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ORIGINAL ARTICLE

Clinical significance of Keap1 in cervical cancer

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Summary

Purpose: *To explore the expression of Kelch-like ECH-associated protein 1 (Keap1) and its clinical significance and function in cervical cancer (CC).*

Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot assays were used to detect the expression of Keap1 in tissues from CC patients as well as CC cells and control cells in vitro. The relationship between Keap1 expression and CC clinicopathologic characteristics was statistically analyzed. Further, effects of Keap1 on the cell proliferation and apoptosis were evaluated through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and flow cytometry assay, respectively.*

Results: *Keap1 expression was downregulated in CC and the expression level of Keap1 was associated with stroma invasion, vascular invasion, parametrial invasion, lymph node metastasis and clinical stage. In in vitro study, upregulation of Keap1 in CC cells by transfection could significantly restrict cell proliferation and promote cell apoptosis.*

Conclusions: *Keap1 was a novel factor involved in CC progression, and constituted a potential biomarker and therapeutic target for the CC patients.*

Key words: *cervical cancer, Kelch-like ECH-associated protein 1 (Keap1), cell proliferation, cell apoptosis.*

Introduction

Cervical cancer (CC) is the second most common malignant tumor in women, of which the squamous cell carcinoma accounts for 80%. There are about 445,000 new cases of CC in the world every year, nearly 270,000 women die of the disease, and over 85% of the deaths occur in developing countries [1,2]. In recent years, the disease appears in younger ages, seriously threatening the life and health of females. Since early CC shows no apparent clinical symptoms, the majority of patients are in advanced stages when diagnosed due to aberrant vaginal bleeding, pelvic pain or dyspareunia, for whom the clinical intervention means are limited [3-5]. Moreover, the prognosis of patients is poorer once they are accompanied with lymph node metastasis. Therefore, exploring the pathogenesis of CC is significantly important for the prevention and treatment of the disease.

Oxidative stress response is a result of the proportion imbalance between production and metabolism of reactive oxygen species (ROS) *in vivo*, which play vital roles in tumors and other diseases [6]. Studies have manifested that the mitochondrial activity was strengthened and metabolism was accelerated in tumor cells during the occurrence and development of tumors. While the growth and proliferation of tumor cells were promoted, massive ROS were produced [6-8]. ROS could induce base modification as well as single and double strand damage, and lead to mutation of nuclear DNA [9]. Besides, the mutation of mitochondrial DNA mediated by histone deletion occurs more easily under the action of ROS [10]. When organisms are exposed to oxidative stress for a long term, multiple signaling pathways would be activated, and phenomena such as instability and mismatching

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of gene expression and activation of oncogenes or inactivation of anti-oncogenes might occur [6]. In addition, ROS-induced protein damage and lipid peroxidation could stimulate the organisms to generate oncogenic active aldehyde [11,12]. Moreover, ROS could affect the mechanism of epigenetic regulation by inducing DNA methylation, thus promoting the expression of oncogenes [13,14].

Kelch-like ECH-associated protein 1 (Keap1) is not only a cytoplasmic protein but also a vital regulatory factor for the signaling pathways of oxidative stress. It acts as an adapter protein combining nuclear factor erythroid 2-related factor 2 (Nrf2) with Cul-3/E3 ligase under normal conditions [15]. According to recent research, Keap1 is abnormally expressed in many types of tumors, implying that it might be involved in the occurrence and development of cancers [16-19]. However, the expression and function of Keap1 in CC was rarely reported. In the present study, the difference in Keap1 expression in the tissue specimens of CC patients was detected, and the influences of Keap1 on the proliferation and apoptosis of CC cells were determined *via* cell experiments *in vitro*, so as to investigate the potential role of Keap1 in the occurrence and development of CC, providing a theoretical basis for the treatment of this disease.

Methods

Tissue specimens collected

The research was approved by the Ethics Committee of People's Hospital of Wuhan University, and informed consent was obtained from patients or their family members. Fresh cancer tissues (n=52) pathologically diagnosed as CC and paired adjacent normal tissues were collected. Chemotherapy, radiotherapy and immunotherapy were forbidden before surgery. The specimens were rapidly placed in cryogenic vials, marked with sample information, cryopreserved in liquid nitrogen until polymerase chain reaction (PCR) assay was performed.

