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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Relationship between Quantitative *GRB7* RNA Expression and Recurrence after Adjuvant Anthracycline Chemotherapy in Triple-Negative Breast Cancer

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Abstract

Purpose: To conduct an exploratory analysis of the relationship between gene expression and recurrence in patients with operable triple-negative breast cancer (TNBC) treated with adjuvant doxorubicin-containing chemotherapy.

Experimental Design: RNA was extracted from archived tumor samples derived from 246 patients with stage I-III TNBC treated with adjuvant doxorubicin-containing chemotherapy, and was analyzed by quantitative reverse transcriptase PCR for a panel of 374 genes. The relationship between gene expression and recurrence was evaluated using weighted Cox proportional hazards model score tests.

Results: Growth factor receptor bound protein 7 (*GRB7*) was the only gene for which higher expression was significantly associated with increased recurrence in TNBC (Korn's adjusted P value = 0.04). In a Cox proportional hazards model adjusted for clinicopathologic features, higher *GRB7* expression was associated with an increased recurrence risk (HR = 2.31; $P = 0.04$ using the median as the split). The 5-year recurrence rates were 10.5% [95% confidence intervals (CI), 7.8–14.1] in the low and 20.4% (95% CI, 16.5–25.0) in the high *GRB7* groups. External validation in other datasets indicated that *GRB7* expression was not prognostic in two adjuvant trials including variable systemic therapy, but in two other trials showed that high *GBR7* expression was associated with resistance to neoadjuvant doxorubicin and taxane therapy.

Conclusions: *GRB7* was associated with an increased risk of recurrence in TNBC, suggesting that *GRB7* or *GRB7*-dependent pathways may serve as potential biomarkers for therapeutic targets. Therapeutic targeting of one or more factors identified which function as interaction nodes or effectors should also be considered. *Clin Cancer Res; 17(22); 7194–203. ©2011 AACR.*

Introduction

Triple-negative breast cancer (TNBC) is defined as breast cancer which lacks expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2/neu protein. Population-based studies indicate that TNBC accounts for about 15% of all breast cancers in the United States and occurs more commonly in younger women, and women of black race or Hispanic ethnicity (1). TNBC is associated with a higher risk of distant recurrence, earlier time to recurrence, and worse prognosis after recurrence (2, 3). About 80% of TNBC are characterized as being of a basal-like breast cancer genotype identified by gene expression profiling (4, 5, 6). A panel of antibodies which includes cytokeratin markers may more accurately classify basal subtypes than relying on ER, PR, and HER2/neu expression alone (7, 8). Although inhibitors of PARP may hold promise (9), therapeutic approaches are currently limited to cytotoxic chemotherapy

In the current study that is the subject of this report, we evaluated gene expression patterns from tumors derived from a cohort of patients with stage I to III breast cancer treated with adjuvant doxorubicin-containing chemotherapy. In addition to conducting gene expression profiling, we defined breast cancer subsets by standard immunohistochemistry (IHC) for ER, PR, and HER2/neu protein expression in a central laboratory (10). We evaluated

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

The study was presented in part as oral presentations on December 11, 2008, at the CTRC-AACR San Antonio Breast Cancer Symposium, San Antonio, TX, and on June 7, 2009, at the 45th Annual Meeting of the American Society of Clinical Oncology Orlando, FL.

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Translational Relevance

Growth factor receptor bound protein 7 (*GRB7)* RNA expression was associated with a significantly increased risk of recurrence in operable triple-negative breast cancer (TNBC) patients treated with adjuvant anthracycline chemotherapy in our analysis, and in external validation studies was associated with resistance to neoadjuvant anthracycline therapy but was not prognostic in operable TNBC patients who received no adjuvant therapy. Growth factor receptor bound protein 7 (*GRB7)* belongs to a small family of mammalian SH2 domain adapter proteins that are known to interact with a number of receptor tyrosine kinases and signaling molecules (including HER1, HER2, and ephrin receptors), with the integrin signaling pathway, and focal adhesion kinase (FAK). *GRB7* shares sequence homology with the *mig-10* gene of *Caenorhabditis elegans*, which is required for migration of embryonic neurons, suggesting an important role in cell motility. These findings suggest that high *GRB7* expression has a role as a potential biomarker for resistance to anthracycline therapy and serve as a therapeutic target in TNBC.

differences in gene expression patterns between triple-negative disease and HR-positive, HER2/neu-negative disease. We also conducted an exploratory analysis evaluating the relationship between gene expression and recurrence within the triple-negative group. Our objectives were to identify RNA expression biomarkers associated with recurrence within the TNBC group and potential therapeutic targets for the TNBC group which may not have been previously recognized.

Materials and Methods

Study population and treatment

The study used tumor specimens and clinical information from patients enrolled on trial E2197 (ClinicalTrials.gov identifier NCT00003519), coordinated by the Eastern Cooperative Oncology Group (ECOG), details of which have been reported elsewhere (11). Briefly, patients were randomly assigned to receive four 3-week cycles of doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 (AC) or docetaxel 60 mg/m^2 (AT). Methods for selection of cases included in the genomic analysis have been previously described (12), and summarized in the CONSORT diagram shown in Supplementary Fig. S1. The characteristics of the sample cohort were comparable with the excluded cohort, also as previously described (12). The clinical protocol was approved by the Institutional Review Boards of all participating institutions and was carried out in accordance with the Declaration of Helsinki, Food and Drug Administration Good Clinical Practices, and local ethical and legal requirements. The use of specimens for this project was approved by the North American Intergroup Correlative Science Committee and by the Northwestern University Institutional Review Board (which oversees the ECOG Pathology Coordinating Office, where the specimens were banked and evaluated).

