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Research Paper

Association of erythropoietin gene rs576236 polymorphism and risk of adrenal tumors in a Chinese population

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Abstract

Erythropoietin (EPO) is a circulating glycosylated protein hormone and has been implicated in the development and progression of non-hematopoietic tissue tumors. The objective of the present study was to determine if the *EPO* rs576236 polymorphism was associated with the risk of adrenal tumors. We genotyped the *EPO* rs576236 polymorphism in a case-control study of 288 adrenal tumor patients and 456 cancer-free controls by using the TaqMan method, and assessed the association between the polymorphism and the adrenal tumor risk by logistic regression. Furthermore, 95% confidence interval (CI) was used to assess the genetic association between the polymorphism and the risk of adrenal tumor. Compared with the TT genotype, the TC genotype had a significantly increased risk of adrenal tumor [adjusted odds ratio (OR) = 1.24, 95% CI = 1.12–2.22]. Furthermore a significantly increased risk of adrenal tumor was found in the combined variant genotypes TC+CC compared with the TT genotype (adjusted OR = 1.17, 95% CI = 1.12–2.21). Our present study suggests that the rs576236 polymorphism of *EPO* confers susceptibility to adrenal tumor in the Chinese population.

Keywords: polymorphism, genetic susceptibility, erythropoietin, adrenal tumor

INTRODUCTION

Adrenal tumors can be functional, subclinical and nonfunctional. Functional adrenal tumors could lead to secondary hypertension or diabetes[1,2]. These tumors are usually detected by the clinical side effects of hormone production or found incidentally on imaging, especially in the case of nonfunctional adenomas[3]. Accumulative epidemiological studies have revealed that cigarette smoking, alcohol consumption and a history of hypertension are associated with adrenal tumors.[4] However, only a small percentage of exposed individuals will eventually develop adrenal tumors in their lifetime, suggesting that genetic susceptibility also plays a role in the development of adrenal tumors.

Erythropoietin (EPO) is produced and secreted into the brain, liver, uterus or other organs and detected in the breath of individuals[5,6]. EPO is mainly produced in renal peritubular interstitial cells when there is a decrease in oxygen saturation and is the principal reg-

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ulator of RBC formation derived from bone marrow progenitor cells. The *EPO* gene is located on chromosome 7q22, which contains 5 exons and 4 introns[7]. The amount of endogenous EPO (eEPO) depends on the concentration of O_2 in the blood. EPO is primarily synthesized in the kidney, although a little amount of others is produced by other tissues such as the liver and brain[8,9]. Several studies demonstrated a direct effect of EPO on the growth and survival of primary tumors. Importance has been attached to the question if EPO receptors (EPO-R) on cancer cells are functional[10–13].

EPO-Rs have also been identified in a wide variety of non-hematopoietic cells. EPO and EPO-R are often expressed in cancer, such as spongioblastoma, lung cancer, breast cancer, gastric cancer, prostatic cancer, liver cancer and so on[14–17]. The mechanism of transitional cell carcinomas of the bladder and renal cancer confirmed that *EPO* could be expressed in the tumor[18,19]. Gene polymorphisms in the *EPO* were detected in patients with cancer in recent studies[20,21]. The role of *EPO* rs576236 polymorphism in adrenal tumor has not been reported. We hypothesize that *EPO* rs576236 polymorphisms are associated with adrenal tumor risk. We investigated the association of *EPO* rs576236 polymorphism and the risk of adrenal tumors through our ongoing, hospital-based, case-control study in the Chinese population.

SUBJECTS AND METHODS

Subjects

The present case-control study included 288 adrenal tumor patients with histopathologically-confirmed adrenal tumor and 456 cancer-free controls. All subjects were genetically unrelated Han Chinese and were enrolled between July, 2009 and July, 2012 by the authors′ affiliated institution. Before enrollment, the trained interviewers gave a standard questionnaire to obtain demographic data and related risk factors by face-to-face interview. Cases with previous cancer, metastasized cancer for other or unknown reason and previous radiotherapy or chemotherapy were excluded. The case-free control subjects were enrolled from those who were treated in the outpatient departments in the hospital. Those who smoked daily for more than 1 year were defined as smokers. Individuals who drank at least 3 times per week were defined as drinkers. In this study, the response rate of the case and control subjects exceeded 85%. After interview, 5 mL venous blood sample was obtained from each subject. Written informed consent was obtained from all participants. The study protocol was approved by the institutional review board of the authors′ affiliated institution.

