RESEARCH COMMUNICATION

# Hypoxia-Inducible Factor 1 Promoter-Induced JAB1 Overexpression Enhances Chemotherapeutic Sensitivity of Lung Cancer Cell Line A549 in an Anoxic Environment

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## Abstract

**The presence of lung cancer cells in anoxic zones is a key cause od chemotherapeutic resistance. Thus, it is necessary to enhance the sensitivity of such lung cancer cells. However, loss of efficient gene therapeutic targeting and inefficient objective gene expression in the anoxic zone in lung cancer are dilemmas. In the present study, a eukaryotic expression plasmid pUC57-HRE-JAB1 driven by a hypoxia response elements promoter was constructed and introduced into lung cancer cell line A549. The cells were then exposed to a chemotherapeutic drug cis-diamminedichloroplatinum (C-DDP). qRT-PCR and western blotting were used to determine the mRNA and protein level and flow cytometry to examine the cell cycle and apoptosis of A549 transfected pUC57-HRE-JAB1. The results showed that JAB1 gene in the A549 was overexpressed after the transfection, cell proliferation being arrested in G1 phase and the apoptosis ratio significantly increased. Importantly, introduction of pUC57-HRE-JAB1 significantly increased the chemotherapeutic sensitivity of A549 in an anoxic environment. In conclusion, JAB1 overexpression might provide a novel strategy to overcome chemotherapeutic resistance in lung cancer.**

**Keywords:** Lung cancer - hypoxia-inducible factor - JAB1 - chemotherapeutic sensitivity - resistance - cisplatin

*Asian Pacific J Cancer Prev,* **13**, 2115-2120

## Introduction

Lung cancer is a leading cause for cancer-related deaths worldwide, developing in more than a million new patients annually (Toh, 2009). Although great efforts have been employed to treat lung cancer in the clinic, 5-year survival rate is only 14% approximately. Low sensitivity of lung cancer cells to chemotherapy is one of the important factors responsible for the poor prognosis of lung cancer. Chemotherapy is a most widely used strategy in the management of lung cancer. In generally, cytotoxic drugs will cause apoptosis in major tumor cells, leaving a small number of survived tumor cells. For limited apoptosis and sublethal damage, it can not initiate normal apoptosis process (Pommier et al., 2004). Thus, it is urgent to clarify the related mechanism and overcome the chemoresistance.

Hypoxia, a common feature of solid tumors, occurs in a wide variety of malignant tumors. It results in chemotherapeutic resistance by depriving oxygen essential for the cytotoxic activities of these agents in tumor cells. In addition, hypoxia may reduce chemotherapeutic sensitivity through one or more indirect mechanisms including proteomic and genomic changes, which in turn results in increased invasiveness and metastatic potential, loss of apoptosis, and disordered angiogenesis, thereby increasing the incidence of chemotherapeutic resistance (Harrison and Blackwell, 2004).

Previously, it was found that hypoxic tumor cells in resting state were comparatively more resistant to chemotherapy in some cases (Harrison and Blackwell, 2004). And, oncogene was markedly up-regulated (Bando et al., 2003), while tumor suppressor gene was significantly down-regulated in a hypoxic condition (Lee et al., 2009).

Hypoxia-inducible factor 1 (HIF-1), a transcription factor in mammal, was first identified by Wang and his colleagues in hypoxia-induced cells in 1993 (Wang and Semenza, 1993). A series of studies have demonstrated that vascular growth factor (VEGF) (Yang et al., 2006), glucose transporter 1 (GLUT1) (Hayashi et al., 2004) genes were significantly increased along with HIF-1 in malignant tumors, which suggested that HIF-1 markedly enhanced the expression of oncogene in an anoxic environment. Further study confirmed that a powerful promoter of hypoxia-responsive element (HIF-1/HRE) was affected in the hypoxic condition (Dachs et al., 1997). Subsequently, it also showed that the HIF-1/HRE significantly promoted the expression of target gene HIF-

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1, leaving no obvious effect on the expressions of other oncogenes in the hypoxic environment (Post and Van Meir, 2001).

