RESEARCH ARTICLE

Association of Single Nucleotide Polymorphisms in the Prostaglandin-endoperoxide Synthase 2 (*PTGS2*) and Phospholipase A_2 Group IIA (*PLA2G2A*) Genes with Susceptibility to Esophageal Squamous Cell Carcinoma

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Abstract

Background: The prostaglandin-endoperoxide synthase 2 (*PTGS2*) and phospholipase A_2 group IIA (*PLA2G2A*) genes encode enzymes that are involved in arachidonic acid and prostaglandin biosynthesis. Dysregulation of both genes is associated with inflammation and carcinogenesis, including esophageal squamous cell carcinoma (ESCC). We therefore hypothesized that there is an association between single nucleotide polymorphisms (SNPs) in these genes and susceptibility to ESCC. Methods: We performed a gene-wide tag SNP-based association study to examine the association of SNPs in *PTGS2* and *PLA2G2A* with ESCC in 269 patients and 269 healthy controls from Taihangshan Mountain, Henan and Hebei Provinces, the rural area of China which has the highest incidence of esophageal cancer in the world. Thirteen tag SNPs in *PLA2G2A* and 4 functional SNPs in *PTGS2* were selected and genotyped using a high-throughput Mass Array genotyping platform. Results: We found a modest increased risk of ESCC in subjects with the *PTGS2* rs12042763 AA genotype (OR=1.23; 95% CI, 1.00-3.04) compared with genotype GG. For *PLA2G2A*, a decreased risk of ESCC was observed in subjects with the rs11677 CT (OR=0.51, 95%CI, 0.29-0.85) or TT genotype (OR=0.51, 95%CI, 0.17-0.96) or the T carriers (CT+TT) (OR=0.52, 95%CI, 0.31-0.85) when compared with the CC genotype. Also for *PLA2G2A*, rs2236771 C allele carriers were more frequent in the control group (*P*=0.02). Subjects with the GC (OR=0.55, 95%CI, 0.33-0.93) or CC genotype (OR=0.38, 95% CI, 0.16-0.94) or the C carriers (GC+CC) (OR=0.52, 95%CI, 0.32-0.85) showed a negative association with ESCC susceptibility. Conclusions: Our results suggest that *PTGS2* and *PLA2G2A* gene polymorphisms may modify the risk of ESCC development.

Keywords: Esophageal SCC - prostaglandin-endoperoxide synthase 2 - phospholipase A_2 - SNPs - susceptibility

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Introduction

For reasons that are not clear, the highest incidence of esophageal cancer, especially squamous cell carcinoma (ESCC), is found in the developing countries, but geographical variation in ESCC incidence within these countries is very striking. The Taihangshan Mountain area, including Linxian in Henan Province, and Cixian, Shexian, Wuan city in Hebei Province, a rural area of China, has the highest incidence of esophageal cancer in the world (>100 per 100, 000 population) (Zhang et al., 2011). Causes for the very high rate of ESCC in the Taihangshan Mountain area have not been established but several studies of ESCC in this area identified several risk factors, including chronic inflammation, cigarette smoking, combined with alcohol consumption, general malnutrition, as well as deficiencies in selenium, zinc, folate, riboflavin, and vitamins A, C, E, and B12 (Tran et al., 2005; Li et al., 2011; Mao et al., 2011). Whether host genetic variation may play a role in the incidence and progression of ESCC in the Taihangshan Mountain area is an important but unresolved research question (Ratnasinghe et al.,

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2004; Abnet et al., 2010; Li et al., 2013a; Li et al., 2013b). Accordingly, we chose to study whether known inflammation-related genetic polymorphisms in the Prostaglandin-endoperoxide Synthase 2 (*PTGS2*) , also commonly known as cyclooxygenase-2 (COX-2) and Phospholipase A_2 group IIA (*PLA2G2A*) genes confer susceptibility to ESCC. *PTGS2* and *PLA2G2A* are two key enzymes for arachidonic acid (AA) and prostaglandin (PG) biosynthesis, and both have been proteins reported to be over expressed in patients with ESCC (Li, et al., 2009; Ren et al., 2013).

