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A genetic variant in *CDKN2A/2B* locus was associated with poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is among the leading causes of cancer related death. Despite of extensive efforts in identifying valid cancer prognostic biomarkers, only a very small number of markers have been identified. Several genetic variants in the 9p21 region have been identified that are associated with the risk of multiple cancers. Here, we explored the association of two genetic variants in the 9p21 region, CDKN2A/B, rs10811661, and rs1333049 for the first time in 273 subjects with, or without ESCC. We observed that the patients with ESCC had a higher frequency of a TT genotype for rs10811661 than individuals in the control group, and this polymorphism was also associated with tumor size. Moreover, a CC genotype for the rs1333049 polymorphism was associated with a reduced overall survival (OS) of patients with ESCC. In particular, patients with a CC (rs1333049) genotype had a significantly shorter OS (CC genotype: 34.5 ± 8.9 months vs. CG+GG: 47.7 ± 5.9 months; p value = 0.03). We have also shown the association of a novel genetic variant in CDKN2B gene with clinical outcome of patients with ESCC. Further investigations are warranted in a larger population to explore the value of emerging markers as a risk stratification marker in ESCC.

KEYWORDS

CDKN2A/B, esophageal squamous cell carcinoma (ESCC), polymorphism, risk marker

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1 | INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the eighth most prevalent cancer globally and the sixth most common cause of mortality, because it is highly aggressive with poor survival rate (Hajian-Tilaki, 2001; Oladi et al., 2015). Thus, the identification of prognostic and predictive biomarkers that can provide an index of risk of developing ESCC, or help in management of patients at high risk, is warranted. It has been reported that genetic polymorphisms of specific genes may be involved in the esophageal carcinogenesis (Hiyama, Yoshihara, Tanaka, & Chayama, 2007). Several genes may contribute to ESCC, such as genes involved in the folate metabolism (Song, Xing, Tan, Wei, & Lin, 2001), carcinogen metabolism, DNA repair (T. Li et al., 2012), cell cycle control, and oncogenes (Meseure et al., 2016). The molecular mechanisms underlying the development of ESCC remained unclear.

Recent genome-wide association studies (GWASs) have illustrated that genetics variants in a region located on chromosome 9p21.3 are associated with multiple cancers (Congrains, Kamide, Ohishi, & Rakugi, 2013). Three genes are located in this region, including cyclin-dependent kinase inhibitors *CDKN2A CDKN2B* and antisense noncoding RNA in the INK4 locus (ANRIL), which have been reported to be an important susceptibility locus for various diseases (Congrains et al., 2013). The expression of the CDKN2B, CDKN2A, and ANRIL genes are coregulated (Cunnington, Santibanej koref, Mayosi, Burn, & Keavney, 2010). This locus has been associated with an increased risk of the cardiovascular disease, and developing several cancers (Congrains et al., 2013). It has been shown that these two genes are abnormally expressed in gastric cancer and ESCC (Congrains et al., 2013; E. B. Zhang et al., 2014). Several factors can influence the levels of CDKN2B and CDKN2A gene expression, including deletion, amplification, and genetic variants. Therefore, the aim of the current study was to examine the association of two genetic variants, rs10811661 and rs1333049 in CDKN2A/B loci with clinical outcomes of ESCC patients.

2 | MATERIALS AND METHODS

2.1 | Patients

Five hundred and ninety individuals with and without ESCC were enrolled from Mashhad University of Medical Sciences. The patients with ESCC were recruited based on the diagnosis of histologically confirmed locally advanced or metastatic ESCC from Omid Hospital of Mashhad University of Medical Sciences, during May/2006–August/2014. The control group was recruited as a part of the Mashhad stroke and heart atherosclerotic disorders cohort study, as described previously (Mehramiz et al., 2016). Individuals had no known history of infectious disease, cancer, myocardial infarction, nor a family history of stroke, and diabetes mellitus. Nine millimeter sections were serially cut from formalin-fixed paraffin-embedded blocks of the patients with ESCC. The study was approved by the local Hospital Ethic Committee of the Mashhad University of Medical Sciences.

