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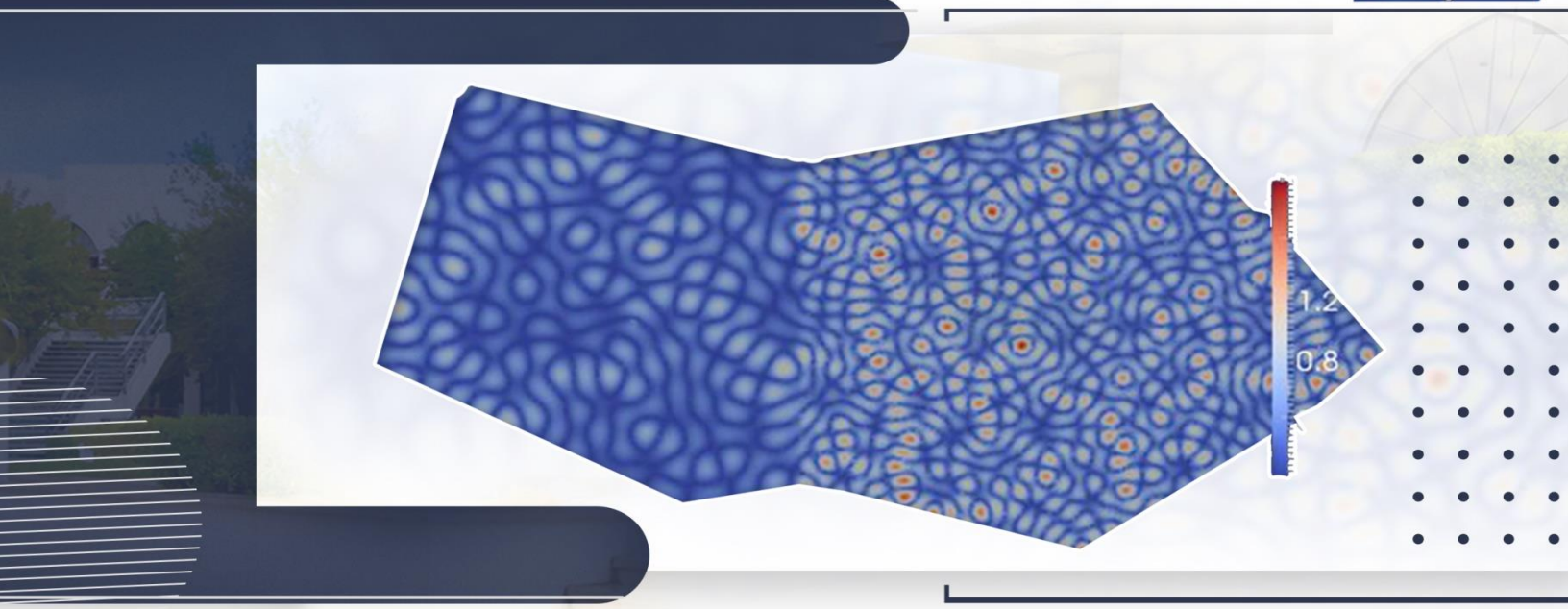

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Expression of Deubiquitinating Enzymes in Lung Cancer Cells with Different REST Status

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A B S T R A C T

The aims of this study were: (1) to screen a panel of lung cell lines for expression of the proteins UCHL1, USP49, USP15, USP4, and USP11. (2) To investigate the correlation of transcript and protein expression level of these DUBs. (3) To find any proteins that discriminate non-small cell lung cancer (NSCLC) from neuroendocrine (NE) SCLC or benign lung carcinoid. (4) To investigate any correlation of DUB proteins expression with lung cancer sub-types that has different amounts of REST protein.

Method: Western blots were performed to detect the expression of DUB expression (USP4, USP11, USP15, USP49, and UCHL1) and REST protein in extracts from normal lung cell lines, SCLC, carcinoid, and NSCLC

Results: we found that the transcript level was in general not a good indicator of DUB protein level. There was a significant positive correlation of REST with DUB proteins from whole-cell extracts for USP15, USP11, USP4, and USP49 in normal lung cell lines. The P values were 0.016, 0.002, 0 .018 and 0 .034 respectively. Also in both normal and NSCLC cell lines total USP15, USP11 and USP49 showed a positive correlation with REST. P values were 0.007, 0.029 and 0.001 respectively. However USP11 protein was highly abundant in REST-deficient SCLC. In contrast, both USP11 and USP15 showed a positive correlation with REST in SCLC and NSCLC nuclear extracts. P values were 0.001 and 0.005 respectively. A positive correlation of DUBs with REST would be consistent with their putative role in stabilizing REST.

