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Immunohistochemical Biomarkers in Ductal Carcinoma *In Situ*

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Abstract

Introduction: Breast ductal carcinoma *in situ* (DCIS) can be defined as a malignant epithelial proliferation with growth limited by the basal membrane of the ductal epithelium, with no evidence of stromal invasion. There has been a trend of trying to subcategorize DCIS based on cell proliferation assays (Ki67) and the expression of hormone receptors and the human epidermal growth receptor (HER-2) as detected by immunohistochemistry, similar to invasive breast carcinomas (IBC). The aims were to evaluate the expression of breast cancer marker proteins in DCIS by immunohistochemistry to better categorize it. **Methods:** 46 biopsies from women with DCIS and IBC Luminal A-like were evaluated by immunohistochemistry staining of proteins already known to be biomarkers in IBC. For controls, normal breast tissue from mammoplasty (n = 3) was used. **Results:** Our results showed an increase of estrogen receptor (ER) and progesterone receptor (PR) expression relative to that in normal tissue samples ($p < 0.0001$). No differences in steroid hormone expression patterns were seen between DCIS and IBC tumors ($p = 0.3145$; $p = 0.7341$, respectively). The proliferation levels of the DCIS and IBC samples were similar as evaluated by the Ki67 labeling index. Only 12.90% of samples showed amplification of HER-2. **Conclusion:** The biology of DCIS is not well understood given the complexity and heterogeneity of the disease, which makes it important to better sub-categorize this tumor, especially considering the possibility of identifying DCIS cases with the potential for recurrence and evolution into IBC.

Keywords

Breast Cancer, Ductal Carcinoma *In Situ*, Immunohistochemistry, Biomarker Proteins, Categorization

1. Introduction

Breast cancer is the most common type of cancer in women and has high mortality rates in Brazil and worldwide [1]. Breast cancer is a heterogeneous disease in which aberrant gene expression confers aggressiveness and a variety of different clinical manifestations [2]. Genomic studies have provided new information about breast cancer heterogeneity, which has allowed its classification into four intrinsic subtypes based on hormone receptor expression: Luminal, which expresses the estrogen receptor (ER) and/or the progesterone receptor (PR); Luminal-HER-2, which is characterized by expression of the estrogen and/or progesterone receptor and the human epidermal growth factor receptor 2; HER-2, which is characterized by HER-2 overexpression but not expression of the two hormone receptors; and triple negative, which does not express any of these three receptors [3] [4]. This classification has been applied to invasive breast carcinoma (IBC) and is important in making therapeutic choices for patients.

Ductal carcinoma *in situ* (DCIS) is a type of noninvasive breast cancer showing malignant epithelial growth limited by the ductal epithelial basement membrane without evidence of stromal invasion. DCIS is considered a preinvasive and heterogeneous disease, which has increased in frequency and clinical relevance following the advent of mammographic screening. Until the 1980s, DCIS represented only 3% to 5% of diagnosed breast carcinomas. Currently, DCIS comprises approximately 20% - 25% of all cases [5] [6] [7]. DCIS treatment is currently variable and may include partial or total mastectomy, radiotherapy and the possibility of hormonal treatment when the tumor expresses ER and/or PR [8] [9] [10] [11]. Weaker risk factors, such as a high body mass index, have been inconsistently associated with the risk of DCIS, and genetic risk factors have been described as being similar to those for IBC: BRCA1 and BRCA2 mutation carriers develop DCIS more frequently and at an earlier age than the general population [12] [13].

The histological classification of DCIS places it into three distinct grades according to the nuclear atypia pattern: high, intermediate or low grade. The presence of comedonecrosis has been associated with high-grade tumors and shorter survival, although there is no evidence of a direct relationship with recurrence [14] [15]. The morphological aspects of DCIS require a multistep succession of histological changes, including a premalignant stage that progresses to preinvasive breast cancer and can sometimes progress to invasive breast cancer [16]. Intrinsic biomarkers of invasive breast carcinoma subtypes have been recently to used subcategorize DCIS based on cell proliferation (Ki67) and ER and HER2 expression as assessed through immunohistochemistry [17] [18]. These biomarkers are already well defined for classifying IBC; however, their roles are still poorly characterized in DCIS.