2.2 | Genotyping

Genomic DNA was extracted from peripheral blood using the QIAamp® DNA Mini-Kit (Qiagen, San Diego, CA) according to the manufacturer's protocol. The concentration and purity of DNAs were assessed by the NanoDrop®-1000-Detector (NanoDrop-Technologies, Wilmington, DE). Genotype analysis of CDKN2A/B-rs10811661 and rs1333049 polymorphisms was carried out using the Taqman®-probes-based assay; polymerase chain reactions were carried out in 12.5 μL total volume, using 10 ng of DNA in the TaqMan® Universal Master Mix with specific primers and probes (C-901792-10 and C-790057-10; Applied Biosystems, Foster City, CA). The ABIPRISM-instrument equipped with the SDS version-2.0 software was used to evaluate the allelic content of the samples (Mehramiz et al., 2016; Oladi et al., 2015).

2.3 | Statistics

Data were analyzed by the SPSS-20 software (SPSS Inc., Chicago, IL). Descriptive statistics of patients with ESCC was reported as the mean and standard deviations for continuous variables, while the frequencies and percentages were used for categorical variables. Genotype and allele frequencies of CDKN2A/B rs10811661 polymorphism were assessed for deviation from the Hardy–Weinberg equilibrium (HWE) by using the Pearson χ distribution. The association between risk of ESCC for the CC and CT genotypes, relative to the risk genotype TT homozygote under recessive genetic model were assessed by logistic regression, adjusting for potential confounders, including; age, sex, body mass index (BMI), and family history. ESCC risk estimates were expressed as the odds ratio and its corresponding 95% confidence interval. The relationship between CDKN2A/B rs10811661 polymorphism and clinic pathological features were assessed through Pearson's chi-square test for categorical variables, and continuous variables were evaluated using Student's *t* tests. Overall survival (OS) was calculated from the day of treatment start to the end point (death or censoring) according to Kaplan–Meier method, and compared by logrank and Wilcoxon tests. The significant prognostic variables in univariate analysis were included in Cox's proportional hazards model. Hazard ratio was assessed to investigate the magnitude and the direction of the effect. All the analyses were two-sided and statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Clinicopathological characteristics of patients

Demographic, clinical, and genetic characteristics of the population are reported in Table 1. Among the patients, 48.6% of patients were female, and 51.4% were male with mean age of 58 ± 11 year

TABLE 1 Clinicopathological features of ESCC patients ($n = 123$)

Variable	Mean ± SD
Age (year)	58 ± 11
BMI (kg/m^2)	19.9 ± 5.2
Weight (kg)	50 ± 14.6
Height (m)	154.6 ± 27.8
Sex (female) *n* (%)	52 (48.6%)
Smoking *n* (%)	26 (24.3%)
Family history *n* (%)	23 (21.5)
TMN classification *n* (%)	
1	22 (20.4%)
2	47 (43.5%)
3	21 (19.4%)
4	17 (15.7%)
Tumor size *n* (%)	
T1	2 (1.9%)
T2	6 (5.6%)
T3	23 (21.5%)
T4	76 (71%)
Nodal status *n* (%)	
N0	72 (67.8%)
N1	34 (32.2%)
Distant metastasis *n* (%)	
M0	89 (83.2%)
M1	18 (16.8%)
Grade *n* (%)	
1	9 (8.4%)
2	39 (36.4%)
3	53 (49.5%)
4	6 (5.6%)

Note. BMI: body mass index; ESCC: esophageal squamous cell carcinoma; M: distance metastasis; N: nodal status; SD: standard deviation; T: tumor size

and BMI of 19.9 ± 5.2. Moreover, in a total of 1.9%, of patients cancer cells grew into the tissue under the epithelium (T1), in 5.6% of ESCC patients, the cancer cells were into the thick layer of muscle, a total of 21.5% was in T3 group which cancer grew into the outer layer of the esophagus, and finally, tumor size in 71% of participants was at T4 status so cancer spread into vicinity tissues. Also in 32.2% of patients the cancer grew into 1 or 2 surrounding lymph node (N1), while 16.8% of cases had M1(the cancer spread to distant lymph nodes or other tissues; Table 1). To evaluate whether the patient characteristics might influence clinical outcome, we analyzed data on progression-free survival (PFS) and OS according to patients' clinic-pathological features. Tumor size, node and metastasis status, and stage were associated with shorter OS and PFS.

