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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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Running title: CDKN2A/B gene polymorphism in ESCC

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Abstract

Esophageal squamous cell carcinoma (ESCC) is among the leading causes of cancer related death. Despite 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 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 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 ESCC patients. Further investigations are warranted in a larger population to explore the value of emerging markers as a risk stratification marker in ESCC.

Key word: Esophageal squamous cell carcinoma, risk marker, CDKN2A/B, polymorphism

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 (1)(2, 3). Thus, 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 esophageal carcinogenesis (4). Several genes may contributed to ESCC, such as genes involved in folate metabolism (5), carcinogen metabolism, DNA repair (6) and cell cycle control and oncogenes (7). The molecular mechanisms underlying the development of ESCC remained unclear.

Recent genome-wide association studies have illustrated that genetics variants in a region located on chromosome 9p21.3 are associated with multiple cancers (8). 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 (8). The expression of the CDKN2B-CDKN2A and ANRIL genes are co-regulated (9). This locus has been associated with increased risk of cardiovascular disease and developing several cancers (8). It has been shown that these two genes are abnormally expressed in gastric cancer and ESCC (8)(10). 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 associations of 2 genetic variants, rs10811661 and rs1333049 in CDKN2A/B loci with clinical outcomes of ESCC patients.

Materials and Methods

Patients

Five hundred and ninety individuals with and without ESCC were enrolled from Mashhad University of Medical Sciences. The ESCC patients were recruited based on the diagnosis of histologically confirmed locally advanced or metastatic ESCC from Omid Hospital of MUMS Medical University, during May/2006-August/2014. The control group was recruited as part of the Mashhad Stroke and Heart Atherosclerotic Disorders (MASHAD) cohort study, as described previously (11). Individuals had no known history of infectious disease, cancer, myocardial infarction, nor a family history of stroke, and diabetes mellitus. Nine-mm sections were serially cut from formalin-fixed paraffin-embedded (FFPE) blocks of the ESCC patients. The study was approved by local Hospital Ethic Committees of Mashhad University of Medical Sciences.

Genotyping

Genomic DNA was extracted from peripheral blood using 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, USA). Genotype analysis of CDKN2A/B-rs10811661 and rs1333049 polymorphisms was carried out using Taqman®-probes-based assay; PCR reactions were carried out in 12.5 µl total volume, using 10 ng of DNA in 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 (11, 12)

Statistics

Data was analyzed by SPSS-20 software (SPSS Inc., IL, USA). Descriptive statistics of ESCC patients was reported as the mean and standard deviations (SD) for continuous variables, while

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 associations 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, family history. ESCC risk estimates were expressed as the odds ratio (OR) and its corresponding 95 % confidence interval (CI). The relationship between CDKN2A/B rs10811661 polymorphism and clinic pathological features were assessed through Pearson's chi-square $\chi 2$ 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 log-rank and Wilcoxon tests. The significant prognostic variables in univariate analysis were included in Cox's proportional hazards model. Hazard Ratio (HR) was assessed to investigate the magnitude and the direction of the effect. All the analyses were two-sided and statistical significance was set at $P<0.05$.

Results

Clinicopathological characteristics of patients

Demographic, clinical and genetic characteristics of the population were reported in Table 1. Among the patients, 48.6% of patients were female, and 51.4% were male with mean age of 58±11yr 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 32.2% of patients the cancer grew into 1 or 2 surrunding lymph node (N1), while 16.8% of cases had M1(the cancer spread to distant lymph nodes or other tissues) (Table 1). In order to evaluate whether the patient characteristics might influence clinical outcome, we analyzed data on PFS and OS according to patients' clinic-pathological features. Tumor size, node and metastasis status, and stage were associated with shorter OS and PFS.

Association of the genetic variant with ESCC

In order 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 3~~, the study included a total of 590 age and sex-matched subjects (92 ESCC patients and 225 healthy controls for rs1333049 and also 68 ESCC patients 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 Hardy-Weinberg equilibrium (HWE) ($P>0.05$). Minor allele frequencies (MAF) 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, 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 poor prognosis in ESCC patients (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 (Log Rank 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).