Several controversies can be found in the literature: a previous study by Tamimi *et al.* showed that the frequency of the Luminal B and HER-2 like pheno-

type was significantly higher in DCIS compared to that in IBC, whereas the Luminal A like phenotype could be found more frequently in IBC [18]. Additionally, Poulakaki *et al.* showed that a majority of DCIS cases had a Luminal B-like phenotype (61.3%) rather than a Luminal A like phenotype (15.2%) [19]. On the contrary, a study by Hammond *et al.* showed that the frequency of the Luminal A like subtype (49.0%) was significantly higher than that of the Luminal B like (9.0%) in DCIS [20].

Overexpression of HER-2 has been associated with increased aggressiveness of tumors and poor survival of women with breast cancer, and its role as well as its expression in DCIS is not well defined. Horimoto *et al.* observed HER-2 gene amplification in 35% of their cohort [21]. However, Polónia *et al.* found HER-2 amplification in 18.9% and 4.4% of high grade and low grade samples, respectively, in DCIS [22]. In addition, changes in the expression patterns of these biomarkers have been controversially associated with the recurrence of DCIS or its evolution into IBC.

Moreover, the results of these previous studies do not present a predefined pattern of biomarker expression in women with DCIS, unlike the well-defined biomarker expression pattern in IBC, and the presented results might be biased, which may justify the difficulty in standardizing a well-defined classification of DCIS. In an attempt to better understand the role of breast cancer biomarkers in this controversial context for DCIS, our group evaluated the expression of these cancer biomarkers by immunohistochemistry in low and high grade cases of DCIS and IBC.

2. Methods

2.1. Samples

This study was approved by the National Ethics Committee and the Ethics Committee of the National Cancer Institute (INCA, Brazil) and was conducted in accordance with the ethical principles involving human studies provided in the Declaration of Helsinki. All samples used were provided with consent from the donors or their legal representatives.

Formalin-fixed paraffin embedded tissue of DCIS (n = 31) and IBC pure Luminal A (n = 15) diagnosed from 2012 to 2018 were randomly chosen from the archives of the INCA Pathology Department. None of the patients had received any chemotherapeutic treatment before the biopsy procedure. Surgical pieces were not used to ensure that no prior chemotherapy treatment has been performed. All diagnoses were duly confirmed on a patient monitoring data platform in the pathology department of the National Cancer Institute. Normal breast ducts coexisting with DCIS have not been evaluated in view of the proximity to the tumor microenvironment and the tendency for protein expression observed in DCIS, which may characterize a study bias. There is still no record of the evolution of DCIS tumors to invasive carcinoma in patients in the evaluated cohort. For controls, formalin-fixed paraffin embedded samples of nor-

mal breast tissue from mammoplasty (n = 3) were used. The clinical features of the patients were obtained from their medical records and included age, tumor size, nuclear grade, and survival time. All the histopathological evaluations were reviewed by two independent observers.

2.2. Immunohistochemistry

Tissue samples were converted into histological sections on previously silanized slides. Evaluation of the cellular atypia pattern was performed by hematoxylin-eosin (HE) staining. The evaluated tumor area was selected by a pathologist, and the *in situ* regions within the invasive tumors were also delineated to allow a better evaluation of the stains. Tissue sections were immunostained with the Polymer Detection System (RE7150-K, Leica Biosystems Newcastle Ltd., Balliol Business Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW, United Kingdom) according to the protocol established by the manufacturer. Primary antibodies were incubated with the tissues for 18 h at 4˚C at different dilutions determined by titration as shown in **Table 1**. For positive controls, tissues suggested by the antibody manufacturer's datasheets were used. The reaction was visualized using diaminobenzidine (DAB), followed by hematoxylin counterstaining. Negative controls were prepared without the primary antibody. HER-2 status was evaluated according to the American Society of Clinical Oncology/College of American Pathologists HER2 Guideline (ASCO/CAP, 2013), and cases classified as 2+ or 3+ were submitted to chromogenic *in situ* hybridization (CISH). The labeling of the ER and PR assays was considered positive when brown/red staining was found on more than 1% of the total cells counterstained with blue/violet hematoxylin [20]. Proliferation was considered high if nuclei that stained positive for Ki67 were seen in >20% of the tumor sample [23]. The positivity of the staining was analyzed in ten random fields and determined by manual counting using Image J software according to the pre-established equation below.

