RESEARCH ARTICLE

Breast Cancer Association with CYP1A2 Activity and Gene Polymorphisms - a Preliminary Case-control Study in Tunisia

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Abstract

The aim of the present study was to evaluate the relative contribution of CYP1A2 isoforms (-3860 G/A, -2467T/delT and -163C/A) in control subjects and breast cancer patients to the metabolism of caffeine in human liver. Restriction fragment length polymorphism analysis of PCR-amplified Fragments (PCR-RFLP) was used for the genotyping of CYP1A2 SNPs and HPLC allowed the phenotyping through the measurement of CYP1A2 activity using the 17X + 13X + 37X/137X urinary metabolite ratio (CMR) and plasma caffeine half life (T1/2). The CYP1A2 -3860A genotype was associated with a decreased risk of breast cancer. In contrast, distributions of the CYP1A2 -2467T/delT or -2467delT/delT and -163A/C or A/A genotypes among breast cancer patients and controls were similar. When the genotype and phenotype relationship was measured by comparing the mean CMR ratios and caffeine half life within the genotype groups between subjects and breast cancer patients, there were no significant differences except for -3860 A, most of them being homozygous for the -3860 G/G SNP and had a significant higher mean CMR ratio and half life than those with -3860 G/A (P=0.02). The results of this preliminary study show a significant association between CP1A2 -3860 G variant and CYP1A2 phenotype which must be confirmed by further large-size case-control studies.

Keywords: Breast cancer - CYP1A2 - gene polymorphism - phenotype

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Introduction

Breast cancer is the most common malignant tumor in women in both developed and developing countries. It was shown that breast cancer incidence in Tunisia is one of the highest in developing countries (Missaoui et al., 2010; 2012). Breast cancer is also the first leading cause of women's cancer death in Tunisia according to the National Board For Family and Population (Ministry of Public Health, Tunisia).

Epidemiological studies have shown that prolonged exposure to estrogens, particularly 17 β-estradiol (E2), is one of the primary risk factors for the development of tumors such as breast cancer tumors (Evans et al., 2003) and genes involved in estrogen metabolism have been reported to mediate that risk (Rebbeck et al., 2007). Hydroxylation of E2 is catalyzed by several enzymes, including CYP450 which exhibits genetic polymorphisms explaining significant differences in metabolic activity and human pharmacokinetics among individuals. Significant effects of heritable factors were observed for breast cancers in a large study of twins performed in three Nordic countries although environment has the predominant role (Ahlbom et al., 1997; Lichtenstein et al., 2000). In addition to genetic polymorphisms, gene-gene interactions, age, gender, gene-environment interactions contribute to susceptibility to cancer risk. Among environmental factors, smoking and dietary exposition to cruciferous vegetables, grapefruit, alcohol and coffee may either induce or inhibit the expression of CYP1A2 (McLeold et al., 2000; Kotsopoulos et al., 2007; Bågeman E et al., 2008; Djordjevic et al., 2010; Arnaud, 2011). Frequent consumption of well-done meat that contains heterocyclic aromatic amines has been reported to elevate the risk of developing human cancers including breast cancers (Zheng et al., 1998; Hein et al., 2000; Williams and Phillips, 2000; DeBruin and Josephy, 2002; Turesky 2004). Increased activity of CYP1A2 has been associated with increased risks of breast cancer in some studies (Hong et al., 2004; Le Marchand, 2005; Shimada et al., 2009; Sangrajrang et al., 2009; Chang-Claude et al., 2010; Khvostova et al., 2012) while a number of other studies investigating the potential associations between CYP1A2 polymorphism and cancer risk have been inconsistent (Agundez, 2004; Long et al., 2007; Qiu et al., 2010; Singh et al., 2011; Lee et al., 2013).

The aim of this study was to compare CYP1A2 genetic polymorphisms and CYP1A2 enzyme activity in

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non smoker Tunisian controls and breast cancer patients. The effect of functional CYP1A2 variants [-3860G/A (rs2069514), -2467T/delT (rs3569413), -163C/A (rs762551)] and their interactions with environmental factors in breast cancer risk was investigated using a hospital-based case-control study. CYP1A2 enzyme activity was measured in breast cancer women and control volunteers using plasma caffeine metabolic ratios as well as caffeine half-life measured using reversed phase HPLC (Caubet et al., 2002). As CYP1A2 activity can be influenced by lifestyle, diet and environmental factors, a questionnaire was filled by each subject.

Materials and Methods

Study subjects

The study included non smoker Tunisian women, 28 healthy women and 20 breast cancer patients from the Farhat Hached Hospital. The protocol was approved by the Ethics Committee of Farhat Hached Hospital. Both oral and written explanations of the study were provided and the subjects gave their written informed consent.

