Vol.57, n.6: pp. 895-899, November-December 2014
http://dx.doi.org/10.1590/S1516-8913201402559
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Protein Expression and Codon 72 Polymorphism of *TP53* Gene in Triple Negative Breast Cancer

Leandra Fiori Lopes[1], Roberta Losi Guembarovski[1], Alda Losi Guembarovski[2], Marina Okuyama Kishima[2], Clodoaldo Zago Campos[3], Daniela Rudgeri Derossi[2], Carolina Batista Ariza[1], Patricia Midori Murobushi Ozawa[1], Carlos Eduardo Coral de Oliveira[1], Bruna Karina Banin-Hirata[1], Glauco Akelinghton Freire Vitiello[1], Sueli Donizete Borelli[4] and Maria Angelica Ehara Watanabe[1*]

[1]Laboratório de Estudos e Aplicações de Polimorfismos de DNA (LEAP); Departamento de Ciências Patológicas; Centro de Ciências Biológicas; Universidade Estadual de Londrina; Londrina - PR - Brasil. [2]Departamento de Patologia, Análises Clínicas e Toxicologia; Centro de Ciências da Saúde; Universidade Estadual de Londrina; Londrina - PR - Brasil. [3]Departamento de Clínica Médica; Centro de Ciências da Saúde; Universidade Estadual de Londrina e Hospital do Câncer de Londrina; Londrina - PR - Brasil. [4]Departamento de Análises Clínicas; Centro de Ciências da Saúde; Universidade Estadual de Maringá; Maringá - PR - Brasil

ABSTRACT

A subgroup of tumor that has received attention is triple-negative breast cancer (TNBC), which presents phenotype of negative estrogen receptor, negative progesterone receptor and has no overexpression of HER2. TP53 acts as a tumor suppressor limiting the proliferation of damaged cells. A polymorphic site (rs1042522) of TP53 encodes either an arginine or a proline amino acid, but its biological significance remains unclear. This study aimed to investigate this variant and its expression in search for a possible involvement in TNBC susceptibility and clinical outcome. Genetic polymorphism was evaluated in 50 patients and 115 controls by PCR based methodology and immunohistochemistry was done with monoclonal antibody. Case-control study showed no positive or negative association (OR= 0.95; CI95%= 0.48-1.89). Comparison of genotypes and clinical outcome showed no significant results. Despite most of patients presented p53 positive staining by immunohistochemistry, there was no significant association in relation to prognostic parameters. Results demonstrated a lack of association between codon 72 polymorphism, susceptibility and prognosis of TNBC. Immunohistochemistry analysis should be done more carefully, since most of the patients had the somatic mutation of p53, which could be an indicator of prognostic value in TNBC.

Key words: breast cancer, TNBC, *TP53*, genetic polymorphism, immunohistochemistry

INTRODUCTION

Estimate data from the National Cancer Institute reveal that has been 52,680 new cases of breast cancer (BC) in Brazil for 2012-2013. It is worth noting that, excluding the type of non-melanoma cancer, the mammary tumor is the most common among women in most regions of Brazil and accounts for a high rate of morbidity and mortality among Brazilian women (Inca 2011). Breast cancer represents a complex and heterogeneous disease that comprises distinct pathologies, histological features and clinical outcome. The status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) have been used as predictive markers to identify a high-risk

*Author for correspondence: maewatuel@gmail.com

phenotype and for the selection of the most efficient therapies (Weigelt and Reis-Filho 2010).

Triple-negative breast cancer (TNBC) is a subtype characterized by the lack of ER, PR, and HER2 expression and is associated with younger age at diagnosis (Dent et al. 2007) and occurs with greater frequency in premenopausal African-American women (Carey et al. 2006). It represents approximately 12–17% of all breast cancers (Foulkes et al. 2010) and encompasses a heterogeneous group of tumors, including, but not limited to, those classified as basal-like. There is an unmet need to better understand the drivers of this breast cancer subtype because the usual antiendocrine and anti-HER2 targeted therapies are ineffective, and traditional cytotoxic chemotherapy seems to be insufficient (Cadoo et al. 2013). The aggressive clinical course, poor prognosis, and lack of specific therapeutic options have intensified the current interest in this subtype of tumor (Cho et al. 2011).

