The Study of WT1 and Cathepsin D in Breast Cancer Cells

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ชื่อวิทยานิพนธ์ การศึกษาโปรตีน WT1 และ Cathepsin D ในเซลล์มะเร็งเต้านม

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บทคัดย่อ

Wilms' tumor 1 (WT1) เป็น transcription factor ในกลุ่ม zinc finger ที่มีบทบาทในกระบวนการทางชีวภาพและการเกิดพยาธิสภาพ เช่น มะเร็ง มีรายงานพบว่ามีการแสดงออกของโปรตีน WT1 ที่เพิ่มขึ้นในมะเร็งเม็ดเลือดขาว (leukemia) มะเร็งปอด มะเร็งรังไข่ และมะเร็งเต้านม การทำงานของโปรตีน WT1 เกี่ยวข้องกับการกระตุ้นหรือยับยั้งกระบวนการต่างๆ โดยขึ้นอยู่กับไอโซฟอร์มของ WT1 ชนิดของเซลล์และโปรตีนที่ร่วมทำงานกับ WT1 จากการศึกษาก่อนหน้าพบว่าเมื่อลดการแสดงออกของโปรตีน WT1 ด้วยเทคนิค siRNA ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 ซึ่งมีตัวรับฮอร์โมนเอสโตรเจนบนผิวเซลล์ พบว่ามีการแสดงออกของโปรตีน Cathepsin D หรือ Cath D ลดลง โดย Cath D เป็นเอนไซม์ย่อยโปรตีนชนิด aspartate ที่ถูกสังเคราะห์ภายในไลโซโซม มีบทบาทเกี่ยวกับการย่อยโปรตีนภายในเซลล์ การรักษาภาวะธำรงดุลของเนื้อเยื่อ การกระตุ้นการทำงานของฮอร์โมนและสารตั้งต้นในเซลล์ ตลอดจนมีบทบาทในการควบคุมการเจริญและการตายของเซลล์ มีหลายการศึกษาพบว่า ในเนื้อเยื่อตัวอย่างของผู้ป่วยมะเร็งเต้านม พบการแสดงออกของ Cath D ที่เพิ่มสูงขึ้น และเป็นตัวระบุการพยากรณ์โรคที่แย่ ถึงแม้ว่าการแสดงออกของ Cath D เกี่ยวข้องกับการกระตุ้นด้วยฮอร์โมนเอสโตรเจน แต่พบว่า ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิดทริปเปิ้ลเนกาทีฟ (TNBC) ซึ่งไม่มีตัวรับฮอร์โมนเอสโตรเจน โปรเจสเตอโรน และ HER2 บนผิวเซลล์ ก็พบการแสดงออกของ Cath D เช่นกัน อย่างไรก็ตาม ความสัมพันธ์ระหว่างโปรตีน WT1 และ Cath D ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด TNBC ยังไม่เคยมีรายงานมาก่อน การวิจัยครั้งนี้จึงสนใจศึกษาบทบาทของโปรตีน WT1 และ Cath D ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิดต่างๆ ในเบื้องต้นได้ศึกษาการแสดงออกของโปรตีน WT1 และ Cath D ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 ซึ่งเป็นกลุ่มที่มี

ตัวรับฮอร์โมนเอสโตรเจนบนผิวเซลล์และในกลุ่มเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด TNBC ได้แก่ MDA-MB-468, MDA-MB-231 และ Hs578T ผลการทดลองพบว่า โปรตีน WT1 และ Cath D มีการแสดงออกที่แตกต่างกันในกลุ่มเซลล์มะเร็งเพาะเลี้ยงทั้ง 2 กลุ่ม กลุ่มมะเร็งเต้านมเพาะเลี้ยงชนิด TNBC พบการแสดงออกของ WT1 ที่มากกว่า Cath D ในขณะที่ Cath D มีการแสดงออกที่เพิ่มขึ้นในกลุ่มเซลล์ที่มีตัวรับฮอร์โมนเอสโตรเจนบนผิวเซลล์ โดยเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MDA-MB-468 ซึ่งมีการแสดงออกของโปรตีน WT1 สูง และมีการแสดงออกของโปรตีน Cath D ที่น้อย ถูกเลือกมาใช้ศึกษาต่อด้วยเทคนิค immunoprecipitation หรือ IP เพื่อทดสอบการจับกันระหว่างโปรตีน WT1 และ Cath D ทั้งยังทำการศึกษาเพิ่มเติมด้วยวิธี molecular docking ผลการศึกษาด้วยวิธีข้างต้น ยืนยันว่าโปรตีน WT1 สามารถจับกับโปรตีน Cath D ได้โดยตรง โดยโครงสร้างดังกล่าวพบว่า active site ของเอนไซม์ Cath D ไม่เข้าจับที่ตำแหน่งย่อยของโปรตีน WT1 จึงสรุปได้ว่า การจับกันของโปรตีนทั้งสองไม่ขึ้นกับบทบาทเอนไซม์ Cath D แต่อย่างไรก็ตาม การทำนายโครงสร้างดังกล่าว ศึกษาที่ pH 7.5 ซึ่งเป็นสภาวะที่พบในไซโทซอลและนิวเคลียสของเซลล์ จึงศึกษาการจับกันของโปรตีนดังกล่าวเพิ่มเติมในสภาวะกรด pH 5 ซึ่งเป็นค่า pH ที่พบได้ในไลโซโซม ด้วยการทำนายประจุโดยใช้ PDB2PQR server ผลการศึกษาแสดงให้เห็นว่า ทั้ง pH 7.5 และ 5 ไม่มีผลต่อการจับกันของ WT1 และ Cath D เนื่องจากบริเวณที่โปรตีนทั้งสองเข้าจับกัน ไม่พบการเปลี่ยนแปลงของประจุ ดังนั้นจึงมีโอกาสพบโครงสร้าง WT1-Cath D ได้ในบริเวณที่มีค่า pH ดังกล่าว

คำสำคัญ: WT1, Cathepsin D, MDA-MB-468, Triple negative breast cancer (TNBC), Protein binding

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ABSTRACT

Wilms' tumor 1 (WT1) is a zinc finger transcription factor that involved in both biological process and pathological condition including cancer. Overexpression of WT1 was reported in leukemia and a variety of solid tumor such as lung cancer, ovarian cancer as well as breast cancer. The effect of WT1 function result in activation or repression role that depended on its isoform, cell type and partner proteins. In MCF-7, ER-positive breast cancer cell line, WT1-related proteins were observed by knockdowned WT1 with siRNA technique and analyzed protein patterns with mass spectrometry. This experiment showed upregulation of cathepsin D (Cath D) when WT1 was downregulated. Cath D is aspartic protease presented mature enzyme in lysosome. It has a role in metabolic degradation of intracellular proteins, tissue homeostasis and activation hormone and precursors in the cell including regulation of cell growth and death program. Various studies have been found Cath D overexpression and proposed as a poor prognostic factor in breast cancer. Although Cath D expression was regulated by estrogen stimulation, but in TNBC which lacking of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression could also express Cath D expression. However, the relationship between WT1 and Cath D in TNBC has never been reported. In this study, the relationship of WT1 and Cath D protein was examined in breast cancer cell lines. Initially, endogenouse protein expression of WT1 and Cath D was observed in MCF-7 ER-positive cell line and TNBC cell lines including MDA-MB-468, MDA-MB-231 and Hs578T. The immunoblotting result showed WT1 and Cath D endogenouse protein expression was different between the groups of cell lines. WT1 protein expression was higher than Cath D in TNBC cell lines while Cath D expression was higher in ER-positive cell line. MDA-MB-468 TNBC cell which showed highest WT1 expression and lowest level of Cath D protein was chosen to clarify WT1-Cath D protein binding by immunoprecipitation (IP) technique. The IP result revealed that WT1 protein could

form complex with Cath D in MDA-MB-468. Molecular docking also confirmed that WT1 could directly bind to Cath D. From WT1-Cath D docking structure, active sites of Cath D enzyme were not presented to WT1 cleavage sites. This result could suggest that WT1 interacted with Cath D with catalytic independent. However, WT1-Cath D binding was predicted at pH 7.5 that presented in cytosol and nucleus of the cell. At pH 5 or acidic compartment where presented in lysosome was further observed by PDB2PQR server for charge prediction. The result showed both pH 7.5 and 5 were not affected to WT1-Cath D interaction because the charge alteration was not presented on WT1 and Cath D interacted surface. Thus, WT1-Cath D binding might find at intracellular compartment where showed above pH.

KEYWORDS: WT1, Cathepsin D, MDA-MB-468, Triple negative breast cancer (TNBC), Protein binding

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LIST OF ABBREVIATIONS AND SYMBOLS

α	=	Alpha
AIF	=	Apoptosis inducing factor
ATCC	=	American Type Culture Collection
β	=	Beta
Bax	=	BCL2-associated X protein
Bcl-2	=	B-cell lymphoma 2
bFGF	=	Basic fibroblast growth factor
Bid	=	BH3 interacting-domain death agonist
BRCA1	=	Breast cancer gene 1
BRCA2	=	Breast cancer gene 2
Cath D	=	Cathepsin D
CK5/6	=	Cytokeratin 5/6
C-terminal	=	Carboxyl-terminus
^{o}C	=	Degree Celsius
DFS	=	Disease-free survival
DNA	=	Deoxyribonucleic acid
ER	=	Estrogen receptor
et al.	=	Et ali (Latin) and others
FOXO	=	Forkhead box protein O 1
g	=	Gram
h	=	Hour (s)
HER2	=	Human epithermal growth factor 2
IGF-I	=	Insulin-like growth factor-I
IGF-IR	=	Insulin-like growth factor-I receptor
IP	=	Immunoprecipitation
kb	=	Kilo base
kDa	=	Kilo Dalton
K	=	Kelvin
KTS	=	Lysine, Threonine, Serine
L	=	Liter

M	=	Molar
mg	=	Milligram
min	=	Minute (s)
ml	=	Milliliter
mM	=	Millimolar
MMPs	=	Matrix metalloproteinase
mRNA	=	Messenger RNA
MW	=	Molecular weight
M6P	=	Mannose-6-phosphate
nM	=	Nanomolar
nm	=	Nanometer
ns	=	Nanosecond
N-terminal	=	Amino-terminus/ amine-terminus
OS	=	Overall survival
PBS	=	Phosphate Buffered Saline
PR	=	Progesterone receptor
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF	=	Transforming growth factor
TNBC	=	Triple negative breast cancer
VEGFR	=	Vascular endothelial growth factor receptor
v/v	=	Volume per volume
WT1	=	Wilms' tumor 1
WT1 (+/+)	=	WT1 17AA+/KTS+
WT1 (+/-)	=	WT1 17AA+/KTS-
WT1 (-/+)	=	WT1 17AA-/KTS+
WT1 (-/-)	=	WT1 17AA-/KTS-
w/v	=	Weight per volume
μ	=	Mu
μl	=	Microliter
μg	=	Microgram
μM	=	Micromolar

11p5	=	Gene located on short arm (p) of chromosome 11 at band/ position 5
11p13	=	Gene located on short arm (p) of chromosome 11 at band/ position 13
xg	=	Relative centrifugal force

CHAPTER 1

INTRODUCTION

Background and rational

Breast cancer is the major threatens health female worldwide. Approximately 1.5 million diagnosed cases of breast cancer were revealed in 2012 and 520,000 cases were identified of deaths rate suffered from breast cancer (1, 2). Numerous factors were related with breast tumor occurrence, such as sex, age, ethnicity, hormone, lifestyle, genetic factor as well as biological molecules. However, early diagnosis of breast cancer are the most considerable strategies to prevent mortality from cancer threaten. In addition, tumor subtype that classified depending on receptor on the cell surface of breast cancer tissue by immunohistochemistry or IHC technique can also provide information for prognosis and therapeutic diagnosis. For instance, estrogen receptor (ER) positive subtype case is medicated with Tamoxifen to block estrogen receptor. In human epithermal growth factor receptor II amplification or HER-2 positive case is treated with Trastuzumab/Herceptin for obstructed HER-2 receptor. However, there is one subtype of breast cancer that lack of the estrogen receptor (ER), progesterone receptor (PR) and human epithermal growth factor 2 (HER-2), called triple negative breast cancer or TNBC. This characteristic result in extremely challenging of the strategic therapy in TNBC patient.

TNBC is typically observed onset at a younger age (45-55 years old women) including with high mean tumor size, higher tumor grade. It has an extremely poor prognosis, relapse of disease and poor survival rate when compared to other breast subtypes (3). Several studies are being developed biomarker that specific target of TNBC subtype. Significantly expressed of molecular biology in breast cancer cell that differed from normal breast were noticed in many studies. High-level of WT1 protein, a zinc finger transcription factor, has been found in leukemia and solid tumor such as stomach, prostate including breast cancer (2, 4). Furthermore, WT1-knockdowned by shRNA technique resulted in mitochondrial damage and lead to apoptosis in several tumor cell which WT1 expressed (5). These evidences showed that WT1 involved in

cell proliferation and inhibited apoptosis of cancer. Therefore, WT1 has an oncogenic role in diverse tumors as well as breast cancer. Qi *et al*. (2012) reported WT1 mRNA high expression can be predicted poor prognostic factor of breast cancer cases and also demonstrated that WT1 was liable upregulated in tumors with high histological grades. Moreover, in ER-negative was higher WT1 expression than ER-positive breast cancer patients (6). This study could explain that estrogen stimulation was independent in tumor cell proliferation in TNBC. However, the tumor survival mechanism in TNBC is greatly complicated. MDA-MB-231, TNBC cell line, were reported that all WT1 isoform-cell sublines including parental cell line expressed MMP-9 that digest extracellular matrix leading to metastasis of cancer cell including vasculogenic mimicry formation in angiogenesis (7). The important role of WT1 in breast cancer not only tumor cell growth and anti-apoptotic function but also invasion and migration properties of WT1 may involve in tumor aggressiveness with its isoform-depending (8). For these reasons, WT1 maybe one of key factors that enhances TNBC aggressiveness. Moreover, previous evidence found WT1 (-/-) or isoform A involved in cell cycle arrest, whereas WT1 (+/+) or isoform D associated with epithelial-mesenchymal transition inducing (9). On insulin-like growth factor-I receptor (IGF-IR) promoter, WT1 without KTS could activate IGF-IR gene while WT1 with KTS was abolish the IGF-IR activity. In addition, when WT1 interacted with p53 wild type, IGF-IR promoter was inhibited. Conversely, IGF-IR expression was stimulated and induced cell transformation and anti-apoptosis when WT1 co-worked with p53 mutation (10). Thus, the effect of WT1 depended on its isoform and partner proteins (4).

Our previous study, WT1 protein expression was knockdown in MCF-7, ER- positive human breast cancer cell line, by using siRNA and related protein expressions were identified by mass spectrometry. Interestingly, after WT1 was silenced, Cathepsin D (Cath D) was significantly upregulated expression when compared with control (Unpublished data). Cath D was reported as mitogenic effect in breast cancer. In many method, such as immunohistochemistry technique, in situ hybridization, cytosolic immunoassay, Northern blot detection and Western blot analysis were showed Cath D overexpression in breast cancer cells (11). Basically, Cath D is an aspartic protease, synthesized at rough endoplasmic reticulum, that has an

important role in both physiological and pathological process including cancer (12). Garcia *et al*. (1996) found Cath D was highly secreted into extracellular compartment resulting in degradation of extracellular matrix components and basement membrane supporting metastasis process (13). High level of Cath D in breast cancer was reported that involve in autocrine and paracrine activity to stimulate growth signaling affecting to tumor growth, invasion and metastasis as well as angiogenesis (14). There were several studies reported the increased levels of Cath D related with a poor prognosis in breast carcinoma, and they had both shorter disease-free survival and overall survival times (15, 16). In ER-positive breast cancer cell line, several studies showed estrogen play an activated role on Cath D promoter by IGF-I (17). Although, IGF-I and another growth factors, such as EGF, TGF were reported associated with Cath D expression (18), but the signaling pathway in absented-hormone receptor breast subtype is greatly complicated. Proteomic result showed Cath D expression when WT1 was transiently decreased expression in MCF-7 ER-positive human breast cancer cell lines. In ER-positive cells, Cath D was activated by estrogen hormone supported breast cancer development. In MDA-MB-231 TNBC cell line, absence of hormonal receptor, was showed Cath D overexpression (19). In addition, Huang *et al*. (2013) revealed Cath D was a key biological marker with high of Ki-67 index among TNBC patients (20).