Jun activation domain-binding protein 1 (JAB1), a coactivator of activator protein 1 (AP-1), interacts with c-Jun and JunD, and selectively potentiates transactivation only by c-Jun, which promoted cell proliferation (Claret et al., 1996). JAB1 is a full-time specific suppressor for E2F1-induced tumor cells apoptosis (Hallstrom and Nevins, 2006). JAB1 and E2F1 co-expression synergistically induced cell apoptosis. In contrast, JAB1 could not synergize with E2F1 to promote cell cycle entry. Meanwhile, E2F1-induced apoptosis and induction of p53 accumulation could not be found in JAB1-depleted cells (Hallstrom and Nevins, 2006).

In this study, we amplified full-length human JAB1 fragment and then subcloned it into pUC57 containing 6×-HIF/HRE sequence to construct eukaryotic expression plasmid pUC57-HRE-JAB1. Subsequently, it was introduced into A549 cells. Following, the mRNA and protein level of JAB1 was investigated by qRT-PCR and western blot, respectively. And, the cell cycle and apoptosis of A549 cells were also assayed in the presence or absence of C-DDP. In view of this, we tried to illuminate the role of JAB1 in the chemotherapeutic resistance of lung cancer cells in an anoxic environment.

## Materials and Methods

*Materials*

Fetal bovine serum (FBS), trypsin/EDTA, Geneticin (G418), T4 DNA ligase, SuperScript II reverse transcriptase, LipofectamineTM 2000, and TRIzol reagent, were purchased from Invitrogen (Carlsbad, CA, USA). Matrigel was provided by BD Transduction Laboratories (CA, USA). C-DDP was a product of F. H. Faulding & Co. Ltd. (Adelaide, Australia).

Cell culture. A549 cells (human lung cancer cell line) were obtained from Shanghai Institutes of Biological Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (100U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% $CO_2$.

Hypoxic culture (Maher et al., 2007). A549 cells were exposed to an anoxic environment (0.3% $O_2$) in a hypoxia glove box (Coylab, Grass Lake, USA) for 24 h. Then the cells were transfected with pUC57-HRE and pUC57-HRE-JAB1. Meanwhile, 2 μg/ml C-DDP was added respectively. In addition, if medium was replaced after the hypoxic exposure, the replaced medium was equilibrated in an anoxic environment for 24 h before use.

*Plasmid construction*

Human JAB1 cDNA (NM. 006837) was amplified by RT-PCR with the oligonucleotides 5'-ATGGCGGCGTCCGGGAGCGG-3' (forward primer) and 5'-TTAAGAGATGTTAATTTGAT-3' (reverse primer) using mRNA isolated from A549 cells as template. The fragment was cloned into eukaryotic expressive vector PUC57 with a 6×-HIF/HRE sequence, and the resulting plasmid was named as pUC57-HRE-JAB1. The orientation and the sequence of the insert was verified by restriction digestion and sequencing.

*Transfection of A549*

A549 were grown to 70% to 80% confluence in 6-well plates. Cells were transfected with either pUC57-HRE (as negtive control) or eukaryotic expressive plasmid pUC57-HRE-JAB1 using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, cells were incubated for 10 h in the medium containing plasmid, then, the transfection solution was removed by pipetting, and cells were allowed to recover for 48h in growth medium. Cells were transferred into growth media containing 400 μg/ml G418 (Roche, USA) and cultured for 14 days to select positive cells. The cells used for the following experiments were named as A549/pUC57-HRE-JAB1 and control cells as A549/pUC57-HRE.

*Quantitative real-time RT-PCR (qRT-PCR)*

Total RNA was extracted from cells with TRIzol reagent and the first-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen). The expression of mRNA for human JAB1 was examined by qRT-PCR using SYBR green-based assays. The primer used for JAB1 is: forward: 5'-GCTGCGGTGCAGTGGCTTCA-3'; reverse: 5'-GTGCCCAATGTGGGAGGCGG-3'. As an internal control, β-actin was analyzed in parallel by using the following primers: forward: 5'-GCGAGGAGAACAAGGG-3'; reverse: 5' - TGG CTG TGC GCAGGT -3'. As an internal control, β-actin was analyzed in parallel by using the following primers: forward: 5' - GTG GGG CGC CCCAGGCACCA -3'; reverse: 5' - CTTCCTTAATGTCACGCACGATTTC -3'.

