Programmed death-1 polymorphisms is associated with risk of esophagogastric junction adenocarcinoma in the Chinese Han population: A case-control study involving 2,740 subjects

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in Programmed cell death 1 (PD-1) gene may contribute to the development of cancer. In this study, we selected PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms and designed a hospital-based case-control study to determine the potential relationship between these functional SNPs in PD-1 gene and esophagogastric junction adenocarcinoma (EGJA) risk. A total of 1,063 EGJA patients and 1,677 controls were enrolled from Eastern Chinese Han population. SNPscan™ genotyping assay was used to analyze the genotyping of PD-1 polymorphisms. We found that PD-1 rs7421861 A>G polymorphism was associated with the development of EGJA. However, PD-1 rs2227982 A>G polymorphism was a protective factor for EGJA. In addition, PD-1 rs36084323 CC homozygote genotype might be associated with a borderline decreased risk of EGJA. In a subgroup analysis, a decreased risk of EGJA in never drinking and never smoking groups was identified. Haplotype comparison analysis suggested that PD-1 T<sub>rs10204525</sub>G<sub>rs2227982</sub>C<sub>36084323</sub>A<sub>rs7421861</sub> haplotype significantly decreased the risk of EGJA. However, T<sub>rs10204525</sub>G<sub>rs2227982</sub>C<sub>36084323</sub>G<sub>rs7421861</sub> haplotype in PD-1 gene may confer risk to EGJA. In conclusion, our study highlights rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms and haplotypes in PD-1 gene, especially within the intron region, are significantly associated with the risk of EGJA. Further case-control studies with larger sample size and detailed gene-environmental data to replicate these findings in different populations are needed to validate our conclusion.

INTRODUCTION

A steady decline of gastric carcinoma (GC) incidence has been observed worldwide, primarily as a result of a reduction in distal GC [1]. However, GC is the fourth most common malignancy and is a relatively higher incidence in Eastern Asian (e.g. China, Korea and Japan). Esophagogastric junction adenocarcinoma
(EGJA) is one of the most rapidly increasing malignancies in North America and Europe and is thought to have different etiology compared to distal GC [2]. Recently, the increasing incidence of EGJA was also identified in Eastern Asian [3, 4]. EGJA is a highly fatal form of malignancies and is a major public health problem in China. The potential risk factors contributing to EGJA are foods preserved by salting, smoking and obesity et al. In addition, it is reported that individual’s genetic background also plays an important role in pathogenesis of EGJA. Although a number of studies have focused on the etiology of EGJA, it is not well understood. Of late, some studies reported the immune system might be implicated in the etiology of EGJA [5, 6].

Programmed cell death 1 (PD-1) gene was found by Ishida Y in 1992 [7]. It is classified as a member of the immunoglobulin superfamily (IgSF). As other inhibitory costimulatory molecules, PD-1 is expressed on many immune cells, such as T cells, exhausted T cells, regulatory T cells (Treg), activated monocytes, B cells, natural killer (NK) cells, dendritic cells (DCs) and natural killer T (NKT) cells [8, 9]. PD-1 protein, a transmembrane glycoprotein, consists of an intracellular domain and an extracellular immunoglobulin V domain. PD-1 protein binds two ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2). Under common physiological conditions, PD-1 interacts with PD-L1 and PD-L2, and then regulates an immune checkpoint. PD-1 may play a very important role in reducing the function of immune system by inhibiting T-cells and up-regulating Treg [10]. Finally, it decreases autoimmunity and results in self-tolerance.

Most recent studies reported several polymorphisms in PD-1 gene may be associated with susceptibility to some human autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes mellitus, and ankylosing spondylitis et al.) [11–16]. Interestingly, accumulating evidences showed that PD-1 single nucleotide polymorphisms (SNPs) were also correlated with susceptibility to human malignancy (e.g., thyroid cancer, breast cancer, cervical cancer, non-small cell lung cancer and gastric cancer et al.) [17–21]. The immune response may be differ greatly among individual tumor hosts, and the potential mechanisms remain unknown. Genetic variations can influence the function of genes and alter the disease phenotypes. Thus, the effect of such functional polymorphisms in immune response genes on cancer risk has attracted our interest. Exploring the relationship of PD-1 SNPs with EGJA risk may be beneficial for providing prevention and personalized diagnosis. Here, we selected PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms and designed a hospital-based case-control study to determine the potential relationship between these functional SNPs in PD-1 gene and EGJA risk.

