ANALYSIS OF OTUB1 EXPRESSION IN HUMAN BREAST ADENOCARCINOMA CELL LINE MCF7 AND HUMAN BREAST EPITHELIAL CELL LINE MCF10A

by

ATHIRAH BINTI ISKANDAR

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (MEDICAL RESEARCH)

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

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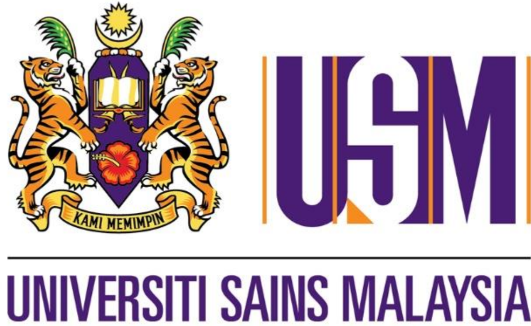

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DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) for the degree of Master of Science in Medical Research. It has not been sent to other universities. With that, this research can be used for consultation and photocopied as reference.

Sincerely,

ATHIRAH BINTI ISKANDAR

(P-IPM0008/18)

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LIST OF SYMBOLS

°	Degree
β	Beta
C	Celsius
CO_2	Carbon dioxide
%	Percentage
cm	Centimeter
g	Gravity
µl	Microliter
µM	Micromolar
µg	Microgram
ml	Milliliter
Kpa	Kilopascal
kb	Kilobase
ng	Nanogram
rpm	Revolutions per minutes
v	Volt
ΔΔCt	Delta delta cycle threshold

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
β-actin	Beta actin
BRCA	Breast cancer gene
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
c-IAP	Cellular inhibitor apoptosis protein
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUBs	Deubiquitinating enzymes
EDTA	Ethylenediamine tetra-acetic acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin-protein ligases
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
HER2	Human epidermal growth factor receptor 2
MCF7	Human breast adenocarcinoma cell line
MCF10A	Human breast epithelial cell line
MDM2	Mouse double minute 2 homolog
OTUB1	Ovarian tumour domain-containing Ub aldehyde-binding protein 1
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
qPCR	Real-time polymerase chain reaction
RT	Reverse transcription
RNA	Ribonucleic acid
RNase	Ribonuclease
RQ	Relative Quantification
SDS	Sodium dodecyl sulphate
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate-EDTA
Ub	Ubiquitin
UPS	Ubiquitin–proteasome system
USA	United States of America

ANALISIS EKSPRESI OTUB1 DI DALAM SEL BARAH PAYUDARA MCF7 DAN SEL EPITHELIUM PAYUDARA MANUSIA MCF10A

ABSTRAK

Proses pasca translasi modifikasi (PTM) protein diketahui memainkan peranan yang penting di dalam fungsi protein. Ubiquitinasi merupakan salah satu proses PTM yang penting di mana molekul ubiquitin ditambah ke protein target oleh enzim pengaktif ubiquitin (E1), enzim konjugat ubiquitin (E2) dan ubiquitin ligase (E3). Proses ini boleh diterbalikkan oleh tindakan enzim khusus yang dikenali sebagai enzim deubiquitinasi (DUBs). OTUB1 merupakan DUBs yang dilaporkan mempunyai peranan penting di dalam permulaan dan perkembangan barah. Untuk menyiasat peranan OTUB1 di dalam barah payu dara, kajian awal dijalankan untuk membandingkan ekspresi OTUB1 di dalam sel barah payudara MCF7 dan sel epithelium payudara manusia MCF10A. Ekspresi OTUB1 di dalam MCF7 dan MCF10A dianalisis menggunakan quantitatif PCR dan nilai pengkuantitian relatif yang diperoleh menggunakan kaedah ΔΔCt. Ujian Mann-Whitney digunakan untuk melihat perbezaan ekspresi OTUB1 di antara MCF7 dan MCF10A. Untuk menyokong kajian qPCR ini, analisis bioinformatik dilakukan menggunakan maklumat mikrodatasusunan daripada Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). Analisis qPCR menunjukkan bahawa ekspresi OTUB1 secara signifikan adalah lebih tinggi di dalam MCF7 berbanding dengan MCF10A (p = 0.037). Penemuan ini disokong oleh analisis bioinformatik daripada maklumat mikrodatasusunan GEO yang terpilih di mana hampir kesemua prob OTUB1 telah mengesahkan bahawa ekspresi OTUB1 lebih tinggi di dalam MCF7 secara signifikan. Justeru, kajian pada masa akan datang perlu dilakukan untuk mendedahkan peranan serta fungsi kompleks OTUB1 dalam jejak laluan berkaitan kanser.

