Relationship between Polymorphisms of Angiotensin-converting Enzyme Gene Insertion/Deletion, Endothelial Nitric Oxide Synthase Gene Intron 4 VNTR and Risk for Cervical Cancer

Jun-Ge Han [1], Hong Jin [2], Lin-Bo Gao [3,Δ], Jian Zhang [4], Xue-Ke Deng [2], Li-Juan Li [1], Chang-Ping Song [3], Tao Wang [3], Lin Zhang [1]

[1.] Department of Forensic Biology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, P.R. China
[2.] Department of Immunology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, P.R. China
[3.] Laboratory of Molecular and Translational Medicine, West China Institute of Women and Children's Health, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China
[4.] Department of Gynecology and Obstetrics, Sichuan Provincial People's Hospital, Chengdu, Sichuan 610041, P.R. China
[Δ] This author contributed equally to this work.
zhanglin@scu.edu.cn; hanjunge@wzmc.edu.cn

Abstract: Background and Aims: Angiotensin-converting enzyme gene (*ACE*) and endothelial nitric oxide synthase gene (*eNOS*) have been reported to be associated with risk for a wide range of cancers. This study evaluated the relationship between polymorphisms of *ACE* insertion/deletion (I/D), *eNOS* intron 4 variable number of tandem repeats (VNTR) and risk for cervical cancer. Methods: Polymorphisms of *ACE* I/D and *eNOS* intron 4 VNTR were analyzed by PCR in 147 cervical cancer cases and 167 healthy control subjects. Statistical analyses were performed with SPSS for Windows. Results: We found that the D/D genotype of *ACE,* compared with the I/I and I/D genotypes, was significantly associated with an increased risk for cervical cancer (D/D vs. I/I: OR = 0.41, 95% CI= 0.18-0.94; D/D vs. I/D: OR = 0.34, 95% CI=0.15-0.78); the 4a/4b genotype of *eNOS*, compared with the 4b/4b genotype, was not associated with increase in the risk for cervical cancer (4b/4b vs. 4a/4b OR = 0.58, 95% CI= 0.31-1.08). The rare variant named 4c allele in *eNOS* intron 4 VNTR was encountered in one patient and one control subject. Conclusions: Our results provide that the *ACE* I/D polymorphisms is associated with the risk for cervical cancer, and there is no enough evidence to show the significant association between the polymorphisms of *eNOS* intron 4 VNTR and the risk for cervical cancer. Further studies with more subjects in diverse ethnic population are necessary to confirm the general validity of our findings.
[Jun-Ge Han , Hong Jin , Lin-Bo Gao , Jian Zhang , Xue-Ke Deng , Li-Juan Li , Chang-Ping Song , Tao Wang , Lin Zhang. **Relationship between Polymorphisms of Angiotensin-converting Enzyme Gene Insertion/Deletion, Endothelial Nitric Oxide Synthase Gene Intron 4 VNTR and Risk for Cervical Cancer.** Life Science Journal. 2012;9(2):100-104] (ISSN:1097-8135). http://www.lifesciencesite.com. 17

Key Words: Polymorphisms, angiotensin-converting enzyme gene, endothelial nitric oxide synthase gene, cervical cancer

1. Introduction

Cervical cancer is the second most common cancer among women worldwide [1-2]. Previously, many risk factors have been reported to be associated with the occurrence of cervical cancer. Among these factors, human papilloma virus (HPV) is regarded as a prime etiologic factor in cervical cancer. And abortion too many times, several sex partners, and prolificacy in early marriage are also identified as risk factors for the development of cervical cancer [3]. Even though the crucial roles of these factors play in the development of cervical cancer, the underlying cellular and molecular mechanisms in the etiopathogenesis of it remain largely unknown.