3.2 | Association of the genetic variant with ESCC

To explore whether there was an association between CDKN2A/2B rs10811661 (C/T) and rs1333049 (C/G) polymorphisms with ESCC, genotyping was performed in all the subjects using DNA extracted from peripheral bloods. Genotyping was successfully performed in the vast majority of DNA samples and no discrepancies were found in the samples analyzed in duplicate (Table 2). As shown in Table 2 and Table 3, the study included a total of 590 age- and sex-matched subjects (92 patients with ESCC and 225 healthy controls for rs1333049, and also 68 patients with ESCC and 205 healthy controls for rs10811661). Table 2 showed the distribution of genotype frequencies of CDKN2A/B rs10811661, and rs1333049 polymorphisms in the whole population, which was in the HWE ($p > 0.05$). Minor allele frequencies for T and C alleles were 0.16 and 0.3 for rs10811661 and rs1333049. The frequencies of CC, CT, and TT genotypes for rs10811661 were 8.9%, 13.2%, and 77.9%, respectively in the ESCC group while these frequencies in control group were 4%, 25.3%, and 70.7%, respectively (Table 2).

We then evaluated the genotype distribution of the CDKN2A/B polymorphism with respect to clinicopathological features of ESCC patients under recessive genetic model (Table 3). This subgroup analysis showed that 70% of women carried a TT genotype, and 80% of patients who had family history had a TT genotype. Based on the recessive genetic inheritance model, we found that the TT genotype of the CDKN2A/B polymorphism was associated with larger tumor size. Moreover, the CDKN2B rs1333049 polymorphism was associated with the poor prognosis in patients with ESCC (Figure 1a-b). In particular, patients with CC genotype had a significantly shorter OS with mean range of 34.5 ± 8.9 months compared to CG+GG genotypes with OS of 47.7 ± 5.9 months (logrank p value = 0.038; Figure 1a). Furthermore, the progression free survival of these cases with TT genotype was 26.9 ± 7.1 months versus CC+CT genotypes with PFS of 36.8 ± 5.5 months (Figure 1b).

4 | DISCUSSION

To the best of our knowledge, this is the first study showing the association of a genetic variant in CDKN2A/B with the poor prognosis of patients with ESCC. Our data demonstrated that patients with a CC genotype had a reduced survival in patients with ESCC. This effect might be due to the function of this gene in the cell cycle (Burdon et al., 2011; Congrains et al., 2013; Dębniak et al., 2005; Sherborne et al., 2010; X. R. Yang et al., 2010). It has been shown that the inactivation of CDKN2A by methylation or ANRIL can suppress the activity of *p15/CDKN2B-p16/CDKN2A-p14/ARF* encoded by these genes (Nielsen, Roos, Emdin, & Landberg, 2001). ANRIL indirectly regulates the cell proliferation via three methylations at histone 3 lysine 27 (3meH3K27) in chromosome 9P21, upfront of CDKN2A/B (Congrains et al., 2013), and recruiting PRC2 and PRC1 complexes to specific loci (Popov & Gil, 2010; Yap et al., 2010). Several GWASs have identified the ANRIL gene as a shared genetic susceptibility locus in different cancers (Yap et al., 2010), and may affect the ANRIL expression (G. Yang, Lu, & Yuan, 2014). However, the potential

TABLE 2 Allele and genotype frequencies of CDKN2A/B rs10811661and rs1333049 polymorphisms

Gene	SNP	Major/minor allele	Major allele homozygote (%)	Heterozygote (%)	Minor allele homozygote (%)	MAF	HWE *p* value
CDKN2A/B	rs10811661	T/C	198(72.7%)	61(22.3%)	14 (5%)	0.16	0.2
		Control (*n* = 205)	ESCC (*n* = 68)	Total (*n* = 273)	Genetic model	*p value*	
	CC	8	6	14	Additive	0.5	
	CT	52	9	61	Recessive	0.1	
	TT	145	53	198	Dominant	0.4	
	rs1333049	G/C	146(22.7%)	130(20.2%)	41(6.4%)	0.3	0.16
		Control (*n* = 208)	ESCC (*n* = 119)	Total (*n* = 327)	Genetic model	*p value*	
	GG	126	20	146	Additive	<0.001	
	GC	76	54	130	Recessive	<0.001	
	CC	23	18	41	Dominant	0.048	

Note. ESCC: esophageal squamous cell carcinoma; HWE: Hardy Wienberg equilibrium; MAF: minor allele frequency; SNP: single-nucleotide polymorphism

