

RESEARCH ARTICLE

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# Polymorphisms in regulatory regions of Cyclooxygenase-2 gene and breast cancer risk in Brazilians: a case-control study

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## Abstract

**Background:** Cyclooxygenase-2 (COX-2) is up-regulated in several types of cancer, and it is hypothesized that COX-2 expression may be genetically influenced. Here, we evaluate the association between single-nucleotide polymorphisms (SNPs) in the COX-2 gene (*PTGS2*) and the occurrence of breast cancer among Brazilian women.

**Methods:** The study was conducted prospectively in two steps: First, we screened the promoter region and three fragments of the 3'-untranslated region of *PTGS2* from 67 healthy Brazilians to identify SNPs and to select those with a minor allele frequency (MAF) of at least 0.10. The MAF of these selected SNPs was further characterized in 402 healthy volunteers to evaluate potential differences related to heterogeneous racial admixture and to estimate the existence of linkage disequilibrium among the SNPs. The second step was a case-control study with 318 patients and 273 controls designed to evaluate *PTGS2* genotype- or haplotype-associated risk of breast cancer.

**Results:** The screening analysis indicated nine SNPs with the following MAFs: rs689465 (0.22), rs689466 (0.15), rs20415 (0.007), rs20417 (0.32), rs20419 (0.015), rs5270 (0.02), rs20424 (0.007), rs5275 (0.22) and rs4648298 (0.01). The SNPs rs689465, rs689466, rs20417 and rs5275 were further studied: Their genotypic distributions followed Hardy-Weinberg equilibrium and the MAFs were not affected by gender or skin color. Strong linkage disequilibrium was detected for rs689465, rs20417 and rs5275 in the three possible pairwise combinations. In the case-control study, there was a significant increase of rs5275TC heterozygotes in cases compared to controls (OR = 1.44, 95% CI = 1.01-2.06; P = 0.043), and the haplotype formed by rs689465G, rs689466A, rs20417G and rs5275C was only detected in cases. The apparent association with breast cancer was not confirmed for rs5275CC homozygotes or for the most frequent rs5275C-containing haplotypes.

**Conclusions:** Our results indicate no strong association between the four most frequent *PTGS2* SNPs and the risk of breast cancer.

## Background

Cyclooxygenases (COXs) are key enzymes in mediating the conversion of free arachidonic acid into prostaglandin H<sub>2</sub>, the precursor of molecules such as prostaglandins, prostacyclin and thromboxanes [1]. Two isoforms of cyclooxygenase (COX-1 and COX-2) are known. The constitutive cyclooxygenase (COX-1) is present in many tissues and synthesizes prostaglandins involved in maintaining normal tissue homeostasis [2]. The inflammatory enzyme COX-2 is not detected in most normal tissues

but can be induced by cytokines, growth factors or tumor promoters. COX-2 catalyzes the synthesis of prostaglandins, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which can affect cell proliferation, apoptosis and angiogenesis [3], contributing to tumor progression. COX-2 is present in several types of solid tumors and, in breast cancer, is associated with parameters of aggressiveness, including tumor size, positive nodal status and lower survival [4,5]. In addition, inhibition of COX-2 by non-steroidal anti-inflammatory drugs has been associated with a protective effect against a variety of cancers [6] and may be effective in the prevention and treatment of breast cancer [7,8].

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The mechanisms involved in the regulation of COX-2 expression remain unclear and may be influenced by genetic variations. The human COX-2 gene, *PTGS2*, is located on chromosome 1 (locus q25.2-q25.3), is 8.3 kb in size, contains 10 exons and produces an mRNA of 4.6 kb. The analysis of the promoter region (PR) reveals the existence of several potential regulatory elements, including a TATA box and transcription binding sites for NF- $\kappa$ B, NF-IL6, AP-1, AP-2, GAS, TBP and cAMP response element. Several genetic variants have been described in regions next to these regulatory sites that may affect enzyme expression [9,10] and contribute to a greater risk of developing cancer.

In addition to variations in the PR, sites in the 3'-untranslated region (3'-UTR) of the gene may also be associated with increased risk of developing cancer. The 3'-UTR of the *PTGS2* gene contains 30 AUUUA elements. Such repetitions generate consensus binding sequences for proteins and inflammatory mediators that regulate the stability and degradation of mRNA [11-13]. These repeats are also present in other genes encoding inflammatory mediators (cytokines and proto-oncogenes) whose mRNAs are very unstable [14]. Genetic variations in the 3'-UTR of the *PTGS2* gene may contribute to increased stability of mRNA and the synthesis of COX-2.

