Investigation of BTLA tagging variants with risk of esophagogastric junction adenocarcinoma

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Abstract

Background: Variants in B and T lymphocyte attenuator (BTLA) gene are likely to affect the function of BTLA protein.

Methods: In the present case-control study, we selected BTLA tagging single nucleotide polymorphisms (SNPs) (rs16859629 T>C, rs1982809 G>A, rs2171513 G>A and rs3112270 A>G) and conducted a case-control study to identify the association of BTLA SNPs with risk of esophagogastric junction adenocarcinoma (EGJA). This study involved 1,236 new incident EGJA cases and 1,540 cancer-free controls.

Results: The genotypes of BTLA SNPs were analyzed using a SNPscan Kit. No association was also found between the BTLA SNPs and the susceptibility of EGJA in overall comparison. In subgroup analyses, the BTLA rs1982809 was found to be associated with an increased susceptibility of EGJA (AA vs. GG: OR\textit{adjusted}=2.09, 95% CI 1.08–4.07, \(P=0.030\); and AA vs. GA/GG: OR\textit{adjusted}=1.99, 95% CI 1.04–3.82, \(P=0.039\)). In haplotype comparison, we identified that TAAG haplotype with the order of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs might increase the susceptibility of EGJA (OR=3.07, 95% CI=1.41–6.71; \(P=0.003\)).

Conclusion: To conclude, this study suggests that BTLA T\textsubscript{rs16859629}A\textsubscript{rs1982809}A\textsubscript{rs2171513}G\textsubscript{rs3112270} haplotype may increase the susceptibility of EGJA. More studies should be conducted to evaluate whether BTLA polymorphisms may influence the susceptibility of cancer in the future.
Introduction

The morbidity of esophagogastric junction adenocarcinoma (EGJA) is promoting rapidly, both in developing and developed countries [1, 2, 3]. EGJA comprise a vital portion esophageal and gastric cancer, with an increasing ratio. It is reported that EGJA is a common fatal tumor in China. EGJA is regarded as an entity with a specific clinical feature and molecular profile. The potential protective factor or a real cause of EGJA is unclear. Thus, an understanding of the potential risk factors influencing the development EGJA biology may be helpful to diagnosis and prognostic assessment for the supervision of EGJA patients.

During the activation of T-lymphocytes, they can express some receptors for receiving various signals. B and T lymphocyte attenuator (BTLA), also named CD272, is a most recently identified and studied member of the immune globulin (Ig) superfamily [4, 5, 6, 7]. BTLA is a glycoprotein and it contains two tyrosine-based inhibitory motifs [8]. During activation, BTLA is not expressed on T helper type 2 (Th2) cells, but Th1 cells. The expression of BTLA on T cells participates in negative regulation of T cell and then leads to an decreased T-lymphocytes proliferation [9]. Recently, many investigations have focused on the relationship of BTLA with inflammation, autoimmune disease and cancer. Shi et al. reported that BTLA-herpes virus entry mediator (HVEM) checkpoint axis might be implicated in the regulation of inflammation in liver [10]. A previous study indicated that the upregulation of BTLA gene expression and soluble BTLA (sBTLA) was validated in thymoma-associated myasthenia gravis [11]. A prognostic investigation showed that the levels of immune checkpoints sBTLA could be considered as a biomarker for unresectable pancreatic adenocarcinoma cases with a poor survival [12]. A functional study identified that IFN-γ level in circulating T-lymphocytes could be promoted by inhibiting BTLA/HVEM pathway [13]. Additionally, Feng et al. [14] and Lan et al. [15] reported that the level of BTLA expression in gastric carcinoma (GC) might be a useful biomarker for the evaluation of GC prognosis.

