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Association of estrogen receptor α gene *Pvu*II and *Xba*I polymorphisms with non-small cell lung cancer

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Abstract. Single nucleotide polymorphisms (SNPs) of the estrogen receptor (ER)-α have been found to be associated with various diseases at significantly different frequencies. However, whether any relationship exists between ER-α polymorphisms and lung cancer remains to be determined. In this study, 84 non-smoking, female, non-small cell lung cancer patients with various stages of disease and 234 cancer-free reference controls were enrolled to examine the association of ER-α polymorphisms in lung cancer. Two restriction SNP sites, *Pvu*II and *Xba*I, in the first intron of the ER-α gene were genotyped by polymerase chain reaction-restriction fragment length polymorphism. The frequencies of the *Pvu*II-*Xba*I haplotypes and genotypes in a Taiwanese population were revealed for the first time. Although the genotypic frequencies of two polymorphic sites of ER-α were in linkage disequilibrium for the lung cancer group (χ^2=50.013, d.f.=4) and reference controls (χ^2=60.797, d.f.=4); and 7 and 8 combined genotypes were present, respectively, the distribution and the major genotypes are different in the two groups (p<0.0001). The p-values for *Pvu*II and *Xba*I genotypes were significantly different between the lung cancer and reference controls. The *PP* genotype presence was found to be significantly lower in the lung cancer group (P=0.005), whereas presence of the *xx* genotype was significantly higher (P=0.042). These findings suggested that the *PP* genotype had a lower risk of lung cancer; whereas the *xx* genotype had a higher risk. In comparison with other studies conducted in various populations, it is of note that the *pX* haplotype frequency of this study was higher than that of other studies, whereas the *px* haplotype was lower. Moreover, the *Xx* genotypic frequency of *Xba*I polymorphisms in the ER-α gene of the reference control group was found to be extremely high, whereas the *xx* genotypic frequency was extremely low. In conclusion, *Pvu*II-*Xba*I polymorphisms of the ER-α gene were found to be associated with the risk, but not cancer severity, of non-small cell lung cancer in a Taiwanese population.

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Introduction

Estrogens affect the growth, differentiation and function of numerous target tissues, including breast, uterus, vagina, ovary, testis, epididymis and prostate (1,2). The biological effects of estrogens are mediated primarily through high-affinity binding to estrogen receptors (ERs) (3). ERs are nuclear receptor proteins that have an estrogen binding domain and a DNA binding domain (4). There are two types of ERs, ER-α and ER-β. Findings of a previous study indicated that ER-β may not have the same physiological function as ER-α but may instead play a cofunctional role that is dependent upon the presence of ER-α (5). ER and the ER-regulated progesterone receptor are of special interest as their protein levels are always elevated in premalignant and malignant cancer cells (6,7). ERs are overexpressed in human breast cancers and associated with differentiated tumors and with a more favorable prognosis (7). The ER is regarded as one of the significant prognostic factors for breast and lung cancers (8-10). It has also been suggested that the combined overexpression of epidermal growth factor

receptor and ER-α in non-small cell lung cancer patients is predictive of poor outcome and, thus, is a valuable prognostic factor (11). Moreover, ER has been used as a target for lung cancer therapy (12). Therefore, inhibition of the synthesis of ER-α has become one of the major strategies for the prevention and treatment of cancer patients (13,14).

The association between genetic polymorphisms in the ER-α gene and the risk of breast cancer has been a subject that has attracted much interest. Variations in a number of DNA sequences in the ER-α gene have been reported (15-17). *Pvu*II and *Xba*I polymorphisms were mapped onto the ER-α gene intron 1 region, located approximately 50 bp apart. *Pvu*II and *Xba*I polymorphisms were found to be correlated to breast cancer incidence at a younger age (15,18,19). Recently, the association of ER-α genetic polymorphisms with lung cancer has attracted much attention since ER acts as a hormone-dependent transcriptional regulator, which may play a significant role in the development of lung cancer (12,20-23). Moreover, the relationship between the ER gene and the risk of lung cancer has also been reported (23,24). However, in light of its potential biological significance in lung cancer, ER expression in the human lung has been understudied (23). Nevertheless, the association between polymorphisms of *Xba*I and *Pvu*II in the ER-α gene and lung cancer risk remain to be determined.

Risk of developing lung cancer is known to be affected by the level of consumption and duration of smoking. The percentage of female smokers in Taiwan is very low; however, lung cancer is currently becoming the first cause of death from cancer in females (25). To exclude the risk factors of smoking and gender for lung cancer, 84 non-smoking, female, non-small cell lung cancer patients and 234 cancer-free reference controls were enrolled to investigate the association between ER-α *Pvu*II-*Xba*I polymorphisms and lung cancer in Taiwan.

