HHS Public Access

Author manuscript

Cancer Genet. Author manuscript; available in PMC 2018 February 09.

Published in final edited form as:

Cancer Genet. 2014 May ; 207(5): 177–187. doi:10.1016/j.cancergen.2014.04.007.

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

Clarissa Torresan[a], **Márcia M.C. Oliveira**[a], **Silma R.F. Pereira**[b], **Enilze M.S.F. Ribeiro**[a], **Catalin Marian**[c], **Yuriy Gusev**[d], **Rubens S. Lima**[e], **Cicero A. Urban**[e,f], **Patricia E. Berg**[g], **Bassem R. Haddad**[h], **Iglenir J. Cavalli**[a], and **Luciane R. Cavalli**[h,*]

[a]Department of Genetics, Federal University of Paraná, Curitiba, PR, Brazil

[b]Department of Biology, Federal University of Maranhão, São Luis, MA, Brazil

[c]Department of Biochemistry, University of Medicine and Pharmacy, Timisoara, Romania

[d]Innovation Center for Biomedical Informatics, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

[e]Breast Unit, Hospital Nossa Senhora das Graças, Curitiba, PR, Brazil

[f]Positivo University, Curitiba, PR, Brazil

[g]Department of Biochemistry and Molecular Medicine, George Washington University Medical Center, Washington, DC, USA

[h]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Abstract

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.

*Corresponding author. lrc@georgetown.edu.

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 neuro-genesis 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 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 ± 10.2 years (range 34–75). The average tumor size was 2.94 ± 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).

The cases were evaluated for diagnostic purposes for estrogen (ER), progesterone (PR), and epidermal growth factor II (HER2) receptor status by imunohistochemistry (IHC) analysis. The monoclonal mouse anti-human estrogen receptor alpha and polyclonal rabbit anti-human progesterone receptor were used for the ER and PR analysis, respectively. For HER2, this analysis was performed using the HercepTest (Dako North America Inc., Carpinteria, CA). ER and PR positivity were considered using a cut-off of 10%, and were observed in 90% and 82% of the cases, respectively (Table 1). HER2 positivity was scored per guidelines of the International Consensus panel (32). Most of the patients were HER2 negative (83.8%); only one case (3.6%) was of the triple negative phenotype (as determined by ER, PR, and HER2 protein expression status by IHC) (Table 1).

DNA isolation

For each case, before the DNA copy number analysis, an H&E section from each tumor block was evaluated by a pathologist to confirm and delineate the presence of tumor tissue. Consecutive 5-μm sections were microdissected for DNA isolation (for TaqMan and array-CGH assays) as we previously described (33). Normal (reference) DNA was prepared from a pool of multiple female donors with no cancer, to minimize the effects of normal interindividual variation (copy number variations) in the copy number analysis by array-CGH. Dual-color FISH analysis was performed in new 5-μm non-microdissected sections that were consecutive from the ones used for DNA analysis.

FISH analysis

FISH hybridization, analysis, and scoring were performed using a standard protocol that we have previously described for the *DLX4* gene (23). Briefly, dual- color hybridization for the *DLX4* and *HER2/NEU* genes was performed using our constructed bacterial artificial chromosome (BAC) clones (BACPAC Resources, Oakland, CA). The *DLX4* probe consisted of a contig of three overlapping BAC clones (RP11-267M22, RP11-298H16, and RP11-479I21), which contained sequences of the *DLX4* gene. The *HER2/NEU* probe was constructed from the clone RP11-62N23. BAC clone DNA was prepared and labeled using nick translation, with biotin-16-dUTP (Roche Applied Sciences, Indianapolis, IN) and digoxigenin-11-dUTP (Roche Applied Sciences) for *DLX4* and *HER2/NEU*, respectively.

The FISH hybridization and detection were performed according to our previous protocol for FFPE samples (23). The scoring for *DLX4* and *HER2/NEU* was performed simultaneously in the same cell, by two independent observers. A minimum of 100 nuclei were evaluated in each case, and only intact, non-overlapping nuclei were scored. Detection of two probe signals was considered as a normal copy number, three signals as a gain, and >3 signals as an amplification. The term “increased copy number” was used for both gain and amplification. To consider a case as having gain or amplification, at least 30% of the nuclei counted in the case had to fall into one of the above categories. It is notable that in our previous FISH analysis of *DLX4* copy number changes in normal cells (from women with no cancer), increased copy number of this gene was observed in up to 4.3% of the cells (23).

TaqMan Copy Number Assays

TaqMan Copy Number Assays (Applied Biosystems, Foster City, CA), were performed in the microdissected DNA isolated from the FFPE tumor sections and from the normal control (pool of DNA from multiple female donors with no cancer). The samples were diluted to 5 ng/μL and amplified in the ABI 7500 HT Real Time PCR instrument (Applied Biosystems) using *DLX4*- and *HER2/NEU*-specific probes and primers. The *RNASE P* gene was used as a reference. The gene sequences were based on the hg19.v9 genomic database (http://genome.ucsc.edu/). The probe locations of the *DLX4*, *HER2/NEU*, and *RNASE P* genes were as follows: 48048179 (17q21.22 region), 37844501 (17q12), and 157907524 (14q11.2), respectively. The *DLX4* and *HER2/NEU* probes were labeled with the fluorescent dye FAM and *RNASE P* with VIC dye. PCR was performed in a total volume of 10 μL in each well of a 96-well plate, which contained TaqMan Universal MasterMix (Applied Biosystems), 5 ng of genomic DNA, and 12.5 picomoles per liter concentration of each primer and probe. PCR conditions included an initial denaturation step of 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. All the reactions were performed in triplicate, and a negative control with no DNA template was included in every PCR run. For all PCR assays, the C_t values were established using SDS 2.2 RQ software (Applied Biosystems), and the copy numbers were normalized against the reference gene (*RNASE P*) and determined by the $2^{-\Delta\Delta Ct}$ method. Copy number changes were only considered for samples that presented with C_t cycles ≤33 and a Z-score value ≥2.65. A twofold increase or decrease in the copy number of *DLX4* and/or *HER2/NEU* in tumor samples in comparison to the normal control was considered as a gain or loss, respectively.

Array-CGH analysis

Array-CGH was performed to identify the copy number alterations in *DLX4* and in other *DLX* and *HOXB* family members, as well as in the *HER2/NEU* gene. An oligonucleotide base 4 × 44K format array-CGH platform (Agilent Technologies, Santa Clara, CA) was used for this assay. The same genomic DNA used for the TaqMan copy number assays was used for the array-CGH hybridizations. The DNA from each case and controls were directly labeled (Array-CGH genomic labeling kit, Agilent Technologies) and hybridized to the array, according to the protocol for the FFPE material that we established in our lab (34,35). Only cases that demonstrated a satisfactory incorporation of labeling, per the Agilent protocol guidelines, were hybridized to the arrays. The arrays were scanned using an Agilent

array scanner, and the data were analyzed using the Feature Extraction (FE) software v10.10 and Genome Workbench version 7.0 software (Agilent Technologies). For each sample, FE gave a $\log_{10}$ ratio (log of tumor processed signal over reference processed signal for each gene) that was imported into Genome Workbench and transformed and viewed as a $\log_2$-based ratio. Outliers detected by the FE were excluded from the analysis. The algorithm ADM-2 and a threshold value of 6.0 were applied with the appropriate filters to analyze the data. Gene amplifications and deletions were considered when the corresponding plotted oligo-probes presented values of $\log_2 > 7/6$ and $\log_2 < 5/6$, respectively. The unsupervised clustering analysis (unsupervised hierarchical clustering (UHC)) of the *DLX*, *HOXB*, and *HER2/NEU* genes was performed using the Multiexperiment Viewer software (MeV 4.8)—a part of the TM4 software package from the Dana-Farber Cancer Institute in Boston, MA. Log_2 values based on DNA copy number changes in these genes were median centered and analyzed using UHC tools. Average dot product was used as a distance measure with clustering performed using average linkage for samples and genes. The results of clustering were visualized as heat maps utilizing MeV visualization tools with a 3-color color scheme where the red color represents values above median, green color represents values below median and black color corresponding to median values.

