Genomic and immunological tumor profiling identifies targetable pathways and extensive CD8+/PDL1+ immune infiltration in inflammatory breast cancer tumors

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Abstract

Inflammatory breast cancer (IBC) is a rare and aggressive form of breast cancer that remains poorly understood at the molecular level. Comprehensive tumor profiling was performed to understand clinically actionable alterations in IBC. Targeted-next-generation sequencing and IHC were performed to identify activated pathways in IBC tumor tissues. siRNA studies examined the impact of IBC genomic variants in cellular models. IBC tumor tissues were further characterized for immune infiltration and immune checkpoint expression by IHC. Genomic analysis identified recurrent alterations in core biological pathways including activating and targetable variants in HER/PI3K/mTOR signaling. High rates of activating HER3 point mutations were discovered in IBC tumors. Cell line studies confirmed a role for mutant HER3 in IBC cell proliferation. Immunological analysis revealed a subset of IBC tumors associated with high CD8+/PD-L1+ lymphocyte infiltration. Immune infiltration positively correlated with an NGS-based estimate of neo-antigen exposure derived from the somatic mutation rate and mutant allele frequency, iScore. Additionally, DNA mismatch repair alterations, which may contribute to higher iScores, occurred at greater frequency in tumors with higher immune infiltration. Our study identifies genomic alterations that mechanistically contribute to oncogenic signaling in IBC and provides a genetic basis for the selection of clinically relevant targeted and combination therapeutic strategies. Furthermore, an NGS-based estimate of neo-antigen exposure developed in this study (iScore) may be a useful biomarker to predict immune infiltration in IBC and other cancers. The iScore may be associated with greater levels of response to immunotherapies such as PD-L1/PD-1 targeted therapies.

Introduction

Breast cancer is the second most common cancer in women. Inflammatory breast cancer (IBC) is the most aggressive and lethal form of primary breast cancer(1, 2). IBC is a clinically and pathologically unique subtype of breast cancer that accounts for up to 5% of breast cancer. However, IBC is the underlying cause of 10% of breast cancer deaths. The median survival of IBC is significantly less than non-IBC (stage IIB IBC 4.75 years vs. stage III non-IBC: 13.4 years)(3). Approximately 85% of IBC patients present with clinical stage IIB or IV disease(4).
IBC is characterized by rapid onset of breast erythema, edema, enlargement, and the characteristic *peau d’orange* appearance of the skin. The inflammatory skin response results from dermal lymphatic invasion, a hallmark of IBC, whereby lymphovascular spaces are occupied by tumor emboli(2). The presence of multiple dermal and stromal tumor emboli are distinguishing pathologic characteristics of IBC(2).

Inflammatory signaling pathways are active in IBC (NF-κB, Cox-2, and JAK2/STAT3)(1, 5), but the exact function of the immune response remains elusive. Immune cells and inflammatory signaling may promote IBC tumor cell proliferation, angiogenesis, and metastasis(1). On the other hand, the presence of cytotoxic tumor-associated lymphocytes has also been associated with a more favorable breast cancer prognosis(6). The contradictory nature of the immune data highlights the necessity of elucidating the relationship between the immune response, IBC tumorigenesis, and subsequent treatments. IBC immunotherapies are currently under investigation in the preclinical setting(2). Given the active IBC immune component and the recent clinical benefit of immune checkpoint inhibitors in cancer treatment(7), IBCs may represent a clinically unique breast cancer population that may benefit from immunomodulating agents.

Primary IBC treatment involves systemic chemotherapy (taxanes and anthracyclines) followed by surgery and radiation(8). *ERBB2* (hereinafter referred to as “HER2”)-positive IBCs may also benefit from trastuzumab, lapatinib, or both(9). Despite advances in the multimodal IBC treatment regimens, IBC remains a therapeutic challenge. Due to the rareness of the disease, IBC remains poorly understood at the molecular level. Previous molecular profiling studies indicate that IBC is a molecularly distinct subtype of breast cancer, providing preliminary insight into clinically relevant pathways that influence IBC tumorigenesis(10). For example, *HER2* is commonly amplified or overexpressed in IBC(11), whereas genes typically overexpressed in non-IBC: TGF-β, progesterone receptor, and the estrogen receptor (ER) are typically downregulated in IBC(12). While previous biomarker studies offer insight into IBC, IBC analyses are typically limited to a subset of target molecules or pathways. Genomic IBC data is sparse and new genomic-based analysis offers the potential to identify a robust set of clinically relevant biomarkers (13).

Recent genomics-based biomarker studies have identified mutations that drive tumorigenesis and provide evidence for genotype-directed therapeutics(14). A prospective breast cancer trial (SAFIR01) recently demonstrated the feasibility of using genomic screenings to identify clinically targetable alterations(15). In this study, pathological analysis revealed subtypes of IBC tumors that are characterized by high levels immune infiltration. To further characterize the
relationship between genotype, tumorigenesis, and the immune response, targeted next-generation sequencing was performed on a panel of cancer-related genes in IBC tumors. The current data provide insight into genomic signatures that drive IBC tumorigenesis and the cell-mediated (T-lymphocyte) immune response. Active IBC pathways range from aberrant HER2/HER3(ERBB3)/PI3K signaling to PD-L1 immune checkpoint signaling, all of which may be amenable to targeted therapeutic intervention (hereinafter ERBB3 is referred to as “HER3”).

