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Incidence of inactive allele CYP2D6*4 among Greek women suffering from hormone-sensitive breast cancer

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Summary

Background: The incidence of CYP2D6*4 among Caucasians is estimated up to 27%, while it is present in up to 90% of all poor metabolizers within the Caucasian population. The hypothesis under question is whether the presence of one or two non-functioning (null) alleles predicts an inferior outcome in postmenopausal women with breast cancer receiving adjuvant treatment with tamoxifen. The aim of the present study is to estimate the incidence of CYP2D6*4, in the Greek population and more precisely among females suffering from breast cancer. *Materials and Methods:* Eighty unrelated mainland Greek female volunteers suffering from hormone-sensitive breast cancer were recruited during their primary handling or follow-up examination in order to provide samples for purification and polymerase chain reaction/ restriction fragment length polymorphism (PCR-RFLP) of genomic DNA derived from buccal swabs. *Results:* The incidence of individuals with at least one present allele*4 within the Hellenic population was estimated to be as high as 30% (n = 24/80), with a 95% confidence interval of 20% to 40%. From the statistical point of view, it can be securely stated that incidence of *4 among Greek women is over 20%. The incidence of homozygous carriers of *4 in the present sample occurred in 8.75%, while the incidence of allele*4 haplotype occurred in 19.4% (n=160). *Conclusion:* Although the outcoming results for Greek women are actually in line with existing data for other European nations, it should be noted, that a routine CYP2D6 testing of women suffering from breast cancer is formally not recommended, as the clinical significance of CYP2D6 phenotype in treatment and outcome of breast cancer remains unclear.

Key words: CYP2D6*4; Breast cancer; Tamoxifen; Caucasian.

Introduction

Cytochrome P-450 2D6 (CYP2D6) is of great clinical relevance, because it represents one of the most important enzymes involved in drug metabolism in general. The gene encoding this enzyme is highly polymorphic, as 93 alleles with varying function have been reported. According to the genotype, pattern individuals can be divided into four phenotype groups: ultra-rapid (UMs), extensive (EMs), intermediate (IMs), and poor metabolizers (PMs). UMs carry three active alleles (duplication or amplification effect); EMs are characterized by the presence of two functional alleles (*1, *2, *9); IMs carry only one active allele, while PMs express two inactive alleles (*3,*4,*5) [1].

In general, Caucasians have a quite higher incidence of the PM phenotype when compared to other races. The studies referring to African populations on the other hand show a wide range of results, with the South-Africans having an incidence of 19%. The lowest frequency is reported within the Asian population. Allele *3 and mostly *4, both of which are non-functional, are mainly responsible for the PM phenotype among Caucasians in general. On the contrary, these alleles are rarely found in the Asian population, explaining the worldwide lowest frequency of PM status in that group [2].

The role of CYP2D6 in the adjuvant treatment of breast cancer is crucial, as this enzyme is mainly involved in the biotransformation of tamoxifen to the potent antiestrogen endoxifen. The aim of the present study is to estimate the incidence of CYP2D6*4, in the Greek population and more precisely within females suffering from breast cancer. Despite the numerous existing studies focusing on the incidence of CYP2D6*4 between Caucasians in general, and among specific European ethnic groups as well, relevant data referring to the Greek race are missing. This is the first country-wide study attempting such an epidemiological screening approach.

Materials and Methods

A total of 80 unrelated mainland Greek female volunteers participated in the study after giving written informed consent. They were all patients suffering from hormone-sensitive breast cancer, that were recruited during their primary handling or follow-up examination at the Second Department of Propaedeutic Surgery of the Medical School in Athens. The study protocol was approved by the Ethics Committee of the Kapodistrian University of Athens Medical School. The patients were informed that the present study was only scheduled for statistical purposes and that the outcoming results would not influence their treatment regimen. The description of the patient's characteristics is presented in Tables 1 and 2.

