Association of microRNA-related gene *XPO5* rs11077 polymorphism with susceptibility to thyroid cancer

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Abstract

Exportin 5 (*XPO5*) is a microRNA (miRNA)-related nuclear export protein, and its disorder may lead to the dysregulation of miRNAs. Recent studies have demonstrated that the aberrant expression of *XPO5* might participate in carcinogenesis in certain cancers. However, there is only limited information of *XPO5* in thyroid cancer (TC) development. In our study, we quantified the expression level of *XPO5* by real-time polymerase chain reaction (PCR) in 64 TC patients' cancer tissues and adjacent normal tissues. After confirming the *XPO5* expression, we evaluated the association between *XPO5* potential functional single-nucleotide polymorphisms (SNPs) and risk of TC in a Chinese population (1140 cases vs 1230 controls). Finally, luciferase assays were performed to investigate the function of the SNP in *XPO5* 3′ untranslation region. The message ribonucleic acid (RNA) levels of *XPO5* were significantly lower in cancer tissues than normal tissues ($P = 0.004$). In SNPs screening, only 1 noble SNP rs11077 was identified in *XPO5* functional region. The results in our case–control study also confirmed that *XPO5* rs11077 was significantly associated with onset of TC (GT/GG vs TT $P = 0.035$, adjusted odds ratio = 1.25, 95% confidence interval = 1.02–1.54). The adverse influence of this polymorphism was mainly observed in age >45 years ($P = 0.028$), female ($P = 0.020$), T1 staging ($P = 0.026$), N1 ($P = 0.038$), metastasis ($P = 0.031$ M0, and $P = 0.035$ for M1), and early stage (I + II) ($P = 0.021$). A following luciferase test revealed the critical role of rs11077 for triggering the *XPO5* expression. Furthermore, patients with G allele of rs11077 showed lower *XPO5* expression level. *XPO5* SNP rs11077 influences the expression of *XPO5*, and this SNP could also be a potential biomarker for the diagnosis of TC in clinical, especially in Chinese population.

Abbreviations: 3′UTR = 3′ untranslation region, miRNA = microRNA, miR-SNP = miRNA-related single-nucleotide polymorphism, PTC = papillary thyroid carcinoma, SNP = single-nucleotide polymorphism, TC = thyroid cancer, XPO5 = exportin 5.

Keywords: polymorphism, susceptibility, thyroid cancer, XPO5

1. Introduction

Thyroid cancer (TC) is generally described as the malignant tumors in endocrine system with rapid growth. Clinically, TC could be further divided into 4 forms: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, medullary TC, and anaplastic TC.[1,2] Previous studies have discovered that both genetic and environmental factors were involved in the onset of TC. The classical genetic studies revealed the close association between TC and oncogenes, such as *RAS*, *MYC*, and *CCND1*.[3–5] With the development of epigenetics, microRNAs (miRNAs) have been discovered to participate in the occurrence and development of TC, which also interact with the alteration of environmental factors.[6,7]

miRNAs are a member of noncoding RNAs with only 19 to 25 nucleotides in length, and miRNAs inhibit the transcription of target genes.[8] In general, miRNAs could be combined to the message RNA (mRNA) of target genes by base pairing in 3′ untranslation region (3′UTR) or through other pathways, such as competitive endogenous RNAs or transcription factors.[9,10] The formation of miRNAs is a complicated biological process. Initially, the miRNA is transcribed to pri-miRNA from some special regions of genome, with the length of approximately 1000 nucleotides. Next, pri-miRNA is spliced into the precursor miRNA (pre-miRNA), with approximately 60 to 70 nucleotides length, in the nucleus. Then, with the help of exportin 5 (*XPO5*), an essential specific nuclear transport factor, the pre-miRNAs finish their transportation into cytoplasm from nucleus.[11] In cytoplasm, the pre-miRNAs are further converted into the mature miRNAs and finally exert their functions.[12]

Recent studies have identified that aberrant expressions of miRNAs were associated with many diseases,[13,14] including cancers.[15] In several previous studies, the dysregulation of miRNAs in TC were confirmed to impact the expressions of target genes and further influenced the imperative cellular

processes, such as proliferation, metastasis, invasion, and apoptosis.[16–18]

It is believed that overexpression of *XPO5* leads to higher activity of miRNAs, while loss of *XPO5* expression would inhibit the nuclear export of pre-miRNAs.[19] Because of the hazardous consequences of target mRNAs and cellular processes induced by small alteration of miRNAs activity or quantity, miRNA-related single-nucleotide polymorphisms (miR-SNPs) of *XPO5* gene were considered as a potential and valuable molecular biomarker in cancer prediction prognosis.[20,21] To date, there is no documented record between the function of *XPO5* and incidence of TC. In this study, we aim to investigate the distinct expression levels between the TC and normal tissues, and then evaluate and quantify the association between *XPO5* miR-SNPs and the susceptibility of TC in a Chinese population.

