Transcriptomic Profiling of Sequential Tumors from Breast Cancer Patients Provides a Global View of Metastatic Expression Changes Following Endocrine Therapy

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Abstract

Purpose: Disease recurrence is a common problem in breast cancer and yet the mechanisms enabling tumor cells to evade therapy and colonize distant organs remain unclear. We sought to characterize global expression changes occurring with metastatic disease progression in the endocrine-resistant setting.

Experimental Design: Here, for the first time, RNAsequencing has been performed on matched primary, nodal, and liver metastatic tumors from tamoxifen-treated patients following disease progression. Expression of genes commonly elevated in the metastases of sequenced patients was subsequently examined in an extended matched patient cohort with metastatic disease from multiple sites. The impact of tamoxifen treatment on endocrine-resistant tumors in vivo was investigated in a xenograft model.

Results: The extent of patient heterogeneity at the gene level was striking. Less than 3% of the genes differentially expressed between sequential tumors were common to all patients. Larger divergence was observed between primary and liver tumors than between primary and nodal tumors, reflecting both the latency to disease progression and the genetic impact of intervening therapy. Furthermore, an endocrine-resistant in vivo mouse model demonstrated that tamoxifen treatment has the potential to drive disease progression and establish distant metastatic disease. Common functional pathways altered during metastatic, endocrine-resistant progression included extracellular matrix receptor interactions and focal adhesions.

Conclusions: This novel global analysis highlights the influence of primary tumor biology in determining the transcriptomic profile of metastatic tumors, as well as the need for adaptations in cell-cell communications to facilitate successful tumor cell colonization of distant host organs. Clin Cancer Res 21(23): 5371-9. ©2015 AACR.

Introduction

The vast majority of breast cancers are steroid receptor positive and depend on estrogen for growth. The use of estrogen receptor modulators, such as tamoxifen and estrogen depletion strategies, including aromatase inhibitors, has been widely successful. Disease recurrence, however, is common, with between 10% and 25% of patients developing extensive local or distant metastases. Most patients with metastases will succumb to their disease, as targeted therapies that provide significant clinical benefit remain elusive. Metastatic breast cancer secondary to endocrine treatment is heterogeneous, ranging from discreet lesions to diffuse multi-organ disease. The dominant site of metastatic involvement can have a significant bearing on clinical outcome. Metastatic disease of the liver has been reported to be a predictor of poor outcome, with a median survival of 18 months (1), whereas metastatic burden confined to the bone is thought to be more indolent (2).

Colonization of the host organ is the ultimate step of a progressive disease path. Most breast cancer cells that enter the circulation and infiltrate distant organs die due to the hostile nature of the host microenvironment (3). For metastatic growth to be established, disseminated breast cancer cells need to survive a period of latency, prolonged exposure to therapy, and subsequently reinitiate growth when appropriate. Evidence of the impact of steroid treatment on tumor progression is emerging. Steroid receptor switching and in particular loss of the progesterone receptor (PGR), an estrogen receptor (ER) target, is observed in approximately 30% of patients (4, 5). Furthermore, gain-of-function mutations in the ESRI gene have been identified in metastatic lesions from endocrine treated patients (6, 7).

The mechanisms required to enable cancer cells to overcome hostile forces and reinitiate growth at distant organs in endocrine-treated patients have not been fully elucidated. Lessons from in vitro studies and murine models have provided important information about discrete tools used by breast cancer cells to prosper at distant metastatic sites. For example, the CXCR4/SDF1 pairing has been identified as a mediator of metastatic cell survival in the bone (8, 9); transcriptional inhibitors of differentiation, Id1...
and Id3, have been associated with lung metastasis (10, 11); and serpins, as metastatic functionaries in the brain (12). However, to date, no global overview of gene expression alterations that are essential for successful colonization of cells at metastatic sites in breast cancer patients has been undertaken.