Although, WT1 overexpression has been found that associated with breast tumor growth in both ER-positive and TNBC. However, in TNBC subtype the role of WT1 and Cath D protein has never been explored. Thus, in this present study, we focused on the role of WT1 and Cath D protein including structural prediction in supporting tumor progression in TNBC cell line.

LITERATURE REVIEW

1. Breast cancer

Breast cancer is currently the most general malignancy among women in worldwide (1). Numerous risk factors were related with breast tumor occur, such as sex, age, ethnicity, hormone and lifestyle, as well as genetic factor. Roughly 5-10% of breast cancer cases result from hereditary that is common present in *BRCA1* and *BRCA2* gene (21). Half of the breast cancer predisposition syndromes are related with mutations in *BRCA1* and *BRCA2* gene. Female with *BRCA1* or *BRCA2* deleted mutations have a significantly higher risk of breast tumor progression (22) .

The breast is divided in two main types depending on breast tissues: glandular tissues and supporting or stromal tissue. Glandular tissues are the glands that produce milk (lobules) and the ducts form tubes that carry breast milk to the nipple while stromal breast tissues comprise fatty and fibrous connective tissues. Most breast cancer initiated in the cells that form the ducts or called ductal breast carcinoma. Some occurring in the cells that form the lobules or lobular breast cancers, while few features originate in the other tissues. Breast cancer can invade and spread outside the breast through blood circulation and lymph duct lead to metastasize to distant organ. Most of the breast malignancies are adenocarcinomas, which found more than 95 percent of breast cancer cases. The main types of breast cancer consist of 1) non-invasive breast cancer: cells that present confinement within the ducts and do not attack fatty and connective tissues around the breast, and 2) invasive breast cancer that cells penetrate the ducts and lobular wall and break through the surrounding fatty tissues and connective breast tissues. Each type of breast cancers is called depend on the site that cancer cell initiation. Ductal carcinoma in situ (DCIS) is the most common kind of noninvasive breast cancer that bordered to the breast ducts. Lobular carcinoma in situ or LCIS that breast cancer cells initiate at the lobule. Infiltrating ductal carcinoma (IDC) is invasive ductal carcinoma, invasive breast cancer types, that initiates in the breast milk ducts and invade the wall of the duct and fatty tissue spreading to distant regions of organ. IDC is the most general type of breast cancer, approximately 80 percentage of breast cancer diagnosis. Invasive lobular carcinoma or infiltrating lobular carcinoma (ILC) is originated in the milk glands or locular but often spreading to other parts of

organ. ILC was found 10-15% of breast cancer cases. In addition, inflammatory breast cancer, mucinous carcinoma, medullary carcinoma, tubular carcinoma, phyllodes tumor and paget's disease of the nipple are less general type in breast cancer. The clinical features of breast tumor are always use grade and stage for helping doctors to intend the appropriated treatment and the accurated prognosis. The TNM staging system uses to describe tumor characteristics in a uniform manner and assists communication among medical personnel. The clinical features were decided by tumor size (T), spread cancer cell to lymph node (N), and spread to distant organs (M). Clinical feature, ultrasound picture, mammography examination, biopsy and MRI image are the diagnostic procedures to detection of breast cancer. Breast cancer therapy relies on the stage and type of breast cancer that lead considering to surgery, radiation, or chemotherapy. Determination of adjuvant treatment for early breast cancer patient relies on the prognostic factors. TNM diagnosis and hormonal receptor status including HER2 expression could prognose the disease progression. Evaluation of microarray-based gene expression profiling has presented breast tumor to be heterogeneous with distinct molecular profiles, clinical outcomes including pathologic features. It can be divided into five classifications: luminal A, luminal B, basal-like, claudin low and HER2-overexpressing following in Table 1 (23). All subtype has different prognoses and responses to adjuvant administration. Hormone receptor status has come to be a routine to aid in estimating the prognosis and treatment. Up to date, molecular prognostic factors have been evaluated to predicting outcome. The carcinoembryonic antigen (CEA), CA 15-3, HER-2, cyclin E, nestin, p53 and Cathepsin D are tumor markers that are often found expression in breast cancer patients. They has an important role in diagnosis, monitoring response to treatment and disease progression (24).

Table 1 Molecular subtype and characteristic of breast cancer (23)

Classification	**Immuno profile**	**Characteristics**	**Example cell lines**
Luminal A	ER+, PR+/-, HER2-	Ki67 low, frequently chemotherapy, responsive endocrine responsive	MCF-7, SUM 185, T47D
Luminal B	ER+, PR+/-, HER2+	Ki67 high, HER2+ are trastusumab responsive, variable to chemotherapy, usually endocrine responsive	ZR-75, BT474
Basal	ER-, PR-, HER2-	Ki67 high, presented of EGFR and/or cytokeratin 5/6, endocrine nonresponsive, often chemotherapy responsive	SUM190, MDA-MB-468,
Claudin-low	ER-, PR-, HER2-	Ki67, claudin-3, claudinin-4 and cludinin-7 low, E-cadherin, Intermediate response to chemotherapy	MDA-MB-231, Hs578T, BT549, SUM1315
HER2	ER-, PR-, HER2+	Ki67 high, Trastusumab responsive, chemotherapy responsive	MDA-MB-453, SKBR3

2. Triple Negative breast cancer (TNBC)

TNBC is a very serious cancer type and often occur in young women including related with a poor prognosis in patients. TNBC is a subclass of breast cancer that manifested estrogens receptor (ER) negative, progesterone receptor (PR) negative and human epidermal growth factor receptor 2 (HER2) negative based on immunohistochemistry (IHC) technique. It is a critical treatment because TNBC lacks of target therapies. Roughly 170,000 breast cancer are TNBC cases and presented 10-20% of invasive breast cancers (25). TNBC patients do not advantage from trastuzumab-based targeted therapies, hormonal therapy, since it is without target receptors. Despite these cases respond to cancer treatment, such as, anthracyclines, taxanes better than other breast cancer subtypes but prognosis in TNBC remains defective (26). The surgery and chemotherapy are common therapies in TNBC patient, however, other targeted therapies are being developed for the case of TNBC, such as mTOR, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR). Hence, patient with TNBC has a poor prognosis that result in shorter disease-free survival (DFS) and overall survival (OS) (27).

3. **Wilms' tumor 1 (WT1)**

WT1 is located on 11p13 chromosome, and act as transcription factor that contain 4 zinc figure proteins. N-terminal of this protein has Cystein 2-Histidine 2 which repeat region of structure for binding with the specific base on the DNA transcription. WT1 encode 10 exons, and forms a 3 kb mRNA. There is an alternative splice at exon 5 (17 amino acid) and exon 9 (3 amino acid, KTS regions) and generate WT1 4 isoforms; -17AA/-KTS (-/-) or isoform A, +17AA/-KTS (+/-) or isoform B, -17AA/+KTS (-/+) or isoform C and +17AA/+KTS (+/+) or isoform D (Figure 1) (4). Each isoform of WT1 showed different effect to the cell, for example, the isoform A affected cell cycle arrest and slow cell growth, whereas expression of the isoform D played a role in inducing an epithelial-mesenchymal transition (9).

The WT1 protein was crucial in urogenital system of developing embryo, and played roles in the nervous system, urogenital system and tissue in hematopoiesis in adult. When WT1 gene was mutated that associated with WAGR syndrome and Frasidr syndrome. However, wild-type WT1 gene played an crucial role in human breast cancer progression (28).

WT1 could either repress or activate cancer cells depending on the cell type, its isoform and the target genes which WT1 interacted. Furthermore, related molecules that interact with WT1 can affect to transcription regulation of gene target. Idelman *et al.* (2003) reported that WT1 interacted with wild-type p53, and inhibited insulin-like growth factor-I receptor (IGF-IR) promoter. Not only WT1 isoforms but p53 status also effect on IGF-IR promoter. They founded WT1 without KTS form leading to activate IGF-IR gene; in addition, p53 mutation also stimulate IGF-IR promoter and induced cell transformation and anti-apoptosis (10). There are many transcriptional targets of WT1 protein besides IGF-IR gene following in Table 2. Moreover, the effect of WT1 transcriptional activity may be described by alteration of WT1 and its binding proteins by cell type-dependent shown in Table 3 (4).

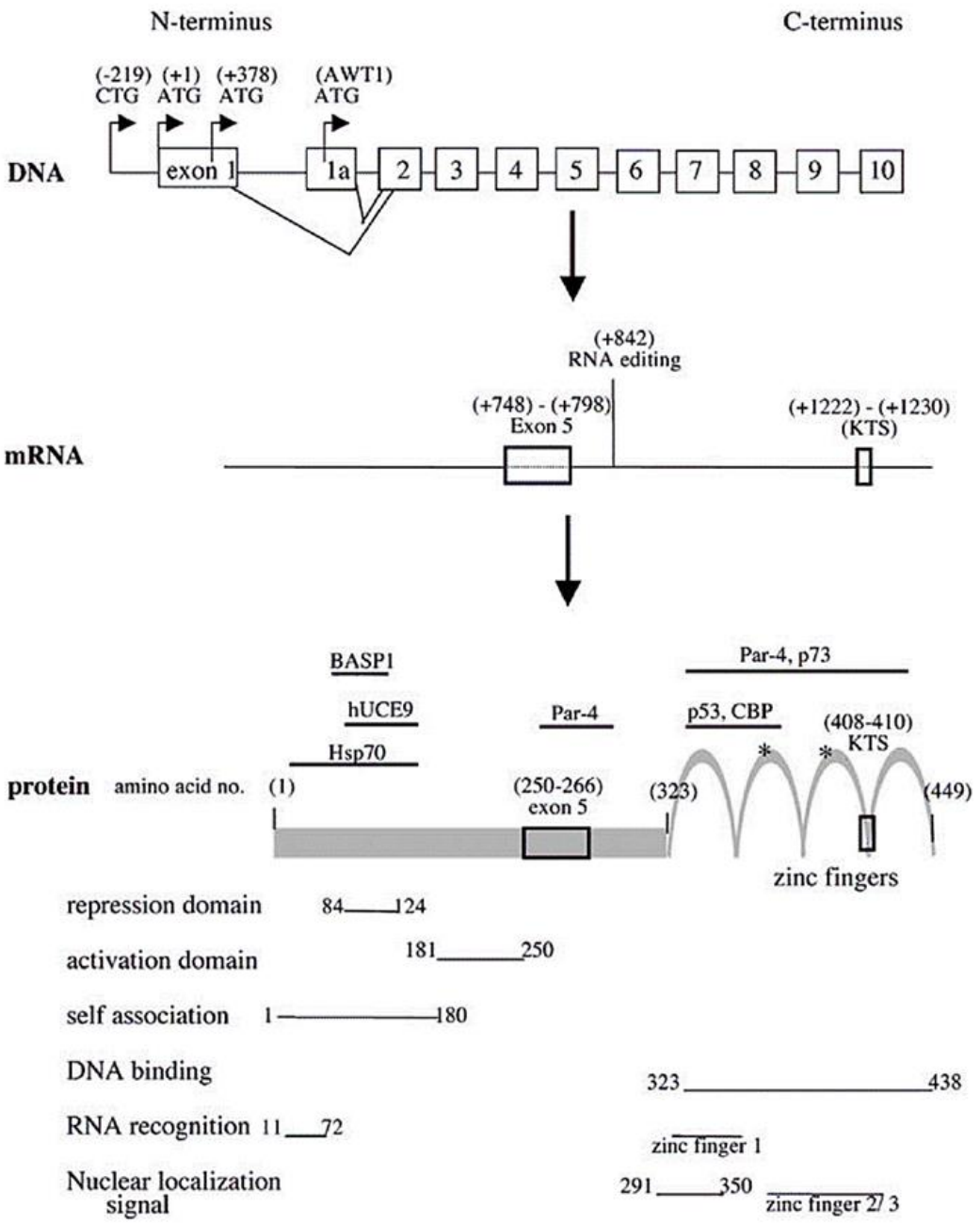


Figure 1 WT1 structure at exons on DNA, mRNA and protein part. (*) presented phosphorylation position, Serine 365 and Serine 393, settled in zinc fingers 2 and 3, respectively (4).

Table 2 Transcriptional targets of WT1 and its reported transcriptional effect (4)

Target	Effect
Growth factor	
- Platelet derived growth factor (PDGF)	Repress or activation
- Transforming growth factor-beta (IGF-β)	Repression
- Colony stimulating factor-1	Repression
- Insulin growth factor II (IGF-II)	Activation or repression
- Amphiregulin	Activation
Receptors	
- Androgen receptor	Repression
- Insulin receptor (IR)	Repression
- Insulin growth factor-I receptor (IGF-IR)	Repression
- Epidermal growth factor receptor (EGFR)	Repression
- Retinoic acid receptor alpha	Repression
Transcription factor	
- c-Myb	Repression
- c-Myc	Activation or repression
- Cyclin E	Repression
- IGFBP-4, Cyclin G1	Activation
- P21	Activation
- Pax-2	Repression
Enzyme	
- Ornithine decarboxylase	Repression
- Human telomerase reverse transcriptase	Repression
Other	
- Syndecan-1	Activation
- E-cadherin	Increased
- Bcl-2	Activation or repression
- Erythropoietin	Activation
- Thrombospondin 1	Repression

Target	Effect
Other (continue) - Wnt-4	 Activation

Previously, WT1 was discovered as a tumor suppressor gene in nephroblastoma, kidney tumor in childhood. Yang *et al*. (2006) has been complied evidence that wild-type WT1 expressed in leukemia and solid human cancer, such as esophageal squamous cell carcinoma, brain tumor, head and neck squamous carcinoma, ovarian carcinoma including breast cancer (4). It featured of oncogene and founded positive around 90% of WT1 expressed, and high of mRNA level in breast tumor patients (29). Consistent with Loeb *et al.* (2001) reported that 87% of primary breast carcinomas could be find WT1 expression, but not in normal breast epithelium (30).

Table 3 Interacting partners of WT1 and their effect (4)

Gene	Effect	WT1 region required for binding (amino-acid residue)	Verification of interaction
P53	Repressor Repressor	Zinc-finger 1 and 2	IP IP
P73	Repressor	Zinc-finger 1-4	IP
Brain acid soluble protein 1 (BASP1)	Repressor	71-101	Affinity chromatography
Creb binding protein (CBP)	Activator	Zinc-finger 1 and 2	IP
Hsp70	Activator	6-180	IP
E1B 55K	Repressor	Zinc-finger 1 and 2	IP
Ciao-1	Repressor	Zinc-finger 1-4	IP, Yeast two-hybrid

Gene	Effect	WT1 region required for binding (amino-acid residue)	Verification of interaction
Par 4	Repressor Activator	Zinc-finger 1-4 245-297	Yeast two-hybrid, IP Protein affinity, IP
Bone marrow Zinc finger 2	Repressor	Zinc-finger 1-4	Affinity chromatography
Ciao-1	Repressor	Zinc-finger 1-4	Yeast two-hybrid, IP
Human ubiquitin conjugating enzyme 9 (hUCE9)	Repressor	85-179	IP, Yeast two-hybrid
U2AF65	RNA processing	Zinc-finger 1-4	IP, Yeast two-hybrid
Orphan nuclear receptor SF-1	Synergistic activation	ND	IP, Yeast two-hybrid, GST pull-down
Four-and-half LIM-domain FHL2	Activator	182-298	IP, Yeast two-hybrid, GST pull-down
WT1-associated protein	ND	C-terminus KTS (-)	IP, Yeast two-hybrid, GST pull-down
WT1	Repressor	N-terminus	Yeast two-hybrid, GST pull-down

Abbreviations: IP stands for immunoprecipitation, ND stands for not determined.

