Gene Expression Analysis of Selected Deubiquitinating Enzymes in Lung Cancer

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تحليل التعبير الجيني لأنزيمات إزالة اليوبيكويتين المختارة في سرطان الرئة

المستخلص:

الغرض من الدراسة: الهدف من هذه الدراسة تصميم بادئات تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي (qRT–PCR) الخاصة بنظائر USP4 و USP15 و USP11 وUCHL1 . وكذلك فحص التعبير الجيني لنظائر DUB في الرئة الطبيعية، وسرطان الرئة ذو الخلايا الصغيرة (SCLC)، وسرطان الرئة ذو الخلايا غير الصغيرة (NSCLC) وخطوط الخلايا المشتقة من سرطان الرئة. بالإضافة إلى ذلك، دراسة العلاقة بين هذه DUBs والأنواع الفرعية لسرطان الرئة التي لها تعبير بروتينREST مختلف.

الطريقة: تم تصميم أزواج البرايمرز لـ QRT–PCR وفحصها باستخدام BLASTA و Netprimer حيث تم استخراج الحمض النووي الريبي RNA من 17 خطًا من خلايا الرئة، 7 خلايا SCLC، و 6 خلايا NSCLC ، و3 رئات طبيعية وخط خلية سرطانية واحدة. بعد التحقق من صحة البرايمرز و RNA عبر سرطان الرئة، تم فحص خطوط الخلايا الطبيعية والسرطانية بواسطة qRT–PCR.

النتائج: كانت بادئات QRT–PCR ذات علاقة نوعيه و خاصة بـ DUB و نظائرها المختارة (USP4 iso1 و 2 و USP15 iso1و2 و USP11 iso1 و UCHL1) حيث تم التعبير عن جميع DUBs على نطاق واسع في خطوط الخلايا المشتقة من الرئة، باستثناء USP4 iso ومن المثير للاهتمام ، أن مستوى نسخ USP15 iso1 و2 في كل من خطوط الخلايا الطبيعية وخطوط خلايا سرطان الرئة كانت متشابهة جدًا. ومع ذلك، في هذه الدراسة، حددنا أيضًا نسخة من USP11 (iso 2) والتي تم التعبير عنها أعلى في معظم خطوط الخلايا من التسلسل المرجعي USP11 (iso1). أخيرًا، وجدنا أنه لا يوجد ارتباط واضح بين مستويات التعبير عن هذه DUBs مع مستوى بروتين REST في سرطان الرئة.

الكلمات المفتاحية: *تفاعل البوليميراز المتسلسل الكمي، بروتين REST، SCLC، NSCLC، DUBs.*

Abstract:

Purpose: The aims of this study were to design quantitative real time polymerase chain reaction (qRT-PCR) primers specific for isoforms of USP4, USP15, USP11 and UCHL1 and to screen for gene expression of these DUB isoforms in normal lung, small cell lung cancer (SCLC), non- small cell lung cancer (NSCLC) and lung carcinoid derived cell lines. Additionally we would look into any correlation of these DUBs with lung cancer sub-types that have different REST expression.

Method: QRT-PCR primer pairs were designed and checked using BLASTA and Netprimer. RNA was extracted from 17 lung cell lines, 7 SCLC, 6 NSCLC, 3 normal lungs and 1 carcinoid cell line. Following primer and RNA validated across lung cancer, normal and carcinoid cell lines were screened by qRT-PCR.

Results: QRT-PCR primers were specific for related DUBs and their selected isoforms (USP4 iso1 and 2, USP15 iso1 and 2, USP11 iso1 and 2 and UCHL1). All DUBs were widely expressed in lung derived cell lines, with the exception of USP4 iso2. Interestingly, the level of USP15 isoform 1 and 2 transcripts in both normal cell lines and lung cancer cell lines were very similar. However, in this study we also identified a transcript of USP11 (iso 2) which was more high expressed in most cell lines than the reference sequence USP11 (iso1). Finally, we found that there was no clear correlation between expression levels of these DUBs with REST protein level in lung cancer.

Keywords: *QRT-PCR, SCLC ,NSCLC, DUBs, REST protein.*

Introduction

Ubiquitin is a highly conserved regulatory protein and it is one of the major post-translational modifications of proteins. The best known function of ubiquitin is in labeling proteins for proteasomal degradation. However, they also control the stability, function, and intracellular localization of a wide variety of proteins. The activation of ubiquitin is achieved by a series of enzymatic reactions that involve coordinated activities of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes (Hershko & Ciechanover, 1998; Pickart & Fushman, 2004). Whilst there is only a single E1 enzyme, several E2 enzymes are able to interact with a specific E3 partner and thus transfer ubiquitin to the target protein. In humans, there are about 600 E3 ubiquitin ligases that ensure the specificity of substrate selection. The range functions for ubiquitylation are partly achieved by the generation of ubiquitin chains assembled through isopeptide bond formation between the carboxy-terminal Gly and any one of seven internal Lysine residues of ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63) (Hicke et al., 2005).