Renin-angiotensin-aldosterone system (RAS) is an important circulation endocrine system in human body, which can adjust blood pressure by action on vascular tension, kidney blood flow dynamics and electrolyte balance, and is closely related with vascular endothelial proliferation and interactions of many cytokines. Angiotensin I-converting enzyme (ACE) is key enzyme of RAS, and can convert angiotensin I (Ang I) of no active T-peptide to angiotensin II (Ang II) of highly vascular activity and stimulating aldosterone secretion and degrade bradykinin. The gene encoding ACE is located on the long arm of chromosome 17 (17q23) that is composed of 26 exons and 25 introns with coding sequence long 4. 3 kb. It has been found that the *ACE* polymorphisms, involves the presence (insertion [I]) or absence (deletion [D]) of a 287-bp sequence of DNA, occurs in intron 16 of the gene. The plasma concentration of ACE in the same individual is constant, but different individual has a lot of difference, the difference in large measure depends on the *ACE*

polymorphisms. Previous studies have shown that the D/D genotype has an increased risk for the development of cancer and the other reproductive system disease, including breast cancer, gastric cancer, prostate cancer and recurrent miscarriage [4-11]. According to these reports, the *ACE* I/D polymorphisms affect the plasma concentrations and activity of AngⅡby affecting the plasma concentrations and activity of ACE. The AngⅡ can promote cell proliferation of human breast cancer and vascular smooth muscle through combination with angiotensin II receptor 1 (AT1R), this could be involved in the mechanism of breast cancer. The combination of AngⅡ and AT1R may also induce increased formation of nitric oxide (NO) and cell adhesion molecules both of which are relevant to tumor growth and metastasis.

Nitric oxide (NO) synthesized with L-arginine and oxygen as the substrates by nitric oxide synthase (NOS) is multifunctional small molecular gas material and has the high free radical activity, and participates in and adjusts multiple physiological and pathological physiology processes. The NOS has 3 kinds of isoforms that comprises endothelial (e), inducible (i), and neuronal (n) NOS. Studies in recent years have found that the expression of eNOS is not only exists in normal tissues, but also in tumor tissues widely, and adjusts endothelial cell damage and bone marrow endothelial progenitor cells mobilization and homing, and participates in the formation of blood vessels through NO generation, which can affect the growth and metastasis of tumors [12]. The gene that encodes eNOS is located on chromosome 7q36 and contains 26 exons and 25 introns with an entire length of 21 kb in humans, and has a 27-bp VNTR polymorphism in intron 4 (intron 4b/a). Studies have shown that the polymorphisms in *eNOS* intron 4 VNTR is associated with the risk for cancers, including breast cancer, prostate cancer and colon cancer [13-16], and the other female reproductive system disease such as recurrent miscarriage [17].

Several epidemiological studies evaluated polymorphisms of *ACE* I/D and *eNOS* intron 4 VNTR in human cancers, but the biologic mechanisms are unexplored. Therefore, in this study, we evaluated the relationship between the polymorphisms (*ACE* I/D, *eNOS* intron 4 VNTR) and the risk for cervical cancer in the population of the Han nationality from southwest China.

2. Material and Methods

2.1 Study population

A total of 314 subjects, comprising 147 patients with cervical cancer (mean age 42.44±8.20) and 167 female control subjects (mean age 48±7.37) who were healthy check-up people, were recruited for this study. These subjects in both groups were unrelated ethnic Han Chinese selected from Chengdu City or the surrounding regions in the Sichuan Province between February, 2009 and June, 2010. Cervical cancer patients were diagnosed by histopathological confirmation in the West China Second University Hospital (Table 1). All participants gave the written informed consent for their participation, and the research protocol was reviewed and approved by the Ethics Committee of Sichuan University.