Single nucleotide polymorphisms (SNPs) in BTLA gene are likely to affect the role of BTLA protein. Some studies have kept a watchful eye on the correlation of BTLA variants with the development of cancer [16, 17, 18]. Fu et al. reported that the frequencies of BTLA rs1844089 and rs2705535 SNPs may alter the risk of breast cancer [17]. In Polish population, it was found that BTLA rs1982809 G>A, a 3' UTR SNP, might be a low-penetrating risk factor for the development of renal cell carcinoma [18]. In addition, another study indicated that BTLA rs1982809 G and rs2705511 C alleles were more frequent in patients with chronic lymphocytic leukemia compared to healthy controls [16]. In view of the vital role in cancer development and progress, we supposed that BTLA SNPs might be correlated with EGJA susceptibility. Here, BTLA tagging SNPs (rs16859629 T>C, rs1982809 G>A, rs2171513 G>A and rs3112270 A>G) were selected. The aim of this study was to identify the association of BTLA tagging SNPs with risk of EGJA.

Materials and methods

Subjects

This study involved 1,236 new incident EGJA patients and 1,540 cancer-free
controls. Among these patients, 393 cases patients diagnosed with EGJA and treated at two affiliated hospitals of Fujian Medical University [Union Hospital (Fuzhou, China) and Fujian Cancer Hospital (Fuzhou, China)] from January 2014 to June 2018. In addition, 843 patients with EGJA were from Jiangsu University People’s Hospital (Zhenjiang, China) from January 2008 to June 2018. Siewert type was used in our study [19]. Here, all EGJA cases included were Siewert type II (their centre within 1 cm proximal and 2 cm distal of the anatomical cardia). All included EGJA cases were diagnosed at the first time with histopathological test. For EGJA cases, the major included criteria were: (a) individuals who did not have a history of other cancers, (b) without any immunological diseases and (c) EGJA patients were not treated with any chemotherapy and/or radiotherapy before the enrolment. We recruited 1,540 cancer-free subjects as controls matching to the EGJA patients by sex, year of birth (±5 year) and ethnicity (Eastern Chinese Han nationality). They were from the hospitals mentioned above for regular health examination. The major included criteria for controls were: (a) cancer-free individuals, (b) without any immunological diseases, (c) sex and age matching to EGJA cases and (d) Han nationality who living in Eastern China. Each participant signed a consent form. The experimental protocol was authorized by the ethics committees of the Jiangsu University.

**Selection of SNPs**

The tagging SNPs of BTLA [from 112458030 to 112504757 in chromosome 3 (extending 5 Kb, upstream and downstream, respectively)] were structured and collected from Chinese populations via Genome Variation Server data. The criteria of tagging SNPs selection were described in our previous studies [20, 21].

**DNA extraction**

Genomic DNA was extracted from the collected blood samples with the Promega DNA Kit (Promega, Madison, USA), according to the explanatory memorandum. A 2-ul DNA was dropped in NanoDrop ND-1000 spectrophotometer (Wilmington, USA) to evaluate concentration and purity of DNA sample.

**Genotyping**

The genotypes of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs were analyzed using a SNPscan Kit (Genesky Biotechnologies Inc., Shanghai, China) as described previously [22, 23, 24]. PCR process was conducted in a 20-ul mixture volume in 96-well plates. ABI 3730xl DNA Analyzer was used to identify the genotype. The data of the sequencing were read by GeneMapper 4.1 (AppliedBiosystems, USA). One hundred and eleven DNA specimens were randomly chosen for repeat genotyping by another person in a blind fashion, and the obtained variants were concordant.

**Statistical method**

For each locus in BTLA gene, an online $\chi^2$ test was used to assess the Hardy-Weinberg equilibrium (HWE) [25]. The Student $t$ test was performed to
deal with continuous variables of demographic characteristics between two groups. And χ² test was harnessed to handle the categorical variables (e.g. different age subgroups, sex, cigarette using and alcohol consumption) and variant distributions of BTLA SNPs between two groups. The haplotypes of BTLA gene were evaluated by SHEsis software [26]. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to evaluate the strength of the correlation of BTLA SNPs with the risk of EGJA. Multiple logistic regression analysis was harnessed to check the distribution of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 genotypes between two groups. Subgroup analyses between the BTLA variants and characteristic variables was also conducted. The adjusted P values, ORs and 95% CIs were calculated by adjustment for age, sex, cigarette using and drinking. A P < 0.05 (2-way tests) was defined as significance in all statistical tests. All statistical analyses described previously were performed in SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Using PS software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize), the power value (α=0.05) was calculated [27, 28]. We also used the false-positive report probability (FPRP) to determine the significant findings [29].

