Genetic variants at 9p21.3 are associated with risk of esophageal squamous cell carcinoma in a Chinese population

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Genome-wide association studies have linked genetic variants at 9p21.3 to the risk of multiple cancers. However, the roles of genetic variants at 9p21.3 in esophageal squamous cell carcinoma (ESCC) development are largely unknown. We evaluated the genetic variants at 9p21.3 reported in cancer genome-wide association studies with a case-control study including 2139 ESCC cases and 2273 controls in a Chinese population, and measured the mRNA expression levels of MTAP, CDKN2A, CDKN2B, and CDKN2B-AS1 in paired ESCC tumor and adjacent normal tissues. We found that the G allele of rs7023329 was significantly associated with a decreased risk of ESCC with a per-allele odds ratio of 0.84 (95% confidence interval, 0.77–0.91; P = 2.95 × 10−5). The rs7023329-G allele was related to a high expression of MTAP (P = 0.020). The rs1679013-C allele had lower expression of CDKN2B compared with adjacent normal tissues (P = 1.24; P = 0.91). Our findings indicate that genetic variants at 9p21.3 may modulate the expression of MTAP and CDKN2B and contribute to ESCC susceptibility. This may further advance our understanding of the 9p21.3 locus in cancer development.
factors have important roles in esophageal carcinogenesis.\(^{\text{3,4}}\) A series of studies have shown that tobacco smoking, heavy alcohol drinking, nutritional deficiencies, and dietary carcinogen exposure may cause this malignancy.\(^{\text{5}}\) There is a strong tendency toward familial aggregation of ESCC in the high incident rates of ESCC, suggesting that genetic susceptibility, in combination with exposure to environmental risk factors, contributes to ESCC.\(^{\text{6}}\) However, the exact mechanism for the carcinogenesis of ESCC is still unclear.

Up to now, three ESCC GWAS\(^{\text{6-8}}\) and relevant pooling analyses\(^{\text{8-11}}\) have identified a number of susceptibility loci associated with ESCC risk in Chinese populations. However, these loci only explain a small fraction of the heritability of ESCC and the other genetic variants associated with ESCC risk need to be further explored. Of interest, the GWAS have shown that genetic variants located at chromosome 9p21.3, consisting of \textit{MTAP}, \textit{CDKN2A}, \textit{CDKN2B}, and \textit{CDKN2B-AS1} genes, are related to multiple cancer risk, including glioma,\(^{\text{12-14}}\) melanoma,\(^{\text{15,16}}\) basal cell carcinoma,\(^{\text{17}}\) nasopharyngeal carcinoma,\(^{\text{18}}\) breast cancer,\(^{\text{19,20}}\) chronic lymphocytic leukemia,\(^{\text{21}}\) and childhood acute lymphoblastic leukemia.\(^{\text{22}}\) This evidence collectively suggests a pleiotropic effect of the 9p21.3 locus on cancer development.

Recently, Song et al.\(^{\text{23}}\) identified eight significantly mutated genes in ESCC, including \textit{CDKN2A} located at the 9p21.3 region, by whole-genome sequencing in 17 ESCC cases and whole-exome sequencing in 71 ESCC cases. Subsequently, Gao et al.\(^{\text{24}}\) confirmed that genes involved in cell cycle and apoptosis regulation, including \textit{CDKN2A}, were mutated in 99% of ESCC cases by sequencing on 113 tumor–normal pairs. These findings highlight the important role of genetic alterations at the 9p21.3 region in the development of ESCC and suggest that 9p21.3 may be a crucial genomic region for the regulation of ESCC development.

In the current study, we undertook a case-control study including 2139 ESCC cases and 2273 controls in a Chinese population, to investigate the role of genetic variants at 9p21.3 in ESCC susceptibility. We also evaluated the mRNA expression levels of \textit{MTAP}, \textit{CDKN2A}, \textit{CDKN2B}, and \textit{CDKN2B-AS1} in paired ESCC tumor and adjacent normal tissues to further explore the genotype–phenotype correlation.

