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## Genes associated with histopathologic features of triple negative breast tumors predict molecular subtypes

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**Abstract** Distinct subtypes of triple negative (TN) breast cancer have been identified by tumor expression profiling. However, little is known about the relationship between histopathologic features of TN tumors, which reflect aspects of both tumor behavior and tumor microenvironment, and molecular TN subtypes. The histopathologic features of TN tumors were assessed by central review and

593 TN tumors were subjected to whole genome expression profiling using the Illumina Whole Genome DASL array. TN molecular subtypes were defined based on gene expression data associated with histopathologic features of TN tumors. Gene expression analysis yielded signatures for four TN subtypes (basal-like, androgen receptor positive, immune, and stromal) consistent with previous studies. Expression analysis also identified genes significantly associated with the 12 histological features of TN tumors. Development of signatures using these markers of

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histopathological features resulted in six distinct TN subtype signatures, including an additional basal-like and stromal signature. The additional basal-like subtype was distinguished by elevated expression of cell motility and glucose metabolism genes and reduced expression of immune signaling genes, whereas the additional stromal subtype was distinguished by elevated expression of immunomodulatory pathway genes. Histopathologic features that reflect heterogeneity in tumor architecture, cell structure, and tumor microenvironment are related to TN subtype. Accounting for histopathologic features in the development of gene expression signatures, six major subtypes of TN breast cancer were identified.

**Keywords** Gene expression · Pathology · Tumor biology · Germline mutation · Breast cancer

## Introduction

Triple negative (TN) breast cancer is a distinct histopathological subtype of breast cancer that accounts for approximately 15 % of all invasive breast cancers [1, 2]. This disease subtype, defined by low or no expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) is treated as a single group clinically. However, TN tumors have been shown to have significant biological heterogeneity that is not captured by clinical subtyping alone [3]. Intrinsic subtypes of breast tumors based on gene expression profiling have been defined using the PAM50 expression microarray. These include two luminal epithelial groups (A and B), a HER2 over-expressing group, a normal-like group, and a basal-like group that is largely TN (80–85 %) [4, 5]. Separately, six subtypes of TN breast cancer (referred to here as Lehmann subtypes) have been defined using combined gene expression microarray data from several studies. These subtypes were classified as

basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) [6].

Independent analyses of microarray data for TN tumors, which had substantial overlap with the original TN study, further suggested that the TN subtypes are driven in part by differences in tumor microenvironment [7]. Specifically, four main gene expression clusters were found, representing a stromal gene signature, a luminal signature, an immune signature, and a basal epithelial signature. Consistent with this model, these gene signatures were not observed in cell lines or xenografted tumors. Thus, the TN subtypes may reflect genomic heterogeneity in tumor cells and/or expression changes resulting from microenvironmental influences. Importantly, these models may have implications for breast cancer treatment and outcome, because tumor microenvironment features, including lymphocytic infiltration and fibrosis, have been associated with breast cancer progression and prognosis [8–12]. Indeed, pathologic complete response rates after neoadjuvant chemotherapy may differ by Lehmann subtype [13], suggesting that TN subtypes may have distinct prognostic implications.

TN tumors exhibit high histologic grade and proliferation rates relative to other breast tumor subtypes [1, 14]. In addition, TN tumors have greater frequencies of several distinguishing histopathologic features including medullary and metaplastic features, pushing borders, fibrous proliferation, lymphocytic infiltrate within the tumor and in lobules adjacent to the tumor, elevated mitotic count, necrosis, and the presence of many syncytial clusters [15–17]. However, the relationship between these features and both TN expression signatures and TN subtypes is not well defined. In this study we identified molecular subtypes of TN tumors based on expression profiles and 11 histopathologic features of TN tumors from the triple negative breast cancer consortium in order to evaluate the relationship between gene expression patterns and key structural and microenvironment features of TN tumors.

## Methods

### Study participants

TNBCC cases ( $n = 704$ ) from ten studies described in detail elsewhere, were included (Table S1) [18–20]. TN breast cancer cases were defined as individuals with ER-negative, PR-negative, and HER2-negative (0 or 1 by IHC) breast tumors. Criteria used for defining ER, PR, and HER2 status varied by study and have been previously described [18–20].

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## Macrodissection, RNA extraction, and gene expression profiling

Tumor samples were either whole 10 micron sections or 1 mm (mm) cores from formalin-fixed paraffin-embedded tumor blocks (Table S1). Whole sections were macrodissected to select the tumor region on the slide, guided by a hematoxylin and eosin (H&E)-stained slide from the same block, reviewed and marked by a single pathologist (D. Visscher). RNA was extracted from all tissue specimens using the Roche High Pure RNA Isolation Kit (Indianapolis, USA). Expression profiling was performed by the Mayo Clinic Medical Genome Facility Gene Expression Core (Rochester, MN, USA) using the Illumina Whole Genome cDNA-mediated annealing, selection, extension, and ligation (DASL) v4.0 assay. RNA samples were run on 12-sample chips in two batches (456 samples and 288 samples, respectively).

## Normalization, data reduction, and batch effect removal

Raw intensity data were exported from the Illumina iScan software (Figs. S1, S2). Given the difference in median expression of per-sample median values between batches (Batch 1 median = 8.1, Batch 2 median = 10.7, Wilcoxon  $p = 1.1 \times 10^{-99}$ ), all subsequent quality control and normalization procedures were performed separately by batch. Median  $R^2$  between 22 replicates was 0.91 (Batch 1 median  $R^2 = 0.91$ , Batch 2 median  $R^2 = 0.99$ ).

After log-2 transformation and exclusion of all universal human reference samples (Batch 1  $n = 10$ , Batch 2  $n = 6$ ), an iterative median quantile normalization (MQN) procedure using cyclic stress reduction separately by batch was performed. First, MQN was applied and a stress measurement was calculated for each sample, equivalent to the median absolute deviation after normalization [21]. The top 10 % of samples with the highest stress was removed, and the MQN and stress calculation were repeated until only 30 % of the original dataset—those with the least stress during repeated normalization remained. This least-stressed dataset was used as a reference for MQN of the full dataset and recalculated stress (Figs. S3, S4). Samples with stress  $\geq 0.5$  were excluded (Batch 1  $n = 27$ , Batch 2  $n = 6$ ); for replicates, the sample with the highest stress was excluded (Batch 1  $n = 16$ , Batch 2  $n = 10$ ). Probes with  $p$ -value of detection  $> 0.05$  were excluded (Batch 1  $n = 713$ , Batch 2  $n = 479$ ).