The *PTGS2* gene is composed of 10 exons and is located on human chromosome 1q25.2- q25.3, encoding for a protein of 604 amino acids. The key role of *PTGS2* in the formation of prostanoids, especially prostaglandins, makes it a strong candidate for conferring susceptibility to epithelial cancers. In recent years, a number of studies have evaluated the correlation between *PTGS2* polymorphisms and cancer risk, including ESCC, however the results remain inconclusive (Zhang et al., 2005; Liu et al., 2006; Salinas et al., 2010; Liang et al., 2011; Wang et al., 2013).

The *PLA2G2A* gene is located on human chromosome 1p35-36, consists of 6 exons, and encodes for a protein of 144 amino acids. *PLA2G2A* is a calcium-dependent phospholipase that hydrolyzes phospholipids into fatty acids. Diverse functions include roles in inflammation, cell growth, signaling, and as potent bacteriocides (Fijneman et al., 2008). The role of *PLA2G2A* in human cancers is complex, acting as either an oncogene (e.g., prostate cancer (Graff et al., 2001; Jiang et al., 2002)) or a tumor suppressor gene (e.g., gastric cancer (Leung et al., 2002; Ganesan et al., 2008; Xing et al., 2011; Wang et al., 2013). In mouse models, *PLA2G2A* was identified as a component of the Mom1 intestinal resistance locus (MacPhee et al., 1995; Cormier et al., 1997; Cormier et al., 2000). In human intestinal cancer the role of *PLA2G2A* remains elusive. Studies have reported both up- and down-regulation of *PLA2G2A* expression in human colorectal cancers. Further, single nucleotide polymorphisms (SNPs) in *PLA2G2A* have been identified as an independent risk factor for fundic gland polyposis in familial adenomatous polyposis (FAP) patients (Yanaru-Fujisawa et al., 2007). However, no studies of *PLA2G2A* polymorphisms and risk of ESCC have been reported.

In this case-control study, we conducted a gene-wide and tag SNP-based association study to determine an association between common variants in the *PTGS2* and *PLA2G2A* genes and susceptibility to ESCC in a high-risk Chinese population.

Materials and Methods

Study Population

From October 2009 to September 2010, a total of 538 Han subjects were recruited consecutively, including 269 unrelated patients, who underwent surgical resection of primary ESCC at the YaoCun Commune-hospital in Linxian, China. At the same time, 269 cancer-free controls were randomly selected from a database of 20, 000 individuals who participated in an upper gastrointestinal cancer screening program for endoscopic examinations in the Taihangshan Mountain area, China. Detailed demographic information including age, gender and smoking status were also collected. The study was approved by the Institutional Review Board of Capital Medical University (Beijing, China), and all subjects gave written informed consent.

Table 1. Tag SNPs Information for the *PTGS2* and *PLA2G2A* Genes in Controls

SNP ID	Genomic position (bp)	Genic positionc	Alleles (major/minor)	MAF[a]	HWE[b] (*P*)
PTGS2					
rs20417	186650321	5'UTR	G/C	0.01	<0.05
rs689466	186650751	5'Near	A/G	0.42	<0.05
rs5275	186643058	3'UTR	T/C	-	-
rs12042763	186651876	5'Near	G/T	0.3	0.33
PLA2G2A					
rs16823215	20297023	3'Near	G/A	0.06	0.29
rs1891320	20297111	3'Near	C/T	0.2	0.06
rs1891321	20297415	3'Near	G/A	0.23	0.52
rs12732308	20298319	3'Near	A/C	0.12	0.09
rs876018	20301927	3'UTR	A/T	0.1	0.18
rs11677	20301964	3'UTR	C/T	0.24	0.19
rs2236772	20304785	Intron4	G/A	0.13	0.14
rs2236771	20304962	Exon4	G/C	0.3	0.85
rs11573162	20305003	Exon4	C/T	0.05	0.08
rs3753827	20305887	Intron2	C/A	0.34	0.07
rs10916685	20309565	3'Near	A/T	0.3	0.74
rs12568139	20311676	5'Near	A/G	0.32	0.22
rs6676409	20315412	5'Near	C/G	0.24	0.97

