Analysis of molecular markers as predictive factors of lymph node involvement in breast carcinoma

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Abstract. Nodal status is the most significant independent prognostic factor in breast cancer. Identification of molecular markers would allow stratification of patients who require surgical assessment of lymph nodes from the large numbers of patients for whom this surgical procedure is unnecessary, thus leading to a more accurate prognosis. However, up to now, the reported studies are preliminary and controversial, and although hundreds of markers have been assessed, few of them have been used in clinical practice for treatment or prognosis in breast cancer. The purpose of the present study was to determine whether protein phosphatase Mg2+/Mn2+ dependent 1D, β-1,3-N-acetylglucosaminyltransferase, neural precursor cell expressed, developmentally down-regulated 9, prohibitin, phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5), phosphatidylinositol-5-phosphate 4-kinase type IIα, TRF1-interacting ankyrin-related ADP-ribose polymerase 2, BCL2 associated agonist of cell death, G2 and S-phase expressed 1 and PAX interacting protein 1 genes, described as prognostic markers in breast cancer in a previous microarray study, are also predictors of lymph node involvement in breast carcinoma Reverse transcription-quantitative polymerase chain reaction analysis was performed on primary breast tumor tissues from women with negative lymph node involvement (n=27) compared with primary tumor tissues from women with positive lymph node involvement (n=23), and was also performed on primary tumors and paired lymph node metastases (n=11). For all genes analyzed, only the PIK3R5 gene exhibited differential expression in samples of primary tumors with positive lymph node involvement compared with primary tumors with negative lymph node involvement (P=0.0347). These results demonstrate that the PIK3R5 gene may be considered predictive of lymph node involvement in breast carcinoma. Although the other genes evaluated in the present study have been previously characterized to be involved with the development of distant metastases, they did not have predictive potential.

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Introduction

Breast cancer is the fifth most common cause of cancer-related mortalities and the second most common form of non-skin-associated cancer worldwide (1). At present, the most important prognostic factors used to guide decisions regarding adjuvant systemic treatment are tumor size, nodal status, tumor clinical stage, hormone receptor [progesterone receptor (PGR) and estrogen receptor (ER)], human epidermal growth factor receptor 2 (HER2) status (2), and histological grade. Various other clinicopathological factors, including proliferation index and novel molecular markers, have been investigated to improve the prediction of clinical outcome (3,4). Despite improvements in risk stratification, the current prognostic factors exhibit moderate accuracy in classifying breast tumors according to their clinical behaviors. In breast cancer, axillary lymph nodes are typically the initial site of metastasis (5). The presence of lymph node metastasis predicts the development of distant metastases and is considered one of the most informative prognostic factors when evaluating patients with breast cancer (6-8).

Several studies have identified correlations between clinicopathological parameters of patients with breast cancer and a high risk of developing lymph node metastases. Among

the parameters most significantly correlated with lymph node involvement are histological grade, tumor size and age (9,10). Furthermore, number and proportion of evaluated sentinel lymph node biopsies (SLNBs) have been correlated with metastases in the axillary lymph nodes (11-13). An SLN is defined as one of the first nodes to collect lymphatic fluid from a malignant tumor and malignant cells (1). The result of the SLNB indicates whether complete axillary lymph node dissection (ALND) should be performed (14), which allows complete evaluation of lymph nodes. However, ALND is associated with significant morbidity and is not associated with a significant increase in patient survival (15). Prognostic and predictive tools are required to more accurately select patients for lymph node dissection and spare large numbers of patients undergoing this procedure when it is not necessary.

The advent of polymerase chain reaction (PCR)-based diagnostic methods, in particular reverse transcription-quantitative PCR (RT-qPCR), made the detection of SLN metastasis (16) and axillary lymph node involvement in breast cancer possible, which resulted in the identification of several molecular markers with significant potential prognosis in relation to risk of axillary and systemic metastases (17-20). The purpose of the present study was to determine whether genes that had been previously described as prognostic markers in breast cancer (21) are also predictors of lymph node involvement in breast carcinoma. The expression of 10 genes [protein phosphatase Mg2+/Mn2+ dependent 1D (PPM1D), β-1,3-N-acetylglucosaminyltransferase (B3GNT7), neural precursor cell expressed developmentally down-regulated 9 (NEDD9), prohibitin (PHB), phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5), phosphatidylinositol-5-phosphate 4-kinase type IIα (PIP4K2A), TRF1-interacting ankyrin-related ADP-ribose polymerase 2 (TNKS2), BCL2 associated agonist of cell death (BAD), G2 and S-phase expressed 1 (GTSE1) and PAX interacting protein 1 (PAXIP1)] was analyzed by RT-qPCR in the primary tumor tissues of patients with and without lymph node involvement, in addition to primary tumors and lymph node metastases of the same patients in order to determine whether these genes have predictive power in relation to risk of axillary metastases.

