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Amplification of the *BP1* homeobox gene in breast cancer

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Abstract

The homeobox gene *BP1* is expressed in over 80% of breast cancers and is associated with tumor progression and invasion. However, the mechanism of *BP1* activation in these tumors remains unknown. Therefore our aim in this study is to assess the amplification status of the *BP1* gene in breast cancer and to determine whether *BP1* protein expression is caused by gene amplification in these tumors. *BP1* amplification and expression were assessed in 36 samples. Twenty primary breast tumors (PBT) and 14 sentinel lymph node (SLN) metastases were analyzed using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), respectively. Because of the close proximity of *BP1* and *HER2/NEU* genes on 17q, correlation between their amplification/expression was also investigated. Increased *BP1* copy number was observed in 33% of the cases, with a frequency of 36% and 29% in the PBT and SLN metastasis, respectively. *BP1* protein was expressed in 91% of the samples: in all of the PBT with increased *BP1* copy number and 65% of PBT with normal copy number. *HER2/NEU* amplification was detected in 22% of the cases. Concordance between *BP1* and *HER2/NEU* copy numbers was found in 68% of the PBT and 90% of the SLN metastasis. In conclusion, we demonstrated that the *BP1* homeobox gene is amplified in breast cancer, both in PBT and SLN metastasis, with a significant correlation with *HER2/NEU* amplification. Considering that *BP1* expression was observed in cases with both increased and normal *BP1* copy number, we conclude that other mechanisms in addition to gene amplification play a role in *BP1* protein expression.

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1. Introduction

The *BP1* gene is a member of the distal-less (DLX) family of homeobox genes and is normally expressed during early hematopoiesis [1]. Several key observations support the hypothesis that *BP1* is an oncogene with a major role in breast cancer, and that its expression correlates with breast cancer progression and invasion. BP1 was detectable in 80% of infiltrating ductal carcinomas, but undetectable in matched normal controls, being the first *DLX* gene to be strongly implicated in breast cancer [2]. BP1 expression was also associated with ER negative tumors [2] and was shown to increase with breast tumor progression [3]. These findings strongly support the involvement of BP1 expression in breast tumor development and aggressiveness. A central question, however, is the mechanism of activation of *BP1* in these tumors. The *BP1* gene is located in an amplicon at the 17q21 region [4] closed to where several known oncogenes have been shown to be amplified in breast cancer [5]. However, to our knowledge, the amplification status of this gene has not been previously investigated. Therefore, the main objective of this study was to determine the amplification status of the *BP1* gene in breast tumors and the possibility that its amplification could be one of the mechanisms leading to the expression of the BP1 protein.

We assessed the *BP1* copy number in archived samples of primary breast tumors (PBT) and sentinel lymph node (SLN) metastasis by fluorescence in situ hybridization (FISH) analysis using a *BP1* specific probe. BP1 protein expression was also investigated in the same set using immunohistochemistry (IHC) so that a direct correlation of *BP1* amplification and protein expression could be made. Because of the close proximity on 17q and the biologic importance of the *HER2/NEU* [6,7] this gene was also evaluated for copy number and protein expression in the same tissue sections.

2. Material and methods

2.1. Sample collection

Formalin-fixed paraffin-embedded (FFPE) sections of 36 untreated breast cancer lesions, including 22 samples of PBT and 14 samples of SLN metastasis were evaluated. In 12 cases, both the PBT and the corresponding SLN lesion from the same patient were available.

The samples were obtained from the tumor banks of the Federal University of Paraná, Curitiba, PR, Brazil ($n = 27$) and the Armed Forces Institute of Pathology (AFIP), Washington DC, USA ($n = 9$), under informed consent and IRB approval (Brazilian Ethics Committee and AFIP-UBNI and UBIF). The tumors were of invasive ductal carcinoma type, grade II and with PBT size ranging from 0.8 cm to 8.5 cm.

The mean age of the patients was 49 years, and the majority of patients were of Caucasian origin. For each case, an H&E section from each tissue block used for the FISH and IHC studies was evaluated by a pathologist who confirmed the presence of invasive cancer and delineated the tissues. Consecutive 5 μm sections were then cut for the FISH and IHC studies.