[a]MAF, minor allele frequency; [b]HWE, Hardy-Weinberg equilibrium; [c]5'Near: the region of 2000bp-5000bp upstream

Tag SNPs selection and genotyping

We used genotype data from the CHB panel (Han Chinese in Beijing) of the phase IIHapMap Project to select tag SNPs within the range of 5000bp upstream of the initiation codon and 5000bp downstream of the termination codon of the *PLA2G2A* gene on the basis of the following criteria: (1) r^2 linkage disequilibrium (LD) statistic >0.8 (Huang et al., 2009; Zhang et al., 2013); (2) minor allele frequency (MAF) >0.1; In addition to 13 tag SNPs in the *PLA2G2A* gene, 4 other tag SNPs (rs20417, rs689466, rs5275and rs12042763) in the *PTGS2* gene were added to the set of markers, based on their functional relevance and significant association with cancer in previous studies (Zhang et al., 2005; Liu et al., 2006; Salinas et al., 2010; Liang et al., 2011; Wang et al., 2013). Detailed information for all the tag SNPs including genomic position, genic position, minor allele frequency, and Hardy-Weinberg equilibrium are shown in Table 1.

DNA extraction

Genomic DNA was extracted from peripheral white blood cells of participants using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. All 17 SNPs were genotyped using MassArray (Sequenom, San Diego, CA) based on allele-specific MALDI-TOF mass spectrometry (Jurinke et al., 2001). DNA from cases and controls was randomly assigned to 96 well plates, and

Table 2. Selected Characteristics and Risk Factors in Subjects with Esophageal Squamous Cell Carcinoma and Controls

Variables	ESCC (n=269)	Controls (n=269)	*P* Value
Age (Years, Mean(SD))	52.0(10.0)	51.0(6.0)	0.08*
Gender, n (%)			0.13#
Male	187 (69.8)	203(75.5)	
Female	82 (30.2)	66 (24.5)	
Smoking, n (%)			0.007#
Yes	169 (62.9)	138 (51.3)	
No	100(37.1)	131 (48.7)	

*t test; #χ^2 test

genotyping blinded to the case or control status of the sample was performed. The call rates for the genotyping of the SNPs were > 95%. The genotypes revealed by MassArray analysis were further confirmed in 5% of the samples by DNA sequencing with an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis

First, Hardy-Weinberg equilibrium was tested for the significance of deviation of genotype distribution in the control group by a χ^2 test. The differences between demographic variables in the ESCC case and control groups were evaluated using the Mann-Whitney test or χ^2 test when appropriate. Pearson's χ^2 test and unconditional logistic regression analysis were used to assess departure from the null hypothesis that cases and controls have the same distribution of genotype counts, in which the association of a SNP with ESCC was adjusted for age, gender, and smoking status, and the odds ratio (OR) with 95% confidence interval (CI) was determined. These analyses were conducted using SPSS software (version 18.0., SPSS Inc, Chicago, IL, USA). Haplotype frequencies were estimated and compared by χ^2 test between the ESCC case and control groups using the program Haploview software (version 4.2., Mark Daly's Laboratory, Broad Institute; http://sourceforge.net/projects/haploview/) (Barrett et al., 2005). Because of multiple testing, the significance level was taken as $P<0.01$, instead of an conservative Bonferroni-like correction of the *P*-values (Rothman, 1990; Perneger, 1999; Wootton et al., 2006). All tests were two-tailed.

Results

Tag SNPs-based association study

The demographic characteristics of the participants for the case-control study are summarized in Table 2. No difference was found for the frequency distribution of gender and age between ESCC cases and controls. The percentage of subjects with a positive smoking history was significantly higher in ESCC cases than in controls. Among 4 selected functional SNPs of the *PTGS2* gene, no variation in rs5275 was found in our samples. Two tag SNPs (rs20417 and rs689466) in the *PTGS2* gene were found not to be meet in Hardy-Weinberg disequilibrium, and thus were excluded from further analyses. Therefore, only 1 out of the 4 original tag SNPs in the *PTGS2* gene were analyzed.

