

## RESEARCH ARTICLE

# Association of Estrogen Receptor Alpha and Interleukin 6 Polymorphisms with Lymphovascular Invasion, Extranodal Extension, and Lower Disease-Free Survival in Thai Breast Cancer Patients

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## Abstract

Breast cancer is the most frequent type of cancer diagnosed among women worldwide and also in Thailand. Estrogen and estrogen receptors exert important roles in its genesis and progression. Several cytokines have been reported to be involved in the microenvironment that promotes distant metastasis via modulation of immune and inflammatory responses to tumor cells. Estrogen receptor genetic polymorphisms and several cytokines have been reported to be associated with breast cancer susceptibility and aggressiveness. To investigate roles of genetic polymorphisms in estrogen receptor alpha (*ESR1*) and interleukin 6 (*IL6*), breast cancer patients and control subjects were recruited from the Division of Head, Neck and Breast Surgery (Siriraj Hospital, Bangkok, Thailand). Polymorphisms in *ESR1* (rs3798577) and *IL6* (rs1800795 and rs1800797) were evaluated by real-time PCR in 391 breast cancer patients and 79 healthy controls. Associations between genetic polymorphisms and clinicopathological data were determined. There was no association between genetic polymorphisms and breast cancer susceptibility. However the *ESR1* rs3798577 CT genotype was associated with presence of lymphovascular invasion (OR=2.07, 95% CI 1.20-3.56, p=0.009) when compared to the TT genotype. *IL6* rs1800795 CC genotype was associated with presence of extranodal extension (OR= 2.30, 95% CI 1.23-4.31, p=0.009) when compared to the GG genotype. Survival analysis showed that *IL6* rs1800797 AG or AA genotypes were associated with lower disease-free survival. These findings indicate that polymorphisms in *ESR1* and *IL6* contribute to aggressiveness of breast cancer and may be used to identify high risk patients.

**Keywords:** Breast cancer - estrogen receptor - interleukin-6 - polymorphisms - prognostic factors

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## Introduction

Breast cancer is the most frequent type of cancer diagnosed among women worldwide (Global Burden of Disease Cancer et al., 2015). In Thailand, breast cancer incidence is increasing and has become the most common cancer among women (Attasara and Buasom, 2010). Accumulation of genetic deregulations contributes to initiation and progression of breast cancer.

Estrogen exerts a significant role in the development of normal breast tissue as well as the initiation and progression of breast cancer. It regulates DNA transcription upon binding estrogen receptor. Interactions between estrogen and its receptor result in stimulation of cell growth in various tissues, especially, breast epithelial tissue (Rayter, 1991; Sommer and Fuqua, 2001). Several estrogen

receptor alpha (*ESR1*) polymorphisms were reported to be associated with breast cancer risk and aggressiveness (Wang et al., 2014; Son et al., 2015). *ESR1* rs3798577 CC genotype located in 3'-untranslated region (UTR) was associated with increased risk of breast cancer (Zhang et al., 2009; Anghel et al., 2010; Son et al., 2015). C allele at this locus was also correlated with recurrence of breast cancer (Anghel et al., 2010).

Interleukin 6 (*IL6*), a proinflammatory cytokine, is involved in inflammatory process and carcinogenesis. This cytokine is produced by a variety of cell types and also several cancer cells (Ataie et al., 2013). Elevated serum *IL6* is associated with poor prognosis in many solid cancers (Heikkila et al., 2008). It can also act as a growth factor in an autocrine or paracrine manner (Weidle et al., 2010). Polymorphisms located at the intron, rs1800795, were

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reported to be associated with levels of *IL6* (Humphries et al., 2001; Vickers et al., 2002; Belluco et al., 2003). CC genotype of rs1800795 was reported to be associated with increased breast cancer susceptibility in Caucasian women (Hefler et al., 2005). In contrast, rs1800795 GG genotype was associated with unfavorable outcome in node positive breast cancer (DeMichele et al., 2003; DeMichele et al., 2009). Heterozygous rs1800797 GA on the 5'UTR of *IL6* was associated with increased risk of breast cancer and presence of rs1800797 GG genotype was correlated with decreased disease-free survival (DFS) (Snoussi et al., 2005; DeMichele et al., 2009).

According to this evidence, polymorphisms in the *ESR1* and *IL6* may contribute to different levels of expression, subsequently result in increased susceptibility and aggressiveness of breast cancer. To assess this hypothesis, correlations between *ESR1* and *IL6* polymorphisms and breast cancer susceptibility and aggressiveness were evaluated.