Materials and methods

Patients. This study was approved by the Human Ethics Committee of Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan. Informed consent to use the tissues for research purposes was obtained from all patients prior to collection. The study participants included 84 non-smoking, female patients, age ranging from 38 to 83 years (median 63.2), who were diagnosed with non-small cell lung cancer in E-Da Hospital, Kaohsiung Armed Forces General Hospital, or Chung Shan Medical University Hospital from January 2006 to July 2009. Of the 84 patients, 27 had clinical stage I, 30 had stage II, 12 had stage III and 15 had stage IV cancer. Another 234 female, non-smoking, cancer-free reference cases, age ranging from 34 to 77 years (median 60.4), were recruited from dental outpatients at Kaohsiung Armed Forces General Hospital or Chung Shan Medical University Hospital. Information regarding gender, age, past histories of smoking and medical illness was obtained from the medical charts or by personal inquiry. Subjects were also excluded if they reported having been smokers.

DNA extraction. The genomic DNA of the lung cancer patients and reference cases were extracted from peripheral blood mononuclear cells using the commercial DNA isolation kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Genomic DNA from control cases was extracted from peripheral blood mononuclear cells. In brief, the tissues were ground prior to extraction. Samples were first lysed in the supplied lysis buffer containing proteinase K, incubated for a suitable period and then the lysates were loaded onto the supplied genomic column. DNA was bound to the column while other cell constituents passed through. Following removal of the remaining contaminants by two wash steps, the purified high-molecular-weight DNA was eluted and precipitated with isopropanol and dissolved in 50 μl distilled water. The quality of the isolated DNA was examined in 1% agarose gel stained with ethidium bromide. The DNA concentration was determined by a spectrophotometer, 2-10 ng of genomic DNA was used for each polymerase chain reaction (PCR).

PCR-restriction fragment length polymorphism (PCR-RFLP). ER-α genotypes were determined using a PCR-RFLP method reported previously (33-35), with certain modifications. The primers used for the analysis were: 5'-CTGCCACCCTATCT GTATCTTTTCCTATTCTCC-3' (forward) and 5'-TCTTTCTC TGCCACCCTGGCGTCGATTATCTGA-3' (reverse). These primers generated a 1.3-kb fragment. The PCR was performed in a thermal cycler (Perkin-Elmer PCR Thermal Cycler, Perkin-Elmer, Wellesley, MA, USA). Each 50 μl of PCR mixture contained 10 ng of DNA, 1X PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 2.5 mM $MgCl_2$, 0.16 mM deoxynucleoside triphosphate, 0.5 μM of each primer and 1.5 units of TaqDNA polymerase. The reaction mixture was initially denatured at 94˚C for 3 min, followed by 36 cycles of 94˚C for 45 sec, 61˚C for 45 sec and 72˚C for 2 min. The PCR was completed by a final extension cycle at 72˚C for 7 min. The product contained a part of intron 1 of the ER-α gene. The PCR products were then digested by the *Pvu*II and *Xba*I restriction endonucleases, respectively. The DNA fragments were separated using 1.5% agarose gel and detected by ethidium bromide staining. *P* and *X*, signified by the absence of restriction sites, yielded a 1.3-kb fragment. *p*, signified by the presence of *Pvu*II restriction sites on the two alleles, was digested into two fragments (0.85 and 0.45 kb). The *x* genotype was digested by *Xba*I into two fragments (0.9 and 0.4 kb). The laboratory staff were blind to the identity of the subjects. Quality control samples were included in genotyping assays.

Statistical analysis. Genotypic distributions were examined for significant departure from the Hardy-Weinburg equilibrium by a goodness-of-fit χ^2 test. The χ^2 test statistical method was employed to evaluate the lung cancer group and reference group differences in the distribution of genotypes. The χ^2 test was also used to test the ER-α *Pvu*II and *Xba*I polymorphisms and cancer severity. Statistical tests were two-sided. These analyses yielded hazard ratios and 95% confidence intervals. $P<0.05$ was considered statistically significant.

Results

High-quality DNA was obtained by the Qiagen spin column method from 84 non-small cell lung cancer patients and 234 cancer-free reference cases. Using PCR-RFLP, two restriction SNP sites, *Pvu*II and *Xba*I, in the first intron of the ER-α gene from the tissue samples of lung cancer

Table I. The statistical analysis of ER-*Pvu*II and *Xba*I polymorphisms with lung cancer risk.

