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Elevated osteopontin and thrombospondin expression identifies malignant human breast carcinoma but is not indicative of metastatic status

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Abstract

Background Our previous characterization of a human breast tumor metastasis model identified several candidate metastasis genes. The expression of osteopontin (OPN) correlated with the metastatic phenotype, whereas thrombospondin-1 (TSP-1) and tyrosinase-related protein-1 (TYRP-1) correlated with the nonmetastatic phenotype of independent MDA-MB-435 cell lines implanted orthotopically into athymic mice. The aim of the present study was to examine the cellular distribution of these molecules in human breast tissue and to determine whether the relative expression level of these three genes is associated with human breast tumor metastasis.

Methods Sixty-eight fresh, frozen specimens including 31 primary infiltrating ductal carcinomas, 22 nodal metastases, 10 fibroadenomas, and five normal breast tissues were evaluated for OPN expression, TSP-1 expression and TYRP-1 expression. Immunohistochemistry was performed to monitor the cellular distribution and to qualitatively assess expression. Quantitative analysis was achieved by enrichment of breast epithelial cells using laser-capture microdissection and subsequent real-time, quantitative PCR.

Results The epithelial components of the breast tissue were the source of OPN and TSP-1 expression, whereas TYRP-1 was present in both the epithelial and stromal components. Both OPN and TSP-1 expression were significantly higher in malignant epithelial sources over normal and benign epithelial sources, but no difference in expression levels was evident between primary tumors with or without metastases, nor between primary and metastatic carcinomas.

Conclusion Elevated expression of OPN and TSP-1 may play a role in the pathogenesis of breast cancer. The multiplex analysis of these molecules may enhance our ability to diagnose and/or prognosticate human breast malignancy.

Keywords: breast carcinoma, immunohistochemistry, metastasis, microdissection, quantitative PCR

Introduction

Breast cancer is a major cause of death among women in the United States and worldwide [1]. While cures are probable if the cancer is detected early and remains localized, many patients will succumb to the disease if the primary tumor metastasizes to secondary organs. Although significant progress has been made in detecting and treating the primary tumor, the ability to predict the metastatic behavior of a patient's tumor and to eradicate or control recurrent disseminated malignancy remain major clinical challenges in oncology.

We have previously characterized an experimental system that enables comparative molecular screening and functional evaluation of candidate breast metastasis-related genes in an isogenic background [2]. Two independent clones of opposing metastatic phenotype were derived from the MDA-MB-435 breast tumor cell line (metasatic M-4A4

FFPE = formalin-fixed, paraffin-embedded; H&E = hematoxylin and eosin; IHC = immunohistochemistry; LCM = laser capture microdissection; OPN = osteopontin; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; qPCR = real-time, quantitative PCR; RT = reverse transcriptase; TSP-1 = thrombospondin-1; TYRP-1 = tyrosinase-related protein-1.

and nonmetastatic NM-2C5 cell lines). When inoculated into the mammary fat pad of athymic mice, both cell lines form primary tumors. While clone M-4A4 aggressively metastasizes to the lung and lymph nodes, the NM-2C5 clone is entirely nonmetastatic. Initial comparative gene expression analysis of the two clones revealed the secreted, integrin-binding protein osteopontin (OPN) [3] to be significantly increased in the metastatic M-4A4 cell line. Conversely, the expression of thrombospondin-1 (TSP-1) [4] and of tyrosinase-related protein-1 (TYRP-1) was markedly overexpressed in the nonmetastatic NM-2C5 cell line [2,5].

OPN is a secreted glycophosphoprotein expressed by a number of cell types, including leukocytes and epithelial lineages, and it has been implicated biologically in bone development, in immune system regulation [6], and in multiple mechanisms through signal transduction via its binding to integrins [7]. OPN has been detected in primary breast tumors and is elevated in the plasma of patients with metastatic breast cancer [8,9], but an association between elevated levels of primary tumor OPN and metastatic burden has not been well established.

TSP-1 is a homotrimeric multidomain glycoprotein synthesized and secreted by numerous cell types, including platelets, vascular smooth muscle cells and keratinocytes [4]. Due to its interaction with a wide variety of proteins, TSP-1 has been implicated in a number of biological processes including coagulation, cell adhesion, the modulation of cell–cell and cell–matrix interactions, control of tumor growth and metastasis, and angiogenesis [10]. Inhibition of tumor cell proliferation has been achieved *in vivo* by systemic treatment with TSP-1 peptide mimetics [11], but a correlation between primary tumor TSP-1 expression and poor prognosis has not been clearly established [12]. While there are reports that these molecules can originate from tumor cells [13], the majority of reports have suggested that the major source of both TSP-1 and OPN is tumor-associated stroma [14] or infiltrating macrophages and lymphocytes [15].