*Western blot analysis*

A549 cells were washed twice with phosphatebuffered saline (PBS) prior to lysis in pre-cooled RIPA lysate (Pierce, USA) containing protease inhibitor cocktail. Protein concentrations were determined by Bradford protein assay (Bio-Rad, USA). The whole cell lysates (50$\mu$g) were separated with 12% SDS-PAGE and transferred to PVDF membrane. Then, the membranes were blocked 4 h with 5% milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween 20). The membrane was then incubated with mouse anti- human JAB1, mouse anti- human Caspase-3 monoclonal antibody, mouse anti- human cleaved Caspase-3 (BD Biosciences, USA) (1: 500), or mouse anti- human β-actin antibodies at 4 °C over night, and HRP-conjugated goat anti- mouse IgG for an additional 1h at room temperature. After washing three times in TBST, enhanced chemiluminescence reagents (ECL) were used for the final detection.

*MTT assay*

A549/pUC57-HRE-JAB1, A549/pUC57-HRE and A549 cells were treated with C-DDP for 48 h. After 20 μl MTT (5 mg/ml) (Sigma, USA) was add to each well, cells were cultured in an anoxic environment (0.3% $O_2$) in a hypoxia glove box at 37 °C for 24 h.Then, the plates were centrifuged and the supernatant was discarded, and 150 μl of DMSO was added. When the formazan crystals

were dissolved, the optical density (OD) at 492 nm was measured using a microplate reader.

*Cell cycle and apoptosis analysis*

Cell cycle and apoptosis of the A549 cells after treatment were determined by FACS. Briefly, A549, A549/ pUC57-HRE, A549/pUC57-HRE-JAB1 cells were sub-cultured in three plates respectively and cultured for 48 h, then washed with PBS and fixed in 70% ethanol and stained with PI solution (50 μg/ml propidium iodide, 1 mg/ml RNase). Cell cycle was determined by a flow cytometry (FACScan, Becton Dickinson, USA). To quantify apoptosis percentage, cells were stained with Annexin-V and PI using a Vybrant Apoptosis Assay Kit (Invitrogen, USA) according to the manufacturer's instructions.

*Statistical analysis*

All data were expressed as mean ± standard deviation (SD). Comparisons between two groups were made with unpaired Student's t-test. Non-parametric comparisons between three or more groups were made with ANOVA followed by Kruskal–Wallis post hoc analysis. $P < 0.05$ was considered statistically significant.

## Results

*JAB1 mRNA and protein level up-regulation after pUC57-HRE-JAB1 transfection into A549 cells in hypoxia*

To investigate whether pUC57-HRE-JAB1 introduction into A549 cells would up-regulate JAB1 mRNA and protein level under hypoxia condition, A549 cells were transfected with the expression plasmid pUC57-HRE-JAB1. The levels of JAB1 mRNA and protein in transfected A549 cells were then analyzed by real-time PCR and western blot. Contrast to the group that transfected with the empty vector, pUC57-HRE, the levels of JAB1 mRNA and protein expression were increased in the group that transfected with the expression plasmid of JAB1 (Figure 1, 2). These results demonstrated that pUC57-HRE-JAB1 introduction could significantly promote JAB1 expression in a hypoxia environment.

*Upregulation of JAB1 inhibited cell proliferation*

After the transfection of pUC57-HRE-JAB1, the cell number began to increase at the second day. However, the cell was significantly less than that in A549 and A549/ pUC57-HRE at the corresponding time point (Figure 3), suggesting pUC57-HRE -JAB1 efficiently inhibited the proliferation of A549 cells.

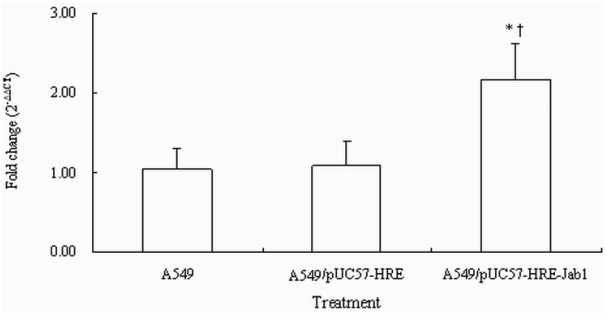


**Figure 1. Effect of pUC57-HRE-JAB1 Introduction on mRNA Level of JAB1 in A549 Cells.** PUC57-HRE-JAB1 introduction significantly increased JAB1 mRNA level compared with the control. And, there was no significant difference between A549 and A549/ pUC57-HRE, suggesting no effect of plasmid pUC57-HRE on the expression of JAB1. *P<0.05 versus A549; †P<0.05 versus A549/pUC57-HRE

