

Possible predictive markers related to micro-metastasis in breast cancer patients

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Abstract. Despite significant advances in micro-metastasis detection methods, little is known about the relationship between micro-metastasis and primary tumors. The purpose of this study was to assess the ability of expression of the breast cancer-related markers, HER-2/neu, COX-2, VEGF and PDGF-B, as a predictor for micro-metastasis. As destination sites for micro-metastasis, we examined the peripheral blood (BD), bone marrow (BM) and sentinel lymph node (SLN) from 53 breast cancer patients. Protein and gene expression of the markers at the primary site were determined by immunohistochemistry (IHC) and quantitative RT-PCR. BD and BM samples were processed using magnetic-activated cell separation and immunocytochemistry. SLNs were examined by hematoxylin and eosin (H&E) staining and IHC. The percentages of patients with micro-metastasis were 24.5% in BD, 56.6% in BM, 26.4% in SLN by H&E and 41.5% in SLN by IHC. COX-2 gene amplification was significantly associated with SLN micro-metastasis by H&E ($P=0.03$). Overexpression of HER-2/neu predicts the presence of SLN micro-metastasis as detected by H&E ($P=0.005$) and COX-2 overexpression predicts the presence of micro-metastasis in BM ($P=0.005$) and SLN by H&E ($P<0.001$) and IHC ($P<0.001$). Similarly, PDGF-B overexpression predicts micro-metastasis in BD ($P=0.002$), BM ($P=0.003$) and SLN by H&E ($P=0.017$), whereas VEGF overexpression predicts only the presence of SLN micro-metastasis by IHC ($P=0.001$). Our results indicate the possible value of using these markers to predict the risk of micro-metastasis in breast cancer.

Introduction

Relapse is a recognized consequence of early tumor cell dissemination in breast cancer patients (1-4). The detection

of micro-metastasis has thus become an issue of significant interest. In the last decade, many new immunological and molecular procedures have been developed to detect micro-metastasis throughout the body (5,6). Much work has also been conducted to determine the genetic and phenotypic characteristics of these cells. Markers are still needed which can indicate the presence or absence of micro-metastasis. Four molecules, two metastasis-associated factors in breast cancer (HER-2/neu and COX-2) and two angiogenesis-associated factors (VEGF and PDGF-B) were examined to determine their relationships (if any) to micro-metastatic spread.

HER-2/neu and COX-2 are widely investigated molecules related to metastasis in breast cancer. Human epidermal growth factor receptor-2 (HER-2) is a cell-surface glycoprotein designated the HER-2 or c-erbB-2 receptor that belongs to the tyrosine kinase receptor family. HER-2/neu is amplified and/or its protein is overexpressed in 15-25% of breast cancers (7-10). It has been associated with poor clinical outcome and, possibly, tamoxifen resistance (11-15). Cyclooxygenase-2 (COX-2) is an enzyme responsible for the conversion of arachidonic acid to prostaglandin E₂ (PGE₂) in three steps (16-19). Overexpression is found in 17-68% of invasive breast cancers and similarly predicts poorer survival rates (20,21).

Both PDGF/VEGF belong to the vascular endothelial growth factor family and have been associated with a worse prognosis and metastatic spread (22-24). Vascular endothelial growth factor (VEGF) is a heparin-binding homodimeric glycoprotein which plays crucial stimulatory roles in tumor angiogenesis. Its release is often triggered by the unusual hypoxic conditions found within tumors (25). The family of Platelet-derived growth factor (PDGF) consists of four gene products that form five dimeric isoforms. In particular, PDGF-B is thought to stimulate tumor growth through tumor stromal cells expressing the PDGF β -receptor in a paracrine fashion through its homodimeric isoform, PDGF-BB (26). There is some speculation that it may play a critical role in metastatic spread by helping disseminating cells adjust to the micro-environments of distant sites (27).