The melanogenic enzymes TYRP-1 and TYRP-2 are well-characterized differentiation antigens recognized by antibodies and T cells of patients with melanoma [16]. The evaluation of TYRP-1 expression in human breast lesions has not been documented to date.

The aim of the present work was to examine the cellular distribution of OPN, of TSP-1 and of TYRP-1 in human breast tissue, and to determine whether the relative expression level of these three genes was associated with malignancy or breast tumor metastasis. To overcome the problem of heterogeneity and stromal cell contamination in homogenized tissue specimens, specific cell types were isolated by laser capture microdissection (LCM) prior to analysis. We used a combination of immunohistochemistry (IHC), LCM and real-time, quantitative PCR (qPCR) analysis of material obtained from a panel of primary breast infiltrating ductal carcinomas and nodal metastases, and compared the target gene expression levels with those found in nonmalignant diseased tissue and in samples of normal breast tissue.

Materials and methods

Tissue samples

Sixty-eight frozen human breast tissue specimens were evaluated by LCM, qPCR and IHC, including 31 primary tumors of infiltrating ductal carcinomas (22 primary tumors with metastasis and nine primary tumors without known metastasis), 22 matched nodal metastases, 10 fibroadenomas and five normal breast tissues (obtained from reduction mammoplasty). In addition, eight tumor-associated stromal components (including fibroblasts, endothelial cells and inflammatory cells) were microdissected from primary tumors without known metastases for analysis.

Breast tissues were obtained from surgical specimens at the John Radcliffe Hospital, Oxford, UK and at the UCSD Medical Center. Samples were obtained in random order from the residue of tissue used for pathological diagnosis that would otherwise have been discarded. Tissues were snap-frozen immediately after resection and were stored in liquid nitrogen until analysis. Four pairs of additional formalin-fixed, paraffin-embedded (FFPE) specimens of primary infiltrating ductal carcinomas and of matched nodal metastasis were also used for IHC only (total of 76 samples for IHC). Histological diagnosis of all samples was performed using H&E-stained cryostat sections (JWR). The histological grade was assigned according to the modified Bloom–Richardson grading scheme [17]. Tissue samples were obtained and processed under approval of the UCSD Institutional Review Board for studying existing tissues (#011246X).

Laser capture microdissection

The total number of samples studied using LCM and qPCR were 76 (68 samples were microdissected to specifically enrich for cells of epithelial origin, and eight samples were microdissected to obtain tumor-associated stroma). Frozen tissues were embedded in Optimal Cutting Temperature Compound (OCT; Sakura Finetek, Torrance, CA, USA) and held at −70°C prior to sectioning. Five-micrometer cryosections were mounted onto uncoated microscope slides, immediately fixed by immersion in acetone for 30 s and washed with RNAse-free water. Staining with H&E (Sigma, St Louis, MO, USA) for 1 min was performed for morphological evaluation. After dehydration in 95% ethanol twice for 1 min and in 100% ethanol for 1 min, sections were cleared in xylene for 2 min and were air-dried. LCM was performed with the PixCell I LCM System (Arcturus Engineering, Mountain View, CA, USA). Following the manufacturer's protocols, approxi-

mately 2–5000 cell equivalents were dissected from each slide for subsequent analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen breast tissues and laser-captured materials using the StrataPrep Total RNA kit (Stratagene, La Jolla, CA, USA). All samples were DNase-treated to remove contaminating DNA and the yield was measured spectrophotometrically. Single-stranded cDNA was synthesized from total RNA using MMLV reverse transcriptase (Retroscript RT kit; Ambion, Inc., Austin, TX, USA).

Real-time, quantitative PCR

Primer sets for OPN, TSP-1, TYRP-1 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, the housekeeping gene) were designed using Primer Express Version 1.5 software (Applied Biosystems, Foster City, CA, USA). The qPCR was performed on an Applied Biosystems PRISM 7700 Sequence Detection System using SYBR Green® I chemistry. Sequence Detection Software version 1.7 was applied to data collection and analyses (Applied Biosystems).

