



A tetranucleotide repeat polymorphism in the *CYP19* gene and breast cancer susceptibility in a Greek population exposed and not exposed to pesticides

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Abstract

Epidemiological studies have suggested that hormones, genetic factors, and environmental agents are significant risk factors in breast carcinogenesis. Some pesticides have the ability to act as xenoestrogens *in vivo*. The *CYP19* gene encodes the aromatase enzyme which is involved in the estrogens biosynthetic pathways. We have assessed the frequency alleles of a (TTTA)_n repeat of *CYP19* gene in breast cancer patients which were either exposed or not exposed to specific pesticides. No differences were observed in the distribution of the alleles between the two groups showing that the polymorphism does not have a significant functional role on the aromatase activity. When compared to healthy control Greek women group, only the (TTTA)₁₀ repeat variant presented a non-significant increased risk in breast cancer susceptibility [odds ratio (OR): 2.46, $P > 0.05$]. Lack of strong association suggests that the polymorphic TTTA short tandem repeat of *CYP19* gene may have not a functional effect on the enzyme's activity and thus its role in the development of breast cancer remains unclear.

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1. Introduction

Epidemiological studies suggest that exposure to some environmental chemicals may play a role in the modulation of the human endocrine system resulting in the development and the progression of certain

types of cancer (Toppari et al., 1996). A variety of occupational chemicals such as certain pesticides have xenoestrogen activity. This means that they have the ability to mimic the action of estrogens in the body and this may be a risk factor for the induction of breast cancer (Bonfeld Jorgensen et al., 1997; Soto et al., 1995). Individual differences in the biosynthesis and metabolism of steroid hormones, arising from alterations in the genes involved in these pathways, may explain varying susceptibilities to breast cancer.

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An important enzyme in the estrogen biosynthesis is the CYP19 aromatase which converts C19 steroids (androstenedione, testosterone) to C18 estrogens (estrone, 17 β -estradiol). A number of pesticides is able to bind to the steroid hormone receptor and/or to affect enzymes that regulate the levels of hormones (Vinggaard et al., 2000). The *CYP19* gene encodes the aromatase enzyme, and genetic variation at this locus has been associated with breast cancer susceptibility (Siegelmann-Danieli and Buetow, 1999). A polymorphic tetranucleotide repeat (TTTA)_n in intron 4 of *CYP19* gene has been identified as a forensic marker (Polymeropoulos et al., 1991) and has recently been associated in several studies with breast cancer risk (Baxter et al., 2001; Haiman et al., 2000; Healey et al., 2000; Kristensen et al., 1998; Miyoshi et al., 2000).

In this case-control study, we investigated whether the genotyping profile for (TTTA)_n repeat polymorphism of the *CYP19* gene is involved in breast cancer susceptibility within Greek women exposed and not exposed to certain pesticides.

2. Materials and methods

2.1. Specimen collection

The data used in this study are from a breast cancer mass-screening programme “Breast cancer epidemiology in Crete and its relation to pesticide exposure” in a high-risk Cretan population. The Venizelion Hospital Mammography Unit, Heraklion, conducted the study during the period 1988–2002, 10651 women participated in the programme. Public breast cancer risk awareness was increased and accessibility criteria were widely publicized through planned presentations at local meetings and media campaigns. All the participants were of comparable age and had similar incidence of risk factors except for the occupational exposure to greenhouse pesticides. They were stratified in two groups: women who had worked at least 10 years in greenhouses, for more than 4 h daily; and women of higher socio-economic status, being residents of larger towns and with non-agricultural occupations. Furthermore, data of the participants were collected by a personal interview. This included age, family history of breast cancer, history of benign breast disease, age at menarche, menopausal status, oral contraceptive use,

menopausal estrogens use, parity, age at first birth, and obesity. Additional inclusion criteria for both groups were: normal blood count, liver function tests and biochemical profile, and Karnofski performance status of 100. Follow-up of these women was carried out until 2002. In a preliminary report on mammographic findings and occupational exposure to pesticides (Dolapsakis et al., 2001) on 1062 of the 10651 women, malignant lesions were found in 19 individuals (1.8%), of which 11 cases from the exposed and 8 cases from the non-exposed group, respectively. From the sample bank of 10651 women, peripheral blood was collected from 202 Greek female patients who underwent mastectomy or breast conserving surgery (1.9%), of which 110 exposed were between 40 and 78 years old (mean age of 55.2 years), and 92 non-exposed were between 37 and 77 years old (mean age of 56.4 years). Healthy controls were 284 Greek women aged 35 to 79 (mean age of 53.1 years) with cancer-free past history and without family history of breast cancer among first-degree relatives. All the specimens were collected at the “Venizelio” General Hospital, Heraklion, Greece, and all the women were residents of Crete. Clinical data (stage, grade, histological subtype, age) were available for all the specimens tested. The ethics committee of the University of Crete and of Venizelio General Hospital had approved this study, and all the patients gave written informed consent.