Quantitative reverse transcription PCR (QRT-PCR) is a relatively new modality used to precisely quantify gene expression levels within cells (28). The use of fluorescence probes allows for precise quantification of the amount of PCR product which is then compared to the quantification levels of a housekeeping gene such as β -actin (29). In order to determine the expression profile at both mRNA level and

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protein level, we combined this with immunohistochemistry (IHC) to assess association of expression of these markers in primary breast cancer tumors with micro-metastasis in the blood (BD), bone marrow (BM) and sentinel lymph nodes (SLNs). The primary end-point was to determine whether the expression of these factors could be used to predict micro-metastatic spread in the above three sites.

Patients and methods

Patients. From April 2003 to March 2004, 53 patients with histology-confirmed breast cancer consented to enter this study at the Department of Surgery, University of Hong Kong Medical Centre. Preoperative examination of the patients showed no evidence of distant metastasis at the time of primary surgery. Blood and bone marrow (BM) samples were collected simultaneously immediately prior to surgery. Primary breast tumors and sentinel lymph nodes (SLNs) were collected during surgery. After surgical dissection, the tumor samples were macroscopically isolated from the specimens. Samples were divided into three parts: one was subjected to routine pathological examination, one was frozen immediately in liquid nitrogen for RNA extraction and one was fixed in formalin and later embedded in paraffin wax. The SLN was divided into two parts: one was subjected to pathological examination and the other was fixed in formalin and later embedded in paraffin wax.

Magnetic-activated cell separation (MACS). Two bilateral bone marrow samples were obtained under general anesthesia from both upper iliac crests of each patient through fine needle aspiration during primary surgery and collected in EDTA anticoagulant. At the same time, blood samples from a peripheral vein were also collected. The blood and bone marrow samples were both subjected to magnetic-activated cell separation (MACS) as previously described (30). In brief, red blood cells were removed and the cell pellet was fixed with a 1/9 mixture of MACS CellFix solution and MACS dilution buffer. The procedures for carcinoma cell enrichment and detection were performed according to the manufacturer's instructions (Miltenyi Biotec, Germany). The fixed nucleated cells were permeabilized with MACS CellPerm solution and the cell pellet was incubated with MACS CK microbeads. After removal of unbound antibodies, the pellet was suspended in staining buffer. The suspension was put through a Nylon mesh to remove cell clumps and then a MACS enrichment column, which was located in a magnet. The enriched cells were eluted using a plunger.

Immunocytochemistry (ICC). Approximately $2-10 \times 10^7$ nucleated cells were enriched and immunostained. The sample slides were made by cytospinning up to 5×10^5 cells. After incubating with 3% H_2O_2 and washing in PBS, the slides were incubated with 1/100 anti-CK-FITC at 4°C overnight. Sections were incubated with 1/300 biotinylated rabbit antibody against mice. Color development was performed with AEC and then counterstained with hematoxylin. Cells were scored as immunocytochemically positive only when showing moderate to strong staining intensity. Positive cells were considered to be breast cancer cells only if they had tumor morphology and lacked hematopoietic characteristics.

Quantitative reverse transcription PCR (QRT-PCR). RNA was extracted from liquid-nitrogen-frozen tissue samples using an SV Total RNA isolation system (Promega, Madison, WI, USA) as specified by the manufacturer's instructions. The quantity and quality of the extracted RNA was confirmed by absorption measurements at 260 and 280 nm using a spectrophotometer (U-1800, HITACHI, Tokyo, Japan). The reagents for first-strand cDNA synthesis were included in the SuperScript III first-strand synthesis system from Invitrogen Inc. (Carlsbad, CA, USA). The final volume of reverse transcription (RT) reaction mixture was 20 μ l, which contained 1 μ g of total RNA, 0.5 μ g Oligo(dT)₂₀, 2 μ l 10X PCR buffer, 5×10^{-5} mmol $MgCl_2$, 2×10^{-4} mmol DTT, 1 μ l 10 mM dNTP mix and 2000 units of SuperScript III RT. The cDNA was synthesized as recommended by the manufacturer. Two units of RNase H were added to the mixture and then the mixture was incubated at 37°C for 20 min.