The optimal primer concentrations for the reference and target genes in the final PCR reaction were determined, and both dissociation curves (ABI Dissociation Curves version 1.0 software; Applied Biosystems, Foster City, CA, USA) and agarose gel electrophoretic analyses were applied to confirm the specificity of PCR products and to check for the presence of primer–dimer formations in the absence of a template. Nontemplate control PCR reactions were always run in triplicate for every gene-specific reaction mix.

Standard curves (cycle threshold values versus template concentration) were prepared for each target and for the endogenous reference (GAPDH) using cDNA from MDA-MB-435 breast cells as the calibrator. For each experimental sample, the amount of target and endogenous reference were determined from the respective standard curves, and then the target amount was divided by the endogenous reference amount to obtain a normalized target value. The sequences of the PCR primer pairs used for each gene are as follows: TYRP-1, 5′-GATGGCAGAGATGATCGGGA-3′ and 5′-AGAAATTGCCGTTGCAGTGAC-3′; TSP-1, 5′-CAGCAGCCGCTTTTATGT-3′ and 5′-CCGAGTATCCCT-GAGCCCTC-3′; OPN, 5′-TTGCAGCCTTCTCAGCCAA-3′ and 5′-GGAGGCAAAAGCAAATCACTG-3′; and GAPDH, 5′-CCACCCATGGCAAATTCC-3′ and 5′-GGGATTTCCATTGATGACA-3′.

Immunohistochemistry

All tissue specimens (68 frozen samples and eight FFPE samples) were evaluated immunohistochemically. Monoclonal antibodies to OPN, to TYRP-1 and to TSP-1 were purchased commercially (OPN [1:100], Chemicon International, Inc., Temecula, CA, USA; TYRP-1 [1:80, clone G-17], Santa Cruz Biotechnology, Inc., CA, USA; TSP-1 [1:320], BD Transduction Laboratories, CA, USA). The TYRP-1 and TSP-1 antibody reactions were optimized using the DAKO LSAB plus kit (DAKO, Carpinteria, CA, USA) for both frozen and FFPE sections. The monoclonal rat anti-human OPN antibody was optimized using the secondary biotinylated rabbit anti-rat antibody (1:600) and streptavidin/horseradish peroxidase (1:400; DAKO). Primary tumors recovered from nude mice inoculated with the M-4A4 and NM-2C5 MDA-MB-435 clones [2] were used as positive controls.

Tumor specimen staining was scored as positive or negative. Positive staining was defined as >50% of the tumor cells or the benign epithelial components examined having distinct cytoplasmic staining. The four additional FFPE archived ductal carcinomas and the matched nodal metastases ($n=8$) were stained with the aforementioned antibodies using the antigen retrieval method now described to assess the utility of the antibodies on archived tissues.

Serial frozen sections were cut for IHC and for LCM/RT-qPCR for concurrent analysis. For frozen tissues, 5 µm cryostat sections (Leica Microsystems, Heidelberger, Germany) were briefly fixed in acetone. Primary antibodies were incubated on slides for 1 hour. After several washings with PBS, secondary antibodies conjugated with streptavidin/horseradish peroxidase were added for 30 min–1 hour. The sections were washed and stained with 3,3′-diamino-benzidine (DAKO) for 5 min and were then counterstained with Gills II hematoxylin (Biochemical Sciences, Inc., Swedesboro, NJ, USA).

For FFPE tissues, 5 µm sections were cut and placed on 3-aminopropyltriethoxysilane-coated slides. After deparaffinization, the endogenous peroxidase was removed by H_2O_2. Antigen retrieval was performed by steam heating for 75 min. After sections were cooled to room temperature, the background staining was reduced by incubation of serum against specific secondary antibody. Slides were incubated with primary antibodies for 1 hour. After washing with deionized water and PBS three times, species-specific secondary antibodies were added and the antibody complex was visualized by 3,3′-diaminobenzidine. The nuclei were counterstained by Gill's II hematoxylin.

Statistical analysis

All statistical analyses used GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Comparison of numerical data was achieved using a two-tailed unpaired *t* test. $P<0.05$ was considered significant.

