until dissolve and store at room temperature

3.10 Destaining gel solution II (5% Methanol;7% Acetic acid)

Mix the reagent as follow:

-	Methanol	25	ml
-	Acetic acid	35	ml
-	ddH_2O to	500	ml

Mix well until dissolve and store at room temperature

3.11 1X Blotting buffer: 1L

Mix the reagent as follow:

-	Trizma base (= 25 mM)	3.03	g
-	glycine (=192 mM)	14.4	g
-	Methanol (20%)	200	ml
-	ddH_2O to	1000	ml

pH should be 8.3; do not adjust

3.12 Blocking buffer: 5% Non Fat Dry Milk or 3% Bovine serum albumin

Mix the reagent as follow:

-	Non Fat Dry Milk	2.5	g
-	Make up in PBS or TBS and sterile filter	50	ml
-	Tween 20 (0.05%)	0.025	ml

Mix well and **freshly prepare before use**

3.13 1X PBS containing 0.1% Tween 20 (Freshly prepare before use)

Mix the reagent as follow:

-	Tween 20	0.5	ml
-	1x PBS	500	ml

Mix well and store at room temperature

3.14 Stripping buffer: 1 L

Mix the reagent as follow:

-	Glycine	15	g
-	SDS	1	g
-	Tween 20	10	ml
-	ddH_2O	1	L

Adjust pH to 2.5

3.15 Transfer buffer

Mix the reagent as follow:

- Trisbase	12.11	g
- Glycine	57.65	g
- Methanol	100	ml
- ddH_2O to	4	L

3.16 Film Developing solution

Mix the reagent as follow:

- Stock Developing solution	100	ml
- Water	400	ml

Store in dark at room temperature (Be able to reuse until color changes to dark tan)

3.17 Film Fixative solution

Mix the reagent as follow:

- Stock Fixative solution	100	ml
- Water	400	ml

Store in dark at room temperature (Be able to reuse until color changes to dark tan)

4. Reagent for agarose gel electrophoresis

4.1 5x TBE buffer

Mix the reagent as follow:

- Tris base	53.0	g
- Boric acid	27.5	g
- 0.5 M EDTA (pH 8.0)	20.0	ml

Add the Ultra-pure water to 1,000 ml

4.2 6x DNA loading dye

Mix the reagent as follow:

- Bromophenal blue	0.025	g
- Xylene cyanol	0.025	g
- Glyceral	3	ml

Add distilled water to a 10 ml final volume, aliquot 1 ml in 1.5 ml centrifuge tube and store at 4°C

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