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Research Article

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Abstract

Our study investigated the role of delta-like 1 (DLL1) in multi-drug resistance of small cell lung cancer. Differentially expressed genes were detected in resistant small cell lung cancer H69AR cells and sensitive small cell lung cancer H69 cells by microarray. DLL1 expression in H69 cells and H69AR cells was further confirmed by RT-PCR and Western blot assay. eGFP-RNA was employed to up-regulate DLL1 expressing in H69AR cells (H69AR-eGFP-DLL1) by transfection. These cells were treated with different chemotherapeutics (ADM, DDP, and VP-16). Cell viability was detected. Cell cycle and apoptosis rate were determined. The DLL1 expression was significantly decreased in H69AR cells when compared with H69 cells. Over-expression of DLL1 increased the sensitivity of H69AR cells to chemotherapy, which was characterized by increase in apoptosis and arrest in G0/G1 phase. Our results demonstrate that up-regulation of DLL1 expression in small cell lung cancer cells may increase their sensitivity to chemotherapeutic agents.

Introduction

Small cell lung cancer (SCLC) cells have rapid cell proliferation resulting in rapid disease progression. Thus, patients usually develop hematological and/or lymph vessel metastasis at initial diagnosis. SCLC is the most malignant lung cancer.

Although 80% of patients with SCLC respond favorably to early chemotherapy and/or radiotherapy, recurrence or disease progression is still present soon after treatment. Drug resistance, especially multidrug resistance (MDR), has been a major cause of failure in chemotherapy of SCLC (Chute et al., 1999; Sandler, 2003). Although 80% of SCLC patients are sensitive to early chemotherapy, relapse is common due to drug resistance. Patients with focal SCLC have a 5-year survival rate of <30%, and those with diffused SCLC have a 5-

year survival rate of 1-2% (Flahaut et al., 2009). Thus, to improve the drug resistance has been a challenge in the clinical treatment of SCLC. Delta-like1 (DLL1) is a single transmembrane glycoprotein belonging to Delta, Serrate, Lag-2 (DSL) family. Human DLL1 is mapped to 6q27 and has a full length of 3.04 kb. The open read frame of DLL1 gene encodes 723 amino acids. DLL1 is one of two ligands of Notch in vertebrates. The binding of DLL1 to Notch may activate Notch signaling pathway, which determines the cell fate and is involved in regulation of growth and development of some tissues. The intracellular domain of DLL1 protein can specifically bind to E3 ubiquitin ligase resulting in DLL1 ubiquitination and endocytosis, and this domain is essential for the activation of Notch signaling pathway (Estrach et al., 2008; Bordonaro et al., 2011; Piazzini et al., 2011). There is evidence showing that DLL1 is closely



related to the growth and differentiation of cancer cells (Huang et al., 2011; Zhang et al., 2011). However, the role of DLL1 in drug resistance of cancer cells, especially SCLC cells, is less investigated. In our pilot study, gene microarray revealed that DLL1 expression was significantly down-regulated in H69AR cells when compared with H69 cells. The present study aimed to confirm the expression of DLL1 in chemotherapy sensitive H69 cells and insensitive H69AR cells and to investigate the influence of DLL1 expression on drug resistance, cell cycle and apoptosis of SCLC cells.

Materials and Methods

Materials: pIRES2-EGFP and competent bacteria were stored in our lab. Human chemotherapy sensitive H69 cells and adriamycin resistance H69AR cells (ATCC, USA), newborn fetal bovine serum, RPMI 1640 (Gibco, USA), cisplatin, adriamycin, etoposide (Pfizer, USA), CCK8 kit, apoptosis detection kit (Shanghai Beyotime Institute of Biotechnology), first strand cDNA synthesis kit, polymerase chain reaction (PCR) kit, DNA marker, DNA purification kit, plasmid extraction kit, reverse transcription kit, restriction endonuclease (TaKaRa, Japan), lipofetamine 2000 (Invitrogen, USA), rabbit anti-human Notch1 monoclonal antibody (Santa Cruz, USA), and goat anti-rabbit IgG (Wuhan Boster, China) were used in the present study.