Results

Immunohistochemical detection of OPN, TSP-1 and TYRP-1 in human breast tissues

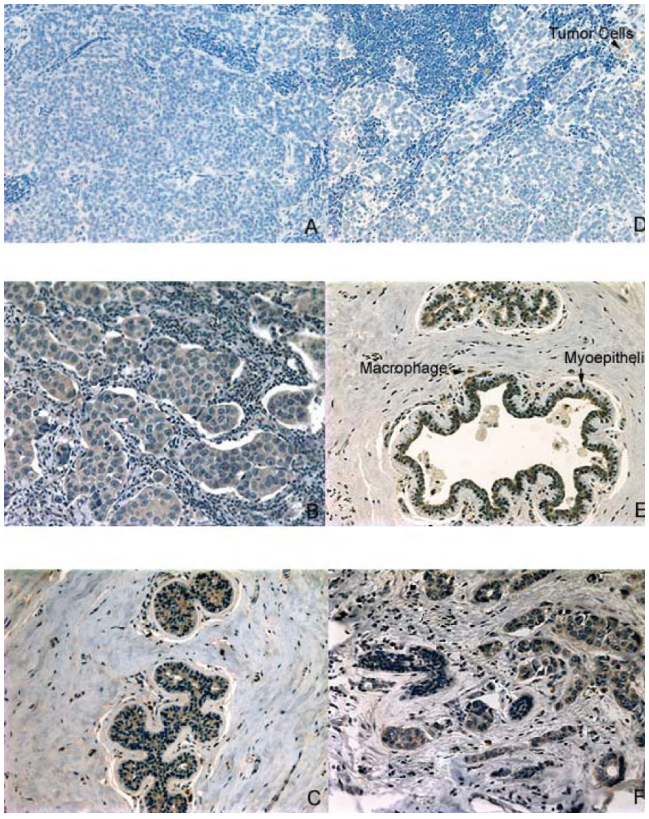
The efficacy and specificity of OPN, TSP-1 and TYRP-1 antibodies in IHC was tested by analysis of frozen tissue

xenografts of known target gene expression [2,5]. Positive cytoplasmic staining was detected with the OPN antibody in nine of 26 (35%) primary breast carcinomas with nodal metastases. A representative example is depicted in Figure 1. Six of 26 (23%) metastatic tissue samples and five of nine (56%) primary tumors without metastases were OPN-positive. Incubation of fibroadenoma and normal breast tissue specimens with the OPN antibody also produced positive staining in the epithelial cells of 53% of nonmalignant cases (five fibroadenomas and three normal breast tissues). OPN was also regularly observed in tumor-infiltrating inflammatory cells and macrophages (Fig. 1). Although difficult to quantitate, a trend of OPN localization towards the epithelial layer but not the myoepithelial layer was observed in normal breast tissue (Fig. 1). Overall, the degree and intensity of OPN expression was qualitatively equal in samples of adenomas, adenocarcinomas and nodal metastases from patients with disseminated disease. The majority of primary carcinomas (66%) that were positive for OPN also had concurrent OPN-positive metastases.

IHC staining of breast tissue sections revealed intracytoplasmic staining of TSP-1 in six of 26 (19%) node-positive primary carcinomas and in 10 of 26 (38%) metastases. In this case, all positive primaries had positive associated metastases, although some metastatic tumors with positive TSP-1 expression had negative TSP-1 stain in their primary tumors. TSP-1 staining was positive in two of nine primary carcinomas without metastases (22%). Very weak staining was observed in fibroadenoma and normal tissues, and no TSP-1 was detected in breast tissue stromal components.

A high percentage of node-positive primary tumors (73%), metastatic carcinomas (73% and 100% concordance between primary tumor and associated metastasis, respectively), primary carcinomas without metastases (89%), benign breast specimens (70%) and normal (60%) breast specimens were positive for TYRP-1 staining (Fig. 1). TYRP-1 staining was confined to the cytoplasm and to the epithelial components of the tissue. The staining achieved with all three antibodies was relatively homogeneous across the tumor in positive cases, and no qualitative differences in staining were apparent at the invasive edge of the carcinomas. No correlation was found between protein expression and tumor grade in the infiltrating ductal carcinomas, and no consistent qualitative differences were revealed between primary tissues with or without associated metastases.