Expressions of ER (*ESR1*, Illumina probe ILMN\_1678535), PR (*PGR*, Illumina probe ILMN\_1811014), and HER2 (*ERBB2*, Illumina probe ILMN\_2352131) were evaluated [22]. The expression values for each of the PAM50 genes, that can be used to define breast cancer subtypes

[luminal A (LumA), luminal B (LumB), basal-like, HER2-enriched (HER2), normal-like (NL)], were correlated with the gene expression centroids for each subtype, using centroid parameters from the “genefu” Bioconductor package (<http://www.bioconductor.org/>). When multiple probes existed for a gene, the correlation between each probe and the corresponding PAM50 gene was included. Each sample was assigned a PAM50 subtype according to the best correlated subtype (Table S2). A total of 78 samples (Batch 1  $n = 51$ , Batch 2  $n = 27$ ) were excluded where the standard deviation of *ESR1* expression was  $\geq 1.5$  from the mean (Fig. S5; Table S2). No further exclusions based on *PGR* or *ERBB2* expression were made (Figs. S6, S7).

Batch effects in the final dataset of 593 samples (Batch 1  $n = 352$ , Batch 2  $n = 241$ ) were corrected by standardization of probes (subtracting the mean expression value and dividing by standard deviation) by study. There was no evidence for batch effects after standardization by 12-sample chip, 96-well plate, study, or batch (Fig. S8), and all samples were combined into a single dataset for subsequent analyses. Lehmann subtypes were determined via hierarchical clustering using Ward’s linkage as implemented in the “hclust” R package, using probes corresponding to 2119 out of the 2188 Lehmann centroid genes [6] for which normalized DASL data were available. When multiple probes were available for a gene, the most variable probe was chosen.

## Agnostic subtype derivation and functional annotation

Hierarchical clustering including the 2158 most variable probes, defined by skewness and interquartile range (Table S3; Fig. S9), was performed using Ward’s linkage as implemented in the “hclust” R package. Consensus clustering was used to determine the number of stable clusters (1000 iterations with average linkage). The optimal number of clusters, representing TN subtypes, was selected as the number at which no substantial increase in the area under the cumulative distribution function was observed. Functional annotation of TN subtypes was performed using gene set enrichment analysis (GSEA) as implemented in GSEA v2.1.0 software using default parameters [23, 24] and a normalized enrichment score (NES)  $\geq 1.5$ . The single probe with the largest interquartile range was used for each gene. Genes within each subtype were tested for enrichment of C2 curated canonical pathways compared to all remaining subtypes using 1000 permutations. A gene signature for these pathways was developed using significance analysis of microarrays (SAM) as implemented in the “samr” R package (<http://cran.r-project.org/>) with 100 permutations. Genes were evaluated for associations with each TN subtype relative to

all TN tumors combined. Among genes significant at a false discovery rate of 0.1 using the Wilcoxon rank sum test, those up- and down-regulated by >3-fold standard deviations from the mean for each feature were selected (Table S4).

### Histopathologic features: review and subtype derivation

H&E slides were available for 310 of the 593 TN tumors included in subtype analyses. Tumors were evaluated for 21 histopathological categories and for 11 histopathological features by a single pathologist (D. Visscher) (Tables S5, S6). Categories of certain tumor features were collapsed due to small numbers as follows: mitotic index (1/2; 3), grade (1/2; 3), trabecular pattern (present; absent), necrosis (present; absent), fibrosis (0/1; 2/3), ductal carcinoma in situ (DCIS present; absent), lobulitis (0/1; 2; 3). Lobular involution was evaluated as previously described [25, 26]. Genes were evaluated for association with individual histopathologic features using SAM with 100 permutations. Among genes significant at a false discovery rate of 0.1 by the Wilcoxon rank sum test, up-regulated genes with a fold change >1.5 and down-regulated genes with fold change <0.67 were selected for each feature (Table S7). A gene signature for phenotype-driven subtypes was identified by SAM. Among genes significant at 10 % FDR, those up- and down-regulated by >3-fold standard deviations from the mean for each feature were selected (Table S8). Lasso-based multinomial regression predicting TN subtype based on histopathologic features was performed with the “glmnet” package in R using cross validation. Per-gene linear regression models were performed using the “lm” function in R to evaluate the multivariate associations between age, histopathologic features, and each of 185 genes from the SAM-derived gene signature. Coefficients with  $p < 0.01$  were considered statistically significant.

### Germline mutation detection

The methods for detecting mutations in 17 breast cancer predisposition genes (*BRCA1*, *BRCA2*, *PALB2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, *RAD50*, *NBN*, *MRE11A*, *XRCC2*, *ATM*, *CHEK2*, *TP53*, *PTEN*, *STK11*, and *CDH1*) in germline DNA samples matched to these TN tumors has been described previously [27]. Briefly, germline DNA samples from TN patients were subjected to custom capture (eArray; Agilent, Santa Clara, CA, USA) for all coding sequences and intron/exon boundaries of coding exons for these genes. Products from each capture reaction were sequenced on a HiSeq 2000 (Illumina, San Diego, CA, USA). Pathogenic mutations were validated by Sanger

sequencing. Mutation screening data were available for 156 TN tumors in this study.

## Results

### Intrinsic subtype classification

Whole genome expression profiling was analyzed for 593 TN tumors from the TNBCC with clinical ER-, PR-, and HER2-negative status. Initially, each sample was assigned an intrinsic breast tumor subtype using published PAM50 gene expression centroids, where 76.7 % was identified as basal-like, 11.6 % as normal-like, 9.1 % as luminal, and 2.5 % as HER2-enriched (Table S2). These findings were consistent with PAM50 distributions among TN tumors from clinical trials and publically available microarray data [7, 28]. We also assigned Lehmann subtypes (denoted with prefix “L-”) using published Lehmann centroid genes in a hierarchical clustering analysis, where 13.2 % was identified as L-BL1, 36.3 % as L-BL2, 11.3 % as L-LAR, 23.8 % as L-IM, 10.1 % as L-M, and 5.4 % as L-MSL.

