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Expression and association of IL-21, FBXL20 and tumour suppressor gene PTEN in laryngeal cancer

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ABSTRACT

Objective: To study the expression of three genes IL-21, FBXL20 and tumour suppressor gene PTEN in laryngeal cancer; analyse the differences in their expression in laryngeal cancer and adjacent tissues; by using pEGFP-N1-IL21 and pGPU/GFP/Neo-FBXL20 expression vectors, to analyse the characteristics in their expression in laryngeal cancer cells outside the body as well as the associations among them.
Methods: The expression of the three genes in laryngeal cancer and adjacent tissues from 30 cases and in normal laryngeal tissues from 20 healthy persons was detected with the RT-PCR; laryngeal cancer cell line (HEp-2 cells) transfection was also performed with the pEGFP-N1-IL21 and pGPU/GFP/Neo-FBXL20 expression vectors we constructed, to detect the mRNA expression of the three genes. Cell proliferation, apoptosis and cell cycle were measured by the MTT assay.
Results: The results of RT-PCR showed that the expression of IL-21 and FBXL20 was up-regulated in laryngeal cancer, while the expression of tumour suppressor gene PTEN was significantly decreased ($p < 0.01$). In HEp-2 cells transfected with pGPU/GFP/Neo-IL-21 and pGPU/GFP/Neo-FBXL20 expression vectors, the mRNA expression of PTEN was restored to some extent ($p < 0.05$); in addition, the ability of HEp-2 cells in proliferation and invasion was also reduced.
Conclusions: IL-21 and FBXL20 genes are important in the occurrence and development of laryngeal cancer; the expression of PTEN gene can suppress laryngeal cancer, and there's a certain association among IL-21, FBXL20 and PTEN.

1. Introduction

Laryngeal cancer is the most common tumour in the head and neck, with an annual incidence accounting for about 2% of global malignant tumours (Chatenoud et al., 2016). As the vast majority of laryngeal cancer cases are derived from cancerous squamous cells in the larynx, laryngeal cancer is also known as laryngeal squamous cell carcinoma (LSCC). The incidence of LSCC in men is much higher than that in women. The men to women ratio is much higher than other head and neck malignant tumours. Most cases are children aged 6–7 (Chatenoud et al., 2016; Forastiere et al., 2017). Smoking and drinking are the most dangerous risk factors for the disease. More than 95% of LSCC patients have a history of smoking and/or alcohol abuse before diagnosis (Steuer et al., 2017). A recent laryngeal cancer study shows that there are currently 142,000 laryngeal cancer patients in the world, and the number is still increasing by 12,500 cases per year (Haapaniemi et al., 2016).

Ubiquitination and inflammatory responses are often associated with cancer (Lipkowitz, 2003). Many studies have shown that ubiquitination and inflammatory factor release are abnormal in cancer patients. FBXL20 gene plays an important role in ubiquitin-mediated cell-regulated protein degradation (Xiao et al., 2015). Interleukin-21 (IL-21) is an important inflammatory response factor secreted by activated CD4+T cells. IL-21 can regulate the proliferation of B cells and thus can to some extent suppress tumours, and its role in cancer treatment has also been widely studied (Korn et al., 2007). However, whether the expression of FBXL20 and IL-21 in laryngeal cancer is associated with

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the expression of tumour suppressor gene PTEN has not been studied.

By using the reverse transcription polymerase chain reaction (RT-PCR) technique and plasmid transfection technique, this article analysed the mRNA expression of FBXL20, IL-21 and PTEN genes in laryngeal cancer and in HEp-2 cells cultured outside the body and explored three potential ties among the three genes, aiming to further understand the pathogenesis of laryngeal cancer and lay a theoretical foundation for the complete cure of laryngeal cancer in the future.

2. Materials and methods

2.1. Cells

HEp-2 cells (Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, CAS) were cultured in an RPMI-1640 medium supplemented with 10% foetal bovine serum regularly.

2.2. Tissue specimens

This study was approved by the ethics committee of XXXX. Subjects participated in this study with informed consent. In XXX hospital, cancer tissues and adjacent tissues from 30 laryngeal cancer patients and normal laryngeal tissues from 20 healthy persons were taken as the tissue specimens.

