CANCER GENOMICS & PROTEOMICS 9: 297-310 (2012)

Review

Breast Cancer and Metastasis: On the Way Toward Individualized Therapy

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Abstract. *Breast cancer (BC) remains the most common cancer type diagnosed in women. Although targeted therapies have improved patient survival for advanced BC, these tumors frequently relapse due to drug resistance mechanisms. A systems biology approach integrates DNA, RNA and protein alterations generated from multidimensional platforms to better understand the mechanisms that regulate the metastatic process. Downstream functional analyses in pre-clinical studies might integrate the role of these aberrations into the cell, leading to discovery of new therapeutic targets. In the present report, we review relevant findings associated with genomic, transcriptomic and proteomic analyses and the contribution of the systems biology concept to the interpretation of these data in the metastatic context. Also, we highlight the importance of re-designing clinical trials towards metastasis prevention for improvement of personalized care.*

In 2012, invasive BC is expected to account for 29% (226,870) of all newly-diagnosed cancer cases and for 14% (39,510) of all cancer deaths in women in USA (1). Although targeted therapies have improved patient survival for advanced BC, these tumors frequently relapse due to drug resistance mechanisms. Some evidence have showed that 30-50% of hormone receptor (HR)-positive BC cases do not respond to tamoxifen therapy (2). HER2-overexpressing BC patients might develop metastases and only 11-34% of metastatic tumors respond to trastuzumab monotherapy (3). Although triple-negative BC (TNBC) patients show about 39% response to chemotherapy, presence of residual disease is associated with early-risk of relapse (4, 5), for which the effects of targeted therapies are currently under investigation in clinical trials (6).

Metastasis is a process characterized by local invasion, intravasion, transport of tumor cells to the parenchyma of other organs, extravasation and establishment of secondary lesions (7). Over the past decade, several reports have provided the scientific community with insights about the molecular mechanisms underlying this process. Evidence indicate that metastasis can originate from genetic and epigenetic alterations in the molecular profile of a subpopulation of cells within the primary tumor, whose behavior is modulated towards a more aggressive phenotype (8). Genetic alterations occur primarily in the DNA sequence (*i.e.* mutations) whereas epigenetic changes are related to the structure of the chromatin and might involve DNA methylation, histone modifications and non-coding RNAs, including microRNAs (9).

A systems biology approach has been employed to explore the functional relationships among genomic, transcriptomic and proteomic alterations during BC progression. In this context, integrative analyses enable studying of the functional role of these aberrations at the cellular level and, therefore, they might represent powerful tools to identify new therapeutic targets as well as prognostic and predictive molecular markers that might be useful to select patients who would beneficiate from a specific treatment. Additionally, these analyses have also been widely employed to identify new cancer biomarkers for early diagnosis and monitoring of response to treatment. Therefore, integrative analyses from multidimensional "omics" technologies have been demonstrated to be important for treating the patient in a personalized way, which might increase the therapeutic efficacy and delay tumor progression (10). Also, clinical trials must be re-designed towards metastasis prevention (11), in order to provide the patient with a personalized therapy in early

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Key Words: Breast cancer, metastasis, individualized therapy, review.

stages of the disease, preventing progression of tumors with high risk of developing metastatic disease.

In this review, we summarize the recent findings related to the identification of potential molecular parameters of BC progression towards metastasis, including the relevance of the interactions of tumor cells with the microenvironment. For these studies, the "omics" technologies have been widely used in an attempt to explore the mechanisms involved in these processes under a systems biology perspective, and, therefore, they have led to the identification of molecular markers required for predicting response to therapy.

Genomics of Metastatic BC

DNA alterations

DNA copy number. Several studies have correlated genetic alterations present in primary tumors with the time in developing metastasis, in an attempt to identify a set of genes that predicts the metastatic ability of BC. Results from array comparative genomic hybridization (aCGH) have demonstrated that copy number alterations (CNA) of primary breast tumors had a higher rate of copy number losses in patients who developed axillary lymph node metastases, compared to those who did not. Metastasizing tumors showed 186 amplified or deleted regions compared to non-metastasizing tumors, including those located on chromosomes 7p, 16q and 18q. Additionally, the gene *TSPAN1* (on 1p34.1) was deleted in metastasizing tumors and might represent an important tumor suppressor gene (12). Oligonucleotide-based Single-nucleotide polymorphisms (SNP) array technology has higher resolution compared to aCGH for mapping CNA and it has been used to identify a copy number signature (CNS) that predicts patient prognostic outcome. Considering those genes whose copy number alterations were significantly concordant with changes in gene expression, this methodology has enabled scientists to define a set of 81 genes that was used to construct a CNS with high accuracy in stratifying primary tumors in subgroups with different times to metastatic relapse (13).

Comparative analyses between primary BCs and metastasis represents other approaches for establishing a copy number signature for BC progression. These analyses have demonstrated that genetic alterations predisposing to the metastatic potential might be detected in the primary tumor. Compared to non-metastatic invasive ductal carcinoma (IDC), both metastatic IDCs and their metastases showed a unique pattern of copy number alterations, including gains at 2p24-13, 2q22-33, 9q21-31, 12q21-23, 17 q23-25 and loses at 11q23-ter, 14q23-31, 20p11-q12, 2q36-ter, 8q24-ter, 9q33-ter, 2p11-q11, and 12q13 (14). Another study has demonstrated frequent copy number variations between axillary lymph node metastasis and primary tumors, including aberrations at 6q15-16, a region containing the gene *PNRC1* (a putative tumor suppressor) and at 9q31.3-33.1, where the genes *DBC1* and *DEC1* (regulators of apoptosis) are located (15).