### Derivation of TN subtypes

We first performed unsupervised hierarchical clustering to identify molecular subtypes of TN breast cancer. Using 2158 highly variable probes, the data were clustered into four groups using the Engelman–Hartigan test (Table 1; Fig. 1a), which was confirmed by analysis of sample classification robustness using consensus clustering (Fig. 1b, c). GSEA revealed a basal-like signature (BL) (50.8 %), a luminal signature (LAR) with overexpression of androgen receptor (AR) and AR transcriptional targets (10.6 %), an immune signature (IM) (16.9 %), and a stromal signature (STR) (21.7 %) (Fig. 2). Tumors defined by PAM50 modeling as LumA, LumB, and HER2-enriched were assigned predominantly to the LAR subtype, while the PAM50 basal-like tumors were divided between the BL (61 %), IM (16 %), and STR (23 %) subtypes. NL tumors were evenly distributed across all four TN subtypes (Table 1). The LAR subtype was also associated with older age (mean = 62.1) compared to non-LAR subtypes (mean = 50.6) ( $p = 1.5 \times 10^{-8}$ ). Enriched signaling pathways among these four subtypes were similar to those seen for comparable groups within the Lehmann classification scheme (Table S9). This is further confirmed by the overlap between agnostic subtypes and Lehmann subtype assignments (Table 1), where roughly 80 % of the BL, LAR, and IM groups and 63 % of the STR group were assigned to analogous Lehmann subtypes. The most notable departure in agreement is the presence of L-IM tumors in the BL and STR groups. When exploring the

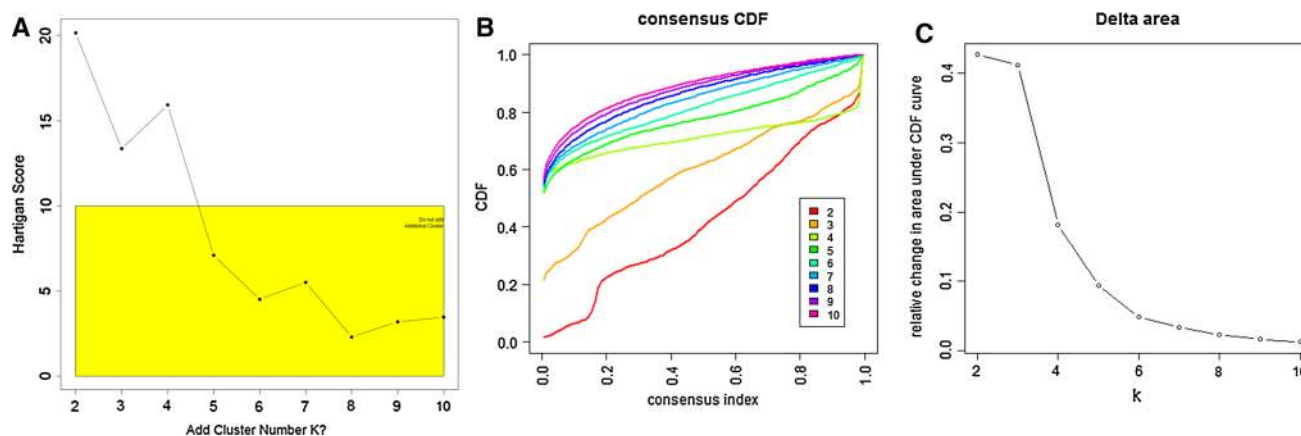
**Table 1** Distribution of intrinsic subtypes and histopathologic features among four agnostic subtypes

	BL <i>n</i> (%) <i>N</i> = 301	LAR <i>N</i> (%) <i>N</i> = 63	IM <i>N</i> (%) <i>N</i> = 100	STR <i>n</i> (%) <i>N</i> = 129	<i>p</i> -value four subtypes	<i>p</i> -value B versus NB <sup>a</sup>	<i>p</i> -value BL, IM, STR
<b>PAM50 subtype</b>							
Basal	278 (92)	0 (0)	74 (74)	103 (80)			
HER2-enriched	1 (0.5)	23 (37)	5 (5)	6 (5)			
Luminal A	0 (0)	15 (24)	2 (2)	2 (1)			
Luminal B	1 (0.5)	7 (11)	2 (2)	5 (4)			
Normal-like	21 (7)	18 (29)	17 (17)	13 (10)	$7.2 \times 10^{-61}$		
<b>Lehmann subtype</b>							
L-BL1	60 (20)	0 (0)	15 (15)	3 (2)			
L-BL2	186 (62)	3 (5)	3 (3)	23 (18)			
L-LAR	2 (0.7)	52 (83)	5 (5)	8 (6)			
L-IM	49 (16)	5 (8)	74 (74)	13 (10)			
L-M	3 (1)	1 (2)	3 (3)	53 (41)			
L-MSL	1 (0.3)	2 (3)	0 (0)	29 (22)	$2.5 \times 10^{-48}$		
	BL <i>n</i> (%) <i>N</i> = 161	LAR <i>N</i> (%) <i>N</i> = 37	IM <i>N</i> (%) <i>N</i> = 55	STR <i>n</i> (%) <i>N</i> = 58	<i>p</i> -value four subtypes	<i>p</i> -value B versus NB <sup>a</sup>	<i>p</i> -value BL, IM, STR
<b>Mitotic index</b>							
1 or 2	23 (14)	17 (46)	10 (18)	12 (21)			
3	138 (86)	20 (54)	45 (82)	46 (79)	$2.7 \times 10^{-4}$	$6.3 \times 10^{-5}$	0.49
<b>Grade</b>							
1 or 2	13 (8)	10 (27)	4 (7)	9 (16)			
3	148 (92)	27 (73)	51 (93)	49 (84)	$6.0 \times 10^{-3}$	$4.3 \times 10^{-3}$	0.21
<b>Trabecular pattern</b>							
Absent	88 (55)	31 (84)	38 (69)	39 (67)			
≥Present	73 (45)	6 (16)	17 (31)	19 (33)	$5.0 \times 10^{-3}$	$9.2 \times 10^{-3}$	0.079
<b>Growth pattern</b>							
Epicenter	107 (66)	13 (35)	33 (61)	38 (66)			
Infiltrative	45 (28)	18 (49)	19 (35)	18 (31)			
Multifocal	9 (6)	6 (16)	2 (4)	2 (3)	0.011	$4.9 \times 10^{-4}$	0.83
<b>Necrosis</b>							
Absent	52 (32)	25 (68)	36 (65)	29 (50)			
≥Present	109 (68)	12 (32)	19 (35)	29 (50)	$3.9 \times 10^{-6}$	$7.5 \times 10^{-3}$	$4.5 \times 10^{-5}$
<b>Fibrosis</b>							
0 or 1	88 (55)	12 (32)	37 (67)	36 (62)			
2 or 3	73 (45)	25 (68)	18 (33)	22 (38)	$7.0 \times 10^{-3}$	$4.4 \times 10^{-3}$	0.22
<b>In situ</b>							
Absent	125 (78)	17 (46)	45 (83)	50 (86)			
≥Present	36 (22)	20 (54)	9 (17)	8 (14)	$2.8 \times 10^{-5}$	$8.5 \times 10^{-6}$	0.31
<b>Lymphoid infiltrate</b>							
0	5 (2)	3 (8)	0 (0)	3 (5)			
1	77 (48)	24 (65)	6 (11)	19 (33)			
2	64 (40)	6 (16)	13 (24)	21 (36)			
3	15 (9)	4 (11)	35 (65)	15 (26)	$1.8 \times 10^{-15}$	$2.0 \times 10^{-3}$	$1.1 \times 10^{-13}$
<b>Lobulitis</b>							
0 or 1	56 (47)	14 (64)	19 (43)	13 (37)			
2	40 (34)	6 (27)	16 (36)	13 (37)			
3	22 (19)	2 (9)	9 (20)	9 (26)	0.59	0.20	0.84

