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A clinically relevant gene signature in triple negative and basal-like breast cancer

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Abstract

Introduction: Current prognostic gene expression profiles for breast cancer mainly reflect proliferation status and are most useful in ER-positive cancers. Triple negative breast cancers (TNBC) are clinically heterogeneous and prognostic markers and biology-based therapies are needed to better treat this disease.

Methods: We assembled Affymetrix gene expression data for 579 TNBC and performed unsupervised analysis to define metagenes that distinguish molecular subsets within TNBC. We used $n = 394$ cases for discovery and $n = 185$ cases for validation. Sixteen metagenes emerged that identified basal-like, apocrine and claudin-low molecular subtypes, or reflected various non-neoplastic cell populations, including immune cells, blood, adipocytes, stroma, angiogenesis and inflammation within the cancer. The expressions of these metagenes were correlated with survival and multivariate analysis was performed, including routine clinical and pathological variables.

Results: Seventy-three percent of TNBC displayed basal-like molecular subtype that correlated with high histological grade and younger age. Survival of basal-like TNBC was not different from non basal-like TNBC. High expression of immune cell metagenes was associated with good and high expression of inflammation and angiogenesis-related metagenes were associated with poor prognosis. A ratio of high B-cell and low IL-8 metagenes identified 32% of TNBC with good prognosis (hazard ratio (HR) 0.37, 95% CI 0.22 to 0.61; $P < 0.001$) and was the only significant predictor in multivariate analysis including routine clinicopathological variables.

Conclusions: We describe a ratio of high B-cell presence and low IL-8 activity as a powerful new prognostic marker for TNBC. Inhibition of the IL-8 pathway also represents an attractive novel therapeutic target for this disease.

Introduction

Different molecular subtypes of breast cancer have been described [1]. The most profound effects on gene expression profiles in breast cancer are related to estrogen (ER), and proliferation status, and to a lesser extent to Human Epidermal Growth Factor Receptor 2 (HER2) status. Not surprisingly, molecular classification and current prognostic signatures mainly reflect these molecular features [2]. However, substantial clinical and molecular heterogeneity remains within current molecular subsets, particularly among ER, progesterone (PgR) and HER2

receptor negative (that is, triple negative breast cancers, TNBC [3]). Furthermore the relationship between clinically defined TNBC and the gene expression profile-based basal-like breast cancer subtype (BLBC) [4] is not fully defined [5]. Some authors use these two terms synonymously given the substantial overlap between the two definitions [6,7]. However, immunohistochemical and molecular profiling studies have shown that only a subset of TNBC express the combination of basal cell markers (for example, CK5 and CK14) that is required for the molecular definition of this disease [5]. The prognostic significance and therapeutic implications of molecular heterogeneity within TNBC remains to be established. From a clinical point of view, further

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understanding of TNBC is important because better prognostic markers and new treatments are needed [8].

The goal of this analysis was to assemble all currently available TNBC gene expression datasets generated on Affymetrix gene chips and search for molecular structures in the data to define gene expression-based subsets within TNBC. We defined metagenes as the average expression of groups of highly co-expressed genes in the data without considering any clinical outcome variable. These metagenes identified several molecular subsets within TNBC, some with good prognosis even in the absence of systemic therapy. Our results also suggest possible new therapeutic strategies for TNBC. This study represents the largest attempt to define clinically important molecular subsets within TNBC [9].

Materials and methods

All analyses were performed according to the REporting recommendations for tumour MARKer prognostic studies (REMARK) recommendations for prognostic and tumor marker studies [10,11] and the respective guidelines to microarray-based studies for clinical outcomes [12]. A respective diagram of the complete analytical strategy and the flow of patients through the study, including the number of patients included in each stage of the analysis, is given in Additional file 1, Supplementary Figure S1. Tissue samples of invasive breast cancer cases (dataset Frankfurt) were obtained with IRB approval and informed consent from consecutive patients undergoing surgical resection between December 1996 and July 2007 at the Department of Gynecology and Obstetrics at the Goethe-University in Frankfurt. Gene expression data have been deposited into the GEO database (accession number GSE31519).

Assembly of TNBC microarray data and definition of metagenes

In order to facilitate pooling of data sets from different laboratories we only used data from a single platform (Affymetrix U133A and U133 Plus 2.0 chips) and included only samples that were defined as triple negative based on the mRNA expression of ER, PgR, and HER2 as previously described [13-15]. To obtain a large enough sample size for discovery it was necessary to pool several datasets. A major concern during this exercise is the possible confounding effect of systematic technical differences that exist between individual datasets. These could lead to false discovery during metagene definition and could also weaken the power of validation. We applied two different strategies to minimize this problem. First, we selected only highly comparable datasets for discovery. We initially identified 579 TNBC from a total of 3,488 publicly available primary breast cancer gene expression profiles representing 28

