THE EFFECT OF OTUB1 OVEREXPRESSION ON HUMAN CERVICAL CANCER HeLa

By

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Dissertation Submitted in Partial Fulfillment Of The Requirement For The Degree Of Master Of Science

UNIVERSITI SAINS MALAYSIA

2015

ACKNOWLEDGEMENT

Alhamdulillah, praise to the Almighty Allah for the successful completion of this dissertation.

I would like to express my appreciation to my supervisor Dr. Nurulisa Zulkifle for introducing me to the methodology of work, and for her passion, support and guidance.

I would like to thank to my co-supervisor Dr. Nor Hazwani Ahmad for the guidance and encouragement in carrying out this project work.

I also wish to express my gratitude to my fellow friends in M.Sc Medical Research (2014/15) and all teaching staff as well as non-teaching staff in IPPT who rendered their help during the period of my project work. This thesis is dedicated to my husband and child who have always stood by me and give me continuous support and strength.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree celcius
µl	Microliter
ANOVA	Analysis of variance
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfide
DNA	Deoxyribonucleic acid
DUBs	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin-protein ligases
FBS	Fetal Bovine Serum
g	Gram
GFP	Green Fluorescent Protein
HCL	Hydrogen chloride
HeLa	Human cervical adenocarcinoma cell line
kPA	Kilopascal
ml	Milliliter

mM	Milimolar
mm	Milimeter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
nm	Nanometer
OTUB1	Ovarian tumour domain-containing Ub aldehyde-binding protein 1
PBS	Phosphate buffer saline
RT-PCR	Real-time polymerase chain reaction
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SPSS	Statistical Package for the Social Sciences
USA	United States of America
USP	Ub-specific protease
UPS	Ubiquitin–proteasome system

KAJIAN KESAN EKSPRESI BERLEBIHAN OTUB1 PADA BARAH SERVIKAL MANUSIA HeLa

ABSTRAK

Protein ubiquitinasi adalah proses regulasi yang mengawal kebanyakan mekanisma fisiologi dan patologi dan yang melibatkan perkembangan tumor. Enzim deubiquitinasi (DUBs) semakin diterima bahawa ia bermutasi pada kanser manusia dan mempunyai peranan sebagai onkogen dan penahan tumor. Kajian terdahulu mendapati DUBs mengawal proses yang berkaitan dengan proliferasi sel dan apoptosis, menjadikan ia sasaran untuk terapi kanser. Dalam kajian ini, kami ingin mengkaji sama ada tumor ovari protease (OTU) iaitu dalam keluarga DUBs mempengaruhi sel proliferasi sel HeLa. Kami melakukan kajian kesan ekspresi berlebihan pada kanser servikal HeLa dan sel L929. Tranfeksi dilakukan menggunakan Lipofectamine™3000 pada kedua-dua sel. Kecekapan transfeksi diukur menggunakan mikroskop fluorescence. Ujian MTT dijalankan untuk melihat proliferasi sel HeLa dan L929. Analisis statistic dijalankan menggunakan ujian ANOVA satu hala. OTUB1 secara signifikan membantut pertumbuhan sel HeLa pada semua masa inkubasi jika dibandingkan dengan sel kawalan. Kesimpulannya, ekspresi berlebihan OTUB1 membantut proliferasi sel HeLa.

THE EFFECT OF OTUB1 OVEREXPRESSION ON HUMAN CERVICAL CANCER HeLa

ABSTRACT

Protein ubiquitination is a highly regulated process that controls multiple physiologically and pathologically relevant mechanisms involved in tumor development. There is growing recognition that mutated deubiquitinating enzyme (DUBs) in human cancers suggesting their roles as oncogenes and tumor suppressors. Previous studies have identified the DUBs regulate processes associated with cell proliferation and apoptosis, and as such represent candidate targets for cancer therapeutics. Here we investigated whether members of the ovarian tumor proteases (OTU) family of DUBs influence the proliferation in HeLa cells. We intended to study the effect of OTUB1 overexpression in cervical cancer HeLa and L929 cells lines. Transfection with Lipofectamine™ 3000 was performed in both cell lines. The transfection efficiency was measured by fluorescence microscopy. MTT assay was performed to evaluate cell proliferation on HeLa and L929 cells. Statistical analysis was performed by using one way ANOVA test. OTUB1 has significantly inhibit the proliferation of Hela cells at all incubation times in comparison to control. In conclusion, overexpression of OTUB1 inhibit the cell proliferation of HeLa.