Tatsumi *et al*. (2008) knockdown WT1 expression by shRNA technique, the result showed mitochondrial damage and lead to apoptosis in several tumor cell which WT1 expressed (5). These evidences suggested that WT1 involved in tumor growth and anti-apoptotic activity. In addition, numerous studies found that highly WT1 expression were not only promote cancer differentiation and anti-apoptosis in breast tumor cells but also contribute to migration and metastasis in high grade of prostate cancer and human ovarian cancer cell (31). Thus, WT1 has an oncogenic role in diverse tumors as well as breast cancer and support cancer aggressiveness. Previous evidence also suggested that WT1 mRNA high expression can be predicted poor prognostic factor of breast cancer cases. Moreover, WT1 level was higher expression in ER-negative than in ER-positive breast cancer patients. They found WT1 expression in basal-like molecular subtype had poorer prognosis than luminal subtype (Figure 2) (6).

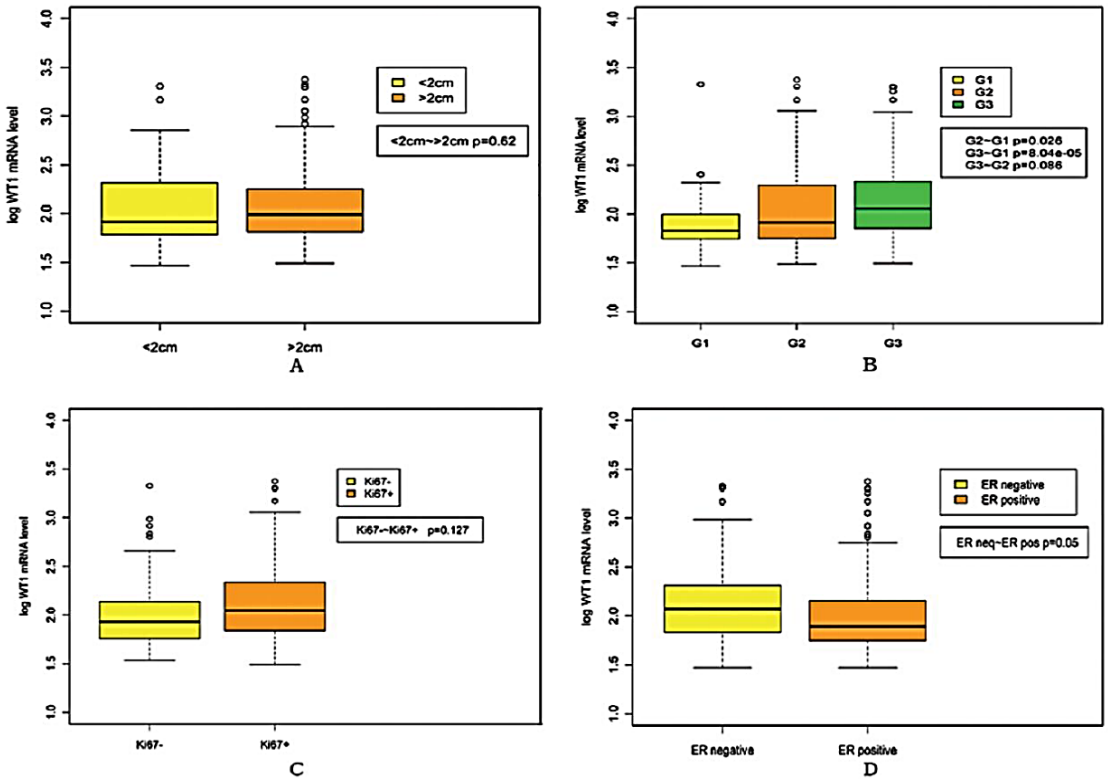


Figure 2 Relationship between WT1 mRNA expression and clinical pathology factors of breast cancer cases. WT1 mRNA expression was comparative among (A) tumor size < 2 cm and > 2 cm groups, (B) G=grading: G1, G2 and G3 demonstration, (C) Basal and Luminal,and (D) ER-negative (-) and ER-positive (+) subtype (6).

4. **Cathepsin**

Cathepsin is one of the lysosomal proteases. They are synthesized in rough endoplasmic reticulum then transport to Golgi stack. Proenzyme is generated from cleavage of preproenzyme and glycosylation. They were transferred to lysosome by mannose-6-phosphate or M6P pathway and activated to mature enzyme. Cathepsin is separated into three subgroups depending on their amino acid at active site; cysteine (Cathepsin B, C, F, H, L, K, O, S, V, W, X, Z), aspartic acid (Cathepsin D, E) and serine (Cathepsin A, G). The characters of human cathepsin members are summarized in Table 4 (32).

Cathepsin is proteolytic enzyme that play a major role in growth factor-binding, and degrade extracellular matrix (ECM). Previous study was reported that cathepsin facilitate invasion growth capability through degrade E-cadherin (33). This characteristic demonstrated that cathepsin promote cancer invasion and metastasis.

Table 4 Characteristics of human cathepsin family members (32)

Cathepsin	Subtype	Endo/ exo peptidase	Expressed tissue	Function
A	Serine	Exopeptiedase	Primary antigen presenting cell, platelets, testis and epididymis	Autophagy, formation of elastic fiber, platelet activation
B	Cysteine	Exo and endo	Endocrine organs, extensively distributed in macrophages, hepatocytes, renal tubules	Protein catabolism, processing of antigens hormone activation and bone change
C	Cysteine	Exo	Widely distributed in tissues	Protein degradation in lysosomes
D	Aspartic	Endo	Extracellular matrix including synovial fluid of cartilage, Endocrine	Protein degradation in lysosome
E	Aspartic	Endo	Immune system	Antigen presentation
F	Cysteine	Endo	Human antigen presenting cells	Antigen presenting
G	Serine	Endo	Polymorphonuclear leukocyte	Digestion of extracellular matrix, immune complex mediated inflammation production of angiotensin II
H	Cysteine	Endo	Ubiquitous in cells and tissues	Endopeptidase activity
L	Cysteine	Endo	Ubiquitously expressed	Antigen presenting, differentiation of keratinocyte, protein turnover

Cathepsin (cont.,)	Subtype	Endo/ exo peptidase	Expressed tissue	Function
K	Cysteine	Endo	skeletal muscle, bone, ovary, heart, placenta, lung, small and large intestine	Bone remodeling
O	Cysteine	Endo	Widely distributed in tissues	Protein turnover and degradation
S	Cysteine	Endo	Spleen and professional human antigen-presenting cells	Proteolysis
V	Cysteine	Endo	Corneal epithelium, thymus gland, testis	Production of enkephalin and neuropeptide Y
W	Cysteine	Endo	T-lymphocytes	Cell-mediated cytotoxicity
X	Cysteine	Endo	Immune system	Phagocytosis, immune responses
Z	Cysteine	Exo	Broadly presented in human tissues	Protein degradation, Neuroinflammation

4.1 Cathepsin D

Cathepsin D (Cath D), a housekeeping enzyme, is a lysosomal aspartyl protease that consists of 412 amino acid residues. It is bi-lobed structure that separated by two catalytic sites consist of aspartyl residues at amino acids 33 and 231 that settled on the 14 kDa and 34 kDa, respectively. Cath D located on chromosome 11p5 and contained nine exons. Its function is protein degradation in lysosome and phagosomes at acidic pH.

Cath D is generated as pre-pro-enzyme at rough endoplasmic reticulum and then cleavage signal peptide to generate pro-Cath D (52 kDa) and glycosylation during transport to Golgi complex. The 52 kDa proenzyme form of is targeted to lysosome by binding with mannose-6-phosphate or M6P receptor. The activation peptide of pro-enzyme is removed, and yield 48 kDa of intermediate Cath D that was cleaved into mature Cath D. It consists of two chain of mature Cath D (14 kDa-light chain and 34 kDa-heavy chain) that cleaved by lysosomal cysteine protease, Cath B and L (34). Generally, Cath D functions involve in secretion, cellular trafficking, post-translation modifications including protein binding partner. For Cath D binding protein, it has been identified in Table 5 (35).

The overexpression of Cath D was high risk of metastatic property and short-lived in breast tumor cases. In cases of primary tumors that high level of Cath D indicated local recurrence or distant metastasis (36). Cath D was overexpressed and secreted by various cancer cell lines such as breast cancer, head and neck tumors, lung cancer, prostatic cancer and etc. (37). In breast cancer, Cath-D is an independent factor of poor prognosis related with metastatic feature (15). High level of Cath D was featured in breast cancer cell; in addition, pro-Cath D is also higher in the tumor microenvironment than normal epithelial or fibroblast cells (11, 38). Several studies supported Cath D stimulate proliferation pf breast cancer cell and fibroblast outgrowth, metastasis including angiogenesis formation (19, 39-41).

Table 5 Binding protein of Cathepsin D (35)

Cath D binding protein (related function)	**Pathological process**
Man-6PR (Cellular trafficking)	Breast cancer
Prosaposin (Cellular trafficking, Secretion)	Breast cancer
Low-density lipoprotein receptor-related protein-1 (LRP1) (Cell development, Cellular trafficking)	Breast cancer
Sortilin (Cellular trafficking)	Colon, pancreas, prostate and ovarian carcinoma
Cystatin C (Cell development)	Brest cancer

Cath D mRNA and protein were upregulated by estrogen and growth factors, (*i.e*. epidermal growth factor (EGF), insulin like growth factor I (IGF-I)) that result in increasing of transcriptional initiation in MCF-7, ER-positive breast cancer cells (18). However, in MDA-MB-231 TNBC cell line also presented overexpression of Cath D (19). Huang *et al.* (2013) revealed Cath D was a significant biological marker with presenting of high of Ki-67 index among TNBC patients (20).

In mitogenic activity, Cath D is a protein ligand that bind to mitogenic receptor in MCF-7, ER-positive breast cancer cell lines, and rat embryo tumor cells (19, 42). Cath D stimulated growth of cancer cells by an autocrine and/ or paracrine mechanism. Previous study reported that the mutated Cath D could function as a mitogen in cancer, endothelial, fibroblast cells, and correspond in athymic nude mice *in vivo* (43). Thus, Cath D presented mitogenic activity in cancer cell and fibroblast cell that independent of its proteolytic activity. It could stimulate fibroblast proliferation by localized at the surface of breast fibroblast and function as mitogenic action or digest component of extracellular matrix to increase cancer invasion by releasing growth factor into blood stream (44). Laurent-Metha *et al*. (2005) suggested that Cath D might

be reliable for supporting proliferation, cancer cell movement and/ or invasion of fibroblast tissue by activation of *ras*/MAPK/ ERKs pathway which promoted cell proliferation (41). The 52 kDa of pro- Cath D form could also auto-activate in acidic (45), and secreted by breast cancer cells into extracellular. It could be endocytosed into cancer cells and fibroblast by paracrine mechanism. The pro-Cath D was captured by fibroblast via mannose- 6- phosphate (M6P) receptor or unidentified cell surface receptor (16). The RAP binding site of LDL receptor- related protein was one of alternative receptor for endocytosis (46). The pro- Cath D endocytosis was partially transported by the mannose-6-phosphate/IGF-II receptor in MCF-7 cells, but in MDA-MB-231, ER-negative, was independent of this receptor (47). Cath D can active in acid milieu, and degrade component of extracellular matrix (ECM) that increasing cancer invasion by release growth factors into blood stream (48). Nonetheless, Cath D acts as mitogen more than a protease resulting in cancer cells extrude the basement membrane, penetrate connective tissue and spread into blood circulation.

In breast cancer metastasis, Cath D overexpressed in rat cancer cell that resulted in increasing of metastatic potential (49). In TNBC cell lines, MDA- MB- 231 was highly metastasis and showed association with high level of Cath D expression in *in vitro* (50). Furthermore, metastatic breast cancer patients showed the high concentration of pro- Cath D in serum. This result was suggested that pro- Cath D is released into blood circulation at least in final stage of cancer (51). The tumorigenesis of Cath D was not only support cancer cell growth, invasion and metastasis but also tumor angiogenesis performed in breast cancer. The 102 patients of invasion breast carcinomas were significantly increased of Cath D expression related with higher vessel counts (52) in order that Cath D might induce endothelial cell proliferation via paracrine mechanism (43). Brizzo *et al.* (1991) proposed that Cath D might encourage angiogenesis in breast cancer cells by presenting of ECM-bound bFGF (53).

In apoptotic function, lysosomal membrane permeabilization release Cath D to the cytosol that impact to cleave Bid to tBid form which activated Bax insert to the mitochondrial membrane leading to cytochrome c releasing into cytosol. Then, pro-caspase 9 and 3 are activated. Furthermore, Cath D also activate Bax which trigger mitochondrial apoptosis inducing factor or AIF releasing (54, 55).

Even through in physiological process lack of Cath D in mice presented anti-apoptotic function. Moreover, thymus of mice had apoptotic feature because of Cath D-knockout in mice model (12). In cancer, highly Cath D expression was presented decreasing of tumor apoptosis than mock-transfected condition of cancer cells in immunohistochemical study (43). Gottlieb *et al.* (2000) demonstrated that wild type of Cath D can inhibit apoptosis of tumor, whereas inactive of Cath D could not function. They suggested that Cath D in tumor cell concerned in its enzymatic role for preventing apoptosis (44). Furthermore, in cancer decreasing of apoptotic cell was found in Cath D group when compared with control group (45).

Objective:

To study of WT1 and Cathepsin D protein in Triple negative breast cancer cell line.

CHAPTER 2

RESEARCH METHADOLOGY

Chemical and reagents

1. Chemicals and reagents for cell cultures

Chemicals/ reagents	M.W.	Source
- Dulbecco's Modified Eagle medium (DMEM)	-	GIBCO BRL
- Fetal bovine serum (FBS)	-	GIBCO BRL
- Hydrocholic acid (HCl)	-	RCI Labscan
- Penicilin-Streptomycin	-	GIBCO BRL
- L-Glutamine (GlitaMAX^{TM})	-	GIBCO BRL
- Sodium bicarbonate ($NaHCO_3$)	84.01	Sigma
- Disodium hydrogen phosphate (Na_2HPO_4)	141.96	Amresco
- Potassium Chloride (KCl)	74.55	Sigma
- Potassium hydrogen phosphate (KH_2PO_4)	136.10	Sigma
- Sodium Chloride (NaCl)	58.44	EMSURE
- Distilled water (DW)		- -

2. Chemicals and reagents for Western Blotting

Chemicals/ reagents	M.W.	Source
- RIPA buffer	-	Pierce
- Bovine serum albumin (BSA) powder	-	Sigma
- Bradford's reagent	-	Bio-Rad
- Tris-base ($C_{14}H_{11}NO_3$)	121.14	Amresco
- 40 percentage of Acrylamide/Bis solution (37.5:1)	-	Bio-Rad
- Ammonium persulfate ($(NH_4)_2S_2O_8$)	228.20	RCI Lascan
- TEMED ($(CH_3)_2NCH_2CH_2N(CH_3)_2$)	116.20	Bio-Rad
- Hydrochloric acid (HCl)	-	RCI Labscan

	Chemicals/ reagents (cont.)	M.W.	Source
-	Sodium Chloride (NaCl)	58.44	EMSURE
-	Glycerol ($C_3H_8O_3$)	92.09	Amresco
-	Sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$)	288.38	Amersco
-	β-Mercaptoethanol ($HSCH_2CH_2OH$)	78.13	Calbiochem
-	Tris-HCl ($NH_2C(CH_2OH)_3HCl$)	157.60	J.T.Beaker
-	Low fat dry milk	-	Fontera Brands
-	MagicMark™ XP Western protein Standard	-	Invitrogen
-	Spectra Multicolor Broad Range Protein Ladder	-	Invirogen
-	Polyoxyethylene (2) Sorbitan Monolaurate (TweenR20)	-	OmniPur®
-	Bromophenal Blue ($C_{19}H_{10}Br_4O_5S$)	669.96	Sigma
-	Ponceau S ($C_{22}H_{16}N_4O_{13}S_4$)	-	Sigma
-	Anti-Rabbit IgG horseradish peroxidase-conjugated Antibody	-	Invitrogen
-	WT1 (C-19) antibody	-	SantaCruz
-	Cathepsin D antibody	-	Cell signaling
-	SuperSignal West Pico chemiluminescence substrate	-	Pierce
-	SuperSignal West Dura chemiluminescence substrate	-	Pierce
-	SuperSignal West Femto chemiluminescence substrate	-	Pierce

Equipment

- Nitrocellulose membrane 0.45 μm	BioRad
- 6-Well plate	Corning
- 24-Well plate	Corning
- 48-Well plate	Corning
- 96-Well plate	Corning
- Cell culture plate (10 cm cell culture dish)	Corning
- Centrifuge tubes	Corning

Research Methodology

The protein expression between WT1 protein and Cath D enzyme was investigated by measuring the endogenous protein level in ER-positive and triple negative breast cancer (TNBC) cell lines. The relationship between WT1 and Cath D protein in TNBC cell line (MDA-MB-468) was clarified by immunoprecipitation (IP). Moreover, interaction result of WT1 and Cath D in silico was predicted by molecular docking.