To clearly define mediators of colonization in endocrine-treated patients, we undertook gene expression analysis from sequential tumor samples. RNAseq analysis was performed on matched primary, nodal, and distant metastatic tumors from breast cancer patients, all of whom had developed a liver metastasis following tamoxifen treatment. At a functional level, studies in xenograft models of endocrine-resistant tumors were undertaken to examine the role of tamoxifen in the development of metastatic disease secondary to therapy resistance. Over-represented pathways identified by the RNAseq analysis were further studied at the transcript and protein level in an expanded cohort of patients with metastases of multiple distant organs.

Materials and Methods

Gene expression and bioinformatic analysis

Following ethical approval, formalin-fixed paraffin-embedded (FFPE) tumor samples were macrodissected to select regions with >70% tumor cells. RNA isolation was performed with High Pure FFPE RNA Micro Kit (Roche) and subjected to duplex specific thermostable nuclease (DSN) treatment. RNA Sequencing was performed by BGI (Hong Kong) using the Illumina HiSeq 2000 with >40 million reads. Insufficient RNA was obtained from the nodal sample of patient 1 for sequencing. Reads were aligned to the hg19 genome using two different types of analysis: one for the differential expression and the other for use with The Cancer Genome Atlas (TCGA) data. For the differential expression analysis, the reads were aligned using TopHat and genes were quantified using HTSeq. For usage with TCGA data, the data were aligned using MapSplice and the genes were quantified using RSEM. Full details of bioinformatics analysis are provided in Supplementary Methods. Data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE58708.

cDNA was synthesized as previously described (13). Gene expression was assessed using TaqMan technology (Applied Biosystems) on the ABI PRISM 7500 platform with the following probes: EPPK1 Hs01104050_s1; COL4A2 Hs01098873_m1; COL4A1 Hs00266237_m1; SPDEF Hs01026050_m1; KRT19 Hs00761767_s1; AHDC1 Hs00210424_m1; NFCOR2 Hs00196955_m1; AKAP8L Hs00205106_m1. EF2B8 Hs00426752_ml was used as an internal control. The comparative C(T (ΔΔC(T)) method was applied to analyze relative mRNA expression levels.

Results

Gene expression profiles of breast cancer patients reflect the histopathology of the original primary tumor
RNA was extracted from matched breast cancer primary, node, and liver metastatic tumor tissue from three tamoxifen-treated luminal breast cancer patients. The patients represented the...
spectrum of the luminal classification, from luminal A (patient 1) to luminal B2 (patient 3; Table 1). Despite the divergent pathology of each of the primary breast tumors (Table 1; Fig. 1A; Supplementary Fig. S1), positive steroid receptor status qualified each of the patients for adjuvant endocrine treatment.

Correspondence analysis of the sequenced RNA from the individual tissue samples revealed that each of the sequential patient samples clustered together, rather than with the metastatic site (Fig. 1B). The relative divergence in gene expression from the primary to the metastatic tumors was reflective of the disease latency period. Patient 1 with a recorded metastasis-free survival period of 11 years had the greatest difference in gene expression profile between the primary and metastatic tumors (Fig. 1B). Patient 2 and patient 3 had shorter disease-free periods and correspondingly substantially less alterations in gene expression, suggesting that some of the genes necessary for successful progression are present in the primary tumor.

RNAseq data from the breast cancer sequential samples were analyzed with breast cancer samples from the TCGA by correspondence analysis of almost 16,000 genes (Fig. 1C). Nodal and metastatic samples clustered in amongst the primary tumors. PAM50 genes were used to classify the breast cancer sequential samples and the TCGA samples into the major breast cancer subtypes. Tumors from patients 1 and 2 straddled the luminal/HER2 border. Interestingly, while the primary tumor from patient 1 was luminal A, the metastatic tumor from this patient was classified as luminal B.

Metastatic gene expression alterations are heterogeneous in endocrine-treated breast cancer patients

To define the changes involved in disease progression, differentially expressed genes (DEG) were identified between matched primary and liver metastatic tumors from each patient. This analysis identified 1,268 upregulated and 2,606 downregulated genes between matched primary and metastatic tumors. Remarkably, less than 3% of DEGs were common to all three patients highlighting the extent of patient heterogeneity (Fig. 2A). Only 31 upregulated and 69 downregulated genes were shared by the 3 patients (Supplementary Tables S1 and S2). The individuality of each patient is evident in the heatmaps of DEGs where each patient elevated their own unique set of genes in the liver metastatic sample (Fig. 2B). Of note, little difference in gene expression was detected between the primary tumor and the nodal metastases, both of which were treatment naive and were resected at the time of initial surgery (Fig. 2B).

