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Invasive ductal breast carcinoma: correlation of AXL and ß-catenin immunohistochemical expression with tumor aggressivity

Carcinoma mamário ductal invasor: correlação da expressão imunoistoquímica de AXL e ß-catenina com a agressividade tumoral

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ABSTRACT

Introduction: Invasive ductal carcinoma corresponds to the most common histological type of the breast, coexisting with different forms of clinical evolution, histological grading, expression of certain tissue markers and genomic profiles that seek a better understanding of the disease.

Objectives: To analyze the correlation of ß-catenin and AXL markers with tumor aggressiveness, with reference to overall survival, tumor progression and histopathological prognostic factors.

Methods: A study of 101 samples of invasive ductal mammary carcinoma was performed. Those with a diagnosis of ductal type, initially submitted to biopsy or definitive surgical treatment, were included. For control purposes, 20 samples of intraductal carcinoma, 35 of breast fibroadenoma and 10 of breast tissue without any alteration were included. Those undergoing neoadjuvant chemotherapy, those without a tumor sample prior to chemotherapy, those lost to follow-up, and those with incomplete data, were excluded.

Results: When the ß-catenin expression was analyzed, it was negative. As for AXL, different degrees of expression were observed without statistical significance between them.

Conclusion: When analyzing invasive ductal breast adenocarcinoma in TMA, there was no correlation in the expression of ß-Catenin and AXL when compared to overall survival, tumor progression and histological grade.

KEYWORDS: Breast ductal carcinoma. Immunohistochemistry. AXL. ß-Catenin.

RESUMO

Introdução: O carcinoma ductal invasor corresponde ao tipo histológico mais comum da mama coexistindo com formas diferentes de evolução clínica, graduação histológica, expressão de determinados marcadores teciduais e perfis genômicos que procuram melhor entendimento da doença.

Objetivo: Analisar a correlação dos marcadores ß-catenina e AXL com a agressividade tumoral, tendo como referência a sobrevida global, progressão tumoral e fatores prognósticos histopatológicos.

Método: Foi realizado estudo de 101 amostras de carcinoma mamário ductal invasor. Foram incluídas aquelas com diagnóstico do tipo ductal, submetidas inicialmente à biópsia ou tratamento cirúrgico definitivo. Incluiu-se para fins de controle 20 amostras de carcinoma intraductal, 35 de fibroadenoma mamário e 10 de tecido mamário sem qualquer alteração. Foram excluídos os submetidos à quimioterapia neoadjuvante, que não tivessem amostra tumoral prévia ao tratamento quimioterápico, que perderam o seguimento, e com dados incompletos.

Resultado: Quando analisada a expressão da ß-catenina, foi negativa. Quanto ao AXL foram observados diferentes graus de expressão sem significância estatística entre eles.

Conclusão: Quando analisados adenocarcinoma mamário do tipo ductal invasor em TMA não houve correlação na expressão de ß-catenina e AXL quando comparados a sobrevida global, progressão tumoral e grau histológico.

PALAVRAS-CHAVE: Carcinoma ductal de mama. Imunoistoquímica. AXL. ß-catenina.

Central Message

Invasive ductal carcinoma corresponds to the most common histological type of the breast, coexisting with different forms of clinical evolution, histological grading, expression of certain tissue markers, and genomic profiles that seek a better understanding of the disease. This study sought to analyze the correlation of ß-catenin and AXL markers with tumor aggressiveness, with reference to overall survival, tumor progression, and histopathological prognostic factors.

Perspective

In view of the current data on breast cancer incidence, prevalence and mortality, it is necessary to search for new markers that can be used as indicators of severity or unfavorable clinical evolution, guiding the use of different treatment modalities, as well as the development of new therapeutic targets. It is known that ß-catenin and AXL are related to the processes of cell regulation and growth, and the aberrant expression of both is related to carcinogenesis. However, when invasive ductal breast adenocarcinoma was analyzed in TMA, there was no correlation in the expression of ß-catenin and AXL when compared to overall survival, tumor progression and histological grade.

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INTRODUCTION

Neoplasms in general occur due to mutations in cellular genetic material, altering several functions and, in this way, cell division is processed abnormally, giving rise to tumors.[1] Breast cancer, like other tumors, is a heterogeneous disease and can be divided into different clinical and histological subtypes.[2] In women, it is the main malignant neoplasm (except for non-melanoma skin tumors). It is important to note that the data published by the National Cancer Institute also warn that its diagnosis occurs, in most cases, in clinical stages III (44.8%) and IV (16.3%),[3] that is, in advanced stages of the disease. This reality is considered worrisome due to the greater need, in these cases, for aggressive and high-cost treatments involving chemotherapy, radiotherapy and radical operations.[4,5]

It should be noted, however, that the process of carcinogenesis is generally very slow, taking several years for abnormal proliferation to give rise to palpable tumor lesions. In 80% of cases, breast neoplasms are painless and only 10% complain of pain without the perception of the tumor.

The predominant histological types of breast carcinomas are ductal and lobular (in situ or infiltrating).

Infiltrating ductal cancer accounts for approximately 75% of cases. Oval, lobulated, or irregular masses with variable contours that can be well-defined, undefined, or even spiculated are reported.[6] It usually manifests as a spiculated, irregular mass associated with dense fibrous tissue in the stroma, giving the tumor a hardened consistency. This growth presents in the form of nodules, which on palpation can reveal adherence to adjacent structures or the chest wall, and retraction of the skin and nipple, distorting the glandular structure.[7]

Invasive lobular imaging can present in a multicentric and bilateral manner. Most patients have only poorly defined local densification or induration, sometimes appearing as a palpable nodular area. In advanced lesions, there may be skin retraction. Regarding the prognosis and pattern of axillary involvement, it presents behavior similar to invasive ductal carcinoma.[8]

TNM staging represents the main prognostic factor considering survival and tumor recurrence; however, histological grading also plays an important role, especially in small tumors without lymph node involvement.

Other prognostic factors have been the subject of research in recent decades. Among them, we can highlight the hormone receptors (estrogen and progesterone) and the human growth factor receptor – 2 (Human Epidermal growth factor Receptor type 2, HER-2). Thus, lesions considered positive estrogen and progesterone receptors predict a good response of tumors to some type of hormonal therapy. However, estrogen receptor (ER) negative tumors have a higher recurrence rate in the first 5 years after diagnosis. On the other hand, HER-2 overexpression is associated with a worse prognosis when the currently available therapeutic modalities are not considered.