Specimen selection, processing, and gene expression analysis

All specimens underwent analysis for tumor grade, and for ER, PR, and HER2/neu protein expression in a central lab as previously described (10). Quantitative RNA expression levels were measured by real-time reverse transcriptase PCR (RT-PCR) using gene-specific primers (13). The panel of 374 genes was assembled by searching the published literature, genomic databases, pathway analysis, and microarray-based gene expression profiling experiments carried out in fresh-frozen tissue to identify genes likely to be associated with prognosis or response to chemotherapy, as previously reported (14).

A total of 246 cases were defined as having TNBC by IHC using the methods described above, of whom 15% had a recurrence. This cohort includes only one patient (who did not recur) whose tumor was HER2/neu positive by the Genomic Health RT-PCR Assay ($\geq$11.5 units), which has been found to exhibit 97% concordance with HER2/neu gene amplification by fluorescent *in situ* hybridization (15). If hormone receptor expression were defined by RT-PCR using the Genomic Health cutoff value for ER $\geq$6.5 units) and PR ($\geq$5.5 units), then there were 258 triple-negative cases (instead of 246; 227 of these are the same) and the estimated slope of continuous *GRB7* (linear effect in log expression level) is 0.692 (instead of 0.637 as in the analysis presented in Fig. 1), indicating that the results would be similar irrespective of which definition was used. The results presented here are based upon cases selection using the

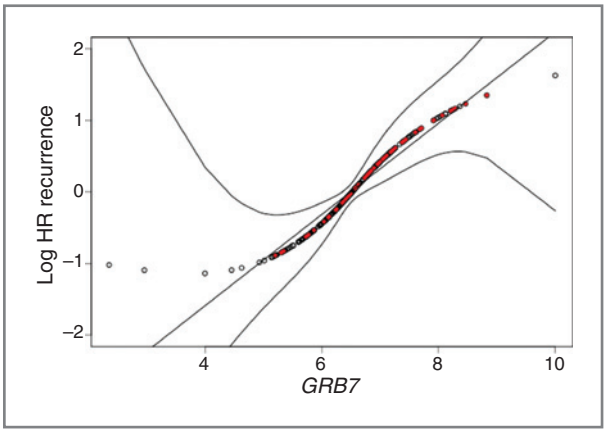

Figure 1. Natural log HR for recurrence risk as a function of quantitative *GRB7* expression without adjustment for other factors. Points in red correspond to recurrences, whereas the curved solid lines are at $\pm$ 2 SE. There was a highly statistically significant association between quantitative expression and recurrence ($P = 0.001$ overall, $P = 0.53$ for nonlinearity.) Straight line is the estimate from the linear model. Each integer on the y-axis scale indicates a 2.72-fold increase in risk of recurrence.

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central immunohistochemical definition for ER, PR, and HER2/neu because this most accurately reflects actual clinical practice, and optimizes the methodology for ER/PR testing by IHC (reflected by somewhat higher concordance with central RT-PCR).

Statistical analyses

The relationship between gene expression and recurrence was evaluated using weighted Cox proportional hazards model score tests used to rank genes by their individual significance for predicting recurrence risk as previously described (14). Recurrence was defined as distant and/or local-regional recurrence of disease. Among the 59 recurrences, 39 were distant and 20 were locoregional. Adjusted *P* values controlling the false discovery proportion (FDP) at 10% or less were computed using algorithm B* in Korn and colleagues (ref. 16; using 500 permutations) and were applied in a step-down fashion (17). A weighted algorithms were used to correct for differential sampling of relapse and nonrelapse cases (18). All *P* values are 2-sided. Differences in gene expression were evaluated between the HR-negative (i.e., triple negative; $N = 246$) and HR-positive, HER2-negative groups ($N = 383$) by a weighted *t* test. Pathway analysis was conducted using Ingenuity Pathway Analysis Version 7.6 on the top 40 genes overexpressed in TNBC versus HR-positive, HER2-negative tumors.