2.3. Construction of pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 expression vectors

pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 expression vectors were constructed by Shanghai GeneChem Co, Ltd, and the oligonucleotides containing the sequence silencing the FBXL20 were inserted into the pGPU6/GFP/Neo-FBXL20 siRNA vector. Sequence of the oligonucleotides is shown as follows: 5′-CACCGC CAAATGCTTAGCCAATCTTTCAAGAGAAGATTGGCTAAGCATTTTTTTG-3′; 5′-GATCCAAAAAACAATGCTTAGCCAATCTTCTCTTGAAAGATTGG CTAAGCATTTC-3′. Fifty milliliter of competent *E. coli* was prepared in a 1.5 mL EP tube and placed in ice bath for 30 min, in which, later, 5 μL of the pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 expression vectors was added (plasmid containing the gene co-expressing the ampicillin and kanamycin), followed by heating at 45 °C for 2 min. Then, they were immediately transferred in the ice bath for 15 min of incubation, and cultured in the LB medium containing the ampicillin and kanamycin at 37 °C for 1 h on a shaker. Thereafter, 200 μL of the culture was extracted and moved into the LB medium containing the ampicillin and kanamycin, and smeared evenly, followed by 18 h of culture in a thermostat incubator at 37 °C. Colonies were selected for the monoclonal culture, from which the plasmid was extracted by using the Plasmid Extraction Kit provided by Qiagen, Germany. The extracted 5 μg of pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 vectors was mixed in 200 μL optimal medium and 10 μL transfection buffer, and placed in the Hep-2 culture medium for transfection.

2.4. Total RNA extraction

Extract total RNA from laryngeal cancer tissues and adjacent tissues with the TRIzol reagent (Invitrogen Life Technologies). Put tissues in 500 mL the TRIzol reagent and ground them; add 0.2 mL chloroform (Tiangen Biotech Co., Ltd., Beijing, China), followed by centrifugation to collect the supernatant; add isopropyl alcohol, turn it upside down to uniformly mix it, stand at room temperature for 5 min, centrifuge at 12,000 rpm for 10 min, discard the supernatant; wash the precipitate with 70% ethanol, centrifuge at 12,000 rpm for 10 min, dry the precipitate, add 60 μL RNA-free water and incubate at 60 °C for 10 min.

2.5. RNA reverse transcription

Purify the total RNA isolated from laryngeal cancer tissues and adjacent tissues with the Invitrogen RNA purification kit. Mix 1 μL DNase with 26 μL total RNA at 37 °C for 30 min; add 3.33 μL DNase inactivation agent, shake evenly and centrifuge at 12,000 rpm for 2 min, then collect the RNA supernatant. Subsequently, measure the mass and concentration of RNA with the Evolution 201 UV–Visible Spectrophotometer. Do reverse transcription with the Invitrogen reverse transcription kit. Aspirate 1 μL RNA, remove genomic DNA with 4 × gDNA wiper Mix; then add 5 × qRT SuperMix II 4 μL at 50 for 15 min to produce cDNA.

2.6. Quantitative real-time PCR (qRT-PCR)

qRT-PCR is carried out with the SYBR Green. Make melting curve analysis after the completion of the reaction to evaluate the mass of the final PCR product. Calculate the C(t) (cycles to threshold) by fixing the background fluorescence in 0.05 unit. Repeat three times for each specimen and calculate the average C (t) level. Calculate ΔC(t) with the formula C(t) - C(t) GAPDH. The n-time increase or decrease calculated by this formula with the ΔΔCt method uses the C(t)GAPDH value as a reference point. Primers used: PTEN upstream primer 5′-CAGCCAAGTCTGT GACTFGCCGTAC-3′, PTEN downstream primer 5′-12CGCTCGAG CAGTCGCTGCAACCATCCA-3′; FBXL20 upstream primer 5′-GTCTTC GTGGATGTCTTGGAG-3′, FBXL20 downstream primer 5′-CAAGACAAGGTTTGAGTCCGTG- 3′; IL21 upstream primer 5′-GAG ATCCAGTCCTGGCAACA-3′, IL21 downstream primer 5′-AGGGAC CAAGTCATTCACATAA-3′.