The frequency of SNPs in the *PTGS2* gene may vary between different ethnic groups [15,16]. No data are available on the frequency of such variant forms in the Brazilian population, either in healthy subjects or in cancer patients. The high rate of racial admixture, with a major contribution from Europeans and Africans in the formation of the Brazilian population, suggests that the variant forms of the *PTGS2* gene may have a high prevalence in Brazil and that their occurrence may lead to haplotypes with different potentials for changes in COX-2 expression.

In the present study, we identified single-nucleotide polymorphisms (SNPs) in the PR and 3'-UTR of the *PTGS2* gene and evaluated their association with breast cancer occurrence among Brazilians.

## Methods

### Experimental Design and Study Population

This study was conducted prospectively in two steps: first, we screened 1.5 kb of the PR and three fragments comprising 1.2 kb of the 3'-UTR of the *PTGS2* gene in 67 healthy Brazilians to identify *PTGS2* SNPs and to select those with a minor allele frequency (MAF) of at least 0.10. The frequency of these selected SNPs was further characterized in 355 other healthy volunteers (comprising a total of 402) to evaluate potential differences in allelic distribution due to heterogeneous racial admixture. We adopted the classification scheme used

in the 2000 Brazilian Census [17], which relies on self-perception of skin color. Accordingly, the individuals were distributed into the following three color groups: white, black and intermediate. The term "color" (*cor* in Portuguese) is preferred to "race" in Brazil because it captures the continuous aspects of phenotypes and also because a racial descent rule is not operational in this country [18]. The color stratification was not intended as an accurate ethnic classification. Instead, our objective was to evaluate potential differences in the frequency distribution of *PTGS2* SNPs to ascertain if an independent population control would be necessary in the case-control study.

The second step was a case-control study, designed to evaluate the genotype-associated risk of breast cancer for the most prevalent *PTGS2* SNPs, i.e., those with at least 0.10 MAF. This case-control study involved 318 women with breast cancer and 273 healthy controls. The patients had a confirmed diagnosis of breast cancer based on histopathological evaluation and were under current treatment at the Brazilian National Cancer Institute. The patients were assigned a recruitment interview when scheduled for routine blood exams. The controls were non-related healthy women with no signs or symptoms of breast cancer who were recruited among patients' escorts, hospital staff and blood donors of the Brazilian National Cancer Institute. The recruitment of both patients and controls occurred between January and October 2008.

All volunteers were informed about the procedures of the study and gave written consent to participate. Patients and controls were interviewed by trained personnel using a questionnaire to determine demographic and lifestyle characteristics. Information on clinical history was obtained from medical records for patients (N = 250) and collected in an additional questionnaire for controls (N = 183). The study was approved by the Ethics Committee of the Brazilian National Cancer Institute (Protocol #116/07).

### SNP Screening and Genotyping

Peripheral blood samples (3 mL) were collected from all subjects (volunteers, cases and controls). DNA was extracted using the DNAzol system (Invitrogen Life Technologies, Carlsbad, USA), following the procedures recommended by the manufacturer and were used to search for SNPs of the *PTGS2* gene (GenBank accession #AY382629). The blood samples were kept at 4°C until DNA extraction, which was performed within 24 h of blood collection.

The genotyping analyses were performed by denaturing high-performance liquid chromatography (dHPLC), using the Wave™ DNA Fragment Analysis System (Transgenomic, Omaha, NE) or by PCR-RFLP (all

