Research Article

Cancer Prevention Research

Germline and Somatic *NF1* Alterations Are Linked to Increased HER2 Expression in Breast Cancer

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Abstract

NF1 germline mutation predisposes to breast cancer. *NF1* mutations have also been proposed as oncogenic drivers in sporadic breast cancers. To understand the genomic and histologic characteristics of these breast cancers, we analyzed the tumors with *NF1* germline mutations and also examined the genomic and proteomic profiles of unselected tumors. Among 14 breast cancer specimens from 13 women affected with neurofibromatosis type 1 (NF1), 9 samples (NF + BrCa) underwent genomic copy number (CN) and targeted sequencing analysis. Mutations of *NF1* were identified in two samples and *TP53* were in three. No mutation was detected in *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *NBN*, *PALB2*, *PTEN*, *RAD50*, and *STK11*. HER2 (ErbB2) overexpression was detected by IHC in 69.2% (9/13) of the tumors. CN gain/amplification of *ERBB2* was detected in 4 of 9 with DNA analysis. By evaluating HER2 expression and *NF1* alterations in unselected invasive breast cancers in TCGA datasets, we discovered that among samples with *ERBB2* CN gain/amplification, the HER2 mRNA and protein expression were much more pronounced in *NF1*-mutated/deleted samples in comparison with *NF1*-unaltered samples. This finding suggests a synergistic interplay between these two genes, potentially driving the development of breast cancer harboring *NF1* mutation and *ERBB2* CN gain/amplification. *NF1* gene loss of heterozygosity was observed in 4 of 9 NF + BrCa samples. *CDK4* appeared to have more CN gain in NF + BrCa and exhibited increased mRNA expression in TCGA *NF1*--altered samples. *Cancer Prev Res; 11(10); 655–64. ©2018 AACR.*

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant hereditary condition caused by germline mutation or genomic deletion of the *NF1* gene. It is rare with a population birth incidence of 1:2–3,000. NF1 is known to be a hereditary neoplasm predisposition syndrome with characteristic features including multiple dermal neurofibromas, café-au-lait macules on the skin, freckles on the intertriginous area, and internal and external large plexiform neurofibromas. Occurrence of several types of benign and malignant neoplasms is increased in these individuals. Women affected with NF1 have moderately increased risk of breast cancer (1–8). Breast cancer lifetime risk for these women is approximately 18%. One study also suggested worsened breast cancer survival in NF1 women compared with age-matched controls (4).

Meanwhile, somatic *NF1* deep deletion and putative driver mutations are found in approximately 2% to 3% of the sporadic breast cancers [The Cancer Genome Atlas Network (TCGA) 2012, TCGA data bank; available from http://www.cbioportal.org; ref. 9; Catalogue of Somatic Mutations in Cancer (COSMIC) database available from http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/; ref. 10]. The association between *NF1* deletion and mammary adenocarcinoma was also demonstrated in *Chaos3* mice (11). However, it is not known whether *NF1* biallelic mutation/deletion is common in breast cancers occurring in individuals with NF1. A wide range of

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

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mechanisms and pathways are known to contribute to mammary oncogenesis and tumor development. Somatic mutations in hereditary breast cancer predisposition genes, such as *BRCA1*, are only found in a small fraction of sporadic breast cancers despite their prominent role in highly penetrant hereditary predisposition to breast cancer. A germline mutation is the first mutation in tumor cells, whereas a somatic mutation develops later and exists in a specific population of cells. Comparing the tumors harboring certain mutations in germline versus somatic (postzygotic) lines may reveal the order of oncogenic events and the dynamics of molecular biological changes. Analyzing the breast cancer specimens with a constitutionally defective *NF1* (i.e., a mutation in germline) as well as sporadic breast cancers harboring an altered *NF1* gene may offer insight into the roles of the *NF1* gene and its encoded protein, neurofibromin, in the landscape of breast cancers.

Neurofibromin is a negative regulator for Ras action by converting GTP-Ras to GDP-Ras in cytoplasm. Two of the downstream signaling pathways activated by GTP-Ras are the PI3K/Akt and Raf/MEK/ERK pathways. Our report characterizes NF1 breast cancer by histology, IHC stain of key proteins in signaling pathways, somatic genomic analysis of tumors with germline *NF1* mutations, as well as TCGA genomic and expression profiles in sporadic tumors with somatic *NF1* alterations.

