RESEARCH COMMUNICATION

Altered Expression of miR-21 and PTEN in Human Laryngeal and Hypopharyngeal Squamous Cell Carcinomas

Jun Liu[1,2], Da-Peng Lei[1,2], Tong Jin[1,2], Xue-Ning Zhao[1,2], Guojun Li[3], Xin-Liang Pan[1,2*]

Abstract

Purpose: The purpose of this study was to examine the expression of mir-21 and phosphatase and tensin homologue (PTEN) in laryngeal squamous cell carcinomas (LSCCs) and hypopharyngeal squamous cell carcinomas (HSCCs), and assess correlations between the two as well as with clinical characteristics of patients. Methods: The expression of mir-21 in tumor and adjacent non-tumor tissues was investigated by real-time RT-PCR. Immunohistochemistry (IHC) was carried out to analyze PTEN protein levels. Results: Mir-21 was up-regulated in LSCCs and HSCCs compared to adjacent non-tumor tissues ($P < 0.05$), and the up-regulated expression of mir-21 was associated with clinical stage ($P = 0.001$), T classification ($P = 0.007$), pathologic differentiation ($P = 0.025$), and lymph node positivity ($P = 0.002$). In contrast, PTEN IHC staining was notably weaker in tumor tissues than in matched non-tumor tissues ($P < 0.05$), and the down-regulated expression of PTEN was correlated with tumor staging ($P = 0.025$), the extent of tumor ($P = 0.017$), and lymph node positivity ($P = 0.040$). Furthermore, the level of mir-21 was reversely correlated with PTEN expression ($P = 0.006$). Conclusion: mir-21 and PTEN might play important roles in the progression of LSCC and HSCC, the two fcators demonstrating a negative correlation.

Keywords: MicroRNA-21 - PTEN - laryngeal SCC - hypopharyngeal SCC - China

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Introduction

Head and neck squamous cell carcinomas (HNSCC), including cancers of the oral cavity, oropharynx, hypopharynx and larynx, constitute the fifth common malignancy worldwide (Jemal et al., 2005). Patients with locally advanced HNSCC have a 5-year overall survival rate hovering approximately 30%, underscoring significant opportunities for improving outcome (Forastiere et al., 2006). Hence, there is a need to acquire deeper understanding of HNSCC biology and to develop predictive molecular signatures, which could improve patient selection for appropriate treatment and guide the development and evaluation of new therapies. In the past decades, researchers have focused largely on the protein-coding genes which were classified into two groups: oncogenes and tumor suppressor genes (Gleich and Salamone, 2002). But recently, MicroRNAs (miRNAs), non-coding RNA gene products, have attracted more attentions from researchers.

MiRNAs are endogenous, evolutionarily highly conserved, single-stranded, about 22-nucleotide-long, small non-coding RNA molecules that regulate gene expression by base pairing with target mRNAs at the 3'-untranslated region, leading to mRNA cleavage or translational repression (Bartel, 2004). Emerging evidence has indicated that miRNAs, as a new family of gene regulators, are involved in various biological processes, including cell proliferation (He et al., 2005), differentiation (Ma and Weinberg, 2008), apoptosis (Jovanovic and Hengartner, 2006), stress resistance, and metabolism (Poy et al., 2004). Moreover, several reports showed that miRNAs participated in human tumorigenesis as tumor suppressors or oncogenes by affecting cell growth and development (Gregory and Shiekhattar, 2005). For example, let-7, miR-15 and miR-16 are down-regulated in lung cancer and leukemia (Takamizawa et al., 2004; Calin et al., 2002), whereas miR-155, miR-21 and miR-31 are over-expressed in some tumor samples or tumor cell lines (Habbe et al., 2009; Liu et al., 2009).

