RESEARCH ARTICLE

Impact of Her-2 Overexpression on Survival of Patients with Metastatic Breast Cancer

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Abstract

Introduction: Breast cancer is a complex and heterogeneous disease which is increasingly important as a public health problem. In Brazil, 57,960 new cases have been estimated to be the burden in 2016 and 2017. Despite advances in early diagnosis and therapy, approximately 20-30% of patients, even with early stage lesions, will develop distant metastatic disease. Tumors with similar clinical and pathological presentations may have differing behavior, so it is important to understand specific biological characteristics. **Objective:** To investigate tumor markers of primary tumors featuring pleural metastasis to identify organ-specific characteristics of metastatic breast cancer. Methods: In a historical cohort study, immunohistochemistry was performed on cell blocks of neoplastic pleural effusions and results were compared with clinicopathological data. **Results:** The median survival time with Her-2 overexpression in malignant pleural effusions was 2.2 months, whereas cases without overexpression survived, on average, for seven months ($p = 0.02$). **Conclusions:** We emphasize that metastases may behave independently of primary tumors, but the present results indicate that therapeutic agents targeting Her-2 overexpression could increase survival in metastatic breast cancer cases.

Keywords: Neoplastic pleural effusion- metastatic breast cancer- Her-2- prognosis

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Introduction

Breast cancer is complex and heterogeneous (Rivenbark et al., 2013) and considered a public health problem (Viale, 2012; Siegel et al., 2013) affecting more females than males (Senkus et al., 2014). In Brazil, 57,960 new cases of breast cancer are estimated to emerge both in 2016 and too 2017 (INCA, 2016). Regarding metastatic breast cancer, estimates diverge, and despite advances in diagnosis and therapy, approximately 20–30% of patients with breast cancer, even early stage, will develop distant metastatic disease (Kennecke et al., 2010; Cadoo et al., 2013).

Pleural effusions often occur in patients with breast cancer (Fentiman et al., 1981; Pokieser et al., 2004), and this clinical condition is associated with poor prognosis (van Galen et al., 2010; Yhim et al., 2010; Santos et al., 2012).

Tumours with similar clinical and pathological presentations may have different biological behaviours, therefore to assist in the characterisation of tumours, researchers have focused on the definition of specific biological characteristics (Goldhirsch et al., 2009; Yersal and Barutca, 2014).

Thus, this study aims to evaluate the expression of tumour markers on invasive breast cancer and malignant pleural effusion (MPE), correlating them with prognosis and survival, helping to understand the specific characteristics of the metastatic site.

Material and Methods

Patient selection

This is a historical cohort study consisting of patients previously diagnosed with breast cancer and an episode of MPE between 2008 and 2014. All cytological samples of pleural effusion were analysed by a senior cytopathologist (JCP). MPE samples diagnosed at the teaching hospital reference in the State of Rio Grande do Sul, whose patients only had breast tumours were included. Regarding losses, cases with no information about the results of histology and immunohistochemistry of breast tumours and the ones with no or poor cellularity sediment formation in the cell block were no included.

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Data collection

The primary breast tumour information was retrieved from medical records. The variables investigated were the date of diagnosis, pathological classification (staging and histology), immunohistochemical panel of breast cancer (hormone receptors oestrogen and progesterone, Ki-67 and Her-2), time until pulmonary progression, outcome, and biochemical information of the pleural effusion such as lactate dehydrogenase (LDH), glucose and total protein. Data were analysed using SPSS® version 18.0, and significance was set at $p<0.05$ with a 95% confidence interval (CI). The survival analysis was performed using log-rank and Kaplan-Meier curves.

Sample processing: Cell block

Pleural effusion (10 ml) was centrifuged (10 min at 1300 rpm), the supernatant was discarded, and then the pellet was fixed for 24 h in a formaldehyde solution (ethanol 99% [85 ml], formaldehyde [10 ml], acetic acid [5 ml]). After removal from the test tube, it was processed histologically and embedded in paraffin, thus creating a pleural liquid paraffin block (Shabaik et al., 2011).

From the cell block, fine cuts on the microtome (4μ) were performed, which were histologically stained with HE (hematoxylin-eosin) to identify neoplastic cells.

To confirm primary breast cancer, IHC (immunohistochemistry) for CK5 /6 markers, calretinin, Gata-3 (Shield et al., 2014), TTF1 and CK7 were held, in addition to immunohistochemistry.

