Polymorphism Analysis of Genes Involved in Xenobiotic Metabolism and Circadian Rhythm in Human Breast Cancer

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Abstract

Individual response to xenobiotic exposures depends on the dynamics of xenobiotic metabolism and the circadian clock system, among other factors. Since these systems are closely related, polymorphisms in their key genes may have an impact on how carcinogenic compounds are metabolized, and therefore on the risk of tumor development. Whereas the mammary gland is exposed to agents that damage DNA, it was considered of high interest to study xenobiotic metabolizing genes (XMG) and clock genes in this tissue. Our aim was to analyze genotype and allele frequencies of polymorphisms in the XMG N-acetyl transferase 2 (NAT2) and glutathione S-transferase T1 (GSTT1), and in the clock genes period 3 (PER3) and CLOCK in human breast tumor samples. As well, it was if these polymorphisms were in linkage disequilibrium. 65 samples were genotyped for polymorphisms in GSTT1 (null), NAT2 (C481T, G590A and G857A), CLOCK (T3111C) and PER3 (length polymorphism), by PCR and PCR-RFLP. For GSTT1, 20% of the samples showed total absence of the gene. When NAT2 genotypes were grouped by their associated acetylator phenotype, 5% of rapid, 49% of intermediate and 46% of slow acetylator phenotypes were indicated. Allele frequencies for CLOCK were T=0.78 and C=0.22; for PER3, they were 0.66 for the 4-repeats allele and 0.34 for the 5-repeats allele. Linkage disequilibrium test indicated evidence of strong linkage between NAT2 and CLOCK (χ^2=13.076; p=0.005). With regard to allele and genotype frequencies, our results are in agreement with those reported for similar populations. The evidence of linkage disequilibrium in our breast cancer samples is interesting and requires further investigation. This work constitutes a first approximation to a combined study of polymorphisms in XMG and clock genes in breast cancer Argentinian patients. Future studies will attempt to address the role of these and other polymorphisms in cancer risk, prognosis and response to treatment.

Keywords

Breast Cancer; Xenobiotic Metabolism; Circadian Rhythm; NAT2; GSTT1; Clock Genes

Introduction

The way individuals respond to drugs, in terms of toxicity and treatment efficacy, and xenobiotics in general is highly diverse. Nowadays, it is well recognized that inherent differences in xenobiotic metabolism may have a considerable influence on the toxicity of these compounds, and their therapeutic efficacy in the case of drugs (Evans and Relling 1999). However, genetic differences in the metabolizing system are not the only cause of variation in the effects of xenobiotics. Numerous studies have demonstrated changes over 24 hours in all the processes determining the availability of drugs (Bruguerolle 1998). Therefore, the way the circadian clock works is essential to define the individual response to xenobiotics exposure.

Xenobiotic metabolizing enzymes (XMEs), coded by a large family of xenobiotic metabolizing genes (XMG), convert endogenous and exogenous compounds into derivatives that can be removed more easily from the body. However, these same enzymes can convert certain metabolites to carcinogens highly toxic and reactive. The existence of pharmacogenetic polymorphisms in XMG has been known for decades, usually associated with aberrations in the expression or enzyme function (Kalow 1962; Kalow 1992). As a consequence, elevated risks of some types of cancers have been associated with certain polymorphisms that cause an impaired ability to inactivate mutagenic molecules (Nebert 1988; W. W. Weber and Nebert 1990; Gonzalez and Nebert 1990).