However, like phosphorylation, ubiquitination is a reversibile modification. The human genome encodes about 79 active deubiquitinating enzymes (DUBs) that play several roles in the ubiquitin pathway (Komander et al., 2009). DUBs may activate ubiquitin proproteins, recycle ubiquitin and regenerate monoubiquitin from unanchored polyubiquitin (Haglund & Dikic, 2005). Importantly, DUBs can also reverse the ubiquitination of target proteins. They can be divided into: (1) ubiquitin C-terminal hydrolases (UCHs), (2) ubiquitin-specific proteases (USPs), (3) ovarian tumour proteases (OTUs), (4) Josephins and (5) JAB1/MPN/MOV34 metalloenzymes (JAMMs). The

UCH, USP, OTU and Josephin families are Cysteine proteases, whereas the JAMMs are zinc metalloproteases (Komander et al., 2009; Reyes-Turcu, et al., 2009). DUBs are emerging as crucial regulators of many tumour suppressors and oncoproteins (Sacco et al., 2010). They can influence the stability of cancer-associated proteins or negatively control ubiquitin mediated signaling. Both oncogenic and tumour suppressive function have been demonstrated for several DUBs. There are numerous examples of DUBs linked to cancer for example USP6 which was the first DUB identified as an oncogene (Onno et al., 1993) and USP4 which is oncoprotein linked to lung cancer (Gray et al., 1995).

Lung cancer is the most common cancer in the UK and the biggest cancer killer worldwide (Reports-Mortality, February 2007). There are two main types of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Whilst NSCLC is more common than SCLC accounting for 75 percent of all lung cancers, both types have a poor prognosis with 5 years survival of 7% - 9% (Reports-Mortality, February 2007). SCLC is characterized by a neuroendocrine phenotype and aggressive disease progression. In contrast, carcinioids of the lung also express neuroendocrine markers, but they are a rare type of relatively benign cancer (Coulson et al., 2003) SCLC have the most common form of neuroendocrine (NE) lung cancer and express slight REST. However, NSCLC do not usually have neuroendocrine gene expression and express REST although this present at variable levels (Coulson et al., 2003).

The repressor element 1–silencing transcription factor (REST) is a transcriptional repressor that restricts the expression of many neuronal genes through interaction with the neuron-restrictive silencer element (NRSE) at the promoter level (Ballas & Mandel, 2005; Chong et al., 1995). REST was originally identified as a crucial transcription factor that repressed expression of neuron-specific genes in non-neuronal cells (Coulson, 2005) and NRSEs were identified in 18 neuron-specific genes (Schoenherr & Anderson, 1995). However, genome-wide analyses have identified REST binding sites in several thousand gene control regions (Gopalakrishnan, 2009) and REST is now known to play essential roles in multiple biological processes and disease states (Westbrook et al., 2005) REST has been demonstrated to have both oncogenic and tumor-suppressor functions in different types of cancer. A splice variant of REST that absences the carboxy terminus has been linked with neuronal cancer and small-cell lung carcinomas which have very low expression of normal REST (Coulson et al., 2000). REST is a labile protein targeted for ubiquitin-dependent proteasomal degradation by interaction with the F-box protein β-SCF^{TrCP} through a phospho-degron in REST. REST is degraded by the E3 ubiquitin ligase SCF^{TrCP} through the G2 stage of the cell cycle to let transcriptional derepression of Mad2 (Guardavaccaro et al., 2008). We therefore reasoned that there may be a DUB that can revere $SCF^{\beta TrCP}$ meditated REST uibiquitintion and this may be altered REST-deficient NE lung cancer. Specific DUBs may have a key role in the stabilisation of REST. The specificity of DUBs and their participation in stabilisation of proteins dysregulated in cancer could make them a potential drug targets (Daviet & Colland, 2008). Here we will focus on four DUBs. The ubiquitin Carboxyl-terminal Hydrolase-L1 gene (UCHL1) and the ubiquitin-specific protease USP15 are two candidates that arose

from library a siRNA screen for DUBs that could increase the stability of REST (Westbrook et al., 2008). USP15 is closely related to two other DUBS: USP4, which has been implicated in lung cancer and USP11. Interestingly, in the screen USP4 did not appear to affect REST stability, whereas USP11 may destabilize REST.

The aims of this study, were three-fold: (i) to design and validate qRT-PCR primers for isoforms of the four candidate DUBs, (ii) to screen for gene expression of these DUB isoforms in normal lung, SCLC, NSCLC and carcinoid-derived cell lines, and (iii) to investigate any correlation of DUB expression with lung cancer sub-types that have different REST expression.

Materials and Methods

Cell culture: four types of human cell lines were used in this study. Group A are normal cell lung lines: normal human bronchial epithelium (NHBE), normal lung fibroblasts (MRC5) and SV40 transformed human bronchial epithelium (BEAS2B)). Group B are small cell lung cancer (SCLC) cell lines NCI-H69, NCI-H345, COR-L88, COR-L47, GLC19, U2020 and Lu-165. Group C are non-small lung cancers (NSCLC): NCI-H460, NCI-H2170, A549, NCI-H322, COR-L23 and NCI-H647. Finally group D was a lung carcinoid line NCI-H727. All cells were maintained in culture medium RPMI + 10 % BCS and were incubated in 5 % CO2 at 37°C. Harvest Cells were by pellet the appropriate number of cells by centrifuged for 5 min at 1500 rpm in a centrifuge tube and removed all supernatant by aspiration. Then the cells washed with PBS and centrifuging for 5 min at 1500 rpm in a centrifuge tube aspirate the PBS.