All subjects refrained from taking coffee, tea, cola drinks, chocolate and any other caffeine-containing beverages and foods for at least 24 h before and throughout the study. They ingested a 140 mg dose of caffeine at 8 am. and a 5 ml blood sample was collected 2, 4 and 6 hours after caffeine administration. Plasma was then keep frozen until used for phenotypes analysis and from another 5 ml blood sample was prepared genomic DNA.

Caffeine phenotyping

The method employed in this study has been already published in detail (Mrizak et al., 2011) and is briefly described. Ammonium sulfate (300 mg) was added to 0.5 ml defrosted plasma sample. After 1 minute vigorous shaking (Vortex), 3 ml of ethyl acetate and isopropyl alcohol (8/2, v/v) were added. The sample was shook again for 2 minutes and then centrifuged at 4000 rpm for 25 minutes and 2.5 ml of the organic phases were collected, dried at 56°C under a flow of nitrogen gas and the residue obtained was dissolved in 100 µl KH_2PO_4 buffer solution.

HPLC (Agilent 1200) separation was performed using two reversed phase columns in series (Hypersil ODS 4×250 mm, 5 µm) and (Eclipse XDB-C18 4.6×150 mm, 5 µm) maintained at 33°C with a direct injection system of 5 µl. A UV diode array was used for the analytic detection. Elution was performed by gradient using two mobiles phases A and B. Phase A solution was composed of water, tetrahydrofurane (THF), acetic acid and acetonitrile (986/3/1/10 v/v respectively). The phase B solution was acetonitrile. The gradient program was chosen as follows: B was set at 0% for the first 20 minutes, increased linearly to 4% at 35 minutes, and then reached 8% from 45 to 50 minutes and return to 0% at 55 minutes.

Plasma caffeine and the concentrations of its metabolites were calculated by CHEMSTATION software using calibrations curves.

Caffeine plasma half life

Plasma caffeine half-life was determined from measurements of plasma levels performed on blood samples taken 2, 4 and 6 hours after caffeine administration. This measurement assess liver ability to metabolize caffeine through CYP1A2 enzyme activity. Plasma extraction and chromatography have been described (Mrizak et al., 2011) and plasma decay curves of caffeine were determined using semi logarithmic values and the method of least squares (Bchir F et al., 2006).

Plasma CMR

Plasma caffeine metabolic ratio is calculated from the peak areas of caffeine (137X) and its major primary metabolites, paraxanthine (17X), theobromine (37X) and theophylline (13X). The caffeine metabolic ratio (CMR) is then measured by calculating the molar ratio of (17X+13 X+37X)/ 137X (Tanaka et al., 1992).

SNP Genotyping

A sample of 5 ml of whole blood was collected from each subject in a K3 EDTA vacutainer tube (purple cap). DNA was extracted from cells using protocols for genomic DNA isolation with the Promega Wizard genomic DNA purification kit (Promega, Italy).

Genotyping was performed using commercially available TaqMan drug metabolism genotyping assays: C__15859191_30 'rs2069514', C__60142977_10 'rs35694136', C___8881221_40 'rs762551' (Applied Biosystems, FosterCity, CA, USA). Briefly, reactions were set up according to the manufacturer's instructions and the samples were run on a Steponeplus Real-Time instrument (Applied Biosystems, Monza, Italy). Allelic discrimination was performed using the SDS software v2.3 (Applied Biosystems). The 25 µl reactions in 96-well plates included 12.5 µl TaqMan Universal PCR Master Mix, No AmpErase UNG (2X), 1.25 µl Drug Metabolism Genotyping Assay Mix (20X) and DNA 11.25 µl (1 ng/µl). Quality-control measures included validation of the results by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping and the blind repeat of 10% of samples for CYP1A2 polymorphic sites (-3860 G/A, -2467T/delT and -163C/A), as previously described (Nakajima et al., 1999). Briefly for the RFLP analysis, all PCR reactions (25 µl) were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Monza, Italy), with each Master Mix (Life Technologies, Monza, Italy) comprising 0.2 mM dNTPs, 1 unit of Taq polymerase, the appropriate concentration of $MgCl_2$ (1.75 and 1.25 mM) and 0.4 µM of each primer (Life Technologies). Variants -3860 G/A, -2467T/delT and -163C/A were identified using DdeI, NdeI and ApaI restriction enzymes purchased from New England Biolabs .

Statistical analysis

Statistical comparisons of the characteristics of the population were made between cases and controls subjects using Fisher's exact test. To investigate whether CYP1A2 genotype was in Hardy-Weinberg equilibrium, distribution of the observed and expected genotype frequencies were compared using Pearson's χ^2 test.

Statistical comparisons of CMR mean values with

CYP1A2 genotype were obtained with the Mann–Whitney U-test and Pearson's χ^2 test was used to test the association between case and control caffeine half-life and genotype frequencies.