**Table 1** continued

	BL <i>n</i> (%) <i>N</i> = 161	LAR <i>N</i> (%) <i>N</i> = 37	IM <i>N</i> (%) <i>N</i> = 55	STR <i>n</i> (%) <i>N</i> = 58	<i>p</i> -value four subtypes	<i>p</i> -value B versus NB <sup>a</sup>	<i>p</i> -value BL, IM, STR
Involution							
Incomplete	66 (52)	8 (0.31)	33 (75)	20 (54)			
Complete	60 (48)	18 (69)	11 (25)	17 (46)	$3.5 \times 10^{-3}$	0.018	0.030
Age [mean (SD)]	50.4 (14.8)	62.2 (13.7)	50.4 (13.2)	51.1 (14.9)	$1.2 \times 10^{-7}$	$1.5 \times 10^{-8}$	0.90

<sup>a</sup> Basal versus non-basal



**Fig. 1** Clustering robustness using 2158 agnostic probes. **a** A plot of Hartigan score values from the Engelman–Hartigan test for two through ten clusters. The *yellow-highlighted area* indicates the threshold below which additional clusters should not be added.

possibility of six TN subtypes (Table S10), no distinguishing pathways or ontologies were observed between two candidate BL subtypes or two candidate STR subtypes (Table S11). Thus, while the four subtypes are similar to those described previously [6, 7], we were unable to duplicate the presence of six subtypes of TN breast cancer in this unsupervised analysis.

### Histopathologic features among TN subtypes

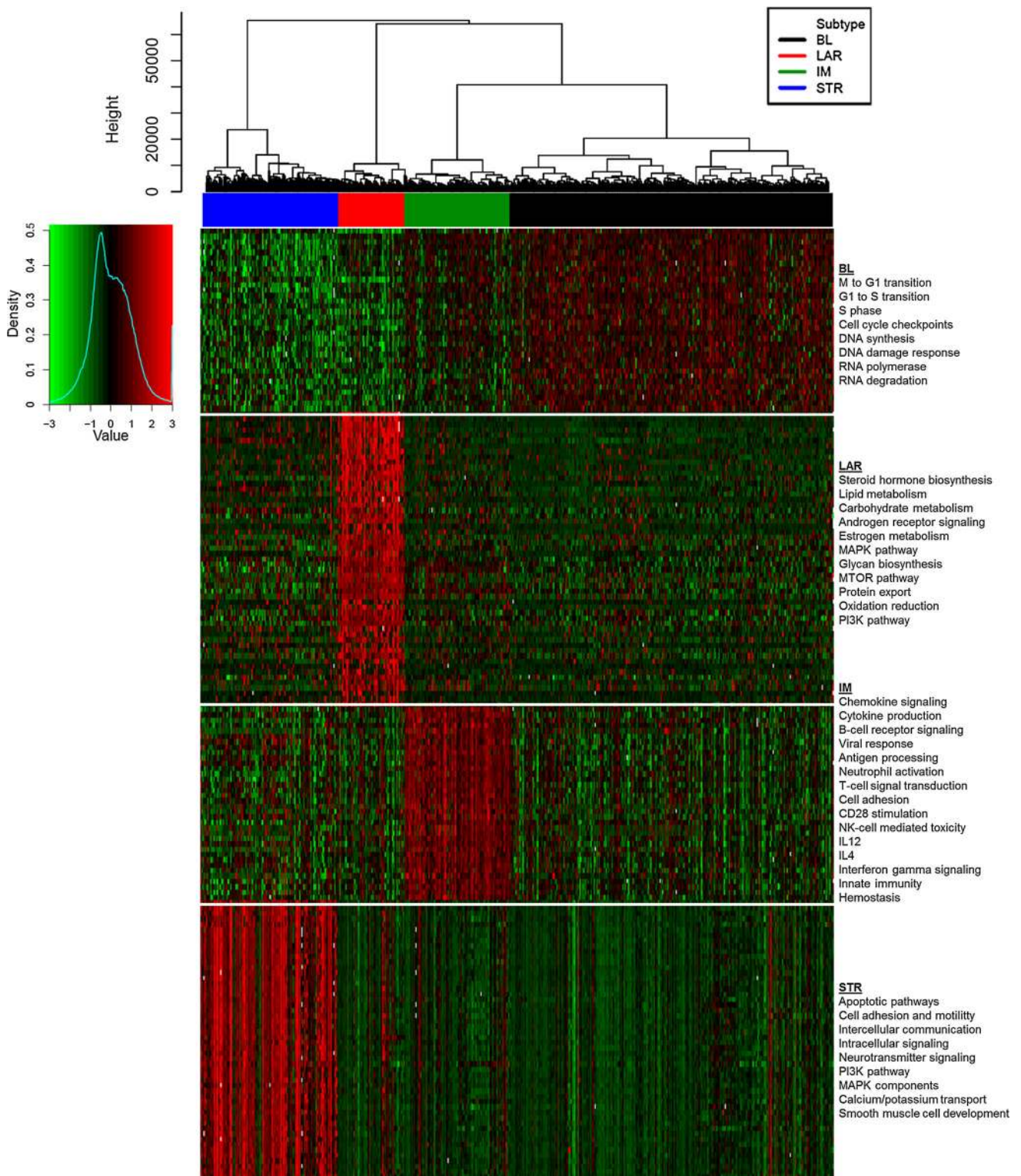
TN tumors have several distinctive histopathologic characteristics that capture differences in tumor cell behavior and the tumor microenvironment. We evaluated 310 TN tumors for 11 histopathological features (Table S5). The TN tumors were most commonly classified as undifferentiated adenocarcinomas (37 %), but several rare tumors including adenoid cystic carcinomas, apocrine adenocarcinomas, metaplastic carcinomas, and medullary carcinomas were also observed (Table S6), with LAR enrichment of apocrine adenocarcinomas and IM enrichment of medullary carcinomas. Significant differences in the distribution of all histopathological features except lobulitis by TN subtype

were observed (Table 1). These associations were driven largely by differences between the LAR subtype and the BL, IM, and STR subtypes (Table 1). When restricting to the BL, IM, and STR subtypes, only necrosis ( $p = 4.5 \times 10^{-5}$ ), lymphoid infiltrate ( $p = 1.1 \times 10^{-13}$ ), and involution ( $p = 0.03$ ) were significantly associated with subtype (Table 1). The degree to which histopathologic features were correlated was variable (Table S12). As expected grade and mitotic index showed the highest correlation ( $r = 0.73$ ). Age was also positively correlated with involution, where breast cancer cases with complete involution were on average 10 years older than those with incomplete involution (complete mean = 60.5 years; incomplete mean = 48.0 years).

