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Correlation of polymorphism C3435T of the *MDR-1* gene and the response of primary chemotherapy in women with locally advanced breast cancer

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ABSTRACT. Primary chemotherapy is a useful strategy for the treatment of locally advanced breast cancer and therefore allows *in vivo* evaluation of the action of cytotoxic drugs and the possibility of accomplishing conservative breast surgeries, as well as the early treatment of metastasis. Mechanisms of resistance to the drugs include the action of protein associated with the efflux of drugs from the intracellular environment hindering their activity; one of the most studied proteins is P-glycoprotein codified by the *MDR-1* gene. The presence of polymorphisms can determine different physiological actions of these proteins, intervening with the

response of the drug's action. We evaluated the presence of single nucleotide polymorphism (SNP) C3435T of the *MDR-1* gene and its correlation with the response to primary chemotherapy using the RECIST criteria. Forty-one Brazilian women with stages II and III breast cancer using the PCR-RFLP analysis were evaluated. Thirty-three patients with the SNP genotype (TT and CT) and eight patients with the wild genotype (CC) were found; there was no statistically significant correlation between the diverse genotypes and the clinical and pathological responses according to the Cramer correlation coefficient (V = 0.14). The parameters: nuclear and histological degree, and estrogens, progesterone and c-erb B2 receptors did not demonstrate a statistical correlation with the SNP C3435T. Patients with complete pathological response (12.5%) showed only the polymorphic genotype and not the wild genotype. The characteristics of miscegenation in our population could explain the absence of the characterization of a sub-group of individuals where the presence of the polymorphic genotype influenced the response to the primary chemotherapy.

Key words: Polymorphism; P-glycoprotein; Chemotherapy; Breast cancer

INTRODUCTION

The treatment of breast cancer has undergone several changes, ever since the concept of radical surgery considered by Halsted, in 1894, up until the application of conservative surgeries by Fisher and Veronesi (Fisher et al., 1985; Veronesi et al., 1986). The use of chemotherapy prior to surgery was initially considered by de Lena et al. (1975) in patients with locally advanced breast cancer; later on, clinical and laboratory evaluation confirmed the systemic nature of breast cancer at the time of its diagnosis as demonstrated by Fisher and Bonadonna (Fisher et al., 1983; Bonadonna, 1989). From these concepts, several authors have widely used primary chemotherapy as a therapeutic modality for the treatment of locally advanced breast cancer (Hortobagyi et al., 1983; Bonadonna, 1989; Valagussa et al., 1990).

The use of the primary chemotherapy for patients with locally advanced breast cancer presents, in principle, the possibility of verifying the *in vivo* response to the therapy, the accomplishment of conservative breast surgeries when radical surgeries would be indicated and the early treatment of micrometastasis (Wolff and Davidson, 2000; Hutcheon and Heys, 2004). However, its use has not demonstrated benefits in the overall survival rate, in the disease-free survival rate and in the long-distance disease-free survival rate (Fisher et al., 1998).

One of the main reasons that can account for the failure of treatment in patients with cancer is the mechanism of drug resistance that can be determined by factors that affect the efflux and influx of drugs across the cell membrane (Terek et al., 2003; Leonessa and Clarke, 2003). Amongst these, the most studied is P-glycoprotein (Pgp), described by Juliano and Ling (1976), responsible for substance elimination by the cell membrane by hydrolysis of ATP and codified by the *MDR-1* gene (Sauna et al., 2001). Some substrates related to the Pgp are widely

used for breast cancer chemotherapy, such as anthracyclines and taxanes (Goldstein, 1996).

Roughly, 28 single nucleotide polymorphisms (SNP) related to the *MDR-1* gene have been described and the most studied is the C3435T located in exon 26 (Brinkmann and Eichelbaum, 2001). Experimental studies have demonstrated that the basal expression of the *MDR-1* gene is weak or absent when associated with TT genotype (Sauer et al., 2002). Hoffmeyer et al. (2000) and Kim et al. (2001) demonstrated that individuals with TT genotype showed low function of Pgp protein, when compared with CC individuals.

Kafka et al. (2003) demonstrated a significant correlation between the occurrence of TT genotype and the complete clinical response in patients with locally advanced breast cancer submitted to primary chemotherapy with anthracycline.

