

RESEARCH ARTICLE

Association of Histopathological Markers with Clinico-Pathological Factors in Mexican Women with Breast Cancer

Bandala Cindy¹, De la Garza-Montaña Paloma¹, Cortés-Algara Alfredo², Cruz-López Jaime³, Domínguez-Rubio Rene², González-López Nelly Judith⁴, Cárdenas-Rodríguez Noemi⁵, Alfaro-Rodríguez A¹, Salcedo M⁶, Floriano-Sanchez E³, Lara-Padilla Eleazar^{2*}

Abstract

Background: Breast cancer (BCa) is the most common malignancy in Mexican women. A set of histopathological markers has been established to guide BCa diagnosis, prognosis and treatment. Nevertheless, in only a few Mexican health services, such as that of the Secretariat of National Defense (SEDENA for its acronym in Spanish), are these markers commonly employed for assessing BCa. The aim of this study was to explore the association of Ki67, TP53, HER2/neu, estrogenic receptors (ERs) and progesterone receptors (PRs) with BCa risk factors. **Materials and Methods:** Clinical histories provided background patient information. Immunohistochemical (IHC) analysis was conducted on 48 tissue samples from women diagnosed with BCa and treated with radical mastectomy. The Chi square test or Fisher exact test together with the Pearson and Spearman correlation were applied. **Results:** On average, patients were 58±10.4 years old. It was most common to find invasive ductal carcinoma (95.8%), histological grade 3 (45.8%), with a poor Nottingham Prognostic Index (NPI; 80.4%). ERs and PRs were associated with smoking and alcohol consumption, metastasis at diagnosis and Ki67 expression ($p<0.05$). PR+ was also related to urea and ER+ ($p<0.05$). Ki67 was associated with TP53 and elevated triglycerides ($p<0.05$), and HER2/neu with ER+, the number of pregnancies and tumor size ($p<0.05$). TP53 was also associated with a poor NPI ($p<0.05$) and CD34 with smoking ($p<0.05$). The triple negative status (ER-/PR-/HER2/neu-) was related to smoking, alcohol consumption, exposure to biomass, number of pregnancies, metastasis and a poor NPI ($p<0.05$). Moreover, the luminal B subtype was associated with histological type ($p=0.007$), tumor size ($p=0.03$) and high cholesterol ($p=0.02$). **Conclusions:** Ki67, TP53, HER2/neu, ER and PR proved to be related to several clinical and pathological factors. Hence, it is crucial to determine this IHC profile in women at risk for BCa. Certain associations require further study to understand physiological/biochemical/molecular processes.

Keywords: BCA - Ki67 - TP53 - HER2/neu - estrogenic receptor - progesterone receptor - Mexican women

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Introduction

Breast cancer (BCa) had an incidence of 1.8 million cases worldwide in 2013, representing a sizeable percentage of the 13.1 million cases of malignancies. It was the second leading cause of cancer deaths for women globally (464,000) and the first cause of disability-adjusted life years (DALYs). The total deaths, 63% occur in developing and 37% in developed countries (Fitzmaurice et al., 2015). BCa incidence has increased steadily in the last few years in Mexico (DGIS/SINAIS, 2010; Bandala et al., 2012; Anaya-Ruiz et al., 2014).

BCa has been the leading cause of mortality from

malignant neoplasms in Mexican women since 2006 (Chavarri-Guerra et al., 2012; Justo et al., 2013; Perez-Santos and Anaya-Ruiz 2013). Several reports have shown that the risk factors of BCa are related to demography, socio-economic status, genetics, lifestyle and reproductive behavior, among other factors (Tirona et al., 2010; Justo et al., 2013).