Detection of DLL1 mRNA expression by real-time fluorescence quantitative PCR: RNA was extracted from cells followed by reverse transcription and real-time fluorescence quantitative PCR. Primers for DLL1 were as follows: 5'-AGGGTGTGATGACCAAC ATGGA-3' (forward) and 5'-TATCGGATGCACTCATCGCA-GTA-3' (reverse). Primers for GAPDH were as follows: 5'-GGAAGGACTCATGACCACAGTCC-3' (forward) and 5'-TCGCTGTI' GAAGTCAGAGGAGACC-3' (reverse). The reverse transcription and PCR were done according to manufacturer's instructions. Experiment was done three times in triplicate, and $RQ=2^{-\Delta\Delta Ct}$ was employed to calculate the expression of DLL1.

Up-regulation of DLL1 expression by transfection with pIRES2-EGFP-DLL1: pIRES2-EGFP-NC and pIRES2-EGFP-DLL1 were independently transfected into H69AR cells. In brief, H69AR cells were seeded into 6-well plate at a density of 5×10^5 /mL 24 hours before transfection. When the cell confluence reached 70%, 500 μ L of Opti-MEM or serum free medium was mixed with 10 μ L of Lipofectamine™ 2000 followed by pipetting. The mixture was kept at room temperature for 5 min. Then, 2 μ g of pIRES2-EGFP-NC or pIRES2-EGFP-DLL1 was mixed with 500 μ L of Opti-MEM or serum free medium followed by pipetting. The Lipofectamine™ 2000 mixture was added to plasmid mixture followed by pipetting. The final mixture was kept at room temperature for 20 min, and then added to 6-well

plate (1 mL/well). After gentle shaking, cells were incubated at 37°C in an environment with 5% CO₂ for 4-6 hours followed by refreshing medium. At 24 hours after transfection, the DLL1 expression was measured; at 48 hours after transfection, the DLL1 protein expression was detected.

Detection of DLL1 protein expression by western blot assay: Total protein was extracted from cells followed by protein quantification with BCA method. Then, 50 μ g of protein was subjected to 10% SDS-PAGE and transferred onto PVDF membrane which was then incubated with 5% BSA/TBST at room temperature for 1 hour. Then, the membrane was treated with rabbit anti-human DLL1 monoclonal antibody (1:200) at 4°C overnight. After washing with TBST thrice, the membrane was treated with HRP conjugated goat anti-rabbit IgG (1:5000) at room temperature for 2 hours. After washing with TBST thrice, visualization was done with ECL.

Detection of chemotherapy sensitivity with CCK8: On the basis of peak serum concentrations of DDP, VP-16 and DOX, cells were treated with chemotherapeutics of 0.01, 0.1, 1 and 10 times of peak serum concentrations. In the negative controls, cells were not treated with drugs. Only medium was included in the blank control group. Experiment was done in quadruplicate. Cells were seeded into 96-well plates (200 μ L/well). After cells were adherent, cells were incubated with different chemotherapeutics for 24 hours. Following addition of freshly prepared CCK8 solution (20 μ L/well), incubation was done at 37°C in an environment with 5% CO₂ for 4 hours. Absorbance (A) was measured at 450 nm with a microplate reader followed by averaging. The cell viability was calculated as follow: Cell viability = $(A_{\text{experiment}} - A_{\text{blank control}}) / (A_{\text{negative control}} - A_{\text{blank control}}) \times 100\%$. Experiment was done three times and average obtained. Then, the IC₅₀ was determined according to the survival curves of different cells treated with distinct drugs.

Detection of apoptosis: Cells in logarithmic growth phase were seeded into 6-well plates followed by incubation at 37°C for 48 hours. Cells were harvested and washed with PBS twice, and then re-suspended in 100 μ L of Annexin V-FITC and binding buffer containing 0.5 μ g of PI (10 mM HEPES pH 7.4, 0.15 M NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). After incubation at room temperature for 15 min, 400 μ L of binding buffer was added followed by flow cytometry analysis.

Detection of cell cycle: Cells in logarithmic growth phase were digested with 0.25% trypsin and 0.02% EDTA followed by washing in PBS twice. These cells were fixed in 75% ethanol for 24 hours, and washed in PBS containing 1% BSA twice. After staining with PI, cells were subjected to flow cytometry. Cell Quest software was employed to determine the proportion of cells in different phases.

