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Oesophageal squamous cell carcinoma in high-risk Chinese populations: Possible role for vascular epithelial growth factor A

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Abstract

Background—Mechanisms involved in wound healing play some role in carcinogenesis in multiple organs, likely by creating a chronic inflammatory milieu. This study sought to assess the role of genetic markers in selected inflammation-related genes involved in wound healing (*interleukin (IL)-1a*, *IL-1b*, *IL-1 Receptor type I (IL-1Ra)*, *IL-1 Receptor type II (IL-1Rb)*, *tumour necrosis factor (TNF)-α*, *tumour necrosis factor receptor superfamily member (TNFRSF)1A*, *nuclear factor kappa beta (NF-κB)1*, *NF-κB2*, *inducible nitric oxide synthase (iNOS)*, *cyclooxygenase (COX)-2*, *hypoxia induced factor (HIF)-1α*, *vascular endothelial growth factor (VEGF)A* and *P-53*) in risk to oesophageal squamous cell carcinoma (OSCC).

Methods—We genotyped 125 tag single nucleotide polymorphism (SNP)s in 410 cases and 377 age and sex matched disease-free individuals from Nutritional Intervention Trial (NIT) cohort, and 546 cases and 556 controls individually matched for age, sex and neighbourhood from Shanxi case-control study, both conducted in high-risk areas of north-central China (1985–2007). Cox proportional-hazard models and conditional logistic regression models were used for SNPs analyses for NIT and Shanxi, respectively. Fisher's inverse test statistics were used to obtain gene-level significance.

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejca.2014.07.022>.

Results—Multiple SNPs were significantly associated with OSCC in both studies, however, none retained their significance after a conservative Bonferroni adjustment. Empiric *p*-values for tag SNPs in *VEGFA* in NIT were highly concentrated in the lower tail of the distribution, suggesting this gene may be influencing risk. Permutation tests confirmed the significance of this pattern. At the gene level, *VEGFA* yielded an empiric significance ($P = 0.027$) in NIT. We also observed some evidence for interaction between environmental factors and some *VEGFA* tag SNPs.

Conclusion—Our finding adds further evidence for a potential role for markers in the *VEGFA* gene in the development and progression of early precancerous lesions of oesophagus.

Keywords

Oesophageal squamous; cell carcinoma; Inflammation; Wound-healing; Genetic marker; Genetics; Inflammation-related events; Vascular endothelial growth factor A; *VEGFA*

1. Introduction

Oesophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death in the world, with a 5-year survival of less than 10% in most countries [1,2]. Oesophageal squamous cell carcinoma (OSCC), the most common type of EC worldwide [3], has a striking geographical variation and the highest rates are observed in the Taihang Mountain range in north-central China and in northeastern Iran on the Caspian littoral. No single dominant risk factor has been identified for this deadly disease in these high-risk regions, although dietary factors, consumption of hot foods and beverages, oral hygiene, socioeconomic status (SES) and genetics have all been implicated as possible risk factors [4,5].

Continuous mucosal irritation and its consequent scarring due to chronic exposure to known risk factors for OSCC, such as hot food and beverages, tobacco and nitrosamines, are believed to induce wound healing mechanisms. This active triggering of wound healing responses, which is not usually self-limiting, can create a chronic inflammatory milieu and eventually lead to dysplasia and tumour development [6,7]. Induction of mediators with carcinogenic properties involved in wound healing/repair pathway may lead to oesophageal malignancy. The interleukin (IL)-1 family (IL-1a, IL-1b, IL-1 Receptor type I (IL-1Ra) and type II (IL-1Rb)), tumour necrosis factor (TNF)- α , tumour necrosis factor receptor superfamily member (TNFRSF)1A (a member of TNF-receptor superfamily), nuclear factor kappa beta (NF- κ B) I and II, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, protein (P)-53, hypoxia induced factor (HIF)1- α and vascular endothelial growth factor (VEGF)A are among many inflammation-related genes involved in the wound healing process. Multiple lines of evidence have been provided about the role of these gene products in different stages of OSCC carcinogenesis [8–16]. The role of germline markers in these genes in OSCC risk, however, has not been well studied.

Using data from the General Population Nutritional Intervention Trials (NIT) cohort study and the upper gastro-intestinal (UGI) Cancer Genetics case-control study (Shanxi Project) conducted between 1985 and 2007 in north-central China, we tested for association between

markers in these 13 inflammation-related genes and occurrence of OSCC in the NIT cohort and Shanxi project individually. Also, data available on OSCC risk factors in these populations provided an opportunity to evaluate possible interaction between genetic markers and some environmental factors.

