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A miR-151 binding site polymorphism in the 3′-untranslated region of the cyclin E1 gene associated with nasopharyngeal carcinoma

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ABSTRACT

Genetic alterations in nasopharyngeal carcinoma (NPC) have been reported in previous works. However, it remains unclear whether polymorphisms within the miRNA-target binding sites are associated with individual NPC risk. In this study, new experimental and computational approaches were developed to assess the polymorphism frequency distribution within the miRNA sites in NPC patients, and to explore its association with NPC risk. We focused on 220 single-nucleotide polymorphisms (SNPs) in the 3′-untranslated regions (3′UTRs) of 32 genes carrying putative miRNA-binding sites by specialized algorithms. A total of 9 candidate genes were selected for further investigation, which were reportedly overexpressed in NPC, including EGFR, COX2, CCNE1, hTERT, MMP2, MMP9, NF-κB VEGF, and WNT3. SNPs in 3′UTRs were genotyped by direct polymerase chain reaction sequencing of the genomic DNA of 24 cases and 24 controls. Then, EGFR rs884225, CCNE1 rs3218073, and MMP2 rs7201 were screened with large samples. Based on the analysis of a series of 167 NPC cases and 171 controls from Guangdong Province, statistically significant associations were found between NPC risk and variant genotypes of CCNE1 rs3218073 for TC + TT (OR = 1.585; 95% CI = 1.023–2.458; $P = 0.046$) and for T-allele (OR = 1.464; 95% CI = 1.012–2.118; $P = 0.042$). In addition, a significant association among rs3218073 genotype TC (OR = 1.959, $P = 0.043$), T-allele (OR = 2.123, $P = 0.006$), and primary tumor (T3–T4) was retrieved. Genotype TC (OR = 1.959, $P = 0.043$) and T-allele (OR = 2.123, $P = 0.006$) of rs3218073 were correlated with increased risk of higher NPC stage (III to IV). In support of the postulation that the 3′UTR SNP directly affected miRNA-binding site, luciferase reporter assay indicated that CCNE1 was a direct target of miR-151, and the rs3218073 T > C change resulted in altered regulation of CCNE1 expression. By contrast, no statistically significant association with NPC risk was found for MMP2 rs7201 and EGFR rs884225 polymorphisms ($P > 0.05$). In conclusion, our data demonstrate that CCNE1 rs3218073 polymorphism located at miRNA-151 binding site is associated with NPC susceptibility and is correlated with NPC stage. These results suggest that CCNE1 rs3218073 polymorphism can be exploited as a novel biomarker for future NPC diagnosis and prognosis.

1. Introduction

MicroRNAs (miRNAs) are endogenous 22 nt small non-coding RNAs that can regulate gene expression by targeting mRNAs through base paring with the 3′-untranslated region (3′UTR) of genes [1], which result in mRNA cleavage or translational repression [2,3]. According to recent genome-wide association studies on human single-nucleotide polymorphisms (SNPs), variations in the non-coding regulatory sites are more likely to be associated with the disease than the coding region variations [4,5]. SNPs in the target site sequence can affect miRNA regulation. Thus, naturally occurring SNPs in target sites are candidates for functional variation that are useful for biomedical applications and evolutionary studies. Since the identification of a SLITRK1 polymorphic RNA-binding site known for its association with muscularity in sheep [6] in 2006, accumulating evidence has shown that polymorphic miRNA-target interactions contribute to cancer development [7].

Hanahan and Weinberg proposed that a tumor demonstrates six characteristics, i.e., self-sufficient growth signals, apoptotic evasion, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, as well as tissue invasion and metastasis [8]. Another study proposed cancer-related inflammation as the seventh feature of tumor-acquired capability [9]. Naso-

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pharyngeal carcinoma (NPC) is a common type of tumor in Asia and northern Africa, especially in Guangdong Province, China. Although genetic alterations in NPC have been reported, it remains unclear whether the polymorphisms within the miRNA-target binding sites are associated with individual NPC risk. Therefore, based on the studies related to genetic susceptibility of NPC and by analyzing the seven hallmarks of tumors in the current paper, new experimental and computational approaches are developed to assess the polymorphism frequency distribution within the miRNA sites in NPC patients, and to explore the association with NPC risk. We found that SNP markers in miRNA target sites with significant differences comprise a novel class of functional polymorphisms that can interfere with miRNA function.