RESULTS

Baseline characteristics

The demographics (age and sex) and major risk factors (smoking and drinking status) of participants are summarized in Table 1. The mean ± SD of age was not significant in the EGJA patients compared with non-cancer controls (P > 0.05). Our study was well-matched by age and gender. Significant difference was observed on smoking status and alcohol consumption between the EGJA patients and the controls (P < 0.001). For PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms, the success rate of genotyping was more than 99%, respectively (Table 2). The minor allele frequency (MAF) in our controls was similar to the data of Chinese population. The distribution of genotype frequencies in controls accorded with Hardy–Weinberg equilibrium (HWE) (Table 2).

Association of PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms with EGJA

The genotype distributions of PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms are summarized in Table 3. The frequencies of PD-1 rs2227982 AA, AG, and GG genotypes were 26.13%, 52.74% and 21.13% in 1,063 EGJA patients and 26.40%, 48.75%, and 24.85% in 1,677 controls, respectively. When compared with the frequency of PD-1 rs2227982 AA genotype, a difference in the frequency of PD-1 rs2227982 GG genotype was found between the EGJA patients and the controls (crude OR = 0.80, 95% CI: 0.64–1.00, P = 0.047). When compared with the frequency of PD-1 rs2227982 AA/AG genotype, there was a difference in the frequency of PD-1 rs2227982 GG genotype between EGJA patients and the controls (crude OR = 0.81, 95% CI: 0.67–0.98, P = 0.026). Adjustment for age, sex, smoking and drinking, there was also difference in recessive genetic model (GG vs. AA/AG: adjusted OR, 0.81; 95% CI, 0.67–0.97; P = 0.024; Table 4).

The frequencies of PD-1 rs7421861 AA, AG, and GG genotypes were 61.67%, 34.39% and 3.94% in 1,063 EGJA patients and 69.65%, 27.12%, and 3.23% in 1,677 controls, respectively. When compared with the frequency of PD-1 rs7421861 AA genotype, there was difference in the frequency of PD-1 rs7421861 AG genotype between EGJA patients and the controls (crude OR = 1.39, 95% CI: 1.17–1.64, P < 0.001). When compared with the frequency of PD-1 rs7421861 AA genotype, there was also difference in the frequency of PD-1 rs7421861 AG/GG genotype between the EGJA patients...
and the controls (crude OR = 1.43, 95% CI: 1.21–1.68, 
P < 0.001). Adjustments for age, sex, smoking and drinking, 
the observed results were not essentially changed (AG vs. 
AA: adjusted OR, 1.39; 95% CI, 1.18–1.65; 
P < 0.001; 
AG/GG vs. AA: adjusted OR, 1.43; 95% CI, 1.21–1.68; 
P < 0.001; Table 4).

The PD-1 rs36084323 T>C polymorphism conferred a borderline statistically decreased risk to EGJA in homozygote genetic model (crude OR = 0.81, 95% CI = 0.66–1.01, 
P = 0.061) and recessive genetic model (crude 
OR = 0.86, 95% CI = 0.72–1.03, 
P = 0.097). When adjusted for age, sex, smoking and drinking, a borderline statistically decreased risk of EGJA was also found in homozygote genetic model (crude OR = 0.82, 95% CI = 0.66–1.02, 
P = 0.074) and recessive genetic model (crude 
OR = 0.86, 95% CI = 0.71–1.03, 
P = 0.097). However, there was no difference in genotype distribution of PD-1 rs10204525 T>C polymorphism among EGJA patients and the controls (Table 4).

