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The glycomic profile of invasive ductal carcinoma of the breast is altered in patients with hypoxic regions: implications for tumor behavior

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Abstract: Hypoxic areas in solid tumors are often associated with poor prognosis and resistance to chemotherapy. The aim of the study was to analyze the expression of galectin-1 (Gal-1), galectin-3 (Gal-3), sialic acid and b1–6 branched glycan structures in hypoxic environment of invasive ductal carcinoma (IDC) of the breast. We performed lectin histochemistry with phytohemagglutinin-L (L-PHA) and *Sambucus nigra* lectin (SNA); and immunohistochemistry for Gal-1, Gal-3, carbonic anhydrase IX, hypoxia-inducible factor, estrogen receptor (ER), progesterone receptor and human epidermal growth factor receptor type-2 for 86 IDC samples. Patients with markers positive for hypoxia were mostly ER-negative ($p = 0.003$) and presented with more nodal invasion than the non-hypoxic group ($p = 0.0439$). Concerning the glycobiological aspects, the hypoxic group expressed more of Gal-3 ($p = 0.0021$) and SNA ligands ($p = 0.0498$), however, there was no association between lectin- and galectin-staining and clinical and histopathological data. Our results suggest a change in the glycomic profile of patients within hypoxic regions of IDC. However, further studies are needed to evaluate the role of lectin- and galectin-ligands in tumor's hypoxic environment, as well as their potential to be used as therapeutic targets. *(Folia Histochemica et Cytobiologica 2014, Vol. 52, No. 2, 96–103)*

Key words: breast cancer; IDC; hypoxia; glycomic profile; galectins; lectin ligands; IHC; histochemistry

Introduction

Breast cancer is the most common cancer among women, accounting for 22% of new cases every year, and the second most common cancer worldwide [1]. Moreover, as with most solid tumors, breast cancers often have central regions of hypoxia which are usually peri-necrotic [2]. Cells that survive in this adverse environment are typically resistant to radiotherapy and chemotherapy and differ from cells in areas with adequate blood supply [3, 4]. Hypoxia-inducible factor 1α (HIF-1α) is the major modulator of hypoxic condition and is constitutively produced under low-oxygen conditions, which typically occurs at distances greater than 180 μm from blood vessel [5]. HIF-1α affects tumor progression and angiogenesis by modulating the expression of genes involved in the adaptation to oxygen- and nutrient-deficiency, leading to increased cell survival, inhibition of apoptosis and migration [6, 7]. Among these genes, carbonic anhydrase IX (CA IX) and glucose transporter 1 (GLUT1) play crucial roles in cellular adaptation, by altering the energy metabolism of neoplastic cells, which begin to show high rates of anaerobic glycolysis [2, 5–7]. The change in metabolic profile is followed by altered expression of enzymes involved in aerobic and anaerobic glycolysis, sugar

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nucleotide transport and carbohydrate synthesis [8]. These modifications increase the amount of flux from the glycolytic pathway to the hexosamine pathway and the N-acetylglucosamine pathway, thereby altering key components of cellular glycosylation, such as substrates for glycosyltransferases [9].

Additionally, hypoxia influences the expression of molecules that change cellular behavior — one of these molecules is galectin 1 and galectin 3 (Gal-1 and Gal-3, respectively), a protein that appears to be pro-neoplastic in the hypoxic microenvironment [10, 11]. However, little is known about the glycomic aspects of breast cancer's hypoxic environment and its possible implications.

We therefore aimed to evaluate Gal-1 and Gal-3 expression and the phytohemagglutinin-L (L-PHA) and SNA ligand profiles by immunohistochemical (IHC) and histochemical staining methods in the invasive ductal carcinoma (IDC) of the breast within and beyond hypoxic areas of tumor tissue. We also evaluated the possible correlations between the expression of these molecules and diagnostic and prognostic parameters and markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type-2 (HER-2).