A QRT-PCR assay was developed to detect the expression of the mRNA of HER-2/neu, COX-2, VEGF and PDGF-B. The expression of the target genes was measured by real-time PCR using the ABI 7700 PRISM sequence detector (PE Applied Biosystems, Foster City, CA, USA). Specific primers and 6-carboxyfluorescein (FAM)-labeled TaqMan® probes were used in combination. Primers were designed to span intron-exon junctions to exclude genomic DNA from amplification. PCR cycles were performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems) that contained Ampli-Taq Gold DNA polymerase, a reaction buffer, dNTP, dUTP and AmpErase uracil-N-glycosylase and Assays-on-Demand gene expression probes (PE Applied Biosystems). PCR was carried out for 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 sec followed by 60°C for 1 min and 68°C for 1 min. PCR amplification of actin mRNA was conducted according to the manufacturer's instructions for the TaqMan actin control reagent.

The amounts of target mRNA in the samples were quantified relative to the control cDNA and standardized with respect to β -actin mRNA. The quantification value of target gene mRNA was described as an amount relative to the control cDNA. As a negative control, 24 normal breast tissue samples were obtained at the time of surgery and studied in order to determine the cut-off value of QRT-PCR assay.

Immunohistochemistry (IHC). Consecutive 4- μ m tissue sections were cut from the paraffin blocks of both SLN and primary tumors and placed on charged poly-L-lysine-coated slides (Menzel-Glaser, Germany). After dewaxing, one set of the SLN slide was stained with hematoxylin and eosin (H&E). A second set was stained with anti-pancytokeratin (Pan-CK) as an immunohistochemical marker to determine whether this improved the sensitivity of the test. Immunohistochemistry was performed using a standard technique of buffer wash and incubation with primary and secondary antibodies using a streptavidin-biotin complex (Dako Corp., CA, USA) and immunoperoxidase with the labeling antigen, diaminobenzidine. Cells were then counterstained with hematoxylin to highlight the nuclei before undergoing fixation and mounting. The number of isolated lymph nodes, the number of metastatic lymph nodes and the size of the metastasis (≤ 2 or >2 mm) were recorded.

Antibody	Nature of determinant	Reagent provided	Source	Dilution
C-erb-B2 onco-protein	Epidermal growth factor receptor 2	Polyclonal, rabbit anti-human	Dako, Inc., Carpinteria, CA, USA	1:250
COX-2 (229)	Membrane-bound enzyme	Monoclonal, mouse anti-human	Zymed Laboratories, Inc., South San Francisco, CA, USA	1:100
VEGF (147)	Vascular endothelial growth factor	Polyclonal, rabbit anti-human	Santa Cruz Biotechnology, Inc., CA, USA	1:100
PDGF-B (Ab-1)	Platelet-derived growth factor-B chain	Polyclonal, rabbit anti-human	Oncogene, San Diego, CA, USA	1:100

For tumor sample slides, antigen retrieval was performed through boiling the tissue sections at 95°C in 10 mmol/l citrate buffer for 10 min. The sections were cooled and treated with peroxidase-blocking reagent for 20 min, rinsed and treated with sufficient primary antibodies (HER-2/neu, COX-2, VEGF and PDGF-B) to cover the entire tissue section for 30 min. The details for the primary antibodies are given in Table I. Sections were rinsed again and treated for 30 min with visualization reagent solution containing both secondary goat anti-rabbit antibody and horseradish peroxidase linked to a common dextran polymer backbone. After rinsing away the excess visualization reagent, the sections were incubated in diaminobenzidine for 10 min to identify the location of immunoprecipitates. Sections were then counterstained with hematoxylin and eosin, and then mounted in Permount. Immunostaining was interpreted using a bright-field Olympus microscope according to the scoring system of the manufacturer as 0, 1+, 2+ and 3+. Scores of 2+ and 3+ were considered as overexpression and scores of 0 and 1+ were considered as low expression. Controls without primary antibody and positive control tissue were included in all experiments to ensure the staining quality.