CHAPTER 1

INTRODUCTION

1.1 Research background

Cervical cancer is a cancer in tissues of the cervix. It is usually a slow growing cancer that may not have symptoms but can be found with regular pap tests. Most cervical cancer cases (83%) occur in developing countries in which they account for 15% of female cancers, compared to 3.6% in developed regions (Gakidou *et al.,* 2008). Cervical cancer is the third most frequent cancer in women after breast and colorectal cancers and is one of the leading causes of cancer death among women in the world (Ferlay *et al*., 2010). According to Globocan statistics (2012) in Malaysia, about 2,145 new cervical cancer cases are diagnosed annually. Cervical cancer ranks as the second cause of female cancer in Malaysia and the second most common female cancer in women aged 15 to 44 years in Malaysia. Compared among the major races, Chinese women had the highest incidence for cervical cancer followed by the Indians and Malays (National Cancer Registry, 2006). Therefore, there is critical need for better targeted therapies for cervical cancer.

OTUB1 is a member of OTU family which are part of deubiquitinating enzymes (DUBs) family. It is establish for its deubiquitinating properties (Messick *et al*., 2008). They play an important role in many physiological and pathological process such as interferon signaling (Huang *et al*., 1995 ;Sass *et al.,* 1995 ; Li *et al.,* 2014). DUBs has been reported regarding an association with cancer as it found mutated in human cancer

suggesting their roles as oncogenes and tumor suppressor genes. DUBs also found to play crucial aspect in regulating cell proliferation (Hussain *et al*., 2009).

There are approximately 100 identified OTU family members of proteins from eukaryotes, viruses and pathogenic bacteria (Balakirev *et al.*, 2003). It is include OTUB1, OTUB2, YOD1 and OTULIN. Previous study has shown that OTUB1 mediates certain types of cancer such as prostate cancer cell invasion through RhoA activation and promotes tumorigenesis in vivo (Iglesias-Gato *et al.*, 2015). OTUB1 also interact with ERα which is key factor involved in the development of breast and endometrial cancers in cells and in vitro (Stanišić *et al.,* 2009). Previous literature shows that OTUB1 from ovarian tumor (OTU) family has been identified as a novel p53 regulator. There are an evidence show that overexpression of OTUB1 cause apoptosis and inhibition of cell proliferations in a p53 dependant manner (Sun *et al*., 2011).

As a result of these finding, OTU family members rise as a promising regulator in cancer associated pathway, human DUBs are increasingly regarded as a potential drug target including cancer and neurodegeneration disease (Pfoh *et al*., 2015). However, most of the research activities in OTU family are still limited. It could be interesting to see overexpression of OTUB1 in cervical cancer using HeLa cell lines as the role of OTUB1 in this disease has still not been fully elucidated.

1.2 Objective of the study

The aim of present study is to study overexpression of OTUB1 could significantly enhance or inhibit proliferation of cancer cells *in vitro*. The specific study were :

1) To transfect and express OTUB1 clones in human cancer cell lines HeLa and mouse fibroblast L929.

2) To assess the effect on proliferation of human cancer cell lines upon overexpression of OTUB1 protein by MTT assay.

1.3 Hypothesis of the study

The expected outcomes of the study were :

1) Overexpression of OTUB1 could significantly promote proliferation of cancer cells in vitro or inhibit proliferation of cancer cells in vitro.

CHAPTER II

LITERATURE REVIEW

2.1 Cancer Statistic

An estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. In year 2012, there were 32.6 million people (over the age of 15 years) alive who had a cancer diagnosed in the previous five years (World Health Organization, 2013).

The most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) (World Health Organization, 2013).

Worldwide, cervical cancer is second only to breast cancer as the most common female malignancy in both incidence and mortality, and results in approximately 275000 deaths annually (Parkin *et al*., 2005). Some 83% of the cases occur in developing countries, where cervical cancer accounts for 15% of female cancers, with a risk before age 65 of 1.5%, while in developed countries it accounts for only 3.6% of new cancers, with a cumulative risk (ages 0–64) of 0.8% (Ferlay *et al.,* 2004).

2.2 Protein Ubiquitination

Protein ubiquitination is a highly regulated process that controls multiple physiologically and pathologically relevant mechanisms involved in tumor development. The degree of ubiquitination of specific proteins is controlled by the concerted actions of E3 ubiquitin ligases, deubiquitinating enzymes (DUBs) and the proteasome (Deshaies RJ & Joazeiro CA., 2009; Komander D *et al*., 2009).

The attachment of ubiquitin to target proteins is mediated by an enzymatic cascade consisting of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-protein ligases) proteins (Fang & Weissman 2004). The existence of a large number (N500) of E3 ligases makes them the main specificity factor in the UPS. Target proteins may be monoubiquitinated or, as in this example, polyubiquitinated. A target protein must be tagged with at least four ubiquitin monomers (forming a polyubiquitin chain) to be recognized by the proteasome. DUBs, are components of the UPS that catalyze the removal of ubiquitin moieties from target proteins or polyubiquitin chains, resulting in altered signaling or changes in protein stability (D'Arcy *et al.,* 2015).

