Knockdown of UBR5 Chemosensitizers Human Laryngeal Carcinoma Cells *In Vitro* through Inhibition of NBS1

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**Abstract**

**Background:** Laryngeal cancer is a common malignant tumor with low chemosensitivity and generally poor response rates. The ubiquitin ligase E3 component n-recognition 5 (UBR5) correlated with prognostic implications in many neoplasm’s, while its role in laryngeal cancer remains to be elucidated.

**Methods:** Immunohistochemistry was performed to measure UBR5 expression in laryngeal cancer and adjacent normal tissues. Differential transcriptional and protein expression were measured using Real-time PCR and Western Blot. The UBR5 gene was silenced with small interfere RNA in laryngeal cancer cells. In addition, proliferation and apoptosis level were measured using MTS assay and flow cytometry. Immunofluorescence was used to analyze DNA damage of UBR5 silencing laryngeal cancer cell induced by chemotherapy.

**Results:** Elevated UBR5 expression is found in laryngeal cancer tissues compared with adjacent normal tissues. After silencing UBR5, cell proliferation and growth *in vitro* were significantly suppressed compared to the control group. In addition, chemotheraphy efficiency in control cells showed a significantly higher *in vitro* proliferation rate than si-UBR5 cells. Mre11 complex subunit protein NBS1 was slightly downgraded after interference with UBR5, while expression level significantly increased after chemotherapy drug process. Silencing or treated with cisplatin (DDP) did not elevate the expression of γ-H2AX in laryngeal cancer cell, while combining UBR5 silencing with DDP treatment significantly increase γ-H2AX expression.

**Conclusion:** Our study has shown that UBR5 is highly expressed in laryngeal carcinoma tissues, and that down-regulation of UBR5 in laryngeal carcinoma cells may reduce sensitivity to chemotherapy.

Together, these findings demonstrate that UBR5 plays a role in regulating sensitivity to chemotherapy in laryngeal cancer, and therefore highlight possible avenues for the development of new therapeutic strategies and targets for the treatment of this disease.

**Keywords:** UBR5; Laryngeal cancer; Chemosensitivity; DNA damage; Apoptosis

**Introduction**

Laryngeal cancer is a common malignant tumor of the head and neck and accounts for 5.7% to 7.6% of all malignant tumors [1]. Approximately 40% of patients present with late stage tumors (stage III-IV) at the time of diagnosis [1,2]. A comprehensive treatment strategy has been developed for laryngeal cancer that includes surgical resection combined with radiotherapy and chemotherapy [3]. However, despite this comprehensive treatment regimen, tumors often demonstrate low chemosensitivity and response rates are generally poor, especially in patients with advanced and recurrent cancer [4]. Moreover, the patient’s quality of life can be severely affected as a consequence of treatment, which can result in difficulties with speech and swallowing [4,5]. Therefore, improved strategies for the prevention and treatment of laryngeal cancer (including the exploration of therapeutic targets) are greatly needed, and this represents an important and pressing issue in the field of biomedical research. The Ubiquitin Proteasome System (UPS) is a regulator of protein homeostasis and cellular signaling. Defective UPS can lead to abnormalities in protein expression, interaction and cellular localization [6]. Of the three known components
of the UPS, the E3 ubiquitin ligases are primarily responsible for determining substrate specificity and ubiquitin chain topology [7]. Recently, targeting E3 ligases as a strategy for cancer treatment has attracted significant interest [6,7]. Although the functions of the E3 ligase family are complex, these proteins warrant detailed study given their potential as a class of biomarkers in laryngeal and other cancers.

The E3 ubiquitin protein ligase UBR5, also known as EDD, is a nuclear phosphoprotein involved in the regulation of DNA damage responses, beta-catenin activity, metabolism, transcription and apoptosis [6]. It has also recently been identified as a key regulator of the UPS in cancer [8].

Elevated UBR5 expression has been shown to mediate metastasis and cisplatin resistance in triple-negative breast cancer, while high UBR5 expression in ovarian cancer is associated with poor prognosis [9,10]. High expression and sustained activation of UBR5 has been associated with chemotherapeutic drug sensitivity in a variety of tumors [10-12]. UBR5, as a key regulator of cancer cell signaling, may therefore play an important role in regulating sensitivity to chemotherapeutics in such cancer types.

