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Increased copy number of the *DLX4* homeobox gene in breast axillary lymph node metastasis

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DLX4 is a homeobox gene strongly implicated in breast tumor progression and invasion. Our main objective was to determine the *DLX4* copy number status in sentinel lymph node (SLN) metastasis to assess its involvement in the initial stages of the axillary metastatic process. A total of 37 paired samples of SLN metastasis and primary breast tumors (PBT) were evaluated by fluorescence in situ hybridization, quantitative polymerase chain reaction and array comparative genomic hybridization assays. *DLX4* increased copy number was observed in 21.6% of the PBT and 24.3% of the SLN metastasis; regression analysis demonstrated that the *DLX4* alterations observed in the SLN metastasis were dependent on the ones in the PBT, indicating that they occur in the primary tumor cell populations and are maintained in the early axillary metastatic site. In addition, regression analysis demonstrated that *DLX4* alterations (and other *DLX* and *HOXB* family members) occurred independently of the ones in the *HER2/NEU* gene, the main amplification driver on the 17q region. Additional studies evaluating *DLX4* copy number in non-SLN axillary lymph nodes and/or distant breast cancer metastasis are necessary to determine if these alterations are carried on and maintained during more advanced stages of tumor progression and if could be used as a predictive marker for axillary involvement.

Keywords Homeobox gene, sentinel lymph node, breast cancer, metastasis, *DLX4*, copy number

Homeobox genes belong to a family of genes that encode transcription factors involved in cellular processes of early development, such as differentiation, morphogenesis, and tissue homeostasis (1). They are evolutionarily highly conserved and contain a common DNA binding site of a 60-amino acid motif encoded by 180 bp homeobox sequences (2), which specifically regulates the expression of downstream target genes (3). Vertebrate homeobox genes can be of two major classes: clustered or *HOX* gene family and non-clustered or orphan homeobox genes, which include the gene families *PAX*, *MSX*, *IRX*, *OTX*, *CDX*, and *DLX* (4). The *DLX* homeobox family is the vertebrate homologue of the *Drosophila distal-less* (DLX) family and is primarily involved in the control of craniofacial, forebrain, and neurogenesis development (5). In humans, this family is composed of seven genes, which are represented by three major gene clusters: *DLX1* and *DLX2*; *DLX5* and *DLX6*; *DLX3*, *DLX4*, and *DLX7* (6).

The *DLX4* gene was first isolated from a human cDNA placenta library and mapped to 17q21.3 (7). This gene is about

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5,761 bp in length and has two splicing variants, BP1 and DLX7, which presumably present different functions (8). In this study, we refer the mRNA and protein expression to the analysis of the *DLX4* splicing variant BP1. *DLX4*, similar to other members of the *DLX* and other homeobox families' members, is reportedly involved in human tumorigenesis, functioning as homeoproteins that can regulate critical cellular processes, such as cell cycle, apoptosis, and cellular transformation (9–12).

BP1 is a repressor of the β-*GLOBIN* gene (13), and the BP1 protein was first demonstrated to be highly expressed in leukemia cells (14). Subsequent studies have shown that BP1 is widely expressed in a variety of other cancers, including lung, ovarian, and prostate (15–18). BP1 was the first member of the *DLX* family to be strongly implicated in breast cancer, with high mRNA expression in several cancer cell lines where it correlated with their in vitro and in vivo tumorigenic potential (19). In clinical breast cancer cases, mRNA and protein overexpression of BP1 were also observed and associated with established poor prognostic factors, such as high histological grade, lymph node positivity, and estrogen (ER) and progesterone (PR) receptor negativity (19–21). In addition, BP1 was demonstrated to play a significant role in breast tumor progression and invasion, and was observed with an increased protein expression in 81% of invasive carcinoma cases, compared with 21% of hyperplasia and 46% of ductal carcinoma in situ (DCIS) cases (20).

A key and essential alteration in cancer development and progression is the deregulation of genes with oncogenic functions, such as *DLX4*. Several mechanisms can lead to their abnormal expression and impact their regular cellular function, including copy number imbalances in specific genomic regions, such as gene amplifications and deletions (22).