2. Methods and materials

This study is a part of the ‘Rare Cancers iSelect Project’. Organised in 2007, this project solicited ideas regarding molecular pathways from investigators in the National Cancer Institute (NCI) Division of Cancer Epidemiology and Genetics (DCEG) and used samples from several NCI supported studies of rare cancers. Over 20,000 individuals from more than 15 different studies were genotyped. High-dimension single nucleotide polymorphisms (SNP) array technology from Illumina was used to develop a custom chip for this project which included over 29,000 SNPs tagging 1316 different genes. Samples from two UGI cancer projects conducted in north-central China over the past 25 years were included in the Rare Cancers iSelect Project; the NIT and Shanxi project. Details of the design of each study have been described previously [17,18].

Briefly, the NITs were two randomised controlled trials, the Dysplasia Trial and the General Population Trial, conducted between 1985 and 1991 in Linxian, Henan Province, an area with very high rates of both OSCC and gastric cardia adenocarcinoma. Over 3000 adults cytologically diagnosed with oesophageal dysplasia were enrolled in the Dysplasia Trial. The General Population NIT enrolled around 30,000 residents of Linxian. The goal of both NITs was to evaluate the role of vitamin/mineral supplementation in preventing UGI cancers in this high-risk population. Follow up of all participants has continued beyond the termination of the original trials to determine cancer incidence and monitor all causes of mortality, and to collect additional data and samples for further aetiological investigations of genetic and environmental risk factors. A 10-ml blood sample was collected from participants in 1999/2000 and stored for genetic and environmental studies. Demographic and risk factor information including age, sex, education, tobacco use, consumption of alcohol, moldy foods, pickled vegetable and other dietary habits, oral hygiene, drinking hot beverages, water source and family history of cancer were gathered from all participants at baseline enrolment, using a detailed questionnaire [17]. During the follow-up period, participants were contacted monthly by either study investigators or village health workers, and all cancer diagnoses were verified by senior Chinese diagnosticians from Beijing. Case ascertainment is considered complete for the NIT and loss to follow-up over this period was less than 1% [19].

Using a case cohort sampling scheme, 546 incident OSCC cases, and 500 disease free individuals frequency-matched for age and gender to the OSCC cases were selected from this cohort for inclusion in the Rare Cancers iSelect Project. Since dysplasia is a well-known precursor of OSCC and the proposed mechanism for any role of polymorphisms in selected genes involved in wound healing would be through induction of dysplasia, including participants in the Dysplasia Trial could obscure the true nature of this process and introduce bias. Thus, we limited our analysis to the individuals from the General Population Trial.

Shanxi project was launched in 1995 and aimed to look for major susceptibility genes involved in the aetiology, prevention and early detection of UGI cancers. There are five components to the Shanxi Study: a tumour/non-tumour study, a high-risk/low-risk population study, a case–control study, a family study and an endoscopy study. Samples from the case–control component of Shanxi project were included in the ‘Rare Cancers iSelect Project’. In the case–control study, information on age, sex, occupation, income, marital status, education, household heating and cooking methods, household exposure to smoke, tobacco smoking, alcohol use, water source, consumption of hot food and other dietary habits, plus family history of UGI cancer was collected using a standardised questionnaire. Six hundred recently diagnosed OSCC patients from five different regions in Shanxi province who had no previous treatment for cancer, had undergone surgery at the Shanxi Cancer Hospital and had confirmed histological diagnosis of OSCC were enrolled in this case–control study. For each case, one control individually matched for age (± 5 years), sex and region was recruited. Blood samples were obtained from study participants at recruitment.

Due to the differences between these two populations, in terms of the distribution of potential confounders, sampling method and study design, each study was analysed separately.

2.1. Analysis of SNPs

Tag SNPs were selected if they had minor allele frequency (MAF) $\geq 5\%$ and were located within 20 kb upstream and 10 kb downstream of the candidate genes. HapMap Han Chinese population was used as a reference group and the Carlson method implemented in the Tagzilla Program was used to select tagging SNPs which provided coverage at an r^2 threshold of 0.8. One hundred and twenty-five tag SNPs for the 13 inflammation-related genes were available in the iSelect marker panel and were used for this analysis. The list of genes, their location and the corresponding number of tagging SNPs are presented in Supplementary Table 1.

2.2. Genotyping platform

All samples were genotyped using the Custom Infinium® BeadChip Assays (iSelect™) developed by Illumina for high-density scanning of specific regions or pathways. All genotyping was done in the Core Genotyping Facility at the NCI, NIH.