Figure 1



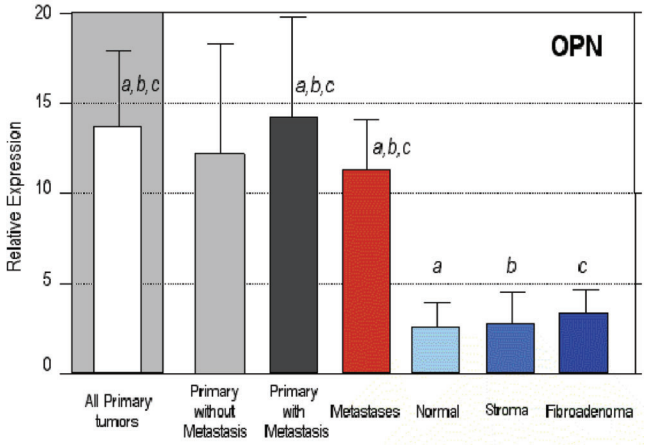
Immunohistochemical detection of osteopontin (OPN), thrombospondin-1 (TSP-1) and tyrosinase-related protein-1 (TYRP-1) in normal and breast tumor cells. **(A)** Negative control (100×), **(B)** cytoplasmic OPN staining of the majority of tumor cells in selected cases (the stroma fibroblasts and endothelial cells did not stain for OPN) (200×), **(C)** TYRP-1 staining in normal breast duct epithelium (staining is specific for the epithelium and not for the surrounding stroma tissue) (100×), **(D)** TSP-1 stained only selected tumor cells (brown signal) and not the majority of the tumor (100×), **(E)** OPN was localized in normal breast duct epithelium but not in myoepithelial cells (100×), and **(F)** TRYP-1 in breast tumor cells (200×).

LCM and qPCR analysis of breast tissues

While providing information on the cellular distribution of target gene expression, the technique of one-color chromogenic IHC staining can result in highly variable data across samples, and it relies on visual 'scoring' that is relatively subjective. To obtain accurate, quantitative information on target gene expression we employed qPCR technology. The selection of specific cell types from the heterogeneous cellular composition of the tissue specimens was attained by LCM. This procedure allowed OPN, TSP-1 and TYRP-1 mRNA analysis in specific cells of interest in each case, eliminating the contribution from normal adjacent cells that could cause difficulties in interpretation.

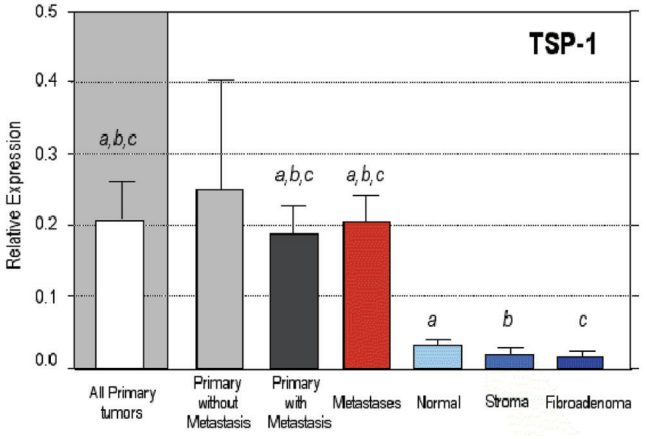
The cells of interest in the majority of cases were those of epithelial origin, but eight specimens were also used for the isolation of tumor-associated stroma (fibroblast, endothelial and inflammatory cells) for comparison. We measured the expression levels of the OPN, TSP-1 and TYRP-1 genes using a quantitative PCR assay (RT-qPCR). This analysis, in line with the findings of the IHC data, confirms that breast tumor cells themselves synthe-

Figure 2



Quantitative analysis of osteopontin (OPN) transcripts in human breast tissue components. Epithelial and stromal components were microdissected from tissue sections and subjected to real-time, quantitative PCR analysis. The levels of OPN transcripts were significantly higher (*a*, *b*, *c*, $P<0.05$) in all primary breast carcinomas (with and without metastases) than in normal breast epithelia, in fibroadenomas and in associated stromal components. Error bars indicate the standard error of the mean.