1. Cell culture

Hs578T, MDA-MB-231, MDA-MB-468 (TNBC cell lines group) and MCF-7 (ER-positive breast cancer cell line) were purchased from ATCC. All cell lines were cultured in DMEM solution by added with ten percent of fetal bovine serum (FBS), 100 μg/ml streptomycin (Invitrogen) and 100 units/ml penicillin then brought up alive cells at 37°C temperature with 5% CO_2.

2. Immunoprecipitation (IP)

MDA-MB-468 breast cancer cell lysate was added 200 µl of 1% NP-40 lysis solution. The cells were spin at 12,000xg for 10 minutes after incubation on ice for 15 minutes. Supernatant was divided as a control and MDA-MB-468 tubes. WT1 antibody at 2.5 µg (stock concentration 200 µg/ml) and one micrograms of IgG stock concentration (1 mg/ml) were dropped into sample and control tubes, respectively. To incubate both tubes were kept overnight at 4ºC. Then, 50% protein-G slurry was loaded about 50 µL and then incubated at 4ºC for 2 hours. After centrifugation at 8,000xg for 2 minutes, 500 µl of 1% NP-40 lysis solution was poured to the tubes, incubate for 5 minutes and centrifuged at 8,000xg, 2 minutes to remove supernatant. The pellets were washed with 500 µl of 1x PBS for 3 times. The binding protein was eluted by using 20 µL of SDS sample buffer prior to heat up at 95ºC for 10 minutes. The eluted binding proteins were run onto 12% SDS-PAGE. Cath D protein was visualized by western blotting with Cath D antibody.

3. Western blot analysis

To lyse cell pellets from cell lines sample were vibrated with Radioimmunoprecipitation assay buffer (RIPA buffer) composing 1% sodium deoxycholate, 25 mM Tris HCl pH 7.6, 150 mM NaCl, and 0.1% SDS (Pierce, Rockford, USA) and then incubation on ice for 10 minutes. Cell lysates were spin at 12,000 rpm at 4°C temperature for 10 minutes. Collected supernatants were determined of protein quantity through Bradford assay (Bio-Rad, Hercules, CA). Samples were put through 12% SDS-PAGE and taken protein to nitrocellulose membranes by electrophoretic process. To block blots were incubated for one hour by 5% low fat dry powdered milk in TTBS that composed of 154 mM NaCl, Tween-20 and 48 mM Tris-base. After that membranes were risen for three times with 1% low fat dry powdered milk in TTBS. The blots were incubated with WT1 antibody (C-19) (1:200 Santa Cruz Bio technology, Santa Cruz, CA, USA), Cathepsin D (1:1,000), Actin (1:1,000) (Cell Signaling Technology, MA, USA) for two hours. Then the membranes were rinsed for three times by washing buffer (1% low fat dry powdered milk in TTBS). Nitrocellulose membrane samples were incubated with a HRP conjugated rabbit IgG at 1:5,000 for one hour and washed three time with 1xTTBS for ten minutes followed detection. WT1 protein was detected with the SuperSignal West Femto Chemiluminescent substrate (Pierce Rockford II, USA). Cathepsin D and β-actin were applied SuperSignal West Dura Chemiluminescent substrate (Pierce, Rockford II, USA). β-actin was performed as an internal control in all experiments.

4. Identification of protein-protein binding by molecular docking

4.1 Protein sequence analysis and prediction

Initially, Cath D cleavage site was studied by publication review from amino sequence of primary structure. Observed amino acid sequence of WT1 protein were noticed and collated to cleavage site of Cath D. Protein sequence was presumed cleavage site of WT1 sequence, moreover, the secondary structure was considered

sample protein can be digested by Cath D was presented with WT1. These procedures could point whether Cath D could cleave WT1. Uniprot was used as data bank for sequence alignment.

4.2 Protein modeling and docking

The structural WT1 protein alignment (449 amino acids) was observed using SWISSMODEL webserver (https://swissmodel.expasy.org) which devoted homology model of three-dimensional protein structure. Then CABSfold server (http://biocomp.chem.uw.edu.pl/CABSfold) was manipulated homological model of WT1 three dimension structure based on amino acid sequence. In the part of Cath D protein (137 amino acids), PDB code: 1LYW that presented Cath D at pH 7.5 in three-dimensional structure was picked from protein database (http://www.rcsb.org). The pKa forecast of WT1 and Cath D three-dimensional protein structure at pH 7.5 were predicted by PDB2PQR package web-server. Molecular dynamics simulations were completed with PMEMD package and AMBER16 force field in AMBER16 via absolute model at 310 Kelvin temperature (37 ^{o}C). The weak-coupling Berendsen algorithm was used for control temperature. Both WT1 and Cath D proteins were simulated for 200 nanoseconds by a timestep of 2 femtoseconds. Cath D structure part, the first 180 nanoseconds were eliminated as an equilibration phase and 10 equidistant snapshots from the last 20 nanoseconds were brought to protein docking analysis. In part of WT1 model, the last snapshot of 200 nanoseconds simulation was selected for examination. ZDOCK tool was used to predict protein-protein interaction between WT1 and Cath D protein. The finest protein binging structure was showed with ZDOCK score that was calculated scoring by using Fast Fourier Transform or FFT algorithm. In addition, electrostatic of significantly statistic for comparisons of potentials was calculated and considered via paired-sample Wilcoxon signed rank test (56).

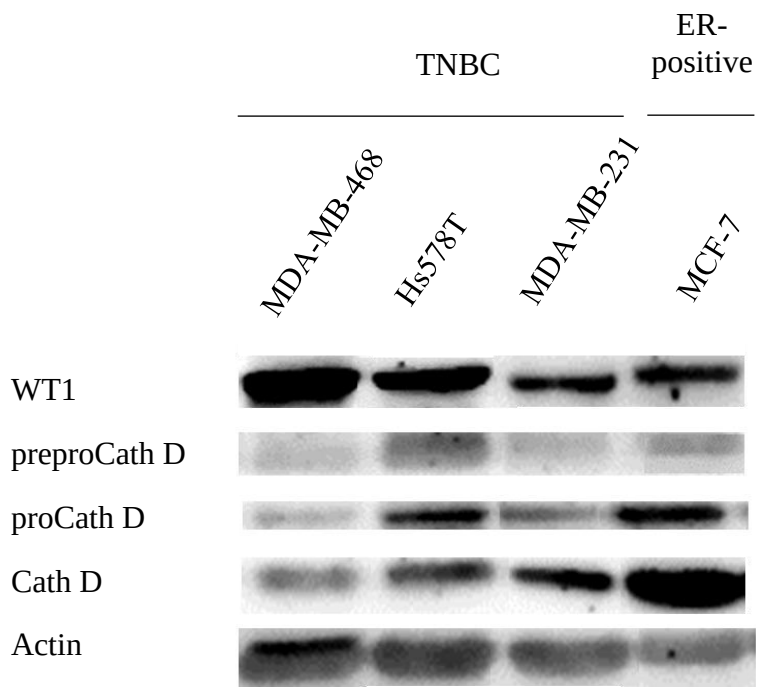
4.3 Charge and pH analysis

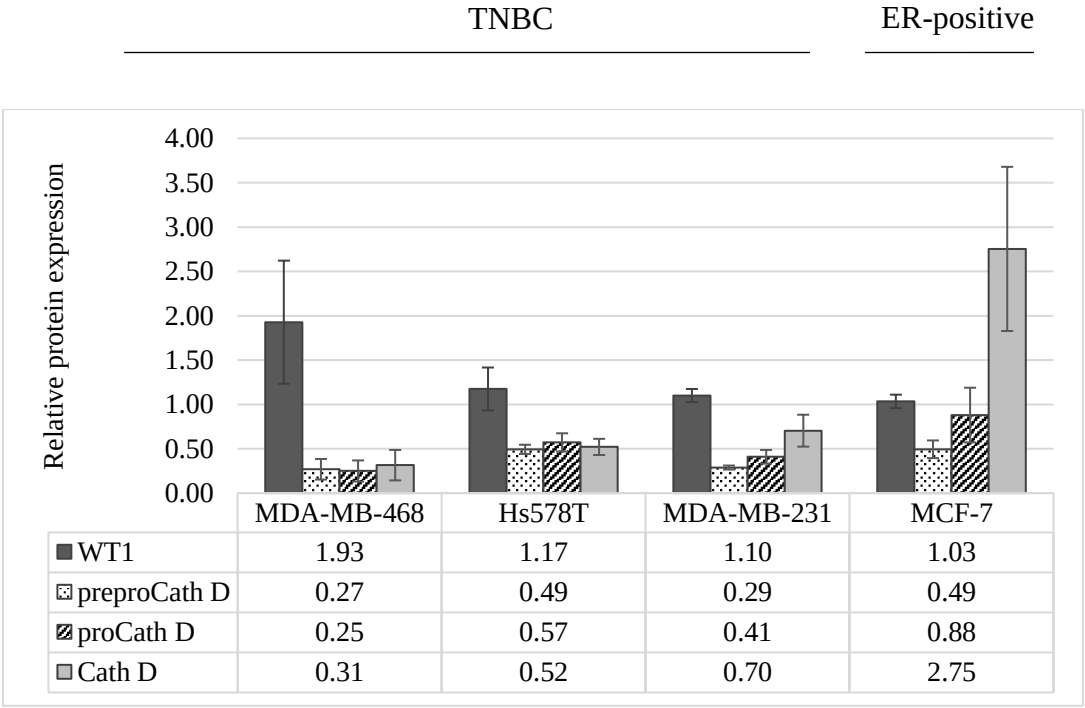
In 3D crystal structure, Cath D at pH 7.5 was chosen for prediction in silico. However, Cath D in acidic environment should further observe to compare when interacted with WT1. Thus, charge and pH of Cath D in acidic environment (pH 5) was analyzed by PDB2PQR server. Charge prediction was analyzed from pKa result and chemical reaction at pH 5 and 7.5 (pH > pKa is deprotonation, pH<pKa is protonation). Interacted position between WT1 and Cath D from protein docking modelling was noticed and considered with charge prediction at pH 5 and 7.5.

CHAPTER 3

RESULTS

Previous report found WT1 associated with TNBC cell aggressiveness (7). In metastatic feature of breast cancer, the researcher found overexpression of Cath D (13). The hyper-secretion result in digestion of extracellular matrix and support cancer growth, invasion and metastasis so it was one of poor prognostic markers in breast tumor case. Cath D is aspartic lysosomal enzyme that hydrolyzed from proCath D at golgi complex. Cath D is matured in lysosome by cleaved Cath D and tagged with mannose-6-phophate signal. To identify the endogenous expression level of WT1 and Cath D, in this finding, the expression of WT1 and Cath D in TNBC group: MDA-MB-468, MDA-MB-231, Hs578T, and ER-positive cell line: MCF-7 were initially observed by immunoblotting (Figure 3). The results showed that in TNBC cell lines, WT1 seemed to show higher expression than Cath D whereas, in ER-positive cell line, the expression level of WT1 was lower than Cath D. The proportion of protein expression in MDA-MB-468 was certainly upregulation of WT1 meanwhile considerably low of Cath D. In Hs578T, Cath D endogenous protein was half expression when compared with WT1 protein. Nonetheless, WT1 and Cath D proteins were equally expressed in MDA-MB-231 cell lines. MCF-7 ER-positive showed highly Cath D expression while low WT1 was displayed in this cell line. Above results showed the overview expression of WT1 and Cath D in the patterns of inverse proportion in TNBC and ER-positive breast cancer cell lines. Nevertheless, the interactive characteristic of these two proteins have never been reported. Thus, protein association was further studied by immunoprecipitation (IP) method and protein docking.





	MDA-MB-468	Hs578T	MDA-MB-231	MCF-7
WT1	1.93	1.17	1.10	1.03
preproCath D	0.27	0.49	0.29	0.49
proCath D	0.25	0.57	0.41	0.88
Cath D	0.31	0.52	0.70	2.75

Figure 3 Endogenous protein expression of WT1 (52 kDa), preproCath D (43 kDa), proCath D (46 kDa) and mature Cath D (28 kDa) in TNBC group: MDA-MB-468, Hs578T, MDA-MB-231 and ER-positive group: MCF-7 breast cancer cell line by immunoblotting method. Actin (42 kDa) was used as a control.

For IP technique, MDA-MB-468 cell lysate, highly WT1 expression and extremely low of Cath D, was precipitated with WT1 antibody. The protein G slurry was loaded to bind WT1 protein complex. The complex proteins were detected by immunoblotting using Cath D antibody. The immunoblotting result could visualize preproCath D (43 kDa), proCath D (46 kDa) and mature Cath D (28 kDa) in WT1 protein complex as shown in Figure 4.

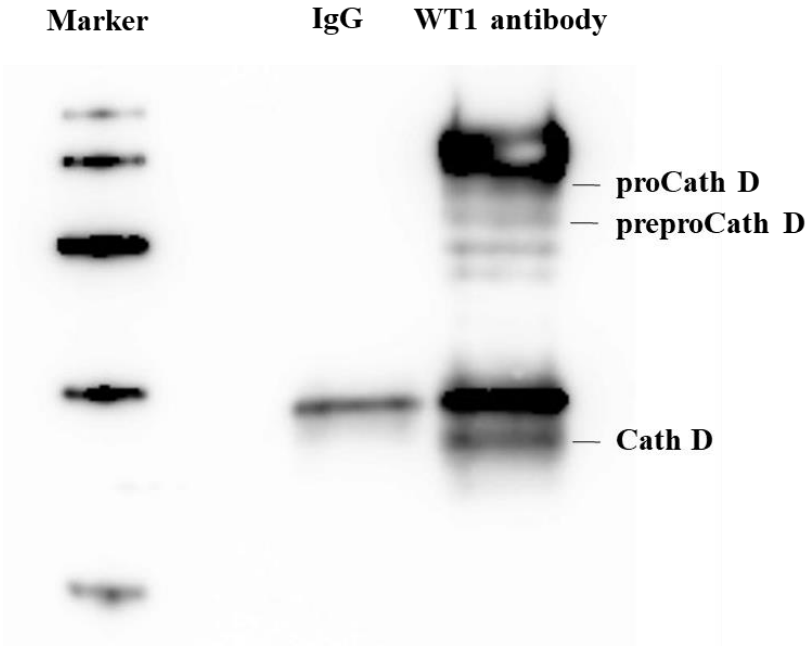


Figure 4 Western blot result of WT1-protein complex. After WT1 antibody was interacted with target proteins. The detection of Cath D (28 kDa), preproCath D (43 kDa) and proCath D (46 kDa) were detected in immunoblotting and IgG was used as a negative control.

