

Research article

Estrogen receptor- α polymorphism in a Taiwanese clinical breast cancer population: a case-control study

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Received: 21 Oct 2003 Revisions requested: 19 Dec 2003 Revisions received: 20 Jan 2004 Accepted: 29 Jan 2004 Published: 26 Feb 2004

Breast Cancer Res 2004, **6**:R180-R186 (DOI 10.1186/bcr770)

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Abstract

Introduction: Receptor-mediated estrogen activation participates in the development and progression of breast cancer. Estrogen receptor (ER)- α polymorphism has been found to be associated with breast cancer and clinical features of the disease in Caucasians. Epidemiologic studies have revealed that age-incidence patterns of breast cancer in Asians differ from those in Caucasians. Genomic data for ER- α in either population is therefore of value in the clinical setting for that ethnic group.

Methods: A case-control study was conducted to establish a database of ER- α polymorphisms in a Taiwanese population in order to compare Western and Taiwanese (Asian) distributions and to evaluate ER- α polymorphism as an indicator of clinical outcome. The ER- α gene was scanned in a Taiwanese clinical breast cancer group (189 patients) and in healthy individuals (177 healthy control individuals). PCR single-strand conformation polymorphism technology was employed and real-time PCR melting curve analysis was performed.

Keywords: estrogen receptor- α , lymph node, metastasis, polymorphism

Results: Three sites of silent single nucleotide polymorphism (SNPs) were found, as reported previously in Western studies, but at significantly different frequencies. Among the three SNPs, the frequency of allele 1 (TCT \rightarrow TCC) in codon 10 was significantly lower in breast cancer patients (32.0%) than in control individuals (40.4%; $P=0.018$). We found that allele 1 (ACG \rightarrow ACA) in codon 594 was less common in breast cancer patients with a family history of breast cancer (5.9%) than in those without such a history (19.6%; $P=0.049$). Individually, both allele 1 in codon 325 (CCC \rightarrow CCG) and allele 1 in codon 594 exhibited a reverse association with the occurrence of lymph node metastasis. Furthermore, incorporation of both SNP markers further increased predictive accuracy.

Conclusions: Our data suggest that ER- α polymorphisms are correlated with various aspects of breast cancer in Taiwan. ER- α genotype, as determined during presurgical evaluation, might represent a surrogate marker for predicting breast cancer lymph node metastasis.

Introduction

Worldwide, breast cancer is the most common cancer among women after skin cancer, and is the second leading cause of cancer death (after lung cancer) in women. Available evidence suggests that breast cancer might result from interactions between genetic elements and a variety of possible environmental factors. Ethnicity also plays a role in risk for breast cancer, with the incidence varying from lowest in certain groups of Asian

women to highest in Caucasian women [1]. Asian-Americans have traditionally had the lowest risk for breast cancer in the USA, although the difference diminishes over a couple of generations [1]. Comparison of incidence-age curves for breast cancer in Asian and Western genomic populations in their native countries reveals an additional interesting difference. Age distributions for East Asian groups exhibit an inverted 'V' shaped curve, with the peak in the age range 40-50 years, contrasting

CI = confidence interval; ER = estrogen receptor; LN = lymph node; OR = odds ratio; PCR = polymerase chain reaction; SNP = single nucleotide polymorphism; SSCP = single-strand conformation polymorphism; Tm, melting temperature.

with the continued increasing incidence beyond age 50 years in Western women. The similar and apparently unique manifestation of breast cancer in genetically similar but geographically separated Asian groups suggests the involvement of an unusual Asian genetic factor.

Breast cancer incidence has increased threefold in Taiwan since 1980 (Annual Report of Cancer Incidence, 1970–1996, Department of Health, Taiwan) [2], leading to the current focus on elucidating risk factors in this area. Thus, in Taiwan special interest has focused on local versus global breast cancer presentation and risk factors because of the following factors: the Asian incidence peak in middle age [3,4]; and the distinctive clinical features of breast cancer patients at younger age in Taiwan, including poor prognosis and weak association of standard pathological factors (e.g. tumor grade, tumor and cell morphology) with disease outcome [5].