Material and methods

Sample collection. Eighty-six samples from IDC patients were selected from the Pathology Department at the Hospital das Clinicas in the Federal University of Pernambuco, Brazil, (HC-UFPE) between 2002 and 2010. The tumor-free edges and 10 reductive mastoplasty biopsies were used as controls. Patients who had inflammatory or medullary carcinoma, or who were less than 30 years of age were excluded from the study. The Medical Records and Statistics Service of HC-UFPE provided the clinical and histopathological information about the patients. The clinicopathological data from patients included in the study is shown in Table 1. The study was approved by the human research ethics committee of the aforementioned institution (CEP/CCS/ /UFPE No 195/09).

Lectin histochemistry. Sections of IDC samples (4-μm thick) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Sections were treated with trypsin, submitted to endogenous peroxidase blocking, and incubated with biotinylated SNA and L-PHA lectins at 20 μg/mL for 2 h at 25°C before incubation for 1 hour with streptavidin-peroxidase. L-PHA recognizes galectins' ligands and SNA recognizes sialic acid that often mask those ligands. The excess reagent was removed in two baths of phosphate-buffered saline (PBS) for 5 min, the peroxidase color reaction was developed with diaminobenzidine-H_2O_2 (DAB) and the nuclei were counter-stained with hematoxylin. Samples of ductal carcinoma in situ of the breast previously known to be positive for lectin histochemistry were used as positive controls, while the negative control was obtained by replacing the lectin with PBS and lectin inhibition with its specific sugar at a concentration of 300 mM [12, 13]. All materials which source was not specified were obtained from SigmaAldrich (St. Louis, MO, USA).

Immunohistochemistry. Sections of IDC samples (4-μm thick) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed in citrate buffer (pH 6), at 95–99°C for 30 minutes, followed by treatment with endogenous peroxidase blocking solution (DAKO, Glostrup, Denmark) at room temperature for 15 min. Nonspecific sites were blocked with 1% PBS bovine serum albumin for 1 h at room temperature. Then sections were incubated with primary antibodies (Table 2) for 2 h at 37°C. The samples were incubated with biotin-free polymer (DAKO) and the peroxidase color reaction was developed by DAB-H_2O_2 (DAKO). Positive controls were performed as indicated by the antibody manufacturer; in the case of negative controls, the primary antibody was replaced with non-specific IgG (DAKO).

Histological evaluation. For each sample, 10 fields were evaluated under the 40 × objective lens by two pathologists and only cells positive for neoplastic markers were counted. For the evaluation of hormone receptors, the scoring system proposed by American Society of Clinical Oncology (ASCO) [15], which evaluates the proportion of stained nuclei and intensity of staining, was used. For the analysis of HER-2 we followed the criteria adopted by the ASCO and the College of American Pathologists Consensus [16], which considers the intensity of membrane staining and the percentage of stained cells. Reaction for CA IX was considered immunopositive when there was any indication of membrane staining, whereas HIF-1α expression was considered positive when there was nuclear staining in more than 10% of the cancer cells analyzed [16]. Stainings for Gal-1, Gal-3, SNA and L-PHA were considered positive when at least 10% of cells showed cellular reactivity, irrespective of cellular location [12, 13, 17]. For the evaluation of stromal cells, 10 fields under the 40 × objective lens were evaluated and any positive stromal cells were included in the counting. The average number of positive stromal cells was compared between hypoxic and non-hypoxic (control) areas.

Statistical analysis. All statistical analyses were performed using GraphPad Prism software version 4.0 (GraphPad, La Jolla, CA, USA). Categorical data were assessed using the chi-square (χ^2) test. Differences were considered statistically significant at $p < 0.05$.

Table 1. Clinical and histopathologic features of invasive ductal breast cancer (IDC) patients with hypoxic and non-hypoxic areas

Parameter	Number and % of IDC CA IX− cells (non-hypoxic group)	Number and % of IDC CA IX+ cells (hypoxic group)	P value
Tumor size			
pT1	20 (30.30%)	2 (10%)	> 0.05
pT2	30 (45.50%)	10 (50%)	
pT3	4 (6.09%)	6 (30%)	
pT4	12 (18.20%)	2 (10%)	
Lymph node metastasis			
Yes	22 (33.33%)	17 (65%)	0.0439
No	44 (66.67%)	3 (35%)	
Histological grade			
1	15 (22.72%)	3 (15%)	> 0.05
2	24 (36.36%)	5 (25%)	
3	27 (40.92%)	12 (60%)	
Immunohistochemical markers			
ER+	22 (46.80%)	4 (20%)	0.0003
PR+	22 (46.80%)	13 (65%)	> 0.05
HER-2+	3 (6.40%)	3 (15%)	> 0.05

