Skeletal Complications of Malignancy

Supplement to ***Cancer***

The Biology of Breast Carcinoma

Judith Clancy Keen, Ph.D.
Nancy E. Davidson, M.D.

The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, Maryland.

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Address for reprints: Nancy E. Davidson, M.D., The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine, 1650 Orleans Street, Baltimore, MD 21231; Fax: (410) 614-4073; E-mail: davidna@jhmi.edu

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The biology of breast carcinoma is complex, with multiple factors contributing to its development and progression. The current review focuses on the role of several critical genes including estrogen receptor, progesterone receptor, retinoic acid receptor-β, epidermal growth factor receptor family members, p53, BRCA1, and BRCA2 as risk factors for the development of disease, predictors of prognosis and response to therapy, and as therapeutic targets. Studies of the biology of these and other genes that contribute to the development and progression of breast carcinoma have had and will continue to have great impact on all aspects of disease management. ***Cancer*** **2003;97(3 Suppl):825–33.** © *2003 American Cancer Society.*
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Breast carcinoma is a leading cause of cancer mortality among women in the Western hemisphere, second only to lung carcinoma. The American Cancer Society estimates that 203,500 new cases in women will be reported and 40,000 women will die of breast carcinoma in the U.S. in 2002 alone. Current estimates suggest in her lifetime, one in eight American women will be diagnosed with breast carcinoma.[1]

Our growing knowledge regarding breast carcinoma biology is having an ever greater impact on clinical management. Distinct characteristics of breast carcinoma can be exploited to help determine lifetime risk of development of the disease, the overall prognosis after a diagnosis of breast carcinoma, and the likelihood of response to specific therapy. In addition, increased understanding of breast carcinoma pathways may enhance our ability to devise targeted approaches to prevention or therapy. Thus, the biology of breast carcinoma can contribute vital information regarding many aspects of the disease.

It is well established that a myriad of factors including steroid hormones and their receptors, peptide growth factors, oncogenes, and tumor suppressor genes play a crucial role in the transformation of the breast.[2–4] This review will focus on selected biomarkers that play a key role in breast carcinoma, including certain steroid receptors (estrogen receptor [ER], progesterone receptor [PR], and retinoic acid receptor [RAR]-β), members of the HER/*erb*B family, and selected tumor suppressor/susceptibility genes (e.g., p53, BRCA1, and BRCA2). Discussion will focus on their function and their possible roles in risk assessment, estimation of prognosis, and prediction of response to therapy, as well as their potential as therapeutic or preventive targets.

Steroid Hormone Receptors

Estrogen receptor

Early menarche, late menopause, and nulliparity are correlated with an increased risk of developing breast carcinoma, suggesting that prolonged exposure to cycling estrogen and progesterone levels con-

tributes to the development of the disease. Removal of endogenous estrogen via oophorectomy decreases the risk of the development of breast carcinoma. Indeed, the earlier the ovaries are removed, the greater the risk reduction.[5,6] In postmenopausal women, the major source of estrogen is androgenic precursors derived from the adrenal glands that are converted into estrogen by the aromatase enzyme in adipose tissues. Postmenopausal women with increased body fat have increased estrogen levels and are more likely to develop breast carcinoma.[4–7] Therefore, increased estrogen exposure via a variety of mechanisms appears to be a critical risk factor in the development of breast carcinoma. The effects of estrogen are mediated at least in part by the ER proteins, α and β.

ER-α and ER-β; are members of the steroid receptor family. In the absence of its ligand, estrogen, ER-α or ER-β forms an inactive complex with HSP 90.[8] Upon ligand binding and dissociation from HSP 90, ER is activated, undergoes a conformational change, dimerizes, and autophosphorylates through intrinsic tyrosine kinases. In this active form, ER dimers bind to recognition sequences termed estrogen response elements (ERE), which are found within the promoter of many genes to regulate gene transcription. Activated ER also can activate the mitogen activated protein kinase (MAPK) pathway, which results in the activation of the AP-1 proteins, fos and jun. Studies have shown that ER binds AP-1 consensus sites through protein-protein interactions with AP-1 proteins to regulate gene transcription.[9–11]