Results

Baseline Characteristics

Table 1 summarizes age, sex, cigarette using and alcohol consumption in two groups. EGJA patients had a mean age of 64.28±8.64 years. The age and sex ratio was not significant between two groups (P=0.408 and P=0.485, respectively). The success rate of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 genotyping was of high quality (> 97%) (Table 2). We present the data of minor allele frequency (MAF) in Table 2. In control group, the frequencies of genotype distribution met HWE (Table 2).

Relationship of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs with EGJA

The genotype distributions and frequencies of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 genotypes are presented in Table 3. In a single SNP analysis, the BTLA rs2171513 G>A genotype frequencies were 62.83% (GG), 32.67% (GA) and 4.50% (AA) in EGJA patients and 63.70% (GG), 32.27% (GA) and 4.03% (AA) in the cancer-free controls. When the BTLA rs2171513 GG genotype was defined as the reference, the BTLA rs2171513 GA genotype was not correlated with the susceptibility for EGJA (GA vs. GG: adjusted OR=1.04, 95% CI: 0.88–1.22, P=0.668); the BTLA rs2171513 AA genotype was not correlated with the susceptibility for EGJA (AA vs. GG: adjusted OR=1.23, 95% CI: 0.83–1.81, P=0.302). In addition, the BTLA rs2171513 GA/AA genotypes did not confer the risk to EGJA in the dominant model (GA/AA vs. GG: adjusted OR=1.06, 95% CI: 0.90–1.24, P=0.497). In the recessive genetic compared model, when the BTLA rs2171513 GG/GA genotypes were defined as a reference, the BTLA rs2171513 AA genotype was not correlated with susceptibility for EGJA (AA vs. GG/GA: adjusted OR=1.21, 95% CI: 0.83–1.78, P=0.327) (Table 3). No association was also found between the BTLA rs3112270 A>G, rs1982809 G>A and rs16859629 T>C SNPs and
the susceptibility of EGJA (Table 3).

**Relationship of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs with EGJA in subgroup analysis**

Table 4 presents the variant frequencies of BTLA rs1982809 SNP in stratification analysis. When we conducted an adjustment for gender, age and alcohol consumption, we identified that the BTLA rs1982809 G>A was associated with an increased susceptibility of EGJA for ever smokers (AA vs. GG: adjusted OR=2.09, 95% CI 1.08–4.07, P = 0.030; and AA vs. GA/GG: adjusted OR=1.99, 95% CI 1.04–3.82, P=0.039). We found that there was no significant association between BTLA rs1982809 G>A SNP and the risk of EGJA in other subgroups.

No association was found between the BTLA rs2171513 G>A, rs3112270 A>G and rs16859629 T>C SNPs and the susceptibility of EGJA in subgroup analyses (data was not shown).

**SNP haplotypes**

Using haplotype constructing software mentioned above [26], we observed twelve BTLA gene haplotypes. We identified that TAAG haplotype with the order of BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs might increase the susceptibility of EGJA (OR=3.07, 95% CI=1.41–6.71; P=0.003). However, other observed BTLA gene haplotypes did not alter the susceptibility of EGJA (Table 5).

**Power calculation and FPRP determining**

Using PS software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize), the power value (α=0.05) was calculated [27, 28]. For BTLA rs1982809 G>A SNP, the power value was 0.631 in AA vs. GG genetic model and 0.589 in AA vs. GG/GA genetic model among ever smokers. In haplotype comparison, T<sub>rs16859629</sub><sub>G</sub><sub>rs1982809</sub><sub>A</sub><sub>rs2171513</sub><sub>G</sub><sub>rs3112270</sub> haplotype could increase the susceptibility of EGJA (power value, 0.830).