Inactivation of Tumor Protein 53 (TP53) tumor-supressor pathway is considered the most common anti-apoptotic lesion in cancers (Vousden and Lu 2002). It is known that TP53 protein effectively acts like tumor suppressor, limits the damaged cell proliferation, and thus protects against malignancy. A polymorphism (rs1042522) at codon 72 in exon 4 encodes either an arginine amino acid (G allele) or a proline (C allele) residue (Matlashewski et al. 1987), with different biochemical properties. Proestling et al. (2012) investigated the impact of this polymorphism on *TP53* key target genes expression in human breast carcinoma. They found that the arginine variant appeared to be a more potent transcription factor and tumor suppressor in human breast cancer than the proline variant *in vivo*. Some studies have reported epidemiological differences in prevalence or prognostic significance of TP53/Arg or TP53/Pro in certain cancer types (Aoki et al. 2009; de Lourdes Perim et al. 2013), but its real role as a susceptibility marker in malignant tumors remains unclear, including in breast cancer.

In this context, the present work aimed to investigate the associations between codon 72 polymorphism and protein expression by immunohistochemistry in the *TP53* gene, in a search for its involvement in susceptibility and progression of TNBC, since this type of neoplasia lacked effective molecular markers and many patients progressed rapidly to a picture of distant metastasis.

MATERIAL AND METHODS

Human subjects

Paraffin embedded tissue samples were obtained from 50 TNBC retrospectively from 10 years diagnosed for breast cancer (Private Laboratory of Pathology, Londrina, Parana State, Brazil and Cancer Hospital of Londrina (HCL), Parana State, Brazil). Clinical staging was determined according to the Union of International Control of Cancer (UICC) classification criteria. Clinicopathological information (tumor size, lymph node involvement and nuclear grade) was obtained for breast cancer patients along with informed consent. For comparison, blood samples from 115 women (neoplasia-free, control group) were collected from the Blood Center of North Parana, Brazil. The protocol of this study was approved by the Institutional Human Research Ethics Committee of the State University of Londrina, Parana, Brazil.

DNA extraction

For polymorphism analyses, the genomic DNA was either isolated from formalin-fixed paraffin embedded samples according to Isola et al. (1994) protocol for the patients, or extracted from the whole blood using a specific Kit (Biopur, Biometrix, Curitiba, PR, Brazil) for neoplasia-free controls. After precipitation with ethanol, all the pellets were dried and resuspended in 50 μL of milli Q water and quantified by Thermo Fisher Scientific NanoDrop 2000c®Spectrophotometer (NanoDrop Technologies, Wilmington, USA) at a wavelength of 260/280 nm.

Genetic polymorphism of *TP53 CODON 72* (rs1042522)

DNA (100 ng) was used for PCR analyses with specific primers for *TP53* codon 72, GenBank accession number U94788 (Table 1). Samples were amplified using the kit buffer plus 1.25 units of Taq polymerase (Invitrogen™, Carlsbad, California, USA). The PCR conditions were: 3 min denaturation at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C for Pro allele and 57°C for Arg allele and 30 s at 72°C with a 10 min for final elongation at 72°C in a thermocycler (PCR-Sprint Hybaid - Guelph, Ontario, Canada). The PCR products were analyzed by electrophoresis on poliacrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method. The *TP53* C (Pro)

allele yielded 178 base pair product, while the G (Arg) allele yielded a 136 base pair product.

Table 1 - Primers sequences and PCR products size for genetic variant analyzed in *TP53*.