RNA extraction

Total RNA was extracted from approximately 5×10^6 cells using the RNeasy plus Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were disrupted by adding RLT buffer with β- mercaptoethanol and precipitated with 70% ethanol before applying to the spin column membranes. Columns were washed with RWI buffer and RPE buffer, the final elution of the total RNA was performed using 30 µl of RNase free water. Total RNA samples were stored at -80°C. The concentration of total RNA was estimated for each sample by using a NanoDrop (The NanoDrop® ND-1000 UV-Vis Spectrophotometer) to measure absorbance at 260 nm and 280 nm. The RNA integrity was confirmed by 1% agarose gel electrophoresis in 0.5× TBE and ethiduim bromide.

cDNA preparation

cDNA synthesis was carried out using the Reverse Transcription System (Promega) and RevertAid H minus M-MuLV Reverse Transciptase (MBI Fermentas). 1µl RNA and 1 µl of oligodT primer were incubated at 70°C for 5 min. Then a mix of 5x reverse transcription buffer (MBI), PCR nucleotide mix, RNasin (Promega) and nuclease free ddH_2O was added to RNA and incubated at 37°C for 5 min. Reverse a transcriptase was added (1.0µl M-MuLV RT) and incubated at 42°C for one hour, followed by 70 °C for 10 mins. Finally, the cDNA was diluted to 100 µl with RNAase free water. The cDNA was amplified using ACTB primers in end-point PCR, (as described below), and Then the products were analyzed by 2% agarose gel electrophoresis.

Primer design and end-point RT- PCR

Seven primer pairs were designed to amplify, USP4 isoform 1 and 2, USP11 isoform 1 and 2, USP15 isoform 1 and2 and UCHL1. Sequences used to design primers were

USP4 iso1 (NM_ 003363.3), USP11 iso1 (NM_004651.3), USP15 iso1 (NM_006313.1) and UCHL1 (NM_004181.4) USP4 iso2 (NM_199443.2), USP15 iso2 (EC gene alternative variant6), USP11 iso2 (Aceview alternative variant bApr07). cDNA or reverse transcriptase-negative control (RT-) were amplified with HotStar Taq using 28 cycles for ACTB and 40 cycles for other genes (95°C for 15min, then cycle of 94°C for 30 sec, 60°C for 30sec and 72°C for 30sec). 2% agarose gels stained with ethidium bromide was used to load PCR products, and visualized under ultraviolet light.

QRT—PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed in triplicate with 1 µl cDNA and 0.25µl each of the forward and reverse primer and IQ SYBR Green Supermix using an IQ5 real time PCR detection system (Bio-Rad). Samples underwent 40 cycles of amplification at 95°C (30 s) and 60°C (30 s). Melt curves analysis was conducted at the end of each run and the quantification cycle (Cq) values for test genes were normalized to the reference gene β-actin (ACTB) using the $2^{-\Delta qt}$ or $2^{-\Delta\Delta qt}$ equations.

Results:

Primer design and validation for deubiquitinating enzyme (DUB) targets.

QRT-PCR is one of the most powerful tools for studying gene expression, particularly as primers can be designed to specific exons or across exon boundaries to determine expression of specific splice variants encoding different isoforms. In this study, primers have been designed to amplify different isoforms (USP4 isoform 1 and 2, USP11 isoform 1 and 2, USP15 isoform 1 and 2, and UCHL1 isoform 1. During the design processes, primers were checked with the BLAST database to ensure that the primers are specific for the target we are interested in, that will be amplified in the RT- PCR reaction. Then all primers were tested on cDNA and RT- samples for one SCLC line (NIC-H69) and one NSCLC line (NCI-H460) (Figure 1). Primers were in each case specific for the cDNA over genomic DNA and amplified the correct sized products. RT-PCR products were successfully amplified in each case except for USP4 iso 2 which did not give any product (data not shown).

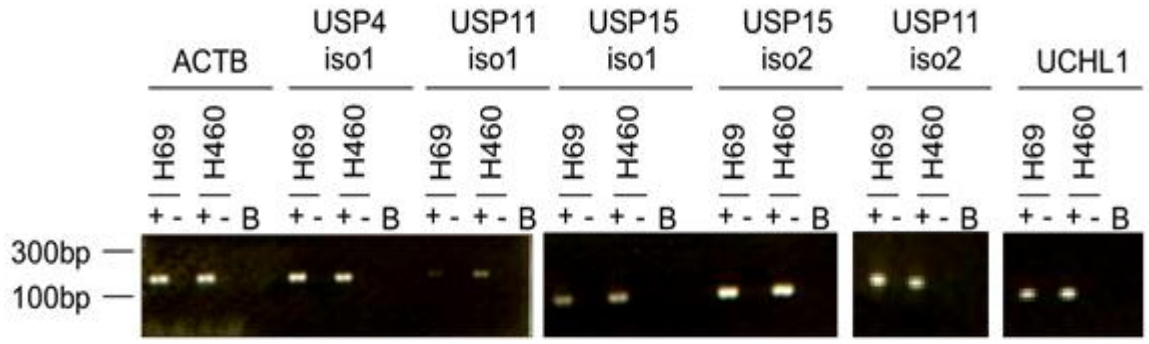

FIG.1. Standard end-point RT-PCR shows primers are specific for cDNA.
The primer pairs (ACTB, USP4iso1, USP11iso1, USP11iso2, USP15iso1, USP15iso2 and UCHL1) were tested with cDNA (+) and RT- (-) samples for two cell lines: NCI-H69 and NCI-H46 a no template control (Blank) (B) was included. 2% agarose gels stained with ethidium bromide was used to load PCR products, and visualized under ultraviolet light.