**Table 1 Nucleotide PRIMER sequences and PCR conditions for genotyping by dHPLC or PCR-RFLP**

| PRIMER Name |      | Nucleotide sequence      | Region    | Identified SNP | Number of cycles | Melting Temperature | Enzyme <sup>#</sup> |
|-------------|------|--------------------------|-----------|----------------|------------------|---------------------|---------------------|
| -1290AG     | F 5' | TGCTGTCATTTCTGTAATGC     | 3' PR     | rs689465       | 34               | 60°C                | <i>PciI</i>         |
| -1195AG     | R 5' | TTTCTCTCCCTGATGCGTGG     | 3'        | rs689466       |                  |                     | <i>AluI</i>         |
| AM1*        | F 5' | GCTGTCAAATCTCCCTTCC      | 3' PR     | rs20415        | 30               | 58°C                |                     |
|             | R 5' | CCACGCATCAGGGAGAGAAA     | 3'        |                |                  |                     |                     |
| AM2*        | F 5' | AACCAAATAATCCACGC        | 3' PR     |                | 30               | 63°C                |                     |
|             | R 5' | CAAGGAGGGGTGAAG          | 3'        |                |                  |                     |                     |
| -765GC      | F 5' | CTTTGTCATCAGAAGGCAGG     | 3' PR     | rs20417        | 35               | 63°C                | <i>AccI</i>         |
|             | R 5' | TAGAGGGTCGAGGAAGTCACG    | 3'        |                |                  |                     |                     |
| AM3*        | F 5' | TTACCTTTCCCGCTCTC        | 3' PR     | rs20419        | 30               | 58°C                |                     |
|             | R 5' | GCGTCGTCATAAACATAAAAC    | 3'        |                |                  |                     |                     |
| AM4*        | F 5' | GCTATGTATGTATGTGCTGC     | 3' PR     |                | 31               | 55°C                |                     |
|             | R 5' | GAAGTGGCTCTCGGAAGC       | 3'        |                |                  |                     |                     |
| AM5*        | F 5' | CGGTATCCCATCAAGGC        | 3' PR     | rs5270         | 31               | 55°C                |                     |
|             | R 5' | CAGTGAGCGTCAGGAGCA       | 3'        | rs20424        |                  |                     |                     |
| 8473TC      | F 5' | CTGTTGCGGAGAAAGGAGTC     | 3' 3'-UTR | rs5275         | 30               | 58°C                |                     |
|             | R 5' | TCAAACAAGCTTTTACAGGTGA   | 3'        |                |                  |                     |                     |
| 9850AG**    | F 5' | CGTTCCTTCTAATTAATGCCCTT  | 3' 3'-UTR | rs4648298      | 34               | 50°C                | <i>AluI</i>         |
|             | R 5' | TGTGTCAAGCACTGTGGTTTTAAT | 3'        |                |                  |                     |                     |
| 10335GA     | F 5' | TTTGGGAAGAGGGAGAAAATGA   | 3' 3'-UTR | rs689469       | 30               | 56°C                |                     |
|             | R 5' | TATGCGAATGTTTCAGTGCC     | 3'        |                |                  |                     |                     |

\* 15; \*\* 16; PR: Promoter Region; 3'-UTR: 3'Untranslated Region

<sup>#</sup> Enzymes used for PCR-RFLP. All other analyses were performed by dHPLC.

enzymes from New England Biolabs). Table 1 summarizes the PCR conditions, the sets of primers and the enzymes used for each analysis.

In the case of dHPLC analysis, all samples with chromatographic profiles suggestive of variation in the gene sequence were analyzed using ABI PRISM-377 equipment (TaqMan, PE Biosystems, Foster City, CA, USA). A portion of controls (10% of the samples) was also analyzed by automatic sequencing, and the results matched completely.

The four SNPs selected for the case-control study were genotyped with the same sets of primers used in the screening step. The SNPs rs689465, rs689466 and rs20417 could not be identified by dHPLC and were genotyped by PCR-RFLP (Table 1).

#### Statistical Analysis

Allelic and genotypic frequencies were derived by gene counting and the adherence to the Hardy-Weinberg principle was evaluated by the chi-square test for goodness-of-fit. The evaluation of pairwise linkage disequilibrium was performed using the Fisher exact test, available online in GENEPOP (<http://genepop.curtin.edu.au/>; [19]), whereas the haplotype patterns were inferred using Haploview (<http://www.broadinstitute.org/haploview>; [20]), based on the algorithm of expectation and maximization. Comparisons of demographic and clinical features and of

genotypic and haplotypic distributions between patients and controls were performed using the chi-square test for proportions. Univariate logistic regression analyses were performed to identify independent factors influencing the risk of developing breast cancer, which was estimated by the odds ratio (OR) with 95% confidence interval (95% CI). The threshold for significance was set at  $P < 0.05$  (Pearson P-value). The clinically relevant factors with independent effects on breast cancer risk (OR and 95% CI  $> 1$ ) were used to create a multivariate final model using the Enter method. All statistical analyses were conducted using SPSS 13.0 for Windows (SPSS Inc., Chicago, Illinois).