Materials and Methods

Recruitment

Women with NF1 and a history of breast cancer were recruited by three Children's Tumor Foundation (CTF)-affiliated neurofibromatosis clinics at Henry Ford Health System, Johns Hopkins University (Baltimore, MD), and Children's National Medical Center (Washington, DC). Additional recruitment advertisements were distributed by CTF newsletters as well as among NF-patient support groups. Participants' archived, formalin-fixed paraffin-embedded (FFPE) breast cancer tumor specimens were collected with informed consent.

IHC assay for archived breast tumor samples from women with NF1 (NF + BrCa)

Hematoxylin and eosin–stained slides were reviewed to select tumor-containing paraffin block with more than 90% tumor. Cases with tumor percentage less than 50% were marked for microdissection to enrich tumor DNA. A tissue microarray was constructed using standard IHC protocol.

All antibodies were obtained from Cell Signaling Technology. Validation process is described in Supplementary S1. IHC staining for the following proteins was performed on the basis of the manufacturer's protocol: pMEK (i.e., phospho-MEK1/2, Ser221, the phosphorylated and activated form of MEK), pErk [i.e., phospo-p44/42 MAPK (Erk1/2; Thr202/Tyr204), the phosphorylated and activated ERK], Erk (i.e., P44/42 MAPK, Erk1/2, the nonphosphorylated ERK), AKT (pan), mTOR, p53, PTEN, and HER2. Staining intensity was graded in 3 tiers, from low to high as 1, 2, and 3. Staining score is the product of the intensity (1, 2, and 3) and the percentage of the cells stained.

Specifically for HER2, immunostained slides were reviewed by light microscopy and the membrane staining intensity pattern and percentage of immunoreactive cells were semiquantitatively assessed and scored as per the ASCO-CAP Guidelines established in 2013 (12). Results were scored as negative (either 0 or 1+ immunostaining), positive (3+ immunostaining), or equivocal (2+ immunostaining).

DNA extraction

Manual DNA extraction protocol (QIAamp DNA Mini Kit, Qiagen) was used for isolation of genomic DNA from the archived breast tumor specimens. DNA yield and purity were evaluated by NanoDrop and TapeStation.

Affymetrix OncoScan FFPE assay

The genomic DNA samples extracted from NF + BrCa specimens were subjected to Affymetrix OncoScan FFPE Assay (Affymetrix) to investigate copy number (CN) variations. Allelic status of individual genes, such as CN gain, loss, or loss of heterozygosity was assigned by Chromosome Analysis Suite (ChAS) Software 3.1 (Affymetrix). Gain is determined by OncoScan as $CN > 2$; loss is $CN < 2$; amplification is $CN \geq 4$. At the single gene level, we obtained the CN of 77 genes known to be amplified in various cancer types (9, 13).

Targeted gene mutation analysis by OneSeq for NF + BrCa samples

The genomic DNA extracted from NF + BrCa specimens was also subjected to tests using OneSeq Constitutional Research Panel developed by Agilent Technologies. Genes known to be associated with hereditary diseases were sequenced using the Agilent SureSelect Focused Exome Panel and analyzed with Agilent SureCall software. The Agilent SureSelect Focused Exome Panel Catalog Kit includes baits for a combination of a CNV backbone and all content from the Focused Exome Panel, targeting hereditary disease-associated genes and regions previously annotated within the HGMD, OMIM, and ClinVar databases. Genes commonly recognized as cancer drivers but not known to be associated with hereditary diseases were not included in this Agilent panel. After the initial variant call, variants were further categorized and curated for pathogenicity based on the following categories: missense, frameshift, nonsense, splice-site altering, variant flagged as "Pathogenic" in dbSNP database, or reported as "hotspot mutation" in cancer somatic genomic database.

Genomic, mRNA, and proteomic dataset analysis on TCGA unselected invasive breast cancers

To examine the effects of somatic *NF1* mutations and deletions, the clinical information as well as somatic mutation, CN, and RNA sequencing V2 RSEM data from TCGA breast invasive carcinoma dataset were downloaded from the cBioPortal (9) and combined with reverse-phase protein array data from The Cancer Proteome Atlas (TCPA; ref. 14). The data were classified by the status of the *NF1* gene: tumors with *NF1* mutation or deletion (i.e., *NF1* altered) were compared with tumors without *NF1* mutation or deletion (labeled as *NF1* nonaltered). The groups were compared using the Mann–Whitney *U* test.

Human research subjects' protection

These studies were conducted in accordance with the U.S. Common Rule and performed after approval by the Institutional Review Board of each participating institution. The investigators obtained informed written consent from the subjects (wherever necessary).