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### Molecular TN subtypes based on histopathology

Given that we were unable to recapitulate the Lehmann subtypes in an unsupervised analysis, we hypothesized that genes related to tumor cell behavior and tumor microenvironment are integral to the identification of TN molecular subtypes. Thus, we next utilized genes associated with 10



**Fig. 2** Agnostic TN subtypes. A heatmap of the 177 genes that were significantly different across four agnostic TN subtypes. *Red* indicates up-regulated genes; *green* represents down-regulated genes compared to the mean. Each of the four subtypes is clustered as shown by the

dendrogram and samples are color coded by subtype: *black* BL, *red* LAR, *green* IM, *blue* STR. Enriched ontologies by GSEA corresponding to each of the four subtypes are listed next to the genes that were significantly up-regulated in clusters



histopathologic features, excluding tumor cell type, in a hypothesis-driven clustering analysis of TN tumors. Within the 310 samples for which histopathologic data were available, we identified 2776 unique probes significantly associated with each of the ten features (Table S7). Hierarchical clustering for all 593 TN tumors using these 2776 probes identified six groups (Table 2; Fig. 3a). This was confirmed by analysis of sample classification robustness using consensus clustering (Fig. 3b, c). The six groups are hereafter referred using the prefix “P-” to distinguish them from the four original TN clusters and the Lehmann subtype assignments.

GSEA showed strong similarities between these six phenotype-driven clusters and the Lehmann subtypes (Table S13; Fig. 4). There was substantial although imperfect agreement between phenotype-driven clusters and Lehmann subtype assignments for all groups except for the P-BL1 subtype ( $\kappa = 0.75$ , 95 % confidence interval 0.70–0.80), where tumors were assigned as L-BL1 (38 %), L-BL2 (38 %), and L-IM (23 %) using Lehmann centroid genes. Breast cancer cases in the P-LAR subtype were older (mean = 62.7) than those in all other subtypes (mean = 50.6) ( $p = 2.1 \times 10^{-7}$ ). Compared to the previous agnostic analysis resulting in only four subtypes, we identified a second BL and a second STR group. The P-BL2 subtype is distinguished from the P-BL1 subtype by the overexpression of cell motility/adhesion and glycolysis/gluconeogenesis pathways, and by the relative lack of expression of inflammation, antigen presentation, and adaptive immunity genes (Fig. 4). The P-STR1 subtype is distinguishable from the P-STR2 subtype largely by the overexpression of a wide range of immunomodulatory pathways, while the P-STR2 subtype is enriched for MAPK components, PI3K, NF $\kappa$ B, and smooth muscle cell development pathways (Fig. 4). Furthermore, overexpression of the CD3, interferon gamma (IFN $\gamma$ ), and IL10Th1 and Th2 cell markers that regulate transcription of innate and adaptive immune response genes including the PD-L1 marker associated with increased T-cell cytotoxic immune response in basal breast cancers [29], was observed among the P-IM, P-BL1, and P-STR1 subtypes (Fig. S10). Finally, AR and AR targets were substantially overexpressed among the P-LAR group of TN tumors ( $n = 58$ , 9.8 %) (Fig. S11). We utilized SAM to identify the probes ( $n = 185$ ) most strongly associated with these six subtypes (Table S8).

### Histopathologic features among pathology-based TN subtypes

The distribution of histopathologic features across the six phenotype-driven subtypes was evaluated to better understand how individual features were associated with each subtype (Table 2). As before, all features were significantly associated

with TN subtypes except for lobulitis (Tables 2, S14). Apocrine adenocarcinomas and medullary carcinomas were again more common among the P-LAR ( $p = 6.9 \times 10^{-6}$ ) and P-IM ( $p = 2.9 \times 10^{-5}$ ) subtypes. In addition, undifferentiated carcinomas were overrepresented among the P-BL1 subtype and underrepresented among the P-LAR subtype ( $p = 8.0 \times 10^{-9}$ ). Mitotic index, grade, growth pattern, necrosis, fibrosis, and lymphoid infiltrate were all significantly associated with non-LAR TN subtypes (Tables 2, S15).

We used a lasso-based multinomial regression model to evaluate the combined effects of histopathologic features and age that best predicted each of the six subtypes (Fig. S12; Table S16). Each subtype had a unique pattern of features that characterized membership. Lymphoid infiltrate was the only variable that was predictive of each subtype, where it was a positive predictor of P-BL1, P-IM, and P-STR1 subtypes and a negative predictor of P-BL2, P-LAR, and P-STR2 subtypes. The presence of necrosis and higher mitotic index were positive predictors of both P-BL subtypes, but these two groups were distinguished by the absence of fibrosis among P-BL1 tumors, the presence of trabecular pattern in P-BL2 tumors, and inverse relationships with lymphoid infiltrate. The two P-STR groups shared no common predictors, where P-STR1 was only predicted by lymphoid infiltrate and absence of DCIS; in contrast, P-STR2 was negatively predicted by grade, mitotic index, and lymphoid infiltrate and positively predicted by fibrosis. Lymphoid infiltrate was the strongest predictor of the P-IM subtype, along with lower rates of necrosis, DCIS, and involution. The P-LAR subtype was distinct in that it was associated with lower grade, older age, and higher rates of DCIS and complete involution.

We then evaluated the multivariate relationships between each gene accounting for histopathologic features and age to identify the features driving the 185-gene signature (Table S17). The majority of genes ( $n = 127$ ) were significantly associated with a single feature in the multivariate models, with no more than three features predicting any single gene ( $p < 0.01$ ). Lymphoid infiltrate and necrosis were the strongest drivers of this 185-gene signature, where lymphoid infiltrate was associated with 35 % of genes and necrosis was associated with 38 % of genes. Age, DCIS, fibrosis, and mitotic index were associated with 12–15 % of genes, while lobulitis, trabecular pattern, and grade had negligible impact on the signature. These patterns further suggest that TN tumor heterogeneity is best described by the integration of histopathologic features and expression patterns of key pathways.