**Discussion**

The incidence of EGJA is increasing in both the East and Western countries. It is reported that altered lifestyle and lower chronic Helicobacter pylori infection may result in an increasing incidence of EGJA [30, 31]. The aetiology of EGJA may be attribute to gene and environment factors. Recent evidence suggested that the variants of immune and inflammatory response related genes could alter the risk of cancer [21, 32, 33, 34, 35]. Considering an important role of BTLA gene in immune, we chose BTLA tagging SNPs (rs16859629, rs1982809, rs2171513 and rs3112270) and explored their effects on the development of EGJA. Here, we identified that BTLA TAAG haplotype with the order of rs16859629, rs1982809, rs2171513 and rs3112270 SNPs might be associated with the development of EGJA.

**BTLA** rs1982809 G>A SNP locates in 3’ UTR, which could participate in post-transcriptional control. Recently, studies have been conducted to identify a potential effect of BTLA rs1982809 locus on the development of malignancy. **BTLA** rs1982809 polymorphism, a 3’ UTR SNP, was found to be associated with the
development of renal cell carcinoma in Polish populations [18]. Another case-control study also found that BTLA rs1982809 polymorphism were associated with chronic lymphocytic leukemia [16]. Subsequently, in the same study, the functional investigation demonstrated that the presence of BTLA rs1982809 G allele was correlated with lower expression of BTLA mRNA in lymphocyte as compared to rs1982809 A allele [16]. In this study, we first studied the relationship between BTLA rs1982809 locus and cancer risk in Asians. We found this SNP might not alter the overall EGJA risk. However, BTLA rs1982809 locus was identified as a risk factor to EGJA in smoking subgroup, which was similar to the previous reports [16, 18]. The results suggested that the role of BTLA rs1982809 G>A polymorphism may be influenced by environmental factors. However, the subjects included in smoking subgroup were related small, these findings may be underpowered. In the future, more case-control studies should be conducted to evaluate whether BTLA rs1982809 G>A polymorphism may inhibit the function of B and T cells and influence the susceptibility of cancer.

In the present case-control study, the BTLA haplotypes were also constructed. We found BTLA T/rs16859629A/rs1982809A/rs2171513G/rs3112270 haplotype might influence the risk of EGJA. However, this rare BTLA haplotypes only altered the susceptibility of a minor fraction of the EGJA patients. We first explore the association of BTLA haplotypes with cancer risk in Asians. Our findings should be verified in the future studies.

It is necessary to acknowledge the limitations in the present case-control study. First, this study was designed as hospital-based. Although the frequencies of genotype distribution in BTLA rs16859629, rs1982809, rs2171513 and rs3112270 SNPs met HWE and the MAFs of these selected SNPs in control group were close to the database for Chinese, the bias might have happened. Second, we only included four risk factors (gender, age, smoking and alcohol consumption). And other potential environment factors (e.g. body mass index, intake of vegetable and fruit, education level and economic income) were not considered. Thus, the potential interactions between gene and these environment factors could not addressed. Third, the participants included were related small in some subgroups, the observations may be insufficient evidence to identify a relationship with a definitive power. Fourthly, in current study, the biological function of BTLA SNPs were not studied. Finally, only four BTLA tagging SNPs (rs16859629, rs1982809, rs2171513 and rs3112270) were selected, which could not fully assess the total hereditary susceptibility in BTLA gene.

To conclude, this investigation suggests that BTLA T/rs16859629A/rs1982809A/rs2171513G/rs3112270 haplotype may increase the susceptibility of EGJA. More studies with multiple environment factors should be carried out to evaluate whether BTLA variants may influence the susceptibility of cancer in the future.
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Author Contribution
All authors contributed significantly to this study.
Conceived and designed the experiments: WT, SC
Performed the experiments: CL, JL, WT
Analyzed the data: MK
Contributed reagents/materials/analysis tools: SC
Wrote the manuscript: MK, WT
Other (please specify): None
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**Table 1** Distribution of selected demographic variables and risk factors in this case-control study

