Role of ubiquitin-specific peptidase 22 in carcinogenesis of human pharyngeal squamous cell carcinoma

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Abstract. Human pharyngeal squamous cell carcinoma (HNSCC) are highly invasive and proliferative and exhibit a poor five-year survival rate, mainly due to poor understanding of HNSCC pathogenesis mechanisms, preventing efficient treatment. Ubiquitin-specific peptidase 22 (USP22) is an important component of cell cycle regulation, as it indirectly affects chromatin structure via histone ubiquitination and regulates activation of gene transcription. In previous studies, silencing of USP22 significantly inhibited tumor cell proliferation. To investigate the expression levels and the role of USP22 in the carcinogenesis of human pharyngeal squamous cell carcinoma, pharyngeal squamous cell carcinoma and adjacent normal tissue samples were collected from four patients. Six pharyngeal squamous cell carcinoma cell lines (SAS, CAL-33, FaDu, HSC-4, UTSCC-5 and UTSCC-8) were also included in this study. The USP22 mRNA and protein expression levels in the patient and cell-line samples were evaluated using quantitative polymerase chain reaction and western blotting analyses. Subsequently, stable USP22 gene silencing in cells was achieved using lentiviral-delivered small interfering RNA (siRNA), and an MTT assay was used to evaluate tumor cell proliferation. Expression levels of cell cycle-associated proteins following USP22 knockdown were assessed using western blot analysis. The results revealed that USP22 was upregulated in pharyngeal squamous cell carcinoma. USP22 knockdown, using lentivirus-delivered siRNA, increased the expression levels of cell cycle proteins P21 and P27, but reduced the levels of phosphorylated retinoblastoma protein, resulting in the inhibition of FaDu cell growth and proliferation. In conclusion, USP22 is involved in the carcinogenesis of human pharyngeal squamous cell carcinoma through regulating tumor cell growth and proliferation.

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Introduction

Cancer of the head and neck is the sixth most common type of cancer worldwide (1). Histopathologically, squamous cell carcinoma accounts for >90% of all head and neck cancers (2,3). The predominant risk factors of head and neck squamous cell carcinoma (HNSCC) are tobacco and alcohol consumption, which exert a synergistic effect (4,5). In addition, studies have demonstrated that human papillomavirus is involved in head and neck cancers (3,6).

The poor prognosis associated with HNSCC is primarily due to local invasion, and regional and/or distant metastatic spread. Surgery, radiotherapy and chemotherapy are conventional approaches for the treatment of HNSCC; however, the five-year survival rate is not satisfactory (1). Certain novel approaches, including minimally invasive surgery (7), targeted molecular therapy (8), gene therapy (9) and immunotherapy (10), have been evaluated in order to identify a more efficient treatment. These novel therapy approaches have been promising, but the majority have remained in the early stages of research. Long-term investigation and safety evaluations are required for clinical application of these approaches in HNSCC treatment. Further investigation of the mechanisms of HNSCC carcinogenesis and identification of key factors and molecules involved in HNSCC development and progress may aid in improving HNSCC treatment and reducing mortality.

Head and neck cancer occurs through a complex multistage process, in which carcinogen exposure, through activities such as cigarette and alcohol use, as well as host genetic susceptibility are involved (11). While a number of risk factors have been identified, mainly determined by epidemiological studies, the underlying mechanisms of HNSCC carcinogenesis remain elusive. Activation of the epidermal growth factor receptor, or signal transducers and activators of transcription signaling pathways, and the subsequent overexpression of oncogenes have been demonstrated to contribute to the development and progression of HNSCC (12). In addition, inactivation of tumor suppressor genes, including p27 (13), p16 (14) and FHIT (15), may also contribute to HNSCC pathogenesis.

