

Original Article

Expression and clinical significance of testin in nasopharyngeal carcinoma

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Abstract: Objective: Our purpose was to investigate the expression of Testin gene and its possible relationship with the clinic pathological features of human nasopharyngeal carcinoma. Methods: The expression of Testin in nasopharyngeal carcinoma tissues were detected by immunohistochemical methods, semi-quantitative reverse transcription polymerase chain reaction and western blot. The correlations of Testin to clinic pathologic features of nasopharyngeal carcinoma were analyzed. Results: The mRNA level of Testin was down-regulated in human nasopharyngeal carcinoma. The positive rate of Testin protein was significantly lower in human nasopharyngeal carcinoma tissues than that in normal tissues. The protein level of Testin was down-regulated in cancers as compared with corresponding normal tissues. Testin expression was positively correlated with the differentiation of nasopharyngeal carcinoma. Meanwhile, differences in gender and age were not significant ($P>0.05$). There was a significant correlation between invasion, distant metastasis and differentiation degree and Testin expression ($P<0.05$ respectively). Conclusion: The decreased expression of Testin gene may play an important role in the development of esophageal squamous cancer. And thus Testin gene might be a novel candidate of tumor-suppressor. It may be an objective marker for prognostic factor and malignant level for nasopharyngeal carcinoma.

Keywords: Testin, nasopharyngeal carcinoma, RT-PCR, immunohistochemistry, western blot

Introduction

The incidence of malignant tumors of head and neck was about 16%~40%. Nasopharyngeal cancer is clinically common malignant tumors of head and neck and is one of the serious hazards to human life and health. Obvious national gathering of nasopharyngeal carcinoma, which prompted nasopharyngeal cancer may be a polygenic diseases related to genetic factors, which may be related to nasopharyngeal carcinoma related chromosomal abnormalities and changes in oncogenes [1].

Tatarelli firstly discovered and identified human testin gene by analysis the structure of bacterial artificial chromosome FRA7G D7S486 Met H in 2000 year [2]. Currently its expression in head and neck malignant tumors rarely reported.

In this study, we observed the testin expression in human nasopharyngeal carcinoma and

normal nasopharyngeal tissues, explored its relationship with the occurrence and development of nasopharyngeal carcinoma and made clinical data analysis of its relationship with clinical pathological features of nasopharyngeal carcinoma.

Materials and methods

Tissue samples

45 cases of biopsy nasopharyngeal new organ-ism has been collected from department of Head and neck surgery, zhongnan Hospital of Wuhan University, from Jan. 2010 to Dec. 2010. All specimens were confirmed by two senior pathology doctors according to pathological diagnosis of nasopharyngeal squamous cell carcinoma, none of all patients receiving radiotherapy, chemotherapy or other cancer treatment resistance. Patients' name, age, sex, and clinical and pathological information were documented. Gathered over 45 cases of normal

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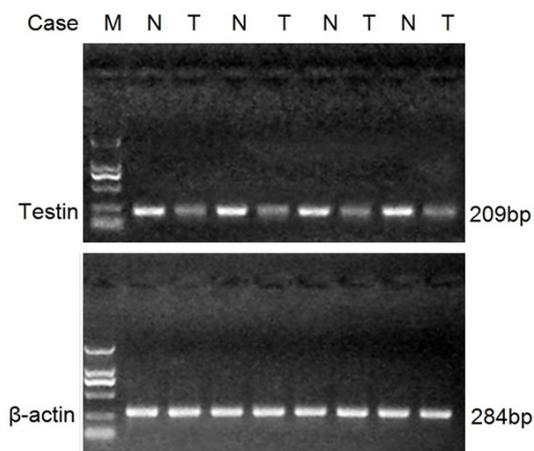


Figure 1. RT-PCR Detection of nasopharyngeal carcinoma TES Expression. T: nasopharyngeal carcinoma; N: normal; M: Marker. In tumor tissue Testin mRNA expression significantly reduced ($P < 0.001$). DNA Marker DL2000: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp.