2. Methods

2.1. Study subjects

The current study was approved by the Institutional Ethics Committee of The Hospital Affiliated to Guizhou Medical University (201674). All subjects who participated in this program received a detailed study description and signed a written informed consent. Considering the high proportion of PTC in total TC patients, 1140 PTC patients and 1230 cancer-free controls were included in our research. All patients were recruited from The Hospital Affiliated to Guizhou Medical University and the Tumor Hospital of Guizhou Province from April 2012 to March 2016. The criteria of PTC diagnosis in this study were based on the histological and cytological examination and pathological classification of PTC according to the criteria of World Health Organization, with no history of other malignant cancer, corresponding radiotherapy, or chemotherapy. The auxiliary data included age, gender, tumor site histological type, depth of invasion, lymph node metastasis, distant metastasis, and clinical tumor node metastasis staging of TC, according to the American Joint Commission on Cancer Staging. All cancer-free controls were enrolled in the same period without genetic relationship to the cases and matched to cases by age (±5 years) and gender.

2.2. Specimen collection

A volume of 5-mL peripheral venous blood was collected from each participant for deoxyribonucleic acid (DNA) extraction by QIAcube HT Plasticware and QIAamp 96 DNA QIAcube HT Kit (Qiagen, Dusseldorf, Germany), following the manufacturer's protocol. The total RNA were extracted from 64 pairs of patients' tumor and adjacent normal tissues from 1140 TC patients by TRIzol reagent (Invitrogen, Carlsbad, CA). All DNAs and RNAs were measured by Nanodrop-2000 spectrophotometer (Thermo, Waltham, MA) for their quality and quantity. For storage, DNAs were stored at $-20°C$, while RNAs were stored at $-80°C$ for further processing.

2.3. RNA detection

Quantitative reverse transcriptase PCR were performed using ABI 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The primer were synthesized by Biolight Tec. Company (Nanjing, China).[22] We adopted glyceralde-hyde-3-phosphate dehydrogenase as the internal control for normalization. The sequences of probes were demonstrated in Supplementary Data, http://links.lww.com/MD/B601 with this manuscript.

2.4. SNP selection criteria and genotyping

The *XPO5* gene single-nucleotide polymorphisms (SNPs) were selected as follows: site in the 5′UTR region, exon, and/or 3′UTR, variant of which could directly influence the expression of XPO5; the minor allele frequency of each SNP should exceed 0.05 in typical Chinese Han population. In addition, we also tended to add any *XPO5* SNPs, which have been reported in any sort of cancers, into our study. After the selection, only 1 eligible SNP rs11077 located on the 3′UTR of *XPO5* remained (Fig. 2).

Genotyping was accomplished via TaqMan SNP Genotyping Assay using ABI 7900HT real-time PCR System (Applied Biosystems). Each control sample was placed in an individual plate to ensure the accuracy of each genotyping. We adopted blind experiment by 2 independent individuals for genotyping assay. More than 10% of samples were randomly selected for further validation, and the results were 100% concordant. The primers and probes for genotyping were also listed in Supplementary data, http://links.lww.com/MD/B601.

2.5. Cell culture conditions

The human normal thyroid follicular epithelial cell line Nthy-ori3-1 cells were purchased from European Collection of Cell Culture and maintained in RPMI 1640 (Gibco; Thermo) with 10% fetal bovine serum (FBS). The Human TC cell lines TPC-1 cells were purchased from American type culture collection, which were maintained in Dulbecco minimum essential medium with 10% FBS. Both cells were maintained in 5% CO_2 at 37°C and supplied with 100 U/mL penicillin, 100 μg/mL streptomycin. All reagents were purchased from Gibco (Thermo).