2. Materials and methods

2.1. Study population and epidemiologic data

We conducted a hospital-based case–control study to assess gene environment interactions in relation to NPC risk. We included 167 NPC patients and 171 healthy individuals selected from the same population living in Guangdong Province. Patients who were admitted to Nanfang Hospital, The First Affiliated Hospital of Southern Medical University (Guangzhou, China), were newly diagnosed and histopathologically confirmed to have NPC between 2005 and 2009 (Table 2). Controls were selected from patients who underwent health examination at Nanfang Hospital during the same period.

Controls had no cancer history and were matched to NPC cases in terms of age, ethnicity, and family history of NPC. Both controls and patients included unrelated subjects. The clinical stages ranged from I to IV, according to the 2002 Union for International Cancer Control tumor classification. The control selection strategy is a feasible and effective approach for molecular epidemiological studies, in which population-based control selection poses a challenge. All patients and controls were interviewed face–to–face by doctors using a structured questionnaire. Blood sample (2 mL) was collected from each participant for laboratory analysis.

2.2. Collection of candidate genes

Based on the studies related to genetic susceptibility of NPC and by analyzing the seven hallmarks of cancer, we collected 32 genes that were well characterized and studied in NPC as candidate genes. These genes are listed in Supplementary Table S1.

We identified the 3′UTR sequences of these candidate genes using the University of California, Santa Cruz genome browser and found the 3′UTR SNPs residing on the miRNA-binding sites by an extensive search in dbSNP. Putative miRNA-binding sites within 3′UTR of each gene were identified by specialized algorithms, namely, MicroInspector, miRnda, Target Scans, and RNAfold webserver. The variant miRNA-binding site was identified by BLAST and BLAST–SNP algorithms.

2.3. DNA extraction and genotyping

Genomic DNA was extracted from whole blood EDTA samples using Wizard DNA Extraction Kit (Promega). The 3′UTR fragments of the candidate genes were amplified using polymerase chain reaction (PCR) and DNA primers specific to these sequences (Supplementary Table S2). All PCR products were then checked for purity and correct size. DNA sequencing was performed with an ABI Prism 3100 Genetic Analyzer with Big Dye Terminator technology (ABI, Foster City, CA, USA).

2.4. Luciferase reporter assay

The entire 531-base pair fragment of CCNE1 3′UTR containing different alleles of rs3218073 was amplified using the primers PF: 5′-gattataga**CCACCCCATCCTTCTCCACCA**-3′ and PR: 5′-gattataga**TCAAAAACAGTATTATCTTT**-3′. Each fragment was respectively cloned downstream of the *Renilla* luciferase gene at the XbaI sites in the pGL-3 promoter plasmid (Promega). To facilitate cloning into the firefly luciferase expression plasmid, the primers were designed to incorporate XbaI site at the 5′end (underlined in the primers above). The constructs were designated as pGL3-CCNE-C (wild-type) and pGL3-CCNE-T (mutant type). HEK293 cells were co-transfected with 30 pmol of either miR-151 mimics or miR-NC and pGL-CCNE using Lipofectamine 2000 (Invitrogen). Transfection efficiency was normalized by co-transfection with a firefly luciferase expressing plasmid. Luciferase activity was measured using the Promega dual-luciferase assay kit, in accordance with the instructions of the manufacturer. Relative protein levels were expressed as *Renilla*/firefly luciferase ratios. Each transfection was twice repeated in triplicates.

