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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 protein ligase E3 component n-recognin 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, chemotherapy 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

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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.Ab70311, 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: CTCGTCTTGATCTACTTTATC. 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'-CTCGTCTTGATCTACTTTATC-3', 5'-GTGTATCAGTTTGCTTTCCAA-3' and 5' CAGTGTTCCTGCCTTCTT-3' respectively. Reverse primers was 5'-GATAAAGTAGATCAAGACGAG-3', 5'-TTGGAAAGCAAACTGATACAC-3' and 5'-AAGAAGGCAGGAACACTGC-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, 5×10^3 cells/well were seeded in a 96-well plate and cultured in a 5% CO_2 incubator at 37°C for 24 h. Add 20 uL/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 instructions. In brief, after transfection with siRNAs, cells were collected and washed 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 1×10^5 transected 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 at 4°C overnight. After that, cells were washed twice and then counterstaining 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). *UBR5* silencing sensitizes DDP in laryngeal cancer.

As shown in Figure 3A, the results indicated that silencing *UBR5* obviously suppressed growth of cells *in vitro*, when compared

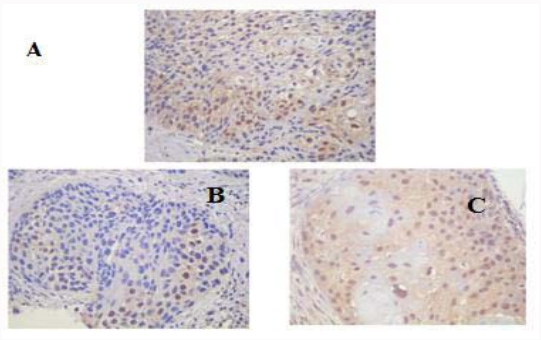

Figure 1: Immunohistochemistry (IHC) staining results suggested that *UBR5* was over expressed in laryngeal specimens detected, while weakly positive staining was observed in the adjacent non-tumor tissues.

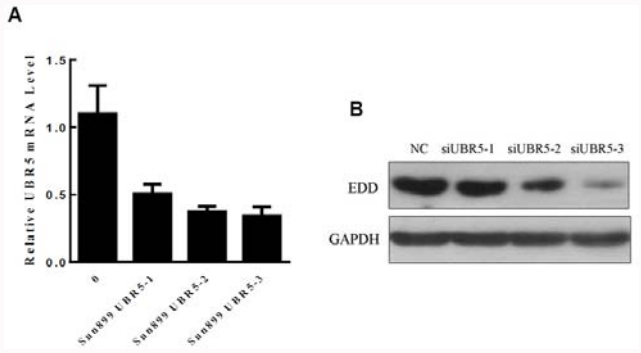

Figure 2: A) Transcriptome efficiency of si-*UBR5*-1, si-*UBR5*-2 and si-*UBR5*-3 were evaluated. B) Protein level indicated a significant decrease of *UBR5* (EDD) expression in three si-groups.

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 si*UBR5* 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, si*UBR5* 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 si*UBR5*+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-string 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 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 sensitize 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 nuclear 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