This study identifies the importance of using a combination of targeted sequencing, immunological analysis, and protein analysis to comprehensively profile human tumors to generate clinically relevant data that provides insight into avenues for therapeutic intervention. Utilizing these approaches we have revealed possible treatment options that may be tailored to the genomic and proteomic background of each cancer. Our study has identified genomic alterations that mechanistically contribute to oncogenic signaling in this aggressive form of breast cancer, and provides a genetic basis for the selection of clinically relevant targeted and combination therapeutic strategies. Furthermore, an NGS-based estimate of neo-antigen exposure developed in this study may be a useful biomarker to predict immune cell infiltration in IBC and other cancers, and this biomarker may be associated with greater levels of response to immunotherapies such as PD-L1/PD-1 targeted therapies.

MATERIALS AND METHODS

Next-Generation Sequencing of IBC Tumor Samples

Sample preparation, library construction and targeted capture, next-generation sequencing, and bioinformatics analyses of tumor and normal samples were performed by Personal Genome Diagnostics (PGDx, Baltimore, MD). A custom hybridization capture based probe library was designed using Agilent SureSelect capture probes (Agilent Technologies; Supplementary Table S1). This panel captures full coding regions of 208 cancer relevant genes and introns of 13 genes to detect the substitution, deletions, copy number changes in the 208 targeted genes and structural rearrangements in 17 genes. Indexed Illumina NGS libraries were prepared from tumor enriched samples specified in SureSelect XT target enrichment system for Illumina Paired-end sequencing library kit. Multiplexed libraries were sequenced using 151-bp paired end runs either on Illumina MiSeq or Illumina HiSeq 2000 for average read depth of >350. IBC tumor tissues were collected from the Fox Chase Cancer Center (Philadelphia, PA). IBC tumor analysis was
performed on formalin-fixed, paraffin-embedded (FFPE) tissues. Tumor tissues, including the cancer stage at diagnosis (and all available clinical information), are described in Supplementary Table S2. A pathologist scored/estimated the percentage of cancer tissue in each tissue sample (Supplementary Table S2).

**Bioinformatic analyses**

Sequencing reads were analyzed and aligned to human genome hg18 with the Eland algorithm in CASAVA 1.7 software (Illumina). Reads were mapped using the default seed-and-extend algorithm, which allowed a maximum of two mismatched bases in the first 32 bp of sequence. Identification of somatic alterations was using the VariantDx (PGDx, Baltimore, MD) next-generation sequencing analysis pipeline that enriched for tumor-specific single-nucleotide alterations and small indels. Briefly, for each position with a mismatch (compared with the hg18 reference sequence using the Eland algorithm), the read coverage of the mismatched and wildtype sequence at that base was calculated. For determination of tumor-specific alterations, tumor were compared and known polymorphisms were removed from the analysis. Potential somatic mutations were filtered and visually inspected, and a candidate-mismatched base was identified as a mutation when distinct paired tags contained the mismatched base and the mismatched base was not present in the matched normal sample.

**Cell Culture**

AU565 Cells [AU-565] (ATCC®CRL-2351™) and BT474 Cells (ATCC®HTB-20™) were purchased from American Type Culture Collection (ATCC; 2011) and expanded upon receipt into vials of low-passage cells for cryopreservation. Cells were passaged for up to 3 months after resuscitation. ATCC characterizes cell through short tandem repeat typing, and re-authentication was not conducted. AU565 cells were cultured in RPMI-1640 and BT474 cells were cultured in Iscove’s Modified Dulbecco Minimum Essential Medium. All media were purchased from Corning and supplemented with 10% FBS (Life Technologies) and antibiotics (Corning). Cells were cultured in a 37°C, 5%CO2 humidified environment.

**siRNA Knockdown**
HER3 (ERBB3) mRNA was knocked down using siRNA-targeting ERBB3, Dharmacon ON-TARGET plus Human ERBB3 (2065) siRNA-SMART pool (cat# L-003127); (Thermo Fisher Scientific) or a scrambled control, Dharmacon ON-TARGET plus Nontargeting siRNA#1; (Thermo Fisher Scientific) with DharmaFECT1 siRNA Transfection Reagent (Thermo Fisher Scientific) as per the manufacturer’s instructions. Cells were harvested at 72 hours for cell counts or protein lysates.

Proliferation

siRNA treated cells were lifted with 0.25% Trypsin/2.21 mM EDTA (Corning) washed and resuspended in PBS for counting. Cell counting and viability were performed on a Bio-Rad TC10 Automated Cell Counter (Bio-Rad). ERBB3 knockdown cell counts were normalized to Scrambled Control Cells. Proliferation were repeated in triplicate. Bar graphs illustrate Mean +/- SEM. Data sets were analyzed using an un-paired t-test with significance at p<0.05.

Western Blot Analysis

Following 72 h treatment with Scrambled Control or ERBB3 siRNA, cell lysates were collected in protein lysis buffer (Cell Signaling) supplemented with PMSF (Sigma-Aldrich), PI cocktail (Sigma-Aldrich), and PhosphoGuard A/B (Therapak). Protein concentrations were determined using the Lowry assay (Bio-Rad) and 30 μg of protein was resolved on 4-15% gels (Bio-Rad). After transfer to PVDF membranes, blots were incubated in LI-COR blocking buffer (LI-COR Biosciences) and probed with primary antibody overnight at 4°C. pERBB3, pS6 (Ser235/236), total S6 and GAPDH antibodies were from Cell Signaling; β- actin antibody was from Sigma-Aldrich and total ERBB3 antibody was from EMD Millipore. Appropriate secondary antibody (LI-COR Biosciences) was utilized to visualize bands on a LI-COR Odyssey® Infrared Imaging System (LI-COR Biosciences). Western blot experiments were repeated in triplicate.