Cell block immunohistochemical of MPE

We used oestrogen receptor (ER) (Mouse Monoclonal Antibody Anti-Human, Clone 6F11, Newcastle UK, 1: 100 Control: neoplastic breast), progesterone receptor (PR) (Mouse Monoclonal Antibody Anti-Human, Clone 16, Newcastle UK, 1: 100 control: neoplastic breast), Ki-67 (Mouse Monoclonal Antibody anti-human, 1: 100 control: palatine tonsil) and Her-2 (Polyclonal Rabbit Antibody anti-human polyclonal, Dako/USA, 1: 2000 control: neoplastic breast) to classify breast tumours. These same tumour markers were performed in the pleural effusion.

Tissue sections were heated (60°C, 30 min), deparaffinised (two washes in xylenes), and rehydrated by successive washes in absolute ethanol and deionised water according to laboratory protocol. For immunohistochemical staining, antigen retrieval was performed at 98°C for 40 min in pH 9.0 TRIS-EDTA. After heating and cooling for 20 min, endogenous peroxidase activity was blocked by immersing the slide in hydrogen peroxide 5% in H_2O (3 × 10 min). The slides were washed twice with phosphate-buffered saline (PBS) and incubated in a solution to block non-specific binding (bovine serum albumin to 1% for 1 h).

A negative control (bovine serum albumin 1%) was substituted for the primary antibody. All primary antibodies and controls were incubated for 1 h at room temperature and then submitted to temperatures of 4°C overnight. Next, the blades were kept at room temperature for 1 h, in sequence, and were washed thrice with PBS.

The sections were incubated with the DAKO AdvanceTM link HRP for 40 min, then washed again with PBS and incubated with DAKO AdvanceTM enzyme HRP for 40 min.

Staining was completed by incubating the specimens with 3,3′-diaminobenzidine+ substrate-chromogen for 5 min. Finally, tissue sections were washed twice with water, and cell nuclei were stained for 20 seconds with hematoxylin-harris.

Sections were washed in deionised water and sequentially immerged for 1 min in 75% ethanol, 90% ethanol, absolute ethanol, and thrice in xylene. Then, slides were mounted in a non-aqueous medium (entelan). Immunohistochemistry slides were photographed under brightfield microscopy using a digital camera (Olympus ® DP2-BSW) and software DP2-BSW.

Technical analysis

The results of the immunohistochemistry of the MPE were submitted to digital image capture in three hotspots (point of maximum concentration of labelled cells) (Swiderska et al., 2015).

The cases were evaluated by two researchers to determine the presence (+) or absence (-) (Tab. 1) of immunostaining. In Her-2 ratings, the indeterminate cases (++) were included in the non-expression group; therefore, the evaluation was qualitative (positive or negative). Additionally, 30% of cases were checked by two experienced pathologists in the area. Cases were considered absent if no reaction was observed in the IHC.

Results

We identified 52 patients with the clinical condition, but nine did not have the necessary information in their medical records or there was no cellularity in the cell

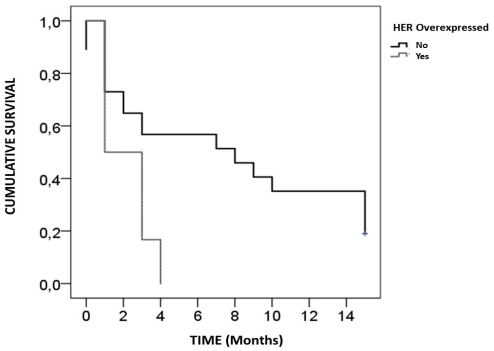

Figure 1. Survival Curve of the Overexpressed Her-2

Table 1. Immunostaining of the Cell

Variables	ER	PR	Ki-67	Her-2
In the cell	Nuclear	Nuclear	Nuclear	Membrane cellular
Positivity	A least 10%	At least 10%	Up to 14%: low; above 15%: high proliferation	Degree varies according to the amount and intensity (0 to 3)

