Journal of Genetics, Vol. 97, No. 1, March 2018, pp. 213–218
https://doi.org/10.1007/s12041-018-0910-3

RESEARCH ARTICLE

Genetic variants of *FOXP1* and *FOXF1* are associated with the susceptibility of oesophageal adenocarcinoma in Chinese population

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Received 6 November 2016; revised 26 July 2017; accepted 1 August 2017; published online 1 March 2018

Abstract. This study aimed to investigate whether the genetic variants of *CRTC1*, *BARX1*, *FOXP1* and *FOXF1* are associated with the development of oesophageal adenocarcinoma (OA) in Chinese population. A total of 744 OA patients and 1138 controls were included in this study. Here we genotyped four SNPs, rs10419226 of *CRTC1*, rs11789015 of *BARX1*, rs2687201 of *FOXP1* and rs3111601 of *FOXF1*. The chi-square test was used to compare the genotype and allele frequencies between the patients and controls. The student's *t*-test was used to compare *FOXP1* expression in the tumour and the adjacent normal tissues. The relationship between genotypes of rs2687201 and *FOXP1* expression was investigated by one-way analysis of variance test. Patients were found to have significantly higher frequency of allele A of rs2687201 and allele C of rs3111601 when compared with the controls (49.2 vs 43.4%, $P = 0.0008$ for rs2687201; 29.1 vs 24.0%, $P = 0.0003$ for rs3111601). There was a significantly higher expression level of *FOXP1* in the tumour than in the adjacent normal tissue (0.0052 ± 0.0021 vs 0.0027 ± 0.0018, $P < 0.001$). Patients with genotype AA were found to have remarkably higher *FOXP1* expression in the tumour than those with genotype CC ($P = 0.01$). To conclude, the varients of *FOXP1* and *FOXF1* genes are functionally associated with OA in Chinese population. With the identification of more susceptible loci, the combined effect of these markers may be helpful for the surveillance of OA.

Keywords. oesophageal adenocarcinoma; susceptibility; polymorphism; Chinese population; *FOXP1* gene.

Introduction

Oesophageal adenocarcinoma (OA) is a lethal disease with rapidly growing incidence and high mortality rate (Sampliner 2005; Hur *et al.* 2013; Kong *et al.* 2014). The prognosis of OA remains unclear, with five year overall survival rate less than 20% (Sampliner 2005; Hur *et al.* 2013; Kong *et al.* 2014). For the primary prevention of OA, it is essential to have a comprehensive understanding of the related risk factors. Previous epidemiological studies have identified several clinical risk factors for OA, including male gender, alcohol intake, smoking, obesity and other environmental factors (Engel *et al.* 2003; Freedman *et al.* 2007). Besides, chronic gastroesophageal reflux disease (GERD) and the premalignant lesion Barrett's esophagus (BE) were also reported to be associated with the risk of OA (Navab *et al.* 2015; Qiao *et al.* 2015; Wolf *et al.* 2015). However, the precise relationship between these risk factors and OA is poorly understood, since only a small part of OA can be explained by these risk factors. Obviously, genetic susceptibility could play a role in the initiation of OA. From this perspective, identification of genetic variants associated with OA could be used to predict the risk of OA in an early stage and thereby increase the prognosis remarkably.

As indicated by the familial aggregation of OA, genetic determinants are promising targets to elucidate the inheritable causes of OA (Trudgill 2002; Sappati Biyyani

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et al. 2007). Using the candidate gene approach, previous genetic association studies have revealed several susceptible genes of OA, including EGF, TNF-β, MGMT, and IL-18 (Doecke *et al.* 2008; Lanuti *et al.* 2008; Babar *et al.* 2012; Menke *et al.* 2012). Recently, genomewide association studies (GWASs) have identified several susceptible variants of OA with high allele frequency (Levine *et al.* 2013; Buas *et al.* 2014). Besides, some loci have been reported to present gene-exposure interactions (Dai *et al.* 2015). Despite these novel findings, few genetic variants have been observed to confer large risk of OA. It seems that numerous genetic risk factors may contribute to the incidence of OA with each of them conferring a small relative risk.

It has been well documented that there could be different modes of linkage disequilibrium (LD) among different populations. Here, replication of these novel loci is warranted to validate their associations with the risk of OA. Recently, the first GWAS on OA revealed three novel susceptible genes including *CRTC1*, *BARX1* and *FOXP1* (Levine *et al.* 2013). In addition, evidence was found that *FOXF1,* which was previously found associated with BE, was also associated with OA (Levine *et al.* 2013). Subsequently, these four genes were replicated in two independent cohorts of OA patients from Germany and Netherlands, respectively (Becker *et al.* 2015; van Nistelrooij *et al.* 2015). To the best of our knowledge, the association between OA and the genetic variants of these four genes remains obscure in the Chinese population. In this study, we aimed to investigate whether the genetic variants of *CRTC1*, *BARX1*, *FOXP1* and *FOXF1* are associated with the development of OA in the Chinese population.