DNA mutations. Mutation analyses have been used to verify the putative role of certain mutations in the function of known oncogenes or tumor suppressor genes throughout metastasis. Comparative analyses between primary and metastatic tumors have shown high frequency of *TP53* mutations during BC metastasis to the brain. The results included mutations that originated from primary tumor and were propagated by clonal expansion or represented completely new alterations in brain metastasis (16).

Single-nucleotide polymorphisms (SNPs) related to the PI3K/Akt signaling pathway have generated interesting findings about the role of these alterations during metastatic progression. The role of this pathway in promoting tumor progression might lead to the assumption that activating mutations of this gene might occur more frequently in the metastasis compared to the primary tumor. Nevertheless, the proportion of patients with activating *PIK3CA* mutations found exclusively in the metastasis was similar to that of patients with activating mutations found uniquely in the primary tumors, suggesting that *PIK3CA* mutations might represent stochastic alterations rather than drivers of metastatic progression (17). Inherited polymorphisms on *PTEN* promoter (a suppressor of PI3K/Akt pathway) have been associated with higher frequency of BC metastasis at diagnosis. Additionally, the gene expression profile in breast tumors from *PTEN* variants carriers, compared to non-carriers showed a set of differentially expressed genes that stratified the patients into two subgroups with different recurrence-free survivals (18).

Genetic variants related to other genes exerting oncogenic roles during BC progression have also been investigated. The polymorphism rs6983267 at 8q24 has been associated with a higher risk for metastasis in inflammatory BC, which might be probably due to de-regulated expression of MYC, since the variant has been suggested to be a cis-regulatory enhancer element of this gene (19).

Interesting findings have been reported by Genome-wide association studies (GWAS), which represent international efforts to identify new markers of BC progression. This approach is based on simultaneous analyses of SNPs in different loci of the human genome and subsequent evaluation of their association with BC risk and prognosis (20). These analyses unraveled the association of the SNP rs3784099 located on chromosome 14 in intron 7 of the *RAD51L1* gene with mortality and recurrence in BC patients. Also, this gene is an established cancer susceptibility gene and encodes a protein essential for DNA repair, which highlights the relevance of these findings (21). Another genome-wide association analysis identified three new BC

susceptibility loci: rs10771399 (at 12p11), rs1292011 (at 12q24), rs2823093 (at 21q21). Two of these alterations lie in regions containing genes related to BC progression: *PTHLH* (12p11), involved in bone metastasis in BC and *NRIP1* (21q21), a regulator or ER signaling and BC cell growth. Although this study has focused on identifying new BC risk loci, these data are suitable for further investigation for elucidating whether these alterations might also be related to metastatic progression (22).

Next-generation sequencing (NGS). Next-generation sequencing (NGS) represents a powerful tool to understing cancer. Differently from array analyses and single-gene sequencing, this approach has focused on whole-genome and has been used to detect chromosomal re-arrangements, somatic mutations and copy number alterations in solid tumors, with a resolution of up to a single base amplification or deletion (23).

This technology has been employed to compare molecular changes between primary breast tumors and brain metastases in order to understand the mechanisms involved in the metastatic process. In a lobular breast tumor, 32 mutations were found in the metastasis. Out of these 32 alterations, 19 *de novo* mutations were detected, since they were not detected in the primary tumor (24). In a basal-like breast patient, 80% of copy number alterations detected in metastasis overlapped with those found in the primary tumor. Additionally, out of the 50 mutations validated, 48 were detected in both primary tumor and brain metastasis and only two *de novo* mutations (genes *SNED1* and *FLNC*) were detected exclusively in the metastasis (25). Altogether, these studies revealed that although additional copy number variations and mutations might occur during the metastatic progression, most of the alterations originated from the primary tumor, are propagated in the metastasis.

Some works have attempted to minimize the bias introduced by genetic tumor heterogeneity, which complicates the understanding of molecular changes associated with tumor evolution. Flow-sorted nuclei technique from primary and metastatic tumors and single-nucleus sequencing (SNS) have been employed to study the population structure of a primary tumor. Copy number variation analyses (CNV) showed that the tumor is composed by distinct subpopulations that share genomic similarities but have also diverged and contain unique attributes compared to each other. Moreover, the results showed that a specific subpopulation might travel to distant sites and expand to establish a metastatic site. According to these data, the authors proposed a mechanism of "punctuated clonal evolution" for metastatic progression, whereby a single clonal expansion forms the primary tumor and seeds the metastasis (26).

DNA methylation. Methylation alterations between primary tumors and metastases have also been reported for groups of known cancer-related genes. A recent work revealed the higher methylation proportion of 12 BC candidate genes in the primary BC compared to normal tissue from the same patient. Out of the 12 genes, *BMP6*, *BRCA1* and *P16* showed higher methylation proportions in the matched lymph node metastasis compared to the normal tissues and, therefore, might represent useful biomarkers for metastasis screening (27).