Materials and methods

Samples and patients. A total of 50 primary tumor samples were collected from 49 patients that underwent segmental resection or mastectomy (1 patient presented with bilateral tumors); 41 samples were obtained from Hospital São Francisco de Assis (Jacareí, Brazil) and 9 were from other hospitals, including Hospital Antoninho da Rocha Marmo, Santos Dumont Hospital and Hospital Pio XII (São José dos Campos, Brazil). Frozen primary breast tumor tissues were collected from women with negative (n=27) and positive (n=23) lymph node status, and, of the positive lymph node cases, primary breast tumors and paired lymph node metastases tissues were acquired from 11 patients. Written informed consent was obtained from all patients during the collection period, and the study was reviewed and approved by the Research Ethics Committee of the University of Taubaté (Taubaté, Brazil) (CEP 554/11). Table I provides a summary of the clinicopathological data from the 50 tumor samples from 49 patients with breast carcinoma related to the status of axillary lymph nodes.

Immediately after surgery, tumor tissue samples were frozen and stored at -80˚C. To ensure consistency, diagnosis of every specimen was made by a single breast pathologist (Center for Diagnostic Medicine, Pathology and Cytology, São José dos Campos, Brazil). Whenever necessary, tissue samples were macrodissected with a scalpel to guarantee that only sections comprised of ≥90% tumor cells were used for RNA isolation and subsequent gene expression analysis. A total of 5 non-tumor breast tissue samples from patients undergoing mammary reduction, which had been histopathologically confirmed as healthy, were used as controls. Histopathological classification was performed according to the International Classification of Disease for Oncology from the World Health Organization (22), and the clinical stage was determined according to the Union for International Cancer Control Tumor-Node-Metastasis (23) classification. The malignancy of carcinoma infiltration was scored according to the Bloom and Richardson grading system (24).

Total RNA isolation, quantification and synthesis of cDNA. Total RNA was extracted from 50 macrodissected primary tumor samples, 11 lymph nodes and 5 healthy breast tissues using the RNeasy® Lipid Tissue Mini kit (Qiagen, São Paulo, Brazil) according to the manufacturer's protocol. RNA samples were purified with 0.1% acetate-ethanol, resuspended in RNase-free ultra-pure water and stored at -80˚C until use. RNA quality was analyzed by 1% agarose gel electrophoresis, and the concentration and quality was measured using a NanoDrop-1000 spectrophotometer v.3.7 (Labtrade, Sao Paulo, Brazil). To avoid DNA contamination, RNA samples were treated with the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol.

cDNA synthesis reactions were carried out in a Peltier Thermal Cycler (MJ-96G; Biocycle Co., Ltd, Hangzhou, China). Total RNA (1 ug) from each sample was reverse transcribed in a 20 μl final volume containing 10 μl 2x first-strand buffer (10 mm $mgcl_2$, and 1 mmde per dntp), 0.5 μl Oligo $(dT)_{20}$, 0.5 μl random primers, 1 μl annealing buffer and 2 μl SuperScript™ III Reverse Transcriptase enzyme (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT was carried out for 50 min at 50˚C, 10 min at 25˚C, followed by 50 min at 50˚C. The reaction mixture was subsequently inactivated for 5 min at 85˚C.

Evaluation of transcript expression by RT-qPCR. RT-qPCR reactions for the 10 study genes and the reference gene (MRLP19) were carried out in duplicate on an ABI Prism 7000 Sequence Detection system (Thermo Fisher Scientific, Inc.) using Platinum®SYBR® Green qPCR SuperMix-UDG (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a total volume of 10 μl according to manufacturer's protocol. Primers were designed using Primer Express software (v3.0; Applied Biosystems; Thermo Fisher Scientific, Inc.) and the sequences are presented in Table II. In order to avoid amplification of contaminating genomic DNA, the primers were placed at the junction between the two exons or in a different exon. RT was carried out for 1 cycle of 95˚C for 10 min, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. A dissociation curve was

included in all experiments. For all primers, amplification curves were constructed with serial dilutions of healthy breast and breast carcinoma cDNA (100, 20, 4, 0.8 and 0.16 ng/μl). Standard curves of the targets and reference genes demonstrated similar amplification efficiencies (>90%). Quantitative data was analyzed using the Sequence Detection system software (v1.0; Applied Biosystems; Thermo Fisher Scientific, Inc.). The details of the gene-specific RT-qPCR assays are presented in Table II.