Besides using histological classification, BCa has been typed by immunohistochemistry (IHC) expression of estrogenic receptors (ERs), progesterone receptors (PRs) and human epidermal receptor 2 (HER2/neu), also known as ErbB2. Seshie et al. (2015) categorized this expression as luminal A (ER/PR+, HER2/neu-), luminal B (ER/PR+,

¹Division of Neuroscience, National Institute of Rehabilitation, SSA, ²Laboratory of Molecular Oncology and Oxidative Stress, Superior Medicine School of National Polytechnic Institute, Mexico City, ³Military School of Graduate of Health, SEDENA, ⁴Gynecology Hospital No. 3, Medical Center "La Raza", IMSS, ⁵Laboratory of Neurosciences (Neurochemistry), National Institute of Pediatrics, SSA, ⁶Unidad de Investigación Médica en Enfermedades Oncológicas, National Center Medical "Siglo XXI", IMSS, Mexico *For correspondence: elarapa@hotmail.com

HER2/neu+), non-luminal (ER-/PR-, HER2/neu+) and triple negative (ER-/PR-/HER2/neu-).

Several markers have been associated with BCa patient prognosis. For instance, the nuclear proliferation marker Ki67 is used to predict response to chemotherapy (Azambuja et al., 2007; Cheang et al., 2009; Yerushalmi et al., 2010; Cuzick et al., 2011; Niikura et al., 2012; Ohno et al., 2013; Von Minckwitz et al., 2013; Shui et al., 2015). CD34, a useful angiogenesis marker, is a transmembrane glycoprotein whose expression is associated with hematopoietic precursors and capillary endothelial cells. The identification of microvessels using CD34 immunohistochemistry can help to identify the most aggressive breast tumors (Hsiao et al., 2010; Maschio et al., 2014). Moreover, alteration of the tumor suppressor gene p53 (also known as TP53) is considered to be a critical step in the development of many human cancers (Lane et al., 1992; Frebourg et al., 1993). Changes in this gene have been detected in a wide range of human tumors including BCa (Nigro et al., 1989). BCa research has focused on patients with primary, node-negative breast disease, and alterations in the p53 gene have been associated with poor prognosis (Thor et al., 1992; Elledge et al., 1993; Thorlacius et al., 1993).

The expression of tumor-specific proteins in mammary glands may be critical to BCa development and the success of chemotherapy treatment (Bandala et al., 2012). Some of these markers have been related to histological grade (Ji et al., 2014; Petric et al., 2014), metastasis (Bandala et al., 2012), lifestyle (León-Hernández et al., 2014; Bandala et al., 2012) and gynecologic-obstetrics (Cárdenas-Rodríguez et al., 2012).

The aim of the present study was to evaluate the IHC profile of Ki67, TP53, HER2/neu, ER and PR in BCa tumors of Mexican women, since these are the most commonly used markers for diagnosis, treatment and prognosis of this carcinoma. We also explored the relation of these parameters to clinical/pathological factors in the population under study.

Materials and Methods

Biological samples

The samples of human mammary carcinomas were obtained from 48 BCa patients diagnosed at the Pathology Service of the Specialty Clinic for Women (Clínica de Especialidades de la Mujer, Secretariat of National Defense, (SEDENA) in Mexico City. Tissue samples were collected during surgery and placed in 10% formalin. The histological classification of the carcinomas as well as the evaluation of non-tumor breast lesions were made according to standard diagnostic procedures and confirmed by two pathologists.

Immunohistochemistry

Tissues were submitted to the conventional histological process for hematoxylin-eosine staining and histopathological diagnosis. Briefly, tissues were embedded in paraffin blocks to obtain 2-3 μ m thick histological cuts, which were mounted on slides (ProbeOn™ Plus Microscope Slides, Fisherband®) and