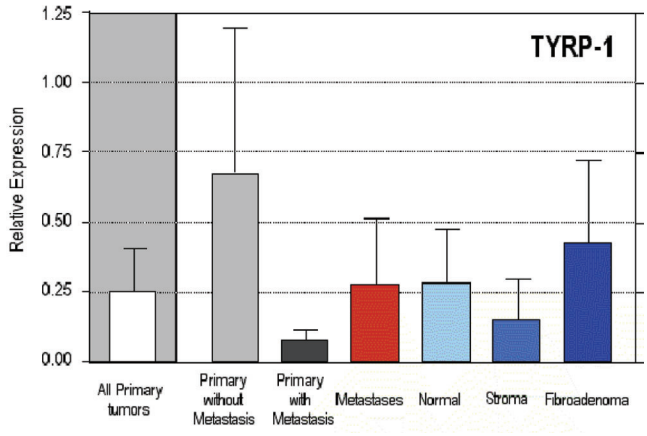
Figure 3



Real-time, quantitative PCR analysis of thrombospondin-1 (TSP-1) transcripts in human breast tissue components. The levels of TSP-1 transcripts were significantly higher (*a*, *b*, *c*, $P<0.05$) in all primary breast carcinomas, with and without metastases, than in normal breast epithelia, in fibroadenomas and in associated stromal components. Error bars indicate the standard error of the mean.

size OPN, TSP-1 and TYRP-1 (in addition to tumor-infiltrating macrophages in the case of OPN). Normal and nonmalignant breast epithelia and stromal elements expressed reduced levels of OPN and TYRP-1 transcripts. Figures 2–4 demonstrate the differential expression pattern and the quantitative expression level of each of the three genes in human breast tissues. Relative to the

Figure 4



Real-time, quantitative PCR analysis of tyrosinase-related protein-1 (TYRP-1) transcripts in human breast tissue components. There were no significant differences in TYRP-1 transcript levels observed between primary breast carcinomas, with metastases or without metastases, and normal breast epithelia, fibroadenomas and associated stromal components. Error bars indicate the standard error of the mean.

GAPDH transcript levels, OPN expression was found to be ~10-fold higher than TYRP-1 expression in all tissues examined. TSP-1 expression was comparable with that of TYRP-1 in malignant cells but was at the limit of detection in normal and benign epithelia and stromal components.

OPN mRNA was found to be present in all microdissected tissue samples examined. Significantly elevated (~5-fold) levels of OPN transcripts were observed in cells of epithelial origin dissected from primary carcinomas and associated metastases over nonmalignant cell sources. All primary tumors combined ($P=0.017$) and all metastases ($P=0.0082$) exhibited significantly elevated OPN expression compared with normal epithelia (Fig. 2). However, there was no difference in OPN expression between primary carcinomas with metastases and those without metastases. Nor was there a quantitative difference in OPN expression between primary carcinomas and matched metastases (Fig. 2).

While trace amounts were detected due to the sensitivity of PCR amplification, thrombospondin transcripts were essentially absent from breast tissue epithelia obtained from normal and benign specimens, and there were no appreciable levels detected in stromal tissue components. However, TSP-1 levels were significantly raised in malignant tissue epithelial sources (Fig. 3). TSP-1 levels were significantly higher (~10-fold) in all primary tumors ($P=0.0017$) and in all metastases ($P=0.0001$) over normal breast epithelia. In line with the OPN patterns of expression, there was no significant difference in TSP-1 levels between

primary tumors with or without metastases, nor between primary carcinomas and associated metastases.

TYRP-1 expression was higher than that of TSP-1 but lower than that of OPN (10-fold to 50-fold). While TYRP-1 transcripts could be detected in all tissues examined (Fig. 4), including malignant and nonmalignant sources, there were no significant quantitative differences revealed between the types of tissue.

Discussion

A greater understanding of the molecular mechanisms involved in metastatic spread is needed to facilitate advances in prognostic evaluation for individual patients, and in the design of therapeutic interventions to inhibit the process. In an effort to establish a methodological framework for analysis of the molecules and the mechanisms involved in this complex multistep process, we previously developed a well-defined experimental system in which the role of candidate genes can be screened and tested [2]. By serial dilution cloning of the MDA-MB-435 tumor cell line and screening by orthotopic implantation into the mammary fat pad of athymic mice, we derived a pair of breast tumor cell lines (M-4A4 and NM-2C5) that originate from the same breast tumor, but that have diametrically opposite metastatic capabilities. Through multiple molecular analyses we have identified and verified a number of genes whose expression correlates with the metastatic phenotype in this xenograft model. The expression of the proteins TSP-1 and TYRP-1 were found to correlate with the nonmetastatic phenotype, whereas OPN was significantly elevated in the metastatic counterpart. These findings prompted us to investigate the occurrence and distribution of these molecules in human breast tissues. The investigative approach was designed to detect and quantify the three molecules in specific breast tissue components, data that were not previously available.