ANALYSIS OF OTUB1 EXPRESSION IN HUMAN BREAST ADENOCARCINOMA CELL LINE MCF7 AND HUMAN BREAST EPITHELIAL CELL LINE MCF10A

ABSTRACT

Post-translational modification (PTM) of protein is known to play a major role in protein function. One of the most important PTM is ubiquitination, in which the ubiquitin molecule(s) are added to a target protein by ubiquitin activating enzymes (E1s), ubiquitin conjugating enzymes (E2s) and ubiquitin ligases (E3s). This process is reversible by the action of specialized enzymes known as deubiquitinases (DUBs). OTUB1 is a DUBs that was reported to has critical roles in cancer initiation and progression. To investigate the role of OTUB1 in breast cancer, this preliminary study was conducted to compare the expression of OTUB1 in human breast adenocarcinoma cell line, MCF7 and human epithelial cell line, MCF10A. Expression of OTUB1 in MCF7 and MCF10A was analysed by quantitative real-time PCR and relative quantification (RQ) value was obtained using ΔΔCt method. Mann-Whitney test was used to evaluate the differences in OTUB1 expression levels between MCF7 and MCF10A. To support our qPCR study, computational analysis was also performed on selected microarray datasets from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). qPCR analysis shows that OTUB1 expression was significantly higher in MCF7 compared to MCF10A ($p = 0.037$). Computational analysis on selected GEO microarray datasets shows that almost all probes corresponding to OTUB1 confirmed that OTUB1 expression is significantly higher in MCF7. Therefore, further studies should be done to uncover the role and function of OTUB1 in cancer-associated pathways.

CHAPTER 1

INTRODUCTION

1.1 Research Background

According to World Health Organization (WHO), breast cancer is among the most commonly diagnosed life-threatening cancer in women worldwide. Among Malaysian women, it is regarded as the most common cancer in all ethnic and age groups. Current breast cancer treatment includes surgery, radiotherapy, hormonal therapy and targeted therapy. However, it is worth noting that there are always adverse effects associated with the treatment. Surgery can cause pain and lymphedema, as well as removal of the entire breast, including the normal tissue. In chemotherapy, hair loss, loss of appetite, fatigue, nausea and vomiting are very common side effects. Meanwhile, hormonal therapy causes headache, aches, hot flashes and night sweats.

In contrast, less side effects were seen in targeted therapy. Targeted therapy can specifically kill cancer cells and usually does not attack the neighbouring healthy cells. Targeted therapy usually involves the use of drugs or other substances that suppress the molecular mechanism in cancer development and progression, for example by blocking the proliferation and promotion of cancer cell cycle regulation, inducing apoptosis or autophagy in cancer cells, and delivering substances that are toxic to cancer cells and kill them. Among the examples of breast cancer targeted drugs that have been developed including trastuzumab that target HER2 proteins (Swain *et al.*, 2015), olaparib for BRCA mutations (Robson *et al.*, 2017) and CDK4/6 inhibitor, palbociclib (Turner *et al.*, 2015). Besides having less side effects than standard chemotherapy drugs, some targeted therapy can also facilitate other types of treatment for better outcome. Therefore, it is crucial to

find a good molecular target to develop targeted drug therapy for more effective treatments and greater survival rates.

Deubiquitinating enzymes or DUBs is a component of the protein ubiquitination system that has been regarded as potential therapeutic target for cancer. In human body, there are at least 101 DUBs that can be categorized into seven families consisting of USP, UCH, OTU, MJD, JAMM, MINDY and ZUFSP. There is growing evidence that the dysregulation of human DUBs often leads to the development of many types of cancer. Some DUBs from USP family such as USP2, USP4, USP7, USP10 and USP29 are reported to regulate p53, one of the important tumour suppressor protein (Kon *et al.*, 2010). Moreover, DUBs are also known to control several cancer signalling pathways, especially those mediated by NFκB, TGF-β and WNT (Jung *et al.*, 2013; Arcy *et al.,* 2015). Apart from signal transduction, some fundamental cellular processes such as cell cycle progression, apoptosis, and response to DNA damage are also regulated by DUBs (Jacq *et al.*, 2013). These information show that altered expression of DUBs is very common in cancer development and progression and thus, DUBs are increasingly regarded as potential targets in cancer therapy (Hussain *et al.*, 2009).

Apart from the well-studied USP family DUBs, OTU family members have recently gaining attention as promising regulators in cancer related pathways. An OTU member known as OTUB1 was reported as a novel p53 regulator (Sun *et al.*, 2012). Many studies also reported that OTUB1 has direct involvement in the development of various malignancies including breast cancer (Karunarathna *et al.*, 2016), ovarian cancer (Wang *et al.*, 2018), lung cancer (Baietti *et al.*, 2016), prostate cancer (Iglesias-Gato *et al.*, 2015), colorectal cancer (Zhou *et al.*, 2014), gastric adenocarcinoma (Weng *et al.*, 2016) and glioma (Xu *et al.*, 2017).

1.2 Problem Statement

Following studies that reported the emerging role of OTUB1 in many types of cancer, there is an increasing interest to study the effect of OTUB1 knockdown or overexpression in cancer cell lines with the aim to develop OTUB1 as targeted cancer therapy. However, due to diverse function of OTUB1, the expression pattern of OTUB1 in breast cancer cell line remain unclear. For instance, interaction of OTUB1 with p53 is reported to increase apoptosis in cancer cell line while OTUB1 and c-IAP partnership would decrease apoptosis (Saldana *et al.*, 2019). Therefore, it is essential to perform a preliminary analysis on the expression of OTUB1 in cancer and normal cells to fully understand the effects of OTUB1 on cancer before proceeding with any therapeutic studies.