Results demonstrated that *PTGS2* rs12042763 genotypes were differentially distributed in ESCC cases (GG, 37.2%; GT, 48.0%; TT, 14.9%) and controls (GG, 50.9%, GT, 41.3%, TT, 7.8%) (χ^2=13.04, *P*=0.001) (Table 3). Multivariate analysis showed that subjects carrying the TT genotype had a 0.23-fold increased risk for ESCC (*P*=0.05; OR, 1.23, 95%CI, 1.00-3.04) compared with subjects carrying the GG genotype.

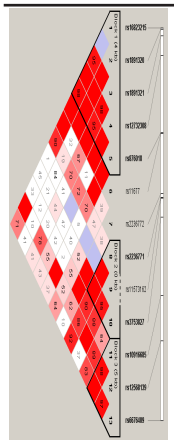
For the *PLA2G2A* gene, 13 tag SNPs met HWE filtering criteria and results showed that 10 tag SNPs did not demonstrate statistically significant associations with ESCC, but 3 tag SNPs were significantly associated

Table 3. Genotype Frequencies of the *PTGS2* and *PLA2G2A* Genes among ESCC Cases and Controls and Their Association with ESCC Risk

Gene	SNP ID	ESCC N (%)	Controls N (%)	*P*1*	*P*2#	OR(95% CIs)#
PTGS2	rs12042763			0.001		
	GG	100 (37.2)	137 (50.9)			1
	GT	129 (48.0)	111 (41.3)		0.08	1.17 (0.74-2.06)
	TT	40 (14.9)	21 (7.8)		0.05	1.23(1.00-3.04)
	GT+TT	169	132	0.001	0.07	1.22 (0.75-2.00)
PLA2G2A	rs11677			0.03		
	CC	175 (65.1)	150 (55.8)			1
	CT	78 (29.0)	107 (39.8)		0.01	0.51 (0.29-0.85)
	TT	16 (5.9)	12 (4.4)		0.03	0.57 (0.17-0.96)
	CT+TT	94	111	0.03	0.01	0.52 (0.31-0.85)
	rs2236771			0.05		
	GG	159 (59.1)	133 (49.4)			1
	GC	85 (31.6)	111 (41.3)		0.03	0.55 (0.33-0.93)
	CC	25 (9.3)	25(9.3)		0.04	0.38 (0.16-0.94)
	GC+CC	110	136	0.02	0.009	0.52 (0.32-0.85)
	rs10916685			0.06		
	AA	155 (57.6)	129 (48.0)			1
	AT	90 (33.5)	115 (42.8)		0.38	0.79 (0.47-1.33)
	TT	24(8.9)	25 (9.3)		0.8	0.89 (0.37-2.15)
	AT+TT	114	140	0.03	0.39	0.81 (0.49-1.32)

*P*1*: χ^2 test; *P*2#, OR and 95%CIs#: Logistic regression and adjusted for age, sex and smoking status.

Table 4. Haplotype Frequencies of PLA2G2A Tag SNPs among ESCC Cases and Controls

LD map SNP ID	Haplotype	Frequencies		χ^2 value	*P* value
		Case	Control		
	Block 1				
	GCGAA	0.52	0.56	1.85	0.17
	GTGAA	0.23	0.2	1.38	0.24
	GCACA	0.12	0.13	0.26	0.61
	ACAAT	0.08	0.06	2.37	0.12
	GCAAT	0.04	0.05	0.1	0.75
	Block 2				
	GC	0.39	0.36	0.92	0.34
	GA	0.37	0.34	0.93	0.34
	CC	0.24	0.3	4.35	0.04
	Block 3				
	AGC	0.28	0.31	1.23	0.27
	TAC	0.26	0.3	1.72	0.19
	AAG	0.24	0.23	0.09	0.77
	AAC	0.22	0.15	7.59	0.006

or have suggestive evidence for association with ESCC risk (Table 3). The genotype frequencies of rs11677 C/T, rs2236771 G/C and rs10916685 A/T in ESCC cases were significantly different from those in controls (Table 3). For rs11677, the genotype frequencies for CC, CT and TT were 65.1%, 29.0%, 5.9%, respectively in the ESCC group, and 55.8%, 39.8%, 4.4% in the control sample (χ^2=7.04, *P*=0.03). Individuals with the CT genotype showed a decreased risk of ESCC compared those with the CC genotype (*P*=0.01; OR, 0.51, 95%CI, 0.29-0.85). The same tendency was also observed in subjects carrying the TT genotype (*P*=0.03; OR, 0.57, 95%CI, 0.17-0.96) and the T carriers (CT+TT) (*P*=0.01; OR, 0.52, 95%CI, 0.31-0.85).

