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Cellular iron metabolism in prognosis and therapy of breast cancer

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Abstract

Despite many recent advances, breast cancer remains a clinical challenge. Current issues include improving prognostic evaluation and increasing therapeutic options for women whose tumors are refractory to current frontline therapies. Iron metabolism is frequently disrupted in breast cancer, and may offer an opportunity to address these challenges. Iron enhances breast tumor initiation, growth and metastases. Iron may contribute to breast tumor initiation by promoting redox cycling of estrogen metabolites. Up-regulation of iron import and down-regulation of iron export may enable breast cancer cells to acquire and retain excess iron. Alterations in iron metabolism in macrophages and other cells of the tumor microenvironment may also foster breast tumor growth. Expression of iron metabolic genes in breast tumors is predictive of breast cancer prognosis. Iron chelators and other strategies designed to limit iron may have therapeutic value in breast cancer. The dependence of breast cancer on iron presents rich opportunities for improved prognostic evaluation and therapeutic intervention.

Keywords

iron; breast cancer; estrogen

Introduction

The study of iron metabolism has largely focused on tissues important to the management of systemic iron, such as the bone marrow, liver, macrophages and duodenum. Such studies have greatly increased understanding of how systemic iron is managed, and identified new proteins and pathways involved in maintaining iron balance. However, all mammalian cells require iron, and many of the proteins and regulatory mechanisms that participate in systemic iron regulation are also present in peripheral tissues such as the breast. Like other metabolic pathways, iron homeostatic mechanisms are frequently altered in cancer. In this chapter we focus on perturbations in iron handling in breast cancer, and how this may be used to prognostic or therapeutic advantage. In Part I, we provide a general overview of breast cancer, and in Part II we discuss the many ways in which malignancy-induced changes in iron metabolism contribute to breast cancer. In Part III we conclude with a discussion on how this information might be leveraged in the management and treatment of breast cancer.

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I. Breast cancer

Breast cancer is a prevalent disease, with over 1,300,000 breast cancer cases and 450,000 breast cancer deaths annually worldwide.[1] In 2012, there were over 225,000 new cases and 39,000 deaths in the US (www.cancer.gov). Factors contributing to risk of breast cancer include family history, parity, age at menarch and age.[2]

1. Breast cancer anatomy, pathology, and clinical staging

The breast is composed primarily of milk-producing glands (lobules), ducts that connect the lobules to the nipple, and stroma (fat, connective tissue, blood vessels and lymphatic vessels). The majority of breast cancers arise in the ducts, but a substantial fraction also arise in the lobules, nipple and other parts of the breast. Ductal cancer that remains confined to the ducts is termed ductal carcinoma in situ (DCIS); it accounts for approximately 20% of new breast cancer cases. The preponderance (70–80%) of breast cancer is invasive ductal carcinoma, in which the cancer has broken through the confines of the duct. A variety of less common histologic types of breast cancer exists; these include inflammatory, comedo, medullary, Paget disease and others.[3]

There are a variety of therapeutic options available to women diagnosed with breast cancer, including surgery, radiation, chemotherapy and hormone therapy. To assist in determining optimal treatment course, several clinical and pathological characteristics are evaluated. In addition to evaluation of the histologic subtype of the tumor, patient evaluation includes an assessment of stage as well as molecular characteristics of the tumor.

To determine clinical stage, a TNM system is used, where T indicates characteristics of the primary tumor (size, inflammatory character, etc.); N evaluates involvement of regional lymph nodes; and M indicates the presence of distant metastases. Through the use of these criteria, patients are divided into stages that range from least (Stage 0) to most advanced (Stage IV), with Stage 0 indicating carcinoma in situ with no metastases and Stage IV indicating the presence of distant metastases.

2. Molecular biology of breast cancer

Molecular characteristics of the tumor play a critical role in selecting the optimal therapy for treating breast cancer patients. Classically, molecular characteristics evaluated in breast tumors include estrogen and progesterone receptor status, and expression of human epidermal growth factor type 2 receptor (HER2/neu). Increasingly, molecular classifiers are also being considered.