## Materials and Methods

### Study population.

The patients were recruited from the Division of Head, Neck and Breast Surgery, Department of Surgery, Faculty of Medicine, Siriraj Hospital (Bangkok, Thailand), from 2002 to 2004. Healthy subjects who visited the division for check-up or other benign conditions were recruited as control subjects. All the patients with pathologically confirmed breast carcinoma fulfilling the selection criteria were asked to be participated in the study. Patients who were diagnosed with breast cancer, aged  $\geq 18$  years and able to provide written informed consent, were included in the study. Patients with history of other cancers were excluded. At recruitment, informed consent was obtained from all the subjects and each participant was interviewed to collect detailed information on demographics and family history of cancer. This study's protocol was approved by the Institutional Review Board of the Siriraj Hospital.

### Genomic DNA isolation

DNA extraction from blood samples was performed using Qiagen Blood Mini Kit (Qiagen, Germany) following the instruction provided. In brief, 20  $\mu$ l of Protease enzyme was mixed with 200  $\mu$ l of blood sample in a 1.5 ml tube. Then, 200  $\mu$ l of Buffer AL was added and mixed thoroughly by vortexing for 15 minutes. The tube containing enzyme, blood and Buffer AL, was incubated at 56°C for 10 minutes. After incubation, 200  $\mu$ l of 70% ethanol was added into the mixture and mixed thoroughly. The mixture was transferred into a spin column and centrifuged at 6000 g for 1 minute. Buffer AW1 was added into the spin column, and the column was centrifuged. Then, buffer AW2 was added, and the column was centrifuged. The isolated DNA was next eluted with DNase-free water. Determination of DNA concentration was conducted utilizing a NanoDrop 1000 spectrophotometer V.3.7 (Thermo Scientific, USA). The DNA was kept at -20°C for further use in genotyping assays.

### Genotyping of polymorphisms in *ESR1* and *IL6* genes

Genotypes of the polymorphisms were evaluated by real-time PCR technique using Taqman® SNP genotyping assay and 7500 real-time PCR instrument (Applied Biosystems, Singapore). Primers and probes used in the real-time PCR were designed and synthesised by Applied Biosystems®. The real-time PCR was conducted following the company's instruction with some alterations. The total reaction volume was 10  $\mu$ l. For all polymorphisms, 5  $\mu$ l of 2X TaqMan® Genotyping Master Mix, 0.25  $\mu$ l of 40X TaqMan® SNP genotyping assay, 2 ng of DNA, and water were added to reach the final volume. Cycling condition composed of pre-PCR read at 60°C for 1 min, holding at 50°C for 1 min, AmpliTaq Gold activation at 95°C for 10 min followed by 40 cycles of 92°C for 15 sec, and 60°C for 1 min with collection of fluorescence signals, and the last step is the post-PCR read at 60 °C for 1 min.

### Statistical analysis

Patients' data on cancer recurrence and death were retrieved through medical record review. The dates of recurrence and death were recorded. The date of last contact was defined as the date of the patient's last visit to the department where they had received breast cancer therapy (Division of Head, Neck and Breast Surgery, Department of Surgery; Division of Oncology, Department of Medicine; and Division of Therapeutic Radiology, Department of Radiology, Siriraj Hospital). Deviation from Hardy-Weinberg equilibrium (HWE) was calculated using online calculator. The URL was <http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html>. The correlations between genotypes and clinicopathological parameters were determined using Chi-square statistics. The DFS analysis endpoint was cancer recurrence/metastasis or breast cancer related death. DFS was defined as the time from diagnosis to the endpoint (recurrence, metastasis, or breast cancer related death), censoring at the date of last contact or non cancer death. The overall survival (OS) analysis endpoint was death from any cause. OS was defined as the time from diagnosis to the endpoint of the study, censoring at the date of last contact. Survival curves were constructed using the Kaplan Meier product limit method and statistical significance was assessed using the log rank test. A multivariate analysis was performed to evaluate the effect of prognostic factors on DFS and OS, using the Cox proportional hazards model. The statistical analyses were conducted using SPSS software version 15.0 (IBM Corp., Armonk, NY, USA).  $P < 0.05$  was considered to indicate statistically significant differences.