Immunohistochemistry

Formalin-fixed paraffin-embedded 5 μm sections were stained for phospho-ErbB3, phospho-S6, CD8, FoxP3 and PD-L1 by immunohistochemistry. Antigen retrieval was performed in the Dako PTLink in Envision FLEX Target Retrieval solution (Dako), high pH for phospho-ErbB3 (Cell Signaling), phospho-S6 (Ser235/236) (Cell Signaling), CD8 (Dako), and PD-L1 (Cell Signaling), low pH for FoxP3 (Abcam). Staining was carried out in a Dako Link 48 Autostainer. The sections
were stained with a primary antibody to phospho-ErbB3 at a 1:500 for 30 minutes, phospho-S6 at a 1:100 for 30 minutes, CD8 (predilute) for 20 minutes, PD-L1 at 1:100 for 30 minutes and FoxP3 at 1:100 for 60 minutes. All other reagents used in the staining process were Dako Evision Flex Reagents (Dako). Hematoxylin (Surgipath/Leica) was used as the counterstain. Image analysis was performed on an Aperio Scanscope XT using Spectrum Software.

RESULTS

Targeted Next-Generation Sequencing Analysis of IBC

Molecular studies of IBC are generally limited and have typically focused on tumor characterization at the mRNA level. While previous gene expression studies provided insight into IBC biology, a complete understanding of IBC requires the analysis of DNA alterations that drive tumorigenesis and progression. In this study, we performed genomic and immunological characterization of 19 IBC tissues. Initial pathological analysis of these IBC tumors identified the characteristic presence of numerous dermal tumor emboli in the skin. Notably, in approximately 50% of tumors, immune cell infiltration was present in the superficial dermis layer and was associated with intra-tumoral and contiguous peri-tumoral desmoplastic stroma (Fig. 1A). High-throughput targeted-next-generation sequencing was used to characterize genetic variants in a panel of 208 cancer-related genes across IBC tissues. The NGS (next-generation sequencing) generated approximately 0.8 Mb of target region sequence and >94% of the target regions were defined by at least 10 reads. Analysis of the types of genetic variants reveal that missense mutations were the most common variant (73%), followed by frameshifts (8%), splice site alterations (6%), nonsense mutations (5.5%), amplifications (5.5%), and In-frame insertions-deletions (3%). In total, NGS identified 391 genetic variants in 19 IBC tissues.

Consistent with reports of inter-tumor heterogeneity in IBC gene expression studies, the NGS revealed allelic heterogeneity between tumors. Both the variant allele class and the frequency of variant alleles differed between tumors (Fig. 1B, 1C). Despite allelic heterogeneity, it is noteworthy that several genes have alterations in multiple IBC samples. Allele frequency is relevant to IBC analysis as the variant frequency is a parameter that may be used to prioritize or identify driver mutations in cancer. In the IBC cohort, the 5 most commonly altered genes are: TP53
(altered in 58% [n=11] of IBC tumors), HER2 (amplified in 53% [n=10] of IBC tumors), ATM (altered in 53% [n=10] of IBC tumors, APC (altered in 37% [n=7] of IBC tumors), and HER3 (altered in 26% [n=5] of IBC tumors). Of the frequently altered genes in IBC, it is noteworthy that genetic alteration of APC, ATM, and HER3 occur at low frequencies in non-inflammatory breast cancer (0.5%, 2.1%, and 1-4%, respectively)(16, 17). Whereas TP53, and HER2 variants occur at elevated frequencies in both IBC and non-inflammatory breast cancer (33% and 30%, respectively)(18).

In total, 47 genes have alterations in at least three independent IBC tumor samples. The allelic frequency differs between tumor samples, and several allelic variants are present at a high frequency in multiple tumors (Fig. 1C). High frequency intratumoral allelic variants (ex. TP53, EGFR, PMS2) may represent trunk mutations present in the bulk of the tumor that occur early in IBC tumorigenesis (Fig. 1C)(19).

Pathway analysis of the NGS data reveals prominent genomic variation of core biologic pathways in multiple IBC tumors (Fig. 1D). Genes with reoccurring variants across IBC tumors grouped into distinct biologic pathways, and the most frequent gene alterations occur in pathways that influence genomic stability/DNA repair, PI3K signaling, chromatin modification, and HER signaling.

**Frequent Genomic Alterations in the PI3K/mTOR Pathway in IBC**

Pathway analysis revealed that IBC tumors are characterized by frequent genomic alterations in the HER/PI3K/mTOR pathway (Fig. 1B). PI3K can promote oncogenic signaling through the activation of the mTOR (mammalian target of rapamycin) pathway. Phosphorylated ribosomal protein-S6 (pS6) is a surrogate marker for mTOR activation and subsequent mTOR-mediated proliferation signals. In order to investigate activity of this pathway in IBC samples, IBC tissues were immunohistochemistry stained for pS6 expression. Ninety-five percent (n=18) of IBC tumors stained strongly positive for pS6, which suggests that the PI3K/mTOR pathway is active in the majority of IBC tumors. Mechanistically, specific genomic alterations may activate the HER/PI3K/mTOR pathway through an increase in oncogene signaling or a decrease in tumor suppressor activity thus contributing to IBC tumorigenesis (Fig. 2A).