MiR-21 is one of the most studied miRNAs in cancers and is up-regulated in many solid tumors, including breast, prostate, lung and stomach carcinomas, glioblastoma, cholangiocarcinoma, and pancreatic endocrine tumor (Cho, 2007). Several recent studies showed that miR-21 was over-expressed in head and neck cancers (Liu et al., 2009; Kimura et al., 2010). As miRNA expression is tissue-specific, the expression signatures could potentially

*[1]Department of Otolaryngology, Qilu Hospital, Shandong University, [2]Key Laboratory of Otolaryngology, Ministry of Health, Jinan, Shandong, China, [3]Department of Head and Neck Surgery, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA *For correspondence: entpanxinl@gmail.com*

suggest biological pathways/mechanisms that differ between the HNSCC subtypes. In the present study, we investigated the expression of miR-21 in laryngeal squamous cell carcinoma (LSCC) and hypopharyngeal squamous cell carcinoma (HSCC), and examined the correlations of its expression with patient clinical characteristics.

Phosphatase and tensin homologue (PTEN) plays an important role in tumorigenesis and reduced PTEN expression is associated with lymph node metastasis, tumor grade, tumor-node-metastasis (TNM) stage, and microvessel density (MVD) (Tamura et al., 1998). Previous reports indicated that PTEN participated in radiosensitivity and chemosensitivity in head and neck cancers (Pattje et al., 2010; Mriouah et al., 2010). In addition, several researchers demonstrated that PTEN was one of the target genes of miR-21 in HCC cell lines (Meng et al., 2007).

The correlation between miR-21 expression and PTEN expression has not been reported in LSCC and HSCC. The correlation may help us understand both function of miR-21 in tumorigenesis and its clinical significance. Therefore, in this study we investigated miR-21 expression and analyzed the correlation between the expressions of miR-21 and PTEN in LSCC and HSCC.

Materials and Methods

Patients and tissue specimens

Pairs of primary tumor tissues and adjacent non-tumor tissues were obtained from 60 patients including 30 LSCC patients and 30 HSCC patients, who were admitted to the Department of Otolaryngology of Qilu Hospital, Shandong University in China from March 2009 to June 2010. All these patients were both male and smokers and drinkers. Patients who had received neoadjuvant chemotherapy or radiation therapy before surgery were excluded from this study. Surgical specimens of the resected tumors and adjacent normal mucosal epithelium tissue, which were at least 2 cm distal to tumor margins, were collected. The resected tissues were divided into two parts, one part was snap-frozen in liquid nitrogen immediately after surgery, and the other was fixed in 10% buffered formaldehyde for pathologic diagnosis and IHC staining studies. Histopathologic diagnosis of LSCC and HSCC was carried out by the Department of Pathology of Qilu Hospital, Shandong University according to the criteria of the World Health Organization. The TNM classification was in accordance with the International Union against Cancer (UICC, 2002) TNM Staging. All samples were collected with informed consent according to the Internal Review and Ethics Boards of the Qilu Hospital.

Real-time RT-PCR

Total RNA enriched with small RNA was isolated from the tissues using TRIzol reagent (invitrogen, USA) according to the manufacturer's instructions. The real-time RT-PCR for miR-21 expression was analyzed by using Taqman miRNA assays and ABI 7900HT Sequence Detection (ABI Applied Biosystems, Foster City, CA). All reactions were performed in a 10μl reaction volume in triplicate. Primers for miR-21 and U6 snRNA were supplied by ABI Applied Biosystems. PCR reaction consisted of an initial denaturation step at 95°C for 30 sec and following amplification by 40 cycles at 95°C for 5 sec, 60°C for 30 sec. The threshold cycle (Ct) is defined as the cycle number at which the fluorescence passed a pre-determined threshold. For expression analysis, the experiment was designed to use the matched non-tumor tissue as the control, and the expression level of miR-21 in tumor tissue was calculated using the equation: amount of target = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{miR\text{-}21} - Ct_{U6})_{tumor} - (Ct_{miR\text{-}21} - Ct_{U6})_{matched\ non\text{-}tumor}$. For the matched non-tumor tissue control sample, $\Delta\Delta Ct$ is zero and $2^{-\Delta\Delta Ct}$ is 1.

