Association of CYP1B1 gene polymorphisms and the positive expression of estrogen α and estrogen β with endometrial cancer risk

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Summary

To investigate the relationship between the CYP1B1 L432V polymorphism, ERα and ERβ positivities and the incidence of endometrial cancer. The relationship between CYP1B1 L432V polymorphism, ERα and ERβ positivities and endometrial cancer was investigated using the allele-specific polymerase chain reaction method to analyze gene polymorphism in exon 3 codon 432 (C-G) of CYP1B1. Our results are as follows: in endometrial cancer cases the prevalence rates of CYP1B1 L432V genotypes C/C, C/G, and G/G were 47.2%, 36.1%, and 16.7%, respectively, and 68.8%, 23.8% and 7.5% in the control group, respectively. The frequencies of CYP1B1 C and G alleles were 65.3% and 34.7% in endometrial cancer patients and 80.6% and 19.4% in the control group. A significant difference was found in the genotype distributions or allele frequencies of CYP1B1 L432V polymorphism between the two groups ($p < 0.05$). Compared with wild-type C/C, the susceptibility of endometrial cancer with homozygotic mutation G/G and heterozygotic mutation C/G increased by 3.235 (95%CI 1.111-9.425) and 2.214 (95% CI 1.067-4.593). Moveover, the positive expression of ERα in genotypes G/G and C/G was higher than in the wild genotype C/C ($p < 0.05$). In conclusion, allelic polymorphism of CYP1B1 L432V increases the risk of endometrial cancer and has a positive correlation with ERα expression.

Key words: Endometrial cancer; L432V polymorphism of CYP1B1 gene; Risk factor; ERα and ERβ.

Introduction

Endometrial cancer is one of the common gynecological malignancies of the female urogenital tract, and its incidence is increasingly becoming significant [1]. However, the genetic basis of this disease is not yet well understood. Studies have shown that endogenous and exogenous estrogens are related to endometrial cancer risk. Estrogen production and metabolism play critical roles in the development and pathogenesis of endometrial cancer [2, 3]. Cytochrome P4501B1 (CYP1B1) is a key enzyme in the estrogen metabolism pathway, which results in the hydroxylation and conjugation of estradiol. CYP1B1 converts estrogen to 4-hydroxylated estrogen, which induces DNA damage [4]. Several polymorphisms of the CYP1B1 gene have been described, of which, four result in amino acid substitutions. Exon 3 contains three polymorphic sites at codons 432, 449, and 453. The amino acid replacement occurs at codon 432, leading to the replacement of Leu →Val [5, 6]. Inherited alterations in the activity of CYP1B1 lead to differences in estrogen metabolism in endometrial cancer, indicating estrogen-mediated carcinogenesis [7]. CYP1B1 polymorphisms have been studied in relation to ovarian, prostate, and breast cancers [8-10]. The present study forwards the hypothesis that polymorphisms of the CYP1B1 gene and the activation of estrogen receptors are significant in the pathogenesis of endometrial cancer. The L432V polymorphism of the CYP1B1 gene in endometrial cancer was investigated. The effect of the CYP1B1 polymorphism on the expressions of estrogen α and estrogen β (ERα and ERβ) were also examined.

Materials and Methods

Research subjects

Seventy-two patients with sporadic endometrial cancer from the Department of Pathology of the Third Hospital in Datong, Shanxi Province, China between April 2000 and June 2005 were used as research subjects. Ages ranged from 38-79 years with a mean age of 59. The histopathological types of these cancers were as follows: 67 cases of endometrioid cancer (9 adenosquamous, 4 adenoacanthoma), two cases of clear cell cancer, and three cases of serous papillary adenocancer. A total of 80 cancer-free control samples were included in the investigation from unrelated, healthy volunteers in the same prefecture. There were no differences between patients and control groups with regards to age, race, family history of cancer, and body mass index (BMI).

DNA extraction

Paraffin-embedded endometrial cancer blocks were cut into 20-40 m sections. After deparaffinization by dimethylbenzene, sections were treated with anhydrous ethyl alcohol. Blood samples for the control groups were collected from healthy women who underwent physical examination prior to collection. DNA was extracted from all endometrial cancer and control samples using a DNA extraction kit (Promega Corporation). Quantity and quality of DNA were measured at 260 and 280 nm with the use of a spectrophotometer.

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Analysis of CYP1B1 polymorphism

To analyze the L432V polymorphism of CYP1B1, each DNA sample was amplified in two separate reactions using one of two 3' primers: 5'-TCC GGG TTA GGC CAC TTC AG-3' or 5'-TCC GGG TTA GGC CAC TTC AC-3'. All reactions included the 5'-ATG CGC TTC TCC AGC TTT GT-3' primers (YingJun Biotechnology Co., Ltd., Shanghai). The 50 μl reactions were comprised of 50 mmol/l polymerase chain reaction (PCR) buffer, 200 μmol/l deoxynucleoside triphosphates (dNTP), 0.4 μmol/l primers, and 1 U Taq polymerase. After initial denaturation at 95ºC for 5 min, the sample underwent 35 cycles at 94ºC for 60 sec 60ºC for 60 sec, and 72ºC for 60 sec before undergoing a final extension at 72ºC for 8 min.