Genome-wide profiling of DNA methylation has been performed in primary breast tumors and revealed genes whose hypermethylation was significantly correlated with relapse-free survival, including *RECK*, *SFRP2* and *ACADL*. Tumor specificity of methylation was confirmed for these genes by sequencing of an independent set of normal/breast tumor samples. Moreover, methylation of *RECK* has been observed in other cancer types and its reduced expression has been associated with worst prognosis (28). Genome-wide analysis has also been employed to characterize the DNA methylation profile of primary BCs with different metastatic potential. A global breast CpG island methylator phenotype (B-CIMP) was identified as an epigenetic profile associated with low risk of metastasis. Parallel gene expression analyses identified genes with both significant hypermethylation and down-regulation in B-CIMP tumors, including those involved in epithelial-mesenchymal transition (EMT), such as *LYN*, *MMP7*, *KLK10* and *WNT6*. Also, the genes in the B-CIMP repression signature showed genes whose differential expression correlated with prognosis across several BC cohorts (29).

Gene expression profile

Gene expression profile of primary breast tumors has been demonstrated to generate a specific gene signature associated with prognostics and outcome. The 70-gene prognostic signature established by van't Veer *et al*. (30) represented a pioneer strategy in identifying a set of gene predictors of short time to develop distant metastases. These genes outperformed conventional parameters for determining tumors more likely to metastasize (*i.e*. as lymph node status), since the patients had no tumor cells in their lymph nodes at diagnosis (30). Another study analyzed 107 primary breast tumors in patients who experienced relapse and revealed a set of 69 differentially expressed genes among tumors that relapsed specifically to the bone and tumors that relapsed elsewhere. Enriched functional pathways associated with this profile included genes that might participate in adhesion processes required for bone invasion (*RND1*, *TSPAN1*, *ANXA9*) and FGF signaling pathway (31).

Comparative analyses between primary tumors and metastasis have revealed that the gene expression profile is maintained throughout the metastatic progression, which replicates the results generated from DNA analyses. For

instance, the 70-gene prognostic signature associated with poor prognosis in primary tumors (described above) was also present in their matched metastases (32). Other works have demonstrated genetic differences between primary tumors and distant metastases. Although gene expression analyses have uncovered few differences between primary tumors and lymph node metastases, distant metastases were distinguished from both groups by high expression of genes involved in the VEGF signaling pathway, including *ANGPTL4* and *ADM* (33).

Proteomics

Protein expression profiling has been used in an attempt to understand how genomic changes might integrate at the protein function. Additionally, this approach might lead to the identification of putative prognostic biomarkers. Mass spectrometry was used to identify differential protein expression between primary breast tumors associated with lymph node-positive (LN+) and lymph node-negative (LN-) states. The results were validated by tissue microarrays (TMAs), which demonstrated that the expression levels of HSP90B1 and DCN were significantly correlated with presence of LN metastasis. Additionally, in an independent cohort of BC patients, high expression of DCN was associated with higher risk for lymph node metastasis and worse overall survival whereas high expression of HSP90B1 was correlated with higher risk of developing distant metastasis, decreased overall survival and predicted better response to hormonal therapy (34).

Protein analyses have been described for 38 invasive ductal carcinomas corresponding to N0, N1 and N2 stages of lymph node metastasis. Differential protein expression for each stage was established by comparing the cancerous to normal breast tissue. As expression levels of caltireculin and tropomyosin alpha-3 chain were commonly up-regulated in the three stages, these proteins might represent potential biomarkers for diagnosis and treatment for different stages of lymph node metastasis. Other proteins showed altered expression in a specific stage and, therefore, they might be used for classification of N staging. Since up-regulation of PDIA3 was only found in the stages N1 and N2, which correspond to BCs that metastasized, this protein might represent a prognostic marker to indicate the metastatic potential of BCs (35).

Genome-wide protein analyses have also been used to identify proteins differentially expressed between triple-negative BCs with different metastatic behaviors. Results have demonstrated that lymph node-positive BC (high metastatic potential) showed higher expression of Stat1 and the HLA II gamma subunit CD74, compared to the lymph node negative samples (low metastatic potential), and, therefore, these proteins were considered potential therapeutic targets specifically for treating metastatic triple-negative BCs (36).

Integrative analyses

During tumor progression, accumulation of "driver" mutations, *i.e.* those that are key mediators of this process, might be paralleled by "passenger" mutations, which occur only due to genomic instability and, therefore, are not related to putative therapeutic targets (37). Considering that cancer arises from an intricate network of DNA, RNA and protein interactions, information generated from genomic, transcriptomic and proteomic analyses might be integrated to improve our ability of identifying molecular "drivers" that exert key roles throughout tumor evolution and, therefore, might represent potential candidates for drug sensitivity prediction and targeted therapy.

In a large cohort of primary breast tumors, integrated analyses of copy number and gene expression have elucidated some mechanisms, whereby genome variation leads to alterations of the tumor expression architecture. The results revealed a limited number of genomic regions that might contain putative driver genes and provided a novel molecular stratification of BC subtypes. Estrogen-positive tumors that showed 11q13/14 amplification correlated with worse outcome compared to those that did not, which might be due to some genes located in this region: *CCND1*, *EMSY*, *PAK1* and *RSF1* (38).