The function of UBR5 in laryngeal carcinoma and its potential role in chemosensitivity remain unclear. In this study, we have performed a screen of UPS-related proteins to identify putative therapeutic targets involved in the pathogenesis of laryngeal cancer. We found that down-regulation of UBR5 expression in laryngeal carcinoma cells results in reduced chemosensitivity, possibly through down-regulation of NBS1 expression, a member of the MRN protein complex. Our study confirms that UBR5 regulates the sensitivity of laryngeal cancer cells to chemotherapy, suggesting that UBR5-related signaling provides a promising avenue of exploration for the development of therapeutic targets for the clinical treatment of this disease.

Methods

Immunohistochemistry (IHC) staining and western blot. Total 100 cases of laryngeal carcinoma tissues and corresponding non-tumor tissues were collected from patients who received curative surgery. The tissue samples were collected and used after obtaining approval from the Ethics Committee of our Institute. Informed consent was obtained from all of the patients who participated in this study according to committee’s regulations. The clinical and tumor tissues were fixed, embedded, sectioned, and deparaffinized. IHC staining was using a Dako Envision System (Dako, USA) following the manufacturer’s protocol. Sections were blocked using serum-free protein block buffer (DAKO, CA, USA) for 30 min, after which they were incubated with anti-UBR5 (1:200, Abcam, USA). The pictures were recorded using a Nikon light microscope, and staining intensity analyzed using Nikon software (Nikon Inc., Melville, NY, USA). Western blot was performed with standard procedure. The following antibodies were used: anti-UBR5 (1:1000, Cat.Ab703311, Abcam, USA), anti-Cleaved Caspase 3 (1:1000, Cat. Ab32042, Abcam, USA), anti-NBS1 (1:1000, Cat.Ab32074, Abcam, USA), anti-ATM (1:1000, Cat.Ab81292, Abcam, USA), anti-ATMIN (1:1000, Cat.Ab191856, Abcam, USA), and anti-GAPDH (1:1000, Cat.Ab181602, Abcam, USA) antibody. RNA Interference and Real-Time Quantitative PCR analysis Small interfere RNAs targeting UBR5 were synthesized and targeting sequences were as follow: CTCGTCCTGTACCTCATTTC. Then si-RNAs were transfected in laryngeal carcinoma cell line. ABI Prism 7900 Sequence Detector (Applied Biosystems) was utilized to realize Real-time PCR reactions. In human laryngeal carcinoma cell line, forward PCR primers of UBR5 and GAPDH was 5'-CTCGTCTGTAGCTACCTTAC-3', 5'-GTGTATCGTGTGGTCTTCCA-3' and 5' CAGTGTCTGCGCCTCCT-3' respectively. Reverse primers was 5'-GATAAGTGATCAAGACGG-3', 5'-TGGGAAAACAACTGATACAC-3' and 5'-AAAGAGCAGGACACTGC-3', respectively.

According to SYBR Green PCR master mix (Applied Biosystems) manufacturer protocols, a total of 10 μL reaction mixture was prepared for each test. Specific PCR operating cycles conditions for UBR5 and GAPDH were performed as follows: denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 20 sec, extension at 68°C for 20 sec, and measurement at 80°C for 20 sec, followed by a final extension at 72°C for 5 min. The relative UBR5 expression quantity was calculated using the 2-ΔΔCt method. Each sample was replicated for three times. MTS Cell Proliferation Assay To measure cell proliferation, 5 x 10^4 cells/well were seeded in a 96-well plate and cultured in a 5% CO2 incubator at 37°C for 24 h. Add 20 μL/well MTS reagent (Cat.Ab197010, Abcam, USA) into each well and incubate for 4 h at 37°C. The OD value of the medium was detected using a spectrophotometer at 490 nm wavelength.

Apoptosis assay

Apoptosis detection assay was performed using Annexin V-FITC Apoptosis Detection Kits (BD, USA) according to the manufacturer’s instruction. In brief, after transfection with siRNAs, cells were washed and collected with phosphate-buffered saline and then resuspended in binding buffer. Then 500 µl cell suspension, 5 µl Annexin V-FITC, and 5 µl propidium iodide solution were added into the test tube. After incubation for 15 min, cell apoptosis was analyzed using a FACS analyzer (BD, USA).

Immunofluorescence 1 x 10^5 transfected cells were plated in each well of a confocal Petri dish. Then cells were fixed by 4% formaldehyde for 30 min and then blocked with BSA at 4°C overnight. After blocking, anti-γ-H2AX (Alexa Fluor 594; red) was added to Petri dish for 4°C overnight. After that, cells were washed twice and then counterstained with 300 nM DAPI for 45 min. The stained cells were examined with Nikon Eclipse E600 fluorescence microscope and photographed with Retiga 1300 Q-imaging camera. The experiment was performed in triplicate.