ER — estrogen receptor; PR — progesterone receptor; HER-2 — human epidermal receptor 2

Table 2. Sources of antibodies and lectins used for immunohistochemical and histochemical staining

Primary antibody/lectin	Source	Dilution/concentration	Clone
ER	Santa Cruz Biotechnology, Dallas, TX, USA	1:100	1D5
PR	Santa Cruz Biotechnology	1:150	1A6
HER-2	DAKO, Glostrup, Denmark	1:400	MIB1
GAL-3	Spring Bioscience, Pleasanton, CA, USA	1:150	9C4
M338	Hybridoma	1:20	–
GAL-1	Santa Cruz Biotechnology	1:100	C8
CA IX	Santa Cruz Biotechnology	1:150	H-120
HIF-1α	Santa Cruz Biotechnology	1:100	H-206
L-PHA	Vector Labs, Burlingame, CA, USA	20 μg/ml	–
SNA	Vector Labs	20 μg/ml	–

ER — estrogen receptor; PR — progesterone receptor; HER-2 — human epidermal receptor 2; Gal-3 — galectin-3; M338 — name given to the hybridoma producing anti-N-terminal Gal-3 antibody; Gal-1 — galectin-1; CA IX — carbonic anhydrase IX; HIF-1α — hypoxia-inducible factor 1; L-PHA — phytohemagglutinin-L; SNA — *Sambucus nigra* lectin

Results

Hypoxic tumor samples are predominantly ER--negative and associated with lymph node invasion

We first evaluated any clinical and histopathological differences between study groups. The 86 samples were subjected to HIF-1α and CA IX immunohistochemistry to confirm the presence of hypoxic areas. Samples of 20 patients presented neoplastic cells positive for hypoxic markers, showing nuclear staining for HIF-1α (Figure 1D) and membrane staining for CA IX (Figure 2A); these patients represented the hypoxic group. Conversely, samples of 66 patients were negative for hypoxic markers and they were considered the control group (non-hypoxic). Interestingly, the control group patients were of younger age (50.5 ± 5.2, mean and SD), whereas the hypoxic group had a higher mean age and standard deviation (58.6 ± 15.0), however, the difference was statistically not significant. We observed a direct association between the hypoxic group and nodal involvement (p = 0.0439). The other clinical and histopathological characteristics are summarized in Table 1.

