行政院國家科學委員會專題研究計畫　成果報告

雌激素代謝基因多型性與乳癌致癌感受性及療效的關係

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中文摘要：

關鍵詞:

乳癌、雌激素代謝、aromatase、荷爾蒙受體、基因多形性、致癌感受性、治療、預後

流行病學及細胞生物學研究已確定雌激素與乳癌發生有關。因此，檢測雌激素平衡的主要變數（包括雌激素的合成、分解代謝及經由雌激素受體調控的組織對雌激素敏感度）與乳癌致癌危險性的關係極為重要。欲研究雌激素平衡在乳癌發生的角色，不但需要評估雌激素代謝過程中個人基因型不同而致的不同乳癌致癌感受性，也要了解雌激素暴露在相關基因與乳癌關聯性上的影響。乳癌的治療包括局部控制與全身性的治療，全身性治療包括化學治療及荷爾蒙治療。只要病人的癌細胞存有雌激素或黃體素受體，即應考慮給予荷爾蒙治療。荷爾蒙治療包括如何拮抗荷爾蒙受體或減少病人荷爾蒙的暴露。後者有在停經婦女 aromatase inhibitor 或未停經婦女卵巢切除。此計畫包含兩部分。第一部份研究是前瞻性對照研究，欲了解雌激素濃度及雌激素受體與代謝酵素基因型和個人乳癌致癌感受性的關係。吾人已收集 300 例乳癌病人白血球及血漿。第二部份以 1989　2000 年在台大醫院手術治療的 500 例原發浸潤性乳癌 DNA 為對象，探討雌激素受體與 aromatase 基因多形性與乳癌病人抗雌激素治療療效的關係。雌激素受體與 aromatase 基因型檢測已完成，與療效的關係分析中。

Abstract in English

Keywords:

breast cancer, estrogen metabolism, aromatase, estrogen receptor,

genetic polymorphism, cancer susceptibility, treatment, prognosis

Both epidemiological and cell biology studies have documented the contribution of estrogen to the development of breast cancer. It is, therefore, important to examine the association between the key variables in estrogen homeostasis (i.e., the synthesis and catabolism of estrogen, and the sensitivity of tissue to estrogen operated via estrogen receptor) and the risk of developing breast cancer. A complete understanding of the etiological role of estrogen in breast carcinogenesis will require studies that evaluate both the genes participating in estrogen metabolism and the extent to which estrogen exposure modifies the associations of these genes with breast cancer risk. The treatments of breast cancer include local control and systemic treatments. The latter includes chemotherapy and hormonal therapy. Hormonal therapy is indicated in cancers with the presence of estrogen receptor or progesterone receptor. Hormonal therapy for breast cancer is aimed at antagonizing the hormone receptor or at lowering the level of hormone. This proposal is composed of two parts. The little estrogen produced in postmenopausal women comes predominantly from aromatization of androgens. Aromatase inhibitor can inhibit the conversion. The first part of the study, a prospective case-control study, is aiming at determining whether estrogen level and genotypic polymorphisms of estrogen receptor and estrogen-metabolizing enzymes are associated with individual susceptibility to breast cancer. So far we have collected DNA and plasma bank of 300 cases. The second part of the study, based on our previous clinical data and DNA bank of 500 primary invasive breast cancers operated at the National Taiwan University Hospital from 1989 to 2000, will determine whether genotypic polymorphisms of estrogen receptor and aromatase are related to response to anti-estrogen treatment in breast cancer patients. Genotyping of these specimens has been completed and survival analysis will be done soon.

目錄

Introduction

Breast cancer is similar to other human cancers in that it arises from a multi-factorial process. Recent attention has focused on genetic predisposition as a risk for developing breast cancer (1,2). More importantly, the influence of reproductive factors strongly supports a hormonal role in cancer etiology (3,4).

