RESEARCH ARTICLE

Effects of Ubiquitin-conjugating Enzyme 2C on Invasion, Proliferation and Cell Cycling of Lung Cancer Cells

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Abstract

The aims of this study were to investigate the influence of ubiquitin- conjugating enzyme E2C (UBE2C) on biological behavior of lung cancer cells. Using MTT, flow cytometry and invasion assays, we detected UBE2C expression and evaluated its biological properties in these cells, including effects on proliferation, the cell cycle profile and invasive capability. Compared with control cells, the UBE2C transfected cells demonstrated increased cellular proliferation (p<0.05). UBE2C transfected cells also had a lower percentage in G1 phase and a higher percentage in S phase (p<0.05). Importantly, the UBE2C transfected cells had a notable enhancement of cell numbers penetrating the basement membrane compared with the control group (p<0.05). Ectopic upregulation UBE2C promoted the growth of lung cancer cells *in vivo*. Furthermore, we found UBE2C increased the expression of cyclin D1 and MMP-2. These results show UBE2C may represent a potential therapeutic target for lung cancer.

Keywords: UBE2C - proliferation - invasion - lung cancer

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Introduction

Lung cancer is the leading cause for cancer-related deaths in the world. The morbidity and mortality of the lung cancer patients in China were increased rapidly in the past decade (Jemal et al., 2008). As lung cancer cells infiltrate into surrounding tissue and metastasize to distant organs, the 5- year survival rate in lung cancer patients was very low (Rizzi et al., 1990). Some lifestyle related habits, such as smoking and alcohol consumption, are closely associated with lung cancer (Bhaskarapillai et al., 2012). Furthermore, the average survival time for squamous cell carcinoma to be better in comparison with the other types of histology (Zahir et al., 2012). However, the pathogenesis of lung cancer is inadequately understood. Therefore, elucidating the molecular mechanism that regulates the initiation and progression of lung cancer is vital to improve survival rate of patients.

The ubiquitin-conjugating enzyme E2C (UBE2C), also named as UBCH10, could promote dissociation of the Mad2 and BubR1 from Cdc20 and activated Cdc20/ APC (Reimann et al., 2001; Hsu et al., 2002). APC is required for the destruction of mitotic cyclin A and allows progression through mitosis and mitotic exit. These results suggest that UBE2C aberrant UbcH10 expression leads to chromosomal instability and further indicate UBE2C is a potential trigger for the initiation of cancer. Substantial evidence has showed abnormal high UBE2C was observed in various human solid cancers including esophageal adenocarcinoma, thyroid cancer, colon cancer, ovarian cancer, breast cancer, hepatocellular carcinoma, and cervix cancer (pallante et al., 2005; Lin et al., 2006; Berlingieri et al., 2007a; 2007b; Ieta et al., 2007; Chen et al., 2010; Bose et al., 2012). In colorectal cancer cells, Bavi et al found UBE2C overexpression promoted cellular proliferation, whereas depletion of UBE2C expression suppressed growth of cells (Bavi et al., 2011). Moreover, Fujita et al reported UBE2C overexpression alternates the cell cycle and accelerate the tumor proliferation in colon cancer (Fujita et al., 2009). These findings confirmed oncogenic role of UBE2C in various cancers. More importantly, higher UBE2C expression was found to be an independent risk factor that influences the postoperative survival time of NSCLC patients (Zhao et al., 2012).

However, to date no experimental evidence exists as to whether UBE2C influence biological behavior of lung cancer cells. To further verify the correlation between UBE2C status and lung cancer progression, we investigate the effects of UBE2C on cell proliferation, cell cycle distribution, and invasion of lung cancer cell lines.

Materials and Methods

Cell culture and transfection

L-78 and SC-1680 cell lines were purchased from American Type Culture Collection (ATCC). L-78 and SC-1680 cells were cultured as fellow. Briefly, cells in RPMI 1640 medium supplemented with 10% fetal bovine

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serum at 37°C in humidified atmosphere containing 5% CO2. By using lipofectamine 2000, lung cancer cells were transfected with the full-length human UBE2C cDNA or a control pcDNA3.1-vector (Kai Nuo, Guang Zhou, China). Selected by G418 (700 μ g/ml) for 3 weeks, stable positive clones were obtained, and Western blots were performed.