Even so, clinical, histological, hormone receptor, and HER-2 parameters cannot always adequately predict the evolution of breast cancer. Thus, additional prognostic factors have been investigated in order to better stratify tumors from the point of view of aggressiveness, progression and recurrence, in order to individualize therapeutic approaches. In this sense, the evaluation of the tumor profile based on molecular findings (Luminal A, Luminal B, HER-2 and basaloid), gene expression (Oncotype Dx, MammaPrint and Predictor Analysis of Microarray) and new tissue biomarkers, including β-catenin and AXL, has been shown to be promising.[9,10]

The aim of this study was to analyze the correlation of β-catenin and AXL markers with tumor aggressiveness, with reference to overall survival, tumor progression, and histopathological prognostic factors

METHOD

This research was approved by the Research Ethics Committee of the HEI, under opinion number 1,999,671 and carried out in partnership with Brigham and Women's Hospital, Harvard Medical School Molecular Oncology and Immunology and Renal Division, Boston, USA, in accordance with the precepts of Resolution 466/12 of the National Health Council/Ministry of Health (CNS/MS). It is an observational, single-center, analytical, and retrospective study.

Sample characteristics

This is the evaluation of 101 samples of invasive ductal breast carcinoma. Those with a ductal diagnosis, initially submitted to biopsy or definitive surgical treatment (segmental resection or modified radical mastectomy) were included; 20 samples of intraductal carcinoma, 35 of breast fibroadenoma, and 10 of breast tissue without any alteration were also included for control purposes. Patients undergoing neoadjuvant chemotherapy, those who did not have a tumor sample prior to chemotherapy, who were lost to follow-up, and with incomplete data were excluded

Histopathological data were collected and tabulated. Clinical evolution was assessed in medical records and divided into 2 groups: good prognosis - when the clinical evolution was favorable, i.e., without evidence of disease progression or recurrence -, and poor prognosis - when the clinical evolution was unfavorable, recognized by the presence of disease progression, recurrence and/or death from the disease during outpatient follow-up. To classify them into these groups, the following were evaluated: disease progression, disease-free survival time, survival time, and outpatient follow-up time.

Materials

Multisample blocks (BMA) were performed and analyzed by immunohistochemistry by the immunoperoxidase technique. Incubation with primary antibodies lasted between 16-20 min at room temperature. Amplification was performed by Ultraview Universal DAB Detection KitÒ. The processing was all performed on an automated Ventana Benchmark UltraTM platform (β-catenin: clone 14/Ventana/prediluted; AXL: polyclonal/ St. John's Laboratory/Dilution 1/100). Internal and external positive controls attested to the fidelity of the reactions.

Statistical analysis

The results were described as means, standard deviations, minimum and maximum values (quantitative variables) or as frequencies and percentages (categorical variables). Fisher's exact test or the chi-square test was used to analyze the association between categorical variables and markers. These same tests were used to compare tumor types in terms of markers. Progression-free and survival time were described by Kaplan-Meier curves. For group comparisons, in relation to these times, the Log-rank test was considered. Values of $p < 0.05$ indicated statistical significance. The data were analyzed with the Stata/SE v.14.1 computer program. StataCorpLP, USA.

RESULT

The analysis presented was based on data from 87 patients with invasive ductal tumor who met all the inclusion criteria. Other subtypes were excluded. Age at diagnosis, tumor staging, disease-free survival, signs of progression, recurrence and/or death from the disease were surveyed. Histopathological factors such as tumor grading, hormone receptor expression, HER2 and the so-called surrogate subtypes based on integrative classification were also analyzed, which are known to be prognostic factors and are closely related to the clinical evolution of the disease. Of the total of 87 patients, 33 (37.9%) were younger than 50 years old and 54 (62.1%) were older than 50. The mean age was 55.7+/-12.8 (32-79).

Clinical stage, evaluation of recipients, subtypes and clinical evolution

From the 8th edition of the AJCC for breast cancer staging, in addition to the traditional TNM clinical staging, the so-called prognostic staging was included, which considers, among others, the tumor grade, evaluation of ER expression, progesterone receptor (PR) and HER2 expression.

TNM Rating

The parameters for cancer staging were according to the TNM classification of the 8th edition of the AJCC. Table 1 details the variables.

TABLE 1 — Tumor grade and staging of breast tumors (n = 87)

Variable	n	Classification	Result*
Tumor type	87	Invasive ductal ca	87 (100)
Degree	87	G1	4 (4,6)
		G2	38 (43,7)
		G3	45 (51,7)
Degree		G1/G2	42 (48,3)
(G1/G2 grouped)		G3	45 (51,7)
T (Tumor Size)	87	T1 (2 cm or less)	14 (16,1)
		T1c	3 (3,4)
		T2	36 (41,4)
		T3	13 (14,9)
		T4	13 (14,9)
		Tier 4a	1 (1,1)
		T4b	2 (2,3)
		T4d	3 (3,4)
		Tx	2 (2,3)
T	85	T1	36 (42,4)
T grouped/excluding Tx)		T2	13 (15,3)
		T3	17 (20,0)
		T4	19 (22,4)
N (metastasis for regional axillary lymph nodes)	87	N0	28 (32,2)
		N1	37 (42,5)
		L2	7 (8)
		N2a	3 (3,4)
		N3	7 (8)
		N3c	1 (1,1)
		Nx	4 (4,6)
N	83	N0	28 (33,7)
(N2/N3 Grouped and excluding Nx)		N1	37 (44,6)
		N2/N3	18 (21,7)
M (Distant metastasis)	87	M0	73 (83,9)
		M1	7 (8)
		Mx	7 (8)
M	87	M0	80 (92,0)
(Mx/M0 grouped)		M1	7 (8,0)
Size (cm)	76		3.6±2.4 (0.8-15)
		<2	15 (19,7)
		2 to 5	45 (59,2)
		>5	16 (21,1)
Clinical stage	85	WOULD	9 (10,6)
		IIA	23 (27,1)
		IIB	18 (21,2)
		IIIA	13 (15,3)
		IIIB	9 (10,6)
		IIIC	6 (7,1)
		IV	7 (8,2)
Clinical stage	85	I	9 (10,6)
(Grouped)		II	41 (48,2)
		III	28 (32,9)
		IV	7 (8,2)

* = Described by mean ± standard deviation (minimum – maximum) or by frequency (percentage)

Grade of breast carcinoma

Histological classification was derived from 3 morphological features, each with a score of 1 to 3. In each block, the first morphological characteristic was sought with tubular structures, with clear and visible lumen;11 the second was nuclear pleomorphism, and the third was mitotic activity, which could be seen preferentially in the periphery of the tumor, where active growth was identified. Several studies have suggested that the histological grade of tumors has a strong correlation with tumor differentiation and its prognosis.[11,12] The Nottingham classification system, which is a modification of the Scarff-

Bloom-Richardson[11,13] classification system, was used to classify the histological grade.[14]

When analyzing tumor differentiation in terms of histological grade in this sample, a predominance of tumors G2 (43.7%) and G3 (51.7%, Table 1) was observed.