Genotyping

Genomic DNA was isolated from leucocytes of peripheral blood and purified by proteinase K digestion and phenol/chloroform extraction. Genotyping of the polymorphism was performed by the MGB TaqMan probe assay from Applied Bios Stems Inc. (Foster City, CA, USA). The sequence of primers and probes for the SNP are available on request. According to the manufacturer′s instructions, amplifications were performed in the 384-well ABI 7900HT Real Time PCR System (Applied Bios Stems). The SDS 2.3 software was used for allelic discrimination (Applied Bios Stems). Positive controls through sequencing and negative controls without DNA were included in each plate to ensure the accuracy of genotyping. About 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Statistical analyses

Student′s *t*-test (for continuous variables) and χ^2-test (for categorical variables) were performed to compare differences in the distributions of demographic characteristics, selected variables, and frequencies of genotypes of *EPO* rs576236 polymorphism between the cases and controls. Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit χ^2-test. The association between *EPO* rs576236 genotypes and the risk of adrenal tumor was estimated by computing odds ratios (OR) and their 95% confidence intervals (CI) from unconditional logistic regression analysis with the adjustment of age, sex, smoking and drinking status. $P < 0.05$ was considered statistically significant and all statistical tests were two sided. All of the statistical analyses were performed with Statistical Analysis System software (9.1.3; SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of the study population

The frequency distributions of selected characteristics of the cases and controls are presented in ***Table 1***. There were no significant differences between the cases and controls in age, sex, smoking and drinking status (all $P > 0.05$). However, there were more subjects with hypertension (67.37%) and diabetes (14.24%) among the cases than among the controls (17.11% and 4.16%, respectively), and these differences were statistically significant ($P < 0.001$ for hypertension and diabetes).

Table 1 **Distribution of selected variables between adrenal tumor cases and controls**

Variables	Cases (n = 288)		Controls (n = 456)		*P*
	N	%	N	%	
Age (years) (mean ± SD)	47.1 ± 12.6		48.3 ± 9.9		0.137
<48	148	51.39	220	48.24	0.404
⩾48	140	48.61	236	51.76	
Sex					
Male	143	49.65	227	49.78	0.972
Female	145	50.35	229	50.22	
Smoking status					
Never	211	73.26	335	73.46	0.952
Ever	77	26.74	121	26.53	
Drinking status					
Never	223	77.43	351	76.97	0.396
Ever	57	22.57	105	23.03	
Hypertension					
No	94	32.63	378	82.89	< 0.001
Yes	194	67.37	78	17.11	
Diabetes					
No	247	85.76	437	95.84	< 0.001
Yes	41	14.24	19	4.16	
Family					
No	228	79.16	387	84.87	0.045
Yes	60	20.83	69	15.13	

*Student's *t*-test for age distributions between cases and controls; two-sided χ^2 test for others selected variables between cases and controls.

Association between the EPO polymorphism and risk of adrenal tumor

The allele frequencies and genotype distributions of *EPO* rs576236 polymorphisms among the cases and the controls are shown in ***Table 2***. All observed genotype frequencies for this polymorphism were in HWE in the controls (χ^2 = 3.03, *P* = 0.08). For the *EPO* rs576236 polymorphism, the frequencies of the TT, CT and CC genotypes were 68.75%, 28.12% and 3.13%, respectively in the cases, and 77.63%, 20.17% and 2.20%, respectively in the controls (*P* = 0.026). When TT genotype was referenced, we found that both CC genotypes were associated with decreased risk of adrenal tumor (adjusted OR = 0.68, 95%CI = 0.64–4.03 for CC) significantly. The TC genotype had a significant increased risk of adrenal tumor (adjusted OR = 1.24, 95% CI = 1.12–2.22). Furthermore, a significantly increased risk of adrenal tumor was found in the combined variant genotypes TC+CC compared with the TT genotype (adjusted OR = 1.17, 95% CI = 1.12–2.21).