We used the Power and Sample Size Calculator (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize) to calculate the power value (α = 0.05) [22]. For PD-1 rs7421861 A>G, the power value was 0.967 in the additive model and 0.990 in the dominant model. For PD-1 rs2227982 A>G, the power value was 0.675 in the homozygote model and 0.607 in the recessive model.

Table 1: Distribution of selected demographic variables and risk factors in EGJA cases and controls

| Variable            | Overall Cases (n = 1,063) | Overall Controls (n = 1,677) | P*  |
|---------------------|---------------------------|-----------------------------|-----|
| Age (years)         | 64.19 (±8.63)             | 63.91 (±10.22)              | 0.451 |
| Age (years)         |                           |                             | 0.165 |
| < 64                | 494                       | 825                         | 0.165 |
| ≥64                 | 569                       | 852                         | 0.165 |
| Sex                 |                           |                             | 0.909 |
| Male                | 759                       | 1194                        | 0.909 |
| Female              | 304                       | 483                         | 0.909 |
| Smoking status      |                           |                             | < 0.001 |
| Never               | 773                       | 1323                        | 0.061 |
| Ever                | 290                       | 354                         | 0.061 |
| Alcohol use         |                           |                             | < 0.001 |
| Never               | 908                       | 1507                        | 0.061 |
| Ever                | 155                       | 170                         | 0.061 |

a Two-sided χ² test and Student t test.

Table 2: Primary information for PD-1 polymorphisms (PD-1 rs10204525 T>C, rs36084323 T>C, rs7421861 A>G and rs2227982 A>G)

| Genotyped polymorphisms | rs10204525 T>C (PD1.6) | rs36084323 T>C (PD1.1) | rs7421861 A>G (PD1.7) | rs2227982 A>G (PD1.9) |
|-------------------------|------------------------|------------------------|-----------------------|-----------------------|
| Chr                     | 2                      | 2                      | 2                     | 2                     |
| Position 37             | 242792321              | 242801596              | 242795350             | 242793433             |
| Region 3'UTR promoter   | 3'UTR                  | Promoter               | Intron 1              | Exon 5                |
| MAF* for Chinese in database | 0.302                  | 0.490                  | 0.165                 | 0.488                 |
| MAF in our controls (n = 1,677) | 0.280                  | 0.496                  | 0.168                 | 0.492                 |
| P value for HWE* test in our controls | 0.888                  | 0.071                  | 0.232                 | 0.309                 |
| % Genotyping value      | 99.01                  | 99.09                  | 99.09                 | 99.09                 |

*MAF: minor allele frequency; 
*HWE: Hardy–Weinberg equilibrium.
### Table 3: The frequencies of **PD-1 rs10204525 T>C, rs36084323 T>C, rs7421861 A>G and rs2227982 A>G polymorphisms in esophagogastric junction adenocarcinoma patients and controls**

| Genotype       | **Overall EGJA case ($n = 1,063$)** | | **Overall Controls ($n = 1,677$)** | |
|----------------|-----------------------------------|---|-----------------------------------|---|
|                | $n$ | %     | $n$ | %     |
| **rs36084323 T>C** |     |       |     |       |
| TT             | 282 | 27.09 | 444 | 26.52 |
| TC             | 521 | 50.05 | 800 | 47.79 |
| CC             | 238 | 22.86 | 430 | 25.69 |
| CT+CC          | 759 | 72.91 | 1,230 | 73.48 |
| TT+CT          | 803 | 77.14 | 1,244 | 74.31 |
| C allele       | 997 | 47.89 | 1,660 | 49.58 |
| **rs10204525 T>C** |     |       |     |       |
| TT             | 544 | 52.36 | 870 | 51.97 |
| TC             | 397 | 38.21 | 672 | 40.14 |
| CC             | 98  | 9.43  | 132 | 7.89  |
| TC+CC          | 495 | 47.64 | 804 | 48.03 |
| TT+TC          | 941 | 90.57 | 1,542 | 92.11 |
| C allele       | 593 | 28.54 | 936 | 27.96 |
| **rs7421861 A>G** |     |       |     |       |
| AA             | 642 | 61.67 | 1,166 | 69.65 |
| AG             | 358 | 34.39 | 454 | 27.12 |
| GG             | 41  | 3.94  | 54  | 3.23  |
| AG+GG          | 399 | 38.33 | 508 | 30.35 |
| AA+AG          | 1,000 | 96.06 | 1,620 | 96.77 |
| G allele       | 440 | 21.13 | 562 | 16.79 |
| **rs2227982 A>G** |     |       |     |       |
| AA             | 272 | 26.13 | 442 | 26.40 |
| GA             | 549 | 52.74 | 816 | 48.75 |
| GG             | 220 | 21.13 | 416 | 24.85 |
| GG+GA          | 769 | 73.87 | 1,232 | 73.60 |
| GA+AA          | 821 | 78.87 | 1,258 | 75.15 |
| G allele       | 989 | 47.50 | 1,648 | 49.22 |