Multi-platform whole-genome microarray analyses has integrated results from copy number variation, gene expression profiling and methylation to identify differentially regulated pathways between a highly metastatic BC cell line and its low metastatic parental cell line. Networks analyses demonstrated that the genes differentially regulated belonged to the EGFR, TGFB1, NF-kb, ERK and MAPK pathways. The robustness of this method was demonstrated by validation experiments, which confirmed that hypermethylated genes correlated with decreased expression in the metastatic, compared to the parental cell line, including the gene stratifin (*SFN*). Interestingly, this approach seemed to refine the results more precisely, since some of these genes also correlated with decreased copy number (*EGFR*) and, therefore, might represent functionally important drivers of metastatic process (39).

Considering that proteins translate effects of genomic alterations into the biological functions of the cell, analyses integrating protein-protein interactions networks with gene-gene co-expression networks might be an important tool to reveal a BC metastasis-specific signature. Data from six different studies have been used in a meta-analysis to build interaction gene-gene networks, whereby pairs of genes might be interconnected based on their correlation expression measures. Moreover, these connections were maintained just in case a protein-protein interaction related to the pair was reported previously, which should improve the selection of functionally more relevant subnetworks. The results generated a consensus network, containing interconnected

genes involved in cell cycle regulation and commonly found in subnetworks throughout all studies. Moreover, the genes interacting in this network were highly expressed in poor prognosis patients, and, therefore, they might represent putative markers for predicting metastatic relapse (40).

The Cancer Genome Atlas (TCGA). Currently, international initiatives have generated an avalanche of information that has expanded the application of integrative analyses in cancer research. Results generated from whole-genome analyses have been submitted in The Cancer Genome Atlas (TCGA) database, which includes copy number aberrations, DNA methylation and mRNA expression (41). These data might be available for integrative analyses of results generated from a single technology platform.

Gene expression profiles of BC patients might be, in this fashion, correlated with other types of genetic changes, such as copy number alterations, reported in the TCGA database. One study using this approach revealed that increased expression of HSP90AA1 and HSP90BB1 - two different isoforms of the heat shock protein (HSP) 90 - might be driven by chromosomal amplification and associated with worse outcome in triple-negative and HER2–/ER+ BC subtypes (42). Another study evidenced that results generated from whole-genome methylation array analysis of primary invasive breast carcinomas have been also validated using independent datasets extracted from TCGA project. The identification of molecular pathways differentially regulated between ER+ and ER– BCs indicated that this approach might lead to a better understanding about the distinctive biological features between these subtypes (43). Therefore, these studies highlight the therapeutic for relevance of integrative analyses, since it allows identification of possible mechanisms whereby a set of genes might contribute to a more aggressive disease in particular cancer subtypes and predict risk of recurrence, contributing to the design of therapies specifically targeting these genes.

Predictive role in drug sensitivity. The identification of molecular predictors of drug sensitivity represents an attempt to increase the therapeutic efficacy in early stages of the disease, which minimizes the chances of residual disease and metastatic relapse after treatment. This strategy allows for identification of the pathways that mediate tumor survival and, therefore, selecting a drug specifically targeting certain components of these pathways.

Molecular characterization of human cancer cell lines has been described as a relevant tool to better understand the mechanisms that render cancer cells susceptible to a particular treatment. The Cancer Cell Line Encyclopedia (CCLE) is a database that comprises information about mutational status, copy number analyses and gene expression profiles of several cell lines. Integrative analyses using these data might provide insights regarding regulatory networks that drive tumor progression and, when coupled with drug sensitivity assays, they represent a robust method for identifing gene predictors of therapeutic efficacy. A recent work using this approach identified the *SLFN11* gene as a predictor of topotecan sensitivity in BC as well as other cell lines (44).

MicroRNA target prediction. The identification of microRNA targets has been extensively explored to unveil their regulatory role in drug resistance and tumor progression towards metastasis, since different microRNAs have been described to exert oncogenic or tumor suppressor functions. Nevertheless, microRNA target prediction remains challenging since a single molecule regulates hundreds of genes. Therefore, some studies have employed integrative analyses in human cancer cell lines to overcome these difficulties, thereby improving our knowledge on how microRNAs exert their biological functions.

In this context, DNA copy number analysis has been demonstrated to be able to identify networks differentially regulated between drug-sensitive and -resistant BC cells when integrated to mRNA and microRNA expression profiles. This strategy resulted in the identification of miR-505 as a tumor suppressor, whose genomic region was found to be deleted in doxorubicin-resistant cells. The suppressive role of miR-505 seemed to be mediated by down-regulation of *Akt3* (an anti-apoptotic gene), a predicted target for this microRNA as evidenced by mRNA profiling coupled with downstream validation studies (45).

Integrative approaches combining target prediction algorithms, microRNA screening and proteomic analyses have uncovered the regulatory role of specific microRNAs in the expression of proteins belonging to a particular oncogenic signaling pathway. This approach led to the identification of miR-124, miR-147 and miR-193a-3p as new tumor suppressors of the EGFR signaling pathway in BC cells (46). Associated with microRNA-regulated gene expression analyses, this strategy also has revealed miR-18a, miR-18b, miR-206, miR-193b and miR-302c as potent ERα-regulating microRNAs, since they repressed known estrogen-induced genes (47).