Table 2. Clinical-Pathological Characteristics of the Study

	N	Minimum	Maximum	Mean	Std. Deviation
Age at diagnosis of breast cancer	43	27	81	53.17	12
Age at diagnosis of pleural effusion	43	28.3	88.8	57.5	12
Age at death	36	28.5	78.7	57.7	11.2
Parameters	N	(%)			
	Histological type				
	Ductal	38	88.4		
	Lobular	3	7		
	Other subtypes	2	4.7		
	Total	43	100		
	Degree	N	(%)		
	1	1	2.3		
	2	9	20.9		
	3	21	48.8		
	Missing	12	27.9		
	Total	43	100		
	Staging T	N	(%)		
	1	6	14		
	2	13	30.2		
	3	6	14		
	4	3	7		
	Missing	15	34.9		
	Total	43	100		
	Staging N	N	(%)		
	0	6	14		
	1	7	16.3		
	2	6	14		
	3	5	11.6		
	Missing	19	44.2		
	Total	43	100		
	ER (%)	PR (%)	Ki-67 (%)	Her-2 (%)	
Tumour markers in breast cancer					
0	10 (23.3)	12 (27.9)	8 (19)	29 (67.4)	
1	33 (76.7)	31 (72.1)	7 (16.7)	14 (32.6)	
2			27 (64.3)		
Total	43 (100)	43 (100)	42 (100)	43 (100)	
For ER, PR and Her-2, no expression of tumour markers: 0; expression: 1. For Ki-67, 0: non-expression; 1: 14% of the cells; 2: above 15% of the cells.					
Classification of subtypes of breast cancer	N	(%)			
1	13	30.2			
2	23	53.5			
3	1	2.3			
4	6	14			
Total	43	100			
Applying the classification of subtypes of breast cancer in the pleural effusion	N	(%)			
1	17	40			
2	7	16			
3	4	9			
4	15	35			
Total	43	100			

Table 3. Expression of Her-2 on Primary Lesions and Pleural Metastases

		Effusion Pleural		
		Non-expression	Expression	
		N (%)	N (%)	
HER-2 Breast cancer	Unexpressed	28 (96.6)	1 (3.4)	p = 0.003
	Expressed	8 (57.1)	6 (42.9)	

block. Therefore, 43 patients with MPE that occurred from 2008–2014 were included in the analysis.

Patients were aged between 27 and 81 years. The average age in each event (diagnosis of breast cancer, pleural effusion and death) is presented in Table 2.

Ductal histology represented 88.4%, totalling 38 cases. There was a predominance of grade 3 in 21 (48.8%) cases. Regarding staging, T2 prevailed, represented by 13 patients (30.2%), and regarding lymph node characteristics, only six (14%) were N0. The clinical and pathological characteristics are shown in Table 2.

Pleural effusion and the tumour markers investigated were ER, PR, Ki-67 and Her-2 on the primary tumour Table 2.

In relation to hormone receptors, eight (80%) patients did not express ER in the primary tumour and continued the inexpression in metastasis, and only two (20%) who did not express ER began to express it in MPE. Although 19 (57.6%) remained without expression, 14 (42.4%) who expressed ER in breast cancer did not express it in metastasis (p = 0.069).

Regarding Her-2 rating, the indeterminate cases (++) were included in the non-expression group. We observed that a substantial change occurred in nine cases: 8 patients that expressed Her-2 in breast cancer did not express it in metastasis, and one patient who did not express it in the primary tumour expressed it in pleural effusion (p = 0.003) (Table 3).

Among the 43 patients, 36 (84%) died. Regarding the patients with overexpression of Her-2 in MPE, the average survival time was 2.2 months (95% CI = 1.103–3.230), in contrast to patients without overexpression, whose median survival time was seven months (95% CI = 5.676–9.676) (p = 0.02) (Figure 1).

The classification of subtypes of breast cancer and pleural effusions, are shown in Table 2.

In the study, 24 (55.8%) patients changed their classification from the primary site compared to the metastatic site. Faced with this change, we found that 22 (61.1%) patients who were luminal A and luminal B changed their classification in metastasis (p = 0.1). We demonstrate the individual characteristics of this cohort in the supplementary material.

The biochemical parameters of pleural effusion did not show statistically significant differences against the outcome (death) when analysed with Levene's Test, which assesses the equivalence between the variances. There was a loss of data for glucose (15 results), LDH (12 results) and total protein (16 results).

The mean glucose pleural effusion of patients who progressed to death (23 patients) was 163 mg/dL and in patients who did not die (five patients) was 115.52 mg/dl (p = 0.47). For the LDH parameter, in patients who progressed to death (26 patients), the average was 438 U/L and in patients who did not progress to death (five patients), it was 324.60 U/L (p = 0.55). For total protein, in patients who progressed to death (23 patients), the average was 4.1 g/dL and in patients who did not progress to death (four patients), it was 4.20 g/dL (p = 0.88).