Estrogens are essential for the development of the reproductive system and play an important role in breast cancer. Many estrogenic effects are mediated through estrogen receptors (ER) alpha and beta. Binding of estrogens to these receptors triggers both genomic and non-genomic effects in mammary epithelial cells. Genomic effects are mediated by binding of activated estrogen receptors to estrogen response elements in the promoter of target genes, which together with the binding of co-factors triggers transcriptional activation or repression. Estrogen-responsive genes include progesterone receptor (PR), cathepsin D, c-myc, cyclinD1 and many others.[4] Non-genomic effects are less well studied, and include modulation of signal transduction and ion channels.[5]

Estrogen receptors are overexpressed in up to 70% of invasive breast cancers[2] and are a key therapeutic target. Tumors that express estrogen receptors are treated with antiestrogens such as tamoxifen, which binds to the receptor itself, and agents that decrease the production of estrogen, such as aromatase inhibitors. These are highly effective therapies, although inherent or acquired resistance is frequently encountered.

Activation of growth factor pathways is also an important pathogenic mechanism in breast cancer. Approximately 20% of breast cancers overexpress HER2 (EGFR2 or ErbB2), a plasma membrane receptor tyrosine kinase. Binding of a ligand to a member of the HER family causes receptor heterodimerization, phosphorylation, and activation of downstream signaling pathways, including the MAPK pathway. Activation of this signaling pathway, in turn, affects growth, proliferation, survival, migration and angiogenesis. Trastuzumab, a monoclonal anti-HER2 antibody, is used in the treatment of patients with tumors that overexpress HER2.[6] Although the mechanism of action of Trastuzumab is still not entirely clear, it appears to affect multiple pathways important to breast tumor growth, including heterodimeric interaction of HER2, host immunity, and angiogenesis

The ras/raf/MEK/MAPK and PI3K/AKT pathways are also important in breast cancer, and Trastuzumab may exert some of its effects by modulating these pathways. Other therapies directed at the MAPK and AKT pathways that are being explored in breast cancer include mTOR inhibitors (e.g. CCI-779, RAD-001, AP23576),[7] which target the mTOR complex downstream of PI3K, and MEK inhibitors (e.g. raf inhibitor sorafenib).[8] A major factor contributing to the difficulty in clinical assessment of breast cancer is the histologic and clinical heterogeneity of the disease. The heterogeneity of breast cancer has led to a search for molecular phenotypes to better evaluate the patient's prognosis and response to therapy. Approximately 10 years ago, initial groundbreaking studies[9,10] tested whether gene expression analysis could be used to identify breast tumor subclasses. These investigators used hierarchical clustering of gene expression derived from microarray analysis of breast tumor tissue to classify tumors into groups with similar expression patterns, termed "intrinsic subtypes". Subtypes identified in this and follow-up analyses[11] are termed luminal A, luminal B, basal and HER2-enriched. Tumor subtypes differ significantly in overall patient survival, with basal-like and HER2-enriched subtypes associated with shortest survival times.[10] When assessed in combination with standard parameters for evaluation of breast cancer patients, intrinsic subtypes predict the risk of recurrence in both untreated and treated patients.[12] Additional analyses performed by the Cancer Genome Atlas network have further examined breast cancer subtype characteristics based on genomic DNA copy number, DNA methylation, exome sequencing, microRNA sequencing and proteomic analysis.[1] This extensive study supports the division of breast cancer into the four intrinsic subtypes and further suggests that breast cancer subtypes are each the result of "convergent evolution," with multiple alternative genetic and epigenetic changes capable of giving rise to each breast cancer subtype.

These results have led to the development of clinical tests for molecular characterization of breast tumor subtypes that can be used in the patient's evaluation. The clinical utility of these tests is currently being evaluated in prospective trials.

One of these tests is the Oncotype Dx assay, which measures the expression of 21 genes (including 5 housekeeping genes used in normalization) in paraffin-embedded tissue obtained at the patient's first surgery. Results are reported as recurrence scores ranging from 0–100. In 3 retrospective studies totaling over 2000 patients, recurrence score was correlated with prognosis in ER+ lymph node negative (LN-) patients.[13–15] The score did not correlate with prognosis in a small study of lymph node positive (LN+) patients;[16] however, a subsequent retrospective analysis of 367 ER+ LN+ patients from a SWOG trial of patients treated with either tamoxifen alone or tamoxifen plus chemotherapy revealed that the recurrence score was prognostic for tamoxifen treated LN+ patients and predicted chemotherapy benefit in patients with high recurrence scores but not those with low recurrence scores.[17] The focus of the first prospective clinical trial of Oncotype Dx (TAILORx trial, Trial Assigning Individualized Options for Treatment [Rx]) has been the evaluation of the benefit of chemotherapy in breast cancer patients whose tumors are ER+