Measurements of gene expression were calculated using the relative ΔΔCq method, in which the mean Cq value of each target gene in each sample is subtracted from the mean Cq value of the reference gene (25). The transcripts of housekeeping genes MRLP19, PPIA, and GAPDH, were quantified as previously described (26), and the MRLP19 gene was selected as an endogenous control, which provided increased accuracy and resolution in the quantitation of gene expression data, facilitating the detection of smaller changes in gene expression than otherwise possible. Normalized expression levels of target gene tumor samples were expressed in fold-change relative to their abundance in a pool of non-tumor breast tissue control samples, which was calculated as follows: $2^{-(\Delta Cq \text{ test sample} - \Delta Cq \text{ control sample})}$.

Statistical analysis. Statistical analyses comparing clinicopathological characteristics (including age, histological type, histological grade, ER, PGR, HER2 and Ki-67 status, and tumor stage and size) with the presence or absence of lymph node involvement were performed using IBM SPSS v20.0 and the χ^2 test. P≤0.05 was considered to indicate a statistically significant difference.

For the analysis of RT-qPCR, the median $2^{-\Delta\Delta Cq}$ values for each analyzed group were compared using Fisher's exact test [performed using GraphPad InStat software v5.0 (GraphPad Software, Inc., La Jolla, CA, USA)] to determine whether the relative fold change was significantly different between the two groups. P≤0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics. A total of 50 frozen primary breast tissues were obtained from 49 patients (1 patient presented with bilateral tumors). The average age of the patients at diagnosis was 57 years (range, 53-60 years). According to clinicopathological data, 53% of patients were lymph node-negative and 47% were lymph node-positive, with the average number of metastatic lymph nodes being 6.04 (range, 1-21). The average age at diagnosis did not differ significantly between those with (53 years) and without (60 years) lymph node metastasis.

Patients were chosen based on sample availability opposed to clinical parameters; therefore, the analyzed tumors represent a variety of pathological characteristics and tumor types. The majority of specimens were from patients with invasive ductal metastasis (84%), predominantly size T1 and T2 (90%). The majority of patients had a positive ER (80%), PgR (78%) and HER2 (50%) status. A total of 2 patients were diagnosed with stage-IV breast cancer. A few months after diagnosis, 4 patients developed distant metastasis and 1 succumbed to the disease. None of the evaluated pathological features differed significantly between groups [primary tumors in women with negative and positive lymph node status: Age, P=0.2047; histological type, P=0.2609; histological grade, P=1.0000; ER status, P=0.6889; PGR status, P=0.4411; HER2 status, P=0.2734; Ki-67 status, P=0.4221; tumor stage, P=0.6492; and tumor size, P=1.0000 (Fisher's exact test, P ≤0.05)]. These results indicate that the clinicopathological characteristics in the analyzed samples are not associated with the presence or absence of lymph node metastasis (Table I).

Table I. Clinicopathological data of 50 tumor samples[a] from 49 patients with primary breast carcinoma associated with the status of axillary lymph nodes.

Features	n	Node-positive n (%)	Node-negative n (%)
Age, years	49		
≤50		9 (18)	5 (10)
>50		14 (29)	21 (43)
Histology	50		
Invasive ductal		21 (42)	21 (42)
Others (*in situ*, lobular)		2 (4)	6 (8)
Grade	46[b]		
1+2		13 (28)	14 (30)
3		10 (22)	9 (20)
ER status	35[b]		
Negative		2 (6)	5 (14)
Positive		11 (31)	17 (49)
PGR status	45[b]		
Negative		4 (9)	6 (13)
Positive		9 (20)	26 (58)
HER2 status	32[b]		
HER2-		4 (13)	12 (38)
HER+		8 (25)	8 (25)
Ki67 status, %	27[b]		
>25		4 (15)	5 (19)
≤25		5 (19)	13 (48)
T stage	48[b]		
T1 + T2		19 (40)	24 (50)
T3 + T4		3 (6)	2 (4)
Tumor size, cm	47[b]		
≤2		10 (21)	11 (23)
>2		13 (28)	13 (28)

[a]One patient presented with a bilateral tumor; [b]complete demographical and cancer-associated information was not available for all patients.