2.3. Quality control

Genomic DNA samples were extracted from peripheral blood using the Trizol method. Study samples must have had 500 ng of total genomic DNA at a concentration of 10 ng/ μ l as determined by Pico Green® to be included in iSelect™ genotyping. Post-genotyping quality control (QC) included evaluation of concordance rates in duplicate samples, SNP call rate, evaluation of concordance within HapMap subjects and Hardy Weinberg Equilibrium (HWE) assessment in non-cases. SNPs with less than 95% completion rate and less than 95% concordance rate, and samples with less than 85% completion rate were excluded. Since both cases and disease free individuals are from the same region in China, population stratification was not a major concern for this analysis.

2.4. Statistical analysis

Continuous and categorical variables other than SNPs are presented as mean ($\pm$ standard deviation) and numbers (percentages), respectively. The Student t-test and the chi-square test were used to test for associations between variables. All analyses were performed at two levels: individual SNPs and at the gene level. Each individual SNP was coded as 0, 1 and 2 reflecting the number of minor alleles. For SNP-based association analyses, we assumed an additive model for allelic effects on risk. We used Cox-proportional hazard models with latent entry and the robust variance estimate to analyse data from the NIT. The time origin for the analyses was time at blood draw, and the exit times were either development of OSCC, loss to follow-up or administrative censoring (set at 31st December 2007). For Shanxi, we used conditional logistic regression models. All models were adjusted for potential confounders including smoking, alcohol consumption, consumption of hot beverages and family history of UGI cancers based on questionnaire data. The Cox-proportional hazard models were additionally adjusted for age and sex.

Multiple comparisons were considered by holding the family-wise error rate (FWER) constant at a probability of at least one type 1 error among the 125 tests performed. We also performed 5000 permutations and looked at the association between these markers and OSCC in each permuted dataset. We shuffled case status, time from blood draw to outcome (OSCC diagnosis, loss to follow-up or administrative censoring) in the NIT and the case status within each matched pair in Shanxi, thus preserving the matching scheme and also the correlation among SNPs. *P*-values from each round of permutations were ranked, and empiric *p*-values were calculated as the fraction of the ranked permuted *p*-values higher than the observed *p*-value for each individual SNP.

In addition to single SNP association, we used Fisher's combination method to test for the joint association of all SNPs within a gene [20]. In this method, minus two times the logarithm of *p*-values for all SNPs in a gene are summed together. When dealing with independent observations, this test statistic should follow a chi-squared distribution with degrees of freedom (df) equal to twice the total number of SNPs [20]. When using all genetic markers, however, given their close proximity and strong linkage disequilibrium (LD), these *p*-values are not independent, and thus this distribution is not appropriate. To overcome this issue, we used permutation tests again to generate an empirical distribution of test statistics under the null hypothesis and ranked the observed *p*-values against this distribution.

We also explored potential interactions between selected environmental irritants to mucosal tissues and tag SNPs for genes with empirical evidence on their role in OSCC aetiology. In this stratified analysis, individuals were grouped based on smoking, drinking habits and consumption of hot drinks into exposed and unexposed categories and the association between these tag SNPs and OSCC was explored within strata of these environmental exposures. We also tested for the presence of interaction between these environmental factors and the individual SNPs by introducing an interaction term into the models. Given the power for such analyses, these results should be regarded as hypothesis generating rather than confirmatory.

All statistical analyses were performed using R v.2.12.2 [21]. All tests of hypothesis were conducted at a confidence level of 0.95 under two-sided alternatives.

3. Results

Four hundred and ten cases and 377 disease free individuals from the NIT General Population Trial and 546 OSCC cases and 556 individually matched controls from Shanxi passed QC measures and were included in these analyses. Overall, 14.8% of the NIT samples and 11.8% of the Shanxi samples did not pass QC post-genotyping.

Compared to participants in Shanxi project, individuals enrolled from the NIT were significantly older, less likely to report drinking and smoking, but more likely to report a positive family history of UGI cancer (p -value < 0.0001). The male to female ratio was lower in the NIT compared to Shanxi; the male to female ratio was 0.8 in NIT and 1.7 in Shanxi (p -value < 0.0001). Participants were on average 63.6 (± 6.3) years old at the time of DNA collection and 57.4 (± 8.4) years old at enrolment in NIT and Shanxi, respectively. The median time from blood draw to OSCC diagnosis was 5.26 years in the NIT. A positive family history of UGI cancer was significantly more common among OSCC cases in both studies (38.5% versus 30.8% in the NIT and 25.3% versus 20.1% in Shanxi). Consumption of hot liquids was associated with a significant increase in the odds of OSCC in Shanxi (p -value < 0.05). Baseline characteristics of participants in both studies are shown in Table 1.