Results

UBR5 expression pattern in human laryngeal cancer tissues to investigate the potential role of UBR5 in laryngeal carcinoma development, we first examined the expression pattern of UBR5 in laryngeal carcinoma tissue samples and the adjacent non-tumor tissues. Immunohistochemistry (IHC) staining results suggested that UBR5 was over expressed in laryngeal specimens detected, while weakly positive staining was observed in the adjacent non-tumor tissues (Figure 1). Silencing of UBR5 in laryngeal cancer cell by using siRNAs Transcriptome efficiency of si-UBR5-1, si-UBR5-2 and si-UBR5-3 were evaluated in Figure 2A, which revealed significant decreased expression in si-UBR5. Protein level also indicated a significant decrease of UBR5 (EDD) expression in si-groups (Figure 2B).

As shown in Figure 3A, the results indicated that silencing UBR5 obviously suppressed growth of cells in vitro, when compared...
Cisplatin has no significant effect on ATMIN and ATM protein expression levels, which is consistent with previous studies. UBR5 silencing increases DNA damage induced by DDP. To further investigate the mechanism that UBR5 silencing sensitizes laryngeal cancer cell to DDP, immunofluorescence was used to analyze DNA damage of UBR5 silencing laryngeal cancer cell induced by DDP. Variant histone H2AX in cell nucleus will transform into γ-H2AX once upon DNA damage. Therefore, the expression of γ-H2AX in nuclear could be used as a surrogate marker of double-strand breaks and other DNA damage. As shown in Figure 5, UBR5 silencing or treated with DDP did not elevate the expression of γ-H2AX in laryngeal cancer cell. However, combining UBR5 silencing with DDP treatment significantly increases γ-H2AX expression.

Discussion

With the incidence of cancer increasing worldwide, acquired resistance to established therapies represents a significant challenge. Consequently, tumor drug resistance is a field of research that is growing substantially [13]. However, the anti-tumor drugs currently available in the clinic represent an extremely diverse molecular arsenal, with widely differing structures and mechanisms of action. Consequently, combating tumor drug resistance is an extremely challenging task. In particular, the emergence of multi-drug resistance in malignant tumors has led to even greater challenges and an even more urgent requirement for research focused on the mechanisms driving drug resistance [14,15]. Laryngeal cancer is a common malignant tumor in otolaryngology-head and neck surgery, and the application of chemotherapeutic drugs plays an important role in it comprehensive treatment [16]. However, drug resistance remains a difficult challenge that limits the efficacy of treatment strategies that rely on chemotherapeutics [17]. It is becoming increasingly clear that UBR5 plays an important role in a diverse range of cancers and consequently this E3 ubiquitin ligase is attracting growing attention as a potential therapeutic target in the cancer field [18]. Cisplatin is a classical alkylating agent and an established antitumor drug. The underlying mechanism of Cisplatin action relies upon the induction of caspase-3-dependent apoptosis [19]. In this study, we found that apoptosis was increased when cisplatin was administered in conjunction with UBR5 silencing; suggesting that down-regulation of UBR5 can promote cisplatin sensitivity in laryngeal carcinoma cells. These finding also suggest the possibility that elevated UBR5 expression may provide a mechanism of chemoresistance in laryngeal cancer, as has previously been shown in other neoplasms [12,20].

The UBR5 gene encodes an important nuclear phosphoprotein involved in the regulation of the DNA damage response, β-catenin activity, metabolism, transcription and apoptosis [21]. Studies have shown that this gene is localized to chromosome 8q22, a region which is disrupted in a variety of cancers. The UBR5 gene encodes a progestin-induced protein belonging to the Homologous to E6-AP Carboxyl Terminus (HECT) family [22,23]. HECT family proteins function as E3 ubiquitin-protein ligases, targeting specific proteins for ubiquitin-mediated proteolysis [24].