Methods

Subjects

The current multicentre case–control study was performed under the approval of local institutional review board. A total of 744 patients and 1138 cancer-free controls were included in the study. All the patients were histopathologically diagnosed as OA at three different clinic centers between April 2006 and September 2015. The 1138 controls were recruited from volunteers participating in a community-based screening programme for GERD. All the controls who have GERD or family history of OA in the first degree relatives were excluded.

While recruiting the subjects, the following baseline characteristics were collected, age, height, weight and smoking status. Body mass index (BMI) was calculated by dividing the weight by the square of the height. Smoking history was classified into never smokers, ex-smokers and current smokers. Pack-years was used to quantify the degree of smoking exposure. Besides, clinical pathologic data of the patients, such as tumour location, survival period and tumour stage, were also collected from the medical records. The 7th edition of TNM staging of the American Joint Committee on Cancer (AJCC) system was applied to determine the clinical stage of tumour (Rice *et al.* 2010). All the subjects gave their informed consent for collecting the blood or tissue samples if applicable. Genomic DNA was extracted from the blood samples with a commercial kit (Qiagen, Tokyo, Japan) following standard protocol.

Genotyping of target variants

TaqMan genotyping assay was carried out for the genotyping of target variants. A total of four SNPs were genotyped, rs10419226 of *CRTC1*, rs11789015 of *BARX1*, rs2687201 of *FOXP1* and rs3111601 of *FOXF1*. The results of genotyping assay were analysed by ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA). Twenty per cent of the samples were randomly selected to validate the quality of the genotyping assay.

Detection of the FOXP1 expression in tissues

Sixty patients with OA were included in the expression analysis, from whom tumour tissue and the adjacent normal oesophageal tissues were obtained during surgery. Total RNA was isolated using a commercial kit (CWBio., Beijing, China) and subsequently reverse transcribed into the cDNA. Quantitative PCR was carried out and the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. All amplification procedures were run in triplicate. A mean value of threshold cycle (Ct) scores was calculated for the determination of relative expression levels of *FOXP1*.

Statistical analysis

SPSS ver. 18.0 (SPSS, Chicago, USA) was used for the data analyses. Baseline characteristics of the cases and controls were compared by the chi-square test or by student's *t*-test. Hardy–Weinberg equilibrium (HWE) test was performed in controls by a one degree of freedom and goodness of fit test. Differences in terms of allele and genotype frequencies between cases and controls were calculated by the chi-square test, with odds ratios (ORs) calculated to determine the risk of OA. The comparison of *FOXP1* expression in the tumour tissues and the adjacent normal tissues was carried out by the student's *t*-test. One-way analysis of varience test was used to analyse the relationship between genotypes of rs2687201 and the tissue expression of *FOXP1*. The statistical significance was set at a P value < 0.05.

Table 1. Characteristics of the patients and controls.

	Patients ($n = 744$)	Controls ($n = 1138$)	P
Age (years)	59.1 ± 11.6	58.8 ± 12.7	0.31
Body mass index (kg/m^2)	23.2 ± 3.7	23.3 ± 3.1	0.24
Ratio of male to female	665/79	1024/114	0.73
Smoking status			
Never smokers	133	250	0.001
Ex-smokers	423	678	–
Current smokers	188	210	–
Pack-years	32.1 ± 5.9	30.8 ± 6.5	< 0.001
Tumour stage		N/A	N/A
I	53 (7.1%)		
II	303 (40.7%)		
III	205 (27.6%)		
IV	183 (24.6%)		

Table 2. Association of the four SNPs with the development of OA.

		Genotype[a]			MAF			
SNPs	MA	Patient	Control	P	Patient	Control	P	Odds ratio (95% CI)[b]
rs10419226	T	60/334/350	43/286/324	0.17	0.305	0.284	0.15	1.11 (0.91–1.31)
rs11789015	G	6/98/640	12/143/983	0.95	0.074	0.073	0.94	1.01 (0.74–1.32)
rs2687201	A	198/336/210	239/509/390	0.001	0.492	0.434	0.0008	1.27 (1.07–1.51)
rs3111601	C	59/315/370	61/423/654	0.008	0.291	0.240	0.0003	1.30 (1.09–1.56)

MA, minor allele; MAF, minor allele frequency; CI, confidential interval.
[a]The three values in the 'genotype' column indicate the numbers of homozygotes with respect to the minor allele, heterozygotes and homozygotes with respect to the major allele, respectively.
[b]Calculated with the minor allele as reference.

