PRECLINICAL STUDY

Aberrant expression of DNA damage response proteins is associated with breast cancer subtype and clinical features

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Abstract Landmark studies of the status of DNA damage checkpoints and associated repair functions in preneoplastic and neoplastic cells has focused attention on importance of these pathways in cancer development, and inhibitors of repair pathways are in clinical trials for treatment of triple negative breast cancer. Cancer heterogeneity suggests that specific cancer subtypes will have distinct mechanisms of DNA damage survival, dependent on biological context. In this study, status of DNA damage response (DDR)-associated proteins was examined in breast cancer subtypes in association with clinical features; 479 breast cancers were examined for expression of DDR proteins γH2AX, BRCA1, pChk2, and p53, DNA damage-sensitive tumor suppressors Fhit and Wwox, and Wwox-interacting proteins Ap2α, Ap2γ, ErbB4, and correlations among proteins, tumor subtypes, and clinical features were assessed. In a multivariable model, triple negative cancers showed significantly reduced Fhit and Wwox, increased p53 and Ap2γ protein expression, and were significantly more likely than other subtype tumors to exhibit aberrant expression of two or more DDR-associated proteins. Disease-free survival was associated with subtype, Fhit and membrane ErbB4 expression level and aberrant expression of multiple DDR-associated proteins. These results suggest that definition of specific DNA repair and checkpoint defects in subgroups of triple negative cancer might identify new treatment targets. Expression of Wwox and its interactor, ErbB4, was highly significantly reduced in metastatic tissues vs. matched primary tissues, suggesting

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that Wwox signal pathway loss contributes to lymph node metastasis, perhaps by allowing survival of tumor cells that have detached from basement membranes, as proposed for the role of Wwox in ovarian cancer spread.

Introduction

Chromosome fragile sites are preferential targets of replication stress, resulting in deletions involving encoded fragile genes [1, 2]. The most commonly altered fragile sites are *FRA3B/FHIT* and *FRA16D/WWOX* at chromosomes 3p14.2 and 16q23.3. Fhit and Wwox protein expression is coordinately lost or reduced in ~2/3 of breast cancers [3] and loss is more frequent in ER/PR negative breast tumors [4]. Coordinate loss of Fhit and Wwox also occurs in ductal carcinoma in situ tissues [5], suggesting early loss during breast carcinogenesis.

Several lines of investigation have focused our interest on expression of DDR-associated proteins in breast cancer and how DNA damage-targeted tumor suppressor loss participates in DDR checkpoint activation and repair processes: (1) Reports of activated DDR checkpoints in preneoplastic lesions, accompanied by allelic *FHIT* deletions [6–8] and loss of expression of Fhit protein in early lesions [9–11], coupled with our knowledge that Fhit expression loss contributes to aberrant DDR [12], suggested that deletions at the *FHIT* gene could activate the DDR and reduction in Fhit protein expression could contribute to neoplastic progression. (2) The discovery that BRCA1/2 deficient breast cancer-derived cell lines were sensitive to killing by inhibitors of PARP enzymatic activity [13, 14], through a synthetic lethal mechanism involving loss of homologous recombination repair (HRR) and blocking of other repair pathways by PARP inhibition [15–17]. (3) BRCA1-deficient breast cancers are mostly triple negative (TN), a subtype associated with defects in some types of DNA repair [18] and endowed with "BRCAness" [19], and Fhit expression is lost in ~90% of BRCA1 and 2-mutated breast cancers [20–22]. Thus, we were interested in how loss of expression of Fhit and Wwox, might contribute to "BRCAness" and to responses to aberrant DDR checkpoints or repair pathways.

Chk2 and Chk1, downstream targets of ATM and ATR, have roles in regulation of checkpoint responses [23, 24] and the absence of expression of wild type ATR or Chk1 leads to accumulation of deletions and rearrangements of fragile sites [25]. The occurrence of DNA double-strand breaks (DSBs) is followed by phosphorylation of histone H2AX by ATM or ATR; the phosphorylated protein, γH2AX, recruits, and localizes DNA repair proteins [26], including BRCA1 [27], to the DNA breaks.

In a study of 837 breast cancers on a tissue microarray (TMA), we observed that TN tumors showed significantly reduced Fhit and Wwox expression [4]. We then analyzed the expression of DDR-associated proteins γH2AX, pChk2, and p53 in these cases and found that increased nuclear expression of γH2AX, pChk2, and p53 was significantly more frequent in TN tumors [GG, CH, REJ, WPW, SC, RTP, CLS, KH, unpublished results]. We have continued similar studies on an independent breast cancer cohort, including all breast cancer subtypes [28] and matched primary and metastatic cases, with linked clinical and follow-up data, to gain a better understanding of the role of activated DDR-associated proteins in breast cancer subtypes and outcomes.

Materials and methods

Construction of the breast cancer TMAs

The studies were approved by the Ohio State University Institutional Review Board for studies of human subjects. Tissue Archive Services and Information Warehouse personnel of the Ohio State University Medical Center, acting as "honest brokers", anonymized all cases by removing identifiers and assigning random numbers before supplying slides, blocks, and linked clinical information to investigators.

The SFBCAS TMA

Breast cancer cases (824) from prior to 1998, were selected from ~4000 women who contributed pathological and clinical outcome data to the National Comprehensive Cancer Network (NCCN) breast cancer database [29]. Selection criteria included all invasive breast cancers for which clinical information, including stage, grade, histologic type, treatment, site of first recurrence, and disease-free and overall survival were available. The parrafin-embedded tissue blocks were reviewed by pathologists (CM and REJ) and 564 were acceptable for the TMA, 303 for which there was tumor only, 226 with tumor and metastases, and 35 with only metastatic tissue. Cores (0.6 mm) from each tumor and metastasis were placed in quadruplicate blocks, with 50 assorted control tissues. The clinical information for the primary breast cancers included histological tumor type, grade and ER, PR, and ErbB2 status of tumors abstracted from clinical records and pathology reports.