The E3 ligase UBR5 is a key regulator of the UPS in both cancer and development. Recent studies have shown that high UBR5 expression promotes cell proliferation in several cancer types. In addition, UBR5 down-regulates pro-apoptotic MOAP-1 in ovarian cancer, suggesting that UBR5 can confer cisplatin resistance and, therefore, may represent an attractive putative therapeutic target for this disease [11,25]. O’Brien et al. [10] found that UBR5 is an adverse

to the control group. In addition, DDP efficiency in control cells showed a significantly higher in vitro proliferation rate than si-UBR5 cells. Percent of apoptosis and Necrosis results suggested a balanced baseline in siUBR5 cells compared with control cells, and that elevated apoptosis and necrosis percent was significantly in si-UBR5 compared with control cells receiving DDP in Figure 3B. After knockdown UBR5 in sun 899 cells, the FACS data showed an insignificant apoptosis rate. As shown in Figure 3C, knockdown of UBR DDP receiving cells significantly increased the number of apoptotic cells compared with control cells.

In addition, we analyzed the expression of the apoptosis marker cleaved caspase 3 (C-caspase 3) in Figure 3D. The C-caspase 3 protein gradually increases correlated with gradually increased apoptotic rate, which is consistent with the flow and MTS results. As expected, siUBR5 cells exhibited increased apoptosis compared with control cells exposed to DDP process. As shown in Figure 3E, intensity of C-caspase 3 expression is significantly higher in siUBR5+DDP cells than control cells. Taken together, these findings suggest that silencing UBR5 contributed to increase apoptosis of laryngeal carcinoma cells when receiving DDP process in vitro. NBS1 may involve in the Role of UBR5 in laryngeal cancer cell UBR5 has been shown to directly interact with numerous proteins implicated in a wide variety of cellular processes. Here, we found that silencing UBR5 decreased the protein expression of NBS1, which acts as a novel participant of DNA double-strand break repair in many cancers.

As shown in Figure 4, the protein level of NBS1 was slightly downgraded after interference with UBR5, while expression level significantly increased after DDP process. UBR5 interference or

Figure 1: Immunohistochemistry (IHC) staining results suggested that UBR5 was over expressed in laryngeal specimens detected, while weakly positive staining was observed in the adjacent non-tumor tissues.

Figure 2: A) Transriptome efficiency of si-UBR5-1, si-UBR5-2 and si-UBR5-3 were evaluated. B) Protein level indicated a significant decrease of UBR5 (EDD) expression in three si-groups.
prognostic factor for serous epithelial ovarian cancer and that it also modulates cisplatin resistance in vitro.

These results identify UBR5 as a new independent prognostic marker for outcome in serous ovarian cancer. In the present study, we found that down-regulating the expression of UBR5 in laryngeal carcinoma cells can enhance sensitivity to cisplatin chemotherapy. Currently, the treatment of laryngeal cancer is predominantly based on surgery combined with radiotherapy and chemotherapy [5]. Such a strategy has the potential to be highly destructive and patients often demonstrate adverse reactions to therapy. To date, research focused on the development of sensitizing agents for laryngeal cancer has largely been lacking [17]. However, UBR5 enhances cell survival and cisplatin resistance in epithelial ovarian cancer, and is a therapeutic target for this disease. In 2008, UBR5 was identified as a new independent prognostic marker for outcome in serous ovarian cancer, suggesting that pathways involving UBR5, including DNA damage responses, may represent new therapeutic targets for chemoresistant disease [10]. Shearer et al. [7] found that UBR5 is highly conserved in metazoans, has unique structural features, and is implicated in the regulation of the DNA damage response, metabolism, transcription, and apoptosis. Hence, UBR5 is a key regulator of a broad range cell signaling events relevant to cancer biology. Interestingly, in our study, we found that NBS1, a member of the MRN complex, is likely
to be involved in the UBR5-dependent regulation of chemosensitivity in laryngeal cancer. In addition, we found that the expression of the DNA damage marker γ-H2AX was significantly increased in cisplatin-treated cells in which UBR5 expression had been suppressed with siRNA, a finding which is consistent with the latest research demonstrating a role for UBR5/ATMIN in promoting DNA damage repair [26,27].

**Conclusion**

Our study has shown that UBR5 is highly expressed in laryngeal carcinoma tissues, and that down-regulation of UBR5 in laryngeal carcinoma cells may reduce sensitivity to chemotherapy. Inhibition of UBR5 expression may result in cell cycle checkpoint defects or abnormalities in NBS1-mediated DNA damage repair. Together, these findings demonstrate that UBR5 plays a role in regulating sensitivity to chemotherapy in laryngeal cancer, and therefore highlight possible avenues for the development of new therapeutic strategies and targets for the treatment of this disease.

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