#### Results

The screening analysis of the PR and 3'-UTR of the *PTGS2* gene in 67 healthy Brazilians revealed the existence of nine SNPs, which occurred with the following MAFs: -1290AG (rs689465, 0.22); -1195AG (rs689466, 0.15); -1131GA (rs20415, 0.007); -765GC (rs20417, 0.32), -604TC (rs20419, 0.015), -163CG (rs5270, 0.02), -62CG (rs20424, 0.007), 8473TC (rs5275; 0.22) and 9850AG (rs4648298; 0.01). The SNP 10335GA (rs689469), which was reported to have a MAF of 0.02 among the Spanish [16], was not found.

The SNPs rs689465, rs689466, rs20417 and rs5275, which showed a higher than 0.10 MAF in this first

**Table 2 Minor allelic frequency (MAF) of *PTGS2* SNPs in Brazilians**

| SNP                | Population    | Alleles (N) | MAF (95%CI)                           | P     | $\chi^2$ |
|--------------------|---------------|-------------|---------------------------------------|-------|----------|
| <b>rs689465 AG</b> | Total *       | 710         | <b>rs689465 G</b><br>0.17 (0.14-0.20) |       |          |
|                    | Men           | 222         | 0.20 (0.15-0.25)                      | 0.273 |          |
|                    | Women         | 488         | 0.16 (0.13-0.19)                      |       |          |
|                    | Whites        | 298         | 0.18 (0.14-0.22)                      | 0.87  |          |
|                    | Intermediates | 248         | 0.16 (0.11-0.20)                      |       |          |
|                    | Blacks        | 164         | 0.16 (0.11-0.22)                      |       |          |
| <b>rs689466 AG</b> | Total *       | 710         | <b>rs689466 G</b><br>0.13 (0.10-0.15) |       |          |
|                    | Men           | 220         | 0.16 (0.12-0.21)                      | 0.221 |          |
|                    | Women         | 490         | 0.12 (0.09-0.15)                      |       |          |
|                    | Whites        | 298         | 0.12 (0.08-0.16)                      | 0.728 |          |
|                    | Intermediates | 246         | 0.13 (0.09-0.17)                      |       |          |
|                    | Blacks        | 166         | 0.15 (0.10-0.21)                      |       |          |
| <b>rs20417 GC</b>  | Total *       | 772         | <b>rs20417 C</b><br>0.30 (0.27-0.33)  |       |          |
|                    | Men           | 242         | 0.28 (0.26-0.38)                      | 0.783 |          |
|                    | Women         | 528         | 0.29 (0.25-0.33)                      |       |          |
|                    | Whites        | 328         | 0.30 (0.25-0.35)                      | 0.949 |          |
|                    | Intermediates | 276         | 0.29 (0.23-0.34)                      |       |          |
|                    | Blacks        | 168         | 0.31 (0.24-0.38)                      |       |          |
| <b>rs5275 TC</b>   | Total *       | 698         | <b>rs5275 C</b><br>0.30 (0.26-0.33)   |       |          |
|                    | Men           | 208         | 0.29 (0.23-0.35)                      | 0.882 |          |
|                    | Women         | 490         | 0.30 (0.26-0.34)                      |       |          |
|                    | Whites        | 296         | 0.27 (0.22-0.32)                      | 0.53  |          |
|                    | Intermediates | 250         | 0.31 (0.25-0.36)                      |       |          |
|                    | Blacks        | 154         | 0.34 (0.26-0.41)                      |       |          |

\* Differences in values are due to missing data (no PCR amplification).  
P  $\chi^2$ : Chi-square test (Pearson P-value); 95%CI: 95% Confidence Interval

population subset, were selected for further characterization in a larger sample (Table 2). No differences were observed in the allelic frequencies due to gender or skin color for any of the SNPs studied. All genotypic distributions followed Hardy-Weinberg equilibrium. Our results indicate strong pairwise linkage disequilibrium involving SNPs rs689465, rs20417 and rs5275 in the three possible combinations (Table 3). In fact, the minor

**Table 3 Pairwise linkage disequilibrium between *PTGS2* SNPs in Brazilians**

| SNP                 | $\chi^2$ | P (Fisher) | [D']* | R2** |
|---------------------|----------|------------|-------|------|
| rs689465 & rs689466 | 3.159    | 0.206      | 99    | 4    |
| rs689465 & rs20417  | infinity | <0.0001    | 77    | 31   |
| rs689466 & rs20417  | 6.860    | 0.032      | 62    | 3    |
| rs689465 & rs5275   | infinity | <0.0001    | 51    | 13   |
| rs689466 & rs5275   | 1.095    | 0.579      | 49    | 2    |
| rs20417 & rs5275    | infinity | <0.0001    | 44    | 18   |

\* D' (degree of imbalance) in module; \*\* R2 (degree of correlation).

alleles of these three SNPs often occurred simultaneously, whereas the rs689466 G allele occurred mostly as an isolated variation.