Table 2 **Genotype and allele frequencies of the *EPO* polymorphism among the case and controls and the association with risk of adrenal tumor**

Genotypes	Cases, n (%)	Controls, n (%)	*P*	OR (95% CI) †
Rs576236				
TT	198 (68.75)	354 (77.63)		1.00 (reference)
TC	81 (28.12)	92 (20.17)		1.24 (1.12–2.22)
CC	9(3.13)	10 (2.20)	**0.026**	**0.68 (0.64–4.03)**
TT	198(68.75)	354(77.63)		1.00 (reference)
TC+CC	90(31.25)	102(22.37)	**0.007**	**1.17(1.12–2.21)**
T	477(82.81)	800(87.72)		1.00 (reference)
C	99(17.19)	112(12.28)	**0.137**	0.67(0.75–3.72)

*Two -sided χ^2 test for the either genotype distributions or allele frequencies between the cases and controls. † Adjusted for age, smoking status, drinking status, and family history of cancer in logistic regression model. OR, odds ratio; CI, confidence interval.

Table 3 **Stratification analyses between *EPO* (rs576236) genotypes and risk of adrenal tumor**

	genotypes					
	TT (n, %)		TC+CC (n, %)			
Variables	Cases	Controls	Cases	Controls	*P*	Adjusted OR (95% CI) †
Total	198(68.8)	354(77.6)	90(31.2)	102(23.4)	**0.007**	**1.17(1.12–2.21)**
Age, years						
<48	102 (68.9)	162 (73.6)	46 (31.1)	58 (26.4)	0.622	1.60 (0.95–2.70)
⩾48	86 (67.7)	168 (85.3)	41 (32.3)	29 (14.7)	**<0.001**	**3.36 (1.75–6.45)**
Gender						
Male	103 (72.0)	154 (67.8)	40 (28.0)	73 (32.2)	0.395	1.00 (0.58–1.72)
Female	95 (65.5)	200 (87.3)	50(34.5)	29 (12.7)	**<0.001**	**3.98 (2.12–7.47)**
Smoking status						
Never	146 (69.2)	273 (81.5)	65 (30.8)	62 (18.5)	**<0.001**	**2.46 (1.51–4.01)**
Ever	52 (67.5)	81 (66.9)	25 (32.5)	40 (33.1)	0.931	1.23 (0.61–2.48)
Drinking status						
Never	153 (68.6)	281 (80.1)	70 (31.4)	70 (19.9)	**0.002**	**2.36 (1.48–3.77)**
Ever	45 (69.2)	73 (69.5)	20 (30.8)	32 (30.5)	0.968	1.08 (0.50–2.34)

*Two-sided $\chi 2$ test for the distributions of genotypes. †Adjusted for age, gender, smoking status and drinking status in logistic regression model.

Stratified analysis of *EPO* polymorphism and the risk of adrenal tumor

In this study, we further evaluated the effect of EPO rs576236 polymorphism on adrenal tumor risk stratified by age, sex, smoking status, pack-years of smoking, and drinking status (***Table 3***). The association between EPO rs576236 polymorphism and adrenal tumor risk appeared stronger in subgroups of older patients (adjusted OR = 3.36, 95%CI = 1.75–6.45), female patients (OR = 3.98, 95%CI = 2.12–7.46), nondrinking (adjusted OR = 2.36, 95%CI = 1.48–3.77), and nonsmokers (adjusted OR = 2.46, 95%CI = 1.51–4.01). These results clearly showed that the *EPO* rs576236 C allele may be associated with reduced adrenal tumor risk.

DISCUSSION

In this hospital-based case-control study, we found that the *EPO* rs576236 polymorphism was associated with the risk of developing adrenal tumor. We found a significantly decreased adrenal tumor risk associated with *EPO* rs576236 genotype, compared with the *EPO* rs576236 T allele. As far as we know, this is the first study confirming the relationship between *EPO* rs576236 polymorphism and the risk of adrenal tumor in Chinese population.

EPO is an important cytokine that stimulates the proliferation, migration, and angiogenesis of vascular endothelial cells[22,23]. In the early 1980s, the cloning of the *EPO* gene allowed the recombinant EPO and analogs to treat patients with anemia. Some subjects have shown that EPO analogs may exert non-hematopoietic effects via direct activation of EPO-R on non-hematopoietic cells, including tumor cells, and that the SNPs in *EPO* could influence *EPO* expression[8,9]. EPO is induced by hypoxia. In tumor tissue or normal renal tissue infiltrated by the tumor, the production of EPO increases[18]. The essential features of *EPO* gene transcription are as follows: decreased oxygen delivery to the kidney due to any cause, say, anemia, results in the activation in specialized renal cortical cells of the transcription factor HIF[24,25]. Protein concentrations of the α-subunit, in contrast to those in β-subunit, are extremely sensitive to oxygen tension, providing the central means by which the HIF complex is regulated[26–28]. Studies have suggested that SNPs in the functional region might influence the expression and function of these genes[29,30]. Therefore, SNPs in the functional region of *EPO* might also impact the expression of *EPO*. In the present study, we observed that individuals carrying C allele were significantly associated with decreased risk of adrenal tumor. One possible mechanism was that the T to C substitution of this polymorphism might increase the transcription and expression of *EPO*.