### Cancer predisposition genes

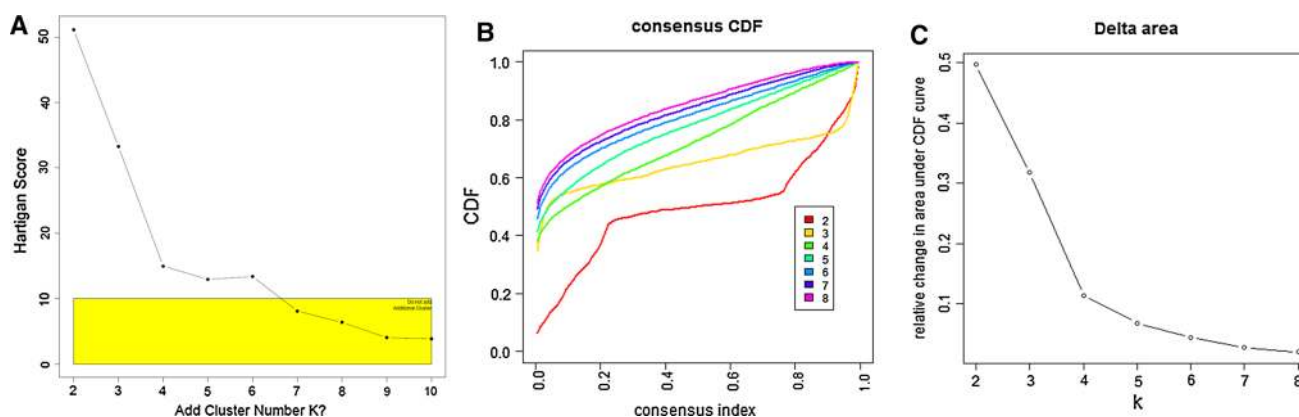
To further characterize these six subtypes in terms of potentially targetable pathways, germline mutations in

**Table 2** Distribution of intrinsic subtypes and histopathologic features among six phenotype-driven subtypes

	P-BL1 <i>n</i> (%) <i>N</i> = 161	P-BL2 <i>n</i> (%) <i>N</i> = 158	P-LAR <i>n</i> (%) <i>N</i> = 58	P-IM <i>n</i> (%) <i>N</i> = 129	P-STR1 <i>n</i> (%) <i>N</i> = 50	P-STR2 <i>n</i> (%) <i>N</i> = 37	<i>p</i> -value
<b>Agnostic subtype</b>							
BL	135 (84)	129 (82)	7 (12)	17 (13)	8 (16)	5 (13)	$3.2 \times 10^{-147}$
LAR	0 (0)	2 (1)	43 (74)	14 (11)	0 (0)	4 (11)	
IM	17 (11)	0 (0)	4 (7)	77 (60)	2 (4)	0 (0)	
STR	9 (5)	27 (17)	4 (7)	21 (16)	40 (80)	28 (76)	
<b>PAM50 subtype</b>							
Basal	155 (96)	146 (92)	4 (7)	84 (65)	47 (94)	19 (51)	$7.0 \times 10^{-47}$
HER2-enriched	3 (2)	1 (1)	16 (28)	9 (7)	2 (4)	4 (11)	
Luminal A	0 (0)	0 (0)	11 (19)	4 (3)	0 (0)	4 (11)	
Luminal B	1 (1)	4 (3)	6 (10)	2 (1)	1 (2)	1 (3)	
Normal-like	2 (1)	7 (4)	21 (36)	30 (23)	0 (0)	9 (24)	
<b>Lehmann subtype</b>							
L-BL1	61 (38)	6 (4)	0 (0)	5 (4)	6 (12)	0 (0)	$5.6 \times 10^{-98}$
L-BL2	61 (38)	133 (84)	7 (12)	4 (3)	2 (4)	8 (22)	
L-LAR	0 (0)	1 (1)	48 (83)	13 (10)	0 (0)	5 (14)	
L-IM	37 (23)	4 (3)	3 (5)	96 (74)	1 (2)	0 (0)	
L-M	1 (1)	14 (9)	0 (0)	8 (6)	37 (74)	0 (0)	
L-MSL	1 (1)	0 (0)	0 (0)	3 (2)	4 (8)	24 (65)	
	P-BL1 <i>n</i> (%) <i>N</i> = 86	P-BL2 <i>n</i> (%) <i>N</i> = 85	P-LAR <i>n</i> (%) <i>N</i> = 37	P-IM <i>n</i> (%) <i>N</i> = 68	P-STR1 <i>n</i> (%) <i>N</i> = 21	P-STR2 <i>n</i> (%) <i>N</i> = 14	<i>p</i> -value
<b>Mitotic index</b>							
1 or 2	8 (9)	10 (12)	18 (49)	14 (21)	1 (5)	11 (79)	$4.8 \times 10^{-12}$
3	78 (91)	75 (88)	19 (51)	54 (79)	20 (95)	3 (21)	
<b>Grade</b>							
1 or 2	4 (5)	4 (5)	12 (32)	7 (10)	1 (5)	8 (57)	$3.2 \times 10^{-10}$
3	82 (95)	81 (95)	25 (68)	61 (90)	20 (95)	6 (43)	
<b>Trabecular pattern</b>							
Absent	45 (52)	46 (54)	33 (89)	49 (72)	11 (52)	12 (86)	$1.8 \times 10^{-4}$
≥Present	41 (48)	39 (46)	4 (11)	19 (28)	10 (48)	2 (14)	
<b>Growth pattern</b>							
Epicenter	62 (72)	58 (68)	11 (30)	42 (63)	16 (76)	2 (14)	$7.1 \times 10^{-6}$
Infiltrative	18 (21)	25 (29)	23 (62)	20 (30)	5 (24)	9 (64)	
Multifocal	6 (7)	2 (2)	3 (8)	5 (7)	0 (0)	3 (21)	
<b>Necrosis</b>							
Absent	25 (29)	22 (26)	26 (70)	47 (69)	11 (52)	11 (79)	$2.6 \times 10^{-10}$
≥Present	61 (71)	63 (74)	11 (30)	21 (31)	10 (48)	3 (21)	
<b>Fibrosis</b>							
0 or 1	60 (70)	46 (54)	8 (22)	41 (60)	16 (76)	2 (14)	$3.3 \times 10^{-7}$
2 or 3	26 (30)	39 (46)	29 (78)	27 (40)	5 (24)	12 (86)	
<b>In situ</b>							
Absent	73 (85)	64 (75)	16 (43)	53 (79)	21 (100)	10 (71)	$3.7 \times 10^{-6}$
≥Present	13 (15)	21 (25)	21 (57)	14 (21)	0 (0)	4 (29)	
<b>Lymphoid infiltrate</b>							
0	0 (0)	4 (5)	4 (11)	0 (0)	0 (0)	3 (21)	
1	20 (23)	59 (69)	28 (76)	10 (15)	0 (0)	9 (64)	
2	49 (57)	21 (25)	2 (5)	23 (34)	7 (33)	2 (14)	