and have not spread to lymph nodes. Patients with low recurrence scores are treated with hormone therapy alone, whereas those with higher scores are treated with chemotherapy in addition to hormones. Mammaprint is a test developed at the Netherlands Cancer Institute that is conceptually related to Oncotype Dx. Mammaprint uses the expression of 70 genes in breast cancer prognosis. The prognostic value of this test is currently being assessed in Europe in the MINDACT trial (Microarray in node negative disease may avoid chemotherapy).[18] A recent comparison of Oncotype Dx, Mammaprint and 2 other similar tests (PAM50-ROR and SET) revealed that all these genomic signature tests were successful in outcome prediction of ER+ breast cancer, and in particular successfully identified a subset of LumA patients with LN- disease who might benefit from adjuvant endocrine therapy alone.[19] Interestingly, <25% of genes are shared between signatures. Although all predictors were statistically independent, combining all signatures only modestly improved predictive performance, perhaps suggesting that gene expression profiling has reached its maximum prognostic power.

The advent of molecular profiling has led to substantial advances in breast cancer prognosis and therapy. However, despite the gains garnered using these unbiased approaches, there is room for improvement in both breast cancer prognosis and therapy. For example, prognostic evaluation in women with LN + disease remains problematic, although a large scale trial has been initiated to test whether Oncotype Dx can be used in this context (RxPONDER (Rx for Positive Node, Endocrine Responsive breast cancer).[20] Women with so-called triple negative breast cancer ($ER^{-}PR^{-}HER2^{-}$) remain a particular therapeutic challenge, since these tumors lack the targets against which frontline breast cancer therapies (tamoxifen and Trastuzumab) are directed. The remainder of this review considers what we have learned about iron and breast cancer, and how hypothesis-driven approaches based on the role of iron in breast cancer can contribute to improving breast cancer prognosis and therapy.

II. Iron and breast cancer

Iron has been viewed as playing two potential roles in cancer. The first is as a tumor initiator: through its participation in Fenton chemistry, iron can enhance the production of DNA-damaging oxygen radicals and give rise to carcinogenic mutations. The second role of iron is as an essential growth factor: iron is in particularly high demand by tumors, and alterations in the pathways of iron acquisition and utilization may be among the key metabolic changes that are the hallmarks of cancer. As described below, there is evidence that iron plays both roles in breast cancer.

1. Dietary and systemic iron and breast cancer

One approach to understanding relationships between iron and breast cancer has been to study the effect of manipulation of systemic iron on tumor induction and growth. An early study demonstrated that excess dietary iron increased tumor incidence in rats treated with the mammary carcinogen 1-methyl-1-nitrosourea.[21] In a larger study, the carcinogen dimethylbenz[a]antracene (DMBA) was used to initiate mammary tumors in rats.[22] Eight weeks later, the rats began a 53 week course of biweekly injections of ferrous sulfate. Iron doubled tumor frequency at 20 weeks, and by 40 weeks, 24/40 iron-treated versus 11/30 control rats were tumor positive ($p=0.001$). Tumors in iron-treated rats were also significantly larger than those in controls. Conversely, in DBA/2 mice implanted with syngeneic M119 breast tumors and then fed diets of low (5 mg/kg diet) or normal (312 mg/kg) iron content, tumor size was reduced in mice fed a low iron diet.[23] Similar results were observed in an MMTV model of spontaneous murine mammary tumors: a low iron diet resulted in a statistically significant reduction in tumor growth rate, with the average tumor growth rate in the normal-iron group at 112%/wk, compared to 62%/wk for the low-iron group ($p=0.02$).[24]