Statistical analysis. Univariate statistical summaries (means and range of 95% confidence intervals for continuous variables and proportions for categorical variables) were used to describe the demographic and clinical characteristics of the samples. For categorical variables, the χ^2 test was used. The gene expression profiles (expression fold above normal) between groups of patients with/without micro-metastasis were analyzed using an independent sample t-test. $P < 0.05$ was considered statistically significant and all the P-values were two-sided.

Results

Determination of micro-metastasis in different homing sites. Micro-metastasis of the blood and bone marrow were detected in 13 (24.5%) and 30 (56.6%) of 53 patients respectively by ICC following MACS. In 14 (26.4%) patients, SLN micro-metastasis was detected by H&E staining. By contrast, 22 (41.5%) had micro-metastasis detected by IHC. In 44 (84%) cases, the results of the two assay methods were the same. In

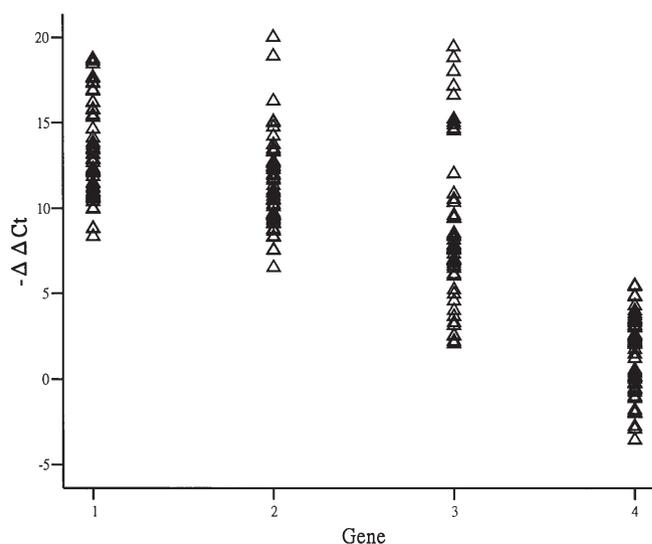


Figure 1. Relative gene expression levels in primary tumors. Real-time RT-PCR analysis of relative gene expression levels of different markers in primary breast cancer. (Gene 1: PDGF-B; 2: COX-2; 3: HER-2/neu; 4: VEGF).

14 (22%) cases, they were positive by both methods and, in 39 (62%), they were negative by both methods.

Gene expression of different markers. For each gene, ΔCt values for normal control and breast tumor tissues were obtained from triplicate reactions. We observed that, in the normal control tissue, HER-2/neu, COX-2, VEGF and PDGF-B had the highest ΔCt values, reflecting the fact that these genes were expressed at the lowest levels in normal tissue. Relative gene expression levels in individual primary tumor samples can be readily derived from real-time RT-PCR data using the equation $2^{-\Delta\Delta Ct}$ (31), and $\Delta\Delta Ct$ is the difference between the mean ΔCt value in normal control samples and the ΔCt value in the experimental primary tumor sample. The values (of $-\Delta\Delta Ct$) for breast cancer-associated genes are shown for the primary tumor samples (Fig. 1). The levels of COX-2 overexpression varied the most and ranged from 1- to 4.9×10^5 -fold above normal. The levels of HER-2/neu and

Table II. Gene expression fold above normal in primary tumors related to presence and absence of BD and BM micro-metastasis.

