Association of Slow Acetylation Profile of *NAT2* with Breast and Gastric Cancer Risk in Brazil

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Abstract. *Background: The N-acetyltransferase 2 (NAT2) gene is a marker for the study of interindividual susceptibility to developing neoplasias. The purpose of this study was to verify a possible association between single nucleotide polymomorphisms (SNPs) of NAT2 and the susceptibility to gastric cancer (GC) and breast cancer (BC) in patients from the North region of Brazil. Materials and Methods: Five SNPs of the NAT2 gene were investigated by direct sequencing. Ancestry was estimated by analysis of a panel with 48 ancestry-informative markers (AIM). Results: Individuals with slow acetylation profile had an increased risk of developing neoplasias up to three times when compared to controls. Conclusion: In this study, slow acetylation profile was found to strongly influence susceptibility to GC and BC.*

Gastric cancer (GC) is the fourth most common type of neoplasias worldwide (1, 2) and breast cancer (BC) has the highest incidence and is the leading cause of female death (2). In Brazil, GC represents the fifth most common type of tumor among the male population and BC is the second most common among the female population (3). Studies of genetic susceptibility to cancer using single nucleotide polymorphisms (SNPs) of the genes that encode the enzymes responsible for the biotransformation of carcinogenic agents may identify the at-risk population and help to clarify the etiology of a given tumor type (4-6). *N*-acetyltransferase 2 gene is a marker for the study of interindividual susceptibility to develop neoplasias; the enzyme NAT2 takes part in the metabolism of carcinogenic agents and SNPs of its gene produce enzymes with different activities, leading to slow or fast acetylation of xenobiotics, such as aromatic and heterocyclic amines (7-9). The purpose of this study was to verify a possible association between SNPS of *NAT2* gene and the susceptibility to GC and BC in patients from the North region of Brazil. An ancestry genomic assay of case and control samples was carried out to estimate the individual inter-ethnic admixture for the volunteers taking part in this study.

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Materials and Methods

Study population. The investigated participants were chosen according to a case control study. The sample cohort consisted of 63 patients from João de Barros Barreto University Hospital, all of them were diagnosed with gastric adenocarcinoma, and 70 patients from Ophir Loyola Hospital, diagnosed with invasive ductal breast carcinoma, both hospitals located in the city of Belém, Brazil. For both groups, risk factors related to the development of neoplasias were established, such as advanced age and gender. For the case group, we also collected other risk factors for the development of neoplasia, such as alcoholism and tobaccoism. The study control population was composed of 89 participants without cancer, living in the North region of Brazil. The protocol used in this study was approved by the Committee of Research Ethics from the João de Barros Barreto University Hospital (protocol number 3505/2004) for gastric cancer samples and by the Committee of Research Ethics from the Tropical Medicine Center of Federal University of Pará (protocol number 043/2008) for breast cancer samples. All patients recruited for this study provided written informed consent to their participation.

Genomic DNA extraction. From each participant, 5 ml of peripheral blood was collected by using EDTA as anticoagulant. DNA extraction was carried out according to the method described by Sambrook *et al.* (10), with modifications. The concentration of DNA was estimated by

the use of a GeneQuant RNA/DNA spectrophotometer (Amersham, Pharmacia Biotech, Cambridge, UK).

Genotyping of NAT2 gene. Five polymorphisms of great importance for the definition of NAT2 profile (C282T, T341C, C481T, A803G and G857A) were investigated by direct sequencing of 986 base pairs, amplified in two reactions, with the following primers: *NAT2*-1F (5'-TTA ATT CTC ATC TCC TGC CAA AGA-3'), *NAT2*-1R (5'-TCA CTC TGC TTC CCA AGA TAA TCA-3'); *NAT2*-2F (5'-ATG GAG TTG GGC TTA GAG GCT AT-3'), NAT2-2R (5'-CTT TGG CAG GAG ATG AGA ATT AAG A-3'). The choice of the primers was made by using the software Primer 3 (11). An amplification was carried out in an ABI Verity thermocycler (Applied Biosystems, Foster City, CA, USA). The standard protocol used: 20 pmol of each oligonucleotide, 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 3 U Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 ng of genomic DNA in a 25 μl reaction volume. Samples were incubated at 95˚C for 3 min, followed by 35 cycles of 94˚C for 2 min, 60˚C for 1 min and 70˚C for 2 min, with a final extension at 70˚C for 30 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gels. PCR products were purified with Purelink kit (Invitrogen Life Technologies). Sequencing was carried out on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed for the identification of SNP by alignment with the reference sequence (GenBank accession X14672). The nomenclature of *NAT2* genotype is given in accordance with that described in 'The Consensus Gene Nomenclature of Human *NAT2* Alleles' (12).