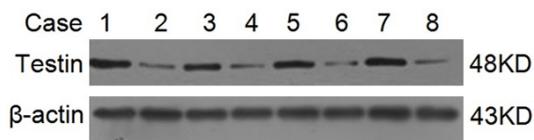


Figure 2. Western blotting detection of Testin in nasopharyngeal carcinoma. 1, 3, 5 and 7: normal tissue, 2, 4, 6 and 8: nasopharyngeal carcinoma.

nasopharyngeal tissues from the above sample, random selection of 10 cases, each sample was divided into two parts, one freezing in -70°C Low temperature in the refrigerator, the remaining tissue samples with conventional paraformaldehyde fixation, paraffin-embedded.

RNA extraction and RT-PCR

Total RNA was extracted from nasopharyngeal cancer tissue and normal tissue using TRIzol Kit according to description in manual. RT-PCR was performed using one-step RT-PCR kit according to the manual. Testin Gene PCR primer sequences are: upstream Primer: 5'-CCCTCAAAGTGCCATGAGT-3' Downstream primers: 5'-TGTGCTCAGCAGATTTGTCC-3' Product length 209 bp, Reference β -actin Primer sequences: upstream Primer: 5'-AGCGAGCATCCCCAAAGTT-3' Downstream primers: 5'-GGGCACGAAGGCTCATCATT-3' Product length was 284 bp, The reaction conditions for: denaturing 94°C , 4 min; Modified 94°C , 30 sec and renaturation

54°C , 30 sec, expansion of 72°C , 25 sec, cycle 40 Extended 72°C , 4 min, 4°C Termination, in order to reduce errors, each sample repeat three times.

Western blotting analysis

Total proteins were extracted from the nasopharyngeal carcinoma specimens for Western blotting analysis. They were analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at room temperature for one hour. It was incubated at room temperature for two hours after added with TES Mouse anti-human antibodies (1:200). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to β -actin.

Immunohistochemical staining

Pathological paraffin blocks of nasopharyngeal carcinoma tissue sections were cut into $4\ \mu\text{m}$ thickness for immunohistochemical staining. They were operated using Immunohistochemical SP Assay Kit (Fuzhou new biological technology development limited company) according to operating instructions. Following deparaffinization, dehydration, and antigen retrieval, the sections were incubated with Mouse anti-human TESTIN Monoclonal antibody (1:200, Santa Cruz Company, United States) at 4°C overnight. After that, they were washed with PBS every time for 2 min, with a total of 3 times. and At the same time, after drop-adding the 2nd antibody, they were incubated at 37°C in water bath for 2 h, and washed with PBS every time for 2 min, with a total of 3 times. After treated with the DAB solution, they were flushed completely, counterstained with hematoxylin, washed with water, treated with dehydration and transparency, mounted on slides and observed under microscope. The results of immunohistochemical staining were scored by the pathologist. Positive degree criterion referenced to Method [3]. All evaluation processes

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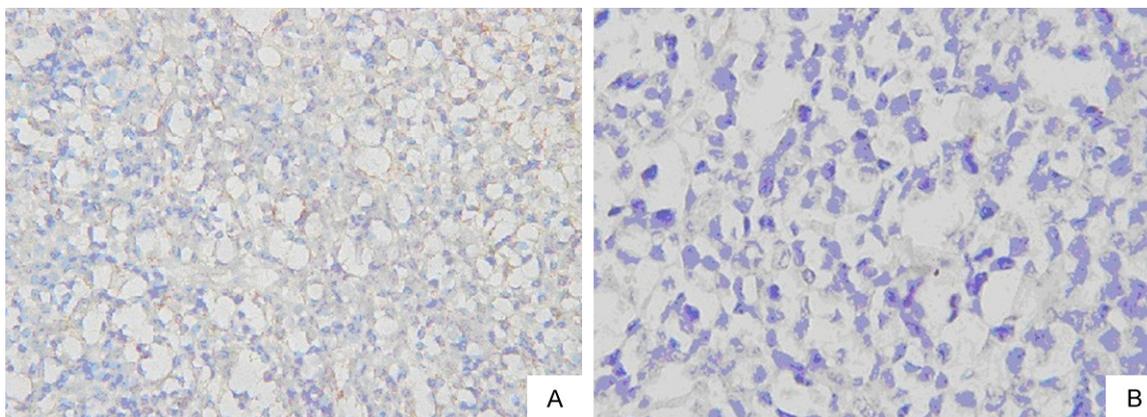