Genes	BD micro-metastasis			BM micro-metastasis		
	Mean	95% CI (Lower; Upper)	Sig. (2-tailed) P-value	Mean	95% CI (Lower; Upper)	Sig. (2-tailed) P-value
HER-2/neu						
(-)	26,923.27	(-108,282.52; 45,943.94)	0.42	6,701.49	(-107,975.75; 9,512.08)	0.09
(+)	58,092.56			55,933.32		
COX-2						
(-)	19,423.79	(-243,925.07; 107,520.84)	0.42	6,294.76	(-130,136.76; 24,639.01)	0.17
(+)	87,625.90			59,043.63		
VEGF						
(-)	7.09	(-5.75; 6.76)	0.87	4.68	(-9.34; 1.28)	0.11
(+)	6.58			8.71		
PDGF-B						
(-)	45,254.95	(-93,752.36; 36,459.84)	0.38	24,362.11	(-99,637.17; 989.04)	0.05
(+)	73,901.21			73,686.18		

Table III. Gene expression fold above normal in primary tumors related to presence and absence of SLN micro-metastasis (detected by H&E and IHC).

Genes	H&E			IHC		
	Mean	95% CI (Lower; Upper)	Sig. (2-tailed) P-value	Mean	95% CI (Lower; Upper)	Sig. (2-tailed) P-value
HER-2/neu						
(-)	15,865.26	(-143,882.11; 2,271.38)	0.05	17,561.40	(-116,197.26; 34,253.67)	0.27
(+)	86,670.62			58,533.20		
COX-2						
(-)	8,453.74	(-199,512.08; -10,207.89)	0.03	9,850.07	(-170,424.36; 43,693.94)	0.23
(+)	113,313.74			73,215.28		
VEGF						
(-)	6.85	(-6.54; 5.67)	0.88	6.85	(-5.74; 5.19)	0.92
(+)	7.28			7.12		
PDGF-B						
(-)	56,668.88	(-47,240.97; 80,460.50)	0.60	50,363.19	(-61,889.71; 52,647.47)	0.87
(+)	40,059.11			54,984.31		

PDGFB overexpression varied from 4- to 4.5×10^5 -fold and 328- to 4.4×10^5 -fold above normal. The level of VEGF overexpression was much lower than the above three genes, varying from 0.08- to 382-fold above normal.

To determine whether the results of the QRT-PCR assay correlated with micro-metastasis, we performed subgroup analysis of individual markers. Tables II and III show the descriptive gene expression fold above normal in primary

tumors related to presence and absence of micro-metastasis in different homing sites (BD, BM, SLN detected by H&E and SLN detected by IHC). Above normal gene expression in primary tumors was much higher in patients with micro-metastasis. This strongly suggests that the distribution of QRT-PCR results is not random but is associated with the presence of micro-metastasis. When conducting subgroup analysis of individual genes, we found a statistically significant difference

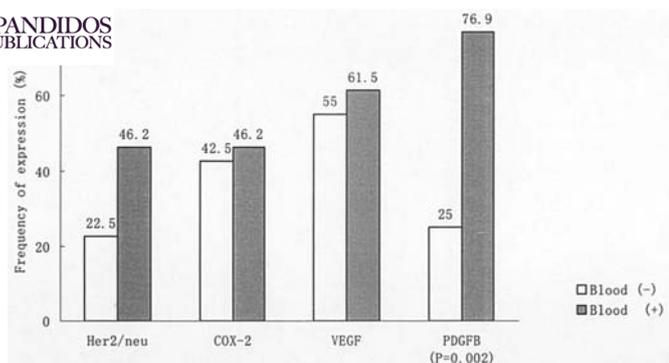


Figure 2. Frequency of protein expression in breast cancer patients with blood micro-metastasis [blood (+)] or not [blood (-)].