Results

IHC assay

Fourteen archived breast cancer tumor specimens were collected from 13 women with NF1. One sample was suspected of poor sample processing and was excluded. Among the remaining 13 specimens from 12 women, 9 (69.2%) stained strongly positive for HER2 (ErbB2; Table 1). The sample histology, hormone receptor status, and IHC staining results for all proteins, that is, pMEK (i.e., phospho-MEK1/2, Ser221, the phosphorylated and activated form of MEK), pErk [i.e., phospo-p44/42 MAPK (Erk1/2; Thr202/Tyr204), the phosphorylated and activated ERK], Erk (i.e., P44/42 MAPK, Erk1/2, the nonphosphorylated ERK), AKT (pan), mTOR, p53, PTEN, and HER2, are provided in Table 1.

CN analysis by Affymetrix OncoScan FFPE assay

Sufficient genomic DNA was obtained from 11 breast cancer specimens from 11 NF1 women. OncoScan FFPE Assay applied to these samples generated 9 datasets that were considered to be of sufficient quality to be analyzed. CN gain on 1q, 8q, and CN loss on 8p were observed as prominent features of these samples, which coincide with the landscape features of general breast cancers from TCGA and arrayMap datasets (15, 16; Supplementary Fig. S2).

By manually examining each sample in NF + BrCa using Affymetrix ChAS software, we identified *ERBB2* CN gain in 2 samples and *ERBB2* CN amplification in 2 samples (Table 2). Only one sample has the CN gain involving the entire 17q arm (CN = 2.33). *NF1* loss of heterozygosity (LOH) was observed in 4 of 9 samples. Many genes have been found to be amplified or overexpressed in various cancers (13). CN status of 77 such genes was examined using ChAS software. CN gain/amplification of the

Table 1. Histology, hormone receptors, HER2 status, and IHC for other proteins

				Phospho-MEK1			Phospho-MAPK (Erk 1/2) P44/42			MAPK (Erk 1/2)			PTEN			AKT			mTOR			p53			HER2/ErbB2	
ID	Histology	ER	PR	Inten-sity	%	Score	Inten-sity	%	Score	Inten-sity	%	Score	Inten-sity	%	Score	Inten-sity	%	Score	Inten-sity	%	Score	Inten-sity	%	Score	Score	Interpretation
1	IDC + DCIS	POS	NEG	1	50	50	3	5	15	1	95	95	1	100	100	1	80	80	1	100	100	3	95	285	3	**POS**
3	IDC	NA	NA	1	50	50	0	0	0	1	70	70	NA			1	100	100	1	100	100	2	85	170	3	**POS**
6	DCIS	NA	NA	1	100	100	3	3	9	2	100	200	1	100	100	2	95	190	2	100	200	1	30	30	2–3	**POS**
8	IDC + DCIS	POS	NEG	1	100	100	3	2	6	1	100	100	1	100	100	2	100	200	1	100	100	3	95	285	3	**POS**
9	IDC + DCIS	POS	POS	1	100	100	0	0	0	2	90	180	1	100	100	1	80	80	1	100	100	1	5	5	1	NEG
2	IDC + DCIS	POS	POS	1	100	100	3	1	3	1	95	95	1	100	100	1	100	100	1	90	90	1	5	5	1	NEG
10[a]	IDC+ DCIS	POS	POS	0	0	0	2	30	60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
4	IDC	POS	POS	1	100	100	3	20	60	2	65	130	0	0	0	2	100	200	3	40	120	2	80	160	3	**POS**
7	IDC	NA	NA	2	100	200	3	30	90	1	40	40	1	100	100	2	100	200	3	90	270	1	2	2	3	**POS**
5	IDC	POS	POS	0	0	0	0	0	0	1	90	90	1	100	100	1	50	50	0	0	0	1	20	20	0	NEG
11	DCIS	NEG	NEG	1	80	80	3	20	60	1	30	30	1	100	100	1	50	50	0	0	0	3	90	270	3	**POS**
12	IDC	NA	NA	2	100	200	3	40	120	2	100	200	1	100	100	1	100	100	1	100	100	3	95	285	3	**POS**
13	DCIS	NA	NA	2	100	200	3	70	210	2	100	200	1	100	100	1	95	95	2	90	180	3	95	285	3	**POS**
14	IDC + DCIS	POS	POS	1	100	100	0	0	0	1	100	100	1	100	100	2	100	200	0	0	0	0	0	0	1	NEG

Abbreviations: DCIS, ductal carcinoma *in situ*; ER, estrogen receptor status; IDC, invasive ductal carcinoma; NA, not available; PR, progesterone receptor status.

[a]Sample excluded because staining pattern suggests poor sample processing leading to false negative results.