$$\frac{\sum_{m=1}^{10} Sc}{\sum_{t=1}^{10} Tc} \times 100 = p$$

Sc represents the number of stained cells in each field, where $s = [1, 2, \cdots, 10]$; *Tc* represents the total cells in each field, where $t = [1, 2, \cdots, 10]$; and *p* represents the mean percentage of positivity.

Table 1. Specifications of the primary antibodies used for breast tumor specimen staining.

Primary antibody	Clone	Staining	Dilution/Manufacturer
ER	1D5	Nuclear	1:2000; DAKO
PR	PgR636	Nuclear	1:2000; DAKO
Her2	6B11	Membrane	1:300; Cell Marque
Ki67	MIB1	Nuclear	1:600; DAKO

2.3. Chromogenic *In Situ* Hybridization (CISH)

The CISH technique was performed on 3-μm-thick sections. The dual-probe assay contains an HER-2 locus-specific probe (black signal) and a control probe specific for the centromere of chromosome 17 (red/pink signal). The procedure was performed using an automated staining system (VENTANA, BenchMark ULTRA, Roche Tissue Diagnostics), and the evaluation of the scoring used at least 40 nuclei from two different areas, with the number of HER-2 and CEP17 signals per nuclei recorded. Only cells with at least one copy of HER-2 and CEP17 were scored. The samples were classified according to the ASCO/CAP CISH criteria: if the staining presented a HER-2/CEP17 ratio larger than 2.2, we defined the sample as HER-2 amplified, and if the HER-2/CEP17 ratio was lower than 1.8, there was no HER-2 gene amplification. If the sample had a ratio between 1.8 and 2.2, it was considered borderline, but these values were not observed in any of the cases. The update of the ASCO/CAP guidelines (2018) provided more rigorous interpretation criteria about HER2 positive patients, although controversial [24]. However, pathologists at the assessed cohort institution still use the ASCO/CAP 2013 guidelines during diagnosis; our group maintained the institution's criteria for assessing HER2 positivity.

2.4. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.01 statistical software (California, USA) available for Windows. Pearson's Chi-squared test was selected for comparison of qualitative variables. T tests and Pearson's correlation coefficient (PCC) were used for comparison of quantitative variables. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Patients and Tumor Characteristics

The clinicopathological characteristics are shown in **Table 2**. A total of 46 biopsies of female patients without any prior chemotherapy treatment who were diagnosed with DCIS or IBC were evaluated in the present study. The tumor size ranged from 0.7 to 6.0 cm without any pattern defined. Among these cases, 31 cases were diagnosed as DCIS and the average patient age was 54.3 years. To better evaluate the cohort, we characterized the nuclear grade of the randomly selected cases, and 6.45% of cases were classified as low grade, 54.84% as intermediate grade and 38.71% as high grade. We also observed necrosis in 80.65% and microcalcifications in 77.42% of the DCIS samples.

Fifteen cases of the Luminal A IBC molecular subtype were evaluated, and the average patient age was slightly higher than that in the DCIS cohort (62.2 years). In this group, 20.0%, 66.67% and 13.33% were classified as histological grades 1, 2 and 3, respectively, and only 20.0% of patients with invasive tumors had necrosis, while 26.67% had tissue microcalcifications.

Table 2. Clinicopathologic characteristics of the cohort.

