RESEARCH ARTICLE

Genetic polymorphisms of microRNA machinery genes predict overall survival of esophageal squamous carcinoma

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Background: MicroRNA (miRNA)-related single nucleotide polymorphisms (miR-SNPs) in miRNA processing machinery genes are implicated in carcinogenesis, as they change the expression profiles of miRNA. Six miR-SNPs in miRNA processing machinery genes, including Dicer (rs3742330), RAN (rs14035), XPO5 (rs11077), TNRC6B (rs9623117), GEMIN3 (rs197412), and GEMIN4 (rs2740348), were evaluated for their association with esophageal squamous cell carcinoma (ESCC).

Methods: The miR-SNP of the miRNA processing genes were genotyped using the polymerase chain reaction-ligase detection reaction (PCR-LDR) assay, while the XPO5 expression levels in ESCC tissues were measured by immunohistochemistry methods.

Results: Patients carrying the rs11077 AA allele exhibited a significantly increased lifespan than AC+CC carriers, as determined by univariate and multivariate analyses (relative risk: 2.490; 95% confidence interval [CI]: 1.225-5.058; $P=.012$). Furthermore, the rs11077 AA genotype displayed a trend for high XPO5 expression in ESCC tissues by immunohistochemistry analysis, and these high XPO5 expression levels were also associated with high survival rates among ESCC patients.

Conclusion: Our results suggested that the miRNA machinery gene expression-associated miR-SNPs would modify cancer outcomes; in this light, XPO5 may be an important new target for ESCC therapy.

KEYWORDS

esophageal squamous cell carcinoma, miR-SNP, survival, XPO5

1 | INTRODUCTION

Esophageal cancer was listed as the sixth most common cause of cancer-related deaths worldwide with an increasing incidence rate.^{1,2} As one of the commonest cancers in north-central China, the majority of esophageal cancers are esophageal squamous cell carcinomas (ESCCs); here, the age-standardized annual incidence rate is >125/100 000.³ Nearly all esophageal cancer patients are diagnosed at advanced stages and they face a poor prognosis; the cumulative mortality is approximately 25% for men and 20% for women.^{4,5} ESCC's clinical characteristics, such as tumor-node-metastasis (TNM) stage and lymph node metastasis, were identified as prognostic factors for esophageal cancer. Moreover, some genetic factors also have a predictive value on esophageal cancer outcomes.^{6,7}

MicroRNA (miRNA) is a noncoding RNA with a nucleotide length of ~22, and it is implicated in various biological processes such as embryonic development, cell differentiation, proliferation, apoptosis, cancer development, and insulin secretion.^{8,9} miRNAs play important roles in cancer development, as they posttranscriptionally regulate the expression of tumor suppressor genes and proto-oncogenes.^{8,10,11} During miRNA processing, the nuclear cleavage of long primary transcripts of miRNAs (pri-miRNAs) by the microprocessor complex, including the RNase III Drosha, could produce a stem-loop intermediate miRNA precursor (pre-miRNA). Pre-miRNAs are transported to the cytoplasm by the nuclear transport factors exportin-5 (XPO5), while RAN, RNase III Dicer, and transactivation-responsive RNA-binding protein (TRBP) process pre-miRNAs to release a 21-bp double-stranded (ds)RNA in the cytoplasm. The RNA-induced silencing

FIGURE 1 XPO5 expression in ESCC tissue with (A) high XPO5 expression; and (B) low XPO5 expression. Original magnification: 200×

complex (RISC) including GEMIN3 and GEMIN4 will select one strand as mature miRNAs and guide these mature miRNAs to their target mRNA sites.^{12–17} miRNA-related single nucleotide polymorphisms (miR-SNPs)—which are defined as single nucleotide polymorphisms (SNPs) in miRNA genes at the miRNA binding site and in the miRNA processing machinery—could modify cancer risk, treatment efficacy, and patient prognosis by modulating miRNA and targeted gene expressions.^{17–22}

In the present study, we genotyped six miR-SNPs of miRNA processing machinery genes, including Dicer (rs3742330), RAN (rs14035), XPO5 (rs11077), TNRC6B (rs9623117), GEMIN3 (rs197412), and GEMIN4 (rs2740348), to evaluate their influence on ESCC.

2 | MATERIALS AND METHODS

2.1 | Tissue specimens and DNA extraction

Blood samples were collected from 128 ESCC patients who underwent tumor resection at the Department of Thoracic Surgery in the Fourth Hospital of Hebei Medical University between 2006 and 2008. The Wizard® Genomic DNA extraction kit (Promega Corporation, Madison, WI, USA) was used for DNA extraction. The program was approved by the Human Tissue Research Committee of the Fourth Hospital of Hebei Medical University. Written informed consent was obtained from all of the patients enrolled in this study.