2.5. Quantitative real-time PCR

Total RNAs from tissues were extracted using Trizol (Invitrogen), following the instructions of the manufacturer. Quantification was performed using the Quantitect SYBR Green PCR Kit (Stratagene, USA) with an MX3005P multiplex quantitative PCR (qPCR) system (Stratagene), according to the recommendations of the manufacturer. Forward (5′- CCTCGGATTATTGCACCATC-3′) and reverse (5′- AGAATTGCTCGCATTTTTGG-3′) primers were used to detect CCNE1 expression. GAPDH was chosen as a housekeeping gene with forward (5′- ATCATCAGCAATGCCTCCTG-3′) and reverse (5′- ATGGACTGTGGTCATGAGTC-3′) primers. The relative mRNA expression levels were calculated using the comparative ΔΔCt method as previously described [10]. The fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$.

2.6. Statistical data analysis

Next, χ^2 test was performed to examine differences in the distribution of wild-type, homozygous, and heterozygous individuals in patients compared with the control group. Logistic regression analysis was applied to calculate odds ratios (ORs) and 95% confidence intervals (CI) for overall NPC. Differences were considered significant when $P \leqslant 0.05$.

3. Results

3.1. Selection of SNPs and genes

A total of 220 SNPs were identified in the miRNA-binding sites. We focused on the nine 9 genes (i.e., EGFR, COX2, CCNE1, hTERT, MMP2, MMP9, NF-κB, VEGF, and WNT3) for further investigation due to their significant overexpressions in NPC (Supplementary Table S3). We evaluated the miRNA-binding site SNPs by assessing higher MAF (Minor Allele Frequency) and lower free energy.

3.2. Screening for functional sequence variations in candidate genes

In order to determine the potential disease-associated variants in the 3′UTR of candidate genes, we sequenced the 3′UTR of the 9 genes randomly selected from 24 healthy controls and 24 NPC patients. However, only 3 targeted SNPs were identified in these genes, which are shown in Table 1 as rs3218073, rs884225, and rs7201 within CCNE1, EGFR and MMP2, respectively.

Table 1
Polymorphisms identified in genes overexpressed in NPC.

Gene	Polymorphism ID	Nucleotide sequence Wild type	Polymorphisms	Targeted miRNA
MMP2	rs7201	GGGGCTGCCC	GGGGCTGACC	miR-548 l
EGFR	rs884225	AGGGGC**A**GCATA	AGGGGC**G**GCATA	miR-103
CCNE1	rs3218073	GTGGCT**C**TCCTCG	GTGGCTTTCCTCG	miR-151

Table 2
Characteristics of NPC patients and control subjects.

	NPC cases (n = 167)		Controls (n = 171)		P
	N	F	N	F	
Age					
⩽35	55	(0.33)	69	(0.40)	0.157
>35	112	(0.67)	102	(0.59)	
Ethnicity					
Han	155	(0.93)	154	(0.90)	0.365
others	12	(0.07)	17	(0.10)	
Family history of NPC					
No	159	(0.95)	168	(0.98)	0.11
Yes	8	(0.05)	3	(0.02)	
Primary tumor (T)					
T1–T2	63	(0.37)			
T3–T4	104	(0.63)			
Lymph node metastasis (N)					
N0–N1	78	(0.47)			
N2–N3					
Distant metastasis (M)					
M0	89	(0.53)			
M1					
Clinical stage					
I–II	150	(0.89)			
III–IV	17	(0.11)			
	53	(0.32)			
	114	(0.68)			

N: number of samples, *F*:frequency of samples.

3.3. Polymorphism in CCNE1 rs3218073 as a risk factor for NPC

Table 3 depicts the genotype distribution and the allele frequencies for rs3218073 in the CCNE1 gene in both NPC patients and controls. Using the chi-square test, the distribution of the TC genotype in NPC patients and controls did not significantly differ from each other. The frequencies of TT, TC, and CC genotypes in the controls and NPC patients were 4%, 41% and 55%, as well as 2%, 32% and 66%, respectively. Using the CC genotype as reference, a significant association was found between TC + TT genotype and NPC risk (OR = 1.585, P = 0.046). Using the C-allele as reference, a significant difference was also observed between T-allele and C (OR = 1.464, P = 0.042).