The association between dietary iron and breast cancer has also been studied in human populations. Case-control studies assessed dietary iron intake in breast cancer patients and matched controls using carefully validated food frequency questionnaires. Several of these studies showed no association between dietary iron intake and breast cancer risk.[25–27] However, a study of over 6000 Chinese participants concluded that animal-derived iron intake (largely heme) was positively associated with breast cancer risk after adjustment for known risk factors and vitamin supplement use (P (trend) < 0.01; OR = 1.49 in the highest vs. lowest quartile, 95% confidence interval [CI] 1.25–1.78).[28] In addition, a recent analysis of 1205 invasive breast cancers in a cohort of 52,128 evaluable women who participated in the prospective Prostate, Lung, Colorectal and Ovarian Cancer Screening trial demonstrated that dietary iron elevated the risk of invasive breast cancer, with a significant trend towards increased risk in quintiles with higher intake (p=0.03).[29] The reason for discrepancies among studies is unclear, but it has been argued that conflicting results may reflect the imprecise relationship between dietary iron intake and body iron stores, particularly in post-menopausal women.[30] Supporting this interpretation, one study observed that although a breast cancer group consumed significantly less iron than the control group, serum iron and proportion of iron overloaded subjects were significantly higher in breast cancer patients (p=0<0.001 and p<0.05, respectively).[31] Modifier genes may also alter susceptibility to dietary iron and complicate analysis. A study of postmenopausal women from the American Cancer Society Prevention II Nutrition Cohort examined associations of polymorphic variants of genes involved in iron-related oxidative stress (Nrf2, NQO1, NOS3, HO-1) and breast cancer risk.[32] They observed that three or more high risk alleles in combination with the highest tertile of iron intake or supplemental iron increased the risk of postmenopausal breast cancer over two-fold compared to subjects with low risk alleles.[32]

Studying the association between hereditary hemochromatosis (HH) and cancer has been another approach used to assess the role of iron in breast cancer. Hereditary hemochromatosis is a genetic disorder characterized by the accumulation of excess iron. It is caused by mutations in several genes, all of which disturb the hepcidin regulatory axis that governs absorption and distribution of iron throughout the body (see [33], [34] for review). Tissues that are particularly susceptible to iron overload in individuals with HH include the liver, heart, and endocrine organs. It is well known that HH increases risk of hepatocellular carcinoma,[35–37] but subjects that carry mutations in HFE may also be at increased risk for extrahepatic cancer, including breast cancer.[38,39] In a recent study, 28,509 individuals in the Melbourne Collaborative Cohort Study were genotyped for the hemochromatosis variant HFE C282Y. Incident cancers in homozygotes were determined from cancer registries. Homozygosity doubled breast cancer risk in women (HR 2.39, 95% CI 1.24,4.61, p=0.01), although there was no increased risk among heterozygotes.[39]

2. Iron uptake and storage and breast cancer

The transferrin pathway is critical to the acquisition of iron by both normal and malignant mammary epithelial cells.[40] In this pathway, diferric transferrin circulating in the bloodstream binds to transferrin receptor 1 (TfR1) on the cell surface. Diferric transferrin bound to TfR is endocytosed, and with the assistance of the STEAP reductases, iron is freed from its association with transferrin, reduced to ferrous iron, and delivered to the cytosol through the action of DMT1. This is a well-conserved pathway, and although not all elements of this pathway have been explicitly studied in breast cells, specimens derived from human breast cancers express all genes in this pathway.[41] Increased display of transferrin receptors is a common theme in many different cancer types, and over 30 years ago, immunohistochemical analysis of normal and malignant breast tissue demonstrated that transferrin receptor was upregulated in 16/22 breast cancer cases.[42] A more recent retrospective analysis of human tumors from 853 cases of invasive breast carcinoma

demonstrated that transferrin receptor is not only expressed in breast tumors, but is a marker of poor prognosis in breast cancer.[43] Thus, high TfR1 expression was associated with poorer response to tamoxifen therapy and shortened breast cancer specific survival[43]. Recent findings that TfR1 associates with and is phosphorylated by Src in breast cancer cells suggest that upregulation of TfR1 may not only enhance iron uptake, but also promote cell survival through participation in signaling pathways.[44]

Such observations have led to the use of anti-transferrin receptor antibodies in tumor targeting and therapy. Although the first generation of anti-transferrin receptor antibodies did not prove therapeutic, there is continued interest in using the transferrin receptor as a targeting ligand for the delivery of therapeutics to multiple cancer types, including breast cancer.[45] Several studies suggest that transferrin receptor targeting can improve delivery of various therapies to breast cancer cells. For example, proof-of-principle experiments studying the delivery of luciferase to MDA-MB-435 breast cancer cells showed 4–10 fold improved targeting efficiency using anti-human TfR scFv -containing liposomes vs untargeted liposomes.[46] Enhanced antiproliferative effect of a holo-Tf targeted doxorubicin-containing nanoparticle against drug resistant MCF7 breast cancer cells,[47] and improved *in vivo* anti-tumor efficacy of an antisense nucleotide targeting HER2/neu following encapsulation in a TfR coated nanoparticle [48] have also been reported. Investigations into the use of transferrin receptor in tumor targeting are ongoing.[45]