Estimates of individual inter-ethnic admixture. The population from the North region of Brazil, the target of this study, is composed of an inter-ethnic admixture of three ancestry populations: African, European and Native American (13). In order to avoid spurious interpretations resulting from the population substructure, we used a panel with 48 AIMs (13). In this way, we estimated the individual inter-ethnic admixture and checked the ancestry for the study's case and control samples.

Statistical analysis. Allele frequencies at individual loci were estimated by the Hardy-Weinberg equilibrium exact test was performed using the GENEPOP software (14). Linkage disequilibrium estimates (D and D`) and the maximum likelihood estimate of haplotype frequencies were calculated with the software PHASE v.2.1 (15). Estimates of Individual inter-ethnic admixture were carried out by using the software Structure v.2.2 (16). All other statistical analyses were performed by using the statistical software SPSS v.12.0 (SPSS, Chicago, IL, USA). Group comparisons for categorical variables were carried out using the χ^2 test, while Student's *t*-test was used for the analysis of continuous variables. Odds ratios (OR) and confidence intervals (95% CIs) were also calculated. Multiple logistic regression analyses were carried out using the backward model. The comparison of ancestry index among the samples was carried out by using the Mann Whitney test. All statistical tests were based on a two-tailed probability, and a *p*-value ≤0.05 was considered significant.

Results

Inter-ethnic admixture estimates in cancer patients and controls. Figure 1 shows the individual parental ethnic contribution of case and control groups estimated through 48 AIMs. Table I lists the average of ancestries between the investigated groups. Based on individual ancestry estimates, it was possible to estimate the averages of such ancestries among the investigated groups, additionally to testing for differences. We found statistical differences for African and European parental contribution when compared between the cancer and control groups; a higher African contribution was detected in the study group with cancer and, in the control group, a higher European contribution was detected ($p<0.001$). The Amerindian parental group was not statistically different when compared to ancestral groups. In order to control the substructure effects, we made use of the individual inter-ethnic admixture estimates as interference factors in statistical analyses of association between genetic markers and two investigated tumor types.

NAT2 gene SNPs frequencies associated with cancer susceptibility. Table II describes the frequencies of the five polymorphisms and their respective haplotypes associated with the acetylation profile in the group of patients with cancer. The most frequent allele was 481T, present in 38% of patient samples, while the least frequent was 857A (10%). All polymorphisms of *NAT2* gene showed a linkage disequilibrium (D'>0.80; $p\leq0.05$). Thirteen (13) haplotypes derived from the five investigated polymorphisms were obtained (Table II). The most frequent haplotype found in association with fast acetylation profile was *NAT2**4 (26%), followed by *NAT2**13 (20%), *NAT2**12 (4%) and *NAT2**11 (3%). As for xenobiotic slow acetylation haplotypes, the most frequent ones found were: *NAT2**5 (37%) and *NAT2**7 (10%). The results of the haplotypes generated show a frequency of 53% for the alleles associated with the fast acetylation profile, while 47% were described as slow acetylation profile. Genotypic ratios found were in accordance with those expected by the Hardy Weinberg equilibrium, in all groups analyzed. Estimate relative risks in the groups with cancer (made up by combining GC and BC) were computed in relation to the control group. The results for separately-investigated polymorphisms showed a significant effect for SNP C282T only. Genotypes from the dominant polymorphism C282T (TT+CT) had a significant association ($p<0.001$; OR=3.076; 95%CI=1.664-5.687) with susceptibility to the different forms of cancer investigated. Separate analysis of the polymorphisms in *NAT2* gene showed that four SNPs (C481T, A803G, G857A and T341C) were not important in the association with the susceptibility to cancer.