Human genome encodes approximately 95 putative DUBs, grouped into five families: Ub-specific protease (USP), Ub C-terminal hydrolase (UCH), ovarian tumour (OTU) domain-containing protease, Machado–Joseph disease (MJD) protease, and JAB1/MPN/Mov34 metalloenzyme (JAMM) (Nijman *et al*., 2005).

The ubiquitin–proteasome system (UPS) has many critical regulatory roles in eukaryotic cellular processes including cell cycle progression, stress response, signal transduction, transcriptional activation, and DNA repair (Ciechanover *et al*., 2000; Ciechanover 2006).

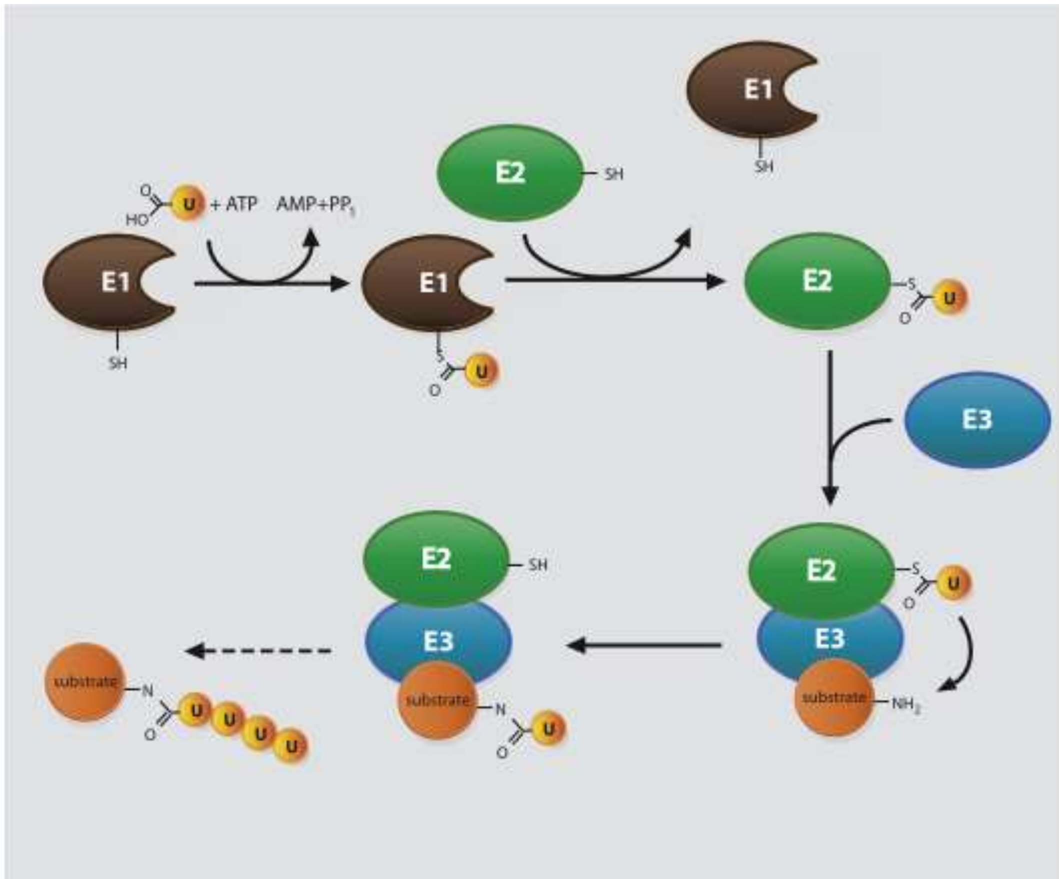


Figure 2.1: Ubiquitination of proteins. Proteins are targeted for degradation by the addition of ubiquitin chains to lysine residues by a process that involves three enzymes, E1,E2 and E3. DUBs catalyze the removal of ubiquitin moieties from target proteins or polyubiquitin chains, resulting in altered signaling or changes in protein stability. (D'Arcy *et al.,* 2015).

2.3 DUB and cancer pathway

Oncogenes and tumor suppressors act at various points along the signal transduction pathway between the plasma membrane and the nucleus affecting mitogenic processes in ways that either enhance or slow cell growth. Given the pervasive role of ubiquitin DUBs and cancer mediated signaling or targeted proteolysis in these pathways, it is not surprising to find that DUBs play critical roles in regulating cell proliferation (Hussain *et al.,* 2009).