1.3 Objective of the Study

This study aimed to compare the OTUB1 gene expression in human breast adenocarcinoma cells MCF7 (cancer cells) and human breast epithelial cell MCF10A (normal cells). At the end of this study, the expression level of OTUB1 in both cells can be compared for the purpose of further studies involving gene manipulation of OTUB1 in cancer cell lines. The specific objectives were:

1. to extract RNA from MCF7 and MCF10A cell lines.
2. to synthesize cDNA using reverse transcription process.
3. to analyze OTUB1 expression in MCF7 and MCF10A by Real-Time Polymerase Chain Reaction (qPCR).
4. to perform a computational analysis and compare OTUB1 expression in MCF7 and MCF10A using selected microarray datasets from Gene Expression Omnibus (GEO) database.

1.4 Hypothesis of the Study

The expression level of OTUB1 in human breast adenocarcinoma cell line MCF7 was higher than human breast epithelial cell line MCF10A.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer statistic

Cancer is regarded as the most common leading cause of death worldwide of all income levels (Ferlay, 2019). In 2018, there were at least 18.1 million new cases and 9.6 million deaths reported (World Health Organization). Globally, in every five men and six women, at least one would develop cancer in their lifetime, and in every eight men and 11 women, at least one would die because of cancer.

The highest cancer incidence is contributed by lung and female breast cancers with 11.6% from the total cancer incidence, which is approximately 2.1 million diagnoses. This is followed by colorectal cancer with 10.2%, which is equal to 1.8 million reported cases. Other top cancers diagnosed are prostate cancer (1.3 million cases, 7.1%), and stomach cancer (1.0 million cases, 5.7%).

In terms of death incidence caused by cancer, lung cancer contributed to the highest number of deaths with 1.8 million deaths (18.4%) followed by colorectal cancer with 881 000 deaths (9.2%). Stomach and liver cancer are responsible for the third leading cause of cancer death, accounting for 783 000 deaths (8.2%), and 782 000 deaths (8.2%) respectively. Figures 2.1 and 2.2 show number of new cases and death caused by cancer in 2018.

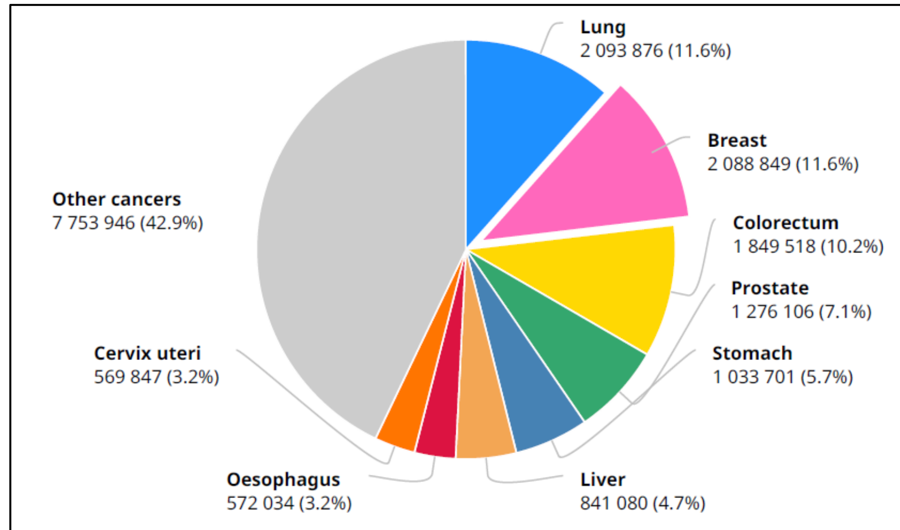

Figure 2.1 Number of new cancer cases in 2018 in both sexes and all ages. Source: GLOBOCAN.

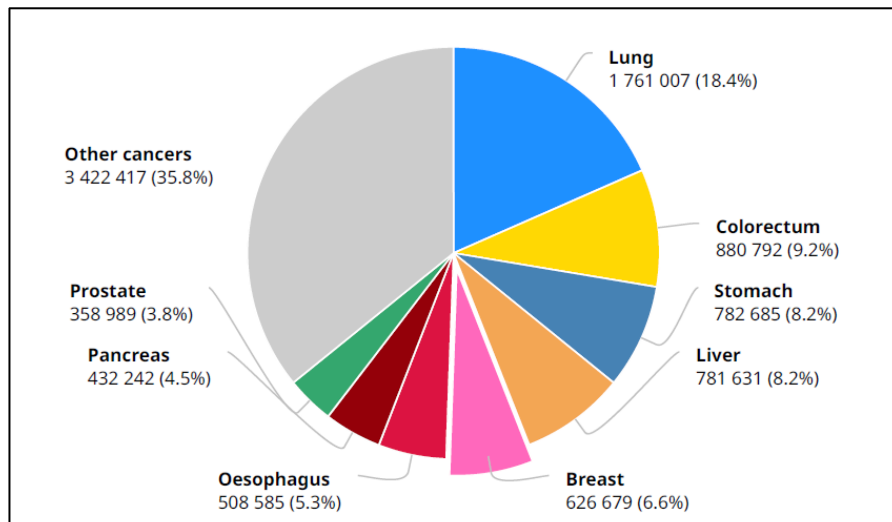

Figure 2.2 Number of deaths caused by cancer in 2018, in both sexes and all ages. Source: GLOBOCAN.