Immunohistochemical analysis

BRCA1, pChk2, γH2AX, p53, Fhit, Wwox, Wwox-interacting proteins (Ap2γ, Ap2α, ErbB4), ErbB3, CK5/6, EGFR immunostainings were performed on TMA sections as described [3–5]. Specific antisera used, staining methods and detection kits are listed in Table 1. Expression of Fhit, Wwox, and CK5/6 was cytoplasmic, Ap2α, Ap2γ, pChk2, and γH2AX nuclear, EGFR, ErbB3, and ErbB4 cytoplasmic and membranous, and BRCA1 nuclear and cytoplasmic. Cytoplasmic expression in $\geq$10% of tumor cells for CK5/6, membranous staining in $\geq$10% of tumor cells for EGFR and nuclear staining in $\geq$10% for Ap2α, Ap2γ, was accepted as positive. Fhit and Wwox staining in tumors was scored according to intensity: absent, highly reduced, reduced staining, or strong expression [4, 5, 30], though final scores were collapsed to two categories (lost/very reduced vs. strong/moderate expression for some statistical modeling); >50% of cells for nuclear γH2AX and >25% of cells for nuclear pChk2, $\geq$50% strong expression of p53, any membrane staining of ErbB3 and ErbB4 was accepted as positive. BRCA1 nuclear expression was scored as < or >90% and reduced cytoplasmic BRCA1, ErbB3, and ErbB4 expression was noted when present. Four pathologists (GG, REJ, WPW, SC) scored the slides and were blinded to the breast cancer subtype; two pathologists (GG, CH) converted scores to numbers, selected cut off values for each marker and entered data into excel and SPSS files. The 423 breast cancers that could be scored were divided into subtypes of breast cancer as defined by their IHC profiles using designated stains [31, 32]. These include luminal A (ER and/or PR positive, ErbB2 negative); luminal B (ER and/or PR positive, ErbB2 positive); ErbB2+++ (ER and PR negative, ErbB2 IHC/FISH+++); basal-like TN (ER, PR, ErbB2 negative and CK5/6 and/or EGFR positive); and TN non-basal (ER, PR, ErbB2, CK5/6, EGFR negative). Since ErbB2 status was not available for some tumors, we also evaluated tumors by ER/PR status: luminal (ER and/or PR positive) vs. not (ER and PR negative), as well as luminal vs. TN vs. ErbB2+++ for ER and PR negative tumors for which ErbB2 status was available.

Statistical analysis

Not all marker or clinical data were available on all subjects, and percentages refer to cases for which data for a specific variable were available. Associations between categorical variables (e.g., marker score data, menopausal status, ER status) were evaluated using chi-square or Fisher exact tests. McNemar tests were used to analyze concordance in marker expression in tumor tissue and matching metastatic tissue.

Relationships of marker expression and clinical features were evaluated in relation to disease subtype using univariate and multivariable logistic regression models. Variable selection in the multivariable models was explored by the leaps and bounds approach [33]. We also assessed potential multivariable models based on the univariate logistic regression models.

Disease-free survival (DFS) was assessed from the time patients were disease-free to date of recurrence and/or death. Patients with metastases at diagnosis were excluded from DFS analyses and only subjects with stage I–III tumors at diagnosis were included. Kaplan–Meier and Cox regression models were used to evaluate DFS, where differences in DFS distributions were evaluated based on

Table 1 Primary antisera and detection kits used in immunohistochemical studies

Primary antiserum	Description	Dilution	Detection kit
Fhit	Rabbit (Huebner lab[a])	1/5000	Vectastain Elite kit (Vector Laboratories, Burlingame, CA)
Wwox	Rabbit (Huebner lab[a])	1/7000	Vectastain Elite kit
Ap2γ	Mouse (Santa Cruz, 6E4/4)	1/75	Universal LSAB+ (Dako, Carpinteria, CA)
Ap2α	Mouse (Santa Cruz, 3B5)	1/75	Universal LSAB+
γH2AX	Rabbit (Bethyl Labs, IHC-00059)	1/500	Vector Impress (Vector Laboratories)
p53	Mouse (Dako, M7001)	1/50	Universal LSAB+
pChk2	Rabbit (Abcam, Ab38461)	1/100	Vector Impress
CK5/6	Mouse (Dako, D5/16 B4)	1/50	Universal LSAB+
EGFR	Mouse (Dako, PharmDx Kit, 2-18C9)	Neat	Dako, Envision Plus, included in kit
ErbB4	Rabbit (Neomarkers, RB-9045—R7)	1/75	UltraTek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories, Cache, Utah,)
BRCA1	Mouse (Calbiochem, MS110)	1/25	Universal LSAB+
ErbB3	Mouse (Neomarkers, H3.105.5)	1/25	UltraTek HRP Anti-Polyvalent Lab Pack

[a] The features of the antiserum have been described in detail [3, 7]

clinical characteristics and marker expression. The P values reported in relation to DFS correspond to log rank tests unless otherwise noted.

Since we simultaneously evaluated multiple markers along with clinical characteristics of interest, we corrected for multiple comparisons using the Benjamini–Hochberg method to control for false discovery rates. Statistical significance was defined by ranking observed P values from smallest to largest and comparing each ith P value to (i/m)*Q, where m = number of P values for that set of analyses and Q is the chosen constraint for false discovery rate (FDR, here set to 0.05). All analyses were conducted using PASW Statistics (SPSS) v. 18 and R version 2.9.0.