Tumor size

The size of the tumor has a high prognostic value and is related to the aggressiveness of the disease. In general, larger tumors are more likely to affect axillary lymph nodes, while smaller tumors are related to a better prognosis, both for disease-free time and overall survival.[11,13]

Among 76 tumors analyzed, the overall mean tumor size was 3.6+/- 2.4 cm, ranging from 0.8 to 15 cm. Most of them (59.2%) between 2-5 cm.

TABLE 2 — Lymph nodes affected in axillary lymphadenectomy (n = 78)

Lymph nodes	n (%)
1-13	4 (5,1)
1-5	2 (2,6)
0-13	2 (2,6)
13-13	2 (2,6)
2-3 (Post-QT)	2 (2,6)
1-9	2 (2,6)
17-18	2 (2,6)
0-8	2 (2,6)
2-9	2 (2,6)
8-16	1 (1,3)
1-15	1 (1,3)
6-6	1 (1,3)
4-14 (Post-QT)	1 (1,3)
1-11	1 (1,3)
9-13	1 (1,3)
7-14	1 (1,3)
4-15	1 (1,3)
2-3	1 (1,3)
2-21	1 (1,3)
0-19	1 (1,3)
1-18	1 (1,3)
0-4	1 (1,3)
1-8	1 (1,3)
1-1	1 (1,3)
1-6	1 (1,3)
7-9	1 (1,3)
1-16	1 (1,3)
3-19	1 (1,3)
34-34	1 (1,3)
14-14	1 (1,3)
0-1(Post-QT)	1 (1,3)
0-7	1 (1,3)
0-2	1 (1,3)
3-3	1 (1,3)
1-12	1 (1,3)
15-18	1 (1,3)
10-12	1 (1,3)
1-10 (Post-QT)	1 (1,3)
0-10, 0--12 and 0--5	1 (1,3)
3-11	1 (1,3)
9-10	1 (1,3)
3-17	1 (1,3)
5-8	1 (1,3)
6-12	1 (1,3)
0-5	1 (1,3)
4-18	1 (1,3)
11-12	1 (1,3)
9-22	1 (1,3)
5-12	1 (1,3)

Lymph nodes

Lymph node status depicts the involvement or not of axillary lymph nodes by neoplastic cells. This is an important prognostic factor for invasive carcinoma, whether for the analysis of overall survival or disease-free survival. In addition, there is a direct correlation between survival time and the number of lymph nodes affected16 (Table 2).

Metastases

Category M1 indicates the presence of metastasis and is sectored by site of involvement (Table 3).

TABLE 3 — Location of distant metastasis (M1)

Variable	n	Classification	n (%)
Local DM	86	No	52 (60,5)
		Yes	34 (39,5)
Variable	n	Classification	n (%)
DM Location	34	Local	4 (11,8)
		Brain	2 (5,9)
		Pulm	2 (5,9)
		Hep/bone	2 (5,9)
		Hep	2 (5,9)
		Local/lym	2 (5,9)
		Bone/pulm/lym	1 (2,9)
		Per	1 (2,9)
		Hep/pulm/lym	1 (2,9)
		Hep/lym	1 (2,9)
		Lym	1 (2,9)
		Bone/lung/brain	1 (2,9)
		Bone	1 (2,9)
		Lung/brain	1 (2,9)
		Bone/brain	1 (2,9)
		Pulm/lym	1 (2,9)
		Local/pulm/lym	1 (2,9)
		Lym/pulm	1 (2,9)
		Location/brain	1 (2,9)
		Brain/location	1 (2,9)
		Bone/lung/hep	1 (2,9)
		Hep/per	1 (2,9)
		Bone/hep/brain/lym	1 (2,9)
		Lym/pulm/bone	1 (2,9)
		Hep/bone/per	1 (2,9)
		Bone/hep	1 (2,9)

Hormone receptors and surrogate subtypes

The evaluation of hormone receptors, HER2 overexpression, Ki-67 proliferative index (determined by immunohistochemistry) and surrogate molecular subtypes show their distribution in Table 4.

TABLE 4 — Expression of receptors and surrogate molecular subtypes of breast carcinoma

Variable	n	Classification	n (%)
HER2	65	Negative	51 (78,5)
		Positive	14 (21,5)
Estrogen	66	Negative	22 (33,3)
		Positive	44 (66,7)
Progesterone	66	Negative	29 (43,9)
		Positive	37 (56,1)
KI67%	66	≤ 14	35 (53)
		> 14	31 (47)
Surrogate molecular subtypes	65	Luminal A	30 (46,2)
		Triple negative	14 (21,5)
		HER2 positive	8 (12,3)
		Luminal B	7 (10,8)
		Luminal /HER2	6 (9,2)
Surrogate molecular subtypes (considering Luminal B/HER2 as HER2 positive)	65	Luminal A	30 (46,2)
		Triple negative	14 (21,5)
		HER2 positive	14 (21,5)
		Luminal B	7 (10,8)

From the results obtained, regarding the analysis of hormone receptor expression, it was found that the highest frequency found was ER+ (66.7%). Regarding the so-called substitute subtypes, the highest frequency was Luminal A (46.2%), triple negative (21.5%), HER2+ (21.5%) and Luminal B (10.8%).

Clinical course

The survival time of the patients was calculated from the diagnosis to the last evaluation in consultation or the date of death related to cancer. Disease-free survival time and total survival were adopted as prognostic indices. Disease-free survival events were considered until a new disease was detected, either due to locoregional recurrence, distant metastasis, or contralateral breast cancer. Total survival events represented survival time to date of last visit (for patients with no evidence of disease) or survival time to death when related to cancer. (Note: Recurrence, distant metastasis, and contralateral metastasis of the patients were confirmed by imaging methods and anatomopathological examinations).

The data obtained on this type of prognosis are summarized in Table 5. Disease-free survival occurred in 67.1%. Tumor progression occurred in 40% of the cases studied. The mean follow-up time was 45.2+/-28.1 months, ranging from 0-87.9 months. Death occurred in 29.9% of the cases.