It is known that breast cancer typically arises in luminal epithelial cells of the mammary gland [6,7]. These cells contain estrogen receptors (ERs), which respond to ovarian estrogen in normal mammary gland development. How estrogens stimulate cell growth is not fully understood, but it is known that estrogen activation of ER results in transcription of various genes that are involved in cellular proliferation. It has been shown that exposure to estrogen correlates with risk for breast cancer, with the risk increasing with duration of exposure [8]. It has been found that ERs are variably present in breast tumors, and that patients expressing ERs are more responsive to hormone treatment [9], making immunohistological assay of ER expression in tumor tissue a widely applied clinical technique. ER- α , which is expressed in luminal epithelium-derived normal or cancerous cells but not in any of the other stromal cell types within the human breast [10], has been proposed to participate in breast carcinogenesis.

Mutation and polymorphism of cancer-associated genes have been found to predict tumor formation and prognosis. The human ER- α gene exhibits low mutational frequency in breast cancer tissue [11]. However, ER- α allelic variants have been associated with breast cancer risk [12–14] in Caucasians, as have certain clinical features including presence of a family history [11] and lymph node (LN) metastasis [14], although no such association was noted in one study [15]. At present the literature contains little information regarding ER- α gene expression, mutational frequency, and allelic variants in breast cancer among Asians, especially those who reside in their native country. Thus, the present study examined ER- α polymorphism in a Taiwanese clinical group of breast cancer patients in order to establish a genetic polymorphism database for the ER- α encoding region of the Taiwanese Asian genome, to compare this distribution with that reported for Western study groups, and to test

for any correlation between ER- α polymorphism and various clinically observable features of breast cancer in Taiwanese women.

Methods

Study population

In the present case–control study, data for female breast cancer patients and control individuals over the period from 2000 to 2002 were evaluated. The breast cancer patients ($n=189$; median age 46.0 ± 10.7 years, range 34–85 years) were diagnosed at the Department of General Surgery, National Cheng Kung University Hospital and Tainan Hospital, Tainan, Taiwan. The diagnosis was confirmed by histologic examination of mastectomy specimens. The control group ($n=177$; median age 43.0 ± 8.8 years, range 19–69 years) included healthy women with no history of any type of cancer or any relatives with a history of breast cancer. An ongoing protocol to collect and store blood samples for future genomic tests had been approved by the institutional review board, and all patients provided written informed consent to participate in that protocol before entering into the present study.

Peripheral whole blood was collected and kept in storage at -70°C until genotyping analysis. Individuals with at least one first-degree or second-degree female relative affected by breast cancer were considered to have a family history. This information was obtained by interview with patients and family members. The clinical characteristics of the 189 breast cancer patients are shown in Table 1.

Screening for estrogen receptor- α variants by single strand conformation polymorphism analysis

In order to identify any mutation or variant sites in the Taiwanese population, our strategy was to screen initial samples for the entire coding region of ER- α using the PCR single-strand conformation polymorphism (SSCP) method. A total of 30 breast cancer patients were screened at this stage and compared with control individuals in order to identify disease-associated variants/mutations. Genomic DNA was extracted from purified white blood cells using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. Genomic DNA (50 ng) was used for each run of PCR-based genotyping. Exons 1–8 of the ER- α gene were amplified by PCR methods, using 10 sets of primers according to the oligonucleotide sequences in 11. Optimal electrophoretic separation for SSCP was conducted in 10% polyacrylamide gel in buffer (90 mmol/l Tris-borate and 2 mmol/l EDTA) at 256 V for 2.6 hours at 4°C . After electrophoresis, the bands on gel were visualized using 3% silver nitrate stain. PCR samples exhibiting varying band shifting patterns were further purified using a High Pure PCR

Table 1**Clinical characteristics of the 189 breast cancer patients in the study**

| Characteristic | Proportion |
|---------------------------------------|------------|
| Tumor type | |
| Infiltrating ductal carcinoma | 86.7% |
| Others | 13.3% |
| Age of onset (years) | |
| <40 | 20.1% |
| ≥40 | 79.9% |
| Familial history | |
| Negative | 90.6% |
| Positive | 9.4% |
| Tumour size | |
| <5 cm | 87.5% |
| ≥5 cm | 12.5% |
| LN metastasis | |
| Absent | 59.0% |
| Present | 41.0% |
| ER expression in breast cancer tissue | |
| Negative | 31.7% |
| Positive | 68.3% |

ER, estrogen receptor; LN, lymph node.

Product Purification kit (Boehringer–Mannheim, Mannheim, Germany) and directly sequenced using a cycle sequencing protocol to determine precise variant sites.