NAT2 genotype associated with cancer susceptibility. Table III shows the distribution of participants according to the characteristics of the haplotypes and their acetylation profiles. For classification purpose, three genotype groups were defined: i) fast genotype, including these with two fast acetylation alleles; ii) intermediate genotype, with one fast

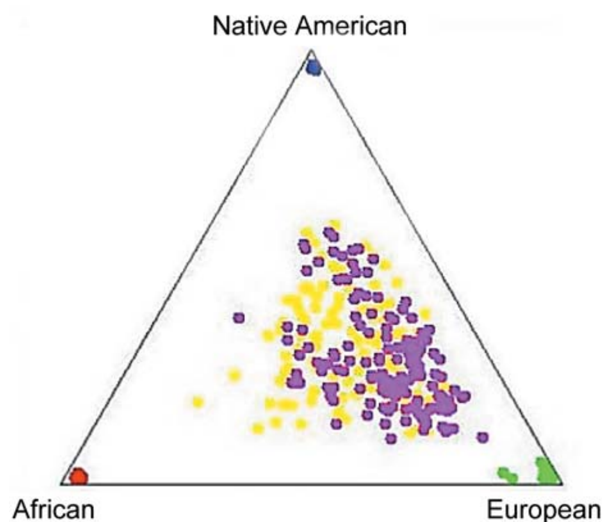

Figure 1. *Individual estimate of inter-ethnic admixture between case and control groups. European, African and Native American ancestries were estimated with the genotyping of the panel of 48 ancestry-informative markers in the groups of patients with cancer (magenta) and controls (yellow). The admixture is estimated by a comparison with subjects from the parental populations: European (green), African (red) and Native American (blue).*

Table I. *Ancestry statistics for patients with cancer and controls.*

Ancestry	Control (n=89)	Cancer (n=133)	*p*-Value[b]
Native American	0.28±0.11	0.30±0.12	0.171
European	0.52±0.12	0.44±0.14	<0.001
African	0.20±0.01	0.26±0.11	<0.001

Data are means±standard deviation for quantitative data. [a]Patients with two different forms of neoplasia: gastric and breast cancer. [b]Mann Whitney test.

acetylation allele and one slow acetylation allele; iii) slow genotype, with two slow acetylation alleles. Genotype *NAT2* *5/*5 was the most predominant (58%) among genotypes for slow acetylation identified in the study group with cancer. A significant association of slow and fast acetylation profile with the susceptibility to develop neoplasias investigated was detected (*p*=0.010; OR=3.054; 95%CI=1.303-7.159) and (*p*=0.041; OR=0.527; 95%CI=0.280-0.973). We showed that individuals with a slow acetylator profile have increased odds of developing neoplasias, up to three times when compared to controls. In this context, we also established that individuals who are homozygotes for fast acetylation have 53% protection against the development of a tumor (GC and BC) compared to those with other genotypes.