Statistical analysis: Statistical analysis was done with SPSS version 13.0 for Windows. Qualitative data were compared with t test or one way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

Results

As shown in Figure 1A, RT-PCR showed the DLL1 mRNA expression in H69AR cells was significantly lower than that in H69 cells ($p = 0.003$). Western blot assay revealed that the DLL1 protein expression in H69AR cells was significantly increased when compared with H69 cells (Figure 1B; $p = 0.000$).

As shown in Figure 2, H69AR cells were transfected with pIRES2-EGFP-NC (a) or pIRES2-EGFP-DLL1 (b) for 48 hours. Fluorescence microscopy showed the transfection efficiency was about 80% (Figure 2A). RT-

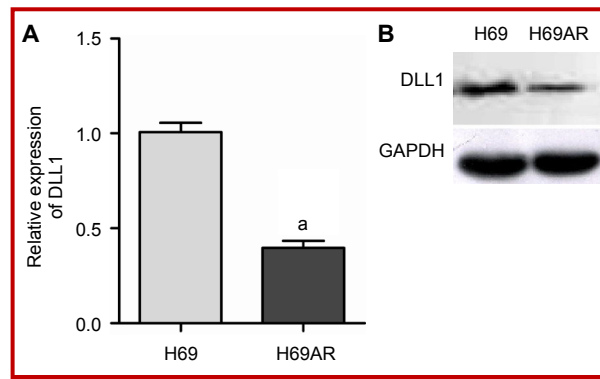


Figure 1: Detection of DLL1 mRNA and protein expressions in H69 cells and H69AR cells by RT-PCR and Western blot assay. ^a $p < 0.01$

PCR and Western blot showed the protein and mRNA expressions of DLL1 in H69AR cells increased significantly after transfection with pIRES2-EGFP-DLL1 (Figure 2B, C; $p = 0.004$).

CKK8 assay showed H69AR cells presented with markedly reduced sensitivity to DDP, ADM and VP-16 when compared with H69 cells (Figure 3A, $p = 0.009$). However, H69AR cells transfected with pIRES2-EGFP-DLL1 displayed dramatically increased sensitivity to DDP, ADM and VP-16 when compared with untransfected H69AR cells and H69AR cells transfected with pIRES2-EGFP-NC (Figure 3B, $p = 0.016$).

Flow cytometry showed the apoptosis rate of H69 cells and H69AR cells was $17.9 \pm 0.7\%$ (Figure 4A) and $2.1 \pm 0.1\%$ (Figure 4B) showing marked difference ($p < 0.01$). The apoptosis rate of pIRES2-EGFP-DLL1 treated H69AR cells was $22.1 \pm 0.2\%$ which was significantly higher than that of H69AR cells and pIRES2-EGFP-NC treated H69AR cells (Figure 4C; $p < 0.01$). These findings suggest that up-regulation of DLL1 expression

significantly increases apoptosis of H69AR cells.

Flow cytometry showed the majority of H69 cells were in G0/G1 phase (Figure 5A), but the proportion of H69AR cells in G2/M phase increased significantly (Figure 5B) when compared with H69 cells ($p < 0.01$). However, the proportion of pIRES2-EGFP-DLL1 transfected H69AR cells in G0/G1 phase (Figure 5D) increased dramatically when compared with untransfected H69AR cells and pIRES2-EGFP-NC transfected H69AR cells (Figure 5C) ($p < 0.01$). These findings suggest that up-regulation of DLL1 expression arrests H69AR cells in G0/G1 phase.