Gene expression. In the present study, the expression of 10 genes (PPM1D, B3GNT7, NEDD9, PHB, PIK3R5, PIP4K2A, TNKS2, BAD, GTSE1 and PAXIP1), which were selected from 58 genes in a previous microarray study (21), was assessed by RT-qPCR in 50 samples from 49 patients

Table II. Details of the gene-specific primers used in reverse transcription-quantitative polymerase chain reaction assays.

Gene	Gene name	Function	Size of PCR product, bp	Primer sequences
P*PM1D*	Protein phosphatase, Mg2+/Mn2+ dependent 1D	Regulators of cell stress response pathways	81	Forward: 5'-TTGGAATATGATTCCACCACAAGA-3' Reverse: 5'-CCATGCTCACCCATCAGGTATTT-3'
B3GNT7	β-1,3-N-acetylglucosaminyltransferase	Cell migration, cell cycle and survival	81	Forward: 5'-CCACGTCCCCTTCATTTTCA-3' Reverse: 5'-TGCCGGTCAGCCAGAAATT-3'
NEDD9	Neural precursor cell expressed developmentally down-regulated protein 9	Regulation of invasion, apoptosis and cell cycle	81	Forward: 5'-AGGCCCCTGACTGTAGCAGC-3' Reverse: 5'-CCTTACCCTGTAGGTGGACGTAATC-3'
PHB	Prohibitin	Negative regulator of proliferation and tumor suppressor	81	Forward: 5'-CCAGCATCGGAGAGGACTATGAT-3' Reverse: 5'-CAAAGCGAGCCACCACTGAC-3'
PIK3R5	Phosphoinositide-3-kinase regulatory subunit 5	Cell growth, proliferation and differentiation	79	Forward: 5'-ACGCTACGTGTTGTGGTCTTTG-3' Reverse: 5'-CCAGCCGCCGAAGGTT-3'
PIP4K2A	Phosphatidylinosito l-5-phosphate 4-kinase type IIα	Regulation of secretion, proliferation and differentiation	65	Forward: 5'-GGCCGAAATGCACAACATC-3' Reverse: 5'-GGGTGATCCCATGACATTCC-3'
TNKS2	TRF1-interacting ankyrin-related ADP-ribose polymerase 2	Promotion of increased telomere length	81	Forward: 5'-AAGATACACTCACCGGAGAAAAGAAG-3' Reverse: 5'-GGAGACCCATGAAATAGCATTCG-3'
BAD	BCL2-antagonist of cell death protein	Regulators of programmed cell death	81	Forward: 5'-CTTTAAGAAGGGACTTCCTCGCC-3' Reverse: 5'-AAGACTCGCGTCCAGCTGG-3'
GTSE1	G2 and S phase-expressed protein 1	Regulator of the DNA damage	63	Forward: 5'-CGGAGAAGCCCAAGAAAGAGAT-3' Reverse: 5'-CCTTCTCAGCTGGGATTTTTGT-3'
PAX1P1	PAX transcription activation domain interacting protein 1	Genome stability and progression mitosis	81	Forward: 5'-AATGGCTTATTTGGCAGGTGC-3' Reverse: 5'-CCAGTTGGTTCTTTACAGATGAGGACT-3'
MRPL19	Mitochondrial ribosomal protein L19	Mammalian mitochondrial ribosomal	70	Forward: 5'-CAGAGATCAGGAAGAGGACTTGGA-3' Reverse: 5'-TCTCGACACCTTGTCCTTCGA-3'