Discussion

The Brazilian population is one of the most heterogeneous population worldwide, with a contribution from three parental groups: Amerindians, Europeans and Africans (13, 17). Therefore, in studies of genetic association to diseases, the addition of a population structure estimate is of great importance aiming at identifying and correcting possible effects of the population substructure (13). In order to avoid spurious interpretations resulting from the population substructure, we used a panel with 48 AIMs (13). This panel has already been used in other studies of genetic association with diseases (18-21). A higher African contribution was detected in the study group with cancer. Kittles *et al*. found a similar population substructure among African Americans for prostate cancer (22). The genomic control by ancestry is able to correct distortions in the analysis of association as evidenced in other work (23, 24). The important role of control genomic ancestry in association studies is clear especially in populations with a high degree of mixing between ethnic groups, such as the Brazilian population. Our results on the association of the *NAT2* gene SNPs with the risk of different forms of cancer investigated suffered no distortion with the use of genomic ancestry. Several studies have revealed the role of genomic ancestry as a risk factor associated with different types of cancer (25-26). A meta-analysis published by Ali *et al*. found strong evidence that specific types of tumors are more prevalent in certain ethnic groups (27). In these studies, five different SNPs were identified through the sequencing of *NAT2* gene (T341C, C481T, A803G, C282T, G857A), which are some of the most important polymorphisms for determining the enzyme's acetylation speed (28, 29). Our study found similar results to association of diseases in the Brazilian population and in the Northern part of the country, which also investigated the *NAT2* gene which found a high frequency of C481T polymorphism (35% and 38%) and low frequency of G857A (4% and 9%) (28,30). The most frequent haplotype found in association with the fast acetylation profile was *NAT2**4 (26%), followed by *NAT2**13(20%), *NAT2**12 (4%) and *NAT2**11 (3%). This higher frequency for the fast acetylation haplotype *NAT2**4 is similar to with another study developed within the same population (19%) (30). In our study, for slow acetylation haplotypes, the most frequent SNP was *NAT2**5 (37%). A study which investigated a Brazilian population found a frequency of 33% for *NAT2**5 and in that study, the authors suggest an association of this haplotype's high incidence with the influence from European and African ancestral groups in the ethnic composition of the Brazilian population, considering the high incidence of NAT2*5 in these two parental populations (17, 29). In that study, the isolated analysis of the polymorphisms in *NAT2* gene showed that only the SNP C282T was important in the association with the susceptibility to cancer. There is no consensus in the literature concerning the individual contribution of C282T

Table II. *Single-nucleotide polymorphisms of N-acetyltransferase 2 gene and haplotypes in patients with cancera from the North region of Brazil.*

Nucleotide	282 T	481 T	803 G	857 A	341 C		
Amino acid	None	None	K268R	G286E	I114T		
Frequency	0.30	0.38	0.27	0.10	0.37		
Haplotype	C282T	C481T	A803G	G857A	T341C	Frequency	Phenotype
*NAT2**4	C	C	A	G	T	0.2556	Fast
*NAT2**5A	.	T	.	.	C	0.1278	Slow
*NAT2**5B	.	T	G	.	C	0.2181	Slow
*NAT2**5C	.	.	G	.	C	0.0113	Slow
*NAT2**5D	.	.	.	.	C	0.0113	Slow
*NAT2**7A	.	.	.	A	.	0.0113	Slow
*NAT2**7B	T	.	.	A	.	0.0865	Slow
*NAT2**7C	T	.	G	A	.	0.0037	Slow
*NAT2**11A	.	T	.	.	.	0.0301	Fast
*NAT2**12A	.	.	G	.	.	0.0263	Fast
*NAT2**12B	T	.	G	.	.	0.0113	Fast
*NAT2**12C	.	T	G	.	.	0.0037	Fast
*NAT2**13A	T	.	.	.	.	0.2030	Fast

[a]Patients with two different forms of neoplasia: gastric and breast cancer.

polymorphism to the genetic susceptibility to cancer; however, this is the most important SNP in the definition of haplotype *NAT2**13, which characterizes the fast acetylation profile and which, in turn, is well-described in the literature to be associated with risk for cancer (31-33). Major SNPs used in the definition of the acetylation profile have already been described with high frequencies in different ethnic groups. Within the Brazilian population the C282T SNP was found at a significantly higher frequency in European descendants (17), and haplotype *NAT2**13 was associated with higher contribution from African ancestry (29,34). These parental populations were particularly significant in our study of comparing the groups of cancer patients and controls.

Different studies involving susceptibility to BC obtained similar results, with greater predominance of fast acetylators (29%) in patients (35). On the other hand, another study with BC found higher frequency of slow acetylators 57% (36). The genotype *NAT2**5/5 was the most predominant (58%) among genotypes for slow acetylation identified in the study group with cancer; in the literature, similar results were found concerning slow acetylation profile (17, 29, 30). We showed that individuals with a slow acetylator profile have probability of developing neoplasia (GC and BC) increased to up to three times when compared to controls (p=0.010; OR=3.054). The findings of several authors were similar to ours for the association of a slow acetylator profile with the risk of developing GC (37, 38) and BC (39-41), separately.