Procedure (NCB/SES)	46/0
Gender (female/male)	46/0
DCIS (n = 31)	
Age (mean ± SD)	54.3 ± 11.6
Necrosis (absent/present)	6/25
Microcalcifications (absent/present)	7/24
Nuclear grade (low/intermediate/high)	2/17/12
IBC (n = 15)	
Age (mean ± SD)	62.2 ± 14.1
Histological type	Luminal A
Necrosis (absent/present)	12/3
Microcalcifications (absent/present)	11/4
Histological grade (1/2/3)	3/10/2

*NCB needle core biopsy, SES surgical excision specimen, SD standard deviation.

3.2. Protein Expression by Breast Cancer and Proliferation Biomarkers

We compared the expression pattern of proteins described as biomarkers of invasive breast tumors across normal breast tissue derived from mammoplasty, DCIS tissues and IBC tissues. The ER expression pattern showed that 96.55% (29/31) of DCIS cases had nuclear positive staining, and the mean percentage of positivity was 87.62%. The results showed a significant increase of ER expression between normal tissue samples (**Figure 1(A)**) and DCIS (**Figure 1(B)**), as shown in **Table 3** ($p < 0.0001$). All patients with IBC (15/15) presented positive nuclear ER staining (**Figure 1(C)**), and the mean percentage of positivity was 89.71%. No significant differences in ER expression patterns ($p = 0.3145$) were observed compared to DCIS samples (**Table 3**).

The PR expression pattern showed that 96.55% (29/31) of the DCIS tissue samples had nuclear positive staining, and the mean percentage of positivity was 59.62%, highlighting a significant increase of PR expression between normal tissue samples (**Figure 1(D)**) and DCIS (**Figure 1(E)**), as shown in **Table 3** ($p < 0.0001$). PR expression in IBC patients presented nuclear staining in 93.33% (**Figure 1(F)**) of samples (14/15), and there were no observed differences in the PR expression patterns ($p = 0.7341$) between the IBC and DCIS samples. Biopsies that presented more than 1% of positivity, on average, were considered positive.

Most of the DCIS cases showed phenotypic characteristics similar to those of the Luminal A like subtype, and these data suggest that changes in steroid receptors (SR) expression patterns could be an early event in the breast tumorigenesis process.

Table 3. Expression of ER, PR, Ki67 and HER-2 in normal, DCIS and IBC tissues.

Parameters	Normal	DCIS	SPR	*P* value (Normal × DCIS)	IBC	*P* value (DCIS × IBC)	SPR
ER							
Negative	3 (100.0%)	2 (6.45%)	0		0		0
Positive	0	29 (96.55%)	80.0% - 100.0%	<0.0001	15 (100.0%)	0.3145	40.0% - 100.0%
PR							
Negative	3 (100.0%)	2 (6.45%)	0		1 (6.67%)		0
Positive	0	29 (96.55%)	10.0% - 100.0%	<0.0001	14 (93.33%)	0.7341	10.0% - 100.0%
Ki67							
Low expression	3 (100.0%)	19 (61.29%)	1.0% - 10.0%		9 (60.0%)		5.0% - 19.5%
High expression	0	12 (38.71%)	30.0% - 80.0%	0.1804	6 (40.0%)	0.9330	21.0% - 30.0%
HER-2							
Negative	3 (100.0%)	27 (87.10%)			15 (100.0%)		
Positive	0	4 (12.90%)		0.5077	0	0.1454	

*DCIS = Ductal carcinoma *in situ*, ER = Estrogen receptor, PR = Progesterone receptor, HER-2 = Human epidermal growth factor receptor, SPR = Staining percentage range.