deparaffinated with heat (60°C) and xylene. Subsequently, the samples were hydrated with a series of alcohols before being put in tridistilled water (high purity) and left for 5 min. Then antigens were recovered with Citrate 20x (2.5 ml in 50 ml of tridistilled water), heated to 120°C for 20 min in a pressure cooker, cooled to room temperature, and then hydrated in tris-buffered saline medium at pH 7.6 (TBS) for 5 min. Afterwards, the endogenous peroxidase was blocked with 3% hydrogen peroxide (H₂O₂) for 5 min (at 6% dilution with methanol starting from 30% H₂O₂), rinsed in TBS and left to stand for 5 min. The samples were then mounted with a Shandon coverplate (Thermo Scientific) before applying the primary antibody (see Table 1) during 30-40 min, followed by a wash with TBS and incubation with Biotin (Dako Cytomation) during 20 min. Slides were washed with TBS before the application of Streptavidin (Dako Cytomation) for 20 min, then washed again before the application of a diaminobenzidine chromogene kit (DAB from Dako Cytomation) at 1.5 ml of diluent plus two drops of aminobenzidine for 5 min. They were then dismantled and stained with Harris hematoxylin for 5 sec before being washed with TBS and mounted with Entellan® resin. Photographs were taken with an Olympus Camedia camera (C-5060 of 5.1 megapixels) in an optic microscope (Olympus BX50) with the 20x/0.70 ∞ /0.17 PLAN APO chromatic objective.

Antibodies

The immunohistochemical assay was conducted with primary (see Table 1) and secondary antibodies for recognizing human antigens. All antibodies were positive to external controls. The percentage of cells positive to Ki67 is about twice that found in the S phase. The number of vessels qualified as having CD34 corresponds to the average number of vessels displayed in 10 fields observed through a high power objective (csf 40x). HER2/neu was evaluated based on the dye affinity of the membrane. There is a table edited by DACO to estimate this parameter.

Ethical aspects

Patients read and signed the informed consent form, as required by the Ethics Commission of the National Institute of Public Health in Mexico. The current protocol was also approved by the Hospital Ethics in Research Commission (ref. no. SI-378).

Statistical analysis

The mean and standard deviation were calculated using the Chi-square test, Fisher exact test, and the Kolmogorov-Smirnov, Pearson and Spearman correlation. Statistical analysis was performed using SPSS v17 for Windows XP (SPSS UK, Ltd, Woking, UK). In all cases $p \leq 0.05$ was regarded as significant.

Results

The average age of the patients included in the study was 58 years, with a range from 31 to 87. The non-gynecologic-obstetric risk factors (Table 2) included the fact that 83.3% of the patients were over 48, 61.7% had a family history of breast cancer, and 80% had a BMI greater than 25

Table 1. Primary Antibodies (Dako Cytomation)

Antibody	Origin	Catalog	Type	Dilution
Estrogenic Receptor clone 1D5	Mo a Hu	M7047	IgG1	0.145833333
Progesterone Receptor clone PgR 636	Mo a Hu	M3569	IgG1	0.145833333
P53 clone DO-7	Mo a Hu	M7001	IgG2b	0.111111111
Ki67 clone MIB-1	Mo a Hu	M7240	IgG1	0.111111111
HER2/neu	Rb a Hu	A0485	-----	0.388888889
CD34 clone QBEnd 10	Mo a Hu	N1632	IgG1	0.180555556

Table 2. Frequency of Non Gynecological and Obstetric Risk Factors for BCa

	Frequency	Percentage (n)
Age (years)	>50	40 83.3% (48)
BCa Familiar History		29 61.7% (47)
BMI (Kg/m2)	<25	9 20 % (45)
	25 a 29	17 37.8% (45)
	>29	19 42.2% (45)
Blood Group	A+	7 14.9% (47)
	B+	2 4.3 % (47)
	O+	38 80.8% (47)
Comorbidity		35 72.9% (48)
Non Physical Activity		46 95.8% (48)
Tobacco Consumption		10 20.8% (48)
Alcohol Consumption		8 16.7% (48)
Biomass Exposition		7 14.6% (48)

kg/m². The predominant blood group was O+ (80.8%), followed by A+ (14.9%) and B+ (4.3%). In 72.9% of patients there was comorbidity, such as diabetes mellitus, HBP or some other metabolic disease. Additionally, 20.8% were smokers, 16.7% consumed alcohol, 95.8% had a sedentary lifestyle, and 14.6% reported having been exposed to biomass.

The gynecologic-obstetric risk factors associated with BCa in the patients of the present study (Table 3) include the following: 52.1% had an early menarche (before turning 12), 97.9% were parous, 59.5% had their first pregnancy after turning 20, 77.1% reported having been pregnant more than 3 times, and 84.8% were no longer menstruating (the mean age of menopause was 41). On the other hand, only 14.6% indicated using hormonal birth control.