MTT assay

The pcDNA3.1-UBE2C transfected cells, pcDNA3.1 transfected cells, and non-transfected cells were seeding in 96-well plates at the density of 1×10^4 cells/well respectively. Cells were incubated at 37°C with 5%CO₂. After they adhered, 50µL MTT stock solution was added to each well, and the cells were incubated for 4h in a 37°C incubator. Each well added to 150µl dimethylsulfoxide and shaken for 10 minutes. The OD values at 570 nm were determined. Each experimental group was included six replicate wells. The experiment was repeated three times. Analysis of cell cycle by flow cytometry

The pcDNA3.1-UBE2C transfected cells, pcDNA3.1 transfected cells, and non-transfected cells were washed three times with PBS, fixed with 75% ethanol and for 25 minutes at 4°C. In the dark, cells were incubated with the DNA-binding dye propidium iodide (50 μ g/ml) and RNase (1.0 mg/ml) for 40 min at 37°C. Lastly, cells were washed and red fluorescence was analyzed by a flow cytometer (BD, Heidelberg, Germany).

Invasion assay

The invasive assay was evaluated by 8- μ m pore size polycarbonate membrane (Corning, MA, USA). Each group (the pcDNA3.1-UBE2C transfected cells, pcDNA3.1 transfected cells, and non-transfected cells) at a density of 1×10⁵ cells/well were added into the upper chamber with 0.2ml serum-free RPMI-1640.0.5ml of 10% FBS medium was covered with the lower chamber. After incubation for 24 hours, cells on the upper chamber were removed, cells on the lower were fixed in methanol and stained with trypan blue.

Western blot detection of UBE2C, cyclin D1, MMP-2 and MMP-9

Lung cancer cells were lysed by a lysis buffer, and lysates were obtained by centrifugation at 4°C. Protein concentrations were evaluated by the Bradford method. Forty µg total protein was subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with UBE2C (1:1500 dilution), cyclin D1 (1:1000 dilution), MMP-2 (1:1200 dilution) and MMP-9 (1:1500 dilution) antibody (Santa Cruz, CA), followed by HRP-conjugated secondary antibody. ECL substrate was used for detection of their expression.

In vivo tumor growth assay

L78-UBE2C (n=3) and SC1680-UBE2C (n=3) cells were implant into the back of the nude mice respectively. As control groups, L78-vector and SC1680-vector cells were handled by the same experimental conditions.

The maximum diameter (a) and the minimum diameter (b) of the tumors were measured, with tumor volume being calculated according to the formula: Tumor volume= $a \times b^2/2$. After 5 weeks of implant, the nude mice were killed and the tumors were excised.

Statistical analysis

Statistical analysis was performed using the SPSS17.0. Comparisons between two groups were conducted by Student's t test. p<0.05 denotes the presence of a statistically significant difference.

Results

UBE2C expression in the lung cancer cell lines L-78 and SC-1680

UBE2C expression was detected in the lung cancer cell lines L-78 and SC-1680. The pcDNA3.1-UBE2C transfected cells and the pcDNA3.1 empty vector-transfected cells were also examined by western blot. As shown in Figure 1, there was no UBE2C expression in pcDNA3.1 empty vector-transfected cells and non-transfected cells, demonstrating that the L-78 and SC-1680 cell lines is UBE2C-deficient and is appropriate for the UBE2C transfected L-78 and SC-1680 cells expressed UBE2C, indicating the construction of stable UBE2C-expressing cell lines.



Figure 1. UBE2C Protein Expression in Lung Cancer Cells



Figure 2. The Influence of UBE2C on the Cell Cycle Profile and Proliferation of L-78 and SC-1680 Cells. (A): a, Mock transfected L-78 cells; b, pcDNA3.1 transfected L-78 cells; c, pcDNA3.1-UBE2C transfected L-78 cells; d, Mock transfected SC-1680 cells; e, pcDNA3.1 transfected SC-1680 cells; f, pcDNA3.1-UBE2C transfected SC-1680 cells. (B): The effect of UBE2C on proliferation of L-78 and SC-1680 cells. (A): L-78 cells; (B): SC-1680