Figure 3. Immunohistochemical results. Testin Positive part is brown-yellow, mainly expressed in the cytoplasm, A: Testin Expression in normal tissues ($\times 200$), B: Testin Expression in nasopharyngeal carcinoma ($\times 400$).

by at least 2 to blinded observation to determine pathology doctors independently.

Statistical analysis

Pairing: a comparison of nasopharyngeal carcinoma and normal tissue Testin Protein expression level (With internal reference standard treatment), SPSS16.0 Software and Companion *t* tests were used for statistical analysis. There are statistically significant differences if $P < 0.05$.

Results

RT-PCR detection

Testin mRNA expression was detected in 45 human nasopharyngeal carcinoma tissue and normal tissue using semi-quantitative RT-PCR methods. Beta-actin was as internal reference. Comparison with normal tissues, 69% (31/45) in nasopharyngeal carcinoma tissue were with testin low expression. There is a significant difference in testin expression of nasopharyngeal carcinoma and normal tissue (**Figure 1**).

Western-blot detection of testin protein

Testin in 45 samples of nasopharyngeal carcinoma tissue and normal tissue were tested with western blotting methods. We found testin protein levels reduced in 70% (14/20) in nasopharyngeal carcinoma ($P < 0.01$, **Figure 2**).

Immunohistochemical results

Testin Positive part is brown-yellow, mainly expressed in the cytoplasm. We found that the

negative rate was 62.2% (28/45), and positive rate was 37.8% (17/45). Concentration of positive expression in cancer cells was in the pulp. The negative rate of testin expression in normal tissues was 11.1% (5/45) and the positive rate was 88.9% (40/45). Statistics have shown that testin expression in nasopharyngeal carcinoma tissue significantly decreased ($P < 0.01$) (**Figure 3**).

Relationship between testin expression and clinical pathological parameters in nasopharyngeal carcinoma

The testin expression was not statistically significant with a gender, age ($P > 0.05$), with or without lymph node metastasis of nasopharyngeal cancer, there is statistical significance with distant metastases and their degree of differentiation ($P < 0.05$) (**Table 1**).

Discussion

Occurrence of nasopharyngeal cancer is a long-term result with more factors, more stage, and more gene role in external and internal mutual causes. The 7q31 is an area of concern in recent years, it is now found in a variety of malignant tumors with high frequency loss of heterozygosity [4]. Testin may be a candidate tumor suppressor gene. It wide expressed in normal tissues and are not expressed in many cancer cell lines or low expression. More studies have found 7q31.2 chromosome region D7S486 high frequency loss of heterozygosity, missing in many human tumors such as colon cancer [5], prostate cancer [6], thyroid cancer [7] and leukaemia [8]. It was found that testin mRNA

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Table 1. The clinic pathologic parameters information in nasopharyngeal carcinoma

Clinical pathological parameters		Number of cases	Testin (-)	P
Sex	Male	36	22 (61.1%)	0.758
	Female	9	6 (66.7%)	
Age	<55	24	15 (71.4%)	0.966
	≥55	21	13 (86.7%)	
Lymph node metastasis	There are	30	23 (76.7%)	0.005
	No	15	5 (33.3%)	
Distant metastasis	There are	20	16 (80%)	0.027
	No	25	12 (48%)	
Degree of differentiation	Well	6	2 (33.3%)	0.001
	Moderate	9	4 (44.4%)	
	Low	20	16 (80%)	
	Is not	10	6 (60%)	

expressed lower in prostate cancer tissue than in normal prostate tissue [9]. Sarti found in 25 cases of breast cancer 36% (9/25) appeared decreased expression of testin [10]. In vitro experiments, testin negative regulatory effect on cell growth, transfection was also found with its tumor suppressor function [11].