| Variable             | Overall Cases (n=1,236) | Overall Controls (n=1,540) | \( p \) \(^*\) |
|----------------------|------------------------|---------------------------|-----------------|
|                      | n                      | %                         | n               | %               |            |
| Age (years)          | 64.28 (±8.64)          |                           | 64.17 (±10.32)  |                 | 0.775       |
| Age (years) < 64     | 568                    | 45.95                     | 732             | 47.53           | 0.408       |
| Age (years) ≥64      | 668                    | 54.05                     | 808             | 52.47           |             |
| Sex                  |                         |                           |                 |                 | 0.485       |
| Male                 | 885                    | 71.60                     | 1,084           | 70.39           |             |
| Female               | 351                    | 28.40                     | 456             | 29.61           |             |
| Smoking status       |                         |                           |                 |                 | 0.087       |
| Never                | 884                    | 71.52                     | 1,146           | 72.73           |             |
| Ever                 | 352                    | 28.48                     | 394             | 27.27           |             |
| Alcohol use          |                         |                           |                 |                 | <0.001      |
| Never                | 1,028                  | 83.17                     | 1,359           | 88.25           |             |
| Ever                 | 208                    | 16.83                     | 181             | 11.75           |             |

\(^*\) Two-sided \( \chi^2 \) test and Student t test
Table 2 Primary information for BTLA targeting SNPs (rs2171513 G>A, rs3112270 A>G, rs1982809 G>A and rs16859629 T>C)

| Genotyped polymorphisms | rs2171513 G>A | rs3112270 A>G | rs1982809 G>A | rs16859629 T>C |
|-------------------------|---------------|---------------|---------------|---------------|
| Chr                     | 3             | 3             | 3             | 3             |
| Position_38             | 112466080     | 112461797     | 112463893     | 112471533     |
| Region                  | 3'UTR         | Promoter      | 3'UTR         | intron_variant|
| MAF<sup>a</sup> in database (1000g- Chinese Han populations) | 0.188         | 0.269         | 0.216         | 0.067         |
| MAF in our controls (n = 1,540) | 0.196         | 0.280         | 0.256         | 0.084         |
| P value for HWE<sup>b</sup> test in our controls | 0.625         | 0.114         | 0.796         | 0.898         |
| % Genotyping value      | 98.34%        | 98.56%        | 98.52%        | 97.48%        |

<sup>a</sup>MAF: minor allele frequency;

<sup>b</sup>HWE: Hardy–Weinberg equilibrium
Table 3 Logistic regression analyses of associations between *BTLA* targeting SNPs (rs2171513 G>A, rs3112270 A>G, rs1982809 G>A and rs16859629 T>C) and the risk of EGJA