Because of the close relation between the risk of breast cancer and exposure to estrogen, it is important to examine the key variables in estrogen homeostasis (i.e., the synthesis and catabolism of estrogen and the sensitivity of tissue to estrogen operated via estrogen receptor). Our previous studies suggest that estrogen play a dual role in breast cancer development, both as a promoter, triggering cell proliferation, and as an initiator, causing DNA damage essential in driving tumorigenesis. The initiating mechanism of estrogen is suggested to involve its metabolite, catechol estrogen, which can be inactivated by Catechol- O- Methyltransferase (COMT) (5). Other candidate genes participating in the estrogen-metabolizing pathway include estrogen receptor (determining sensitivity of tissue to estrogen), *CYP1B1* (involved in estrogen hydroxylation), and aromatase (CYP19). In addition, a complete understanding of the etiological role of estrogen in breast tumorigenesis will require studies that evaluate both the genes participating in estrogen metabolism and the extent to which estrogen exposure modifies the associations of these genes with breast cancer risk.

The successful treatment of early breast cancer necessitates good local control by surgery and/or radiation therapy, and elimination of systemic micrometastasis by adjuvant treatments including chemotherapy and hormonal therapy. Hormonal therapy for breast cancer is aimed at antagonizing the hormone receptor or at lowering the level of hormone. Tamoxifen, an antagonist of estrogen receptor, has been used as an adjuvant therapy for primary breast cancer, while aromatase inhibitor can inhibit the production of estrogen in postmenopausal women.

The genetic polymorphisms of genes participating in the estrogen-metabolizing pathway may be associated with the susceptibility to breast cancer development and/or the response to hormonal therapy.

Specific Aim

This study is composed of two parts. The first part of the study is a prospective case-control study aiming at determining whether estrogen level and genotypic polymorphisms of estrogen receptor and estrogen-metabolizing enzymes, including COMT, CYP1B1, CYP19, are associated with individual susceptibility to breast cancer. DNA and plasma bank of 500 breast cancer cases and 500 controls will be collected. The second part of study, based on our previous DNA bank of 503 breast cancer patients, will determine whether genotypic polymorphism of estrogen receptor and aromatase are related to response to anti-estrogen treatment in breast cancer patients.

Background and Significance

Estrogen Synthesis

The P450 aromatase (cyp19) enzyme catalyzes the conversion of androgen to estrogen. The little estrogen that is produced in postmenopausal women comes predominantly from aromatization of adrenal and ovarian androgens in extragonadal tissues such as the liver, muscle, and fat tissues (6). A [TTTA]n tetranucleotide repeat polymorphism within intro 4 of CYP 19 has been analyzed for possible associations between the repeat number and breast cancer susceptibility (7-11). Seven alleles have been reported.

Catabolism of Estrogens

Estrogens are metabolized by cytochrome p450 1A1 into a variety of compounds, via two major pathways. The 2-hydroxyestrone (2-HE) and 4-hydroxyestrone (4-HE) are formed by one pathway, and 16*-hydroxyestrone (16-*) is formed by the other. Among three major components, 2-HE is not dangerous, because it fails to show up as a mutagen in cell culture studies or as a carcinogen in animals. Instead, the other two are dangerous. The 16-* promotes breast cancer, and it has been shown that cancerous breast tissue from women harbors much more 16-* than does normal breast tissue. On the other hand, the 4-HE, if not inactivated, is mutagenic, since the metabolites of 4-HE, i.e. catechol 4-HE, is able to react with DNA to form depurinating adducts. These adducts are lost from DNA by cleavage of the glycosidic bond, leaving apurinic sites, subsequently resulting in mutation (12). To overcome this metabolites, in extrahepatic tissues, O-methylation catalyzed by COMT is a major contributor to the inactivation of catechol estrogens (13).

CYP1B1 exceeds CYP1A1 in its catalytic efficiency as E2 4-hydroxylase (14,15). Given the carcinogenic potential that has emerged for 4-hydroxyestradiol (13), CYP1B1 assumes a special role as the principal enzyme producing this catechol estrogen. The CYP1B1 gene contains three exons, but only exons 2 and 3 encode the protein. In this study, we will focus on exon 3 because it encodes the heme binding domain, which is essential for the catalytic function of CYP 1B1. Three polymorphic sites have been reported at nucleotides 1294G→C, 1347T→C, and 1358A→G(16).

Estrogen Receptor

In specific cells and tissues containing estrogen receptors, estrogen binds to the receptor, and this ligand–receptor complex binds to and activates specific sequences in the regulatory region of genes. These genes in turn regulate cell growth and differentiation. The human ER gene (ESRα) is localized to chromosome 6q25.1 and is

over 140 kb in size, containing 8 exons (17). The ER gene contains several polymorphic sites, some have shown associations with breast cancer risk (18).