TABLE 5 — Clinical evolution

Variable	n	Classif	n (%)
Disease-free survival	85	No	28 (32,9)
		Yes	57 (67,1)
Progression	85	No	51 (60)
		Yes	34 (40)
Follow-up (months)	85		45.2 ± 28.1 (0 – 87.9)
Death	87	No	61 (70,1)
		Yes	26 (29,9)
Follow-up death (months)			46.5 ± 27.5 (1.9 – 103)

R Rating

The R classification demonstrated the absence or presence of residual tumor after surgical treatment. The definitions were: Rx, when the presence of a residual tumor cannot be evaluated; R0, in the absence of residual tumor; R1, with microscopic residual tumor, and R2, with macroscopic residual tumor.

TABLE 6 — Residual tumor after surgery

Variable	n	Classification	n (%)
R	87	0	68 (78,2)
		1	14 (16,1)
		2	5 (5,7)

Evaluation of the association between markers and demographic and clinical variables

Tables 7 to 9 present the frequencies and percentages according to the combinations of the classifications of each variable and each marker. The p-values of the statistical tests are also presented (Note: The percentages were calculated in relation to the total in the lines).

TABLE 7 — Tissue marking pattern for AXL and ß-catenin

Variable	n	Classification	n (%)
AXL	87	Negative	19 (21,8)
		Weak	19 (21,8)
		Moderate	14 (16,1)
		Strong	14 (16,1)
		Inconclusive*	21 (24,1)
AXL (excluding inconclusive)	66	Negative	19 (28,8)
		Weak	19 (28,8)
		Moderate	14 (21,2)
		Strong	14 (21,2)
β-catenin	87	Negative	66 (75,9)
		Inconclusive**	21 (24,1)
β-catenin (excluding inconclusive)	66	Negative	66 (100)

*Inconclusive without fragment on the slide (SF) (n = 11); inconclusive without fragment tumor (ST) (n = 10). ** Inconclusive SF (n = 11); inconclusive TS (n = 9); inconclusive (n= 1)

TABLE 8 — Distribution of the AXL by age, histological grade and clinical stage

Variable	Classif	n	AXL				p*
			Negative	Weak	Moderate	Strong	
Age (years)	< 50	24	10 (41,7)	6 (25)	4 (16,7)	4 (16,7)	0,377
	≥ 50	42	9 (21,4)	13 (31)	10 (23,8)	10 (23,8)	
Degree	G1/G2	31	6 (19,4)	11 (35,5)	7 (22,6)	7 (22,6)	0,420
	G3	35	13 (37,1)	8 (22,9)	7 (20)	7 (20)	
Stadium	I	6	3 (50)	1 (16,7)	0 (0)	2 (33,3)	0,296
	II	32	8 (25)	13 (40,6)	6 (18,8)	5 (15,6)	
	III	21	6 (28,6)	4 (19,1)	5 (23,8)	6 (28,6)	
	IV	6	2 (33,3)	0 (0)	3 (50)	1 (16,7)	
T	T1	10	4 (40)	2 (20)	2 (20)	2 (20)	0,383
	T2	31	7 (22,6)	13 (41,9)	5 (16,1)	6 (19,4)	
	T3	10	3 (30)	3 (30)	2 (20)	2 (20)	
	T4	14	5 (35,7)	0 (0)	5 (35,7)	4 (28,6)	
N	N0	21	7 (33,3)	8 (38,1)	1 (4,8)	5 (23,8)	0,433
	N1	27	7 (25,9)	7 (25,9)	8 (29,6)	5 (18,5)	
	N2/N3	15	4 (26,7)	3 (20)	5 (33,3)	3 (20)	
M	M0	60	17 (28,3)	19 (31,7)	11 (18,3)	13 (21,7)	0,204
	M1	6	2 (33,3)	0 (0)	3 (50)	1 (16,7)	
PDM	No	39	13 (33,3)	12 (30,8)	7 (18)	7 (18)	0,699
	Yes	26	6 (23,1)	7 (26,9)	7 (26,9)	6 (23,1)	
Subtypes	Triple negative	14	5 (35,7)	6 (42,9)	0 (0)	3 (21,4)	-
	Luminal A	30	7 (23,3)	8 (26,7)	8 (26,7)	7 (23,3)	
	HER2 positive	7	3 (42,9)	2 (28,6)	1 (14,3)	1 (14,3)	
	Luminal B	7	2 (28,6)	2 (28,6)	3 (42,9)	0 (0)	
	Luminal B/HER2	6	2 (33,3)	1 (16,7)	2 (33,3)	1 (16,7)	
Subtypes/ grouped	Triple negative	14	5 (35,7)	6 (42,9)	0 (0)	3 (21,4)	0,475
	Luminal A	30	7 (23,3)	8 (26,7)	8 (26,7)	7 (23,3)	
	HER2 positive	13	5 (38,5)	3 (23,1)	3 (23,1)	2 (15,4)	
	Luminal B	7	2 (28,6)	2 (28,6)	3 (42,9)	0 (0)	
Size (cm)	< 2	8	3 (37,5)	2 (25)	1 (12,5)	2 (25)	0,340
	2 to 5	39	10 (25,6)	15 (38,5)	6 (15,4)	8 (20,5)	
	> 5	10	5 (50)	0 (0)	3 (30)	2 (20)	

Results described by frequency (percentage); * = Fisher's exact test or chi-square test, p < 0.05

TABLE 9 — Distribution of ß-catenin by age, histological grade and clinical stage

Variable	Classif	n	Negative	p*
Age (years)	< 50	24	24 (72,7)	0,614
	≥ 50	42	42 (77,8)	
Degree	G1/G2	31	31 (73,8)	0,803
	G3	35	35 (77,8)	
Stadium	I	6	6 (66,7)	0,826
	II	31	31 (75,6)	
	III	22	22 (78,6)	
	IV	6	6 (85,7)	
T	T1	10	10 (58,8)	0,217
	T2	30	30 (83,3)	
	T3	11	11 (84,6)	
	T4	14	14 (73,7)	
N	N0	21	21 (75)	0,694
	N1	27	27 (73)	
	N2/N3	15	15 (83,3)	
M	M0	60	60 (75)	1
	M1	6	6 (85,7)	
PDM	No	39	39 (76,5)	1
	Yes	26	26 (74,3)	
Subtypes	Triple negative	14	14 (100)	-
	Luminal A	30	30 (100)	
	HER2 positive	8	8 (100)	
	Luminal B	7	7 (100)	
	Luminal B/HER2	6	5 (83,3)	
Subtypes/groups	Triple negative	14	14 (100)	-
	Luminal A	30	30 (100)	
	HER2 positive	13	13 (92,9)	
	Luminal B	7	7 (100)	
Size (cm)	< 2	8	8 (53,3)	0,044
	2 to 5	38	38 (84,4)	
	> 5	11	11 (68,8)	

Results described by frequency (percentage); * = Fisher's exact test or chi-square test, $p < 0.05$

Progression-free time analysis – AXL and β-catenin markers

For each of the markers, the null hypothesis that the progression-free time curves were the same for all marker classifications was tested, vs. the alternative hypothesis of different curves. Figure 1 shows the Kaplan-Meier curves for progression-free time (overall) and for each of the classifications of the markers compared. The p-values of the statistical tests (Log-rank test) are also presented.