External validation

We evaluated the relationship between *GRB7* expression and outcomes in several publicly available datasets, which used various methods to examine gene expression, including populations with operable disease (5, 19, 20) in which we evaluated the relationship between *GRB7* expression and prognosis, and in 2 neoadjuvant data sets in which we evaluated the relationship between *GRB7 e*xpression and response to anthracycline and taxane-containing neoadjuvant chemotherapy (21, 22). From studies in which the depositing investigators had annotated their samples as "Basal-like" we used these designations as an approximation for TNBC (5, 23). The studies used included the following: (i) Bonnefoi and colleagues data (GSE6861; ref. 21): All patients received neoadjuvant chemotherapy. TNBC status was assigned to samples negative for ER and PR and nonamplified for HER2 as follows: Analysis of the probe for ESR1 (g4503602_3p_at) and ERBB2 (Hs.323910.2.A1_3p_a_at) revealed a clear bimodal distribution. The higher expressing samples for each probe were considered to be ER positive and HER2 amplified, respectively. The ER-negative, HER2 nonamplified sample set was then further curated to remove samples expressing high levels of PR (g4505766_3p_at). This yielded 76 cases which we designated TNBC, of whom 32 had a pathologic complete response. (ii) Tabchy and colleagues data (GSE20271; ref. 22): The data provided by these investigators were annotated for ER, PR, and HER2 status. Using their annotation, we selected the ER-negative, PR-negative and HER2-negative samples (58 samples) as our TNBC set. Analysis of the expression levels of ER, PR, and HER2 revealed one sample (GSM508138) with very high HER2 levels (within the range of the tumors annotated as HER2-amplified, and the fifth highest level of HER2 in the dataset overall using the probe 210930_s_at for ERBB2). This sample was excluded from our analysis, leaving 57 we considered TNBC. Of these 57, there were 12 pathologic complete response (pCR) and 45 patients with residual disease (RD). Data from these 57 samples were presented in the manuscript, however the data were also analyzed without excluding the suspicious HER2-high sample, and the difference in GRB7 levels between the pCRs and the RDs remained significant ($P < 0.05$). (iii) NKI295/Van de Vijver data (23): Data provided by these investigators were already annotated by breast tumor molecular subtype. 46 cases annotated as "basal" by Van de Vijver and colleagues were considered TNBC for the purpose of our analysis. There was no difference in recurrence rate between *GRB7*-high and *GRB7*-low tumors ($N = 46$, 24 of whom experienced recurrence). We also examined outcomes for the subset of these patients who received systemic chemotherapy ($N = 18$) and there was no difference in outcomes by *GRB7* levels. (iv) Wang and colleagues data (GSE2034; ref. 19): The data provided by these investigators included ER status. We used the expression values of the probe for ERBB2 (216836_s_at) to exclude tumors likely to be ERBB2-amplified. This left 51 tumors we considered TNBC. There were 17 relapses. (v) Parker and colleagues (ref. 5): This study included several independent datasets. The specific data we analyzed were obtained from Supplementary Tables S1 and S4 in the online Supplementary Material for this article. These data consisted of RT-PCR gene expression data on 279 tumors, designated "UBC-PCR" in Supplementary Table S4. The investigators annotated 61 of the samples as basal like. We examined the relationship between GRB7 and recurrence-free survival in 55 of these samples (3 samples lacked follow-up data for recurrence-free survival and 3 samples lacked expression data for GRB7). There were 26 relapses. To summarize, in the Parker and colleagues study (5), we analyzed the basal-like tumors from the 289 patient qRT-PCR dataset presented in the Supplementary Material. In the remaining studies, we used the gene expression levels reported by the probes for ER, PR, and ERBB2 to define subgroups that were negative for ER and PR and not ERBB2 amplified. Samples without follow-up and one sample annotated as Basal-like but with very high levels of ERBB2 mRNA were excluded. This approach provided 285 cases for further analysis, broken down as follows: 76 cases (32 pCRs; ref. 21), 57 cases (12 pCRs; ref. 22), 46 cases (24 recurrences; ref. 23), 51 cases (17 recurrences; ref. 19) and 55 cases (26 recurrences; ref. 5).

For the neoadjuvant datasets, a major issue was the relative insensitivity of the Affymetrix probesets compared the sensitivity of the RT-PCR assay used in our analysis. The RT-PCR assay for *GRB7* detected differences across a 190-fold range among TNBC cases in our study, whereas the Affymetrix X3P probe used in the study by Bonnefoi and colleagues had a dynamic range of 3-fold in the TNBC samples, and the Affymetrix U133A probe used in the study

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by Tabchy and colleagues had a dynamic range of only 5-fold. To try to understand the inefficiency of these probesets we used the Affy package of Bioconductor to extract the raw probe-level data from each microarray used in the Bonnefoi study. This permitted us to individually analyze the signal values for each of the 11 probes that make up the *GRB7* probeset across 160 microarrays. In general, the correlation coefficient between the individual probes and the aggregate value reported for *GRB7* in each array was relatively good, with Pearson correlation coefficients ranging from 0.79 to 0.93. However, when we stratified the data into *ERBB2*/*GRB7* amplified (i.e., high *GRB7* mRNA) and nonamplified (i.e., low *GRB7* mRNA), an interesting difference emerged. For the high *GRB7* expressors, the performance of the individual probes remained good, with Pearson Correlation coefficients ranging from 0.80 to 0.91). However, in the low *GRB7* expressors (of which the TNBC samples in this study are a substantial subgroup) the correlation between the individual *GRB7* probes the overall value reported for the *GRB7* probeset was significantly reduced, with Pearson's correlation coefficients ranging from −0.38 to 0.29). From this analysis, we concluded that although the *GRB7* probeset on these arrays works very well when *GRB7* mRNA is abundant, at lower *GRB7* levels it suffers from a substantial amount of noise, resulting in a substantial loss of linearity. For this reason, our validation studies have not assumed that the signals in this range are drawn from a Gaussian distribution, and we have used the rank-based Mann–Whitney *U* test to attempt to discern whether there are differences in outcome depending on *GRB7* levels.