Table 2. CN, allelic status, and sequencing variants of targeted genes in NF + BrCa samples

							HER2/NEU/ *ERBB2*			*NF1*			*TP53*			12q15 *YEATS4, MDM2, DYRK2*		12q14.1 *CDK4*	12p13.32 *CCND2*	12p12.1 *KRAS*
ID	Quality	Total CN Aberr.	LOH %	Genome changed %	Aberr. cells %	Ploidy	IHC	CN	LOH	CN	LOH	Tumor V	Germline V	CN	LOH	Tumor V	CN	LOH	CN	CN	CN
1	0.0799	75	0.93	15	Homo	2	POS					Het, G>T, chr17:29662050	Het, c.5943+1G>T	1.5							
3	0.0899	197	23.3	57.6	NA	NaN	POS	8.67		1.67	LOH	ND	NA	1.67	LOH	Het TrnV C>T, R to Ter; rs121913344	1.67	LOH	2.67		
6	0.102	128	17.4	21.7	30	2	POS					ND	NA	1.5							
8	0.157	93	13.3	20.6	Homo	2	POS		LOH	1.5	LOH	ND	Het c.1733T>G (p.Leu578Arg)	1.5	LOH	Hom, T>C, H to R, rs786201838 confirmed somatic	2.5			2.5	2.5
9	0.206	82	16.7	60.6	NA	NaN	NEG	2.33	LOH	2.33	LOH	NS	Het Del exon 37 and 39		LOH		2.33	2.33	2.33	2.33	
2	0.0863	99	28.8	40.9	NA	NaN	NEG	2.33				NS	Het SpliceV, c.1260+1604A>G				2.33	2.33	2.33	2.33	
4	0.0922	36	9.55	13.3	60	2	POS					ND	Het TrnV/SpliceV in exon 21 (c.2621_2634dup AGGGTTCTATGATT)								
7	0.104	199	17.3	75.8	40	3	POS	25			LOH	Hom, TrnV, Chr17: 29657433TC>T	NA		LOH	Het TrnV, TA>T, Chr17:7578266 confirmed somatic	3	3			
5	0.0984	29	1.95	10.8	Homo	2	NEG					ND	Het TrnV c.1541_1542delAG				2.5	2.5	2.5	2.5	

NOTE: Quality, the quality and general assessment of the genome in cells given by OncoScan; Aberr., aberrations; NA, not available; Homo, homogeneous when aberrant cells are 100%; ploidy, the most likely ploidy state and is assigned as the median CN state of all markers; NaN, the percentage of aberrant CNs cannot be determined; POS, positive; NEG, negative; CN, copy number called by OncoScan; LOH, loss of heterozygosity called by OncoScan; Tumor V, the variant was identified by tumor tissue sequencing using OneSeq; Germline V, the variant was identified in the lymphocytes of the subject; these samples with germline mutations were published by Wang and colleagues, 2017.

Variant description, described in terms of changes in cDNA nucleotide, amino acid, GRCh37/hg19 genomic coordinates, and its reference SNP ID number "rs#"; Hom, homozygous; Het, hyeterozygous; TrnV, nonsense or frameshift variant; SpliceV, splicing variant.

ND, not detected; and NS, variant detected is not significant as it is not expected to affect function.

following genes appeared to be much more prominent in NF + BrCa than those in TCGA METABRIC datasets: *YEATS4*, *MDM2*, and *DYRK2*, 55% (5/9) versus 10% (251/2,509); *CCND2*, 44% (4/9) versus 10% (251/2,509); *CDK4* 55% (5/9) versus 7% (176/2,509), and *KRAS*, 44% (4/9) versus 8% (201/2,509; Table 2). ChAS chromosome view showed that the CN gain of these genes is mostly a result of CN gain of the entire chromosome 12 or the long arm of chromosome 12. Statistical analysis is not applied for the comparison because of the small sample size of NF + BrCa and the different methods from which the TCGA CN datasets were generated.

Targeted gene sequencing analysis

OneSeq Constitutional Research Panel (targeted at hereditary diseases) sequencing generated limited data from the DNA extracted from archived tumor specimens. Identifiable pathogenic variants of *NF1* and *TP53* gene are included in Table 2. This OneSeq panel did not identify any pathogenic variant of other genes related to hereditary risk for breast cancers (*ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *NBN*, *PALB2*, *PTEN*, *RAD50*, and *STK11*). Many known somatic tumor-driver gene mutations are not covered by this panel. Variants without convincing evidence of pathogenicity for cancer were filtered out and are not included in this report.