1 Introduction

Ubiquitin (Ub) is a highly conserved protein composed of 76 amino acid and plays an important role in protein-degradation. Ubiquitin is essential in many cell-mechanisms and biological processes, including the cell cycle, growth control and prevention of neurodegeneration (Amerik & Hochstrasser, 2004). It is also involved in the organizing and stability of many proteins. Ubiquitin does not degrade proteins by itself, but it works just as a mark of proteins that will then be degraded by the 26S proteasome. The ubiquitin- protein conjugation requires three types of enzymes, ubiquitin activating enzymes (E1) which is activated the C-terminal glycine in ATP, then the activated ubiquitin transmitted to cysteine residue of ubiquitin conjugating enzymes (E2) and finally catalyzed by ubiquitin ligase (E3) enzymes to which the substrate protein is specifically bound (Haglund & Dikic, 2005).

Similar to phosphorylation, ubiquitination is a reversible process. Hydrolysis of ubiquitin or ubiquitin chains from proteins are catalyzed by deubiquitinating enzymes (DUBs) in cells (Komander, Clague, & Urbe, 2009).

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In mammals there are about one hundred DUBs, which can be divided into five sub-classes based on their ubiquitin-protease domains. These are ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs). DUBs can play an important role in regulating many tumour suppressors and oncoproteins (Sacco, 2010). There are a large number of DUBs which are linked to tumors such as USP4 which was identified as oncoprotein related to lung cancer (Gray *et al*., 1995).

Ubiquitin is becoming an interesting target to find biomarkers or drugs for a variety of diseases. These drugs might prevent protein-degradation or could activate the system to damage proteins which we do not need.

Lung cancer is the leading cause of cancer death between both women and men in the worldwide and has poor prognosis. It is divided in small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC accounts for approximately 20%, while NSCLC accounts for 75% of all lung cancers. Early detection and diagnosis are the most important factors in determining the outcome in lung cancer patients (Bührens, Amelung, Reymond, & Beshay, 2009). Both SCLC and lung Carcinioid express neuroendocrine markers, whereas SCLC is the most aggressive neuroendocrine lung tumors, Carcinioid lung cancer is a benign cancer (Reports-Mortality., February 2007). Although NSCLC don't have neuroendocrine gene, they express repressor element 1-silencing transcription factor (REST) at different levels (Coulson, Ocejo-Garcia, & Woll, 2003).

The repressor element-1 silencing transcription factor, which is also known as neuron restrictive silencer factor (REST/NRSF) is a transcriptional suppression factor that acts as repressor. REST plays an essential role in many biological processes in human medulloblastomas REST is overexpressed (Coulson, 2005). REST is an interactor of the F-box protein β-TrCP and is degraded by the ubiquitin ligase SCF β-TrCP through the G2 phase of the cell cycle to allow transcriptional derepression of Mad (Guardavaccaro, 2008; Westbrook, Martin, & Schlabach, 2005). It shows interaction with a number of proteins, many of which are required for its repressor function, including histone deacetylases HDAC1 and HDAC2 There is a splice variant of REST that has associated with SCLC (Coulson & *et al*, 2003).

We, therefore, thought there might be a DUB that can revere SCFβTRCP mediated REST ubiquitation and this may be changed REST-deficient in neuroendocrine lung cancer. Specific DUBs may have a role in the REST - stabilization. The specificity of DUB in stabilization of proteins dysregulated in cancer could make them potential drug targets (Daviet & *et al*., 2008). Therefore, we here will focus on five DUBs. The ubiquitin Carboxyl-terminal Hydrolase-L1 gene (UCHL1) and the ubiquitin-specific protease USP49 and USP15 are three candidates that arose from library a siRNA screen for DUBs that may reverse REST ubiquitination. USP4 and USP11 DUBs are closely related to USP15 (Westbrook & *et a*l., 2008).

The aims of this project were (1) to screen a panel of lung cell lines for expression of the proteins UCHL1, USP49, USP15, USP4 and USP11. (2) To investigate the correlation of transcript and protein expression level of these DUBs. (3) To find any proteins that discriminate non-small cell lung cancer (NSCLC) from neuroendocrine (NE) SCLC or benign lung carcinoid by compare the results which is might be identified a potential protein biomarkers. (4) To investigate any correlation of DUB protein expression with lung cancer sub-types that has different amounts of REST protein.