Having detected all three proteins immunohistochemically in normal, benign and malignant breast epithelia, the quantitation of OPN, TSP-1 and TYRP-1 mRNA was achieved using LCM and qPCR. This technique allows for the accurate procurement of cells from specific microscopic regions of tissue sections, and therefore enables the molecular genetic analysis of pure populations of malignant breast cells in their native tissue environment [18]. This technical advance helps one to avoid the variation and subjectivity associated with IHC, and to overcome the limitations of data interpretation caused by tissue heterogeneity and normal stromal cell contamination in homogenized tissue specimens. Coupled with qPCR, LCM can reveal significant differences in specific gene expression that are not accessible with relatively insensitive IHC alone.

We have demonstrated that human breast epithelia can themselves synthesize OPN and, to a lesser extent, TSP-1 and TYRP-1 *in vivo*, and that elevated OPN and TSP-1 levels are indicative of malignancy. Significant OPN expression has been reported in a number of breast epithelial cell lines [19,20], and so it is known that cells of breast epithelial origin can synthesize OPN in certain conditions. But it is possible that *in vitro* OPN expression is a phenomenon of cell culture or that OPN-positive cells have a selective growth advantage during extended passage. Synthesis of OPN was also observed in infiltrating lymphocytes and macrophages, a factor that would seriously compromise efforts to quantify OPN in homogenized tissue specimens. Such analysis would highlight tumors with a high level of infiltrating inflammatory cells rather than evaluate the levels of the marker molecule(s) in the proliferative epithelia of a lesion.

As observed in IHC, tumor-infiltrating inflammatory cells were presumably the stromal source of OPN. Transcript levels in normal and fibroadenoma epithelia were equal to those found in isolated stroma, indicating that the scattered infiltrating macrophages (not lymphocytes) have relatively high OPN expression. The presence of inflammatory infiltration is a serious problem in the molecular analysis of tissue samples if the tissue is homogenized prior to analysis. The presence of OPN in inflammatory cells highlights the advantage of microdissection in this analytical context.

High levels of OPN in breast tumor cell lines appear to correlate with a more aggressive *in vivo* phenotype [2,20]. Transfection of OPN expression constructs into cells producing noninvasive mammary epithelial tumors has been reported to convert them to a malignant phenotype [8,21]. Furthermore, transfection of antisense OPN constructs or OPN-targeting ribozymes into transformed tumorigenic cells can result in impaired anchorage-independent growth, as well as in diminished tumor-forming ability *in vivo* [22,23]. OPN is also typically overexpressed in human tumors, including breast cancer [15,24]. However, the source of OPN in primary breast tumors was originally thought to be infiltrating macrophages rather than tumor cells themselves [15].

By applying *in situ* hybridization, Tuck and colleagues identified breast tumor cells as a source of OPN [13]. Using a semiquantitative scoring system to evaluate immunohistochemical staining, these investigators found a correlation between OPN positivity (defined as >33% of tumor cells in a section staining for OPN) in lymph node-negative primary breast carcinomas and patient survival, suggesting that tumor cells with elevated OPN levels may behave differently with regard to disease progression [13]. Another recent study showed immunohistochemically that while OPN protein is expressed weakly in normal breast tissue, OPN is expressed strongly (>50% tumor cell staining) in pregnant/lactating tissue and in the majority (66%) of breast carcinomas. These investigators also found that the presence of OPN on tumor cells is correlated with patient demise [25].

Our LCM-mediated transcript detection acts as an important confirmation of breast tumor cell OPN synthesis because IHC analysis does not rule out the possibility that secreted proteins such as OPN and TSP-1 are sequestered by the tumor cells from the local environment. Experiments with cell lines show that tumor cells can bind soluble OPN and can migrate towards an OPN concentration gradient [8,19]; sequestration from the extracellular matrix *in vivo* is thus pertinent. In the present study, the epithelial layer appeared to express more OPN than the myoepithelial cells, suggesting some OPN accumulation or induction via the myoepithelial layer or via stromal interactions. Unfortunately, the resolution of LCM does not enable the selection of epithelial cells from myoepithelial cells and so we were unable to confirm this quantitatively.