**Association of PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms with EGJA in different stratification groups**

Table 5 summarizes the genotype frequencies of **PD-1 rs10204525 T>C polymorphism** in the stratified analysis by gender, age, alcohol consumption and smoking status. There was no significant difference in genotype distribution of **PD-1 rs10204525 T>C polymorphism** among EGJA patients and the controls in any subgroup.

The genotype frequencies of **PD-1 rs36084323 T>C polymorphism** in the stratified analysis by gender, age, alcohol consumption and smoking status are summarized in Table 6. In never smoking group, after adjustment for gender, age, alcohol consumption and smoking status by logistic regression analysis, the **PD-1 rs36084323 CC genotype was associated with a significantly decreased risk of EGJA compared with the TC/TT genotype [CC vs. TC/TT: adjusted OR = 0.80, 95% CI 0.65–0.99, $P = 0.043$ (Table 6)]. In never drink group, after logistic regression analysis, the **CC genotype of PD-1 rs36084323 T>C polymorphism** was also associated with a significantly decreased risk of EGJA compared with the **TT genotype [CC vs. TT: adjusted OR = 0.78, 95% CI 0.62–0.99, $P = 0.037$ (Table 6)].

In the stratified analysis by gender, age, alcohol consumption and smoking status, the genotype frequencies of **PD-1 rs7421861 A>G polymorphism** are summarized in Table 7. After adjustment by logistic regression analysis, we found that **PD-1 rs7421861 A>G polymorphism was associated with a significantly increased risk of EGJA in**
In the stratified analysis by gender, age, alcohol consumption and smoking status, the genotype frequencies of PD-1 rs2227982 A>G polymorphism are summarized in Table 8. After adjustment by logistic regression analysis, the PD-1 rs2227982 GG genotype was associated with a decreased risk of EGJA compared with the AG/AA genotype in three groups [≥ 64 years subgroup: GG vs. AG/AA: adjusted OR = 0.72, 95% CI 0.56–0.93, P = 0.011; never smoking group: GG vs. AG/AA: adjusted OR = 0.74, 95% CI 0.60–0.92, P = 0.008 and never drinking group: GG vs. AG/AA: adjusted OR = 0.76, 95% CI 0.62–0.93, P = 0.008 (Table 8)]. Additionally, compared with the PD-1 rs2227982 AA genotype, the PD-1 rs2227982 GG genotypes were also associated with a significantly decreased risk of EGJA in never drink group [GG vs. AA: adjusted OR = 0.76, 95% CI 0.60–0.97, P = 0.024 (Table 8)].