Next, it was tested whether the primers were selective between closely related DUBs by amplifying cloned cDNA from the plasmids pEGFP-USP15, pEGFP-USP4 and pEGFP-USP11 using the appropriate primers (USP15 iso1, USP4 iso1 and USP11 iso1 respectively) by qRT-PCR. As shown in Figure 2 the primers were selective for the intended DUBs. For example product from pEGFP-USP15 template was only amplified with the USP15 iso1 primer pair. Therefore we concluded that the primers were specific for DUBs.

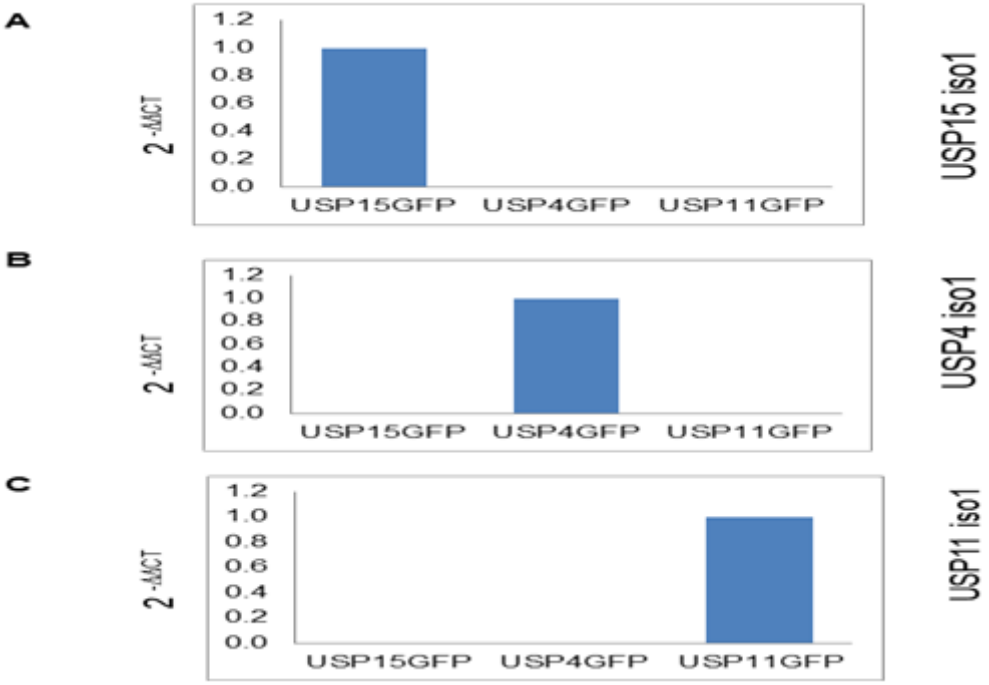

FIG.2. Specificity of RT-PCR primer pairs for DUB paralogs. The plasmids pEGFP-USP15, pEGFP-USP4 and pEGFP-USP11 were amplified by qRT-PCR with the USP15 iso1, USP4 iso1 and USP11 iso1 primer pairs. **A-** USP15 iso 1 primer pair. **B-** USP4 iso1 primer pair. **C-** USP11 iso1 primer pair. Expression data is shown for mean of three replicates normalized to the intended template gene.

Determination of relation Primer Efficiency for qRT-PCR

Estimation of relative transcript amounts using SYBR green by the $2^{-\Delta\Delta qt}$ method relies on the assumption that the amount of product doubles each cycle. The most common method for the calculation of the amplification efficiency of a qRT-PCR reaction requires preparation of a series of serial dilutions of the sample and creation of a standard curve, whereby efficiency is estimated from the slope of the standard curve. This method was used to test the efficiency of all primer pairs and an example of the standard curve derived for USP4 iso1 is shown in Figure 3. A high R^2 value of > 0.98 for qRT-PCR and

acceptable amplification efficiencies of 90-105 %, except for UCHL1, which had an amplification efficiency of 115.4%. One explanation for the high efficiency observed with the UCHL1 primers (Table 1) would be amplification of nonspecific products. SYBR green based qRT-PCR has been used and amplification of non-specific products will increase the signal product. To confirm that we were amplifying a single product, melt curves were analyzed at the end each qRT-PCR reaction. Representative examples for each primer pair are shown in Figure 4. In each case a single major peak was seen with little evidence of non-specific amplification and primer dimer.