These data demonstrate the successful application of integrative analyses for tumor microRNAs targeting prediction in order to understand how they might exert their biological functions in BC. These findings have therapeutic implications, since they could guide the development of new treatments based on microRNA expression in the future.

Functional genomics

Although integrative analyses have improved our ability to identify significant molecular aberrations in BC patients, results from these approaches require downstream functional assays in order to distinguish “drivers” and “passengers” events occurring throughout metastasis.

In vitro models. Short-hairpin RNAs and small-interfering RNAs (siRNAs) introduction in cell lines enable the use of loss-and-gain of function to evaluate the biological roles of the alterations observed in patients in the cellular context (48). Considering that cyclin D1b expression is associated with poor prognosis in BC patients, effects of its knockdown by siRNA has confirmed its biological relevance, since it led to increased apoptosis as well as inhibition of proliferation and transformation of BC cells. The role of *COX2* in brain metastasis was evidenced upon its knockdown in human brain endothelial cells (HBECs), which reduced the transmigration of BC cells through the blood-brain barrier (BBB) (49).

Cell lines transfected with microRNAs have validated the regulatory role of these molecules in several biological processes. This approach confirmed miR-519c as a putative suppressor of ABCG2 expression, which contributes to mitoxantrone resistance in BC cell lines (50). Ectopic expression of miR-128 has evidenced its therapeutic role since it improved chemotherapeutic sensitivity of breast tumor-initiating cells (TICs) *via* repression of Bmi-1 and ABCC5 genes (51). The tumor suppressor role of miR-451 has been demonstrated since its overexpression restored the growth-inhibitory effects of tamoxifen in endocrine-resistant cells *via* suppression of 14-3-3ζ, a protein whose expression correlates with early relapse of ER+ BCs (52).

Other studies have demonstrated the role of microRNAs in metastatic progression. Transfection studies with miR-203 have demonstrated its ability to reduce migratory and invasive properties of BC cell lines by suppressing SNAI2 expression, which is a mediator of the epithelial-mesenchymal transition (EMT) required for cellular acquisition of metastatic potential (53). The expression of miR-200c has also been described as a therapeutic approach to suppress EMT, since it decreases the expression of genes related to cytoskeletal organization, such as *FHOD1* and *PPM1* (54).

In vivo models. Although tri-dimensional cell culture systems have improved the ability of mimicking the real situation *in vivo*, animal models are definitely required for further investigation, since they offer the opportunity to investigate other important aspects related to tumor progression, such as interaction of the tumor cells with the surrounding microenvironment. In this context, *in vivo* models have been demonstrated to be useful in identifying genes directly related to the metastatic process.

For this purpose, pleural effusion of patients with advanced disease might be injected into mice for consecutive rounds of *in vivo* selection of cells that preferentially infiltrate the brain. Gene expression profiling of these cells and clinical tumors has identified *COX2*, *EGFR* and *ST6GALNAC5* as putative mediators of metastatic process. Functional validation of these genes included the knockdown of *ST6GALNAC5* in the brain metastatic cells, which resulted in inhibition of their adhesion to a monolayer of brain endothelial cells *in vitro* (55).

In vivo models also represent a successful approach for functional validation of genes potentially related to metastasis. In mouse models of BC bone metastasis, knockdown of the genes osteopontin (*OPN*), bone sialoprotein (*BSP*) (56), resulted in reduced bone-metastatic lesions. Silencing of heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) indicated the therapeutic role of heparin-like polysaccharides (57) in reducing bone metastasis by interfering with TGFB-induced IL-11 expression. Another xenograft model has elucidated the critical role of the Myc-Skp2-Miz1 transcriptional complex in RhoA-mediated cancer metastasis, since the knockdown of each gene reduced RhoA protein expression and BC metastasis to the lung (58).

Functional screening. In addition to their importance in validation studies, *in vitro* and *in vivo* models also represent relevant tools when used as the first attempt to identify new therapeutic targets. For these studies, the identification of a potential therapeutic target might be carried out by analyzing the effects of specific microRNAs and interference RNA (siRNA) transfections on cellular behavior.

Co-transfection of a microRNA library with a luciferase reporter plasmid carrying the 3' UTR sequence of estrogen receptor α (ERα) mRNA, has identified miR-22 as a pivotal supressor of ERα-expression in a cell-based system (59). Human BC cell lines transfected with a microRNA library and subsequently treated with TRAIL have led to discovery of microRNAs regulating TRAIL-induced apoptosis. Caspase-3 activation assays indicated let-7c and miR-7 as potential therapeutic targets since they enhanced TRAIL-induced apoptosis (60).

Small-interfering library screening has suggested polo-like kinase-1 (PLK1) as a potential therapeutic target for the treatment of TNBC. Suppression of PLK1 by siRNA in a TNBC cell line specifically reduced the tumor-initiating cells (TIC) subpopulation, whose presence has been associated with metastatic relapse of these tumors after chemotherapy (61). Similar approaches have unraveled the role of lemur tyrosine kinase-3 (LMTK3) as a potential mediator of ER+ activation in BC (62). Additionally, targeting of insulin receptor (InsR) and insulin-like growth factor-I receptor (IGF-IR) (63) has been reported as a novel strategy to reduce resistance to endocrine therapy.