Approximately 70–80% of all breast tumors express ER-α protein and therefore are termed ER positive (ER+). These tumors tend to grow more slowly, are better differentiated, and are associated with a slightly better overall prognosis.[4] Thus ER expression is one of a few prognostic factors, along with axillary lymph node status, tumor size, and histologic grade and subtype.[4] More important, the detection of ER-α in breast carcinoma cells is an important indicator of potential response to endocrine therapy. A number of endocrine strategies currently exist to deplete the ligand estrogen (oophorectomy or luteinizing hormone-releasing hormone [LHRH] analogues in premenopausal women or aromatase inhibitors in postmenopausal women), interfere with ligand-receptor interaction (selective ER modulators such as tamoxifen and raloxifene), or destroy the ER (selective ER destroyers such as fulvestrant or ICI 182, 780). The molecular effects of these strategies are being understood more and more. For example, tamoxifen, raloxifene, and fulvestrant can reduce the expression of cell cycle proteins including cyclin D1 and cyclin E and inhibit the phosphorylation of the retinoblastoma (Rb) gene, a major target of the cyclin-associated kinases that are critical in cell cycle progression and cellular proliferation.[12,13] Selective ER modulator (SERM) treatment, estrogen withdrawal, or aromatase inhibition results in tumor shrinkage, decreases the numbers of cells in S-phase, and induces markers of cellular apoptosis.[14–17] Clinically, this is manifested by the observation that tumors expressing ER-α protein are the most likely to manifest a response to endocrine therapy; those lacking ER-α seldom respond.

SERMs also have been used prophylactically in women with a high risk of breast carcinoma to reduce the risk of development of disease. Indeed 5 years of tamoxifen use has been shown to reduce the risk of developing breast tumors by up to 50% in high-risk women. It is interesting to note that this strategy reportedly is beneficial only in reducing the development of ER+ tumors. Tamoxifen does not appear to have an impact on the development of ER-negative (ER-) tumors.[18]

Given the central role of ER-α in defining response to endocrine therapy for breast carcinoma, there is great interest in determining mechanisms for its absence of expression in some breast carcinoma patients. A number of studies have shown that the loss of ER expression in ER- tumors seldom is the result of mutations, deletions, loss of heterozygosity, or polymorphisms within the gene. Instead, it has been shown that ER gene expression occasionally is silenced through reversible epigenetic modifications including histone deacetylation and DNA methylation. The presence or absence of acetyl groups on histone tails (primarily H3 and H4) can govern chromatin structure and gene transcription. However, histone protein modification is not the only method of gene silencing but likely interacts with a second mechanism, DNA methylation. CpG dinucleotides are dispersed throughout the genome, but are more highly clustered within gene promoter regions. Cytosine residues located 5′ of guanine residues can be modified by the addition of methyl groups mediated by the DNA methyltransferase (DNMT) proteins. DNA methylation within the promoter and first exon of genes is correlated with gene silencing and a lack of gene expression.[19–23] The ER-α promoter contains a CpG island within its promoter and first exon that is methylated in ER- human breast carcinoma cell lines. Furthermore, histones isolated from these cell lines are deacetylated, suggesting a dual mechanism of methylation and histone deacetylation for ER silencing in these cells.[21,24–26] Because these posttranscriptional modifications are reversible, the treatment of cells with epigenetically silenced genes with histone deacetylase (HDAC) inhibitors including Trichostatin

A (TSA) or DNMT inhibitors (such as 5-Aza-2′-deoxycytidine) should result in expression from the intact gene. As predicted, the treatment of ER- human breast carcinoma cell lines with TSA or 5-Aza-2′-deoxycytidine results in reexpression of ER mRNA and functional protein, suggesting that epigenetic mechanisms may be important mechanisms for the absence of hormone response.[21,24–26]

For several decades, it was believed that there was a single ER gene. In 1996, a second ER, termed ER-β, was cloned first from the rat and subsequently from the human.[27,28] ER-α and ER-β are structurally similar, sharing key features of the steroid receptor family. Although their overall sequence homology is only approximately 30%, there is high homology within the DNA and hormone-binding domains at 95% and 53%, respectively. This domain-specific homology suggests that ER-α and ER-β are likely to share similar DNA and ligand-binding function, but the low overall homology may indicate that their global effects differ.

Similar to ER-α, ER-β is expressed in a variety of tissues including mammary gland, uterus, ovary, prostate, epididymus, testis, pituitary, kidney, thymus, bone, and central nervous system.[29] Within normal mammary tissues, ER-β is highly expressed in the epithelial and stromal layers. Investigation into ER-β regulation and its role in breast carcinoma remains in its early stages. Preliminary studies suggest that ER-β expression is readily detectable in ductal carcinomas in situ and lobular carcinomas in situ but drops dramatically in late-stage tumors.[30–38] Studies published to date have to our knowledge relied primarily on reverse transcriptase-polymerase chain reaction-based techniques because reliable antibodies against ER-β are not yet widely available. The role of ER-β in cancer in general and breast carcinoma in particular still needs to be determined.

