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Expression and significances of MiRNA Let-7 and HMGA2 in laryngeal carcinoma

F.-C. SONG, Y. YANG, J.-X. LIU

Department of Otolaryngology Head and Neck Surgery, Tianjin Union Medical Center, Tianjin, China

Abstract. – OBJECTIVE: High mobility group protein A2 (HMGA2) is a reported new oncogene been regulated by tumor suppressor microRNA Let-7. HMGA2 has become a hot topic in fundamental and clinical research of laryngeal carcinoma. The aim of the current study is to investigate the molecular mechanism of Let-7 and HMGA2 in oncogenesis and progression of laryngeal cancer.

PATIENTS AND METHODS: We used quantitative RT-PCR to detect the expression of miRNA Let-7a and HMGA2 mRNA from 59 pairs of fresh laryngeal cancer tissues and adjacent tissues collected for the laryngeal cancer patient. The expression of HMGA2 protein was detected by western blot method.

RESULTS: There is a negative correlation between low expressed miRNA Let-7a and high expression of HMGA2 mRNA in human laryngeal cancer ($p < 0.05$). The expressions of miRNA Let-7a and HMGA2 have a significant difference in patients with clinical stage I-II and clinical stage III-IV, patients with well-differentiated tumor and patients with poorly differentiated tumor, patients with lymph node metastasis and patients without lymph node metastasis. Spearman correlation analysis of miRNA Let-7a and HMGA2 mRNA showed expression of miRNA Let-7a is negatively correlated with HMGA2 expression.

CONCLUSIONS: The down-regulation of miRNA Let-7a and up-regulation of HMGA2 promote the invasion and metastasis of laryngeal cancer.

Key Words:
Laryngeal neoplasms, Let-7 miRNA, HMGA2, Human.

Introduction

Laryngeal cancer is a common malignant tumor occurs in head and neck, which are accounts for 5.7% to 7.6% in systemic malignant cancers. The morbidity of laryngeal cancer has increased significantly in recent years[1,2]. The majority patients with laryngeal cancer are among 40 to 60 years. Though the rational combination of surgery, radiotherapy, chemotherapy and biological treatment therapies have greatly improved the prognosis of laryngeal cancer[3], the long-term survival rate of patients with laryngeal cancer still not increased significantly. Once occurring the lymph node metastasis, the 5-year survival rate of laryngeal cancer patients may be less than 50%, and the 5-year survival rate may be less than 20% when distant metastasis occurred[4]. So far the pathogenesis of laryngeal cancer is still not completely understood, therefore increasing the survival rate of patients, illustrating the metastasis mechanism of laryngeal cancer at the molecular level, and seeking for ideal molecular bio-marker for early diagnosis and prognosis are the current focus of laryngeal cancer. With the development of tumor genome research, many aberrant expressed genes have been found in laryngeal cancer, while the potential gene therapy target is still rare. High-mobility group protein A2 (HMGA2) is a tumor-associated protein consisted by 109 amino acids[5]. Reports indicated[6-10] that HMGA2 may play important role in tumor angiogenesis and epithelial-mesenchymal transition process, which are closely associated with tumor cells proliferation and cancer metastasis. These researches indicate HMGA2 is a new oncogene[9] and it could be a promising biomarker and target for cancer therapy. However, the mechanism of HMGA2 in oncogenesis and tumor progression is still unclear. miRNA Let-7 is firstly found in nematode and it can regulate the differentiation and proliferation of nematode cells. So far, there were Let-7a, Let-7b, Let-7c, Let-7d, Let-7e, Let-7F, Let-7G, Let-7i and miR-98 been reported as subtypes of Let-7 in human[11]. Studies have reported an expression of Let-7 decreased significantly in liver cancer[12], ovarian cancer[13], esophageal

Corresponding Author: Yang Yang, MD; e-mail: yangyangtjin@163.com

cancer[14], oral cancer[15], head and neck cancer[16]. Restoring Let-7 expression can prevent oncogenesis, which indicates that Let-7 may be a member of tumor suppressor gene. Studies have demonstrated negatively regulate Let-7 miRNA could modulate HMGA2 expression[14,15,17]. The researchers confirmed Let-7 exert its' effect through binding the 3'UTP of HMGA2 mRNA[18]. In this study, we preliminary investigated the suppressing of miRNA Let-7 to HMGA2 in patients with laryngeal cancer, and provide useful information of miRNA Let-7a and HMGA2 in the clinical diagnosis and treatment of laryngeal cancer, especially in the Chinese population.