Statistical analysis

A sample size power calculation was performed, considering a medium effect size of 0.5: A paired *t* test, two-sided, with a significance of 0.05, would give a power of 0.8 with a sample size of 33.36 pairs of PBT and SLN metastases (36,37). The chi-square (χ^2) and Fisher exact tests were used to compare the results of *DLX4* and *HER2/NEU* copy number changes observed in the FISH and TaqMan copy number assays in both the PBT and SLN metastatic group, and to test the nonrandom involvement and the homogeneity distribution of the number of DNA copy number changes of these genes in the lesions studied. The dependence of the variations in the *DLX4* and *HER2/NEU* copy number changes observed in the SLN metastasis in function to the ones observed in the PBT was verified by regression coefficient (b) analysis, as well as the variations of the alterations in the *DLX* and *HOX* gene families (observed by array-CGH) as a function of the variations of the *HER/NEU* gene. The correlation coefficient (r) was used to verify the dependence of the changes of *DLX4* and *HER2/NEU* obtained by FISH and TaqMan.

The clinical-histopathological data of the parameters from the patients (age, tumor size, tumor grade and ER, PR, and HER2 receptors) were analyzed by the Student *t* test and Fisher exact test; the significance level was $P < 0.05$.

Results

FISH and TaqMan copy number assays

Copy number assessment of the *DLX4* and *HER2/NEU* genes by either FISH or TaqMan Copy Number Assay was successfully performed in all the paired samples of PBT and SLN metastasis in this study. Seventy-three percent (27 of 37) of the paired cases were analyzed by FISH and 75.7% (28 of 37) by TaqMan Copy Number Assay.

FISH analysis showed *DLX4* increased copy number in 29.6% (8 of 27) of both PBT and SLN metastasis. TaqMan Copy Number Assay showed increased copy number for *DLX4* in 17.8% (5 of 28) of the PBT and 10.7% (3 of 28) of the SLN metastasis. The results obtained from both methods in the PBT and SLN metastasis analysis were not statistically significant ($\chi_1^2=1.05$, $P > 0.30$, and $\chi_1^2=3.07$, $P > 0.05$, respectively), which indicated a homogeneous distribution of the data. Therefore, the total number of samples with DNA copy number results were combined, and the final frequency of *DLX4* increased copy number in the PBT and SLN metastasis was 21.6% (8 of 37) and 24.3% (9 of 37), respectively (Table 2). This difference was not statistically significant ($\chi_1^2=0.08$, $P > 0.70$). Figure 1 shows a representative case (case 22), with increased copy number for the *DLX4* gene in both the PBT and SLN metastasis by both FISH and TaqMan assays.

For the *HER2/NEU* gene, FISH analysis showed increased copy number in 18.5% (5 of 27) and 33.3% (9 of 27) of the PBT and SLN metastasis, respectively. TaqMan Copy Number Assay showed increased copy number for *HER2/NEU* in 14.3% (4 of 28) of both the PBT and SLN metastasis. As it was verified for *DLX4* analysis, the results for *HER2/NEU* obtained from both methods in the PBT and SLN metastasis were not statistically significant (Fisher exact test $P = 0.73$ and $\chi_1^2=2.77$, $P > 0.05$, respectively). Therefore, the final frequency of *HER2/NEU* increased copy number in the PBT and SLN metastasis was 16.2% (6 of 37) and 24.3% (9 of 37), respectively. This difference was not statistically significant ($\chi_1^2=0.76$, $P > 0.30$).

Increased copy number of the *DLX4* and *HER2/NEU* genes in both of the lesions was more frequent than losses (77.2% (17 of 22) and 71.4% (15 of 21), respectively). The difference between the groups was not statistically significant ($\chi_1^2=0.19$, $P > 0.50$); however, considering the random occurrence of increased copy number and losses, a statistically significant difference was observed ($\chi_1^2=10.26$, $P < 0.01$).

Finally, *DLX4* and *HER2/NEU* copy number status were concordant in 81.1% (30 of 37) of the PBT and in 89.2% (33 of 37) of the SLN metastasis. Most of the lesions with concordant results for these genes presented with normal copy number. The DNA copy number alterations of both genes observed in SLN metastasis were evaluated in function of their DNA copy number alterations in PBT in the lesions studied by the regression analysis. The results showed that the alterations in the *DLX4* gene observed in the SLN metastasis were dependent on the *DLX4* alterations in the PBT ($b = 0.69 \pm 0.10$, $t = 6.9$, $P < 0.001$) and in all the lesions ($b = 0.41 \pm 0.09$, $t = 4.56$, $P < 0.001$). For the *HER2/NEU* gene, however, this dependence was not observed ($b = 0.19 \pm 0.14$, $t = 1.36$, $P > 0.10$). We also verified the dependence of *DLX4* and *HER2/NEU* gene copy number alterations in both lesions by correlation analysis and observed a non-significant correlation coefficient value ($r = 0.01$, $t = 0.12$, $P > 0.90$) (Table 2), indicating that the variations of *DLX4* and *HER2/NEU* DNA copy number were not dependent.

Array-CGH

Copy number changes of *DLX4* and *HER2/NEU*—Array-CGH was performed in seven pairs of PBT and matched SLN metastases and two unpaired SLN metastatic lesions (total of 16 lesions). Copy number alterations for the *DLX4* gene were observed in four paired cases and in the two unpaired SLN metastases; *DLX4* amplification was observed in both PBT and matched SLN metastasis in two cases (cases 14 and 22) (Figure 1) and one unpaired SLN metastasis (case 7). For the *HER2/NEU* gene, copy number changes were observed in five paired cases and in the two unpaired SLN metastases; *HER2/NEU* amplification was observed in both the PBT and SLN metastasis from four cases (cases 14, 17, 20, and 22) and in the unmatched SLN metastases (cases 3 and 7). The significant $\log_2$ ratios for *DLX4* were tabulated, and a linear regression analysis was performed with the significant values for $\log_2$ for each lesion. No significant value was observed, which indicated that *DLX4* copy number changes were not dependent on the *HER2/NEU* copy number changes (b = 0.44, $t = 2.00$, $P > 0.05$).

Copy number changes of other *DLX* and *HOX* homeobox gene families—The copy number results for other homeobox genes of the *DLX* family (*DLX3*, *DLX5*, and *DLX 6*) and for genes of the *HOXB* family cluster (*HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXB13*) were assessed in 16 cases (7 paired PBT and SLN metastases and 2 unpaired SLN metastases). The average number of cases with copy number alterations observed in the *DLX* and *HOXB* family genes were 9.25 ± 3.34 and 9.6 ± 2.45, respectively, a difference that was not statistically significant ($t = 0.34$, $P > 0.70$). In the *DLX* gene family, *DLX3* (only gains) and *DLX5* (gains and losses) were the genes with the highest copy number changes, which were observed in 81% and 68.7% of the cases, respectively. *DLX6* and *DLX2* presented mostly losses, which were observed in 56% and 25% of the cases, respectively. In the *HOXB* family, *HOXB7* and *HOXB1* were the genes with a higher frequency of alterations, in 87.5% and 75% of the cases, respectively. The alterations in the other members of this family ranged from 31–69%. Figure 2 shows the amplification of the 17q21.32 region, where a cluster of the *HOXB* genes is located in both PBT and SLN metastasis from case 17.