In vivo RNAi screening has been described as an innovative methodology to identify new functionally relevant tumor suppressors. In this approach, human mammary epithelial cell lines transduced with a genome-wide shRNA library are injected into mouse mammary fat pads and genomic DNA sequencing of the tumors enables identification of those genes whose silencing led to tumor formation. The results revealed integration of shRNA

targeting leukemia inhibitory factor receptor gene (*LIFR*) in tumors from multiple mice, suggesting this gene as a putative tumor suppressor (64).

Metastasis and microenvironment

Tumor-microenvironment interactions strengthen the invasive potential of BC, promoting BC progression and metastasis. Therefore, efforts have been made to discover molecular mediators of these interactions, which might represent new targets for anticancer therapy.

The hepatocyte growth factor (HGF) has been described as an important mediator of angiogenesis, which is necessary for BC progression. Primary breast endothelial cells (BRENCs) have been demonstrated to induce EMT in breast epithelial cells co-cultured in a 3D environment and these interactions seemed partially mediated by HGF production (65). Furthermore, HGF has also been described as a potent regulator of resistance to antiangiogenic drugs, such as VEGFR2 inhibitors. Accordingly, carbozantinib (a dual VEGFR2/MET inhibitor) suppressed tumor growth and metastases in a human BC xenograft model (66). The establishment of bone metastases has been reported to be mediated by HGF originating from the microenvironment, which increases the invasive potential of bone-metastatic cells by activation of the β-catenin/Wnt pathway (67).

Other mediators of tumor-microenvironment interactions include sphingosine-1-phosphate (S1P), a lipid produced by the enzyme sphingosine kinase 1 (SphK1). Inhibitors of SphK1 have been described to suppress angiogenesis and lymphangiogenesis in a murine model of BC metastasis (68). Interestingly, both processes were inhibited not only around the primary tumor but also in lymph nodes distant from the tumor. Consequently, inhibition of SphK1 reduced the occurrence of lymph node and lung metastases, suggesting S1P and SphK1 as potential therapeutic targets for metastatic disease.

Blood-based biomarkers

Frequently, biopsies from tumor tissue cannot be provided in necessary amounts for detailed molecular characterization, which restricts its use for monitoring the response of the patient during the course of treatment. Moreover, the detection of blood-based biomarkers represents an innovative approach for diagnosis and prognosis, enabling for selection of those patients who might benefit from an innovative therapy.

Circulating cell-free nucleic acids. The presence of nucleic acids in the blood might be associated with apoptosis and necrosis of tumor cells in the cancer microenvironment. Secretion has been described as another source of cell-free DNA and active release also might contribute to the amount of circulating nucleic acids. Also, circulating tumor cells (CTCs) and tumor cells present in micrometastases at distant sites (including bone marrow), are thought to release nucleic acids into the blood (69).

Circulating cell-free DNA. Specific circulating cell-free DNA alterations have been detected in the blood of metastatic BC (MBC) patients, such as differential levels compared to healthy individuals, epigenetic modifications and mutations in tumor suppressors and oncogenes. Circulating DNA from the plasma of MBC patients with amplified *HER2* gene in the primary tumor has been shown to predict the response of these patients to trastuzumab in combination with chemotherapy. Patients that had decreased levels of HER2 DNA in the plasma showed better response to treatment and higher overall survival (70).

Serum levels of methylated gene promoter 14-3-3-σ have been demonstrated to distinguish metastatic BC patients from both healthy controls and disease-free patients, who did not develop metastases. Moreover, MBC patients that had a continuous decline of the levels of methylated 14-3-3-σ gene were associated with better prognosis compared to those who showed increased levels throughout successive cycles of chemotherapy. These data suggest 14-3-3-σ to be a putative biomarker for metastasis screening and monitoring of response to treatment (71).

In a recent study, blood-based DNA analyses have demonstrated high accuracy for detecting *PIK3CA* mutations present in the primary breast tumor of patients with metastatic disease. Interestingly, this methodology also led to the identification of *PIK3CA* alterations related to tumor progression, since discordance for *PIK3CA* mutational status was observed between primary early-stage tumor and the blood testing after recurrence (72).

Sequencing of entire genes using blood DNA might be required to improve the detection of progression-related mutations, identifying biomarkers of disease progression and monitoring of response to treatment. Tagged-amplicon deep sequencing (TAm-Seq) of plasma DNA has been described as a powerful technique for sequencing large genomic regions in an attempt to identify and monitor mutations in tumor suppressors and oncogenes. Plasma DNA sequencing of a relapsed ovarian cancer patient led to the identification of a *de novo EGFR* mutation, which had not been found in tumor biopsies collected at the time of the initial surgery. Also, this methodology enabled to determine the origin of metastatic relapse in a patient with synchronous primary tumors. During the treatment of a MBC patient, the detection of 10 plasma tumor-specific mutations correlated with the clinical course, since they showed marked decrease after systemic treatment and increase in parallel to disease progression (73).