2 Materials and Methods

Cell Culture. In this study, four types of human lung cell lines have been used; Normal cell lung lines were normal human bronchial epithelium (NHBE), normal lung fibroblasts (MRC5), SV40 transformed human bronchial epithelium (BEAS2B and SV40-HBE), SV40 transformed Normal lung fibroblasts (MRC5VA) and SV40 transformed embryonic lung fibroblast (WI38-VA13). Small cell lung cancer (SCLC) cell lines Lu-165, GLC19, NCI-H69,

NCI-H345, COR-L88, COR-L47 and U2020. A lung carcinoid line NCI-H727. Finally non-small lung cancers (NSCLC): NCI-H460, NCI-H322, NCI-H647, NCI-H2170, COR-L23 and A549. All cells were maintained in RPMI culture medium with 10 % BCS and were incubated in 5 % CO2 at 37°C.

Protein extraction: whole cell extracts were prepared by rinsing cells in PBS before they were lysed in 1x laemmli buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol and made up to 50 ml) and heated at 1100C for 10 minutes with vortexing every 2 minutes. Then they were diluted 1:5 in laemmli buffer. Nuclear and cytoplasmic protein fractions were extracted by scraping cells in 5 ml of ice cold phosphate- buffered saline (PBS) and collection by centrifuging the pellet was resuspended in 500 µl NP40 (150mM NaCl, 1% NP-40, 50mM Tris pH7.5, 1.5mM EDTA ,2 ug/ml Aprotonin, 2µg/ml Leupeptin, 1mM PMSF, H20). Samples were incubated on ice for 10 min with vortexing. To pellet nuclei samples were centrifuged at 16,000 xg for 5 min and the supernatant collected as the cytoplasmic fraction. To obtain the nuclear extract the cell pellet was resuspended in 40 µl Dignam buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M Nacl, 1.5 mM EDTA and protease inhibitors 0.5 mM DTT) and incubated in a cold room for 40 mins with vortexing at 2000 rpm. Supernatant was collected as the nuclear fraction. All protein extracts were quantified by BCA assay.

Western Blotting: Western blots were performed to detect the expression of seven proteins expression (USP4, USP11, USP15, USP49, UCHL1, and REST) in extracts from normal lung cell lines, SCLC, carcinoid and NSCLC. 10 µg of whole cells protein extract, 5 µg of cytoplasmic protein fraction or 2.5 µg of nuclear protein fraction was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using either 8 % or 10% polyacrylamide gel depending on molecular weight of protein of interest. Gels were run for 1 hour at 200 V. Perfect protein and Rainbow molecular weight markers were loaded in first and last lane of each gel for size standardization. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) for 60 min at 21-22 V in transfer buffer (14.4 gm Glysine, 3.03 gm Tris , 200 ml methanol and mad up to 1000 ml). Membranes were cut to three pieces at 75 kDa and 150 kDa. The first piece from 20-75 kDa was blotted with anti actin and tubulin. The second piece from 75-150 kDa was blotted with anti-DUBs (USP4, USP15 and USP11). The third piece from 150-225 kDa was blotted with anti- REST. Nitrocellulose membranes were blocked with 5% nonfat dried milk (NFDM) in TBST (TBS 0.1% Tween 20) for all proteins except REST, which was blocked with 3% NFDM in phosphate- buffered saline (PBS) to block nonspecific sites. Then blots were incubated with primary antibody: mouse monoclonal anti Beta-Actin (1:10000, Abcam), mouse monoclonal anti-Tubulin (1:10000) were from Sigma. Rabbit anti-USP11 (1:1000), rabbit anti-USP15 (1:1000) and rabbit anti-USP4 (1:2000) were from Bethyl. Polyclonal rabbit anti-REST (1:2000) was from Millipore, and polyclonal goat anti-USP49 (1/200). Polyclonal rabbit anti-UCHL1 (1:1000) was from Abcam. The USP4, USP11, USP15, USP49 and UCHL1 primary antibodies were diluted in TBST with 5% NFDM and incubated overnight at 40C. While the REST antibody was diluted in TBS with 3 % NFDM and incubated overnight at 40C. We used anti-mouse with actin and tubulin as secondary antibodies. Anti-rabbit for all DUBs and REST protein. Proteins were quantified using Odyssey® Infrared Imaging. Then all proteins normalized to actin.