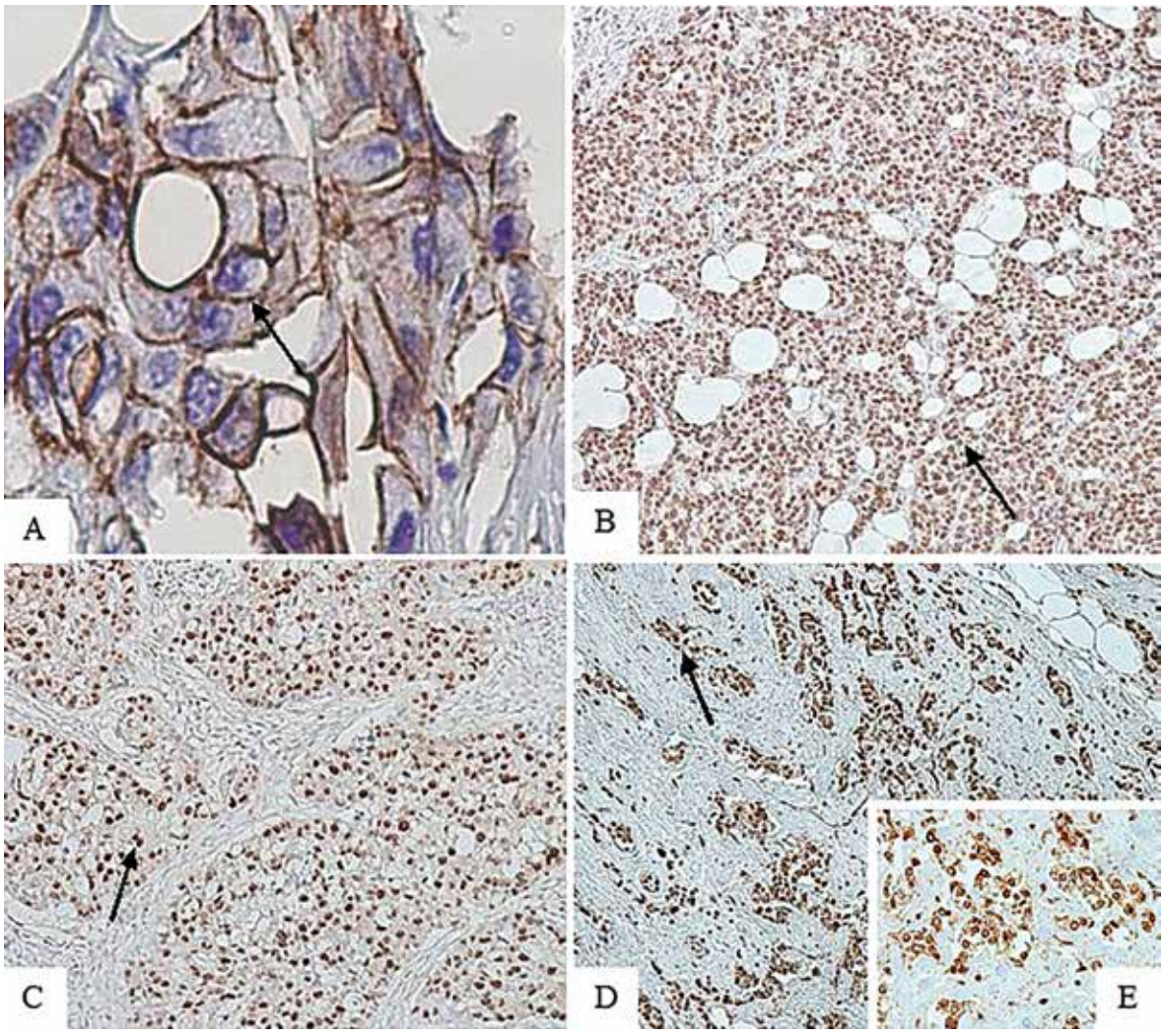

Figure 1. Immunohistochemical expression of markers used in the study of breast invasive ductal carcinoma. **A.** Her-2 reveals intense membrane staining (arrow). Positive nuclear immunoreactivity for estrogen receptor (**B**, arrow), progesterone receptor (**C**) and HIF-1α (**D–E**). The figure presents representative cases of the study. Magnification: A and E × 400; B, C and D × 100

In relation to markers predictive of treatment response (ER, PR and HER-2; Figure 1), we did not observe any significant difference between analyzed groups ($p > 0.05$). However, an inverse association between positive ER and CA IX immunoexpression in the hypoxic group was observed ($p = 0.003$). There were no significant correlations between CA IX and PR and HER-2 immunoreactivities in the hypoxia group ($p > 0.05$).

Increased expression of Gal-3 and SNA ligands in hypoxic samples of IDC

The glycomic analysis of analyzed samples revealed that SNA staining revealed more cases in the hypoxic group than in the control non-hypoxic group ($p = 0.0498$), while there was no significant difference in L-PHA ligands between groups ($p = 0.39$) (Figure 2). There was no positive staining for Gal-1 in neoplastic cells of either of the two studied groups.

In an attempt to find possible associations between hypoxia, tumor histopathologic parameters and Gal-1 immunoreactivity in stromal cells, cells positive for Gal-1 were counted in 10 randomly selected fields at the 40 × objective lens in the control and hypoxic samples. However, no differences were established (data not shown).