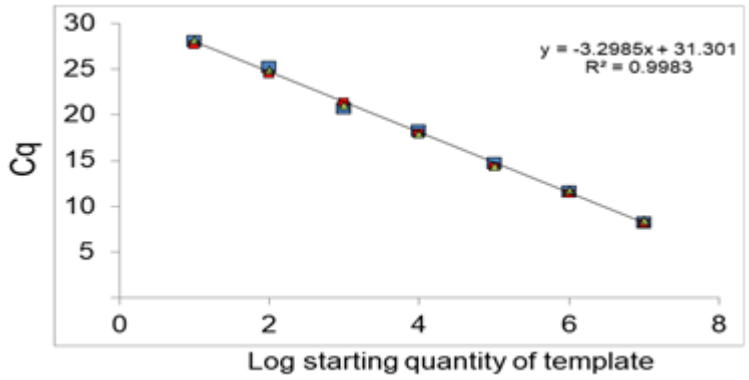

FIG.3. Standard curve with C_q for 3 independent replicates plotted against the log of the starting quantity of plasmid pEGP-USP4 for each dilution. A slope of -3.3 corresponds to template doubling in each cycle.

Table1. QRT-PCR primer efficiency. An R^2 value close to 1.00 indicates linearity across the template concentration range and efficiency of primers should be 100% (+ / - 10%) to use the $2^{-\Delta\Delta ct}$ method to most accurately estimate target abundance in cDNA samples.

Primers pair	Sequence ccession	Template	R^2	Efficiency
USP15 iso1	NM_006313.1	USP15 Plasmid	0.99	98.6 %
USP15 iso2	EC gene alternative variant6	GLC19 cDNA	1.00	104.4 %
USP4 iso1	NM- 003363.3	USP4 Plasmid	0.99	100.9 %
USP11 iso1	NM- 004651.3	USP11 Plasmid	0.98	91.8 %
USP11iso2	Aceview alternative variant bApr07	COR-L88 cDNA	1.00	102.5%
UHCL1	NM_004181.4	COR-L88 cDNA	0.98	115.4 %

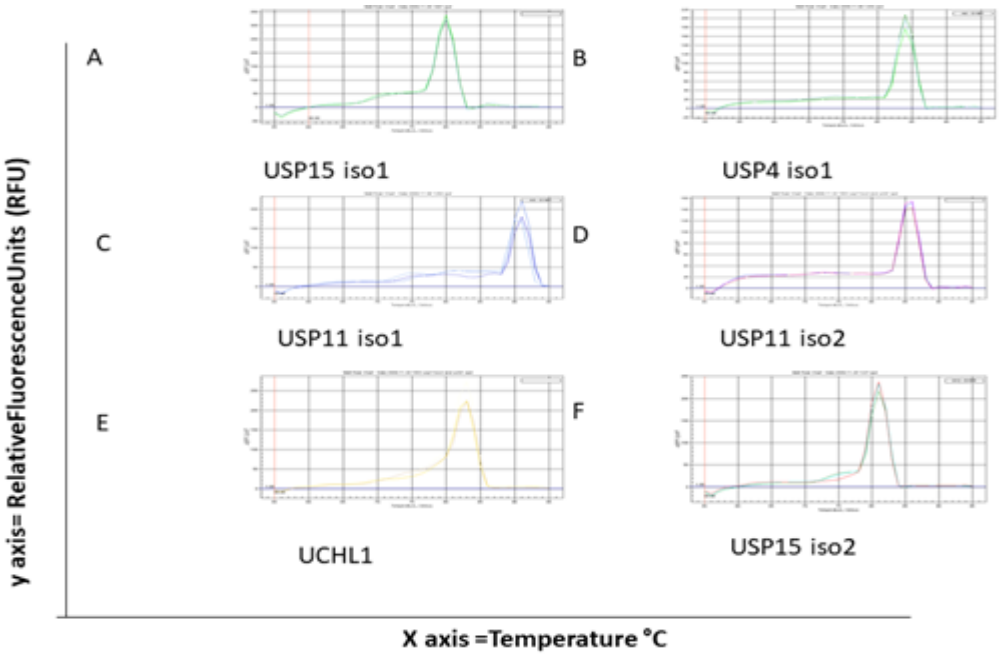

FIG.4. Example of representative melt curves for each primer pair on amplification of lung cell lines cDNA using SYBR® Green. The cDNA showing highest amplification was selected and the melt curve for three replicates is shown in each case:
A): USP15 iso1 primers for COR-L23 cDNA. **B):** USP4 iso1 primers for NCI-H460 cDNA. **C):** USP11 iso1 primers for NCI-H727 cDNA. **D):** USP11 iso2 primers for NCI-H460 cDNA. **E):** UCHL1 primers for U2020 cDNA**. F):** USP15 iso2 primers for COR-L88 cDNA.

Expression of DUB transcripts in lung cancer cell lines

To examine expression in lung cancer cells, RNA was prepared from 17 cell lines. Of these 7 cell lines were small cell lung cancer (SCLC), NCI-H345, NCI-H69, Lu165, COR-L88, COR-L47, U2020 and GLC19. One cell line was a lung carcinoid (NCI-H727). Six were non -small lung cancers (NSCLC), NCI-H460, NCI-H2170, A549, NCI-H322, CORL23 and NCI-H647. Lastly, three normal lung derived cell lines were used: normal human bronchial epithelial cells (NHBE), normal lung fibroblasts (MRC5), and SV40- transformed human bronchial epithelial cells (BEAS2B). *To check the quantity and purity of RNA, a Nanodrop was used to measure the absorbance at 260nm and 280nm. Pure RNA should* have A260:A280 ratio of greater than 1.8. A ratio of between (1.9–2.1) was obtained for all RNA extracted from the 17 cell lines. RNA has a maximum absorbance at 260 nm and this is used to calculate the concentration of RNA in each sample which had the range between 0.9- 2.8 µg/µl. Representative scans for RNA samples are shown in Figure 5.