2.6. Construction of XPO5 3′UTR-reporter plasmid and luciferase assay

The human *XPO5* 3′UTR sequences with different alleles of rs11077 T/G were synthesized and constructed into psiCHECK-2 vector (Promega, Madison, WI) by Biolight Tec. Company. Allele plasmids were confirmed by DNA sequencing.

For transfection, 1×10^6 cells were seeded in 24-well culture plate. Cells were transfected by lipofectamine-2000 transfection reagent with 0.5 mg constructed luciferase reporter plasmids. Twenty-four hours after transfection, all cells were washed with phosphate-buffered saline and lysed with 1 passive lysis buffer. Luciferase activity was determined with a dual luciferase report assay system (Promega), following the manufacturer's protocol.

2.6.1. Statistical analysis. The statistical analyses were performed in SAS software (version 9.13, SAS Institute Inc, Cary, NC). SNP distribution was tested by χ^2 test to meet the Hardy–Weinberg equilibrium. Difference in the distribution of demographic characteristics, clinical feature, and frequencies of genotypes between cases and controls were calculated by Student t test or χ^2. Unconditional univariate and multivariate logistic regression analyses were done to estimate adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs). The distinction in RNA expression test between cancer and corresponding normal tissues was performed by paired Student t test. All P values were 2-sided, with 0.05 as significance level.

Demographic characteristics and clinical features.

Variables	Cases (n = 1134)		Controls (n = 1228)		P^*
	n	%	n	%	
Age, y					0.754
≤45	590	52.0	631	51.4	
>40	544	48.0	597	48.6	
Sex					0.846
Male	355	31.3	389	31.7	
Female	779	68.7	839	68.3	
Topography					
T1	885	78.0			
T2	138	12.2			
T3	98	8.6			
T4	13	1.2			
Lymph node					
N0	695	61.3			
N1	439	38.7			
Metastasis					
M0	1113	98.2			
M1	21	1.8			
Grade					
I + II	963	84.9			
III + IV	171	15.1			

* Two-sided χ^2 test for the frequency distributions of selected variables between cases and controls.
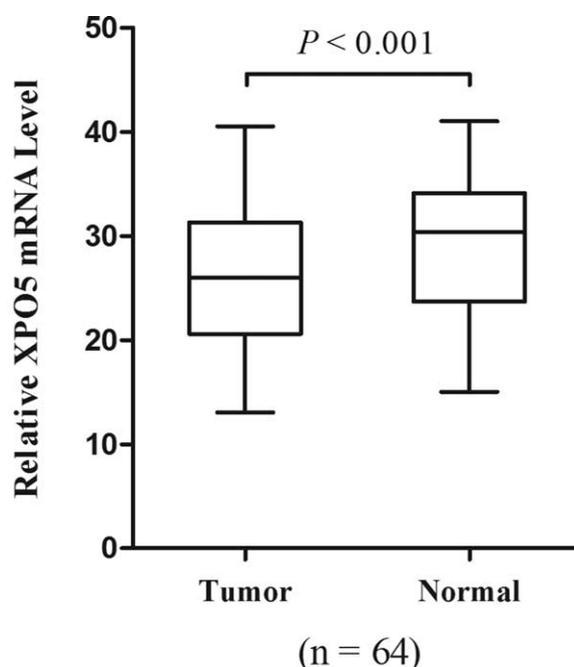

Figure 1. Expression of *exportin 5* message RNA in thyroid cancer patients' cancerous and noncancerous tissues. N = 64, $P < 0.001$. RNA, ribonucleic acid.

3. Results

3.1. Characteristics of study participants

The frequency distributions of cases and controls are summarized in Table 1. Basic characteristics of TC patients were as follows: cases and controls were similar in age ($P = 0.754$) and sex ($P = 0.846$). For patients, 885 (78.0%) were in T1 stage, 138 (12.2%) in T2, 98 (8.6%) in T3, and 13 (1.2%) in T4. A total of 695 patients (61.3%) were diagnosed without lymph node metastasis, compared with 439 N1 patients (38.7%). In addition, only 21 patients (1.8%) showed distant metastasis. For pathological staging, 963 patients were diagnosed as early stage (I + II) and 171 in advanced stage (III + IV), respectively.

3.2. Expression of XPO5 mRNA in thyroid cancer and corresponding normal tissues

The expression levels of *XPO5* mRNA in TC and adjacent normal tissue samples are shown in Fig. 1. Compared with normal tissues, the mRNA level of *XPO5* was significantly lower ($P < 0.001$) in TC tissues in 64 newly diagnosed patients.