Considering that several of the homeobox genes from the *HOXB* family that presented with DNA copy number changes in this study were mapped at 17q21, in close proximity to the *HER2/NEU* gene, regression analysis (of the significant array-CGH $\log_2$ ratios) was performed, together with other members of the *DLX* family, to determine if these changes were dependent on *HER2/NEU* changes (i.e., a coefficient of regression was calculated independently for each gene with *HER2/NEU*). For the *HOXB* family members, a dependence on *HER2/NEU* changes was observed for *HOXB3*: b = 0.54, $t = 2.49$, $P < 0.05$, *HOXB7*: b = 0.87, $t = 3.32$, $P < 0.01$, *HOXB9*: b = 1.02, $t = 2.79$, $P < 0.05$, and *HOXB13*: b = 0.53, $t = 3.004$, $P < 0.01$. For the *HOXB2* gene, the regression coefficient was negative but close to the significance limit (b = 0.57, $t = 2.06$, $P = 0.06$). For the other *HOXB* genes, *HOXB1*, *HOXB4*, *HOXB5*, *HOXB6*, and *HOXB8*, the regression coefficients were not significant, which indicated that their DNA copy number changes were not dependent on the *HER2/NEU* changes. For the *DLX* family, the only member that was dependent on *HER2/NEU* alterations was *DLX5*, for which the value of the regression coefficient was

significant and negative (b = −0.31, $t = 2.40$, $P < 0.05$), which indicated an inverse dependence on *HER2/NEU*. The coefficient of regression for the other *DLX* family members, *DLX2*, *DLX3*, and *DLX6*, was not significant, which indicated that their copy number changes were not dependent on *HER2/NEU* gene changes.

Unsupervised clustering analysis (i.e., UHC) was performed based on copy number changes in these genes (i.e., array-CGH $\log_2$ ratios used previously for the regression analysis) (Figure 3). *DLX4* was clustered separately from *HER2/NEU*. Interestingly, based on the $\log_2$ values, most of the paired samples were clustered next to each other, with the exception of one case (case 18).

Evaluation with clinical-histopathological parameters from the patients

DLX4 and *HER2/NEU* increased copy number was evaluated with the following clinical and histopathological parameters: age, tumor size, tumor grade, ER, PR, and HER2 protein expression status. Only the data obtained from the FISH and TaqMan copy number assays (grouped data) were used for this analysis, considering that they were performed in a larger number of paired samples.

The mean age and tumor size of the patients with *DLX4* increased copy number in at least one lesion was 53.2 ± 10.4 and 2.9 ± 1.5, respectively, in comparison with 54.2 ± 10.6 and 3.0 ± 2.0 for the cases with no *DLX4* increased copy number. The Student *t* test did not show any significant difference among these groups for age or tumor size ($t = 0.28$, $P > 0.70$ and $t = 0.15$, $P > 0.80$, respectively). The same occurred for the mean age and tumor size with *HER2/NEU* increased copy number ($t = 1.08$, $P > 0.20$ and $t = 1.19$, $P > 0.20$, respectively).

For tumor grade, patients with *DLX4* and *HER2/NEU* increased copy number presented a lower number of grade II and I+III tumors as compared with patients with no increased copy number; however, differences between these grade groups (grades I and III considered together) were not significant (Fisher exact test, $P = 0.68$ for *DLX4* and $P = 1.0$ for *HER2/NEU*).

Finally, for ER, PR, and HER2 expression status (as measured by IHC), the Fisher exact test also did not show significant differences between the groups of patients with or without *DLX4* increased copy number (Fisher exact test, $P = 1.0$, 0.21, and 0.18 for ER, PR, and HER2, respectively). The same was observed between the groups of patients with and without *HER2/NEU* increased copy number (Fisher exact test, $P = 1.0$, 0.55, and 0.16 for ER, PR, and HER2, respectively).

Discussion

With wide implementation of population-based screening methods, breast cancers are now often detected in the early stages with no regional lymph node involvement, conferring a 5-year survival time 98% to the vast majority of patients; however, for patients with regional nodal metastases, the 5-year survival can be as low as 40% (38). Therefore, finding molecular markers of invasive or metastatic potential in early-stage lesions, when distant

axillary and/or hematogenic metastases are not present, would have considerable impact on patient survival, emphasizing the importance of early detection (39,40). Several clinical and histological parameters, such as age, tumor size, histological grade, lympho-vascular invasion, steroid, and HER2 receptor status, are considered as strong predictors for recurrence (41). In addition, the molecular prognostic signatures, based on gene expression profiling of primary tumors, have also been employed to predict metastasis and recurrence risk (42); however, the axillary lymph node status still remains one of the most important predictors of disease-free and overall survival for early-stage breast cancer patients (39,40,43,44). To date, the best indication of axillary metastasis is the presence of tumor cells in the SLN (45,46).

In this study, we have evaluated these axillary metastatic lesions by characterizing the copy number changes of the *DLX4* homeobox gene, which was previously reported to be involved in breast cancer metastasis (20,21,23–25). Our analysis, which used three different methods to assess DNA copy number (FISH, TaqMan Copy Number Assay, and array-CGH) performed in paired samples of PBT and SLN metastasis demonstrated increased copy number of the *DLX4* gene in 21.6% and 24.3% of the lesions, respectively. The difference in frequencies between these lesions was not statistically significant, demonstrating that there is no preferential increase in the copy number of *DLX4* in either the PBT or SLN metastasis. These findings were also observed in our previous study in a smaller number of paired samples of PBT and SLN positive lesions (23); however, in this study, we demonstrated that the *DLX4* alterations in the SLN metastasis were dependent on the ones observed in the PBT, which, interestingly, was not observed for the *HER2/NEU* gene.

Several models of the molecular evolution from the primary tumors to a metastatic state have been proposed, underlying the complexity of breast tumor progression and the presence of multiple sub-clones with different metastasis capacity in the primary tumors (47–50). Although a high molecular heterogeneity and disparities are reported in molecular profiles among these lesions (51), our results are in agreement with several other genomic, transcriptomic, and proteomic studies (33,52–56), demonstrating that primary and metastatic lesions do not present divergent molecular profiles. Of interest are our findings based on the genome-wide copy number analysis obtained by array-CGH (data not shown) in these same samples investigated for *DLX4*. A remarkable similarity in the genomic profiles was obtained between the matched primary tumors and the SLN metastasis.