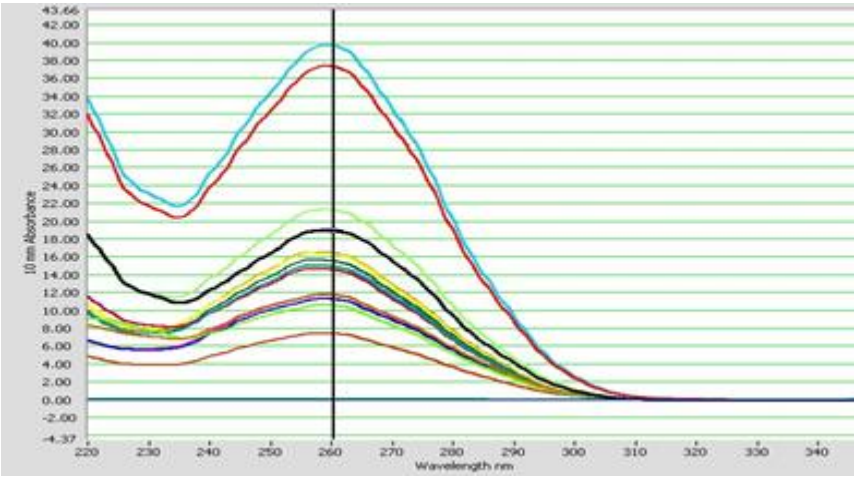

FIG.5 Absorbance scans used to determine the purity and concentration of RNA extracted from the 17 cell lines.

To establish the integrity of RNA, 5 µl of total RNA were loaded on 1% agarose gel with ethidium bromide. As shown in Figure 6A the 18S and 28S ribosomal RNA bands were clearly observable in all RNA samples. No low molecular or degradation products were seen except in NCI-H2170 RNA which was a little degraded related to other samples. Next cDNA was synthesized and amplified by end point PCR with ACTB primers and the correct product was observed for each sample Figure 6 B.

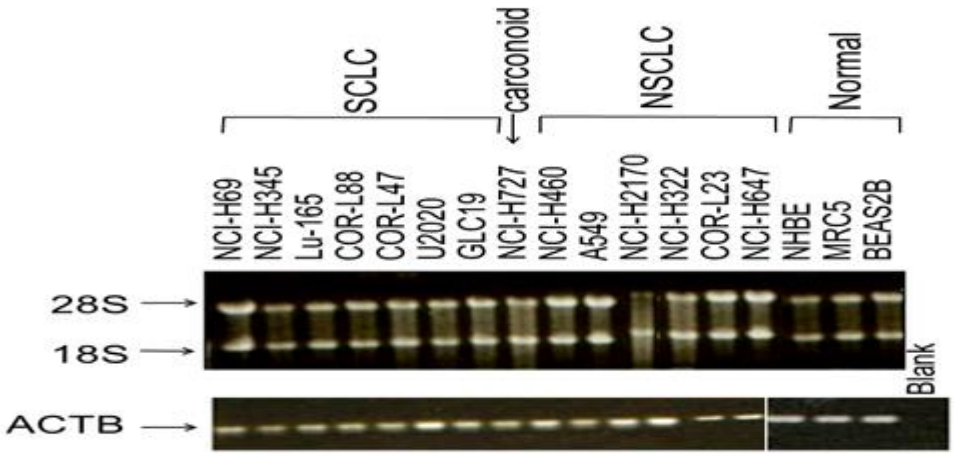

FIG.6. Quality control of RNA and cDNA prepared from lung cancer and normal lung cell lines. **A -** Gel images illustrate the quality of RNA by loading on a 1% agarose gel with ethidium bromide. **B-** Amplification of cDNA by RT-PCR using RNA extracted from lung cancer cells and ACTB primers. PCR products were resolved on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

To quantify gene expression of the DUBs, we screened cDNA from the cell panel (SCLC, NSCLC and normal) cell lines with all six primers and normalized to ACTB as a reference gene in each case (Figure7). There are two reference sequence of USP4 for different splice variants. But we found that only isoform 1 was detected and isoform 2 was not detected. In contrast, there is a single reference cDNA sequence of USP15, but there is also predicted alternative splice variant. In fact both were expressed at similar levels in cell lines tested and regression analysis showed that these correlated well with each other (R^2= 0.63). For USP11 there is also a single reference sequence and a predicted alternative splice variant. In fact this variant (iso2) was expressed more highly in most cells and had little correlation with isoform 1 (R^2 =0.058).Finally, UCHL1 had a variety of expression across the cell lines.