Discussion

To the best of our knowledge, this is the first study showing the association of a genetic variant in CDKN2A/B with poor prognosis of patients with ESCC. Our data demonstrated that patients with a CC genotype had a reduced survival in ESCC patients. This effect might be due to the function of this gene in the cell-cycle (8, 13-16). 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 (17). ANRIL indirectly regulates cell proliferation via three methylations at histon 3 lysine 27 (3meH3K27) in chromosome 9P21, upfront of CDKN2A/B (8) and recruiting PRC2 and PRC1 complexes to specific loci (18, 19). Several genome-wide association studies have identified the ANRIL gene as a shared genetic susceptibility locus in different cancers (18), and may affect ANRIL expression (20). However, the potential mechanisms underlying the role of these genetic variants in ESCC still remains to be elucidated (21). Several studies have shown a high ANRIL expression in ESCC patients (22). Furthermore, inactivation of the CDKN2A gene can be due gene mutations, homozygous deletion and promotor methylation (23). Frequent homozygous and heterozygous deletions are reported in CDKN2A and CDKN2B locus in some cancers, including ESCC (24).

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Cunnington et al., demonstrated that ANRIL expression was strongly associated with genetic variants in the CDKN2A/B promoter (9). Suzuki et.al suggested that intragenic point mutations of CDKN2A and CDKN2B rarely happen in primary esophageal tumors (25). Studies have reported several mutations in p53 in esophageal carcinoma (26, 27). 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 (28). Conversely, Shete and colleagues revealed that polymorphisms in *CDKN2A/B* were associated with grade IV and grade II/III astrocytomas but not with oligo II/III (29). Similarly, Walsh et al., showed that genetic variants in CDKN2B were associated with low-grade astrocytomas (30) although it was associated with higher risk for astrocytic tumors in all grades, including glioblastomas (31). Dębniak et.al. investigated the potential value of CDKN2A as a breast cancer susceptibility gene (32). They found that the CDKN2A A148T variant may contribute to early-onset breast cancer in Poland (32). Similarly, Antoniou et al., showed the association of the rs1011970, near CDKN2A/CDKN2B, with increased risk of breast cancer (33). Moreover, Driver and colleagues 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 (34). 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 TNM stage and tumor size. Their results suggested that ANRIL expression was an independent predictor for overall survival (10). Aberrations in CDKN2A gene were also reported with poor prognosis in renal clear cell carcinoma (35) and ESCC (36-41). In particular, Shen et al., 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 lymph node metastasis and the expression of these genes was lower in dysplasia than in normal esophageal epithelium (36). Another large scale study analyzed 9p21 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 (38). Gu et al., 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 (40). They reported that genetic variants in CDKN2A may be associated with ESCC and several other tumors (40). In line with these observations (37-42), our data showed an association between CDKN2A/B rs1333049 and clinical outcome of ESCC patients, supporting further studies in a larger population.

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347 **Fig. 1. Kaplan-Meier survival curves. A)** Overall Survival (OS) and B) Progression-Free
348 Survival (PFS) based on different genotypes of CDKN2B rs1333049 polymorphism. *P*-values
349 were calculated with the log-rank test.

Table 1 Clinicopathological features of ESCC patients (n=123)

Variable		Mean±SD
Age (year)		58±11
BMI(kg/m²)		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%)

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	

Abbreviation: SNP, Single nucleotide polymorphism; MAF, Minor Allele Frequency; HWE, Hardy Wienberg Equilibrium; ESCC, Esophageal cancer

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 (y)		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

Data are reported as mean±SD.$^+$*p value* for rs1333049=0.03.* *p value* for rs10811661=0.002.

Abbreviation: BMI, Body Mass Index; M, distance Metastasis; N, Nodal status; T, Tumor size.