Circulating cell-free RNA. Tumor-associated mRNAs in plasma have been associated with a more aggressive phenotype. BC patients with *cyclin D1* mRNA expression

detected in plasma at diagnosis have been associated with shorter overall survival, identifying patients with poor outcome in a group of good-prognosis tumors. Moreover, the presence of *cyclin D1* mRNA also correlated with poor response to tamoxifen therapy (74). Presence of *Bmi-1* mRNA in the plasma of patients with primary breast carcinomas has been associated with poor prognosis, since Bmi-1 expression correlated with advanced stages of disease and shorter overall survival (74).

Although results from blood-based microRNAs biomarkers remains exploratory, their predictive value has been highlighted in studies that have demonstrated differential expression levels of specific microRNAs before the treatment (baseline) in responder *versus* non-responder patients. In this context, miR-210 has been described as a putative predictor of HER2+ BC response to trastuzumab (75), since patients that showed residual disease at the end of the treatment showed higher baseline levels of this microRNA compared to those that achieved complete pathological response (pCR). Early-stage BC patients who would benefit from neoadjuvant chemotherapy (NCT) might be identified by analyzing the baseline levels of miR-122, considering the differential expression of this microRNA between patients who had metastatic relapse compared with those who did not (76).

Protein expression profiling. The protein expression profile also has led to the identification of putative serum biomarkers. The screening of serum from 64 primary BC patients unraveled a 21-candidate protein biomarker signature associated with the risk of developing metastasis. This list included several cytokines (IL-6, IL-18) involved with cell migration, infiltration and angiogenesis, events characteristic of metastatic behavior. The results were validated in an independent cohort, since this signature led to the classification of patients into high- *versus* low-risk for progressing towards MBC (77). In another study, the levels of 25 cytokines were assessed in serum from 200 BC patients, with equal proportions of positive and negative lymph nodes. The results revealed eotaxin, MCP-1 and IP-10 as putative lymph node metastasis markers, since their decreased expression levels were significantly correlated with patients who had more positive lymph nodes. Other cytokines were significantly correlated with the ER and HER-2 status of the tumors, unraveling the role of inflammation in the progression of BC (78).

Differently from this proteomic screenings, other studies have focused on the protein expression analyses of specific molecules. High serum HER-2 extracellular domain (ECD) levels have been associated with a worse disease-free survival and overall survival in primary operable BC patients, thus representing a useful biomarker that might provide prognostic and predictive information besides enabling the monitoring of the effects of therapy during the course of treatment (79). Serum amyloid A (SAA) protein expression has been demonstrated to gradually increase with tumor progression as the levels of this protein were significantly higher in the blood of BC stage IV patients compared to those in stage I. Moreover, the expression of this protein also indicated presence of lymph node metastases and distant metastases (80). Assessment of apoptosis-related proteins in serum of BC patients has demonstrated that pre-treatment MIF levels tended to be higher in patients that did not respond to neoajuvant chemotherapy, unraveling the predictive role of this protein in BC (81).

Circulating tumor cells. The presence of circulating tumor cells (CTCs) in the blood of BC patients has been also investigated as a putative biomarker of disease progression, response to therapy and might be applied to management of early-stage disease, minimal residual disease (MRD) and metastatic tumors. The prognostic value of CTCs as biomarkers has been suggested in clinical trials. Baseline CTC count in 267 metastatic BC patients on first-line chemotherapy predicted progression-free survival (PFS) and overall survival (OS), since higher levels of CTCs at baseline levels and before cycle 2 of chemotherapy were associated with worse PFS and OS (82). Results from other studies indicated the predictive value of CTCs in MBC patients undergoing different therapies. Compared to low baseline CTC counts, high CTC counts were associated with little benefit from endocrine therapy, even if the patients were eligible for this treatment based on the receptor status of their primary or metastatic tumors. Similarly, patients with high CTC counts showed small benefit from chemotherapy alone and demonstrated greater benefit from combination therapy (83).

Some reports have showed that detection of methylated genes might correlate with the presence of CTCs in the blood of MBC patients and, therefore, high numbers of CTCs and circulating methylated DNA might both be associated with more aggressive cancer phenotypes (83). Methylated *APC* and *GSTP1* genes also have been correlated with CTCs in blood and associated with prognosis of patients with advanced BC (84). These data suggest that CTCs might also be analyzed in parallel to cell-free DNA and whether this approach is more powerful to predict prognosis of MBCs, compared to each strategy alone, remains to be explored.

Epithelial-to-mesenchymal transition (EMT). Circulating tumor cells might show a dedifferentiated phenotype, corresponding to the expression of epithelial-to-mesenchymal transition (EMT) and stemness markers. These characteristics might enhance the ability of CTCs to disseminate from primary breast tumors in early stages of the disease and to survive to conventional therapies, leading to treatment failure. The CTCs from patients with primary breast tumors have been

demonstrated to express PIK3α, Akt2 and Twist1 (EMT markers) as well as ALDH1, Bmi-1 and CD44 (stemness indicators). As these markers were also detected in lymph node-negative patients, the detection of CTCs in the bloodstream might be considered more accurate in predicting dissemination of primary BC cells compared to lymph node status (85). Comparison of other EMT-related markers expression in CTCs (Twist and vimentin) between early-stage and MBC patients have demonstrated that the EMT phenotype is more prevalent in advanced stages of the disease, since metastatic patients showed higher proportion of CTCs expressing these markers compared to early-stage patients (86).