2.2 | Genotyping of miR-SNPs

The miR-SNP of the miRNA processing genes including XPO5 (rs11077), RAN (rs14035), Dicer (rs3742330), TNRC6B (rs9623117), GEMIN3 (rs197412), and GEMIN4 (rs2740348) were genotyped as described previously, with the corresponding primers and probes.^{21,22} Briefly, the polymerase chain reaction (PCR)-ligase detection reaction (LDR) assay was performed to amplify the DNA fragments flanking miR-SNPs using the sequence provided in the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/snp/>). Ligation was performed using the different probes that were matched to the miR-SNPs, while the ligated products were separated using the ABI PRISM Genetic Analyzer 3730XL (Applied Biosystems, Foster City, CA, USA), which was used to detect the length difference of ligated products.

2.3 | Measurement of XPO5 levels in ESCC tissue

The ESCC tissue was first immunostained with an anti-XPO5 antibody (Abcam plc, Cambridge, UK) at a dilution of 1:100 at 4°C overnight; it was subsequently incubated with a biotinylated secondary anti-mouse immunoglobulin (Ig)G antibody at room temperature for 1 hour. After being incubated with horseradish peroxidase (HRP)-conjugated streptavidin, the staining of ESCC tissue was developed with 3,3-diaminobenzidine (DAB).

The stained slides were semi-quantified by two pathologists who were blinded to the SNP data using HSCORE (19). Briefly, we calculated the score based on the estimates of the percentage of positively stained ESCC cells in each of five intensity categories (0, 1+, 2+, 3+, and 4+). The HSCORE represents the sum of the percentages of each tissue multiplied by the staining intensity, as follows: $HSCORE = (i+1)\pi$, where $i=1, 2, 3$, and 4 and π varies from 0% to 100%. High expression

TABLE 1 Univariate analysis for clinical characteristics with overall survival of ECSS patients

Characteristics	Cases	5-year survival rate (%)	P value
Gender			
Male	89	49.4	.424
Female	39	53.8	
Age (years old)			
≤55	55	56.4	.376
>55	73	46.6	
Tumor length			
≤5 cm	78	53.8	.283
>5 cm	50	46.0	
Tumor location			
Up-part	16	50.0	.920
Middle-part	87	49.4	
Low-part	21	57.1	
Muliple	4	50.0	
Clinical stages			
0	2	100.0	.000
I	10	50.0	
II	74	63.0	
III	42	26.2	

TABLE 2 Univariate analysis of miR-SNP with ESCC overall survival

SNP	Genotype	Case	5-year survival rate(%)	P value
rs3742330				
(Dicer)	AA	58	41.4	.074
	AG+GG	70	58.6	
rs14035				
(RAN)	CC	87	50.6	.915
	CT+TT	41	51.2	
rs11077				
(XPO5)	AA	117	53.8	.006
	AC+CC	11	18.2	
rs9623117				
(TNRC6B)	TT	115	50.4	.885
	CT+CC	13	53.8	
rs197412				
(GEMIN3)	TT	61	49.2	.579
	CT+CC	67	52.2	
rs2740348				
(GEMIN4)	GG	96	49.0	.346
	CG+CC	32	56.3	

was defined as a score of >100% and low expression was defined as a score of <100% (Figure 1A and B).

2.4 | Statistical analysis

The χ^2 test was used to analyze dichotomous values, such as the presence or absence of any individual SNP in ESCC patients and healthy controls. The distribution of XPO5 expression referring to each rs11077 genotype was also compared with the χ^2 test. Survival curves were calculated using the Kaplan-Meier method, and a multivariate survival analysis was performed using a Cox proportional hazards model. Statistical analyses were performed using the SPSS 18.0 software (IBM Corporation, Armonk, NY, USA). For all statistical tests, $P < .05$ was considered to represent a statistically significant difference.

3 | RESULTS

We genotyped these six miR-SNPs of the miRNA processing machinery genes including XPO5 (rs11077), RAN (rs14035), Dicer (rs3742330), TNRC6B (rs9623117), GEMIN3 (rs197412), and GEMIN4 (rs2740348) in 128 ESCC patients and 120 healthy controls to evaluate the impact of these miR-SNPs on ESCC risk. No difference in allele distribution frequency of these miR-SNPs could be found between controls and ESCC patients by the χ^2 test. We subsequently assessed the prognostic value of these miR-SNPs on the overall survival of postoperative ESCC patients.