3 Results

3.1 Expression of DUBs.

DUB expression was tested by immunoblotting in lung cancer and then, correlate with mRNA, which we measured previously in the first rotation by qRT-PCR to investigate any correlation between transcript-protein levels. To test whether there was any correlation between DUB protein and transcript levels, DUB proteins in whole lung cancer cell lines and normal lung cell lines was examined using immunoblot analysis to detect one protein within a mixture of others. In this study, whole cell protein extracts were used from twenty different lung cell lines: six normal cell lines (three epithelial and three fibroblast cell lines), seven SCLC, six NSCLC and one lung carcinoid cell line. We investigated expression for five DUBs USP15, USP11, USP4, USP49 and UCHL1 that may reverse REST ubiqutination. Replicate gels were run for several reasons. Firstly, USP15, USP11 and USP4 proteins migrate with similar molecular weights 100-120 kDa. Secondly, USP49 and UCHL1 required a different percentage of gel because they had small molecular weight. REST protein was detected from the same gel as USP11. REST protein was detected in both normal and NSCLC, while it was absent in all SCLC and carcinoid lung cell lines as shown in figure 1. USP11 and UCHL1 proteins were overexpressed in SCLC compare with normal and NSCLC lung cell lines. Also, there was a variety of USP4 and USP49 expression in different cell lung lines. USP15 protein was more in normal lung cell lines compare with lung cancer cell lines.

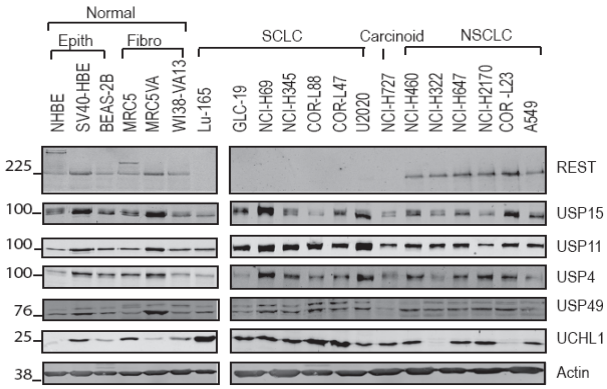

Figure (1). Immunoblot analysis of different DUB protein in whole cell protein lysate from 20 cell lung cell lines. Blots demonstrate REST, USP15, USP11, USP4, USP49 and UCHL1 protein in normal epithelial and fibroblast (Epith, Fibro); small cell lung (SCLC); carcinoid and non-small lung cell cancer (NSCLC). Actin protein was used as control to correct for protein loading. All proteins were quantified using Odyssey and normalized to actin then expressed to relative a sample of NCI- H647 which was analyzed on every gel.

Next, we quantified proteins using Odyssey and correlated to mRNA from previous project which measured by qRT-PCR. Overall, there was not a good correlation between DUB proteins and their transcript levels in the 17 lung cell lines (table1). The exceptions were UCHL1 protein, which has some evidence of correlation in normal and NSCLC lung cell lines, but it was lost in SCLC (figure 2). Interestingly, USP11iso 2 had some correlation in NSCLC. This may be the isoform which is transcript.

	Lung cell line sub-type			
DUB transcript	normal	SCLC	NSCLC	all
USP4 iso1	0.0771	0.4648 (-ve)	0.1762	0.059
USP11 iso1	0.035	0.048	0.0185	0.0003
USP11 iso2	0.0022	0.034	**0.3769 (+ve)**	0.0317
USP15 iso1	**0.9811 (+ ve)**	0.0026	0.1604	0.0105
USP15 iso2	0.5038 (-ve)	0.0031	0.0329	0.0213
UCHL1	**0.46 (+ve)**	0.0151	**0.2951 (+ve)**	0.0087

Table (1). Correlation of DUB protein-transcript levels. R^2 values are shown for lung cell lines estimate between USP4 iso1, USP11 iso1 and 2, USP15 iso 1 and 2, and UCHL1 transcript determined by qRT-PCR and whole cell lung protein extracts in 17 lung cell lines.