Ubiquitin-specific peptidase 22 (USP22) is a member of the deubiquitinating enzyme (DUB) gene family and exhibits low

expression in a variety of normal human tissues (16). As a type of DUB, USP22 is the predominant component of the human Spt-Ada-Gcn5-acetyltransferase complex and is required for activating transcription and cell cycle progression by regulating ubiquitination and inducing the acetylation of certain histones (17). This may further activate transcription and translation of a number of cancer-associated genes (17). Overexpression of USP22 was identified in tumors derived from different tissues, as determined by DNA microarray analyses (18). In addition, the expression levels of USP22 were highly correlated with risk of tumor metastasis and patient prognosis (19). Silencing of USP22, accomplished with small interfering RNAs (siRNAs), significantly inhibited tumor cell proliferation and cell cycle arrest (20,21). At present, the pattern of expression and the involvement of USP22 in pharyngeal squamous cell carcinoma is largely unknown. Therefore, in the present study, the expression levels and role of USP22 in pharyngeal squamous cell carcinoma tissues and tumor cell lines were investigated.

Materials and methods

Materials. Four sets of pharyngeal squamous cell carcinoma tissues and normal tissues adjacent to the cancer cells were collected from patients with pharyngeal squamous cell carcinoma (the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China) and stored at -80°C for isolation of total RNA and proteins. The SAS, CAL-33, FaDu, HSC-4, UTSCC-5 and UTSCC-8 human pharyngeal squamous cell carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). TRIzol, Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). MTT and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 6-, 24- and 96-well plates were purchased from Corning Inc. (Acton, NY, USA). The reverse transcription kit and 5-bromo-2-deoxyuridine (BrdU) incorporation cell proliferation kit were supplied by Promega (Madison, WI, USA). Retinoblastoma (Rb), phospho (p)-Rb, P21, P27 and tubulin antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polymerase chain reaction (PCR) primers for USP22 were synthesized by Shanghai Invitrogen Corporation (Shanghai, China). The green fluorescence protein, enhanced chemiluminescence (ECL) kit and lentivirus expression systems were purchased from Guangzhou Yongnuo Biotechnology Limited Company (Guangzhou, China). The research protocol was approved by the Clinical Research Ethics Committee of the Sun Yat-sen University of Medical Sciences (Guangzhou, China) and all patients provided written informed consent.

Culture and passage of human pharyngeal squamous cell carcinoma cell lines. Pharyngeal squamous cell carcinoma cell lines were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C with 5% CO_2 and a relative humidity of 95%. The cells were passaged every 2-4 days and cells in the exponential growth phase were used in the experiment.

Evaluation of cell viability using an MTT assay. FaDu cells in the exponential growth phase ($3x10^4$ cells/ml) were seeded into 96-well plates. Subsequently, 10 μl MTT (5 mg/m1) was added into each well and the cells were incubated for 4 h. The supernatant was subsequently removed and DMSO (100 μl/well) was added to terminate the reaction. The absorbance value at 570 nm was measured using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Western blot analysis. The frozen pharyngeal squamous cell carcinoma tissues and the adjacent normal tissues were homogenized and digested using radioimmunoprecipitation assay lysis buffer (Sigma Aldrich). The lysate was centrifuged at 4°C and 14,000 g for 30 min, whereupon the supernatant was collected and the protein quantities measured using the Bradford method. FaDu cells with and without USP22 knockdown, as determined by lentiviral-delivered siRNA, were cultured in six-well plates for 48 h in order to achieve 80% confluence. The cells were then collected and lysed. Protein quantitation was determined with the Bradford method. Subsequently, the proteins were transferred to polyvinylidene difluoride membranes and electrophoresed at 100 V for 1 h. The membranes were blocked with 0.5% (w/v) non-fat milk at 4°C for 1 h and incubated with primary antibodies (mouse anti-USP22 and -Tubulin; 1:1,000 dilution; Sigma Aldrich) overnight. The membrane was washed twice with 0.1% (v/v) Tris-buffered saline and Tween 20, and incubated with secondary antibodies (goat anti-mouse polyclonal IgG; 1:1,000 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. The membrane was developed using ECL, subsequent to being washed three times.