Purification and polymerase chain reaction/ restriction fragment length polymorphism (PCR-RFLP) of genomic DNA was derived

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Table 1. — *Cohort descriptives I.*

	Total		CYP2D6=*4 No		Yes	
	N	%	N	%	N	%
Grade	14	17.5	11	19.6	3	12.5
I						
II	51	63.8	32	57.1	19	79.2
III	15	18.8	13	23.2	2	8.3
Histological type						
Papilar	1	1.3	1	1.8	.	.
Lobular	8	10	6	10.7	2	8.4
Mixed	2	2.5	2	3.6	.	.
Ductal	68	85.0	47	83.9	21	87.5
Hybrid	1	1.3	.	.	1	4.2

Table 2. — *Cohort descriptives II.*

			CYP2D6=*4	
		Total	No	Yes
ER- status	N	80	56	24
	Mean	0.7	0.7	0.8
	Median	0.8	0.8	0.8
	Min	0.2	0.2	0.2
	Max	1.0	1.0	1.0
PR-status	N	80	56	24
	Mean	0.6	0.6	0.7
	Median	0.7	0.6	0.7
	Min	0.0	0.0	0.0
	Max	1.0	1.0	1.0
Age (years)	N	80	56	24
	Mean	53.6	54.1	52.4
	Median	53.0	53.5	50.5
	Min	30.0	30.0	40.0
	Max	88.0	88.0	75.0

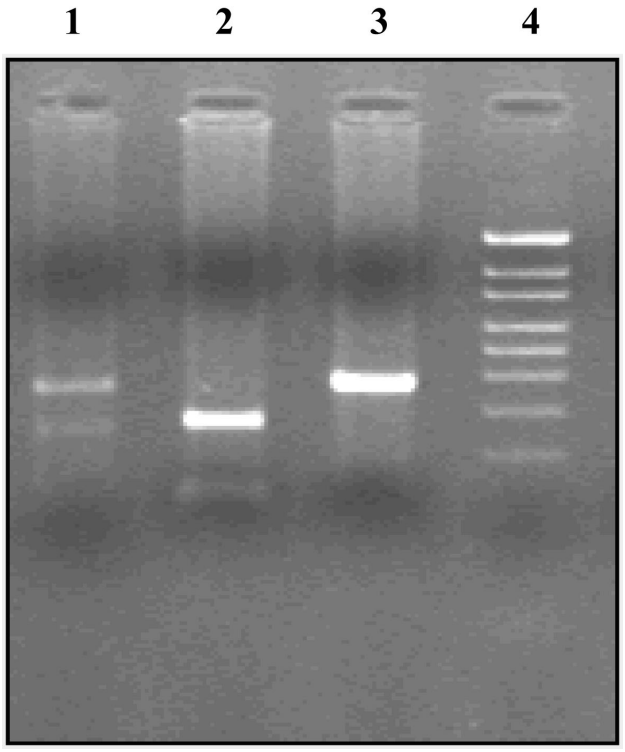

Figure 1. — Gel picture, CYP2D6*4 polymorphism.
Lane 1 – IM genotype (intermediate metaboliser -334, 230, and 104 base pairs).
Lane 2 – EM genotype (extensive metaboliser -230 and 104 base pairs).
Lane 3 – PM genotype (poor metaboliser -334 base pairs).
Lane 4 – 100 base pair ladder as marker.

from buccal swabs that was performed. The samples were collected with cotton swabs. The swab was scraped firmly against the inside of each cheek several times and was set to air dry. All individuals were informed to avoid consuming food or drink within 30 minutes prior to the collection of the sample. Each dry swab material was placed in a two-mL micro- centrifuge tube, where 300 µL PBS and 25 µL proteinase K solution was added. It followed a mix by vortexing 2x5 seconds and incubation for ten minutes at 56°C. The swab was at that point removed and 300 µL buffer B3 were added. The solution was vigorously vortexed and the sample was incubated at 70°C for another ten minutes. In order to adjust the DNA binding conditions 300 µL of 96%-100% ethanol were added to each sample and the new solution was once again mixed by vortexing. At that point 600 µL of the samples were transferred from the two-mL micro-centrifuge tubes into NucleoSpin Tissues Columns and was centrifuged at 12,000 x g for one minute. The prior added ethanol binds the DNA on to the column membrane. The flow-through was discarded and the columns were placed back into the collection tube. The silica membrane was initially washed after adding 500 µL of buffer BW and centrifuging for one minute at 4,500 x g. The second wash was performed with an addition of 600 µL buffer B5 to the column and centrifugation at 14,000 x g for two minutes. The flow-through was once more discarded. In order to remove the residual ethanol, the NucleoSpin Tissue Column were then placed into a new collection tube and were incubated with an open lid for one to two minutes at 70°C. In the next step and in order to elute highly pure DNA, the columns were placed into a 1.5-mL micro-centrifuge tube where 80 mL of pre-warmed elution buffer BE (70°C) were added. The solution was incubated for one minute and then centrifuged at 12,000 x g for one more minute. The quantitative measurement of the amount of isolated DNA could then be performed with photometry.