N-acetyl transferase 2 (NAT2) and glutathione S-transferase T1 (GSTT1) are phase II XME, reported to be polymorphic. GSTT1 is involved in detoxifying carcinogens and reactive intermediates, and plays an important role in protecting against oxidative stress. A deletion of both copies of the GSTT1 gene results in

the complete loss of mRNA and protein product. Individuals lacking GSTT1 genes have a higher incidence of bladder, breast, lung and colorectal cancer (Jancova, Anzenbacher, and Anzenbacherova 2010). NAT2 is involved in the biotransformation of aromatic amines and hydrazines. Several missense and silent substitutions have been described in the coding exon of NAT2, responsible for the acetylator phenotype. NAT2 *4 allele is considered as the wild-type due to the absence of such mutations, and represents the rapid acetylator phenotype. NAT2*5, *6 and *7, among others, define alleles associated with slow acetylation. The different acetylator phenotypes are predisposing factors for the sensitivity of individuals to toxicity; in fact, NAT2 slow acetylation genotype has been associated with risk of developing non-Hodgkin lymphoma, lung, colon, liver and bladder cancer (Agúndez 2008).

The metabolism of xenobiotics is influenced by the circadian clock, as well as the rest of the physiological functions. The central clock is located in the suprachiasmatic nuclei of the hypothalamus, and peripheral clocks are located in many tissues (Schibler, Ripperger, and Brown 2003; Levi and Schibler 2007). The genes that control circadian functioning are well conserved among mammals, and include CLOCK, BMAL1, PER and CRY families and a large array of complementary genes (Sehgal 2004).

A small set of clock-controlled genes (CCG) is expressed in multiple organs, encoding key regulators of cell cycle progression and apoptosis (Fu and Lee 2003; Zhao and Lee 2010). Based on this evidence, Fu and Lee (Fu and Lee 2003) suggested that the circadian clock may function as tumor suppressor. In addition, "the circadian genes hypothesis" has also been proposed (Hoffman et al. 2009), which suggests that genetic variants of clock genes can affect individual susceptibility to cancer, given the results of epidemiological studies (Zhu et al. 2005; Zhu et al. 2006; Zhu et al. 2008; Hoffman et al. 2010; Dai et al. 2011; Chu et al. 2008).

CLOCK is a transcription factor with an essential role in the feedback loop involved in the circadian clock operation. Single nucleotide polymorphism T3111C, located in the 3'-UTR region of CLOCK could affect the phenotype by disrupting microRNA binding sites. Furthermore, it seems to affect both stability and half-life of the mRNA (Mignone et al. 2002). A correlation between polymorphisms in CLOCK and breast cancer risk has been reported (Hoffman et al. 2010), with apparent effect modification by estrogen receptor or progesterone receptor status. Period3 (PER3) belongs to the Period gene family and is another central component in the clockwork mechanism. PER3 gene has a length polymorphism (VNTR, variable number tandem repeats), consisting of 4 or 5 copies of a 54 bp repeated sequence. As a result, a protein with an indel of 18 amino acids is obtained. The least number of amino acids that are phosphorylation substrates, in the short variant, may have a functional impact on the phosphorylation-dependent activity, such as on stability and translocation of PER3 to the nucleus. Genotypes 4/5 and 5/5 in this gene have been associated with an increase in breast cancer risk among premenopausal women (Zhu et al. 2005).

It is noteworthy that transcription factors CAR, PPARa, AhR/Arnt and PARbZip are regulated by the clock, generating circadian expression patterns in many XMG, which changes the way that xenobiotics are metabolized over 24 hours (Lim et al. 2006; Gachon and Firsov 2011). Given the close relationship between the circadian clock and metabolic processes, it is possible that polymorphisms in key genes of both systems have an impact on how carcinogenic compounds are metabolized, and therefore on the risk of tumor development. For Argentina, breast cancer is the most common cancer in women, a feature that is repeated throughout the world (GLOBOCAN - IARC 2008). Whereas the mammary gland is exposed to agents that damage DNA, it was considered interesting to study XMG and clock genes in this tissue. Our work aim at analyzing the genotype and allele frequencies of polymorphisms in the XMG NAT2 and GSTT1, and in the clock genes CLOCK and PER3 in human breast tumor samples. It was also investigated if these polymorphisms were in linkage disequilibrium, in order to get information about possible combinations of alleles at different loci that could be related to breast cancer. Currently, there is no conclusive evidence that associate the polymorphisms with increased/decreased risk of disease. Moreover, allelic frequencies can differ between populations. This study is one of the first to evaluate the allelic and genotypic frequencies of these genes, for an Argentinian population of women with breast cancer, constituting a basis for future association studies.