Serum Estrogen Concentrations

Studies of the relation between serum estrogen concentrations and the risk of breast cancer in premenopausal women have had conflicting results, most likely because the measurements were made at various times during the menstrual cycle. However, in some epidemiologic studies, low serum estrogen concentrations were associated with a low risk of breast cancer, and conversely, high concentrations were associated with a high risk (19). In several large studies, postmenopausal women in whom breast cancer subsequently developed had higher serum concentrations of free estradiol than women in whom breast cancer did not develop (20,21).

Modification of Risk by Estrogen Exposure

Ethnic differences in genetic polymorphism related to cancer risk may also be attributable to difference in exposure "dosage" of carcinogens among populations. Specific genotypes against/susceptible to cancer risk commonly reveal their protective/promotion effect mainly for subjects exposed to a "normal dose" of procarcinogens. For example, the greatest incremental lung cancer risk (seven times) from the "susceptible" CYP1A1 genotype was seen in light smokers, whereas heavy smokers with this genotype had less than twice the risk of heavy smokers without the genotype (22). The differences in frequency distribution of genetic polymorphism and the serum estrogen levels suggest the necessity of conducting local epidemiologic study to examine the role of estrogen-metabolizing gene in relation to breast cancer.

Hormone Therapy for Breast Cancer

The presence of ERs in tumor tissue indicates adjuvant hormone therapy and is an important prognostic factor that correlates with higher survival rates and lower risk of relapse (17,23). Approximately half of all ER+ and nearly all ER- breast tumors fail to respond to anti-estrogen treatment (17).

Aromatase catalyzes the conversion of androgen to estrogen in peripheral tissue, which is the only source of estrogen in postmenopausal women. Five years of Tamoxifen after surgery, radiation and/or adjuvant chemotherapy is currently the standard of care for breast cancer patients with positive hormone receptor. One randomized trial demonstrated that postmenopausal breast cancer patients receiving anastrozole, an aromatase inhibitor, had longer disease-free survival than women receiving Tamoxifen. Another trial showed letrozole, another aromatase inhibitor, can reduce recurrence and improve disease-free

survival after five years of Tamoxifen in postmenopausal women.

Methods

This proposal is composed of two parts of studies. The first part is a prospective, case-control study aiming at determining whether polymorphisms of estrogen-metabolizing genes are associated with elevated risk for breast cancer in Taiwanese women, and whether the association between genotype and risk may be modified by estrogen exposure. About 500 breast cancer patients are expected to be enrolled in three years. An experienced research nurse was assigned to administer a structured questionnaire to both case and control subjects. The information collected included all recognized risk factors. For perimenopausal breast cancer patients or patients having undergone hysterectomy, we will determine the menopausal status with the examination of estradiol and follicular stimulating hormone (FSH).

The second part is aiming at determining the prognostic significance of polymorphisms of ER and aromatase, in breast cancer patients. We have already collected DNA specimens from about 500 patients since 1995. All breast cancer patients have pathologically confirmed primary breast carcinoma, and all were diagnosed and treated at the National Taiwan University Hospital. Adjuvant therapy was given based on the following well-established prognostic factors: axillary lymph node status, tumor size, histologic grading, hormonal receptors and menopausal status. These patients have been regularly followed up in our clinic, after they underwent surgery and adjuvant therapy. An experienced research nurse will obtain follow-up from patients' charts and tumor registry records.

Laboratory Analyses

A 10-ml sample of peripheral blood collected in acetate-citrate dextrose was obtained twice from each breast cancer patient before treatment and from each control subject. Blood was drawn between 8:00 a.m. and 2:00 p.m. on day 13 and on any other day in a menstrual cycle, and serum was immediately frozen to -20℃ and stored in liquid nitrogen at -190℃ until the assays were performed. The buffy coats of these specimens were prepared immediately and stored at -80℃ until extraction of the genomic DNA. Genomic DNA was obtained by conventional proteinase K extraction and stored at -80℃.