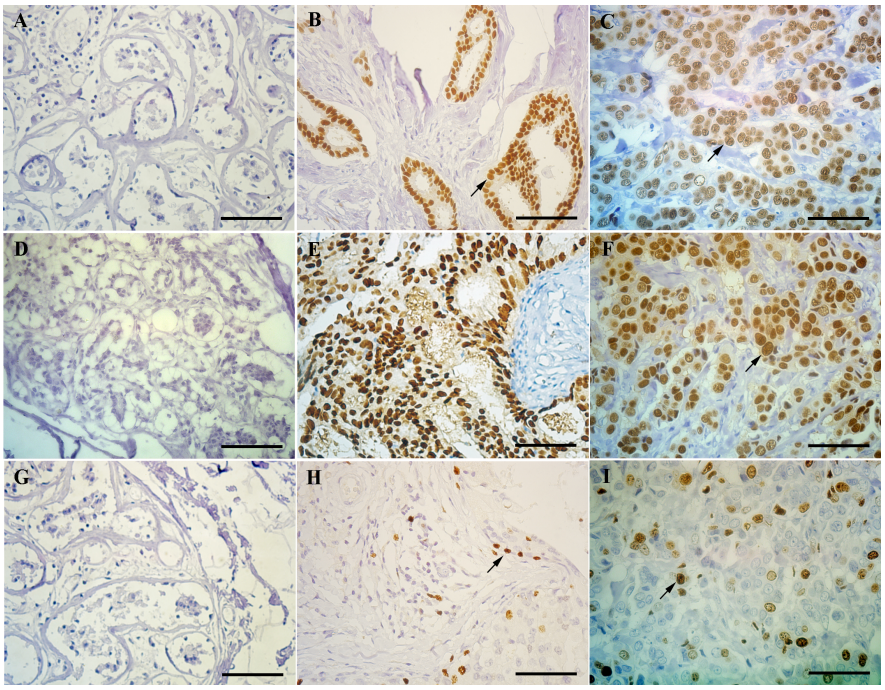

Figure 1. Immunohistochemical staining of ER, PR and Ki67 in normal breast, DCIS and IBC tissues. Nuclear ER expression was slightly increased in DCIS (B) and IBC (C). Nuclear staining of PR was increased in DCIS (E) and IBC (F). A small increase in proliferation could be observed given the nuclear labeling of Ki67 in DCIS (H) and IBC (I) samples. No nuclear staining was observed for ER (A), PR (D) and Ki67 (G) in normal breast tissue. Scale bar: 100 μm. ×400 magnification. Arrows: indicate positive labeling.

The nuclear antigen Ki-67 is commonly used to measure the proliferation rate of many tumors, including breast tumors, and could be a key element of progression of the disease [25]. The Ki-67 expression pattern showed that 61.29% (19/31) of the DCIS tissue samples had low expression and 38.71% (12/31) had high expression (**Table 3**), and there was no significant difference compared with the proliferation of normal tissue ($p = 0.1804$). Similar findings were observed when evaluating IBC tissue samples: 60.0% (9/15) had low expression of the Ki67 antigen and 40.0% (6/15) had high expression of the Ki67 antigen, and no significant differences were observed ($p = 0.9330$) compared with the rates of proliferation of the DCIS samples (**Table 3**). The cut-off used for evaluating the proliferation rate was 20%. A small increase in Ki67 nuclear labeling was observed in DCIS and IBC tissues compared to that in normal tissue (**Figure 1(H)**, **Figure 1(I)** and **Figure 1(G)**, respectively). There was no direct correlation among ER, PR and Ki67 expression (data not shown).

For HER-2 evaluation, the standard expression immunohistochemical classification (ASCO/CAP 2013) was used to quantify incomplete, weak and scarce staining in less than 10% (score 0) and more than 10% (score 1+) of the tumor cell membranes; circumferential and incomplete staining in more than 10% or complete staining in less than 10% (score 2+); and uniform and intense coloration (score 3+). None of the normal tissues evaluated showed HER-2 membrane labeling (**Figure 2(A)**). Only 12.90% of the DCIS samples (4/31) had homogeneous and complete membrane labeling (**Figure 2(B)**) for HER-2 (score 3+).

All samples with scores 2+ (3/31) and 3+ (4/31) for the HER-2 receptor were then subjected to Chromogenic *in situ* Hybridization (CISH) evaluations, which corroborated the immunochemical procedure: only score 3+ samples (4/31) showed amplification of HER-2 (**Figure 2(C)**). Interestingly, all DCIS cases that had experienced HER-2 amplification also had a mean cell proliferation rate of 40% or greater and presented with characteristics of an invasive tumor in adjacent areas, although the number of patients was too small to establish a correlation. No IBC samples were HER-2 labeled (all IBC cases evaluated were Luminal A).