Through IHC analysis of histopathological markers (Figure 1), immunostaining of the nucleus was observed for ER, PR, Ki67 and TP53, while HER2/neu was identified in the cell membrane and CD34 stained endothelial cells. The frequency of these markers found among the patients herein studied (Table 4) was 58.3% positive for ER, 56.2% positive for PR, 97.9% positive for Ki67, 56.3% negative for HER2/neu, and 56.5% showed 1 to 20 blood vessels with CD34. Of the total number of patients, in 47.9% the left breast was affected, in 39.6% the right breast, and in 12.5% both breasts. The most frequent histological type was invasive ductal carcinoma (95.8%), with 45.8% presenting tumor grade 3, followed by 35.4% with grade 2 and 18.8% with grade 1. Metastasis was found in 42.5% of patients. A poor NPI was predominant, shown by 80.4% of patients.

ER was associated with smoking (p=0.04), alcohol consumption (p=0.05), metastasis (p=0.02), PR (p=0.001), HER2/neu (p=0.05) and Ki67 (p=0.01). There was a tendency of association between ER and a high level of

Table 3. Frequency of Gynecological and Obstetric Risk Factors for BCa

	Frequency	Percentage (n)
Menarche	<12 años	25 52.1% (48)
Oral Contraceptives		7 14.6% (48)
Nulliparity		1 2.1% (48)
Age of first pregnancy	<20 años	17 40.5% (42)
Number of pregnancies	>3	37 77.1% (48)
Menopause		39 84.8% (46)

Table 4. Frequency of Histopathological Markers Identified in Mexican Patients Diagnosed with BCa

	Frequency	Percentage (n)
Estrogenic Receptor	Positive	28 58.3% (48)
Progesterone Receptor	Positive	27 56.2% (48)
TP53	Positive	31 64.6% (48)
Ki67	Positive	47 97.9% (48)
HER2/neu	Positive	21 43.7% (48)
CD34	1-10 blood vessels	25 56.5% (45)
	11-20 blood vessels	20 43.5% (45)

triglycerides (p=0.09). PR was associated with smoking (p=0.009), alcohol consumption (p=0.05), metastasis (p=0.01), Ki67 (p=0.02) and urea (p=0.01). There was of association between HER2/neu (p=0.08) and creatine (p=0.09). The marker of Ki67 proliferation was also associated with HER2/neu (p=0.02), TP53 (p=0.001) and a high level of triglycerides (p=0.05). On the other hand, TP53 was associated with a poor NPI and showed a tendency of association with the consumption of alcohol (p=0.07) and with comorbidity involving HBP and some metabolic disorders (p=0.09). The HER2/neu marker was also associated with the number of pregnancies (p=0.005) and tumor size (p=0.04). Finally, the CD34 marker was related to smoking (p=0.05) and showed a tendency of association with a sedentary lifestyle (p=0.09) and a high level of triglycerides (p=0.09).

The condition of triple negative (ER-/PR-/HER2/neu-) showed an association with smoking (p=0.02), consumption of alcohol (p=0.006), exposure to biomass (p=0.04), number of pregnancies (p=0.04), metastasis (p=0.05), and a poor NPI (p=0.003), as well as a tendency of association with triglycerides (p=0.09). On the other hand, the condition of triple positive (ER+/PR+/HER2/neu+) was associated with histological type (p=0.007), tumor size (p=0.03), and high cholesterol (p=0.02). Tumor size was associated with a family history of BCa (p=0.005), histological type (p=0.01), presence of comorbidity (p=0.02), the age of the first pregnancy (p=0.02), high cholesterol (p=0.05) and blood group (p=0.04), and there was a tendency to association with