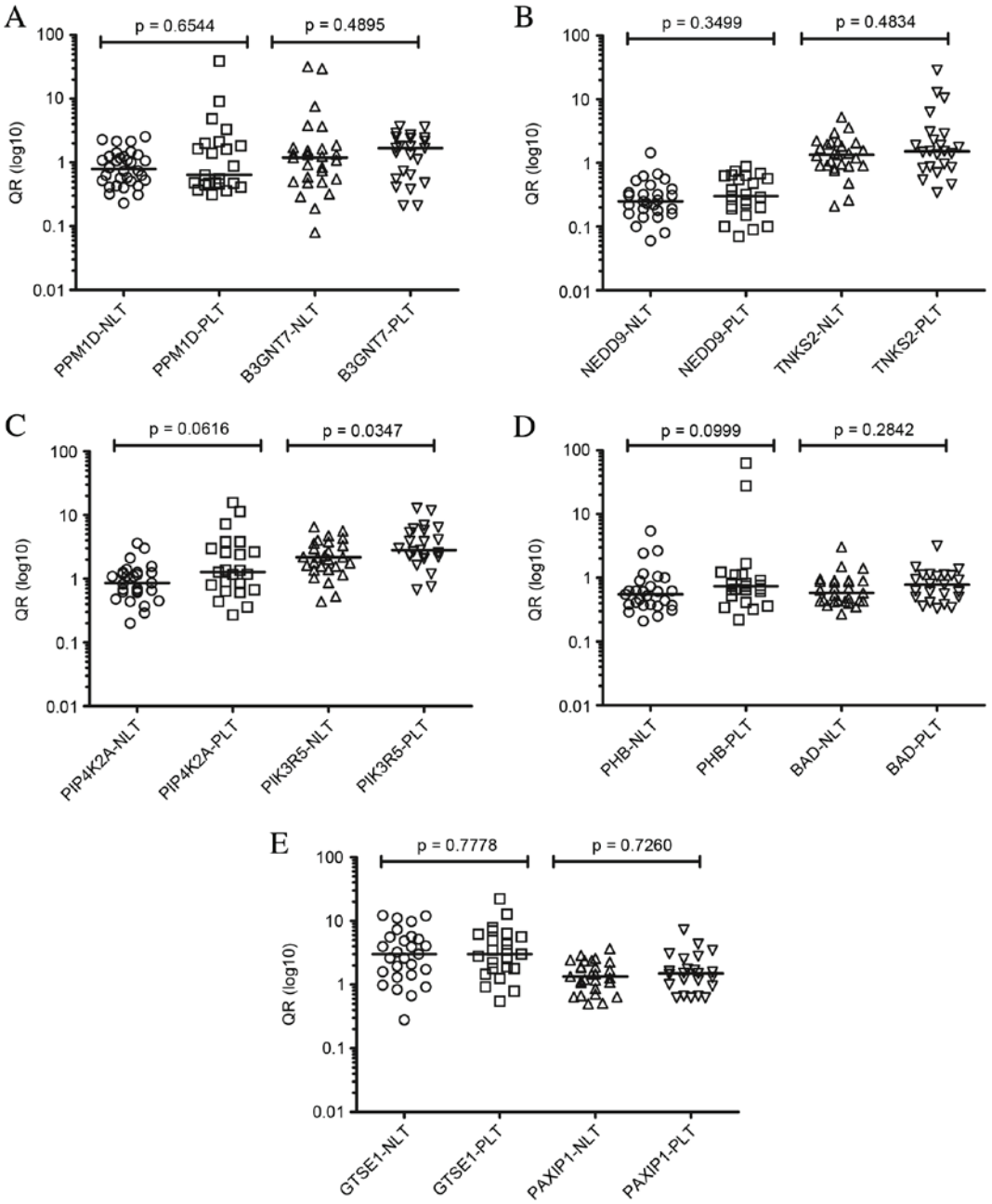

Figure. 1 Comparison between levels of expression by reverse transcription-quantitative polymerase chain reaction of the transcripts (A) PPM1D and B3GNT7, (B) NEDD9 and TNKS2, (C) PIP4K2A and PIK3R5, (D) PHB and BAD and (E) GTSE and PAXIP1 of primary tumors with and without lymph node involvement (analysis 1) with respective P-values. QR, relative quantification; NLT, negative lymph node tumor; PLT, positive lymph node tumor; PPM1D, protein phosphatase Mg2+/Mn2+ dependent 1D; B3GNT7, β-1,3-N-acetylglucosaminyltransferase; NEDD9, neural precursor cell expressed developmentally down-regulated 9; TNKS2, TRF1-interacting ankyrin-related ADP-ribose polymerase 2; PIP4K2A, phosphatidylinositol-5-phosphate 4-kinase type IIα; PIK3R5, phosphoinositide-3-kinase regulatory subunit 5; PHB, prohibitin; BAD, BCL2 associated agonist of cell death; GTSE, G2 and S-phase expressed 1; PAXIP1, PAX interacting protein 1.

with breast cancer with lymph node metastasis in two comparisons: i) Primary tumors without lymph node involvement (n=27) compared with primary tumors with lymph node involvement (n=23) according to the clinicopathological data (analysis 1); ii) and primary tumor (n=11) samples compared with corresponding lymph node metastases (n=11) (analysis 2).