2.2. FISH analysis of the BP1 and HER2/NEU genes

FISH analysis and scoring were performed using a standard protocol that we have previously described [8,9]. For the *BP1* gene, we developed a FISH probe consisting of a contig of 5 overlapping bacterial artificial chromosome (BAC) clones (RP11-723D2, RP11-347L6, RP11-479I21, RP11-298H16, RP11-267M22) containing sequences of the *BP1* gene (BACPAC Resources, Oakland, CA). For the HER2 probe, we used the BAC clone RP11-62N23 (BACPAC Resources, Oakland, CA). BAC clone DNA was prepared and labeled using nick translation, as previously described [10].

Briefly, the BP1 and HER2 probes were labeled with biotin-16-dUTP (Roche Applied Sciences, Indianapolis, IN) and digoxigenin-11-dUTP (Roche) respectively, using nick translation. The biotin-labeled probe was visualized with avidin conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA) and the digoxigenin-labeled probe with a mouse anti-digoxin antibody (Sigma, Saint Louis, MO), followed by detection with a goat anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Sigma, Saint Louis, MO). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and embedded in antifade to reduce photobleaching. Correct chromosomal localization of the BAC clones to 17q21 was confirmed using standard FISH mapping to normal human chromosomes [10].

The two probe sets showed high quality hybridization signals on both metaphase chromosomes and interphase nuclei. For each clone, two distinct signals were detected in the majority of the metaphase and interphase cells analyzed. No additional non-specific signals were detected. The efficiency of the probe for use with FFPE material was further confirmed by analyzing areas with normal breast morphology, or normal lymph node sections, from each case analyzed. In these normal tissues, the cells displayed two copies for each of the two probes, thus demonstrating their specificity.

Digital image acquisition was performed using a 63X objective mounted on a Leica DMRBE microscope (Leica, Wetzlar, Germany) equipped with optical filters for DAPI Fluorescein and TRITC (Chroma Technologies, Brattleboro, VT) and a cooled charge coupled device (CCD) camera (Photometrics, Tucson, AZ). The IPLab software package (Scanalytics Inc, Fairfax, VA) was used for the image acquisition and processing. Slides were scored by two independent observers. A minimum of 100 nuclei were evaluated in each case. Only intact non-overlapping nuclei were scored. Detection of 2 FISH signals was considered as normal copy number, 3 signals as gain, and 6 signals as high-level gain or amplification. To characterize a tumor as having gain or amplification, at least 30% of the nuclei counted must fall into one of the above categories.

2.3. Immunohistochemistry (IHC) analysis of BP1 and HER2/NEU proteins

IHC was performed as we have previously described, using a BP1 specific antibody (Rabbit Anti-BP1 Polyclonal Antibody, Novus Biologicals, Inc., Littleton, CO) that we have shown to be optimal for BP1 analysis by IHC [3]. An anti-human monoclonal antibody was used against HER2/NEU (Vector). We used a standard protocol for IHC assays (the ABC method) for both the BP1 and the HER2/NEU proteins. A given cell was considered positive for BP1

expression if distinct chromogen coloration was consistently seen in its cytoplasm or nucleus in at least two duplicates of the same IHC analysis. A case was considered positive if more than 5% of its entire cell population showed distinct BP1 immunoreactivity [3]. For HER2/NEU analysis, specimens were considered negative for protein expression when presenting scores of 0 or 1+ and positive when presenting 3+. Score 2+ was considered equivocal and required FISH analysis for clarification [11].

2.4. Statistics analysis

Contingency tables (2x2) were created for FISH (amplified/gain vs. normal for both *BP1* and *HER2/NEU* genes) and IHC (positive vs. negative protein expression) across the 2 sets of breast lesions analyzed (PBT and SLN). Fisher's exact tests were used to compare the distributions of the FISH (or IHC) results between the PBT and SLN groups. The estimated Spearman's rank-order correlation coefficient (r) between the BP1 and HER2/NEU were calculated for FISH and IHC, and tests of H0: r = 0 were performed.

3. Results

Results from FISH and IHC analyses for both *BP1* and *HER2/NEU* genes are summarized in Table 1. Increased copy number (gain and/or amplification) of the *BP1* gene was found in 12/36 (33%) of the breast lesions analyzed: 8/22 (36.3%) PBT and 4/14 (28.6%) SLN metastases. This difference was not statistically significant ($p = 0.73$). In 12 cases, it was possible to analyze both the PBT and the corresponding SLN metastasis from the same patient. Among these pairs, 8/12 (66.7%) showed concordant results (same gene copy number status in both PBT and SLN) for *BP1* gene copy number. In contrast, IHC analysis showed that the BP1 protein was expressed in most cases (29/32, 91%): in 17/20 of the PBT (85%) and in all the 12 SLN metastasis. There was no statistically significant difference between the IHC results in the 2 groups at 5% level ($p = 0.27$).