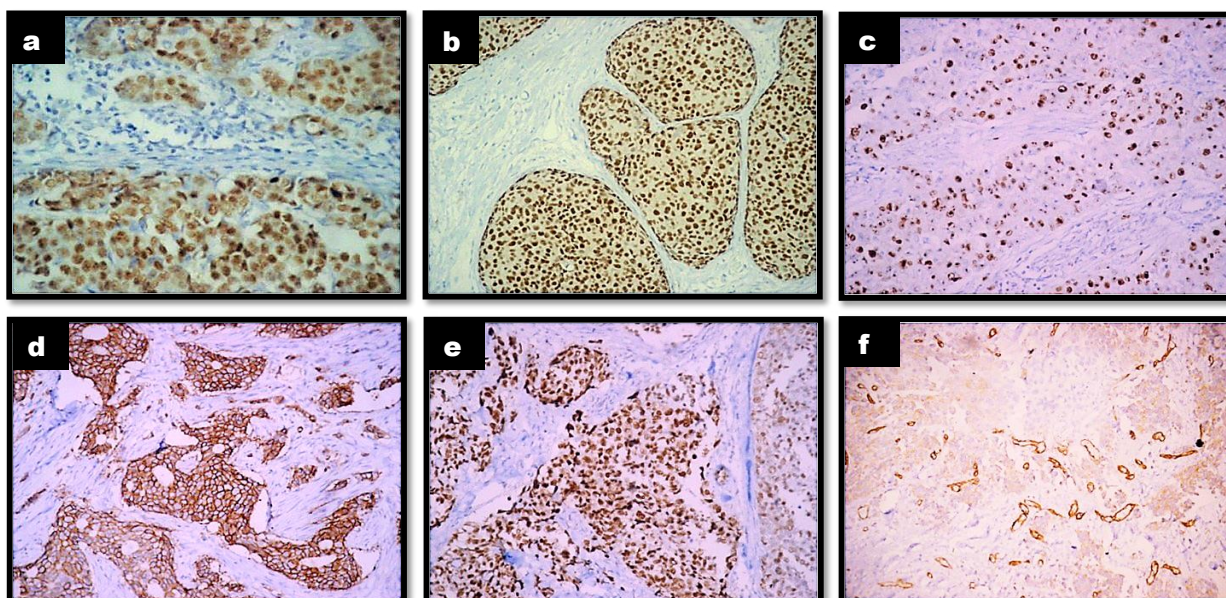


Figure 1. Histopathological Markers Identified in Biopsies of Patients with BCa. a) Estrogenic receptor, b) Progesterone receptor, c) Ki67, d) HER2/neu, e) TP53 and f) CD44

a high level of triglycerides ($p=0.07$). Histological type was associated with the BMI ($p=0.004$), high cholesterol ($p=0.05$) and the O+ blood group ($p=0.0001$), and there was a tendency to association with menopause ($p=0.06$) and alcohol consumption ($p=0.07$). Histological grade was associated with alcohol consumption ($p=0.03$) and the tumor stage (TNM) ($p=0.02$). A poor NPI was associated with metastasis (0.001), tumor stage (TNM) (0.02), histological grade ($p=0.001$) and the number of pregnancies ($p=0.02$). Additionally, the tumor stage (TNM) was associated with the BMI ($p=0.01$). Metastasis showed an association with menopause ($p=0.01$) and smoking ($p=0.007$). Finally, the blood group of patients was associated with their BMI ($p=0.005$), family history of BCa ($p=0.001$), age of menarche ($p=0.02$), and age of menopause ($p=0.001$).

An analysis was made of the correlations between each of histopathological markers considered herein, including ER, PR, TP53, Ki67 and HER2/neu, and also between these parameters and other risk factors. With respect to ER, a correlation was found with PR ($p=0.0001$; $\rho=0.822$), Ki67 ($p=0.005$; $\rho=-0.400$), a high level of triglycerides ($p=0.05$; $\rho=0.521$) and high cholesterol ($p=0.07$; $\rho=0.498$). PR showed a correlation with Ki67 ($p=0.001$; $\rho=-0.463$), a high level of triglycerides ($p=0.003$; $\rho=0.727$) and high cholesterol ($p=0.003$; $\rho=0.727$). TP53 was associated with the age of the first pregnancy ($p=0.01$; $\rho=0.378$). Ki67 was related to the age at which BCa was diagnosed ($p=0.02$; $\rho=-0.326$), the age of menopause ($p=0.01$; $\rho=0.360$), and a high level of triglycerides ($p=0.05$; $\rho=-0.525$). TP53 also demonstrated a tendency to correlation with high cholesterol ($p=0.09$; $\rho=-0.470$). Finally, CD34 was correlated with high cholesterol ($p=0.05$; $\rho=0.535$).