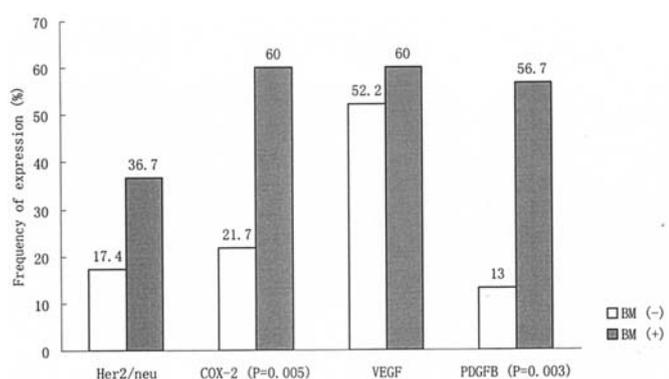


Figure 3. Frequency of protein expression in breast cancer patients with BM micro-metastasis [BM (+)] or not [BM (-)].

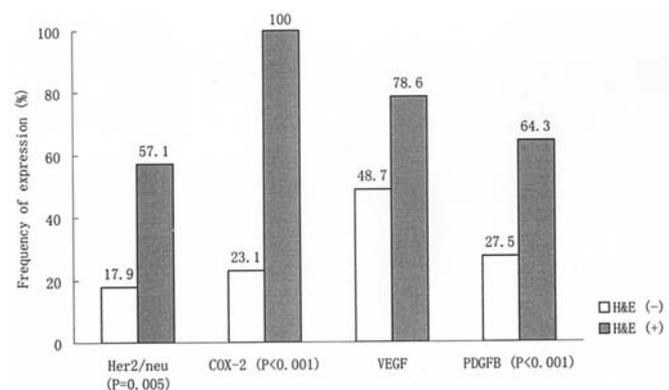


Figure 4. Frequency of protein expression in breast cancer patients with SLN micro-metastasis [H&E (+)] or not [H&E (-)] by H&E.

in COX-2 expression between the presence and absence of SLN micro-metastasis by H&E (P=0.03, 95% CI = -199,512.09 to -10,207.89). Also, HER-2/neu and PDGF-B expression levels correlated highly with micro-metastasis in SLN and BM separately (P=0.05).

Protein expression of different markers. All markers included in this study were mainly identified in the cytoplasm of breast cancer cells. The expression of different markers in primary breast cancer was 26.4% for HER-2/neu, 43.4% for COX-2,

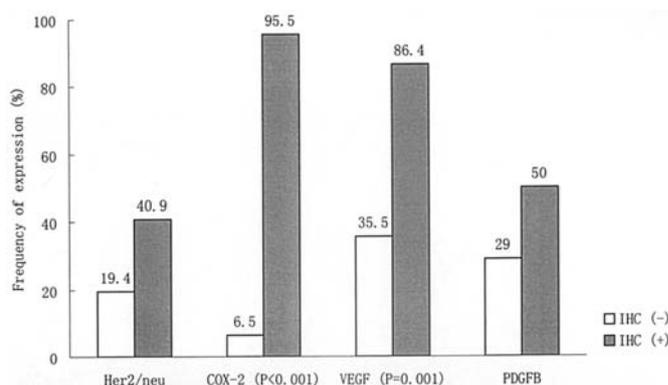


Figure 5. Frequency of protein expression in breast cancer patients with SLN micro-metastasis [IHC (+)] or not [IHC (-)] by IHC.

56.6% for VEGF and 37.7% for PDGF-B respectively. To determine the value of multimarker analysis, we evaluated the frequency of protein overexpression in patients with and without micro-metastasis in different homing sites (Figs. 2-5). Overexpression of HER-2/neu predicts the presence of SLN micro-metastasis as detected by H&E (P=0.005) and COX-2 overexpression predicts the presence of BM (P=0.005), SLN by H&E (P<0.001) and IHC (P<0.001). Similarly, PDGF-B overexpression predicts BD (P=0.002), BM (P=0.003) and SLN by H&E (P=0.017), whereas VEGF overexpression predicts only the presence of SLN by IHC (P=0.001).

Discussion

Micro-metastasis is considered to be the missing link between a primary tumor and formation of metastasis. Micro-metastasis of cancer cells in local and distant organs, such as peripheral blood, BM and SLN, generally results in recurrence even after a curative operation (32). For breast cancer, in particular, the detection of micro-metastasis in BM has been suggested as a potential prognostic factor for survival (33-36). Improvements in the sensitivity and specificity of detection methods may yield more important information in the future.