TABLE 3 Genotype distribution of CDKN2A/B rs10811661 SNP (under recessive model) and rs1333049 SNP (under dominant model) with respect to clinicopathological features of ESCC patients

		rs10811661		rs1333049		
		CC+CT (*n* = 11)	TT (*n* = 56)		GG+GC(*n* = 74)	CC(*n* = 18)
Age (year)		50.5 ± 9	50.2 ± 9		54 ± 10	54 ± 4
Sex (Women %)		29%	71%		85%	15%
BMI (Kg/m^2)		25.7 ± 5	25.3 ± 5		21.9 ± 6	20.9 ± 4
Positive Smoking habit (%)		33%	77%		80.4%	19.6%
Family history of ESCC (%)		20%	80%		70%	30%
Clinical measures (reported as %)						
M	M0	20	80	M0	83.9	16.1
	M1	33.3	66.7	M1	71.4	28.6
N	N0	22.2	77.8	M0	75.5	24.5
	N1	22.2	77.8	M1	95.7	4.3*
T	T(1–2)	53	46.2	T(1–2)	71.4	28.6
	T(3)	12.2	87.8**	T(3)	85.5	14.5
Stage	0–2	23.5	76.5	0–2	79.6	20.4
	3	8.3	91.7	3–4	85.5	14.8
	4	37.5	62.5			
Grade	0–2	23.4	76.6	0–1	86.1	13.9
	3	0	100	2–3	77.5	22.5

Note. BMI: body mass index; ESCC: esophageal squamous cell carcinoma; M: distance metastasis; N: nodal status; SNP: single-nucleotide polymorphism; T: tumor size

*p *value* for rs1333049 = 0.03.

**p *value* for rs10811661 = 0.002.

mechanisms underlying the role of these genetic variants in ESCC still remains to be elucidated (C. H. Li & Chen, 2013). Several studies have shown a high ANRIL expression in patients with ESCC (Chen et al., 2014). Furthermore, the inactivation of CDKN2A gene can be due to gene mutation, homozygous deletion, and promotor methylation (Gamieldien et al., 1998). Frequent homozygous and heterozygous deletions are reported in CDKN2A and CDKN2B locus in some cancers, including ESCC (Maesawa et al., 1996).

Cunnington et al. (2010) demonstrated that the ANRIL expression was strongly associated with genetic variants in the CDKN2A/B promoter. Suzuki et al. (1995) suggested that intragenic point mutations of CDKN2A and CDKN2B rarely happen in primary esophageal tumors. Studies have reported several mutations in p53 in esophageal carcinoma (Biramijamal et al., 2001; Sepehr et al., 2001). However a case-control study conducted on 380 ESC and 380 controls in China population showed no association between ANRIL rs2151280 T/C with risk of ESC (Kang et al., 2015). Conversely,