Of the 125 tagging SNPs, eight SNPs (four in *VEGFA*, one in *TNF- α* , and three in *IL-1R1*) in the NIT, and five SNPs (one in *iNOS*, two in *TNFRSF1A* and three in *VEGFA*) in Shanxi showed nominally significant associations with risk to OSCC. None of these SNPs, however, retained statistical significance when the FWER was considered. The covariate-adjusted hazard ratio (HR)/odds ratio (OR) estimates for OSCC of the nominally significant SNPs are listed in Table 2. The covariate-adjusted HR/OR estimates for OSCC of the rest of the SNPs are presented in Supplementary Table 2. We further looked at the distribution of observed p -values for SNPs within each gene to check if any statistical signal in the combination of SNPs within each gene was apparent in these data. In the NIT, the observed p -values for tested SNPs were not distributed uniformly for SNPs in the *VEGFA* gene (as should occur if the null hypothesis were universally true), and were mostly concentrated in the lower tail. Positions of the 16 tagging SNPs for *VEGFA* gene structure in NIT are shown in Fig. 1. We used permutation test to check if this enrichment pattern in empirical significance could just be due to chance alone using permutation-based tests as presented in Fig. 2. As seen in Fig. 2, the observed $-\log(p\text{-values})$ for tagging SNPs for *VEGFA* in NIT falls in the upper boundary of the permuted $-\log(p\text{-values})$, indicating some true signal in this collection of SNPs. Using Fisher's combination method, markers in *VEGFA* yielded a permutation significance level (p -value) of 0.03. Supplementary Figure 1 presents the distribution of the permuted Fisher's test statistics for *VEGFA* gene in the NIT, noting the location of the observed test statistic. In Shanxi, the empirical distribution of these SNPs did not suggest any statistically significant association with OSCC. Likewise, the empirical gene-level p -value for *VEGFA* using Fisher's inverse test statistics was not significant ($p = 0.2$).

The distributions of observed p -values for tagging SNPs in the other 12 genes were uniform in both studies, as expected under the null hypothesis. Permutation testing did not show any signal for any of these 12 candidate genes. At the gene-level, none of these candidate genes achieved empirical significance using permutation testing. Gene-level p -values obtained using the Fisher's inverse test statistics are listed in Table 3. Figs. 3 and 4 represent box plots of the permuted Fisher's inverse test statistics, over imposed by the observed Fisher's inverse test statistic.

Since *VEGFA* gene was the only gene that was statistically significantly associated with OSCC risk, we further evaluated whether risk of OSCC associated with these SNPs was different in people with different exposure status to available environmental risk factors (e.g. drinking hot liquid, consuming alcohol and smoking tobacco). There were several nominally significant qualitative interactions between these tag SNPs and the environmental factors assessed in the NIT. For example, for individuals drinking liquids at normal temperature (versus those drinking hot liquids), the hazard of OSCC associated with each variant allele at rs10434 increased by 2.0 (95% confidence interval (CI): 1.0, 3.9) fold. In the NIT, only 12.4% of women reported alcohol drinking, no women reported smoking and consumption of hot liquids among women was significantly less than men. Thus, we further restricted these analyses to men. The estimated HRs (95% CI) associated with the nominally significant SNPs in different subgroups of environmental exposures are presented in Table 4. As can be seen in the table, effects of the significant SNPs were generally more pronounced in men.

4. Discussion

In these two samples from a population-based cohort and a case-control study in north-central China, a high-risk region for OSCC, SNPs in *IL-1R1*, *TNF- α* and *VEGFA* genes showed nominally significant associations with OSCC in the NIT, and SNPs in *iNOS*, *TNFRSF1A* and *VEGFA* genes were significantly associated with OSCC in Shanxi. At the gene level, we observed a significant association between several markers in the *VEGFA* gene and OSCC risk, but only in the NIT. We also observed different effects of these SNPs on OSCC risk in people with different exposure to drinking hot liquids, consuming alcohol and smoking tobacco. We did not observe any statistical evidence for a significant association between the other 12 genes and OSCC in either study.

Chronic mucosal irritation by environmental insults, such as hot liquids, has been proposed to predispose to OSCC [22]. It is believed that continuous mucosal irritation can induce wound-healing responses and form a micro-inflammatory milieu. This repeated physiologic response to an environmental irritant could become maladaptive over the long term [23], resulting in non-healing wounds where the tissue remains in a chronic inflammatory state over long periods of time [24]. Chronic inflammatory milieu of non-healing wounds further promotes malignant transformation through different mechanisms involved in cellular homeostasis. Prolonged and progressive angiogenesis, a hallmark of inflammation, can induce carcinogenesis by increasing the vasculature (through the angiogenic switch) and providing an environment rich in micronutrient and growth factors, which supports tumour growth [24,25]. *VEGFA* is the key mediator of angiogenesis under normal and physiologic

conditions [25]. Thus, any factor causing dysregulation in VEGFA expression may be involved in disease process.