When comparing patients with primary tumors without lymph node involvement (n=27 samples) with patients with lymph node involvement (n=23 samples) (analysis 1), no statistically significant difference was detected for the majority of the genes evaluated. Only the *PIK3R5* gene exhibited increased expression in primary tumor samples with lymph node involvement compared with primary tumors without involvement (P=0.0347). The *PIP4K2A* gene demonstrated a tendency of increased expression in primary tumors with lymph node involvement compared with those without impairment. For all assessed genes, expression in primary tumors (n=11 samples) compared with paired lymph node metastases (n=11 samples) (analysis 2) did not demonstrate any significant differences (P≤0.05). Data from analyses 1 and 2 are presented in Figs. 1 and 2, respectively.

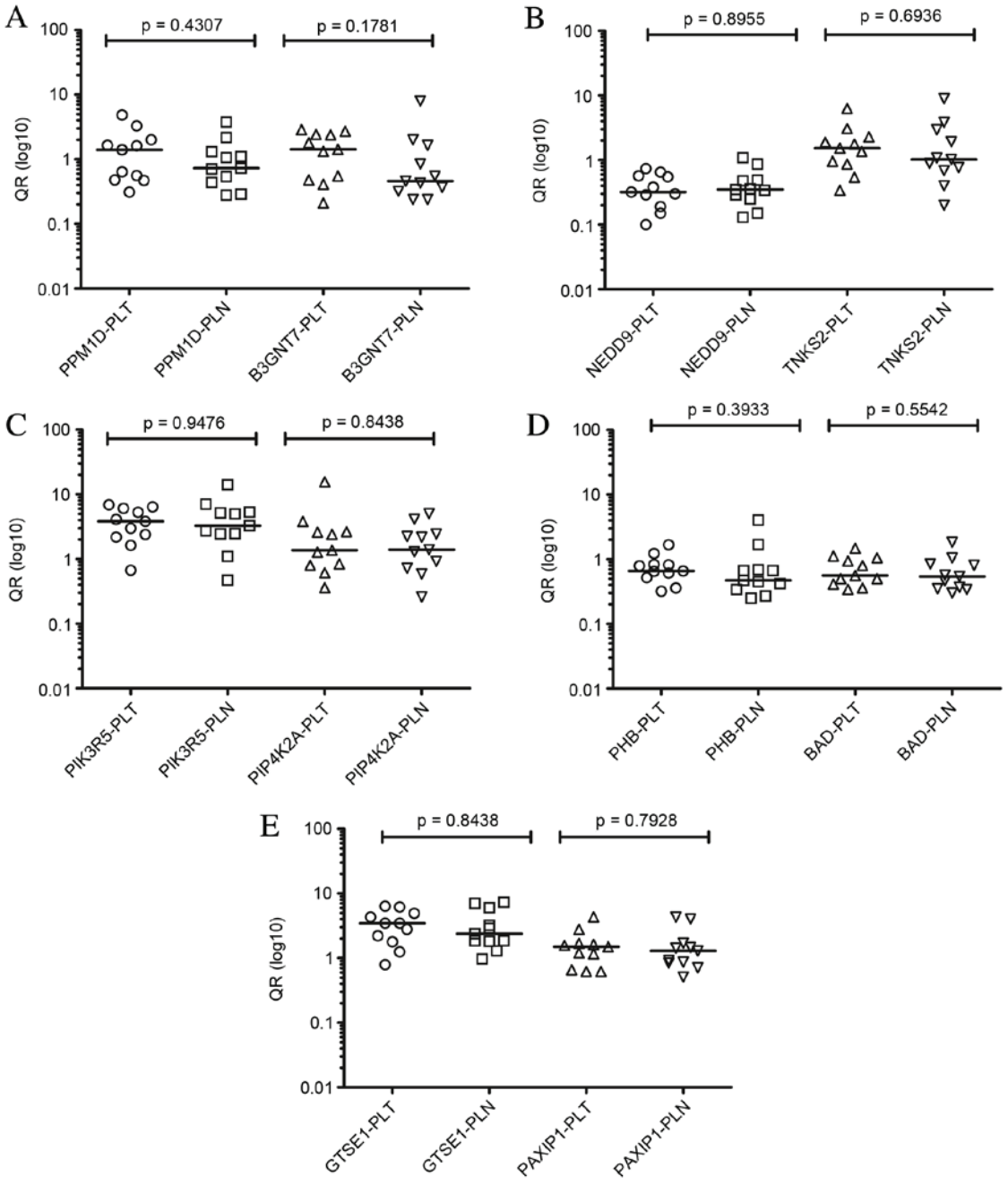

Figure. 2 Comparison between levels of expression by reverse transcription-quantitative polymerase chain reaction of the transcripts (A) PPM1D and B3GNT7, (B) NEDD9 and TNKS2, (C) PIP4K2A and PIK3R5, (D) PHB and BAD and (E) GTSE and PAXIP1 of primary tumors and paired lymph node metastases (analysis 2) with respective P-values. QR, relative quantification; PLT, positive lymph node tumor; PLN, positive lymph node; PPM1D, protein phosphatase Mg2+/Mn2+ dependent 1D; B3GNT7, β-1,3-N-acetylglucosaminyltransferase; NEDD9, neural precursor cell expressed developmentally down-regulated 9; TNKS2, TRF1-interacting ankyrin-related ADP-ribose polymerase 2; PIP4K2A, phosphatidylinositol-5-phosphate 4-kinase type IIα; PIK3R5, phosphoinositide-3-kinase regulatory subunit 5; PHB, prohibitin; BAD, BCL2 associated agonist of cell death; GTSE, G2 and S-phase expressed 1; PAXIP1, PAX interacting protein 1.

Discussion

Attempts have been made to characterize factors that may predict an increasing risk of nodal involvement, which is the most significant independent prognostic factor in breast cancer and remains the most important feature for defining risk category. Identification of genes involved in the stabilization of metastasis in the lymph nodes may increase the understanding of the metastatic process (27). Differentially-expressed genes may represent those involved in the initiation of metastasis, which alter angiogenesis, cell motility and invasion, therefore allowing primary tumor cells with metastatic potential to disseminate (28). Furthermore, determining the status of these genes may provide key information to establish the potential of these markers as predictors of lymph node involvement. Hence, these markers would serve as molecular targets against which novel therapeutics could be developed to prevent the early stages of metastasis. The identification of molecular markers may spare women at low risk of lymph node metastasis from unnecessary surgical procedures, including ALND, and the

ensuing complications of lymph node disruption. In addition, this may allow identification of the 8-10% of node-positive women diagnosed by SLNB as node-negative (29), thus leading to a more accurate prognosis and delineation of a specific treatment for each patient (30). However, based on literature, the data are preliminary and controversial. To date, in spite of assessment of hundreds of markers, few have been used in clinical practice for treatment or prognosis in breast cancer.