Twenty-one percent of IBC tumors contained mutations in PIK3CA (Fig. 1B), and all of the reported PIK3CA mutations occur in previously characterized regions of mutational hotspots(20). In addition to the genetic variants that may potentiate oncogenic signaling in the PI3K/mTOR pathway, the IBC tumors also harbored mutations that may disrupt the activity of tumor suppressor genes in the PI3K/mTOR pathway. NGS analysis revealed mutations that may
lead to a loss of function in the tumor suppressors \textit{TSC1}, \textit{TSC2} and \textit{PTEN}, all of which are important negative regulators of the PI3K/mTOR pathway. Forty percent of IBC tumors harbor a mutation or a deletion in \textit{TSC1} or \textit{TSC2} (Fig. 1B). NGS analysis of \textit{PTEN} revealed one deletion event in the IBC tumor cohort (Supplementary Table S3A-S3C). Taken together, the gain-of-function alterations in oncogenes, and loss-of-function mutations in tumor suppressors may contribute to the high activity level of the PI3K/mTOR pathway in IBC tumors (Fig. 2A).

\textbf{Frequent \textit{HER3} Hotspot Mutations in IBC}

In agreement with previous studies, \textit{HER2} was amplified in 53\% of IBC tumors (21). Moreover, \textit{HER3} was mutated in 26\% (n=5) of IBC tumors, which is higher than \textit{HER3} mutational rates reported for gastric cancer (12\%), colon cancer (11\%), and non-inflammatory breast cancer (1-4\%)(17, 22). Interestingly, 4 of the 5 \textit{HER3} alterations occur in regions of mutational hotspots that are located in the extracellular domain or the kinase domain of \textit{HER3} (Fig. 2B). It is also noteworthy that 4 of the 5 \textit{HER3} mutations co-occur with \textit{HER2} amplification (Fig. 1B)(17).

The high activity of the HER/PI3K pathway across IBC tumors and the relatively high \textit{HER3} mutation rates in IBC tumors indicate that \textit{HER3} may play a functional role in IBC. To determine if \textit{HER3} mutants influence downstream signaling, we examined IBC tumors for the presence of activated (phosphorylated) \textit{HER3} (Fig. 2C, 2D). Immunohistochemistry (IHC) analysis reveals elevated levels of phosphorylated \textit{HER3} in IBC tumor samples with \textit{HER3} mutations (Fig. 2D). A subset of IBC tumors, with high levels of phosphorylated-\textit{HER3}, simultaneously possess an amplified \textit{HER2} and a mutant \textit{HER3} (Fig. 2D).

To further elucidate the role of \textit{HER3}, we carried out functional assays in breast cancer cell lines. The lack of specific \textit{HER3} mutant/\textit{HER2} Amplified IBC cells lines prompted the use of AU565 and BT474 which possess the desired \textit{HER2}/\textit{HER3} status (AU565 [\textit{HER3} mutant (kinase domain); \textit{HER2} amplification] and BT474 [\textit{HER3} wild-type; \textit{HER2} amplification]). In cell lines AU565 and BT474, transfection of \textit{HER3}-targeted siRNA led to a decrease in both the phosphorylated and unphosphorylated forms of \textit{HER3}. The \textit{HER3}-targeted siRNA also produced a significant drop in proliferation in AU565 cells but not BT474 cells (Fig. 2E), which suggests that mutant \textit{HER3} signaling may contribute to the proliferative phenotype of breast cancer cells. The scrambled siRNA control did not alter the proliferation or the levels of phosphorylated \textit{HER3} in either cell line (Fig. 2E). Western blot analysis of phosphorylated ribosomal protein S6 (S6), a surrogate marker of mTOR activity, revealed that phosphorylated S6 levels decrease in response to \textit{HER3}-
targeted siRNA in AU565 cells but not in BT474 cells (Fig. 2E). In both AU565 and BT474, the levels of unphosphorylated S6 did not change following transfection of the HER3-targeted siRNA (Fig. 2E), which suggests that elevated levels of phosphorylated S6 may be due to an increase in upstream HER signaling pathways.

**Subset of IBC tumors are characterized by extensive immune infiltration**

Recent studies suggest the importance of immune cells in the progression and treatment of cancer, and we sought to define the nature of the immune response in the current IBC cohort. Pathological examination of IBC tissues identified a subset of IBC tumors associated with infiltration of immune or inflammatory cells. To further investigate the nature of these infiltrates, CD8 (cytotoxic T cell marker) and FOXP3 (T regulatory cell marker) IHC staining was performed on tissues to characterize T cells populations present in each tumor. Twelve IBC tumors had sufficient tissue for IHC analysis. Infiltrates stained minimally for FoxP3+ Tregs (Fig. 3A). IHC staining identified the majority of infiltrating cell populations as CD8+ cytotoxic T cells (Fig. 3B). CD8+ T cells typically localized in associated intra-tumoral and contiguous peri-tumoral desmoplastic stroma. Each tumor was scored by a pathologist as high CD8+ (infiltrating cells occupying ≥5% of tumor area occupied by tumor cells; Fig. 3B) or low CD8+ (infiltrating cells occupying <5% of tumor area occupied by tumor cells; Fig. 3C). High levels of CD8+ infiltration were observed in 5/12 tumors, and frequently presented as cell aggregates. Remaining tumors generally displayed low levels of single cell spreads of CD8+ cells across tissues, which was further confirmed by image analysis of average size of cellular aggregates in each tissue (Table 1).