IHC staining

IHC staining was performed to study altered protein expression in the 60 tumor samples and 60 matched adjacent normal tissues. Briefly, approximately 4-μm sections of routine formalin-fixed and paraffin-embedded material were deparaffinized with xylene and rehydrated in graded alcohols and distilled water. Antigen retrieval was carried out by microwave treatment of the slides in sodium citrate buffer (pH 6.0) for 25 min. To quench the endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide in methanol for 10 min. After blocking the non-specific binding with 10% normal goat serum for 15 min, the sections were stained with rabbit polyclonal antibody against PTEN (1:50, Boster, China). The antibody was incubated overnight at 4°C and then washed in buffer. After washing, the tissue sections were then reacted with the biotinylated secondary antibody for 15 min and finally incubated with peroxidase-conjugated streptavidin at room temperature for 15 min. Color development was done with the 3, 3'-diaminobenzidine tetrachloride. All slides were counterstained with hematoxylin stain solution. For negative controls, the primary antibody was replaced by normal goat serum. The slides were examined with a standard light microscope independently by two pathologists who had no knowledge of any patient's clinicopathological data. Brown staining in nuclei and cytoplasm was considered as positive immunoreactivity, and was evaluated as percentage staining over the whole preparation. The proportion of PTEN immunostaining cells varied from 0% to 100%, and a four-grade scoring system was used to evaluated the degree of immunostaining: score 0, < 5%; score 1, 5% to 25%; score 2, 25%- 50%; and score 3, > 50% of cells with immunostaining. Low expression was defined as either score 0 or score 1, and high expression was defined as score 2 or score 3.

Statistical analysis

Statistical analysis was performed using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). The relative expression of miR-21 was presented as mean ± standard deviation (SD). The differences of miR-21 expressions between tumor and matched adjacent normal tissue were assessed by the Paired-Student's t test and the differences of PTEN expressions were analyzed by Wilcoxon Singed Ranks Test. Differences between two groups were examined by the Independent Samples t Test

Table 1. Correlations Between Mir-21 Expression and Clinical Characteristics in LSCC and HSCC Patients

Characteristics	Relative expression of mir-21		
	No.	Mean ± SD	P
Clinical Stage			0.001
I-II	21	2.93 ± 1.77	
III-IV	39	5.37 ± 2.57	
T classification			0.007
T1-T2	26	3.41 ± 2.02	
T3-T4	34	5.30 ± 2.73	
Differentiation			0.025
Well	16	3.12 ± 2.11	
Mediate	21	4.67 ± 1.96	
Poor	23	5.35 ± 3.05	
Node metastasis			0.002
N0	35	3.68 ± 2.03	
N1-N2	25	5.69 ± 2.87	

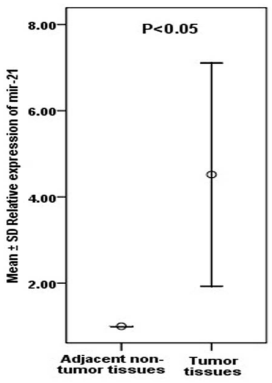

Figure 1. Difference in Expression Levels of miR-21 Between LSCC and HSCC and Matched Adjacent Non-tumor Tissues (Error Bar diagrams with mean and SD)

or Mann-Whitney U test. Multiple group comparisons were analyzed using One-way ANOVA or Kruskall-Wallis test. The criterion for statistical significance was set at $P < 0.05$.

Results

Up-regulated MiR-21in LSCC and HSCC compared to the non-tumor tissues

The mean of relative expression of miR-21 ($2^{-\Delta\Delta Ct}$ in tumor samples and in non-tumor tissue control samples was 4.52 (SD, 2.59) and 1.00 (SD, 0), respectively (Figure1). The Paired-Student's t test showed that miR-21 expression level was significantly higher in tumor specimens than that in the non-tumor tissues ($P < 0.05$). The mean of the relative expression of miR-21 was 5.37 in advanced tumors and 2.93 in early tumors. The difference of the expression of miR-21 between the advanced tumors and the early tumors was statistically significant ($P = 0.001$). In addition, the over-expression of miR-21 was also associated with the extent of tumor ($P = 0.007$), pathological features of tumor (($P = 0.025$) and lymph node positivity ($P = 0.002$) (Table1).