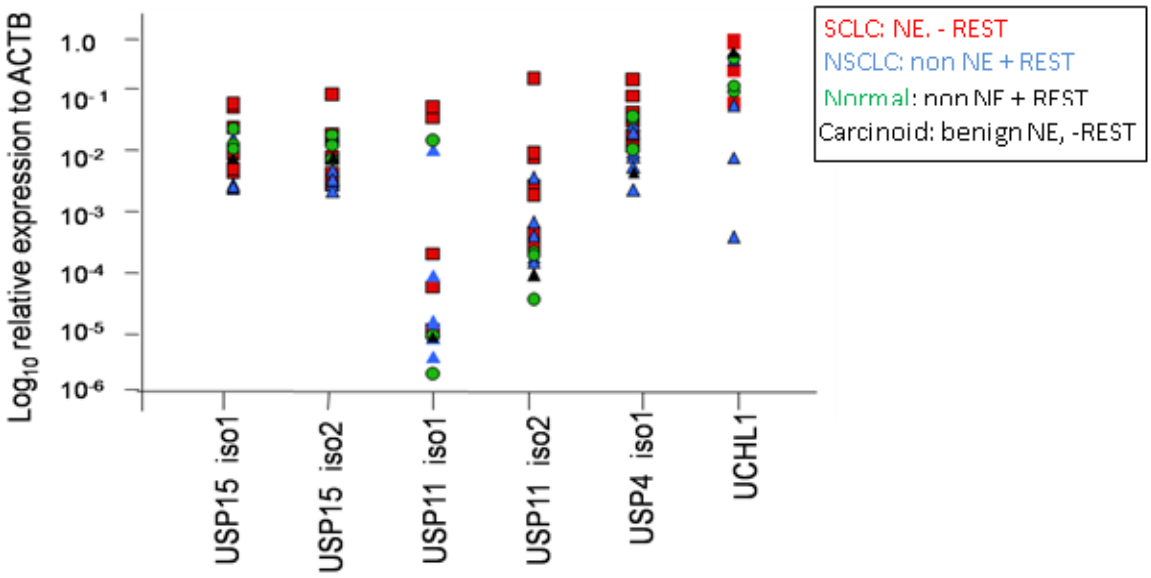

FIG.7. The gene expression of six DUB transcripts in lung cancer and normal lung cell lines. USP15 iso1, USP15 iso2, USP11 iso1, USP11 iso2, USP4 iso1 and UCHL1 were amplified a panel of 14 lung cancer (SCLC, NSCLC, Carcinioid) and three normal lung cell lines by qRT-PCR and the means of three technical replicates normalized to ACTB is shown in each case ($2^{-\Delta\Delta ct}$).

The association of USP transcript levels was also tested with specific lung cancer types as shown in table 2. The relative fold-expression in NE cell lines compared to non-NE cell lines was highest for USP11 iso 2 and UCHL1 (Table 2). Interestingly, although, like SCLC, the carcinoid cell line lacks REST and expresses neuroendocrine genes; its expression of DUB transcripts was generally lower than SCLC, with the exception of UCHL1. USP11 iso2 was also over expressed in some non-NE NSCLC compared to normal lung cells and so appeared to be more generally elevated in cancer.

Table 2. The relative expression of DUB transcripts in different lung cell types, the mean $2^{-\Delta\Delta ct}$ value was compared for different groups of cell lines.

Cell line compared	USP15 iso1	USP15 iso2	USP11 iso1	USP11 iso2	USP4 iso1	UCHL1
NE/non-NE	2.77	2.60	4.58	61.54	3.01	38.23
SCLC/carcinoid	3.74	3.15	4381.19	703.24	16.33	57.51
cancer / non-cancer	1.03	1.14	1.76	243.36	1.30	82.25
SCLC/normal	1.65	1.75	2.97	387.52	2.07	148.80
NSCLC/normal	0.31	0.42	0.35	7.77	0.41	4.62

REST expression in the cell lines was also compared to DUBs based on table 2. There is no indication in SCLC or carcinoid, where REST protein is not detectable, that expression of DUBs, which might increase its stability, is altered. REST protein is variable in NSCLC cell lines, but regression analysis revealed the best correlation for REST was with UCHL1 (R2 = 0.35).

Discussion:

It is clear that a sense of balance among ubiquitination and deubiquitination procedures plays a key role in regulating the destiny of proteins within cells. The altered of balance of ubiquitination and deubiquitination may lead to the aberration of intracellular processes including cell cycle progression, transcriptional activation, signal transduction, antigen presentation, apoptosis oncogenesis, preimplantation, and DNA repair (D'Andrea & Pellman, 1998). In this study, gene expression of candidates DUBs associated with REST stability was examined. QRT-PCR was used to measure DUB expression owing to its wide dynamic range for quantification and its precision for accurate evaluation of gene expression and quantification of splice **iso**forms. We have, therefore, designed primers specific to DUBs and used these to amplify cDNA from 17 lung cell lines by RT-PCR. The data shows a wide range of gene expression in SCLC, NSCLC, carcinoid and normal cell lines.

The expression of these DUBs has not previously been investigated by qRT-PCR in lung cancer. However, DNA microrarry data is available from a study of lung tumour samples. In this study, just one isoform of USP4 was identified. USP4 isoform 2 did not give any product when the primer pair was tested by RT-PCR. According to Bhattacharjee et al., (2001) (Figure 8), there was a slightly increased expression of USP4 in carcinoid compared to normal lung tissue and in another report based on human lung cancer tissue (Gray et al., 1995), it was found that USP4 had high gene expression levels in small cell lung tumors and adenocarcinomas of the lung, which may lead to suggestion of a possible oncogenic role lung for USP4 in neoplasia. In addition, in a further study of cell lines rather than primary tissue, USP4 protein levels were slightly but constantly

reduced in cell lines derived from small cell tumors therefor the expression and role of USP4 in cancer remains contarvsial (Frederick et al., 1999) .