Cell culture and transfection

The CC cell lines (siHa) together with normal human embryonic kidney cell lines (293T) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). After cells were resuscitated, they were inoculated into the Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% newborn fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin and cultured in an incubator with 5% CO_2 at 37°C. After the cells were fused, they were digested with 0.25% trypsin and subcultured. When there were a certain number of cells in the logarithmic growth phase, they were ready for use.

The transfection was performed according to the lipofectamine™2000 instructions (Invitrogen, Carlsbad, CA, USA) after 24-h transfection by LV-negative control (LV-NC) and LV-Keap1. Cells were divided into two groups for further study based on the difference in Keap1 expression.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration was determined through an ultraviolet spectrophotometer. Then, the total RNA was reversely transcribed into complementary DNA (cDNA) according to the steps in the instructions of the RT kit (TaKaRa, Tokyo, Japan), and PCR was conducted with the synthesized cDNAs as the templates and β-actin as the internal reference. Reaction conditions were as follows: 95°C for 10 min, 95°C for 30 s, 95°C for 5 s, 61°C for 30 s and 72°C for 5 min, for a total of 50 cycles. The relative expression level of Keap1 was calculated based on 2-ΔΔCt. Keap1: Forward 5'-CTGGAGGATCATACCAAGCAGG-3', Reverse 5'-GGATACCCTCAATGGACACCAC-3'; Glyceraldheyde 3-phosphate dehydrogenase (GAPDH): Forward 5'-AGCCACATCGCTCAGACAC-3', Reverse 5'- GCCCAATACGACCAAATCC-3'.

Western blots analysis

The siHa cells were harvested after different treatments, and the concentration of the protein sample was measured by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Later, 20 μg of sample were loaded for sodium dodecyl sulfate salt-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was sealed with 5% skim milk powder at 4°C overnight, added with Keap1 antibody (1:1000) for incubation at 4°C overnight and washed in Tris-buffered saline Tween 20, followed by incubation with corresponding secondary antibody on a shaking table for 1 h, routine membrane washing and color development using chemiluminescent reagent. Finally, the relative expression level of protein was calculated with GAPDH as an internal reference.

Cell proliferation

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (0.5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added into the medium of siHa cells in LV-NC group and LV-Keap1 group, and incubated at 37°C for 12, 24, 48 and 72 h separately. Then the supernatant was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added into each well and shaken gently for 10 min for complete dissolution. After that, the absorbance at 450 nm was measured.

Cell apoptosis

According to the instructions of the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit, the cells in the culture plates in LV-NC group and LV-Keap1 group were digested with 0.25% trypsin and then collected. After resuspension, the cells were incubated with Annexin V-FITC and PI in the dark at room temperature for 30 min. Finally, the percentage of apoptotic cells was determined using a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Statistics

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by *post hoc* test (least significant difference). Chi square was performed for the association between Keap1 and clinicopathologic parameters of patients, and the influence of Keap1 on cells was performed with a Student's t-test or F-test. All p values were two-sided and $p<0.05$ was considered significant and analyzed by Prism 6.02 software.

Results

Keap1 was downregulated in CC tissues

The expression level of Keap1 was detected in CC tissues and para-cancer normal tissues. It was worth emphasizing that all tissues were pathologically confirmed to ensure that there were no cancer cells in control tissues. From the PCR results, we found that Keap1 was significantly downregulated in CC tissues, and Keap1 expression in para-cancer tissues was significantly higher than that in CC (Figure 1A).

The relationship of Keap1 with clinicopathologic features

The CC patients were divided into high-expression group and low-expression group according to the median expression level. The association between Keap1 expression and clinicopathologic features of patients was analyzed (Table 1). The expression level of Keap1 was associated with tumor stroma invasion, vascular invasion, parametrial invasion, lymph node metastasis and clinical stage.

Table 1. Keap1 expression and clinical features of patients with CC

Features	*NO.*	*Keap1*		*p*
		high	*low*	
No.	70	35	35	
Age(years)				0.1503
<50	33	20	13	
≥50	37	15	22	
Menopause				0.4403
Yes	48	22	26	
No	22	13	9	
Gravidity				0.3319
<3	29	12	17	
≥3	41	23	18	
Tumor diameter (cm)				0.0534
<4	39	24	15	
≥4	31	11	20	
Keratinization				0.4734
Yes	36	20	16	
No	34	15	19	
Stromal invasion				0.0275
Superficial stroma	9	8	1	
Deep stroma	61	27	34	
Vascular invasion				0.0033
Negative	29	21	8	
Positive	41	14	27	
Parametrial invasion				0.0001
Negative	40	29	11	
Positive	30	6	24	
Lymph node metastasis				0.0041
Negative	22	17	5	
Positive	48	18	30	
Clinical stage				0.0027
I+II	15	13	2	
III+IV	55	22	33	