Ubiquitination of oncoproteins and tumour suppressors can promote their destabilization by targeting them for degradation (e.g., K48-linked poly-ubiquitination specifies proteasomal degradation), or regulate their activity (activation or inactivation). Activation here may refer to a variety of processes like translocation to the nucleus (e.g PTEN and FOXO), or engagement in signalling protein interaction networks (TRAF6, RIP1). Specific DUBs implicated in tumourigenesis. Previous study by Iglesias-Gato *et al*., 2015 demonstrated that OTUB1 is overexpressed in prostate cancer suggests a role for OTUB1 in tumorigenesis and invites additional exploration of its mechanisms of action.

The ubiquitin proteasome pathway is intricately involved in nearly all aspects of cell biology. Ubiquitination is covalent attachment of the small protein modifier ubiquitin to a substrate protein is involved in virtually all cellular processes by mediating the regulated degradation of proteins. Deubiquitination is have been shown to play a role in the cleavage of ubiquitin from translational precursors and in the maintenance of free ubiquitin levels within the cell by the deubiquitinating enzymes (or DUBs). However, DUBs can also remove both monoubiquitin and polyubiquitin chains from proteins, or can trim the distal

ubiquitin from polyubiquitin chains. Consequently, these activities can potentially antagonize the functions of ubiquitination within the cell (Komander et al, 2009). Deubiquitination plays an equally important regulatory role as well as ubiquitination. There is a growing list of human cancers in which direct mutational alterations in DUBs has been observed. Much more work is required in order to fully appreciate the role of deubiquitination in malignancy (Hussain *et al.,* 2009).

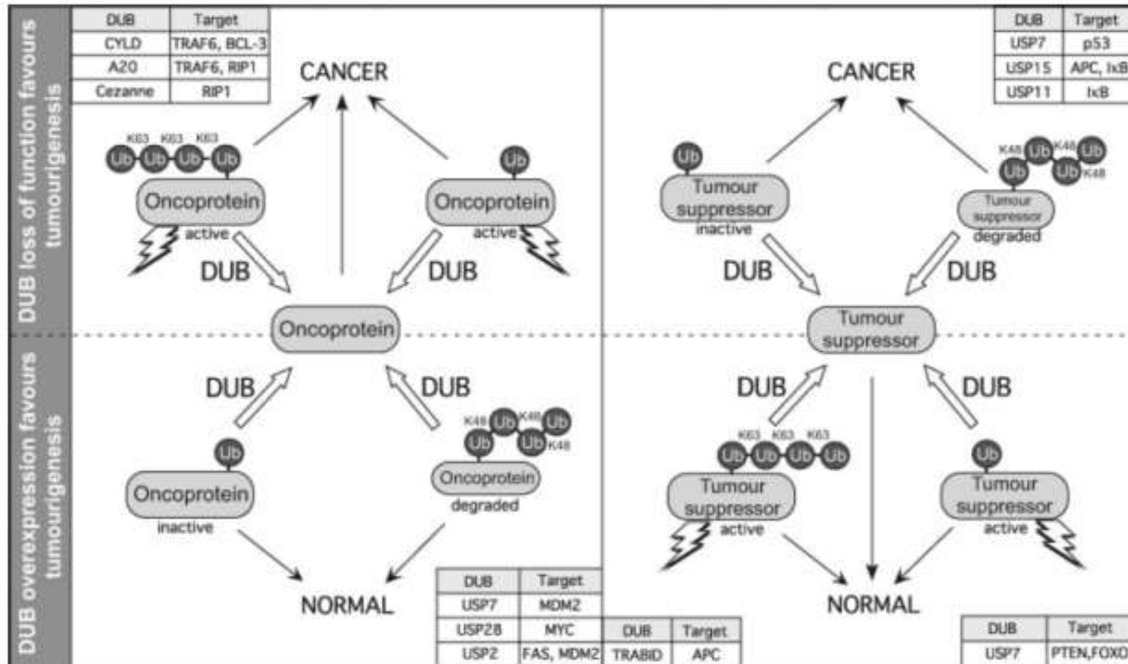


Figure 2.2. Deubiquitinases are important regulators of oncogenes and tumour suppressors. Both overexpression and loss of function of DUBs can promote cancer (Sacco *et al*., 2010).

2.4 OTUB1

Ovarian-tumor-domain-containing proteases (OTUs) are part of the deubiquitinating enzymes (DUBs) family (Makarova *et al*., 2000 ; Edelmann M, 2009). One of the most recently recognized DUBs is the OTUs. This family mainly comprises a group of putative cysteine proteases including OTUB1, OTUB2, A20 and yeast OTU1 (Edelmann M, 2009).