| Genotype                  | EGJA case (n=1,236) | Controls (n=1,540) | Crude OR (95%CI) | P       | Adjusted OR \(^a\) (95%CI) | P       |
|---------------------------|---------------------|--------------------|------------------|---------|-----------------------------|---------|
| rs2171513 G>A            |                     |                    |                  |         |                             |         |
| GG                       | 754                 | 985                | 1.00             | 1.00    |                             | 1.00    |
| GA                       | 392                 | 489                | 1.05 (0.89-1.23) | 0.580   | 1.04 (0.88-1.22)             | 0.668   |
| AA                       | 54                  | 56                 | 1.26 (0.86-1.85) | 0.241   | 1.23 (0.83-1.81)             | 0.302   |
| GA+AA                    | 446                 | 545                | 1.07 (0.91-1.25) | 0.404   | 1.06 (0.90-1.24)             | 0.497   |
| GG+GA                    | 1,146               | 1,474              | 1.00             | 1.00    |                             | 1.00    |
| AA                       | 54                  | 56                 | 1.24 (0.85-1.82) | 0.269   | 1.21 (0.83-1.78)             | 0.327   |
| A allele                 | 500                 | 601                |                  |         |                             |         |
| rs3112270 A>G            |                     |                    |                  |         |                             |         |
| AA                       | 639                 | 782                | 1.00             | 1.00    |                             | 1.00    |
| AG                       | 472                 | 641                | 0.90 (0.77-1.06) | 0.197   | 0.90 (0.77-1.06)             | 0.192   |
| GG                       | 95                  | 107                | 1.09 (0.81-1.46) | 0.582   | 1.10 (0.82-1.48)             | 0.538   |
| AG+GG                    | 567                 | 748                | 0.93 (0.80-1.08) | 0.330   | 0.93 (0.80-1.08)             | 0.333   |
| AA+AG                    | 1,111               | 1,423              | 1.00             | 1.00    |                             | 1.00    |
| GG                       | 95                  | 107                | 1.14 (0.85-1.52) | 0.380   | 1.15 (0.86-1.53)             | 0.343   |
| G allele                 | 662                 | 855                |                  |         |                             |         |
| rs1982809 G>A            |                     |                    |                  |         |                             |         |
| GG                       | 668                 | 846                | 1.00             | 1.00    |                             | 1.00    |
| GA                       | 461                 | 586                | 1.00 (0.85-1.17) | 0.964   | 1.00 (0.85-1.17)             | 0.984   |
| AA                       | 76                  | 98                 | 0.98 (0.72-1.35) | 0.911   | 1.00 (0.85-1.37)             | 0.980   |
| GA+AA                    | 537                 | 684                | 0.99 (0.85-1.16) | 0.941   | 1.00 (0.86-1.16)             | 0.979   |
| GG+GA                    | 1,129               | 1,432              | 1.00             | 1.00    |                             | 1.00    |
| AA                       | 76                  | 98                 | 0.98 (0.72-1.34) | 0.917   | 1.00 (0.73-1.36)             | 0.983   |
| A allele                 | 613                 | 782                |                  |         |                             |         |
| rs16859629 T>C           |                     |                    |                  |         |                             |         |
| TT                       | 1,028               | 1,265              | 1.00             | 1.00    |                             | 1.00    |
| TC                       | 158                 | 231                | 0.84 (0.68-1.05) | 0.122   | 0.84 (0.67-1.04)             | 0.106   |
| CC                       | 13                  | 11                 | 1.45 (0.65-3.26) | 0.363   | 1.39 (0.62-3.13)             | 0.426   |
|        |     |     |     |            |     |     |     |
|--------|-----|-----|-----|-----------|-----|-----|-----|
|        | 171 | 14.26 | 242 | 16.06 | 0.87(0.70-1.08) | 0.197 | 0.06(0.70-1.07) | 0.166 |
| TT+CT  | 1,186 | 98.92 | 1,496 | 99.27 | 1.00 | 1.00 |     |
| CC     | 13 | 1.08 | 11 | 0.73 | 1.49(0.67-3.34) | 0.332 | 1.43(0.64-3.21) | 0.389 |
| C allele | 184 | 7.67 | 253 | 8.39 |     |