Results

Breast cancer characteristics

Table 2 lists the characteristics of cases included in the SFBCAS TMA. Due to missing clinical data, primarily

Table 2 Characteristics of the SFBCAS TMA cases (n = 423)

	Number of patients
Race	
Caucasian	368
African American	48
Other	4
Menopause status	
Pre-menopausal	150
Post-menopausal	227
Grade	
I	33
II	175
III	174
ER status	
Negative	102
Positive	307
PR status	
Negative	135
Positive	268
ErbB2 status	
Negative	234
3+ IHC or FISH+	77
TN	
No	340
Yes	47
Basal	
No	266
Yes	33

Table 2 continued

	Number of patients
ER/PR status	
ER and PR neg	99
ER+, PR+, or both	301
Subtype	
Luminal A	179
Luminal B	47
ErbB2+++	29
TN	47
Age at diagnosis	
≤40	47
41–50	135
>50	238
Histology	
Invasive ductal	313
Invasive lobular	27
Mixed ductal lobular	35
Other	48
Death	
No	398
Yes	22
Cause of death	
N/A (alive)	398
Cancer-related	20
Other causes	2
Recurrence[a]	
No	245
Yes	85
Type of 1st recurrence[a]	
In situ	4
Local/Regional	19
Distant	60
Type unknown	2
Metastatic tissue type	
Lung nodule	1
Lymph node(s)	105
Skin	3
N/A (no mets tissue)	314

[a] Does not include 93 cases that were never disease-free, those unknown if ever disease-free and those with missing recurrence information

ErbB2+++ status, subtype could be determined in only 302 cases. Most cancers 60% (179/302) were classified as luminal A, 15% (47/302) were luminal B, 10% (29/302) ErbB2+++, and 15% (47/302) TN. Combining luminal A and B groups (ER and/or PR positive) allowed classification of more tumors: 80.4% (312/388) were ER and/or PR positive, 7.5% (29/388) ErbB2+++, 12.1% (47/398) TN. Among the 47 TN cases, CK5/6 and EGFR expression

scores were available in all but 4. Of the remaining 43, 33 (76.8% showed a basal-like phenotype as determined by EGFR and CK5/6 staining. The TMA analyses included 109 subjects with both tumor tissue and metastatic tissue; 96% of metastatic tissues were from lymph nodes (Table 2).

As noted in earlier studies, there were significant differences in the ages at diagnosis by breast cancer subtype; the median age for TN cases was 47 years, while for luminal A, B, and ErbB2+++ median ages were 55, 51, and 52 years, respectively ($P = 0.003$).

DDR-associated protein expression in breast cancer

The SFBCAS TMA

There were data on 423 breast cancer subjects, including biochemical markers determined by IHC and integrated clinical data (Table 2 for summary). We assessed associations among pairs of proteins in this TMA (Supplementary Table S1): pChk2 and γH2AX expression were highly positively correlated only with each other ($P < 0.00001$); BRCA1 nuclear expression was significantly positively correlated with expression of cytosolic ErbB3 ($P = 0.00004$) and with Fhit and Wwox expression (each $P = 0.02$), indications of an intact HRR pathway in cancers expressing nuclear BRCA1. Expression of the transcription factor Ap2γ was highly, positively associated with expression of pChk2 and p53 ($P < 0.001$).

The DDR checkpoint proteins, γH2AX, pChk2, and p53, were expressed more frequently in TN and basal-like cancers vs. other subtypes (Fig. 1 for illustration of results, Fig. 2 for representative photos of IHC results). As observed by others [19], we find that TN tumors frequently show reduced expression of BRCA1. The bar graph in Fig. 1 shows that highly positive BRCA1 expression (90% of cells with nuclear expression) was rare in the TN tumors

(Table S2 lists expression scoring results for specific markers relative to clinical features). By univariate modeling, BRCA1 nuclear expression was significantly, inversely associated with ER/PR negativity ($P = 0.0004$) and specifically with TN breast cancer subtype ($P = 0.009$, Table 3). p53 expression was also highly correlated with TN and basal-like subtypes ($P < 0.00001$).