TCGA data analysis of unselected breast cancer specimens

HER2 status. In the TCGA and TCPA breast invasive carcinoma dataset, the HER2 expression, measured by RNA-seq and protein array (RPPA), was significantly lower in tumors with *NF1* mutation/deletion (categorized as "*NF1* altered") compared with tumors without such alteration ($P < 0.001$). In this analysis, "*NF1* altered" is defined as the samples with deleterious mutations or heterozygote CN loss or homozygous CN loss; "*NF1* unaltered" is defined as CN neutral or with CN gain/amplification. However, when we separated the samples with HER2 positive from those with HER2-negative status, we discovered a divergent pattern based on *NF1* alteration status. Among tumors with *ERBB2* amplification reported by FISH, the *ERBB2* mRNA and protein were significantly overexpressed in *NF1* mutated/deleted tumors compared with the *NF1* unaltered tumors, $P = 0.005$ and $P = 0.0402$, respectively. Among *ERBB2* nonamplified tumors identified by FISH, HER2 mRNA, and protein expression were significantly lower in *NF1*-mutated/deleted tumors, $P < 0.001$ and $P < 0.001$, respectively (Fig. 1; Supplementary Tables S1 and S2).

Only 375 of 960 TCGA samples with complete sequencing data have HER2 FISH status. To further verify the above divergent *ERBB2* expression pattern, we repeated this analysis on 960 samples with *ERBB2* CN data generated by tumor next-generation sequencing. We define the "CN gain" as $\log_2$ (ratio = CN in tumor/CN in normal tissue) > 1 and ≤ 2, that is equivalent to $2 < \text{CN} \leq 4$; "CN amplification" as $\log_2$ (ratio) > 2, that is equivalent to CN > 4. Once again, in tumors with *ERBB2* CN gain or amplification, *ERBB2* mRNA and protein were significantly overexpressed in *NF1* mutated/deleted tumors compared with unaltered tumors, $P < 0.001$ and $P < 0.001$, respectively. Among tumors without *ERBB2* gain or amplification, *ERBB2* mRNA and protein expression were significantly lower in *NF1*-mutated/deleted tumors, $P < 0.001$ and $P < 0.001$, respectively (Supplementary Tables S1 and S2).

However, after removing *ERBB2* CN gained, this *ERBB2* overexpression phenomenon is no longer significant on *ERBB2*-amplified samples at mRNA or protein level, $P = 0.0645$ and $P = 0.1552$, respectively. The effect of altered *NF1* in tumors with *ERBB2* CN not gained or nonamplified remains the same, $P < 0.001$ (Supplementary Tables S1 and S2).

Genes on chromosome 12. Because several genes on chromosome 12 (*YEATS4*, *MDM2*, *DYRK2*, *CCND2*, *CDK4*, and *KRAS*) appeared to have more CN gain in NF + BrCa samples, we examined them in TCGA datasets. In TCGA samples, mRNA expression of *DYRK2* and *CDK4* on the long arm of chromosome 12, that is, 12q, was both elevated in *NF1* mutated/deleted samples ($P < 0.001$, multiple test $P < 0.001$; Supplementary Table S1). mRNA of *YEATS4* and *MDM2*; however, were decreased ($P = 0.045$ and $P < 0.001$, respectively, with multiple test $P = 0.805$ and $P < 0.001$, respectively). *KRAS*, the gene on the short arm, 12p12.1, showed preferential CN gain/amplification (33% in *NF1* altered vs. 23% in *NF1* nonaltered; $P < 0.001$). The mRNA of *KRAS* showed overexpression ($P = 0.0097$) in TCGA *NF1* altered samples; however, it failed the multiple testing (0.174; Supplementary Table S1).

Akt pathway. We examined a Ras responder, Akt in TCGA samples. The *NF1* altered samples showed increased level of Akt proteins ($P = 0.0047$, $P = 0.0424$ after correction for multiple testing; Supplementary Table S2), which appeared to be primarily contributed by higher mRNA expression of *AKT1* only ($P < 0.001$; Supplementary Table S1). However, the levels of Akt phosphorylation at residues T308 and S473 did not appear to show significant difference by *NF1* status (Fig. 2; Supplementary Table S2).

MEK–ERK pathway. MEK–ERK pathway is another major responder to Ras signaling. The *NF1* altered did not show a preferentially elevated phosphorylation of the MEK1 protein. Furthermore, the MEK/ERK pathway downstream effector, Erk1/2 protein residues T202/Y204 were slightly less phosphorylated in *NF1* altered samples ($P = 0.0098$). However, the association is not significant after correcting for multiple testing ($P = 0.0879$; Fig. 2; Supplementary Table S2).