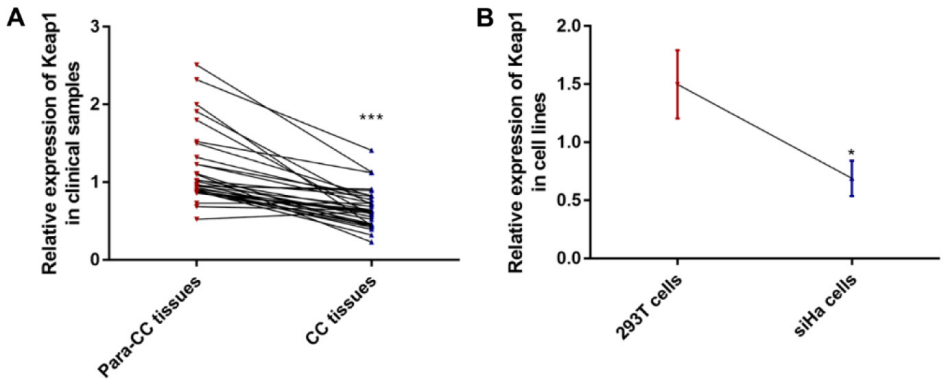

Figure 1. The expression of Keap1 was measured in clinical tissues **(A)** and cell lines **(B)** by qRT-PCR (*p<0.05, ***p<0.001).

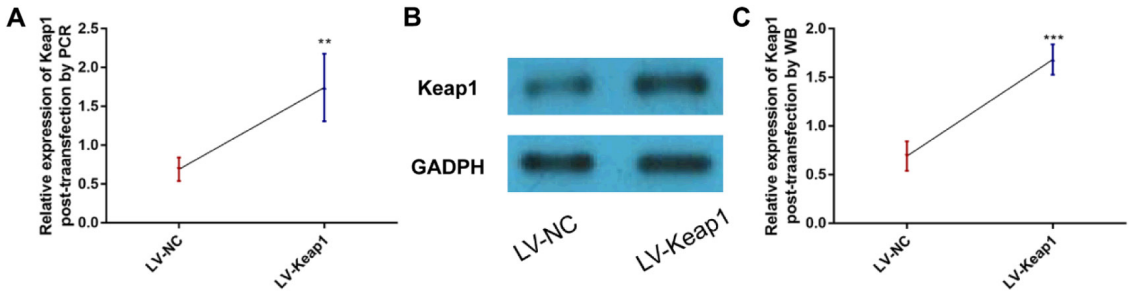

Figure 2. The expression of Keap1 after different transfection was measured in cells *in vitro* by qRT-PCR **(A)** and Western blot **(B-C)** (**p<0.01, ***p<0.001).

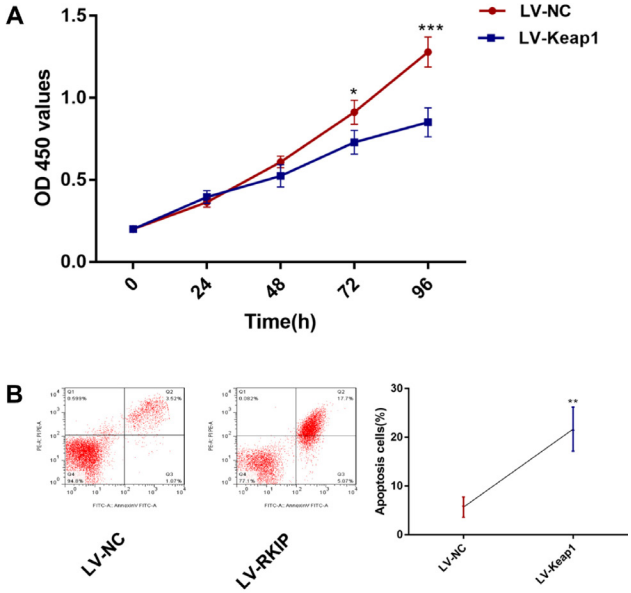

Figure 3. Cell proliferation and apoptosis detected using MTT **(A)** and flow cytometry assay **(B)**, respectively (*p<0.05, **p<0.01 and ***p<0.001).