**Materials and Methods**

**Study subjects.** Esophageal squamous cell carcinoma cases were consecutively recruited from the Cancer Hospital of Jiangsu Province (Nanjing, China) and The Affiliated Huaiyan First People’s Hospital of Nanjing Medical University (Huaiyan, China). All of the cases were histopathologically confirmed and the cases were excluded if they had a cancer history. A total of 2139 incident ESCC cases were included in this study. After being frequency-matched to the cases on age (5-year interval) and gender, 2273 controls were selected from cancer-free participants in a community-based screening program for non-infectious diseases in Jiangsu province. All of the subjects were self-reported ethnic Han Chinese. After signing informed consent, each subject was interviewed face-to-face with a standard questionnaire including information about demographic information and relevant risk factors, such as tobacco smoking and alcohol consumption. Each participant donated a \(\approx 5\)-mL venous blood sample. The characteristics of cases and controls are shown in Table S1. The cases had higher proportions of smokers and drinkers as compared with controls. This study was approved by the institutional review board of Nanjing Medical University (Nanjing, China).

**Polymorphism selection strategy and genotyping assays.** Genetic variants were selected mainly from the published GWAS that reported significant association between genetic variants located at chromosome 9p21.3 and the risk of various types of malignant tumor, including glioma (rs4977756, rs1412829),\(^{\text{12,13}}\) melanoma (rs7023329),\(^{\text{15,16}}\) basal cell carcinoma (rs2151280),\(^{\text{17}}\) nasopharyngeal carcinoma (rs1412829),\(^{\text{18}}\) breast cancer (rs1011970),\(^{\text{19,20}}\) chronic lymphocytic leukemia (rs1679013),\(^{\text{21}}\) and childhood acute lymphoblastic leukemia (rs3731217).\(^{\text{22}}\) In addition, a recent study explored the associations between common genetic variants in this region and multiple tumors, and confirmed that rs3731239 was significantly associated with ESCC in Chinese patients.\(^{\text{25}}\)

We filtered the above reported variants according to the following items: (i) only one variant was selected as multiple one were in LD at \(r^2 > 0.2\) (1000 Genomes Project data of Chinese population, http://browser.1000genomes.org/index.html); (ii) those variants with a minor allele frequency \(< 5\%\) in the Chinese population were excluded. Finally, we included rs7023329, rs3731217, rs3731239, rs1011970, and rs1679013 as tag SNPs to be genotyped in this study.

Genotyping was carried out using the TaqMan allelic discrimination assay on the ABI 7900 system (Applied Biosystems, Foster City, CA, USA). Two negative controls were included in each 384-well plate for quality control. Detailed information regarding the primers and probes is shown in Table S2. Technicians were blinded to the status of subjects (case or control) when carrying out the genotyping. The genotypes were called using the SDS 2.3 Allelic Discrimination Software (Applied Biosystems, Foster City, CA, USA).

**RNA extraction, reverse transcription, and quantitative real-time PCR assay.** Total RNA was extracted from ESCC tumor and adjacent normal tissues using TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The concentration of the RNA was measured by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and the absorbance values at 260 and 280 nm were acquired. All of the samples were of good quality, and 500 ng RNA was reverse transcribed to cDNA by using the PrimeScript RT Master Mix (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions. \textit{MTAP}, \textit{CDKN2A}, and \textit{CDKN2B} mRNA expression levels were tested using the Hot Taq PCR Reaction Mix (BioSteed BioTechnologies, Nanjing, China), while quantitation of \textit{CDKN2B-AS1} transcripts was completed using SYBR Green labeling (TaKaRa, Kyoto, Japan) in accordance with the manufacturer’s instructions.
Table 1. Summary of associations between genetic variants at 9p21.3 and esophageal squamous cell carcinoma risk in 2139 cases and 2273 controls in a Chinese population