The effect of TSP-1 expression on tumor growth and metastasis has been examined *in vivo* by orthotopic inoculation of TSP-1-overexpressing MDA-MB-435 cells. A dose-dependent inhibition of spontaneous pulmonary metastases was reported, but this was paralleled by an inhibition of primary tumor growth [26]. TSP-1 has also been implicated in the phenomenon of concomitant tumor resistance. This refers to the ability of some large primary tumors to hold smaller, distant tumors in check, preventing their progressive growth. Volpert and colleagues demonstrated this phenomenon using tumors formed by the human fibrosarcoma line HT1080 in nude mice, and obtained data indicating that it was caused by secretion of TSP-1 by the primary tumor [27]. Some of the properties attributed to TSP-1 are conflicting, however; TSP-1 can be both adhesive and antiadhesive *in vitro*, can stimulate or inhibit angiogenesis, and can inhibit or enhance proteolytic enzyme activity. The composition of the matrix, the availability of cytokines and proteases, and the expression of various receptors in a given cellular environment might explain these opposing functions.

Our findings in the MDA-MB-435 metastasis model are in agreement with TSP-1 playing a role in the reduction of metastatic potential [2]. Clinically, TSP-1 expression is inversely correlated with tumor grade and survival rate in a number of cancers [28,29], most probably because of the anti-angiogenic effect of TSP-1. A recent study confirms this to be the case in breast cancer, where expression of TSP-1 in stroma adjacent to ductal carcinoma *in situ* is lost in cases with more aggressive histological features [30]. There are also reports, however, that fibroblast TSP-1 can induce matrix metalloproteinase-9 in breast tumor cells and gastric cancer tumor cells, and this in turn may lead to a more invasive phenotype [14,31]. In the present study, TSP-1 was not expressed differentially in the stroma of benign tissue specimens or malignant tissue specimens, nor in the epithelial component of breast tumors with or without metastases. The increased levels observed in malignant breast epithelia suggest a shift in the balance between the anti-angiogenic and pro-invasive effects of TSP-1 during tumor progression. The precise biological role of TSP-1 in human tumor progression awaits more definitive study.

In the present study, TYRP-1 mRNA was present, but at very low levels, in breast tumor tissue, in associated stroma and in normal breast tissues. However, IHC reveals that TYRP-1 protein staining is easily detectable in many samples. The melanogenic enzymes tyrosinase, TYRP-1 and TYRP-2 are well-characterized differentiation antigens recognized by antibodies and T cells of patients with melanoma. TYRP-1 is a type I membrane protein expressed by melanomas and normal melanocytes that was originally characterized as an oxidase in the melanin biosynthetic pathway. Clinically, TYRP-1 is thought to be important as a melanoma antigen that is highly antigenic to cytotoxic T cells, providing a potential target for melanoma vaccine development [16]. TYRP-1 appears to be less expressed in advanced primary lesions and metastases of human cutaneous melanoma and uveal melanoma [32]. Primary melanoma samples express TYRP-1 transcripts at higher levels than melanoma metastases [33], and IHC analysis of malignant melanocytic lesions has shown that the invasive cells of primary melanomas are TYRP-1-negative [34].

Our finding that TYRP-1 is also expressed in the poorly metastatic or noninvasive cell lines NM-2C5 [2], T47D, MCF7 and DU145 [5], which are derived from human breast cancers and prostate tumors that are nonmetastatic, indicates that this molecule is not exclusively expressed in cells of the melanocytic lineage. It has long been known that the sera of patients who suffer from melanoma or mammary carcinoma show higher tyrosinase activity than normal sera [35] but, to our knowledge, this is the first report of TYRP-1 expression in human breast duct epithelium. The biological function of and any clinical significance of TYRP-1 expression in normal or malignant breast carcinomas are presently unknown.

Conclusion

We have quantitatively analyzed transcripts of OPN, of TSP-1 and of TYRP-1 in microdissected human breast tissues. Elevated levels of OPN and TSP-1 expression in primary and metastatic carcinomas suggest that these genes may be necessary for, but not sufficient for, dissemination. Tumor cells that synthesize matrix-associated factors such as OPN and TSP-1 may increase their ability to independently manipulate their surroundings, encouraging invasion and migration. These studies suggest that the quantitative analyses of OPN and/or TSP-1 may be clinically useful in identifying malignant lesions over benign lesions but they are not, as yet, promising markers for the evaluation of primary tumors for metastatic propensity.

Competing interests

None declared.

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