Upregulation of Keap1 affected cell destiny

To further explore the effects of Keap1 on the biological function of CC cells, CC cell line (siHa) was harvested to performed *in vitro* experiments. In PCR results, we found that the expression of Keap1 in siHa cells also showed low expression status as in the tissue (Figure 1B). Based on it, we established the high Keap1 expressed cells by transfecting LV-Keap1 on siHa cells. PCR and western blot experiments confirmed the reliability of LV-Keap1 transfection (Figure 2).

In MTT assay, we found that the proliferation ability of SiHa cells with high expression of Keap1 was significantly weaker than that of the control group with the passing of time. From the third day, there was a significant difference in the proliferation rate between the two groups (Figure 3A), while in AV/PI apoptosis test Keap1 showed an excellent promoting effect on apoptosis, and the apoptosis rate of siHa cells with high expression of Keap1 was significantly increased (Figure 3B). These results suggested that Keap1 may inhibit the development of CC by interfering with tumor cell proliferation and apoptosis.

Discussion

The mortality rate of CC, a common gynecological malignant tumor in the clinic, ranks second among all the cancers in women [1]. Clinical studies had indicated that the occurrence and development of CC was a multivariate process, and oxidative stress response had close correlations with the occurrence

and progression of tumors. The action mechanism of Keap1 as a newly discovered tumor suppressor protein has not been clarified yet. According to previous reports, Keap1 could bind to Nrf2 as a crucial regulatory factor for oxidative stress response, regulate the expressions of various antioxidant enzymes and phase II detoxifying enzymes in cells and exert important effects in the responses of organisms to exogenous stimuli and oxidative damage [20]. Normally, Keap1 is able to form heterodimers by binding to the Neh2 domain of NRF2 through double glycine repeat (DGR), enhance the effect of E3 uibiquitin ligase (Cul-3) on Nrf2 and accelerate the degradation of NRF2, thus maintaining the expression of NRF2 at a low level in the cytoplasm. Once the amount of NRF2 transferred from the cytoplasm to the nucleus is maintained steady, it would regulate the production of peroxiredoxin and sustain the stability of cells. Exogenous stimuli such as carcinogenic factors and oxidative stress could alter the conformation of Keap1, attenuate the Nrf2-binding ability, reduce the ubiquitination of Nrf2 and increase the expression level in the cytoplasm and the amount of nuclear import. Moreover, it could interact with ARE after conjugating with small Maf protein in the nucleus, so as to initiate the transcription of peroxiredoxin. After the organisms return to a stable state at the end of intracellular oxidative stress response, Keap1 is capable of inhibiting Nrf2. Besides, the inhibition and activation of the Keap1-Nrf2 signaling pathway was under strict control. Once the balance is destroyed by endogenous or exogenous stimuli, Nrf2 would be overexpressed, and the expression of downstream genes would be abnormally active, which not only stimulates the growth of cancer cells but also enhances the resistance of cancer cells to radiotherapy and chemotherapy drugs, thereby facilitating the occurrence and progression of cancers.

In this study it was proved that the expression of Keap1 declined in tumor tissues compared with that in para-carcinoma tissues of CC patients. Further analysis of pathological characteristics indicated that Keap1 expression was associated with stroma invasion, vascular invasion, parametrial invasion, lymph node metastasis and clinical stage. The expression of Keap1 was also decreased in different degrees in other malignant tumors, which was consistent with the experimental results in this study. The reason was that the mutation of somatic cells might result in decrease in Keap1 expression level in tumor tissues. Padmanabhan et al [18] discovered that the mutations of G430C and G364C in DGR can change the conformation of Keap1. Moreover, OTHA confirmed that gene mutation emerges in the major domain of Keap1 in the tumor tissues and cell lines of lung cancer. The mismatching, deletion or insertion of gene in the functional areas could not only induce functional changes in Keap1 but also causes weakened expression of Keap1 [21]. Based on above conclusions, it was concluded that the decline in Keap1 expression in the tumor tissues of CC patients might be induced by mutations. In *in vitro* study, the transfection of LV-Keap1 could reduce the expression level of Keap1 in cells, thus restricting the proliferation and promoting the apoptosis of CC cells.

Conclusions

Our results showed that Keap1 could limit the progression of CC, and Keap1 might become a new diagnostic index and therapeutic target for CC.

Conflict of interests

The authors declare no conflict of interests.

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