In the present study, immunohistochemical method is for qualitative analysis and RT-PCR and western blotting methods are for semi-quantitative analysis. We found 70% (14/20) appeared lower testin levels in 45 patients with nasopharyngeal carcinoma. RT-PCR method and immunohistochemistry revealed that in nasopharyngeal carcinoma tissue expression of testin significantly reduced. These suggested that testin decreased or missing expression may be associated with development of nasopharyngeal carcinoma, findings was similar to that in other malignant tumors.

Testin Expression in nasopharyngeal cancer decline mechanism has not yet been clarified. In recent years, studies have found that lead Testin the primary mechanism for gene inactivation may be the promoter region hypermethylation. Up to now, it has been found in a large number of tumor cells and inactivation of tumor suppressor genes in the excessive methylation in the promoter region of the gene-related. Tobias [12] suggested CpG Island hypermethylation was one of the inactivating reasons, they detect 10 cases of ovarian cancer and 21 Kinds of tumor cells, they found that 7 Testin 5'-End CpG Island hypermethylation, at

the same time in breast cancer cells ZR-75 was found in the Testin frameshift mutations of Exon. Above results prompted promoter region CpG Island methylation in nasopharyngeal carcinoma maybe one of testin gene inactivation mechanism, other mechanisms are yet to be further studied.

We further examined the relations of testin expression and clinical pathological features, it showed that there was no significant relationship between testin expression of nasopharyngeal carcinoma and patient gender, age. With or without lymph node metastasis, there was correlation between distant

metastasis of nasopharyngeal carcinoma and its degree of differentiation. Differentiation of tumor associated with a variety of internal and external causes, which contained a variety of abnormal expression of genes. We found testin expressed low in the differentiation of nasopharyngeal carcinoma, the results suggested testin maybe involved in the differentiation of nasopharyngeal carcinoma, but the specific mechanisms to be studied further.

Tumor invasion and metastasis are the main causes of death in cancer patients. Testin protein c-ends with a series of cytoskeletal proteins (such as Mena, VASP, Zyxin) interactions, causing actin accumulation, increasing adhesion among cells, reducing the movement of cells, thus inhibiting the tumor occurrence. Therefore, testin low expression will lower the adhesion between cells, increase the likelihood of cancer. Griffith [13] found that overexpression of testin can cause increase in cell adhesion, decrease in cell movement. Therefore, testin decline in gene expression can lead to cell adhesion, thus increasing the occurrence and spread of tumors.

C-terminal of Testin protein can interact with the cytoskeleton proteins (such as Mena, VASP, zyxin, etc.) and to increase cell adhesion and reduce the cell motility, thus inhibit the occurrence of tumor [14, 15]. Clinical data analysis of this study found that testin expression fall on inhibitory effect of weakening of nasopharyngeal carcinoma, eventually causing the occurrence, development and metastasis of naso-

pharyngeal carcinoma, which further illustrated that there was relationship between testin and invasion, metastasis and development of nasopharyngeal carcinoma.

In a word, the occurrence of nasopharyngeal carcinoma is a long-term combined action result of multi-factors, multi-stages and multi-genes. Only detection of testin can not diagnosis of such cancer or predict the outcome of this cancer. However, reduced testin expression in nasopharyngeal carcinoma was associated with invasiveness and metastasis in nasopharyngeal carcinoma. Future work should explore its value of biology mark and target point for the gene treatment.

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Disclosure of conflict of interest

None.

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