The immunohistochemical detection of Gal-3, was found in a higher number of cases in the hypoxic group compared with the non-hypoxic group ($p = 0.0021$), which was accompanied by a change in cytoplasmic and nuclear localization of Gal-3, with its increased nuclear localization in the hypoxic group (Figure 3B). Figure 2 summarizes the percentage of hypoxic and

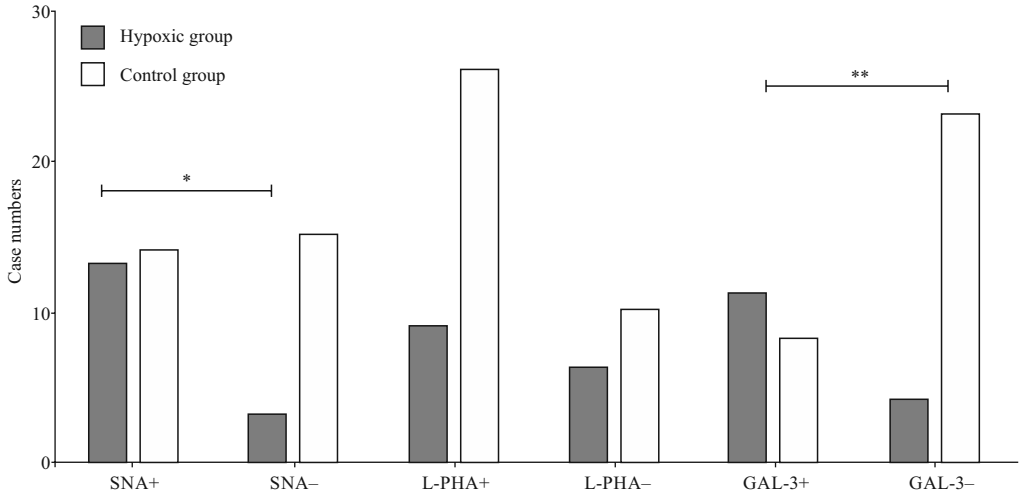

Figure 2. IDC percentage for the glycan markers, SNA, L-PHA, Gal-3 and M338 by study group. SNA lectin and anti-Gal-3 were positive in a higher number of cases in the hypoxic group compared with the control non-hypoxic group. SNA — *Sambucus nigra* agglutinin; L-PHA — phytohemagglutinin lectin; M338 — name given to the hybridoma producing anti-N-terminal Gal-3; Gal-3 — galectin-3

non-hypoxic IDC cases positive and negative for SNA, L-PHA and Gal-3 staining.

When we used M338 (antibody against N-terminal region of Gal-3), we found increased cytoplasmic staining with or without nuclear staining in the hypoxic group, however, the number of positive cases for this antibody was very low (data not shown). The subcellular localization analysis was also performed for SNA and L-PHA (Figure 3C and D, respectively). In the case of SNA, we noticed a marked tendency towards changing the cell staining pattern from the cytoplasmic and membranous, to the cytoplasmic only in the hypoxic group. The analysis of L-PHA subcellular staining did not show a significant change between hypoxic and nonhypoxic groups, only a small increase in cytoplasmic staining and a slight decrease in cytoplasm and membrane co-staining.

Discussion

The tumor microenvironment consists of highly complex cellular and molecular networks that contribute to the hallmarks of cancer, including evasion of cell death, self-sufficiency in secretion of growth signals, insensitivity to anti-proliferative signals, tissue invasion and metastasis, limitless replicative potential, and angiogenesis [18]. These processes can be affected by hypoxia, which alters the expression of genes [2] including those which encode galectin-1 and galectin-3, proteins which appear to modulate many of the tumor characteristics and play a key role in tumorigenesis [19].

In the IDC samples, we observed that patients positive for CA IX had more aggressive clinical characteristics than previously reported [20]. CA IX is principally regulated at the transcriptional level and its expression is stimulated by hypoxia through HIF-1, which binds to the hypoxia responsive element (HRE) in the CA IX promoter to increase transcription [21]. Carbonic anhydrase IX confers an acidic microenvironment compatible with tumor cell viability and proliferation and has been associated with necrosis, high tumor grade, higher relapse rates and worse overall survival in patients with breast carcinoma [22, 23].