Materials and Methods

Samples

This study included 65 DNA samples from female patients with breast cancer, between 30 and 85 years

old, who were previously analyzed for NAT2, GSTT1 and GSTM1 polymorphisms (Pavicic, Laguens, and Richard 2009). All patients were Argentinian citizens. Briefly, samples were obtained from a Clinic Centre in La Plata City, Buenos Aires. Each patient signed an informed consent, approved by the Ethics Clinic Committee. Breast tumor tissue samples were obtained from biopsies or surgery, between 1997 and 2004, prior to radio- and/or chemotherapy. Those tissues that showed 85% or more of abnormal cellular composition according to routine histopathological techniques, were considered tumoral. DNA extraction was performed as previously described (Pavicic, Laguens, and Richard 2009).

XMG Analysis

Polymorphisms in the genes NAT2 and GSTT1 of the phase II of detoxification system were detected by PCR and PCR-RFLP. Most of the samples were already genotyped in previous studies of our group (Pavicic, Laguens, and Richard 2009); here the reactions in those samples were employed that could not be processed before because of lack of DNA or problems with the techniques. As a result, this study included a different set of samples that those previously published. All the genotypes (published and unpublished) were included in the current combined analysis of genetic polymorphisms in XMG and clock genes.

1) GSTT1 Gene

GSTT1 gene deficiency resulted from the deletion of the locus (null allele). The detection was performed by duplex PCR with CYP1A1 as an internal positive control, in a final volume of 15 µl with 1X buffer, 50 ng DNA, 1.5 mM MgCl2, 200 µM dNTPs, 0.25 µM and 0.12 µM primers for GSTT1 and CYP1A1, respectively, 0.45 U Taq Platinum Polymerase (Invitrogen, Life Technologies), and H2O up to 15 µl. GSTT1 forward primer 5'-TCCTTACTGCTCACAGTCCT-3' and GSTT1 reverse primer 5'-TCACCGGATCATGGCCAGCA-3' were applied for amplification of 490 bp fragment; CYP1A1 forward primer 5'-GGACTGCTGCCATCTCAGCTT-3' and CYP1A1 reverse primer 5'-CAGCATCTGGAATTGGTGCTC-3', were used to amplify the internal control with a product of 312 bp. PCR reactions were carried out using an initial denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension at 72°C for 5 min. Verification of PCR products, and subsequent identification of genotype, were

performed using 2% agarose gels stained with GelRed (Biotium Inc., CA, USA.)

2) NAT2 Gene

By PCR-RFLP, we studied the polymorphisms C481T (dbSNP ID: rs1799929), G590A (dbSNP ID: rs1799930) and G857A (dbSNP ID: rs1799931). The alleles defined by these substitutions, called *5, *6 and *7, respectively, are associated with slow acetylator phenotypes (University of Louisville 2012).

We amplified a 578 bp fragment, in a final volume of 20 µl, with 1X buffer, 50 ng DNA, 1.5 mM MgCl2, 200 µM dNTPs, 0.25 µM of each primer, 0.6 U of Taq Platinum Polymerase and H2O up to 20 µl. PCR parameters were as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min of annealing at 58°C and 1 min of extension at 72°C, ending with an extension of 5 min at 72°C. Primers used were: forward 5'-GATTCTATAGAGAGGAGCAC-3' and reverse 5'-GGATCCAGCTCCCAGTGATG-3'. After checking amplification in 2% agarose gels, we proceeded to digest 5 µl of PCR product in a final volume of 15 µl, using 5U of restriction enzyme in an appropriate buffer for KpnI (C481T) and TaqI (G590A). In the case of BamHI (G857A), 2.5 µl of PCR were digested in a final volume of 10 µl, also using 5U of enzyme. Incubation time was 5 hours at 37°C for KpnI and BamHI, and at 65°C for TaqI. Digestion products were visualized independently, in 4% low melting point agarose gels, stained with GelRed (Biotium Inc., CA, USA.).