Genotyping by LightCycler® real-time PCR assay

Based on the initial screening results, three sets of primers and probes were subsequently designed to determine frequency variation by real-time PCR combined with melting curve analysis using the LightCycler® system (Roche Diagnostics, Basel, Switzerland). PCR was performed using 2 µl LightCycler® FastStart Enzyme mixture (LightCycler®-DNA Master Hybridization Probes Kit) with 3 mmol/l MgCl₂ and 0.5 µmol/l each of sense and antisense primers. For genotype detection, 3 pmol of each hybridization probe was added to the reaction mixture. Each hybridization probe pair consisted of two oligonucleotides: the sensor probe, labelled at the 3' end with LightCycler® LC Red 640 as an acceptor fluorophore; and the anchor probe, labelled at the 5' end with fluorescein as a donor. The amplitude of the emitted fluorescence of the acceptor fluorophore from the LC Red 640 probes was recorded. The position and sequences of the primers and hybridization probes are listed in Table 2. PCR was performed for 40 cycles of 5 seconds at 95°C, 15 seconds at 55°C and 15 seconds at 72°C. After the final cycle, melting temperature (T_m) analysis of all samples and controls was performed over the range 37–77°C. Genotypes were determined by amplicon T_m (Table 2).

Statistical analysis

χ² testing was employed to assess the influence of polymorphism status on features of breast cancer. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using unconditional logistic regression. Statistical analysis was performed using SPSS software (version 9.0 for Windows; SPSS Inc., Cary, NC, USA).

Results

The encoding region of the ER-α gene of an initial 30 randomly selected breast cancer patients was screened for mutation or variant sites by PCR-SSCP and DNA sequencing. This stage of testing revealed no novel mutations but it did reveal the presence, in the Taiwanese population studied, of three silent single nucleotide polymorphisms (SNPs) that have previously been reported in Western study groups. Specifically, these SNPs are as follows: codon 10 (TCT → TCC), codon 325 (CCC → CCG) and codon 594 (ACG → ACA). Then, with primers constructed using data from this initial screening (thus recognizing only these three SNPs), the remainder of the patient and control groups were analyzed with respect to the distribution of these three SNPs by real-time PCR coupled with melting curve analysis. T_m values were used to indicate specific allele types. In total, 189 patients with breast cancer and 177 healthy control individuals were examined. The observed numbers of individuals with different genotypes showed that all three SNPs fitted the Hardy–Weinberg equilibrium for both control and patient groups (*P* > 0.05).

Table 3 presents the genotypic and allelic frequencies within the Taiwanese group studied, indicating the following: the frequency of allele 1 in codon 10 is significantly less in cancer patients (32.0%) than in control individuals (40.4%; *P* = 0.018); the frequency of allele 1 in codon 325 is lower in cancer patients (52.1%) than in control individuals (58.2%), although the difference was not statistically significant (*P* = 0.099); and the frequency of allele 1 in codon 594 is lower in cancer patients with a family history (5.9%) than in those without a family history (19.6%) – a difference of 14% (*P* = 0.049).

ER-α genotypes were compared with selected clinical breast cancer features, including age at onset, tumor size, and LN metastasis. The only significant correlation was found for LN metastasis, as indicated by the ORs presented in Table 4. Genotype frequencies exhibited different distributions in the presence and absence of LN metastasis, with statistical significance for codon 325 (*P* = 0.013); statistical significance was almost achieved for codon 594 (*P* = 0.062). The estimated risk was threefold lower for individuals who were 11 homozygotic in codon 325 (OR 0.3, 95% CI 0.11–0.82) or twofold lower for those who were 01 heterozygotic in codon 594 (OR 0.5, 95% CI 0.22–0.98) than for the corresponding