Discussion

The current contribution explores protein expression in BCa tumor biopsies taken from 48 Mexican women. The

following histological markers were explored: ER, PR, HER2/neu, Ki67, TP53 and CD34. Analysis was carried out to find the possible relation of the expression of each protein with the others and with socio-demographic and gynecologic-obstetric factors.

The average age of the Mexican women herein diagnosed with BCa (over 48) coincides with reports for Mexico and other Latin American countries by various authors (Rummel et al., 2012; Keyhani et al., 2013; Justo et al., 2013; Villarreal-Garza et al., 2013; Floriano-Sánchez et al., 2014; Ji et al., 2014; Doval et al., 2015). For these patients, the frequency of early menarche, the age of the first pregnancy, the number of pregnancies, early menopause, and the history of taking oral birth control are similar to that reported by Gates et al. (2012) and by other authors (Cardenas-Rodriguez et al., 2012; Amadou et al., 2014; Floriano-Sanchez et al., 2014; Doval et al., 2015). Neither a family history of BCa or nulliparity were common in the patients of the current contribution. The scarcity of nulliparous women in the study is probably due to cultural factors that lead most women in Mexico to be multiparous.

Regarding lifestyle factors, sedentariness, overweight/obesity and comorbidities were very frequent in the patients herein analyzed, while smoking and alcohol consumption were not. These data are congruent with those reported by other authors (Gates et al., 2012; Floriano-Sanchez et al., 2014; Amadou et al., 2014; Maschio et al., 2014).

The O+ blood group predominated, followed by group A+, also coinciding with that reported in other studies of BCa in Hispanic populations. However, studies from Caucasian populations report that group A+ is more common for BCa patients. This is easily explained by the fact that O+ is the most common blood group in the Hispanic population, whereas the A+ group is more frequent in the Caucasian population. Nevertheless, there are contradictory reports on this question in regard to BCa (Gates et al., 2012; Zhang et al., 2014; Abobaker

and Kamil, 2014).

The detection of a BCa tumor was more frequent in the left breast, similar to that reported by other authors (Keyhani et al., 2013; Ji et al., 2014). The most common histological type was invasive ductal carcinoma, also similar to many other reports (Rummel et al., 2012; Cardenas-Rodríguez et al., 2012; Keyhani et al., 2013; Petric et al., 2014). Stage 3 was the most commonly detected tumor stage in the present study, which coincides with some studies (e.g., Petric et al., 2014) but differs from others that have found a greater presence of stage 1 or 2 (Rummel et al., 2012; Keyhani et al., 2013; Justo et al., 2013; Ji et al., 2014).

A poor NPI was determined in the majority of patients herein studied, coinciding with other reports on the Mexican population (e.g., Bandala et al., 2012) but contrasting with studies conducted in European populations, where a good NPI was most commonly identified (e.g., Bates et al., 2014). Metastasis at the moment of diagnosis was 10% more frequent in the present study than what Cardenas-Rodríguez et al. (2012) or Maschio et al. (2014) reported, and similar to that reported by Leon-Hernández et al. (2014).

The frequency of ER+ in the current sample of BCa patients was lower than that reported by several authors: 12.9% lower versus Keyhani et al. (2013), 19.7% versus Rummel et al. (2012), 4.7% versus Maschio et al. (2014), 3.9% versus Doval et al. (2015), and 13.7% versus Ji et al. (2014). Meanwhile, the frequency of PR+ presently detected was lower than that reported by several authors (10.8% versus Gates et al., 2012; 8.5% versus Keyhani et al., 2013; 16.9% versus Ji et al., 2014) and higher than that reported by some others (1.2% versus Maschio et al., 2014; 3% versus Doval et al., 2015). Pervaiz et al. (2015) found an association between ER+ and histological grade, while PR+ was associated both with this factor and tumor size. Floriano-Sanchez et al. (2014) found a significant relation of ER+ with alcoholism and PR+.