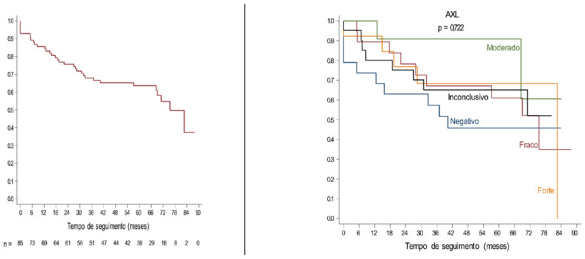

FIGURE 1 — Kaplan-Meier curves for progression-free time (overall)

Survival time analysis – AXL and β-catenin markers

Figure 2 shows the Kaplan-Meier curves for survival time (overall) and for each of the classifications of the markers compared. The p-values of the statistical tests (Log-rank test) are also presented.

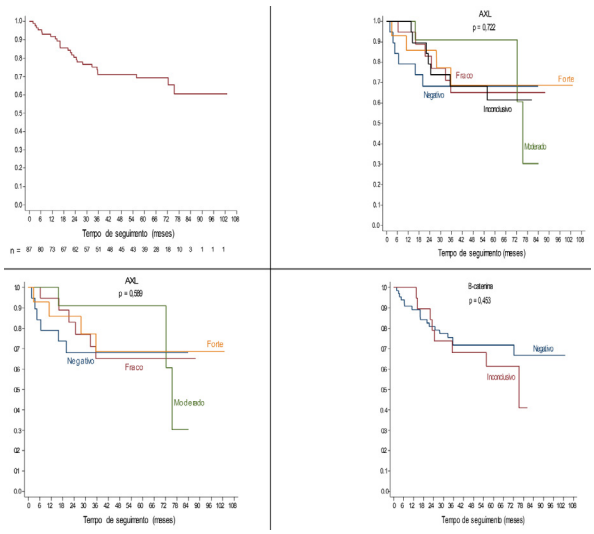

FIGURE 2 — Kaplan-Meier curves for survival time (overall): comparison of the groups defined by tumor type in relation to the AXL and β-catenin markers

For each comparison of groups and each of the markers, the null hypothesis that the distributions on the marker ratings were equal in the groups under comparison was tested, vs. the alternative hypothesis of different distributions (Tables 10 and 11)

TABLE 10 — AXL marking pattern by tumor group: A) invasive, intraductal, fibroadenoma and non-tumor control; B) ductal and non-ductal controls; C) Rogue and controls

	AXL	Group			
		Ductal	Intraductal	Non-tumor	Fibroadenoma
A	Negative	19	2	2	1
		28,8%	28,6%	40,0%	7,1%
	Weak	19	2	0	1
		28,8%	28,6%	0,0%	7,1%
	Moderate	14	2	1	6
		21,2%	28,6%	20,0%	42,9%
	Fort and	14	1	2	6
		21,2%	14,3%	40,0%	42,9%
	Total	66	7	5	14

p = 0.259 (chi-square test, $p < 0.05$)

	AXL	Group		
		Ductal	Intraductal	Non-tumor/fibroadenoma
B	Negative	19	2	3
		28,8%	28,6%	15,8%
	Weak	19	2	1
		28,8%	28,6%	5,3%
	Moderate	14	2	7
		21,2%	28,6%	36,8%
	Strong	14	1	8
		21,2%	14,3%	42,1%
	Total	66	7	19

p=0.182 (chi-square test, p<0.05)

	AXL	Group	
		Ductal	Intraductal / non-tumour / fibroadenma
C	Negative	19	5
		28,8%	19,2%
	Weak	19	3
		28,8%	11,5%
	Moderate	14	9
		21,2%	34,6%
	Strong	14	9
		21,2%	34,6%
	Total	66	26

p = 0.130 (chi-square test, $p < 0.05$)

TABLE 11 — ß-catenin labeling pattern by tumor group: A) invasive, intraductal, fibroadenoma and non-tumor control; B) ductal and non-ductal controls; C) rogue and controls

A	ß-catenin	Group			
		Ductal	Intraductal	Non-tumor	Fibro adenoma
	Negative	66	7	6	19
		75,9%	53,9%	60,0%	54,3%

p = 0.074 (chi-square test, p < 0.05)

B	ß-catenin	Group		
		Ductal	Intraductal	Non-tumor/fibroadenoma
	Negative	66	7	25
		75,9%	53,9%	55,6%

p = 0.033 (chi-square test, p < 0.05)

C	ß-catenin	Group	
		Ductal	Intraductal / non-tumour / fibroadenma
	Negative	66	32
		75,9%	55,2%

p = 0.011 (Fisher's exact test, p < 0.05)

DISCUSSION

Several factors are associated with a higher risk of developing breast cancer, including age, since 75% of all breast carcinomas occur in women over 50 years of age. The hereditary factor also contributes to an increased risk of between 1.5-3%. In addition, aspects related to diet and lifestyle habits also compete, since women with a body mass index greater than or equal to 31.1 have a 3.5 times higher risk of developing the disease when compared to those with a BMI less than or equal to 22.[6,2,17-19]

When other age groups are considered, especially those classified as "young" (defined as aged between 25 and 40 years), some particularities are observed in the aspects of presentation of this cancer and in the related risk factors, which deal, for example, with biological and hormonal characteristics, directly influencing the specific tumor pattern.[20]

Studies with women in the age group ranging from 30-50 years indicate that the age groups up to 35 years have worse survival when compared to other more advanced groups, but still in the premenopausal range[21,22] demonstrated the same for the group between 36 and 40 years old, also with a higher frequency of distant metastases. These data can be justified by studies that relate breast carcinoma in younger patients to a worse prognosis,[23,24] often because they have a larger tumor size at diagnosis, with a lower degree of histological differentiation, greater lymph node involvement, and diagnosis in more advanced stages.[25-28]

Younger patients also have a frequency of more aggressive molecular phenotypes,[27] such as HER2+ and triple negative, and a lower frequency of tumors with a more favorable prognosis, such as Luminal A.[29] Finally, and considering the aspects described above, it is observed that patients under 40 years of age also had a higher rate of death from breast cancer (80%) when compared to older patients (49%) in a 15-year follow-up period.[25]

ß-catenin

In the interpretation of the data obtained, all reactions related to it were considered negative, both in the tumor group analyzed and, in the controls, contrary to the data in the literature. This fact led us to interpret these results as immunostaining failures.