Discussion

The metastatic condition, in particular, pleural metastasis, gives a poor prognosis for patients, as it reflects short life expectancy depending on the type and primary tumour stage (Antunes et al., 2003; Shaw and Agarwal, 2004; Ozyurtkan et al., 2010; Santos et al., 2012).

It is here emphasised that the cohort of patients had a mean interval of four years between the diagnosis of breast cancer and the occurrence of an episode of MPE, and as previously discussed (van Galen et al., 2010), minor interval primary breast cancer and the occurrence of MPE suggests a poor prognosis, reflecting a more aggressive disease or treatment failure.

The biochemical parameters in pleural effusion (glucose, LDH and protein) in our study showed no difference in the prognosis of patients. This is in agreement with other studies (van Galen et al., 2010); however, it is contrary to one study (Ozyurtkan et al., 2010), which found differences in biochemical patterns (lower glucose values, LDH and total protein showed poorer prognosis of patients regarding three-month survival). The results showed that the association between pH, glucose and survival time occurred due to an accumulation of the final products of glycolysis in the pleural space caused by extensive tumour deposits.

We showed a change in Her-2 expression in nine cases, unlike what was previously observed (Koo et al., 2010), in which 100% agreement with the expression of this marker in breast cancer and its metastases was expressed. The study by Vincent-Salomon et al., (2002) also showed a change/no Her-2 expression after performing preoperative chemotherapy, in which nine of 11 tumours maintained the Her-2 overexpression in metastases and, in the other two patients, in which the primary tumour had low levels of Her-2 expression, staining was not observed in the secondary tumour (one lung and one liver tumour). In the same study, the authors did not identify tumours with Her-2 overexpressing only in metastasis, diverging from this study, which showed overexpression exclusively in pleural effusion (Vincent-Salomon et al., 2002). Four cases had a mean survival of 2.2 months, representing patients with a worse prognosis (p = 0.02), corroborating

previous (Rasbridge et al., 1994) data, in which, after treatment with chemotherapy, there was a modification of the Her-2 status (17% overexpressed and 8% did not express). Treatment cannot be evaluated in our study, due to its retrospective nature.

Although studies (Masood and Bui, 2000; Niehans et al., 1993; Barnes et al., 1988) have suggested that Her-2 expression remains at the metastatic site, surveys have shown that there is a change in this expression. We agree with previous authors (Vincent-Salomon et al., 2002) who have supposed a transient change in cell metabolism related to treatment, or even cells that acquire the ability to invade the lymph system and may be biologically different, including by acquiring vascular invasion capacity and growing far from the site of origin.

This hypothesis confirmed the importance of performing biopsies on the metastatic site to target specific therapies according to the new tumour entity. According to the study Kaufman et al. (2009) proposed a targeted therapy for patients with advanced breast cancer considering the Her-2 expression in the metastatic site, and showed that patients with specific targeted therapy survived longer (Kaufman et al., 2009).

The review of Mustacchi et al., (2015) showed that target specific therapy for Her-2 overexpression improved survival time in both the early phase and metastatic breast cancer. However, some patients who responded well to treatment, relapsed, which could be explained by different molecular mechanisms that contribute to drug resistance (Nahta et al., 2006; Mustacchi et al., 2015), which may be related to intratumoural heterogeneity, referring to cell morphology, proliferation rate, metatastisar ability, drug sensitivity, dependence signs of growth, tumour initiation, which are recognised as an important characteristic of most malignant tumours (Condeelis and Pollard, 2006; Kalluri and Zeisberg, 2006; Calbo et al., 2011).

We emphasise that metastases can behave independently of the primary tumour, therefore, despite knowing that the treatment of breast cancer metastatic focuses on prolonging the progression-free survival and improving the quality of life, this work show that new targeted therapeutic agents increase survival in metastatic breast cancer. Where we demonstrated that the Her-2 overexpression in metastatic pleural cancer reflects a worse prognosis.

Ethical aspects

This work was in accordance with the Declaration of Helsinki, respecting the ethical and legal aspects, and was approved by the Committees for Research Ethics in the reference Institutions (ISCMPA and UFCSPA) under the opinions 193.243/2013 and 252.516/2013, as recommended by the National Resolution CNS 466/2012.

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