2.2 Breast cancer

Globally, breast cancer is among the most commonly diagnosed cancer in women, accounting for approximately one in four of all newly diagnosed cancer cases. Moreover, breast cancer is also reported as the most common cancer in 154 of the 185 countries listed in Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2018. From all the new breast cancer cases diagnosed worldwide in 2012, more than 600,000 (39%) new cases were reported in Asia alone (Fan *et al.*, 2015). Breast cancer is also regarded as the most common cancer in all ethnic groups and all age groups among Malaysian women. Thirty out of 100 women who suffer from cancer in Malaysia are breast cancer patients (Lim and Yahaya, 2002). Based on the study by Hisham and Yip (2004), more than half of the breast cancer patients in Malaysia were Malay (53.8%) followed by Chinese (27.1%), Indian (9.6%), other ethnic group (7.7%) and foreigners (1.9%).

There are several ways of treating breast cancer such as surgery, radiotherapy, hormonal therapy and targeted therapy. Surgery is done by removing the cancer tissue as well as some surrounding normal tissue. Sometimes, the entire breast is removed in a procedure called mastectomy. Surgery is not only invasive but women who want to recreate a bulge to replace the removed breast must undergo another surgery for breast reconstruction. Radiotherapy is a treatment with high doses of radiation to destroy the cancer cells. The patients usually experience fatigue, redness, skin irritation and dryness at the targeted area. For chemotherapy, anti-cancer drugs are given to the patients intravenously or in the form of pills to stop or reduce the proliferation rate of cancer cells. It is considered a systemic therapy, in which drugs are spread throughout the body to treat cancer cells. Therefore, chemo drugs not only rapidly attack growing cancer cells, they also affect healthy growing cells. In chemotherapy, patients may experience sides effects such as loss of appetite, nausea and vomiting (Toprak *et al.*, 2018). Several blood forming

cells in bone marrow are also damaged. Thus, patients experience fatigue, easily bleed and bruise, and are susceptible to infections.

Some breast cancer growth is stimulated by the hormones in the body such as progesterone or estrogen. These types of cancers are known as hormone receptor-positive cancers. Hormone therapy works by blocking the hormones from acting on cancer cells or decreasing the hormone levels. Fulvestrant is one of the drugs that block and permanently degrades the estrogen receptor on breast cancer (Osborne *et al.*, 2004). However, patients who are taking this drug experience some side effects such as headache, bone pain, hot flashes and night sweats.

In contrast to chemotherapy, targeted therapy does not attack all the cells including healthy cells. Targeted cancer therapy involves drugs or other substances that block the proliferation and promotion of cell cycle regulation of the cancer cells. Besides, targeted therapy also induces apoptosis or autophagy and destroying the cancer cells by delivery of toxic substances specifically to cancer cells (Padma, 2015). Examples of breast cancer targeted drugs that have been developed include trastuzumab, which targets HER2 proteins (Swain *et al.*, 2015), olaparib for BRCA mutations (Robson *et al.*, 2017) and CDK4/6 inhibitor, palbociclib (Turner *et al.*, 2015).

Targeted therapy usually have less s side effects than chemotherapy drugs (Vega *et al.,* 2012) and some targeted therapy can facilitate other treatments for better result. Therefore, it is crucial to find a good target to develop targeted drug therapy for more effective treatments and greater survival rates.

2.3 Protein Ubiquitination

Ubiquitin is a small molecule of 76-amino acid polypeptide encoded on multiple genes. It is highly conserved among many species and expressed in all eukaryotes. Ubiquitination is a process of attaching this ubiquitin molecules to other protein (substrate) through a complex, specific and highly regulated process (Fang and Weissman, 2004). Three enzymes are involved in catalyzing the ubiquitination process; E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and two types of E3 ubiquitin ligase called HECT and RING. These three enzymes are known to work in a hierarchical manner. In ubiquitination process, the ubiquitin molecules are firstly activated by E1 through an ATP dependent step. E1 forms a thiol ester bond between its active cysteine and the ubiquitin's C terminus before transferring this activated ubiquitin to the E2's active site cysteine residue. Lastly, the ubiquitin is transferred to the substrate (or other ubiquitin)'s lysine residue to form a mono- or polyubiquitin chain on the substrate (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). The ubiquitin functions as a 'tag' or label' for its substrate, in which every ubiquitinated substrates is assigned to perform specific function according to the type of ubiquitin chain. For example, substrates that are ubiquitinated with four ubiquitin molecule (polyubiquitination) at their Lys48 destroy themselves through proteasomal degradation, while substrates that are ubiquitinated at their Lys63 will enter DNA damage process (Mcclurg and Robson, 2015; Chen, 2016; He *et al.*, 2016).

Ubiquitination is a reversible process. Apart from E1, E2 and E3, a set of enzymes known as deubiquitinases (DUBs) is specifically tasked to counter protein ubiquitination (D'Arcy *et al.,* 2015; Suresh *et al.*, 2015; Coyne and Wing, 2016; Pinto-fernandez and Kessler, 2016). The DUBs function to remove the covalent attachment between ubiquitin

molecules or ubiquitin-substrates thus rescuing the substrates from their fates assigned by ubiquitination process.