Figure 3. The Influence of UBE2C on the Invasive Capability of L-78 and SC-1680 Cells. (A): L-78 cells; (B), pcDNA3.1 empty vector transfected L-78 cells; (C), pcDNA3.1-UBE2C transfected L-78 cells; (D), SC-1680 cells; (E), pcDNA3.1 empty vector transfected SC-1680 cells; (F), pcDNA3.1-UBE2C transfected SC-1680 cells



Figure 4. Cyclin D1, MMP-2 and MMP-9 Protein Levels in L-78 and SC-1680 Cells. UBE2C induce the expressions of cyclin D1 and MMP-2. However, UBE2C has little influence on MMP-9 expression. (A), pcDNA3.1-UBE2C transfected group; (B), empty vector group; (C), Control group

Effects of UBE2C expression on the cell cycle and proliferation in L-78 and SC-1680 cells

Cell cycle analysis showed that the L-78 and SC-1680 cells expressing UBE2C had less cells in G1 phase than the pcDNA3.1 empty vector groups and the non-transfected groups (p<0.05). Furthermore, UBE2C-transfected groups had more cells in S phase compared with the pcDNA3.1 empty vector groups and the non-transfected groups (p<0.05).

These results suggest enhancement UBE2C expression influence the cell cycle distribution of L-78 and SC-1680 cells and UBE2C overexpression cause G1 phase shortened and prolonged S phase prolonged (Figure 2A).

MTT assay was performed to study the effect of UBE2C on proliferation of L-78 and SC-1680 cells. Four days after seeding, the OD values of the pcDNA3.1-UBE2C expressing L-78 and SC-1680 cells were significantly higher than that of the cells in the pcDNA3.1 empty vector groups and the non-transfected groups (p<0.05) (Figure 2B). Moreover, there was no significant difference between the pcDNA3.1 empty vector groups and the non-transfected groups. Indicating that the vector had no effect on proliferation of L-78 and SC-1680 cells.

Effect of UBE2C expression on the invasion of L-78 and SC-1680 cells

Transwell experiments were used to detect invasiveness in the pcDNA3.1-UBE2C expressing L-78 and SC-1680 cells, the pcDNA3.1 empty vector groups and the nontransfected groups. As shown in Figure 3, the number



Figure 5. The Expressions of UBE2C, Cyclin D1 and MMP-2 were Up-regulated in L78-UBE2C (B,F,J) and SC1680-UBE2C (D, H, L) Cells Compared with the Control Groups (A, E, I; C, G, K); Tumor volume in L78-UBE2C (M) and SC1680-UBE2C (N) cells was significantly larger than those in control groups. *<0.05

of L-78 cells penetrating through the membrane of the chamber was significantly more in the pcDNA3.1-UBE2C transfected cells than that in the empty vector groups and the non-transfected groups (p<0.05), while there was no significant difference between the empty vector groups and the non-transfected groups (p>0.05). Similar results were obtained in SC-1680 cells, the invasive ability of pcDNA3.1-UBE2C transfected groups was stronger than that in the empty vector groups and the non-transfected groups was stronger than that in the empty vector groups and the non-transfected groups (p<0.05), suggesting that the findings were not75.0 idiosyncratic to L-78 cells. These results suggest that UBE2C expression increase the invasive ability of L-78 and SC-1680 cells.

Impact of UBE2C expression on cyclin D1, MMP-2 and MMP-9 protein expression

Western blot analysis revealed that the pcDNA3.1-25.0 UBE2C transfected L-78 and SC-1680 cells displayed significantly higher levels of cyclin D1 and MMP-2 than the pcDNA3.1 and non-transfected cells (p<0.05; Figure 4). Enhanced expression of cyclin D1 contributed to cell cycle transformation from G1 to S. This finding elucidated the role, at least in part, of UBE2C in proliferation of lung cancer cells. Furthermore, UBE2C may regulate the expression of the MMP-2, which may be correlated with the increased invasiveness capacity of the UBE2C transfected cells.