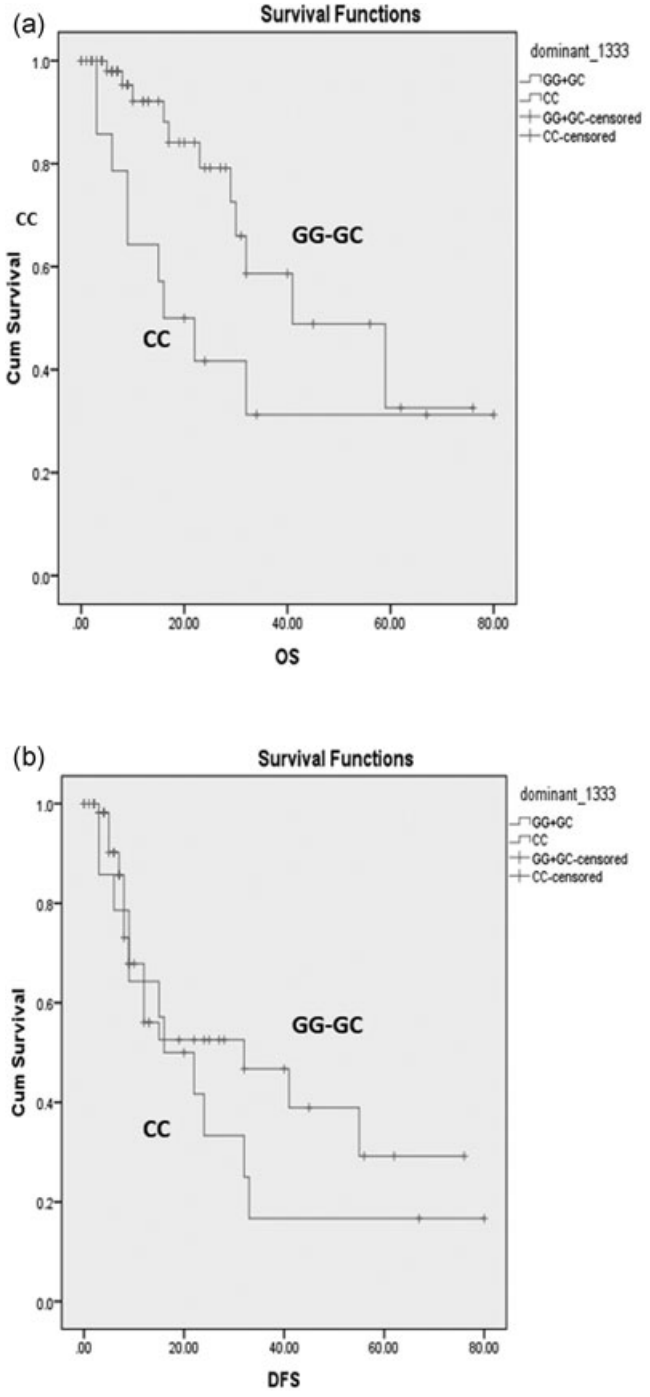

FIGURE 1 Kaplan–Meier survival curves. (a) Overall Survival (OS) and (b) Progression-free survival (PFS) based on different genotypes of CDKN2B rs1333049 polymorphism. *p*-values were calculated with the logrank test

Shete et al., revealed that polymorphisms in *CDKN2A/B* were associated with grade IV and grade II/III astrocytomas, but not with oligo II/III (Shete et al., 2009). Similarly, Walsh et al., showed that genetic variants in CDKN2B were associated with low-grade astrocytomas (Yung et al., 2014) although it was associated with the higher risk for astrocytic tumors in all grades, including glioblastoma (Claus et al., 2015). Dębniak et al., investigated the potential value of CDKN2A as a breast cancer susceptibility gene (Dębniak et al., 2007). They found that the CDKN2A A148T variant may contribute to early-onset breast cancer in Poland (Dębniak et al., 2007). Similarly, Antoniou et al., showed the association of the rs1011970 near CDKN2A/CDKN2B, with increased risk of breast cancer (Antoniou et al., 2012). Moreover, Driver et al., conducted a large-scale case–control study evaluating several polymorphisms within 13 genes involved in the cell cycle pathway with the risk of breast cancer. This study revealed a significant relationship between four genetics variants in the region of CDKN2A/2B and breast cancer risk (Driver et al., 2008). Another study in a cohort of 120 gastric cancer patients showed the higher expression of ANRIL in these tumor, which was significantly correlated with a higher tumor node metastasis stage and tumor size. Their results suggested that the ANRIL expression was an independent predictor for overall survival (E. B. Zhang et al., 2014). Aberrations in CDKN2A gene were also reported with poor prognosis in the renal clear cell carcinoma (Kawada et al., 2001) and ESCC (Cheng et al., 2016; Gu et al., 2013; Hu et al., 2004; W. Q. Li et al., 2014; Shen, Mei, Qiu, & Shi, 2016; J. Zhang et al., 2015). In particular, Shen et al. (2016) recently showed that CDKN2A, CDKN2B, FSCN1, and HOMER3 are candidate cancer-associated genes and may play a tumorigenic role in ESCC. This revealed that a homozygous deletion of CDKN2A or CDKN2B was associated with the lymph node metastasis, and the expression of these genes was lower in dysplasia than in normal esophageal epithelium. Another large scale study analyzed 9p21 single-nucleotide polymorphisms (SNPs) from eight GWASs, including studies of ESCC, gastric cancer, pancreatic cancer, renal cell carcinoma, lung cancer, breast cancer, bladder cancer, and prostate cancer. They identified several genetic variants in this region associated with the risk of multiple cancers including ESCC, suggesting that this region may contribute to a shared susceptibility across different cancer types (W. Q. Li et al., 2014). Gu et al. (2013) investigated 203 tagging SNPs of 22 genes on 9p21.3 (19.9–32.8 Mb) in eight case-control studies: thyroid cancer, endometrial cancer, renal cell carcinoma, colorectal cancer, colorectal adenoma, gastric cardia adenocarcinoma, osteosarcoma as well as in ESCC. They reported that genetic variants in CDKN2A may be associated with ESCC and several other tumors (Gu et al., 2013). In line with these observations (Cheng et al., 2016; Gu et al., 2013; Hu et al., 2004; W. Q. Li et al., 2014; Tajbakhsh et al., 2016; J. Zhang et al., 2015), our data showed an association between CDKN2A/B rs1333049, and clinical outcome of patients with ESCC, supporting further studies in a larger population.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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