Gel electrophoresis genotype analysis

Each of the CYP1B1 genes from the PCR was electrophoretically separated on 2% agarose gels using 180 V at ambient temperature. The products were then visualized by ethidium bromide staining under UV light.

The DNA samples were homozygous wild-type and mutated-type genotypes. For genotype confirmation, some of the PCR products were subjected to direct sequencing.

ERα and ERβ immunohistochemistry

The 72 patients with endometrial cancer were also studied by immunohistochemical analysis. Anti-ERα and anti-ERβ antibodies were used to identify the expressions of these receptors in cancerous endometrium using standard immunohistochemical techniques. Paraffin-embedded endometrial cancer blocks were cut into 5 μm sections and dried at room temperature. After deparaffinization, sections were treated with 2% hydrogen peroxidase in methanol for 20 min to inactivate endogenous peroxidase. After blocking with 3% normal goat serum for 10 min, sections were incubated overnight with ERα and ERβ antibodies at 1:75 dilution in PBS at 4ºC under a humid chamber. Sections were washed with PBS and incubated with secondary antibodies for 30 min. Immunostaining was done using avidin-biotin peroxidase method with diaminobenzidine (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing) as the chromogen. This was followed by counterstaining with hematoxylin, thoroughly washed with tap water, and airdried.

Positive controls with sections known to contain the protein as well as negative controls without the usage of the primary antibody were performed to ensure that specific immunoreactivity was analyzed. Expressions of ERα and ERβ were observed in the cell nucleus. Expression levels of ERα and ERβ were quantified according to the percentage of positive cells from five randomly seleced view fields with 100 cells counted per field. The results were judged by two independent pathologists as - (no positive staining, and < 10% positive cells), + (10%-60%), or + + (> 60%).

Statistical analysis

Analyses were performed using the Statistical Package for the Social Sciences program (SPSS). Chi-square analysis was used to test for differences in genotype and allele frequencies of the polymorphism between endometrial cancer and control samples as well as between the stages and grades of cancer. The relative risk associated with a particular genotype or allele was estimated by calculating odds ratios (ORs), along with 95% confidence intervals (CIs).

Table 1. — *Genotypic frequency of L432V polymorphism of the CYP1B1 gene in endometrial cancer patients and controls.*

Genotype	Amino acid	Cancer (n = 72) (%)	Control (n = 80) (%)	OR (95% CI)	χ^2	*p* value
C/C	Leu	34 (47.2)	55 (68.8)	1.00		
C/G	Leu/Val	26 (36.1)	19 (23.8)	2.214 (1.067~4.593)		
G/G	Val	12 (16.7)	6 (7.5)	3.235 (1.111~9.425)	$\chi^2 = 7.644$	$p = 0.022$*

*$p < 0.05$.

Table 2. — *Allele frequency of L432V polymorphism of the CYP1B1 gene in endometrial cancer patients and controls.*

Allele	Amino acid	Cancer (n = 144) (%)	Control (n = 160) (%)	OR (95% CI)	χ^2	*p* value
C	Leu	94 (65.3)	129 (80.6)	1.00		
G	Val	50 (34.7)	31 (19.4)	2.213 (1.315~3.727)	9.133	$p = 0.003$*

*$p < 0.01$.

Results

The polymorphism of CYP1B1 L432V in the 72 patients with endometrial cancer and 80 control subjects was analyzed by the PCR method. Table 1 shows the frequency of distribution of the genetic polymorphism of CYP1B1 L432V. Genotype-specific ORs were estimated with the assumption that the genotype frequencies in the controls were consistent with the Hardy-Weinberg equilibrium. The distributions of genotypes on codon 432 were significantly different between endometrial cancer patients and the control group ($\chi^2 = 7.644$, $p = 0.022$). Of the endometrial cancer patients, 16.7% showed 432G/G, and 7.5% of the controls showed this genotype, while 36.1% of the endometrial cancer patients showed 432C/G compared with 23.8% of controls. The relative risks of 432G/G and 432C/G were calculated as 3.235 and 2.214, respectively, in comparison with the wild-type.

Table 2 shows that the frequencies of individuals carrying the G allele were 34.7% and 19.4% for endometrial cancer patients and controls, respectively. The allele frequency distribution on codon 432 were significantly different between the endometrial cancer patients and the controls ($\chi^2 = 9.133$, $p = 0.003$). When the adjusted ORs were calculated, patients with G allele revealed a 2.213-fold higher risk of endometrial cancer than those with C allele. The higher frequency of patients with G/G genotype or G allele indicates that a person carrying this genotype or allele has an increased risk for endometrial cancer.