Up-regulation UBE2C expression promoted tumor growth in vivo

As shown in Figure 5 B and D, UBE2C expression was increased in tumors transplanted with L78-UBE2C and SC1680-UBE2C cells compared with that in L78-vector (L78-pcDNA3.1-vector) and SC1680-vector (SC1680-pcDNA3.1-vector) groups. Furthermore, compared with control groups, immunoreactivity of cyclin D1 and MMP-2

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in tumors injected with L78-UBE2C and SC1680-UBE2C cells were significantly stronger (Figure 5E-L, p<0.05). We also found tumors transplanted with L78-UBE2C and SC1680-UBE2C cells had larger volume than that of animals injected with L78-vector and SC1680-vector cells (Figure 5M-N, p<0.05). Therefore, our results indicated that up-regulation of UBE2C expression promoted tumor growth in vivo.

Discussion

As the important role in regulating cell cycle, UBE2C is considered as an oncogene in a variety of cancers and its overexpression is associated with proliferation of tumors cells (Okamoto et al., 2003; Jiang et al., 2008; Troncone et al., 2009; Jiang et al., 2010). Taking into account the correlation between UBE2C and chromosomal instability, further study demonstrates the involvement of UBE2C in tumorigenesis. By immunohistochemical staining, Perrotta et al found UBE2C is a potential tool for diagnosis and therapy of lung cancer (perrotta et al., 2012). Similarly, higher UBE2C expression was associated with a shorter postoperative survival time of non-small cell lung cancer (NSCLC) patients and may play an important role in NSCLC carcinogenesis (Zhao et al., 2012). Previous study has established a connection between UBE2C expression and TNM stage in lung cancer. Moreover, UBE2C was positively correlated with the Ki-67 expression, suggesting UBE2C had a high correlation with proliferation of lung cancer cells.

In this study, we reported influence of UBE2C in biological behavior of lung cancer cells. Firstly, we found that UBE2C expression was low in lung cancer cell lines L-78 and SC-1680. Thus, we used these cells to explore the role of UBE2C in lung cancer cells. After transfected with UBE2C cDNA, expression of UBE2C in pcDNA3.1-UBE2C transfected L-78 cells and SC-1680 cells had significant enhancement, indicating these cells were stably expressing UBE2C. MTT assays and flow cytometry were used to investigate the effect of UBE2C on the proliferation and cell cycle in L-78 cells and SC-1680 cells. MTT assay showed UBE2C expression promoted proliferation of lung cancer cells. Flow cytometry demonstrated that cells transfected with UBE2C had a lower percentage of G1 phase cells and a higher percentage of cells in S phase compared with the other groups. However, in colorectal carcinoma, specific small-interfering RNA against UBE2C caused cells to be arrested at the G2/M phase. The different categories of cancers explained the discrepancy in cell cycle progression. Therefore, following studies were focused on detecting the expression of cyclin D1, which is a regulator of cell cycle. The result demonstrated that cyclin D1 expression was increased in L-78 cells and SC-1680 cells after transfection with UBE2C.

This may explain, at least in part, the cause of the cell cycle change in cells transfected with UBE2C. Lastly, in lung cancer cells xenograft model, tumors injected with L78-UBE2C and SC1680-UBE2C cells were larger than those control groups. Moreover, compared with L78-vector and SC1680-vector cells, expression levels of cyclin D1 and MMP-2 in tumors injected with L78-

UBE2C and SC1680-UBE2C cells were significantly higher. These results confirmed the vital roles of UBE2C in tumorigenesis of lung cancer.

Transwell invasion assay was executed to evaluate the effect of UBE2C expression on the invasive ability of L-78 cells and SC-1680 cells. We found cells transfect with UBE2C had a much higher rate of penetrating through the membrane than the control cells, indicating that UBE2C enhanced the invasive capability of lung cancer cells. Many studies have revealed that matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) (Bernhard et al., 1994; Nagase et al., 1999; Westermarck et al., 1999) correlate with the invasion ability of cancer cells. Therefore, we investigated the effect of UBE2C on MMP-2 and MMP-9 expression in L-78 cells and SC-1680 cells. Our results indicated that L-78 cells and SC-1680 cells that transfected with UBE2C had higher levels of MMP-2 protein than that in control groups, indicating UBE2C may induce the expression of MMP-2 and improve the invasion ability in L-78 cells and SC-1680 cells.

In summary, our results collectively suggest that the UBE2C gene is capable of promoting proliferation and invasion of lung cancer cells. Therefore, UBE2C may play a pivotal role in development and invasion of lung cancer. These studies provide important implications for novel strategy of treating lung cancer.

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