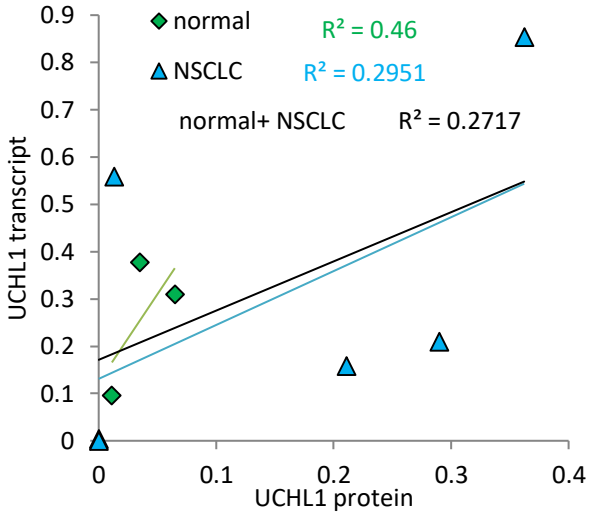

Figure (2). Example of transcript-protein levels correlation. UCHL1 Whole cell protein extracted from normal (n=3) and NSCLC (n=6) lung cell lines. UCHL1 transcript was quantified by qRT-PCR and UCHL1 protein was quantified by immunoblot. Both UCHL1 transcript and protein were normalized to Actin as the reference gene/ protein.

3.2 Determination of Correlation Induvial DUB Protein-REST Levels

Afterwards, we tested whether there was any correlation between total DUB protein and REST protein in a panel of lung cell lines. Looking for DUB levels may be related to REST. We found that there was a significant positive correlation for all DUBs except for UCHL1 (P= 0.31) in normal lung cell lines (table 2). However, this association was reduced in NSCLC for all DUBs except USP49 (P= 0.029) (figure 3).

Table (2). Correlation between DUB and REST protein levels. R^2 values are shown the correlation of DUBs (USP4, USP11, USP15, USP49 and UCHL1) and REST protein in a panel of 20 lung cell lines.

DUB	Lung cell line sub-type				
	Normal	SCLC	NSCLC	all	SCLC + NSCLC
USP4	**0.7871 (+ve)**	N/D	**0.0823 (+ve)**	0.063	0.097
USP11	**0.9345 (+ve)**	N/D	0.0007	0.118	**0.394 (+ve)**
USP15	**0.8018 (+ve)**	N/D	**0.3132 (+ve)**	0.0265	**0.532 (+ve)**
USP49	**0.7157 (+ve)**	N/D	**0.7343 (+ ve)**	0.0002	**0.656 (+ve)**
UCHL1	**0.2525 (+ve)**	N/D	0.066 (–ve)	0.2122 (-ve)	0.022(-ve)