Other members of the *DLX* family, *DLX2*, *DLX3*, *DLX5*, and *DLX6*, were also evaluated in relation to their copy number status by array-CGH. The genes with a higher frequency of increased copy number were *DLX3* and *DLX5*, followed by *DLX6* and *DLX2*. These different members of the *DLX* family have been demonstrated to play diverse roles in tumorigenesis. *DLX2*, *DLX3*, and *DLX5* were reported to be mostly involved in hematological malignancies (57–59), although *DLX5* has been shown to be up-regulated in solid tumors, such as lung, endometrial, uterine, and ovarian cancers (60–62). Recently, in the triple-negative breast cancer cell line MDA-MB-231, it was shown that *DLX2*, *DLX5*, and *DLX6* were associated with metastatic potential when induced by the protein ENDOTHELIN 1 (ET1) (63). In vitro treatment of these cells with ET1 resulted in positive expression of both *DLX5* and *DLX6*, but loss of *DLX2*, in the lung and bone metastasis. A

preliminary evaluation of these genes in clinical breast cancer cases also showed inhibition of *DLX2* and induction of *DLX5* expression when compared to normal breast tissue. In our study, we observed by array-CGH loss of *DLX2* in 18.8% of the cases, both in the PBT and SLN metastasis and both gains and losses of *DLX5*, in 31.3% and 37.5% of the cases, respectively. An evaluation of their respective expression levels in these cases should be performed to determine whether the copy number alterations observed correspond to alterations in mRNA or protein expression.

Members of the homeobox family *HOX* are also often highly overexpressed in breast cancer and reportedly involved in the regulation of tumor growth and metastasis (11,64). In our study, we evaluated DNA copy number changes of the *HOXB* gene cluster, due to its location on 17q21. *HOXB7* was the member of this family most frequently altered, with higher frequencies of amplification in both PBT and SLN metastasis. This gene has been previously shown to be overexpressed in primary tumors and distant metastasis from clinical breast cancer cases (65). Interestingly, *HOXB7* has been shown to be overexpressed and involved in the process of EMT in placenta and endometrium tissue (66,67), and in epithelial breast cancer cells accompanied by acquisition of aggressive properties of tumorigenicity, migration, and invasion (27,68). Alterations of this gene are also reportedly involved in tumor progression and metastasis promotion in other types of tumors, including pancreatic and lung cancer (65,69,70). Overexpression of *HOXB13*, also observed with increased copy number in our cases, has been correlated with the migration and invasion capacity in ER-positive breast cancer cells resistant to tamoxifen (71). Distant metastasis from these patients also displayed high HOXB13 expression, suggesting a role for *HOXB13* in tumor dissemination and survival. In breast cancer, these two *HOXB* genes are commonly associated with aggressive tumor phenotypes (11,71). Of note in our UHC analysis, these two genes clustered near each other and in the same major cluster of *DLX4*.

DLX4 maps to the chromosome 17q21 region, within the 17q amplicon, which is mostly driven by *HER2/NEU* amplification (72,73). This is a very complex and heterogenous amplicon where the molecular variations targeting other associated breast cancer genes and their clinical implications remain largely unknown (74,75). Therefore, it is critical to investigate the copy number and expression of these genes in relation to *HER2/NEU*, since they may present independent biological value to breast cancer progression. In our study, the evaluation of *DLX4* and *HER2/NEU* by the DNA copy number assays demonstrated that alterations in these genes occur independently (r = 0.01, $t = 0.12$, $P > 0.90$ by FISH and TaqMan Copy Number Assay and b = 0.44, $t = 2.00$, $P > 0.05$ by array-CGH). These data suggest that increased copy number of *DLX4* in breast cancer is not a result of a co-amplification mechanism associated with *HER2/NEU*. Linear regression analysis based on the array-CGH data was also performed for the *DLX* and *HOXB* family members in relation to *HER2/NEU* changes. *DLX2*, *DLX3*, and *DLX6* did not present any dependence on the *HER2/NEU* changes, but *DLX5* presented a significant and inverse dependence (b = −0.31, $t = 2.40$, $P < 0.05$). For the *HOXB* family, a dependence of *HER2/NEU* changes was observed for *HOXB3*, *HOXB7*, *HOXB9*, and *HOXB13*. For *HOXB1*, *HOXB4*, *HOXB5*, *HOXB6*, and *HOXB8*, the regression coefficients were not significant, which indicated that their DNA copy number changes were not dependent on *HER2/NEU*.

In conclusion, our study supports the involvement of *DLX4* and other homeobox genes in the process of axillary breast cancer metastasis. Increased *DLX4* copy number was observed with non-significant different frequencies in both the PBT and corresponding SLN metastasis; however, regression analysis showed that *DLX4* (but not *HER2/NEU*) alterations in the SLN metastasis were dependent on the ones observed in the PBT, indicating that they occur early during breast tumor progression in the cell populations of the primary lesions and in the "very first" site of cancer metastasis outside the breast, before distant metastasis occurs. 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. Studies should also be pursued in the evaluation of primary tumors from patients with negative axillary nodes, to evaluate the value of *DLX4* as a predictive molecular marker for axillary lymph node involvement. Finally, copy number alterations of *DLX4* should be investigated, coupled with gene and protein expression and, mechanistically, in assays that modulate DLX4 expression, to determine if they functionally play a role in conferring breast cancer metastasis-related capacity both in vitro and in vivo. These analyses would ultimately reveal the potential role of *DLX4* as an additional biological marker that can contribute in the future to the improvement of the selection criteria for patients who might undergo SLN biopsy or axillary sampling, and/or its role as a target for the development of therapeutics to prevent the early stages of breast cancer metastasis.

Acknowledgments

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for providing scholarship for C.T., M.M.C.O., and S.R.P, respectively. This study was partially supported by an American Cancer Society–Research Scholar Award (no. 118650-RSG-10-034-01-CCE) and The Nina Hyde Center for Breast Cancer Research (Georgetown University) to L.R.C. The Histopathology and Tissue Shared Resource and Genomics and Epigenomics Shared Resource from Lombardi Comprehensive Cancer Center (LCCC), partially supported by the NIH/NCI grant P30-CA051008, were utilized in this study.

References

1. Magli MC, Largman C, Lawrence HJ. Effects of HOX homeobox genes in blood cell differentiation. J Cell Physiol. 1997; 173:168–177. [PubMed: 9365517]
2. Scott MP, Tamkun JW, Hartzell GW. The structure and function of the homeodomain. Biochim Biophys Acta. 1989; 989:25–48. [PubMed: 2568852]
3. Svingen T, Tonissen KF. Hox transcription factors and their elusive mammalian gene targets. Heredity (Edinb). 2006; 97:88–96. [PubMed: 16721389]
4. Popovici C, Leveugle M, Birnbaum D, Coulier F. Homeobox gene clusters and the human paralogy map. FEBS Lett. 2001; 491:237–242. [PubMed: 11240134]
5. Bendall AJ, Abate-Shen C. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene. 2000; 247:17–31. [PubMed: 10773441]
6. Panganiban G, Rubenstein JL. Developmental functions of the Distal-less/Dlx homeobox genes. Development. 2002; 129:4371–4386. [PubMed: 12223397]
7. Quinn LM, Johnson BV, Nicholl JB, et al. Isolation and identification of homeobox genes from the human placenta including a novel member of the Distal-less family, DLX4. Gene. 1997; 187:55–61. [PubMed: 9073066]
8. Fu S, Stevenson H, Strovel JW, et al. Distinct functions of two isoforms of a homeobox gene, BP1 and DLX7, in the regulation of the beta-globin gene. Gene. 2001; 278:131–139. [PubMed: 11707330]