The next step was a case-control study, conducted to examine the association between SNPs rs689465, rs689466, rs20417 and rs5275 and the occurrence of breast cancer. Cases and controls were first compared with regard to the distribution profile of clinical aspects potentially implicated in the risk of developing cancer and that could interfere as confounding factors on the analysis of the risk associated to *PTGS2* SNPs (Table 4). A significant difference between cases and controls was found only for age (OR = 1.72, 95% CI = 1.24-2.39; P = 0.001).

The genotypic distributions of *PTGS2* SNPs in cases and controls are shown in Table 5. Our results indicate a significant difference in the distribution of rs5275 genotypes, with a higher frequency of heterozygotes among cases than among controls (OR = 1.44, 95% CI = 1.01-2.06; P = 0.043). To control possible confounding variables that may have influenced the observed association measures, a multivariate regression model was built. The best model, combining the effects of age and the rs5275 SNP, showed a higher risk for rs5275 heterozygotes (OR<sub>adjusted</sub> = 1.43, 95% CI = 1.00-2.06; P = 0.049). The analysis of rs5275 SNP under recessive or dominant models did not confirm the risk association for breast cancer (data not shown).

The distribution of *PTGS2* haplotypes among cases and controls was also examined (Table 6). All genotype information was included in the analysis, and the haplotype inference could be obtained for 302 cases and 264 controls. No significant difference in the haplotypic distribution was observed between patients and controls (P = 0.99, Fisher exact method). The three less frequent haplotypes appeared to be differently expressed between cases and controls. However, it was not possible to calculate the OR between cases and controls due to the absence of these haplotypes in one of the groups. An adequate evaluation of their impact on cancer risk would require a much larger sample. To further evaluate the impact of the rs5275 SNP on the risk of developing

**Table 4 Impact of clinical and demographic characteristics on the risk of breast cancer in Brazilian women**

| Characteristic                 | Category            | Cases | Controls | OR   | 95%CI       | P $\chi^2$   |
|--------------------------------|---------------------|-------|----------|------|-------------|--------------|
| <b>Age*</b> (years)            | < 48                | 147   | 163      | 1    |             |              |
|                                | ≥ 48                | 171   | 110      | 1.72 | (1.24-2.39) | <b>0.001</b> |
| Missing data                   |                     | 0     | 0        |      |             |              |
| <b>Menarche (Age)</b>          | < 12                | 120   | 93       | 1    |             |              |
|                                | ≥ 12                | 120   | 74       | 1.25 | (0.84-1.86) | 0.258        |
| Missing data                   |                     | 78    | 106      |      |             |              |
| <b>Menopause (Age)**</b>       | ≤ 54                | 98    | 62       | 1    |             |              |
|                                | ≥ 55                | 10    | 5        | 0.79 | (0.25-2.42) | 0.68         |
| Missing data                   |                     | 127   | 103      |      |             |              |
| <b>First Birth (Age)</b>       | ≤ 30                | 161   | 103      | 1    |             |              |
|                                | ≥ 31 or nulliparous | 75    | 68       | 1.41 | (0.93-2.13) | 0.09         |
| Missing data                   |                     | 82    | 102      |      |             |              |
| <b>Fertile Time‡</b>           | < 35                | 45    | 35       | 1    |             |              |
|                                | ≥ 35                | 60    | 27       | 1.73 | (0.83-3.42) | 0.089        |
| Missing data                   |                     | 3     | 5        |      |             |              |
| <b>Use of contraceptives †</b> | < 2                 | 110   | 105      | 1    |             |              |
|                                | ≥ 2                 | 92    | 59       | 1.49 | (0.97-2.27) | 0.065        |
| Missing data                   |                     | 116   | 109      |      |             |              |
| <b>Use of HRT<sup>0</sup></b>  | no                  | 79    | 43       | 1    |             |              |
|                                | yes                 | 25    | 20       | 0.68 | (0.32-1.44) | 0.276        |
| Missing data                   |                     | 4     | 4        |      |             |              |
| <b>Use of NSAIDs</b>           | no                  | 187   | 154      | 1    |             |              |
|                                | yes                 | 31    | 13       | 1.96 | (0.99-3.88) | 0.053        |
| Missing data                   |                     | 100   | 106      |      |             |              |
| <b>BMI</b>                     | Underweight: ≤ 18.4 | 9     | 1        | 4.13 | (0.50-33.6) | 0.28         |
|                                | Normal: 18.5 - 24.9 | 98    | 45       | 1    |             |              |
|                                | Overweight: ≥ 25.0  | 159   | 63       | 1.15 | (0.73-1.83) | 0.55         |
| Missing Data                   |                     | 52    | 164      |      |             |              |
| <b>Smoking Habit</b>           | no                  | 174   | 83       | 1    |             |              |
|                                | yes                 | 98    | 38       | 1.23 | (0.77-1.94) | 0.42         |
| Missing data                   |                     | 46    | 152      |      |             |              |
| <b>Color</b>                   | White               | 131   | 120      |      |             | 0.619‡       |
|                                | Intermediate        | 125   | 96       |      |             |              |
|                                | Black               | 62    | 57       |      |             |              |
| Missing data                   |                     | 0     | 0        |      |             |              |