In order to investigate more broadly the association of alterations to expression of DDR proteins in TN breast cancers, a DDR score was devised, based on frequency of altered or activated expression of nuclear BRCA1, γH2AX, pChk2, Fhit, p53; i.e., proteins involved in recognition of DNA breaks (γH2AX), in checkpoint activation in response to breaks (pChk2, p53), or DNA repair (Fhit, BRCA1). The number of deleteriously expressed markers was summed, where reduced nuclear BRCA1 or Fhit, or elevated γH2AX, p53, or pChk2 were considered deleterious. The median number of markers with deleterious expression was 2. TN cancers had a median of 3 aberrantly expressed markers vs. non-TN cancers (median = 3 vs. 2, $P < 0.0001$). Across the 279 tumors with data for all 5 markers, only 4 had deleterious expression of all 5 markers (2 luminal, 2 TN); 17 had no deleterious expression of any markers (all luminal) (data for stage I–III cases with recurrences summarized in Table S3). Based on logistic regression models, DDR score (0 vs. 1 vs. 2 vs. 3 vs. 4 vs. 5) was significantly associated in the univariate setting with TN status ($P < 0.00001$). Analyses of the DDR protein scores supported a collapsing of the scores into two groups: 0–1 vs. 2–5; this collapsed grouping of 0–1 vs. 2–5 was also significantly associated with TN status (OR = 6.2, $P = 0.003$, Table 3). By this assay, TN cancers were 6× more likely to exhibit aberrant expression of 2 or more DDR-associated proteins. The distribution of scores between TN and non-TN subjects is shown in the box plot in Fig. 3.
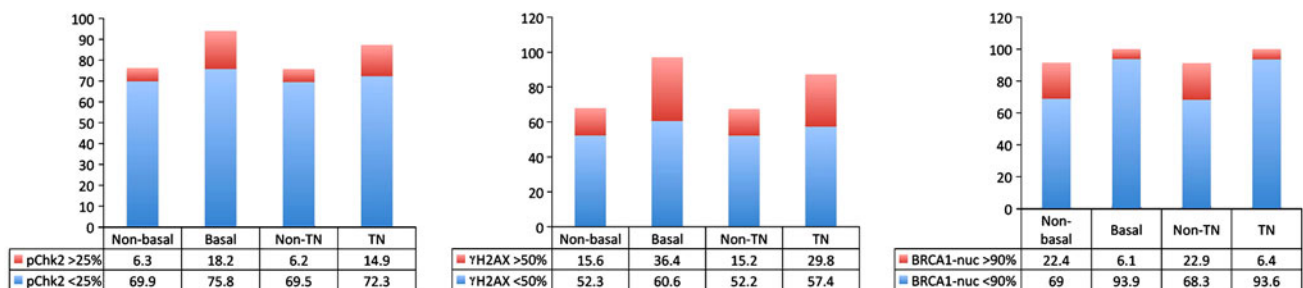
Fig. 1 Graphical representation of expression of DDR proteins in TN cancers. In studies of the breast cancers on the SFBCAS TMA, DDR proteins were expressed more frequently in TN and basal-like tumors. The numbers to the lower left of each graph represent the cut-off percentages for scoring a tumor positive or negative; i.e., for pChk2, tumors were scored positive if >25% of nuclei were positive for pChk2. For γH2AX the cut-off was at 50% and for BRCA1 at 90%. Because percentages were calculated using denominators that included cases for which scores were not available, the percentages will not necessarily sum to 100% across the expression groups for individual markers
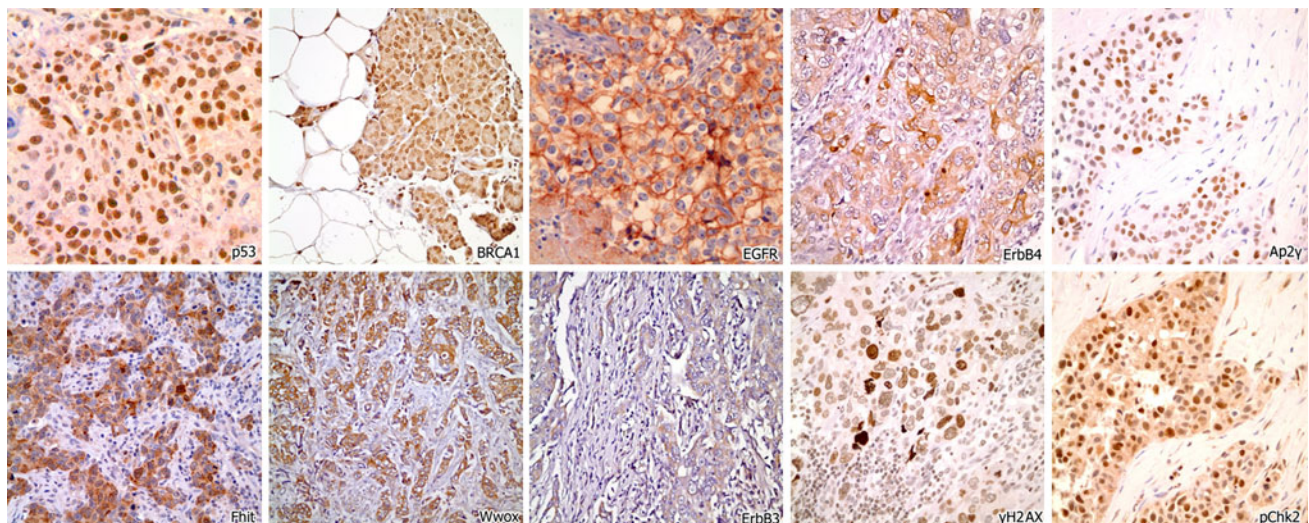
Fig. 2 Expression of specific proteins in breast cancers by IHC analysis. Photographs (×400) were taken of immunohistochemical detection of each of the specific proteins assessed on the TMA and representative photographs are shown for each protein

Table 3 Univariate associations of markers with subtype and stage

Marker	TN vs. other		Basal vs. other		Stage II/III vs. I	
	OR est	*P* value	OR est	*P* value	OR est	*P* value
Ap2γ	4.53	**0.00005**	4.75	**0.0003**	1.01	0.98
CK5/6	5.67	**<0.00001**	10.61	**<0.00001**	1.32	0.40
pChk2	1.52	0.08	1.64	0.051	0.79	0.57
γH2AX	1.33	0.12	1.42	0.08	1.15	0.38
BRCA1 nuc	0.20	**0.009**	0.20	**0.03**	0.57	0.037
ErbB3 cyto	0.29	**0.0002**	0.36	**0.007**	0.56	**0.016**
ErbB3 membr	0.27	**0.007**	0.40	0.07	1.02	0.92
ErbB4 cyto	2.18	**0.03**	2.53	**0.035**	0.98	0.95
ErbB4 membr	2.1	**0.02**	2.2	**0.03**	0.80	0.35
Fhit	0.11	**0.0003**	0.17	**0.005**	0.47	**0.001**
Wwox	0.28	**0.0002**	0.28	**0.001**	0.66	0.16
p53	6.1	**<0.00001**	6.2	**<0.00001**	1.95	0.043
EGFR	11.0	**<0.00001**	29.4	**<0.00001**	1.81	0.063
DDR score (0–1 vs. 2+)	6.2	**0.003**	4.4	**0.017**	2.25	**0.005**