Down-regulated PTEN in LSCC and HSCC compared to the non-tumor tissues

We analyzed the protein expression of PTEN in

Table 2. Correlations Between PTEN Expression and Clinical Characteristics in LSCC and HSCC Patients

Characteristics	PTEN No, (%)		
	L-PTEN	H-PTEN	P
Clinical Stage			0.025
I-II	6 (28.6)	15 (71.4)	
III-IV	23 (59.0)	16 (41.0)	
T classification			0.017
T1-T2	8 (30.8)	18 (69.2)	
T3-T4	21 (61.8)	13 (38.2)	
Differentiation			0.093
Well	4 (25.0)	12 (75.0)	
Mediate	12 (57.1)	9 (42.9)	
Poor	13 (56.5)	10 (43.5)	
Node metastasis			0.040
N0	13 (37.1)	22 (62.9)	
N1-N2	16 (64.0)	9 (36.0)	

L-PTEN, Low expression of PTEN; H-PTEN, High expression of PTEN; High expression, ≥25% of cells with positive staining; Low expression, <25% of cells with positive staining

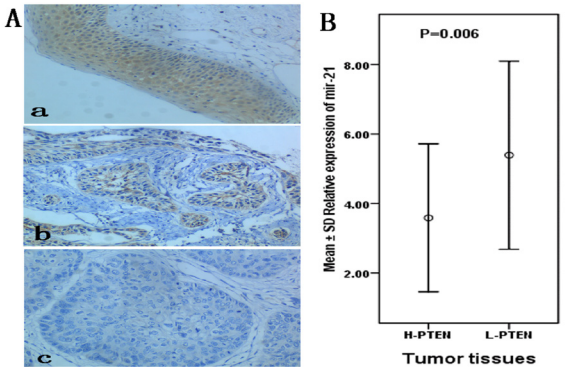

Figure 2. PTEN Expression. (a) low PTEN expression (IHC staining, ×200) in adjacent non-tumor tissues; (b) High PTEN expression (IHC staining, ×200) in tumor tissues with low miR-21 expression; (c) Low PTEN expression (IHC staining, ×200) in tumor tissues with high miR-21 expression; (B) Difference in expression levels of miR-21 between L-PTEN and H-PTEN in tumor tissues

paraffin sections of LSCC and HSCC samples by IHC staining. PETN staining was observed both in nuclei and cytoplasm, where cytoplasmic staining was predominant in most cases. The IHC staining analyses indicated that PETN was positively expressed in 88.3% (53/60) of adjacent non-tumor tissues (Figure2). However, PETN could be only detected in 48.3% (29/60) of the tumor tissues. The difference of expression of PTEN between tumor and matched adjacent non-tumor tissues was statistically significant ($P < 0.05$). In addition, the down-regulation of PTEN was correlated with tumor staging ($P = 0.025$), the extent of tumor ($P = 0.017$), and lymph node positivity ($P = 0.040$), while the differences of PTEN expression among different pathological differentiation samples were not significant ($P = 0.093$) (Table2).

Correlation between up-regulated miR-21 and down-regulated PTEN

The mean of the relative expression of miR-21 was higher (mean, 5.39 and SD, 2.71) in tumors with low expression of PTEN than that (mean, 3.59 and SD, 2.13)

in tumors with high expression of PTEN. The data analysis indicated that there was a statistically significant difference in the expression of miR-21 between the tumors with high and low expression of PTEN in LSCC and HSCC (P = 0.006).

Discussion

MiR-21 and PTEN play important roles in development of various types of malignant tumors. Several studies have proved that miR-21 was over-expressed in HNSCC both in vitro and in vivo by using different experimental methods of assays including microarray, Northern blotting, and real-time RT-PCR (Tran et al., 2007; Hui et al., 2010). However, miR-21 could potentially have different biological pathways/mechanisms in tumorigenesis that differ between the HNSCC subtypes as miRNA expression could be different in each of these different tumor sites. In most of studies, however, few of such stratification analysis by tumor sites to meaningfully address different expressions of miR-21 has been performed in these tumor subsites of HNSCC including LSCC and HSCC. In the present study, 30 patients with LSCC and 30 patients with HSCC were taken for such analysis to study the expression of miR-21 and PTEN in LSCC and HSCC.