Group	Gene genotypes, n (%)									P-value
PvuII	*PP*	*Pp*	*pp*							
Lung cancer (n=84)	3 (3.6)	60 (71.4)	21 (25.0)							0.005
Reference controls (n=234)	40 (17.1)	132 (56.4)	62 (26.5)							
XbaI	XX	Xx	xx							
Lung cancer (n=84)	9 (10.7)	48 (57.1)	27 (32.1)							0.042
Reference controls (n=234)	23 (9.8)	166 (70.9)	45 (19.2)							
PvuII and *XbaI*	*PPXX*	*PPXx*	*PPxx*	*PpXX*	*PpXx*	*Ppxx*	*ppXX*	*ppXx*	*ppxx*	
Lung cancer (n=84)	3 (3.6)	0	0	3 (3.6)	45 (53.6)	12 (14.3)	3 (3.6)	3 (3.6)	15 (17.9)	<0.0001
Reference controls (n=234)	13 (5.6)	21 (9.0)	6 (2.6)	10 (4.3)	110 (47.0)	12 (5.1)	0	35 (15.0)	27 (11.5)	

Table II. Presence of ER-α *Pvu*II and *Xba*I polymorphisms in samples of different stages of lung cancer.

Genotype	Stage I	Stage II	Stage III	Stage IV
PPXX	1	1	0	1
PPXx	0	0	0	0
PPxx	0	0	0	0
PpXX	0	1	1	1
PpXx	17	18	4	6
Ppxx	4	3	3	2
ppXX	1	1	0	1
ppXx	0	1	1	1
ppxx	4	5	3	3

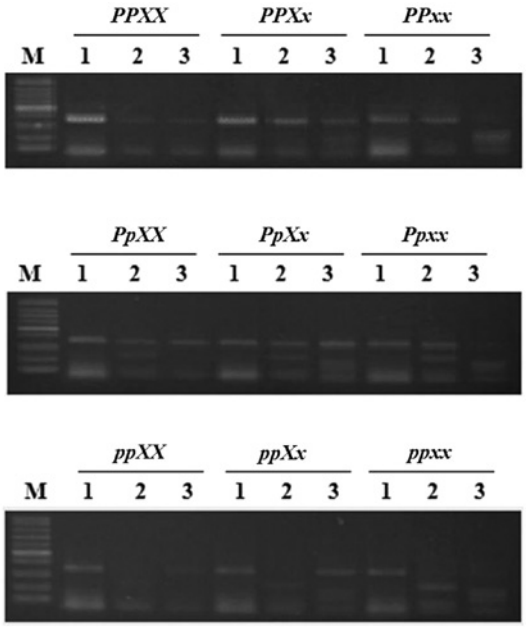

Figure 1. The DNA fragments indicating different genotypes separated on an agarose gel. M, marker standard; lane 1, uncut DNA (1.3 kb); lane 2, *Puv*II digestion (0.85 and 0.45 kb); lane 3, *Xba*I digestion (0.9 and 0.4 kb).

patients were genotyped. Fig. 1 shows the DNA fragments indicating different genotypes separated on an agarose gel.

The allele frequencies were in Hardy-Weinberg equilibrium in the groups studied. Table I shows detailed statistical analysis results of ER-α *Pvu*II and *Xba*I polymorphisms with lung cancer risk. Among the cases with *Pvu*II genotyped in this study, approximately 28.6% (24/84) of the lung cancer group and 43.6% (102/234) in the reference control group were homozygous (*PP* and *pp* genotypes), while 71.4% (60/84) of the lung cancer cases and 56.4% (132/234) of the reference controls were heterozygous (*Pp* genotype). For *Xba*I, approximately 41.4% (36/84) of the lung cancer group and 29.1% (68/234) in the reference control group were homozygous (*XX* and *xx* genotypes), while 57.1% (48/84) of the lung cancer cases and 70.9% (166/234) of the reference controls were heterozygous (*Xx* genotype). The p-values for *Pvu*II and *Xba*I genotypes were significantly different between the lung cancer group and

Table III. Comparison of the *Pvu*II-*Xba*I haplotype frequencies of the human estrogen receptor-α gene in various ethnic groups.

