Original Paper

The Functional Variant in the 3'UTR of PTPRT with the Risk of Esophageal Squamous Cell Carcinoma in a Chinese Population

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Key Words

Genotype • miR-218 • 3'-UTR • Tumor growth • Metastasis

Abstract

Background/Aims: PTPRT is an essential tumor suppressor that plays crucial roles in regulating the mechanisms of tumorigenesis. Polymorphisms in PTPRT have been reported associated with human longevity, but their association with the risk of esophageal squamous cell carcinoma (ESCC) has not been found so far. In this study, we focused on the miRNAs associated SNPs in the 3'-UTR of PTPRT to investigate the further relationship of the SNPs with miRNAs among Chinese ESCC patients. **Methods:** We performed case-control study including 790 ESCC patients and 749 cancer-free controls. Genotyping, real time PCR assay, cell transfection and the dual luciferase reporter assay were used in our study. **Results:** We found that patients suffering from smoking exposure, drinking exposure and the history of cancer indicated to be the susceptible population by comparing with controls. Besides, SNP rs2866943 in PTPRT 3'-UTR was involved in the occurrence of ESCC by acting as a protective factor while rs6029959 acting a risk factor. SNP rs2866943 was also could be regulated by miR-218 which caused a down-regulation of PTPRT in patients with CT and TT genotype. Furthermore, the carriers of CT and TT genotype presented a small tumor size as well as the low probability of metastasis. **Conclusion:** Our findings have shown that the SNP rs2866943 in PTPRT 3'-UTR, through disrupting the regulatory role of miR-218 in PTPRT expression, rs2866943 in PTPRT might act as a protective factor in the pathogenesis of ESCC.

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Introduction

Esophageal carcinoma is one of the most deadly malignant tumors in the world. It has become the sixth leading cause of death and the eighth most frequently diagnosed cancer [1]. Esophageal carcinoma is divided into three types including squamous carcinoma (ESCC); adenocarcinoma and undifferentiated carcinoma based on pathological diagnosis [2]. ESCC is the most common subtype with a poor treatment and survive rate [3, 4]. Age, diet, genetic background and environmental factors are thought to be associated with risk of ESCC [5, 6]. Although many people are exposed to these risk factors, only a few individuals develop ESCC in their lifetimes, suggesting that genetic variation may contribute to esophageal carcinogenesis. Traditional treatment on ESCC including chemotherapy, radiation therapy and esophageal resection is not enough comparing with the increasing number of people suffering with ESCC [7-9]. Improving results with the candidate gene approach have led to its growing acceptance as a potentially useful method for investigating genetic risk factors for ESCC among Chinese.

Protein Tyrosine Phosphatase, Receptor type, T (PTPRO) is a member of the protein tyrosine phosphatase (PTP) family [10]. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation [11]. This PTP possesses an extracellular region, a single transmembrane region, and two tandem intracellular catalytic domains, and thus represents a receptor-type PTP. The extracellular region contains a meprin-A5 antigen-PTP (MAM) domain, Ig-like and fibronectin type III-like repeats [12]. Recently researchers have identified a heterozygous c.4094C-T transition in the PTPRT gene, resulting in a thr1365-to-met (T1365M) substitution at a highly conserved residue in the second tyrosine phosphatase catalytic domain which was highly associated with the pathogenesis of cancer [13, 14]. Wang et al. reported that the mutated tyrosine phosphatases are tumor suppressor genes, regulating cellular pathways that may be amenable to therapeutic intervention [15].

MiRNAs are small, non-coding RNA molecules of 19-25 nucleotides which have been reported to play important roles by regulating cell differentiation, proliferation, migration and apoptosis [16]. MiRNAs negatively regulate their target genes expression at the posttranscription level through binding to 3' untranslated regions (UTRs) of their targets message RNAs [17, 18]. miRNAs could also regulate the 3'UTR region who harboured the single-nucleotide polymorphisms (SNPs), SNP, as the most common human genetic variations, have been proved to be significantly related to the occurrence of diseases including gastric cancer [19]. More and more studies have provided evidences that SNPs located in the miRNA (miRSNPs) binding sites through affecting the binding of miRNAs with the target genes resulted in reduction or increase in the target mRNA translation, and thus being associated with the susceptibility to cancers [20, 21]. Researchers have found that the rs2910164 polymorphism in the sequence of miR-146a precursor may influence the susceptibility to gastric cancer in the Chinese population as well as SNPs rs4143815 and rs4819388 in the 3'UTRs of B7-H1 and B7-H2 genes were significantly related to the occurrence of gastric cancer [22-24].