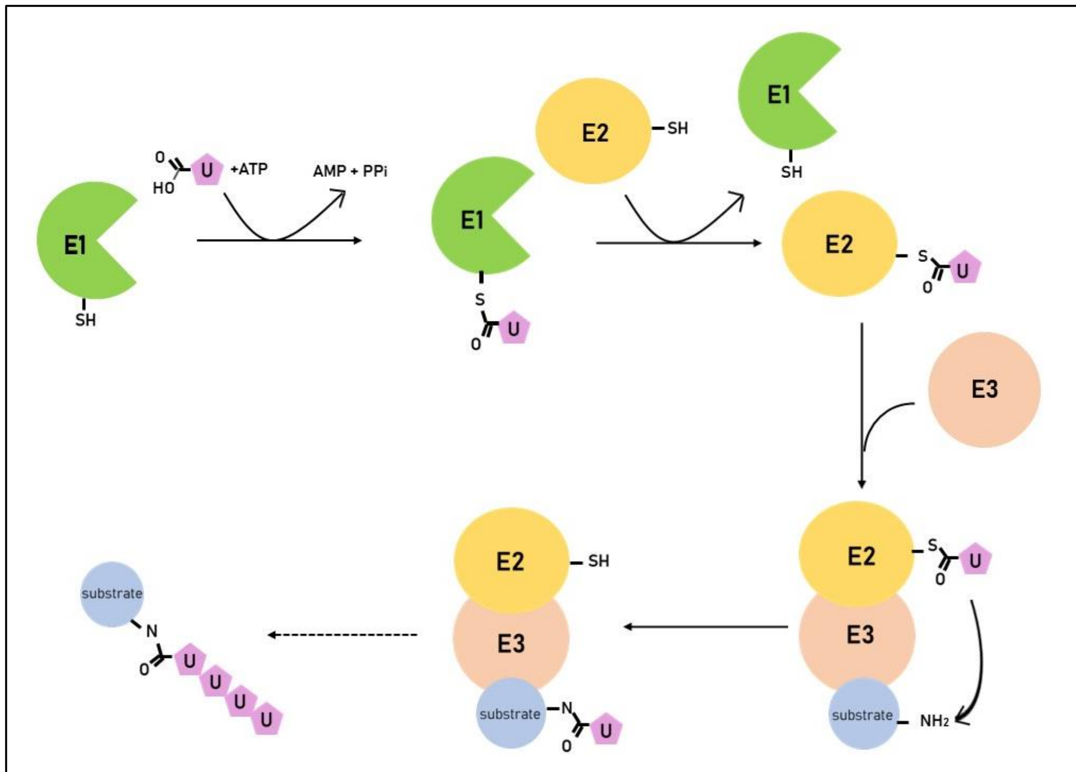

Figure 2.3 Process of ubiquitination. Ubiquitination occurs as a result of the sequential action of three different enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3). (Extracted from Maupin-Furlow (2014)).

2.4 The Deubiquitinating Enzymes

The ubiquitin system is catalyzed by two E1s, about 35 E2s and more than 600 E3s to confer specificity for substrate ubiquitination and diversity to ubiquitin chains (Chaugule and Walden, 2016). To counter the highly specific ubiquitination process, 101 deubiquitinases (DUBs) from various families are involved in the de-ubiquitination process. DUBs can be categorized into seven families based on sequence and structural similarities which are ubiquitin-specific proteases (USPs), ovarian tumor domain proteases (OTUs), ubiquitin carboxy-terminal hydrolases (UCHs), JAB1/MPN/MOV34 domain associated metallopeptidases (JAMMs), Machado-Joseph disease protein domain proteases (MJDs), and newly identified motif interacting with Ub-containing novel DUB family (MINDYs) and zinc finger with UFM1 specific peptidase domain (ZUFSPs) (Nijman *et al.*, 2005; Mevissen and Komander, 2017). USPs, UCHs, OTUs, MJDs and the novel MINDYs and ZUFSPs families belong to thiol proteases, while JAMMs, are Zn^{2+} metalloproteases (Arif *et al.*, 2016; Kwasna *et al.*, 2018). These DUBs vary in size, characteristics, specificity and have different spectrum of substrates or interacting protein (Mevissen and Komander, 2017).

The most essential role of DUBs is their ability to specifically remove ubiquitin from target proteins (Fraile *et al.*, 2012). Since ubiquitination govern nearly every process in human cellular system, DUBs have a great influence on many biological processes and cellular pathways (He *et al.*, 2016; Kee and Huang, 2016). Recently, DUBs have been the subject of intensive research closely linked to human disease (Mevissen *et al.*, 2013). This is supported by Clague and colleague (2012) who reported that many of the DUBs are predicted to have implications in human diseases such as neurological diseases and cancer. In relation to cancer, abnormality in DUB signalling and activity can play important roles in cancer development, progression and metastasis. This is because DUBs

are known to regulate cellular growth, survival and homeostasis, which are closely related to cancer, through multiple pathways including epigenetics. (Mcclurg and Robson, 2015). Studies by Luise *et al.* (2012) show that many DUBs are associated with tumours due to alterations in gene or protein expression levels. For example, various breast cancers showed an increase in expression level of USP7, UCH37, OTUD6B, VCPIP1 and COPS5. Meanwhile, oncogenic protein USP6 is found to be overexpressed in primary aneurysmal bone cyst (ABC) (Ye *et al.*, 2010). Therefore, exploration of the in-depth mechanism by which DUBs regulate cancer-associated pathways could provide new insight in cancer therapeutics strategy.