\* 48 years old is the median of cases + controls; \*\* Menopausal status: postmenopausal: cases n = 108, controls: n = 67; premenopausal: cases: n = 83, controls n = 103; ‡ Age of Menopause - Age of Menarche (only for women in menopause); † For at least 2 years; <sup>0</sup>HRT: Hormonal Reposition Therapy (only for women in menopause); NSAIDs: Non-Steroidal Anti-Inflammatory Drugs; BMI: Body Mass Index at diagnosis (patients) or at recruitment (controls), BMI = weight (Kg)/height<sup>2</sup> (m<sup>2</sup>); OR: Odds Ratio; 95%CI: 95% Confidence Interval; P from Chi-square test (Pearson p-value); ‡ P from Fisher test.

breast cancer, the haplotypes were separated into groups according to the presence of the rs5275 *T* or *C* allele. No risk association was found for rs5275 *C*-containing haplotypes when considered as a combined group (OR = 1.09, 95% CI = 0.83-1.43; P = 0.5). Taken together, the results appear to indicate no association between *PTGS2* SNPs (in their most frequent haplotypes) and the risk of breast cancer.

## Discussion

In the past five years, several studies have aimed to evaluate the impact of *PTGS2* SNPs on the risk of developing

different types of cancer [10,15,16,21-46]. However, most of these studies evaluated only one or a few SNPs at a time, sometimes with no clear selection criteria. Zhang et al. [10] were the first to perform a screening strategy to identify the most frequent *PTGS2* SNPs. This approach was also preferred in our case, due to the heterogeneity of the Brazilian population and to the consequent hazards of using frequency data obtained elsewhere.

In our screening strategy, we evaluated 1.5 kb of the PR and 1.2 kb of the 3'-UTR, which encompass the most important regulatory sites of *PTGS2* expression [9,10,13]. The focus on the regulatory regions of the

**Table 5 Genotypic distribution of *PTGS2* SNPs in cases and controls**

| SNP      | Population | N*  | Genotypic Distribution N (Freq) |            |           | P $\chi^2$   |
|----------|------------|-----|---------------------------------|------------|-----------|--------------|
| rs689465 |            |     | AA                              | AG         | GG        |              |
|          | Cases      | 290 | 202 (0.69)                      | 83 (0.28)  | 5 (0.03)  | 0.927        |
|          | Controls   | 244 | 172 (0.70)                      | 67 (0.27)  | 5 (0.03)  |              |
| rs689466 |            |     | AA                              | AG         | GG        |              |
|          | Cases      | 289 | 224 (0.77)                      | 62 (0.21)  | 3 (0.02)  | 0.968        |
|          | Controls   | 245 | 190 (0.77)                      | 51 (0.21)  | 3 (0.02)  |              |
| rs20417  |            |     | GG                              | GC         | CC        |              |
|          | Cases      | 308 | 157 (0.51)                      | 127 (0.41) | 24 (0.08) | 0.731        |
|          | Controls   | 264 | 129 (0.49)                      | 117 (0.44) | 18 (0.07) |              |
| rs5275   |            |     | TT                              | TC         | CC        |              |
|          | Cases      | 294 | 125 (0.42)                      | 149 (0.51) | 20 (0.07) | <b>0.046</b> |
|          | Controls   | 244 | 120 (0.49)                      | 99 (0.41)  | 25 (0.10) |              |