Results

Characteristics of triple-negative population

The characteristics of patients with TNBC are shown in Table 1. Most patients were 65 or younger (93%), had tumors with poor histologic grade (90%) who were associated with negative axillary lymph nodes (81%), and occurred in white subjects (87%). When compared with 60 patients who had HR-negative, HER2-positive disease, patients with TNBC were younger (41% vs. 27% less than 46, $P = 0.04$) and more likely to have negative axillary nodes (81% vs. 66%, $P = 0.01$), but were otherwise similar with regard to tumor size, tumor grade, and race.

Table 1. Characteristics of triple-negative patient population included in this analysis

	n (%)
Total	246
Age, y	
≤45	101 (41)
45–65	128 (52)
>65	17 (7)
Central grade	
Well/moderate	25 (10)
Poor	221 (90)
Positive axillary node	
0 positive	200 (81)
1 positive	29 (12)
2–3 positive	17 (7)
Tumor size, cm	
≤2	116 (47)
>2 to ≤5	120 (49)
>5	10 (4)
Race	
White	214 (87)
Black	27 (11)
Other	5 (2)

Genes associated with increased recurrence in triple-negative disease

We evaluated the relationship between gene expression and recurrence in the 246 patients with TNBC. There were 6 genes significantly associated with recurrence (adjusted value of $P < 0.05$), including 1 gene for which increased expression was associated with increased recurrence, and 5 with decreased recurrence. To show the relationship between gene expression and recurrence, we show the HR/SD, which is the HR for a difference of one SD of the log expression level of the gene, where the SD refers to the distribution of log expression levels of the gene in the sample. This is a measure of effect that is invariant to rescaling (or shifting) the log expression levels (and invariant to the base used in the logs). The only gene associated with increased recurrence was *GRB7* ($P = 0.04$, estimated HR/SD of gene expression = 1.74). Genes associated with decreased recurrence included *APOC1* (apolipoprotein C1; $P = 0.03$; HR/SD = 0.59), *ESR2* (estrogen receptor β; $P = 0.03$; HR/SD = 0.53), *PIM2* (*PIM2* oncogene, HR/SD = 0.59; $P = 0.04$), *CD68* (macrophage antigen, HR/SD = 0.67; $P = 0.05$), and *BIRC3* (baculoviral IAP repeat 3; $P = 0.05$; HR/SD = 0.60). There were only 6 genes whose expression correlated ($r > 0.4$) with *GRB7* (found on chromosome 17q12), including *ERBB2* ($r = 0.70$; HR/SD = 1.30, chromosome 17q21.1), *DDR1* (discoidin domain receptor tyrosine kinase 1; $r = 0.53$; HR/SD = 1.32, chromosome 6p21.3), *KRT19* (keratin 19; $r = 0.49$; HR/SD = 1.56, chromosome 17q21.2), *ERBB3* ($r = 0.48$; HR/SD = 1.18, chromosome 12q13), *GPR56* (G protein-coupled receptor 56; $r = 0.48$; HR/SD = 1.29, chromosome 16q13) and *PHB* (prohibitin; $r = 0.42$; HR/SD = 1.01, chromosome 17q21). Expression levels were evaluated for genes located on chromosome 17q, including *ERBB2*, *GRB7*, *PHB*, and *KRT19*, and were significantly lower for *ERBB2* and *GRB7* in TNBC compared with HER2/neu overexpressing breast cancer (Supplementary Fig. S2).

Relationship between GRB7 expression and recurrence as a continuous or categorical variable

To further characterize the relationship between *GRB7* expression and recurrence in TNBC, we evaluated this relationship as a continuous variable using a spline model for the log HR as shown in Fig. 1. *GRB7* RNA expression

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Table 2. Multivariate model evaluating relationship between GRB7 expression and recurrence (HRs and 95% CIs)

	Model I	Model II	Model III
Age ≤45 vs. >65	0.61 (0.21–1.79)	0.49 (0.17–1.42)	0.49 (0.17–1.46)
Age 45–65 vs. >65	0.81 (0.29–2.26)	0.67 (0.25–1.84)	0.63 (0.22–1.78)
Nodes 1 vs. 0	2.37 (1.39–4.03)	2.04 (1.17–3.57)	2.27 (1.32–3.92)
Nodes 2–3 vs. 0	1.83 (0.83–4.05)	1.57 (0.65–3.83)	1.96 (0.86–4.47)
Grade poor vs. moderate/well	1.53 (0.56–4.19)	1.62 (0.53–4.95)	1.43 (0.51–3.98)
Tumor size >2 vs. ≤2 cm	1.93 (1.09–3.41)	1.97 (1.10–3.55)	1.95 (1.09–3.47)
GRB7 $x + 2$ vs. x		3.41 (1.78–6.53)[a]	
GRB7 High vs. low			2.31 (1.30–4.11)[b]

[a] $P = 0.002$.
[b] $P = 0.004$.