P values reflecting assessments of univariate associations of each marker were calculated from univariate logistic regression models, where the odds ratio estimates (OR est) are the exponential of the parameter estimates for that variable in the model

Tumor suppressors and interacting proteins in association with breast cancer subtypes

As shown in Table 3, expression of various proteins was significantly associated with subtype groups in the univariate setting: lost or reduced Fhit and Wwox expression was strongly associated with TN tumors (*P* = 0.0003 and 0.0002), as were absence of ErbB3 cytosolic expression and positive membrane expression of ErbB4 (Table 3). Other significant factors for TN status in the univariate setting were: Ap2γ (*P* = 0.00005), reduced BRCA1 nuclear expression (*P* = 0.009) and p53 (*P* < 0.00001) expression.

When protein expression markers were evaluated in the multivariable setting in relation to TN status (Table 4), four markers remained significant: reduced Wwox and Fhit (*P* = 0.002 and 0.03), and p53 and Ap2γ positivity (both *P* = 0.004). Overall, Wwox, Ap2γ, and p53 remained significant across multivariable models for each of the subtype groupings (ER/PR negative, TN, and basal-like).

Primary tumors and metastases

In analyses for level of agreement between markers in tumor vs. metastatic tissue, expression of several proteins were significantly discordant, as shown in Table 5 (Fig. 4
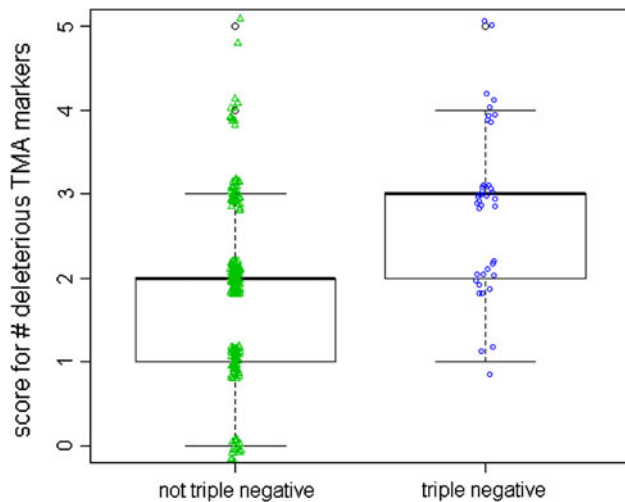
Fig. 3 Boxplot of DDR scores, for number of aberrantly expressed DDR-associated proteins in TN vs. non-TN breast cancers. The DDR-associated proteins assessed were: altered or activated expression of nuclear BRCA1, γH2AX, pChk2, Fhit, p53; i.e., proteins involved in recognition of DNA breaks (γH2AX), in checkpoint activation in response to breaks (pChk2, p53), or in DNA repair (Fhit, BRCA1)

Table 4 Multivariable logistic regression modeling

Marker	TN vs. other		Basal vs. other	
	OR est.[a]	P value	OR est.[a]	P value
Intercept	0.29	0.02	0.14	0.002
Ap2γ	4.5	**0.004**	4.6	**0.005**
ErbB3 cyto	0.45	0.07	0.50	0.14
ErbB3 membr	0.26	0.07	0.35	0.17
Fhit	0.20	**0.03**	0.30	0.12
Wwox	0.22	**0.002**	0.27	**0.01**
p53	4.1	**0.004**	3.0	**0.03**

[a] OR est from these models reflect the exponential of the multivariable logistic regression model parameter estimates. In looking at the models of TN vs. other, and basal-like vs. other, these same variables that are statistically significant in the multivariable models shown above were also significant factors in multivariable models using an all subsets approach to variable selection (leaps and bounds method [33]). The only difference was that in the all subsets methods, the ErbB3 cytoplasmic score was also a significant identified marker for basal-like vs. other. Also, in the all subsets method, the addition of the Fhit score did not contribute much to the overall model in determining TN vs. other

for representative photographs of protein expression in matched primary and metastatic sections). Expression of Wwox was highly significantly reduced in the metastatic tissues ($P < 0.0001$) vs. the primary tumor tissue; loss of cytosolic and membrane ErbB4 expression was also associated with occurence of metastases ($P = 0.002$ and 0.004), as was BRCA1 nuclear expression ($P = 0.003$).

Table 5 Protein expression differences in primary tumors vs. metastases

| Protein	Cases with tumor and metastatic tissue		
	Tumor tissue	Metastatic tissue	P value*
ErbB4 cyt			
0	36	60	**0.002**
1	70	46	
ErbB4 membr			
0	65	85	**0.004**
1	41	21	
BRCA1 nucl			
0	14	4	**0.003**
1	72	67	
2	10	25	
BRCA1 cyt			
0	14	26	0.038
1	81	69	
Wwox			
1–2	26	87	**<0.00001**
3–4	62	1	

* P value from McNemar's test to evaluate paired categorical outcomes and a measure of agreement between the tumor and metastatic tissue expression profiles; ErbB3, Ap2α/γ, pChk2, γH2AX, Fhit, p53, and EGFR did not show significant differences in expression in primary tumor vs. metastasis. ErbB2 data was not available for the metastases. There were differences in frequencies of specific subtypes among the non-metastatic (luminal 83%, ErbB2+++ 7%, TN 10%) vs. metastatic (luminal 72%, ErbB2+++ 10%, TN 18%) primary cancers

Associations of markers and outcomes

For a subset of 330 patients, there was recurrence data available; 60 distant and 19 locoregional. Patients who developed in situ recurrences or had metastases at diagnosis were excluded from the disease-free status analyses. The resulting cohort used in the analyses was 305 non-metastatic breast cancer subjects with data available on disease-free and recurrence status; 79 subjects had a recurrence and the median follow-up was 98 months (range: 4–242 months); only 8 subjects had <12 months follow-up.