Angiogenesis is considered a rate-limiting step, which is required for tumour development, progression and metastatic spread [25,26]. Evidence for the occurrence of an angiogenic switch prior to tumour development comes from both mouse models and studies in human premalignant lesions [26]. In the UGI tract, the angiogenic switch and VEGFA expression (a potent inducer of the angiogenic switch) have been reported to be early events in the development of OSCC, oesophageal adenocarcinoma (EAC) and gastric cancer (GC) [12,27–29]. Also, a stepwise increase in VEGFA expression has been reported in premalignant lesions of both EAC and GC [30,31].

The *VEGFA* gene, located on chromosome 6, spans a 14 kb coding region, and has eight exons and seven introns [32]. Polymorphisms in this gene have been evaluated in cancers in which angiogenesis is believed to play a fundamental role. Markers in the promoter, 5'- and 3'-untranslated regions (UTR) of *VEGFA* have been shown to affect gene expression, mRNA concentration, protein production and cancer risk [22,33–35]. Several studies have reported a role for these polymorphisms in gastrointestinal cancers including colon cancer, GC and EAC [35–37].

In the NIT, we found a protective effect for the minor allele at three SNPs in this gene: rs3025039, rs699947 and rs8333053. The hazard for OSCC associated with each additional minor allele at these SNPs decreased 18–23%. The role of rs3025039 (C/T), located in the 3'-UTR of the *VEGFA* gene, in different cancers has been extensively studied. The T allele has been linked to decreased VEGFA plasma levels [22,33] and an increased or decreased risk of cancer. While a protective effect for the T allele has been reported in breast cancer [22,38,39], Bae et al. reported an increased risk of colon cancer with this allele in women <55 years of age [37]. A higher risk for oesophageal adenocarcinoma has been reported to be associated with rs3025039 [35,40].

We also saw a 21% decrease in the risk of OSCC for each A allele at rs699947. This SNP is located in the 5'-UTR region but its functional role has not been well defined. VEGFA expression levels have been reported to depend on alleles at this marker [40,41]. This same SNP has been reported to decrease the risk of colon cancer in Korean women [42].

We observed a protective effect for rs8333053 (located 13.5 kb downstream of the gene), and risk effect for rs833060, which lies almost 5 kb downstream of the gene, and OSCC risk in NIT. Currently, there is little information about any functional role for these SNPs in the literature.

Previous studies have provided solid evidence for the role of *VEGFA* in OSCC. Kitadai et al. showed that tumour cells and dysplastic cell lines express VEGFA, and that this expression correlates with lesion depth [12]. This suggests VEGFA plays a role in the development and progression of early precancerous lesions. Although as described above, the exact biological effects of these markers are not clear, the empirically significant association between markers in the *VEGFA* gene and OSCC using Fisher's combination method provides some further evidence for a role for VEGF in OSCC development. Fisher's combination method

has the advantage of using a pooled p -value for all SNPs studied in a single gene (to assess gene-level significance) instead of relying on single SNP associations. SNP associations alone are often deemed insufficient to understand disease mechanisms [20], and may fail to distinguish SNPs contributing only small increments of risk. Additionally, analysis of individual SNPs does not address the possible joint action of multiple SNPs within a gene or multiple genes [20,43].

Our exploratory results of gene-environment interaction provided some evidence for the existence of multiple qualitative interactions between the studied SNPs and drinking hot liquids, alcohol consumption and cigarette smoking. It seems polymorphisms in *VEGFA* gene may have different effects on OSCC risk depending on environmental factors. This may be yet another example of the complex nature of cancer, emphasising the interplay of inherited germline markers, epigenetic changes and environmental exposures contributing to cancer susceptibility. An interesting observation in the subgroup analyses was the elevated hazard of OSCC among men who consume alcohol and those who did not drink hot liquid associated with each variant allele at rs833069. Although no carcinogenic role has been reported for this intronic SNP, this SNP is in perfect LD with rs2010963 ($r^2 = 0.9$, $D' = 1$), a marker located in the 5'-UTR region of the gene which has been associated with several cancers. Like rs699947, the functional roles reported for this SNP remain unclear and range from affecting protein translation efficacy, decreasing expression and poor vascular density to increasing expression and inducing angiogenesis [34,41]. Higher risk of GC has been reported for this SNP among ever smokers, never drinkers and non-white participants in a study by Guan et al. [36].