*Effect of pUC57-HRE -JAB1 on cell cycle and apoptosis of A549 cells*

The FACS result showed that the cell number was significantly increased in $G_0/G_1$ and decreased in $G_2/M$ phase after the transfection of pUC57-HRE-JAB1. Further, PI in the A549/ pUC57-HRE-JAB1 was significantly decreased compared with that in the A549 and A549/ pUC57-HRE ($P<0.05$), suggesting the pUC57-HRE -JAB1 introduction arrested the cell cycle mainly in $G_0/G_1$ phase (Figure 4, Table 1).

In the presence of C-DDP, the cells were significantly increased in $G_0/G_1$ and decreased in $G_2/M$. The cells were further increased in $G_0/G_1$ and decreased $G_2/M$ after treatment of pUC57-HRE-JAB1 introduction in combination with C-DDP. The PI in A549 cells was also significantly down-regulated after the treatment of pcDNA-HRE-JAB1 introduction in combination with C-DDP (Figure 4, Table 1).

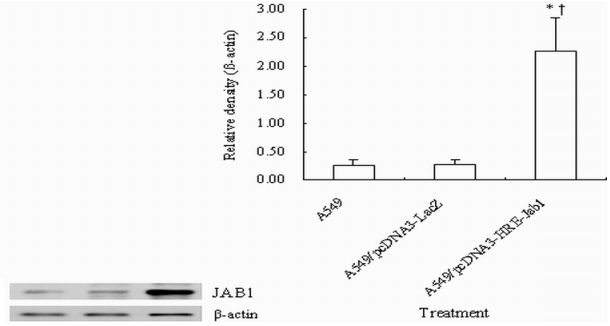


**Figure 2. Effect of pUC57-HRE-JAB1 Introduction on Protein Expression of JAB1.** The expression of JAB1 protein after pUC57-HRE-JAB1 introduction significantly higher than that in the control. However, the simple pUC57-HRE introduction could not up-regulate the expression of JAB1 protein. *P<0.01versus A549; †P<0.01versus A549/ pUC57-HRE

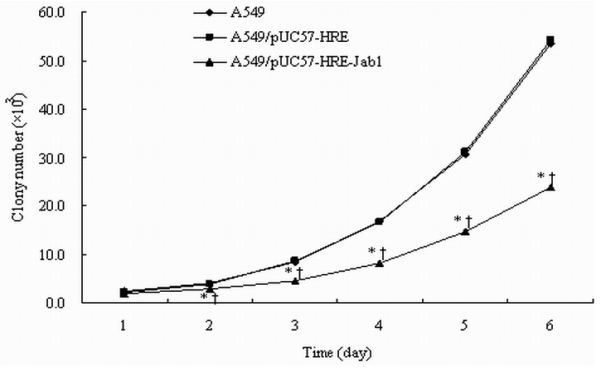


**Figure 3. pUC57-HRE-JAB1 Introduction Suppressed the Cell Proliferation.** Cells were digested by 0.1% trypsin, washed twice with PBS and stained with trypan blue. Finally, the live cells were counted. From the second day after the transfection, the cell number after pUC57-HRE-JAB1 introduction was significantly less than that in the A549 and A549/ pUC57-HRE. *P<0.01versus A549; †P<0.01versus A549/ pUC57-HRE

**Table 1. Effect of pUC57-HRE-JAB1 Introduction on Cell Cycle Distribution of A549 Cells**

| Treatment | G0/G1 (%) | S (%) | G2/M (%) | PI (%) |
|---|---|---|---|---|
| A549 | 50.6 ± 3.8 | 40.8 ± 2.5 | 8.6 ± 1.5 | 49.1 ± 5.2 |
| A549/pUC57-HRE | 50.3 ± 2.7 | 39.7 ± 1.8 | 10.0 ± 1.4 | 49.2 ± 3.8 |
| A549/ pUC57-HRE -JAB1 | 59.9 ± 4.5* | 35.3 ± 2.4 | 4.8 ± 1.0* | 38.3 ± 3.6* |
| A549+C-DDP | 61.3 ± 5.5* | 36.4 ± 3.9 | 2.3 ± 0.5* | 38.1 ± 4.3* |
| A549/ pUC57-HRE +C-DDP | 61.1 ± 5.4* | 36.2 ± 3.4 | 2.7 ± 0.6* | 37.6 ± 5.1* |
| A549/ pUC57-HRE -JAB1+C-DDP | 72.6 ± 6.2** † ‡ | 27.3 ± 2.5* † | 1.1 ± 0.2** † ‡ | 27.6 ± 2.3** † ‡ |