Progesterone receptor

The PR gene also is a member of the nuclear receptor superfamily. Two isoforms of the PR, PRA and PRB, are encoded by the same gene, utilizing two distinct transcriptional start sites and yielding proteins that differ with regard to their animo terminal regions and biologic activities.[39] Although both PRA and PRB are highly expressed in normal tissues, PRB protein concentrations reportedly are elevated in breast carcinoma. This results in a decrease in the PRA:PRB ratio that is believed to be an important parameter for progesterone-mediated functions.[40–42]

Similar to ER-α, PR status is a good predictor of tumor responsiveness to therapy. Nearly 50% of all ER+ tumors also are reported to be PR+ and approximately 75% of these ER/PR+ tumors respond positively to endocrine therapy.[3] ER+, PR- tumors are reported to be less responsive to therapy, perhaps suggesting that PR may be necessary for positive therapeutic outcomes with hormone therapy. Alternatively, because ER is a key transcription factor for the activation of PR, lack of PR expression in these ER+/PR- cells also could suggest that the estrogen response pathway may not be functional in these tumors.[41] To our knowledge only a small fraction of tumors are ER-/PR+ ($< 5\%$) and they demonstrate an intermediate response to endocrine therapy.[3]

Similar to ER, PR expression also is regulated by epigenetic modulation.[25] Methylation and/or acetylation of a CpG island within the PR promoter region also is important for PR expression. However, studies have shown that demethylation alone using 5-Aza-2′-deoxycytidine is not sufficient for reactivation of PR, suggesting that ER-mediated chromatin remodeling of the locus involves several mechanisms in conjunction with demethylation and is critical for the faithful expression of PR.[43] Chromatin reorganization using both HDAC inhibitors and demethylating agents results in the reexpression of PR.[26] It still is unclear whether this is a direct effect on the PR promoter or an indirect effect mediated by enhanced expression of the ER.

Retinoic-acid receptor

All three RARs (RAR-α, RAR$-\beta$, and RAR$-\gamma$) are highly expressed in normal mammary epithelial tissues. Similar to other members of the nuclear receptor superfamily including ER and PR, RARs are ligand-activated receptors that regulate gene transcription through interactions with retinoic acid response elements (RAREs) found within gene promoter regions. When activated, RARs form homodimers or heterodimers with the retinoid X receptors (RXR–α, RXR$-\beta$, and RXR$-\gamma$). They function as tumor suppressor genes inhibiting proliferation and inducing cell differentiation and apoptosis.[44] These antiproliferative and apoptotic effects may be regulated by inhibition of the cell cycle, arresting cells in the G_1-S-phase.[45] RAR-β expression is high in normal mammary epithelial cells, but is down-regulated at both the mRNA and protein levels in malignant tumors including tumors of the lung, head and neck, esophagus, ovary, prostate, and breast.[46–52]

Mechanisms underlying the loss of RAR-β expression are an active area of research. Loss of heterozygosity at 3p24, the chromosomal region encoding RAR-β, is detected in primary breast tumors, suggesting that one mechanism of loss may be mediated by allelic deletion.[53,54] Recent studies have shown that RAR-β also is under epigenetic regulation by methylation and histone deacetylation of the promoter re-

gion, resulting in gene silencing.[44,54–58] In a study of both primary breast carcinomas and breast carcinoma cell lines, TSA induced the reexpression of RAR-β2, even in the absence of retinoic acid, a known inducer of RAR-β2 expression. This suggests that chromatin remodeling is a critical mechanism for the regulation of RAR-β2 expression that can be exploited with novel therapeutic approaches.[59]

Peptide Growth Factors and Their Receptors

A number of peptide growth factors and their receptors have been implicated in normal mammary development and carcinogenesis. These include members of the HER/*erb*B, tumor growth factor-β (TGF-β), and insulin-like growth factor families. Here we focus on the HER/*erb*B family to illustrate how these families might contribute to the development of breast carcinoma and be exploited clinically.