3.4. Prognostic significance of rs3218073 polymorphism in CCNE1 gene

Regarding prognostic parameters, we analyzed the relations between the rs3218073 polymorphisms and the clinical characteristics of the tumor (including primary tumor size, lymph node metastasis, and clinical stage) in NPC patients. The genotype distribution and allele frequencies of the CCNE1 rs3218073 polymorphisms among NPC patients are shown in Table 4.

Significant differences were observed in the genotype and allele frequencies of the rs3218073 polymorphism in primary tumor size (T1 to T2 and T3 to T4 stages) and clinical stage (stages I to II and III to IV). Compared with genotype CC or C-allele, genotype TC (OR = 1.959; 95% CI = 1.015–3.783; P = 0.043), genotype TC + TT (OR = 2.286; 95% CI = 1.194–4.375; P = 0.011), and T-allele (OR = 2.123; 95% CI = 1.219–3.698; P = 0.006) were associated with later primary tumor (T3–T4). Genotype TC + TT (OR = 2.014; 95% CI = 1.024–3.962; P = 0.041) and T-allele (OR = 1.950; 95% CI = 1.088–3.492; P = 0.023) in rs3218073 showed an increased risk of higher stage tumor (III to IV). However, no significant differences in the genotype or allele distribution of CCNE1 polymorphism rs3218073 were observed for lymph node metastasis.

3.5. Genotyping of MMP2 rs7201 and EGFR rs884225

The rs7201 genotypes associated with the MMP2 gene in the case group (AA 56%, AC 30%, and no homozygous CC) were not less frequent in the control group (AA 54%, AC 41%; OR = 0.690, P = 0.135). No significant difference was observed in the distributions of A- and C-alleles (80% vs. 20%; OR = 0.769; P = 0.209) between cases and controls. No association between NPC and rs7201 polymorphism was revealed with P > 0.05, according to variant genotype distribution.

Meanwhile, for the rs884225 polymorphisms in the EGFR gene, the distribution of polymorphism was not significantly different between the cases and controls (AA, AG, and GG genotypes; 33%, 50%, and 17% vs. 37%, 41%, and 22%; P > 0.05), with the frequency of the variant G-allele being similar in cases and in controls (42% vs. 43%, P = 0.839). Compared with the AA genotype, the AG + GG genotype was not associated with a significant risk of NPC (OR = 1.188; 95% CI = 0.759–1.859; P = 0.451) (data not shown).

Table 3
Comparison of genotype of rs3218073 polymorphism between NPC patients and controls.

	NPC cases (n = 167)		Controls (n = 171)		OR	95% CI	P
	N	F	N	F			
Genetype							
CC	91	(0.55)	112	(0.66)	1		
TC	69	(0.41)	55	(0.32)	1.544	0.985–2.420	0.057
TT	7	(0.04)	4	(0.02)	2.514	0.611–7.587	0.363[a]
TC + TT	76	(0.45)	59	(0.34)	1.585	1.023–2.458	0.046*
Allele							
C	251		279		1		
T	83		63		1.464	1.012–2.118	0.042*

OR: odds ratio CI: confidence interval F: Frequencies.
The chi-square test was used to determine whether significant differences (P-value) were observed when patients was compared to control subjects.
N: number of samples, *F*: frequency of samples.
[a] Using continuity correction chi-square test.
* $P \leqslant 0.05$.

Table 4
Correlation of rs3218073 variant with clinical parameters of NPC cases.