The goal of the present study was to demonstrate the correlation between SNP C3435T of the *MDR-1* gene and the clinical and pathological responses in Brazilian patients with stages II and III breast cancer who were submitted to primary chemotherapy.

MATERIAL AND METHODS

This was a longitudinal and prospective study, where 41 patients were selected between July 2004 and July 2006 and submitted to biopsy of the breast for diagnostic confirmation of invasive ductal carcinoma, with only unilateral tumors, in stages II and III according to criteria proposed by the UICC, 6th edition. All patients had been submitted to three cycles of neoadjuvant chemotherapy, with 21-day intervals, according to the regimen: 5-fluorouracil (500 mg/m^2), epirubicin (75 mg/m^2) and cyclophosphamide (500 mg/m^2) on day one. For each cycle, the patients were evaluated according to RECIST's criteria (Therasse et al., 2000).

In the present study, responsive patients (RP) were considered to be those who showed complete or partial response; nonresponsive patients (NRP), those with steady illness and progression. The complete pathological response was considered as absence of invasive neoplasia or *in situ* neoplasia (Sataloff et al., 1995). This study was approved by the Research Ethics Committee of the Santa Casa Hospital of São Paulo.

Extraction of DNA and polymerase chain reaction

Peripheral blood samples were collected from the patients in sterile tubes containing EDTA, where the genomic DNA was extracted and later amplified by polymerase chain reaction (PCR) to obtain the fraction of 249 bp that included the polymorphic region C3435T located in exon 26, using the primers sense 5'-ATG GGC TCC GAG CAC ACC TG-3' and antisense 5'-AGG CAG TGA CTC GAT GAA GGC-3' (Invitrogen™ Life Technologies, Carlsbad, CA, USA). We used an Eppendorf PCR thermocycler (Eppendorf GAC 22331, Hamburg, Germany) with the following standardization: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 7 min.

Digestion with restriction enzyme

The identification of SNP C3435T of the *MDR-1* gene was determined by diges-

tion with the restriction enzyme *Sau3*AI (New England BioLabs, USA), using 1 µL of enzyme and 3 µL of the PCR product in proper drain plug and incubating the reaction mixture at 37°C for 3 h. The genotype was identified by electrophoresis on a 2.5% agarose gel and comparing it with a 50-bp marker stained with ethidium bromide and visualized under ultraviolet light.

The enzyme *Sau3*AI promoted the cleavage of DNA producing specific bands observed on the gel, where CC genotype was disclosed as two fragments of DNA of 158 and 91 bp, the TT demonstrated only one fragment of 249 bp and the CT displayed three fragments of 91, 158 and 249 bp.

The photographic documentation was performed using a digital camera, Canon model Rebel XT (Digital Canon EOS Rebel XT, EF-S 18-55, Japan).

Statistical analysis

For statistical analysis the Microsoft Excel®, version 2003, software was used to build our database and the Statistical Software Package SPSS®, version 2003 (SPSS Incorporation, Chicago, IL, USA), software was used for elaboration of the statistical tests used for the Cramer correlation, non-parametric test and function statistics of the chi-square test. A P value of <0.05 was considered statistically significant.

RESULTS

The measure of the tumors prior to the chemotherapy varied from 2.5 to 15 cm with an average of 5.79 cm; after 3 cycles of chemotherapy, the largest diameter of the tumors varied from 0 to 8.5 cm with an average of 3.59 cm. The clinical response showed the following distribution: 25 patients were classified as RP and 16 as NRP. The distribution of the genotypes disclosed 16 patients with CT genotype, 17 with TT and 8 with CC.

The incidence based on race demonstrated, for white individuals, 10 patients with CT genotype, 11 patients with TT and six with CC; and for black individuals, 6 patients with CT, 6 with TT and 2 with CC.

The correlation between RP and NRP who carried the polymorphic alleles (CT and TT) or wild allele (CC) was not statistically significant, as shown in Table 1. The comparison between the neoplastic axillary involvement and the diverse alleles did not demonstrate a statistically significant correlation, as shown in Table 2.

The histological and nuclear grades did not show a significant correlation with the CT/TT and CC genotypes (with V = 0.1216 and 0.0461, respectively).