Quantitative polymerase chain reaction (qPCR) assay. Total RNA was isolated from tissues and cancer cell lines using TRIzol and was then reversely transcribed to cDNA, using RNA reverse transcription kits (Fermentas, Waltham, MA, USA). PCR was performed using the following USP22 primers: Forward 5'-GTGGCACAGTCTCGGCTCAC-3' and reverse 5'-TGGCTCACGCCTATAATCCC-3' under the following conditions: 94°C for 5 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec and 25 amplification cycles; 72°C for 10 min. The PCR products were analyzed using electrophoresis in 2% agarose gels.

Construction and siRNA transfection. The following USP22 interference sequences were designed using the DEQOR program (22): si-USP22-1,5'-GCAAGGCCAAGTCCTGTAT-3' and si-USP22-2, 5'-GGAGAAAGATCACCTCGAA-3'. Loops were added to the two fragments to form a complementary structure. The complementary fragment was then inserted into the pLL3.7 vector (a specific RNA interference vector containing a U6 promoter; Forevergen, Guangzhou, China.) following digestion with *Xho*I and *Hpa*I restriction enzymes (Fermentas). Clones were isolated and sent for DNA sequencing to select vectors containing siRNA.

The constructed interference vectors pLL-USP22-1-siRNA and pLL-USP22-2-siRNA, as well as the corresponding control vector (without siRNA fragments), were mixed with packaging plasmids (pGag/Pol, pRev and pVSV-G, respectively; Fermentas), and co-transfected into FaDu cells (Invitrogen Life Technologies) with Lipofectamine™ 2000. The successfully

transfected cells were identified using enhanced green fluorescent protein fluorescence (Forevergen). Following confirmation of successful transfection, the cells and supernatant were collected at 72 h and centrifuged at 40,000 g for 120 min. The virus-infected cells were then subjected to G418 screening (Mediatech Inc., Manassas, VA, USA) to obtain laryngeal squamous carcinoma cells with stable USP22 interference.

Statistical analysis. SPSS statistical software 19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Values are presented as the mean ± standard deviation. One-way analysis of variance was used to compare the mean values of multiple groups and $P<0.05$ was considered to indicate a statistically significant difference.

Results

USP22 mRNA and protein expression levels in pharyngeal squamous cell carcinoma tissues. Four pairs of pharyngeal squamous cell carcinoma tissues and the adjacent normal mucosa tissues were included to evaluate USP22 protein and mRNA expression levels using western blot and qPCR analyses. As shown in Fig. 1, the expression levels of USP22 protein and mRNA were increased in human pharyngeal squamous cell carcinoma tissues (T) compared with adjacent normal mucosal tissues (N).

Detection of USP22 protein and mRNA expression levels in pharyngeal squamous cell carcinoma cell lines. Protein and mRNA were isolated from six pharyngeal squamous cell carcinoma cell lines (SAS, Cal-33, FaDu, HSC-4, UTSCC-5 and UTSCC-8) and analyzed using western blot and qPCR analyses. As shown in Fig. 2, USP22 protein and mRNA were detected in all six pharyngeal squamous cell carcinoma cell lines. Among the six cell lines, the FaDu line exhibited the highest USP22 protein and mRNA expression levels.

RNA interference-mediated USP22 gene silencing. To investigate the role of USP22 in pharyngeal squamous cell carcinoma, two siRNA fragments, si-USP-22-1 and si-USP-22-2, were designed to be used in the FaDu cancer cell line. The siRNA was delivered by a lentiviral vector to silence the USP22 gene. The stably silenced cells were selected as determined by GFP fluorescence. As shown in Fig. 3A, the cells were successfully transfected with the virus. Western blotting and qPCR results demonstrated that the si-USP-22-1 and si-USP-22-2 fragments effectively inhibited the expression of USP-22 protein and mRNA, compared with the scrambled control (Fig. 3B).