The IP result in MDA-MB-468 TNBC cell line revealed that WT1 protein could form complex with Cath D but could not suggest whether these two proteins were direct or indirect binding. To clarify whether the WT1 could directly bind to Cath D the molecular docking bioinformatic tools for predicting the structure of the intermolecular complex formed between two or three molecules, was chosen to circumvent this issue.

Previous finding, target site of Cath D enzyme was considered by literature review for check whether Cath D involve with WT1 depend on hydrolyze function in TNBC. There are various native proteins presented to be cleaved by Cath D, such as kallistatin, insulin, as a catalase fibronectin (57-59) (Figure 5). In addition, we found that Cath D aspartic protease could perform through a site of aromatic amino acids (tyrosine, tryptophan, phenylalanine) and hydrophobic group (methionine, leucine, isoleucine, valine, phenylalanine) of the target proteins.

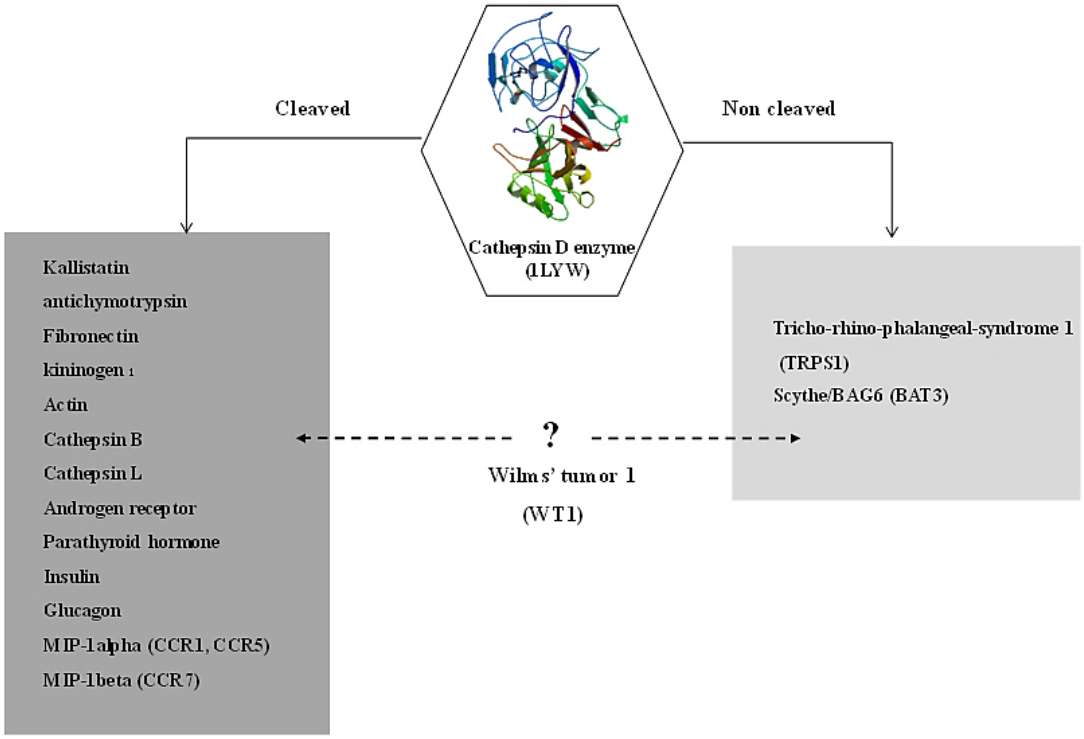


Figure 5 Proteins that are able to be cleaved and not cleaved by Cath D (56-58)

Moreover, we further studied secondary structure that Cath D can hydrolyze by program PSIPRED analysis. This database showed amino acid with secondary structure of selected protein. A pink cylinder implied helix structure and yellow arrow stands for sheet structure. The coil structure was represented by the line. The result manifested Cath D not only cleaved above amino group but also hydrolyzed all types of secondary structure, such as helix, sheet, coil. For instance, kallistatin, a human macrophage inflammatory protein 1 alpha (MIP-1 alpha) and human serine proteinase inhibitor, have aromatic amino acids and hydrophobic group. The black

arrows indicate Cath D cleaved site which are at the helix, sheet and coil structure (Figure 6).

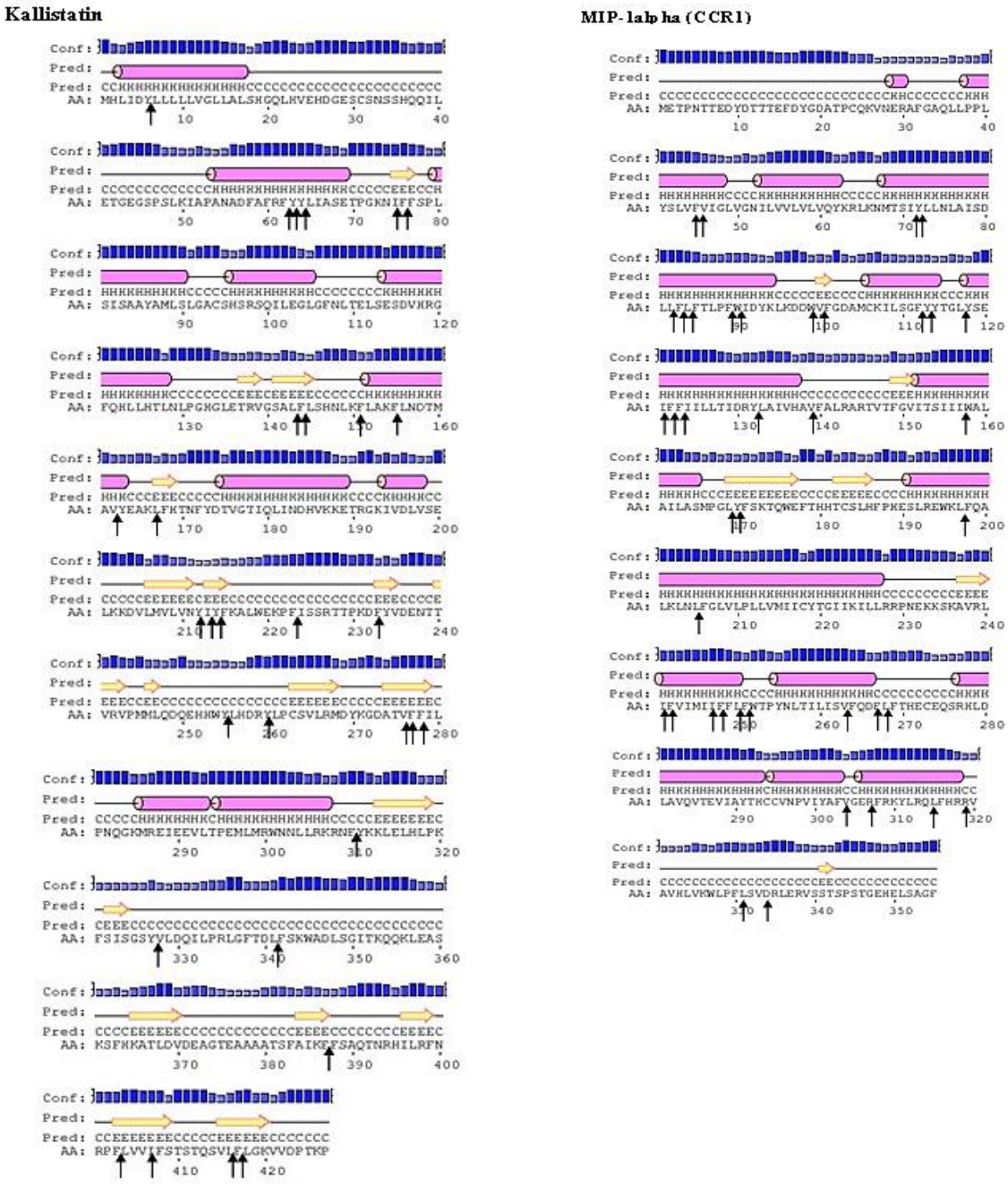


Figure 6 Native proteins with cleavage site (black arrow) of Cath D lysosomal enzyme. The secondary structures of these proteins were hunted by PSIPRED database. A pink cylinder implies helix structure. A yellow arrow represents sheet structure, and a line means the coil structure.

We also studied a cleavage site of Cath D enzyme in WT1 protein sequence. The arrows were marked at Cath D cleaved site on the coil structure of WT1 protein (Figure 7). Thus, WT1 has a target site for Cath D protein.

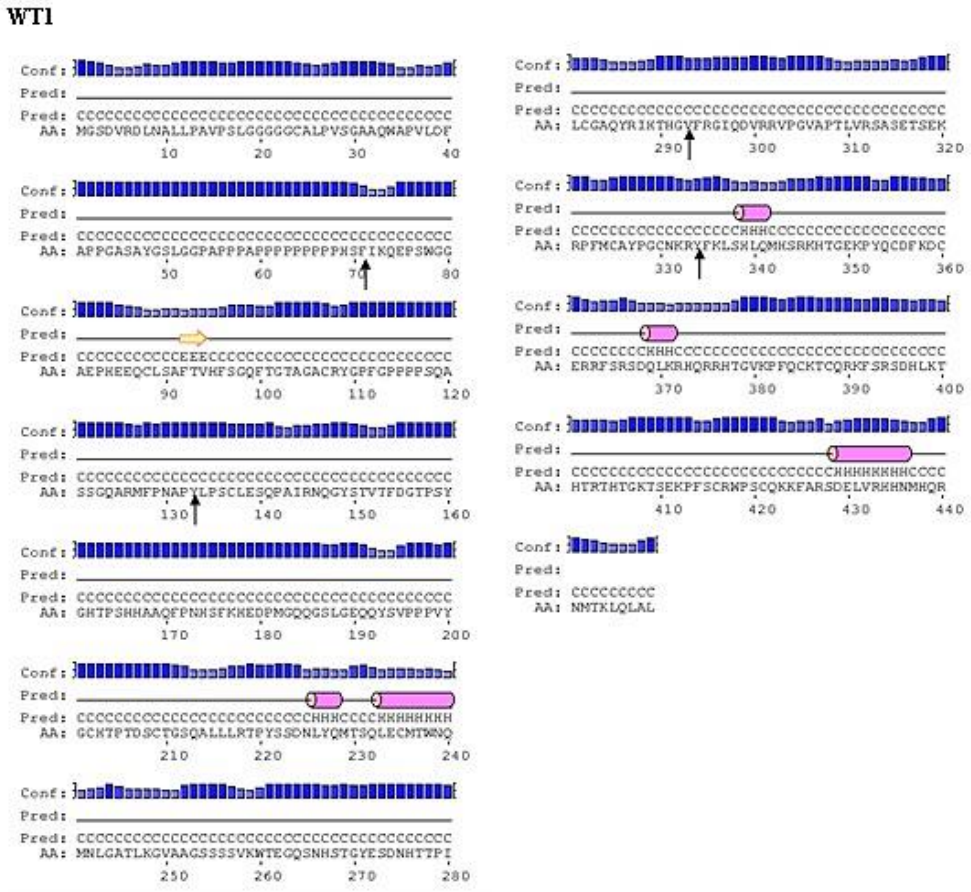


Figure 7 WT1 protein sequence with presented Cath D cleavage site (black arrow). WT1 protein was search from PSIPRED database. A pink cylinder represents helix structure. A yellow arrow denotes sheet structure, and a line indicates the coil structure.

Noticeably, TRPS1 has a role as repressor GATA-mediated transcription, and BAT3 as a nucleo-cytoplasmic shuttling chaperone protein were found that they can bind with Cath D in breast cancer cell (60). When we observed a Cath D cleaved site on TRPS1 and BAT3 protein, these proteins presented Cath D cleaved site (Figure 8). These data suggested that protein which has cleavage site of enzyme may not be appeared hydrolysis since enzyme will bind to other sites which are

not an active site of protein. Consequently, it could be indicated WT1 may form a complex with Cath D without cleavage of WT1 itself; nevertheless, molecular docking was chosen to confirm in protein binding examination.

Zinc finger transcription factor Trps1 (TRPS1)

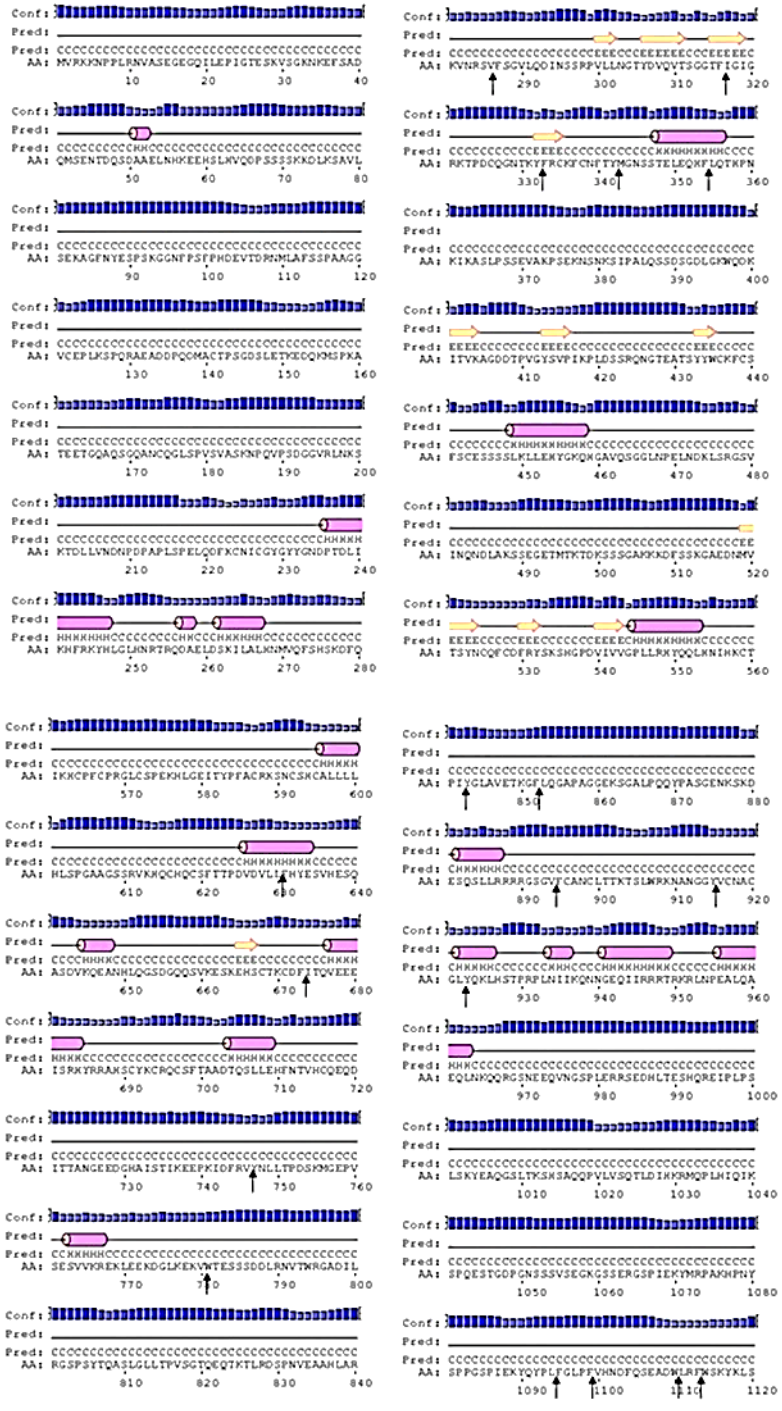


Figure 8 Protein sequence of zinc finger transcription factor Trps1 or TRPS1. This protein was investigated to be able to bind the Cath D without protein hydrolysis. A black arrow points the cleavage site.