3.3. Effects of XPO5 rs11077 and clinical features in TC patients

Because of the lower expression of *XPO5* in TC, we focused on SNPs that were supposed to be closely related to the *XPO5* expression. After screening, only rs11077 remained in our study. Then we performed a case–control study to quantify the association between rs11077 polymorphism and TC risk, and the results are demonstrated in Table 2. In dominant model, there is no significant differences among TT, GG, or GT genotypes. However, GT/GG genotype individuals display a significant hazardous effect compared to those with TT genotype ($P = 0.035$, adjusted OR = 1.25, 95% CI = 1.02–1.54) in codominant model. Meanwhile, distinction between individuals with G and T alleles

was also profound in allele comparison with $P = 0.024$, adjusted OR = 1.25, 95% CI = 1.03 to 1.51. Furthermore, the trend test of rs11077 confirmed the impact of G allele in the susceptible of TC (P trend = 0.024).
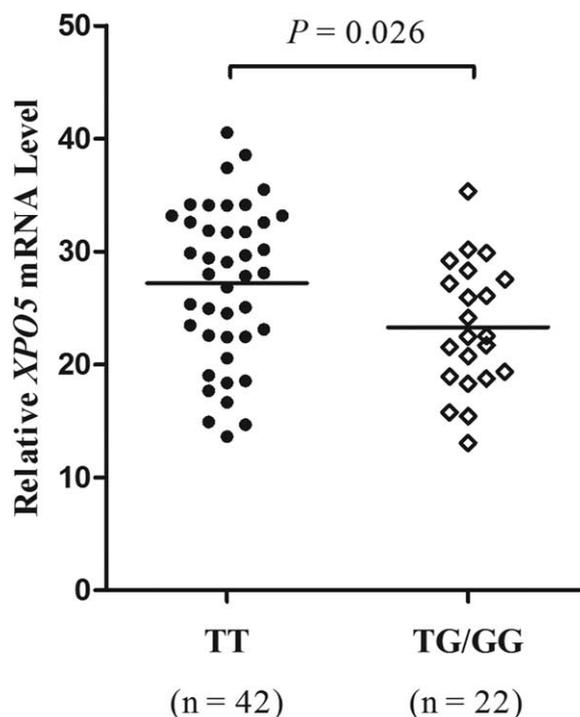

Figure 2. Association between variants of *exportin 5* 3′ untranslated region microRNA-related single-nucleotide polymorphism rs11077 and exportin 5 message RNA levels in thyroid cancer. TT (N = 42) versus TG/GG (N = 22) genotypes, $P = 0.028$. RNA, ribonucleic acid.

Table 2

Distribution of rs11077 polymorphisms and the association with thyroid cancer.

	Cases		Controls			
Genotypes	n = 1134	%	n = 1228	%	P^*	Adjusted OR (95% CI)[†]
TT	907	80.0	1023	83.3		
GT	210	18.5	194	15.8	0.065	1.22 (0.99–1.52)
GG	17	1.5	11	0.9	0.153	1.74 (0.81–3.74)
GT/GG	227	20.0	205	16.7	**0.035**	**1.25 (1.02–1.54)**
T allele	2024	89.2	2240	91.2		
G allele	244	10.8	216	8.8	**0.024**	**1.25 (1.03–1.51)**
P trend					**0.025**	

CI = confidence interval, OR = odds ratio.
* Two-sided χ^2 test.
[†] Adjusted for age and sex in logistic regression model.
Bold values signifies $P < 0.05$.

3.4. Stratified analysis of the effect of XPO5 rs11077 polymorphism in clinical features in TC

We carried out the stratification analysis in all participants to gain a more comprehensive insight into the effect of *XPO5* rs11077 in TC. Among the discussed variables in Table 3, the hazardous influence of this polymorphism mainly showed in age >45 years ($P = 0.028$, adjusted OR = 1.39, 95% CI = 1.04–1.87), female group ($P = 0.020$, adjusted OR = 1.36, 95% CI = 1.05–1.67), T1 staging ($P = 0.026$, adjusted OR = 1.29, 95% CI = 1.03–1.61), N1 ($P = 0.038$, adjusted OR = 1.22, 95% CI = 1.01–1.48), metastasis ($P = 0.031$, adjusted OR = 1.27, 95% CI = 1.05–1.51 for M0; and $P = 0.035$, adjusted OR = 1.24, 95% CI = 1.02–1.54 for M1), and early stage (I+II) ($P = 0.021$, adjusted OR = 1.29, 95% CI = 1.04–1.61).