3.3. Correlation of the DCIS Grade and Expression of Molecular Characteristics

We compared the expression of breast cancer biomarker proteins to the tumor grade (**Table 4**) to understand the role of each of these proteins in DCIS biology. All low-grade DCIS samples showed expression of steroid hormones (ER and PR positive) and did not show HER-2 amplification. Interestingly, 1 patient with low-grade DCIS (50.0%) had high levels of cell proliferation.

Most of the cohort tissue samples evaluated were of intermediate grade (17/31): all of these biopsies were positive for ER and PR, while only 1 (5.88%) had HER-2 amplification. However, 4 DCIS samples showed high proliferation levels (23.53%), which corroborates the tendency of low aggressiveness of DCIS tumors of this grade; however, these tumors were more aggressive than low grade tumors.

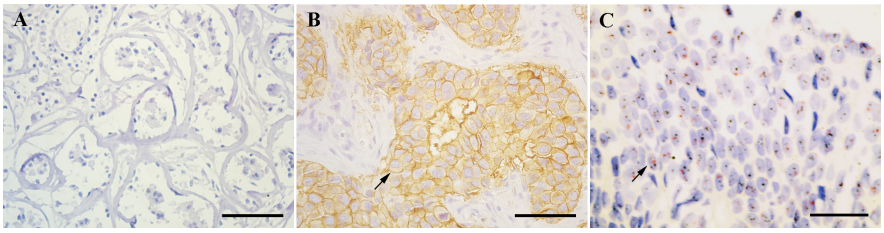

Figure 2. HER-2 expression in DCIS tissues. No HER-2 membrane staining was observed in normal breast tissue (A). Tissue with an HER-2 score 3+ (B) was submitted to the CISH technique to evaluate HER-2 gene amplification (C). Scale bar: 100 μm. ×400 magnification. Arrows: indicate positive labeling and HER-2 amplification.

Table 4. Relationship of the molecular characteristics and DCIS grade.

Grade (DCIS)	ER^+/ER^-	PR^+/PR^-	Ki67 (High/Low)	HER-2 (+/−)
Low (2/31)	2/0	2/0	1/1	0/2
Intermediate (17/31)	17/0	17/0	4/13	1/16
High (12/31)	10/2	10/2	7/5	3/9

In high-grade DCIS tumors, variations in the protein expression patterns related to molecular characteristics were observed. Interestingly, two patients (16.67%) had characteristics similar to the triple negative subtype of IBC tumors (2/12): no expression of steroid hormones (ER and PR negative), no HER-2 amplification and high proliferation rates. We also observed that most patients with high-grade DCIS had high levels of the proliferation index (7/12, 58.33%). Furthermore, HER-2 amplification was observed in 25% of the high-grade samples (3/12). These findings suggest a tendency towards greater aggressiveness associated with high grade DCIS tumors. No relationship was observed between the presence of necrosis and microcalcifications with the DCIS grade (data not shown).

4. Discussion

DCIS is frequently described as a noninvasive lesion as well as a preinvasive lesion of breast cancer and is defined as a neoplastic proliferation of epithelial cells with varying degrees of cytologic atypia that are confined to the mammary ductal-lobular system. The mortality rate is low in women with DCIS, with approximately 1.0% to 2.6% dying as a result of IBC carcinoma 8 to 10 years after diagnosis of DCIS. However, studies have shown that misdiagnosis of DCIS may lead to omission of surgery and, consequently, recurrence as well as an increase in IBC tumors after 30 years to 14% to 53%. Moreover, the invasive recurrence rate after 15 years in women treated with surgery alone can lead to an incidence of 28%, with mortality reaching 18% [26]-[31], and fifty percent of DCIS recurrences actually present as an invasive cancer [32].