OTUB1 was the first member of OTU family to be confirmed for its deubiquitinating properties. It is located at chromosomal position 11q13.1, and is ubiquitously expressed in human tissues such as in kidney tissue (Messick *et al.,* 2008 ; Zhang *et al.*, 2012).

OTUB1 is thought to play an important role in many physiological and pathological processes of human being. The OTUB1 gene product is identified to be involved in the control of cell division and differentiation of the cystoblast into an oocyte and nurse cells (Huang YZ et al. 1995; Sass *et al.,* 1995).

Although widely expressed, OTUB1 was specifically implicated in mediating lymphocyte antigen responsiveness through affecting the stability of the lymphocyte-specific E3 ligase GRAIL (gene related to anergy in lymphocytes) in CD4+ T-lymphocytes (Soares L *et al.,* 2004). Moreover, OTUB1 was also found in Lewy bodies of the brain on mass spectrometry, and may be involved in the pathogenesis of neurodegenerative disorders (Xia Q *et al.,* 2008).

OUT DUB name	Mutation/translocation	Published abnormalities/protein level	Oncomine	
			Upregulation	**Downregulation**
OTUB1	None reported	None reported	Bladder/lung,prostate, HNSCC, breast	Brain, HNSCC, testis, cervical, sarcoma
A20	Chromosomal deletions and inactivating mutations found in several lymphoma subtype. 2 out 11 lung cancer	Study showing overexpression in Hodgkins and anaplastic B-cell lymphomas with downregulation in other lymphoma types	HNSCC, luekemia, lung, brain, cervical.	Bladder, ovary, lung, lymphoma, sarcoma
Cezanne	None reported	None reported	Liver, myeloma	Ovarian
TRABID	1/202 kidney cancer	None reported	Brain, testiscular, leukemia Oesophagus, liver	Brain, leukemia, liver, testicular, bladder

Table 1.1: Example of OTU DUBs including OTUB1 that have mutation and altered expression in cancer cell lines, listed from COSMIC (Catalogue of Somatic Mutations in Cancer) and Oncomine database. (Sacco *et al.*, 2010)

2.5 Therapeutic application of ubiquitination

Key cancer-associated proteins whose levels are tightly controlled by the ubiquitin proteasome include p53, p27, cyclins and BCL2 family members. The enzymes involved in conjugation and deconjugation of ubiquitin to protein substrates include an activating ATP dependent ubiquitin enzyme (E1), an ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligases (E3s) that often form multi-component complexes key for substrate recognition, and deubiquitinases (DUBs) that cleave ubiquitin from protein substrates. In humans, there are just a few E1 enzymes, around 40 E2 enzymes, over 500 E3 ligases (most commonly RING and HECT domain E3s) and around 100 DUBs, the majority belonging to the ubiquitin-specific protease (USP) sub-family (Lipkowitz & Weissman 2011, Budhidarmo *et al*., 2012, Jacq *et al*., 2013).

These enzymes have major regulatory roles in normal cellular processes, both within and independently of the ubiquitin-proteasome, including DNA repair, maintaining genomic stability and transcription. Aberrant expression of a number of DUBs and E3s has been linked to cancer (Lipkowitz & Weissman 2011 ;Clague *et al.,* 2013). As a consequence, many of these enzymes are generating extensive interest as targets for the treatment of cancer (Marsh 2015).

Several DUBs have been implicated in various diseases, including neurological disorders, infectious diseases and cancer. A genome-wide RNAi (RNA interference) screen of the catalytically active human USPs in cancer-relevant cellular models and phenotypic assays was performed to identify potential USP targets in cancer (Colland, F. 2006).

2.6 Targeting p53 as anticancer therapy

p53 plays a critical role in tumor suppression mainly by inducing growth arrest, apoptosis, and senescence, as well as by blocking angiogenesis. In addition, p53 generally confers the cancer cell sensitivity to chemoradiation. Thus, p53 becomes the most appealing target for mechanism-driven anti-cancer drug discovery. The approaches currently undertaken to target p53 and its regulators with an overall goal either to activate p53 in cancer cells for killing or to inactivate p53 temporarily in normal cells for chemoradiation protection. The compounds that activate wild type (wt) p53 would have an application for the treat- ment of wt p53-containing human cancer. Likewise, the compounds that change p53 conformation frommutant to wt p53 (p53 reactivation) or that kill the cancer cells with mutant p53 using a synthetic lethal mechanismcan be used to selectively treat human cancer harboring a mutant p53. The inhibitors of wt p53 can be used on a temporary basis to reduce the normal cell toxicity derived from p53 activation. Thus, successful development of these three classes of p53 modulators, to be used alone or in combination with chemoradiation, will revolutionize current anticancer therapies and benefit cancer patients (Wang & Sun 2010).