*Pvu*II and *Xba*I haplotypes (%)				Subject no.	Country/region	Author group (Refs)
PX	*Px*	*pX*	*px*			
20.5	24.8	24.8	29.9	234	Taiwan	This study
18.7	26.5	0.3	54.5	238	Japan	Kobayashi *et al* (44)
18.3	22.3	0	59.4	2226	Japan	Yamada *et al* (45)
9.5	20.6	22.0	47.9	73	Japan	Niino *et al* (46)
7.9	35.9	10.2	46.0	551	Japan	Kazama *et al* (47)
6.8	28.4	12.4	52.4	125	China	Lin *et al* (48)
18.5	22.3	2.3	57.7	598	Korea	Han *et al* (49)
8.6	30.4	13.4	47.6	219	Korea	Koh *et al* (50)
6.2	30.7	10.6	52.5	268	Korea	Lee *et al* (51)
33.7	13.3	0	53.0	454	Denmark	Bagger *et al* (52)
36.1	10.9	0	53.0	1100	Netherlands	Van Meurs *et al* (53)
50.0	13.6	0	36.8	19	United States (African)	Van Meurs *et al* (53)
33.5	9.2	1.2	56.1	206	United Kingdom	Albagha *et al* (54)
40.9	5.7	1.3	52.1	610	Italy	Becherini *et al* (55)
35.6	9.5	0	54.9	662	Canada	Patel *et al* (56)
17.3	24.4	10.9	47.4	64	Poland	Jakimiuk *et al* (57)

reference controls. Although the genotype frequencies of the two polymorphic sites of ER-α were in linkage disequilibrium for the lung cancer group (χ^2=50.013, d.f.=4) and reference controls (χ^2=60.797, d.f.=4), and 7 and 8 combined genotypes were present, respectively, the distribution and the major genotypes were found to be different in the two groups (p<0.0001). This is the first study to show the haplotypes and allele frequencies of *Pvu*II and *Xba*I polymorphisms in the ER-α gene of a Taiwanese population. Furthermore, Table II lists the presence of ER-α *Pvu*II-*Xba*I polymorphisms in samples of various stages of lung cancer. Analysis by the Fisher's exact test suggests that there was no association between the ER-α *Pvu*II and *Xba*I polymorphisms and cancer severity.

Discussion

The ER-α gene has been associated with elevated lung cancer risk (12,20,21,26-28). However, the association between polymorphisms of *Xba*I and *Pvu*II in the ER-α gene and the risk of developing lung cancer remains unknown. With the exception of lung cancer, a number of other diseases including breast cancer (15,19,29), Alzheimer's disease (30), obesity (31,32), multiple sclerosis (33), endometriosis (34), uterine leiomyomas (35) and bone mineral density (32,36), have been evaluated for possible linkage with the two *Pvu*II and *Xba*I polymorphism loci in the ER-α gene. Various ER-α genotypes may determine the differences in ER-α expression rather than impact on the final effects of ER-α (37). In China, breast cancer patients who carried the *pp* genotype of *Pvu*II in the ER-α gene had an earlier onset of the disease than women carrying the *PP* or *Pp* genotype (16). In Norway, no difference was found in the allele frequency ratio of the *Pvu*II polymorphism between 360 breast cancer patients and 672 controls. However, the frequency of allele *x* of the *Xba*I polymorphism in breast cancer patients was 1.4 times higher than that in the controls (38-40).

The induction of cell proliferation by estrogens has been found in breast adenocarcinomas, and since the presence of ER has been demonstrated in lung tumors, a similar role of estrogens in the development of lung cancer has been proposed. ERs reportedly inhibit proliferation and invasion of breast and lung cancer cells (7,28). Extranuclear forms of ER play a role in promoting downstream signaling for hormone-mediated proliferation and survival of breast as well as lung cancers and offer a new target for anti-tumor therapy (22). Findings of a study in Japan suggested that ER-α expression and the absence of ER-β expression are associated with a poorer prognosis among non-small cell lung cancer patients (23). In the present study, the frequencies of *Pvu*II-*Xba*I haplotypes and genotypes in a Taiwanese population were revealed for the first time. Consistent with previous studies regarding the ER-α gene as a susceptibility gene for lung cancer (23), an association between the *Pvu*II-*Xba*I polymorphisms and non-small cell lung cancer was proposed. Previous studies have suggested that *PP* homozygotes are protective against endometriosis, adenomyosis and leiomyomata (41), whereas *XX* homozygotes are protective against breast cancer and endometriosis (42,43). Our results demonstrated that presence of the *PP* genotype is significantly lower in the lung cancer group, whereas prescence of the *xx* genotype is significantly higher (Table I). These findings suggest that the *PP* genotype has a lower risk of lung cancer, whereas the *xx* genotype has a higher risk. Our results also demonstrated that the frequencies of *Pvu*II-*Xba*I haplotypes are evenly distributed as 20.5, 24.8, 24.8 and 29.9% for *PX*, *Px*, *pX* and *px*, respectively. In comparison with other studies conducted in different populations, it is of note that the *pX* haplotype frequency of this study is higher than that of other frequencies, whereas the *px* haplotype is lower (Table III).