3.5. The relationship between XPO5 rs11077 genotype and expression of XPO5

Here, we explore the connection between the different genotyping and the expression of *XPO5* in a clinical setting. As shown in

Fig. 2, the *XPO5* mRNA expression in patients with TG/GG genotype was significantly lower than in patients with TT (23.28 ± 1.20 vs 27.19 ± 1.07, mean ± standard error of mean, $P = 0.026$). These results further suggested that different genotypes could influence expression of *XPO5*.

3.6. Effects of XPO5 3′UTR rs11077 on gene regulation

The functional effects of rs11077 in different alleles were tested by luciferase assay. After transient transfection, the plasmid with T allele showed a higher luciferase activity compared to G plasmid ($P = 0.023$ in Nthy-ori3-1 cell line and $P = 0.002$ in TPC-1 cell line) as shown in Fig. 3. These results also implied that variants of rs11077 might be more sensitive in TC cells than normal tissue cells for their potential function of *XPO5* expression.

4. Discussion

We have investigated the relationship between *XPO5* and TC in this study. The first important finding of our study was that

Table 3

Association between rs11077 and thyroid cancer in a dominant model.

	TT (case/control)		GT/GG (case/control)			
Variables	n	%	n	%	P^*	Adjusted OR (95% CI)[†]
Age, y						
≤45	484/528	82.0/83.7	106/103	18.0/16.3	0.455	1.12 (0.83–1.51)
>45	423/495	77.8/82.9	121/102	22.2/17.1	**0.028**	**1.39 (1.04–1.87)**
Sex						
Male	280/311	78.9/80.0	75/78	21.1/20.0	0.713	1.07 (0.75–1.53)
Female	627/7112	80.5/84.9	152/127	19.5/15.1	**0.020**	**1.36 (1.05–1.76)**
Topography						
T1	703/1023	79.4/83.3	182/205	20.6/16.7	**0.026**	**1.29 (1.03–1.61)**
T2	111/1023	80.4/83.3	27/205	19.6/16.7	0.368	1.23 (0.78–1.93)
T3	83/1023	84.7/83.3	15/205	15.3/16.7	0.804	0.93 (0.52–1.65)
T4	10/1023	76.9/83.3	3/205	23.1/16.7	0.555	1.48 (0.40–5.46)
Lymph node						
N0	586/1023	84.3/83.3	109/205	15.7/16.7	0.536	0.92 (0.72–1.19)
N1	321/1023	73.1/83.3	118/205	26.9/16.7	**0.038**	**1.22 (1.01–1.48)**
Metastasis						
M0	890/1023	80.0/83.3	223/205	20.0/16.7	**0.031**	**1.27 (1.05–1.51)**
M1	17/1023	81.0/83.3	4/205	19.0/16.7	**0.035**	**1.24 (1.02–1.54)**
Grade						
I+II	766/1023	79.5/83.3	197/205	20.5/16.7	**0.021**	**1.29 (1.04–1.61)**
III+IV	141/1023	82.5/83.3	30/205	17.5/16.7	0.892	1.03 (0.67–1.57)

CI = confidence interval, OR = odds ratio.
* Two-sided χ^2 test.
[†] Adjusted for age and sex in logistic regression model.
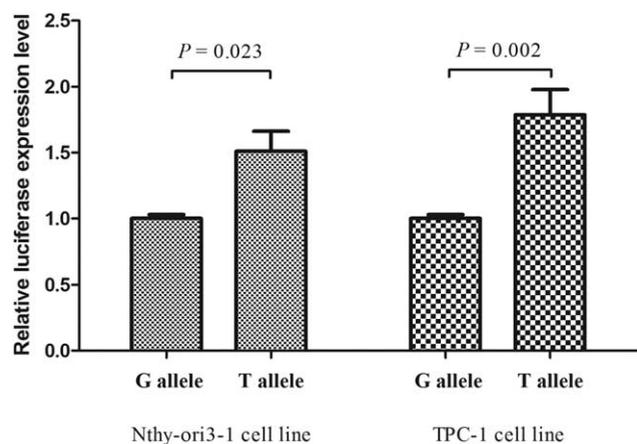Bold values signifies $P < 0.05$.