**SNP haplotypes**

Using SHESIS software (http://analysis.bio-x.cn/myAnalysis.php) [23], we constructed six haplotypes (Table 9). We found that TCGA haplotypes with the order of PD-I rs10204525 T>C, rs2227982 A>G polymorphisms in gene position significantly decreased the risk of EGJA (OR = 0.83, 95% CI = 0.71–0.96; P = 0.015). However, TGCG and other haplotypes with the same order of PD-I SNPs in gene position significantly increased the risk of EGJA (OR = 22.19, 95% CI = 5.27–93.56; P < 0.001 and OR = 2.50, 95% CI = 1.73–3.60; P < 0.001).
Table 5: Stratified analyses between PD-1 rs10204525 T>C polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable          | PD-1 rs10204525 T>C (case/control) a | Adjusted OR b (95% CI); P |
|-------------------|--------------------------------------|--------------------------|
|                   | TT        | TC        | CC        | TT        | TC        | CC        | TC / CC   | CC vs. (TC/TT) |
| Sex               |           |           |           |           |           |           |           |            |
| Male              | 384/621   | 289/476   | 71/94     | 1.00      | 0.96 (0.79–1.17); P: 0.704 | 1.20 (0.86–1.67); P: 0.294 | 1.04 (0.86–1.25); P: 0.700 | 1.24 (0.90–1.72); P: 0.194 |
| Female            | 160/249   | 108/196   | 27/38     | 1.00      | 0.79 (0.58–1.08); P: 0.140 | 1.08 (0.63–1.85); P: 0.776 | 0.89 (0.66–1.19); P: 0.422 | 1.23 (0.73–2.08); P: 0.433 |
| Age               |           |           |           |           |           |           |           |            |
| <64               | 245/442   | 187/310   | 50/71     | 1.00      | 1.04 (0.82–1.33); P: 0.725 | 1.26 (0.85–1.87); P: 0.260 | 1.13 (0.90–1.42); P: 0.283 | 1.27 (0.86–1.86); P: 0.226 |
| ≥64               | 299/428   | 210/362   | 48/61     | 1.00      | 0.80 (0.64–1.01); P: 0.055 | 1.10 (0.73–1.66); P: 0.640 | 0.88 (0.71–1.09); P: 0.237 | 1.24 (0.83–1.84); P: 0.291 |
| Smoking status    |           |           |           |           |           |           |           |            |
| Never             | 385/679   | 300/534   | 68/108    | 1.00      | 0.94 (0.78–1.13); P: 0.505 | 1.08 (0.78–1.50); P: 0.644 | 1.01 (0.84–1.21); P: 0.926 | 1.14 (0.83–1.57); P: 0.419 |
| Ever              | 159/191   | 97/138    | 30/24     | 1.00      | 0.84 (0.60–1.18); P: 0.321 | 1.60 (0.89–2.88); P: 0.115 | 0.97 (0.71–1.33); P: 0.849 | 1.74 (0.98–3.08); P: 0.058 |
| Alcohol consumption |           |           |           |           |           |           |           |            |
| Never             | 464/774   | 339/610   | 83/121    | 1.00      | 0.89 (0.74–1.05); P: 0.171 | 1.11 (0.82–1.50); P: 0.508 | 0.96 (0.82–1.14); P: 0.660 | 1.20 (0.89–1.61); P: 0.230 |
| Ever              | 80/96     | 58/62     | 15/11     | 1.00      | 1.11 (0.68–1.79); P: 0.680 | 2.22 (0.91–5.43); P: 0.080 | 1.26 (0.80–1.99); P: 0.328 | 2.14 (0.89–5.10); P: 0.088 |

aThe genotyping was successful in 1063 (97.74%) EGJA cases, and 1677 (99.82%) controls for PD-1 rs10204525 T>C; bAdjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model.

DISCUSSION

EGJA is considered as a separated carcinoma entirety of upper digestive tract malignancies [24]. Although the incidence of GC is declining, the incidence of EGJA rapidly increases in both western (e.g. North America and Europe) and eastern Asian countries [2–4, 25]. Thus, EGJA is one of the most prevalent malignancies worldwide. The etiology of EGJA is very complicated. Accumulating evidences demonstrated that SNPs in some immune response genes might be associated with risk of cancer [6, 26–28]. In consideration of the vital role of PD-1 in tumor immunology, we chose PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms to examine their potential roles in EGJA. In the present study, we found that PD-1 rs2227982 A>G and rs36084323 T>C polymorphisms might decrease the risk of EGJA. However, PD-1 rs7421861 A>G might be a risk factor for EGJA. In addition, we found TGGT rs10204525 rs2227982 rs36084323 rs7421861 haplotypes significantly decreased the risk of EGJA. On the contrary, TGGT rs10204525 rs2227982 rs36084323 rs7421861 and other haplotypes increased the risk of EGJA.