ranged from a low of 2.4 to as high as 10 units, which corresponds to about a 190-fold difference in RNA expression between the highest and lowest values. There was a highly significant relationship between the risk of recurrence and *GRB7* expression ($P = 0.001$). The median value of 6.5 was chosen for the additional categorical analyses, which falls on the maximal slope of the curve. The estimated HR for high versus low expression based on the median split was 2.24 ($P = 0.006$). If tertile splits were used, the difference between the low and intermediate groups was not significant (HR = 0.85; 95% CI, 0.39–1.88), but was significant for high versus intermediate is (HR = 2.41; 95% CI, 1.27–4.59; $P = 0.007$). Therefore, the median split was used to define *GRB7* as a categorical variable in subsequent analyses. Higher *GRB7* expression was also associated with significantly higher risk of recurrence in the subset of 60 patients with ER/PR-negative, HER2/neu-positive disease (HR for high vs. low expression = 1.75; 95% CI 1.02–3.00; $P = 0.04$).

Relationship between GRB7 expression and clinical variables

To evaluate the relationship between *GRB7* expression and clinical variables, we compared the clinical characteristics of patients with high versus low expression levels (supplementary Table S1). There were no significant differences in any clinical characteristic examined between patients who exhibited high versus low *GRB7* expression levels, except for fewer patients over 65 years of age with high GRB7 expression (3% vs. 10%, $P = 0.03$).

Multivariate analysis: relationship between GRB7 expression and recurrence

To evaluate the relationship between *GRB7* expression and recurrence adjusted for clinicopathologic variables, Cox proportional hazards models were fit to examine the joint effects of factors on recurrence rates, as shown in Table 2. The models included age, nodal status, centrally determined tumor grade, and tumor size. In Model I, which did not include *GRB7* expression, features associated with an increased risk of recurrence included one positive axillary lymph node (vs. none) and large tumor size (>2 cm). Model II added *GRB7* as a continuous linear variable to Model I; *GRB7* $x + 2$ versus x was used for the HR (corresponding to an approximately 4-fold increase in gene expression), where x is an arbitrary value of *GRB7* (comparable with the analysis of recurrence score as continuous variable in the report by Paik and colleagues (13). In this model, *GRB7* expression was a highly significant predictor for recurrence (HR = 3.41; 95% CI, 1.78–6.53; $P = 0.002$). Model III added *GRB7* as a dichotomous variable (high vs. low, using the median split) to model I. In this model, there was also a significant relationship between *GRB7* expression and recurrence (HR = 2.31; 95% CI, 1.30–4.11; $P = 0.004$).

Pathway analysis of differentially expressed genes

Comparing gene expression in TNBC with HR-positive, HER2-negative disease revealed 269 genes (73%) with significantly different expression ($P < 0.0001$). The top 40 genes showing significantly higher expression and lower expression in the TNBC group are shown in Supplementary Table S2 and S3, respectively. The top 40 genes showing higher expression in the TNBC group included genes associated with nucleosome assembly (*CENPA*), kinase activity (*TTK*), invasion (*CTSL2*), DNA damage response (*CHEK1*), transcriptional regulation (*MYBL2*), transmembrane amino acid transport (*SLC7A5*), transcription (*FOXM1*), cell division (*CDC20*, *KIFC2*, *AURKB*, *PLK1*), and the cell cycle (*KIFC1*, *DEPDC1*, *CDCA8*). Pathway analysis was done including the 40 top genes showing higher expression in TNBC and showed substantial interaction between the proteins that they encode (Fig. 2). Some of the encoded proteins seemed to serve as nodes for interaction, including cytoplasmic proteins such as the antiapoptotic protein survivin (*BIRC5*), kinases involved in mitosis such as Aurora Kinase B (*AURKB*) and in the cell cycle such as cyclin-dependent kinase 1 (*CDC2*), and nuclear transcription factors such as forkhead box M1 protein (*FOXM1*) and Myb-related protein B (*MYBL2*).