In this study, we focused on the SNPs in the 3'UTR of PTPRT which has not been reported before. By using the bioinformatics software (<http://www.bioguo.org/miRNASNP/>), we obtained all the SNPs which could regulate by different miRNAs as candidate SNPs and further investigated the allele distribution in a case-control study.

Materials and Methods

Study subjects

The hospital-based case-control study consists of 790 patients newly diagnosed with ESCC and 749 cancer-free controls. All the subjects were genetically unrelated Han Chinese recruited from the the People's Hospital of Kunshan City, China between January 2007 and December 2012. Patients with other

hematological disorders, previous history of cancers, radiotherapy and chemotherapy were excluded. The cancer-free control subjects from the same geographic area showed no evidence of genetic relationship with the cases. The patients were classified according to World Health Organization classification. This study was approved by Ethics Committee of the First People's Hospital of Kunshan City, and every patient had written informed consent. The clinical feature of all the cases and controls were presented in Table 1.

Genotype

We extracted genomic DNA from peripheral whole blood of every validation subject by using QIAamp DNA blood mini kits (Qiagen, Germany) according to the manufacturer's instructions. Genotyping was performed with the TaqMan SNP Genotyping Assay. The PCR reactions were carried out in a total volume of 5 µL containing TaqMan Universal Master Mix, 80X SNP Genotyping Assay Mix, Dnase-free water and 10-ng genomic DNA. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The 384-well ABI 7900HT Real Time PCR System

Real time PCR assay

Real time polymerase chain reaction (RT-PCR) was performed to determine whether the C to T mutation changed the expression level of PTPRT. The amplification conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 40 seconds, and 72°C for 30 seconds, and finally 4°C for 30 minutes for cooling.

Cell lines and cell culture

ESCC cell lines and TE-1 and Eca-109 were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, USA) and grown in humidified 5 % CO₂ at 37°C. MiR-16 mimics and normal control were obtained from Genepharma (Shanghai, China). The transfection was conducted by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Prediction of miRNAs binding to the SNP

Bioinformatics analysis (<http://www.bioguo.org/miRNASNP/>) was used to predict the related SNPs in the 3'UTR of PTPRT which could be regulated by different miRNAs.

Construction of luciferase-based reporter plasmids

All the fragment of the 3'UTR containing either C or T allele was amplified. The PCR production was cloned into the pGL3-promoterless luciferase-based plasmid (Promega, CA, USA) at the cloning site between KpnI and XhoI. The amplified fragment was verified by DNA sequencing.

Dual-luciferase reporter assay

The 3'-UTR sequence of PTPRT predicted to interact with miR-218 or a mutated sequence with the predicted target sites were inserted into the KpnI and XhoI sites of pGL3 promoter vector (Genscript, Nanjing, China). For reporter assay, cells were plated onto 24-well plates and transfected with 100 ng of pGL3-PTPRT wild, pGL3-PTPRT mutant and miR-218 mimics, respectively by using Lipofectamine 2000 (Invitrogen Corp, CA, USA). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected to normalize the differences in transfection efficiency. Transfection was repeated three times in triplicate.

Statistical analysis

The association between rs2866943 and rs6029959 genotypes and the risk of ESCC was evaluated by calculating the odds ratios (ORs) and their 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. Stratification analysis was performed according to the clinical characteristic and risk classification to determine the genotype distribution in cases and controls as well as their association with the risk of ESCC. The difference of the expression levels of PTPRT with three genotypes and the difference of the relative luciferase activities between the wild and mutant genotype were evaluated by independent-sample t test. All statistical tests were two-sided and P < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS 13.0 (SPSS Ltd.) and SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). The graphs were generated by Graphpad Prism 5.0 (Graphpad Software, Inc.).