Patients and Methods

The study was approved and registered by the Ethics Committee of Tianjin Union Medical Center in January 2012 (approval number: A2012TJ202). The Ethics Committee approved relating screening, treatment, and data collection of these patients; all subjects signed written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

Study Subjects

59 pairs of fresh laryngeal cancer tissues and adjacent tissues (0.5 cm-1.0 cm from the cancerous tissues) were collected from patients in Tianjin Union Medical Center from July 2012 to November 2015. The collected tissues were all confirmed by pathological examination; the adjacent laryngeal epithelium tissues were also confirmed normal. All specimens were preserved in liquid nitrogen with 30 minutes after surgical removal and then stored at -80°C refrigerator. The medical data of the patients were collected. Screened patients were all confirmed diagnosed as laryngeal cancer first time; they did not receive any radiotherapy, chemotherapy or immunotherapy when resected samples were collected.

Total RNA Extraction

50 mg resected tissue was prepared for total RNA extraction. After taken from the liquid nitrogen, samples were crushed immediately and then were moved into a 2 ml of sterile Eppendorf tube. 1ml Trizol lysis was added cells and the samples were centrifuged at 4°C at 12000 g for 10 mins. The upper contents were removed to a 1.5 ml EP without RNase. Phenol/chloroform were used for deproteinization, ethanol was used to precipitate RNA, DEPC water was added to dilute these RNAs. The purity of extracted RNA was tested by ultraviolet spectrophotometer. The OD 260/OD 280 ratio value should be greater than 1.8. The agarose gel electrophoresis of RNA was also conducted to confirm the purity of RNA.

Reverse transcription of RNA (RT-PCR)

The total microRNA was extracted by the microRNA Extraction Kit (miRNeasy Mini Kit, Qiagen, Hamburg, Germany). miRNA Let-7a was measured by reverse transcription-polymerase chain reaction (RT-PCR) method. Briefly: 1 μg of total microRNA was transcript. The quantitative PCR was performed using ABI 7500 Sequence Detection System (Life Technologies, New York, NY, USA); the expression of miRNA Let-7a was normalized by miRNA U6. The primers were designed and synthesized by TIANGEN Biotech (Beijing, China). The objective miRNA was amplified with the following conditions: 95°C for 30 s, 95°C for 5 s, 60°C for 34 s (40 cycles). The RT-PCR of HMGA2 mRNA was performed according to standard procedure described before[19]. β-actin served as control, mRNA was amplified with following conditions: 95°C for 30 s, 60°C for 20 s (40 cycles) and 60°C for 15 s. The relative expression of miRNA Let-7a and HMGA2 were calculated by Schmittgen method: $F = 2^{-\Delta ct}$, Δct =ct miRNA Let-7a (mRNA HMGA2) -ct miRNA-U6 (mRNA β-actin). CT means the number of cycles experienced by the fluorescent signals reached the threshold inside the reactor.

The used sequences of primers were:
U6: 5'-GCTTCGGCAGCACATATACTAAAAT -3'
miRNA Let-7a (F): TGAGGTAGTAGGTTGTGTGGTT
HMGA2 (F): 5'- TTCAGCCCAGGGACAACC-3'
HMGA2 (R): 5'-GCTGCTTTAGAGGGACTCTTGT-3'
β-actin (F): 5'- CATCCTGCGTCTGGACCTGG-3'
β-actin (R): 5'-TAATGTCACGCACGATTTCC-3'

Western Blot Analysis

After taken from the liquid nitrogen, samples were crushed immediately and then homogenized on ice with RIPA buffer [50 mM TRis (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, Sterile Solution] containing protease inhibitor and phosphatase inhibitor. The supernatants were collected and centrifuged at