2.7 Regulations of p53 by OTUB1

The ubiquitin (Ub) proteasome system plays a pivotal role in the regulation of p53 protein stability and activity. p53 is ubiquitinated and destabilized by MDM2 and several other Ub E3s, whereas it is deubiquitinated and stabilized by Ub-specific protease (USP)7 and USP10.

According to study by Sun *et al.*, (2011) OTUB1 is a novel p53 regulator. OTUB1 directly suppresses MDM2-mediated p53 ubiquitination in cells and *in vitro*. Overexpression of OTUB1 drastically stabilizes and activates p53, leading to apoptosis and marked inhibition of cell proliferation in a p53-dependent manner.

Under physiological conditions, p53 is maintained at low levels primarily by the oncoprotein MDM2. MDM2 also promotes p53 ubiquitination and degradation through the proteasome system (Haupt et al, 1997; Kubbutat et al, 1997). Together, their results suggest that OTUB1 have a novel function in regulating p53 stability and activity.

CHAPTER III

MATERIALS AND METHODS

3.1 Introduction

The major requirement is to maintain an aseptic work area that is restricted to cell culture work. The procedure must be performing in a designated cell culture including sterile handling, incubation, and storage of cell cultures, reagents, and media. The simplest and most economical way to provide aseptic conditions is to use a cell culture hood (biosafety cabinet). All material need in this study including glassware and plasticware were sterelize. Autoclavable materials were autoclaved at 121 °C for 30 minutes at the pressure of 100 kPA prior to use.

3.2 Preparation of Cells

3.2.1 Cell line

Two cell lines were chosen in this study which is HeLa cell lines and L929 mouse fibroblast. L929 mouse fibroblast was selected as it represent normal cell. Both cell lines were maintain in the same medium. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) medium was used to culture HeLa (American Type Culture Collection ATCC, USA) and L929 (Life technology, USA) in suspension and supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, USA), 1% (v/v) Penicillin-Streptomycin (Gibco, USA).

Centrifugation was done at 500 × g for 10 minutes after the collection of cells in log phase of growth.

3.2.2 Thawing of HeLa Cells

The cryopreserved HeLa and L929 cells were taken out from a liquid nitrogen tank and immediately soaked in a water bath at 37°C until cells become semi fluid. The cells were transferred into a 15ml tube (BD Biosciences, USA) containing 5 ml of prewarmed complete DMEM (Gibco, USA) and mixed gently. The cells suspension was centrifuged at 500 x g for 10 minutes. The supernatant of DMSO then discarded and the pellet was resuspended with pre-warmed complete DMEM growth medium. The cells suspension was transferred into 25 cm2 tissue culture flask. Then the cells suspension was incubated at 37°C in 5% CO2. Cells were routinely checked under inverted microscope to determine the confluence of cells growth. The cells were subculture when the cells reached 70 to 80% confluent of cell growth.

3.2.3 Subculturing of Cell lines

HeLa and L929 cells were detached from the flask by incubating 1 ml of trypsin express (Gibco, USA) for 5 to 10 minutes in 5% CO_2 air at 37 °C. Then the flask was gently tapped to detached the cells from the wall of flask. The floating cells were transferred into the 15 ml tube and centrifuged for 10 minutes at 500 × g. The supernatant was discarded and the pallet was resuspended with complete DMEM growth medium. The cells were transferred into flask and incubated in an incubator at 37 °C in 5% CO_2.

3.3 pEGFP-N1-OTUB1 Vector Information

pEGFP-N1 is used to insert genes of interest, OTUB1. It encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. pEGFP-N1 encodes the GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. The MCS in pEGFP-N1 is between the immediate early promoter of CMV (P CMV IE) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neor), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in E. coli. The pEGFP-N1 backbone also provides a pUC origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