Altogether, these data support the idea of improving CTCs capture efficiency by using a panel of mesenchymal markers to detect these cells in the blood. Accordingly, a recent work has demonstrated that circulating tumor cells might escape from EpCAM-based methodology due to EMT (87).

Disseminated tumor cells (DTCs) in bone marrow. Detection of disseminated tumor cells (DTCs) in the bone marrow (BM) at diagnosis has been associated with poor prognosis in BC patients. Nevertheless, BM aspiration is an invasive method and, therefore, some studies have addressed the feasibility of CTCs detection as an alternative approach to indicate BM occult micrometastases. Some of these studies have demonstrated low concordance between CTCs in peripheral blood and DTCs in bone marrow, which might be attributed to inaccurate procedures for selection of CTCs and different mechanisms of dissemination between CTCs and DCTs (87-89). Other studies demonstrated that both CTCs and DCTs demonstrated similar accuracy in providing clinically relevant information (90, 91). In conclusion, more studies are required to bypass technical issues and to provide a deeper knowledge about the mechanisms of cancer dissemination. These results might provide a better explanation about these discordances and confirm whether CTCs might be applied as a replacement method for BM aspiration to detect BM DTCs.

CTCs and treatment failure. Discordance between CTCs expression and primary/metastatic sites has been described in several studies, including ER and HER-2 expression, for which the primary tumor or metastatic tissue were negative and CTCs were positive (92, 93). Discordances have been also found after comparing presence of ER and PR expression between CTCs and the primary tumor. These results might be explained by further clonal evolution of CTCs from the time of diagnosis and acquisition of more EMT-like characteristics compared to primary tumors/metastases (94). Therefore, therapies initially based on the characteristics of the primary tumor might not be effective in late stages of the disease, when CTCs that escaped from therapy can further disseminate and seed new metastases, leading to treatment failure.

Moreover, cellular heterogeneity has been described for CTCs, with ER+ and ER- CTCs coexisting in the same sample (94). Accordingly, single CTC transcriptional profiling has unraveled the metastatic cell diversity of CTC populations, which might guide a selection of a multidrug regimen targeting different subpopulations of CTCs, since a therapy designed for only one CTC population might facilitate grow and spreading of other subpopulations for advanced BC patients (95).

Future directions: a systems biology approach for individualized therapy

BC progression involves a complex regulatory network that enables tumor cells to disseminate from the primary tumor and travel to distant organs, giving rise to lymph node and distant metastases. Comparative genomic profiling of metastases and primary tumors has provided insights about the molecular mechanisms related to the aggressive phenotype of metastatic cells. These results have indicated that metastases might result from the clonal expansion of a subpopulation of cells within the primary tumor whose invasive potential was acquired by mutations, copy number aberrations (CNA) and epigenetic modifications. Nevertheless, most of the mutations in a tumor might be considered "passengers", which makes difficult to identify the "drivers" of BC metastasis.

A "systems biology" approach might be implemented to integrate data generated from multidimensional platforms (*i.e.* DNA, RNA and protein profiles) and improve our ability to distinguish drivers from passenger alterations throughout BC metastatic progression. Moreover, functional assays might lead to a better understanding over how genomic and proteomic alterations integrate into the biological functions within a cancer cell. Consequently, this approach would guide us to design rational therapeutic strategies for specifically targeting molecular pathways, which are altered in individual patients, expanding the concept of "personalized therapy" for BC. The feasibility of these analyses might improve our ability to select the patients who would benefit from a certain therapy.

Combined with non-invasive methods of monitoring response to therapy, such as blood-based biomarker analyses, the personalized therapy requires increased implementation of new drugs to be tested in clinical trials. Nevertheless, the efficacy of these drugs in pre-clinical studies might not be replicated in clinical trials if we do not change our way of performing clinical research. Most of the drugs demonstrated to be efficient in animal models might not show the same efficacy in clinical trials because the patients enrolled in these studies have already a more advanced disease. A new clinical trial design is needed to test for metastasis prevention compounds, since treatment of an established metastatic disease has high risk of failure. Patients with high risk of

developing metastases might be enrolled in clinical trials focused on metastasis prevention instead of shrinkage of a pre-existent metastatic tumor (11).

Conclusion

BCM involves a complex regulatory network composed by DNA, RNA and protein interactions. For this reason, a systems biology approach must integrate these results to improve our understanding about the molecular mechanisms that drive tumor progression. Functional assays have been demonstrated to be robust methods to validate the results generated from integrative genomics. Along with these evidence, a re-design of clinical trials towards metastasis prevention might be relevant for the development of personalized therapy for metastatic disease in the future.

Acknowledgements

This work was supported in part by Komen for the Cure Catalyst Award KG090341 (AMG), American Cancer Society Research Scholar Grant 121329-RSG-11-187-01-TBG (AMG), and National Cancer Institute through The University of Texas MD Anderson's Cancer Center Support Grant (P30 CA016672).

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Received August 28, 2012
Accepted August 31, 2012