Table 1. Frequency distributions of selected variables in patients and cancer-free controls. *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls

Variables	Cases (n = 790)		Controls (n = 749)		P*
	N	%	N	%	
Age (years)					
≤ 60	431	54.6	411	54.9	0.901
> 60	359	45.4	338	45.1	
Gender					
Male	449	56.8	442	59.0	0.387
Female	341	43.1	307	40.9	
Smoking exposure					
Negative	216	27.3	422	56.4	0.030
Positive	574	72.7	327	43.6	
Drinking exposure					
Negative	248	31.4	521	69.6	< 0.001
Positive	542	68.6	228	30.4	
Family history of cancer					
Negative	373	47.2	411	54.9	0.003
Positive	417	52.8	338	45.1	

Results

Subject characteristics

The characteristics of the 790 ESCC patients and 749 healthy controls are summarized in Table 1. No statistically significant differences were observed between cases and controls in terms of sex and age (both $P > 0.05$). Patients suffering from smoking exposure, drinking exposure and the history of cancer indicated to be the susceptible population by comparing with controls.

The miRSNPs in the PTPRT gene 3'-UTR

In order to investigate the miRNA associated SNPs in the 3'UTR of PTPRT, we first found all the SNPs from the SNP databases NCBI db SNP BUILED 129 and ENSEMBL v58 in the 3'-UTR of PTPRT gene with the minor allele frequency(MAF) >0.05 . We then used bioinformatic softwares Diana-Micro, MicroInspector, miR-anda, miRNAmap, RNAhybrid to predict miRNAs that can bind to the PTPRT 3'-UTR. The miRNASNP database was also applied to explore the miRNAs which could also bind to the 3'-UTR of patients harbored the SNP. As shown in Table 2. Finally, we obtained 23 SNPs in the 3'UTR which could be regulated by different miRNAs. The positions of the SNPs in PTPRT as well as the variants were listed. Further genotyping was performed to detect the distribution of allele gene of the 23 SNPs in our research.

Correlation of rs2866943 and rs6029959 with gastric cancer

We detected all the 23 SNPs genotype frequencies in 790 ESCC cases and 749 controls. Only two SNPs (rs2866943 and rs6029959) indicated a significant difference in ESCC patients with the T-allele carriers and C-allele carrier, respectively; however, the other SNPs presented no difference (data not shown). As listed in Table3, Chi-square statistical analysis results showed that the genotypes of rs2866943 were in Hardy-Weinberg equilibrium distribution pattern in the healthy control group ($P = 0.42$). Further, logistic regression analysis results revealed that The CT genotype and TT genotype presented a significant decreased risk of ESCC

Table 2. SNPs located in the PTPRT gene 3'-UTR and the predicted miRNAs. Chr = chromosome; UTR= Untranslated Regions

SNP	chr	3'UTR position	Associated miRNA	Allele*
rs1065155	20	3145-3165	miR-874	A/G
rs112700109	20	3341-3360	miR-2110	C/T
rs113281274	20	1338-1363	miR-1262	A/C
rs114291605	20	1338-1363	miR-140-3p	T/G
rs116838271	20	8050-8074	miR-574	A/C
rs2664587	20	6616-6637	miR-3694	C/T
rs2866943	20	6693-6710	miR-218	C/T
rs3787281	20	1622-1642	miR-26a/b	A/G
rs3787282	20	3564-3586	miR-1297	A/G
rs41278132	20	5376-5397	miR-3180	C/T
rs45474194	20	6699-6724	miR-1321	A/G
rs55683344	20	3630-3648	miR-876-3p	C/T
rs60102878	20	1160-1181	miR-1291	A/G
rs6029959	20	2465-2488	miR-142-5p	A/C
rs6072621	20	4835-4855	miR-3153	A/G
rs6102660	20	596-618	miR-431/942	C/T
rs6102662	20	6474-6499	miR-548a	C/G
rs6102663	20	3654-3679	miR-361-5p	A/G
rs67506870	20	1789-1811	miR-3928	A/G
rs73119823	20	731-752	miR-3154	G/T
rs73269502	20	3107-3129	miR-345	A/G
rs7352413	20	4245-4270	miR-3656	G/T
rs73907079	20	1105-1121	miR-30a/b/c/d	C/G