PD-1 rs7421861 A>G polymorphism locates on intron 1, where a lot of regulatory components and splicing control elements may interact with it [29]. Some epidemiological studies focused on the effect of PD-1 rs7421861 A>G locus on the development of multiple cancers; however, the results remained inconsistent. Several case-control reported that PD-1 rs7421861 A>G polymorphism was not associated with cancer risk [19, 27, 28, 30]. However, Ge et al. found PD-1 rs7421861 A>G polymorphism might increase the risk of overall colorectal cancer [31]. Recently, a meta-analysis indicated that PD-1 rs7421861 A>G was correlated with a borderline statistically increased risk of overall cancer [32]. In the present study, we found PD-1 rs7421861 A>G polymorphism might be associated with the susceptibility of EGJA, which was similar to the findings of the previous studies [31, 32]. This study did not examine the potential effect of this polymorphism on regulating the expression of PD-1 in EGJA patient blood samples. However, in the future, we will conduct a further study to assess whether PD-1 rs7421861 A>G polymorphism is associated with the inhibited activation of T cells in EGJA patients, which may impede the surveillance mechanism of immune system.

PD-1 rs2227982 A>G polymorphism, a SNP in Exon 5, encodes a Val to Ala substitution in the extracellular domain of PD-1 receptor during protein synthesis, which influences the sequence, and may alter the function of PD-1 protein. In this study, we found that PD-1
rs2227982 A>G polymorphism was associated with the decreased risk of EGJA risk. Ren et al. reported that PD-1 rs2227982 A>G polymorphism was associated with the development of breast cancer [19]. In addition, several studies reported there was no significant association between PD-1 rs2227982 A>G polymorphism and cancer (e.g. esophageal squamous cell carcinoma, colorectal cancer, breast cancer and non-small cell lung cancer) [27, 28, 30, 31]. A recent meta-analysis suggested that PD-1 rs2227982 A>G polymorphism might be not associated with the risk of overall cancer. However, we found that only five case-control studies with relatively small sample sizes were included in this pooled analysis. The current evidence of the relationship might be very limited.

In this study, we constructed haplotypes to explore the potential inherited patterns of haplotype. We found that PD-1 T_{rs10204525}G_{rs2227982}C_{rs6084323}A_{rs7421861} haplotype significantly decreased the risk of EGJA. However, T_{rs10204525}G_{rs2227982}C_{rs6084323}G_{rs7421861} haplotype in PD-1 gene might significantly increase the risk of EGJA. We first studied the relationship of haplotypes in PD-1 rs10204525 T>C, rs2227982 A>G, rs6084323 T>C and rs7421861 A>G polymorphisms with EGJA susceptibility. Compared PD-1 T_{rs10204525}A_{rs2227982}G_{rs6084323}A_{rs7421861} haplotype, we also found that A→G variation in PD-1 rs7421861 locus might significantly inverse the risk of haplotype to EGJA.

In addition, some limitations in this case-control study should be acknowledged. All participants were enrolled in three hospitals from Eastern Chinese Han population. Although the genotype distributions of PD-1
rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms in controls were consistent with HWE and the MAF in the selected controls was very close to the data of Chinese (Table 2), the non-cancer controls might not well-represent the whole Chinese population. As well, only four important SNPs (MAF ≥ 0.05) in PD-1 gene were selected, which might not be enough to determine the total genetic susceptibility in PD-1 gene. Future, a fine-mapping study with larger sample sizes, multiple centers and detailed risk factors is need to confirm these primary findings.

In summary, our study highlights rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms and haplotypes in PD-1 gene, especially within the intron region, may be associated with the risk of EGJA in Eastern Chinese Han population. Our primary findings suggest that PD-1 genetic variation may be beneficial for the exploration of Eastern Chinese subjects genetically susceptible to EGJA.