There are various protein-protein docking tools such as, ZDOCK, Hex, Clus-Pro, Fire Dock, SwarmDock, etc. In this experiment, ZDOCK (http://zdock.umassmed.edu.) was chosen to investigate the binding of WT1 and Cath D complex since it was a high predictive accuracy on protein–protein docking benchmarks and provided a good complex structure within the top ten ranked complexes. Moreover, it was quickly and easily produced structural models of protein–protein complexes and available to all academic. 3D crystal structure of Cath D at pH 7.5 (code: 1LYW) from RCSB PDB protein data bank was used to run molecular dynamics simulation and H-chain light of mature Cath D was selected for docking test. In terms of WT1 protein preparation, it has never been reported the crystal structure in data bank, so WT1 structure was constructed by using Swissmodel and CABS-fold servers. Molecular dynamics simulation, was carried out for the structure refinement of both Cath D and WT1 protein at 37℃ atmosphere. The refined structures were then adopted as initial inputs for protein-protein docking so as to examine the atomistic view of WT1-Cath D protein complex. The 200 ns simulated Cath D and 200 ns simulated WT1 structures were exploited as a predicted representative for the protein complex. ZDOCK analysis proposed ten ranging of the best ZDOCK scoring that presented possible structure of protein-protein interaction (Figure 9A). This structure prediction suggested that Cath D could directly bind to WT1 (Figure 9B). From WT1-Cath D model, four positions of WT1 protein (TYR133, ILE72, VAL293 and TYR334) that found as digest targets of Cath D enzyme did not interface with catalytic site of Cath D (Aspartic acid, D295) (Figure 10). This bioinformatics tool suggested that Cath D bind WT1 without cleavage.

(A) ZDOCK score

Complex ranging	ZDOCK score
1	1832.310
2	1723.653
3	1707.034
4	1706.250
5	1701.687
6	1655.740
7	1649.277
8	1626.163
9	1620.625
10	1613.716

(B) Structure model of WT1 and Cath D protein binding

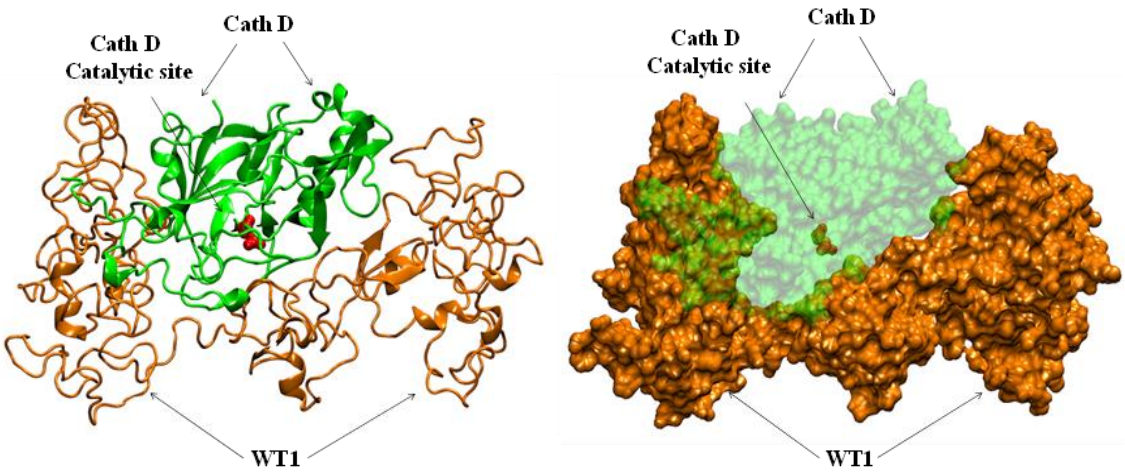


Figure 9 (A) 10th range of the best ZDOCK score of WT1-Cath D binding that ZDOCK sever proposed. (B) Proposed structure of Cath D-WT1 protein complex. The green and orange structures are the Cath D (at pH 7.5) and WT1 respectively. The model distinctly presented that if WT1 and Cath D could form a protein complex of the other region but not Cath D catalytic site (D295, red spot).

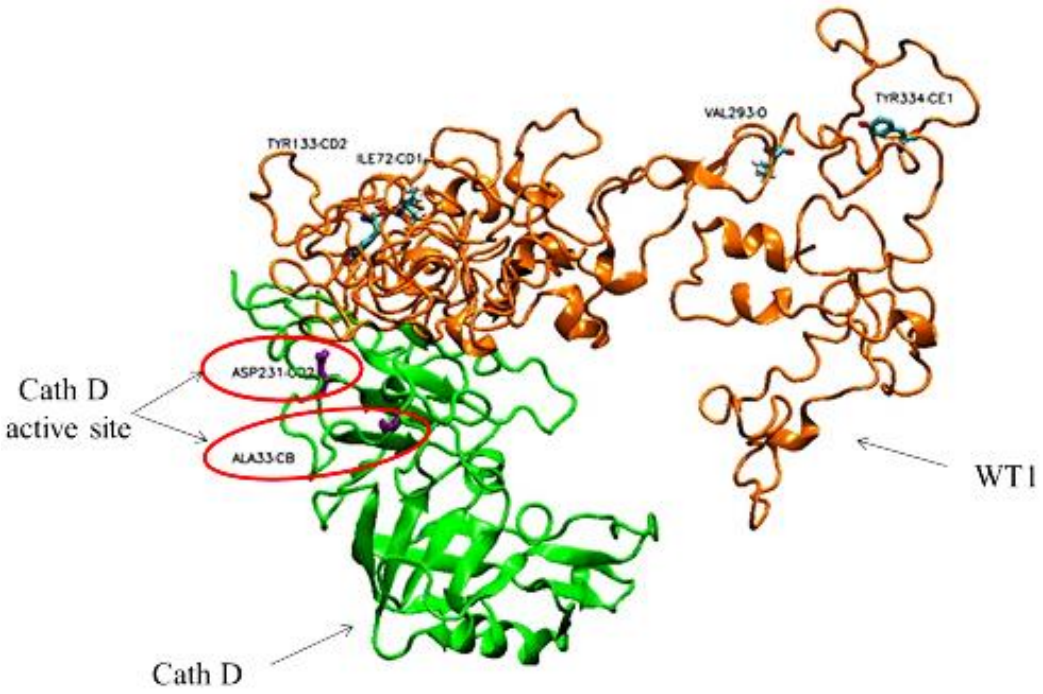
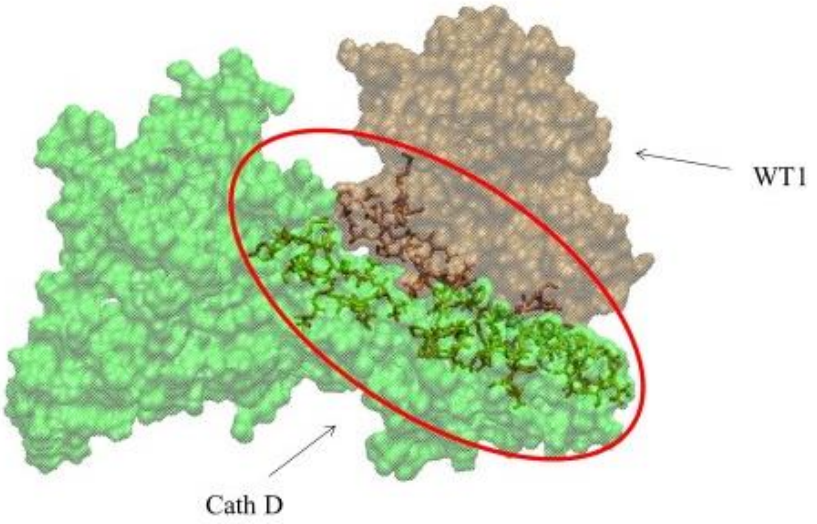
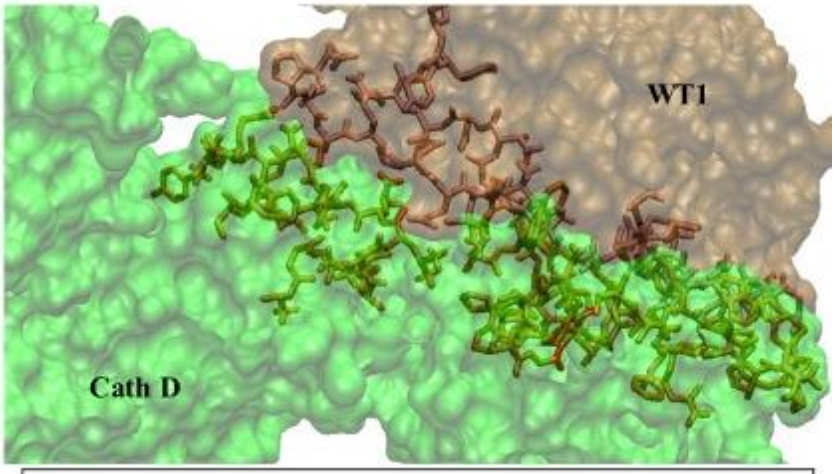


Figure 10 Cleavage sites of WT1 protein: TYR133, ILE72, VAL293 and TYR334. This data was based on primary structure analysis. Active site of Cath D is purple: ASP231, ALA33. WT1 protein is brown and Cath D enzyme is green, TYR=Tyrosine, ILE=Isoleucine, VAL=Valine, ASP=Aspartic acid, ALA=Alanine.

However, Cath D 3D crystal structure at pH 7.5 from PDB data bank was chosen to process molecular dynamics simulation and docked with WT1 protein. Neutral pH could find in cytosol and nucleus environment (pH 7.2-7.5) whereas Cath D synthesis was matured in lysosome at pH 5-5.5 (61). Thus, Cath D in acidic environment was additional observation with WT1 protein. pKa and chemical reaction from PDB2PQR server were used to predict charge of amino acid residue at pH 5 and 7.5. Alteration of charge at WT1-Cath D interacted site when pH was changed can predict ability of protein-protein binding. For interacted sites, amino acid positions of Cath D protein at 1-13, 53-56 and 67-87 were interfaced with amino acid at 38-60, 110-130 and 210-228 of WT1 (Figure 11).





The position of amino acid at interaction sites of proteins at pH 7.5	
Cath D	**WT1**
1-13 53-56 67-87	38-60 110-130 210-228

Figure 11 The position of amino acid at interacted sites of WT1 protein (brown) and Cath D protein (green) at pH 7.5. Amino acid position of Cath D protein at 1-13, 53-56 and 67-87 were interacted with amino acid positions in 38-60, 110-130 and 210-228 of WT1.

The result showed that charge was altered when pH was varied that showed as gray highlight in Table 6. However, charges of amino acid position on WT1-Cath D interaction site were not change at natural and acidic pH (Table 6). This result suggested that at pH 7.5 and pH 5 may has no effect on WT1-Cath D binding.

Table 6 pKa, chemical reaction and charge prediction of WT1 protein and Cath D protein at pH 7.5 and 5, respectively. The gray highlight presented amino acid position which altered chemical reaction and charge. D=Deprotonation, P=Protonation

- WT1 protein

RESIDUE of WT1 protein	**pKa**	**pKmodel ligand atom-type**	**pH 7.5**		**pH 5**	
			Chemical reaction	**Charge**	**Chemical reaction**	**Charge**
ASP 4 A	2.69	3.80	D	-1	D	-1
ASP 7 A	1.45	3.80	D	-1	D	-1
ASP 39 A	1.96	3.80	D	-1	D	-1
ASP 155 A	0.78	3.80	D	-1	D	-1
ASP 180 A	-0.11	3.80	D	-1	D	-1
ASP 207 A	**6.38**	**3.80**	**D**	**-1**	**P**	**0**
ASP 224 A	2.81	3.80	D	-1	D	-1
ASP 274 A	3.73	3.80	D	-1	D	-1
ASP 299 A	3.57	3.80	D	-1	D	-1
ASP 356 A	3.72	3.80	D	-1	D	-1
ASP 359 A	2.91	3.80	D	-1	D	-1
ASP 368 A	1.22	3.80	D	-1	D	-1
ASP 396 A	1.95	3.80	D	-1	D	-1
ASP 429 A	2.33	3.80	D	-1	D	-1
GLU 75 A	3.43	4.50	D	-1	D	-1
GLU 82 A	4.57	4.50	D	-1	D	-1
GLU 85 A	3.79	4.50	D	-1	D	-1

RESIDUE of WT1 protein (cont.)	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
GLU 86 A	4.31	4.50	D	-1	D	-1
GLU 139 A	2.65	4.50	D	-1	D	-1
GLU 179 A	4.05	4.50	D	-1	D	-1
GLU 190 A	2.87	4.50	D	-1	D	-1
GLU 234 A	3.88	4.50	D	-1	D	-1
GLU 262 A	3.54	4.50	D	-1	D	-1
GLU 272 A	2.28	4.50	D	-1	D	-1
GLU 316 A	2.07	4.50	D	-1	D	-1
GLU 319 A	1.44	4.50	D	-1	D	-1
GLU 350 A	2.97	4.50	D	-1	D	-1
GLU 361 A	2.29	4.50	D	-1	D	-1
GLU 411 A	**5.07**	**4.50**	**D**	**-1**	**P**	**0**
GLU 430 A	2.98	4.50	D	-1	D	-1
C- 449 A	1.97	3.20	D	1	D	1
HIS 69 A	4.93	6.50	D	0	D	0
HIS 84 A	**6.52**	**6.50**	**D**	**0**	**P**	**1**
HIS 95 A	4.07	6.50	D	0	D	0
HIS 162 A	2.95	6.50	D	0	D	0
HIS 166 A	**6.1**	**6.50**	**D**	**0**	**P**	**1**
HIS 167 A	**5.77**	**6.50**	**D**	**0**	**P**	**1**
HIS 174 A	**6.51**	**6.50**	**D**	**0**	**P**	**1**
HIS 178 A	**6.34**	**6.50**	**D**	**0**	**P**	**1**
HIS 203 A	**6.08**	**6.50**	**D**	**0**	**P**	**1**
HIS 267 A	**6.04**	**6.50**	**D**	**0**	**P**	**1**
HIS 276 A	**6.23**	**6.50**	**D**	**0**	**P**	**1**
HIS 289 A	**5.78**	**6.50**	**D**	**0**	**P**	**1**
HIS 291 A	**6.21**	**6.50**	**D**	**0**	**P**	**1**
HIS 339 A	**6.09**	**6.50**	**D**	**0**	**P**	**1**

RESIDUE of WT1 protein (cont.)	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
HIS 343 A	**6.31**	**6.50**	**D**	**0**	**P**	**1**
HIS 347 A	**6.52**	**6.50**	**D**	**0**	**P**	**1**
HIS 373 A	**6.01**	**6.50**	**D**	**0**	**P**	**1**
HIS 377 A	**6.26**	**6.50**	**D**	**0**	**P**	**1**
HIS 397 A	4.66	6.50	D	0	D	0
HIS 401 A	**5.39**	**6.50**	**D**	**0**	**P**	**1**
HIS 405 A	**5.74**	**6.50**	**D**	**0**	**P**	**1**
HIS 434 A	**6.01**	**6.50**	**D**	**0**	**P**	**1**
HIS 435 A	**6.08**	**6.50**	**D**	**0**	**P**	**1**
HIS 438 A	**5.48**	**6.50**	**D**	**0**	**P**	**1**
LYS 73 A	10.67	10.50	P	1	P	1
LYS 177 A	10.99	10.50	P	1	P	1
LYS 248 A	10.44	10.50	P	1	P	1
LYS 259 A	10.34	10.50	P	1	P	1
LYS 320 A	10.48	10.50	P	1	P	1
LYS 332 A	10.34	10.50	P	1	P	1
LYS 336 A	10.41	10.50	P	1	P	1
LYS 346 A	10.33	10.50	P	1	P	1
LYS 351 A	10.34	10.50	P	1	P	1
LYS 358 A	11.81	10.50	P	1	P	1
LYS 371 A	10.44	10.50	P	1	P	1
LYS 381 A	11.99	10.50	P	1	P	1
LYS 386 A	10.45	10.50	P	1	P	1
LYS 391 A	10.87	10.50	P	1	P	1
LYS 399 A	9.34	10.50	P	1	P	1
LYS 408 A	10.23	10.50	P	1	P	1
LYS 412 A	14.14	10.50	P	1	P	1
LYS 423 A	10.38	10.50	P	1	P	1