The investigation of the presence of CYP2D6*4 was performed using PCR-RFLP. For successful PCR, about five µl (200 ng) of DNA extract and 45 µl of PCR mix - including the two specific primers- were incubated under specific conditions. The used forward and reverse primers for CYP2D6*4 genotyping had the following nucleotide sequences: GCTTCGCCAACCACTCCG (CYP2D6-f) and AAATCCTGCTCTTCCGAGGC (CYP2D6-r). The 45 µl PCR mix contained five µl PCR-buffer w/o Mg, one µl dNTPs, 1.5 µl MgCl2, one µl of each of the primers 2D6-f and 2D6-r, 0.5 µl *Taq*-polymerase and 40 µl of H_2O. Thermocycling conditions were as follows: initial denaturation at 95°C for five minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 60 seconds. The terminal elongation was performed at 72°C for five minutes. If the PCR was successful (PCR product of 334 base pairs, checked by 2% agarose gel electrophoresis), 15 µl of the product was diluted with five volumes of distilled water and stored at 4°C. The PCR-product was then digested using the restriction endonuclease BstNI. The final digestion mix contained

Table 3. — *CYP2D6 genotyping results.*

CYP2D6	n	%
*4/*4	7	8,75
wt/*4	17	21.25
wt/wt	56	70.0

Table 4. — *Binomial proportion of CYP2D6*4.*

Proportion	0.3000
ASE	0.0512
95% lower conf. limit	0.1996
95% upper conf. limit	0.4004

15 μl of PCR product, five μl of NEB buffer, one μl of BstNI, and 29 μl H_2O (total mix volume 50 μl) and was incubated at 60°C for one hour. The digestion products were further analyzed on a 10% acrylamide gel electrophoresis, together with a 100-bp DNA weight marker. The excepted electrophoresis patterns and their interpretation are presented in Figure 1.

Statistical analysis

Sample size estimation was based on the assumption that the true incidence of allele *4 would approximately be 20%. The majority of the existing literature evidence that the incidence of allele *4 among healthy Caucasian women ranges from 18% to 21% [3, 4]. Thus with 80 patients (160 examined alleles), the expected level of confidence would be ± 7%. Asymptotic 95% confidence intervals where used in order to assess the level of accuracy for the point estimates, while hypothesis testing was used to test several alternatives.

Results

In the present study, the incidence of individuals with at least one present allele*4 within the Hellenic population was estimated to be as high as 30% (n = 24/80), with a 95% confidence interval of 20% to 40%. With this mean, it can be securely stated that incidence of *4 among Greek women is over 20%. Furthermore, the incidence of homozygous carriers of *4 in the present sample occurred on 8.75% (Tables 3 and 4), while the incidence of allele*4 haplotype occurred in 19.4% (n=160).

Discussion

The main question remaining to be answered is whether the routine use of CYP2D6 genotyping should be introduced in the adjuvant setting of tamoxifen or not. Focused on these issues while designing the present study, the sample consisted exclusively of Greek female patients with hormone-sensitive breast cancer.

A number of studies have estimated the incidence of CYP2D6 phenotype and the distribution of CYP2D6 alleles within Caucasians. Few of them are restricted to simple phenotype prediction as summarized in Table 5 [5], while others have specifically focused on the incidence of allele*4 in various European ethnic groups.

Table 5. — *Incidence of poor metabolizers (PM) within Caucasians [5].*

Population	PMs (%)
British	8.9
Swiss	10
German	7.7
Polish	8.3
Croatian	3.0

Table 6. — *Ethnic studies.*

Population	n	*4 incidence (%)	Population	n	*4 incidence (%)
Croatian [27]	200	14.0	Greek [21]	283	17.8
Croatian [26]	144	11.4	Italian [23]	350	15.3
Czech [12]	223	22.9	Norwegian [15]	118	20.0
Danish [19]	240	18.1	Polish [10]	145	23.1
Danish [20]	325	20.6	Polish [11]	300	23.0
Dutch [16]	756	18.4	Russian [18]	290	18.2
Estonian [13]	151	21.5	Russian [17]	204	14.4
Finnish [32]	302	12.8	Sardinian [22]	250	16.8
Finnish [31]	122	11.1	Spanish [30]	290	16.6
French [25]	514	18.6	Spanish [28]	258	12.2
French [24]	171	14.9	Spanish [29]	105	13.8
German [4]	589	20.7	Swedish [8]	281	24.4
German [14]	195	19.5	Swedish [9]	248	23.0

The activity of the CYP2D6 enzyme can be easily measured in vivo after the oral administration of a probe drug that is mainly CYP2D6 metabolized, such as dextromethorphan, debrisoquine or sparteine. The consequent estimation of the ratio of metabolite to parent drug concentration indicates the CYP2D6 metabolic status [6]. Regarding the detection of CYP2D6*4, it should be mentioned that, the standard nomenclature of the *4 allele is based on the presence of the 1846G>A defining variant. Furthermore, other haplotype variants could also be present [7].