By using immunohistochemical labeling techniques, similar to those applied in this study, researchers evaluated the correlation of β-catenin and its prognostic value in breast cancer, demonstrating that this adhesion molecule was present in smaller quantities in diseased breast tissue when compared to normal. In that same study, the researchers also found a positive correlation between β-catenin expression that was higher in less advanced stages of breast cancer, both from the point of view of histological grading and TNM classification.[30]

According to Li et al.[31] research that seeks to correlate the expression of β-catenin with the evolution of breast cancer is very controversial in the results. They investigated it in different subtypes of breast cancer and their respective clinical significance, resulting in more frequent and intense expression of β-catenin in the nucleus and cytoplasm of invasive tumor cells, while membranous expression, which was characteristic of normal cells, was reduced. The expression of β-catenin in both the nucleus and cytoplasm is indicative that the Wnt signaling pathway is active. However, in the present study, the slides that could be analyzed did not present any specific marking pattern.

When the aberrant expression of β-catenin was analyzed, i.e., when the adhesion molecule was expressed in greater quantities in the nucleus and cytoplasm and compared to the subtypes of breast cancer, 78.95% of those with triple-negative subtype had aberrant expression of the molecule, which may indicate a possible relationship between β-catenin and this subtype of the neoplasm. An important hypothesis suggested by the study is that the discrepancies between the results of previous studies may be related to the lack of subcellular localization of the β-catenin expression site, since the older studies focused on the expression of the molecule in the cytoplasmic membrane.[31] Again, in this study, even when evaluating the correlated group (triple-negative tumors), no marking was observed.

Still on triple-negative breast cancer, Xu et al.[32] investigated through in vivo and in vitro assays the tumor behavior of breast cancer cells and the role played by β-catenin in this process. According to the authors, activation of the Wnt signaling pathway is more intense in triple-negative breast cancer and is associated with reduced overall survival of patients. The researchers observed that, through the silencing and knock-out of β-catenin, there was a reduction in the tumor cell population and in the expression of genes related to stem cells. In general, the data obtained in the research suggest that β-catenin is necessary for the development of triple-negative breast cancers because it controls several tumor-related properties, such as migration,

chemosensitivity, and anchorage-independent growth.[32]

In the same sense, another study blocked the Wnt/β-catenin signaling pathway, evaluating the expression of genes related to the expression of tumor stem cells (CSC-related genes) and how this signaling pathway correlates with the occurrence of metastasis and tumor formation in vivo and in vitro. The researchers hypothesized that Wnt/β-catenin should regulate the self-renewal and migration of stem cells, thus enabling the occurrence of metastasis and systemic spread of breast cancer. Again, the authors demonstrated more intense activation of the signaling pathway in neoplastic tissues, in addition to relating this activation to a worse prognosis and greater potential for metastasis, since this greater activation was shown to be linked to greater activity of genes linked to tumor stem cells. Thus, through a Wnt1 knockout , the signaling pathway was suppressed as expected and the stem cell population decreased.

Sun et al.[33] evaluated the expression of ALDH1A1, β-catenin and their combined expressions in breast cancer patients treated with cyclophosphamide. Again, cytoplasmic expression of β-catenin was more related to breast cancer, in addition to being more expressed in tumors with higher expression of ALDH1A1. When analyzed in isolation, increased expression of cytoplasmic ALDH1A1 and β-catenin were both associated with lymph node metastasis and a worse clinical prognosis, especially in patients treated with cyclophosphamide.[33] In the present study, when analyzing a group with similar characteristics (with lymph node metastases and/or a subgroup with poor prognosis), no markings related to β-catenin expression were observed.

In addition to triple-negative tumors, in a previous study, the location of β-catenin expression in patients with another important subtype of breast cancer, HER2-positive, was evaluated. It was observed that those with β-catenin expression more prominently located in the membrane, had significantly higher neoplasm-free survival and overall survival. The study also evaluated the behavior of β-catenin and HER2 expression in terms of cell exposure to stress (induced with cadmium administration). This induction caused β-catenin and HER2 to change their location towards the cytoplasm and perinuclear regions, a fact that has already been demonstrated in the literature and which relates the subcellular location of β-catenin to the worse prognosis of the tumor.[34] Again, when selecting a similar sample of overexpressed HER2 tumors, in this study no markings related to β-catenin were observed.

When considering tumor sites other than the breast, β-catenin expression has been shown to be related to a worse prognosis and lower overall survival in cases of renal cell carcinoma and squamous cell carcinoma in the lung. In renal carcinomas, β-catenin has been shown to be an independent prognostic factor related to a 4-fold higher risk of mortality and can even be used as an auxiliary tool in the risk stratification of these patients.[35,36]

Still on renal carcinomas, Kovacs et al.[36] investigated in 488 patients diagnosed with renal cell carcinoma the possible relationship between β-catenin expression as a specific biomarker of survival and concluded that cytoplasmic expression of β-catenin may represent an independent poor prognostic factor related to a higher risk of disease progression after surgical treatment and tumor-related death.

As discussed in the studies above and based on data widely validated in other researches, immunohistochemistry, despite being a relatively simple and available method, has certain particularities that must be respected in the different stages of material processing, since the evaluation of the results is directly influenced by factors such as the initial fixation of the specimen, the appropriate choice of antibodies to be used for analysis, as well as and, finally, the interpretation or reading of the slides.[37]

Studies similar to the one developed - which also report on failures of immunostaining methods, or even situations in which immunohistochemistry did not contribute to the resolution of diagnostic problems - often have some factors in common. According to Leong and Wright,38 among the main reasons for immunohistochemistry not contributing to the final diagnosis included, for example, the nonspecific results of certain stains in some tumors and inadequate fixation of specimens.[38] Other authors also pointed to inadequate fixation in formaldehyde as one of the main complicating factors, although in different percentages in the global assessment compared to other failures related to immunostaining. These differences may be due to the evolution of techniques over the years (more recently done in an automated manner), as well as advances in antigenic recovery mechanisms that are currently performed more specifically for each antibody, partially reducing the importance of tissue preservation and antigenic epitopes in the quality of immunohistochemical reactions.