For the rs2236771 SNP, we also observed a decreased risk of ESCC associated with the C allele, with an OR of 0.55 (95%CI, 0.33-0.93; *P*=0.03) for the GC genotype, an OR of 0.38 (95%CI, 0.16-0.94; *P*=0.04) for the CC genotype as well as an OR of 0.52 (95%CI, 0.32-0.85, *P*=0.009) for the C carriers (GC+CC) when compared with the GG genotype.

Univariate analysis showed that the T carriers (AT+TT) of rs10916685 SNP had a trend towards significant association with ESCC (χ^2=7.04, *P*=0.03) compared with AA genotype carriers. However, no significant difference in the patients and controls was found when analyzed by multivariate logistic regression.

Haplotype analysis of PLA2G2A polymorphisms

We identified three haplotypes involving the 13 tag SNPs in the *PLA2G2A* gene. The haplotype frequency results are presented in Table 4. A significant difference in haplotype frequencies was observed between ESCC patients and controls with AAC for block 3 (rs10916685_rs12568139_rs6676409) (*P*=0.006).

Discussion

In an area of high risk for ESCC in China, we investigated the association of *PTGS2* and *PLA2G2A* tag SNPs with ESCC risk. For the *PTGS2* gene, among 4 SNPs studied, an association with 1 SNP was found (Table 3). Although the association of *PTGS2* gene polymorphisms with ESCC has been studied extensively among Asian, Caucasian and other ethnic populations (Zhang et al., 2005; Liang et al., 2011; Wang et al., 2013), our data are the first report linking the rs12042763 G/T polymorphism in the promoter region of *PTGS2* gene with susceptibility to ESCC. This novel finding may be due to the expanded scope of the screening for tag SNPs within the range of 5000bp upstream of the initiation codon of the *PTGS2* gene. Our results showed that subjects who carried the rs12042763 GT genotype had a modest increased risk of ESCC. The *PTGS2* promoter region contains several key cis-acting regulatory elements, including a cAMP-responsive element, and binding sites for nuclear factor-B, nuclear factor-IL-6, and stimulatory protein-1 (SP1), suggesting that a complex array of factors is involved in its regulation (Tazawa, et al., 1994). The rs12042763G/T variant is not located within any of these transcription factor binding sites; however, it has recently been shown that aspirin users who are homozygous for the major G allele at rs12042763 had the OR of 0.60 developing prostate cancer compared to asprin nonuser (Salinas, et al., 2010). It is noteworthy that we identified the rs12042763 TT genotype as having a modest association with ESCC susceptibility. These findings add to evidence for the role of the *PTGS2* gene in esophageal carcinogenesis and warrant further studies focusing on functional association.

To our knowledge, no study has investigated the role of *PLA2G2A* polymorphisms in ESCC. We found that 2 tag SNPs had a statistically significant association with ESCC risk. *PLA2G2A* can serve as an enzyme that releases free fatty acids and lysophospholipids through catalysis of membrane phospholipids at the sn-2 position (Fijneman, et al., 2008). The resulting product, arachidonic acid, leads to synthesis of prostaglandins via the PTGS (PTGS1 and *PTGS2*) pathways. The PTGS pathways regulate cellular proliferation and apoptosis and have been reported to mediate diverse biological activities including inflammation, mitogenesis and tumour metastasis (Graham et al., 2008).