*P<0.05, ** P<0.01 versus A549; †P<0.05 versus A549/pcDNA-HRE-JAB1; ‡P<0.05 versus A549+C-DDP; PI= (S+G2M)/ (G0/G1+S+G2M)

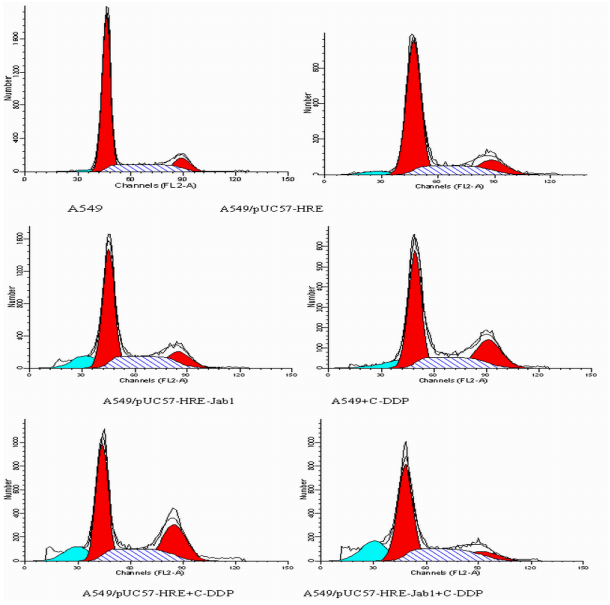


**Figure 4. Effect of pUC57-HRE-JAB1 Introduction on Cell Cycle**

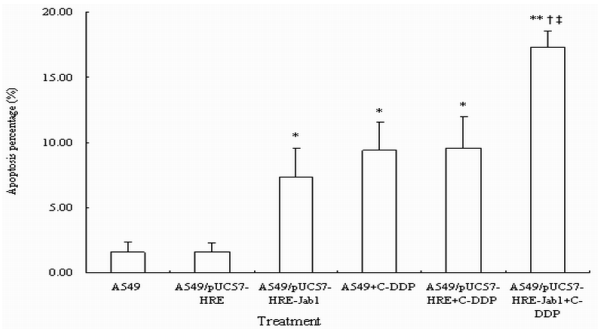


**Figure 5. pUC57-HRE-JAB1 Introduction Promoted the Cell Apoptosis.** PUC57-HRE-JAB1 introduction significantly increased the apoptosis of A549 cells. But the introduction of pUC57-HRE could not promote the cell apoptosis. C-DDP could also markedly induce the cell apoptosis. However, the additional pUC57-HRE introduction could not further increase the cell apoptosis percentage. Interestingly, the pUC57-HRE-JAB1 introduction further enhanced C-DDP-induced apoptosis effect in the A549 cells. *P<0.05, ** P<0.01 versus A549; †P<0.01 versus A549/ pUC57-HRE -JAB1; ‡P<0.01 versus A549+C-DDP

pUC57-HRE-JAB1 introduction significantly increased the apoptosis of A549 cells. Meanwhile, C-DDP markedly induced the cell apoptosis. Further, pUC57-HRE -JAB1 introduction significantly amplified C-DDP-induced apoptosis effect in the A549 cells (Figure 5).

*pUC57-HRE-JAB1 introduction increased the protein expression of Caspase-3 and cleaved Caspase-3 in A549 in a hypoxia condition*

The western blot result showed that pUC57-HRE-JAB1 introduction significantly increased the expression of Caspase-3 protein in A549 in the absence or presence of C-DDP. For cleaved Caspase-3 is a crucial signal molecule for the initiation of apoptosis, thus pUC57-HRE-JAB1 promoted the cell (Figure 6).