* Adjusted for age, sex, smoking, status of Chronic hepatitis B virus infection and drinking; Bold values are statistically significant (P < 0.05)
**Table 5** Stratified analyses between *BTLA* rs1982809 G>A polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable          | BTLA (case/control) a | rs1982809 | Adjusted OR b (95% CI); P |
|-------------------|-----------------------|-----------|--------------------------|
|                   | GG        | GA        | AA          | GG       | GA vs. GG | AA vs. GG | GA/AA vs. GG | AA vs. (GG/GA) |
| Sex               |           |           |             | GG       |          |          |            |                |
| Male              | 488/605   | 328/412   | 49/61       | 1.00     | 0.99(0.82-1.20); P: 0.925 | 1.01(0.68-1.49); P: 0.981 | 0.99(0.83-1.19); P: 0.937 | 1.01(0.68-1.49); P: 0.966 |
| Female            | 180/241   | 133/174   | 27/37       | 1.00     | 1.02(0.75-1.37); P: 0.916 | 0.99(0.58-1.69); P: 0.983 | 1.01(0.76-1.34); P: 0.932 | 0.99(0.59-1.66); P: 0.962 |
| Age               |           |           |             | GG       |          |          |            |                |
| <64               | 304/391   | 205/287   | 40/51       | 1.00     | 0.92(0.73-1.17); P: 0.502 | 1.00(0.64-1.56); P: 0.996 | 0.93(0.75-1.17); P: 0.553 | 1.04(0.67-1.60); P: 0.876 |
| ≥64               | 364/455   | 256/299   | 36/47       | 1.00     | 1.07(0.86-1.33); P: 0.545 | 0.97(0.61-1.53); P: 0.896 | 1.06(0.86-1.30); P: 0.609 | 0.94(0.60-1.48); P: 0.801 |
| Smoking status    |           |           |             | GG       |          |          |            |                |
| Never             | 487/606   | 325/424   | 50/81       | 1.00     | 0.95(0.79-1.15); P: 0.600 | 0.78(0.54-1.14); P: 0.199 | 0.93(0.77-1.11); P: 0.392 | 0.80(0.56-1.15); P: 0.229 |
| Ever              | 181/240   | 136/162   | 26/17       | 1.00     | 1.13(0.83-1.54); P: 0.449 | 2.09(1.08-4.07); P: 0.030 | 1.22(0.91-1.64); P: 0.193 | 1.99(1.04-3.82); P: 0.039 |
| Alcohol consumption |        |           |             | GG       |          |          |            |                |
| Never             | 563/737   | 378/524   | 63/90       | 1.00     | 0.95(0.80-1.13); P: 0.543 | 0.92(0.66-1.30); P: 0.639 | 0.94(0.80-1.11); P: 0.493 | 0.94(0.68-1.32); P: 0.725 |
| Ever              | 105/109   | 83/62     | 13/8        | 1.00     | 1.40(0.91-2.17); P: 0.126 | 1.56(0.61-3.99); P: 0.350 | 1.42(0.94-2.16); P: 0.098 | 1.37(0.54-3.44); P: 0.504 |

aThe genotyping was successful in 1,205 (97.49%) EGJA cases, and 1,530 (99.35%) controls for *BTLA* rs1982809.

bAdjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;

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Table 6 BTLA haplotypes frequency (%) and the association between BTLA haplotypes and risk of EGJA

| Haplotypes | Case | Control | Crude OR (95%CI) | P     |
|------------|------|---------|------------------|-------|
|            | n    | %       | n                | %    |                |
| TGGA       | 1159 | 48.64   | 1459             | 48.41| Reference      |
| TAGG       | 407  | 17.08   | 518              | 17.19| 0.99(0.85-1.15)| 0.887 |
| TGAA       | 283  | 11.88   | 350              | 11.61| 1.02(0.85-1.21)| 0.843 |
| CGGA       | 136  | 5.71    | 175              | 5.81 | 0.98(0.77-1.24)| 0.856 |
| TGAG       | 120  | 5.04    | 154              | 5.11 | 0.98(0.76-1.26)| 0.88  |
| TGGG       | 71   | 2.98    | 105              | 3.48 | 0.85(0.62-1.15)| 0.309 |
| TAGA       | 70   | 2.94    | 87               | 2.89 | 1.01(0.73-1.40)| 0.938 |
| TAAA       | 69   | 2.9     | 79               | 2.62 | 1.10(0.79-1.53)| 0.575 |
| CAGG       | 34   | 1.43    | 57               | 1.89 | 0.75(0.49-1.16)| 0.192 |
| TAAG       | 22   | 0.92    | 9                | 0.3  | 3.07(1.41-6.71)| 0.003 |
| CAGA       | 7    | 0.29    | 19               | 0.63 | 0.46(0.19-1.11)| 0.076 |
| Others     | 5    | 0.21    | 2                | 0.07 | 3.15(0.61-16.26)| 0.149 |

With the order of BTLA rs16859629 T>C, rs1982809 G>A rs2171513 G>A and rs3112270 A>G in gene position.