The prevalence of the CYP2D6*4 allele, as estimated in the present study, complies with the Hardy-Weinberg equilibrium and is in line with the majority of published results for other European ethnicities of Caucasian origin. More precisely, the following frequencies have been reported among different Caucasian ethnicities: 24.4% - 23% in Swedes [8, 9], 23.1% - 23,0% in the Polish [10, 11], 22.9% in Czechs [12], 21.5% in Estonians [13], 19.5% and 20.7% in Germans [4, 14], 20.0% in Norwegians [15], 18.4% in Dutch [16], 14.4% - 18.2% in Russians [17, 18], 18.1% and 20.6% in Danish [19, 20], 17.8% in Greeks [21], 16.8% in Sardinians [22], 15.3% in Italians [23], 14.,9% - 18.6% in French [24, 25], 1.4% - 14% in Croatians [26, 27], 12.2%, 13.8% and 16.6% in Spanish [28-30] and 11.1% - 12.8% in Finish [31, 32]. The examined population and the concomitant incidence of CYP2D6*4 of the aforementioned reported studies are presented in Table 6.

Table 7. — *Multi-ethnic studies.*

Population	n	*4 incidence (%)
European [3]	672	18.9
European [33]	157	17.2
French	25	16.0
French Basque	24	20.8
Sardinian	28	21.4
North Italian	14	14.3
Tuscan	8	18.8
Orcadian	16	12.5
Adygei	17	8.8
Russian	25	20.0
Mediterranean [34]	247	16.0
Sardinian	48	12.5
Central Italians	31	12.9
Alps	28	19.64
Basques	38	21.05
Southern Spaniards	51	17.65

A special report should be also made on three large studies that analyzed the allele*4 incidence in multi-ethnic European cohorts (Table 7). Marez *et al*. analyzed 672 individuals of European origin and estimated the incidence of allele*4 to be as high as 18.9%. Further details regarding the sample composition are not available [3]. Similar results with an allele*4 incidence of 17.2% are also reported in the study of Sistonen *et al*. performed in a sample of 157 Europeans individuals [33]. The authors have additionally reported the respective frequencies in every ethnic group being part of their cohort. In one further large study within six populations of the Mediterranean region, the prevalence of allele*4 occurred in 16%. A further subanalysis of the allele*4 frequencies in each ethnic group has also been reported [34].

Four studies provide data about the incidence of CYP2D6*4 allele within Caucasians suffering from breast cancer. Bonanni *et al*. determined the CYP2D6 genotype in hysterectomized women participating in the Italian chemoprevention trial of tamoxifen. The frequency of the CYP2D6 *4/*4 genotype was statistically significant higher (9%) in women who developed breast cancer (n=46) than in the control group (n=136). The authors assumed that the expression of the inactive allele*4 may consist of a predisposing factor for breast cancer. A strong bias of their study is due to a lack of group-matching of the follow-up period and the risk factors associated with breast cancer as well [35].

Two further studies performed between Spanish individuals are giving conflicting results. Fernandez-Santanter *et al*. genotyped 96 breast cancer Spanish patients and compared them to 100 healthy control subjects. The incidence of allele*4 was 13.5% *vs.* 22% in patients and controls, respectively. Their results supported a statistically significant association between wild type CYP2D6 *vs.* homozygous *4 genotype and breast cancer risk [36]. This data is conflicting with the results of another study among Spanish individuals published by Ladona *et al*. Their cohort consisted of 151 breast cancer patients and 187 healthy controls. The authors supported an inverse relationship between CYP2D6 activity and breast cancer risk. The prevalence of heterozygous CYP2D6 (wt/*4) genotype was higher between individuals with breast cancer (26.7% *vs.* 17.2%, $p = 0.037$) [37].