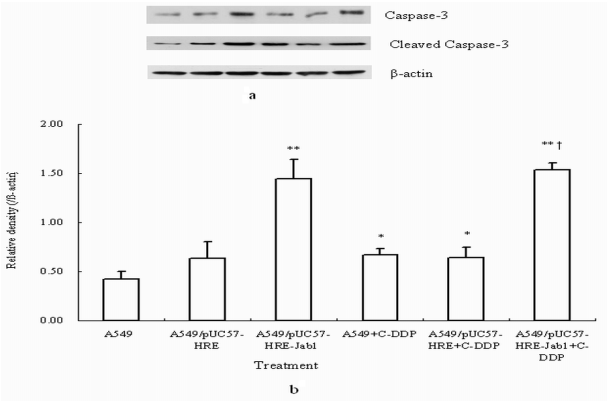


**Figure 6. pUC57-HRE-JAB1 Introduction Promoted Cell Apoptosis.** For cleaved Caspase-3 is a crucial signal molecule for the initiation of apoptosis, we detected the expression of Caspase-3 and cleaved Caspase-3 protein in A549 prior to and after the transfection of pUC57-HRE-JAB1. Western blot result suggested that the pUC57-HRE-JAB1 introduction significantly overexpression of Caspase-3 and cleaved Caspase-3 in A549. And, the pUC57-HRE-JAB1 introduction also further enhanced the apoptosis effect in the presence of C-DDP. *P<0.05, ** P<0.01 versus A549; †P<0.01 versus A549+C-DDP

*pUC57-HRE-JAB1 introduction increased sensitivity of A549 to C-DDP in a hypoxia condition*

To study the effects of JAB1 on the sensitivity of A549 cells to chemotherapeutic agent, we respectively transferred pUC57-HRE-JAB1, pUC57-HRE into the A549 cells using Lipofectamine™ 2000 in a hypoxia environment. Then MTT assay was used to analyze the response of the transfected cells to C-DDP.

Before the transfection, C-DDP significantly suppressed the growth of the A549 cells (P<0.05) (Figure 7). After the transfection, the cells became more susceptible to C-DDP compared with pre-transfection (P<0.05) (Figure 7), suggesting the up-regulation of JAB1 enhanced C-DDP-induced growth inhibition.

## Discussion

An imbalance between oxygen supply and consumption

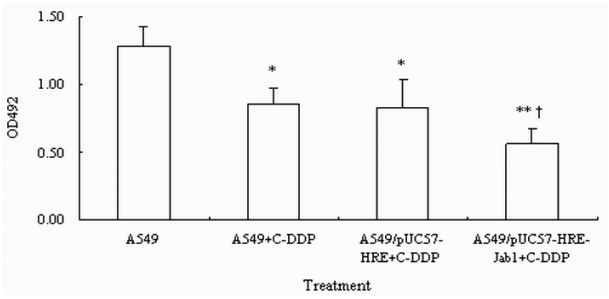


**Figure 7. Effect of pUC57-HRE -JAB1 Introduction on the Sensitivity of A549 to C-DDP.** Prior to the transfection, 2 μg/ml C-DDP significantly suppressed the growth of the A549 cells. After the transfection, the cells became more susceptible to C-DDP and the cell growth was further suppressed significantly. *P<0.05, ** P<0.01 versus A549; †P<0.05 versus A549+C-DDP

will result in the occurrence of tissue hypoxia due to active cell proliferation and an inefficient vascular supply, which is associated with the development of tumor and resistance to chemotherapy (Yasuda, 2008; Lara et al., 2009).

Previously, enhancement of chemotherapeutical susceptible genes by transfection was used to reduce the chemotherapeutical resistance of tumors. Although it did work in some degree, it could not obtain satisfactory results for low tranfection efficiency and target specificity.