Table 2**PCR primers and hybridization probes used in estrogen receptor- α genotyping by real-time PCR combined with melting curve analysis**

| Polymorphism site | Melting temperature (°C) | Oligonucleotide sequences |
|-------------------|--------------------------|--|
| Exon 1/codon 10 | | |
| TCT | 56 | Primers for PCR reaction: Sense: 5'-GGTTTCTGAGCCTTCTGCCCTG-3' (301-322) Antisense: 5'-AGGCCGGTCTGACCGTAGA-3' (593-575) Hybridization probes: Sensor: 5'-ACACCAAAGCATCCGGGATGred-3' (377-396) Anchor: 5'-fluoresceinCCCTACTGCATCAGATCCAAGGGAACG-3' (398-424) |
| TCC | 64 | |
| Exon 4/codon 325 | | |
| CCC | 58.5 | Primers for PCR reaction: Sense: 5'-ACCTGTGTTTTTCAGGGATACGA-3' (336-357) Antisense: 5'-GCTGCGCTTCGATTCTTAC-3' (705-686) Hybridization probes: Sensor: 5'-GCTGAGCCCCGATACTCTAred-3' (554-573) Anchor: 5'-fluoresceinCCGAGTATGATCCTACCAGACCCTTCA-3' (576-602) |
| CCG | 65.5 | |
| Exon 8/codon 594 | | |
| ACG | 61 | Primers for PCR reaction: Sense: 5'-CTGTGTCTCCACCTACAG-3' (337-356) Antisense: 5'-GGGTAAAATGCAGCAGGGATT-3' (641-621) Hybridization probes: Sensor: 5'-TCCCTGCCACAGTCTGAGAGCred-3' (575-595) Anchor: 5'-fluoresceinCCCTGGCTCCACACGGTTCAG-3' (597-618) |
| ACA | 67 | |

00 homozygote. Taking the two polymorphic sites together, we noted that the greater the frequency of allele 1, the lesser the likelihood of LN metastasis. Patients in the category with genotypes 01:11/11:01/11:11 exhibited the lowest OR of 0.19 (95% CI 0.05-0.71). Because of the limited number for genotype 11:11 ($n=3$), this subgroup was combined with patients carrying 01:11/11:01. Our results demonstrated that a combination of the two SNP markers may increase accuracy in predicting LN metastasis.

Finally, we compared the known global geographical distributions of ER- α polymorphism in codons 10, 325 and 594. Inspection reveals that only exons 1 and 4 are significantly different in comparison with reported Western genomic studies. Comparison of the data indicates the following. The frequency of allele 1 in codon 10 is lower in Taiwan (32.0%) than in the USA (44.9%), England (41.0%) and Australia (51.0%). The frequency of allele 1 in codon 325 in Taiwan (52.1%) matches that in Korea (50.0%), and is much more common than is found in USA, England, Australia and Portugal (approximately 20%). Finally, the frequencies of codon 594 in Taiwan, USA and Australia are approximately equal (allele 1: 18.5%, 19.0% and 24.0%, respectively). Thus, the Taiwanese population exhibited a distinct pattern of ER- α polymorphism.

Discussion

Substantial evidence has been reported that indicates that ER participates in mammary gland tumorigenesis, and thus ER is among the genes that affect breast cancer susceptibility. Breast cancer associated ER- α polymorphisms were surveyed in previous studies [11-16]. Somatic mutation of the ER- α gene has been identified [17], but ER- α germ-line mutation rarely occurs in breast cancer patients. In agreement with observed low mutation rates, the present study found no novel mutations. Unexplained differences between Asian and Western breast cancer symptomatology and demographics led us to consider whether unknown genetic factors within the Taiwanese genome are involved, prompting us to conduct the present PCR analysis of ER- α polymorphism.

Initial ER- α screening was conducted in 30 consecutive breast cancer patients unselected for age or family history. PCR primers used in the initial screening were from a US study conducted in Caucasians [11]. Although the initial PCR-based genotyping was able to detect new mutations, none was found. However, the initial screening revealed the presence in the Taiwanese population of three SNPs – in codons 10 (TCT \rightarrow TCC), 325 (CCC \rightarrow CCG) and 594 (ACG \rightarrow AGA) – that were previously reported for USA, UK, Australian and Portuguese populations. Initial screening

Table 3**Genotypic and allelic frequencies of estrogen receptor- α in the Taiwanese population: breast cancer group versus control group and breast cancer cases in the presence versus the absence of a family history**