ICC on cytokeratin fragments is one of the best approaches for micro-metastasis detection in BD and BM (37-39). Braun *et al* (35) in an analysis of the BM of 200 and 500 patients without cancer and with breast cancer respectively detected cytokeratin positive cells in only 1% of control patients but in 30-40% of breast cancer patients. Immunomagnetic procedures such as MACS should enhance the sensitivity and consistency of this procedure. We detected rates of 24.5 and 56.6% respectively for BD and BM, which is consistent with the 20-52% and 16-80% respectively quoted in most studies (40-47).

For detection in SLN, H&E or immunochemical staining is typically used. IHC for SLN generally tends to yield more positive results than H&E; micro-metastasis detected by IHC is approximately 10-20% in specimens that are negative according to H&E (48-52). We used both approaches and detected rates of 26.4 and 41.5% with H&E and IHC respectively. This is consistent with the rates previously detected of 14-32% by H&E and 22-45% by IHC (48-52). Previous research has provided a basis for selecting the factors for study. However, research in general relating biochemical factors to

metastasis is still quite limited. We know PDGF-B is a potent osteotropic factor that stimulates the functions of both osteoclasts and osteoblasts (53). Yi *et al* (54) have established an animal model that suggests that MCF-7/neu-derived human PDGF-B plays a causative role in the development of osteosclerotic bone metastasis in breast cancer. *In vivo* studies have also reported that elevated levels of PDGF in the circulation and increased expression of PDGF in tumors correlate with increased metastases, lower response to chemotherapy, and shorter survival for patients with breast cancer (55-57). Our research found PDGF-B to predict micrometastasis not only in BM but in BD and SLN (by H&E) as well.

HER-2/neu overexpression on occult metastatic cells in BM predict a poor clinical outcome for breast cancer patients (58). Furthermore, Braun *et al* (58) found that HER-2/neu might be important for the growth and survival of disseminated tumor cells. According to our research, HER-2/neu was only related to SLN (by H&E).

VEGF is known to have a broad stimulatory role in neo-angiogenesis and is a potent regulator of breast carcinogenesis (59). Numerous studies have confirmed this in a clinical setting (60-64). VEGF-C and -D overexpression have been also detected in lymph node-positive breast tumors (65). This may be related to its overexpression being predictive of a higher micro-metastatic rate in SLN (by IHC).

Increased concentrations of PGE₂, a major product of COX-2, have been reported in human breast cancer and in experimental murine mammary tumor models (66). COX-2 expression has also been proved to associate with angiogenesis and lymph node metastasis in breast cancer patients (21). Our research finds COX-2 is associated with both BM and SLN (by H&E) micro-metastasis and QRT-PCR detects differences in amplification levels between non-SLN by H&E positive and negative groups. This finding could indicate that COX-2 expression in human breast cancer might be an early event in tumor progression, in accordance to colorectal and gastric cancer, in which COX-2 plays a role in tumorigenesis and is considered to be an early event (16).

To the best of our knowledge, this is the first study to suggest an association between primary tumors and micro-metastasis in different homing sites from breast cancer patients. The current study provides clinical evidence that the over-expression of HER-2/neu, COX-2 and VEGF is associated with the presence of micro-metastasis in SLN while that of PDGF-B is related to micro-metastasis in blood and bone marrow, suggesting that this may be of value for the diagnosis of micro-metastasis in different homing sites.

Our preliminary findings suggest that COX-2 as detected by QRT-PCR is associated with SLN as detected by H&E and that protein overexpression as detected by IHC can predict micro-metastatic spread. Further work may lead to additional insight-improving prediction of early tumor cell dissemination and help to stratify breast cancer patients at high-risk of metastasis for different treatment as well as identify those at low-risk for whom no further systemic therapy is warranted.

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