Proliferation of the FaDu cell line is reduced following RNA interference-mediated USP22 gene silencing. To investigate whether RNA interference-mediated USP22 gene silencing affected the proliferation of the FaDu cell line, the MTT assay was used to analyze the viability of FaDu cells after 48 h of culture. As shown in Fig. 4A, the viability of FaDu cells with silenced USP22 was significantly reduced compared with the scrambled control groups ($P<0.05$). The BrdU incorporation assay was used to address whether USP22 silencing inhibited cell proliferation and further reduced the number of FaDu cells. As shown in Fig. 4B, USP22 gene silencing inhibited
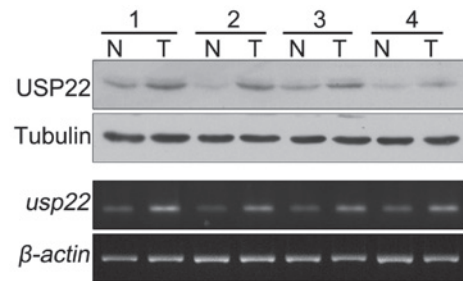

Figure 1. USP22 expression levels in pharyngeal squamous cell carcinoma tissues were higher than those in the adjacent normal mucosal tissues. The top panel shows western blot results of USP22 in pharyngeal squamous cell carcinoma tissues (T) and adjacent normal mucosal tissues (N). Tubulin served as a loading control. The bottom panel reveals quantitative polymerase chain reaction results of USP22 mRNA in pharyngeal squamous cell carcinoma tissues (T) and adjacent normal mucosal tissues (N) where β-actin served as a loading control. USP22, ubiquitin-specific peptidase 22.
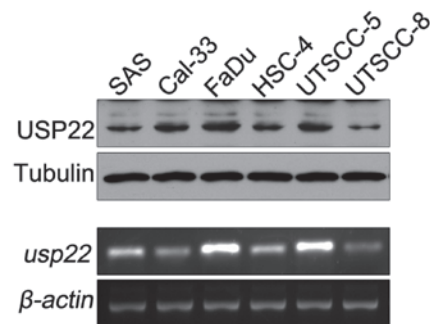

Figure 2. Expression levels of USP22 in different pharyngeal squamous carcinoma cell lines. The top panel shows the expression levels of USP22 protein in pharyngeal squamous cell carcinoma cell lines (SAS, Cal-33, FaDu, HSC-4, UTSCC-5 and UTSCC-8), detected using western blot analysis. Tubulin served as a loading control. The bottom panel shows the mRNA expression levels in pharyngeal squamous carcinoma cell lines, identified using quantitative polymerase chain reaction analysis. β-actin served as a loading control. USP22, ubiquitin-specific peptidase 22.

the proliferation of FaDu cells. This demonstrated that RNA interference reduced the number of cells through the suppression of cell proliferation.

RNA interference affects the cyclin-dependent kinase inhibitor protein (CDKI)/Rb signaling pathway. To elucidate the underlying mechanism by which USP22 gene silencing resulted in inhibition of FaDu cell proliferation, the expression levels of key cell cycle regulatory proteins, including Rb, p-Rb, P21 and P27, were evaluated using western blotting. As compared with the scramble control group, the P21 and P27 CDKIs were significantly upregulated, whereas Rb phosphorylation was markedly reduced in USP22 siRNA-transfected FaDu cells (Fig. 5). The total Rb protein and internal reference tubulin protein levels remained unchanged. These results suggested that inhibition of FaDu cell proliferation by USP22 gene silencing was mediated through the CDKI/Rb signaling pathway.