BP1 protein was expressed in all the PBT that showed an increased copy number of the *BP1* gene as well as in 11/17 (65%) of the cases that presented normal BP1 copy number (2 copies) by FISH (Fig. 1). No statistically significant association was detected between copy number changes and protein expression for BP1 in the combined samples ($r = 0.1284$; $p = 0.46$).

HER2/NEU FISH analysis was performed in 32 of the samples (22 PBT and 10 SLN). *HER2/NEU* amplification was observed in 7 samples (32%), and all 7 were primary tumors. In the 22 PBT analyzed for both *BP1* and HER2/NEU copy number, 15 (68%) had concordant findings for both genes, whereas in the 10 SLN cases, 9 (90%) had concordant findings ($r = 0.4731$, $p = 0.01$) (Figs. 2 and 3). In 25% of the samples, increase copy number was observed for both *BP1* and *HER2/NEU* genes. Of note, three samples (#14 PBT, #21 PBT, and #21 SLN) presented gain/amplification of *BP1* but no gain/amplification of *HER2/NEU* gene).

IHC for the HER2/NEU protein was performed on all the samples tested for *HER2/NEU* amplification by FISH. HER2/NEU protein was expressed in 22% (7/32) of the samples, and there was concordance between gene and protein status in 75% (24/32) of the lesions ($r =$

0.4101; $p = 0.026$). IHC expression between the BP1 and HER2/NEU proteins however, did not show significant correlation ($r = 0.1735$; $p = 0.58$).

4. Discussion

This is the first report showing that the *BP1* homeobox gene is amplified in breast cancer: one third of the tumors analyzed had an increased copy number of the *BP1* gene. BP1 protein expression, measured by IHC analysis, was observed in all the cases that showed increased copy number (gain or amplification) of the *BP1* gene, however this correlation was not statistically significant. BP1 expression was also detected in cases with no *BP1* copy number alteration. These findings suggest that additional mechanisms other than gene amplification may be responsible for BP1 protein expression.

Gene amplification is a common mechanism of activation, and can result in overexpression of oncogenes in various malignancies, including breast cancer [12,13]. In the 17q21 chromosomal region, where the *BP1* gene maps, there is a known amplicon in breast cancer [4,5], in close proximity where numerous genes are highly overexpressed, including members of other homeobox gene families, such as the *HOX* gene family. The *HOXB7* gene, found to be overexpressed in breast cancer cell lines [14–17], was amplified in 10% of PBT in a large series of cases studied by Hyman et al. [17]. These authors suggested that DNA amplification could be a mechanism for overexpressing *HOXB7*. Other *HOX* genes, such as *HOXA5*, *HOXA7*, and *HOXC10* have also been shown to be amplified in breast cancer [18]. Although the effect of gene copy number changes on protein expression levels is largely unknown, several studies have explored gene expression changes in specific amplicons [12,13,19].

HER2/NEU amplification in breast tumors is tightly associated with HER2/NEU protein overexpression. In our study, we observed a significant correlation between *HER2/NEU* amplification and protein overexpression in 75% of the cases. In contrast, some authors have found that amplification of other genes in breast cancer, such as *TOP2A* or *EGFR*, are not necessarily associated with high levels of its corresponding protein expression [20,21].

We observed that BP1 was expressed in all PBT that showed increased copy number of the gene. Some of the genes mapped to 17q21 are up regulated along with the *HER2/NEU*. *TOP2A* amplification, for instance, though considered to be an independent event from *HER2/NEU* amplification, is more frequently seen in tumors with *HER2/NEU* amplification [13,22,23].

In this study, 25% of the breast lesions analyzed presented increased copy number for both *BP1* and *HER2/NEU* genes. In 2 PBT samples and one SLN sample, however, an increase in *BP1* copy number was observed with no corresponding alteration in the *HER2/NEU* gene copy number. Additional samples need to be investigated to determine if this was a non-random event and if these genes are amplified in an independent manner.

BP1 positive expression was previously found in breast cancer cases that were HER2/NEU positive by IHC [3]. Of the 7 cases that showed HER2/NEU positive expression, BP1 expression was studied in 6 and all 6 were positive. Recently, HER2/NEU protein

overexpression was found to be predictive of SLN metastasis in clinically negative nodes [24]. Patients whose tumors were HER2/NEU positive had approximately a 50% higher risk of developing SLN metastasis than those whose tumors did not expressed this marker.