RESIDUE of WT1 protein (cont.)	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
LYS 424 A	9.89	10.50	P	1	P	1
LYS 444 A	10.32	10.50	P	1	P	1
ARG 6 A	12.71	12.50	P	1	P	1
ARG 108 A	13.7	12.50	P	1	P	1
ARG 126 A	13.86	12.50	P	1	P	1
ARG 145 A	12.74	12.50	P	1	P	1
ARG 218 A	12.82	12.50	P	1	P	1
ARG 287 A	13.21	12.50	P	1	P	1
ARG 295 A	12.26	12.50	P	1	P	1
ARG 301 A	13.49	12.50	P	1	P	1
ARG 302 A	12.69	12.50	P	1	P	1
ARG 312 A	14.93	12.50	P	1	P	1
ARG 321 A	11.57	12.50	P	1	P	1
ARG 333 A	11.93	12.50	P	1	P	1
ARG 345 A	12.19	12.50	P	1	P	1
ARG 362 A	12.33	12.50	P	1	P	1
ARG 363 A	12.32	12.50	P	1	P	1
ARG 366 A	14.08	12.50	P	1	P	1
ARG 372 A	12.38	12.50	P	1	P	1
ARG 375 A	12.44	12.50	P	1	P	1
ARG 376 A	12.05	12.50	P	1	P	1
ARG 390 A	12.4	12.50	P	1	P	1
ARG 394 A	12.23	12.50	P	1	P	1
ARG 403 A	12	12.50	P	1	P	1
ARG 417 A	13.29	12.50	P	1	P	1
ARG 427 A	13.17	12.50	P	1	P	1
ARG 433 A	12.29	12.50	P	1	P	1
ARG 440 A	12.12	12.50	P	1	P	1

RESIDUE of WT1 protein (cont.)	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
N+ 1 A	7.48	8.00				

- Cath D protein

RESIDUE of Cath D protein	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
ASP 27 A	3.46	3.80	D	-1	D	-1
ASP 47 A	4.17	3.80	D	-1	D	-1
ASP 56 A	2.86	3.80	D	-1	D	-1
ASP 67 A	1.62	3.80	D	-1	D	-1
ASP 69 A	2.78	3.80	D	-1	D	-1
ASP 82 A	3.25	3.80	D	-1	D	-1
ASP 106 A	2.49	3.80	D	-1	D	-1
ASP 126 A	4.91	3.80	D	-1	D	-1
ASP 137 A	3.03	3.80	D	-1	D	-1
ASP 184 A	2.22	3.80	D	-1	D	-1
ASP 205 A	3.42	3.80	D	-1	D	-1
ASP 218 A	**5.18**	**3.80**	**D**	**-1**	**P**	**0**
ASP 229 A	**5.06**	**3.80**	**D**	**-1**	**P**	**0**
ASP 231 A	3.67	3.80	D	-1	D	-1
GLU 6 A	2.83	4.50	D	-1	D	-1
GLU 12 A	3.32	4.50	D	-1	D	-1
GLU 75 A	4.66	4.50	D	-1	D	-1
GLU 109 A	3.02	4.50	D	-1	D	-1
GLU 119 A	3.17	4.50	D	-1	D	-1
GLU 122 A	2.51	4.50	D	-1	D	-1
GLU 138 A	3.94	4.50	D	-1	D	-1
GLU 141 A	3.38	4.50	D	-1	D	-1

RESIDUE of Cath D protein	pKa	pKmodel ligand atom-type	pH 7.5		pH 5	
			Chemical reaction	Charge	Chemical reaction	Charge
GLU 155 A	4.22	4.50	D	-1	D	-1
GLU 161 A	3.98	4.50	D	-1	D	-1
GLU 183 A	3.52	4.50	D	-1	D	-1
GLU 239 A	3.13	4.50	D	-1	D	-1
HIS 104 A	7.13	6.50	P	1	P	1
LYS 4 A	10.37	10.5	P	1	P	1
LYS 15 A	10.45	10.50	P	1	P	1
LYS 25 A	10.47	10.50	P	1	P	1
LYS 53 A	10.44	10.50	P	1	P	1
LYS 84 A	12.77	10.50	P	1	P	1
LYS 87 A	11.36	10.50	P	1	P	1
LYS 98 A	9.96	10.50	P	1	P	1
LYS 118 A	12.8	10.50	P	1	P	1
LYS 144 A	11.45	10.50	P	1	P	1
LYS 162 A	10.52	10.50	P	1	P	1
LYS 172 A	12.26	10.50	P	1	P	1
LYS 176 A	10.42	10.50	P	1	P	1
LYS 179 A	10.35	10.50	P	1	P	1
LYS 188 A	11.47	10.50	P	1	P	1
LYS 194 A	10.37	10.50	P	1	P	1
ARG 7 A	15.04	12.50	P	1	P	1
ARG 36 A	11.16	12.50	P	1	P	1
ARG 66 A	13.02	12.50	P	1	P	1
ARG 97 A	13.52	12.50	P	1	P	1
ARG 140 A	13.58	12.50	P	1	P	1
ARG 223 A	13.41	12.50	P	1	P	1
ARG 230 A	12.79	12.50	P	1	P	1
ARG 234 A	12.17	12.50	P	1	P	1
N+ 1 A	7.81	8.00				

CHAPTER 4

DISCUSSION

WT1, zinc finger transcription factor play an oncogenic role, was overexpressed in breast cancer patient that associated with proliferation and suppress apoptosis of tumor cell (31). High level of WT1 mRNA was found in breast cancer and correlated with poor prognosis. Interestingly, ER-negative cases were found higher expression of WT1 protein than in ER-positive breast cancer patients. Furthermore, WT1 overexpression also related with poor prognostic factor occurring in TNBC cases (6). To investigate WT1 related proteins, WT1-silenced by siRNA technique in MCF-7, ER-positive human breast cancer cell line was observed in previous study. Upregulated Cath D protein, a lysosomal aspartic protease, was presented when detected by proteomics analysis (unpublished data). Interestingly, in cancer issues Cath D was revealed overexpression in tumor cell and presented a role in invasion and metastasis (36). Immunohistochemistry found high level of Cath D in breast cancer when compared to the normal breast tissue (60). Thus, these evidence line can suggest that Cath D associated with breast cancer cell development and aggressiveness. However, the relation between WT1 and Cath D has never been reported. Remarkably, Robinson *et al*. (2005) revealed that overexpressed-WT1 was significantly in malignant mesothelioma tissue by using immunohistochemistry examination (62). In the same tumor, Hosako *et al*. (2012) found decreasing of Cath D expression by proteomic analysis (63). These reports may imply the relationship between WT1 and Cath D protein expression in cancer. In this research, the endogenous protein of WT1 and Cath D were studied in breast cancer cell lines by immunoblotting. We noted that WT1 and Cath D expression in breast cancer cell lines depend on their cell types. Thus, in this study TNBC (MDA-MB-231, MDA-MB-468, Hs578T cell lines) and ER-positive (MCF-7) human breast cancer cell lines were observed WT1 and Cath D expression. The result suggested that WT1 had higher expression than Cath D in TNBC cell lines. In ER-positive breast cancer cell line, WT1 protein showed lower level than Cath D which was overexpressed. These inverse correlation between WT1 and Cath D seemed to be subtype specific. For further investigation of relationship between WT1 and Cath

D, MDA-MB-468 TNBC cell line containing WT1 overexpression with low level of Cath D protein, was chosen as an *in vitro* model by immunoprecipitation technique or IP. This experiment revealed that WT1 presented with Cath D protein. This IP result could suggest that WT1 complexed with Cath D protein in MDA-MB-468 however the physical protein binding is uncleared. ZDOCK web server was used to explain structural of WT1-Cath D interaction. ZDOCK analysis was confirmed that WT1 directly bind Cath D protein in silico docking. Moreover, cleavage sites on WT1 protein, investigated by literature review based on primary protein structure, were not presented to active site of Cath D enzyme. This finding suggested that WT1 could interact with Cath D by catalytic independent. Consistently, various studies revealed that Cath D concerned to support tumor development without enzymatic role (43). Furthermore, previous studies that treated HOMECs cell line with Cath D and pepstatin A, inhibitor Cath D enzyme, in epithelial ovarian cancer, HOMECs cell could present migration and invasion (64). These results strongly supported that not only enzymatic role but also non-hydrolase of Cath D could promote breast tumor aggressiveness.

Above computational docking result was predicted WT1 with Cath D at pH 7.5. This neutral pH could find in cytosol and nucleus compartment (61). Niksic *et al*. (2003) revealed that WT1 could localize between nucleus and cytoplasm (65) meanwhile Cath D has been reported that involved in cytosol (post-translational modification, cellular trafficking secretion) and nucleus (nuclear transcription factor) in ER-positive breast cancer cell (60). This issue can imply that WT1-Cath D interaction may function in cytoplasm and/or nucleus. Nevertheless, WT1-Cath D binding in acidic compartment where presented mature Cath D and appropriated for Cath D enzymatic function is not clarified. In this research, pKa and chemical reaction result from PDB2PQR server were used to predict charge of amino acid of WT1 and Cath D at pH 5 and 7.5. The result revealed that charges were altered when pH was varied except charge on interaction site of WT1-Cath D binding. This study suggested that at pH 7.5 and pH 5 may has no effect on WT1-Cath D binding. However, at vary pH may result in charge alteration of amino acid interacted area. Thus, the effect, structural and functional of WT1-Cath D binding have to further investigate in vary pH.

CHAPTER 5

CONCLUSIONS

In this research, WT1 and Cath D endogenous protein was observed in ER-positive and TNBC breast cancer cell lines. Western blot analysis showed that WT1 was higher expression than Cath D in TNBC cell lines while Cath D expression was higher in ER-positive cell line. This endogenous protein detection could imply that inverse correlation between WT1 and Cath D seemed to be subtype specific. Protein relation from IP and ZDOCK prediction were clarified that WT1 directly bind with Cath D in MDA-MB-468 TNBC cell line. Furthermore, structure modelling and amino acid sequence observation were showed that WT1 interact with Cath D with catalytic independent. However, pKa and charge analysis from PDB2PQR server revealed that charge at interacted surface of both proteins were not altered at acidic (pH 5) and neutral (pH 7.5) environment. Although at pH 5 and 7.5 were no effect on WT1-Cath D binding, cellular compartment where WT1-Cath D function have to further investigate.

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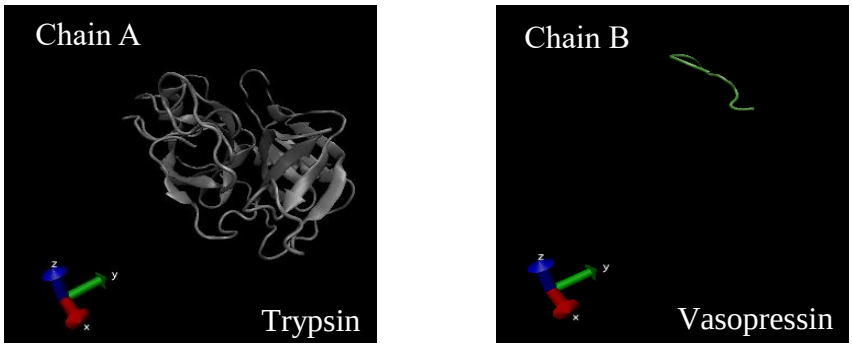
APPENDIX

ZDOCK tool validation

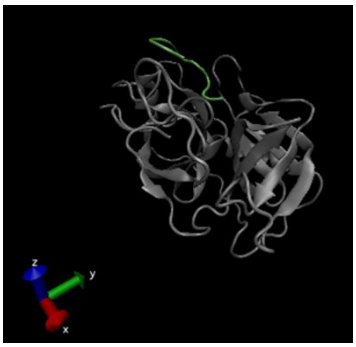
Structural protein-protein binding from ZDOCK web-server was validated with PDB protein data bank that accumulated biological protein interaction in a crystal structure, gold standard for the structure explanation. The best complex of protein binding from ZDOCK prediction was showed similarly binding position with crystal structure database. This validated step could reflect that ZDOCK is an efficient tool for protein-protein interacted prediction.

The step of validation ZDOCK tool with crystal structure sample from PDB database

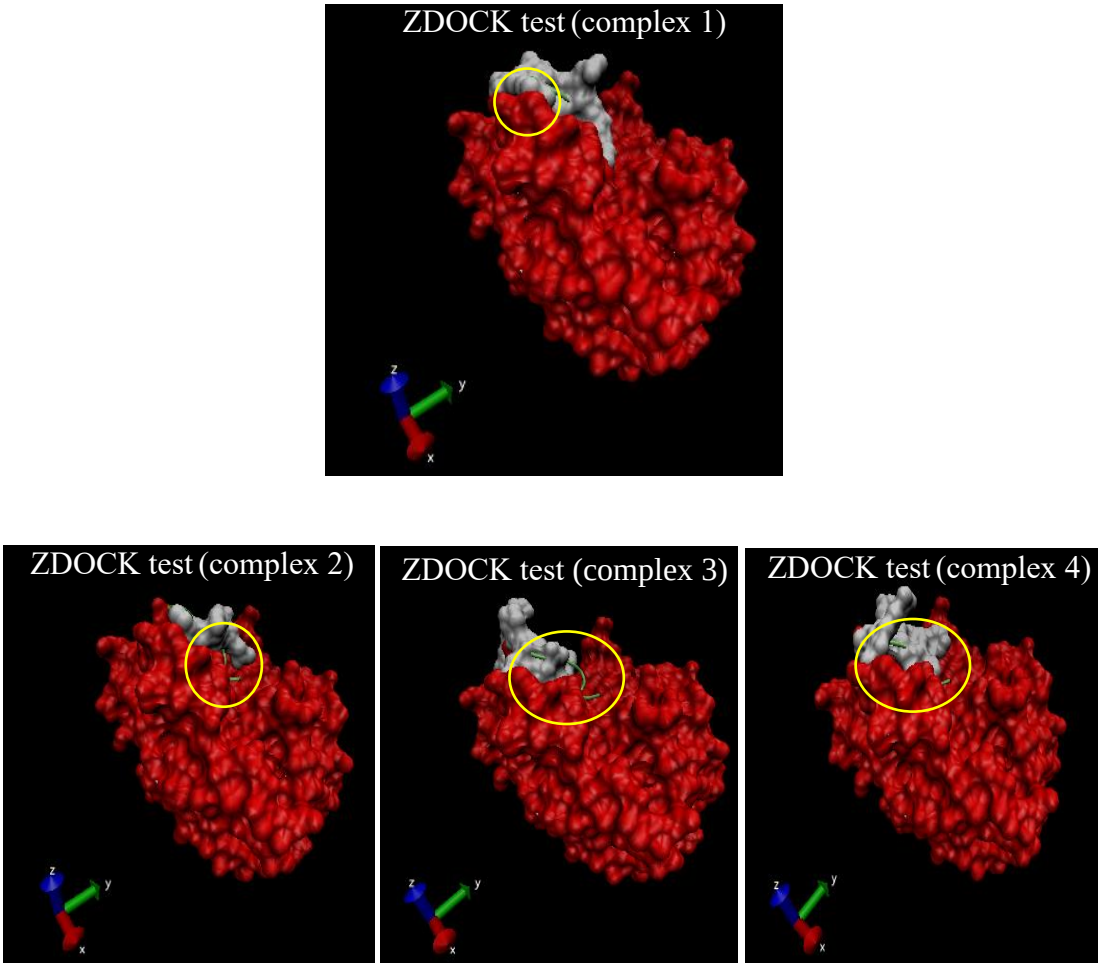
Sample: PDB code - 1YF4 (Crystal Structure of trypsin-vasopressin complex)