Disease-free survival of TN cancers was significantly worse than for luminal subtypes (see Fig. 5), as expected from earlier studies (note that ErbB2+++ cases are from before introduction of trastuzamab treatment). DFS for TN cases vs. all other subtypes was also significantly worse ($P = 0.0001$) (Table 6). DFS of cases with reduced Fhit was significantly worse and this figure remained significant when Fhit negative and mild expressers were combined together vs. moderate plus high expressers ($P = 0.007$, Fig. 6). DFS of cases with membrane ErbB4 expression
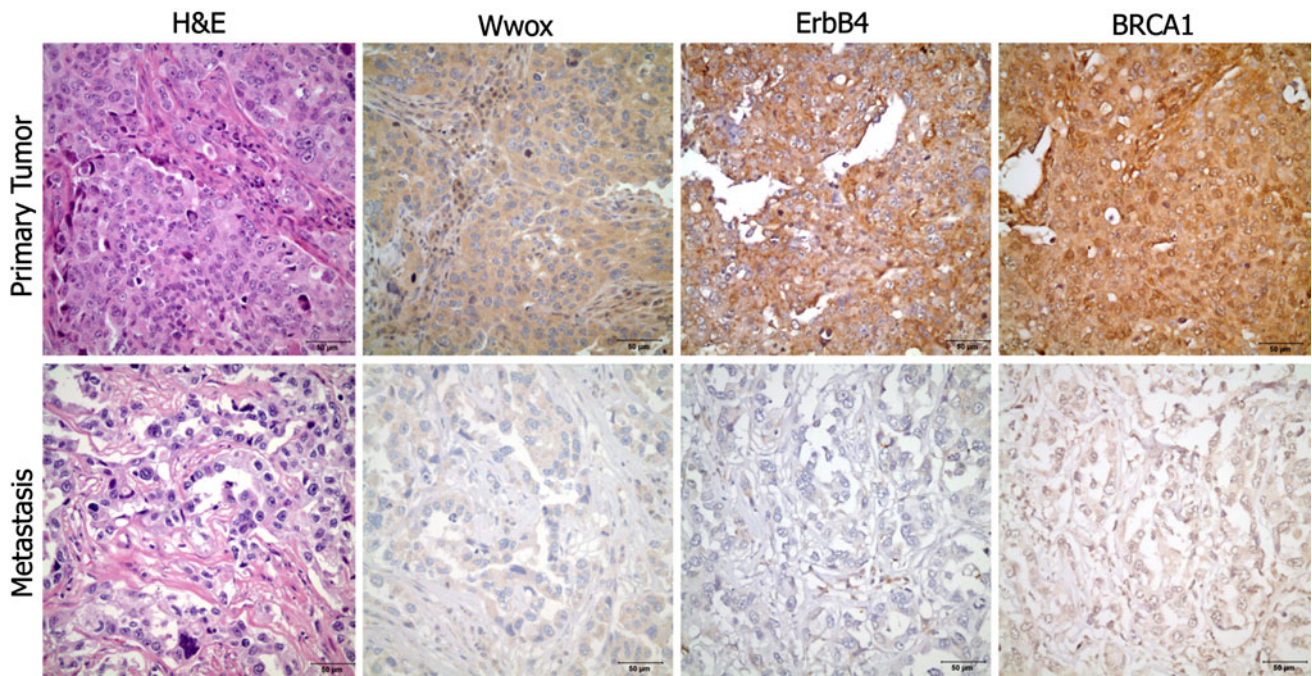
Fig. 4 Differentially expressed proteins in primary and metastatic breast cancers. Photographs (×400) of differentially expressed markers in matched primary and metastatic lymph node cancer tissue from a single patient
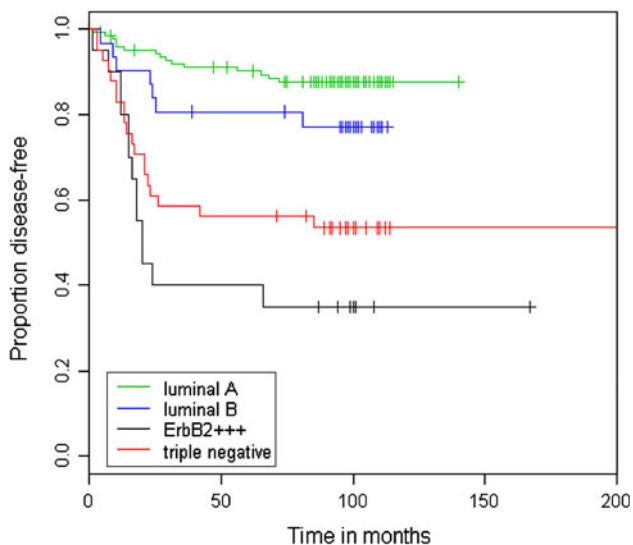
Fig. 5 DFS of patients with TN breast tumors. DFS of patients with TN breast tumors cohort was significantly worse than luminal subtypes or the other subtypes grouped together (not shown); also DFS for basal-like tumors was worse than other subtypes grouped together (not shown). Note that this TMA represents cases treated before the introduction of trastuzamab for ErbB2+++ cases

Table 6 DFS in stage I–III cases at diagnosis

Group	N	# of events	HR	P value
Luminal A	125	15	Ref.	–
Luminal B	31	7	1.98	0.14
ErbB2+++	20	13	8.1	**<0.00001**
TN	41	20	5.0	**<0.00001**
Luminal	215	34	Ref.	–
ErbB2+++	20	13	6.3	**<0.00001**
TN	41	20	3.8	**<0.00001**
Luminal	215	34	Ref.	–
ErbB2+++/TN	81	42	4.2	**<0.00001**
Not TN	235	47	Ref.	–
TN	41	20	2.89	**0.0001**
Not basal-like	243	53	Ref.	–
Basal-like	29	12	1.96	0.042

Ref reference group; i.e., the hazard ratios (HR) are in reference to this group. For example, if HR = 2, cases in that group are twice as likely to have an event than those in ref group; estimates from a Cox regression model

was also significantly worse than others ($P = 0.0013$, not shown).