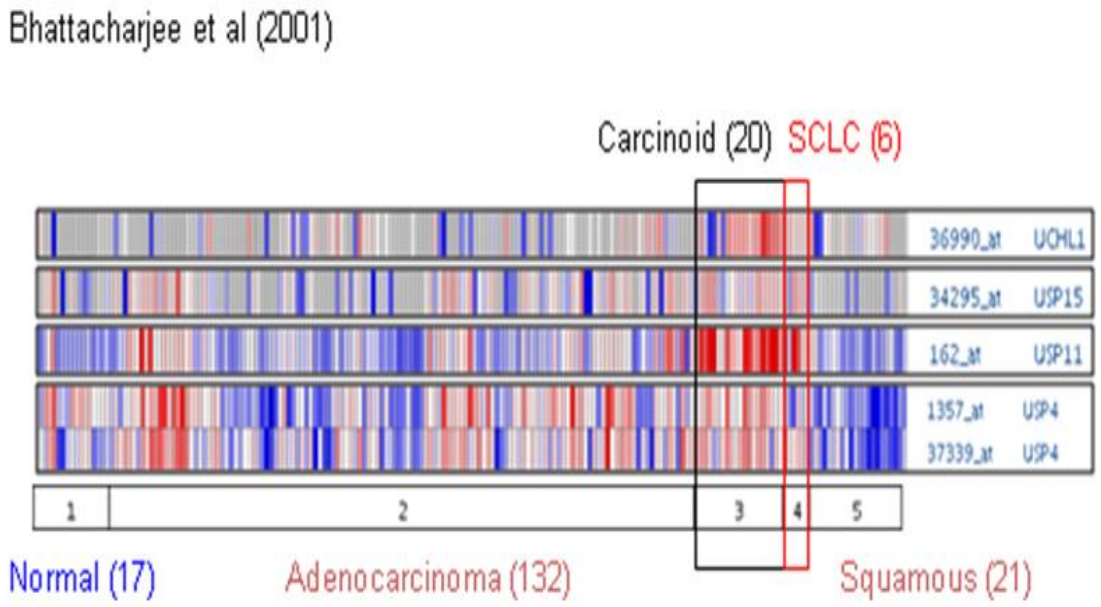

FIG.8. Oncoming (compendia Bioscience) was used for analysis and visualisat of microarray DNA from the study of Bhattacharjee et al, (2001).

To our knowledge there is little published information available on alternative USP11 splice variants isofoms. Two different isoforms of USP11 had been identified in this study. Both isoforms have 21 exons but they differ only in that they have a different exon 1. This is results in the delection of a DUSP domain in isoform 2 which may affect USP11 protein. (Figure 9). Both isoforms were amplified in lung cancer as well as normal cell lines but USP11 iso1 showed a highly variable expression and was expressed at a very low level in most cell lines, while USP11iso2 had much high expression in all cell lines compared to isoform 1. In agreement with our data, Bhattacharjee, showed USP11 to be over expressed in SCLC and carcinoid NE lung tumors Figure 9. It was added to this data by showing that the major isoform in most cases is splice variant 2 rather than the published reference sequence.

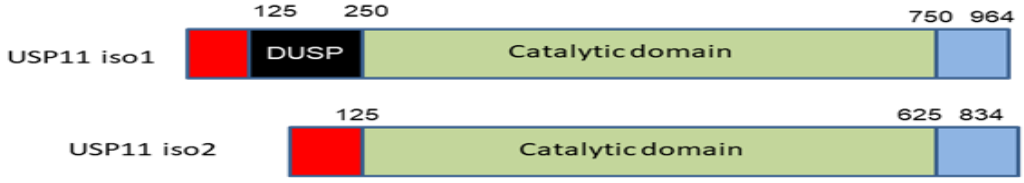

FIG.9. Diagramatic scheme showing the difference between USP11 isoform 1 (NM_004651.3) and isoform 2 (Aceview alternative variant bApr07).

Microarray data from Bhattacharjee study showed that USP15 had a variable expression level in SCLC and carcinoid. Both USP15 iso1 and 2 showed a similar expression level and pattern across cell lines. Both had the highest expression level in the GLC-19 cell line (SCLC), while the lowest expression was in A549 and NCI- H647 cell lines (NSCLC). UCHL1 had more expression in carcinoid and SCLC according to Bhattacharjee et al., 2001. Also in our results, UCHL1 had high expression in carcinoid and in some SCLC. According to Sacco and *et al* UCHL1 expression in esophageal squamous cell carcinoma and pancreatic carcinoma and has been describe in NSCLC. UCHL-1 is also involved in neural cell apoptosis (Sacco et al., 2010).

In this experiment, qRT-PCR was used to study gene expression of DUBs in lung cancer but because qRT-PCR could not tell us about DUBs activity or protein level, it can be suggested that the next step would be using Westren blot to look at DUB protein level.

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