Results

Demographic data of the participants

The baseline characteristics of the participants are summarized in table 1. The mean age of the patients was 59.1 ± 11.6 years (range, 45–74 years) and 89.4% of them were male. Compared with the controls, patients were found to have a significantly higher incidence of former or current smokers than the controls. There was no statistical difference between cases and controls regarding age, ratio of male to female and BMI.

Most tumours were located at the oesophagogastric junction (45.4%) or at the distal oesophagus (41.7%). The majority of the tumours were moderately (37.9%) or poorly (46.3%) differentiated. Stage II was the most common tumour stage (42.1%) and 36.9% of the patients were found to have positive lymph nodes, and 6.4% of the patients had distant metastasis. After surgery, the mean overall survival period was 46.9 ± 8.9 months (range, 39 – 73 months) and the five year overall survival rate was estimated at 18.6%.

Contribution of the genetic variants to the development of OA

The four variants were genotyped for all participants successfully. HWE test showed no remarkable difference in the genotype frequency of the controls. Compared with the controls, OA patients were found to have significantly higher genotype AA of rs2687201 and genotype CC of rs3111601 (26.6% vs 21.0%, $P = 0.001$ for rs2687201; 7.9% vs 5.4%, $P = 0.008$ for rs3111601) (table 2). Besides, patients were found to have significantly higher allele A of rs2687201 and allele C of rs3111601 than that of the controls (49.2% vs 43.4%, $P = 0.0008$ for rs2687201; 29.1% vs 24.0%, $P = 0.0003$ for rs3111601). The OR values were 1.27 for rs2687201 and 1.30 for rs3111601, respectively. As for the allele or genotype frequencies of rs10419226 and rs11789015, no significant difference was found between the patients and the controls.

Relationship between genotypes of rs2687201 and FOXP1 expression

There was a significantly higher expression level of *FOXP1* in the tumours than in the adjacent normal tissues (0.0052

Table 3. Tissue expression of *FOXP1* and its relationship with the genotype of rs2687201.

Expression of *FOXP1*	Genotypes of rs2687201			*P*
	AA (n = 17)	AC (n = 24)	CC (n = 19)	
Tumours	0.0073 ± 0.0028	0.0048 ± 0.0022	0.0035 ± 0.0015	0.01
Normal tissue	0.0031 ± 0.0022	0.0028 ± 0.0017	0.0023 ± 0.0012	0.19

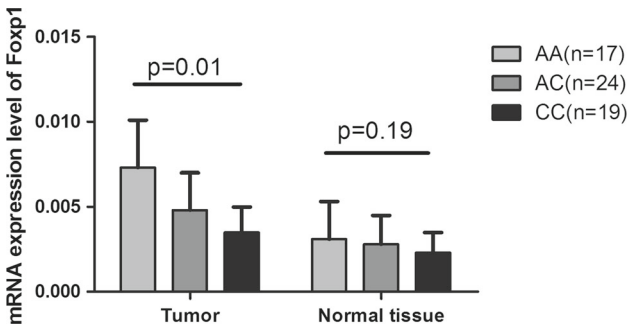

Figure 1. The tissue expression of *FOXP1* in patients with different genotypes of rs2687201. Patients with genotype AA had remarkably higher *FOXP1* expression in the tumour tissues than those with genotype CC (P = 0.01). In the adjacent normal tissues, there was no significant difference among different genotypes regarding the *FOXP1* expression (P = 0.19).

± 0.0021 vs 0.0027 ± 0.0018, P < 0.001). As shown in table 3, patients with genotype AA were found to have a remarkably higher *FOXP1* expression in the tumours than those with genotype CC (P = 0.01) (figure 1). However, no significant difference was found among patients with different genotypes regarding the *FOXP1* expression in the adjacent normal tissues.

Discussion

New genetic variants associated with the risk of OA have been recently revealed through GWASs (Levine *et al.* 2013; Buas *et al.* 2014). The Barrett's and Esophageal Adenocarcinoma Consortium performed a GWAS in 1516 patients with OA and 3209 healthy individuals from the European ancestry (Levine *et al.* 2013). In total, variants at four different loci showed genomewide significant association (Levine *et al.* 2013). Becker *et al.* (2015) replicated these loci in the OA patients from German, and confirmed that *FOXP1*, *BARX1* and *FOXF1* are genetic risk loci for the development of OA. While in another replication study performed in Netherlands (van Nistelrooij *et al.* 2015), only *CRTC1* and *BARX1* were validated to be associated with OA. Considering that there could be a significant ethnical or regional differences regarding the frequencies of genetic variants, we here investigated the effects of those reported variants in the Chinese population. In the current multicentre case–control study, we demonstrated for the first time that rs2687201 of *FOXP1* and rs3111601 of *FOXF1* are associated with the risk of OA in Chinese population.