PLA2G2A has been identified as a tumor resistance gene for cancer of the small and large intestine in mice (Cormier, et al., 1997; Cormier et al., 2000; Fijneman, et al., 2008) but it does not behave like a classical tumor suppressor gene. In human cancers *PLA2G2A* is proposed to act as an oncogene in prostate cancer (Graff, et al., 2001; Jiang et al., 2002), but in contrast *PLA2G2A* expression is positively associated with survival in late stage gastric cancer patients (Leung et al., 2002; Ganesan et al., 2008; Xing et al., 2011; Wang et al., 2013). These conflicting findings may reflect differing microenvironments and different genetic pathways present in different tissues (Birts et al., 2010).

A polymorphic site in exon 3 of *PLA2G2A* was reported previously in a genetic study on familial adenomatous polyposis (FAP) (Tomlinson et al., 1996). Our current study observed a decreased risk of ESCC for carriers of the heterozygous CT (OR=0.51), homozygous TT genotypes (OR=0.57) and T carriers (OR=0.52) at the rs11677 polymorphism in the 3' untranslated region (UTR)

of the *PLA2G2A* gene. The 3'UTR region of genes is commonly associated with mRNA stability, thus affecting gene expression and translation efficiency. The binding of microRNAs to the 3'UTR of mRNAs is critical for regulating mRNA levels and protein expression (Chen et al., 2008). When a SNP is located in the target sequence of a microRNA, base variation can alter microRNA activity by affecting binding affinity. The C/T polymorphism at rs11677 has been identified as located within the *PLA2G2A* 3'UTR complementary to the seed region of miR-187 (Landi et al., 2008). Our case-control study validated the importance of this target SNP in human ESCC, with potential functional significance for in-depth functional characterization.

For the rs2236771 SNP, which is located in exon 4 of the *PLA2G2A* gene we observed a decreased risk of ESCC that is associated with the heterozygous GC (OR=0.55) and homozygous CC genotypes (OR=0.38) as well as with an OR of 0.52 for the C carriers (GC+CC) when compared with the GG genotype. The rs2236771 G/C coding variant causes synonymous mutations at position 32 of the *PLA2G2A* enzyme, which should not directly influence biological function, but perhaps may still exert some effect on translation efficiency or on protein structure.

The rs10916685 SNP located in the promoter region of the *PLA2G2A* gene, which creates a hepatocyte nuclear factor-3 (HNF-3) binding site, may result in changed transcriptional activity of the *PLA2G2A* gene. We did not observe a significant association with risk for ESCC when analyzed by multivariate logistic regression. A further larger sample is needed to verify this univariate association.

More importantly, except for the above explored several possibilities how the candidate tag SNPs could relate to the *PLA2G2A* gene biological functions. One of the most likely situation that these tag SNPs are simply in LD with actual causal mutations, which have yet to be identified (Plenge et al., 2010). Finally, construction of haplotypes using the *PLA2G2A* gene tag SNPs increased our power to evaluate the role of variation in the *PLA2G2A* gene in ESCC risk. Haplotypes of AAC for block 3 (rs10916685_rs12568139_rs6676409) showed a consistent association with an increased risk for ESCC (P=0.0059).

We note some limitations in our study. Selection bias may have occurred because our ESCC cases were from YaoCun hospital in Linxian, whereas the control group was selected from the high-risk screening population in the Taihangshan Mountain. However, cases and controls are both Han ethnic groups, and the genotype frequencies we analyzed among the control group fit the Hardy-Weinberg law. In addition, we selected 4 tag SNPs of the *PTGS2* gene based on their functional relevance and having been significantly associated with cancer in previous studies. Among them, no variation was found in the polymorphic site of rs5275 in our study, and rs20417 and rs689466 failed Hardy-Weinberg criteria (P<0.05) and were excluded from further analyses. For the *PLA2G2A* gene, we did not analyze the association between gene-environmental factors, such as age, gender and smoking on the risk for ESCC because of the relatively small population sample sizes when divided into subgroups. In addition to small sample sizes in the subset analyses, the functional significance of these sequence polymorphisms is lacking.

In conclusion, these results contribute further evidence that *PLA2G2A* and *PTGS2*, two key inflammatory enzymes involved in the arachidonic acid pathway may contribute to esophageal cancer susceptibility and highlight the need for additional research.

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