A genetic variant in CHRN3–CHRNA6 increases risk of esophageal squamous cell carcinoma in Chinese populations

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Abstract
Nicotinic acetylcholine receptors are important regulators of smoking behavior and tobacco carcinogenesis. We studied the association of the CHRN3-A6 variant rs13280604 in relation to esophageal squamous cell carcinoma (ESCC) in Chinese populations. Two independent case–control studies were conducted. The first case–control study, consisted of 866 ESCC patients and 1621 healthy controls from Northern China, and the second case–control study consisted of 853 ESCC patients and 860 unrelated controls from Southern China. A logistic regression model was used to evaluate the associations of rs13280604 with cancer risk. We found that Rs13280604 GG/AG genotypes were significantly associated with increased risk for ESCC in both case–control studies from Northern [odds ratio (OR), 1.42, 95% confidence interval (CI), 1.19–1.70, P = 1.1 × 10⁻⁴], Southern China (OR, 1.56, 95% CI, 1.26–1.93, P = 5.2 × 10⁻⁵), and the combined population of both studies (OR, 1.44, 95% CI, 1.26–1.65, P = 8.7 × 10⁻⁸), respectively. Our results suggest that this CHRN3-A6 variant confers susceptibility to ESCC risk. However, future larger studies are needed to validate our finding.

Introduction
Esophageal cancer is one of the most common cancers worldwide, with >480,000 new cases and 400,000 deaths occurring each year (1). China is among the highest risk areas of esophageal squamous cell carcinoma (ESCC) in the world. The fact that the incidence of esophageal squamous cell carcinoma (ESCC) varies dramatically among regions of the world and this variation changed over time suggests that multiple risk factors (both genetic and environmental) likely contribute to the development of the disease. Although the etiology is not well understood, extensive evidence indicates smoking and alcohol drinking as the major global risk factors for ESCC. In the high risk areas, ESCC risk is thought to be highly related to diet, include consumption of nitrosamines-contaminated food, vitamin and micronutrient deficiencies, and consumption of hot beverage and food (2,3).
Tobacco use is a pervasive public health issue, especially in China. Several twin studies unequivocally suggest for moderate to high genetic influence on nicotine dependence (4,5). The addiction-relevant effects of nicotine are directly derived through antagonism of nicotinic acetylcholine receptors (nAChRs) in central and peripheral nervous system. In cancer cells, the cancer microenvironment and distant organs, nAChRs act as central regulators of a complex network governing growth, angiogenesis and neurogenesis, in a cell-type-specific manner (6). Emerging evidence reveals that chronic exposure to tobacco-specific compounds upregulates cancer-stimulatory nAChRs and desensitizes cancer-inhibitory nAChRs, resulting in an increased risk of carcinogenesis (6–9).

Evidence of an association of genes encoding nAChRs with lung cancer was recently emphasized by a meta-analysis of genome-wide association studies (10). The variant rs6474412 in CHRNA3–CHRNA6 (CHRNB3–A6), the gene cluster encoding nAChR subunits α6–β3, were significantly associated with lung cancer in European descent (10). R6474412 showed perfect linkage disequilibrium (LD) with rs13280604 in European descent (10). Another six polymorphisms (rs1451240, rs10958725, rs4736835, rs10958726, rs4950 and rs6474415) in CHRNA3–A6 have also been associated with smoking behavior in several independent studies, suggesting their role in nicotine dependence and possible smoking carcinogenesis (11–13). We analyzed patterns of pair-wise LD of the above-mentioned variants in the HapMap and found that they were in strong or even perfect LD (r² > 0.8) in the CEU (Utah residents of northern and western European ancestry) samples and had similar distribution of genotype frequencies in the CEU and CHB (Han Chinese in Beijing, China) samples (data not shown). Recently, a nonsynonymous CHRNA5 rs16969968 single nucleotide polymorphism (SNP) was found in a recent meta-analysis to be the most replicated signal in the CHRNA5/CHRNA3/CHRNB4 gene cluster for nicotine dependence (14), while the association of this SNP with risk of ESCC in Chinese population has not been evaluated.

Given the roles of rs13280604 and rs16969968 in nicotine dependence as stated above, both SNPs are likely candidate biomarkers for susceptibility to ESCC. However, studies evaluating rs13280604 and rs16969968 variants in relation to ESCC are scarce. In the study, we selected rs13280604 as the ‘representative’ marker for CHRNA3–A6 region because it is in strong LD with these SNPs previously identified and rs16969968 for CHRNA5 region to evaluate the impact of these two variants on risk of ESCC, among two independent Chinese populations.