Results

For this study 20 patients with pathologically confirmed breast cancer and 28 healthy controls were recruited. Subject characteristics are presented in table 1 . It was confirmed that all subjects did not drink any beverages containing caffeine 24hr before coffee ingestion and throughout the study period and they have never smoked. There was also no significant difference for BMI between the cases and the controls. In contrast, control subjects were significantly better educated than breast cancer patients.

The relationship between the CYP1A2 genotypes and breast cancer risk is shown in Table2.

The expected genotype frequencies were not significantly different from the observed frequencies in breast cancer patients and controls when separately considered, indicating that they were in Hardy-Weinberg equilibrium.

The incidence of CYP1A2 -3860A indicated a decreased risk of breast cancer associated with this CYP1A2 genotype. By contrast, distributions of the CYP1A2 -2467T/delT or -2467delT/delT and -163A/C or A/A genotypes among breast cancer patients and controls were similar, indicating no overall effect of these polymorphisms on breast cancer risk.

The genotype and phenotype relationship was measured by comparing the mean CMR ratios (17X+13X+37X/137X) within the genotype groups and the caffeine half life between subjects and breast cancer patients. There were no significant differences in mean CMR ratios between the different SNP genotype groups in breast cancer patients and controls with the exception of -3860 A for which most of them are homozygous for the -3860 G/G SNP and had a significant higher mean CMR

Table 1. Characteristics of Sample Population

	Cases n= 20(%)	Controls N= 28(%)	p value
Gender			
Male	0(0)	0(0)	ND
Female	20(100)	28(100)	
Age (years)			P=0.0659
<60	17	28	
60+	3	0	
BMI			P=0.8564
Normal Range (18.5-24.99 kg/m^2)	8(40)	13(46)	
Underweight (<18.50 kg/m^2)	1(5)	0	
Overweight (25-29.99 kg/m^2)	10(50)	13(46)	
Obese (>29.99 kg/m^2)	1(5)	2(8)	
Instruction			P<0.0001
None	11(55)	0	
Elementary school	6(30)	2(8)	
Secondary school	3(15)	23(82)	
University	0	3(10)	
Smoking status			
Current	0	0	
Never smoking	20(100)	28(100)	ND
Alcohol drink			
Current	0	0	
Never	20(100)	28(100)	ND
Passive smoke			P<0.0001
Daily	9(45)	28(100)	
Less than daily	11(55)	0	

* Fisher's exact test

Table 2. CYP1A2 Genotype Frequencies in BC Cases and Controls and Risk of BC

CYP1A2 genotype	Cases		Controls		
	Observed Number	Expected[a]	Observed Number	Expected[a]	Pearson's χ^2 (P value)
-3860 G/G	19	19	21	21	
G/A	1	1	6	5	2.08(0.037)
-2467 T/T	11	11	18	18	
T/ delT	5	6	6	5	
delT/delT	1				0.71(0.48)
-163 C/C	2	3	4	5	
C/A	11	9	14	13	
A/A	5	6	9	9	1.79(0.07)

[a]According to Hardy–Weinberg law

Table 3. Relationship between CYP1A2 Genotype and Phenotype (Caffeine Metabolic Ratio) in BC Cases and Controls and Risk of BC Associated with the CYP1A2 Phenotype

CYP1A2 genotype	Cases				Controls				
	Observed Number	CMR[a] Mean±SD	N(%)[b]	Half life	Observed Number	CMR[a] Mean±SD	N(%)[b]	Half life	Pearson's χ^2 (P value)
–3860 G/G	16	3.07±1.77	8(50)	4.57±2.50	21	2.30±0.95	10 (48)	5.58±3.59	0.02(0.89)
–3860 G/A	1	2.13	1(100)	8.20	6	2.55±1.52	3 (50)	3.26±1.73	0.88(0.35)
–2467 T/T	11	3.51±1.97	6(55)	4.66±2.66	18	2.31±0.99	8 (44)	6.02±3.71	0.28(0.60)
–2467 T/ delTordelT/delT	6	2.15±0.46	2(33)	4.92±2.67	6	2.27±0.78	4 (66)	2.68±1.41*	1.33(0.25)
–163 C/C	2	2.56±1.34	1(50)	1.52±0.66	4	1.97±0.62	1 (25)	6.96±5.83	0.38(0.54)
–163 C/A or A/A	16	3.15±1.80	8(50)	4.83±2.56*	23	2.27±0.94	10 (43)	4.67±2.90	0.16(0.69)

Statistical comparisons of CMR mean value: Mann–Whitney U-test,*CYP1A2 –163 C/C *vs* –163 C/A or A/A p<0.05; CYP1A2 –2467 T/T *vs* –2467 T/ delT or delT/delT p < 0.05; [a]This caffeine metabolic ratio (CMR)=(17X+13X+37X/137X); [b]N(%) Above median value

ratio and half life than those with –3860 G/A (P=0.02)

These results demonstrated a significant association between CP1A2 -3860 G variant and CYP1A2 phenotype. Because of the small number of subjects recruited for this preliminary study, this association must be interpreted with caution.