2.5 OTUB1

The OTU family comprises a group of cystine protease homologous to the ovarian tumor gene product of *Drosophila Melanogaster* (Makarova *et al*., 2000). In the human genome, OTU domains exist in at least 18 genes (Mevissen *et al.*, 2013). Examples of deubiquitinating enzymes in OTU family are OTUB1, OTUB2, OTUD1, OTUD3, OTUD4, OTUD5, OTUD6A, OTUD6B, OTUD7, YOD1, OTULIN, A20, Cezanne, Cezanne2, TRABID and VCPIP1 (Coyne and Wing, 2016). OTUB1, the most well-known member in the OTU family was discovered in 2003 (Balakirev *et al.*, 2003). This protein is reported to have the ability to cleave the iso-peptide bond within polyubiquitin chains, with a preference target to polyubiquitin chain joined by Lys48 (Edelmann *et al.*, 2009).

2.6 OTUB1 in cancers

A growing number of studies demonstrate that OTUB1 has a critical role in cancer development and progression by regulating apoptosis, therapeutic resistance, proliferation, migration and invasion in several cancer types (Saldana *et al.*, 2019). However, it should be noted that OTUB1 might have a complex effect in different types of malignancies because it regulates the expression and activity of many substrates and participates in multiple cell signalling pathways (Nakada *et al.*, 2010).

According to Baietti *et al* (2016), the deubiquitinase OTUB1 promotes lung cancer formation and is associated with poor patient prognosis. In breast cancer, OTUB1 prevents the proteasomal degradation of FOXM1 upon epirubicin treatment and regulates the expression of FOXM1. FOXM1 is known to be involved in therapeutics resistance across many tumour types, thus, its stabilization by OTUB1 could enhance the proliferative rate and treatment resistance of breast cancer cells (Karunarathna *et al.*, 2016). Overexpressed OTUB1 in ovarian cancer also interacts with FOXM1, which suggests promotion of tumorigenesis and tumour progression (Wang *et al.*, 2016).

In gastric adenocarcinoma patients, cells expressing higher levels of OTUB1 are correlated with adverse clinicopathological features and lower survival rates. *In vitro* experiments further suggest that OTUB1 promotes tumour progression by enhancing tumour cell migration and invasion (Weng *et al.*, 2016). In addition, research by Zhou *et al.* (2014) demonstrate that OTUB1 promotes the migration, invasion, and metastasis of colorectal cancer cells *in vitro* and *in vivo,* and could potentially be developed as a metastasis marker and prognostic factor in colorectal cancer. This study is also supported by Liu *et al.* (2014).

It is also reported that OTUB1 expression is elevated in hepatocellular carcinoma and plays an important role in tumour recurrence, tumour cell migration and invasion (Ni

et al., 2017). Research done by Iglesias-Gato *et al.* (2015) shows that OTUB1 protein is highly expressed in prostate cancer and induces cell invasion through a RhoA-mediated mechanism.

2.7 OTUB1 as p53 regulator

p53 is a tumour suppressor that plays a crucial role in maintaining genome stability and preventing organisms from getting cancer (Oren, 2003). The main function of p53 is to act as a transcription factor to activate or repress many genes, including genes that are involved in the three major DNA damage responses which are DNA damage repair, cell cycle arrest and apoptosis (Vogelstein *et al.*, 2000). Loss of p53 function through genetic mutations or alterations in the p53 regulatory networks is observed in most human cancers (Soussi *et al.*, 2000).

While a majority of the studies point to a tumorigenic role for OTUB1, research published by Sun *et al.,* (2012) indicates that OTUB1 promotes apoptosis in osteosarcoma and lung non-small cell carcinoma cell lines in a p53-dependent manner. They discovered that OTUB1 is directly bound and weakly deubiquitinates p53, which may further enhance OTUB1-mediated p53 stabilization. Apart from that, OTUB1 also inhibits MDM2-mediated ubiquitination of p53 in a non-canonical manner to promote its stabilization. In this case, OTUB1 blocks MDM2-medited p53 ubiquitination by binding with UbcH5, an E2 conjugating enzyme for MDM2. The inhibition of UbcH5 prevents ubiquitin conjugating step in ubiquitination of p53 and thus, rescues p53 from proteasomal degradation.

Generally, MDM2 is an oncoprotein that functions to maintain a low p53 expression level in human cells. It does so by binding with the p53 N-terminal transactivation domain, directly inhibiting its transcriptional activity (Chen *et al.*, 1993).

Besides direct inhibition, MDM2 also regulates p53 levels by promoting ubiquitination on p53 that leads to its degradation through the proteasome system (Haupt *et al.*, 1997; Kubbutat *et al*., 1997). Using these two mechanisms, MDM2 efficiently suppresses cell growth arrest and apoptosis mediated by p53. However, some DUBs such as OTUB1 can counteract the ubiquitination process and rescue the p53 from proteasomal degradation and thus, promote apoptosis in cancer cells (Hock *et al.*, 2011; Yuan *et al.*, 2011).