9. Hung YC, Ueda M, Terai Y, et al. Homeobox gene expression and mutation in cervical carcinoma cells. Cancer Sci. 2003; 94:437–441. [PubMed: 12824890]
10. Song Y, Dang C, Fu Y, et al. Genome-wide analysis of BP1 transcriptional targets in breast cancer cell line Hs578T. Int J Biol Sci. 2009; 5:1–12. [PubMed: 19119308]
11. Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. Nat Rev Cancer. 2010; 10:361–371. [PubMed: 20357775]
12. Trinh BQ, Barengo N, Naora H. Homeodomain protein DLX4 counteracts key transcriptional control mechanisms of the TGF-beta cytostatic program and blocks the antiproliferative effect of TGF-beta. Oncogene. 2011; 30:2718–2729. [PubMed: 21297662]
13. Chase MB, Fu S, Haga SB, et al. BP1, a homeodomain-containing isoform of DLX4, represses the beta-globin gene. Mol Cell Biol. 2002; 22:2505–2514. [PubMed: 11909945]
14. Haga SB, Fu S, Karp JE, et al. BP1, a new homeobox gene, is frequently expressed in acute leukemias. Leukemia. 2000; 14:1867–1875. [PubMed: 11069021]
15. Neufing PJ, Kalionis B, Horsfall DJ, et al. Expression and localization of homeodomain proteins DLX4/HB9 in normal and malignant human breast tissues. Anticancer Res. 2003; 23:1479–1488. [PubMed: 12820413]
16. Hara F, Samuel S, Liu J, et al. A homeobox gene related to Drosophila distal-less promotes ovarian tumorigenicity by inducing expression of vascular endothelial growth factor and fibroblast growth factor-2. Am J Pathol. 2007; 170:1594–1606. [PubMed: 17456765]
17. Tomida S, Yanagisawa K, Koshikawa K, et al. Identification of a metastasis signature and the DLX4 homeobox protein as a regulator of metastasis by combined transcriptome approach. Oncogene. 2007; 26:4600–4608. [PubMed: 17260014]
18. Schwartz AM, Man YG, Rezaei MK, et al. BP1, a homeoprotein, is significantly expressed in prostate adenocarcinoma and is concordant with prostatic intraepithelial neoplasia. Mod Pathol. 2009; 22:1–6. [PubMed: 18931648]
19. Fu SW, Schwartz A, Stevenson H, et al. Correlation of expression of BP1, a homeobox gene, with estrogen receptor status in breast cancer. Breast Cancer Res. 2003; 5:R82–R87. [PubMed: 12817998]
20. Man YG, Fu SW, Schwartz A, et al. Expression of BP1, a novel homeobox gene, correlates with breast cancer progression and invasion. Breast Cancer Res Treat. 2005; 90:241–247. [PubMed: 15830137]
21. Yu M, Yang Y, Shi Y, et al. Expression level of beta protein 1 mRNA in Chinese breast cancer patients: a potential molecular marker for poor prognosis. Cancer Sci. 2008; 99:173–178. [PubMed: 17999690]
22. Tang YC, Amon A. Gene copy-number alterations: a cost-benefit analysis. Cell. 2013; 152:394–405. [PubMed: 23374337]
23. Cavalli LR, Man YG, Schwartz AM, et al. Amplification of the BP1 homeobox gene in breast cancer. Cancer Genet Cytogenet. 2008; 187:19–24. [PubMed: 18992636]
24. Man YG, Schwartz A, Levine PH, et al. BP1, a putative signature marker for inflammatory breast cancer and tumor aggressiveness. Cancer Biomark. 2009; 5:9–17. [PubMed: 19242057]
25. Fu Y, Lian Y, Kim KS, et al. BP1 homeoprotein enhances metastatic potential in ER-negative breast cancer. J Cancer. 2010; 1:54–62. [PubMed: 20842225]
26. Fu J, Qin L, He T, et al. The TWIST/Mi2/NuRD protein complex and its essential role in cancer metastasis. Cell Res. 2011; 21:275–289. [PubMed: 20714342]
27. Zhang L, Yang M, Gan L, et al. DLX4 upregulates TWIST and enhances tumor migration, invasion and metastasis. Int J Biol Sci. 2012; 8:1178–1187. [PubMed: 23091415]
28. Stevenson HS, Fu SW, Pinzone JJ, et al. BP1 transcriptionally activates bcl-2 and inhibits TNFalpha-induced cell death in MCF7 breast cancer cells. Breast Cancer Res. 2007; 9:R60. [PubMed: 17854498]
29. Trinh BQ, Ko SY, Barengo N, et al. Dual functions of the homeoprotein DLX4 in modulating responsiveness of tumor cells to topoisomerase II-targeting drugs. Cancer Res. 2013; 73:1000–1010. [PubMed: 23222298]

30. Urban, CA. Master's thesis. Curitiba, Brazil: Federal University of Parana; 2001. Efficacy of the association of lymphoscintigraphy-gama-camera and blue dye in the identification of the sentinel lymph node in breast cancer.
31. Compton, CC.Byrd, DR.Garcia-Aguilar, J.Kurtzman, SH.Olawaiye, A., Washington, MK., editors. AJCC cancer staging atlas A companion to the seventh editions of the AJCC Cancer Staging Manual and Handbook. 2nd. New York: Springer; 2013.
32. Goldhirsch A, Wood WC, Coates AS, et al. Strategies for sub-types–dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer. Ann Oncol. 2011; 22:1736–1747. [PubMed: 21709140]
33. Cavalli LR, Urban CA, Dai D, et al. Genetic and epigenetic alterations in sentinel lymph nodes metastatic lesions compared to their corresponding primary breast tumors. Cancer Genet Cytogenet. 2003; 146:33–40. [PubMed: 14499694]
34. Torresan, C., Santos, SC., Camps, J., et al. Genomic profiling of sentinel lymph node breast cancer metastasis. Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; Apr. 17–21 2010; Philadelphia: AACR. 2010. Abstract nr 327
35. Torresan, C., Santos, SC., Camps, J., et al. Molecular characterization of breast tumor cells in sentinel lymph nodes. Proceedings of the American Society of Clinical Oncology (ASCO) - The 2010 Breast Cancer Symposium; Oct. 1–3, 2010; Washington, DC: ASCO. 2010. Abstract nr 15
36. Cohen, J. Statistical power analysis for the behavioral sciences. second. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
37. Kenny David, A. The two group design. In: Kenny, D., editor. Statistics for the social and behavioral sciences. Boston: Little, Brown; 1987. p. 215
38. Welch HG, Schwartz LM, Woloshin S. Are increasing 5-year survival rates evidence of success against cancer? JAMA. 2000; 283:2975–2978. [PubMed: 10865276]
39. Cavalli LR. Molecular markers of breast axillary lymph node metastasis. Expert Rev Mol Diagn. 2009; 9:441–454. [PubMed: 19580429]
40. Cavalli LR, Ellsworth RE, Klein C, et al. Breast axillary lymph node metastasis. Int J Breast Cancer. 2011:304697. [PubMed: 22482057]
41. Yoshihara E, Smeets A, Laenen A, et al. Predictors of axillary lymph node metastases in early breast cancer and their applicability in clinical practice. Breast. 2013; 22:357–361. [PubMed: 23022046]
42. Espinosa E, Gámez-Pozo A, Sánchez-Navarro I, et al. The present and future of gene profiling in breast cancer. Cancer Metastasis Rev. 2012; 31:41–46. [PubMed: 22124734]
43. Howard JH, Bland KI. Current management and treatment strategies for breast cancer. Curr Opin Obstet Gynecol. 2012; 24:44–48. [PubMed: 22123219]
44. van der Leij F, Elkhuizen PH, Bartelink H, et al. Predictive factors for local recurrence in breast cancer. Semin Radiat Oncol. 2012; 22:100–107. [PubMed: 22385917]
45. Bassi KK, Seenu V, Srivastava A, et al. Role of axillary sampling in the era of sentinel lymph node biopsy: a critical review. Indian J Cancer. 2012; 49:66–73. [PubMed: 22842171]
46. Vidal-Sicart S, Valdés Olmos R. Sentinel node mapping for breast cancer: current situation. J Oncology. 2012; 2012:361341.
47. Nguyen DX, Massagué J. Genetic determinants of cancer metastasis. Nat Rev Genet. 2007; 8:341–352. [PubMed: 17440531]
48. Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer. 2009; 9:302–312. [PubMed: 19308069]
49. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta. 2010; 1805:105–117. [PubMed: 19931353]
50. Comen EA. Tracking the seed and tending the soil: evolving concepts in metastatic breast cancer. Discov Med. 2012; 14:97–104. [PubMed: 22935206]
51. Stoecklein NH, Klein CA. Genetic disparity between primary tumours, disseminated tumour cells, and manifest metastasis. Int J Cancer. 2010; 126:589–598. [PubMed: 19795462]