MATERIALS AND METHODS

Subjects

A total of 2,740 participants were recruited in this case-control study. Among them, 280 EGJA patients were enrolled from the Affiliated Union Hospital of Fujian Medical University and Fujian Medical University Cancer Hospital from January 2014 to May 2016. In addition, 783 EGJA patients were recruited from the Affiliated People’s Hospital of Jiangsu University from January 2008 to November 2016. All cases with histologically confirmed EGJA were enrolled in the present study. EGJA patients who had a history of another malignancy or received prior chemoradiotherapy were excluded. At the same time, 1,677 subjects who underwent health check in these hospitals were recruited as non-cancer controls. Controls were matched with EGJA cases in terms of gender and age. All participants were unrelated Eastern Chinese Han population. Each participant signed the written informed consent. The study was approved by the Review Boards of Jiangsu University (Zhenjiang, China) and Fujian Medical University (Fuzhou, China), in accordance with the Declaration of Helsinki. Two experienced doctors interviewed each individual and collected the relevant risk factors and demographic variables. The corresponding data are summarized in Table 1. Each study participant provided an ethylenediamine tetraacetic acid (EDTA)-anticoagulated intravenous blood sample after an overnight fast. The approved guidelines were used as experimental protocol.
Selection of SNPs

The tagging polymorphisms among the gene region of PD-1 (upstream and downstream of gene extending 5 Kb, respectively) were collected from the CHB population via an internet the HapMap Project (http://hapmap.ncbi.nlm.nih.gov/index.html.en) and were conducted with Haploview 4.2 software with the criterion of pairwise linkage disequilibrium (LD) $r^2$ threshold of 0.8 between polymorphisms (with a minimum LD of $r^2 > 0.8$) [35]. Finally, SNPs with a HWE $P \geq 0.05$, MAF $\geq 0.05$ and call rate $\geq 95\%$ in the CHB population were included [36]. PD-1 rs36084323 T>C polymorphism locates on the promoter of PD-1 gene, which region may influence the transcription of PD-1. Thus, in this study, we also included this important SNP. The primary

Table 8: Stratified analyses between PD-1 rs2227982 A>G polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable                  | PD-1 rs2227982 A>G (case/ control) a | Adjusted OR b (95% CI); $P$ |
|---------------------------|--------------------------------------|-----------------------------|
|                           | AA AG GG                             | AA AG GG                    | GG (GG vs. (AG/ AA)) |
| Sex                       |                                       | 1.00                        | 1.00                   |
| Male                      | 188/315 393/572 165/304              | 1.12 (0.89–1.39); $P: 0.333$| 1.10 (0.89–1.35); $P: 0.399$|
| Female                    | 84/127 156/244 55/112                | 0.87 (0.62–1.23); $P: 0.433$| 0.90 (0.65–1.24); $P: 0.513$|
| Age                       |                                       | 0.87 (0.63–1.20); $P: 0.383$| 0.99 (0.77–1.29); $P: 0.948$|
| <64                       | 126/212 253/426 103/185              | 0.93 (0.71–1.22); $P: 0.597$| 0.93 (0.71–1.23); $P: 0.625$|
| ≥64                       | 146/230 296/390 117/231              | 1.13 (0.87–1.45); $P: 0.359$| 1.10 (0.89–1.35); $P: 0.666$|
| Smoking status            |                                       | 0.87 (0.63–1.20); $P: 0.383$| 0.99 (0.77–1.29); $P: 0.948$|
| Never                     | 188/351 415/638 151/332              | 1.11 (0.90–1.37); $P: 0.339$| 1.09 (0.89–1.34); $P: 0.422$|
| Ever                      | 84/91 134/178 69/84                  | 0.84 (0.58–1.22); $P: 0.365$| 0.90 (0.63–1.28); $P: 0.542$|
| Alcohol consumption       |                                       | 0.94 (0.61–1.46); $P: 0.788$| 1.06 (0.83–1.35); $P: 0.666$|
| Never                     | 224/387 481/739 182/379              | 1.04 (0.85–1.26); $P: 0.714$| 0.91 (0.74–1.11); $P: 0.732$|
| Ever                      | 48/55 68/77 38/37                    | 0.99 (0.59–1.87); $P: 0.961$| 1.00 (0.88–1.14); $P: 0.988$|

The genotyping was successful in 1063 (97.93%) EGJA cases, and 1677 (99.82%) controls for PD-1 rs2227982 A>G; b Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model.

