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’ untranslated 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’ untranslated 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’ untranslated 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 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.\textsuperscript{[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.\textsuperscript{[19]} Because of the hazardous consequences of target miRNAs 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.\textsuperscript{[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 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. Both were collected from 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).\textsuperscript{[22]} We adopted glyceraldehyde-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% CO2 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 XPOS 3’UTR-reporter plasmid and luciferase assay

The human XPO5 3’UTR sequences with different alleles of rs11077 TG 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 \(\chi^2\) 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 \(\chi^2\). 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.
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).

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Table 1
Demographic characteristics and clinical features.

| Variables | Cases (n = 1134) | Controls (n = 1228) | P* |
|-----------|------------------|---------------------|----|
| Age, y    | n %              | n %                |    |
| ≤45       | 590 52.0         | 631 51.4           | 0.754 |
| >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         | 906 73.6           |    |
| T2        | 138 12.2         | 189 15.4           |    |
| T3        | 98 8.6           | 97 8.0             |    |
| T4        | 13 1.2           | 10 0.8             |    |
| Lymph node|                 |                    |    |
| N0        | 695 61.3         | 747 60.6           |    |
| N1        | 439 38.7         | 481 39.4           |    |
| Metastasis|                 |                    |    |
| M0        | 1113 98.2        | 1186 96.3          |    |
| M1        | 21 1.8           | 32 2.7             |    |
| Grade     |                  |                    |    |
| I+II      | 963 84.9         | 1031 84.0          |    |
| III+IV    | 171 15.1         | 247 16.0           |    |

* Two-sided χ² test for the frequency distributions of selected variables between cases and controls.

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

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.
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...

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**Table 2**

| Genotypes | Cases n=1134 | Controls n=1228 | P | Adjusted OR (95% CI) |
|-----------|-------------|----------------|---|---------------------|
| TT        | 907         | 1023           | 0.065 | 1.22 (0.99–1.52) |
| GT        | 210         | 194            | 15.8 | 1.20 (0.99–1.29) |
| GG        | 17          | 11             | 0.9  | 1.07 (0.81–1.41) |
| GT/GG     | 227         | 205            | 16.7 | 0.035               |
| T allele  | 2024        | 2240           | 91.2 | 1.25 (1.02–1.54) |
| G allele  | 244         | 216            | 8.8  | 0.024               |
| P trend   |             |                |      | 0.025               |

CI = confidence interval, OR = odds ratio.
Two-sided χ² test.
Adjusted for age and sex in logistic regression model.
Bold values signify P < 0.05.

**Table 3**

| Variables          | TT (case/control) n | % | TT (case/control) n | % | P | Adjusted OR (95% CI) |
|--------------------|---------------------|---|---------------------|---|---|---------------------|
| Ages, 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             | 623/711             | 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                 | 101/1023            | 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             | 768/1023            | 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 χ² test.
Adjusted for age and sex in logistic regression model.
Bold values signify P < 0.05.
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 TGGG 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, 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-or3-1 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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