Analysis of enriched KEGG pathway terms was performed for the DEGs of each patient and highlighted a number of important pathways that were unique to each patient (Supplementary Fig. S2 and S3). The luminal A tumor from patient 1 was highly endocrine dependent with strong expression of both ER and PGR. The large divergence from primary to metastasis on correspondence analysis as well as the large number of differentially expressed genes is indicative of the multiple adaptations that were required for successful tumor progression and colonization. Following endocrine treatment, steroid receptor status remained strong; however, alterations in growth factor signaling networks, including MAPkinase, Jak-STAT, and ErbB signaling pathways were...
observed (Supplementary Fig. S2). As protein function cannot always be directly inferred from gene expression analysis, expression of phosphoproteins from these pathways was examined and confirmed in matched primary and metastatic tumors from a luminal A patient (Supplementary Fig. S4). In contrast with patient 1, the luminal B1 primary tumor from patient 2 displayed limited endocrine dependence, with low steroid receptor expression and no HER2. Limited divergence on correspondence analysis and relatively low number of gene alterations suggest that the genes required for metastatic progression may have been present in the primary tumor. Pathways that were altered in the liver metastatic tumor include DNA replication and cell cycle (Supplementary Fig. S2), also confirmed at the protein level (MCM4; Supplementary Fig. S4). The luminal B2 tumor from patient 3 was strongly positive for ER, PGR, and HER2. From correspondence analysis, genes from the primary, node and liver metastasis clustered with the HER2 subtype using the PAM50 classification. Alterations in the WNT signaling pathway observed in the metastasis, at both the gene and phosphoprotein level (Supplementary Fig. S2 and S4), signify tumor adaptation to endocrine therapy and activation of the epithelial to mesenchymal transition network.

Of note, the mTOR pathway, which is a known predictor of endocrine resistance (14), was found to be elevated in the liver metastases of patients 1 and 3 at the transcript level in comparison with the primary tumor (Supplementary Fig. S2). Expression levels of phospho-mTOR and phospho-P70S6 kinase in matched primary and metastatic tumors from endocrine-treated patients confirmed activation of the mTOR pathway (Supplementary Fig. S5).

Figure 1. Molecular profiling of matched tumor samples from 3 heterogeneous breast cancer patients. A, IHC staining of classic biomarkers in primary breast tumor tissue (top) and H&E staining of matched liver metastatic tumor tissue (bottom) showing strong heterogeneity between the 3 patients. ER, estrogen receptor; PGR, progesterone receptor; H&E, hematoxylin and eosin staining. B, correspondence analysis of RNAseq data from primary (P), nodal (N), and metastatic liver (M) tumors of the 3 patients. Patient heterogeneity is evident and tumors do not cluster based on site of origin. Patient 1 shows the largest divergence between primary and metastatic tumors. C, correspondence analysis of RNAseq data from the primary, nodal, and metastatic samples from the 3 patients (n = 8) and breast cancer patient samples from the TCGA (n = 840). Each dot or shape represents an individual tumor, colored based on PAM50 profiling.
Figure 2.
Matched primary and metastatic tumors display altered expression of genes involved in cell–cell interactions and cancer pathways. A, Venn diagrams showing DEGs for each individual patient between their primary and metastatic tumors. Left, genes upregulated in metastatic tumor relative to matched primary tumor. For all of these genes, expression in a normal liver sample was less than the primary tumor. Right, genes downregulated in metastatic tumor relative to matched primary tumor. DEGs exhibited a fold change $>1.5$ and $>50$ counts per million. B, heatmaps displaying the 1268 upregulated (left) and 2,606 downregulated (right) DEGs from part A. Each patient displayed a distinct pattern of gene expression as highlighted. C, network maps showing enriched KEGG pathways from the upregulated (left) and downregulated (right) DEGs. Details of pathways unique to each patient are provided in Supplementary Figs. S2 and S3. Enriched KEGG pathways common to all 3 patients include a number of cancer pathways and also cell interaction pathways such as focal adhesions and ECM receptor interactions.
Figure 3.
Tamoxifen contributes to the development of metastasis secondary to endocrine resistance. A, representative in vivo bioluminescence images of mice following orthotopic injection of luciferase expressing endocrine resistant breast cancer cells. (Continued on the following page.)
Cell-to-cell communication is a common pathway in liver metastasis