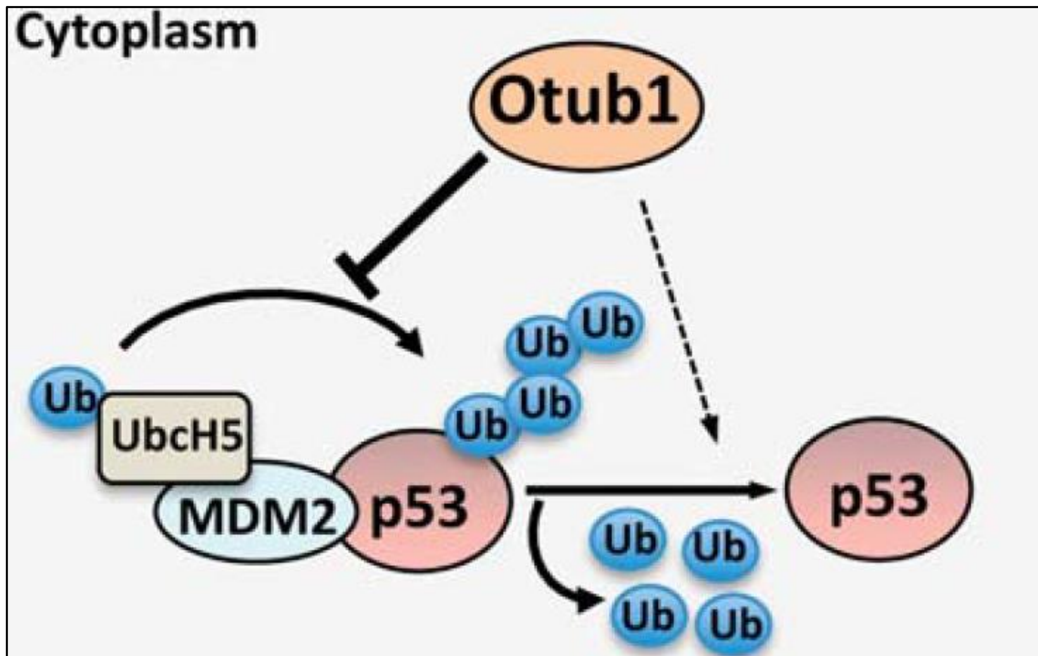

Figure 2.4 A schematic diagram for OTUB1 regulation of p53 ubiquitination. Otub1 suppresses p53 ubiquitination in the cytoplasm primarily by non-canonical inhibition of UbcH5/MDM2 activity, while it may also possess weak canonical DUB catalytic activity to deubiquitinate p53. Arrow indicates activation, whereas bar indicates inhibition (Sun *et al*., 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

MCF7 and MCF10A were purchased from American Type Culture Collection (ATCC), USA. Dulbecco's Modified Eagle Medium (DMEM), DMEM/Nutrient Mixture F-12 (DMEM/F12), FBS, penicillin-streptomycin, horse serum, recombinant human epidermal growth factor (hEGF), insulin and trypsin express were from Gibco, USA. Hydrocortisone and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich, USA. All plasticware were from Greiner Bio-One, USA.

All primers and oligos were commercially synthesized by IDT, USA. TRIzol reagent was from Life Technologies, USA. Agarose, DNA 1kb ladder and loading dye were purchased from Thermo Fisher Scientific, USA. Ethidium bromide and TAE buffer were obtained from Sigma Aldrich, USA. QuantiNova Reverse Transcription kit and QuantiNova SYBR Green PCR kit were from Qiagen, USA. Tissue culture flasks and dishes were from JET Biofil, China. Other plasticware were from Greiner Bio-One, USA.

3.2 Methods

3.2.1 Cell Culture

A proper aseptic area, technique and sterile handling were required for cell culture work. Biosafety cabinet was sanitized using 70% alcohol as well as other materials and equipment such as media bottles, pipette and tips boxes. Autoclavable materials were autoclaved at 121 °C for 30 minutes at 100 kPa prior to use.

3.2.1.1 Maintaining Cells

MCF7 human breast cancer cell lines and MCF10A human breast epithelial cell lines were chosen for this study to represent cancer cells (MCF7) and normal cells (MCF10A). MCF7 was grown in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Meanwhile, MCF10A were maintained in DMEM/F12 in suspension and supplemented with 5% (v/v) horse serum, 20 ng/ml of hEGF, 0.5 µg/ml of hydrocortisone, 10 µg/ml of insulin and 1% (v/v) penicillin-streptomycin.

3.2.1.2 Thawing of Cells

The cryopreserved MCF7 and MCF10A in liquid nitrogen tank were taken out and immediately submersed in a water bath at 37°C until cells become semi fluid. The cells were transferred into 5 ml of pre-warmed complete DMEM or DMEM/F12 in 15 ml tube and were mixed gently. The cells suspension was centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended with pre-warmed complete media. The cell suspension was transferred into 25 cm^2 tissue culture flask and incubated at 37°C in 5% CO_2. Cells were routinely checked under inverted microscope to observe the confluency of cells growth. The cells were subcultured when they reached 70 to 80% confluency.

3.2.1.3 Sub-culturing of Cells

Media was removed from the flasks and MCF7 and MCF10A cells were detached from the flask by incubating the cells in 3 ml of trypsin express for 5 to 10 minutes in 5% CO_2 air at 37 °C. Then, the flask was gently tapped to detach the cells from the flask's wall. 5 ml complete medium was added to the trypsinized cells. The cells suspension was transferred into the 15 ml tube and centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded, and the pellet was resuspended with complete media. The cells were

transferred into a new 25 cm^2 tissue culture flask and incubated in humidified incubator (Thermo Fisher Scientific, USA) at 37 °C in 5% CO_2.