Genotype	Clinical parameters				OR	95%CI	*P*
Primary tumor (T)	T1–T2 (n = 63)	*F*	T3–T4 (n = 104)	*F*			
CC	42	0.66	49	0.47	1		
TT	0	0	7	0.07			
TC	21	0.33	48	0.46	1.959	1.015–3.783	0.043*
CT + TT	21	0.33	56	0.54	2.286	1.194–4.375	0.011*
Alleles							
C	105	0.83	146	0.70	1		
T	21	0.17	62	0.30	2.123	1.219–3.698	0.006**
Lymph node metastasis (*N*)	N0–N1 (n = 78)	F	N2–N3 (n = 89)	F			
CC	45	0.57	46	0.52	1		
TT	2	0.02	5	0.07	2.446	0.451–13.26	0.439[a]
TC	31	0.38	38	0.43	1.199	0.640–2.246	0.570
CT + TT	33	0.40	43	0.48	1.275	0.691.2.351	0.436
Alleles							
C	121	0.78	130	0.73	1		
T	35	0.22	48	0.27	1.276	0.773–2.107	0.338
Stage	I–II (n = 53)	F	III–IV (n = 114)	F			
CC	35	0.66	56	0.49	1		
TT	0	0	7	0.06			
TC	18	0.34	51	0.45	1.771	0.894–3.597	0.095
CT + TT	18	0.34	58	0.51	2.014	1.024–3.962	0.041*
Alleles							
C	88	0.83	163	0.71	1		
T	18	0.27	65	0.29	1.950	1.088–3.492	0.023*

OR: odds ratio CI: confidence interval F: frequencies.
The chi-square test was used to determine whether significant differences (*P*-value) were observed when patients was compared to control subjects.
[a] Using continuity correction chi-square test.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.

3.6. Effect of SNP rs3218073 interaction between miR-151 and 3'UTR of CCNE1

A luciferase-based reporter was constructed to evaluate the effect of miR-151 direct binding to the putative target site on the 3'UTR of CCNE1. To substantiate the assumption that miR-151 can directly repress CCNE1, the reporter construct pGL3-vector or pGL3-CCNE-C and pGL3-CCNE-T was co-transfected with miR-151 mimic, miR-NC and miR-151 inhibitor or inhibitor-NC to HEK293 cells. Luciferase activity was then assayed. As shown in Fig. 1, for pGL3-CCNE-C construct, miR-151 mimic significantly lowered luciferase activity compared with miR-NC. By contrast, miR-151 inhibitor increased luciferase activity in HEK293 cells compared with inhibitor-NC. There was no different luciferase activity observed between the pGL3-vector and pGL3-CCNE-T constructs. These findings support the hypothesis that miR-151 directly targets CCNE1 expression, and the variant allele alters the dependence of CCNE1 3′UTR, thus allowing increased CCNE1 expression in the presence of this variant allele.

3.7. Altered CCNE1 mRNA expression level of tumor tissues with different genotypes

Tumor tissues were collected to match the DNA genotype, with 10 samples of CC, 10 samples of TC, and 2 samples of TT. qPCR was performed too validate the hypothesis that individuals carrying variant T-allele (TT + TC) increased CCNE1 expression level. The results showed that the relative expression level significantly increased in the group of individuals carrying T-allele, compared with that of group without T-allele (CC) (Fig. 2). The results further confirmed that rs3218073 played an important role during CCNE1 expression targeted by miR-151.

4. Discussion

The discovery that a 3′UTR polymorphism affects miRNA targeting has revealed a novel functional mechanism, by which non-coded polymorphisms can contribute to the risk of common disease [11]. Given that 30% of human genes are regulated by miRNAs, miRNA regulation can be implicated in human diseases at unexpectedly high risk levels [12]. The functions of genetic variants of the miRNA-binding sites in tumorigenesis have been reported in several studies [13,14]. In the present study, we hypothesized that several genetic variants within miRNA-binding sites in genes overexpressed in NPC (including EGFR, COX2, CCNE1, hTERT, MMP2, MMP9, NF-κB, VEGF, and WNT3) can modulate the NPC risk. To test this hypothesis, we sequenced the 3′UTR of the 9 genes to determine the potential functional polymorphisms with small samples and screened 3 polymorphisms, namely, EGFR rs884225, CCNE1 rs3218073, and MMP2 rs7201. Case-control study and reporter assay, followed by validation test of qPCR, were performed to evaluate the individual and joint associations of the polymorphisms with NPC. The results demonstrated that rs3218073 polymorphism in CCNE1 gene was associated with NPC risk, and was especially correlated with increased risk of higher NPC stage; furthermore, rs3218073 played an important function during CCNE1 expression targeted by miR-151.