52. Suzuki M, Tarin D. Gene expression profiling of human lymph node metastases and matched primary breast carcinomas: clinical implications. Mol Oncol. 2007; 1:172–180. [PubMed: 19383293]
53. Santos SC, Cavalli IJ, Ribeiro EM, et al. Patterns of DNA copy number changes in sentinel lymph node breast cancer metastases. Cytogenet Genome Res. 2008; 122:16–21. [PubMed: 18931481]
54. Li J, Gromov P, Gromova I, et al. Omics-based profiling of carcinoma of the breast and matched regional lymph node metastasis. Proteomics. 2008; 8:5038–5052. [PubMed: 19003862]
55. Ellsworth RE, Field LA, Love B, et al. Differential gene expression in primary breast tumors associated with lymph node metastasis. Int J Breast Cancer. 2011:142763. [PubMed: 22295210]
56. Pentheroudakis G, Spector Y, Krikelis D, et al. Global microRNA profiling in favorable prognosis subgroups of cancer of unknown primary (CUP) demonstrates no significant expression differences with metastases of matched known primary tumors. Clin Exp Metastasis. 2013; 30:431–439. [PubMed: 23124598]
57. Ferrari N, Palmisano GL, Paleari L, et al. DLX genes as targets of ALL-1: DLX 2,3,4 down-regulation in t(4;11) acute lymphoblastic leukemias. J Leukoc Biol. 2003; 74:302–305. [PubMed: 12885948]
58. Tan Y, Timakhov RA, Rao M, et al. A novel recurrent chromosomal inversion implicates the homeobox gene Dlx5 in T-cell lymphomas from Lck-Akt2 transgenic mice. Cancer Res. 2008; 68:1296–1302. [PubMed: 18316591]
59. Xu J, Testa JR. DLX5 (distal-less homeobox 5) promotes tumor cell proliferation by transcriptionally regulating MYC. J Biol Chem. 2009; 284:20593–20601. [PubMed: 19497851]
60. Pedersen N, Mortensen S, Sørensen SB, et al. Transcriptional gene expression profiling of small cell lung cancer cells. Cancer Res. 2003; 63:1943–1953. [PubMed: 12702587]
61. Maxwell GL, Chandramouli GV, Dainty L, et al. Microarray analysis of endometrial carcinomas and mixed mullerian tumors reveals distinct gene expression profiles associated with different histologic types of uterine cancer. Clin Cancer Res. 2005; 11:4056–4066. [PubMed: 15930340]
62. Tan Y, Cheung M, Pei J, et al. Upregulation of DLX5 promotes ovarian cancer cell proliferation by enhancing IRS-2-AKT signaling. Cancer Res. 2010; 70:9197–9206. [PubMed: 21045156]
63. Morini M, Astigiano S, Gitton Y, et al. Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231. BMC Cancer. 2010; 10:649. [PubMed: 21108812]
64. Sun M, Song CX, Huang H, et al. HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis. Proc Natl Acad Sci U S A. 2013; 110:9920–9925. [PubMed: 23716660]
65. Chen H, Lee JS, Liang X, et al. Hoxb7 inhibits transgenic HER-2/neu-induced mouse mammary tumor onset but promotes progression and lung metastasis. Cancer Res. 2008; 68:3637–3644. [PubMed: 18463397]
66. Quinn LM, Kilpatrick LM, Latham SE, et al. Homeobox genes DLX4 and HB24 are expressed in regions of epithelial-mesenchymal cell interaction in the adult human endometrium. Mol Hum Reprod. 1998; 4:497–501. [PubMed: 9665637]
67. Quinn LM, Latham SE, Kalionis B. A distal-less class homeobox gene, DLX4, is a candidate for regulating epithelial-mesenchymal cell interactions in the human placenta. Placenta. 1998; 19:87–93. [PubMed: 9481790]
68. Wu X, Chen H, Parker B, et al. HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition. Cancer Res. 2006; 66:9527–9534. [PubMed: 17018609]
69. Yuan W, Zhang X, Xu Y, et al. Role of HOXB7 in regulation of progression and metastasis of human lung adenocarcinoma. Mol Carcinog. 2012; 53:49–57. [PubMed: 22911672]
70. Nguyen Kovochich A, Arensman M, Lay AR, et al. HOXB7 promotes invasion and predicts survival in pancreatic adenocarcinoma. Cancer. 2013; 119:529–539. [PubMed: 22914903]
71. Shah N, Jin K, Cruz LA. HOXB13 mediates tamoxifen resistance and invasiveness in human breast cancer by suppressing ERalpha and inducing IL-6 expression. Cancer Res. 2013; 73:5449–5458. [PubMed: 23832664]

72. Luoh SW. Amplification and expression of genes from the 17q11 approximately q12 amplicon in breast cancer cells. Cancer Genet Cytogenet. 2002; 136:43–47. [PubMed: 12165450]

73. Kauraniemi P, Kallioniemi A. Activation of multiple cancer-associated genes at the ERBB2 amplicon in breast cancer. Endocrine Relat Cancer. 2006; 13:39–49.

74. Staaf J, Jönsson G, Ringnér M, et al. High-resolution genomic and expression analyses of copy number alterations in HER2-amplified breast cancer. Breast Cancer Res. 2010; 12:R25. [PubMed: 20459607]

75. Jacot W, Fiche M, Zaman K, et al. The HER2 amplicon in breast cancer: topoisomerase IIA and beyond. Biochim Biophys Acta. 2013; 1836:146–157. [PubMed: 23628726]