Discussion

In recent years, some studies have shown that Notch1 receptor and its ligand DLL1 play important roles in the growth, differentiation, proliferation and apoptosis of cancer cells. Notch signal transduction is dependent on binding of Notch to its ligand (Chen et al., 2010; Bridges et al., 2011; de Antonellis et al., 2011; Han et al., 2011; Oon and Harris, 2011; Groth and Fortini, 2012). To date, a total of 5 Notch ligands have been identified in humans including DLL1, DLL3, DLL4, JAG1 and JAG2 (Jubb et al., 2012). One of the earliest described non-canonical ligands for Notch is Delta-like 1 (Dlk-1), also known as Pref-1, or FA-1 (Smas and Sul, 1993; Bachmann et al., 1996), whose predominant role is inhibiting adipogenesis (Wang et al., 2006). Delta-like 1 homolog (DLK1) is expressed in animals from birds to mammals extensively in immature cells, and is down-regulated during fetal development, which implicates that DLK1 plays an important role in fetal development and growth. However, DLK1 is only detected in selected adult tissues and several tumor cells including NB, gliomas, small-cell lung carcinoma, and leukemia (Kim, 2010). Studies have confirmed that DLL1 is able to inhibit the proliferation of cancer cells and promote their differentiation (Kolev et al., 2005; Begum et al., 2012). In addition, DLL1 may serve as a potential target against cancer stem/progenitor cells of hepatocellular carcinoma (Xu et al., 2012) and as a serum marker for hepatoblastoma in young infants (Falix et al., 2012). Moreover, the increased DLK1 expression in gliomas was found to be associated with oncogenic activities (Yin et al., 2006). However, little is known about the relationship between DLL1 and drug resistance of cancers.

MicroRNAs (miRNAs) are small un-translated RNAs that can inhibit expression of target genes through translational inhibition or transcriptional silencing (Denli et al., 2004). Bioinformatics analysis predicts that about 30% of all the protein-coding genes are targets of miRNAs (Berezikov et al., 2005). The miR-34 family members share high sequence homology (Bommer et al., 2007) and are associated with a variety of tumors

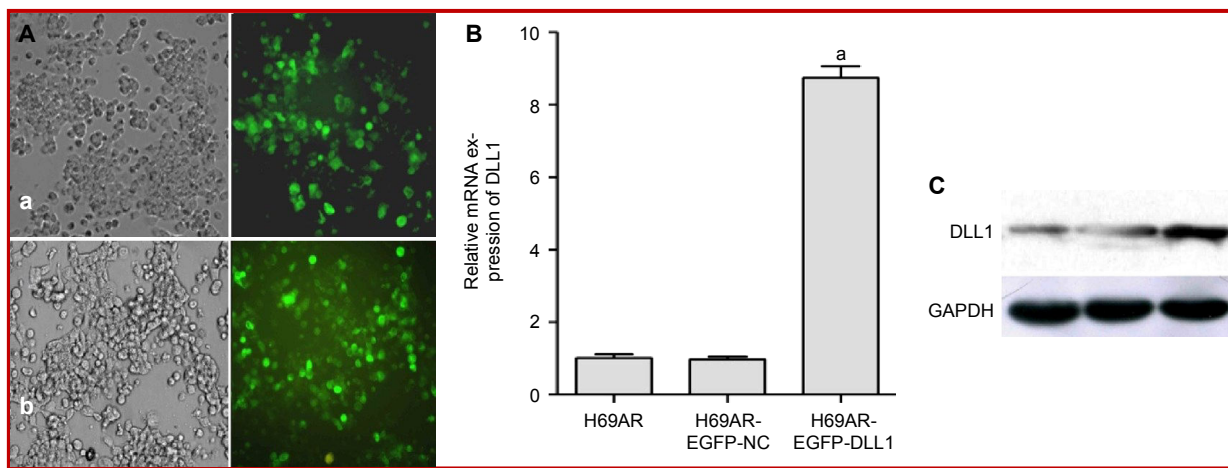


Figure 2: Up-regulation of DLL1 expression by transfection with pIRES2-EGFP-DLL1. (A) H69AR cells were transfected with pIRES2-EGFP-NC (a) or pIRES2-EGFP-DLL1 (b) for 48 hours, and transfection efficiency was determined under fluorescence microscope. Protein (B) and mRNA (A) expressions of DLL1 increased significantly in H69AR cells after pIRES2-EGFP-DLL1 transfection. ^a $p < 0.01$.