2.2. DNA extraction

Genomic DNA was extracted from whole blood using proteinase K followed by phenol extraction and ethanol precipitation. DNA was resuspended in 50 μ l TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Working stocks were prepared by 10-fold dilution in double distilled H₂O.

2.3. Polymerase chain reaction (PCR) methods

PCR assays were performed by introducing 100 ng of genomic DNA in a PCR reaction mixture to a 50 μ l total reaction volume. Amplification parameters were: 2 min for initial denaturation at 94 °C; amplification for 35 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; final extension step at 72 °C for 10 min. The oligonucleotide primer sequences for the amplification of the (TTTA)_n repeat (used to a final

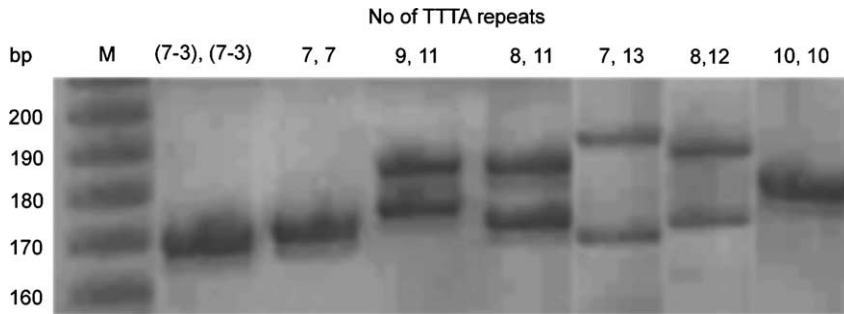


Fig. 1. Representative examples of the $(TTTA)_n$ short tandem repeats of the *CYP19* gene. M: 10 bp DNA molecular size standard.

concentration of 0.3 μ M) were previously described by Polymeropoulos et al. (1991). PCR assays were carried out in a PTC-100 programmable thermal controller (MJ Research Inc., USA).

2.4. Electrophoresis

PCR products were denatured and then analyzed by 6% polyacrylamide gel electrophoresis with a 10 bp DNA ladder and silver stained. The lengths of homozygous products of the TTTA repeats were measured by cycle sequencing and these numbers of repeats were used as size standards. Gels were scanned on an Agfa SnapScan 1212u (Agfa-Gevaert N.V., Belgium). PCR product length was calculated by digital imaging using the Adobe Photoshop 6.0 software (Adobe Systems Inc., USA).

2.5. Statistical analysis

Chi-square test with estimation of odds ratios (OR) with 95% confidence intervals or Fisher's exact test were used to compare genotype distribution between cases and controls. All statistical calculations carried out by the SPSS 8.0 (SPSS Inc., USA) programme.

3. Results

We assayed 110 genomic DNA samples extracted from blood of breast cancer patients who had occupational exposure to pesticides, 92 samples of sporadic breast cancer patients without known or occupational exposure to pesticides, and 284 aged and sex matched controls. Eight different alleles of

CYP19 gene $(TTTA)_n$ repeat were detected, as shown in Fig. 1, with sizes ranging from 168 to 195 bp: $(TTTA)_{7-3}$ (168 bp), $(TTTA)_7$ (171 bp), $(TTTA)_8$ (175 bp), $(TTTA)_9$ (179 bp), $(TTTA)_{10}$ (183 bp), $(TTTA)_{11}$ (187 bp), $(TTTA)_{12}$ (191 bp), and $(TTTA)_{13}$ (195 bp). The shorter allele $(TTTA)_{7-3}$ had three bases (TCT) deletion 50 bp upstream of the $(TTTA)_n$ repeat. The number of individuals with different genotypes, observed both in breast cancer or control groups, gave a good fit to the Hardy–Weinberg equilibrium.

The distribution of the $(TTTA)_n$ repeat alleles between the two breast cancer study groups (exposed and not exposed to pesticides) did not show any significant over-representation ($P = 0.912$). Moreover, statistical analysis did not reveal any significant differences in the genotype prevalence between the two breast cancer populations ($P = 0.849$). Homozygosity and heterozygosity were detected in contiguous frequencies with 79.6% and 80.0% heterozygotes of 110 exposed and of 92 non-exposed breast cancer cases, respectively. In the absence of over-representation of a certain allele or a specific genotype of the *CYP19* $(TTTA)_n$ repeat between the two breast cancer patient groups, they were analyzed as one (total breast cancer cases) population, and the results were compared with those of the control group.