Discussion

Head and neck cancer is the sixth most common type of cancer in humans. The majority of cases of hypopharyngeal head and
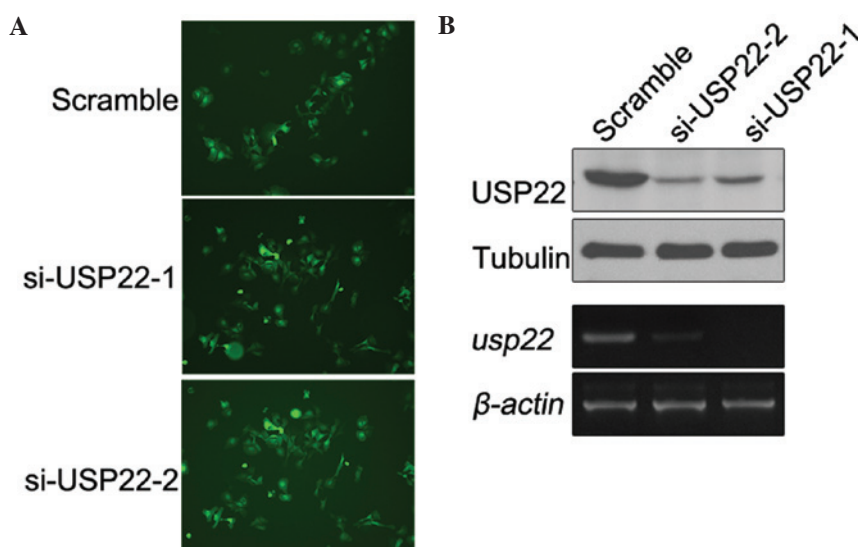
Figure 3. RNA interference mediated by USP22 gene silencing. (A) Enhanced green fluorescent protein-positive cells were observed under a fluorescence microscope (magnification, x200). (B) Western blot analysis of USP22 protein expression levels in transfected cell lines (top panel). Tubulin served as a loading control. Quantitative polymerase chain reaction analysis of USP22 mRNA expression levels in FaDu transfected cell lines (bottom panel). β-actin served as a loading control. USP22, ubiquitin-specific peptidase 22.
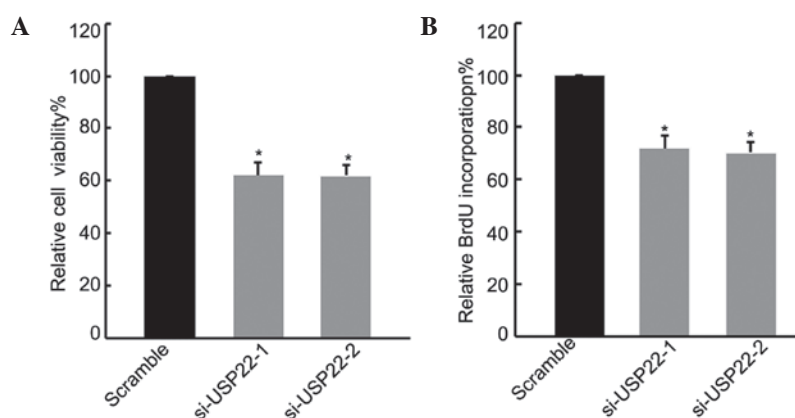

Figure 4. RNA interference inhibited the growth and proliferation of FaDu pharyngeal squamous cell carcinoma cells. (A) Cell viability was measured using the MTT assay. (B) Cell proliferation was measured using a BrdU incorporation assay. The data are presented as mean ± standard deviation (n=3). *P<0.05, compared with the control. USP22, ubiquitin-specific peptidase 22; BrdU, 5-bromo-2-deoxyuridine; si, small interfering RNA.

neck cancer are squamous cell carcinomas, which have a high invasion and growth rate. Currently, surgery, radiotherapy and chemical therapy are used in treatments for hypopharyngeal head and neck tumors. However, the five-year survival rate remains low, which is mainly due to poor understanding of HNSCC pathogenesis mechanisms, preventing efficient treatment (23). In the present study, ubiquitin hydrolase USP22 levels were found to be upregulated in pharyngeal squamous cell carcinoma samples as compared with those in normal adjacent tissue samples. The FaDu pharyngeal squamous cell carcinoma cell line was also observed to express high levels of USP22. Following USP22 knockdown by lentiviral vector-mediated small RNA interference, the expression levels of the cyclin proteins P21 and P27 in FaDu cells were increased, the p-Rb expression levels were reduced and cell proliferation was inhibited. According to these observations, USP22 may be considered a key protein in the carcinogenesis of pharyngeal squamous cell carcinoma. Modification of
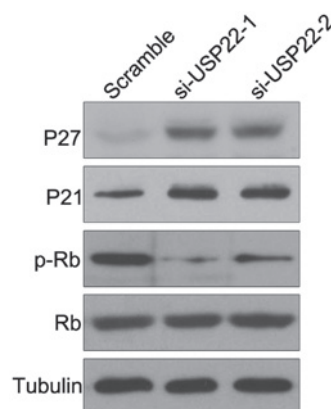