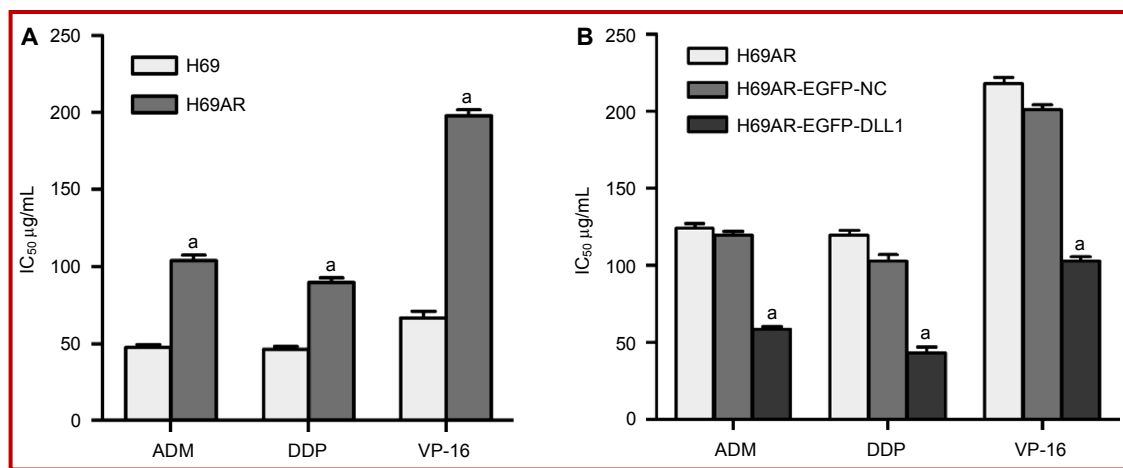


Figure 3: Chemotherapeutics sensitivity of cells: (A) CCK8 was used to determine the sensitivity of H69 cells and H69AR cells to DDP, ADM and VP-16. (B) H69AR cells transfected with pIRES2-EGFP-DLL1 presented with significantly increased sensitivity to DDP, ADM and VP-16 when compared with untransfected H69AR cells and H69AR cells transfected with pIRES2-EGFP-NC. ^a $p < 0.05$. DDP: Cisplatin; ADM: Adriamycin; VP-16: Etoposide

(Lodygin et al., 2008). Among these, miR-34a is one of the earliest known miRNA tumor suppressor and is directly transactivated by p53 (Chang et al., 2007; He et al., 2007). Ectopic miR-34a expression induces apoptosis, cell cycle arrest, and differentiation or reduces migration (Chen and Hu, 2012). de Antonellis found that DLL1 was a target of miR-34a in medulloblastoma (de Antonellis et al., 2011). In their study, results showed miR-34a can specifically act on Notch ligand DLL1 causing impairment on the proliferation of CD15+/CD133+ cancer cells, which promotes differentiation of medulloblastoma cells (de Antonellis et al., 2011). Huang et al., 2011 found that to selectively stimulate DLL1-Notch signaling pathway could recover the T cell functions and inhibit the growth of cancer. In addition, there is evidence

showing that up-regulation of Notch ligand DLL1 in B16 melanoma cells could inhibit the angiogenesis resulting in inhibition of cancer growth (Zhang et al., 2011). Pang et al investigated the bioeffects of miR-34a in depth in a series of studies. In 2010, they found that miR-34a could bind to the 3' un-translated regions of Notch1 and forced expression of miR-34a altered the expression of Notch1 protein as well as Notch signaling. In addition, forced expression of miR-34a suppressed the invasiveness of HeLa and JAR cells, which was attributed to the regulation of expression of urokinase plasminogen activator through Notch (Pang et al., 2010). More recently, they observed that miR-34a reduced cell proliferation and invasiveness, at least, partially through its inhibitory effect on DLL1 in choriocarcinoma (Pang et al., 2013).