Clock Genes Analysis

CLOCK and PER3 polymorphisms were detected by PCR and PCR-RFLP; and all tumor samples were processed for this study.

1) CLOCK Gene

SNP T3111C (dbSNP ID: rs1801260) was detected by PCR amplification of a region of 221 bp, with subsequent digestion with Bsp 1286I, as described in Lattuada et al. (2004).

2) PER3 Gene

PER3 length polymorphism (Accession No. AB047536) consists of 4 or 5 copies of a repeated sequence of 54 bp, located in exon 18. The detection of allelic variants was performed by PCR, using the primers forward 5'- TGGTCCCAGCAGTGAGAGT

-3' and reverse 5'-CCAGATGCTGCTCTACCTGAACC -3'. The reaction conditions were as follows; in a final volume of 15 μl: 1X buffer, 50 ng of DNA, 0.25 mM of each primer, 200 μM dNTPs, 1.5 mM MgCl2, 0.45 U of Taq Platinum Polymerase and H2O up to 15 μl. PCR cycling consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, annealing at 57°C for 1 min and elongation at 72°C for 1 min, with a final extension at 72°C for 5 min. The products (261 bp and 315 bp) were visualized in 2% agarose gels, stained with GelRed (Biotium Inc., CA, USA.).

Statistical Methods

Genotype and allele frequencies were calculated by simple gene counting. Population analyses (Hardy-Weinberg and Linkage Disequilibrium) were carried out using the software Arlequin 3.5 (Excoffier and Lischer 2010).

Results

The present study evaluated the genotype distribution for the xenobiotic metabolizing enzymes GSTT1 and NAT2, and for the clock genes CLOCK and PER3, in 65 breast tumor samples from argentinian women.

Polymorphism analysis could be performed in 60 to 62 samples, of the 65 available samples. Linkage disequilibrium analysis was performed only with samples in which genotypes were complete for all genes (55 samples). The results of genetic analysis are summarized in Tables I-III.

TABLE I DISTRIBUTION OF CASES ACCORDING TO GSTT1, NAT2, CLOCK AND PER3 GENOTYPES

Polymorphisms		Cases N (%)
GSTT1	+/+; +/null	48 (80)
	null/null	12 (20)
NAT2	Wild-type homozygotes	3 (5)
	Heterozygotes	30 (49)
	Slow homozygotes	28 (46)
CLOCK	T/T	36 (58)
	T/C	25 (40)
	C/C	1 (2)
PER3	4/4	27 (44)
	4/5	27 (44)
	5/5	7 (12)

Tumor samples were in Hardy-Weinberg equilibrium for all loci, except for GSTT1 due to the limitations of the detection technique.

For GSTT1, 20% of the samples showed total absence of the gene (genotype null/null). When NAT2 genotypes were grouped by their associated acetylator

phenotype, 5% of rapid acetylator phenotypes, 49% of intermediate phenotypes, and 46% of slow acetylator phenotypes were found. The frequency of the CLOCK homozygous T/T genotype was 58%, the heterozygous genotype T/C was 40% and the homozygous genotype C/C was 2%. The observed allele frequencies were T = 0.78 and C = 0.22. In the case of PER3, 44% of the samples were homozygous 4/4, an equal percentage was heterozygous 4/5, and the remaining 12% was homozygous 5/5. Allele frequencies were 0.66 for the 4-repeats allele and 0.34 for the 5-repeats allele (Tables I and II). Published allele frequencies for the genes under study are shown in Table II as reference.