Table 9: PD-1 haplotype frequencies (%) in cases and controls and risk of esophagogastric junction adenocarcinoma

| Haplotypes                     | Case (n=2,126) | Control (n=3,354) | Crude OR (95% CI) | $P$ |
|--------------------------------|---------------|-------------------|-------------------|-----|
| $T_{rs10204525}A_{rs2227982}T_{rs6084323}A_{rs7421861}$ | 1000 49.24   | 1644 49.07      | 1.00              |     |
| $C_{rs10204525}G_{rs2227982}C_{rs6084323}G_{rs7421861}$ | 354 17.43    | 544 16.24       | 1.07(0.92–1.25)   | 0.394|
| $T_{rs10204525}G_{rs2227982}C_{rs6084323}A_{rs7421861}$ | 347 17.09    | 688 20.54       | **0.83(0.71–0.96)** | **0.015**|
| $C_{rs10204525}G_{rs2227982}C_{rs6084323}A_{rs7421861}$ | 198 9.75     | 374 11.16       | 0.87(0.72–1.05)   | 0.150|
| $T_{rs10204525}A_{rs2227982}C_{rs6084323}A_{rs7421861}$ | 29 1.43      | 48 1.43         | 0.99(0.62–1.59)   | 0.977|
| $T_{rs10204525}G_{rs2227982}C_{rs6084323}A_{rs7421861}$ | 27 1.33      | 2 0.06          | **22.19(5.27–93.56)** | <0.001|
| Others                        | 76 3.74      | 50 1.49         | **2.50(1.73–3.60)** | <0.001|
information of PD-1 functional SNPs is summarized in Table 2.

DNA extraction and genotyping

Peripheral blood sample was collected and stored at −20°C. Using the Promega Genomic DNA Purification Kit (Promega, Madison, USA), the genomic DNA was carefully extracted from lymphocytes. The obtained genomic DNA was frozen at −80°C. SNPscan™ genotyping assay (Gnensky Biotechnologies Inc., Shanghai, China) was used to analyze the genotyping of PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms. For quality control, 110 DNA samples (4%) were randomly selected. The genotypes of PD-1 rs10204525 T>C, rs2227982 A>G, rs36084323 T>C and rs7421861 A>G polymorphisms were reanalyzed by different laboratory technicians. The reproducibility was 100%. The success rate of PD-1 genotyping is shown in Table 2.

Statistical analysis

Age was expressed as the mean ± standard deviation (SD). And we used Student’s t-test to calculate the differences for continuous variables between EGJA patients and controls. An internet-based calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to determine the deviation of genotype frequencies from HWE. Chi-square test (χ²) was conducted to compare the categorical variables (e.g. age, sex, smoking status, and drinking) and the genotype distributions between EGJA patients and non-cancer controls. Multivariate logistic regression was used to obtain the crude/adjusted odds ratios (OR) and their 95% confidence intervals (CI) for the relationship of PD-1 gene with EGJA risk. The SAS software (Version 9.4; SAS Institute Inc., Cary, NC, USA) was used to analyze all data. SHESIS online software ([http://analysis.bio-x.cn/myAnalysis.php], Bio-X Inc., Shanghai, China) was harnessed to construct the haplotypes of PD-1 gene [23]. The criterion of statistical significance was defined as P < 0.05 (two-tailed). We performed the Power and Sample Size Calculator (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize) to assess the power value of the study (α = 0.05) [22].

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CONFLICTS OF INTEREST

The authors have no potential financial conflicts of interest.

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