CD8+ immune infiltrates may serve as an important immune regulator of tumor growth, but tumor or infiltrating cells may express immune checkpoint inhibitors that prevent the activation of a tumor targeting immune response. Negative regulation of CD8+ T cells may explain the high level of CD8+ TILs at the periphery of IBC tumors (Fig. 3D). Recent studies highlight the importance of PD-L1 (Programmed cell Death receptor-Ligand 1) mediated immune resistance in tumor progression. This immune checkpoint signaling molecule inhibits T-cell activation and its expression has been previously reported in both tumor and infiltrating immune cells. In order to explore the possible role of PD-L1 in IBC, we IHC stained IBC tissues for PD-L1 (Fig. 3D, 3E). Evaluation of PD-L1 staining demonstrated low-intensity tumor cell staining in 3/12 tumors studied and high-intensity tumor cell staining in 1/12 tumors (Fig. 3F). Notably all immune
cell infiltrates stained positively for PD-L1 (Fig. 3F). Intensity of PD-L1 staining on immune infiltrates was generally high with IHC scores typically ranging from 2+ to 3+ (Fig. 3D).

**Mutation rates correlate with extent of immune cell infiltration in IBC**

In order to further understand the differences in levels of immune cell infiltration between IBC tumors, NGS data was analyzed in tumors with high and low levels of immune infiltrate (Due to limited tissue availability 12 of 19 IBC samples were available for IHC analysis). Notably, somatic mutation rates were significantly higher in high infiltration vs low infiltration tumors (Fig. 4A, p<0.05). We rationalized that this correlation between somatic mutation rate and immune cell infiltration may be related to the exposure of tumor neo-antigens to the immune system. We next proposed that a score (iScore) that combines the somatic mutation rate and average mutant allele frequency may better represent the level and variability of neo-antigens throughout the bulk of the tumor. The iScore is calculated by multiplying the average mutant allele frequency by the total number of somatic mutations in the tumor. Comparison of iScores between high infiltration and low infiltration tumors demonstrated high significance in the association between levels of CD8+ infiltration and iScore in IBC tumors (Fig. 4B, p<0.01), suggesting that the iScore may be a reliable NGS-based estimate of immune infiltrate in IBC tumors. It is noteworthy that, when compared to the somatic mutation rate alone (Fig. 4A), the iScore more significantly distinguishes tumors with either high or low levels of CD8+ cell infiltration (Fig. 4B).

NGS data reveals that more than 90% of IBC tumors harbor mutations in genes that contribute to “Genome Stability & DNA repair” (Fig. 1D). We proposed that alterations in DNA repair pathways may explain the variations in somatic mutation rates among IBC tumors (ranging from 6-78 mutations/megabase; Supplementary Table S3C). Interestingly, 43% of IBC tumors possess alterations in at least one gene in the DNA mismatch repair (MMR) pathway (Fig. 5). Tumors with higher somatic mutation rates typically were associated with more frequent alterations in MMR genes and often harbored multiple mutations in the MMR pathway (Fig. 5). NGS revealed mutations in the following MMR pathway members: PMS2 (nonsynonymous mutations), MSH2 (nonsynonymous mutations), MSH6 (nonsense mutation and frameshift), and MLH1 (nonsense mutation). It is noteworthy that specific IBC mutations in MSH2 and PMS2 have also been reported in an inherited disorder, Lynch syndrome, which is linked to higher rates of colon cancer(23) and characterized by mutations in the DNA MMR pathway(24). In addition, alterations in epigenetic...
signaling pathways may contribute to MMR in IBC. The chromatin remodeling genes ARID1A and ARID1B also have a role in DNA MMR(25). Genetic alterations in either ARID1A or ARID1B are found in 6 of 19 IBC tumors (Fig. 5), indicating that aberrant epigenetic signaling may disrupt DNA repair and ultimately contribute to IBC progression. It is noteworthy that ARID1A variants occur in 26% of the current IBC cohort, whereas only 2% of non-IBC tumors harbor mutations in ARID1A(16).

In IBC samples, nonsense mutations and frameshifts in MMR genes are likely to disrupt the function of a wild-type protein, however, nonsynonymous mutations may also disrupt protein function. For example, the nonsynonymous PMS2 mutation (D575G) which occurred in one IBC sample is located within a protein domain (“level” domain) which interacts with MSH6 and is important to PMS2 function(26). The functional characterization of IBC variants in MMR genes warrants further study. Albeit a limited size IBC tumor cohort, high immune cell infiltration positively correlates with the presence of MMR gene alterations in comparison to IBCs with low immune cell infiltration (Fig. 3C).

**DISCUSSION**

In this study, we performed targeted NGS to profile a panel of 208 cancer-associated genes in 19 IBC tumors. Although the current study is based on a small cohort, the targeted exome analysis provides compelling evidence that specific biologic pathways and somatic variants may influence IBC tumorigenesis and the concomitant immune response. The genomic profiling may also provide a clinically relevant means to stratify patients into cohorts that may benefit from targeted therapeutics.

NGS data reveals somatic alterations in pathways that regulate HER/PI3K signaling, chromatin modification, and DNA repair in IBC. The biologic significance of these pathways is supported by independent studies demonstrating that PI3K, HER2, and DNA repair genes are altered in IBC tumors(27). In our cohort, the most abundant somatic alterations occur in genes that contribute to HER/PI3K/mTOR signaling, and IHC indicates that downstream effector pathways are active in a majority of IBC tumors.