2.7. Cell proliferation experiment

Detect cell proliferation with the MTT assay and repeat the experiment for three times. Culture log phase cells in a 96-well plate. Divide these cells into pGPU6/GFP/Neo-FBXL20 siRNA group, pEGFP-N1-IL21 group and HEp-2 cell group, each group containing 6 replicates. Add the MTT reagent (20 μL/mL) into the cell culture plate at four time points (d1, d2, d3, and d4) to measure cell viability in each well. After additional 4 h incubation, remove the medium and then add 150 μL DMSO solution into each well. Calculate the absorbance in each well at 590 nm using a microplate reader (Bio-Rad, Hercules, CA, USA), with the wavelength of 620 nm as a reference, and draw a growth curve.

2.8. Statistical analysis

The mean ± the standard error of the mean (SEM) was used for the statistics of the results. Measurement data were compared by using Student's *t*-test, Fisher's exact test, or ANOVA. $\alpha = 0.05$ was set as the inspection level.

3. Results

3.1. Expression of IL-21, FBXL20 and tumour suppressor gene PTEN in laryngeal cancer tissues, adjacent tissues and normal tissues

IL-21 is an inflammatory factor that is often highly expressed at the lesion. The RT-PCR results showed that the expression of IL-21 in laryngeal cancer tissues was highly up-graded than that in normal tissues ($p < 0.05$, Fig. 1A). The expression was also slightly up-regulated in adjacent tissues, but it was not significant. It may be

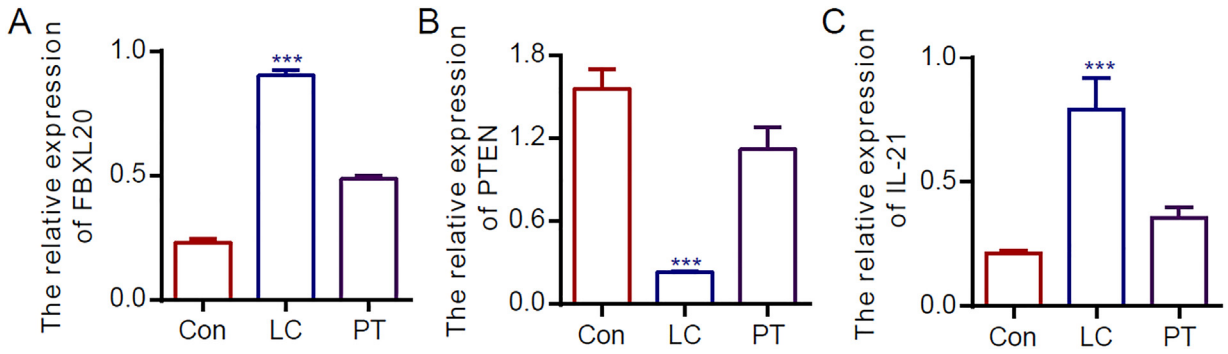

Fig. 1. The expression of FBXL20, PTEN, and IL-21 in normal tissues (Con), laryngeal cancer tissues [8], and adjacent tissues (PT).

because subtle immune responses also occurred in adjacent tissues or IL-21 slightly gathered in adjacent tissues after blood transport. The expression of FBXL20 was significantly elevated in laryngeal cancer tissues, while low in adjacent tissues and in normal tissues ($p < 0.05$, Fig. 1B). On the contrary, tumour suppressor gene PTEN had a low expression level in laryngeal cancer tissues but a high level in adjacent tissues and normal tissues ($p < 0.05$, Fig. 1C). These results indicated the underlying correlation of the high expression of FBXL20 in laryngeal cancer patients with the biological changes of cells, including proliferation and migration and that PTEN may suppress the FBXL20 expression.