The association between *VEGFA* tagging SNPs and OSCC risk observed in NIT was not observed in the Shanxi case-control study. Only one SNP, rs69947, showed a significant association with OSCC in both studies. However, the direction of the effect was reversed; while the minor allele was associated with a 21% decrease in the hazard of OSCC in the NIT, it was associated with a 24% increase in the odds of OSCC in the Shanxi case-control study. This flip-flop should not be a great surprise since the association between this SNP and OSCC in both studies was not significant after controlling for multiple comparisons. As a result, the exact effect size and the direction of the association seen in either study may not reflect any true biological mechanism. Although *VEGFA* had the smallest p -value at the gene-level in Shanxi, the p -value for *VEGFA* did not reach a level of formal statistical significance. Aside from limited power in Shanxi and potential false positive findings in the NIT, differences in study design and limitations inherent in case-control studies, including prevalence-incidence bias and referral admission rate bias, gene-environment and gene-gene interaction might explain this lack of consistency [44].

This study has some important limitations. First, we had only limited coverage of the genes studied. For example, despite the relatively good coverage provided by markers on the iSelect panel, some SNPs in the promoter region of *VEGFA* (such as rs1570360) were neither genotyped nor in strong LD with the SNPs that were evaluated. Second, incomplete coverage of genes in the pathway is another limitation of this study. Third, this study relied on questionnaire-based environmental exposure information rather than biological measurements of tissue irritation assessment and other potential triggers of the inflammatory

response. Finally, the modest sample size of both studies limited our power to investigate interactions between genes and environment and between genes and genes in any thorough manner.

One of the strengths of this study is our use of a novel mechanism for considering suspected risk factors for OSCC in this high-risk population. We used a systemic approach to examine mechanistically-plausible genes involved in a key biological pathway. The genes were selected based on an innovative approach which links two substantially supported notions: an association between OSCC and environmental mucosal irritants such as hot drinks that has been shown in different settings, and the role of inflammation in cancer. The data presented here came from relatively large population-based studies in high-risk population for OSCC. To our knowledge, this is the first study on *VEGFA* polymorphisms and OSCC risk. While there is solid evidence for the role of *VEGFA* gene expression in OSCC aggressiveness and prognosis, more investigation on the role of *VEGFA* in OSCC risk is needed.

In conclusion, we found some evidence for a potential role for markers in the *VEGFA* gene in OSCC risk in the NIT and also suggestive evidence of interaction between environmental factors and markers in *VEGFA* but we failed to replicate these findings in a case-control study conducted in Shanxi. This inconsistency does not necessarily nullify a possible role for *VEGFA* in the aetiology of OSCC but rather warrants further investigation in larger studies with compatible study designs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Position of the 16 tag single nucleotide polymorphism (SNP)s for *vascular endothelial growth factor A (VEGFA)* gene (top) and the pairwise linkage disequilibrium (LD) structure of the genotyped markers in 377 unrelated controls in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan, China, 1985–2007. Plots were produced using the `snp.plotter` package within R version 2.12.2. The dashed line presents the $-\log_{10}$ (empirical p -value) for the overall test for an individual SNP.

Fig. 2. Distribution of the observed p -values of the *vascular endothelial growth factor A (VEGFA)* tag single nucleotide polymorphism (SNP)s in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, China, Henan, 1985–2007. The grey lines and the black line represent the permuted and the observed p -values, respectively. Dashed lines are the 95% confidence limits and the median of the permuted p -values.

Fig. 3. Box plots of the permuted Fisher's inverse test statistics of the 12-inflammation-related genes, over imposed by the observed Fisher's inverse test statistics (black dots) in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan, China, 1985–2007.

Fig. 4.

Box plots of the permuted Fisher's inverse test statistics of the 12-inflammation-related genes, over imposed by the observed Fisher's inverse test statistics in Shanxi (black dots) in the Shanxi case-control study (Shanxi), Shanxi, China, 1995–2007.

Table 1

Baseline characteristics of study participants in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan and Shanxi case-control study (Shanxi), Shanxi, China (1985–2007).