Our data identifies frequent HER2 amplification and the occurrence of HER3 point mutations and truncations in IBC tumors. HER2 and HER3 form a heterodimer upon ligand binding that leads to the formation of a phosphorylated HER2/HER3 heterodimer with potent oncogenic signaling capabilities(28). HER2 expression is necessary for downstream events in mutant HER3 oncogenic signaling(17) and the co-expression of HER2/HER3 is a common event in breast
Notably, HER3 is altered in 26% of IBC tumors, which is higher than the HER3 mutation rates reported in other tumors (1-12%)(17, 22). Variants typically occurred in known mutational hotspot regions in the kinase and extracellular domains of HER3 and frequently co-occurred with HER2 amplification. In our study, tumors harboring HER3 mutations were associated with higher levels of tumoral expression of phosphorylated HER3, which provides further evidence that HER3 mutation may be associated with constitutive HER3 activation in IBC. Other studies have demonstrated that somatic HER3 mutations may promote ligand-independent oncogenic signaling and increased sensitivity to HER3 targeted therapies (17). Cell line studies also suggest a role for mutant HER3 in IBC. siRNA-mediated knockdown of HER3 decreased proliferation and reduced phospho-S6 expression in the HER3 mutant/HER2 amplified breast cancer cell line (AU565) but not in the HER3 wild-type/HER2 amplified BT474. This may indicate that HER3 dependence varies between genetically distinct breast cancer cells, however; further investigation is needed to explore the mechanistic role of HER3 mutation in IBC and other cancers. Taken together, the co-occurrence of HER2 amplification and HER3 mutations may contribute to the oncogenic HER2/HER3 signaling in IBC tumors and have implications for treatment with HER targeted therapies.

Clinical responses to a small molecule HER2 inhibitor (lapatinib) and HER2 targeted antibodies (trastuzumab and pertuzumab) have been demonstrated in HER2 amplified IBC tumors(30, 31). However, HER2 blockade can promote the upregulation of HER3, which compensates for impaired HER2 signaling, and promotes downstream activation of the PI3K pathway(32). Trastuzumab treatment is recommended for HER2-positive IBC(13), but IBC tumors commonly acquire resistance to HER2 therapy(33). As demonstrated in prior studies, aberrant HER3 activity, including HER3 mutation, may contribute to anti-HER2 therapeutic resistance, and thus the combination of HER2 and HER3 inhibitors/antibodies may be required to disrupt downstream oncogenic signaling in IBC tumors(32). HER3-targeted therapies (Patritumab and LJM716) are currently in clinical development(34) and prior studies have demonstrated that these drugs may be effective in combination with HER2 therapies(17, 34). The high frequency of HER3 mutations in IBC supports further investigation of the efficacy of HER3 targeted drugs in this cancer.

PIK3CA, a kinase that functions downstream of HER and other oncogenes, plays a role in proliferation and cell survival and is frequently mutated in breast cancer. NGS analysis identifies frequent variants in the PI3K pathway including PIK3CA hotspot mutations and predicted loss of function mutations in TSC1 and TSC2 tumor suppressors. The activity of mTOR is negatively regulated by the tumor suppressor proteins TSC1 and TSC2. Given the abundant mTOR
activity and frequent alterations in the pathway, IBCs may benefit from targeted disruption of PI3K/mTOR activity. The potential benefit of an mTOR-targeted therapy was demonstrated by the BOLERO-3 trial, in which the mTOR inhibitor, everolimus, was shown to provide a clinical benefit in the treatment of trastuzumab-resistant, HER2-positive breast cancer(35). While PIK3CA activating-mutations are associated with better response to PI3K targeted therapies, they are also associated with a poor response to HER2-targeted therapies in breast cancer(36), raising the possibility that PIK3CA status should be taken into account with respect to the effectiveness of HER-targeted therapies in IBC. Conversely, the HER2/HER3 status may impact the efficacy of PI3K inhibitors. For example, the PI3K-inhibitor, XL147, inhibits cell growth in wild-type HER2 breast cancer cells, but not in HER2-amplified breast cancer cells(37). HER-2 amplified breast cancer cells became susceptible to XL147 following the knockdown of HER3 or adjuvant treatment with trastuzumab or lapatinib(37). Collectively, the interplay of genetically altered cancer pathways raises important considerations for treatment of IBC and other cancers.