An alternative mechanism of iron acquisition that is less well studied than the transferrin pathway is mediated by lipocalin−2 (24p3, LCN2, NGAL). This pathway also appears important in breast cancer. Lipocalins are a family of proteins that bind small hydrophobic ligands. Their shared characteristic is an eight-stranded antiparallel beta barrel that forms the ligand binding site.[49] Lipocalin−2, a member of this family, ligates bacterial catecholate-type ferric siderophores such as ferric-enterobactin, the primary siderophore of enteric bacteria.[50] LCN2 also ligates siderophore-like molecules synthesized by eukaryotic cells.[51, 52] LCN2 binds to specific receptors on the cell surface (24p3R, megalin),[53] and if LCN2 is complexed with ferric siderophore, it can deliver iron.[54] However, 24p3R can also bind LCN2 that is complexed to an iron-free siderophore. Internalization of the iron-free siderophore-LCN2 complex can lead to iron efflux and cell death.[52, 54] Thus, the cellular effect of LCN2 is dependent on whether its associated siderophore contains iron or is iron-free.

LCN2 is upregulated in a number of cancers, including breast cancer.[55] Overexpression of LCN2 in MCF7 breast cancer cells increases proliferation[56] and increases tumor angiogenesis.[57] In addition to its effects on primary breast tumors, LCN2 over-expression enhanced the migration and invasion of 4T1 murine breast cancer cells *in vitro* and more than tripled the formation of lung metastases *in vivo*.[58] Consistent with a role of LCN2 in breast cancer, knockout of *Lcn2*, the gene encoding murine LCN2, suppressed mammary tumor formation in mice: tumors induced in both MMTV-ErbB2 (V664E) [55] and MMTV-PyMT[59] models of spontaneous breast cancer were delayed in onset, multiplicity, and size when crossed with an *Lcn2* knockout mouse.[38,41] Surprisingly, however, no correlation between LCN2 expression and breast tumor aggressiveness was observed when LCN2-deficient mice and MMTV-PyMT mice were crossed into a FVB/N background.[60] The explanation for this discrepancy is unclear, although the authors speculated that weak expression of the *BDH2* gene (responsible for synthesis of a eukaryotic 2,5-DHBA siderophore) in FVB/N mice might prevent iron from being effectively utilized by the LCN2 pathway in tumors in this genetic background.

Analysis of LCN2 expression in human breast cancer prognosis indicates that LCN2 expression is associated with shorter disease-specific survival and may predict response to

therapy in human primary breast cancer.[61,62] In a retrospective immunohistochemical analysis of LCN2 expression in tissue microarrays from 652 biopsies of breast cancer patients who subsequently underwent neoadjuvant chemotherapy, LCN2 was detected in 42% of breast carcinomas. Although LCN2 expression did not correlate with the response rate of the overall population, expression was associated with higher response rates to neoadjuvant chemotherapy in defined patient subsets, including low risk subgroups with small tumors, hormone receptor positive tumors, and node-negative patients. High staining intensity correlated with decreased disease-free survival in the entire cohort and subgroups. Multivariate analysis revealed that LCN2 expression was an independent prognostic factor for disease-free survival.

It should be noted that LCN2 has additional effects apart from its role in iron scavenging and delivery that may also contribute to its pro-tumorigenic effects. For example, LCN2 promotes the activity of MMP9, a protease involved in tumor invasion. Thus, LCN2 may contribute in multiple ways to breast cancer.[63]

Ferritin, which functions as an intracellular iron storage protein as well as exhibiting a number of other functions,[64–66] has variously been reported to be increased or decreased in breast cancer, which may be a reflection of breast cancer heterogeneity.A recent report suggested that human breast cancer cells with a more differentiated phenotype express low levels of ferritin, whereas those with a more aggressive mesenchymal phenotype (MDA-MB-231) express higher levels of ferritin.[67] Downregulation of ferritin in MDA-MB-231 triple negative breast cancer cells using microRNA miR200b increased sensitivity to the chemotherapeutic agent doxorubicin, presumably by increasing intracellular oxidative stress and possibly by simultaneously increasing redox cycling of the drug. Sensitization to carmustine, an anti-cancer alkylating agent, was also observed following delivery of siRNA to ferritin H in MCF7 cells with cationic liposomes.[68] These results suggest that it may be possible to leverage alterations in breast cancer iron metabolism to enhance effects of conventional chemotherapy.