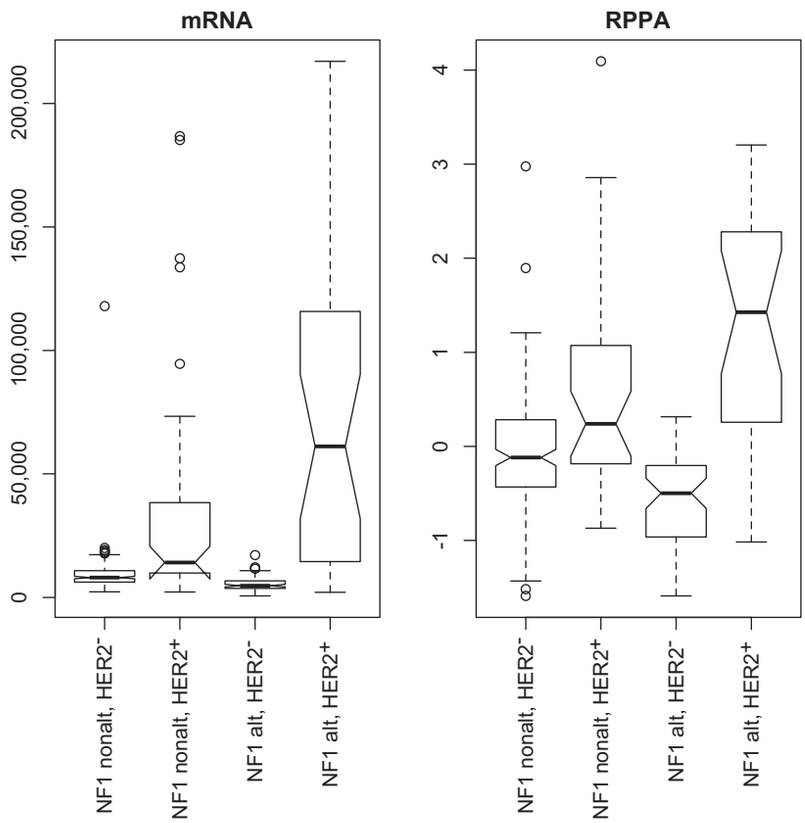

Figure 1.
Expression of *ERBB2* mRNA and its protein product HER2 in TCGA breast-invasive carcinoma dataset. The samples are stratified by their *NF1* gene status and the presence of HER2 amplification.

Expression of other selected genes and proteins. In comparison with *NF1* unaltered samples, total protein level of mTOR is decreased ($P < 0.001$, $P = 0.0031$ after correction for multiple testing); p53 protein level is slightly increased ($P < 0.001$; Fig. 2; Supplementary Table S2).

Discussion

To our knowledge, this is the first study exploring the genomic and histologic features of breast cancer harboring germline defects in the *NF1* gene. By interrogating the histology, genomic and proteomic profiles of tumor samples with germline versus somatic *NF1* mutations, we aimed at uncovering previously uncharacterized consequences of *NF1* deficiency in breast cancer.

Our analysis revealed an unusual pattern of HER2 expression both in the breast tumor samples with germline *NF1* mutations and with somatic *NF1* mutations. Our small cohort of breast cancer with germline *NF1* mutations (NF + BrCa) demonstrated HER2 overexpression by IHC in a majority of the samples (69%, 9/13). The *ERBB2* CN amplification ($n \geq 4$, generated by Affymetrix OncoScan) alone is found in 22% (2/9) and CN low-level gain ($4 > n > 2$) is found in 22% (2/9), which echoes the findings in the Finnish study showing HER2 amplification by FISH analysis in 31% (8/26) of the tumors from NF1 patients (3). However, our 9 tumor samples' IHC HER2 positivity was discordant with the *ERBB2* CN that 4 of the 6 IHC strong positive samples were with no detectable *ERBB2* gene gain or amplification. Chromosome 17 polysomy did not appear to be responsible for the *ERBB2* CN gain or amplification in the NF + BrCa samples. One sample had CN gain involving the entire 17q arm. FISH was not performed on these samples. This discordance between IHC and CN amplification may be due to the heterogeneity of the tissue, that is, the CN gain being present only in a small percentage of cells and consequently below the limit of detection in next-generation sequencing and microarray analyses that use genomic DNA extracted from homogenized tumor tissue. The overexpression observed by HER2 IHC staining may also be a result of enhanced transcription or translation rather than genomic CN gain or amplification. In comparison, IHC and FISH results' discordance has been seen in a much smaller scale in TCGA unselected breast cancer samples. Approximately 80% of the HER2 overexpression is due to *ERBB2* gene amplification detected by FISH (9, 17, 18). Our NF + BrCa data suggest that HER2 may be preferentially overexpressed in *NF1* deficient breast tumor via some mechanisms in addition to HER2 CN gain or amplification.