To this end, efforts have been made to develop a molecular signature of breast tumors that differs between patients with and without lymph node metastasis. Certain studies have detected a considerable number of genes differentially-expressed in the two groups (31-33). By contrast, a number of research groups have been unable to develop molecular signatures predictive of lymph node metastasis (33-36). Similarly, a previous study failed to detect an effective power in the molecular signature of primary breast tumors associated with lymph node metastasis (27). This study, through evaluation of 41 samples of primary tumors without lymph node involvement and 35 samples with lymph node involvement by microarray analysis, identified only 13 differentially-expressed genes that correctly classified 90% of negative lymph nodes and only 66% of lymph node positive tumors (27). The authors suggested that a single molecular classifier for lymph node metastasis may not exist for several factors, including the paucity of cells within the primary tumor with metastatic potential, tumor heterogeneity, effect of the microenvironment or inherited host susceptibility to metastasis (27).

In the present study, the expression of only 1 gene (PIK3R5) of 10 analyzed was significantly different in analysis 1, with increased expression in samples of primary tumors with lymph node involvement compared with primary tumors without lymph node involvement. This gene serves a role in cell growth, differentiation, proliferation, motility, survival and intracellular transport, and its route is associated with the progression of melanoma (37,38). The *PIK3R5* gene is associated with inhibition of autophagy (promoting tumor growth) (39) and certain authors have suggested that autophagy also works as a cytoprotective mechanism (40). In the current study, the *PIP4K2A* gene exhibited a tendency for increased expression in primary tumors with lymph node involvement compared with those without impairment. This gene is involved in various processes, including cell proliferation, differentiation and motility (41). Myhre *et al* (41) suggested that *PIP4K2A* affects the metastatic process in breast cancer after observing that it was highly expressed in tumors in patients who developed distant metastases compared with patients without metastasis. Increasing the number of samples within a similar study may potentially confirm the predictive value of this gene. Based on these results, the present study observed that of the 10 genes analyzed, only *PIK3R5* may be considered a predictor of lymph node involvement in breast carcinoma, and that although other genes have been characterized to be involved with the development of distant metastasis (21), they did not have predictive potential. The results obtained in the current study are consistent with several studies in the literature that failed to obtain a molecular signature with predictive power (27,35,36,42). Based on these results, it may be concluded that tumor prognosis is independent of the presence or absence of lymph node involvement.