In addition, we observed that the association between *EPO* rs576236 polymorphism and adrenal tumor risk could be present in elderly patients. Female patients may be exposed to fewer risk factors than male patients, such as tobacco smoking and heavy drinking, but female patients′ estrogenic hormone may also play a role. Our results also showed that the risk associated with the rs576236 polymorphism was more pronounced in non-smokers and non-drinkers. We have found that the risk effect of this SNP was only significant in the

subgroups of non-smokers and non-drinkers. Smoking and drinking were established risk factors of adrenal tumor;[4] therefore, the small effect of this SNP might be overwhelmed by smoking and drinking. However, it should be noted that since our sample size was relatively small, especially in female cases, our findings might be false positive.

This study showed that hypertension and diabetes were risk factors of adrenal tumor. In stratified analysis, we observed that the increased risk associated with *EPO* rs576236 polymorphism was more pronounced in healthy subjects, which indicated that genetic effects might be overwhelmed by environmental effects. We also found that the effect of *EPO* rs576236 polymorphism on the risk of adrenal tumor was more remarkable in people without family history of cancer, suggesting that the polymorphism effect might be overwhelmed by the effect of other inherited genetic factors.

Several limitations about this study should be noted. First, as our study was a hospital-based design, we could not rule out the possibility of selecting subjects who might have been associated with a particular genotype. Second, our sample size was moderate, and the statistical power of the study was limited, especially for interaction analyses and subgroups. Third, *EPO* levels were not measured in our study. However, it was known that SNP in the *EPO* promoter region significantly increased the transcriptional activity.

In conclusion, we found that the *EPO* rs576236 polymorphism was susceptible to adrenal tumor and the variant C allele of *EPO* rs576236 was associated with decreased adrenal tumor risk. A survey of the interactions between genetic and non-genetic risk factors, including dietary habit and environmental factors, should be performed to evaluate their contribution to the risk of adrenal tumor.

References

[1] Gordon RD, Stowasser M, Tunny TJ, Klemm SA, Rutherford JC. High incidence of primary aldosteronism in 199 patients referred with hypertension. *Clin Exp Pharmacol Physiol* 1994;21:315–8.

[2] Schwartz GL, Turner ST. Screening for primary aldosteronism in essential hypertension: Diagnostic accuracy of the ratio of plasma aldosterone concentration to plasma renin activity. *Clin Chem* 2005;51:386–94.

[3] Barzon L, Boscaro M. Diagnosis and management of adrenal incidentalomas. *J Urol* 2000;163:398–407.

[4] Liu Y, Wang C, Ding ZY, Chen HJ, Ran XW, Huang H. Prognosis of patients with adrenocortical carcinoma. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2012;43:293–6.

[5] Maiese K, Li F, Chong ZZ. Erythropoietin in the brain: Can the promise to protect be fulfilled? *Trends Pharmacol Sci* 2004;25:577–83.

[6] Caprara C, Grimm C. From oxygen to erythropoietin: Relevance of hypoxia for retinal development, health and disease. *Prog Retin Eye Res* 2012;31:89–119.

[7] Maiese K, Li F, Chong ZZ. New avenues of exploration for erythropoietin. *JAMA* 2005;293:90–5.

[8] Sasaki R, Masuda S, Nagao M. Erythropoietin: Multiple physiological functions and regulation of biosynthesis. *Biosci Biotechnol Biochem* 2000;64:1775–93.

[9] Yasuda Y, Hara S, Hirohata T, Koike E, Yamasaki H, Okumoto K, et al. Erythropoietin-responsive sites in normal and malignant human lung tissues. *Anat Sci Int* 2010;85:204–13.