Table 1.Characteristics of the 147 cervical cancer patients

Characteristics	Patients, n	Patients, %
Clinical stages		
Carcinoma in situ	33	22
Non- carcinoma in situ	114	78
Pathological type		
Squamous cell carcinoma	83	56
Adenocarcinoma	7	5
Gland squamous cell carcinoma	4	3
Other types	6	4
Not classification	47	32

2.2 Genotyping

All subjects of controls and 68 patients with cervical cancer donated approximately 2ml venous blood immediately following admission to the hospital, and were interviewed to obtain demographic and clinical information. Blood samples were collected in sterile tubes with EDTA-Na_2 anticoagulants, and stored at –20°C. 79 patients with cervical cancer donated tumor tissue from the surgery department, and stored at –70°C. Genomic DNA was extracted from the stored blood and the tumor tissue by using a commercial extraction kit (Bioteke Corporation, Beijing, China) according to the manufacturer's instructions.

Polymorphisms of *ACE* I/D and *eNOS* intron 4 VNTR were identified on the basis of PCR amplification of the respective fragments. Primer sequences [18] determine polymorphisms of *ACE* I/D were tested by PCR. The PCR procedure was denaturation at 94℃ for 5 minutes, then 35 cycles of 94℃ for 30 seconds, 70℃ for 90 seconds, 72℃ for 90 seconds, and finally by 10 minutes at 72℃ for final extension. The genotypes were identified as follows: I/I, a single band of 597 bp; I/D, two bands of 319 and 597 bp; and D/D, a single band of 319 bp. The D allele in heterozygous subjects was preferentially amplified, therefore this maybe misclassify the I/D genotype as the D/D genotype. For fear of this misclassification, a second independent PCR was done with a primer pair that recognized insertion-specific sequences [19] and the identical PCR conditions, because this reaction yields a 335 bp fragment just in the presence of the I/I and I/D genotype but not in the D/D genotype. The

27-bp intron 4 VNTR polymorphisms of *eNOS* was analyzed by using primers as previous research published [20]. The thermocycling procedure was consisted of initial denaturation at 94℃ for 5 minutes, 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 60℃for 40seconds, extension at 72℃ for 40 seconds, and a final extension at 72 for 5 minutes. We detected two variants that the intron 4b (five 27-bp repeats) and the intron 4a (four 27-bp repeats) in individuals carrying genotype 4b/4b, 4a/4b or 4a/4a. In this study, we also discovered a rare variant named 4c (six 27-bp repeats) in the *eNOS* in one patient carrying 4a/4c and one control subject carrying 4b/4c [12].

The PCR products were distinguished on 5% polyacrylamide gel and visualized by silver staining to determine the genotypes. The PCR products of the two loci with different genotypes were confirmed by the direct sequencing method.

2.3 Statistical Analysis

Genotype and allele frequencies of *ACE* I/D and *eNOS* intron 4 VNTR were obtained by Modified-powerstate standard edition software. Hardy-Weinberg equilibrium was tested with a goodness of fit chi-square test (with one degree of freedom) to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. Genotype and allele frequencies of the two loci were compared between cervical cancer patients and control subjects by the chi-square test, and odds ratios (ORs) and 95% confidence intervals (CIs) were used to evaluate the relative risk conferred by a particular allele or genotype. The demographic and clinical data of the groups were compared by the chi-square test and the Student's *t*-test. Statistical significance was assumed at the $P < 0.05$ level. All data were analyzed by the SPSS for Windows software package version 13.0 (SPSS Inc., Chicago, IL).

3. Results

The genotype and allele frequencies of ACE gene I/D and eNOS gene intron 4 VNTR in the cervical cancer patients and control subjects are shown in Table 2 and Table 3. The genotype frequencies of the two loci in the control subjects were all in agreement with the Hardy-Weinberg equilibrium. We found that the D/D genotype of *ACE*, compared with the I/I and I/D genotypes, was significantly associated with an increased risk of cervical cancer (D/D vs. I/I: OR = 0.41, 95% CI= 0.18-0.94; D/D vs. I/D: OR = 0.34, 95% CI=0.15-0.78); the 4a/4b genotype of *eNOS*, compared with the 4b/4b genotype, was not associated with increase in the risk of cervical cancer (4b/4b vs. 4a/4b OR = 0.58, 95% CI= 0.31-1.08). A new variant named 4c allele in *eNOS* intron 4 VNTR was encountered in one patient and one control subject.