NAT2 is predominantly expressed in the liver, gastrointestinal tract and colon; although mRNA is detected in several other tissues at basal levels (8, 42). The metabolic profile of the enzyme can be associated with the susceptibility to several tumors types in different organs, depending on the expression variation of the enzyme in such organ (43). Hein *et al*. published a review mentioning molecular studies of *NAT2* gene in association with different forms of neoplasia (43). In their work, the influence of the acetylation profile variation on the increase of the risk of developing several types of tumor is clear. Moore *et al*. carried out a global meta-analysis and showed the great influence of a slow acetylation profile on bladder cancer (44).

Our findings strengthen studies which associate the slow acetylation profile with susceptibility to different forms of cancers lung (45), breast (40, 41), bladder (46, 47), cervical (48), gastric (37, 38), head and neck (43) and prostate (43). In the literature, much is discussed about the role of *NAT* acetylator profile in carcinogenesis. The metabolic role of the enzyme, such as N- and O-acetylation, can be modulated by SNPs, mainly, which will change the speed of metabolism of potentially carcinogenic agents (32, 49, 50). Another predominant and modulating factor of the risk of developing cancer is the type of exposure of individuals or populations, such as to heterocyclic and aromatic amines, resulting from several lifestyle habits, mainly including cigarette smoking, ingestion of well-done meat and alcohol (50-52). In this study, it was not possible to collect data concerning expositive to carcinogenic agents for the control group. The use of a panel of 48 AIMs allowed us to estimate the individual and global ratios of ancestral population contributions in the case and control samples. Ancestry genomic control was effectively important for this investigation once significant differences were found concerning ethnicity among those with and without neoplasias; based on such estimates, it was possible

Table III. *Characterization of N-acetyltransferase 2 genotype and preliminary definition of the acetylation profile of patients with cancer*[a] *and controls*.

Genotype NAT2	Total (n=222) (%)	Controls (n=89) (%)	Cancer[a] (n=133) (%)	*p*-Value[b]	OR (95% IC)[b]
No allele for slow acetylation	76 (34.2)	38 (41.6)	38 (28.6)	0.041	0.527 (0.280-0.973)
*4/*4	18 (23.7)	9 (23.7)	9 (23.7)		
*11/*4	3 (4.0)	0	3 (7.9)		
*11/*11	1 (1.3)	1 (2.6)	0		
*11/*12	1 (1.3)	0	1 (2.6)		
*11/*13	2 (2.6)	0	2 (5.3)		
*12/*12	5 (6.6)	5 (13.2)	0		
*12/*4	9 (11.8)	5 (13.2)	4 (10.5)		
*12/*13	2 (2.6)	0	2 (5.3)		
*13/*13	8 (10.6)	6 (15.8)	2 (5.3)		
*13/*4	27 (35.5)	12 (31.5)	15 (39.4)		
One allele for slow acetylation	106 (47.8)	42 (48.3)	64 (48.1)	0.922	0.971 (0.540-1.747)
*4/*5	46 (43.4)	25 (59.4)	21 (32.8)		
*4/*7	7 (6.6)	2 (4.8)	5 (7.8)		
*5/*11	3 (2.8)	1 (2.4)	2 (3.1)		
*5/*12	4 (3.8)	2 (4.8)	2 (3.1)		
*5/*13	33 (31.1)	8 (19.0)	25 (39.1)		
*7/*12	4 (3.8)	2 (4.8)	2 (3.1)		
*7/*13	9 (8.5)	2 (4.8)	7 (11.0)		
Two alleles for slow acetylation	40 (18.0)	9 (10.1)	31 (23.3)	0.010	3.054 (1.303 -7.159)
*5/*5	24 (60.0)	6 (66.7)	18 (58.1)		
*5/*7	15 (37.5)	3 (33.3)	12 (38.7)		
*7/*7	1 (2.5)	0	1 (3.2)		

[a]Patients with two different forms of neoplasia: gastric and breast cancer. bp value and odds ratio (OR) adjusted by ancestry.

to control the ancestry effect on the association of *NAT2* gene with susceptibility to cancer. This is the first study to be carried out in the North region of Brazil, which examined the effect of population substructure associated with the susceptibility to neoplasia. Only the C282T polymorphism was importantly associated with the neoplasias studied. In this study, the strong influence of a slow acetylation profile on the susceptibility to GC and BC was clearly shown.

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