3. Iron efflux pathways and breast cancer

Approximately 10 years ago, ferroportin was identified as an iron efflux pump in vertebrate cells.[69–71] Ferroportin plays an important role in control of systemic iron by delivering iron from the enterocyte to transferrin as well as by mediating the efflux of iron from macrophages following catabolism of effete red blood cells. Ground-breaking experiments revealed that hepcidin, a circulating peptide hormone synthesized by the liver, binds to ferroportin and triggers its degradation;[72](reviewed in[73]). This mechanism both prevents intestinal absorption of iron and limits iron recycling when iron stores are high.[72] The expression of hepcidin is transcriptionally controlled by cytokines, particularly IL6, as well as by BMP signaling.[33,74]

The ferroportin-hepcidin axis was initially identified as a regulator of systemic iron, but these proteins are also expressed in peripheral tissues, including breast tissue, where they may establish a microclimate of iron within the macroenvironment of the organism as a whole. Our group observed that ferroportin is expressed in normal mammary epithelial cells, and that its levels are sharply reduced in breast cancer cells.[75] Similar results were reported by an independent group.[76] Somewhat unexpectedly, we found that breast cells also express hepcidin, with cancer cells expressing higher levels than non-cancer cells. As expected from its iron efflux function, the reduction in ferroportin mediated by either reduced ferroportin or increased hepcidin was associated with a concomitant increase in the labile iron pool in breast cancer cell lines. Levels of ferroportin appear to be important contributors to breast tumor growth: when ferroportin was restored to normal levels by transfection of ferroportin into MDA-MB-231 breast cancer cells, growth of tumor xenografts was substantially

slowed. The relevance of this pathway to human breast cancer was revealed by immunohistochemical analysis of samples from human breast cancer patients, which showed a similar reduction in ferroportin protein levels in malignant breast cancer specimens when compared to normal breast. Most importantly, microarray analysis from over 500 breast cancer patients revealed that ferroportin expression was significantly correlated with prognosis of breast cancer patients: for example, the 10 year probability of distant metastasis-free survival was over 90% in patients whose tumors expressed high levels of ferroportin and low levels of hepcidin, and approximately 70% in patients whose tumors expressed low ferroportin and high hepcidin. In addition to its potential clinical utility, this observation offers insights into additional roles of iron, and suggests that expression of ferroportin and hepcidin may affect metastasis as well as growth of breast tumors.

4. Unique aspects of the role of iron in breast cancer

In addition to these roles -- shared among many cancer types -- iron may play a unique role in breast cancer through its interaction with estrogen. Lifetime exposure to estrogen is a known risk factor for breast cancer.[77] Contributors to lifetime exposure include use of oral contraceptives, hormone replacement therapy, body mass index, age at menarch and menopause, and parity.[78] Although the contribution of estrogen to breast cancer risk is multifactorial, one pathway involves redox cycling of estrogen metabolites. For example, the estrogen metabolite 4-hydroxyestradiol is elevated in malignant breast tissue compared to non-neoplastic mammary tissue,[79] and treatment with 4-hydroxyestradiol induces cancer in rodent models.[80] SNP haplotype evaluation of enzymes involved in the redox cycling of estradiol has been proposed as a predictive model of breast cancer risk.[78] Redox cycling of estradiol is important because it can directly produce depurinating DNA adducts.[81] In addition, redox cycling of estrogen can damage DNA by generating superoxide,[82] which can in turn participate in the iron-dependent Fenton reaction to generate the DNA-damaging hydroxyl radical. Iron-dependent reactions appear to contribute to estrogen-mediated DNA damage and tumor formation. Thus, iron promoted[83] and the chelator deferoxamine attenuated[84] formation of oxidized DNA bases produced by redox cycling of estrogens.[85] Importantly, estrogen and iron exert combined effects in stimulating proliferation of breast cancer cells in tissue culture,[86] and an iron-rich diet accelerated estrogen-mediated tumor formation in hamsters.[84] Superoxide produced by estrogen redox cycling has also been shown to mobilize iron from ferritin, which may serve as a feed-forward mechanism by which estrogens increase DNA damage.[87] It has been suggested that iron may interact with estrogen in two different ways to promote breast tumor growth: low iron may stimulate HIF-1α activation and consequently promote angiogenesis, whereas high iron may increase oxidative stress.[88]