Estrogen receptor genotyping

Detection of the *XbaI* and *PvuII* restriction endonuclease sites in intron 1 was facilitated by PCR amplification of a region spanning the sites with oligonucleotide

primers (forward primer, 5'-ATC-CAG-GGT-TAT-GTG-GCA-ATG-AC-3'; reverse primer, 5'-ACC-CTG-GCG-TCG-ATT-ATC-TGA-3'). Separate reactions will be performed for the *XbaI* and *PvuII*. Restriction sites is indicated by a +, whereas the absence of the sites are denoted by a -. The allele–specific TaqMan PCR assay was used to double check the RFLP data.

Three bi-allelic polymorphisms from the *ESRα* gene were genotyped using PCR followed by restriction enzyme digestion. Each of the tested polymorphisms was a sequence variant: exon 1, codon 10 (TCT to TCC); exon 4, codon 325 (CCC to CCG); and exon 8, codon 594 (ACA to ACG). PCR amplification of the exon 1 and 4 polymorphic regions used oligonucleotide primers previously reported by Iwase *et al* (24). while primers for exon 8 amplification were specifically designed (forward, GAGGAGACGGACCAAAGCCAC; reverse, GCCATTGGTGTTGGATGCATGC). Oligonucleotides for PCR amplification were supplied by Geneworks (Rundle Mall, Adelaide, Australia) and restriction enzymes used for each RFLP digestion were supplied by Genesearch (Arundel, Australia).

For the exon 1 polymorphism, PCR amplification was performed in a 20µl mM dNTPs, 1×PCR buffer, 4 mM $MgCl_2$ and DNA polymerase. PCR conditions were 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 64°C and 90 sec at 72°C; followed by 5 min at 72°C. Following amplification, 10 µl of PCR product were digested with MspI for 3 hr at 37°C. Samples were then loaded into an ethidium bromide-stained 2% agarose gel for genotype determination. Genotypes were determined as follows: wild-type, 275 and 9 bp; variant , 198, 77 and 9 bp; heterozygote, 275, 198, 77 and 9 bp (the 9 bp fragment was not detectable on a 2% agarose gel).

For the exon 4 polymorphism, PCR amplification was performed in a 20 µl reaction mixture containing 50 to 100 ng genomic DNA, 0.5 µM each primer, 0.2 mM dNTPs, 1×PCR buffer, 4 mM $MgCl_2$ and DNA polymerase. PCR conditions were 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 57°C and 90 sec at 72°C; followed by 5 min at 72 °C. Following amplification, 10µl of PCR product were digested with HinfI for 1 hr at 37°C. Samples were then loaded into an ethidium bromide-stained 2% agarose gel for genotype determination. Genotypes were determined as follows: wild-type, 119 bp; variant, 98 and 21 bp; heterozygote, 119, 98 and 21 bp.

Finally, exon 8 PCR amplification was performed in a 20 µl reaction mixture containing 50 to 100 ng genomic DNA, 0.25 µM each primer, 0.2 mM dNTPs, 1×PCR buffer, 3.75 mM $MgCl_2$ and DNA polymerase. PCR conditions were 2 min 30 sec at 94

°C; 5 cycles of 45 sec at 94°C, 1 min at 69°C and 2 min at 72°C; followed by a further 30 cycles of 30 sec at 94°C, 30 sec at 67°C and 45 sec at 72°C; with a final 5 min at 72°C. Following amplification, 10 µl of PCR product were digested with BtgI overnight at 37°C. Samples were then loaded into an ethidium bromide-stained 5% ultra-high-resolution agarose gel for genotype determination. Genotypes were determined as follows: wild-type, 227 bp; variant, 129 and 98 bp; heterozygote , 227, 129 and 98 bp.

Aromatase genotyping

CYP19 genotyping analysis was performed as follows (25): PCR fragement was generated using primers, 5'-GTCTATGAATATGCCTTTTT-3', and 5'-GTTTGACTCCGTGTGTTTGA-3'. The forward primer was 5'-labeled with a fluorescent dye (FAM) for automatic analysis. Amplification will be performed in a final volume of 50 µl containing 40 ng of genomic DNA, 1.5 nits of Taq polymerase (Life Technologies, Inc.), 1.5 mM MgCl2, 200 µM of each deoxynucleotide triphosphate, 0.3 µM of each primer, and 5 µl of 10×PCR buffer (500 mM KCl and 200 mM Tris-HCl) and water to a total volume of 50 µl. Thermal cycling consists of an initial 4–min denaturation step (94°C), followed by 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 1 min) , and a final elongation step(72°C, 5 min). A 5% Long Ranger /6M urea gel was used for rapid fragment detection using the ABI Prism 377 DNA sequencer. Amplified products were determined relative to Gene Scan-500 size standard using Genescan and software. Homozygotes alleles (17,19,20,26) detected by microsatellite analysis were sequenced to confirm repeat length.