We also utilized matched adjacent non-tumor tissue as control to minimize variations in gene expressions caused by individuals. As found in overall mixed tumor sites of HNSCC, our results also showed that the expression of miR-21 was up-regulated in LSCC and HSCC compared to their matched adjacent non-tumor tissues, and that over-expression of miR-21 was not only associated with tumor clinical stage and T classification, but also correlated with lymph node positivity and tumor pathological differentiation. In agreement with our findings, previous reports have shown that miR-21 is up-regulated in many other solid tumors, and is correlated to tumor progression. For example, the increased miR-21 contributes to the development of glioblastomas (Chan et al., 2005) and hepatocellular cancer (Meng et al., 2007). Furthermore, miR-21 correlates with advanced tumor staging, lymph node metastasis, and poor prognosis in breast carcinomas (Yan et al., 2008). In addition, over-expression of miR-21 in colon cancers indicates poor survival and poor therapeutic effect (Schetter et al., 2008). In the present study, our results suggested that LSCC and HSCC with over-expression of miR-21 may be more aggressive and miR-21 may play an important role in the progression, invasion and metastasis of human LSCC and HSCC.

In the current study, we also investigated the expression of PTEN and analyzed the correlation between the expression of PTEN and the clinical characteristics in LSCC and HSCC. Our results indicated that the expression of PTEN in LSCC and HSCC was lower than that in the adjacent normal tissues. Although K. Guney et al showed that down-regulation of PTEN was not associated with clinical characteristics of LSCC (Guney et al., 2007), our results indicated that the down-regulation of PTEN was associated with certain clinical characteristics of LSCC and HSCC. Thus, future well-designed studies with larger sample sizes are needed to confirm this conflicting result.

It has been showed that the miRNA-mediated gene regulation is quite complex, in that one miRNA might regulate multiple target genes, and could be able to act as efficient regulators of tumor-related genes in tumors (Bartel, 2004). Therefore, it is critical to identify the target genes of the altered miRNA to understand the mechanism by which the altered miRNA is involved in tumor pathogenesis. To date, at least 3 genes have been identified as targets of miR-21 including PTEN, TPM1 and PDCD4 (Meng et al., 2007; Frankel et al., 2008; Li et al., 2009). The previous study has showed that the expression of PTEN could be inhibited by miR-21 in HCC cell lines (Meng et al., 2007). In our study, we also analyzed the relationship between up-regulated miR-21 and down-regulated PTEN in LSCC and HSCC. Our results demonstrated that the expression of miR-21 was significantly higher in tumors with low PTEN expression than in those with high PTEN expression. Thus, miR-21 expression was inversely associated with the expression of PTEN in LSCC and HSCC. These results suggested that PTEN may be also the target of miR-21 in LSCC and HSCC and miR-21 may be involved in tumorigenesis of LSCC and HSCC at least partly through the PTEN pathway.

Previous reports have showed that miRNAs enhance malignant phenotype of tumor by repressing expression of various tumor suppressor genes simultaneously (Chen, 2005; Wu et al., 2007). Therefore, in addition to those genes mentioned above, we speculated that there might be other target genes of miR-21 in LSCC and HSCC which still have been uncovered. Because one miRNA can target a set of coding genes rather than a single one, therapies based on miRNA interference could be very potent in cancer treatment by targeting multiple molecular pathways. Our results showed that miR-21 was up-regulated in LSCC and HSCC and overexpression of miR-21 indicated more aggressive tumor phenotypes. Therefore, miR-21 could be a novel therapeutic target for future treatment of human LSCC and HSCC.

In summary, miR-21 is up-regulated and PTEN is down-regulated and their expressions are reversely correlated in human LSCC and HSCC. Our results suggest that miR-21 and PTEN might play an important role in the progression of LSCC and HSCC. However, the exact mechanism by which miR-21 and its possible target genes (PTEN, TPM1 and PDCD4) are involved in the development of these subsites of HNSCC warrants further in vitro and in vivo studies.

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