Treatment of patients with DCIS is currently variable, consisting of partial or total mastectomy, which may be followed by radiotherapy and/or endocrine

therapy. In this context, a more effective classification of DCIS tumors becomes essential for treatment optimization. This study attempted to evaluate the differences of the clinicopathological features and molecular characteristics of DCIS compared to those the subtype Luminal A of IBC tumors to understand the biology of DCIS and the role breast tumor biomarkers play in this tumor.

The degree of cellular atypia is determined histologically, leading to assignment into three grades (low, intermediate and high) based on the degree of nuclear atypia [33]. In the present study, hematoxylin-eosin staining was performed to observe the cohort's morphological characteristics. Two patients with low nuclear grade DCIS (with occasional mitoses) were observed. Twelve biopsies (~42%) had frequent mitoses, large nuclei, and irregular chromatin and were therefore classified as high-grade DCIS. Previous studies have shown that high-grade DCIS has heterogeneity in 12% - 50% of cases and is considered a high-risk factor for recurrence. These cases represented approximately 42% - 53% of the DCIS cases, and the cohort used in the present study is consistent with the literature in this regard [12] [15] [34] [35] [36]. Seventeen DCIS biopsies showed neither low nor high grade morphological characteristics and were classified as intermediate grade [37].

Recently the "intrinsic subtypes" of IBC have been used to categorize DCIS tumors. The expression of ER, PR, Ki67 and HER-2 is usually used to discriminate different molecular subtypes of IBC (Luminal A, Luminal B HER-2 negative or positive, HER-2 positive and triple-negative (TN)). Similar molecular phenotypes have been proposed for DCIS tumors using immunohistochemistry surrogate markers. Previous studies have indicated by immunohistochemical staining that 49% of DCIS tumors could be classified as Luminal A (ER positive, Ki67 low), 8.7% as Luminal B/HER-2 negative (ER positive, Ki67 high), 17% as Luminal B/HER-2 positive (ER positive, HER-2 positive), 16% as the HER-2 subtype (ER negative, HER-2 positive) and 7% as triple negative (ER negative, PR negative, HER-2 negative). These findings are contrary to the frequency of the appearance of these proteins in IBC tumors, among which a higher proportion of TN appears (14% - 24%) and a lower percentage of the HER-2 positive subtype (6% - 7%) has been described [18] [38] [39] [40] [41].

In the present study, 58.1% of DCIS (18/31) samples showed ER-positive nuclear labeling with low proliferation levels (Ki67 low) while 25.8% (8/31) showed high levels of Ki67 and ER expression. Only three patients had simultaneous ER and HER-2 expression (9.7%); one was HER-2 positive and ER negative (3.2%), and two had no positive labeling for either breast cancer biomarker protein (6.4%), presenting characteristics similar to the TN subtype of IBC, suggesting that there are variations in the expression patterns of mammary tumor marker proteins in DCIS tumors.

ER is one of the most intensively studied biological markers in breast cancer, and ER status may predict the response to endocrine therapy. A previously published review evaluated 36 studies that examined the ER expression rates in

DCIS and showed that the mean ER expression rate was 68.7% (range: 49% - 96.6%). Our results showed that 96.55% (29/31) of the DCIS tissue samples were ER positive, and the mean of percentage of cells staining was 87.62% (range: 0% - 100%; standard deviation = ±0.25), with the variation of the mean trending toward higher values but within the previously described range.

PR can also be considered crucial in prognosis and disease-free survival as well as in predicting the response to endocrine therapy, and 28 papers evaluated by the same previous work showed that the PR expression rate was 59.6% (range: 40% - 83.3%) [42]. The results of the present study found that 96.55% (29/31) of the DCIS tissue samples were PR positive, and the mean of percentage of cells staining was 59.62% (range: 0% - 100%; standard deviation = ± 0.36). The PR expression rate shows a wide range of variation in the expression of breast cancer biomarker proteins, although the mean of positivity corroborated the literature data. Therefore, the expression of steroid receptors in non-invasive DCIS patients observed in the present study suggests a trend towards the Luminal A subtype when recurrence to invasive tumors occurs. However, the cohort evaluated in the study is small and consists of samples from a single center, which reveals a limitation of the study and leads to the need for more comprehensive studies.