Moreover, we found an inverse correlation at the level of IHC staining between ER and CA IX positivity — a fact that can be related to the predominant tumor subtype and menopausal status of patients with a possible poor prognosis. These data agree with previous results obtained by Tan et al. [24] and other authors [25–27]. Furthermore, CA IX expression in hypoxic regions is regarded as an indicator of resistance to chemotherapy and hormonal therapy, and may therefore be a target in hypoxia-specific therapies [28, 29].

The significant decrease in ER staining in the hypoxic group, which is associated with poor prognosis [30], could relate to the fact that HIF-1α induces ER proteasomal degradation in hypoxic microenvironment, and decreases its transcription by reducing the RNApol II recruitment in the promoter region of ESR1 *locus* [31].

In the IDC samples, Gal-1 staining was observed in the tumor-associated stromal cells, but no signifi-

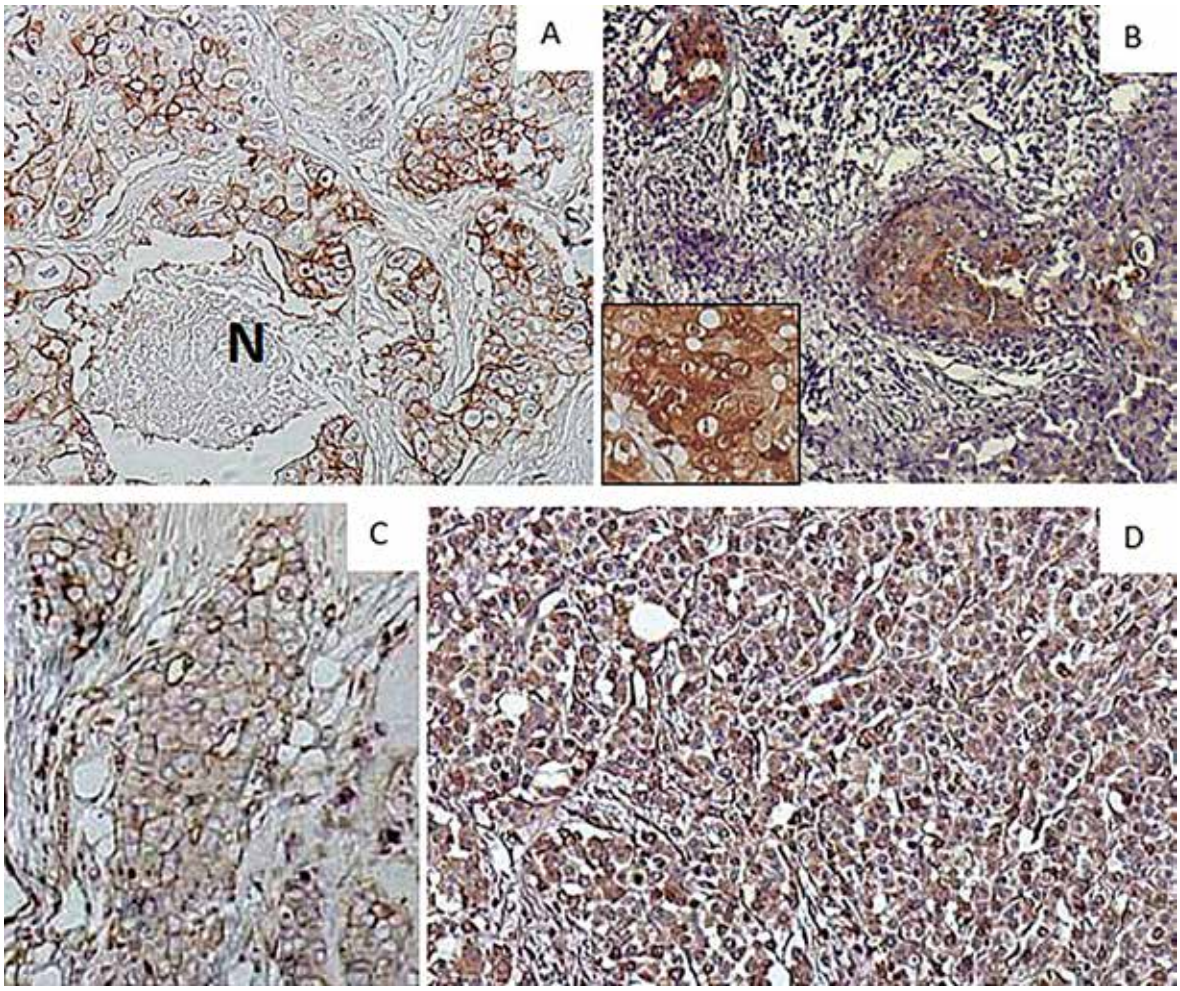

Figure 3. Lectin- and antibody-staining in the hypoxic group of breast invasive ductal carcinoma. **A.** CA IX reactivity was present exclusively in cell membranes (N, necrotic core). **B.** Anti-gal-3 immunoreactivity intensely present in cytoplasm and nuclei (nuclear staining is highlighted in the inset). **C.** SNA immunopositive reaction in the cytoplasm is characteristic of O-glycosylation. **D.** L-PHA staining is present in cell membranes and cytoplasm. Abbreviations as for Table 2. Magnification: A, B and D × 100; C × 200

cant difference between the hypoxic and the control group was found. This is in contrast to glioblastoma, where neoplastic expression of Gal-1 is associated with clinical outcome and hypoxic regions [32]. One of the major roles of Gal-1 in cancer progression is immunosuppression, which facilitates a pro-tumorigenic environment, thus, Gal-1 expression in stroma appears to be inversely proportional to the level of CD3 staining, *i.e.* presence of T lymphocytes [33, 34]. In head and neck cancers, the role of Gal-1 under hypoxic conditions represents a pivotal link between hypoxia and tumor-immune privilege [33]. However, one has to be careful with the interpretation of this data, because Gal-1 staining was found in parenchymal and not stromal cells in the aforementioned study.