The distribution of each allele in breast cancer cases and controls is shown in Table 1. The shorter and longer alleles were more frequent among control subjects with frequencies 34.7%, 14.3%, 37.1%, 2.6%, and 1.1% for the $(TTTA)_{7-3}$, $(TTTA)_7$, $(TTTA)_{11}$, $(TTTA)_{12}$, and $(TTTA)_{13}$, respectively. On the other hand, the alleles with 8, 9, and 10 repeats of the tetranucleotide sequence were observed with 10.4%, 1.2%, and 1.5% frequencies, respectively, among

Table 1
CYP19 intron 4 (TTTA)_n repeat alleles in breast cases and controls

(TTTA) repeat number	Number of alleles (frequency)		P-value	OR (95% CI)
	Controls	Cases		
7–3	197 (0.347)	138 (0.342)	0.014	0.985 (0.765–1.267)
7	81 (0.143)	55 (0.136)	0.062	0.955 (0.662–1.376)
8	49 (0.086)	42 (0.104)	0.230	1.125 (0.300–4.214)
9	5 (0.009)	4 (0.010)	0.557	1.406 (0.404–4.888)
10	4 (0.007)	7 (0.017)	0.122	2.460 (0.716–8.460)
11	211 (0.371)	146 (0.361)	0.048	0.973 (0.760–1.245)
12	15 (0.026)	9 (0.022)	0.159	0.844 (0.366–1.947)
13	6 (0.011)	3 (0.007)	0.249	0.703 (0.175–2.827)

breast cancer cases. The (TTTA)₁₀ repeat of *CYP19* gene was the only allele which could be associated with a high risk for the development of breast cancer. It was over-presented in cancer cases with a frequency of 1.5% as compared to 0.5% among controls but this difference failed to reach statistical significance ($P = 0.122$, OR: 2.46, 95% CIs: 0.716–8.460).

An increased frequency of (TTTA)₁₀ repeat homozygotes among breast cancer case, as compared to controls, was also observed. In fact, among controls there were no carriers of the (TTTA)₁₀ genotype while 2 of the 202 breast cancer cases (one from the exposed and the other from the non-exposed group) were carriers of this genotype with a frequency of 0.046 of the homozygotes among patients. Heterozygosity among cases and controls was observed in 78.7% and 78.1% of the individuals, respectively.

4. Discussion

The tetranucleotide (TTTA)_n tandem repeat polymorphism of the *CYP19* gene which has a functional role in the endogenous estrogen production has been studied in order to identify the low-penetrance genetic loci correlated with increased breast cancer risk. We tried to determine whether the exposure to certain pesticides, in combination with a specific genotype of the examined *CYP19* polymorphism, might represent a risk factor for the development of breast cancer. As some pesticides may mimic the action of estrogens, the activity of the enzymes which are responsible for the homeostatic balance of hormones could pos-

sibly be affected by the exposure to these chemical compounds.

In this study, we carried out a genetic distribution analysis of *CYP19* (TTTA)_n alleles in two well-defined populations of breast cancer patients either exposed or non-exposed to certain pesticides. According to a cohort study within the Cretan population, the incidence for breast cancer was 2.1% and 1.5% for the exposed and non-exposed groups, respectively (Dolapsakis et al., 2001). This difference was not statistically significant. However, compared to the general female population of Crete, the exposed group may have higher risk of incidence for a number of lesions, which are risk markers for subsequent invasive breast cancer. When we examined the association of the incidence of breast cancer with the tetranucleotide repeat polymorphism of *CYP19* gene in exposed and non-exposed breast cancer patient groups, no significant differences in the distribution of alleles between the two groups were found. However, this may be due to the small number of patients in each group. This, to our knowledge, is the first report that tries to correlate a genetic polymorphism of the aromatase gene with the exposure of individuals to pesticides. Lack of association between certain alleles and exposure shows that the (TTTA)_n repeat polymorphism may not have a direct effect on the aromatase activity.

As we did not find any association between the exposed and non-exposed breast cancer patient groups and the polymorphism examined, we pooled the data for these two groups and compared the distribution of alleles of the pooled group with cancer-free population (age and sex matched) in order to test the (TTTA)_n repeat as a breast cancer risk factor. In the present

study, no association between the allelic frequencies of *CYP19* gene and breast cancer could be observed.

The most interesting finding of this study was the over-representation of the (TTTA)₁₀ allele, with 2.5% and 1.7% carriers and alleles frequencies among patients, with two of the carriers homozygotes. In the control group, the frequency of the carriers was 1.4%, and no individuals with the homozygotic genotype were found. The (TTTA)₁₀ allele carriers had a 2.46-fold higher risk in the development of breast cancer. Although this difference did not reach statistical significance which may be due to limited sample size, our results are consistent with previous reports. Thus, in a previous study on Caucasian women, Baxter et al. (2001) reported an increased frequency of the (TTTA)₁₀ allele in cancer cases (1.5%) versus controls (0.2%). However, no homozygotes were found for this allele. Moreover, in a recent meta-analysis, Healey et al. (2000) reported a significant association of the (TTTA)₁₀ allele with breast cancer among English women (1.3% in breast cancer patients versus 0.8% in controls).