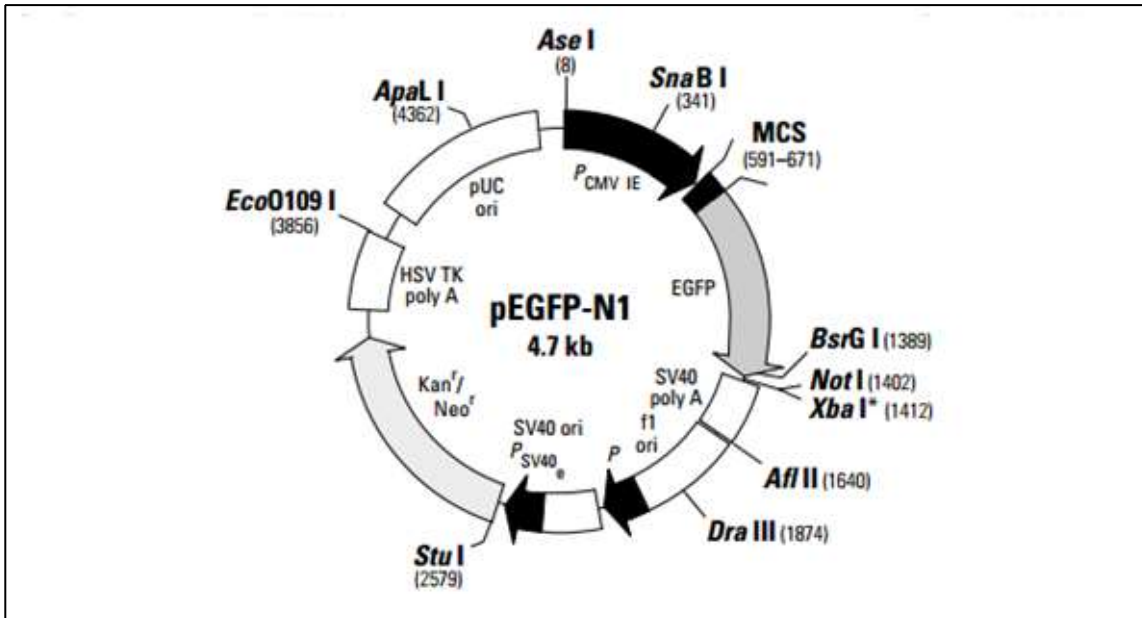


Figure 3.1 Vector information of pEGFP-N1-OTUB1

3.4 Transfection using Lipofectamine ™ 3000

Cells were transfected at high cell density. One day before transfection, Cells were plated 0.5-2 x 10x 5 cells in 500 µl of growth medium without antibiotics so that cells will be 90 to 95% confluent at the time of transfection for high efficiency, high expression levels, and to minimize cytotoxicity. Complexes were prepared using a DNA (µg) to Lipofectamine™ 3000 (µl). Opti-MEM medium were used to dilute Lipofectamine™ 3000 and DNA before complexing. Add diluted DNA to diluted Lipofectamine™ 3000 Reagent (1:1 ratio). DNA concentration should contain 0.1 ng/ml in each well. But DNA stock available For HeLa cells is 495 ng/ml. So each well of treated cell contain 0.2 µl of DNA. In L929 cells, 683.3 ng/ml were used and each well of treated cell were contain 0.15 µl of DNA. Incubate for 5 minutes at room temperature. After 5 minute incubation, combined diluted DNA with diluted Lipofectamine™ 3000. Mix gently and incubate at 37°C for 4 days and continue with viability assays.

3.5 Transfection efficiency analysis

Proliferation of HeLa cells and L929 cells in response to the OTUB1 overexpression were were identified by fluorescence microscopy for expression of the OTUB1. Transfections were identified by using Green Fluorescent Protein (GFP) as reporter gene.