Uncontrolled cell proliferation is one of the cancer hallmarks, and G1 to S-phase transition is the most common reported cell cycle abnormality in tumors [15]. The CCNE1 gene encodes cyclin E, a G1 cyclin essential for S-phase entry, which has an important function in oncogenesis. Previous studies showed that polymorphisms in CCNE1 increase the risk of ovarian cancer [16]. miRNAs have been implicated in cyclin E regulation. For example, miR-34c targets and represses cyclin E expression in lung cancer [17]. In head

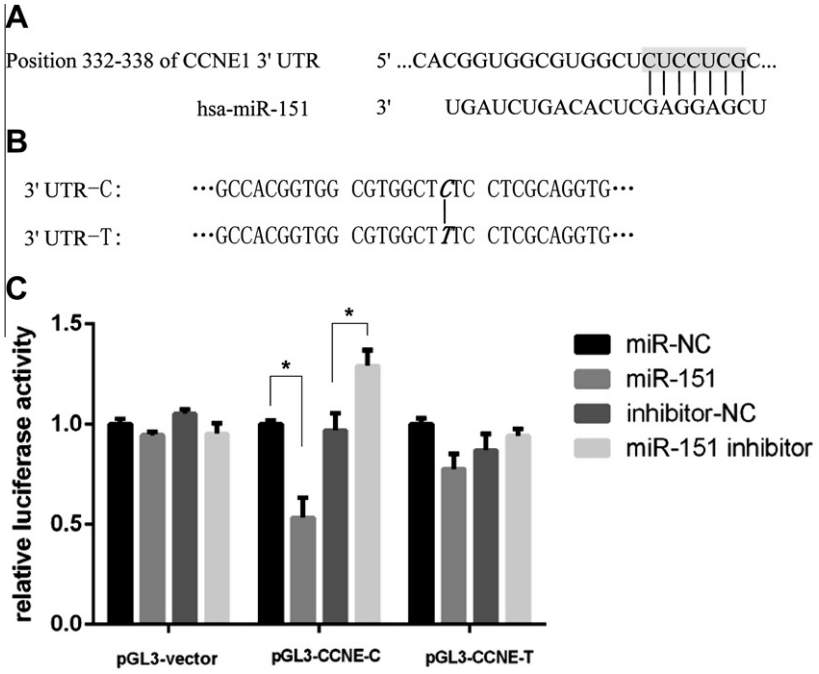

Fig. 1. Effect of the putative miR-139 binding site derived from the CCNE1 3′UTR on luciferase expression. (A) Schematic of the potential miR-151 binding site containing CCNE1 3′UTR. M: SNP rs2239680 T/C. (B) Schema of the constructs harboring different alleles of the -151 binding site. (C) Luciferase activity in HEK293 cells transfected with miR-NC, miR-151 mimics, inhibitor-NC, and miR-151 inhibitor with pGL3-vector or pGL3-CCNE-C and pGL3-CCNE-T 36 h post-transfection. Luciferase activity was measured using the Promega dual-luciferase assay kit, according to the instructions of the manufacturer. Data represent mean ± SD of the three independent experiments, and each experiment was performed in triplicate. $^{*}P < 0.05$.