## Results

### Correlation between *ESR1* and *IL6* and breast cancer susceptibility

Genotyping was performed in 391 breast cancer patients and 79 healthy subjects. Mean age at diagnosis of breast cancer group was 53.73 (52.58-54.88) years, range 21-90 years. Mean age of control group was 45.44 (41.91-48.96) years, range 23-73 years. Genotype distributions of *IL6* rs1800795 polymorphisms among controls were

**Table 1. Distribution of Different Polymorphisms among Healthy Controls and Breast Cancer Patients**

Polymorphisms	Controls, n (%)	Cases, n (%)	Odds ratio <sup>a</sup> (95%CI)	p-value
<b>IL6 rs1800795</b>				
GG	55 (70.5)	243 (62.5)	ref	
CG	3 (3.8)	9 (2.3)	1.02(0.56-1.86)	
CC	20 (25.6)	137 (35.2)	1.39(0.16-12.16)	0.22
<b>IL6 rs1800797</b>				
GG	76 (96.2)	376 (96.9)	ref	
AG	3 (3.8)	11 (2.8)	1.71(0.21-14.00)	
AA	0	1 (0.3)	N/A	0.815
<b>ESR1 rs3798577</b>				
TT	30 (38.5)	143 (36.7)	ref	
CT	39 (50.0)	171 (43.8)	1.05(0.56-1.96)	
CC	9 (11.5)	76 (19.5)	1.55(0.62-3.86)	0.239

<sup>a</sup>Age adjusted odds ratio.

deviated from HWE ( $p < 0.001$ ). Distributions of *IL6* rs1800797 and *ESR1* rs3798577 among control were in HWE ( $p = 0.863$  and  $p = 0.490$ , respectively). There was no difference in genotype distribution among healthy controls and breast cancer patients. After adjusted for age, there was no association between genotype and breast cancer susceptibility. The distributions of *ESR1* and *IL1* polymorphisms were summarized in Table 1.

*Correlation between genotypes and clinicopathological parameters*

In this analysis, the patients with in situ carcinoma or presence of metastatic disease at diagnosis were excluded. Table 2 summarized demographic data of 354 patients. Most of the patients had invasive ductal carcinoma. 48.3% of the patients had T2 tumor. Axillary nodal metastasis was present in 57.06%. Univariate analysis revealed that *ESR1* rs3798577 CT genotype was associated with presence of lymphovascular invasion (OR=2.07, 95%CI 1.20-3.56,  $p = 0.009$ ) when compared to TT genotype. *IL6* rs1800795

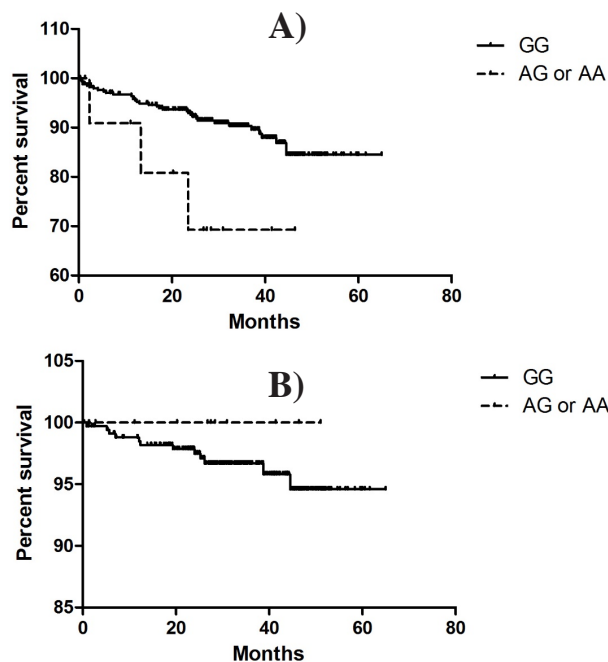
**Table 2. Clinicopathological Parameters and Demographic Data of Breast Cancer Patients**

Characteristics	Breast cancer patients, n(%)
<b>Age at diagnosis, years</b>	
≤50	151 (42.7)
>50	203 (57.3)
<b>Tumor type</b>	
Invasive ductal carcinoma	315 (89.0)
Invasive lobular carcinoma	21 (5.9)
Invasive papillary carcinoma	14 (3.95)
Mucinous carcinoma	4 (1.1)
<b>Tumor size, mm</b>	
≤20	151 (42.7)
>20-≤50	171 (48.3)
>50	32 (9.0)
<b>Axillary nodal metastasis</b>	
No	202 (57.1)
Yes	152 (42.9)
<b>Stage at diagnosis</b>	
I	106 (29.9)
II	160 (45.2)
III	88 (24.9)
<b>Histological grading</b>	
Well-differentiated	47 (13.3)
Moderately-differentiated	208 (58.8)
Poorly-differentiated	86 (24.3)
Unknown	13 (3.7)
<b>ER</b>	
Negative	77 (21.8)
Positive	277 (78.3)
<b>PR</b>	
Negative	81 (22.9)
Positive	272 (76.8)
<b>HER2</b>	
Negative	271 (76.6)
Positive	78 (22.0)

CC genotype was associated with presence of extranodal extension (OR= 2.30, 95%CI 1.23-4.31,  $p = 0.009$ ) when compared to GG genotype.