Another unique relationship between iron and breast cancer is mediated by lactoferrin. Lactoferrin is an 80kDa glycoprotein found in high concentrations in breast milk, other exocrine secretions, and a number of tissues, including neutrophils, where its release exerts antimicrobial activity.[89] Lactoferrin is expressed in non-lactating breast tissue as well as in many breast cancers.[90,91] In addition to a secretory form, there is a splice variant of lactoferrin that is cytosolic and can enter the nucleus.[92] Lactoferrin belongs to the transferrin family and binds two ferric ions with very high affinity. Very low (below 4th quartile) expression of lactoferrin was associated with poor prognosis in one study of breast cancer.[93] In tissue culture, addition of exogenous lactoferrin[94, 95] or adenovirus-mediated expression of lactoferrin[96] inhibits the proliferation of breast cancer cells. Injection of recombinant adenovirus containing lactoferrin into EMT6 murine tumors induced apoptosis and inhibited tumor growth in mice, suggesting that lactoferrin may have therapeutic benefit in at least some breast cancers.[97] The anti-proliferative effect of lactoferrin may be mediated by its ability to bind iron, since recombinant lactoferrin would be expressed in its non iron-binding

form, and iron deprivation retards cancer growth. However, the role of iron in the effects of lactoferrin was not examined in this study.

Lactoferrin may play a different role in triple negative breast cancers. A proteomic analysis identified lactoferrin as up-regulated in triple negative breast tumors ($ER^{-}PR^{-}HER2^{-}$) when compared to HER2 positive ($ER^{-}PR^{-}HER2^{+}$) tumors.[98] A recent study unexpectedly revealed that addition of recombinant iron-saturated lactoferrin to cultured ZR-75 breast cancer cells downregulated ER, PR and HER2 in a proteasome-dependent fashion.[99] Lactoferrin also stimulated proliferation and invasiveness. Interestingly, none of these effects were observed with apo-lactoferrin. However, the specific role of iron in effects of lactoferrin was not investigated. Microarray analysis followed by detailed reporter gene analysis revealed that lactoferrin enhanced invasiveness of MDA-MB-231 and MDA-MG-468 breast cancer cells through transcriptional induction of endothelin−1 (ET-1), a secreted protein previously shown to promote breast cancer progression.

III. Iron and breast cancer prognosis and therapy

The findings described above serve as the foundation for ongoing approaches designed to leverage the relationship between iron and breast cancer for improved prognosis and therapy.

There are several opportunities to apply iron metabolism to improving breast cancer prognosis. As discussed above, decreased ferroportin gene expression in breast cancer is associated with a significant reduction in disease-specific survival.[75] The prognostic value of ferroportin and hepcidin is independent of traditional breast cancer risk factors, indicating that measurement of ferroportin and hepcidin may capture prognostic information not currently evaluated during clinical decision-making. A striking finding was that 40% of patients with a favorable prognosis profile (high ferroportin and low hepcidin transcript levels) had disease that had spread to their lymph nodes (LN+). This suggests that analysis of ferroportin and hepcidin may enable the identification of a subgroup of patients with favorable prognosis among LN+ patients, who are generally considered a high-risk group. The ability to differentiate prognostic groups among LN+ patients might spare some women unneeded chemotherapy.

The prognostic value of “iron genes” likely extends beyond ferroportin and hepcidin. Based on analysis of the literature and gene ontogeny lists, 61 genes with functions related to iron metabolism were selected and analyzed for their association with breast cancer patient survival.[41] Almost half of the genes in this group were significantly associated with distant metastasis-free survival of breast cancer patients, which is a much larger fraction than would be expected by chance ($p<0.02$). Further analysis revealed that most of the prognostic information contained in these genes could be captured using 16 of the 61 genes. This 16 gene set was termed the “iron regulatory gene signature” (IRGS), and included genes with known associations with breast cancer risk (e.g. *transferrin receptor 1, ferroportin*) as well as genes with no known association with breast cancer (e.g. *dcytb*). Importantly, the IRGS was able to separate homogeneously treated LN+ patients into groups with significantly different prognosis ($p=0.006$), again suggesting that measurement of “iron” genes may be useful in evaluating LN+ as well as LN- patients.