Materials and methods

Study population

A hospital-based case–control study design was employed. The subjects were recruited from two independent study sites—Northern China and Southern China. The Northern China case–control set consisted of 866 ESCC cases, and 1621 healthy controls from Shandong Province. The cases were newly diagnosed and histologically confirmed during 2009–2012, recruited from Qilu Hospital of Shandong University, Shandong Tumor Hospital and Institute and the Second Hospital of Shandong University. Controls were healthy volunteers who presented to the physical examination center of Qilu Hospital during the same period. Demographic information, including age, sex, smoking, and alcohol drinking, was collected at an interview conducted by trained medical students or doctors. Those who had been smoking or drinking <1 year in their lifetime were defined as never smoker and never drinker; otherwise, they were classified as ever smoker and ever drinker. The Southern China case–control set consisted of 853 newly diagnosed with histologically confirmed ESCC patients and 860 healthy controls from Guangdong Province. Cases were recruited from Sun Yat-Sen University Cancer Center during 2005–2012 and health controls were recruited during the same period from physical examination centers of several large comprehensive hospitals within Guangdong Province. Age, sex, smoking and alcohol drinking status were obtained from medical records for cases or physical examination for controls.

The study was approved by the Ethics Committees of Qilu Hospital of Shandong University and Sun Yat-Sen University Cancer Center, and all participants gave informed consent.

DNA extraction and SNP genotyping

Genomic DNA from blood samples was extracted using a commercial DNA extraction kit (Tiangen Biotech, Beijing, China). The rs13280604 and rs16969968 were genotyped by the 5’ nuclease cleavage assay (TaqMan method), using an Applied Biosystems 7500 Sequence Detection System in both populations. Allelic discrimination was determined by analysis using the SDS 1.4 software (Applied Biosystems, Foster City, CA). Genotyping was performed by laboratory personnel blinded to case–control status at two time points. Ten percent of the samples were randomly chosen for re-testing, and the results were in 100% concordance with those of the initial assays. For genotyping, because we found that the minor allele frequency (A allele) for rs16969968 was approximately 3%, which was rare in Chinese population, we did not include this specific SNP to continue to perform further analysis in this study.

Statistical analysis

Demographic characteristics were compared between cases and controls using the chi-square test. Deviation from Hardy–Weinberg equilibrium of rs13280604 was assessed in the controls using a goodness-of-fit chi-square test. Unconditional logistic regression model was used to estimate crude and age, sex, smoking, alcohol drinking and study site-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for genotype-specific risk. Stratification analyses were performed on age, sex, smoking and alcohol drinking status, to evaluate whether the associations varied between these subgroups. A likelihood ratio test was performed to evaluate the significance of interaction, by comparing the full model containing the interaction term with the model with only the main effects. All tests were two-sided with a P value <0.05 taken to be statistically significant. Statistical analyses were performed with SAS (version 9.2, SAS Institute, Cary, NC).

Results

Table 1 provides the demographic characteristics of the subjects. Significant difference in smoking and alcohol drinking status was observed across case and control groups in both study sites—as expected, smoking and alcohol drinking prevalence were significantly higher in the cases than the controls. The observed frequency differences in age and sex between cases and controls were adjusted for in subsequent logistic regression analysis.

No deviation from Hardy–Weinberg equilibrium was observed in either control group or combined controls (P > 0.05). Table 2 shows the association analysis results of rs13280604, in each case–control group and the combined group. In the Northern Chinese population, rs13280604 GG/AG genotypes were significantly associated with increased risk for ESCC (adjusted OR = 1.42, 95% CI = 1.19–1.70, P = 1.1 × 10⁻⁴). The significant association was confirmed in the independent study in the southern Chinese population (adjusted OR = 1.55, 95%
CI = 1.25–1.92, P = 5.1 × 10⁻⁶). We then performed a pooled analysis of the Northern and Southern Chinese ESCC cases and controls and revealed a 1.44-fold risk for ESCC (95% CI = 1.26–1.65, P = 8.7 × 10⁻⁴) associated with rs13280604.

To explore possible subgroup effects, we further analyzed the effect of rs13280604 on cancer risk, stratified by age, sex, smoking and alcohol drinking status. Overall, no interaction between rs13280604 and the stratification variables was found (P > 0.05).