Table 1. Correlation between the frequencies of CT/TT and wild (CC) allele polymorphisms and responsive (RP) and nonresponsive patients (NRP), submitted to primary chemotherapy.

Allele polymorphism	RP	NRP	Total
CT + TT	19	14	33
CC	6	2	8
Total	25	16	41

Cramer's correlation coefficient; V = 0.1415.

Table 2. Correlation between the frequencies of SNP C3435T and neoplastic axillary involvement (LN).

SNP	0 LN	1 to 4 LN	≥5 LN	Total
CT	8	3	5	16
TT	8	2	7	17
CC	5	2	1	8
Total	21	7	13	41

Cramer's correlation coefficient; V = 0.2077.

The distribution of the patients according to expression of the estrogen, progesterone and c-erb B2 receptors did not demonstrate a significant correlation with the CT/TT and CC genotypes (with V = 0.1107, 0.0853 and 0.0853, respectively).

Complete pathological response was verified in 5 patients, with 3 patients having the CT genotype and 2 the TT genotype; no patient had the CC genotype.

DISCUSSION

Despite the diverse chemotherapy regimens used for the treatment of breast cancer, the rates of complete clinical response range between 17 and 80% (Bonadonna et al., 1990; Fisher et al., 1997) and the complete pathological response is considered to be between 5.9 and 13% (Bonadonna et al., 1990; Semiglazov et al., 1994). In the present study, 25 (60.98%) patients were classified as RP and 5 (12.9%) showed complete pathological response.

The search for new prognostic and predictive markers is important for the development of even more specific and individualized treatments, optimizing results and reducing the collateral effects (Kafka et al., 2003; Ayers et al., 2004; Tripathy, 2005).

In this context, we decided to analyze the significance of SNP C3435T of the *MDR-1* gene and its correlation with the clinical and pathological responses in Brazilian patients with breast cancer submitted to primary chemotherapy, through the RFLP technique whose use has been widely reported in the literature due to its simplicity (Cavaco et al., 2003; Balram et al., 2003).

In the present study, the general distribution of the genotypes was 19.5% for CC individuals, 39.04% for CT and 41.46% for TT; other authors (Rodrigues et al., 2005) have found distribution patterns with 27.9% for CC, 52.2% for CT and 20.3% for TT. The distribution according to race demonstrated that 51.21% of the SNP individuals were of the white race and 29.26% were of the black race; other authors have demonstrated that the incidence of the SNP was detected in 43.3% of Euro-American individuals and 69.5% of Afro-Americans (Kim et al., 2001).

The incidence of SNP C3435T of the *MDR-1* gene did not correlate, in this study, with cancer staging, the presence of axillary metastasis and histological and nuclear grades, similar to the findings of various authors (de La Torre et al., 1994; Chevillard et al., 1996; Kafka et al., 2003).

The expression of estrogen, progesterone and c-erb B2 receptors was not correlated with SNP C3435T of the *MDR-1* gene, as has been demonstrated by other authors (Dexter et al., 1998; Kafka et al., 2003).

The clinical response did not show a significant correlation with SNP C3435T, similar

to the findings of Kafka et al. (2003). The pathological response did not show a significant correlation in our study, findings conflicting with the literature that demonstrates a frequent association between TT genotype and complete pathological response (Kafka et al., 2003), although it allowed us to verify that such finding was attributed to the polymorphic genotypes (CT/TT) and not to the wild genotype (CC). The association found by Kafka et al. (2003) makes sense when we take into consideration previous studies that have described the diverse functional character of the alleles associated with SNP C3435T, where TT genotype was correlated with reduced expression and functional status of Pgp protein and, therefore, reduced cellular elimination and blood concentration of chemotherapeutic drug (Hoffmeyer et al., 2000; Kim et al., 2001).

Thus, the expression of the polymorphic genotype would cause greater bioavailability of the drug and optimization of its cytotoxic effect.

The miscegenation of our sample, which showed a peculiar distribution, could explain the fact of not characterizing a sub-group of polymorphic individuals where we could demonstrate a statistically significant association with the clinical and pathological responses to primary chemotherapy. More population studies must be carried out in order to determine the true role of SNP C3435T in the treatment of breast cancer.

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