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Distinct role of endothelial nitric oxide synthase gene polymorphisms from menopausal status in the patients with sporadic breast cancer in Taiwan

Chien-Hung Chen[a], Szu-Hsien Wu[b], Yang-Ming Tseng[c], Ming-Feng Hou[d,***], Li-Yu Tsai[a,*], Shih-Meng Tsai[e,**]

[a] Department of Medical Laboratory Science and Biotechnology, College of Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Division of Plastic Surgery, Department of Surgery, Taipei Veterans General Hospital and Yang Ming University, Taipei, Taiwan
[c] Department of Pathology and Laboratory Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan
[d] Department of Surgery, Faculty of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Department of Public Health and Environmental Medicine, Faculty of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

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ABSTRACT

Breast cancer has a high incidence in Taiwanese women and worldwide. Previous studies have indicated that NO has multiple independent roles in carcinogenesis; genetic polymorphisms in the endothelial nitric oxide synthase (*eNOS*) gene could modify its transcription and endogenous NO production. Previous studies have reported conflicting results for the relationship between polymorphisms in the *eNOS* gene and breast cancer risk. Estrogen levels are associated with eNOS expression; accordingly, variation in estrogen levels may contribute to the discordant results. Therefore, in this study, the effects of *eNOS* polymorphisms on breast cancer susceptibility were examined in terms of menopausal status in Taiwanese women. Three *eNOS* polymorphisms (−786T > C, VNTR, and 894G > T) were genotyped in 283 patients with breast cancer (139 premenopausal and 144 postmenopausal) and 200 cancer-free controls (100 premenopausal and 100 postmenopausal) by PCR-RFLP. There was a significantly higher breast cancer risk in premenopausal women carrying 894G > T T than in those with the 894G > T GG genotype; however, postmenopausal women carrying 894G > T T had a lower risk of developing breast cancer. In addition, based on a binary logistic regression analysis, interaction effects of these polymorphisms differed according to menopausal status. The relationship between *eNOS* polymorphisms and breast cancer hazard depended on menopause status, especially for the 894G > T polymorphism, which may provide an explanation for previous conflicting results.

1. Introduction

Breast cancer has the highest incidence and is the fourth leading cause of mortality in Taiwanese women [1]. The risk factors for breast cancer include high caloric intake and a high-fat diet, early menophania and late menopause, obesity, high stress, and environmental pollution. In addition, studies have shown that there are significant genomic differences between patients with breast cancer in Taiwan and western countries [2], indicating the need for additional breast cancer studies in Taiwan.

Nitric oxide (NO) has important physiological and pathological functions [3]. It not only functions in autocrine and paracrine signal transduction [4], but also leads to increased tumor growth [5]. Pervin et al. showed that NO levels are higher in invasive breast tumors than in benign or normal tissues, and are positively correlated with tumor grade, indicating that NO has multiple independent roles in carcinogenesis [6,7]. NO is synthesized by nitric oxide synthase (NOS). One NOS type, endothelial nitric oxide synthase (eNOS), is expressed in human breast tumors [8].

The *eNOS* gene is located on chromosome 7q36.1. Lacchini et al. [9] indicated there were three genetic polymorphisms in *eNOS* that could modify its transcription and endogenous NO production: a single nucleotide polymorphism (SNP) −786T > C (rs2070744) in the promoter region, a 27-bp variable nucleotide tandem repeat (VNTR) in intron 4, and another SNP 894G > T (rs1799983) in exon 7. eNOS transcriptional activity is reduced by 50% in −786T > C C allele carriers, thus inhibiting mRNA synthesis and decreasing protein expression [10,11]. VNTRs regulate eNOS expression via small

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: mifeho@kmu.edu.tw (M.-F. Hou), tsliyu@kmu.edu.tw (L.-Y. Tsai), tsaism@kmu.edu.tw (S.-M. Tsai).

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interference RNA (siRNA); therefore, endothelial cells from individuals with four repeats express lower quantities of siRNA, leading to higher *eNOS* mRNA expression than that in individuals with five or more repeats [12,13]. Furthermore, the 894G > T SNP, retards the entrance of inactive eNOS to the caveolae and decreases enzyme activity and NO production [14,15].

Associations between *eNOS* polymorphisms and breast cancer have been widely investigated; however, the results of these studies were inconclusive [16–21]. The conflicting results may be explained by differences among races or other sampling effects. In Taiwan, previous studies have demonstrated associations between *eNOS* polymorphisms and recurrent pregnancy loss [22], early-onset colorectal cancer [23], metabolic syndrome [24], cardiovascular diseases [25], and erectile dysfunction [26], but the association between *eNOS* polymorphisms and breast cancer has not been determined. Several studies have demonstrated that estrogen influences the eNOS system, including increased eNOS expression and elevated NO production in cultured endothelial cells [8,27]. NO also modifies human estrogen receptor structure by *S*-nitrosylation, which in turn impairs the DNA-binding activity of the estrogen receptor and blocks estrogen-dependent gene transcription [28]. Therefore, we conducted a case-control study to evaluate the effect of *eNOS* polymorphisms on breast cancer susceptibility in Taiwanese women with different menopausal statuses.

2. Materials and methods

2.1. Study subjects

A total of 139 premenopausal and 144 postmenopausal women with diagnosed breast cancer, aged 29–80 years, were included in the study. Additionally, healthy female volunteers (100 premenopausal and 100 postmenopausal women) served as control subjects, ranging in age from 42 to 65 years. Information about the menopausal status of patients and controls was obtained from a questionnaire. The patients and controls were nonsmokers, did not take any dietary vitamin C or E supplements, hormones, or oral contraceptives, and did not consume alcohol. None of the subjects had concomitant diseases, such as diabetes mellitus, rheumatoid arthritis, liver disorders, or any other malignancies. The study was approved by the Ethical Committee of Kaohsiung Medical University, and informed consent was obtained from all participants (KMUH-IRB-9950394).