Table IV. Comparison of the *Pvu*II-*Xba*I allele frequency of the human estrogen receptor-α gene with other studies.

Gene	Genotypes, n (%)			Subject no.	Country/region	Author group (Refs)
*Pvu*II	*PP*	*Pp*	*pp*			
	40 (17.1)	132 (56.4)	62 (26.5)	234	Taiwan	The present study
	43 (24.0)	71 (39.7)	65 (36.3)	179	Japan	Kitawaki *et al* (41)
	371 (16.7)	1058 (47.5)	797 (35.8)	2226	Japan	Yamada *et al* (45)
	6 (8.2)	32 (43.8)	35 (47.9)	73	Japan	Niino *et al* (46)
	114 (20.7)	255 (46.3)	182 (33.0)	551	Japan	Kazama *et al* (47)
	37 (21.5)	88 (51.2)	47 (27.3)	172	Japan	Wang *et al* (58)
	12 (15.0)	36 (45.0)	32 (40.0)	80	Japan	Kikuchi *et al* (59)
	16 (12.8)	56 (44.8)	53 (42.4)	125	China	Lin *et al* (48)
	36 (16.4)	98 (44.7)	85 (38.8)	219	Korea	Koh *et al* (50)
	44 (16.4)	110 (41.1)	114 (42.5)	268	Korea	Lee *et al* (51)
	33 (21.2)	77 (49.4)	46 (29.5)	156	Italy	Massart *et al* (35)
	134 (22.0)	299 (49.0)	177 (29.0)	610	Italy	Becherini *et al* (55)
	132 (19.9)	333 (50.3)	197 (29.8)	662	Canada	Patel *et al* (56)
	11 (17.2)	32 (50.0)	21 (32.8)	64	Poland	Jakimiuk *et al* (57)
	25 (25.0)	48 (48.0)	27 (27.0)	100	Greece	Georgiou *et al* (60)
*Xba*I	*XX*	*Xx*	*xx*			
	23 (9.8)	166 (70.9)	45 (19.2)	234	Taiwan	The present study
	78 (3.5)	642 (28.8)	1506 (67.7)	2226	Japan	Yamada *et al* (45)
	7 (9.6)	32 (43.8)	34 (46.6)	73	Japan	Niino *et al* (46)
	20 (3.6)	159 (28.9)	372 (67.5)	551	Japan	Kazama *et al* (47)
	12 (7.0)	56 (32.8)	103 (60.2)	171	Japan	Wang *et al* (58)
	8 (6.4)	32 (25.6)	85 (68.0)	125	China	Lin *et al* (48)
	11 (5.0)	75 (34.2)	133 (60.7)	219	Korea	Koh *et al* (50)
	14 (5.2)	62 (23.1)	192 (71.1)	268	Korea	Lee *et al* (51)
	31 (19.9)	70 (44.9)	55 (35.2)	156	Italy	Massart *et al* (35)
	117 (19.2)	280 (45.9)	213 (34.9)	610	Italy	Becherini *et al* (55)
	82 (12.4)	307 (46.3)	273 (41.2)	662	Canada	Patel *et al* (56)
	4 (6.25)	22 (34.4)	38 (59.4)	64	Poland	Jakimiuk *et al* (57)

Moreover, the *Xx* genotype frequency of *Xba*I polymorphisms in the ER-α gene of the reference control group is extremely high, whereas the *xx* genotype frequency is extremely low (Table IV). The allele frequencies in the reference group of this study were in Hardy-Weinberg equilibrium. It is unclear as to whether the allele frequencies in other studies were in Hardy-Weinberg equilibrium and, thus, this was the cause of discrepancy. Further studies with regard to other ethnic groups are required to confirm whether our findings are applicable to different populations other than Taiwanese populations.

In conclusion, as demonstrated in this study, the *Pvu*II and *Xba*I polymorphisms of the ER-α gene were associated with the risk, but not cancer severity, of non-small cell lung cancer in a Taiwanese population. However, regarding the association of the ER-α gene polymorphisms with non-small cell lung cancer, our results were based on a relatively modest sample size. The biological pathway for *Pvu*II and *Xba*I that may affect oncogenesis remains unknown, although it is known that the intronic polymorphism of the ER-α gene may affect its protein synthesis and function as a modulator of the ligand estrogen (7,41). Moreover, with the exception of the ER-α *Pvu*II and *Xba*I polymorphisms, there may also be numerous confounding factors between the groups, such as smoking and family histories and occupational exposures. Nevertheless, our findings should be applied to other ethnic groups and should be confirmed using larger scale samples as well as other aspects and various factors with non-small cell lung cancer.

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