Epidermal growth factor receptor family

The HER or *erb*B proteins are members of the subclass I of the receptor tyrosine kinase (RTK) superfamily. This subgroup of RTKs contains four members: epidermal growth factor receptor (EGFR/*erb*B1/HER-1), *erb*B2/*neu*/HER-2, *erb*B3/HER-3, and *erb*B4/HER-4. These transmembrane proteins share a similar structure but only 25–30% overall homology. There are at least 25 known ligands that can bind HER family members including epidermal growth factor (EGF), TGF-α, amphiregulin, heparin-binding EGF (HbEGF), β-cellulin, epiregulin, cripto-1, neuregulin, and heregulin.[60] Upon ligand binding and activation, HER proteins form homodimers or heterodimers comprised of different combinations of family members.[60] It is interesting to note that, to our knowledge, a specific ligand for HER-2 has not been identified; rather, HER-2 frequently is the preferred partner for other ligand-bound HER molecules.

In vitro and in vivo, both HER-1 and HER-2 have been shown to play a clear role in neoplastic transformation.[61–64] The HER-2/*neu*/*erb*B2 protein is overexpressed in approximately 25% of invasive breast tumors, usually because of gene amplification.[65] In some studies, HER-2 overexpression has been reported to be correlated with poor prognosis, but not with tumor size, degree of differentiation, or metastatic potential, suggesting that HER family members may play a role in overall outcome, but not in the pathway leading to the transformed state.[66–68] It is unclear what role HER-3 or HER-4 play in normal mammary cells, but HER-3 protein often is overexpressed in breast tumors in conjunction with HER-B2. This finding suggests the possibility that HER-2-HER-3 dimers may play a role in these tumors.[69]

Because overexpression of HER-1 and HER-2 characterizes a significant fraction of breast carcinoma cases, there has been great interest in developing therapies targeting the HER family members. ZD1839 is a member of the anilinoquinazoline class of RTK1 that initially was developed as a HER-1 inhibitor; however, in vitro, ZD1839 also is reported to be a very effective HER-2 inhibitor as well.[70] Mechanistically, ZD1839 decreases HER-1 and HER-2 expression by interfering with phosphorylation of PI3K, activation of AKT, and phosphorylation of the MAPK cascade.[71–73] ZD1839 also inhibits cell cycle progression by downregulating key proteins in cell cycle progression including cyclin D1, Cdk4, $p27^{Kip1}$, and Cdk2. This results in inhibition of proliferation and induction of apoptosis in HER-2-positive cell lines.[56,72–75] It is interesting to note that the p85 subunit of PI3K associates with HER-3 and not with HER-2, suggesting that the functional dimer includes HER-3 as well in tumors that overexpress HER-2.[71] Clinical trials of ZD1839 as a single agent currently are underway for women with advanced breast carcinoma.

Trastuzumab is a monoclonal antibody that was raised against the ectodomain of HER-2 that blocks cell proliferation, inhibits cell growth, and induces apoptosis in breast carcinoma cells.[76,77] Therefore, the presence of overexpressed HER-2 serves as a good predictive factor of clinical response to trastuzumab. Trastuzumab inhibits both PI3K activation of the AKT pathway and activation of the MAPK pathway. Cells treated with trastuzumab accumulate in the G_1 phase of the cell cycle, suggesting that trastuzumab inhibits cell proliferation via a G_1 –S-phase block.[78–80] The activity of trastuzumab alone and with cytotoxics has been established in women with advanced breast tumors overexpressing HER-2.[75,80] Preclinical studies suggest that ZD1839 and trastuzumab may work synergistically to inhibit tumor progression via inhibition of both the AKT and MAPK pathways. A combination of ZD1839 and trastuzumab enhanced apoptosis and tumor regression in tumor cells overexpressing HER-2 compared with tumor cells that were negative for HER-2.[72,75,81] A Phase I clinical trial of the combination currently is in progress.

The nature of the cross-talk between the ER and HER pathways also is of great interest. Studies of ER, EGF receptor (EGFR), and HER-2 in breast carcinoma suggest that ER expression is inversely correlated with EGFR or HER-2 expression. The possibility that HER-2 overexpression is associated with tamoxifen resistance has been suggested by some but not all studies. For example, serial samples of primary breast tumors during a course of neoadjuvant endocrine therapy suggest that HER-2-overexpressing tumors do not demon-

strate the same decrease in Ki-67 as observed in HER-2-negative tumors.[82] If confirmed, these findings could have implications for the ability of HER-2 to serve as a predictive marker for endocrine therapy and could support clinical trials of combinations of anti-HER and endocrine therapy.