	NIT		Shanxi				
	Disease free individuals, <i>n</i> = 377		OSCC*, <i>n</i> = 410	Total, <i>n</i> = 787	Control, <i>n</i> = 556	OSCC*, <i>n</i> = 546	Total, <i>n</i> = 1102
Age [†] , mean (standard deviation (SD))	63.3 (7.5)		63.9 (7.0)	63.6 (7.3)	57.4 (8.2)	57.4 (8.6)	57.4 (8.4)
Sex, <i>n</i> (%)							
Male	167 (44.3)		186 (45.4)	353 (44.8)	345 (62.1)	343 (62.8)	688 (62.4)
Female	210 (55.7)		224 (54.6)	434 (55.2)	211 (37.9)	203 (37.2)	414 (37.6)
Hot drink consumption, <i>n</i> (%)							
No	296 (78.5)		322 (78.5)	618 (78.5)	142 (25.5)	77 (14.1)	219 (19.9)
Yes	81 (21.5)		88 (21.5)	169 (21.5)	414 (74.5)	469 (85.9)	883 (80.1)
Family history of upper gastro-intestinal (UGI) cancer, <i>n</i> (%)							
No	116 (30.8)		158 (38.5)**	274 (34.8)	444 (79.9)	408 (74.7)**	852 (77.3)
Yes	261 (69.2)		252 (61.5)	513 (65.2)	112 (20.1)	138 (25.3)	250 (22.7)
Consumption of alcohol, <i>n</i> (%)							
No	95 (25.2)		96 (23.4)	191 (24.3)	304 (54.7)**	288 (52.7)	592 (53.7)
Yes	282 (74.8)		314 (76.6)	596 (75.7)	252 (45.3)	258 (47.3)	510 (46.3)
Smoking Tobacco, <i>n</i> (%)							
No	116 (30.8)		118 (28.8)	234 (29.73)	235 (42.3)	227 (41.6)	462 (41.9)
Yes	261 (69.2)		292 (71.2)	553 (70.3)	321 (57.7)	319 (58.4)	640 (58.1)

* OSCC, oesophageal squamous cell carcinoma.

[†] Age at the time of DNA collection in NIT and age at enrolment in Shanxi.

** *P*-value < 0.05.

Table 2

Hazard ratio (HR)/odds ratio (OR) (95% confidence interval (CI)) for oesophageal squamous cell carcinoma associated with nominally significant markers in selected inflammation-related genes and in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan and Shanxi case-control study (Shanxi), Shanxi, China (1985–2007).*

Gene	Single nucleotide polymorphism (SNP) (major, minor allele)	Minor allele frequency (MAF) [†]	HR/OR (95% CI) ^{**}	Crude <i>P</i> -value
NIT				
NIT <i>Interleukin (IL)1-R1</i>	rs3917296 (A, G)	0.1	0.7 (0.6, 0.9)	0.02
	rs17026654 (T, C)	0.3	1.3 (1.0, 1.5)	0.04
	rs3771202 (G, C)	0.2	1.3 (1.0, 1.6)	0.04
<i>Tumour necrosis factor (TNF)-α</i>	rs1800629 (G, A)	0.1	0.5 (0.3, 0.8)	<0.01
<i>Vascular epithelial growth factor A (VEGFA)</i>	rs3025039 (C, T)	0.1	0.8 (0.6, 0.9)	0.04
	rs699946 (A, G)	0.2	1.3 (1.0, 1.6)	0.04
	rs833060 (G, T)	0.2	1.3 (1.0, 1.6)	0.05
	rs833053 (T, C)	0.3	0.8 (0.7, 1.0)	0.05
	rs699947 (C, A)	0.2	0.8 (0.6, 1.0)	0.05
Shanxi				
<i>VEGFA</i>	rs833052 (C, A)	0.2	1.3 (1.1, 1.6)	0.01
	rs699947 (C, A)	0.2	1.2 (1.0, 1.5)	0.05
<i>NOS2A (inducible nitric oxide synthase (iNOS))</i>	rs944725 (C, T)	0.2	1.3 (1.0, 1.6)	0.05
<i>Tumour necrosis factor receptor superfamily member (TNFRSF)1A</i>	rs2234649 (T, G)	0.1	0.7 (0.5, 0.9)	0.02
	rs11064145 (T, G)	0.2	0.9 (0.6, 0.9)	0.04

* All models were adjusted for age, gender, family history of upper gastro-intestinal (UGI) cancer, consumption of hot drinks, smoking and drinking alcohol.

[†] MAF (minor allele frequency) in disease free individuals in the NIT and controls in Shanxi.

^{**} The reported estimates are HR for each risk allele (minor allele) in NIT and OR for each risk allele (minor allele) in Shanxi, respectively.

Table 3

Empirical gene-level *P*-value of the 13 inflammation-related genes in relation to oesophageal squamous cell carcinoma risk in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan and Shanxi case-control study (Shanxi), Shanxi, China (1985–2007).

Gene	# of single nucleotide polymorphism (SNP)s	<i>P</i> -value*	
		NIT	Shanxi
<i>Hypoxia induced factor (HIF)-1α</i>	5	0.99	0.44
<i>Interleukin (IL)1A</i>	3	0.98	0.91
<i>IL1B</i>	8	0.49	0.82
<i>IL1-R1</i>	14	0.09	0.69
<i>IL1-R2</i>	15	0.95	0.65
<i>Nuclear factor kappa beta (NF-κB)1</i>	8	0.83	0.76
<i>NF-κB2</i>	3	0.22	0.78
<i>NOS2A (inducible nitric oxide synthase (iNOS))</i>	20	0.51	0.37
<i>PTGS2 (cyclooxygenase (COX)2)</i>	3	0.28	0.94
<i>Tumour necrosis factor (TNF)-α</i>	12	0.15	0.64
<i>Tumour necrosis factor receptor superfamily member (TNFRSF)1A</i>	11	0.90	0.35
<i>P53</i>	7	0.94	0.63
<i>Vascular endothelial growth factor (VEGF)A</i>	16	0.03	0.24

Nominally significant HRs are bolded in the Table.