N: Number of examined samples (with available PCR amplification);  
 \* Differences in sample sizes for cases and controls are due to available data in each category. Freq: Frequency; P  $\chi^2$ : P from Chi-square test (Pearson p-value).

gene is justified by the fact that *PTGS2* mRNA is very unstable [13], and an increase in its stability promotes COX-2 expression in colon cancer cells [13]. In addition, *in vitro* studies indicate possible functional effects of the SNPs rs689466 and rs20417 on *PTGS2* promoter activity (evaluated by a luciferase gene reporter system) [9,10] and on COX-2 activity (evaluated by PGE2 production in human monocytes) [47]. Although screening the entire *PTGS2* gene would be theoretically preferable because variants in the coding and non-coding regions could tag other functional SNPs, previous reports involving these variants suggest no significant effect on COX-2 activity [48] or on cancer risk [16,21,25,28-30,32,33,35-43,45,46]. The only exception is a recent publication by Zhao *et al.* [49], which suggests an increased risk of esophageal squamous cancer associated to SNP rs20432 that is located in intron 5. This positive association, however, is not confirmed in other types of cancer [16,21,25,29,33,36,45].

In our screening analysis, we found nine SNPs, five of which showed very low MAFs (approximately 0.01): rs20415, rs20419, rs5270, rs20424 and rs4648298. These MAFs are in accordance with previous reports involving different populations [15,16,50]. The SNPs rs689465, rs689466, rs20417 and rs5275 were selected for the case-control study and appear to be the most frequent SNPs in other Western populations [16,21,23,25,51-58].

The present work is the first study on the frequency of *PTGS2* SNPs among Brazilians, who are one of the world's most heterogeneous populations as a result of extensive interethnic crosses over the last 500 years

**Table 6 Haplotype distribution in patients and controls and association with breast cancer risk**

|              | Haplotype | Cases      |          | Controls   |          | OR   | 95%CI     | P $\chi^2$ |
|--------------|-----------|------------|----------|------------|----------|------|-----------|------------|
|              |           | N          | F        | N          | F        |      |           |            |
|              | AAGT      | 284        | 0.47     | 248        | 0.47     | 1    |           |            |
| 1            | AAGC      | 72         | 0.12     | 63         | 0.12     | 0.99 | 0.68-1.45 | 0.92       |
|              | GACC      | 60         | 0.1      | 48         | 0.09     | 1.09 | 0.72-1.62 | 0.68       |
|              | AACC      | 54         | 0.09     | 42         | 0.08     | 1.12 | 0.72-1.73 | 0.65       |
|              | GAGC      | 6          | 0.01     | 0          | 0        |      |           | 0.03*      |
|              | Total     | 192        | 0.32     | 153        | 0.29     | 1.09 | 0.83-1.43 | 0.5        |
| 2            | AGGT      | 66         | 0.11     | 53         | 0.1      | 1.08 | 0.72-1.62 | 0.68       |
|              | AAGT      | 30         | 0.05     | 26         | 0.05     | 1.00 | 0.58-1.75 | 1          |
|              | GAGT      | 24         | 0.04     | 26         | 0.05     | 0.80 | 0.45-1.44 | 0.46       |
|              | AGCT      | 0          | 0        | 5          | 0.01     |      |           | 0.02*      |
|              | GAGT      | 0          | 0        | 5          | 0.01     |      |           | 0.02*      |
|              | Others    | 8          | 0.01     | 12         | 0.02     | 0.58 | 0.23-1.44 | 0.23       |
|              | Total     | 128        | 0.21     | 127        | 0.24     | 0.88 | 0.65-1.18 | 0.4        |
| <b>Total</b> |           | <b>604</b> | <b>1</b> | <b>528</b> | <b>1</b> |      |           |            |

The haplotype combining the predominant alleles was used as a reference. Group 1 was formed by any haplotype containing the rs5275 C allele and group 2 included all the other haplotypes. The haplotypes with less than 1% frequency (Others) are not listed. The impact on breast cancer risk was calculated for the two groups, considering the Odds Ratio (OR) and the 95% Confidence Interval (95%CI) P: Pearson P-value; N: Number of haplotypes. F: Frequency of haplotypes. \* Fisher Exact Probability Test (two-tailed). OR was not calculated because of N = 0.

between autochthonous Amerindians, European colonizers and Africans [59-61]. Studies based on population-specific alleles, blood groups and electrophoresis of protein markers have outlined the hazards of equating color or race with geographic ancestry in Brazilians [18,59-63]. Thus, the stratification of our population into three groups based on self-reported skin color (white, intermediate and black) was not intended for ethnic classification but to evaluate potential differences in the frequency of *PTGS2* SNPs due to heterogeneous racial admixture. Because no significant difference in the genotype distribution was detected for the four SNPs among the color groups, either in the general population or among patients (data not shown), no population control or stratification based on continental-specific alleles was necessary in the case-control study.