3.2.1.4 Freezing Cells

Cryopreservation was done to preserve MCF7 and MCF10A stocks. Cells were trysinized, detached, collected and pelleted by centrifugation at 2000 rpm for 5 minutes. Then, the cell pellet was resuspended in 1 ml fresh growth media. DMSO was added to the cell suspension at a final concentration of 10 % to prevent formation of ice crystals and fragmentation of the cell membrane. Finally, the cell stock was transferred into cryovials and stored at -80°C.

3.2.2 Molecular Biology

3.2.2.1 RNA Extraction

MCF7 and MCF10A were grown in a 3.5 cm culture dish (10 cm^2) until reached 80% of confluency. Total RNA was isolated from cells using TRIzol reagent according to manufacturer's protocol. In brief, growth media were aspirated and replaced with ice-cold PBS to wash cells. 1 ml TRIzol reagent was added directly to the cells to lyse the cells and was pipetted up and down several times to homogenize the cells lysate. The cell lysate was transferred to 1.5 ml Eppendorf tube and incubated at room temperature for 5 minutes. Then, 0.2 ml of chloroform was added, and the tube was shaken vigorously before being centrifuged at 12 000 × g for 15 minutes at 4 °C to extract RNA from the upper translucent aqueous layer. The aqueous layer was transferred to fresh tube and 0.5 ml isopropyl alcohol was added to precipitate RNA. The mixture was vortexed and incubated at room temperature for 10 minutes. Then it was centrifuged at 12 000 × g for 10 minutes at 4 °C. The precipitated RNA was pelleted, washed with 1 ml of 75 % ethanol

and centrifuged again at 7500 × g for 5 minutes at 4 °C. The supernatant was removed completely, and the pellet was left to air dry until it became translucent. RNA pellet was then resuspended in RNase-free water and incubated in heat block at 57 °C for 13 minutes. Then, the tubes were placed on ice and the RNA concentration were measured using Nanodrop 2000c (Thermo Fisher Scientific, USA).

3.2.2.2 Checking RNA Integrity

RNA integrity of the extracted RNA was checked by using gel electrophoresis. 1.0 % (w/v) agarose gels were prepared by adding 0.5 g of agarose into 50 ml 1x TAE buffer and boiled in a microwave until completely dissolved. Gel mixture was left to cool before adding 2.5 μl of liquid ethidium bromide. Gel mixtures was poured into the casting tray and allowed to solidify for 1-2 hours at room temperature. After that, the gel is submerged in 1x TAE buffer in an electrophoresis tank. 2 μl of RNA suspension were mixed with 0.5 μl loading dye before being loaded into the wells. The electrophoresis was performed at 90 V for 45 minutes before visualising RNA bands using UV Transilluminator and Infinity Image Software (Vilber Lourmat, France).

3.2.2.3 cDNA synthesis

Reverse transcription of RNA was performed on Thermal Cycler (Bio-Rad, USA) using QuantiNova Reverse Transcription kit. The synthesis of cDNA was done using the RNA template extracted in 3.2.2.1. The process of cDNA synthesis comprises of two main steps; genomic DNA (gDNA) elimination and reverse transcription reaction. The gDNA reaction mixture was prepared as in Table 3.1 in Eppendorf tube, incubated at 45 °C for two minutes and placed on ice immediately. Then, reverse transcriptase and reverse transcription was added to the mixture for the second reaction (reverse transcription reaction). The details for the second reaction are shown in Table 3.2. The mixture was

then incubated to anneal at 25 °C for three minutes, at 45 °C for 20 minutes for reverse transcription and inactivation of reaction at 85 °C for five minutes.

Table 3.1 Composition of genomic DNA elimination.

Components	**Volume**
gDNA Removal Mix	2 µl
RNA Template	Variable
RNase-free water	Variable
Total reaction volume	15 µl

Table 3.2 Composition of reverse transcription reaction.

Components	**Volume**
Reverse Transcriptase	1 µl
Reverse Transcription Mix	4 µl
RNA Template	14 µl
Total reaction volume	20 µl

3.2.2.4 Verification of cDNA synthesis

Verification of cDNA synthesis was achieved by amplification of house-keeping gene, β-actin. β-actin forward (5’ AGAGCTACGAGCTGCCTGAC 3’) and reverse (5’ AGCACTGTGTTGGCGTACAG 3’) primers were commercially synthesized. The reaction mixture is as shown in Table 3.3 and PCR reaction was set up as in Table 3.4. The PCR product was then subjected to electrophoresis. Agarose gel was prepared as mentioned in 3.2.2.2. Then, 10 µl of RNA suspension with 2.5 µl loading dye was loaded into each well. 5 µl of 1kb plus ladder were loaded into the first lane to serve as a marker to determine the size of the PCR product. Electrophoresis was performed at 100 V for 45 minutes before visualizing RNA bands using UV Transilluminator and Infinity Image Software (Vilber Lourmat, France).

Table 3.3 Compositions of PCR reaction.

Components	**Volume**
cDNA template	1 µl
Primer (β-actin)	0.5 µl
Taq 2x Master Mix	12.5 µl
RNase-Free Water	11 µl
Total reaction volume	25 µl

Table 3.4 Reaction steps in PCR.

Step	**Time**	**Temperature**	**Cycle**
Initial	30 seconds	95 °C	1
Denaturation	30 seconds	95 °C	
Annealing	60 seconds	55 °C	30
Extension	2 minutes	68 °C	
Final Extension	5 minutes	68 °C	1