as compared with the CC genotype. Besides, the T carrier also indicated an increased risk ($P=0.015$). The PTPRO rs6029959 C allele was also shown to be a risk allele. Logistic regression analyses indicated that individuals with the rs6029959 AC genotype was significantly associated with gastric cancer risk ($OR=1.50$, 95 % $CI=1.24-2.99$; $P=0.0001$). Individuals having the rs6029959 CC genotype had an OR of 2.47 (95 % $CI=1.05-1.32$; $P=0.0001$) for ESCC compared with individuals having the rs6029959 AA genotype. All ORs were adjusted for sex, age, and smoking status, drinking history or family cancer history.

The effect of rs2866943 and rs6029959 on the regulatory role of miRNAs in PTPRT expression
Since the SNP rs2866943 and rs6029959 were predicted to locate in the binding site of miR-218 and miR-142-5p, respectively. We hypothesized that the expression of PTPRT might be regulated by these miRNAs, which can be impacted

by rs2866943 or rs6029959. To test whether or not the inhibitory role of these miRNAs impacted by the two SNPs, we first detected the expression level of PTPRT expression level in patients harbored the CC, CT and TT genotypes as well as the patients with AA, AC and CC genotypes. We found that patients with CT or TT genotypes presented a decreased level of PTPRT by comparing with the patients with CC genotype; however there was no difference in rs6029959 (Fig. 1A and 2A). We then constructed pGL3 vectors including the allele-specific binding sequences, and then co-transfected it with miR-218 or miR-142-5p as well as the controls in ESCC cell lines including TE-1 and Eca109 (Fig. 1B and 2B). As presented in Figure 1C, D and 2C, D, we found that the expression of T-allele-specific pGL3 construct was significantly suppressed by miR-218 ($P=0.003$). However, the expression of C-allele-specific pGL3 construct was not inhibited by miR-142-5p ($P=0.324$). These findings suggested that the inhibitory affection of the TT or CT genotype of PTPRT might be regulated by miR-218.

Stratified analyses of association between PTPRT polymorphism and ESCC risk
Then, we did stratified analysis of the association of the rs2866943 genotypes with the clinicopathological parameters of ESCC (Table 4). We found a significant association of the

Table 3. Genotype frequencies of the PTPRT rs2866943 and rs6029959 polymorphism among ESCC cases and controls. ^aThe ORs, 95% CIs and *P* value were calculated after adjusting for age, gender, parental smoking, drinking and family cancer history

Genotype	Cases (<i>n</i> =790)		Controls (<i>n</i> = 749)		OR (95% CI) ^a	<i>P</i> Value ^a
	<i>N</i>	%	<i>N</i>	%		
rs2866943						
CC	374	47.4	401	53.5	1.00	0.0001
CT	328	41.5	324	43.3	0.78 (1.20-1.72)	
TT	88	11.1	24	3.2	0.93 (1.11-1.94)	
T carrier	416	52.7	348	46.5	0.82 (1.32-1.53)	0.015
rs6029959						
AA	355	45.0	422	56.4	1.00	0.0001
AC	381	48.2	301	40.2	1.50 (1.24-1.99)	
CC	54	6.8	26	3.4	2.47 (1.05-1.32)	
C carrier	435	55.0	327	43.6	1.58 (1.12-1.53)	0.0001

Table 4. Stratified analysis of rs2866943 genotype with clinicopathological parameters of ESCC. *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls

Feather	Genotype				CT vs CC P Value	TT vs CC P Value	T carrier vs CC P Value
	CC	CT	TT	T carrier			
Age (years)							
≤ 60	204	179	48	227	0.99	1.00	0.99
> 60	170	149	40	189			
Gender							
Male	202	194	53	247	0.17	0.29	0.13
Female	172	134	35	169			
Differentiation grade							
Well	186	159	43	202	0.91	0.97	0.90
Moderate	124	109	29	138			
Poorly	64	60	16	76			
Tumor Size(cm)							
≤3cm	172	226	55	281	0.001	0.005	0.001
>3cm	202	102	33	135			
Tumor Number							
Solitary	191	160	41	201	0.66	0.45	0.52
Multiple	183	164	47	211			
Tumor Capsular							
Incomplete	201	192	49	241	0.20	0.74	0.24
Complete	173	136	39	175			
Metastasis							
Yes	290	136	29	165	0.001	0.001	0.001
No	84	192	59	251			

rs2866943 genotypes with the tumor size, and tumor metastasis,. Compared with the CC homozygote, the carriers of CT and TT genotype presented a small tumor size as well as the low probability of metastasis.