Tumor Suppressor Genes and Breast Carcinoma Susceptibility Genes

p53

Somatic cell mutation in the p53 nuclear phosphoprotein is observed in approximately 20–30% of primary breast carcinoma cases.[83,84] Although these mutations are found scattered throughout the entire gene, the majority of mutations are confined to a 200-amino acid span containing 1 of 4 conserved core domains and result in decreased DNA binding affinity and decreased gene transactivation.[83,85–88] In the majority of p53-negative tumors, a missense mutation of one allele is associated with deletion of the second allele. Tumors with p53 mutations are more likely to be highly invasive, poorly differentiated, high-grade breast tumors. It is hypothesized that p53 mutations may precede the development of tumors with fully malignant and invasive phenotypes.[89] Therefore, mutant p53 has been suggested to be a biomarker predicting risk for subsequent breast carcinogenesis.[89–93] The ER has been shown to physically associate with the amino terminus of p53 to form complexes containing p53 and MDM2.[93–96] It is interesting to note that ER-α protects p53 from MDM2-mediated degradation, suggesting that ER-α signaling results in the up-regulation of p53 mRNA and protein and stabilizes expression to mediate G_1 cell cycle arrest.[93,95,97–99] However, overexpression of ER-α has been reported to mediate the overexpression of MDM2 and decrease p53 transcriptional activity.[96,100] This may be a potential mechanism leading to neoplastic transformation of the cell and suppression of p53 with increased cellular proliferation through lack of control at critical cell cycle checkpoints.[96,100]

Germline p53 mutation also serves as a risk factor for breast carcinoma development as part of the Li–Fraumeni syndrome. Although quite rare, Li–Fraumeni is a dominant inherited cancer syndrome that manifests itself with a high rate of early-onset breast carcinoma as well as multiple other tumor types.[101] p53 mutations have been identified in nearly 60% of families with this disease, suggesting that loss of p53 may be a critical parameter in the development of multiple carcinomas. Fibroblasts isolated from patients with Li–Fraumeni syndrome have not been reported to exhibit permanent G_1 or G_2 cell cycle arrest, suggesting that a loss of p53 results in the loss of cell cycle checkpoint control, which may be responsible for the increased cellular proliferation.[102,103]

BRCA1/BRCA2

Hereditary breast carcinoma is reported to account for a small proportion of all breast carcinoma cases. Germline mutations in two breast carcinoma susceptibility genes, BRCA1 and BRCA2, have been implicated in a fraction of these via an autosomal dominant inheritance mechanism. It is interesting to note that although these genes are important in hereditary breast carcinoma, they have not been found to be associated with the development or progression of sporadic breast carcinoma. Tumorigenesis in women with BRCA1 or BRCA2 mutations requires the loss or inactivation of the remaining wild-type allele, resulting in expression of a nonfunctional protein and a loss of cell cycle control and DNA repair mechanisms.[104] BRCA1 and BRCA2 apparently function to regulate DNA repair and gene transcription and maintain genome integrity. Women with a mutation in 1 of these genes are reported to have an approximately 60–80% risk of developing breast carcinoma in their lifetime.[105] Although hundreds of mutations are found scattered throughout these genes, some mutations are more prevalent and have a higher penetrance than others. Some of these "hotspot" mutations are more highly expressed in particular ethnic groups. For example, three mutations (BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT) are found to have a high penetrance in the Ashkenazi Jewish population.[106,107] Screening for and detection of BRCA1/BRCA2 mutations may be helpful in determining the overall risk for the development of breast carcinoma, especially in families with hereditary cases. Individuals who are mutation carriers may wish to undertake different surveillance strategies, chemoprevention interventions, or surgical prophylaxis for carcinomas of the breast and ovary.

Conclusions

The molecular mechanisms and changes therein leading to the development and progression of breast carcinoma are extremely complex. The biology of breast carcinoma can be exploited to determine risk, overall prognosis, and response to specific therapy. BRCA1, BRCA2, and p53 are genes that are reported to be involved in hereditary breast carcinoma. Individuals with mutations in these genes, usually leading to a truncated and nonfunctional protein, are reported to be at a higher risk of developing breast carcinoma in their lifetime. Some of these mutations are correlated with early onset of disease, whereas others are associated with increased overall lifetime risk. Therefore,

testing for mutations in these genes can contribute critical information regarding the risk of developing breast carcinoma. The evaluation of certain molecular markers such as ER and PR expression in individual tumors also may contribute to the determination of prognosis in patients with breast carcinoma. Several genes also are reported to be predictors of clinical outcome with current therapy. For example, the presence of ER and/or PR is reported to predict response to endocrine therapies such as SERMs and selective estrogen down-regulators (SERDs), whereas HER-2 overexpression predicts the response to trastuzumab. Finally, knowledge of the biology of these and other genes and their molecular changes can lead to the development of novel agents for the treatment and prevention of breast carcinoma.

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