In our previous study, we demonstrated for the first time that the *DLX4* gene is amplified in breast cancer (23), which could be one of the mechanisms that leads to its mRNA and/or protein overexpression. Both amplification of *DLX4* and protein overexpression of its isoform BP1 were observed in the primary tumors as well as in their corresponding lymph node metastasis that were analyzed. Overexpression of BP1 was additionally shown to be present in lymph node metastasis in the immunohistochemistry analysis of inflammatory tumors, a rare but extremely aggressive form of breast cancer (24). Additional evidence found in breast cancer cell line models has implicated BP1 in the metastatic process (21,25). Interestingly, BP1 has been reported to play a critical role in epithelial–mesenchymal transition (EMT) (26,27) as well as in tumor resistance to several therapeutic agents (12,28,29)—mechanisms commonly associated with metastatic tumors.

In this study, based on the observed role of *DLX4* in tumor progression and invasion, our main goal was to evaluate its involvement in the early stages of the axillary lymph node metastatic process by determining its DNA copy number status in sentinel lymph node (SLN) metastatic lesions, the first metastatic site of the breast. Fluorescence in situ hybridization (FISH), TaqMan Copy Number Assay, and array comparative genomic hybridization (array-CGH) assays were performed in a group of 37 paired samples of SLN metastasis and primary breast tumors (PBT) from patients with invasive breast cancer, and evaluated in relation to their clinical and histopathological parameters. In addition, considering the proximity of *DLX4* to the *HER2/NEU* gene in the 17q region, copy number status of this gene was also determined to verify if *DLX4* amplification is a separate event or a consequence of a co-amplification mechanism due to its chromosomal location. The copy number status of other *DLX* genes (*DLX2* (located at 2q31.1), *DLX3* (17q21.33), and *DLX5* and *DLX6* (7q21.3)) and of the *HOXB* gene family (*HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXB13*—all located in the 17q21 region) was also determined in both the PBT and SLN metastasis analyzed.

Materials and methods

Sample characterization

A total of 74 (37 pairs of PBT and SLN metastasis from the same patient) formalin-fixed paraffin-embedded (FFPE) samples of invasive breast cancer were analyzed for DNA copy number alterations of the *DLX4* and *HER2/NEU* genes. The samples were obtained from the pathology tumor banks of Clinical Hospital and Hospital Nossa Senhora das Graças (HNSG), Curitiba, PR, Brazil during 1999–2008 from patients who underwent surgery for primary tumor removal, before any cancer treatment. The FFPE samples were collected from the tumor bank of the aforementioned hospitals and transferred to Georgetown University, Washington, DC, under patient informed consent and through the IRB approval of Georgetown University, of the hospitals involved, and the National Review Board of Ethics in Research (CONEP-Brazil). The SLN biopsy was performed by both lymphoscintigraphy and injection of blue dye and radiocolloid with intraoperative gamma-probe. The accuracy rate of SLN identification using these methods in the aforementioned hospitals is approximately 94% (30). The SLN biopsies were investigated for tumor cells intraoperatively by standard frozen section analysis. Each FFPE block was uniformly analyzed for its diagnosis and the presence of tumor cells by an experienced breast cancer pathologist at Georgetown University. Only FFPE blocks that contained tumor tissue were processed for tissue sectioning and were used in the DNA copy number assays.

The clinical characteristics and follow-up information of the patients are presented in Table 1. A total of 31 patients presented with invasive carcinoma of the ductal type and 6 of the lobular type. Tumors were classified according to the TNM system (31), and were of grade I, II, and III in 9%, 79%, and 12% of the patients, respectively. The majority of the patients were Caucasian, with an average age of 53.8 $\pm$ 10.2 years (range 34–75). The average tumor size was 2.94 $\pm$ 1.85 cm (range 0.7–8.0). Most of the patients were treated with classical chemotherapy regimens (cyclophosphamide, methotrexate, and fluorouracil (5FU) (CMF), or fluorouracil, adriamycin, and cyclophosphamide (FAC)) and tamoxifen. Clinical follow-up information (of at least 5 years or the occurrence of death) was obtained for 89.2% of the patients. The majority of the patients are alive with no evidence of disease (NED). Recurrence to distant organs occurred in 21.2% of the patients, including lung, bone, endometrium, and liver. Among these patients, 57% are not alive and 42.8% are alive with the disease (as of the last follow-up in November 2013).