Figure 5. Inhibition of FaDu pharyngeal squamous cell carcinoma cell proliferation by the USP22 siRNA via cyclin-dependent inhibitor protein/Rb signaling pathway. Western blot analysis revealed upregulation of P27 and P21, as well as downregulation of p-Rb in USP22 siRNA-transfected FaDu cells, compared with a scramble control. Tubulin served as a loading control. USP22, ubiquitin-specific peptidase 22; p-Rb, phosphorylated retinoblastoma protein; siRNA, small interfering RNA.

USP22 may provide a novel approach for efficiently treating this type of carcinoma.

The USP22 gene is located on chromosome 17 and consists of 14 exons. The product of the USP22 gene is a type of DUB (16). USP22 has been widely analyzed in a number of human cancer types and has been shown to indirectly affect chromatin structure via histone ubiquitination (H2A and H2B), thereby regulating the transcriptional activation of numerous genes and affecting a wide range of biological functions (17,24). Therefore, USP22 is an important component of cell cycle regulation. Zhang *et al* (17) found that interference with USP22 expression significantly inhibited tumor cell proliferation by cell cycle arrest in the G1 phase. Ovaa *et al* (25) observed that USP22 promoted proliferation and immortalization of human lymphocytes, mainly due to high USP22 expression levels and the resulting effects on cell cycle gene regulation. In addition, Lee *et al* (16) demonstrated that USP22 exhibits periodic expression in mouse embryogenesis, suggesting that USP22 may be involved in the regulation of cell growth and development. Recent studies have shown that USP22 modifies telomeric repeat-binding factor 2, a telomeric DNA binding protein, to affect the balance of telomeres (26,27). In conclusion, USP22 is a cell-signaling molecule important in cell proliferation and differentiation.

Previous studies have demonstrated that USP22 is involved in the tumorigenesis and progress of particular types of cancer, exhibiting a specific expression pattern. As determined by expression analysis using microarray, two studies by Glinsky (28,29) revealed that USP22 expression levels were increased in the majority of tumors and were associated with solid tumor metastasis, drug resistance and patient prognosis. Thus, USP22 was classified as a stem cell marker gene, similar to BMI-1 and cyclinB1 (28). Abnormal USP22 expression levels are considered to be an indicator of malignant transformation and a possible predictor of tumorigenesis and infiltration (30).

In the present study, to the best of our knowledge, USP22 was observed for the first time to be upregulated in squamous cell carcinoma of the pharynx. USP22 may be a key protein involved in the tumorigenesis of pharyngeal squamous cell carcinoma. The results demonstrated that USP22 knockout promoted the upregulation of the cell cycle-associated proteins P21 and P27, thereby inhibiting Rb phosphorylation and pharyngeal squamous cell proliferation. This biological activity of USP22 is consistent with studies demonstrating that USP22 affects chromatin structure and regulates gene transcription activation (31). This is also concurrent with studies revealing the effect of USP22 on the proliferation of pharyngeal squamous cell carcinoma by regulating the cell cycle (32). However, the present study has certain limitations. For example, whether USP22 upregulation in pharyngeal squamous cell carcinoma is associated with poorer prognosis or other clinical parameters remains elusive. To answer these questions, more detailed analysis is required. In conclusion, the results of the present study demonstrated that USP22 is a key protein involved in pharyngeal squamous cell carcinoma, which may provide novel methods for the treatment of this disease.

Acknowledgements

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