3.2. pGPU6/GFP/Neo-FBXL20 siRNA plasmid transfection increased the expression of PTEN

HEp-2 cells were cultured outside the body and transfected with pGPU6/GFP/Neo-FBXL20 siRNA plasmids. Then by RT-PCR, the expression of IL-21, FBXL20 and tumour suppressor gene PTEN in the transfected cells group and in the HEp-2 cells control group was detected. In HEp-2 cells transfected with pGPU6/GFP/Neo-FBXL20 siRNA plasmids, the expression of PTEN was significantly picked up compared with the cultured tumour cells, while the expression of FBXL20 was significantly decreased, indicating that the plasmids could suppress the FBXL20 expression, and the expression of IL-21 was also significantly increased ($p < 0.05$, Fig. 2). The RT-PCR results generated by the in vitro cultured cells indicated that the expression of PTEN inhibited the expression of FBXL20 in HEp-2 cells, and the increased expression of IL-21 was associated with the decreased expression of FBXL20. In order to eliminate some uncertainties, Sanger-PCR sequencing was performed on the above RT-PCR specimens. The sequencing results showed that there were neither other DNA mutations affecting gene promoters, nor abnormal changes in gene methylation. Therefore, it was believed that the up-regulation of PTEN may occur at the transcriptional level, while the up-regulation of FBXL20 may be associated with the development and metastasis of laryngeal cancer.

3.3. pEGFP-N1-IL21 plasmid transfection inhibited the expression of FBXL20

HEp-2 cells were transfected with the pEGFP-N1-IL21 plasmids. Then by RT-PCR, the expression of IL-21, FBXL20 and tumour suppressor gene PTEN in the transfected cells group and in the control group was detected. In HEp-2 cells transfected with pEGFP-N1-IL21 plasmids, the expression of PTEN was not significantly different from that of cultured tumour cells, while the expression of FBXL20 was decreased to some extent, indicating that the high expression of IL-21 to some extent inhibited the expression of FBXL20. The expression level of IL-21 was also very high

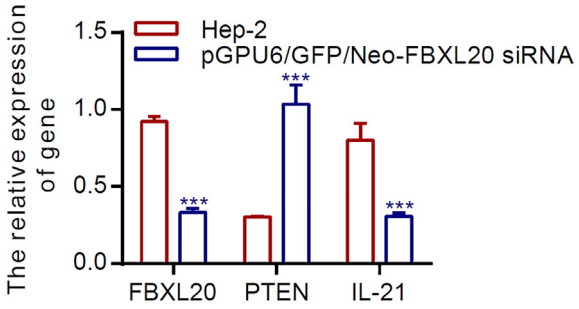

Fig. 2. Expression levels of FBXL20, PTEN and IL-21 in HEp-2 cells before and after transfection with pGPU6/GFP/Neo-FBXL20 siRNA plasmids.

($p < 0.05$, Fig. 3). The above results suggested that the high expression of IL-21 may also suppress the expression of FBXL20. However, the expression of IL-21 was already very high in laryngeal cancer tissues and was just not great enough to reach the threshold of inhibiting FBXL20. The expression of PTEN was not regulated by IL-21.

3.4. pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 plasmid transfection could suppress the growth of laryngeal cancer cells

Besides the finding that the inhibition of FBXL20 could affect the expression of IL21 and PTEN, by using the MTT method, we also found that pGPU6/GFP/Neo-FBXL20 siRNA plasmid transfection could suppress the growth and proliferation of HEp-2 cells. The same phenomenon was also found in HEp-2 cells after pEGFP-N1-IL21 plasmid transfection. After transfected by the two kinds of plasmids, cancer cells had to take a longer time to grow to a same OD, and the pGPU6/GFP/Neo-fbxl20 siRNA plasmids had better performance (Fig. 4, $p < 0.05$).

4. Conclusions

Laryngeal cancer remains the leading cause of cancer deaths around the world. Large numbers of studies have demonstrated the important role of PTEN, FBXL20 and IL-21 in the occurrence of laryngeal cancer. Xiao et al. found that the knockout of Fbw7 and p53 may enhance the invasion and metastasis of adenocarcinoma cells, and the allograft developed into the adenocarcinoma in high malignancy (Falchetti et al., 2005). IL-21 was highly expressed in liver cancer tissues with severe immune responses (Cheng et al., 2013). The deletion of PTEN could induce rectal cancer with an extremely poor prognosis, whereas highly expressed PTEN inhibited the occurrence and development of rectal cancer (Slattery et al., 2010).