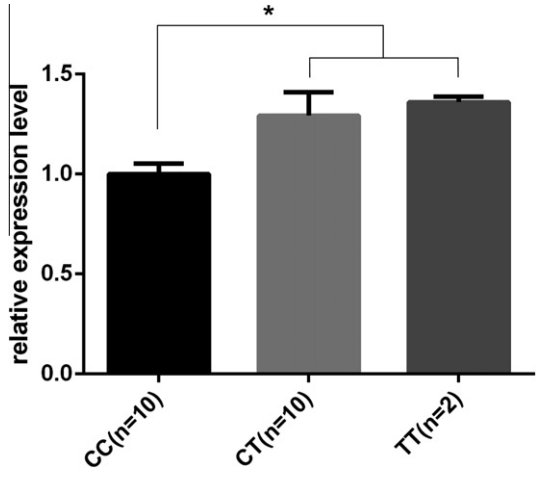

Fig. 2. CCNE1 mRNA expression levels of tumor tissues with different genotypes by qPCR. Data represent mean ± SD of each sample group. Each experiment was performed in triplicate. $^{*}P < 0.05$, CT + TT compared with CC.

and neck cancer, protein kinase C-α deregulates cyclin E expression by suppressing miR-15a [18]. Furthermore, the miR-16 family can trigger cell accumulation in G0/G1 by silencing multiple cell cycle genes, including CCND3, CCNE1, and CDK6 [19].

The current study is the first to investigate rs3218073 polymorphism within the CCNE1 gene and its association with NPC development, especially correlated with increased risk of higher NPC stage. In this study, individuals carrying the T-allele were at a significantly increased risk of NPC and tumor staging compared with those individuals with the C-allele. In addition, compared with CC homozygote and TC heterozygote variants, TT homozygote variant showed the highest OR (2.514) and the highest *P* value (0.363). This result can be attributed to the TT distribution frequency (4%) that is significantly lower than those of TC (41%) and CC (55%). However, the mechanisms underlying the association between T-allele polymorphism and NPC risk remain to be elucidated in future studies. The "seed region" of miR-151 is predicted to bind to a target site containing the same SNP within the CCNE1 gene. When T substitutes C, miRNA-151 is predicted to bind less tightly to the CCNE1 3′UTR.

According to previous studies, miR-151 is up-regulated in NPC [20], esophageal squamous cell carcinoma [21], colon cancer [22] and acute lymphoblastic leukemia [23], but is down-regulated in chronic myeloid leukemia [24]. In the present study, luciferase reporter assay supported the hypothesis that miR-151 can directly target CCNE1 expression, and that the variant allele can alter the dependence of CCNE1 3′UTR, thus allowing increased CCNE1 expression in the presence of this variant allele. The mRNA expression of tumor tissues by qPCR further confirmed that rs3218073 played an important function during CCNE1 expression targeted by miR-151. Given the difficulties involved in collecting normal tissues, especially the paired normal-tumor tissues, validation test has yet to be performed to confirm the earlier conclusion of higher CCNE1 expression with NPC. Therefore, miRNA-151 expression and functional analysis of miRNA-151 in rs3218073 within the CCNE1 gene by luciferase assay, site-directed mutagenesis, or RNA interference assay are necessary in further characterizing the molecular mechanisms underlying the observed associations.

In this study, we also evaluated the association of NPC with two SNPs, namely, rs884225 and rs7201 in the EGFR and MMP2 genes, respectively. Both rs7201 and rs884225 exhibited no association with increased NPC risk. The rs7201 SNP is reportedly associated with small vessel infarcts in stroke, and is identified as an independent risk factor by multivariable logistic regression analysis [25]. However, few studies have investigated the association between EGFR rs884225 polymorphisms and human cancer risk. Choi et al. [26] reported that the rs884225 variant genotypes were not associated with lung cancer risk.

In conclusion, our data demonstrate that CCNE1 rs3218073 polymorphisms located at miRNA-151 binding sites are associated with NPC susceptibility and are correlated with NPC stage. These results suggest that CCNE1 rs3218073 polymorphism can alter the targeting ability of miRNA-151, making it a novel biomarker for NPC diagnosis and prognosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.024.

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