The absence of a signature for lymph node metastasis may be assigned to biological properties of primary tumors, including the nature and number of cells within the primary tumor with metastatic potential. Several studies have reported the presence of small subpopulations of cells with full metastatic potential in localized regions of the primary tumor and that the genetic signatures from these rare cells could be masked by the majority of tumor cells that do not have full metastatic capacity (43,44). These studies do not exclude the ability of the gene expression signatures derived from primary tumors to predict which tumors may metastasize (27,45,46).

In addition, evaluation of gene expression differences between primary breast tumors and matched metastatic lymph nodes should allow genes involved in the metastatic process to be identified. However, in the majority of studies, the status of assessed genes is determined only at the primary tumor, and to the best of our acknowledge, few studies have been published in the literature regarding the evaluation of gene expression, both by microarray and RT-qPCR, which compare primary breast tumors and paired lymph node metastases in breast cancer (47-50).

Contrary to those results (27,43-46), Feng *et al* (47) hypothesized that metastases in the lymph nodes must originate from a fraction of metastatic cells from primary tumors, and genes differentially-expressed between the primary tumor and corresponding axillary metastasis must serve a key role in metastasis in breast cancer. This study performed a microarray analysis of 21,000 well-characterized genes, and 79 genes with differential expression in 14/26 cases analyzed distinguished primary tumors and corresponding lymph node metastasis samples, establishing a pattern of changes in gene expression associated with the metastatic process (47). Despite identifying similarities between primary breast and paired lymph node metastases, Ellsworth *et al* (50) detected 51 genes that were differentially-expressed between these two groups; 13 of these genes with higher expression in lymph node metastasis are largely involved in signal transduction, transcription and immune response. This study detected similar classes of genes involved in the comparison of primary tumor with matched lymph node metastases to those obtained by Feng *et al* (47). However, additional studies observed contradictory results that support a model in which genes involved in changes in extracellular matrix stability are critical to the early metastatic process, while those involved in immune response, signal transduction and proliferation are important for colonization at the secondary site (48,49).

In the present study, the expression analysis of 10 genes from primary tumors and corresponding lymph node metastases (analysis 2) was conducted. The results did not detect significant differences in the expression of any of the evaluated genes in each group. These results are consistent with the hypothesis that only a fraction of cells, which are phenotypically and biologically heterogeneously localized in certain regions of the primary tumor, have higher metastatic potential (27,51). This may explain why changes in expression of specific genes with predictive potential, including *PIK3R5*, cannot be detected in the primary tumor; molecular alterations of these rare cells are able to be masked by cells of the primary tumor that lack a high metastatic capacity. An additional factor

that may explain the inability to detect the differential expression of this gene between primary tumors and corresponding lymph nodes is that it could only be detected if the tumors of the majority of these patients were already involved in the metastatic process. In the current study, only 2/11 patients with positive lymph nodes presented with distant metastases. Presently, for therapeutic purposes, it is assumed that the molecular phenotype of the primary tumor is the same as that for lymph node involvement; however, it should be noted that there is great heterogeneity in tumors, and that, over time, affected lymph nodes may acquire novel biological characteristics and different forms of invasion, blood or lymph, thus leading to failures in treatment (52).

In conclusion, the present study identified that *PIK3R5* exhibited differential expression between node-positive and node-negative primary tumors. This gene serves a role in cell growth, differentiation, proliferation, motility, survival and intracellular transport, and the results of the current study demonstrate that it should be considered as a predictor of lymph node involvement in breast carcinoma. Although the majority of the evaluated genes have been characterized in previous studies as prognostic markers involved in the development of distant metastases, they did not have predictive potential in the present study. Further studies with a larger number of samples are required to confirm these results, and novel molecular markers are necessary to effectively discriminate patients with and without the propensity to develop lymph node metastasis, therefore sparing low-risk women from the morbidities associated with surgical evaluation and reducing the false-negative rate associated with SLNB.

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