Table 2.The genotype frequency of ACE gene I/D and eNOS gene intron 4 VNTR in cervical cancer patients and controls

Gene	Genotype	Patients, n (%)	Controls, n (%)	OR (95% CI)	*P* value
ACE	D/D	9 (6.1)	25 (15.0)	1.00 (reference)	
	I/I	60 (40.8)	68 (40.7)	0.41 (0.18-0.94)	0.032
	I/D	78 (53.1)	74 (44.3)	0.34 (0.15-0.78)	0.009
eNOS	4b/4b	117 (80.1)	145 (86.8)	1.00 (reference)	
	4a/4b	28 (19.2)	20 (12.0)	0.58 (0.31-1.08)	0.081
	4a/4a	0	1 (0.6)		
	4b/4c	0	1 (0.6)	-	-
	4a/4c	1 (0.7)	0	-	-

Abbreviations: OR=odds ratio; CI=confidence interval

Table 3. The allele frequency of ACE gene I/D and eNOS gene intron 4 VNTR in cervical cancer patients and controls

Gene	Allele	Patients, n (%)	Controls, n (%)	OR (95% CI)	*P* value
I/D	I	198 (67.3)	210 (62.9)	1.00 (reference)	
	D	96 (32.7)	124 (37.1)	0.82 (0.59-1.14)	0.24
eNOS	4b	262 (89.7)	311 (93.1)	1.00 (reference)	
	4a	29 (9.9)	22 (6.6)	1.57 (0.88-2.79)	0.13
	4c	1 (0.3)	1 (0.3)	1.19 (0.07-19.07)	0.90

Abbreviations: OR=odds ratio; CI= confidence interval

4. Discussion

There are many research papers about the relationship between polymorphisms of *ACE* I/D, *eNOS* intron 4 VNTR and the risk for human cancers. No study concerning the effect of the polymorphisms (*ACE* I/D, *eNOS* intron 4 VNTR) on cervical cancer has been reported. In this study, we analyzed the influence of the polymorphisms (*ACE* I/D, *eNOS* intron 4 VNTR) on individual susceptibility to cervical cancer. The alleles frequency of the two loci in the population of the Han nationality from southwest China was similar to those reported in previous studies, the

frequencies of the D allele (37.1%) and 4b allele (93.1%) were nearly identical to those observed among the Chinese women in control subjects [6, 21-22].

We found that the D/D genotype of *ACE* was significantly associated with an increased risk for cervical cancer (D/D vs. I/I: OR = 0.41, 95% CI= 0.18-0.94; D/D vs. I/D: OR = 0.34, 95% CI=0.15-0.78). This positive result was similar to the most previous studies that the D/D genotype of *ACE* has an increased risk for the development of cancer, including breast cancer, gastric cancer and prostate cancer [4-10]. The possible mechanism is that due to the absence of a 287-bp sequence of DNA, the D/D genotype of ACE gene loses the regulation of the ACE gene expression, increasing the plasma concentrations and activity of ACE and leading to the plasma concentrations and activity of Ang Ⅱ increase, which can promote the development of tumor. And the results may show that the *ACE* polymorphisms may exist in populations with enriched genetic susceptibility and may contribute to human cancer susceptibility and progression.