Even so, even with the evolution of techniques related to the different stages in the immunostaining process, basic care with the material is essential and must be respected from the beginning of the process. In this sense, in general, formaldehyde (4%) is used as the main solution in the fixation process, containing formaldehyde (4%). In this step, the main molecular modification induced by formaldehyde is the formation of cross-links between proteins and each other or between protein and tissue nucleic acids.[39] The formation of these bonds is possibly one of the factors responsible for the masking of epitopes by altering the three-dimensional conformation of proteins; however, the secondary structure of the fixed proteins is still maintained in the same way as it was prior to fixation.[40]

The formation of cross-links by formaldehyde is a slow and necessary process, requiring at least 24-48 h to be completed. Shorter fixation times interrupt the process and may lead to alternative forms of fixation, as occurs, for example, in the fixation process by coagulation during the dehydration of the tissue by alcohol in the step that follows the fixation process itself. Thus, these different fixation mechanisms can result in a mixture between the formation of de facto cross-links and fixation by coagulation, which is one of the main factors responsible for failures and variabilities observed in immunostaining processes.

After the period of fixation in formaldehyde, the tissue is usually embedded in paraffin. This process has as stages dehydration in an alcoholic solution, washing with xylene solution and then incubation in a hot incorporation medium, which is usually done with paraffin.

In general, all fixation processes tend to reduce the antigenic capacity of tissues. In the case of formaldehyde, the use of enzymes or heat-induced epitope retrieval can recover unavailable sites, depending on the epitope sought and the antibody used. Since the interpretation of immunostains is done in a semi-quantitative way, it is important to minimize variabilities in the intensity of reactions that are related to both the fixation process and antigenic processing or retrieval. To this end, the need for rigorous standardization in the care of the specimen from the time it is obtained to the immunostaining phase is emphasized.

Delays in fixation, for example, result in increased proteolytic degradation and, depending on the antigen used, may determine absence or weak reaction due to reduced tissue immunoreactivity. In addition, proteolysis often causes nonspecific bonds between unrelated molecules. This fact may have contributed to the absence of labeling in the evaluation of β-catenin expression in this study. To this end, the fixation process should preferably begin immediately after the surgical removal of the tissue (in a period of less than 30 min). Organs and solid tumors must be incised to ensure faster fixation. If a delay in the clamping process is unavoidable, refrigeration of the part is recommended.

The formaldehyde fixation process begins in the peripheral portions of the tissue as the solution penetrates the material and, if this process is interrupted by early removal of the material (less than 24 h), there may be mixed fixation of the tissue, initially given by formaldehyde and, secondarily, by alcohol. Thus, the formation of cross-links will occur only at the margins while towards the center, the process of fixation by coagulation by alcohol may occur (during the dehydration phase) or, still, the central portions may remain unfixed. As a result, in certain sections, immunostains may be erroneously more intense in the center or periphery, depending on the antibody used and whether the recovered epitope was used or not.

To avoid similar situations, samples of surgical specimens intended for immunohistochemistry should preferably be processed in thin slices (about 3 mm) and fixed for a period of 24 h to ensure optimization in the formation of cross-links throughout the tissue. For small fragments (such as biopsies) in which fixation for 24 h is not always feasible. A minimum period of 100 min must then be respected, as at least half of the maximum binding capacity of formaldehyde has already been reached.

On the other hand, it is worth noting that prolonged fixation processes in formaldehyde solution can also lead to weak or absent reactions, which will largely depend on the individual susceptibility of the epitopes to be analyzed. An excess of cross-links, as well as the presence of contaminants in the solution, possibly contributes to this and can lead to irreversible damage to some epitopes.

As a possible means of avoiding this situation, when handling tissues that have remained for a prolonged fixation time, it is recommended to process the material in 3 separate blocks, exposing them to different and progressively longer times of action to proteases or heat-induced recovery of epitopes. The slice that presents the best marking pattern should be used for reading and interpretation. In addition, artifices such as exposure to high concentrations of antibodies, long incubation periods or even signal amplification can also be useful in this process. However, it is worth remembering that all these mechanisms can recover some epitopes, but they often increase background noise. Therefore, the best thing to do is always to avoid excessive setting periods beyond 48 h.

Finally, with regard to the artifacts resulting from the processing of the tissue, some points should be highlighted. Firstly, if the alcoholic solution is repeatedly used, tissue dehydration may occur improperly since the solution may lose its dehydrating capacity, resulting in weak or absent staining of cells. At the same time, non-specific reactions, especially in the periphery of the sections, may occur.

In addition, both the presence of contaminants in xylene and variations in the temperature of incorporation into paraffin can be deleterious and contribute to wide variations in immunostaining. However, it is known that this tissue processing exerts less influence on immunostaining mechanisms when compared to the formaldehyde fixation step.

AXL

The tyrosine kinase receptor family (Tyro 3, Axl, MerTK) is so defined because each member of this family has 1 extracellular combination of 2 immunoglobulin-like domains and 2 type III fibronectin repeats, 1 transmembrane portion and 1 intracellular region with intrinsic tyrosine kinase activity. Increased expression of AXL has been frequently detected in various types of neoplasms and its role in maintaining tumorigenesis has been well recognized. Thus, AXL has been related to tumor

growth and dissemination through positive effects regarding cell survival, proliferation, migration, and tumor invasion. In addition, AXL signaling is also involved in other processes involving mechanisms ranging from cell differentiation, protection of blood vessels against injury, removal of apoptotic cells, hematopoiesis, platelet aggregation, and regulation of pro-inflammatory cytokine production.[41-43]

In this study, the analysis of AXL expression in invasive ductal carcinoma of the breast was performed with the presence of controls that were composed of normal breast tissue, fibroadenoma (benign lesion) and intraductal carcinoma (lesion in situ). In addition, the analysis of AXL expression in the tissues was categorized semi-quantitatively into different patterns, defined as absent, weak, moderate, and strong. Thus, when considering the marking percentages among all tumor samples evaluated, the following distribution was obtained: negative (28.8%); weak (19%); moderate (21.2%); and strong (14%).

Although there was no statistical significance in the different analyses when the respective AXL expression patterns were subdivided and correlated with specific prognostic factors or with the different control groups, it was still possible to observe some percentage differences that are worth mentioning as follows.