FIGURE 2 Significant survival differences for ESCC patients between the AA and AC+CC genotypes of rs11077**TABLE 3** Multivariate analysis of ESCC survival associated predictors with COX hazards model

Factors	Relative risk	95%CI	P value
rs11077	2.490	1.225-5.058	.012
Clinical stages	2.134	1.394-3.267	.000

TABLE 4 Associate of rs11077 genotype with XPO5 expression

XPO5			
rs11077	Low-expression	High-expression	P value
AA	13	38	.070
AC+CC	5	4	

The Kaplan-Meier method was first performed to identify the clinical characteristics and ESCC stage associated with ESCC survival, as well as to display the association between ESCC stage and ESCC survival using the log-rank test (Table 1). As for the miR-SNPs (Table 2), the rs11077 of the XPO5 gene was identified as a predictive marker for postoperative ESCC patients with the AA allele, as these patients tended to have a high 5-year survival rate (53.8% for AA patients and 18.2% for AC+CC patients; $P = .006$), as illustrated by the log-rank test. The survival curve of AC+CC patients decreased dramatically when compared with those with an AA genotype (Figure 2). The multivariate analysis was performed with a Cox proportional hazards model that included the ESCC outcome predictors from Tables 1 and 2; the rs11077 of the XPO5 gene was identified as an independent predictor of ESCC outcomes (relative risk: 2.490; 95% confidence interval [CI]: 1.225-5.058; $P = .012$) (Table 3).

The relationship between XPO5 expression and the rs11077 genotype was evaluated in ESCC tissues. The HSCORE of XPO5 was

calculated; the rs11077 genotype-based XPO5 expression in ESCC patients is listed in Table 4. The rs11077 AA genotype displayed a trend toward higher XPO5 expression when compared with the AC+CC genotype; this finding was of borderline statistical significance ($P=.070$). A survival analysis related to XPO5 expression was performed; it was determined that patients with high XPO5 expression levels had a longer lifespan than did those patients with low XPO5 expression levels (5-year survival rate: 57.1% vs 33.3%, respectively; $P=.045$).

4 | DISCUSSION

A number of cancer risks and outcomes were identified and found to be associated with the miR-SNP in the miRNA machinery genes.^{17–22} As such, we evaluated the relationships between the miR-SNP of miRNA machinery genes and ESCC in the present study. The miR-SNP of rs11077 located in the 3'UTR of the XPO5 gene was found to be associated with the overall survival of postoperative ESCC patients. A functional study also showed that the AA genotype of rs11077 seems to be correlated with high XPO5 expression levels, and that this high XPO5 expression level is associated with longer survival times among ESCC patients.

As an important miRNA machinery gene, XPO5 mediates the nuclear export of dsRNA, including pre-miRNAs, viral hairpin RNAs, and transfer (t) RNAs; therefore, to regulate global miRNA expression, XPO5 also interacts with Dicer and it is further implicated in cell-cycle control.^{23–25} The miR-SNP of rs11077 in the XPO5 gene has been associated with the risk of developing colorectal cancer and renal carcinoma, and it also appears to be related to the outcomes of multiple myeloma and non-small cell lung cancer patients.^{17,18,20,26,27} The true mechanism underlying the rs11077 SNP in the 3' UTR of the XPO5-modified ESCC survival rate remains unclear. This SNP might affect mRNA stability and subsequently alter the expression of XPO5; however, the genes or miRNAs that modulate XPO5 expression through this site have not yet been specified. The Renilla luciferase assay had previously shown that the CC type of rs11077 was associated with reduced Renilla expression,¹⁸ which is consistent with our immunostaining results, whereby CC+AC genotypes tended to exhibit low XPO5 expression levels. Knocking down XPO5 expression resulted in the reduction of global miRNA levels, thus enhancing carcinogenesis.^{28,29} Furthermore, a mutated and inactive XPO5 also reduces miRNA processing and decreases target gene inhibition; in this way, the restored XPO5 seems to act as a tumor suppressor to reverse the impaired export of pre-miRNA.³⁰ We also found that the low XPO5 expression levels were linked with shorter survival times among ESCC patients; thus, our data implied that rs11077 was responsible for changes in XPO5 expression, and that altering XPO5 expression might mediate ESCC outcomes by modulating global miRNA expression.

To our knowledge, this is the first study to investigate the associations between the miR-SNP of the microRNA processing machinery genes and ESCC survival. XPO5, a miRNA machinery gene, is thus an important new target for ESCC therapy.

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