Immunohistochemical staining of estrogen and progesterone receptor

Five-micron sections of paraffin-embedded tissue will be dewaxed, rehydrated to water, and immersed in 0.01M Tris-EDTA at pH 8.1. Epitope retrieval and IHC staining with monoclonal anti-ER and anti-PR (Novocastra, UK) are performed on an automated immunostainer in accordance with the manufacturer's instruction.

Results and Discussion

Our original proposal was composed of two parts. The first part of the study is a prospective case-control study. This part is to determine whether high estrogen level is a risk factor of breast cancer and whether estrogen level and total estrogen exposure modifies the estrogen-associations of genotypic polymorphisms of estrogen receptor and multiple metabolizing genes with breast cancer susceptibility. Ethnic differences in genetic polymorphism related to cancer risk may be, at least in part, attributable to difference in exposure "dosage" of carcinogens among populations. To dissect the biologic pathway of estrogen metabolism, hormone levels should be recorded and plasma should therefore be collected from each woman at the same time during menstrual cycle. In this study, we start to collect plasma which was not collected in our previous studies. To the best of our knowledge, there have been no studies reporting whether estrogen level modifies the associations of genotypic polymorphisms with breast cancer risk. The present proposal will be the first study to investigate this aspect. Three hundred cases have been enrolled by now.

The second part of the study was aiming at determining the prognostic significance of polymorphisms of estrogen receptor and estrogen-metabolizing genes in breast cancer patients. We have collected more than 500 cases of DNA specimens since 1989. Since Tamoxifen, an estrogen antagonist, and aromatase inhibitors are the current hormone therapy for breast cancer, the prognostic significance of genotypic polymorphism of ER, PR and aromatase (CYP19) was explored. We have completed genotyping of CYP19 in 503 breast cancers (table 1) and ER *Pvu* genotyping in 518 breast cancers (wt/wt:58.7%, wt/vt:34.4%, vt/vt:6.9%). The immunohistochemical stainings of ER and PR were also reviewed (Table 3) and re-stained if necessary. Survival analysis will be done soon.

Table 1. Allele Frequency of CYP19 polymorphism

	number	frequency
6	1	0.001
7	537	0.534
8	7	0.007
9	1	0.001
10	12	0.012
11	375	0.372
12	71	0.071
13	2	0.002
Total	1006(503cases)	1

Table 2. Genotype Frequency of ER polymorphism

	ER Genotype	
	number	frequency
wt/wt	304	0.587
wt/vt	178	0.344
vt/vt	36	0.069
Total	518	1

Table 3. Immunohistochemical staining of ER & PR

	ER	PR
0 (-)	156	157
1 (+,＜10%)	11	22
2 (+,10-30%)	16	26
3 (+,30-50%)	25	29
4 (+,50-70%)	32	43
5 (+,＞70%)	219	183
尚未判定	59	58
Total	518	518

成果自評

In addition,to the best of our knowledge, there have been no studies reporting whether estrogen level modifies the associations of genotypic polymorphisms with breast cancer risk. The present proposal will be the first study to investigate this aspect.

The estrogens in postmenopausal women arise only from non-ovary origin and are converted via aromatase. The aromatase inhibitors have demonstrated equivalent or superior efficacy over that of tamoxifen in metastatic disease. A randomized trial investigated the benefit of an aromatase inhibitor, letrozole, in postmenopausal women after five years of Tamoxifen therapy for early breast cancer. Due to the unexpected and considerable difference in the rate of recurrent events favoring letrozole, this study was terminated early. The results of our second part of the study, aimed to determine whether genotypic polymorphism of estrogen receptor and aromatase are related to response to anti-estrogen treatment in breast cancer patients, should be very important.

Since we can not get the grant support for the second and third year, we only collected three hundred cases of DNA and plasma, and only completed the genotyping of ER and CYP19 of 500 cases. However, we will try to get grant support to continue our project.

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