HER2/neu was positive in 43.7% of cases herein studied, higher than that reported by various authors (24.7% higher versus Gates et al., 2012; 21.2% versus Keyhani et al., 2013; 20.7% versus Doval et al., 2015; 9.1% versus Ji et al., 2014, which was identified by FISH), but lower than that found by others (9.6% versus Rummel et al., 2012; 13.3% versus Maschio et al., 2014). A positive HER2/neu was related to the number of pregnancies, PR+ and the tumor size, coinciding with that observed by Keyhani et al. (2013), while Ji et al. (2014) found a relation of HER2/neu with histological grade, ER+, PR+ and TP53.

The condition of triple negative (ER-/PR-/HER2/neu-) showed an association with smoking, alcohol consumption, exposure to biomass, number of pregnancies, and with malignancy parameters like metastasis, coinciding with the results reported by Leon-Hernandez et al. (2014). An association was also found presently between this triple negative condition and a poor NPI. Dent et al. (2007) reported that triple negative patients had a lower survival rate, which was associated with histological grade 3 and a greater tumor size. On the other hand, Doval et al. (2015) found an association between tumor grade, histological type, tumor stage, early menopause, and women under

50 years of age. Kurshumliu et al. (2014) observed that a poor NPI was related to overexpression of HER2/neu.

In the current contribution the condition of triple positive (ER+/PR+/HER2/neu+) was associated with histological type, similar to the finding of Zheng et al. (2015), as well as with tumor size and high cholesterol (p=0.02). Ki67 was expressed in almost all patients studied presently (97.9%), similar to other reports (Keyhani et al., 2013; Ji et al., 2014; Petric et al., 2014; Maschio et al., 2014). However, the majority of the aforementioned authors utilized low/moderate/high as the classification of expression for this marker, whereas in the present study the analysis of Ki67 expression was not carried out in this way. Ki67 expression was associated with HER2/neu, similar to what Keyhani et al. (2013) and Ji et al. (2014) reported, and was also related to ER+, PR+ and a high level of triglycerides.

The expression of TP53 in the current contribution was greater than that reported by two authors (31.3% greater versus Keyhani et al., 2013; 9.6% versus Maschio et al., 2014) and similar to the results of Cárdenas-Rodríguez et al. (2012). The expression of TP53 found presently was associated with a poor NPI and Ki67+, the latter coinciding with the findings of Keyhani et al. (2013), who also mentioned an association between tumor grade and CD34 expression.

Regarding CD34, we evaluated this parameter as 1-10 vessels or 11-20 vessels, whereas other authors classified it as low/moderate/high. The present results show a greater frequency for 1-10 vessels, which coincides with Keyhani et al. (2013), who found that a lower expression was more frequent. CD34 is important because of its demonstrated relation to other angiogenic factors (VEGF and HIF-1). Moreover, it has proven to be an important factor in BCa progression and its abundance in breast tumors has prognostic and predictive value (Maschio et al., 2014). CD34 was herein associated with smoking and a high level of cholesterol. On the other hand, Keyhani et al. (2013) found a relation between CD34 and HER2/neu in an Iranian population, and Maschio et al. (2014) reported that this marker was related to hormonal therapy, ER, HER2/neu, Ki67, radiotherapy and metastasis in a Brazilian population.

Although the current analysis had some limitations, such as a possible inherent bias due to the retrospective nature of the analysis, the evidence herein presented unequivocally establishes that the histopathological markers of ER, PR, HER2/neu, TP53 and Ki67 cannot be omitted when analyzing BCa patients, since these are closely linked to diagnosis, prognosis and treatment, as well as being predictive in their relation with risk factors for BCa.

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