TABLE II OBSERVED ALLELE FREQUENCIES FOR GSTT1, NAT2, CLOCK AND PER3 IN BREAST TUMOR SAMPLES. PUBLISHED FREQUENCIES FOR OTHER POPULATIONS ARE PLACED AS REFERENCE

Alleles		Observed frequencies	Published frequencies	
GSTT1	null/null	0.200	0.110-0.212	(Garte et al. 2001; Bailliet et al. 2007; Moore et al. 2004; Cotignola et al. 2013)[1]
NAT2	*4	0.295	0.226-0.512	(Garte et al. 2001; Bailliet et al. 2007)[2]
	*5	0.303	0.250-0.460	
	*6	0.352	0.061-0.285	
	*7	0.049	0.029-0.201	
CLOCK	T	0.780	0.740-0.770	(Ciarleglio et al. 2008; Casiraghi et al. 2010)[3]
	C	0.220	0.230-0.260	
PER3	4	0.660	0.630-0.660	(Ciarleglio et al. 2008; Casiraghi et al. 2010)[3]
	5	0.340	0.340-0.370	

[1] Published frequencies for Caucasians, native Amerindians from Argentina and Paraguay, and populations from Córdoba and Buenos Aires (Argentina)

[2] Published frequencies for Caucasians and native Amerindians from Argentina and Paraguay

[3] Published frequencies for European Americans and a population from Buenos Aires (Argentina)

Linkage disequilibrium test performed by Arlequin software (Table III), between all loci in Hardy-Weinberg equilibrium, indicated evidence of strong linkage between NAT2 and CLOCK (χ^2 = 13.076; p = 0.005).

TABLE III LINKAGE DISEQUILIBRIUM TEST. THE TABLE SHOWS x^2, p AND DEGREES OF FREEDOM FOR EACH PAIR OF LOCI

Pairs		
NAT2/CLOCK	χ^2	13.076
	p	**0.005**
	d.f.	3
NAT2/PER3	χ^2	3.590
	p	0.309
	d.f.	3
CLOCK/PER3	χ^2	0.110
	p	0.740
	d.f.	1

Discussion

Numerous studies have demonstrated that environmental and estrogen-related exposures play a role in breast cancer etiology (Kocabas 2002). There is evidence that several polymorphisms in enzymes involved in the metabolism of endogenous and exogenous compounds, increase the risk of developing cancer (Dunning et al. 1999; Autrup 2000). Moreover, genetic variants in key genes of the circadian clock may also alter the individual cancer susceptibility, through the regulation of CCG involved in proliferation and cell cycle control. Under this hypothesis, a number of studies have investigated the association between clock genes polymorphisms and certain types of cancers (Zhu et al. 2005; Zhu et al. 2006; Zhu et al. 2008; Hoffman et al. 2010; Dai et al. 2011; Chu et al. 2008).

Although the risk of breast cancer due to genetic alterations in the genes analized here may not be as intense as the risk due to high penetrance genes, such as BRCA1 and BRCA2, they affect a higher percentage of individuals, leading to a higher impact in the population as a whole (B. L. Weber and Nathanson 2000). However, analyses done with mixed racial American population have exhibited conflicting results.

GSTT1 deletion frequency varies among different populations (Garte et al. 2001). The prevalence of the GSTT1 null genotype is lower among Caucasians (10–20%) compared with Asians (50–60%) (Nelson et al. 1995). NAT2 slow acetylator phenotype prevalence also varies worldwide. It is estimated that it is present in 10-20% of Asians, 35% of African Americans, 65-90% of individuals of Middle Eastern descent, and about 55% of whites (Ambrosone et al. 2011). At the present time, it is accepted that the association between polymorphisms of NATs and cancer susceptibility exists, but this relation is modest and generally involves genes of phase I and II simultaneously.

Bailliet et al. (2007) determined genotype and allele frequencies of several XMG, in a sample of healthy individuals from 8 Native American populations from Argentina and Paraguay, identified as Amerindians. As regards NAT2, the allelic frequencies obtained differ from those reported by Bailliet et al., probably due to different ethnic origin of samples.