| SNP                | Cases, n (%) | Controls, n (%) | OR (95% CI)† | P-value† |
|--------------------|-------------|----------------|-------------|---------|
| rs7023329          | n = 2121    | n = 2239        | 1.00 (reference) |        |
| AA                 | 706 (33.29) | 576 (25.73)     | 0.84 (0.77–0.91) | 2.95 × 10⁻⁵ |
| AG                 | 950 (44.79) | 1129 (50.42)    | 1.12 (0.97–1.29) | 0.118  |
| GG                 | 465 (21.32) | 534 (23.85)     | 0.76 (0.45–1.18) | 0.312  |
| T allele           | n = 2120    | n = 2239        | 1.00 (reference) |        |
| AT                 | 1325 (64.38) | 1421 (63.89)    | 1.00 (reference) |        |
| GT                 | 331 (15.85) | 381 (17.11)     | 0.88 (0.75–1.03) | 0.112  |
| TT                 | 12 (0.57)   | 11 (0.49)       | 1.20 (0.52–2.75) | 0.667  |
| C allele           | n = 2058    | n = 2224        | 0.99 (0.89–1.11) | 0.909  |
| rs3731217          | n = 2058    | n = 2224        | 1.00 (reference) |        |
| TT                 | 1325 (64.38) | 1421 (63.89)    | 1.00 (reference) |        |
| GG                 | 641 (31.15) | 715 (32.15)     | 0.95 (0.83–1.08) | 0.423  |
| G allele           | n = 2088    | n = 2227        | 1.00 (reference) |        |
| rs1011970          | n = 2088    | n = 2227        | 0.90 (0.77–1.05) | 0.183  |
| GG                 | 1745 (83.57) | 1835 (82.40)    | 0.99 (0.89–1.11) | 0.909  |
| GT                 | 331 (15.85) | 381 (17.11)     | 0.88 (0.75–1.03) | 0.112  |
| TT                 | 12 (0.57)   | 11 (0.49)       | 1.20 (0.52–2.75) | 0.667  |
| C allele           | n = 2120    | n = 2182        | 1.12 (1.01–1.24) | 0.039  |
| rs1679013          | n = 2120    | n = 2182        | 1.10 (1.03–1.18) | 0.035  |
| TT                 | 1247 (58.54) | 1366 (62.60)    | 1.00 (reference) |        |
| TC                 | 778 (36.53) | 739 (33.87)     | 1.07 (0.94–1.21) | 0.322  |
| CC                 | 105 (4.93)  | 77 (3.53)       | 1.44 (1.06–1.96) | 0.020  |
| C allele           | n = 2058    | n = 2224        | 0.99 (0.89–1.11) | 0.909  |

†Derived from logistic regression with an adjustment for age, sex, and smoking and drinking status. CI, confidence interval; OR, odds ratio.

Results

Genotyping call rates were >95% for all five SNPs and the observed genotype frequencies were all in agreement with HWE in the controls (Table S3). The genotype distributions of the five SNPs between the cases and the controls are summarized in Table 1. In the additive model, the G allele of rs7023329 was significantly associated with a decreased risk of ESCC with a per-allele OR of 0.84 (95% CI, 0.77–0.85); in the codominant model, individuals carrying either AG or GG genotype showed a significantly reduced ESCC risk (OR = 0.68 and 0.72, respectively) compared with those with AA genotype. The C allele of rs1679013 was associated with an increased risk of ESCC with a per-allele OR of 1.12 (95% CI, 1.01–1.24; P = 0.039) in the additive model, and in the codominant model, individuals carrying CC genotype showed an increased ESCC risk (OR = 1.44, 95% CI, 1.06–1.96; P = 0.020) compared with those with TT genotype. After conditioned on each other for these two SNPs, the association results for both variants changed little; rs1679013 and rs7023329 might be independent loci for ESCC susceptibility (Table S4). Furthermore, subgroup analyses stratified by age, gender, and smoking and drinking status, were carried out for the association of two SNPs (rs7023329 and rs1679013) and ESCC risk. As shown in Table 2, there were no significant differences between subgroups (P > 0.05 for heterogeneity tests) for associations of rs7023329 and rs1679013 with ESCC risk. However, no significant associations were observed for the other three SNPs with ESCC risk (additive P = 0.581, 0.909, and 0.183 for rs3731239, rs3731217, and rs1011970, respectively) (Table 1).