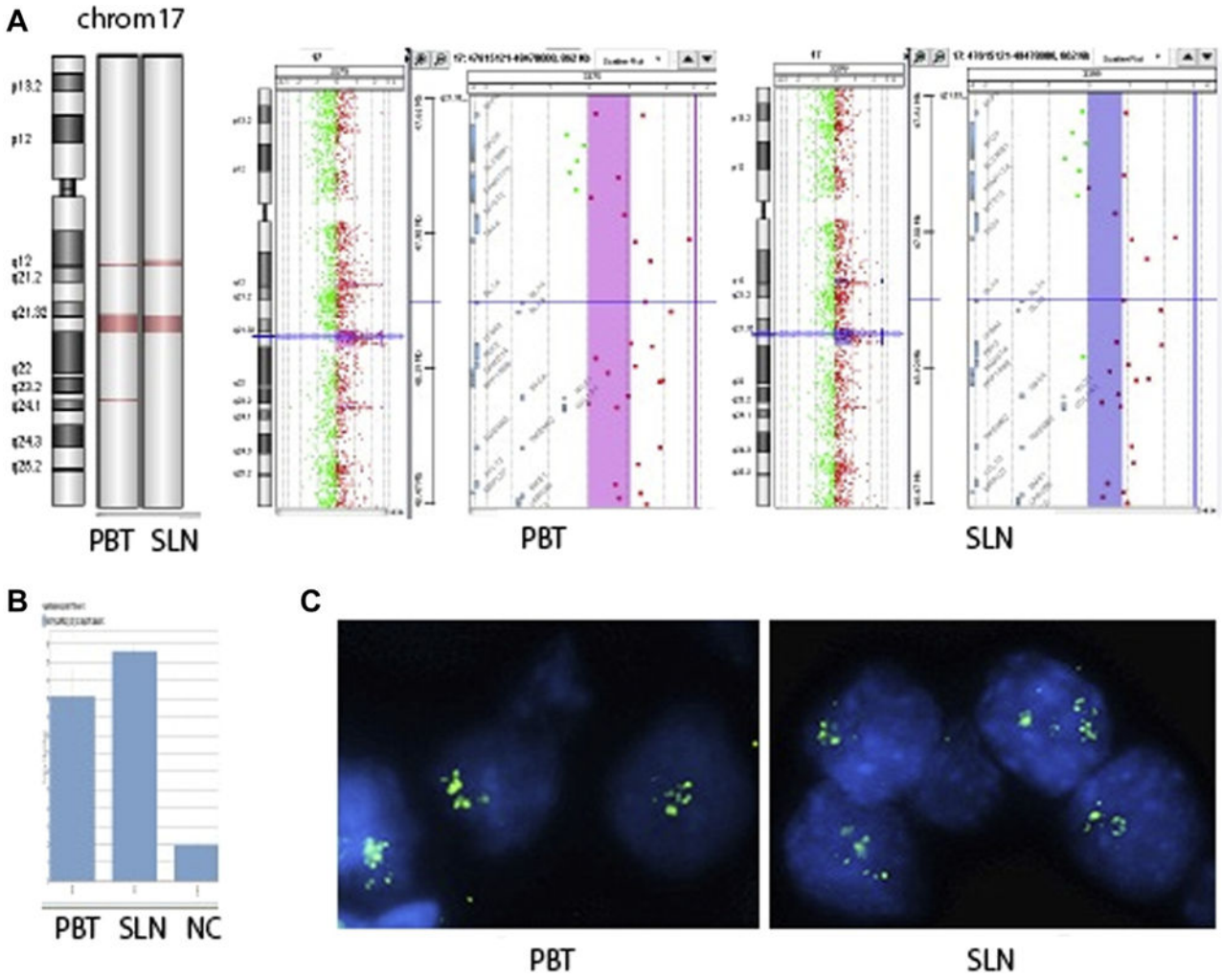

Figure 1.

Increased copy number of the *DLX4* gene demonstrated by array-CGH (A), TaqMan Copy Number Assay (B), and FISH (C) analysis in both PBT and SLN metastasis from the same patient (case 22). (A) (left side) Ideogram of chromosome 17 showing three and two amplified regions in 17q in the PBT and SLN metastasis, respectively. (A) (right side) A detailed image of the region 17q21, showing amplification of the *DLX4* gene by array-CGH analysis, in both PBT and SLN metastasis (blue bars). (B) TaqMan Copy Number Assays in the same case showing high level of *DLX4* amplification in both PBT and SLN metastasis when compared to the normal control (NC). (C) Representative interphase nuclei from this case showing multiple signals of the *DLX4* probe in the two lesions analyzed (green signals).

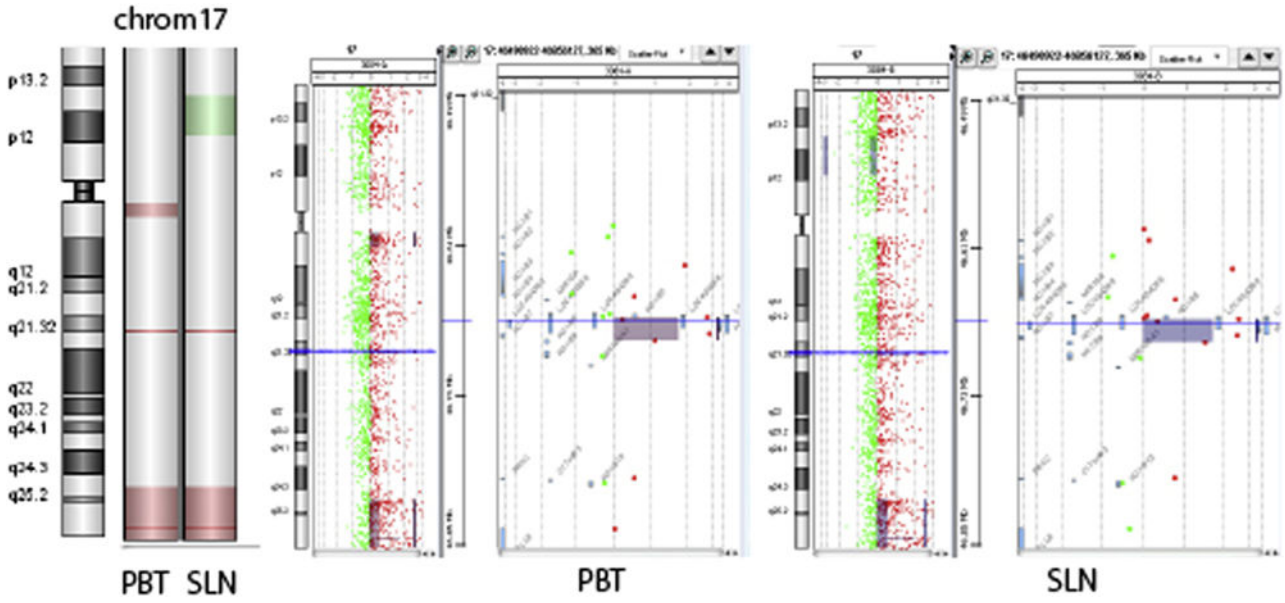

Figure 2.

Array-CGH analysis of the paired lesions of case 17. Ideogram of chromosome 17 showing amplification of three and two regions on 17q on the PBT and SLN metastasis, respectively, and one region of loss on 17p in the SLN metastasis (left side). A detailed image of the region 17q21.32, showing amplification of the *HOXB* genes cluster in the lesions analyzed (right side, blue bars).

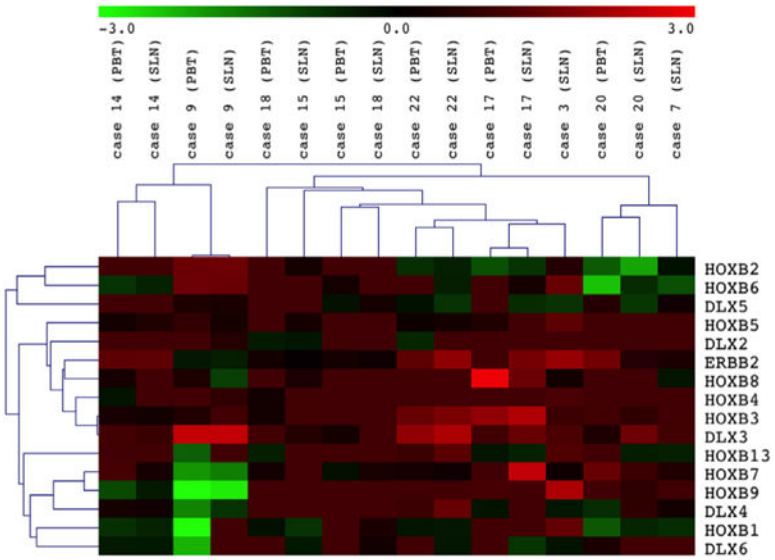

Figure 3.