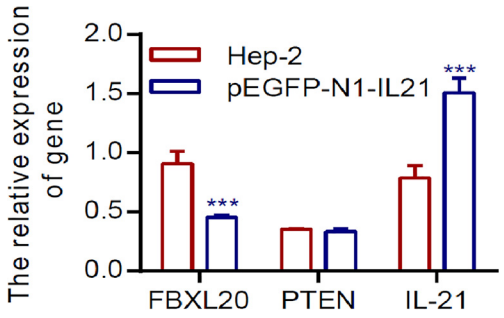

Fig. 3. Expression levels of FBXL20, PTEN and IL-21 in HEp-2 cells before and after transfection with pEGFP-N1-IL21 plasmids.

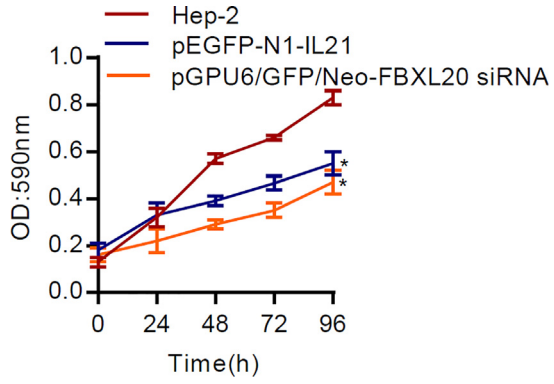

Fig. 4. pGPU6/GFP/Neo-FBXL20 siRNA and pEGFP-N1-IL21 plasmid transfection suppressed the growth of laryngeal cancer cells.

In this study, the proliferative activity of plasmid-transfected HEp-2 cells was significantly reduced than original HEp-2 cells. PTEN and FBXL20 are critical to the regulation of the biological events, and the dysregulation of PTEN is usually concomitant with the poor outcomes (Ortegamolina and Serrano, 2013). When PTEN was highly expressed, the proliferation of HEp-2 cells was significantly reduced, indicating that PTEN to some extent changed the cell function. FBXL20 is a protein that regulates ubiquitination and is an important factor in protein degradation. A previous study showed that FBXL20 initiates the metastasis of invasive colon cancer (Zhu et al., 2012). In addition, according to the experimental results, the inhibiting mechanism of pGPU6/GFP/Neo-FBXL20 siRNA plasmids in HEp-2 cell proliferation was probably because the reduced expression of FBXL20 elevated the expression of PTEN. And the inhibitory effect of pEGFP-N1-IL21 was probably because the highly expressed IL-21 immune factors stimulated local immunity and thereby inhibited cancer cells. In addition, the function of FBXL20 and PTEN was not fully explained in this experiment. But a large number of studies have confirmed that FBXL20, PTEN and IL-21 are closely associated with the activation of the Wnt signalling pathway in cancer tissues (Laguë et al., 2008; Zhang et al., 2018; Zhu et al., 2012). In this study, it was also observed that when comparing to the control group, PTEN was upregulated in the pGPU6/GFP/Neo-FBXL20 siRNA transfection group, suggesting that PTEN, FBXL20 and IL-21 were all likely to exist in the Wnt signalling pathway.

PTEN is a tumour suppressor with many mutations have been found in more than 50% of malignant tumours (Nuala et al., 2016; Zhao et al., 2017). Previous evidence has shown that PTEN may also enhance the apoptosis in addition to its inhibitory effect on the growth of cells. RT-PCR showed that the expression of p53 significantly decreased in the HEp-2 cell line but obviously increased in the cells transfected with pGPU6/GFP/Neo-FBXL20 siRNA plasmids, indicating the importance of PTEN in laryngeal cancer cells. However, the mechanism behind it deserves further study.

In summary, our study showed that FBXL20 and IL-21 genes were overexpressed in human laryngeal cancer. In addition, the inhibition of FBXL20 expression could effectively block the proliferation of laryngeal cancer cells, possibly regulated by PTEN. The expression of FBXL20, IL-21 and PTEN was associated with laryngeal carcinoma. Nevertheless, future work is needed to further prove the association and the relevant mechanism.

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