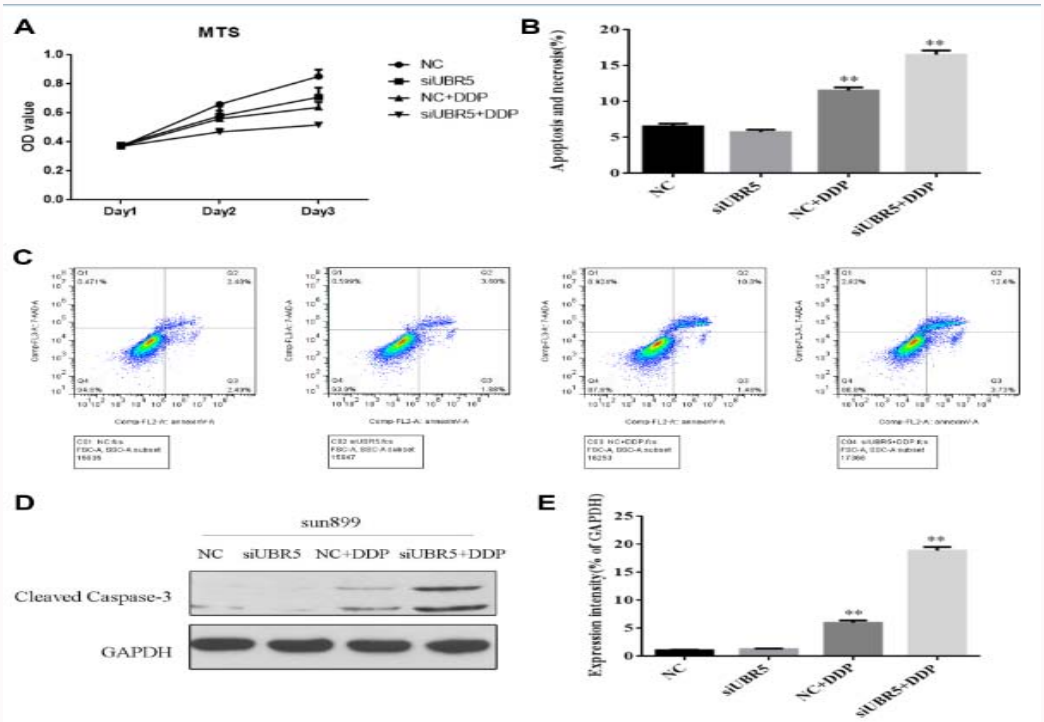

Figure 3: A) Silencing *UBR5* significantly suppressed growth of cells in vitro compared to the control group. B) Percent of apoptosis and necrosis results suggested a balanced baseline in si*UBR5* cells compared with control cells, while that elevated apoptosis and necrosis percent was significantly in si-*UBR5* compared with control cells receiving DDP. C) Knockdown of *UBR5* DDP receiving cells significantly increased the number of apoptotic cells compared with control cells. D) Expression of the apoptosis marker C-caspase 3 was increased and correlated with gradually increased apoptotic rate. E) Intensity of GAPDH expression is significantly higher in si*UBR5*+DDP cells than control cells.

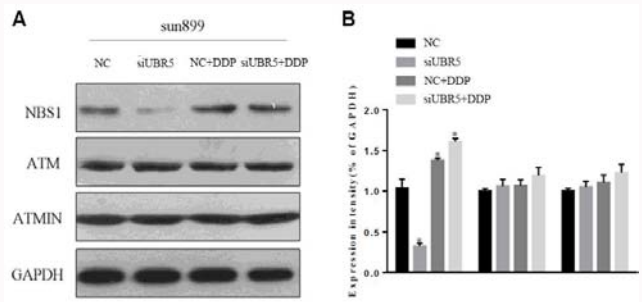

Figure 4: A) Silencing *UBR5* decreased the protein expression of NBS1, which acts as a novel participant of DNA double-string break repair in many cancers. Protein level of NBS1 was slightly downgraded after interference with *UBR5*, while expression level significantly increased after DDP process. *UBR5* interference or cisplatin has no significant effect on ATMIN and ATM protein expression levels, which is consistent with previous studies. B) GAPDH expression intensity baseline.

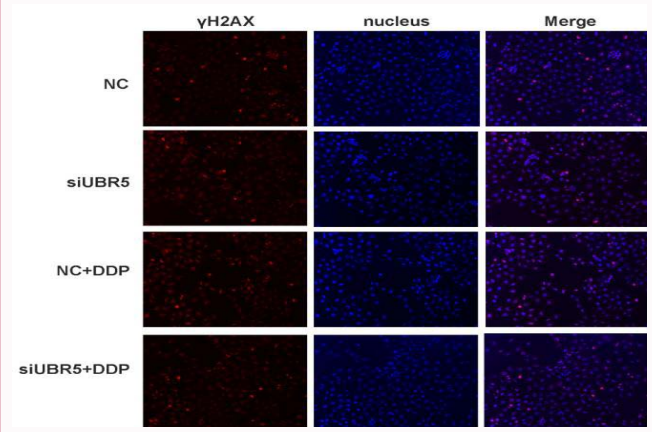

Figure 5: Immunofluorescence indicated *UBR5* silencing or treated with DDP did not elevate the expression of γ-H2AX in laryngeal cancer cell, while combining *UBR5* silencing with DDP treatment significantly increase γ-H2AX expression.

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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