Heat map of the UHC of samples and genes based on DNA copy number values. Red color represents $\log_2$ values above median; green color represents $\log_2$ values below median; and black corresponds to median $\log_2$ values.

Table 1

Clinical characteristics and follow-up information for the group of patients studied

Patient no.	Age (y)	Tumor size (cm)	Pathology	Tumor grade	ER	PR	HER2	Year of surgery	Years of DFS	Recurrence	Status
1	56	3.5	ILC	NA	POS	POS	POS	2005	8	No	NED
2	58	1.0	IDC	II	POS	NA	NEG	2000	NA	NA	NA
3	73	3.0	IDC	II	POS	POS	POS	2005	5	No	NED
4	59	2.0	IDC	I	POS	POS	NEG	2003	10	No	NED
5	37	2.0	IDC	II	POS	POS	NEG	2002	11	No	NED
6	59	2.0	IDC	II	POS	POS	POS	2004	2	No	NA
7	48	0.7	ILC	II	POS	POS	NEG	2000	13	No	NA
8	44	1.2	IDC	II	POS	POS	NEG	2001	12	No	NED
9	54	2.9	IDC	II	POS	POS	NEG	2001	12	No	NED
10	56	1.5	IDC	I	POS	POS	NEG	2005	8	No	NED
11	42	5.5	IDC	II	POS	NEG	NEG	2002	11	No	NED
12	75	2.5	IDC	II	POS	NEG	NEG	2000	9	No	NA
13	34	2.8	IDC	II	NA	NA	NEG	2001	5	No	NA
14	48	5.5	IDC	II	POS	POS	NEG	2000	13	No	NED
15	45	4.5	ILC	II	POS	POS	NEG	2008	5	Breast, liver	AWD
16	45	1.5	IDC	II	POS	POS	NEG	2007	6	No	NED
17	53	1.8	IDC	II	POS	NEG	NEG	2004	9	No	NED
18	61	8.0	IDC	III	POS	POS	NEG	2008	1	Endometrium	Death
19	34	2.0	IDC	I	NEG	POS	NEG	2004	5	No	NA
20	55	3.0	IDC	II	NA	NA	NEG	2006	NA	NA	NA
21	69	1.8	IDC	NA	NA	NA	NEG	2007	6	No	NED
22	58	2.0	IDC	III	NA	NA	POS	2006	4	No	NA
23	54	5.5	IDC	II	NA	NA	POS	2008	5	No	NED
24	35	3.2	IDC	II	POS	POS	NEG	2006	7	No	NED
25	55	2.5	ILC	NA	NA	NA	NEG	2007	2	Bone	Death
26	67	1.4	IDC	II	POS	POS	NEG	2007	6	No	NED
27	65	1.3	IDC	II	POS	POS	NEG	2007	5	No	NED
28	66	0.8	IDC	III	POS	POS	NEG	2004	9	No	NED

Patient no.	Age (y)	Tumor size (cm)	Pathology	Tumor grade	ER	PR	HER2	Year of surgery	Years of DFS	Recurrence	Status
29	60	1.0	IDC	II	NA	NA	NEG	2000	NA	NA	NA
30	53	8.0	ILC	II	POS	POS	NEG	2006	7	Liver, bone	AWD
31	64	5.5	IDC	II	POS	POS	NEG	2000	4	Lung	Death
32	59	2.0	IDC	II	NEG	NEG	NEG	2005	8	No	NED
33	47	4.5	IDC	II	POS	NA	NEG	2001	6.5	Bone	Death
34	57	3.0	IDC	I	POS	POS	NEG	2008	NA	NA	NA
35	48	3.0	IDC	II	POS	POS	NEG	2005	7	No	NED
36	54	1.5	IDC	II	NEG	NEG	POS	2005	7	Breast	AWD
37	47	5.0	ILC	II	POS	POS	POS	2004	9	No	NED

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; DFS, disease-free survival; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; POS, positive; NEG, negative; NED, no evidence of disease; AWD, alive with disease; NA, not available (lost follow-up).

Table 2

Summary of *DLX4* copy number changes observed in the 37 pairs of PBT and corresponding SLN metastases analyzed

		DNA copy number changes	
Patient no.	**Lesion**	***DLX4***	***HER2/NEU***
1	PBT	Normal	Normal
	SLN	Normal	Normal
2	PBT	Amplification	Amplification
	SLN	Amplification	Amplification
3	PBT	Normal	Normal
	SLN	Gain	Gain
4	PBT	Gain	Normal
	SLN	Normal	Normal
5	PBT	Normal	Normal
	SLN	Normal	Normal
6	PBT	Amplification	Amplification
	SLN	Amplification	Amplification
7	PBT	Normal	Normal
	SLN	Normal	Normal
8	PBT	Normal	Normal
	SLN	Normal	Normal
9	PBT	Gain	Gain
	SLN	Normal	Normal
10	PBT	Normal	Normal
	SLN	Normal	Normal
11	PBT	Amplification	Normal
	SLN	Amplification	Normal
12	PBT	Normal	Normal
	SLN	Normal	Normal
13	PBT	Normal	Normal
	SLN	Normal	Normal
14	PBT	Normal	Normal
	SLN	Normal	Normal
15	PBT	Normal	Normal
	SLN	Normal	Normal
16	PBT	Gain	Normal
	SLN	Normal	Gain
17	PBT	Normal	Normal
	SLN	Normal	Normal
18	PBT	Normal	Normal
	SLN	Normal	Normal
19	PBT	Normal	Normal

		DNA copy number changes	
Patient no.	**Lesion**	***DLX4***	***HER2/NEU***
	SLN	Gain	Gain
20	PBT	Normal	Normal
	SLN	Gain	Gain
21	PBT	Normal	Normal
	SLN	Normal	Gain
22	PBT	Amplification	Amplification
	SLN	Amplification	Amplification
23	PBT	Amplification	Amplification
	SLN	Amplification	Amplification
24	PBT	Normal	Normal
	SLN	Normal	Normal
25	PBT	Normal	Normal
	SLN	Normal	Normal
26	PBT	Normal	Normal
	SLN	Normal	Normal
27	PBT	Normal	Gain
	SLN	Normal	Normal
28	PBT	Normal	Normal
	SLN	Normal	Normal
29	PBT	Normal	Normal
	SLN	Normal	Normal
30	PBT	Normal	Normal
	SLN	Normal	Normal
31	PBT	Normal	Normal
	SLN	Normal	Normal
32	PBT	Normal	Loss
	SLN	Normal	Normal
33	PBT	Loss	Loss
	SLN	Gain	Normal
34	PBT	Normal	Normal
	SLN	Normal	Normal
35	PBT	Loss	Normal
	SLN	Loss	Loss
36	PBT	Normal	Loss
	SLN	Normal	Normal
37	PBT	Loss	Loss
	SLN	Loss	Loss