[10] Golfam M, Samant R, Eapen L, Malone S. Effects of radiation and total androgen blockade on serum hemoglobin, testosterone, and erythropoietin in patients with localized prostate cancer. *Curr Oncol* 2012;19:e258–63.

[11] Han ZG, Yu TT, Shan L. [expression of erythropoietin and erythropoietin receptor in non-small cell lung cancer and its correlation with microvessel density]. *Zhonghua Zhong Liu Za Zhi* 2012;34:605–8.

[12] Lin YT, Chuang HC, Chen CH, Armas GL, Chen HK, Fang FM, et al. Clinical significance of erythropoietin receptor expression in oral squamous cell carcinoma. *BMC Cancer* 2012;12:194.

[13] Zhang C, Duan X, Xu L, Ye J, Zhao J, Liu Y. Erythropoietin receptor expression and its relationship with trastuzumab response and resistance in her2-positive breast cancer cells. *Breast Cancer Res Treat* 2012;136:739–48.

[14] Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, et al. Erythropoietin regulates tumour growth of human malignancies.*Carcinogenesi*s 2003;24:1021–9.

[15] Giatromanolaki A, Fiska A, Pitsiava D, Kartalis G, Koukourakis MI, Sivridis E. Erythropoietin receptors in endometrial carcinoma as related to hif1{alpha} and vegf expression. *In Vivo* 2009;23:699–703.

[16] Kuster O, Simon P, Mittelbronn M, Tabatabai G, Hermann C, Strik H, et al. Erythropoietin receptor is expressed in meningiomas and lower levels are associated with tumour recurrence. *Neuropathol Appl Neurobiol* 2009;35:555–65.

[17] Sinclair AM, Rogers N, Busse L, Archibeque I, Brown W, Kassner PD, et al. Erythropoietin receptor transcription is neither elevated nor predictive of surface expression in human tumour cells. *Br J Cancer* 2008;98:1059–67.

[18] Papworth K, Bergh A, Grankvist K, Ljungberg B, Rasmuson T. Expression of erythropoietin and its receptor in human renal cell carcinoma. *Tumour Biol* 2009;30:86–92.

[19] Wu P, Zhang N, Wang X, Zhang C, Li T, Ning X, et al. The erythropoietin/erythropoietin receptor signaling pathway promotes growth and invasion abilities in human renal carcinoma cells. *PLoS One* 2012;7:e45122.

[20] Percy MJ, McMullin MF, Lappin TR. Sequence analysis of the 3′ hypoxia-responsive element of the human erythropoietin gene in patients with erythrocytosis. *Biochem Mol Med* 1997;62:132–4.

[21] Jeong KH, Lee TW, Ihm CG, Lee SH, Moon JY. Polymorphisms in two genes, il-1b and ace, are associated with erythropoietin resistance in korean patients on maintenance hemodialysis. *Exp Mol Med* 2008;40:161–6.

[22] Yamaji R, Okada T, Moriya M, Naito M, Tsuruo T, Miyatake K, et al. Brain capillary endothelial cells express

two forms of erythropoietin receptor mrna. *Eur J Biochem* 1996;239:494–500.

[23] Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc Natl Acad Sci U S A* 1990;87:5978–82.

[24] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-pas heterodimer regulated by cellular o2 tension. *Proc Natl Acad Sci U S A* 1995;92:5510–4.

[25] Ratcliffe PJ. Understanding hypoxia signalling in cells–a new therapeutic opportunity? *Clin Med* 2006;6:573–8.

[26] Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, et al. Mitochondrial complex iii is required for hypoxia-induced ros production and cellular oxygen sensing. *Cell Metab* 2005;1:401–8.

[27] Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, et al. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic hif-alpha activation. *Cell Metab* 2005;1:393–9.

[28] Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M, et al. Oxygen sensing requires mitochondrial ros but not oxidative phosphorylation. *Cell Metab* 2005;1:409–14.

[29] Theodoropoulos GE, Gazouli M, Vaiopoulou A, Leandrou M, Nikouli S, Vassou E, et al. Polymorphisms of caspase 8 and caspase 9 gene and colorectal cancer susceptibility and prognosis. *Int J Colorectal Dis* 2011;26:1113–8.

[30] Putra AC, Tanimoto K, Arifin M, Hiyama K. Hypoxia-inducible factor-1alpha polymorphisms are associated with genetic aberrations in lung cancer. *Respirology* 2011;16:796–802.