Although at the gene level, patient heterogeneity was very apparent in the endocrine-resistant metastatic tumors, a number of functional pathways were common to all three patients (Fig. 2C). Alterations in several cancer pathways, including small cell lung carcinoma, renal cell carcinoma, and pathways in cancer were observed. Of interest, pathways important in cell to cell interaction were represented in gene sets that were both enhanced and suppressed in the metastatic liver. Alterations in genes important in ECM receptor interactions and focal adhesion indicate the importance of communication between the tumor and the host liver for successful colonization.

Endocrine treatment contributes to the development of metastasis secondary to endocrine resistance

Of the sequential tumor samples sequenced here, primary and nodal tumors were biopsied at the same time. Liver tumors were biopsied following a latency period and also following treatment intervention. To determine the impact of endocrine treatment on the development of distant metastasis in endocrine-resistant tumors, we used a xenograft model. Endocrine-resistant cells were implanted into the mammary fat pad of immune-compromised mice in the presence of estrogen (Fig. 3A). Once established, the primary tumors were resected. Local disease recurrence occurred in animals treated and untreated with the SERM tamoxifen. Distant metastasis, however, was only established in the tamoxifen-treated group (Fig. 3A). Metastatic disease was observed in the bone, lung, and liver (Fig. 3B). Expression of the proliferation marker, Ki-67 and keratin 19 (KRT19), a marker of disseminated cancer cells, which was elevated in all of the patient liver metastases, was found to be enhanced in the xenograft liver metastatic tissue compared with the primary (Fig. 3B). These data demonstrate that tamoxifen has the potential to contribute to the development of metastatic disease progression in endocrine-resistant tumors. Analysis of the patient RNAseq data revealed that the majority of the identified upregulated DEGs were in fact elevated in metastatic tumors relative to both matched primary and nodal tumors (Fig. 3C). These data suggest that for the majority of these genes, expression did not increase when the cells first moved away from the primary site to the node but did increase following treatment with tamoxifen, the latency period, and intravasation of the distant organ. Remarkably, of the common DEGs displaying this pattern of expression (Supplementary Table S3), almost half have defined roles in cell-to-cell communication and cell structure (Supplementary Fig. S6).

Cellular communication and cell structure proteins are evident at other metastatic sites

An extended cohort of matched primary and metastatic patient samples was used to examine the RNA and protein expression of shared genes from the RNAseq data. The patient cohort included a range of metastatic sites, including the liver, lung, bone, and peritoneum of endocrine-treated patients (Supplementary Table S4). Real-time PCR confirmed the elevated expression of genes in liver metastases relative to matched primary tumors (Fig. 3D). Increases in gene expression were also detected at other metastatic sites (Fig. 3D).

At the protein level, IHC analysis of matched primary and metastatic tumors revealed strong keratin 19 protein expression, consistent with that observed in the metastatic tumors from the tamoxifen-treated xenografts (Fig. 3E). Protein expression of the cellular communication proteins, lamin B2 (LmNB2), KIF12, and envoplakin (EVPL) were confirmed in the metastatic tumors of the endocrine-treated patients. Greater expression of these proteins was found in the metastatic tumor cells in comparison with the surrounding normal host tissue (Fig. 3E). Furthermore, analysis of unmatched metastatic brain datasets revealed enhanced expression of our defined 31 gene set in the metastatic tumors compared with normal brain tissue (Supplementary Fig. S7). Taken together, these data support a role for cellular communication in breast cancer colonization of distant organs following treatment resistance.