Initially, when comparing the AXL expression pattern between the tumor group and controls, there was a predominance of negative or weak markings in the tumor group (58%), while in the control groups the predominance was moderate or strong (70%). These data are in contrast to the information available in the literature, because according to some authors, AXL is widely distributed in solid tumors, including breast carcinoma, and its levels correlate with a higher occurrence of metastases.[44,45] In addition, several studies have shown that aberrant AXL expression is linked to the activation of oncogenesis signaling pathways, including the PI3K/Akt and/or MAPK/Erk pathways, and that, in addition, they are among the main pathways involved in the maintenance of aggressive characteristics of tumor cells, including drug resistance and metastatic behavior.[46]

In analyses involving clinical aspects and histopathological prognostic factors related to breast cancer, when the age factor was initially considered, it was observed that in the group of younger patients (<50 years) there was a predominance of negative or weak expression patterns (67%).

Regarding the evaluation of tumor size (T), it was observed that, for smaller tumors, AXL expression predominated as a negative or weak pattern, with a frequency of 60% in T1 tumors and 64.5% in T2-weighted tumors. For T3 and T4 tumors, there were no percentage differences between the existing labeling patterns, which limits the interpretation of these data.

In the same sense, when correlating lymph node involvement with AXL expression, it was observed that in the group of patients without lymph node involvement (N0), the predominant patterns were negative or weak (71%). For the subgroups with axillary lymph node involvement (N1, N2 and N3) there were no percentage differences in expression.

When considering the degree of tumor differentiation, among the other anatomopathological prognostic factors described, it was observed that, in poorly differentiated tumors (G3), the highest percentage was of negative or weak patterns (60%). In the well-differentiated (G1) and moderately differentiated (G2) groups, no percentage differences were observed.

In contrast to these data, Ahmed et al.[47] suggested that increased expression of AXL is associated with greater aggressiveness of breast cancer, given, among others, by a lower degree of tumor differentiation and greater lymph node involvement. However, Jin et al.[48] did not find any significant correlation between AXL expression and some of the prognostic factors described above, which include age, tumor size, and lymph node metastases. Nevertheless, these authors reported a significant correlation between AXL expression and tumor histological grade, as well as with ER and PR expression.[48]

In the analysis of the AXL expression pattern correlated with the surrogate molecular subtypes, it is noted that in the tumor subtypes considered to have a more aggressive biology (triple negative and HER2+) there is a higher percentage of negative and weak expression patterns (78% in the triple negative and 71% HER 2+) when compared to the moderate and strong expression patterns. Regarding the luminal subtypes, no percentage differences were observed in these distributions. These data are contradicted by some authors who relate AXL expression mainly with the triple-negative subtype (TNBC) and, due to this, it has been considered as a marker of this subtype of breast carcinoma.

These findings are also confirmed by analyses of RNA sequencing data that revealed that only the TNBC subtype expresses high levels of AXL. However, the analysis of AXL expression levels in different patient samples led to conflicting data.[48]

Other authors evaluated AXL expression at the protein level, using AXL antibodies in samples of human breast tumors, correlating this information with clinical data. These analyses revealed that AXL expression can be detected in several molecular subtypes, as found in the present study, and the expression levels may be related to clinical evolution. Thus, in patients with the HER2+ subtype, in whom the existence of co-localized AXL/HER2 protein complexes in the plasma membrane is at high levels, a higher occurrence of metastases in the lung and brain is observed.[49]

Finally, considering the clinical outcome and the progression or not of the disease, which is characterized by local recurrence or evolution with metastases in patients with favorable clinical outcome, i.e., without signs of disease progression,

the predominant expression pattern was negative or weak (64.1%). Those with an unfavorable clinical outcome, i.e., with evidence of disease progression, did not show percentage differences in these patterns. Thus, again, these findings are partially discordant with previous publications that correlate high expression of the AXL protein with unfavorable clinical outcome in all subtypes of breast carcinoma. On the other hand, low levels of AXL expression were associated with longer survival, which, in part, is in agreement with the percentages identified in this study.[49] However, other researchers have not found any association between AXL expression and disease-free survival or overall survival.[48] These findings show, so, associations in conflicts between AXL expression and the biological behavior of breast cancer, as also observed in previous publications. D'Affonso et al.[50] investigated 569 cases of breast cancer and suggested that there is no relationship between AXL expression and the level of ER expression; Berclaz et al.[51,] suggested that AXL expression was significantly associated with ER level, thus, AXL expression was confined to ER-positive tumors, however not all ER-positive tumors expressed the AXL protein. In this sense, these authors hypothesized that ER regulates AXL activation, also inhibiting cell apoptosis by overexpression of Bcl-3 (anti-apoptotic gene), which would lead to some of the characteristics of aggressive behavior observed in these tumor cells.[51]

Therefore, according to some data in the literature, as well as considering the results of this study, the relationship between AXL expression and the specific prognostic factors of invasive ductal mammary carcinoma, as well as the expression of AXL as a possible independent prognostic factor when related to clinical outcomes, remains contentious. Therefore, the mechanisms underlying these relationships remain not completely understood.

Final considerations, limitations, and future perspectives

In view of the current data on breast cancer incidence, prevalence and mortality, it is necessary to search for new markers that can be used as indicators of severity or unfavorable clinical evolution, guiding the use of different treatment modalities, as well as the development of new therapeutic targets. Major limitations of this study were that it was retrospectively evaluated, through the collection of data from medical records and histological blocks, of patients diagnosed with breast cancer treated at a single university hospital. Searches were carried out to collect the respective paraffin blocks with the tumor sample (biopsy or surgery). It is noteworthy that at this stage there was a significant loss of samples, either due to the fragmentation of data in medical records, the absence of an adequate clinical segment, the lack of corresponding histopathological blocks, or the fact that they were insufficient for the analyses that are intended to be performed. A positive point is that in the planning of the work, multisample blocks were made (from tumor histopathological material) to perform the immunohistochemistry technique using β-catenin and AXL antibodies, but controls composed of normal breast tissue, benign breast lesion (fibroadenoma) and tumor in situ (intraductal carcinoma).

Despite the results conflicting with the data available in the literature, it is known that β-catenin and AXL are related to the processes of cell regulation and growth, and the aberrant expression of both is related to carcinogenesis. In addition, some studies analyzing the expression pattern of these markers, correlating them with response patterns in different types of malignant neoplasms, suggest a possible predictive and prognostic role of both as potential biomarkers of aggressiveness, as well as possible targets for anticancer therapies. New efforts should be directed to further clarify the relationship between these and other markers so that, in the near future, new predictors of disease evolution and target-specific therapeutic agents can be developed, further individualizing breast cancer treatment so that a new horizon of greater survival and better quality of life is revealed.

CONCLUSIONS

When invasive ductal breast adenocarcinoma was analyzed in TMA, there was no correlation in the expression of ß-catenin and AXL when compared to overall survival, tumor progression, and histological grade.

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Supervision: Ana Maria Waaga-Gasser
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Writing (review and editing): All authors

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