*Survival analysis*

Median follow-up time was 30.97 months (range, 0.30-148.93 months). During follow-up period, there were 14 locoregional recurrences, 27 distant metastases, and 12 breast cancer- related mortalities. DFS and OS by clinicopathological parameters and genotypes were summarized in Table 3. As expected, several clinicopathological parameters were correlated with



**Figure 1. Patient Survival Following Diagnosis by *IL6* rs1800797 Polymorphisms.** A) AA or AG genotypes were correlated with lower DFS ( $p = 0.037$ ). B) No correlation between *IL6* rs1800797 polymorphisms and OS ( $p = 0.547$ )

**Table 3. DFS and OS by Clinicopathological Parameters and Genotypes**

Parameters	DFS				OS			
	Cases, n	Event	5-year survival, %	p-value	Cases, n	Event	5-year survival, %	p-value
Age at diagnosis								
≤50 years	151	21	79.1	0.076	151	5	95.9	0.999
>50 years	203	17	87.6		203	7	93.9	
Tumor size								
≤ 20 mm	136	3	96.1	<0.001	136	0	100	0.004
>20 mm	218	35	75.7		218	12	91	
Tumor grade								
Well or moderately differentiated	255	21	87.6	0.001	255	6	97.3	0.023
Poorly differentiated	86	17	68.2		86	6	83.1	
Lymphovascular invasion								
Absence	252	17	90.5	<0.001	252	6	97.3	0.146
Presence	92	20	65		92	5	87.4	
Perineural invasion								
Absence	256	25	86.7	0.537	256	7	96.9	0.398
Presence	36	5	78.4		36	2	87.4	
Estrogen receptor								
Negative	77	18	60.1	<0.001	77	7	82.2	0.001
Positive	277	20	90.7		277	5	98	
Progesterone receptor								
Negative	81	18	66.8	<0.001	81	9	81.3	<0.001
Positive	272	20	89.4		272	3	98.7	
HER2								
Negative	271	19	89.6	<0.001	271	5	97	0.001
Positive	78	19	59.9		78	7	85.3	
Axillary nodal metastasis								
Absence	202	14	88.4	0.006	202	4	97.8	0.084
Presence	152	24	78.1		152	8	90.4	
Extranodal extension								
Absence	128	16	78.4	0.931	128	5	90.5	0.643
Presence	88	12	81.7		88	5	91.8	
ESR1 rs3798577								
TT	132	16	81.3	0.527	132	4	94.5	0.772
CT or CC	222	22	85.5		222	8	95.2	
IL6 rs1800795								
GG	222	19	84.3	0.762	222	7	96.5	0.595
CG or CC	130	19	83.5		130	5	95.3	
IL6 rs1800797								
GG	340	35	84.5	0.037	340	12	94.6	0.547
AG or AA	12	3	69.3		12	0	100	

DFS and OS. *IL6* rs1800797 AG or AA genotypes were associated with lower DFS (Figure 1). There was no significant correlation between genotypes and time to metastasis or OS. However, after multivariate analysis, only positive HER2 was significantly associated with lower DFS (HR=2.72, 95%CI 1.02-7.26, p= 0.046). Negative progesterone receptor was associated with lower OS (HR=12.77, 95%CI 1.78-91.80, p=0.011).

## Discussion

In the present study, we evaluated the association between polymorphisms of *ESR1* and *IL6* with breast cancer susceptibility/aggressiveness in Thai breast cancer patients. No association between genetic polymorphisms of *ESR1* and *IL6* and breast cancer risk was observed. *ESR1* rs3798577 CT genotype was associated with presence of lymphovascular invasion when compared to TT genotype. *IL6* rs1800795 CC genotype was associated with presence of perinodal invasion. *IL6* rs1800797 AG or

AA genotypes was associated with lower DFS.

Genotype distributions of *ESR1* rs3798577 polymorphisms were comparable to the previous reports in Korean and Chinese population (Zhang et al., 2009; Wang et al., 2014; Son et al., 2015). Association between breast cancer risk and *ESR1* polymorphisms could not be demonstrated in this study, similar to the previous reports in Chinese population by Wang et al. and Zhang et al. (Zhang et al., 2009; Wang et al., 2014). No study supported the association between *ESR1* polymorphisms and the presence of lymphovascular invasion.