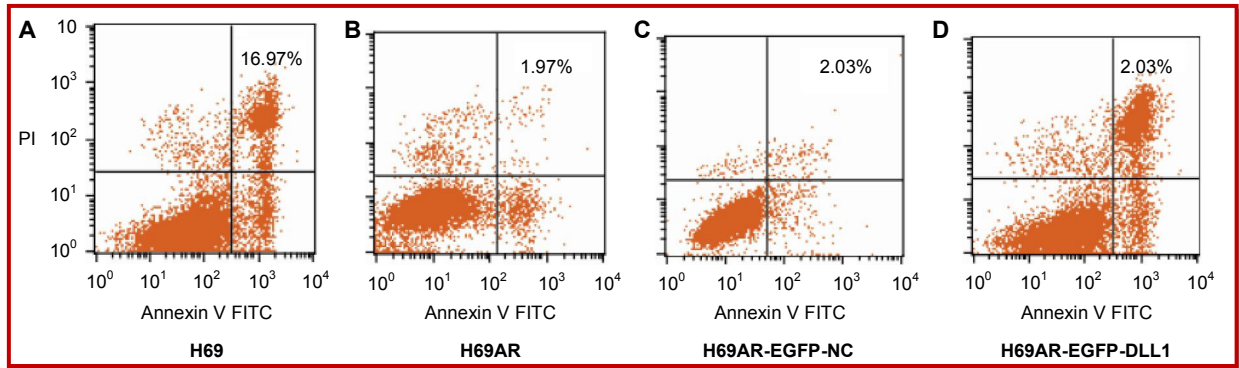


Figure 4: Cell cycle of H69 cells and H69AR cells with and without transfection. A: H69 cells; B, H69AR cells; C, H69AR-EGFP-NC transfected cells; D, H69AR-EGFP-DLL1 transfected cells

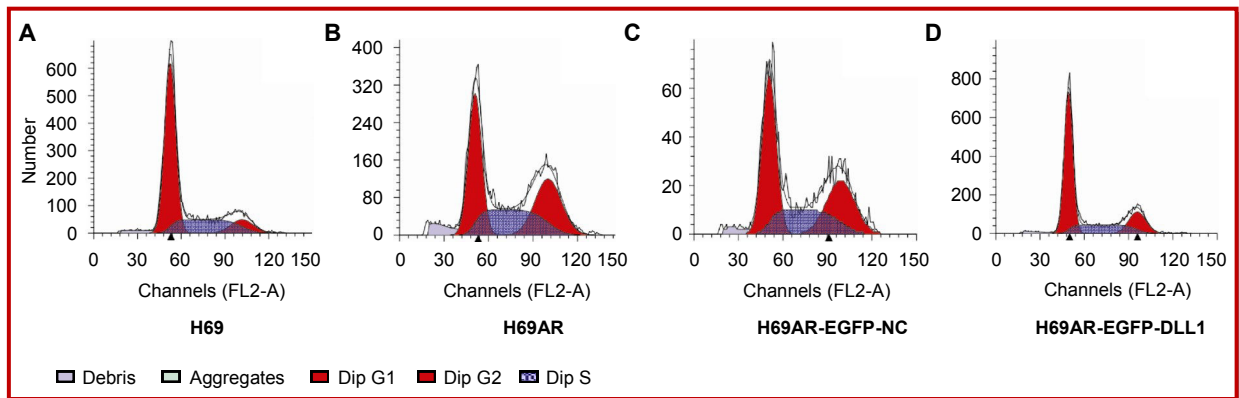


Figure 5: Cell cycle of H69 cells and H69AR cells with and without transfection. (A) H69 cells; (B) H69AR cells; (C) H69AR-EGFP-NC transfected cells; (D) H69AR-EGFP-DLL1 transfected cells

Our pilot study with microarray revealed that the DLL1 expression in resistant H69AR cells was significantly lower than that in sensitive H69 cells. In the present study, the mRNA and protein expressions of DLL1 were further determined in H69AR cells and H69 cells, and results were consistent with those from microarray. Thereafter, H69AR cells were transfected with pIRES2-EGFP-DLL1 to over-expression DLL1 in these cells. Results showed pIRES2-EGFP-DLL1 treated cells presented with increased sensitivity to chemotherapeutics, elevated apoptosis and arrest in G0/G1 phase. These findings suggest that DLL1 is closely related to the drug resistance of SCLC, but the specific mechanisms are required to further study in depth. Our findings provide evidence for future investigation on mechanisms underlying the drug resistance of SCLC.

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