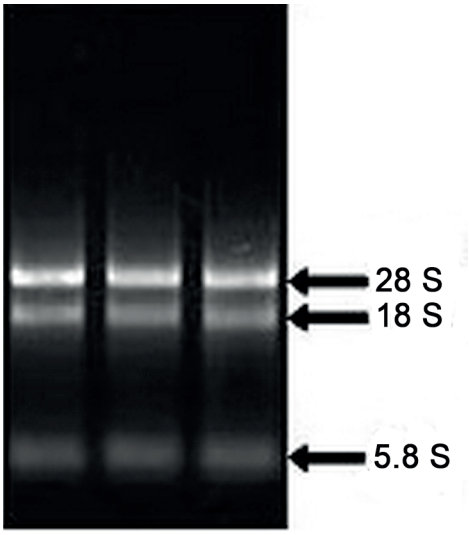

Figure 1. The agarose gel electrophoresis of total RNA, results showed isolated total RNA had good quality.

12000 g for 25 minutes at 4°C. Protein samples were electrophoresed on 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to PVDF membrane. Nonspecific reactivity was blocked in 5% nonfat dry milk in TBS/T for 1 hour at room temperature. The membrane was then treated with primary antibodies of HMGA2 (1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight; Then cultured with horseradish peroxidase-labeled with a secondary antibody (1:2000, Zhongshan Bio Tec, Beijing, China) at 37°C for 2 hours. The protein was colored by DAB and captured by Gel imaging system (Amersham, San Diego, CA, USA). The gray value was measured. β-actin served as control.

Statistical Analysis

The relative expression data of miRNA Let-7a and HMGA2 in laryngeal carcinoma and adjacent tissues were distributed skewed, the median and interquartile range were used to describe the central tendency of data. SPSS 19.0 (Chicago, IL, USA) was used for statistical analysis. The paired sample Wilcoxon rank sum test was used for comparison between relative expression of miRNA Let-7a, HMGA2 in laryngeal carcinoma and adjacent normal tissue. The correlation was analyzed by Spearman correlation analysis. The miRNA Let-7a relative expression between different clinical pathological factors was compared by Mann-Whitney U test or ANOVA test, the Bonferroni correction was used for post-hoc test for ANOVA. $p < 0.05$ was considered as statistical significance.

Results

Demographic information: there were 59-tumor tissue and adjacent tissues of patients with laryngeal cancer were collected finally in our study. Demographic data showed there were 17 cases of supraglottic laryngeal carcinoma, 33 cases of lottis carcinoma and 9 cases of subglottic laryngeal carcinoma; 42 cases were male and 17 cases were female. The average age of these patients was 63.52± 6.8 years, with 33 cases of them below 60 years. Pathological results confirmed they all have squamous cell carcinoma of the larynx, 28 cases were highly differentiated and 31 cases were low differentiated. Lymph node metastasis occurred in 17 cases and 42 cases have no lymph node metastasis. TNM stage showed 6 cases have stage I, 26 cases have stage II, 19 cases have stage III and 8 cases have stage of IV.

Transcribed RNA quality

Agarose gel electrophoresis was used to measure the integrity of isolated total RNA. The ultraviolet gel imaging system showed 3 strip bands: 28 s, 18 s bands and 5.8 s; the light of 5.8 s band was light, and ratio of the brightness of 18 s bands and the 28 s bands was 1:1.5 to 1:2, which showed the degradation of total RNA is less and the quality is good (Figure 1).

miRNA Let7-7a Expression in Laryngeal Cancer Tissues and Adjacent Normal Tissues

In this study, $2^{-\Delta\Delta Ct}$ assay was used to calculate the relative expression of miRNA leta-7a. Results showed that, as a tumor suppressor gene, the expression of miRNA- Let7-7a in laryngeal carcinoma tissue was significantly lower than that in adjacent normal tissue; The expression of miRNA- Let7-7a in tumor tissue was almost 5 times lower than that of normal tissue (0.423 *vs.* 2.63, $p < 0.0001$, Table I).

Expression of miRNA Let-7a and its Relationship with Clinical Pathological Factors in Laryngeal Carcinoma

We analyzed the expression of miRNA Let7-7a in laryngeal carcinoma with different clinical

Table I. Expressions of miRNA - Let7-7a in laryngeal carcinoma tissue and adjacent normal tissue (N=59).