The eNOS is a key enzyme for synthesizying NO and plays an important role in tumor growth and angiogenesis [23]. NO is an important endothelium-derived relaxing factor. Studies have indicated that NO participates in the development and transfer process of tumorigenesis and plays a dual role, on the one hand NO has promoting tumor effect through regulating the cell proliferation related gene expression and promoting the formation of tumor blood vessels, on the other hand NO plays antitumor function through inducing tumor cell apoptosis [24]. Although no significant association of the polymorphisms in *eNOS* intron 4 VNTR and the risk of cervical cancer was observed (4b/4b vs. 4a/4b: OR = 0.58, 95% CI= 0.31-1.08), some researches suggested that the 4a genotype of *eNOS* has an increased risk for the development of cancer, including prostate cancer and colon cancer [16-17]. This may be relevant to the different ethnic population or the regional difference, and be relevant to the distribution and express of the eNOS in cervical cancer by the other polymorphisms in *eNOS* (eg. G894T, T786C) [25-26], and also be relevant to the relatively small subjects in this study. A rare variant named 4c allele in *eNOS* intron 4 VNTR polymorphisms was encountered in one patient and one control subjects, which indicates that the complex relationship exists between the polymorphisms in *eNOS* and cervical cancer, and further studies with more subjects in diverse ethnic populations are necessary to confirm the general validity of our findings.

There may be some limitations in this study. First, the study subjects were nearly from ethnic Han Chinese, and the results should not be extended to other ethnic populations because of genetic heterogeneity. Second, study materials in previous researches about polymorphisms on human cancers are nearly blood samples, some of our samples were the tumor tissues besides blood. To our knowledge, genomic instability may, and most commonly, result from gross chromosomal changes, such as translocations or amplifications, which leads to chromosomal instability. Genomic instability may also present itself through alterations in the length of short repeat stretches of coding and non-coding DNA, resulting in microsatellite instability (MSI). The MSI often refers to simple repeat sequence (1-4bp) increase or lost. In this study the two loci we selected do not belong to the MSI, and the sample differences between tumor tissues and blood can be neglected to affect our results.

In conclusion, the *ACE* I/D polymorphisms is associated with the risk for cervical cancer in the population of the Han nationality from southwest China, which indicates that it may be used as a genetic marker for cervical cancer. There is no enough evidence to show the significant association between the polymorphisms of *eNOS* intron 4 VNTR and the risk for cervical cancer. These findings should be further validated by larger, preferably prospective, studies with more diverse ethnic groups.

Acknowledgements:

This study was supported by the Key technology R&D Programs of Science and Technology Commission Foundation of Sichuan Province (No. 2010SZ0109).

Corresponding author:
Prof. Zhang Lin
Department of Forensic Biology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, P.R. China
Tel: +86-28-85469033; fax: +86-28-85405541
E-mail address: zhanglin@scu.edu.cn (L. Zhang)

References

1. Shi JF, Qiao YL, Smith JS, et al. Epidemiology and prevention of human papillomavirus and cervical cancer in China and Mongolia. Vaccine 2008; 26:53-9.
2. Schiffman M, Castle PE, Jeronimo J, et al. Human papillomavirus and cervical cancer. Lancet 2007; 370:890-907.
3. Zhou q, Huang MZ, Huang S, et al. Meta-analysis of the influential factors on cervical cancer among married women in China. China oncology 2011; 21 (2):125-9.
4. Arzu Y, Sebahat T, Raziye K, et al. Association between the polymorphism of the angiotensin-converting enzyme gene and tumor size