Transcription factor binding site searching by MatInspector software (Genomatix GmbH, Munich, Germany) revealed that *ESR1* rs3798577 T allele served as binding site for forkhead box transcription factor (FOXPI) and helicase-like transcription factor (HLTF). *ESR1* rs3798577 C allele may serve as Sex determining region Y-box5 (SOX5) binding site. FOXPI is one of the members of forkhead or winged helix group of transcription factors. It exerts important roles in proliferation, differentiation,

Association of *ERα* and *IL6* Polymorphisms with Lymphovascular Invasion and Lower Survival in Thai Breast Cancer Patients and also malignant transformation (Myatt and Lam, 2007). A study in breast cancer cell line indicated that FOXP1 might act as coregulator of *ESR1* (Fox et al., 2004). Expression of FOXP1 is associated with expression of *ESR1* and correlated with favorable outcome of breast cancer (Fox et al., 2004) (Rayoo et al., 2009). The presence of potential binding site for FOXP1 on 3'UTR of *ESR1* gene might suggest possible role of FOXP1 in regulation of *ESR1* expression. SOX5 is a transcription factor involved in the modulation of embryonic development and in the determination of the cell fate. This transcription factor binds to *ESR1* promoter. In-vitro experiment demonstrated that this transcription factor increased activity of *ESR1* promoter in osteoblastic-like cell line but not in breast cancer cell line (Lambertini et al., 2003). There was no data regarding the association between *ESR1* and helicase-like transcription factor, a member of SWI/SNF family involved in chromatin remodeling.

Genotype distributions of *IL6* rs1800795 in the current study were deviated from HWE and different from the previous reports in Chinese population (Chen et al., 2015; Liu et al., 2015). This may due to wrong genotype discrimination by real-time PCR, but sequencing of representative samples confirmed that the genotyping was accurate. We could not demonstrate the association between the polymorphisms and breast cancer risk similar to the previous reports by Chérel et al. (2009) and Gonzalez et al. (2006). However, CC genotype was correlated with presence of perinodal invasion.

Study in HeLa cells showed that the rs1800795 G allele is expressed at 60% higher levels than the rs1800795 C allele, and is more responsive to inducers such as IL1 and endotoxin. In addition, circulating *IL6* levels were 68% higher in individuals with GG as opposed to the CC genotype (Fishman et al., 1998). Consistent with the report by Belluco et al. which demonstrated that circulating levels of *IL6* are higher in colorectal cancer patients with GG genotype, compared to those with CG or CC genotype (Belluco et al., 2003). However, in cardiovascular disease, levels of circulating *IL6* were higher in the patients with CC than those with GG genotype (Jones et al., 2001; Bruunsgaard et al., 2004). Transcription factor binding site search showed that GLI family zinc finger 3 (GLI3), which plays an important role in embryogenesis and PR domain containing 16 (MEL1) which is important in the pathogenesis of myelodysplastic anemia and acute myeloid leukemia, could bind to *IL6* rs1800795 G allele and C allele, respectively. However, the polymorphisms locate in intron and there was no data that support the regulation of *IL6* expression by these transcription factors. The rs1800795 polymorphisms may not directly act as functional polymorphisms.

The frequencies of *IL6* rs1800797 were vary rare, similar to the previous data in Chinese population (Pan et al., 2011; Gao et al., 2014) but different from the previous reports in Caucasian population (Slattery et al., 2007; DeMichele et al., 2009). In the current study, AA or AG genotypes were associated with lower DFS. Subgroup analysis of positive estrogen receptor patients in our study showed the same result. In contrast, in the study conducted by DeMichele et al., the patients with positive

estrogen receptor status who had GG genotype, had lower DFS (DeMichele et al., 2009). Although previous studies showed that the patients with GG genotype had higher serum levels of *IL6* (Brull et al., 2001; Villuendas et al., 2002), no transcription factor could be identified to explain the mechanism of gene regulation.

Polymorphisms of *ESR1* and *IL6* may contribute to breast cancer susceptibility or aggressiveness as low penetrance gene or by linkage disequilibrium with other functional polymorphisms. The small sample size in the current study may result in not having enough power to demonstrate the difference in genotype distribution among breast cancer patients and control groups. Longer follow-up time is required to demonstrate the impact of these polymorphisms on DFS and OS.

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