3.4 Viability assays

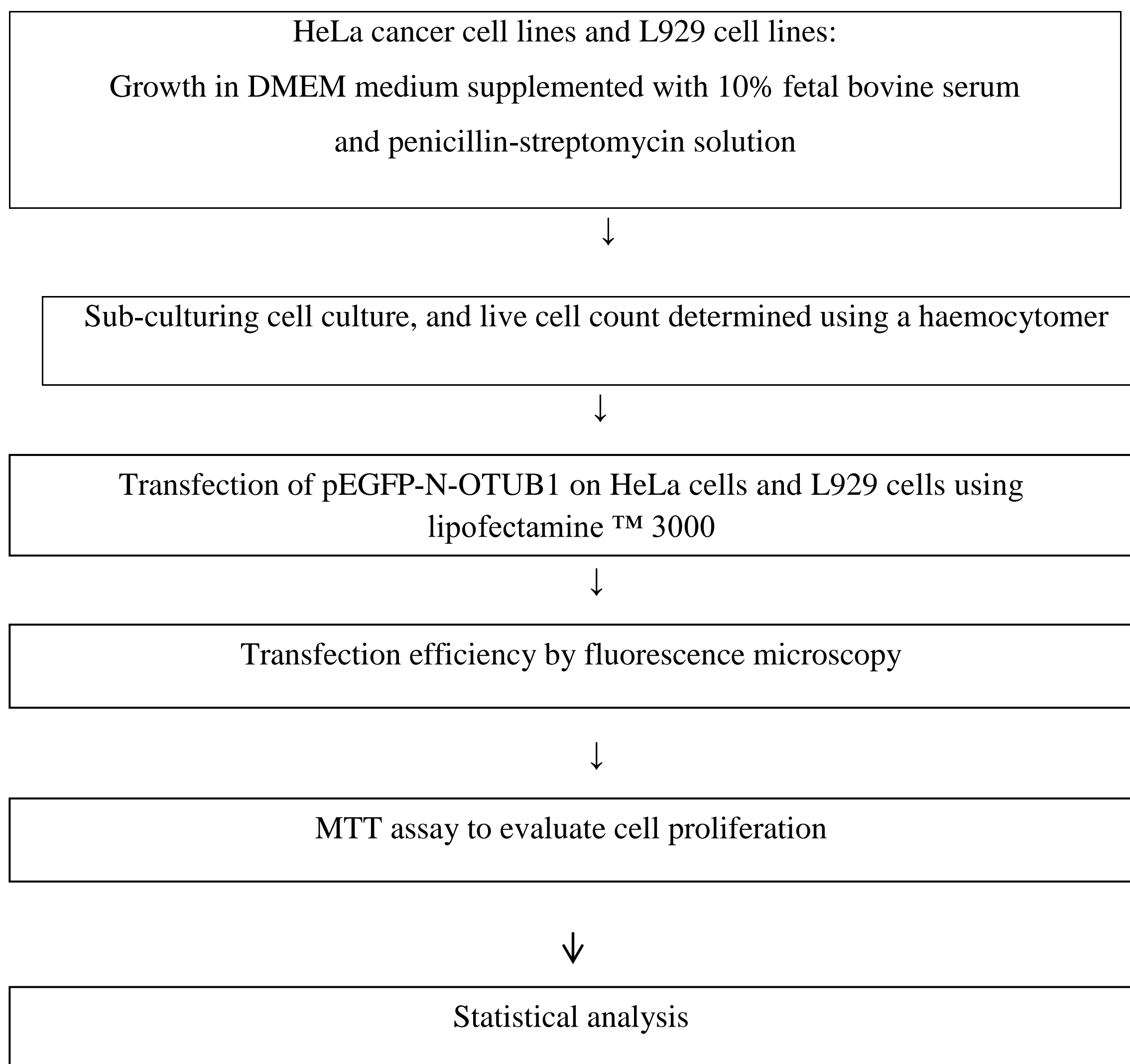
3.4.1 Reagent Preparation

Proliferation of HeLa cells and L929 cells in response to the OTUB1 overexpression was assessed by using MTT assays and the cell number determined by using standard microplate absorbance readers. The protocol of MTT assay was performed according to the method of developed by Mossman T (1983). 12 mM MTT stock solution were prepared by adding 1 mL of sterile PBS to one 5 mg vial of MTT (Component A). The solution were mix by vortexing or sonication until dissolved. Some particulate material that will not dissolve is removed by filtration or centrifugation. Component B were prepared by add 10 mL of 0.01 M HCl to one tube containing 1 gm of SDS. The solution gently dissolves by inversion or sonication. Once prepared, the solution should be used promptly. Cell were treated with complex Opti-MEM medium, Lipofectamine ™ 2000, Lipofectamine ® 2000 Reagent. Cell were analysed in 0 hour, 12 hour, 24 hour and 48 hour. Cells seeded at densities between 5000 to 10,000 cells per well. The medium were removed and replace with 100 µL of fresh culture medium. 10 µL of the 12 mM MTT stock solution were added to each well. 10 µL of the MTT stock solution also were added to 100 µL of medium of negative control. Microplate were incubate for 4 hours. After that 100 µL of the SDS HCl solution were added to each well and mix thoroughly using the pipette. Microplate were incubate again at 37°C for 4 to 18 hours . Samples were mix again using a pipette and absorbance were read at 570 nm.

3.5 Statistical Analysis

The representative data were presented as mean ± SEM. Statistical analysis was performed using IBM SPSS Statistic Version 2.0. The comparison between control and treated was tested for significance using one way ANOVA. Differences at $p < 0.05$ were considered to be statistically significant.

3.6 Flow chart of study



CHAPTER IV

RESULT

4.1 Transfection of pEGFP-N-OTUB1

4.1.1 Transfection of pEGFP-N-OTUB1 on HeLa cells

HeLa cancer cell lines were transfected with plasmid containing pEGFP-N1-OTUB1 in 96 well plates at 70% confluent. Cells were transfected by vectors with Lipofectamine™ 3000 following the manufacturer's protocol. Transfection were performed using an appropriate concentration of DNA and 5 µL of Lipofectamine™ 3000 Reagent. Clones were identified by fluorescence microscopy for expression of the OTUB1. Transfections were identified by using Green Fluorescent Protein (GFP) as reporter gene.