SNP rs2687201 is located 75 kb downstream of the 5′-untranslated region of the *FOXP1* gene. Previous studies have reported the role of the transcription factors *FOXP1* and *FOXP2* in the development of lung and oesophagus (Koon *et al.* 2007; Shu *et al.* 2007). *FOXP1* was suggested as a potential therapeutic target in cancer (Koon *et al.* 2007). Besides, the FOX family is observed to be overexpressed in oesophageal cancer (Levine *et al.* 2013). Comparably, in this study, the tissue expression of *FOXP1* was found significantly higher in the tumours than in the adjacent normal tissues. Expression quantitative trait locus (eQTL) analyses based on the public database suggested that rs2687201 may be associated with altered *FOXP1* expression levels in oesophageal mucosa (Consortium 2015). In the current study, we further investigated the regulatory role of rs2687201 in the tissue expression of *FOXP1* in OA patients. Genotype AA of rs2687201 was indicative of significantly higher *FOXP1* expression in the tumours. Previously, *FOXP1* was reported to encode a transcription factor regulating oesophagus development and acts as therapeutic target in cancer (Koon *et al.* 2007; Shu *et al.* 2007). The high expression of *FOXP1* was observed to confer a poor prognosis in lymphomas or hepatocellular carcinoma (Wlodarska *et al.* 2005; Zhang *et al.* 2012). Further, investigation on the relationship between the *FOXP1* expression and the long-term survival of OA patients is warranted to illustrate the prognostic role of *FOXP1* in this disease. Moreover, the precise functional effects of rs2687201 or linked variants on expression levels of *FOXP1* remain to be determined by more *in vivo* cellular experiments.

Several SNPs in *FOXF1* have been reported to be associated with the risk of OA. (Levine *et al.* 2013). The rs3111601 of *FOXF1* replicated in this study is in LD with the SNP rs9936833 that has been identified as a susceptible variant of OA. Su *et al.* (2012) firstly found rs9936833 of *FOXF1* can contribute to the genetic susceptibility to Barrett's esophagus. Dura *et al.* (2013) provided evidence that rs9936833 could also increase OA susceptibility in Caucasians. In the GWAS performed by Levine *et al.* (2013) four additional SNPs of *FOXF1* in the region 16q24, including rs3111601, were found to have more significant *P*

values than rs9936833. To our knowledge, the causal variant at this region is yet to be fully investigated, and further fine mapping of the region surrounding rs3111601 may be helpful to reveal the role of *FOXF1* in the development of OA.

Although rs10419226 of *CRTC1* and rs11789015 of *BARX*1 gene were reported as susceptible variants of OA (Levine *et al.* 2013; van Nistelrooij *et al.* 2015), in this study, we failed to validate the association of these two SNPs with OA. Through the Ensembl gene annotation system (Aken *et al.* 2016), we found that the minor allele frequency (MAF) of rs11789015 of *BARX1* is 0.07 in the Chinese but 0.30 in the European population. By contrast, the MAF of rs3111601 of *FOXF1* is 0.24 in the Chinese and 0.30 in the European population. And the MAF of rs2687201 of *FOXP1* is 0.44 in the Chinese and 0.32 in European population (Aken *et al.* 2016). Here, we speculated that the lack of replication of rs11789015 of *BARX1* could be partially resulted from the ethnic heterogeneity. However, we cannot entirely exclude the association between these two genes and the risk of OA in the Chinese population. Further investigation is warranted to determine whether other polymorphisms of *CRTC1* and *BARX1* gene might be responsible for the reported association with OA.

The findings of this study shed light on the role of the genetic polymorphism in OA. We confirmed that the *FOXP1* and *FOXF1* genes are associated with the risk of OA in the Chinese population. Besides, rs2687201 is evidenced as a putative functional variant that can affect the expression of *FOXP1* in patients with OA. However, variants of these two genes can only explain limited variance of OA as indicated by the low OR ranging from 1.26 to 1.31. Therefore, more susceptible variants of OA await to be explored. The functional role of these susceptible genes needs to be explored to clarify the aetiology of OA.

Acknowledgement

We sincerely thank Dr Haozhong Hu for his assistance in the proof reading of our manuscript.

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Corresponding editor: Dhavendra Kumar