In univariate analyses, the following factors/markers had a P value <0.20 and were considered in the multivariable model: stage, grade, breast cancer subtype, ErbB3 (cytoplasmic), ErbB4 (membrane), Fhit and p53; grade was highly correlated with stage ($P < 0.00001$) and with breast cancer subtype ($P < 0.00001$). To avoid multicolinearity issues, grade was excluded from the final multivariable model. Ultimately, the three significant factors in the model for DFS were stage ($P = 0.00014$), breast cancer subtype ($P < 0.0001$), and ErbB4 membrane score ($P = 0.0065$).
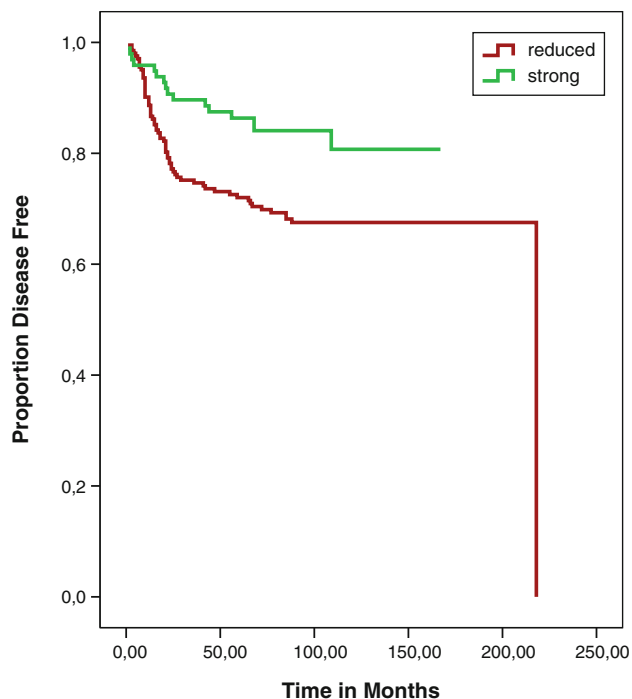
Fig. 6 DFS of breast cancers with low or no Fhit expression vs. cancers with moderate or high Fhit expression ($P = 0.008$)

As described above, we created a DDR marker-based score that reflected the number of DNA damage response proteins with aberrant expression in each cancer. DFS of patients differed significantly depending upon the number of deleterious markers exhibited as shown in Table S3 and Fig. 7. Our analyses are limited by the large percentage of censored observations and the constraint that subjects needed to have data available on all five markers used to generate the DDR score.

Discussion

Associations among marker proteins

BRCA1 nuclear expression was strongly, positively associated with ErbB3 cytoplasmic expression, as well as with Fhit and Wwox expression; Fhit loss was correlated with expression of p53, and strongly, positively correlated with expression of cytoplasmic ErbB3 and ErbB4; p53 was most strongly correlated with expression of Ap2γ; and Wwox loss was most strongly correlated with loss of expression of ErbB3 cytoplasmic protein and with Fhit loss. The unexpected strong correlation of Fhit expression with cytoplasmic ErbB4 and Ap2α, both Wwox interactors, and correlation of Wwox and Fhit with cytosolic ErbB3, warrant mechanistic studies in breast cancer-derived cell lines.
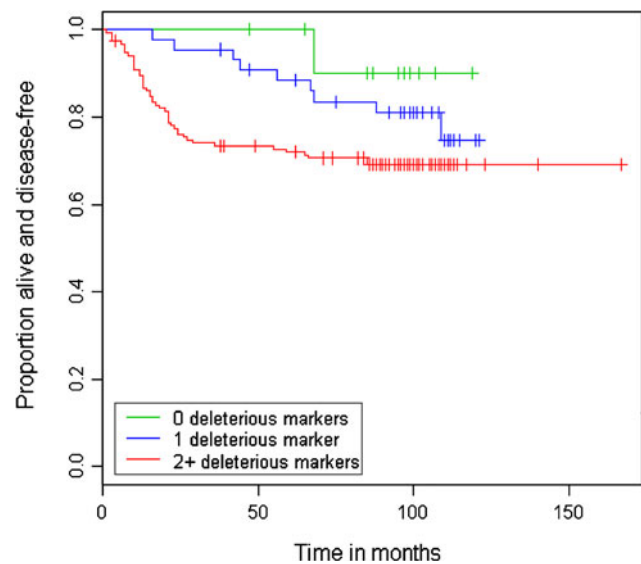
Fig. 7 DFS of patients based on breast cancer expression levels of DDR proteins. The DDR-associated proteins assessed and used in this DFS analysis were altered or activated expression of nuclear BRCA1, γH2AX, pChk2, Fhit, p53; i.e., proteins involved in recognition of DNA breaks (γH2AX), in checkpoint activation in response to breaks (pChk2, p53), or DNA repair (Fhit, BRCA1). It is likely that subtype is the driver of these differences because the DDR proteins tend to be most frequently altered in TN cancers, though the analyses are limited by the relatively large percentage of censored observations and the small numbers of cases in some subgroups, as illustrated in Table S3