* Empiric *P*-values are calculated as the fraction of the ranked permuted Fisher's inverse test statistics ($-2\sum(\log(P\text{-values}))$) that are as high or higher than the observed test statistics.

Hazard ratio (95% confidence interval) of oesophageal squamous cell carcinoma for nominally significant *vascular endothelial growth factor A (VEGFA)* tag single nucleotide polymorphism (SNPs) by subgroups of hot liquid consumption, drinking alcohol and smoking cigarette in the General Population Nutrition Intervention Trial (NIT) cohort, Linxian, Henan, China (1985–1991).*

SNP (major, minor allele)	Adjusted hazard ratio (HR) (95% confidence interval (CI)) [†]					
	Hot liquid consumption		Drinking alcohol		Smoking cigarette	
	Yes (n = 618)	No (n = 169)	Yes (n = 191)	No (n = 596)	Yes (n = 234)	No (n = 553)
rs833060 (G, T)	1.2 (0.9,1.6)	1.5 (1.0,2.5)	2.0 (1.3,3.2)	1.1 (0.8,1.4)	1.3 (0.9,1.9)	1.2 (0.9,1.6)
rs699947 (C, A)	0.8 (0.6,1.1)	0.6 (0.4,1.0)	0.7 (0.4,1.1)	0.8 (0.6,1.1)	1.1 (0.7,1.7)	0.7 (0.5,0.9)
rs833069 (T, C)	1.1 (0.8,1.4)	1.6 (1.1,2.5)	1.6 (1.0,2.5)	1.1 (0.8,1.4)	1.3 (0.9,1.9)	1.2 (0.9,1.5)
rs10434 (G, A)	1.0 (0.8,1.4)	2.0 (1.0,3.9)	0.9 (0.5,1.4)	1.3 (0.9,1.8)	0.9 (0.6,1.4)	1.3 (1.0,1.9)
rs3025010 (T, C)	1.0 (0.8,1.3)	0.5 (0.3,0.9)	0.9 (0.5,1.4)	0.9 (0.7,1.1)	1.2 (0.8,1.7)	0.8 (0.6,1.0)
rs9369421 (T, C)	1.0 (0.7,1.4)	0.8 (0.5,1.3)	1.4 (0.8,2.5)	0.9 (0.7,1.1)	1.5 (1.0,2.3)	0.8 (0.6,1.1)
Men						
	Yes (n = 309)	No (n = 44)	Yes (n = 137)	No (n = 216)	Yes (n = 234)	No (n = 119)
rs833060 (G, T)	1.4 (1.0,2.0)	4.4 (1.2,16.1)	2.4 (1.4,4.2)	1.2 (0.8,1.8)	1.3 (0.9,1.9)	2.1 (1.2,3.9)
rs699947 (C, A)	0.9 (0.6,1.3)	0.5 (0.1,1.6)	0.5 (0.3,0.9)	1.1 (0.7,1.7)	1.1 (0.7,1.7)	0.5 (0.3,1.0)
rs833069 (T, C)	1.1 (0.8,1.6)	5.3 (1.2,23.2)	1.9 (1.0,3.5)	1.1 (0.7,1.7)	1.3 (0.9,1.9)	1.5 (0.7,2.9)
rs10434 (G, A)	0.7 (0.5,1.1)	5.3 (1.5,18.3)	0.9 (0.5,1.5)	0.9 (0.5,1.4)	0.9 (0.6,1.4)	1.0 (0.5,2.0)
rs3025010 (T, C)	1.1 (0.8,1.5)	0.2 (0.1,0.8)	0.8 (0.4,1.3)	1.1 (0.7,1.7)	1.2 (0.8,1.7)	0.5 (0.3,1.0)
rs9369421 (T, C)	1.0 (0.7,1.5)	1.5 (0.5,4.1)	1.9 (0.9,3.7)	0.8 (0.5,1.2)	1.5 (1.0,2.3)	0.4 (0.2,0.8)

Nominally significant HRs are bolded in the Table.

* All models are adjusted for age, place of residence and family history of upper gastro-intestinal cancers. Each model was also additionally adjusted for other covariates not stratified for.

[†]HR for each risk allele (minor allele) in the NIT.