Discussion

In the present study, we investigated the relationship between miRSNPs within the 3'-UTR of PTPRT gene and the risk of ESCC. We observed that PTPRT rs2866943 CT or TT genotype is associated with significantly decreased ESCC risk compared with CC genotype and the increased risk of CC and AC genotypes comparing with AA genotype of SNP rs6029959.

Fig. 1. Suppression of PTPRT of patients with CT/TT genotypes of SNP rs2866943 was regulated by miR-218. **A:** The expression level of PTPRT was determined by RT-PCR in patients with CC, CT and TT genotypes. **B:** Bioinformatics predicted the binding site between the miR-218 with PTPRT and the mutation types were conducted into the pGL3 plasmid as presented. **C, D:** Cells were co-transfected with miR-218 mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the TE-1 cell line (panel C) while the right indicated Eca109 (panel D) cell lines. Data was presented as the mean $\pm$ SEM. * indicates a significant difference ($P < 0.05$).

Fig. 2. MiR-142-5p could not regulate the 3'UTR of PTPRT with SNP rs6029959. **A:** The expression level of PTPRT was determined by RT-PCR in patients with AA, AC and CC genotypes. **B:** Bioinformatics predicted the binding site between the miR-142-5p with PTPRT and the mutation types were conducted into the pGL3 plasmid as presented. **C, D:** Cells were co-transfected with miR-142-5p mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the TE-1 cell line (panel C) while the right indicated Eca109 cell lines (panel D). Data was presented as the mean $\pm$ SEM. * indicates a significant difference ($P < 0.05$).

We further found that the SNP rs2866943, locating in the binding sites of miR-218 through disrupting the inhibitory role of miR-218 on PTPRT expression, played an important role in the development of ESCC.

Protein tyrosine phosphatases (PTPs) are known to be signaling molecules that regulate a variety of cellular processes including differentiation, cell proliferation, mitotic cycle and oncogenic transformation [25]. Emerging evidence has revealed that mutations in PTPs play vital roles in tumorigenesis [26]. PTPRT, also known as PTPp, is a member of the PTPs family. Recently, it has been reported that PTPRT is the most frequently mutated PTP gene in colorectal cancers (CRCs). PTPRT normally acts as a tumor suppressor and over-expression of PTPRT inhibits CRC cell growth [15]. It has been also reported that PTPRT knockout mice are highly susceptible to carcinogen azoxymethane-induced colon tumor [15]. Considering about the tumor suppressor gene function of PTPRT, our founding indicated that the C>T SNP might be acted as protective factor in the pathogenesis of ESCC, however, the CT and TT genotypes could be down-regulated by miR-218. Numerous microRNAs have been reported to be associated in cancers; however, the consequence of miRNA deregulation in cancer is still unknown for many miRNAs [27]. As for the miRNAs focused in this study, miR-218 expression was found to be increased in multiple cancers. Increased miR-218 was associated with tumor growth and metastasis which was consistent with our results [28, 29].

In summary, we reported the first evidence that the SNP rs2866943 in PTPRT 3'-UTR was involved in the occurrence of ESCC by acting as a protective factor while rs6029959 acting a risk factor. SNP rs2866943 was also could be regulated by miR-218 which caused a down-regulation of PTPRT in patients with CT and TT genotype. This SNP was also found to be related to the clinicopathological features of ESCC, suggesting it may have important roles in preventing tumor development. Our results support the hypothesis that genetic variants interrupting miRNAs mediated regulation tumor suppressors would be involved in esophageal cancer etiology.

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Disclosure Statement

None declared.

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