The incidence in breast cancer development was not associated with other (TTTA)_n alleles (7–3 to 9 and 11–13 repeats of the tetranucleotide). Haiman et al. (2000), besides reporting an increased frequency of (TTTA)₁₀ allele, also found a non-significant increase of the (TTTA)₁₂ allele frequency. Recently, Kristensen et al. (1998) observed an over-representation of the (TTTA)₁₂ allele among a US population. The shorter allele (TTTA)₇, a high-risk allele according to Siegelmann-Danieli and Buetow (1999), was found among Caucasian women with 13/14 carriers being homozygotes. Young et al. (2000), however, did not find any evidence of association between any of the alleles of the short tandem repeat polymorphism and susceptibility to breast cancer among Caucasian men.

Although in the present study the (TTTA)₁₀ allele was over-represented in patients compared to the controls, its frequency was rare in the general population and a strong association was not established. It is not clear if the polymorphism of the *CYP19* gene could be useful as biomarker for breast cancer susceptibility as different genetic variants of the gene exist that may affect the activity of aromatase. Larger molecular epidemiology studies are required to establish the role of the aromatase gene in breast carcinogenesis.

References

- Baxter, S.W., Choong, D.Y., Eccles, D.M., Campbell, I.G., 2001. Polymorphic variation in *CYP19* and the risk of breast cancer. *Carcinogenesis* 22, 347–349.
- Bonefeld Jorgensen, E.C., Autrup, H., Hansen, J.C., 1997. Effect of toxaphene on estrogen receptor functions in human breast cancer cells. *Carcinogenesis* 18, 1651–1654.
- Dolapsakis, G., Vlachonikolis, I.G., Varveris, C., Tsatsakis, A.M., 2001. Mammographic findings and occupational exposure to pesticides currently in use on Crete. *Eur. J. Cancer* 37, 1531–1536.
- Haiman, C.A., Hankinson, S.E., Spiegelman, D., De Vivo, I., Colditz, G.A., Willett, W.C., Speizer, F.E., Hunter, D.J., 2000. A tetranucleotide repeat polymorphism in *CYP19* and breast cancer risk. *Int. J. Cancer* 87, 204–210.
- Healey, C.S., Dunning, A.M., Durocher, F., Teare, D., Pharoah, P.D., Luben, R.N., Easton, D.F., Ponder, B.A., 2000. Polymorphisms in the human aromatase cytochrome P450 gene (*CYP19*) and breast cancer risk. *Carcinogenesis* 21, 189–193.
- Kristensen, V.N., Andersen, T.I., Lindblom, A., Erikstein, B., Magnus, P., Borresen-Dale, A.L., 1998. A rare *CYP19* (aromatase) variant may increase the risk of breast cancer. *Pharmacogenetics* 8, 43–48.
- Miyoshi, Y., Iwao, K., Ikeda, N., Egawa, C., Noguchi, S., 2000. Breast cancer risk associated with polymorphism in *CYP19* in Japanese women. *Int. J. Cancer* 89, 325–328.
- Polymeropoulos, M.H., Xiao, H., Rath, D.S., Merrill, C.R., 1991. Tetranucleotide repeat polymorphism at the human aromatase cytochrome P-450 gene (*CYP19*). *Nucleic Acids Res.* 19, 195.
- Siegelmann-Danieli, N., Buetow, K.H., 1999. Constitutional genetic variation at the human aromatase gene (*Cyp19*) and breast cancer risk. *Br. J. Cancer* 79, 456–463.
- Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* 103 (Suppl. 7), 113–122.
- Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette Jr., L.J., Jegou, B., Jensen, T.K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J.A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., Skakkebaek, N.E., 1996. Male reproductive health and environmental xenoestrogens. *Environ. Health Perspect.* 104 (Suppl. 4), 741–803.
- Vinggaard, A.M., Hnida, C., Breinholt, V., Larsen, J.C., 2000. Screening of selected pesticides for inhibition of *CYP19* aromatase activity in vitro. *Toxicol. In Vitro* 14, 227–234.
- Young, I.E., Kurian, K.M., MacKenzie, M.A., Kunkler, I.H., Cohen, B.B., Hooper, M.L., Wyllie, A.H., Steel, C.M., 2000. A polymorphic tetranucleotide repeat in the *CYP19* gene and male breast cancer. *Br. J. Cancer* 82, 1247–1248.