Finally, Topic *et al.* compared the incidence of inactive allele*4 between breast cancer patients and healthy volunteers from Croatia [26]. The prevalence of CYP2D6*4 occurred in 18.4% among breast cancer subjects (28/152 tested alleles) *vs.* 11.4% among control individuals (33/288 tested alleles). The reported difference was furthermore not statistically significant, hence no association between CYP2D6 genotype and breast cancer risk could be safely supported .

In reference to the Greek population, the only previous existing study investigating the prevalence of various CYP2D6 genotypes within healthy Greeks has been published by Arvanitidis *et al.* [21]. In a total of 283 healthy subjects, 92 were detected to be carriers of the inactive allele*4. Eight of them were estimated to be homozygous (3.2%), while the frequency of allele*4 itself occurred in 17.84% (101/566 tested alleles). The present results, although exclusively based on breast cancer patients, are actually in line with those of Arvanitidis *et al*, so that no etiological relationship between CYP2D6 genotype and breast cancer risk among Greek women could be assumed. The extraction of such a conclusion is actually not safe and strongly biased, as the two cohorts were completely and independently analyzed and consisted of non-matched individuals.

The present authors refer to a prior review research of their institution, that evaluates the clinical implication of the non-functional allele *4 in breast cancer, always in regards to tamoxifen therapy [38]. The results were conflicting and quite inconclusive. Three former reports showed a favorable outcome in CYP2D6*4 carriers with ER+ breast cancer. These findings are actually opposed to the basic assumption and could not be supported by any other later study [39-41].

The great volume of published studies shows a clear negative relationship between intermediate/poor CYP2D6 metabolizing status and the outcome of ER+ breast cancer. This main hypothesis has also been supported from studies that have exclusively focused on allele*4 alone [35, 42-45]. Interestingly, the presence of inactive CYP2D6 alleles and in particular allele*4 has also been associated with lower circulating serum levels of tamoxifen metabolites. In that mean, the benefit of tamoxifen treatment is strongly limited in this patient group [46, 47]. On the other hand, the acceptance of such a negative impact of allele*4 in the course of breast cancer is not

unique. In a large population study published by Abraham *et al*., no association between CYP2D6 phenotypes (allele*4 included) and survival in breast cancer patients under tamoxifen treatment could be proven. Based on their results, the authors argued against CYP2D6 testing in the clinical setting [48].

Although the current recommendations for breast cancer treatment do not support a CYP2D6 screening prior to tamoxifen treatment, the interpretation of every existing result should be made with respect to the special parameters of each population.

The estimated incidence of CYP2D6*4 among Greek females suffering from breast cancer is quite high in comparison to previous presented results for other ethnic groups of Caucasian origin. The present cohort included only women with hormone positive cancers, in an effort to maximize the accuracy of the present results for this patient group, in which actually the administration of tamoxifen is absolutely indicated. The outcoming results are in accordance to the Hardy-Weinberg equilibrium and can be considered as highly reliable given Greece's consistent and homogeneous population.

In the present study, the testing for CYP2D6 allele*4 was performed using germline DNA extracted from hosts' buccal-derived sample. In a large number of published studies that doubt about the clinical significance of allele*4 in the treatment with tamoxifen, the genotyping procedure was performed at paraffin-fixed cancer tissue. It should be mentioned that tumor DNA may show significant differences from germline DNA due to "loss of heterozygosity" during cancer progression, a fact that depicts a strong bias of all these studies.

The cost effectiveness parameter is of high importance, given the long required time of tamoxifen administration in relation to the strong financial limitations of the Greek health system in an area of financial crisis. In patients where impaired function of CYP2D6 is expected, due to the presence of one or two *4 alleles, dose adjustment or other therapy regime should be considered. In patients which have been found to be intermediate or poor metabolizers, caution should be also given in any potential CYP2D6 inhibitors that may be occasionally co-prescribed due to other medical reasons [49]. If the administration of tamoxifen should be continued, dose reduction or alternative medication for the handling of co-morbidities might be indicated. Finally, patients that are not likely to benefit from a treatment with tamoxifen should also not be exposed to its possible various side and adverse effects [50, 51].

Conclusion

CYP2D6*4 is the most frequent allele associated with loss of enzymatic activity among Caucasians. The present study, performed on an ethnic basis, focused only on women with hormone-sensitive breast cancer, a patient group in which the administration of tamoxifen is absolutely indicated. The outcoming results for Greek women are actually in line to existing data of other European nations. Nevertheless it should be noted, that a routine CYP2D6 testing of women suffering from breast cancer is formally not recommended, as the clinical significance of CYP2D6 phenotype in treatment and outcome of breast cancer remains unclear.

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