Discussion

The ability of selected tumors to adapt and evade endocrine therapy is well established. Enhanced expression of plasticity networks (4, 15, 16), as well as mediators of EMT (17, 18), in the primary tumor has been associated with treatment failure. Information about changes that occur with tumor progression on treatment, however, is not readily available. Here, we describe the first global transcriptomic analysis of sequential primary and metastatic tumor samples from endocrine-resistant breast cancer patients.

Several initiating events of tumor adaptability have been proposed, including the degree of intra-tumor heterogeneity (19, 20) as well as the presence of stem cell populations (21). Though the source of the treatment adaptability is not addressed in this study, the resultant mediators that enable the successful colonization of the tumor at a distant site are elucidated. Here, global mapping of gene expression alterations that occur in metastatic breast cancers revealed a significant degree of patient...
divergence in gene expression was observed in the ER-positive tumors of patient 1 and the metastasis-free survival period of >10 years. Differences in gene expression suggest that the primary tumor underwent significant alterations over an extended period of time to successfully develop liver metastasis. In contrast, the low ER-positive, PGR/HER2-negative tumor (patient 2) displayed relatively little alteration in gene expression between the primary and the metastatic tumors. These data suggest that the primary tumor from patient 2 may harbor metastatic progression genes (8), which could have altered functions at the primary and metastatic sites. The extent of divergence in gene expression between primary and metastatic tumors would therefore appear to be inversely related to the aggressiveness of the primary tumor.

Signal pathways enriched at the metastatic site were also patient specific and closely related to the biology of the primary tumor. The ER/PGR/HER2-positive patient (patient 1) displayed elevations in plasticity networks, including the WNT signaling pathways, whereas the ER/PGR-positive, HER2-negative patient (patient 1) had enhanced growth factor signaling. Increases in de-differentiation signals and in particular induction of steroid receptor/growth factor receptor cross-talk have been well described as mechanisms of tumor adaptability to endocrine treatment (16, 25, 26). Furthermore, elevations in key components of the mTOR pathway were observed in both patient 1 and patient 3 in metastatic liver tissue. Activation of the mTOR pathway has previously been associated in endocrine resistance (14, 27) and elevated expression of p-mTOR, p-4EBP1, and p-p70S6K has been reported in metastatic tumors in comparison with matched primary tissue (28).

The impact of sequential endocrine therapy in the treatment of endocrine-resistant disease in patients, however, remains to be fully clarified. Though several clinical studies suggest that some metastatic patients respond to continued endocrine treatment, a subset of patients shows little or no benefit (29–31). Here, using endocrine-resistant xenografts to model sequential treatment strategies, we found that the development of distant metastasis was dependent on continued tamoxifen treatment. Though the molecular mechanisms driving this continued resistance have not been described in date, ex vivo studies demonstrating gain-of-function mutations in breast metastatic patient tumors following endocrine treatment suggest that ER may remain an important player (7). In this study, the majority of DEGs were elevated in the metastatic tumors relative to both the primary and the node, with little alteration observed between the primary and the matched nodal tissue. The gene expression changes that occur during the latency period, under the pressure of treatment are therefore those that are important for the establishment of distant metastatic disease.

Though several elegant studies have used in vitro and in vivo models to determine key players in breast cancer colonization of distant organs, including the bone, lung, and brain (8–12), there is little information about signaling networks important in metastatic liver disease. In this study, common pathways elevated in liver metastatic tumors included pathways in cancer, ECM receptor interactions, and focal adhesions. Extracellular matrix proteins have previously been shown to be important in colon cancer liver metastasis (32), moreover enhanced Claudin 2-extracellular matrix interactions have been demonstrated in liver-aggressive breast cancer in vivo models (33). The ability of breast tumor cells to communicate with and affect host cells would therefore appear to be essential to its ability to colonize and survive at a distant site.

Elucidation of differential gene expression in metastatic tumors is a key step to understanding the complex mechanisms controlling tumor adaptability. Here, we define gene expression alterations that occur in endocrine treated metastatic liver tumors. These global studies build on model systems and clinical observations, which along with further metastatic sequencing studies will enable rational personalized treatment strategies to be developed for endocrine-resistant breast cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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