We have recently shown that there was an association between BP1 positivity and positive lymph node status (*in preparation*). Future studies should include analysis of both BP1 and HER2/NEU protein expression in breast cancer patients with known lymph node status, to determine the prognostic ability of BP1 alone or in combination with HER2/NEU.

In summary, we have demonstrated that the *BP1* homeobox gene is amplified in breast cancer (one third of the cases in our study), with significant correlation with *HER2/NEU* amplification. BP1 expression was observed in all PBT with increased copy number of the *BP1* gene, as well as in 65% of the PBTwith normal *BP1* copy numbers, indicating that other mechanisms in addition to gene amplification play a role in BP1 protein expression. Further studies investigating the role of amplification of *BP1* in breast cancer will be important to determine if *BP1* can be considered a new and independent prognostic marker in the 17q region.

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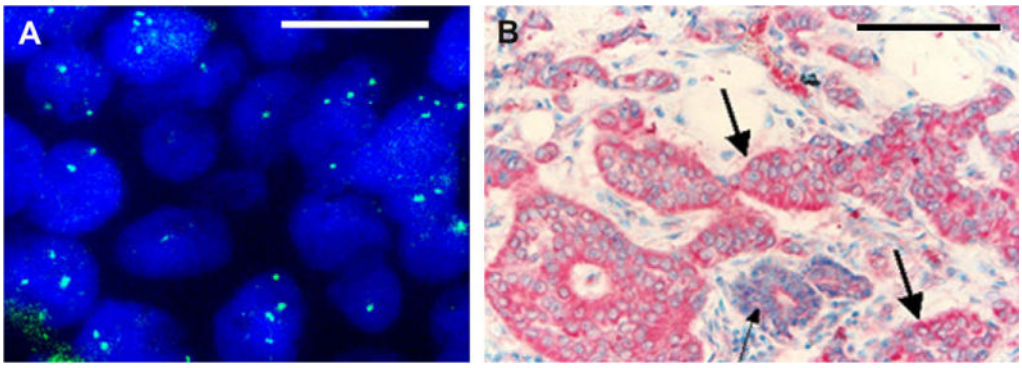

Fig. 1.

Formalin-fixed, paraffin-embedded (FFPE) section from a representative primary breast tumor sample showing: A: Amplification of *BP1* gene (green signals) by FISH (100×; magnification bar is 100 μm); B: Immunostaining for BP1 (red). Thick arrows identify invasive lesions with strong BP1 expression. Thin arrows identify normal area with no or weaker BP1 expression (40×; magnification bar is 2.5 mm).

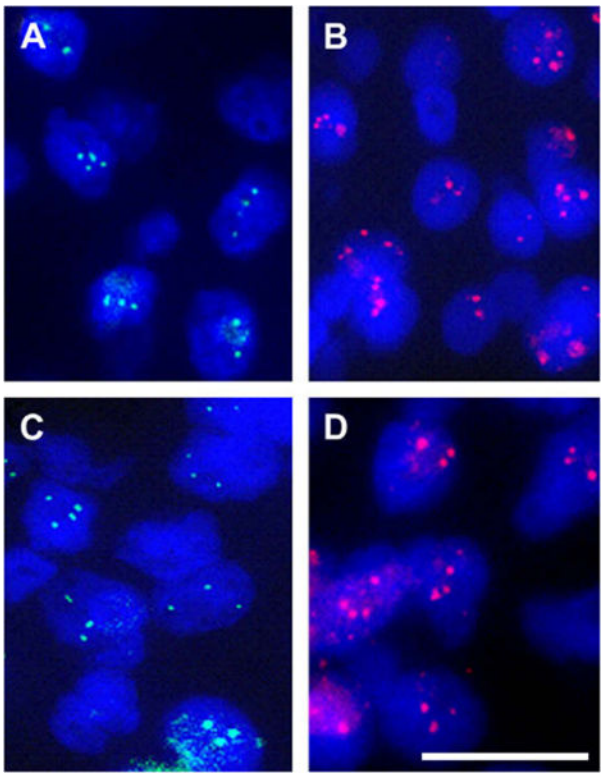

Fig. 2.

FISH analysis of formalin-fixed, paraffin-embedded (FFPE) sections of a pair of primary breast tumor (A and B) and corresponding sentinel lymph node metastasis (C and D) showing hybridization for the *BP1* (green signals) and *HER2/NEU* (red signals) genes. Note the detection of increased copy number for both genes in both of the lesions (100x; magnification bar is 100 μm).