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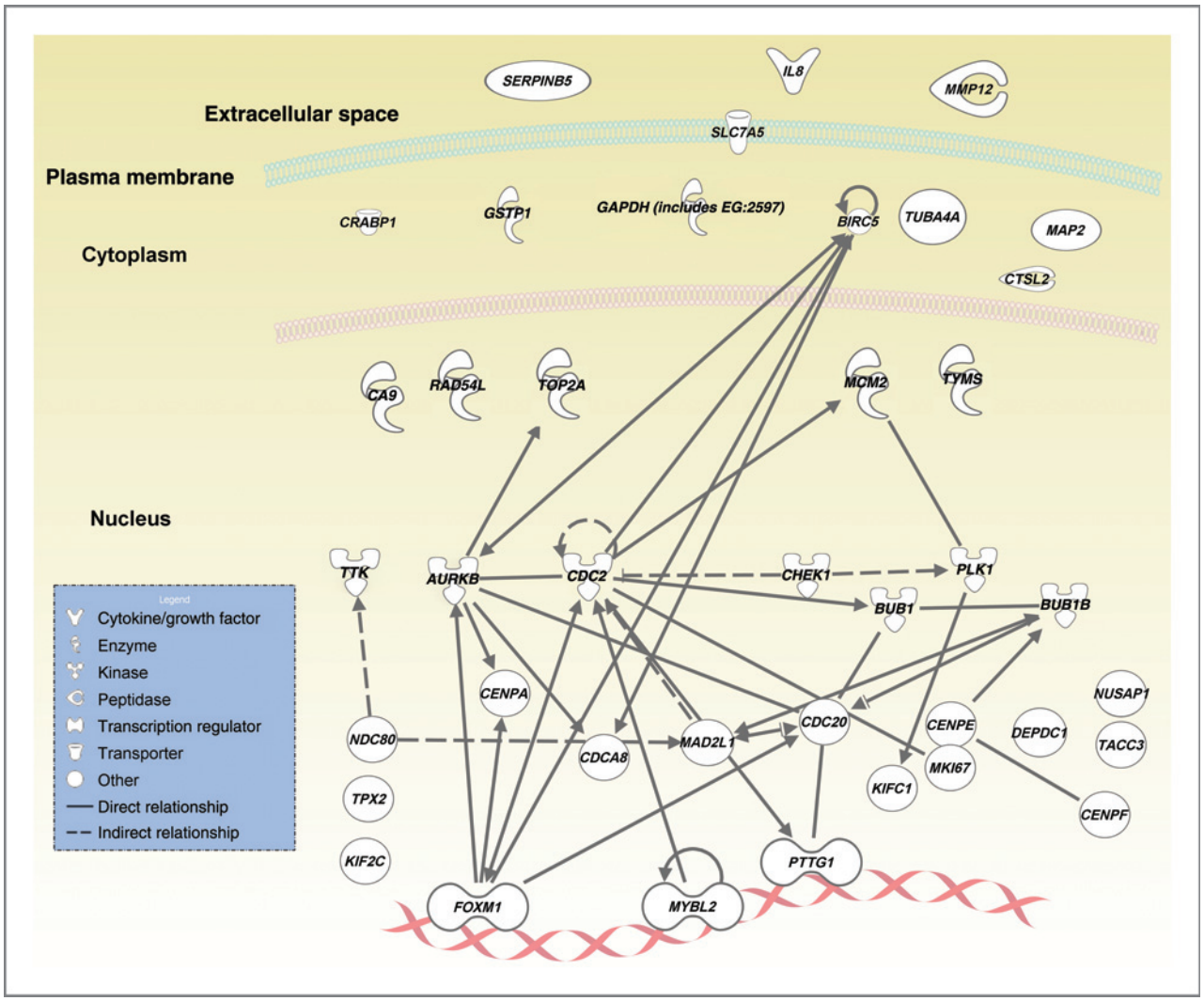

Figure 2. Pathway analysis of genes highly expressed in TNBC compared with HR-positive, HER2-negative breast cancer. The nodes of the network are the proteins encoded by the genes overexpressed in TNBC and their shapes represent their biological activities as shown in the inset. The nodes are connected by edges which indicate direct and indirect biological relationships between these proteins. Highly connected nodes may represent therapeutic targets, inhibition of which could attenuate signaling throughout the network.

Validation in other data sets

We evaluated the relationship between *GRB7* expression and outcomes in several publicly available datasets, which used various methods to examine gene expression. With regard to prognosis, we found no relationship between *GRB7* expression and recurrence in patients with node-negative breast cancer who received no adjuvant chemotherapy (excluding patients with high *ERBB2* and ER expression; ref. 19), and in patients with basal breast cancer subtype with node-negative or -positive disease (some of whom received adjuvant chemotherapy; data not shown; refs. 5, 20). With regard to prediction, *GRB7* expression level was evaluated in 76 patients with TNBC treated with neoadjuvant sequential epirubicin and docetaxel-containing chemotherapy (21). There was no significant difference in the proportion with pCR who had an elevated *GRB7* expression at or above the median compared with below the median [13 of 38 (34%) vs. 19 of 38 (50%), the Fisher exact test: $P = 0.17$]. On the other hand, patients who did not have a pCR had significantly higher median *GRB7* expression (Mann–Whitney *U* test for difference between medians: $P = 0.0397$, Fig. 3A), which was consistent with our findings indicating an association between higher GRB7 expression and resistance to adjuvant anthracycline therapy (given with or without concurrent docetaxel). Likewise, in a second neoadjuvant dataset in which 12 of 57 patients (21%) with TNBC had a pCR after treatment with neoadjuvant 5-flourorucaracil, doxorubicin, and cyclophosphamide (FAC) alone or preceded by paclitaxel (T/FAC; ref. 22), median *GRB7* expression levels were significantly higher in the nonresponders ($P = 0.0044$, Mann–Whitney *U* test, Fig. 3B).