Around the selected variants at 9p21.3 region, there are four important genes: MTAP, CDKN2A, CDKN2B, and CDKN2B-AS1 (Fig. 1). We next sought to determine whether rs7023329 and rs1679013 genotypes were associated with expression of these genes by quantitative RT-PCR analysis of ESCC tumors and adjacent normal tissues (Figs 2, S1). We observed that the protective allele rs7023329-G was related to a high expression of MTAP and CDKN2B, whereas the C allele of rs1679013 was associated with a decreased expression of CDKN2B than did non-carriers (P = 0.035) among adjacent normal tissues (Fig. 2a,b). We found that CDKN2B was also significantly downregulated in ESCC tumor tissues compared with adjacent normal tissues (P = 3.50 × 10⁻⁵; Fig. 2c) while CDKN2B-AS1 was significantly upregulated (P = 3.34 × 10⁻³; Fig. 2d). These findings indicate that genetic variants at 9p21.3 may modulate the expression of MTAP and CDKN2B and contribute to ESCC susceptibility.

Discussion

In this study, we investigated the associations of genetic variants at 9p21.3 with the risk of ESCC in a Chinese case-control study with 2139 cases and 2273 controls. We found that the G allele of rs7023329 was significantly associated with a reduced risk of ESCC, whereas the C allele of rs1679013 was associated with an increased risk of ESCC. Further genotype-phenotype analysis confirmed that the protective allele rs7023329-G was related to a high expression of MTAP, which might increase the role of tumor suppressor MTAP. We also found that the carriers of the risk allele rs1679013-C had lower
Table 2. Stratified analyses of association between rs7023329 and rs1679013 and esophageal squamous cell carcinoma risk in 2139 cases and 2273 controls in a Chinese population

| Variables                          | P-value† | Cases (OR: 95% CI) | Controls (OR: 95% CI) | OR (95% CI) |
|-----------------------------------|----------|--------------------|-----------------------|-------------|
| Variables                          | P-value† | Cases (OR: 95% CI) | Controls (OR: 95% CI) | OR (95% CI) |
| Age, years                         | 0.0146   | 1.29 (1.09-1.52)   | 1.10 (0.96-1.26)      | 1.09 (0.94-1.27) |
| Gender: Female                     | 0.161    | 1.32 (1.08-1.61)   | 1.12 (0.94-1.32)      | 1.12 (0.91-1.36) |
| Smoking: Never                     | 0.091    | 0.85 (0.72-0.99)   | 0.84 (0.73-0.95)      | 0.84 (0.73-0.95) |
| Drinking: Never                    | 0.019    | 0.81 (0.66-0.99)   | 0.75 (0.65-0.86)      | 0.75 (0.65-0.86) |

**Note:**
- † Derived from additive model using logistic regression analyses with an adjustment for age, sex, smoking, and drinking status.
- ‡ P-values were from heterogeneity test based on chi-square test.
patients. These data suggest that genetic variants in the MTAP/CDKN2A/2B/2B-AS1 cluster may modulate ESCC susceptibility through regulating expression levels of genes in the cluster. Although we evaluated the possible mechanism by genotype–phenotype analysis and bioinformatics annotations, it is not enough to illustrate the exact biological function between these variants and ESCC. Further functional evaluations are warranted to further clarify these findings.

In summary, in a relatively large case–control study in a Chinese population, we found two independent variants in 9p21.3 (rs7023329 and rs1679013) associated with ESCC risk and provided additional evidence for the role of 9p21.3 locus in ESCC development. Further work is needed to validate our findings in diverse ethnic populations and to clarify the functional variants at 9p21.3 contributing to the development of ESCC.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CI confidence interval
ESCC esophageal squamous cell carcinoma
GWAS genome-wide association studies
HWE Hardy–Weinberg equilibrium
LD linkage disequilibrium
MTAP methylthioadenosine phosphorylase
OR odds ratio

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig S1. Genotype–phenotype correlation and differential expression analysis between esophageal squamous cell carcinoma tumor and adjacent normal tissues.

Table S1. Comparison of characteristics between esophageal squamous cell carcinoma cases and controls.

Table S2. Primers and probes used for genotyping.

Table S3. Selected SNP information and genotyping results.

Table S4. Multivariate regression analysis of rs7023329 and rs1679013 with esophageal squamous cell carcinoma risk.