| | ER- α genotypes | | | ER- α | |
|--|-----------------------------|-----------|-----------|-----------------------------|------------|
| | 00 | 01 | 11 | 0 | 1 |
| Exon 1, codon 10 (TCT \rightarrow TCC) ^a | | | | | |
| Breast cancer | $\chi^2 = 4.949, P = 0.026$ | | | $\chi^2 = 5.566, P = 0.018$ | |
| Cases ($n = 189$ subjects/378 alleles) | 91 (48.1) ^d | 75 (39.7) | 23 (12.2) | 257 (68.0) | 121 (32.0) |
| Controls ($n = 177$ subjects/354 alleles) | 69 (39.0) | 73 (41.2) | 35 (19.8) | 211 (59.6) | 143 (40.4) |
| Family history in case group | $\chi^2 = 0.387, P = 0.534$ | | | $\chi^2 = 0.421, P = 0.517$ | |
| Presence ($n = 17$ subjects/34 alleles) | 12 (70.6) | 1 (5.9) | 4 (23.5) | 25 (73.5) | 9 (26.5) |
| Absence ($n = 163$ subjects/326 alleles) | 76 (46.6) | 70 (43.0) | 17 (10.4) | 222 (68.1) | 104 (31.9) |
| Exon 4, codon 325 (CCC \rightarrow CCG) ^b | | | | | |
| Breast cancer | $\chi^2 = 2.910, P = 0.088$ | | | $\chi^2 = 2.723, P = 0.099$ | |
| Cases ($n = 189$ subjects/378 alleles) | 43 (22.7) | 95 (50.3) | 51 (27.0) | 181 (47.9) | 197 (52.1) |
| Controls ($n = 177$ subjects/354 alleles) | 25 (14.1) | 98 (55.4) | 54 (30.5) | 148 (41.8) | 206 (58.2) |
| Family history in case group | $\chi^2 = 1.020, P = 0.312$ | | | $\chi^2 = 1.055, P = 0.304$ | |
| Presence ($n = 17$ subjects/34 alleles) | 4 (23.5) | 11 (64.7) | 2 (11.8) | 19 (55.9) | 15 (44.1) |
| Absence ($n = 163$ subjects/326 alleles) | 38 (23.3) | 76 (46.6) | 49 (30.1) | 152 (46.6) | 174 (53.4) |
| Exon 8, codon 594 (ACG \rightarrow ACA) ^c | | | | | |
| Breast cancer | $\chi^2 = 0.003, P = 0.957$ | | | $\chi^2 = 0.003, P = 0.956$ | |
| Cases ($n = 189$ subjects/378 alleles) | 128 (67.7) | 52 (27.5) | 9 (4.8) | 308 (81.5) | 70 (18.5) |
| Controls ($n = 177$ subjects/354 alleles) | 117 (66.1) | 55 (31.1) | 5 (2.8) | 289 (81.6) | 65 (18.4) |
| Family history in case group | $\chi^2 = 3.605, P = 0.058$ | | | $\chi^2 = 3.877, P = 0.049$ | |
| Presence ($n = 17$ subjects/34 alleles) | 15 (88.2) | 2 (11.8) | 0 (0.0) | 32 (94.1) | 2 (5.9) |
| Absence ($n = 163$ subjects/326 alleles) | 107 (65.6) | 48 (29.5) | 8 (4.9) | 262 (80.4) | 64 (19.6) |

Data are expressed as n (%). ^aAllele 0, TCT; allele 1, TCC. ^bAllele 0, CCC; allele 1, CCG. ^cAllele 0, ACG; allele 1, ACA.

Table 4**Estimated risk for lymph node metastasis with estrogen receptor- α genotypes**

| Genotype | LN metastasis ($n = 156$) | | P | OR (95% CI) |
|-------------------------|-----------------------------|----------------------|-------|------------------|
| | Absent ($n = 92$) | Present ($n = 64$) | | |
| Single site | | | | |
| Codon 10 ^a | | | | |
| 00 | 44 (54%) | 37 (46%) | 0.194 | 1.0 (reference) |
| 01 | 35 (63%) | 21 (37%) | | 0.7 (0.36–1.43) |
| 11 | 13 (68%) | 6 (32%) | | 0.6 (0.19–1.59) |
| Codon 325 ^b | | | | |
| 00 | 16 (50%) | 16 (50%) | 0.013 | 1.0 (reference) |
| 01 | 43 (53%) | 38 (47%) | | 0.9 (0.39–2.00) |
| 11 | 33 (77%) | 10 (23%) | | 0.3 (0.11–0.82) |
| Codon 594 ^c | | | | |
| 00 | 56 (53%) | 49 (47%) | 0.062 | 1.0 (reference) |
| 01 | 32 (71%) | 13 (29%) | | 0.5 (0.22–0.98) |
| 11 | 4 (67%) | 2 (33%) | | 0.6 (0.10–3.26) |
| Dual sites | | | | |
| codon 325:codon 594 | | | | |
| 00:00 | 12 (43%) | 16 (57%) | 0.001 | 1.00 (reference) |
| 01:00 or 00:01 | 29 (49%) | 30 (51%) | | 0.78 (0.31–1.92) |
| 01:01 or 11:00 or 00:11 | 35 (71%) | 14 (29%) | | 0.30 (0.11–0.79) |
| 01:11 or 11:01 or 11:11 | 16 (80%) | 4 (20%) | | 0.19 (0.05–0.71) |