Discussion

CYP1A2 activity was measured in 48 non smoker Tunisian women using CMR ratio of caffeine metabolites and caffeine half life in relation with SNP genotyping.

Both measurements of CMR and caffeine half life reflect the activity of CYP1A2. Other metabolic pathways involved in the first step of caffeine metabolism and not mediated by CYP1A2 such as the 8-hydroxylation of caffeine into trimethyluric acid is a minor pathway with about 1% excretion of the caffeine dose (Callahan et al., 1982). Caffeine half life thus reflect a better measurement of CYP1A2 activity than CMR as dimethylxanthines concentrations are quantitatively more dependent on other major pathways with 8-hydroxylations leading to dimethyluric acid and N-acetyl-transferase 2 enzyme activities producing AFMU (5-Acetylamino-6-formylamino-3-methyluracil), one of the most important caffeine and paraxanthine (67%) metabolite (Arnaud, 2011). As a consequence, caffeine is the most commonly used probe for NAT2 phenotype determinations (Grant, Tang and Kalow, 1984). Epidemiological studies have suggested that the NAT1 and NAT2 acetylation polymorphisms modify risk of developing cancers such as breast cancers (Hein et al., 2000).

Measurements performed on the two groups of women show that there was no significant difference between healthy women and women bearing breast cancer. Mean plasma caffeine half lives in these two groups were almost identical suggesting that the enzymatic activity of CYP1A2 as well as NAT are not significantly different.

This study thus failed to demonstrate a polymorphism of CYP1A2 in these two groups of women suggesting that environment factors or the small number of recruited volunteers did not allow the observation of trends. This preliminary study need to be completed by an extended larger clinical study. In a meta-analysis of published case-control studies, the same three polymorphisms were selected to evaluate their association with the risk of development of a cancer. It was shown a statistically significant higher risk for lung cancer for 2467T/delT and for carriers of the C-allele (163 C>A) there was an increased overall risk of developing a cancer in Caucasian population (Zhenzhen et al., 2013). Another meta-analysis performed in 46 case-control studies with a total of 22,993 cancer cases and 28, 420 healthy controls showed that the A allele of 163C>A polymorphism may increase the risk of breast cancer while this association was not found in other cancers (Tian et al., 2013).

Another study performed on 295 pre and postmenopausal women established relationships between CYP1A2 activity and risk factors for breast cancers showing that some associations were contrary to the hypothesis such as increased CYP1A2 activity may be associated with increased risk for breast cancer (Hong et al., 2004).

Our results obtained in Tunisian women are in agreement with a previous population-based case-control study of 1090 cases and 1183 controls of the Shanghai Breast Cancer Study, where it was shown in a subset of 236 study subjects that there was no association between CYP1A2 genotypes and breast cancer risk in Chinese women, although the C- and A-allele (163 C>A) polymorphism was found to be related to CYP1A2 activity (Long et al., 2006; Long et al., 2007). Another study performed on 411 BRCA1 mutation carriers in Canadian population (170 cases and 241 controls) showed that CYP1A2 genotype alone did not affect breast cancer risk (Kotsopoulos et al., 2007). In North Indian women as well, CYP1A2 polymorphisms do not play a significant role in breast cancer susceptibility (Singh et al., 2011).

However, in a female population of the Novosibirsk district including patients with breast cancer (n=335), and healthy women (n=530), the frequency of allelic variants of CYP1A2 showed that the genotype C/C of the CYP1A2 gene have an increased risk of development of breast cancer (Khvostova et al., 2012). CYP1A2 polymorphism was also suggested to modify susceptibility to breast cancer in 570 Thai women with histopathology confirmed breast cancer and 497 controls (Sangrajrand et al., 2009).

Breast cancer is a complex polygenic disease in the etiology of which genetic factors are known to play an important role but the effects of CYP1A2 polymorphisms are not yet clearly established and need further investigations.

Individual patients may differ significantly in the metabolism of drugs and xenobiotics and genetic factors explain part of the differences. Pharmacogenetic analysis can be used to predict the drug metabolizing potential of patients before starting therapy. A major challenge is thus to identify genetic polymorphisms which are relevant for a particular therapy and to determine the clinical consequences in relation with the choice of the drug and the dosing (van Schaik, 2008). For the cytochrome P450 system, which catalyzes oxidative reactions, many new genetic polymorphisms have been identified in the last past years. In this preliminary study it was shown a relationship between 3860 G/G and CYP1A2 activity but this observation must be confirmed with further large-sized case-control study.

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