	Cancer tissue	Adjacent tissue
Numbers	59	59
miRNA Let7-7a (median (interquartile range)	0.423 (0.288-0.738)	2.63 (1.678-4.5556)
95% CI	0.457-0.663	2.600-3.428
p (Wilcoxon matched-pairs signed rank test)	< 0.0001	

pathological factors such as age, gender distribution, tumor location and TNM stage etc. Results showed low expressed miRNA Let7-7a in laryngeal carcinoma was significantly correlated with low differentiation ($p < 0.0001$), lymph node metastasis ($p = 0.0268$) and higher TNM stage (stage III and IV, $p < 0.0001$). The detailed data are listed in Table II.

HMGA2 expression in laryngeal cancer tissues and adjacent normal tissues

The relative expression of HMGA2 mRNA was also calculated by $2^{-\Delta\Delta Ct}$ assay. Results showed the expression of HMGA2 mRNA in laryngeal carcinoma tissue was significantly higher than that in normal tissue. The median value in laryngeal carcinoma tissue was 0.759 while the median value in adjacent normal tissue was 0.0851 (Table III).

The Relative Expression of miRNA Let-7a and HMGA2 mRNA in Laryngeal Carcinoma was negatively correlated

Spearman correlation analysis of miRNA Let-7a and HMGA2 mRNA showed miRNA Let-7a expression is negatively correlated with HMGA2 mRNA (r =-0.4612, 95% CI of -0.6461 to -0.2251; p =0.002); The downregulation of miRNA Let-7a in laryngeal carcinoma could cause up-regulation of HMGA2 expression.

Western Blot Results

In 59 laryngeal cancer tissues, the positive rate of HMGA2 expression was 74.0% (37/50), and 14.0% in 50 adjacent normal tissues. The HMGA2 expression in laryngeal cancer tissues was higher than adjacent normal tissues, and the difference was significant ($p < 0.05$). Figure 2 showed the difference of HMGA2 expression in different tissues.

Discussion

Let-7a is a representative member of Let-7 miRNA family. As an endogenous non-coding single strand RNA, the transcription of Let-7a is independent. The miRNA Let-7a negatively regulates the mRNA translation at the transcriptional level. Our results showed that compared with adjacent

Table II. Analysis of miRNA Let7-7a in laryngeal carcinoma with different clinical pathological factors.

		N	miRNA Let7-7a expression (median (interquartile range)	F-value	p-value
Tumor location	supraglottic laryngeal carcinoma	17	0.523 (0.273-0.932)	0.321	0.727
	glottic carcinoma	33	0.356 (0.269-0.611)		
	subglottic laryngeal carcinoma	9	0.456 (0.382-0.681)		
Gender	male	42	0.428 (0.322-0.751)		0.526
	female	17	0.378 (0.269-0.751)		
Age (yr)	≤60	33	0.356 (0.263-0.596)		0.1447
	>60	26	0.428 (0.345-0.789)		
Differentiation	high	28	0.708 (0.453-0.998)		< 0.0001
	low	31	0.342 (0.256-0.400)		
Lymph node metastasis	yes	17	0.346 (0.267-0.418)		0.0268
	no	42	0.523 (0.293-0.776)		
TNM stage	I + II	32	0.613 (0.441-0.976)		< 0.0001
	III + IV	27	0.288 (0.246-0.346)		

Table III. Expressions of HMGA2 mRNA in laryngeal carcinoma tissue and adjacent normal tissue (N=59).

