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Original article

Gender-associated expression of tumor markers and a small gene set in breast carcinoma

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ABSTRACT

Breast carcinomas in both genders share pathological features, although differences in incidence, prognosis and survival are reported. Expression of 33 genes was investigated in male and female breast carcinomas in association with ER, PR, HER-2/neu and EGF-receptor. Among 98 male breast cancers, 82 were ER+ and 78 were PR+. ER and PR protein levels were greater in males compared to females, although no differences were observed in ESR1 and PGR expression. A difference was observed in binding affinities of PR but not ER between genders. No differences were observed in HER-2/neu, EGFR protein, or patient age. Expression of NAT1, TBC1D9, IL6ST, RABEP1, PLK1 and LRBA was elevated in carcinomas of males compared to those of females, in which ER status appeared to be related to expression. Overexpression of protein products of these genes represents novel molecular targets for development of gender-specific therapeutics and companion diagnostics.

Introduction

Breast carcinomas in men represent only ~1% of those diagnosed annually with 450 deaths [1]. Although breast carcinomas in each gender share pathological features, notable differences occur regarding incidence, prognosis and survival. We and others (e.g. [2,3]) analyzed estrogen receptors (ER) and progestin receptors (PR) in male breast cancers, reporting a propensity for their expression compared to breast lesions from female patients. Later investigations demonstrated that hormone receptor status plays a role in treatment of both male and female breast cancer (cf. [4]). Tamoxifen, an adjuvant treatment for female breast cancer [5,6], was used in male breast cancer, presumably due to high numbers of ER-positive biopsies [7]. However, tamoxifen use in male patients appears to have poor compliance due to side effects [8].

Most genetic studies in male breast cancer involved patients with a family history with BRCA1 and BRCA2 mutations (e.g. [9–12]). The androgen receptor (e.g., [13,14]) and tumor suppressor PTEN (e.g., [15]) have been studied, but few investigations of the transcriptome have been conducted. A notable investigation performed gene expression profiling of 66 male breast cancers, using Illumina bead arrays, and utilized two external gene expression datasets [16]. Products of the HLA and NAT1 genes were validated by tissue arrays containing 220 male breast cancers using immunohistochemistry [16]. These investigators categorized tumors into two distinct subtypes, luminal M1 and luminal M2 with differences in tumor biology and clinical outcome, and reported that the gene NAT1 was correlated significantly with increased patient survival [16].

Callari et al. [17], utilized microarray analysis of 37 ER-positive male and 53 ER-positive female breast cancer tissues to identify nearly 1000 genes differentially expressed between male and female breast cancer populations. Further examination suggested a prominent role for androgen receptors in male breast cancer with PR and ERBB2 (HER-2/neu) having minor roles [17]. Both studies hypothesize that male and female breast cancers are very different biologically and are likely to have differing susceptibility to treatments and clinical implications [16,17].

The long-term goal of our investigations is to identify novel molecular targets for development of gender-specific therapeutics and companion diagnostics. We describe expression of 33 candidate genes in male and female breast carcinomas in association with gene expression and protein product levels of currently employed biomarkers; ER, PR, HER-2/neu and EGF-receptor.

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Materials and methods

Tumor marker analysis

Investigations are approved by the Human Subject Protection Program Institutional Review Board, University of Louisville. A unique IRB-approved Database and Biorepository composed of de-identified tissue specimens previously collected under standardized, stringent conditions [18] were used. De-identified specimens of primary breast carcinomas collected from 1988 to 1996 were examined using REMARK criteria [19]. Highly relevant to our studies (e.g., [20,21]), analyses of ER and PR were performed by FDA-approved methods quantifying biomarkers under stringent quality control (e.g., [18,22]) unlike the majority of reports that used immuno-histochemistry prior to release of CAP/ASCO Guidelines [23]. The content of carcinoma cells in a tissue section, expressed as percent of the total cell population, was estimated by visual inspection of an intact tissue section stained with H & E. Patients were treated with the standard of care at the time of diagnosis.

Tumor marker results for 98 male breast cancers and 18,366 female breast carcinomas were identified from our IRB-approved comprehensive database containing de-identified results and clinical information (Table 1). ER and PR were quantified using either radio-ligand binding (NEN/DuPont) or enzyme immunoassay (Abbott Labs) [18,24,25]. Cut-off values approved by the FDA discriminating positive from negative expression of receptor proteins were 10 fmol/mg protein for ligand binding assay and 15 fmol/mg protein for EIA. Ligand binding assays also provided measurements of ligand affinities of either ER or PR proteins. HER-2/neu levels were determined by either ELISA (Oncogene Science) or EIA (Triton Biosciences), which quantified the levels of the membrane-bound oncoproteins in breast carcinoma cells. EGF-receptor levels were quantified by an in-house radio-ligand competition assay that also assessed binding affinities of membrane-bound receptors [26]. Cathepsin D was measured quantitatively by EIA [27].