In our previous study, we confirmed that cationic liposome-mediated gene transfection of p16INK4a and p14ARF genes into human lung cancer cell line A549 significantly enhanced the apoptosis of cells and chemotherapeutic sensitivity, whereas it did not achieve the desired therapeutic effect (Xie et al., 2005). The increased p16INK4a immunopositivity in HSIL and SCC appears in line with the integrated existence of the hrHPV and may provide more insightful information on risk of malignant transformation of cervical squamous intraepithelial lesions than mere hrHPV detection (Cheah, et al., 2012). The main reasons may be that these genes have a dual role in cell cycle regulatory pathway, either inducing cell apoptosis, or blocking cell cycle progression, while the latter is obviously unfavorable for killing cancer cells completely.HIF, an oxygen-sensitive heterodimeric transcription factor that promotes the expression of genes containing hypoxia response element (HRE) (Semenza, 1999; Semenza, 2000). HIF-1 binds to hypoxia-responsive element (HRE) in the enhancer region of its target genes and initiates gene transcription when mammalian cells are subjected to hypoxia. The core consensus sequence for HRE has been identified as (A/G) CGT (G/C) (Semenza et al., 1996). Down-regulated HIF-1α expression induced by siRNA could effectively suppress the growth of transplanted ESCC in vivo. HIF-1αsiRNA could enhance the cytotoxicity of cisplatin, which suggests that a combination of these two agents may have potential for therapy of advanced ESCC (Liao et al., 2012). Several studied have shown that HIF-1/HRE can be successfully used as hypoxia-regulated tool for gene therapy in a hypoxic environment (Ruan et al., 2001; Post and Van Meir, 2001). Thus, we subcloned JAB1 fragment and constructed the pUC57-HRE -JAB1 plasmid, and then introduced it into A549 cells to investigate the effect of JAB1 on the chemotherapeutic resistance.

Recently, JAB1 has been confirmed to be one specific apoptotic gene for tumor cells. And, it does work via regulating elongation factor E2F1 directly. E2F family genes are terminal factors in the "p16-CDK4/6-cyclin D-Rb-E2F" cell cycle control pathway, and E2F1 is also a p14ARF upstream regulator, which can enhance the ability of p53 to promote apoptosis by promoting the expression of p14ARF.

In 1996, Claret et al. screened out JAB1 from a human lymphocyte cDNA library using a yeast two-hybrid system (Claret et al., 1996). Then a subunit of COP9 signalosome (CSN) was found to be an autoploid of JAB1 in Arabidopsis thaliana (Tomoda et al., 2002). JAB1 could specifically interact with p27kip1 and enable p27kip1 to shuttle and move to the cytoplasm, thereby reducing intracellular p27kip1 content via accelerating its degeneration (Kouvaraki et al., 2003; Shintani et al., 2003). Studies have furthermore shown that JAB1 was expressed in many tumors such as pancreatic cancer (Kouvaraki et al., 2006), lung cancer (Osoegawa et al., 2006), lymphoma (Wang et al., 2007), and etc. It has been reported that p27kip1 level was significantly reduced after the introduction of Jab1 gene into breast cancer cells (Kouvaraki et al., 2003). Further, the reduced expression of p27kip1 was closely related to the proliferation activity and invasiveness of laryngeal squamous cell carcinoma (LSCC) (Tamura et al., 2001). And, the proliferation was associated with the development and prognosis of the tumor. In fact, the role of JAB1 in apoptosis is disputable. Liu et al. found that co-expression of JAB1 and Bcl-Gonad short form (BclGs) synergistically induces apoptosis of HeLa cells (Liu et al., 2008). JAB1 could compete with Bcl-XL/Bcl-2 to bind to BclGs, thereby promoting the cells apoptosis. Reversely, RNAi-mediated knock-down of JAB1 gene markedly reduced the proapoptotic activity of BclGs.

In the present study we also found that the overexpression of JAB1 in A549 cells significantly increased the cells apoptosis in the absence or presence of C-DDP, suggesting the apoptotic promotion role of JAB1. And, the cell apoptosis was arrested at $G_0/G_1$ phase, which was consistent to the study of Tomoda et al. in some degree (Tomoda et al., 2004). They found that Jab1-/- embryonic cells lacking other CSN components expressed comparatively higher levels of p53, p27, and cyclin E, thereby resulting in impaired proliferation and accelerated apoptosis. However, the amount of Jab1-containing small subcomplex in Jab1+/- mouse embryonic fibroblast cells was decreased, they proliferated poorly, and the cell cycle was delayed in the progression from $G_0$ to S phase compared with the wild-type cells. All the findings suggested that Jab1 might control cell cycle progression and cell survival via regulating multiple cell cycle signaling pathways (Tomoda et al., 2004).

In summary, as a specificity factor for E2F1-induced apoptosis, JAB1 overexpression enhances chemotherapeutic sensitivity of lung cancer cell line A549 in an anoxic environment, which might be associated with the increased cell apoptosis. Importantly, it might have the potential to improve the efficacy of chemotherapy in the treatment of lung cancer in the clinic.

## Acknowledgements

This study was supported by a grant of Natural Science Foundation of China (No. 30801366).

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