Upon further dissection of the sporadic breast cancer TCGA genome, RNA expression and protein expression data, we discovered an intriguing phenomenon that when *ERBB2* CN gain or amplification presents with

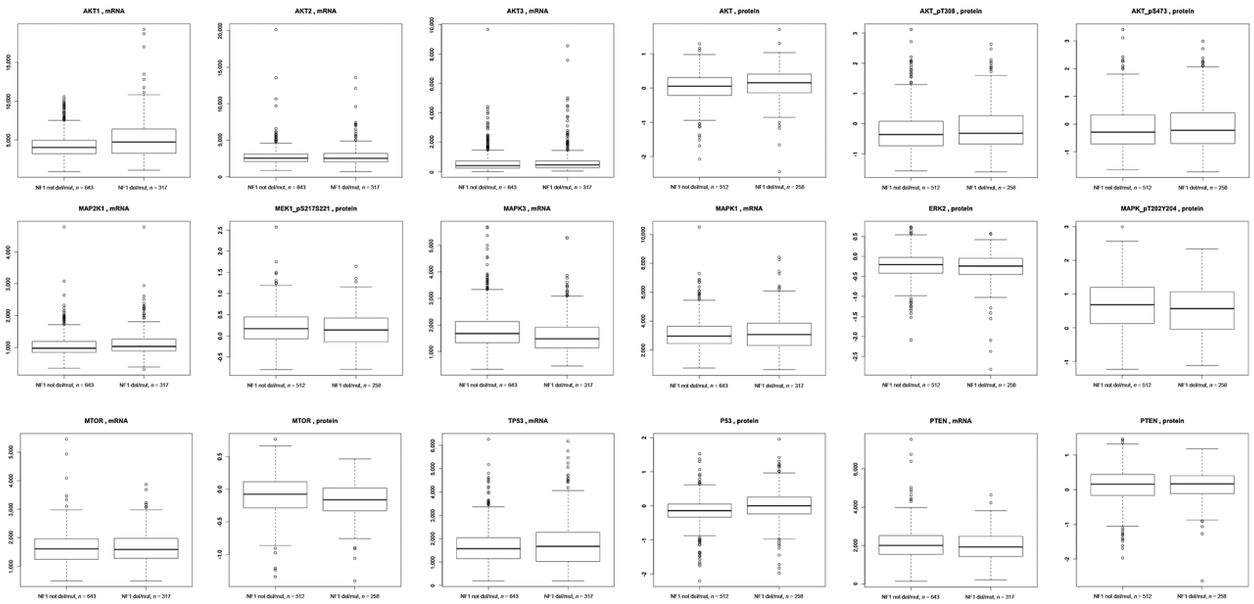

Figure 2.
Protein and mRNA levels of selected signaling molecules in the TCGA breast-invasive carcinoma dataset. The samples are stratified by their *NF1* gene status.

defective or deficient *NF1*, the HER2 expression is much more pronounced compared with normal *NF1*. On the contrary, when *ERBB2* is without CN gain, the *NF1* deficiency or defect surprisingly associates with relatively lower HER2 expression. Our analysis was based on correlating *NF1* mutations or deletions with HER2 expression. Because *NF1* and *ERBB2* genes are located in close proximity on chromosome 17, deletions of the *NF1* gene may also span the *ERBB2* gene. Indeed, the samples with *NF1* deletion and no gain or amplification of *ERBB2* mostly contained loss of *ERBB2* CN, which is likely to explain the decrease in HER2 expression. In contrast, when *ERBB2* amplification is detected by FISH or sequencing, the deletion of the *NF1* gene does not extend to *ERBB2*, allowing higher expression of HER2. It therefore seems that the defective *NF1* associates with either upregulation of the HER2 expression or a survival benefit for HER2 overexpression at least when excessive *ERBB2* copies are present. This may also be the case in the absence of *ERBB2* gain/amplification but the overlapping chromosomal deletions mask the effect.

Both germline and somatic alterations of the *NF1* gene associate with an increase in HER2 expression in breast cancer. Also, *ERBB2* amplifications are overrepresented in breast tumors of NF1 patients, as demonstrated in this study and also described previously (3). It therefore seems that *NF1* deficiency and HER2 overexpression act synergistically to provide the cancer cells an evolutionary benefit. Further molecular and biochemical studies at the cellular level are needed to reveal the nature of NF1–HER2 interaction.