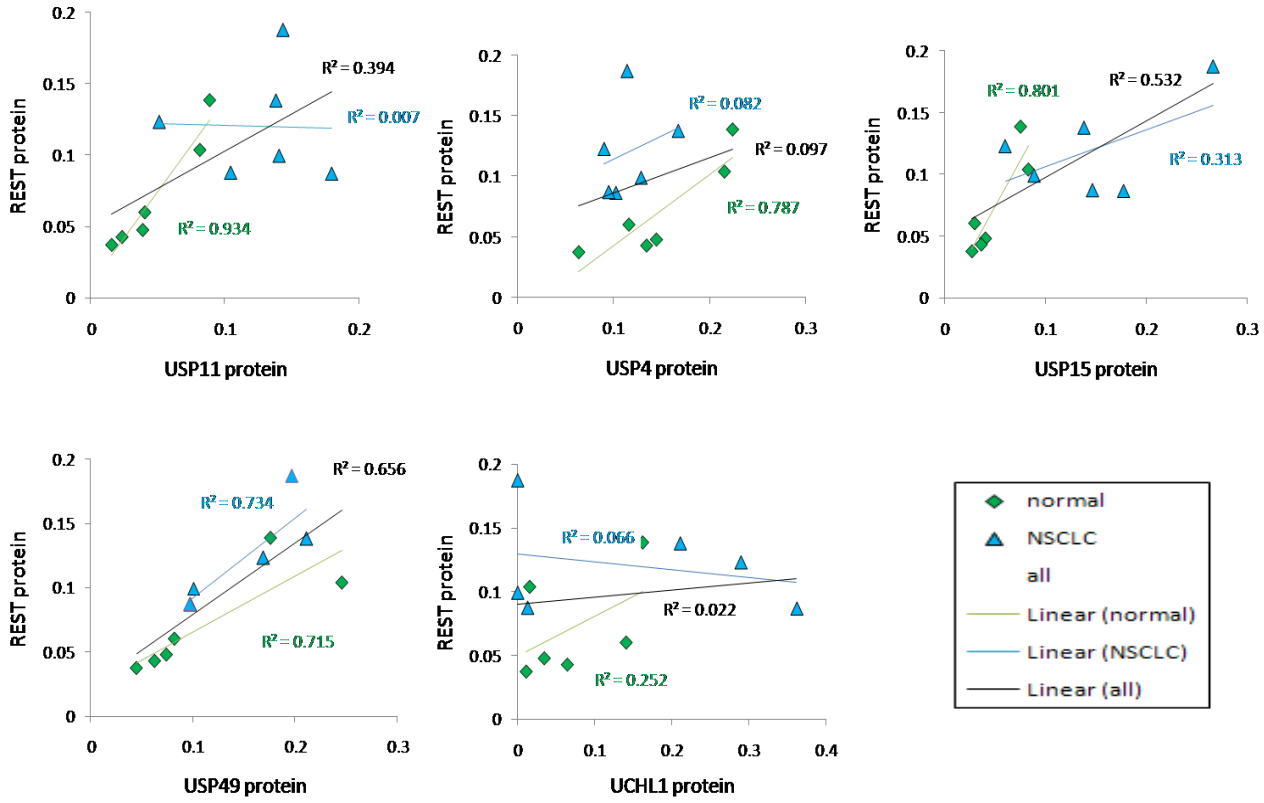

Figure (3). Correlation between DUB and REST protein levels. R^2 values are shown the correlation between whole cell DUB protein (USP11, USP4, USP15, USP49 and UCHL1) and REST protein. All DUB protein was normalized to actin then expressed to relative a sample of NCI- H647 which was analyzed on every gel.

3.3 Determination of DUB Distribution Between Nuclear and Cytoplasmic Protein Extracts from Lung Cell Lines

To determine whether these DUBs were predominately associated with cytoplasm or Nucleus in lung derived cell lines we prepared fractionated cell. It found some evidence all DUBs in the nuclear fraction except UCHL1 in all cell lines (figure 4). Immunobloting for REST was carried out twice, once when the fractions were fresh (a), and REST (b) was detected after freezing and thawing samples. There were some correlation between USP11 and REST in nuclear fraction therefore; we tested USP11 expression in normal lung cell lines in cytoplasmic and nuclear fractions (figure 4 B). Also, there was associated between USP11 and REST proteins in nuclear fraction.

3.4 Correlation of DUB and REST Protein in Fractionated Cells

Tested the correlation between DUBs (USP11, USP15 and USP49) and REST protein in cytoplasmic and nuclear fractions was performed. It had found that, a positive correlation of USP11 (P= 0.001) and USP15 (P= 0.005) with REST protein in SCLC and NSCLC nuclear extracts rather than in cytoplasm fraction. Also, there was positive correlation in SCLC with USP49 protein as shown in (table 3).