Gene	Primer Sequence	PCR Product
TP53 (rs1042522)	**Arg (G allele)**	
	5'-TCCCCCTTGCCGTCCCAA-3'	136 bp
	5'-CTGGTGCAGGGGCCACGC-3'	
	Pro (C allele)	
	5'-GCCAGAGGCTGCTCCCCC-3'	178 bp
	5'-CGTGCAAGTCACAGACTT-3'	

Immunohistochemical staining

Immunohistochemical staining of breast tumor sections was performed following the standard protocols. Briefly, 5 μm of paraffin sections were heated at 56°C, deparaffinized in xylene and rehydrated through a series of graded alcohols. Antigens were retrieved by briefly boiling the sections in sodium-citrate buffer (10 mM). Non-specific endogenous peroxidase activity was quenched by 10 min incubation with methanol / H_2O_2 (93% / 7%). Tissue sections were then incubated with primary anti-p53 antibody (Spring Biocience, Pleasanton, CA, USA), diluted 1:800 in PBS / 1% BSA at 4°C overnight. After PBS washes, the sections were incubated with biotinylated secondary anti-rabbit IgG (Bio SB, Santa Barbara, CA, USA). The immunoreaction products were detected using the streptavidin–biotin and DAB chromogen (Sigma-Aldrich, St Louis, USA). Counter staining was performed with Gill's hematoxylin and slide was assembled in Canada balsam. For negative controls, sections were incubated without primary antibody and did not show any detectable signals. Analyses were made with at least two pathologists in independent analyses.

Statistical analysis

The association study between the patients and controls was performed using contingency tables to calculate the odds ratios (OR) with a confidence interval (CI) of 95%. For *TP53* in which the three genotypes were identified, a 3x2 contingency table was constructed, with the considered wild type genotype as reference (OR=1.0) to determine the OR value for heterozygotes and rare genotypes using DPP Braile Biomedical (http://www.braile.com.br). Also, the rare and heterozygotes for *TP53* were grouped for the presence of at least one allelic variant, considering the small number of mutants homozygous individuals. The statistical analyses for the immunohistochemical and clinical and histopathological parameters were realized using the SPSS Statistics 17.0 software for correlation tests (SPSS Inc., Chicago, Illinois, USA). A p value $\leq$ 0.05 was considered statistically significant.

RESULTS

The median age of the patients was 54 ±13 years old. Although specific clinicopathological characteristics for some patients were not available, 83% of the patients had nuclear grade in stages II or III, 51% had lymph node involvement and the mean tumor size was 3.5 cm.

Genetic Polymorphism and Clinicopathological Characteristics Analyses

TP53 (rs1042522) polymorphism was analyzed in 50 TNBC patients and in 115 neoplasia-free controls. The genotype frequency was 62% (n=31) and 61% (n=70) for GG homozygote, 32% (n=16) and 29.5% (n=34) for GC heterozygote and 6% (n=3) and 9.5% (n=11) for CC rare homozygote in the patients and controls, respectively (Table 2). The case-control study showed an absence of positive or negative association: OR = 0.95; CI95% = 0.48-1.89. When comparing the genotypes of *TP53* and parameters of clinical outcome, there was no significance with the following: tumor size (p= 0.742, rho= 0.048), lymph node involvement (p= 0.778, rho= 0.047) and nuclear grade (p= 0.742, rho= 0.50).

Table 2 - Genotype distribution and case-control association study for *TP53* genetic variant.

		Controls (n=115)	Patients (n=50)	OR	IC	p value (χ^2)
TP53 rs1042522	GG	70 (61%)	31 (62%)	1.00	—	—
	GC	34 (29.5%)	16 (32%)	1.12	0.55-2.30	0.754
	CC	11 (9.5%)	3 (6%)	0.60	0.16-2.26	0.742
	GC+CC	45 (39%)	19 (38%)	0.95	0.48-1.89	1.000

Immunohistochemistry and clinicopathological characteristics analyses

For most of the samples (88%) the immunohistochemical staining for TP53 protein was done, since this parameter was a prognostic

indicator performed in clinical routine. In the sample of TNBC, 70% of patients (n=31) were positive for this protein (the mutant form). This information was used to perform the associations with genotype analysis (p= 0.764) and also with clinical outcome. Results showed no statistical significance: tumor size p= 0.787; lymph node involvement p= 0.286 and nuclear grade p= 0.524.