Candidate gene selection

To obtain a clinically relevant set of candidate genes to assess breast carcinomas in association with patient gender, we reasoned that a gene should be present in various expression profiles despite differences in methodology. As described [20,28], GenBank Accession numbers (NCBI) of genes deciphered using LCM-procured carcinoma cells and those of other reports using intact tissue as described earlier [29–38]were entered into the UniGene database (National Center for Biotechnology Information (NCBI)), which separates GenBank sequences into non-redundant sets of gene-oriented clusters. UniGene identifiers were compiled into Microsoft® Access and analyzed collectively. Comparisons identified 32 candidate genes appearing in at least three signatures, (EVL, NAT1, ESR1, GABRP, ST8SIA1, TBC1D9, TRIM29, SCUBE2, IL6ST, RABEP1, SLC39A6, TPBG, TCEAL1, DSC2, FUT8, CENPA, MELK, PFKP, PLK1, XBP1, MCM6, BUB1, PTP4A2, YBX1, LRBA, GATA3, CX3CL1, MAPRE2, GMPS, CKS2 and SLC43A3). PGR was included due to its role in breast carcinoma (e.g. [18]).

Gene expression analyses

Prior to expression analyses, histological examination was performed on sections from cancer tissues using hematoxylin and eosin to evaluate structural integrity (Fig. 1). Only specimens retaining structural integrity and a significant component of carcinoma cells were investigated. On average, there were 58% carcinoma cells in male breast cancers utilized in gene expression analyses.

As previously described [20,28,39], RNA was isolated from tissue sections of 12 male and 233 female patients using RNeasy® Mini kits (Qiagen) and analyzed with Agilent RNA 6000 Nano Kits and the Bioanalyzer™ Instrument (Agilent Technologies, Palo Alto, CA). cDNA was prepared in 250 mM Tris–HCl buffer, pH 8.3 containing 375 mM KCl, and 15 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.1 M DTT (dithiothreitol, Invitrogen), 10 mM dNTPs (Invitrogen), 20 U/reaction of RNasin™ ribonuclease inhibitor (Promega, Madison, WI) and 200 U/reaction of Superscript™ III RT (reverse transcriptase, Invitrogen) with 5 ng T7 primers. qPCR reactions contained Power Sybr™ Green PCR Master Mix (Applied Biosystems, Foster City, CA), forward/reverse primers and cDNA. qPCR reactions were performed in triplicate with duplicate wells in each 384-well plate. Relative gene expression levels were determined using the $\Delta\Delta Ct$ method with ACTB for normalization and Universal Human Reference RNA (Stratagene, La Jolla, CA) as the calibrator.

Table 1
Characteristics of the patient population and tumor marker status employed in this study.

	Male breast cancer cohort		Female breast cancer cohort	
	N (%)	Mean (std dev)	*N* (%)	Mean (std dev)
Age at diagnosis		63.1 (12.4)		61.4 (14.4)
ER+	79 (80.6)		13,347 (72.9)	
ER−	19 (19.4)		4968 (27.1)	
ER level (fmol/mg protein)		161.0 (195.9)		146.0 (241.4)
PR+	79 (81.4)		12,039 (67.9)	
PR−	18 (18.6)		5697 (32.1)	
PR level (fmol/mg protein)		325.9 (572.6)		170.9 (368.9)
HER-2/neu level (hnu/mg protein)	16	1321.8 (1371.6)	1394	3635.5 (10,424.0)
HER-2/neu+	9 (56.3)		730 (52.4)	
HER-2/neu−	7 (43.8)		664 (47.6)	
EGFR level (fmol/mg protein)	21	153.4 (628.0)	26	341.8 (5334.4)
Cathepsin D (pmol/mg protein)	19	51.2 (30.7)	973	57.9 (41.5)

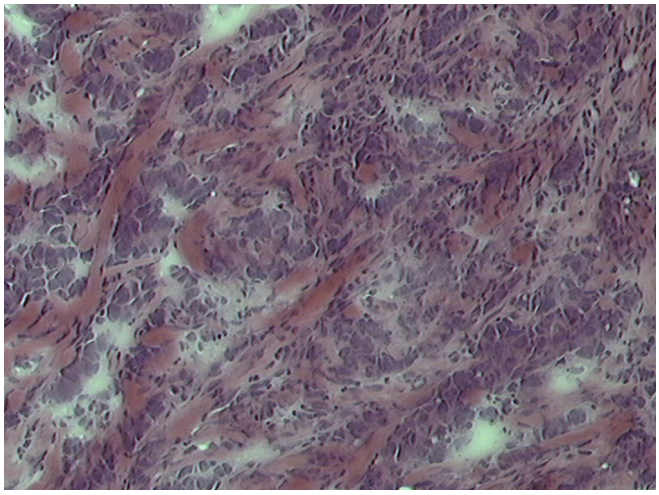

Fig. 1. A representative H&E stained tissue section of a breast carcinoma from a 44 yo male patient utilized to assess structural integrity and cell content. The carcinoma exhibited an ER level of 73 fmol/mg protein (Kd = 7.6 E-11 M) and a PR of 14 fmol/mg protein (Kd = 7.1 E-10 M) with a HER-2/neu oncoprotein level of 19 hnu/mg protein.