**Table 2** continued

	P-BL1 <i>n</i> (%) <i>N</i> = 86	P-BL2 <i>n</i> (%) <i>N</i> = 85	P-LAR <i>n</i> (%) <i>N</i> = 37	P-IM <i>n</i> (%) <i>N</i> = 68	P-STR1 <i>n</i> (%) <i>N</i> = 21	P-STR2 <i>n</i> (%) <i>N</i> = 14	<i>p</i> -value
3	17 (20)	1 (1)	3 (8)	34 (51)	14 (67)	0 (0)	$4.0 \times 10^{-29}$
Lobulitis							
0 or 1	27 (40)	30 (55)	15 (65)	19 (39)	7 (44)	4 (5)	
2	26 (38)	19 (35)	4 (17)	19 (39)	3 (19)	4 (5)	
3	15 (22)	6 (11)	4 (17)	11 (22)	6 (38)	0 (0)	0.13
Involution							
Incomplete	36 (51)	36 (59)	7 (27)	36 (71)	10 (63)	2 (22)	
Complete	34 (49)	25 (41)	19 (73)	15 (29)	6 (38)	7 (78)	$2.6 \times 10^{-3}$
Age [mean (SD)]	49.8 (14.4)	49.3 (14.1)	62.7 (15.1)	52.4 (14.0)	49.7 (14.5)	55 (14.8)	$4.2 \times 10^{-8}$



**Fig. 3** Clustering robustness using 2776 pathology-based probes. **a** A plot of Hartigan score values from the Engelman–Hartigan test for two through ten clusters. The yellow-highlighted area indicates the threshold below which additional clusters should not be added. **b** The

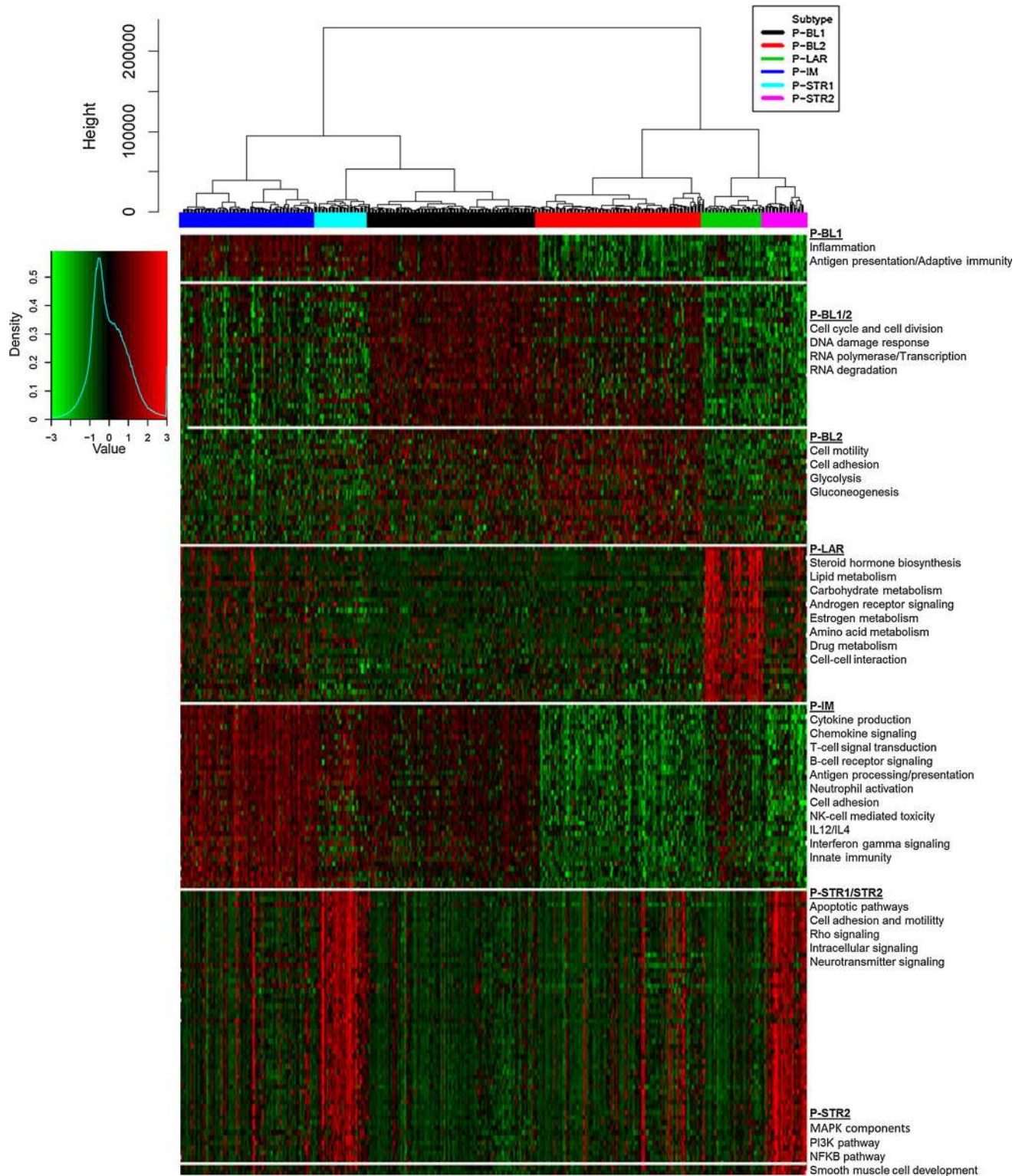
consensus cumulative distribution functions (CDF) for each solution from two to ten clusters. **c** The delta area, or the relative change in the area under the consensus CDF curve for each solution from two to ten clusters

breast cancer susceptibility genes were evaluated by TN subtype [27]. The distribution of pathology-based TN subtypes in the 156 TN tumors with mutation screening data was comparable to the overall group of 593 tumors (Table S18). A total of 21 (13.5 %) pathogenic mutations were identified in six DNA repair genes: *BRCA1*, *BRCA2*, *BARD1*, *RAD50*, *BRIP1*, and *PALB2* (Table S18), which is consistent with the 14.6 % of TN cases found to carry germline mutations in other larger studies [27, 30]. The mean age at diagnosis for women with germline mutations (mean = 40.3 years) was significantly lower than for women without germline mutations (mean = 48.1 years) ( $p = 0.0013$ ). Consistent with the observation that DNA repair pathways are integral to the P-BL1 and P-BL2 subtypes, these two subtypes had the highest proportion of individuals with germline mutations (17.8–22.5 %). Interestingly, although the number of P-STR2 tumors screened was small ( $n = 9$ ), a relatively large proportion of these tumors contained mutations in *BRCA1* and *PALB2*