Interestingly, in our study Gal-3 was significantly overexpressed in hypoxic IDC samples, with an increase in nuclear Gal-3 staining, which is associated with more aggressive tumors and worse prognosis [35]. On the other hand, decreased expression of cytoplasmatic Gal-3 is also associated with progression of human breast cancer [36, 37]. Furthermore, Gal-3 plays an important role in the regulation of Wnt/β-catenin signaling — a key pathway in development, tissue homeostasis, and tumor growth [38].

In some specific environments, such as necrotic areas, Gal-3 and its ligands are overexpressed in glioblastoma in hypoxic regions known as pseudopalisades [39], as well as in regions around necrotic areas from ductal carcinoma *in situ* of the breast, as demonstrated in a previous study by our group [12]. Another interesting result was the low immunopositivity of staining with M338 antibody which recognizes N-terminal region of Gal-3, a fact which could be

relate to N-terminal cleavage performed by matrix metalloproteinases, as well as phosphorylation in hypoxic regions [40, 41].

Regarding glycomic markers, we found increased expression of SNA ligands in the hypoxic group. Since SNA recognizes sialic acid residues [42] the increased monosaccharides' staining reflects their higher expression at the cell surface, which reduces interactions of tumor cells with the extracellular matrix, thereby facilitating metastasis and promoting immune-escape, associated with a poor prognosis in breast cancer [43]. Furthermore, L-PHA-negative cases were SNA-positive, suggesting that L-PHA might not recognize its ligand (branched β1,6 oligosaccharides) owing to steric impediment promoted by sialic acid; this was indeed the case in B-cell lymphomas, where L-PHA ligands were masked by sialic acid in an α2–6 linkage [44] that is recognized by SNA.

To our knowledge this is the first report to analyze the glycomic profile in the hypoxic environment of breast cancer, and correlate data with diagnostic and prognostic markers. However, further studies are needed to evaluate the role of lectin- and galectin-ligands in tumor's hypoxic environment, as well as their potential to be used as therapeutic targets.

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