The correlation of the L432V polymorphism of the CYP1B1 gene with ERα and ERβ expressions and the clinical pathological data of endometrial cancer tissue are shown in Table 3. The 432G/G showed a significant correlation with ERα and ERβ positive expressions. Of the samples, 83.3% and 84.6% of those with 432G/G and 432C/G were ERα positive, whereas 55.9% of the sam-

Table 3. — *Correlation between the genotypes of L432V of the CYP1B1 gene and ERα and ERβ receptor expressions*.

	n (%)	Genotype of L432V of CYP1B1 gene C/C	C/G	G/G	χ^2	*p*
ERα						
Positive	51 (70.8%)	19 (55.9%)	22 (84.6%)	10 (83.3%)		
Negative	21	15	4	2	6.977	0.031*
ERβ						
Positive	12 (16.7%)	2 (5.8%)	6 (23.1%)	5 (41.7%)		
Negative	60	32	20	7		
Total	72	34	26	12	8.370	0.015*

*$p < 0.05$.

ples with 432C/C were ERα positive ($\chi^2 = 6.977$, $p = 0.031$). Of the samples, 41.7% and 23.1% of those with 432G/G and 432C/G, respectively, were ERβ positive compared with only 5.8% with 432C/C ($\chi^2 = 8.370$, $p = 0.015$). No significant correlations were found with any of the polymorphisms of CYP1B1 in terms of clinical stage, pathological types of cancer, histological grade, age, family history of cancer, BMI, *et al*. (data not shown).

Discussion

Endometrial cancer is the most common gynecological malignancy of the female genital system. The global incidence of endometrial cancer has significantly increased. Estrogen can stimulate the hyperplasia of the endometrium, and hormone replacement therapy may increase the risk of endometrial cancer, which indicates that steroid hormones are related to endometrial cancer [11]. Studies have shown that the estrogen levels of endometrial cancer patients are relatively high, whether the source of estrogen is exogenous or endogenous. Estrogens have been shown to greatly contribute to the growth and development of endometrial cancers [12, 13].

In the human cytochrome P4501B1 (CYP1B1) gene located at the 2p21-22 region, the length of DNA is 12 kb, and it consists of three exons and two introns. The length of mRNA is about 5.2 kb. The open reading frame starts in the second exon, which is 1629 bp in length and encodes a protein with 543 amino acids [14]. Six polymorphisms of CYP1B1 have been described, in which the amino acid replacement of Leu →Val at codon 432 of exon 3 (C-G mutation) may influence the catalytic function towards procarcinogens and estrogen. This then contributes to endometrial cancer susceptibility in humans [15]. Studies have shown that CYP1B1 is a key enzyme in the metabolism of 17β estrodial ($E\text{-}_2$) [16]. CYP1B1 was found to be highly expressed in estrogen-related tissue, such as the breast, uterus, and ovary. This indicates that CYP1B1 plays an important role in the metabolism and maintenance of the balance of estrogen; 4-hydroxy estrogens, converted from estrogens by CYP1B1, have genotoxic effects and are mainly responsible for the estrogen-induced initiation of cancer [17, 18].

In the present study, a significant difference in the genotypic distributions and allelic frequencies of the codon 432 region of CYP1B1 was found between endometrial cancer patients and healthy controls in a Datong population. The presence of a mutant G allele revealed a 2.213-fold higher risk of having endometrial cancer than allele C. The relative risk was calculated at 3.235-fold compared with wild-type C/C. The higher frequency of patients with G/G genotype or G allele indicates that a person carrying this genotype or allele has an increased risk for endometrial cancer. Mutant genotype 432G/G may increase the effects of exogenous or endogenous estrogen, increase the sensitivity of estrogen, or increase the catalysis of 4-hydroxy estrogen. These results are in agreement with studies done on ovarian and prostate cancers. Goodman *et al*. [5, 19] reported an association between these cancers and the Val^{432} of CYP1B1. Cecchin *et al*. [20-22], however, did not find any association of ovarian and colorectal cancer with codon 432.

The 432G/G also showed a significant correlation with ERα and ERβ positive expression, indicating that heterozygous or homozygous mutations of 432G increase the exposure of estrogen and genotoxic 4-hydroxy estrogen conversion. Thus, 432G appears to be of importance in the pathogenesis of endometrial cancer. Previous studies have shown that the 432G/G genotype has a positive correlation between ER and the risk for endometrial and breast cancers, although some studies have shown no association between this type of polymorphism and cancer risk [23-25].

The correlation between the 432G/G genotype of CYP1B1 and the stages and grades of endometrial cancer were also analyzed. No association for cancer risk was found at any site. This lack of association with the stage and grade has also been shown to be true with other CYP gene polymorphisms in endometrial cancer, such as CYP1A1, CYP19, CYP17, and CYP2D6 [18, 26, 27].

The L432V polymorphism on the CYP1B1 gene is correlated with susceptibility to endometrial cancer, since polymorphisms are inherited. Thus, the 432G or Val^{432} variant CYP1B1 enzyme, which can lead to enhanced hydroxylation activity, may be responsible for inter-individual differences in endometrial cancer risk associated with carcinogens. These findings suggest that the mutant genotype of CYP1B1 might be a risk factor for endometrial cancer.

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