Crystal structure of chain A (trypsin) and chain B (vasopressin)



Binding of Crystal structure of chain A (trypsin) and chain B (vasopressin)

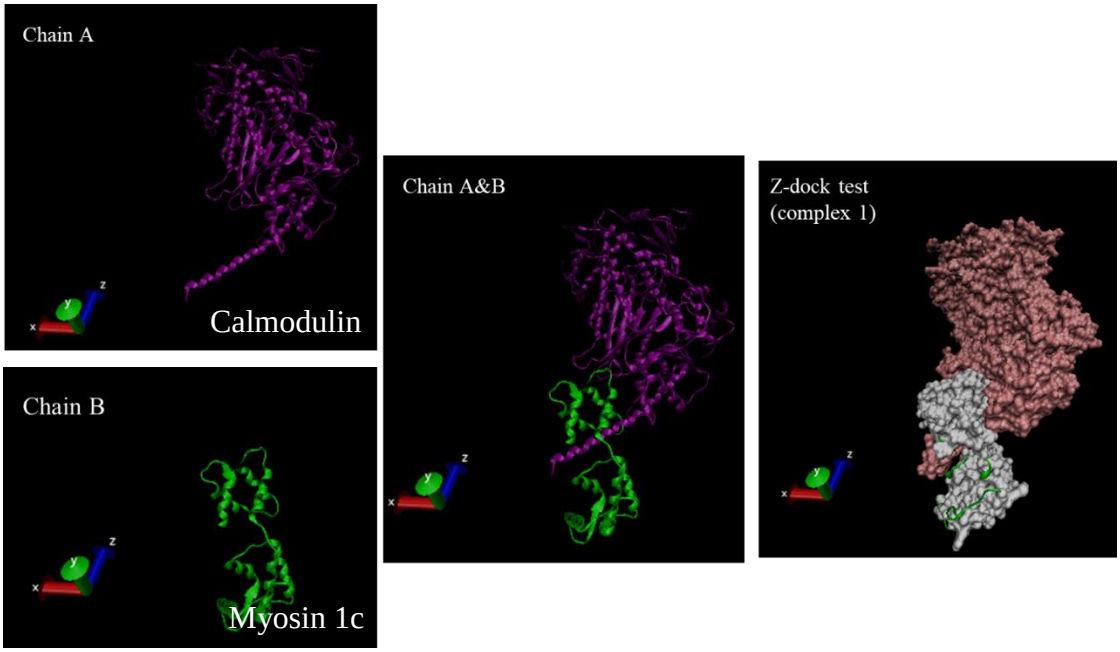


ZDOCK prediction results

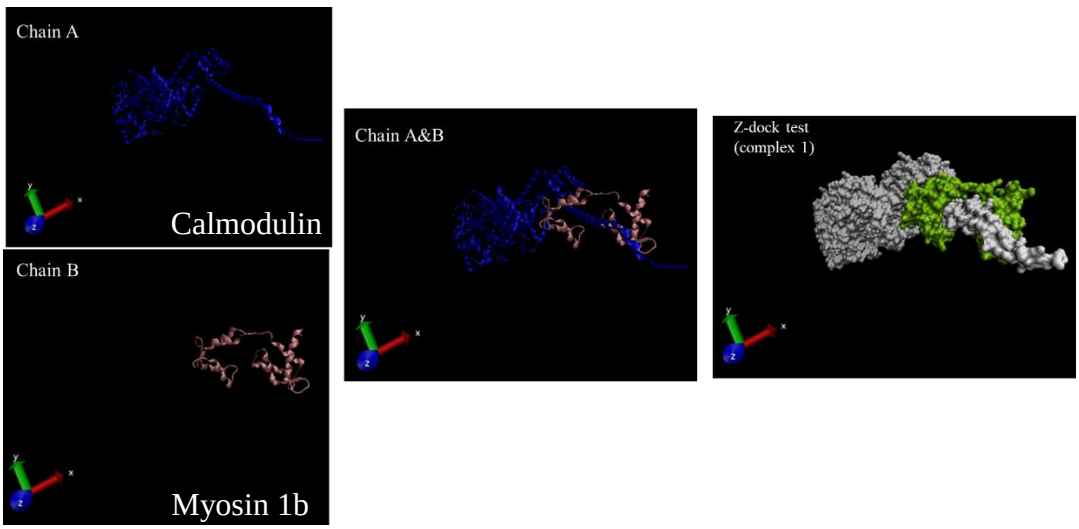
ZDOCK result proposed complexes of chain A (trypsin) and chain B (vasopressin) binding. Complex 1 is the best complex that ZDOCK result rearranged. The best ZDOCK structure showed that ZDOCK protein binding position were similar with crystal structure (protein docking model from ZDCOK result can cover almost area of crystal structure). The yellow circle displayed position of protein interaction that ZDOCK tool uncovered crystal structure presentation.

The best structural ZDOCK result of various proteins interaction with crystal structure from PDB database

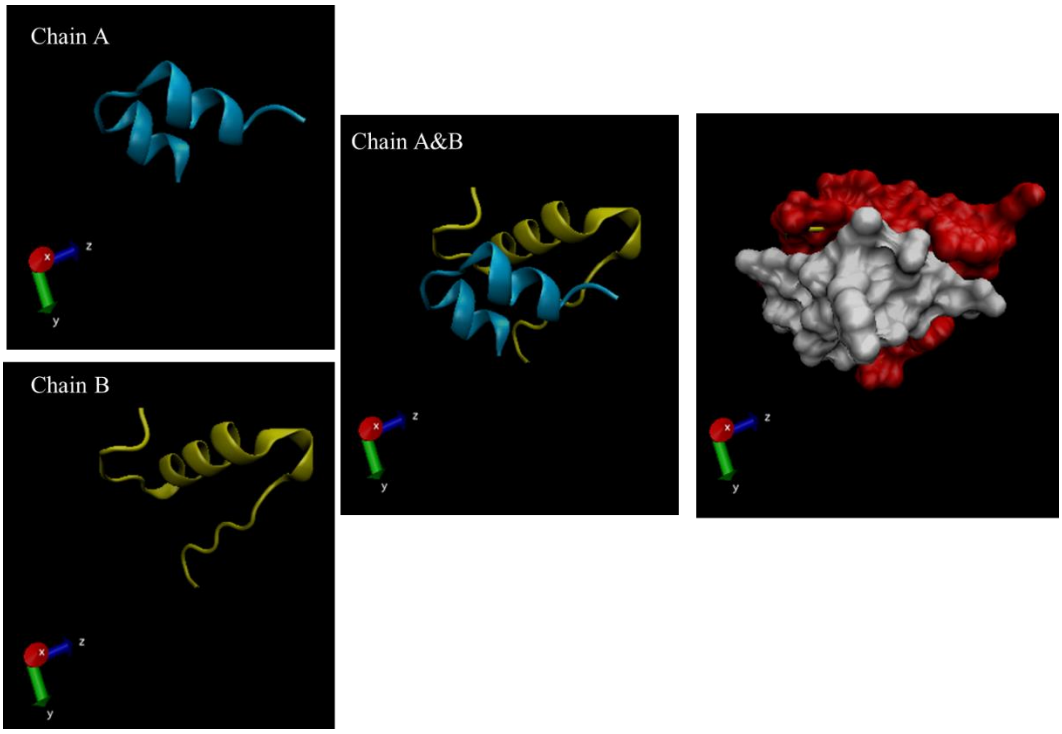
- PDB code: 4BYF (Crystal structure of human Myosin 1c in complex with calmodulin in the pre-power stroke state)



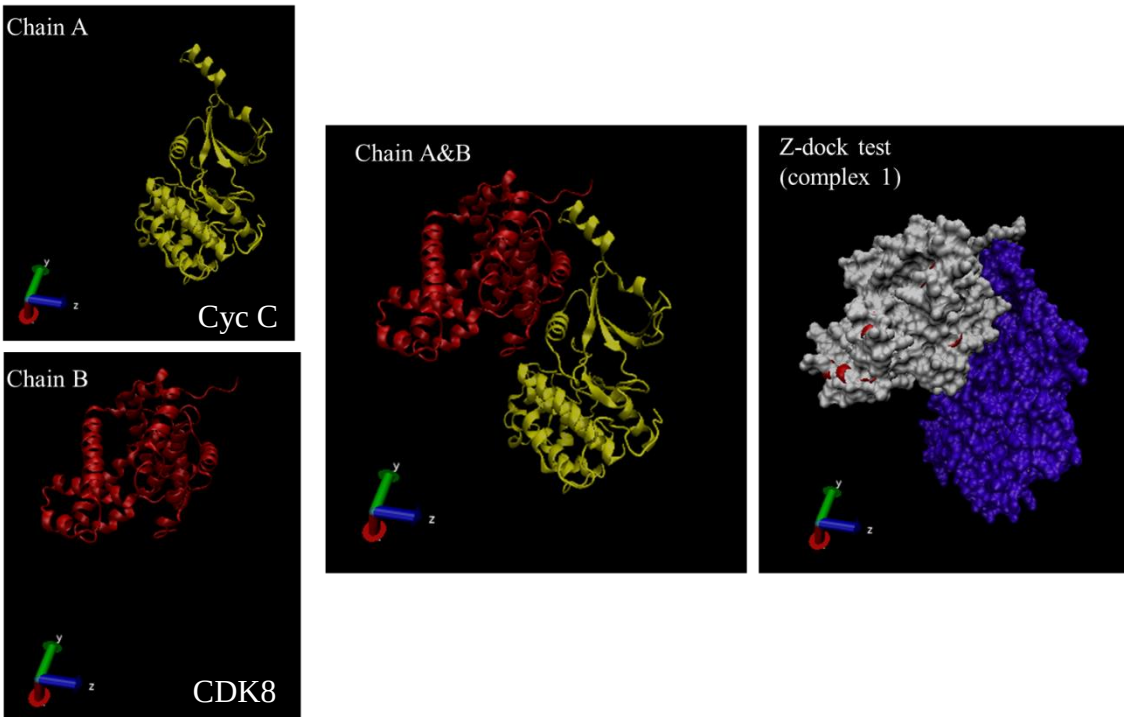
- PDB code: 4L79 (Crystal Structure of nucleotide-free Myosin 1b residues 1-728 with bound Calmodulin)



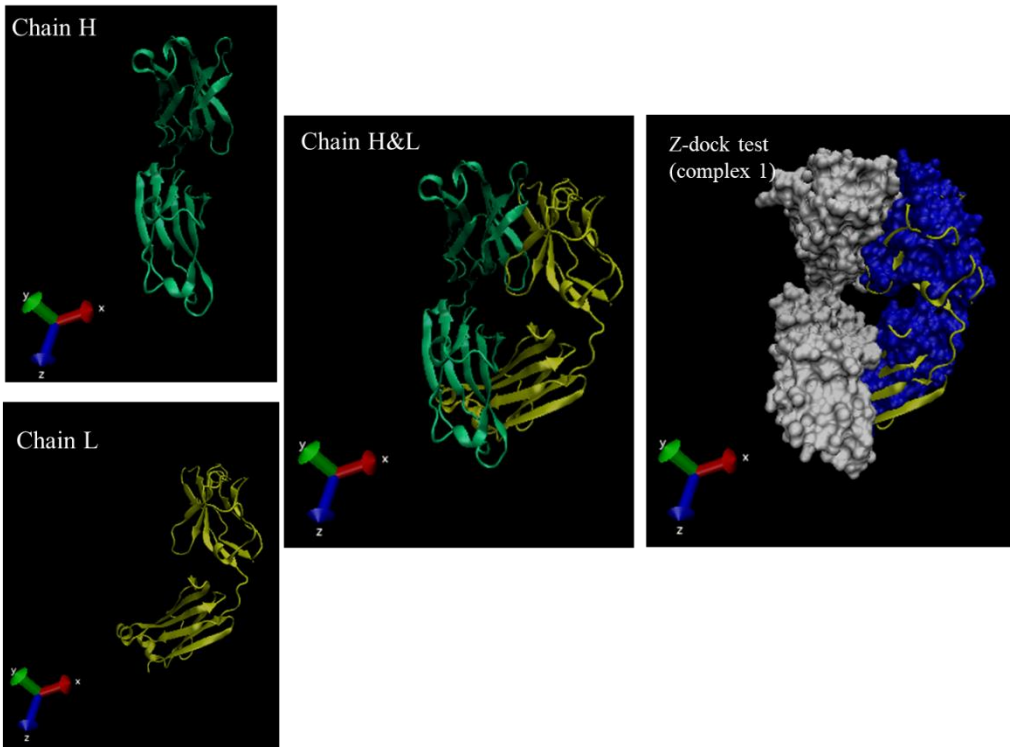
- PDB code: 3I40 (Human insulin)



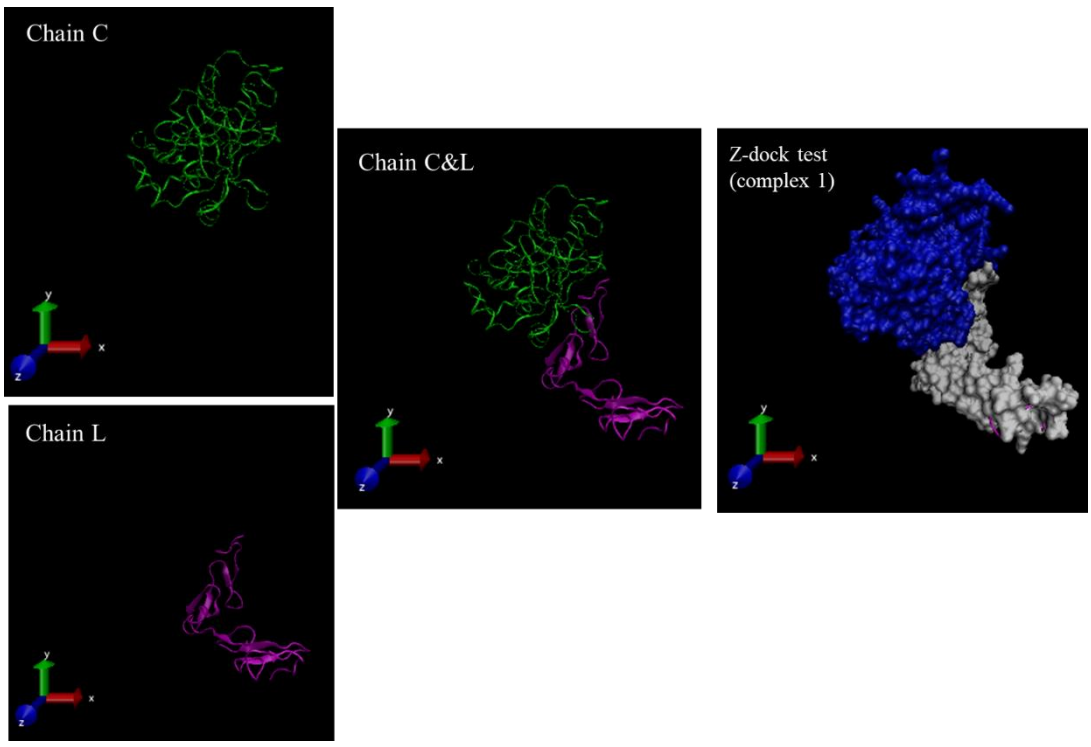
- PDB code: 3RGF (Crystal Structure of human CDK8/Cyc C)



- PDB code: 4NKI (Crystal structure of a Fab)



- PDB code: 1AUT (Human activated protein C)



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