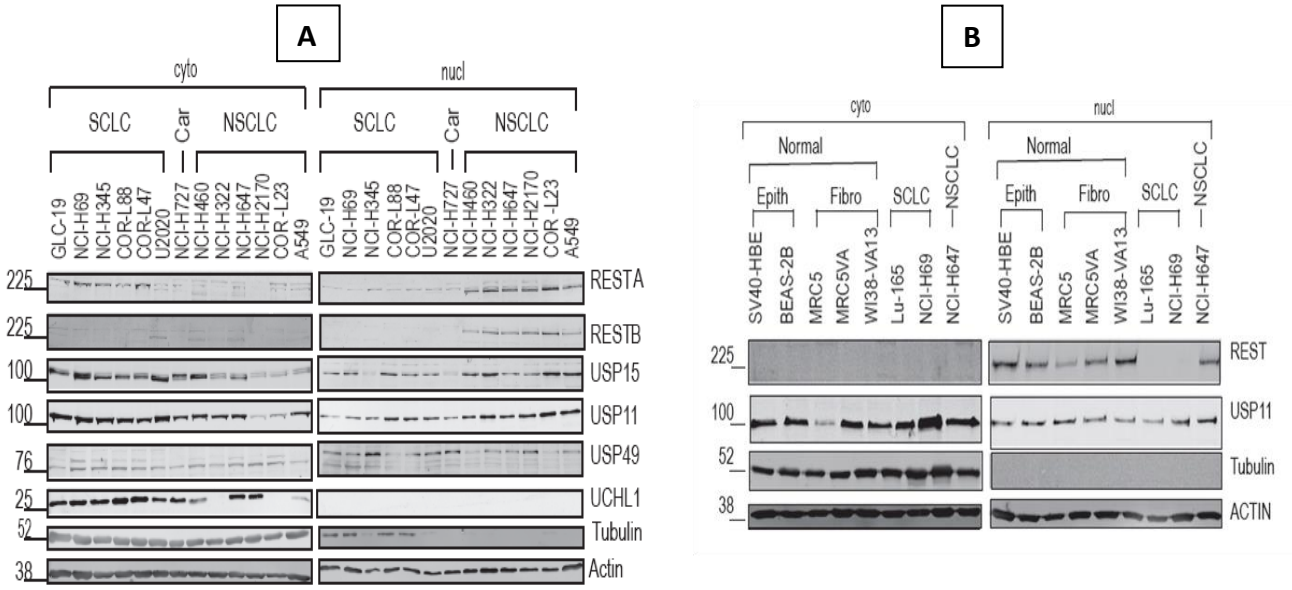

Figure (4). Enrichment of DUB proteins in cytoplasmic or nuclear fractions of cells. All DUB proteins (USP15, USP11, USP49 and UCHL1) and REST protein were tested in cytoplasmic (cyto) and nuclear (nucl) fractions of; A) SCLC, NSCLC and carcinoid (Car) lung cell lines. B) In normal lung epithelia (Epith) and fibroblast (Fibro) lung cell lines. Blotted with actin for normalization and tubulin to check fractionation**.**

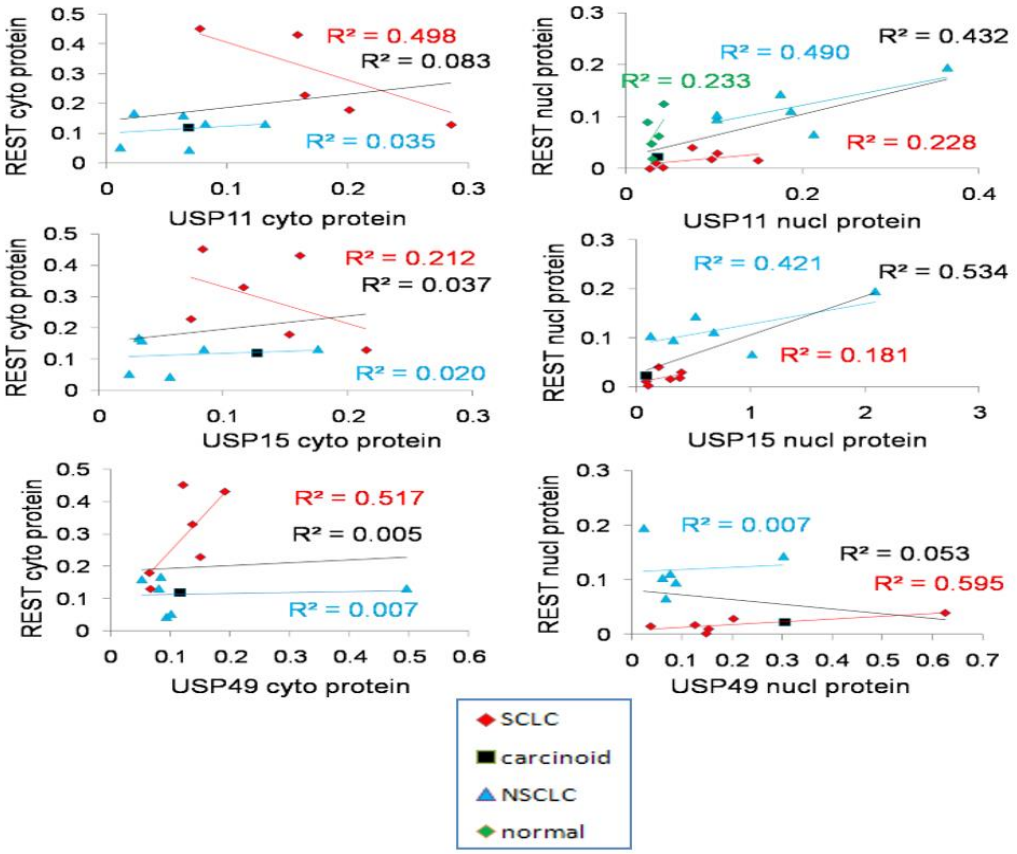

Figure (5). The correlation between DUB and REST proteins in cytoplasmic (cyto) and nuclear (nucl) fractions. All DUB proteins were normalized to Actin and then expressed relative to NCI- H647 on the same gel.