As shown in Table 3, the association between rs13280604 and ESCC risk was more pronounced in men (adjusted OR in pooled subjects = 1.47, 95% CI = 1.25–1.72, P = 1.4 × 10⁻⁶), young adults (age ≤ 60 years-old) (adjusted OR in pooled subjects = 1.67, 95% CI = 1.38–2.03, P = 2.6 × 10⁻⁴), and never-drinkers (adjusted OR in pooled subjects = 1.53, 95% CI = 1.28–1.81, P = 1.7 × 10⁻⁴). The increased ESCC risk accompanied by GG/AG genotypes was similar between ever-smokers (adjusted OR in pooled subjects = 1.44, 95% CI = 1.20–1.74, P = 9.8 × 10⁻³) and never-smokers (adjusted OR in pooled subjects = 1.46, 95% CI = 1.20–1.78, P = 1.4 × 10⁻⁴).

### Discussion

In this study, we found that there is a significant increase in risk for ESCC associated with rs13280604. This increased risk was consistent between individuals from both Northern China and Southern China. Moreover, this variant reached marginal genome-wide significance in the pooled association for cancer risk with ESCC risk (P = 8.7 × 10⁻⁴), suggesting that this genetic variant in the CHRN3-A6 cluster modulate individual susceptibility to ESCC in Chinese population. Rs13280604 is located in the first intron of the CHRN3-A6 gene and is classified as having minimal biding evidence according to the Regulome SNP program (Version 1.1, http://regulomedb.org/). Although this SNP itself is not likely to be a functionally regulatory SNP, future functional analysis is needed to understand its potential role in CHRN3-A6 activity and more work are expected to identify the causal SNPs within the region.

In the previous meta-analyses of genome-wide association studies by Thorgeirsson et al., (10) allele T of rs6474412 in CHRN3-A6, which is in perfect LD with allele A of rs13280604, exhibited a weak association with increased risk for lung cancer (OR = 1.09, P = 0.04). In our study, the association of rs13280604 with ESCC was much stronger but in the opposite direction to the findings from the study by Thorgeirsson et al. (10). This difference could be related to organ specific carcinogenesis by tobacco. Tobacco that contains multiple compounds and nicotine is responsible for the majority of addictive properties of tobacco smoking. Nicotine-derived nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosornicotine (NNN) are the most potent tobacco-specific carcinogens. NNK and NNN...
have been found to account for the observed associations of tobacco smoking with lung and esophageal cancers, respectively (15,16). NNK and NNN are strong agonists for nAChRs, inducing carcinogenesis via α7nAChR and heteromeric α-βnAChRs, respectively (17,18). Therefore, it is biologically conceivable that CHRNA3-A6, which encodes heteromeric nAChR subunits α6-β3, confers different susceptibility to lung cancer and esophageal cancer. This discrepancy may also be attributed to inherent differences between various populations, who may differ in demography, primary risk exposure, socioeconomic status, dietary factors and clinical characteristics of the included cases. Furthermore, the genetic effect on cancer risk might be influenced by other genes or environmental factors through gene-gene and/or gene-environment interaction. This discrepancy could also be due to other possibilities. First is the limited sample size. The previous study by Thorgerisson et al. consisted of 4403 lung cancer patients and thus had the power to detect weak effect of risk allele on cancer risk. Second, the previous study was in European descents and the racial difference could be a major effect modifier of the association. As a matter of fact, rs13280604 and rs6474412 are in perfect LD in European descents but are in strong LD \((r^2 = 0.89)\) in Chinese (HapMap). Third, in the previous study, the OR was calculated without adjustment for any confounding factors, particularly smoking behaviors. This could also partly explain the discrepancy. Further studies in different populations should help to clarify the discrepancy in question.

Limitations of this study should be concerned when interpreting the results. First, we could not evaluate factors such as family history of cancer and dietary factors. Since only limited exposure variables have been provided for analysis, bias could remain because of possible unmeasured or unknown confounders. Second, smoking is self-reported by the study subjects, which raises the possibility of misreporting and recall bias. In addition, in the study, we had no detailed information on smoking duration and intensity, which were required to calculate smoking pack-year. This limits our ability to assess the involvement of smoking level in the association. Third, the hospital-based case–control study design may introduce selection bias. Fourth, our results are not generalizable to other races. Future studies are expected to evaluate the associations in populations with different racial background.

In conclusion, this is the first study that confirms the association between rs13280604 in CHRNA3-A6 and risk of ESCC in Chinese populations. Further research is required to establish replication in different ethnic populations, and future studies on other smoking-related cancer, such as lung and head and neck cancers, are expected to validate the association of this genetic variant with risk of smoking-related cancers in Chinese population.

### Funding

National Natural Science Foundation of China (30671956 and 81270238) and National Natural Science Foundation of Shandong Province (ZR2012HM083).

### Acknowledgements

The authors are grateful to Drs. Shikun Wang and Yuanna Du for suggestions and for skilful technical assistance.

Conflict of Interest Statement: None declared.

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