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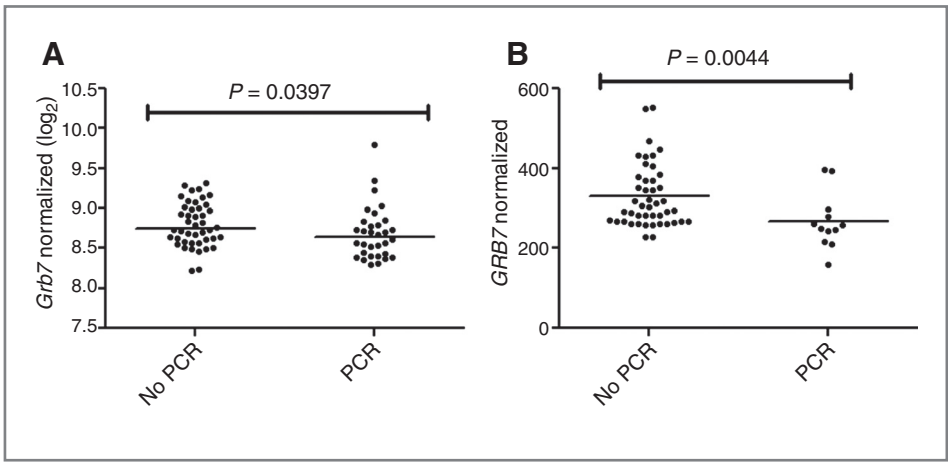

Figure 3. External validation showing significantly higher median *GRB7* expression levels in patients who did not have a pathologic complete response to therapy compared with those who did for the study reported by Bonnefoi and colleagues (3A; ref. 21) and Tabchy and colleagues (B; ref. 22).

Discussion

We conducted an exploratory analysis evaluating the relationship between recurrence and a panel of genes in 246 patients with stages I to III TNBC who received standard doxorubicin-containing chemotherapy and were followed for at least 5 years. Quantitative RT-PCR was used to measure RNA extracted from paraffin-embedded tumor specimens for a panel of 374 rationally selected genes. All samples were centrally evaluated for ER, PR, and HER2 expression by IHC in a standardized and rigorous manner and confirmed to be triple negative (24). *GRB7* was the only gene for which higher expression was found to be associated with a significantly elevated risk of recurrence, suggesting that *GRB7 m*ay serve as an important biomarker in TNBC, and that perhaps *GRB7* or *GRB7*-dependent pathways may serve as therapeutic targets. Similar to Oncotype DX recurrence score (RS) in ER-positive breast cancer, the relationship between *GRB7* expression and recurrence was evident when evaluated as a continuous variable or dichotomous variable adjusted for other covariates, and the relative risk elevation was comparable. For example, a 50 unit increase in RS (which has a range of 0–100) was associated with a 2- to 8-fold increase ($P < 0.001$) in risk of distant recurrence in ER-positive disease treated with tamoxifen in the B14 trial (25), and 2.1-fold increase ($P = 0.06$) in ER-positive disease treated with adjuvant chemotherapy plus tamoxifen in the E2197 trial (12), whereas a 2 unit increase in *GRB7* expression (range, 2.4–8) was associated with a 3.4-fold increase ($P = 0.002$) in the risk of recurrence in TNBC evaluated in this dataset. When evaluated in 4 other publicly available datasets, although *GRB7* expression was not associated with recurrence in patients who received no adjuvant chemotherapy, median *GRB7* expression levels were significantly higher in patients with TNBC who failed to achieve a pCR after preoperative anthracycline and taxane therapy. Although there was no specific *GRB7* expression threshold predictive of response in these relatively small neoadjuvant trials, the significantly higher median expression levels in nonresponders are nevertheless consistent with a relationship between *GRB7* expression and sensitivity to anthracycline and/or taxane therapy. In addition, Ramsey and colleagues have recently reported an association between high *GRB7* protein expression and recurrence in the presence or absence of adjuvant chemotherapy (26), providing additional independent evidence supporting our findings.

GRB7 belongs to a small family of mammalian SH2 domain adapter proteins that are known to interact with a number of receptor tyrosine kinases and signaling molecules (including HER1, HER2, and ephrin receptors), with the integrin signaling pathway, and with focal adhesion kinase (FAK; refs. 27, 28). *GRB7* shares sequence homology with the *mig-10* gene of *Caenorhabditis elegans*, which is required for migration of embryonic neurons, suggesting an important role in cell motility (29). *GRB7* is also included in the 21-gene signature in ER-positive disease (25), and in the 512 intrinsic gene set (4) and PAM50 gene set (5), indicating other evidence that it may be an important biomarker. *GRB7* is located on the same amplicon as the *ERBB2* gene and thus usually coamplified in HER2/neu-overexpressing breast cancers, and we confirmed that *GRB7* expression levels were significantly lower in HER2/neu non-overexpressing tumors. Although *GRB7* expression was correlated with *ERBB2* expression within the TNBC group ($r = 0.70$) *GRB7* but not *ERBB2* expression was significantly associated with recurrence in this population, supporting its role as a prognostic marker in this setting. Supporting its potential as a therapeutic target, several inhibitors of *GRB7* have been developed, some of which have been shown to potentate the effects of cytotoxic therapy and trastuzumab (30–33). In addition, inhibitors of *GRB7*-dependent pathways such as FAK, ephrins (34), and integrins (35) offers additional therapeutic potential. Evaluation of a highly specific *GRB7* peptide inhibitor (G7-18NATE) in a panel of 4 TNBC cell lines revealed that *GRB7* inhibition significantly impaired migration and invasion, reduced colony formation in 3-dimensional culture by promoting apoptosis, and synergistically sensitized TNBC cell lines to doxorubicin and docetaxel (O. Giricz; submitted for publication). Taken together, these findings *GRB7* as a key mediator of migration, invasion, colony growth, and chemoresistance of TNBC, and suggest that in addition to serving as a