DDR-associated proteins and breast cancer subtypes

Our studies show a previously unreported feature of TN breast cancers. Three proteins of the activated DDR checkpoint, γH2AX, pChk2, and p53, are more frequently expressed in this group of neoplasms with 67% of pChk2 positive cases being γH2AX positive; and expression of BRCA1 and Fhit, associated with aberrant DNA damage repair, are highly significantly reduced in TN cancers. Interestingly, Fhit and BRCA1 are reduced in expression together in 70.9% of cases. We do not know if γH2AX and pChk2 expression together indicate an intact-activated checkpoint or if the 70% of γH2AX positive cases that are negative for pChk2 expression are evidence of a crippled checkpoint. It would be useful to examine subtype specific breast cancer cell lines with persistently activated checkpoints for sensitivity to inhibitors of Chk2 and Chk1. In addition, since absence of BRCA1 and Fhit are not correlated with activation of the checkpoint proteins, it may be that the BRCA1 and/or Fhit-deficient TN cancers are more likely to be sensitive to PARP inhibitors, as expected from previous studies [13, 14, 17].

So why are TN breast cancers, including the basal-like subgroup, more likely to show evidence of activated checkpoints, deleted fragile tumor suppressors, and DDR defects? It could be related to the cell type of origin for

these cancers. Basal-like breast tumors are enriched in breast cancer stem cells [34–37], and p53 and BRCA1 proteins may have roles in stem cells [36, 37]. Presumably stem cells, including cancer stem cells, have a longer history of cell divisions than differentiated cells, providing many more opportunities for exposure to replication stress followed by repair defects and genome instability. TN cancers do show evidence of more genomic alterations than other breast cancer subtypes [38]. It has even been suggested that reduced expression of BRCA1 and Fhit may be hallmarks of stem cells [37, 39]. The persistent expression of DDR-associated proteins observed in this study in TN cancers probably has an important role in development of these cancers.

In a relevant example, Asakawa et al. [40] studied human breast cancer biopsies and showed that treatment with DNA damaging drugs induced nuclear foci of γH2AX, conjugated ubiquitin, and Rad51 in a substantial fraction of cases. The presence of BRCA1, γH2AX, or Rad51 foci before treatment was inversely correlated with tumor response to chemotherapy. DDR competence was further evaluated by considering all four repair indicators together. A high DDR score significantly correlated with low tumor response. The results "suggested importance of evaluation of DDR competence to predict breast cancer chemosensitivity…" [40].

Tumor suppressors and interactors in breast cancer subtypes

The most striking finding concerning Wwox expression was that nearly 100% of metastatic tissues showed lower Wwox expression than the matched primary tumors. This is of particular interest because recent studies have shown that siRNA-mediated knockdown of endogenous Wwox in ovarian cancer cells increased adhesion to fibronectin and WWOX-transfected cells in suspension culture displayed a proapoptotic phenotype. The authors proposed that Wwox acts as an ovarian tumor suppressor by modulating the interaction between tumor cells and the extracellular matrix and by inducing apoptosis in detached cells, suggesting a role for Wwox loss in peritoneal dissemination of ovarian cancer cells [41, 42]. Reduction of Wwox expression in metastatic tissues suggest a similar role for Wwox loss in lymph node metastasis. Expression of the Wwox-interacting protein, ErbB4, in membrane and cytosolic forms, was also significantly reduced in the metastatic lesions. Sundvall et al. [43] have reviewed aspects of ErbB4 expression and prognostic significance in breast cancer and have shown that absence of Wwox protein was associated with the number of cancer-positive axillary lymph nodes.

Ap2α and γ transcription factors have been implicated in control of proliferation, differentiation, and apoptosis of normal breast epithelium and in breast cancer. It is possible that the association of Ap2γ with TN cancers is related to the role of Ap2γ in proliferation of the mammary gland precursor cells. A recent study [44] showed that the WWOX gene is a transcriptional target of the Ap2γ transcription factor, suggesting an autoregulatory loop involving Wwox and Ap2γ proteins; thus, perhaps Wwox loss in TN cancers permits nuclear Ap2γ localization and upmodulation of proliferative activity.

Clinical associations

In this study, DFS of cases with ErbB4 membrane positivity and reduced Fhit was significantly worse than others, though expression of membrane ErbB4 has been associated with favorable overall survival in previous studies [45, 46]. Our current results showed that expression of both cytoplasmic and membrane ErbB4 was more frequent in TN/basal-like tumors, and the membrane form was associated with worse DFS. Altogether, the results imply that assigning a singular "good or bad" role for ErbB4 in breast carcinogenesis is not straightforward [43].

Aqeilan et al. [46] assessed the clinical significance of the Wwox-ErbB4 association in >500 breast cancers. Loss of Wwox expression was associated with unfavorable outcome, co-expression of membrane ErbB4 and Wwox was associated with favorable outcome, compared with cases with membrane ErbB4 and no Wwox immunoreactivity. In our study, Wwox was lost in nearly 100% of the metastases, and reduced cytoplasmic ErbB4 was significantly associated with metastatic tissues. Other investigators have reported that the ErbB4 cytosolic form is associated with apoptosis and favorable outcomes [45, 47], results that seem consistent with our observation of loss of cytosolic ErbB4 in metastatic tissues. Thus, there was a strong association of loss of the Wwox signal pathway with breast cancer lymph node metastasis, a finding that is likely tied to the role of expression loss in allowing survival of tumor cells that have detached from basement membranes [41].

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