The Ki67 nuclear antigen is commonly used to assess proliferation rates of many types of tumors, including breast cancer, and may be an important element in disease progression [25]. A previous study evaluated the proliferation profiles of DCIS tumors and showed low expression rates of Ki67 (10.9% - 15.5%). A few studies associated high proliferative activity with positive HER-2 expression (44.29% ± 3.42%) as well as the presence of comedonecrosis and other architectural patterns in DCIS tumors. In addition, these studies related high proliferative activity with high-grade DCIS lesions [43] [44] [45] [46] [47].

Our results showed that the mean of percentage of Ki67 nuclear staining was 38.68% (range: 1% - 80%; standard deviation = ±0.22), and all of the DCIS HER-2 positive tumors showed high Ki67 expression, although there was not a direct correlation between high Ki67 expression and HER-2 positivity. Additionally, no association was observed between the proliferation levels and the occurrence of necrosis, microcalcifications or nuclear grade. In addition, there was no difference between the nuclear Ki67 expression patterns of DCIS and IBC tumors.

In addition to steroid receptors, HER-2 is one of the most extensively studied biomarkers in DCIS. Studies have found HER-2 to be of great importance in diagnosing IBC; however, its importance in DCIS is poorly described and still needs to be further elucidated [48]. A previous study also suggested that overexpression of HER-2 or amplification of the gene in DCIS and invasive tumors with DCIS components may be involved in the transition from DCIS to IBC [49]. Several studies investigated HER-2 expression rates by subtype and found major expression in the comedonecrosis subtype [43] [50] [51]. In addition, the

relationship between HER-2 amplification and high-grade DCIS tumors has also been previously described [21].

Our results showed that all HER-2 positive samples presented with necrosis and a high nuclear grade, corroborating the findings in the literature. A few studies showed HER-2 to be inversely correlated with ER and PR expression [48] [50] [51] [52]. Another study showed that DCIS ER positive tumors often overexpress HER-2 protein without gene amplification [20]. Only 25% (1/4) of the HER-2 positive samples in the present study did not show steroid hormone expression, and the other HER-2 positive samples (75%; 3/4) were also ER and PR positive. In a retrospective study, HER-2 was positive in 20% of DCIS cases [53], but our results found that 12.90% (4/31) of the DCIS samples had HER-2 amplification.

HER-2 is an established negative prognostic factor in IBC. HER-2 expression has been observed more frequently in DCIS than in IBC, and this phenomenon is paradoxical in understanding the role of HER-2 in aggressive invasive disease, and the molecular mechanism that leads to the loss of HER-2 expression during the progression of DCIS to IBC is still unclear and needs to be better elucidated [20] [42] [48] [54] [55] [56] [57]. In this context, a better investigation of the receptor in DCIS is necessary to better clarify the relationship between HER-2 amplification and HER-2 expression, and describing its functionality is crucial to narrow the classification of breast tumors, which would contribute to the treatment of the disease.

5. Conclusion

The biology of DCIS is still not well understood given its complexity and heterogeneity. Our results suggest that the predominant expression of steroid hormones in DCIS may be related to the fact that they are earlier, less aggressive and well differentiated tumors and could be associated to an evolutionary trend towards IBC-like Luminal A in recurrent DCIS. Our results also showed that high-grade DCIS shows a higher frequency of other markers, suggesting that this tumor may have an evolutionary process analogous to that of invasive tumors, and these findings suggest the possibility of identifying cases that would tend to become invasive.

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Author Contributions

IP: designed the research study, conducted the experiments, performed the interpretation and quantification of experimental data and wrote the manuscript; FRR: selected similar tumor regions on patients' slides and quantified the expe-

rimental data; PVF: contributed to the random selection of patients, separated the tissue bank samples from the INCA patients and standardized the antibodies; EA: designed the research study and critically revised the manuscript. All the authors read and approved the final manuscript.

Ethical Approval

This study was approved by the independent institutional advisory committee on September 23rd, 2015 (protocol 1241052). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding this study.

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