of breast cancer in premenopausal patients. Tohoku J EXP.med 2006; 210:109-16.
5. Gonzalez Zuloeta Ladd AM, Vasquez AA, Tabatabaei FAS, et al. Angiotensin converting enzyme gene insertion/deletion polymorphism and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005; 14:2143–6.
6. Koh WP, Yuan JM, Sun CL, et al. Angiotensin I-converting enzyme (ACE) gene polymorphism and breast cancer risk among Chinese women in Singapore. Cancer Res 2003; 63:573–8.
7. Carl-McGrath S, Ebert MP, Lendeckel U, et al. Expression of the Local Angiotensin II System in Gastric Cancer May Facilitate Lymphatic Invasion and Nodal Spread. Cancer Biology & Therapy 2007; 6(8):1-9.
8. Sugimoto M, Furuta T, Shirai N, et al. Influences of chymase and angiotensin I-converting enzyme gene polymorphisms on gastric cancer risks in Japan. Cancer Epidemiol Biomarkers Prev 2006; 15:1929-34.
9. Röcken C, Lendelkel U, Dierkes J, et al. The number of the lymph node metastasis in gastric cancer correlates with the Angiotensin I-converting enzyme gene insertion/deletion polymorphism. Clin Cancer Res 2005; 11:2526–30
10. Medeiros R, Vasconcelos A, Costa S, et al. Linkage of angiotensin I-converting enzyme gene insertion/deletion polymorphism to the progression of human prostate cancer. J Pathol 2004; 202:330–5
11. Al Sallout RJ, Sharif FA. Polymorphisms in NOS3, ACE and PAI-1 genes and risk of spontaneous recurrent miscarriage in the gaza strip. Med Princ Pract 2010; 19:99-104.
12. Xie K， Huang S. Contribution of Nitric Oxide-mediated Apoptosis to Cancer Metastasis Inefficiency. Free Radio BiolMed 2003; 34(8):969-86.
13. Yeh CC, Santella RM, Hsieh LL, et al. An intron 4 VNTR polymorphism of the endothelial nitric oxide synthase gene is associated with early-onset colorectal cancer. Int J Cancer 2009; 124(7):1565-71.
14. Yang J, Ambrosone CB, Hong CC, et al. Relationships between polymorphisms in NOS3 and MPO genes, cigarette smoking and risk of post-menopausal breast cancer. Carcinogenesis 2007; 28:1247-53.
15. Medeiros R, Morais A, Vasconcelos A, et al. Endothelial nitric oxide synthase gene polymorphisms and genetic susceptibility to prostate cancer. European Journal of Cancer Prevention 2002; 11:343-50.
16. Silvia F, Michael H, Hermann B, et al. Effect Modification by Smoking on the Association between Genetic Polymorphisms in Oxidative Stress Genes and Colorectal Cancer Risk Cancer Epidemiol Biomarkers Prev 2009; 18:2336-38.
17. Tempfer C, Unfried G, Zeillinger R, et al. Endothelial nitric oxide synthase gene polymorphism in women with idiopathic recurrent miscarriage. Hum Reprod 2000; 16:1644-7.
18. Lindpaintner K, Pfeffer MA, Kreutz R, et al. A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. N Engl J Med 1995; 332:706 -11.
19. Mitsushige Sugimoto, Takahisa Furuta, Naohito Shirai, et al. Influences of chymase and angiotensin I-converting enzyme gene polymorphisms on gastric cancer risks in Japan. Cancer Epidemiol Biomarkers Prev 2006; 15:1929-34.
20. Wang XL, Sim AS, Badenhop RF, et al. A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. Nat Med 1996; 2:41–5.
21. He Y, Yang DZH, Yu H, et al. Research on correlativity of 27 bpVNTR and G894T polymorphisms of endothelial nitric oxide synthase gene and coronary heart disease in Henan Han population. China Journal of Modern Medicine 2008; 18:523-7.
22. Xu YSH, Zhang M, Song DP, et al. The Distribution of Tandem Repeat Polym orphism of eNOS Gene in Kunming Han Nationality. Journal of Kunming Medical College 2007; 2:27-30.
23. Grande M, Carlstrom K, Stege R, et al. Estrogens increase the endothelial nitric oxide synthase (eNOS) mRNA level in LNCaP human prostate carcinoma cells. Prostate 2000; 45:232-7.
24. Wink DA, Hanbauer I, Grisham MB, et al. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. Curr Top Cell Regul 1996; 34: 159-87.
25. Nakayama M, Yasue H, Yoshimura M, et al. T-786-->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999; 99:2864–70.
26. Yoshimura M, Yasue H, Nakayama M, et al. A missense Glu298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. Hum Genet 1998; 103:65–9.

3/5/2012