(22.2 %). In contrast, only 5.9 % of the P-IM subtype had mutations in *BRCA1* and *BRCA2*. No mutations were identified in either the P-LAR or the P-STR1 subtypes. In combination with the histopathologic features described above, these patterns of germline mutations in DNA repair genes provide further evidence for the heterogeneity of TN tumors.

## Discussion

In this analysis of 593 TN tumors, we identified six distinct TN molecular subtypes by expression profiling and showed that these subtypes are characterized by genes related to histopathologic features that reflect heterogeneity in tumor architecture, cell structure and behavior, and tumor microenvironment. While our six phenotype-driven subtypes were largely similar to those described by Lehmann, et al. when comparing subtype-specific pathway enrichment,



**Fig. 4** Phenotype-driven TN subtypes. A heatmap of the 185 genes that were significantly different across the six phenotype-driven TN subtypes. *Red* indicates up-regulated genes; *green* represents down-regulated genes compared to the mean. Each of the six subtypes is clustered as shown by the dendrogram and samples are color coded by

subtype: *black* P-BL1, *red* P-BL2, *green* P-LAR, *blue* P-IM, *cyan* P-STR1, *purple* P-STR2. Enriched ontologies by GSEA corresponding to each of the six subtypes are listed next to the group of genes that were significantly up-regulated in clusters

there were differences between the two classification schemes. In particular, differences between the two BL subtypes and two STR subtypes in the current study were dependent on the integration of signaling pathways associated with pathology features. Specifically, the up-regulation of immune pathways was a distinguishing feature of both the P-BL1 and P-STR1 subtypes, consistent with lymphocytic infiltration histopathological patterns across the six TN subtypes. However, mitotic index, grade, growth pattern, necrosis, fibrosis were also driving factors in the differences in histopathological patterns between these subtypes.

Basal-like breast cancers are known to have higher grade and mitotic index and have greater presence of necrosis, tumor infiltrating lymphocytes, and incomplete involution compared to non-basal breast cancer subtypes [16]. We validate this in the current study by showing that these differences exist between the P-LAR and basal-like subtypes. In addition, we show that these features are heterogeneous among basal-like subtypes. In particular, mitotic index and grade were low among the P-IM and P-STR2 subtypes, necrosis was substantially lower among P-IM and P-STR tumors, complete involution was highest among the P-STR2 subtype, and lymphoid infiltrate was highly variable across all basal-like subtypes. We further show for the first time that unique combinations of these features can be used to predict TN molecular subtype.

In a recent study of germline cancer predisposition gene mutations in TN cases, mutations in genes involved in homologous recombination (HR) accounted for 81 % of all non-*BRCA1/2* mutations identified [27], indicating that the perturbation of HRDNA repair may be particularly important for TN breast carcinogenesis. In light of this, it is not surprising that the 21 mutations identified in the 156 women included in the current study were all found in six HR genes (*BRCA1*, *BRCA2*, *BRIP1*, *BARD1*, *PALB2*, *RAD50*) [31]. While it is known that DNA repair mutations are associated with the basal breast cancer molecular subtype [32], our data suggest that this effect may be limited to the development of the P-BL1, P-BL2, and possibly P-STR2 subtypes of TN breast cancer through disruption of the HR pathway.

A better understanding of the immune cells associated with each of these subtypes is particularly important considering the prognostic implications of lymphocytic infiltration in highly proliferative tumors such as TN breast cancers. The presence of T regulatory cells and tumor-associated macrophages has been correlated with negative effect on survival in pancreatic, liver, colorectal, and breast cancers [33–38]. In contrast, better overall and disease-free survival has been associated with increased infiltration of CD8+ cytotoxic T cells in colorectal, ovarian, hepatocellular, and breast cancers [39–42]. Cytokines associated with T helper cells, Th1 (IFN- $\gamma$ /CD3+) and Th2 (IL10/

CD3+) cells have also been used to show that the relative levels of Th1/Th2 cells have prognostic implications for ovarian, non-small cell lung, and breast cancer [43–45]. Further studies that explore the extent to which these individual cell types, and the immune processes they represent, influence specific TN subtypes are necessary.

Another important implication of the identification of specific pathways up-regulated in TN subtypes is the possibility for the development of targeted therapies. The PD1/PD-L1 pathway is a key inhibitor of the immune system that favors tumor progression [46–48]. PD-L1 expression is up-regulated specifically in basal breast tumors, and high expression has been associated with better metastasis-free and breast cancer-specific survival in basal breast tumors [29]. Clinical trials of melanoma, renal, lung, prostate, and bladder cancers investigating the use of anti-PD1 and anti-PD-L1 agents suggest that blocking the PD1/PD-L1 pathway may restore anti-cancer immunity [49–51] in a number of tumors. The knowledge that PD1 and PD-L1 are up-regulated in specific subtypes of TN breast cancer allows for more focused and hence better-designed clinical trials of anti-PD1/PD-L1 therapy in breast cancer and TN breast cancer. Beyond PD1/PD-L1, these data may identify additional targetable pathways that may lead to the development of new therapies specific to TN breast cancer subtypes.

In conclusion, we have shown that TN tumors are highly heterogeneous, as determined by both gene expression and pathology. The integration of these data with other genomic and epidemiologic data is integral to a better understanding of these aggressive tumors. The utilization of tools such as targeted sequencing and DNA copy number variation in combination with gene expression and pathology will help to further define TN subtypes and to identify potentially targetable biomarkers. It is also of particular importance to characterize the implications of these TN subtypes for response to treatment, disease progression, and survival. Further studies are necessary to explore the full extent of heterogeneity in TN tumors and the associated clinical implications.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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