An additional finding was that several signatures are likely to be embedded within the IRGS. Thus, the group of patients with favorable prognosis based on their expression of ferroportin and hepcidin did not substantially overlap the group of patients with favorable prognosis based on their expression of transferrin receptor and HFE. These results suggest that there are multiple iron pathways whose expression affect breast cancer patients prognosis, which

represents an opportunity for future discovery. A systems biology approach may be useful in unraveling the contribution of these genes to tumor growth and metastases.[100, 101]

Changes in iron metabolism may extend beyond the tumor cells themselves to cells in the tumor microenvironment, presenting an additional diagnostic or therapeutic target. The tumor microenvironment contains many cells essential to tumor growth, including endothelial cells, stromal cells and macrophages. Alternatively polarized macrophages (M1 versus M2) exhibit different profiles of expression of iron-related genes.[102,103] A recent proteomic analysis identified ferritin light chain (FTL) as an independent prognostic marker in node-negative breast cancer, and showed that FTL was stored in tumor-associated macrophages.[104]

Information on iron metabolism in breast cancer may also be used to improve breast cancer therapy. An exciting direction grows out of the observation that levels of both lactoferrin and ET-1 are significantly elevated in the tumors of women with triple negative breast cancer when compared to women with ER+ breast cancer.[99] Since ET-1 receptor antagonists are being evaluated in clinical trials, these results suggest that it may be possible to identify women who are good candidates for ET-1 receptor antagonist therapy by measuring lactoferrin and ET-1 in their tumors. This work also identifies ET-1 receptor antagonists as new potential therapies that may upregulate ER and HER2 in triple negative breast cancers and sensitize these treatment-refractory breast tumors to tamoxifen or trastuzumab.

The requirement for iron in breast cancer growth suggests that iron chelators represent a viable approach to breast cancer therapy. Several iron chelators are currently under clinical or pre-clinical development in the treatment of cancer, including breast cancer. Examples of iron chelators with activity in breast cancer include salicylaldehyde isonicotinoyl hydrazone derivatives,[105] Dp44mT,[106,107] and desferri-exochelin.[108] Iron chelators may also provide new insight into iron-dependent pathways in cancer cells. One example is the Wnt pathway. Dysregulation of the Wnt pathway is observed in many tumors, including breast tumors.[109,110] Wnt is a secreted ligand that binds to frizzled receptors on the cell surface (see [111] for review). In the canonical pathway, binding of Wnt triggers a signal transduction cascade that frees the transcription factor β–catenin from its association with a destruction complex, enabling the translocation of β–catenin to the nucleus and activation of downstream genes. A screen for inhibitors of the Wnt pathway recently identified N-((8-hydroxy-7-quinolinyl)(4-methylphenyl)methyl)benzamid (HQBA), an iron chelator, as a top hit.[112] HQBA was shown to bind ferrous iron with very high affinity (dissociation constant 1.2 × 10(−19)M) and exhibited tumor cell cytotoxicity *in vitro*. *In vivo*, HQBA inhibited tumor growth in two genetically engineered mouse models of mammary cancer: MMTV-Wnt1 and MMTV-PyMT.

Conclusion

Despite major advances, breast cancer remains a clinical challenge. Improved understanding of metabolic changes that occur in this disease may benefit both prognosis and therapy. A large body of laboratory and clinical evidence indicates that iron is closely associated with breast cancer growth and metastasis. Breast cancer cells increase levels of intracellular iron through multiple pathways, including increased uptake and decreased efflux. The dependence of breast cancer on iron presents rich opportunities for improved prognostic evaluation and therapeutic intervention.

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Abbreviations

BDH2	3-hydroxybutyrate dehydrogenase
BMP	bone morphogenetic protein
DCIS	ductal carcinoma in situ
DHBA	dihydroxybenzoic acid
DMBA	dimethylbenz[a]anthracene
DMT1	divalent metal transporter 1
ER	estrogen receptor
ET-1	endothelin 1
HFE	hereditary hemochromatosis protein
HH	hereditary hemochromatosis
HO-1	heme oxygenase 1
HER2/neu	human epidermal growth factor type 2 receptor
LCN2	lipocalin 2
LN	lymph node
MAPK	mitogen activated protein kinase
MEK	MAPK Erk kinase
MMP9	matrix metallopeptidase 9
mTOR	mammalian target of rapamycin
MMTV	mouse mammary tumor virus
Nrf2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H dehydrogenase quinone 1
NOS3	nitric oxide synthase 3
PI3K	phosphatidylinositol 3 kinase
PR	progesterone receptor
PyMT	polyoma virus middle T antigen
SNP	single nucleotide polymorphism
STEAP	six transmembrane epithelial antigen of prostate
SWOG	National Cancer Institute-supported clinical trials cooperative group, formerly Southwest Oncology Group
TfR1	transferrin receptor 1
Wnt	wingless-type MMTV integration site

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