	Cancer tissue	Adjacent tissue
Numbers	59	59
HMGA2 mRNA (median (interquartile range)	0.759 (0.478-1.000)	0.0851 (0.0596-0.186)
95% CI	0.6698-0.9032	0.1064-0.1683
p (Wilcoxon matched-pairs signed rank test)	< 0.0001	

normal tissues, the Let-7a was down-regulated in laryngeal cancer tissues, and this outcome is consistent with Long's report[20]. As a member of high mobility group proteins (HMG), HMGA2 could change the structure of DNA through binding it and further, regulate the DNA transcription. HMGA2 also could bind transcription factors directly to regulate the DNA transcription. HMGA2 expresses highly in many malignant tumors and is closely associated with clinical stage and lymph node metastasis. Our research showed the HMGA2 mRNA and protein were all up-regulated in laryngeal cancer tissues compared with adjacent normal tissues. Invasion and metastasis are the main characteristics of malignant cancer. Epithelial-mesenchymal transition (EMT) is an important hypothesis of tumor metastasis[21,22]. Epithelial-mesenchymal transition could promote the production of cancer stem cells and the apoptosis of tumor cells[23-25]. Many reported have indicated the EMT is highly associated with gastric carcinoma, liver cancer, pancreatic carcinoma, melanoma and related drug resistance[26-28]. HMGA2 plays important roles in metastasis through regulating the transcription of EMT-regulatory protein. The inhibitors of epithelial marker, E-cadherin, include Snail, Twist and Slug; HMGA2 could up-regulate the inhibitors expression and the down expressed E-cadherin could induce EMT[29]. HMGA2 could also combine with the promoter of Snail or combine with the Smad to activate the transcription of Snail[30]. Thuault et al[31] confirmed that Let-7 is also involved in the negative regulation of EMT and up-regulation of Let-7d could suppress the migration ability of OECM-1 (the cell line of oral squamous carcinoma) effectively. Our study revealed that miRNA Let-7a was down-regulated in laryngeal cancer tissues compared to adjacent normal tissues and the expression of Let-7 was lower in advanced laryngeal cancer compared with the early stage laryngeal cancer. The expression of Let-7a was further decreased with the progression of the tumor. On the contrary, the expression of HMGA2 was up regulated. The down-regulated Let-7a caused up-regulation of HMGA2, thus promotes EMT and facilitated the metastasis and invasion of laryngeal cancer. These results suggest Let-7a and HMGA2 might serve as the biomarkers of laryngeal cancer. Researches have demonstrated the Let-7 and HMGA2 were reciprocal negatively regulated in ovarian cancer[32], retinoblastoma[19], leiomyosarcomas[33] and laevicellulare[34]. The HMGA2 regulates Let-7 via two ways in cancer progression: one through Let-7 targeted deletion of 3' terminal non-coding region of HMGA2 gene in some benign tumors; another way occurred in some cancers such as lung cancer, in which Let-7 could bind with HMGA2 gene and the HMGA2 mRNA is degraded or the transcription is inhibited, so that the HMGA2 expression is down-regulated[35]. Consistent with the above report, our research showed that HMGA2 and Let-7a were negative correlation in laryngeal cancer. However, this preliminary conclusion still needs further validation with a larger sample size. In addition, the patients with laryngeal cancer are more complicated

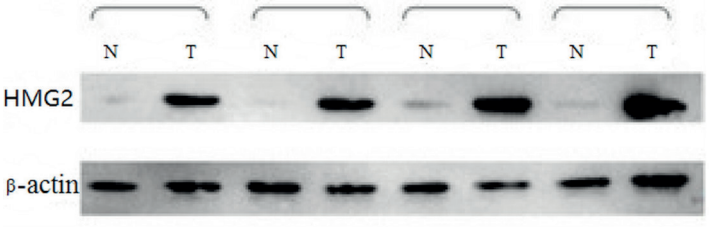

Figure 2. Western blot. The figure represents the positive expression of HMGA2 in laryngeal cancer tissue but relatively lower expression in adjacent tissues.

in the actual clinical practice, whether the present conclusion will consistent when the patients received radiotherapy and chemotherapy? Whether the conclusion is inconsistent with late prognosis of the patients with laryngeal cancer? Or whether miRNA Let-7a level could serve as an evaluation index for curative effect? These all needs further study design and verification.

Conclusions

Our report suggests that the down-regulation of miRNA Let-7a and up-regulation of HMGA2 promote the invasion and metastasis of laryngeal cancer and Let-7a is negatively regulate HMGA2 expression in laryngeal cancer. However, more researches are still needed to verify this conclusion.

Conflicts of interest

The authors declare no conflicts of interest.

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