The majority of IBC tumors harbor mutations in genes that contribute to genome stability and DNA repair. Genetic alterations in DNA repair pathways may disrupt the fidelity of DNA repair and thus contribute to the development and progression of IBC. Notably, in our study, we have detected frequent alterations in the MMR pathway in IBC tumors that correlate with higher somatic mutation rates and greater levels of immune infiltration. The occurrence of MMR gene variants in IBC has not been previously reported and thus the role that these genetic alterations play in IBC progression is less clear. In Lynch syndrome, a hereditary syndrome associated with high rates of colorectal cancer development, somatic variants in DNA MMR genes (PMS2, MSH2, MSH6, and MLH1) leads to microsatellite instability (MSI)(24). Prior studies have also identified that MSI is associated with elevated TIL levels that may be related to the neoantigens that arise from errors in MMR(38). Furthermore, high somatic mutation rates have also been linked to higher tumor antigenicity in other cancers through the production of immune-response-eliciting neoantigens(39). Somatic mutations play a significant role in neoantigen production that triggers a T-cell response(40). Alterations in the MMR pathway are typically associated with the generation of frequent somatic point and frameshift mutations that may disrupt protein function and create immunogenic neoantigens(39). It is tempting to speculate that alterations in the MMR pathway in IBC may have similar links to somatic mutation rate and levels of immune infiltration, however, further mechanistic studies are required to support this hypothesis.
To further characterize immunogenicity in IBC, we developed an NGS-based estimate of antigen exposure, the iScore. The iScore takes into account both the somatic mutation rate and average mutant allele frequency for each individual tumor. The iScore more significantly characterized TIL levels than somatic mutation rates alone, which suggests that mutant allele frequency may be an important factor for the estimation of tumor antigenicity and TIL levels. Since somatic mutations contribute to neoantigen production and a TIL response, the average mutant allele frequency of the somatic mutations may serve as a quantitative measure of neoantigen exposure and the subsequent TIL response(39). Furthermore, incorporation of mutant allele frequency accounts for the prevalence of DNA variants throughout the bulk of the tumor. The importance of TILs in breast cancer is highlighted by an analysis of breast cancer stroma that reveals a correlation between a favorable prognosis and the expression of CD8+ T cell-related genes(41). In addition, a cohort analysis of breast cancer patients revealed a positive correlation between CD8+ T cell density and improved clinical outcome(42, 43). While the iScore is significantly associated with TIL infiltration in IBC tumors, there are also many other factors that may play a role in controlling this process and as such further multivariate analysis is required in a larger cohort to better characterize this relationship.

TILs may disrupt tumor progression in IBC, but an optimal anti-tumor response requires the presence of functional T-cells. TILs are often in a state of T-cell exhaustion, a T-cell phenotype that is defined by poor effector function(44). T-cell exhaustion is typically associated with the expression of immune checkpoint inhibitors, such as PD-L1 on tumor cells and/or TILs(45, 46). Within the context of the breast cancer microenvironment, upregulation of PD-L1 may contribute to immune evasion(47). High levels of PD-L1, detected in tumors in this study, may negatively regulate T cells thus preventing the activation and migration of CD8+ T cells into the tumor. The presence of CD8+ T-cells is a strong predictor of response to anti-PD-L1 therapy(48), and PD-L1 expression is also predictive of a clinical response to anti-PD-L1 therapies(46, 49). It is noteworthy that a response to PD-L1 therapy is strongly associated with immune cell PD-L1 expression, which is characteristic of IBC rather than tumor cell PD-L1 expression(49). Based on clinical relevance of immune checkpoint therapy in tumors with high levels of TILs and PD-L1(46, 49), TIL/PD-L1 positive IBC tumors may be suitable for anti-PD-L1 therapies. A therapeutic PD-L1 blockade may reverse T-cell exhaustion, and may also promote the interferon-gamma-mediated recruitment of additional T-cells at the tumor site(50).

In summary, comprehensive tumor profiling identifies somatic variants and other factors that may influence IBC tumorigenesis, the concomitant immune response, and subsequent avenues for therapeutic intervention.
amplified/HER3-mutant IBC genotype may promote cell proliferation through the downstream activation of the PI3K/mTOR pathway, which suggests that HER-targeted therapies in combination with other HER/PI3K/mTOR therapies should be further explored in IBC treatment. The iScore may serve as a surrogate biomarker of neo-antigen exposure to the immune system. The iScore may also be a useful tool to predict neoantigen exposure in other tumor types, however, a larger cohort of patients will be necessary to determine the clinical utility of the iScore in IBC and other tumors. Given the robust immune component of IBC, the presence of PD-L1-positive IBC immune infiltrate suggests that IBCs may benefit from therapies that disrupt PD-L1 signaling. Clinical studies will be required to further understand the implications of each tumor background with respect to the proposed treatment options. Overall this study highlights the importance of comprehensive tumor profiling that incorporates genomics, immunological analysis, and other approaches to provide greater insight into the ideal strategy for personalized treatment in IBC and other cancers.

REFERENCES:


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**High TILs** | **Low TILs**
FIGURE LEGENDS

Figure 1. Cellular and molecular characterization of IBC tumors A, Pathological analysis of IBC tumors identified the characteristic presence of numerous dermal tumor emboli in the skin. Notably, in approximately 50% of tumors, immune cell infiltration was present in the superficial dermis layer (Left Panel: arrow note immune cell infiltration; H&E stain, 10X magnification) and was associated with lymphatic invasion (Left Panel: dashed circle highlights lymphatic space occupied by tumor cells; H&E stain, x20 magnification). B, Genes with somatic alterations in at least 3 of 19 IBC tumor samples. Genes are sorted by their putative biologic function and mutation rate is listed for each gene. The most abundant somatic mutations are found in genes that function in HER signaling, the PI3K pathway, and Genomic stability and DNA repair. C, Variant allele frequency among IBC tumors. Overall, TP53 variants occur are found at high relative frequency in IBC tumors. However, heterogeneity exits among tumor samples and certain tumors possess variants that are more abundant than TP53. D, Prevalence of somatic alterations in core biologic pathways. In the current IBC cohort, the most frequently altered pathways are Genomic stability DNA repair, PI3K signaling, and Chromatin modification.