Figure 3. Luciferase assays of *exportin 5* 3′ untranslated region microRNA-related single-nucleotide polymorphism rs11077 different alleles for their functional behaviors.

expression of *XPO5* in TC tissues was significantly lower than *XPO5* in normal tissues. Further case–control study showed that individuals with *XPO5* rs11077 TG/GG genotype were more prone to TC. Also, the TG/GG genotypes correspond to a lower expression of *XPO5* mRNA. These results suggested that *XPO5* miRNA-SNP rs11077 could be regarded as a potential biomarker for TC prediction via its functional impact to *XPO5* expression.

XPO5, a member in the importin-β family of nucleocytoplasmic transport factors, is widely acknowledged as a mediator of nuclear export of siRNA and miRNA. By its feature of Ran-guanosine triphosphate (GTP)-dependent dsRNA-binding protein, *XPO5* transports the pre-miRNAs from nucleus to cytoplasm via a GTP-dependent process. After exportation, pre-miRNAs further undergo the successive mature process and finally transformed as functional miRNAs in cells.[23,24] In recent study, *XPO5* has also been identified to protect pre-miRNAs against nuclear degradation.[25] Knockout of *XPO5* could trigger an overall decrease of miRNA's expression and lead to the occurrence, progression, and metastasis of cancers.[26] In this study, we investigated the differential expression of *XPO5* between tumor tissues and adjacent normal tissues, and the results confirmed decrease trend of *XPO5* expression in cancers. Thus, *XPO5* potentially participates in the onset of TC, as *XPO5* plays tumor-suppressive roles in TC cells. Our findings were consistent with experiments in colorectal cancer cells and hepatocellular carcinoma cells.[26,27]

Evidences have shown that alteration of *XPO5* by genomic mutations, aberrant expression, or other mechanisms could significantly influence the final protein expression.[27] We found that the *XPO5* miRNA-SNP rs11077 G allele was associated with lower expression of *XPO5* in cancer tissues from TC patients. Besides, regarding its special physical location in 3′UTR of *XPO5*, rs11077 was also hypothesized to regulate the *XPO5* expression level. Previous research predicted the potential pathways including rs11077 for *XPO5* regulation, and miRNA-617/miRNA-5002-3p was reported to lose/gain the binding ability when G allele existed.[28] In this study, we verified these predictions by luciferase assay in Nthy-ori3-1 and TPC-1 cell line. The results of luciferase showed a significant distinction of promoting/enhancing abilities between T and G alleles of rs11077. However, after interference or overexpression of miRNA-617 or miRNA-5002-3p, the results of luciferase assay

did not present any variation (data shown in Supplementary data, http://links.lww.com/MD/B601). It is possible that miRNA-617 or miRNA-5002-3p might not be a major regulator for rs11077 in *XPO5* expression, at least in TC; there might be some other unknown regulating mechanisms for *XPO5* rs11077 in TC cells.

In addition, it is well established that several proteins, especially IMP family members, protect against cellular metastasis process in TC. As described in current studies, miRNA-21 and miRNA-221 were overexpressed in TC cells, which potentially bound PDCD4 and TIMP3 mRNA as their targets, respectively.[29–31] Following these pathways, higher level of *XPO5* expression might lead to an upregulation of these oncogenic miRNAs, which were partially consistent with the impact of the rs11077 G allele within the lymph node invasion N1 and metastasis M1 in our stratification analysis. These results suggested that miRNA-SNP rs11077 is not only a potential biomarker for TC susceptibility, but also distant metastasis and lymphatic invasion indicator in clinical diagnose of TC.

There are some limitations in this study. First, all participants in the case–control study were enrolled from hospital, and this process bears inherent selecting and information biases. Second, confounders for TC risk, such as tobacco and alcohol consumption, were not available in this study. In addition, although more than 1000 TC patients were included in our study, the statistical power of our study was still limited, especially in our stratification analysis. Finally, the explicit function of rs11077 on the expression of *XPO5* in TC was still not clear.

5. Conclusion

Our study identified that *XPO5* had lower expression in TC tissues. Besides, an miRNA-SNP rs11077 T > G in 3′UTR of *XPO5* was strongly associated with the expression of *XPO5*. Furthermore, this miRNA SNP could be a potential biomarker for susceptibility of TC for its significant adverse effect in G allele. Further epidemiological studies with large sample size, clinical prognosis, and functional investigation of this SNP were fervently expected.

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