In the present study, there was no association between rs689465, rs689466 or rs20417 and the occurrence of breast cancer. The results for rs689466 and rs20417 are in accordance with previously published data [23,25,39]. This is the first report on rs689465 and the risk of breast cancer.

Our results show an increase in the frequency of rs5275 TC heterozygotes among patients compared to controls, with an apparent increased risk of breast

cancer development after adjustment for age differences. The borderline significance of this association, however, limits its confidence. The number of subjects in our case-control study was initially calculated considering the allele frequencies in the general population and a possible 2-fold increase in the rs5275 MAF among patients, with a significance level of 5% and an error level of 20%. Although the actual sample size was larger than first estimated to ensure statistical power, it was still small for the evaluation of inheritance models or for the study of the less frequent haplotypes.

A review of the literature concerning the impact of rs5275 on the risk of breast cancer shows conflicting results. Langsenlehner *et al.* [24] found that carriers of the rs5275 C allele in the Austrian population were more frequent among breast cancer patients (34.8%) than among age-matched controls (29.9%;  $P = 0.018$ ), with an increased risk of breast cancer in rs5275CC homozygotes (OR = 2.1; 95% CI = 1.3-3.3;  $P = 0.002$ ). These results, however, were not corroborated by other authors. Vogel *et al.* [34] found no association between rs5275 genotype and breast cancer susceptibility, which was confirmed in three independent large studies [25,28,39]. Cox *et al.* [25], combining data from three separate studies in the American population ( $N = 5144$ ), indicated that women homozygous for the rs5275 C allele have a 20% lower risk of breast cancer than those homozygous for the T allele (OR = 0.80, 95% CI = 0.66-0.97) [25]. This reduced risk was confirmed by Zhu *et al.* [64] in a meta-analysis, which, however, did not include the large number of individuals in the work by Abraham *et al.* [28] and by Dossus *et al.* [39]. Taken together, these studies appear to suggest no strong influence of rs5275 SNP on breast cancer risk.

The present work indicates that variants in the PR and in the 3' UTR of *PTGS2* do not appear to greatly influence breast cancer risk, as the apparent risk association found for rs5275 SNP was limited to heterozygotes with a low OR value and borderline significance. However, the apparently negative results do not exclude potential low risks (i.e., OR < 1.5), whose detection with high level of statistical significance ( $P < 0.001$ ) would require large individual studies or meta-analysis ( $N > 6000$ ). Our data also highlight the existence of various *PTGS2* haplotypes that have not been thoroughly studied and should be considered for further evaluation of risk association with cancer development and/or progression.

## Conclusion

Our results indicate no strong association between the four most frequent *PTGS2* SNPs and the risk of breast cancer.

## Acknowledgements

We thank all volunteers and patients who participated in this study. We also would like to thank Dr. Guilherme Suarez-Kurtz for the use of laboratory facilities.

This work was supported by grants from Fundação Carlos Chagas Filho de Amparo à Pesquisa no Rio de Janeiro - FAPERJ (E-26/170.721/2007, E-26/110.374/2007) and from INCT para Controle do Câncer (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq 573806/2008-0, FAPERJ E26/170.026/2008). D. N. Piranda received a scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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## Authors' contributions

DNP and RVJ were involved with the rationale, study design, development of genotyping protocols, data analysis and preparation of the manuscript. DNP, JSFV and LMA conducted the recruitment and the all the laboratory procedures. AB and RVJ were responsible for statistical analysis. All authors approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

Received: 6 January 2010 Accepted: 8 November 2010

Published: 8 November 2010

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#### Pre-publication history

The pre-publication history for this paper can be accessed here:  
<http://www.biomedcentral.com/1471-2407/10/613/prepub>

doi:10.1186/1471-2407-10-613

**Cite this article as:** Piranda *et al*: Polymorphisms in regulatory regions of Cyclooxygenase-2 gene and breast cancer risk in Brazilians: a case-control study. *BMC Cancer* 2010 **10**:613.

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