Several mechanisms other than gene amplification have been proposed to regulate HER2 expression, including transcription factors, catecholamine receptor, and nuclear receptor coactivator (19–22). Kannan and Tainsky described Ras activating mutation that led to cell transformation by activation of transcription factor AP-2α (23). Later, Kannan discovered that anomalous abundance of AP-2α is accompanied by elevated levels of HER2 protein in a mammary epithelial cell line (24). HER2-positive breast cancer has long been recognized as a unique category with well-established targeted treatment options (25, 26). However, resistant tumors still exist. Understanding the mechanism of enhanced HER2 transcription may offer novel therapeutic approaches.

NF1 LOH was not consistently observed in this small cohort of breast cancer with a germline heterozygous *NF1* mutation. This is in contrast to the most common malignant neoplasm in NF1, malignant peripheral nerve sheath tumor (MPNST), where biallelic *NF1* loss is a common feature. Although *NF1* LOH may not be essential to NF + BrCa, observing it in as many as 4 of 9 tumors suggests an oncogenic role of *NF1* LOH in breast cancer development. Next-generation whole-exome sequencing of the tumor sample may not be comprehensive enough to identify all *NF1* mutations, partly because of the heterogeneity of the tumor tissue, the wide spectrum of the *NF1* alterations (including deep-intron splice variants and rearrangements), the large gene size and the complex gene structure (27). Because of these limitations, it is possible that some biallelic *NF1* defects in the sample were not detected. In addition, because NF1 is associated with an approximately

2-fold lifetime risk of breast cancer, half of the breast cancer cases would occur even without the *NF1* mutation. Therefore, it is not surprising that no biallelic loss of *NF1* was observed in 5 of 9 tumors. These NF + BrCa samples analyzed can be "sporadic" in nature without being driven by *NF1* germline mutation. Some of the NF + BrCa specimens do not have sequencing data from a matched sample representing germline tissue. Therefore, the somatic versus germline origin of the mutation identified in tumor tissue cannot be determined in all samples.

It is known that various *NF1*-deficient tumor cells and cell lines in human or animals, such as MPNST, hematologic malignancies (AML, CMML, and JMML), melanoma, or optic glioma, demonstrate mostly activated Raf/MEK/ERK pathway and sometimes PI3K/Akt pathways (28–35). Clinically, NF1 plexiform neurofibroma has shown favorable responses to selumetinib, an MEK inhibitor (36). Investigating these pathways in breast cancer may yield useful information for future treatment. Our small cohort study was retrospective in nature and only archived FFPE tumor sample was available to be analyzed. It is known that formalin cross-links peptides may mask epitopes and impair the sensitivity of IHC assay. The age of storage may also cause the tissue section to lose its antigen reactivity. In addition, the protein phosphorylation is a dynamic process where prolonged time between resection and fixation can change the phosphorylation status in the tissue. IHC analysis of the archived NF + BrCa samples did not yield any signs of MEK–ERK or Akt pathway activation. We proceeded with analyzing samples in TCGA database. The expression and phosphorylation patterns of unselected breast cancers did not support an activated MEK–ERK pathway either. However, Akt expression was increased in *NF1* altered samples, contributed by *AKT1*, but the Akt phosphorylation showed no difference.

The percentage of mutation or shallow CN loss of the *TP53* gene in NF + BrCa was not deviated from the sporadic cancer in TCGA. No additional mutations in the hereditary risk genes were found. Many common breast cancer gene mutations in sporadic breast cancers were not tested in this small cohort.

The inherent limitation of this study is secondary to the small sample size of NF + BrCa, which was deemed exploratory in nature. More samples are needed to characterize the mutations and CN variations in contrast to sporadic breast tumor samples using comparable sequencing and array methodology. Limited tumor material and funding restricted the opportunity to a thorough molecular investigation with FISH or in-depth somatic tumor exome sequencing with attention to cancer driver genes. Targeted tumor sequencing results could not be confirmed with Sanger sequencing at this time, either.

This study demonstrates that both germline and somatic *NF1* mutations associate with an increase in HER2 expression, resulting from either gene amplification or transcriptional upregulation. Furthermore, this is the first genetic characterization of breast cancer in NF1, providing basis for future mechanistic studies on breast cancer and NF1.

Disclosure of Potential Conflicts of Interest

J.O. Blakeley is a consultant/advisory board member for Abbvie. No potential conflicts of interest were disclosed by the other authors.

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