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Table 3. Potential targets in TNBC identified in this analysis

Gene	Protein name	Protein function	Drugs
AURKB	Aurora Kinase B	Binds microtubule K fibers near kinetichores	AZD1152, VX-680, AT9283
PLK1	Polo-like Kinase 1	Regulates G_2–M transition	BI6727, ON01910
KIFC1	Kinesin Family Member C1	Microtubule motor activity	ARRY-250, ispinesib, SB743921
CHEK1	CHK1 Checkpoint Homologue	Regulates G_2–M checkpoint	AZD7762, PF0477736
FOXM1	Forkhead Box M1	G_1–S and G_2–M cell-cycle phase progression; mitotic spindle integrity	Siomycin A
CDC2	CDK1	G_1–S and G_2–M cell-cycle phase progression	Flavopiridol

prognostic or predictive biomarker, *GRB7*-dependent pathways may prove to be important therapeutic target in TNBC.

Although *GRB7* was the only gene for which increased expression was associated with an increased risk of recurrence, increased expression of several other genes was found to be associated with a significantly lower risk of recurrence, including CD68, a macrophage antigen likely reflecting host cells infiltrating the tumor. CD68 is also one of the 16 tumor-associated genes in the Oncotype DX recurrence score for HR-positive disease, in which higher expression is likewise associated with a more favorable outcome. This suggests the importance of host response, or specific antigens eliciting a host response, that may play an important role in determining prognosis in TNBC.

The analysis of differential gene expression comparing TNBC with HR-positive, HER2-negative disease provided some additional information suggesting potential therapeutic targets which are summarized in Table 3. These include inhibitors of Aurora Kinase B (36–38), polo-like kinase 1 (39, 40), kinesin family member C1, checkpoint kinase 1 (41), and forkhead box M1 (42). The pathway analysis indicates that some of these proteins (encoded by their genes) may be particularly vulnerable targets because they serve as nodes for interaction or transcriptional effectors, including Aurora Kinase B (*AURKB*), cyclin-dependent kinase 1 (*CHEK1*), forkhead box M1 (*FOXM1*), and myb-related protein B (*MYBL2*). Other reports have likewise confirmed the important role of some of the genes. For example, Thorner and colleagues reported a significant association between the G/G genotype of a nonsynonymous MYBL2 germ line variant (rs2070235, S427G) and an increased risk of basal-like breast cancer (OR = 2.0; 95% CI, 1.1–3.8); they also showed that MYBL2 is involved in cell-cycle control, and that its dysregulation contributes to increased sensitivity specifically to DNA topoisomerase II inhibitors (43).

There are several notable strengths of this analysis. First, this is one of the largest training sets specifically evaluating gene expression in a uniformly treated cohort of patients with TNBC; inadequate sample size is recognized as a major limitation of previous studies (44). Second, the TNBC group was defined by standard immunohistochemical methods commonly used in clinical practice but done in a standardized and rigorous manner in a central laboratory. Third, we evaluated a limited panel of candidate genes that were rationally selected because of their known or postulated association with prognosis or response to chemotherapy rather than a genome-wide approach, and used a standardized RT-PCR method that brings precision and large dynamic range. This offers the potential to reduce the likelihood of identifying falsely positive associations by enriching for candidate genes likely to be associated with recurrence, and provides confidence in assuring reproducibility of the identified genes and the method of measuring their expression. In addition, stringent statistical methods were used to control false discovery (17). Some of the analyses were also adjusted for clinicopathologic variables to explore whether specific genes, such as *GRB7*, provided information beyond standard clinicopathologic measures. Finally, we carried out external validation in 4 independent data sets and confirmed that high *GRB7* expression was associated with resistance to doxorubicin and taxane therapy, but did not provide prognostic information in the absence of systemic chemotherapy.

There were also several limitations of this analysis. A fundamental premise of our study is that increased gene transcription, as reflected by RNA expression levels, may identify potential therapeutic targets, biomarkers predictive of clinical behavior or response to therapy, or both. However, altered transcription may reflect an effect rather than a cause of the malignant phenotype. In addition, searching for activating gene mutations, oncogenes, or inactivated tumor suppressor genes may be a more fruitful strategy for therapeutic targeting (45). On the other hand, there is a clear precedent for effectively targeting pathways in breast cancer that are not associated with discernable activating mutations, as exemplified by antiestrogen therapy.

In conclusion, we identified several genes that are novel therapeutic targets in TNBC, and which may have potential clinical utility. Validation in preclinical systems will be required for drug development, and additional validation in other clinical datasets will be required for clinical application.

Disclosure of Potential Conflicts of Interest

B.H. Childs, D. Brassard, and S. Rowley have employment (other than primary affiliation, e.g., consulting) from Sanofi-Aventis; S. Shak, F.L. Baehner, and R. Bugarani have employment (other than primary affiliation, e.g., consulting) from Genomic Health. S. Shak has ownership interest (including patents) in Genomic Health. No potential conflicts of interest were disclosed by other authors.

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