Table 1
Clinical characteristics of patients with breast cancer by menopausal statuses.

Parameters	Premenopausal patients (n = 139)		Postmenopausal patients (n = 144)	
Ages (y, mean ± SD)	42.4 ± 5.6		59.0 ± 6.9	
Cancer sites, N (%)				
Left breast	73	(52.5%)	74	(51.4%)
Right breast	64	(46.0%)	68	(47.2%)
Bi-sites	2	(1.4%)	2	(1.4%)
Pathological diagnosis, N (%)				
Ductal carcinoma	122	(87.8%)	129	(89.6%)
Lobular carcinoma	9	(6.5%)	6	(4.1%)
Ductal and Lobular carcinoma	1	(0.7%)	1	(0.7%)
Other neoplasms	7	(5.0%)	8	(5.6%)
Histology differentiation, N (%)				
Well	12	(8.6%)	10	(6.9%)
Moderate	89	(64.0%)	97	(67.4%)
Poor	30	(21.6%)	33	(22.9%)
Unknown	8	(5.8%)	4	(2.8%)
Tumor sizes (cm), N (%)				
≤ 2	83	(59.7%)	77	(53.5%)
> 2-5	41	(29.5%)	49	(34.0%)
> 5	5	(3.6%)	9	(6.3%)
Unknown	10	(7.2%)	9	(6.3%)
Lymph-node metastasis				
Positive	44	(31.7%)	57	(39.6%)
Negative	95	(68.3%)	87	(60.4%)
Stages for TNM system, N (%)				
In situ	10	(7.2%)	6	(4.2%)
Stage I	62	(44.6%)	56	(38.9%)
Stage II	37	(26.6%)	48	(33.3%)
Stage III	26	(18.7%)	33	(22.9%)
Stage IV	4	(2.9%)	1	(0.7%)
Estrogen Receptor, N (%)				
Positive	100	(71.9%)	89	(61.8%)
Negative	38	(27.3%)	55	(38.2%)
Unknown	1	(0.7%)	0	(0.0%)
Progesterone Receptor, N (%)				
Positive	97	(69.8%)	64	(44.4%)
Negative	41	(29.5%)	80	(55.6%)
Unknown	1	(0.7%)	0	(0.0%)
HER-2, N (%)				
Positive	42	(30.2%)	53	(36.8%)
Negative	94	(67.6%)	91	(63.2%)
Unknown	3	(2.2%)	0	(0.0%)

SD = standard deviation; HER-2 = human epidermal growth receptor 2.

2.2. eNOS genotyping (−786T > C, VNTR, and 894G > T)

Blood samples from 483 subjects were added to sterile tubes containing sodium EDTA. Leukocyte DNA was extracted from buffy coat samples using a modified phenol-chloroform extraction method. The DNA was dissolved in a Tris-EDTA buffer and stored at 4 °C. The *eNOS* polymorphisms (−786T > C, VNTR, and 894G > T) were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (PTC-200 Peltier Thermal Cycler; MJ Research, Waltham, MA, USA). In the PCR-RFLP assay, two primers were used for PCR amplification and one restriction enzyme was used for the assay. The SNPs, primers, restriction enzymes, and PCR conditions were as follows: −786T > C, primers: forward 5′-ATGCTCCCA CCAGGGCATCA-3′ and reverse 5′- GTCCTTGAGTCTGACATTAGGG-3′, PCR conditions: denaturation, 94 °C for 1 min, annealing, 61 °C for 1 min, extension, 72 °C for 1 min for 40 cycles, followed by a final extension at 72 °C for 5 min; VNTR, primers: forward 5′-AGGCCCTAT GGTAGTGCCTTG-3′ and reverse 5′-TCTCTTAGTGCTGTGGTCAC-3′, PCR conditions: denaturation, 94 °C for 45 s, annealing, 58 °C for 45 s, extension, 72 °C for 45 s for 35 cycles, followed by a final extension at 72 °C for 5 min; 894G > T, primers: forward 5′-AAGGCAGGAGACA GTGGATGGA-3′ and reverse 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′, PCR conditions: denaturation, 95 °C for 1 min, annealing, 61 °C for 1 min, extension, 72 °C for 1 min for 30 cycles, followed by a final extension at 72 °C for 5 min.

For SNP -786T > C, the T allele has no *Ngo*MIV cleavage site, whereas the PCR product is cleaved into two fragments 204 bp and 33 bp long, respectively, in the presence of the C allele. For SNP 894G > T, the T allele has no *Ban*II cleavage site, whereas the PCR product is cleaved into two fragments 163 bp and 85 bp long, respectively, in the presence of the G allele. The genotypes of the PCR products were confirmed by a DNA sequence analysis. In each experiment, DNA samples from the subjects, together with two or three previously sequenced DNA samples as quality controls (1 for each genotype), were concomitantly amplified by PCR-RFLP.

2.3. Statistical analysis

Numeral data are expressed as means ± SD. The *eNOS* genotype distributions for the cases and controls were tested for adherence to the Hardy–Weinberg equilibrium by performing the χ^2 test. The frequencies and odds ratios (OR) with 95% confidence intervals (CI) for *eNOS* polymorphisms (−786T > C, VNTR, and 894G > T) in patients with breast cancer and controls were evaluated by the χ^2 test as well. The analysis for the interaction between menopausal status and gene polymorphisms for the risk of breast cancer were performed by the logistical model. Meantime, the interaction effects of the genetic

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