DISCUSSION

It is known that *TP53* is a tumor suppressor that is mutated in the majority of human cancers and its function is to arrest the cellular proliferation in response to a variety of cellular stresses, including DNA damage, hypoxia and activated oncogenes. The TP53 protein is at the center of cell regulatory pathways influencing the transcription and activity of several replication and transcription factors. In this study, a *TP53* codon 72 polymorphism (rs1042522) was analyzed in 50 TNBC patients and 115 controls (neoplasia-free), which showed the frequency of 6% in the cases and 9.5% in controls, respectively for rare genotype CC, with no positive or negative association with tumor susceptibility (OR= 0.95; CI95%= 0.48-1.89) (Table 2). Significant associations between codon 72 polymorphism and risk of cancer have been reported, although the results regarding most cancers, including breast cancer, remain inconclusive (Weston and Godbold 1997; Papadakis et al. 2000).

The breast cancer lesions presented a significant over-representation of *TP53* GG homozygosity (62%) compared to *TP53* CC homozygosity (21%) (Papadakis et al. 2000). Although in this study the homozygous GG were similar, the homozygous CC was only 6% (Table 2), which reflected different frequencies between the distinct samples or even ethnics groups. Eltahir et al. (2012) evaluated the associations of *TP53* codon 72 polymorphism with different cancers and found that breast carcinoma patients most prominently showed excess of homozygous GG when compared to the controls. Results from Al-Qasem et al. (2012) indicated that the G allele of codon 72 polymorphism was a potential risk factor, whereas the GC (heterozygosis) form is a protection factor against breast cancer among Saudi women. Surekha et al. (2011) reported that *TP53* codon 72 polymorphism might predispose the development of breast cancer as well as to bad prognosis. Damin et al. (2006) found that the GG genotype was significantly associated with an increased risk for breast cancer (OR= 2.9; CI95%= 1.43–3.6; p < 0.002). They observed no correlation between the genotype distribution and specific prognostic predictors for the disease outcome. In the present study, neither the genotypes in homozygosity or heterozygosity of this genetic variant were associated with TNBC susceptibility (Table 1). TNBC prognosis did not show any significant correlations with the clinical parameters of tumor progression: tumor size (p= 0.742; rho= 0.048), lymph node involvement (p= 0.778; rho= 0.047) and nuclear grade (p= 0.742; rho= 0.50).

Generally, there are conflicting data about the associations between the *TP53* polymorphism in codon 72 and risk to develop breast cancer. However, Ma et al. (2011) reported a meta-analysis, which provided strong evidence that the *TP53* codon 72 polymorphism was not associated with the risk to develop breast cancer. The present results corroborated these authors, as there was no association of this poymorphism and susceptibility or progression of TNBC; besides, these samples were composed by a specific molecular subtype of breast cancer. Seventy percent of the patients who had the results of immunohistochemistry were positive for TP53 staining, having a mutant form of this suppressor gene, since normal TP53 has a short half-life and is", changing the words "had, therefore, not detected by this methodology. These results were in accordance to Calza et al. (2006) reported that *TP53* mutations occurred in 65% of basal-like breast cancer, which was closely related to TNBC subtype.

The information of protein expression was used to perform the associations with genotype analysis (p= 0.764) and clinical outcome. This showed no statistical significance (tumor size p= 0.787; lymph node involvement p= 0.286 and nuclear grade p= 0.524). The findings of Kikuchi et al. (2013), differently from these results, indicated that TP53 overexpression was associated with unfavorable characteristics and prognosis and appeared to be a significant prognostic factor in the patients with other molecular subtype of breast cancer, luminal/HER2-negative. Zhang et al. (2013) also reported high Ki-67 labeling index and high TP53 labeling index as the risk predictors of relapse for TNBC (P<0.05). Therefore, although the present sample size was relatively small, it consisted of a specific molecular subgroup of breast tumors, which reinforced the lack of

association between *TP53* polymorphism of codon 72 and susceptibility or clinical outcome in TNBC. The results of immunohistochemistry should be considered more carefully, since although there was no association with prognostic parameters, most of the patients had the somatic mutation of *TP53*, which could be an indicator of prognostic value in TNBC pathogenesis.

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Received: November 11, 2013;
Accepted: February 18, 2014.