^aGenotype 00, TCT/TCT; genotype 01, TCT/TCC; genotype 11, TCC/TCC. ^bGenotype 00, CCC/CCC; genotype 01, CCC/CCG; genotype 11, CCG/CCG. ^cGenotype 00, ACG/ACG; genotype 01, ACG/ACA; genotype 11, ACA/ACA. LN, lymph node.

was followed by more rapid real-time PCR and melting curve analysis for the rest of the study population.

The frequencies of the three studied ER- α SNPs exhibited a different pattern from that in Western study groups. Comparison of the local Taiwanese ER- α genotype in breast cancer patients with findings from other countries indicates the following. Allele 1 in codon 10 is less frequent in Taiwan than in the West. The frequencies of allele 1 in codon 325 are similar between Asian areas, including Taiwan and Korea, and greater than frequencies in the West. Finally, frequencies of allele 1 in codon 594 are approximately equal for Taiwan, USA and Australia. We conducted the comparison in order to clarify the relation between demographics and ER- α polymorphism. Demographically, the greatest variance between the Asian and Western groups was found in codon 325. The frequency of allele 1 in codon 325 was much greater in the Asian population studied here than in Western populations; this finding, together with the relatively low incidence of breast cancer in Taiwan, suggests that this SNP is protective against breast cancer.

In terms of practical utility, the relation between codon 325 and 594 variants and probability of LN metastasis deserves further consideration as a clinical indicator during presurgical evaluation, at least in the Taiwanese population. Such a test is of interest because lymphatic invasion is associated with local recurrence and disease progression, and LN metastasis is considered an important indicator when deciding whether chemotherapy should be given [18–21]. Various studies of LN metastasis have considered factors such as intrinsic genetic factors involving cell mobility, vascular invasion and angiogenesis. Large tumor size of the primary breast cancer is known to increase the incidence of LN metastasis [22]. In the present study, tumor size was approximately equal by genotypes and alleles for all three SNPs. However, data presented here show that there is a negative correlation between allele 1 in codons 325 and 594 and LN metastasis, indicating that presence of allele 0 and absence of allele 1 may be independent parameters for node positivity (Table 4). Our observation is in agreement with a Portuguese study based on ER- α codon 325 PCR analysis of excised cancer tissue samples [16]. We further observed that allele 1 in codon 594 and allele 1 in codon 325 independently and additively decrease risk for LN metastasis (Table 4). To our knowledge, the link between silent polymorphisms and phenotypes is unclear. One of the possibilities might be that the silent polymorphism is in linkage with another genetic mutation that directly affects breast cancer phenotype. The other possibility might be that the nucleotide composition at the silent polymorphic site could alter the gene expression level of ER- α , thus leading to the association to LN metastasis in breast cancer.

In conclusion, ER- α polymorphisms in a Taiwanese clinical breast cancer group (189 breast cancer patients and 177 control individuals) were established using PCR SSCP and real-time PCR technology with LightCycler[®] melting curve analysis of peripheral blood. The same three SNPs reported in Western studies were found in the Taiwanese population studied, but at different frequencies than in Western studies. Small but statistically significant correlations were found between allele distribution, and individual and familial manifestation of breast cancer. Because of the limited sample size in the present study, our finding of a correlation between LN metastasis and allele 1 of codons 325 and 594 will require further confirmation. This is planned as part of our future work, because SNP determination from peripheral blood represents a highly feasible and noninvasive option for preoperative evaluation.

Competing interests

None declared.

Acknowledgements

This research was supported by medical research grants NCKUH 92-075 from National Cheng Kung University Hospital and NSC-90-2314-B-006-134 from National Science Council of Taiwan. The authors gratefully thank Miss Shu-Fen Lu for technical assistance.

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