Table (3). The correlation between DUB and REST proteins in cytoplasmic (cyto) and nuclear (nucl) fractions. R^2 values of the correlation of REST- DUB proteins.

Sub-type (CYTO)	Normal	SCLC	NSCLC	all	SCLC+ NSCLC
USP4	not done	not done	not done	not done	not done
USP11	Not detected	0.4989 (-ve)	0.0354	0.0832	0.0832
USP15	Not done	0.2123 (-ve)	0.209	0.0379	0.049
USP49	Not done	**0.5175 (+ve)**	0.0076	0.0058	0.004
UCHL1	Not done	0.1417	0.0008	0.3277	0.359
Sub-type (NUCL)	Normal	SCLC	NSCLC	all	SCLC+ NSCLC
USP4	not done	not done	not done	not done	not done
USP11	0.233	**0.228 (+ve)**	0.4908 (+ve)	**0.4326 (+ve)**	**0.6732 (+ve)**
USP15	not done	**0.1811 (+ve)**	0.4212 (+ve)	**0.534 (+ve)**	0.0514
USP49	not done	**0.5958 (+ve)**	0.007	0.007	0.007
UCHL1	not done	not detected	not detected	not detected	not detected

4 Discussion

Studies of protein expression in human cancers have led to identification of many polypeptides as markers, or as useful tool for diagnostic. In this study, the expression of USP15, USP11, USP4 and UCHL1 proteins in a panel of 17 lung cell lines was measured. We compared data with the transcript of these DUBs, which we had measured previously. We found that there was not a good correlation between the difference DUB isoforms transcript and protein levels. There were some evidences of correlation between USP11iso 2 transcript and USP11 protein. This suggests that it could be most USP11, which was expressed is USP11 iso2, but we cannot predict the level. Our data suggests that estimation of transcript is not a good indication of DUB protein level. There was agreement of our data with research done recently by Shebl and *et al* (Shebl *et al.*, 2010) on cytokines in HPV which shows that, the correlation between gene and protein levels were variable among different cytokines. Whereas, our data were in disagreement with study by Celis and Coworkers (Celis *et al*., 2000) microarray study in bladder cancer, they found a good correlation between protein and transcript levels.

This may result of translation- modification or could result of protein -ubiquitination which could influence the stability of protein. A significant correlation was reported between whole cell DUB proteins) (USP15, USP11, USP4 and USP49) and REST protein in normal lung cell lines and in cancer cell lines (NSCLC and carcinoid) except with UCHL1 protein. This may due to UCHL1 and REST proteins are little in the same comportment because when we test UCHL1 in nuclear and cytoplasm fraction we found that, UCHL1 protein was manly in cytoplasm while REST protein was manly in nuclear. USP11 has an important role in regulation and stabilizing the HPV-16E7 by reducing ubiqutination (Lin, 2008)and it also controls an IkB (Yamaguchi, 2007). It has been identified of USP15 in regulation of E6 protein (Vos, 2009) and it is also implicated in stabilization of APC (Huang, 2009).

When we compare the correlation between whole cell, cytoplasm and nuclear protein, it was obvious that USP11 (P= 0.001) and USP15 (P= 0.005) showed a significant positive correlation with REST in SCLC and NSCLC nuclear fraction. The significant correlation of these DUBs with REST may be consistent with their putative role in stabilizing REST. The specificity of DUB in stabilization of proteins dysregulated in cancer could make them potential drug targets.

5 Conclusions

In summary the transcript level was in general not a good indicator of DUB protein level. There was a significant positive correlation of REST with DUB proteins from whole cell extracts for USP15, USP11, USP4 and USP49 in normal lung cell lines. Also in both normal and NSCLC cell lines total USP15, USP11 and USP49 showed positive correlation with REST. However, USP11 protein was highly abundant in REST -deficient SCLC. In contrast, both USP11 and USP15 showed a positive correlation with REST in SCLC and NSCLC nuclear extracts. A positive correlation of DUBs with REST would be consistent with their putative role in stabilizing REST.

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Conflict of Interest: The author declare that there are no conflicts of interest.

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