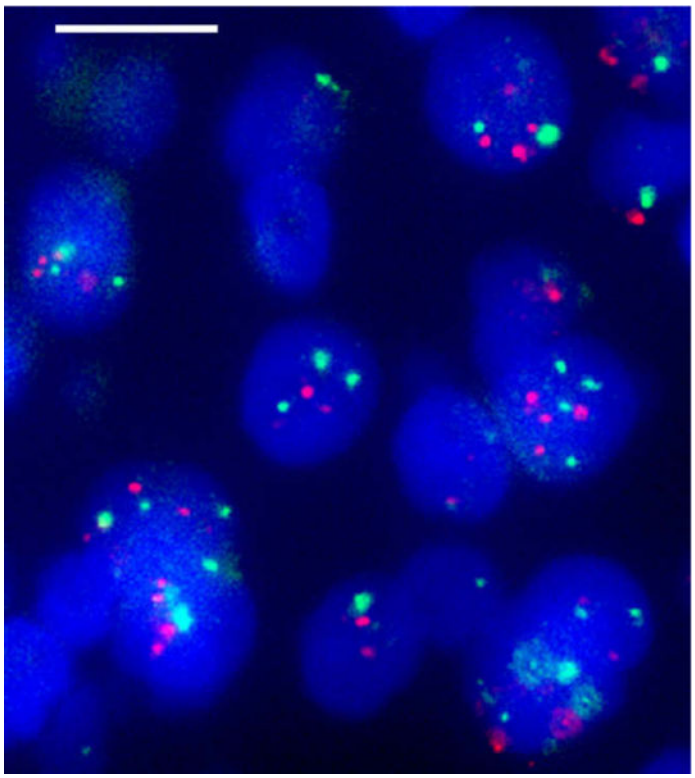

Fig. 3.

FISH analysis of a formalin-fixed, paraffin-embedded (FFPE) section of a representative primary breast tumor sample showing increased copy number for the *BP1* (green signals) and *HER2/NEU* (red signals) genes (magnification bar is 100 μm).

Table 1

Primary breast tumor (PBT) and sentinel lymph node (SLN) metastasis analyzed by FISH and IHC for *BP1* and *HER2/NEU* gene copy number and protein expression, respectively

PATIENT#	TISSUE	FISH *BP1*	IHC BP1	FISH *HER2/NEU*	IHC HER2/NEU
1	PBT	Normal	pos	Gain	pos
2	PBT	Gain	pos	Amplification	pos
3	PBT	Amplification	pos	Amplification	pos
4	PBT	Normal	pos	Normal	neg
5	PBT	Normal	pos	Amplification	pos
6	PBT	Normal	neg	Gain	neg
7	PBT	Normal	pos	Normal	neg
8	PBT	Normal	neg	Amplification	neg
9	PBT	Normal	pos	Amplification	neg
10	PBT	Normal	pos	Normal	pos
11	SLN	Normal	pos	–	–
12	SLN	Gain	–	–	–
13	PBT	Amplification	pos	Gain	neg
	SLN	Normal	pos	–	–
14	PBT	Amplification	pos	Amplification	neg
	SLN	Gain	pos	Gain	neg
15	PBT	Gain	pos	Normal	neg
	SLN	Normal	pos	Normal	neg
16	PBT	Normal	pos	Normal	neg
	SLN	Normal	pos	–	–
17	PBT	Amplification	pos	Amplification	pos
	SLN	Normal	pos	Normal	neg
18	PBT	Normal	neg	Normal	neg
	SLN	Normal	pos	Normal	neg
19	PBT	Normal	pos	Normal	neg
	SLN	Normal	pos	Normal	neg
20	PBT	Gain	–	Gain	pos

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PATIENT#	TISSUE	FISH *BP1*	IHC BP1	FISH *HER2/NEU*	IHC HER2/NEU
	SLN	Gain	pos	Gain	neg
21	PBT	Normal	pos	Normal	neg
	SLN	Normal	pos	Normal	neg
22	PBT	Amplification	–	Normal	neg
	SLN	Amplification	–	Normal	neg
23	PBT	Normal	pos	Normal	neg
	SLN	Normal	pos	Normal	neg
24	PBT	Normal	pos	Normal	neg
	SLN	Normal	pos	Normal	neg

Abbreviations: pos, positive; neg, negative; –, result not obtained.