Figure 2. Genetic alterations in the HER/PI3K pathway contribute to aberrant signaling and cell proliferation. A, Schematic representation of the HER/PI3K pathway. Each pathway member is color coded to represent the frequency of IBC tumors that harbor a somatic variant in each respective gene. The majority of IBC tumors (95%) stain positive for phosphorylated S6, a surrogate marker for PI3K/mTOR activity. B, Schematic overview of the HER3 gene and the location of HER3 mutations among the IBC cohort. Mutations occur in hotspot regions located in the extracellular domain or the Tyrosine kinase domain (TKD). C, IHC (pHER3) staining of IBC tumor cells (x10 magnification). D, HER3 mutant IBC tumors display elevated levels of phosphorylated-HER3 (pHER3). Immunohistochemistry and pathology scoring were used to quantify pHER3 levels in IBC tumors. Results from the pathology scoring are represented in the graph, and the IHC score represents the pHER3 level. IBC tumor samples with amplified HER2 are noted in the graph, and “*” indicates a predicted truncation mutation in HER3. E, HER3-targeted siRNA disrupts proliferation in breast cancer cells. Transfection of HER3-targeted siRNA leads to a drop in protein levels of HER3, which is concomitant with significant decrease in proliferation in the breast cancer cell line AU565 but not BT474 (”*” p≤0.01). Transfection of a scrambled-siRNA control vector did not affect proliferation in
either cell line. In response to the HER3-targeted siRNA, phosphorylated S6 levels decrease in AU565 cells but not in BT474 cells. \textit{HER2} and \textit{HER3} status is noted for each cell line.

\textbf{Figure 3.} Pathological analysis of lymphocytes in IBC tumors \textbf{A}, Minimal staining for FOXP3+ Tregs within immune infiltrates. IHC staining of Treg cells (FoxP3) in IBC tumors (x10 magnification). \textbf{B}, Representative image of an IBC tumor with high levels of CD8+ T cells (infiltrating cells occupying ≥5% of tumor area occupied by tumor cells; x10 magnification). Note that the images in panel A and panel B are taken from successive tissue sections from the same tumor, which demonstrates the high levels of CD8+ cells relative to FoxP3 cells. \textbf{C}, Representative image of an IBC tumor with a low level of CD8+ T cells (infiltrating cells occupying <5% of tumor area occupied by tumor cells (x10 magnification). \textbf{D}, Intensity of PD-L1 staining on immune infiltrates was generally high with IHC scores typically ranging from 2+ to 3+. \textbf{E}, When detectable in IBC tumor cells, PD-L1 was expressed at low levels. \textbf{F}, Immune infiltrates stain highly positive for the immune checkpoint inhibitor PD-L1. Immunohistochemistry and pathology scoring were used to quantify PD-L1 levels in tumor cells and lymphatic cells.

\textbf{Figure 4.} The mutation rate and the iScore correlate with high levels of TILs. \textbf{A}, Mutation rate distinguishes between IBC tumor with high or low TIL levels (p=0.0449). \textbf{B}, The iScore combines the average mutant allele frequency and the mutation rate, and the iScore significantly associates with TIL levels (p=0.0090). The iScore is more significantly associated with TIL levels than solely the mutation rate.

\textbf{Figure 5.} IBC tumors with high TIL levels frequently harbor mutations in genes that play a role in the DNA MMR pathway. The mutation type, and the pathologist score (TIL levels) are listed for each of the 12 evaluable tumors. High immune cell infiltration positively correlates with MMR gene alterations in comparison to IBCs with low immune cell infiltration.
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**Figure 1**

- A: 10X and 20X magnification images of breast cancer tissue.
- B: Heatmap showing gene expression and variation across different pathways.
- C: Scatter plot comparing gene expression across different samples.
- D: Bar chart showing percentage of tumors with variant allele frequency across different categories.
**Frequency of Tumors Harboring an Allelic Variant of a Particular Gene**

- 0-10%
- 11-20%
- 21-30%
- 31-40%
- 41-50%
- 51-60%

**Phospho-S6 Positive** (3+): 95% IBC Tumors

**Figure 2**

**Phosphorylated HER3 Levels in IBC Tumors**

**Proliferation: 72hrs Post-Transfection of HER3-SiRNA**

**Pathologist IHC Score (pHER3/pERBB3)**

**HER2 Amplified**

**HER3 Mutant**

**HER3 Wild-type**
Figure 3

**A** FoxP3

**B** CD8

**C** CD8AB

**D** PD-L1 Expression: Immune Infiltrate

**E** PD-L1 Expression: IBC Tumor Cells

**G** PD-L1 Expression in Tumor and Immune Cells

- **Tumor Cells**
- **Lymphatic Cells**

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**Legend:**
- **High TILs:** S012-1579, S012-4469, S012-351, S012-285A, S012-8030, S011-4558, S012-2449, S011-5475, S011-3378, S011-6906
- **Low TILs:** S012-756, S011-4160, S012-2499
Figure 4

A. Mutation Rate and TIL Levels

Mutations Rate

HIGH TIL | LOW TIL

p = 0.0449

B. iScore and TIL Levels

iScore (Mut Rate x Avg Freq)

HIGH TIL | LOW TIL

p = 0.0090
TIL Levels Positively Correlate with Alterations in the DNA MMR Genes

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Figure 5
Genomic and immunological tumor profiling identifies targetable pathways and extensive CD8+/PDL1+ immune infiltration in inflammatory breast cancer tumors

Christopher A Hamm, Diarmuid Moran, Kakuturu Rao, et al.

Mol Cancer Ther Published OnlineFirst April 19, 2016.