In another study conducted in the province of Cordoba, Argentina, with the aim to analyze risk factors for bladder cancer, researchers detected 16% and 11% of GSTT1 deletion in cases and controls,

respectively (Moore et al. 2004). Moreover, Cotignola et al. (2013) informed a 21,2% of GSTT1 deletion in 105 patients from Buenos Aires, Argentina, diagnosed with prostate cancer. In our study, 20% of samples with complete absence of GSTT1 was found. Although this percentage is close to reported values for other populations in Argentina, and for Caucasians in general, it represents one of the highest frequencies reported for the deletion in populations with similar ethnic origins.

Argentinian population is highly heterogeneous because of the ethnic admixture of people from Europe, Africa and the native Amerindians. This admixture is spread along the country, with predominance of certain groups depending on the region. This heterogeneity means that, at present, genetic contributions of Native Americans, Europeans and Africans to the general population vary between different regions (Motti et al. 2009).

As in the case of XMG, clock genes also differ in their allelic frequencies between different populations in the world. Ciarleglio et al. (2008) conducted a large study with population samples of African Americans, European Americans, Chinese and Africans. Our results are highly similar to those reported for European Americans. Furthermore, for the allele of 4-repeats of PER3, Nadkarni et al. (2005) showed that European populations and African Americans have intermediate allelic frequencies, between 0.6 and 0.7 approximately.

In another study conducted in Argentina, Casiraghi et al. (2010) compared two local populations, one of healthy individuals and the other in which people were affected by bipolar disorder. The frequencies obtained for the control group (n = 39) were 0.63 for the 4-repeats allele and 0.37 for the 5-repets allele of PER3, and 0.77 for T allele and 0.23 for C allele of CLOCK. The frequencies obtained in our samples from patients with breast cancer are consistent with those reported by Casiraghi et al., and in general, with those observed in large population groups.

In summary, NAT2 allelic frequencies observed in this study were different from those reported in the literature; it is thought that it is related to ethnic causes. As regards GSTT1, CLOCK and PER3, the frequencies observed in our breast tumor samples were within the frequency ranges published, as shown in Table II. Stratifying the samples according to tumor or patient characteristics may be a strategy to find differences in gene frequencies that affect cancer development,

instead of analyzing the samples as a whole.

The linkage disequilibrium test performed in this study indicated a strong linkage between NAT2 and CLOCK. Up to date, there are no scientific publications that evaluate the impact of allelic variants of circadian genes and XMG together in the context of tumor development. It may be interesting to test, in a larger sample, if certain loci are in linkage disequilibrium in the population under study. This would indicate that some allelic combinations occur more frequently than other in cancer patients, thus having an impact on cancer development.

A key limitation to arrive at more meaningful conclusions in this study is the low number of samples analyzed. Future studies should include a greater number of cases and appropriate controls, in order to survey the impact of polymorphisms in cancer development. In addition, it will be required to incorporate patient information.

Conclusions

This work constitutes a first approximation to a combined study of polymorphisms in XMG and clock genes in breast cancer Argentinian patients. A potential role for polymorphisms in breast cancer risk, prognosis and response to treatment merits further investigation. The possibility to identify individuals at increased risk would provide a useful tool in the fields of public health and preventive medicine. Given the central role of the circadian clock mechanisms in cell proliferation, cell cycle regulation, and apoptosis, and also the importance of the xenobiotic metabolizing system in absorption, distribution, metabolism and elimination of carcinogen compounds, a large amount of human genetic polymorphisms in XMG and clock genes are under characterization; and some of them show a correlation with risk of cancer, while the others remain equivocal and demand more investigation.

Analyses of other polymorphisms of clock genes and XMG, as well as of mRNA expression in tumor tissues involving a large number of individuals are being carried out in our laboratory.

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