individual datasets (Additional file 2, Supplementary Table S1). We excluded 13 datasets contributing 185 TNBC cases from the discovery cohort because they did not fulfill our criteria of comparability of the microarray data (for details see Additional file 4, Supplementary Methods Section 1 and Additional file 1, Supplementary Figure S2). The final discovery cohort to identify metagenes included 394 TNBC from 15 datasets (cohort-A). The 185 samples excluded from discovery were retained as a validation set (cohort-B) to assess correlations between various metagenes and between metagenes and clinical outcome (Additional file 1, Supplementary Figure S1). This strategy maximized the integrity of metagene discovery at the cost of possibly reducing the power of the validation study. The two cohorts did not significantly differ with respect to age, tumor size and histological grade. However, the validation cohort-B contained a larger number of lymph node positive patients and a higher proportion of fine needle aspiration (FNA) samples. Follow-up data were available for 2,348 of the total 3,488 samples and 327 of the 579 TNBC samples. Since the number of patients with follow-up in validation cohort B was too small ($n = 30$ of 185) an additional independent validation cohort-C [16] ($n = 76$) was included to assess the prognostic value of the metagenes (Additional file 1, Supplementary Figure S1). The patient characteristics of the discovery and validation cohorts are given in Table 1. For analysis of normal tissue a dataset from a benign breast was used (Additional file 2, Supplementary Table S1).

Unsupervised analysis, without input of clinical variables, was performed to identify metagenes that were defined as the arithmetical average expression of highly correlated genes. Gene clusters were selected with either a minimal membership of 10 genes and a minimal correlation threshold of 0.7, or a minimum of 25 genes and a correlation of 0.6, respectively (for details see Additional file 4, Supplementary Methods Section 2). We also employed a screen to remove genes that showed data-set bias. The dependence of the expression levels of the metagene probesets on the dataset vector was analyzed using the Kruskal-Wallis statistic (Additional file 4, Supplementary Methods Section 3). Only *Stroma* and *Hemoglobin* metagenes displayed a bias for FNA samples that reflect frequent contamination of these types of samples with blood and the lack of stromal elements compared to core needle or surgical biopsies (Additional file 1, Supplementary Figure S3 and Additional file 4, Supplementary Methods). Therefore, these two metagenes were analyzed only in surgical biopsies.

No systematic bias was observed between the U133A and U133 Plus2.0 arrays, which differ only in the spatial feature size of the probesets (for details see Additional file 4, Supplementary Methods Section 4). Both

Table 1 Clinical data of TNBC patients from the finding-cohort-A and the validation cohorts-B and -C

Parameter	Status	Finding cohort-A (n = 394)	Validation cohort-B (n = 185)	P-value (Chi ²) B vs A	Validation cohort-C (n = 76)	P-value (Chi ²) C vs A
Lymph node status	LNN	240	36		44	
	Node pos.	68	60	< 0.001	32	0.001
	<i>n.a.</i>	86	89		0	
Age	≤ 40 yr	63	25		10	
	41 to 50 yr	91	41		17	
	51 to 60 yr	76	39		13	
	> 60 yr	79	35	0.87	36	0.003
	<i>n.a.</i>	85	45		0	
Tumor size	≤ 2 cm	85	29		11	
	> 2 cm	224	122	0.068	62	0.035
	<i>n.a.</i>	85	34		3	
Histological grade	grade 3	227	110		62	
	grade 1 and 2	82	46	0.57	14	0.18
	<i>n.a.</i>	85	29		0	
Biopsy method	surgical	346	130		76	
	core	19	22		0	
	FNA	29	33	< 0.001	0	0.009
Five-year DFS	no event	202	24		49	
	event	95	6	0.25	26	0.69
	<i>n.a.</i>	97	155		1	

metagene distributions and “Centroid methods” were used to classify subtypes of TNBC as given in Additional file 4, Supplementary Methods Sections 8 and 9).

Survival analysis

Relapse free survival (RFS) was preferentially used as a clinical endpoint for event free survival (EFS). Only if RFS was not available in some datasets was it replaced by distant metastasis free survival (DMFS). Details on used endpoints, Kaplan-Meier and Cox regression analysis are given in Additional file 4, Supplementary Methods Section 5. Optimized cutoffs for dichotomizing of metagene scores to plot survival curves were derived from the discovery cohort and were applied without modification to the validation cohorts (Additional file 4, Supplementary Methods Section 6). All *P*-values are two-sided and 0.05 was considered as a significant result. Analyses were performed using the R software [17] and SPSS version 17.0 (SPSS Inc. Chicago, IL).

Results

Identification of subsets of TNBC based on metagene expression profile

In our discovery cohort we identified 16 clusters of correlated genes by unsupervised methods whose expression

values were averaged as metagenes (Figure 1). As expected, no cluster of genes correlated with ER, PgR, and HER2 status [4] were identified. In contrast the identified metagenes presented in Table 2 included the basal-like phenotype [4], an apocrine/androgen receptor signaling signature [18,19], five signatures related to different types of immune cells [4,20-25], a stromal signature [26,27], the claudin-CD24 signature [28,29], markers of blood [30] and adipocytes [4], as well as an inflammatory signature [31-33] and an angiogenesis signature [23,34]. These phenotypes corresponded to previously described gene signatures that have also been used to define subsets of TNBC in a recent smaller study [9]. The angiogenesis signature (VEGF metagene) has been described very recently as a “hypoxia signature” associated with poor outcome and expressed in distant metastases [34]. As shown in Figure 1, we observed the highest correlation between different types of immune cell metagenes. Similar relationships between the metagenes were detected in the validation cohort-B (Figure 1) and -C (Additional file 1, Supplementary Figure S4). The presence of B-lymphocytes in the tumor is the primary source of the expression of the B-Cell metagene that is largely composed of immunoglobulin genes [20,22]. In contrast, immunohistochemical analyses of IL-8 expression and analysis of

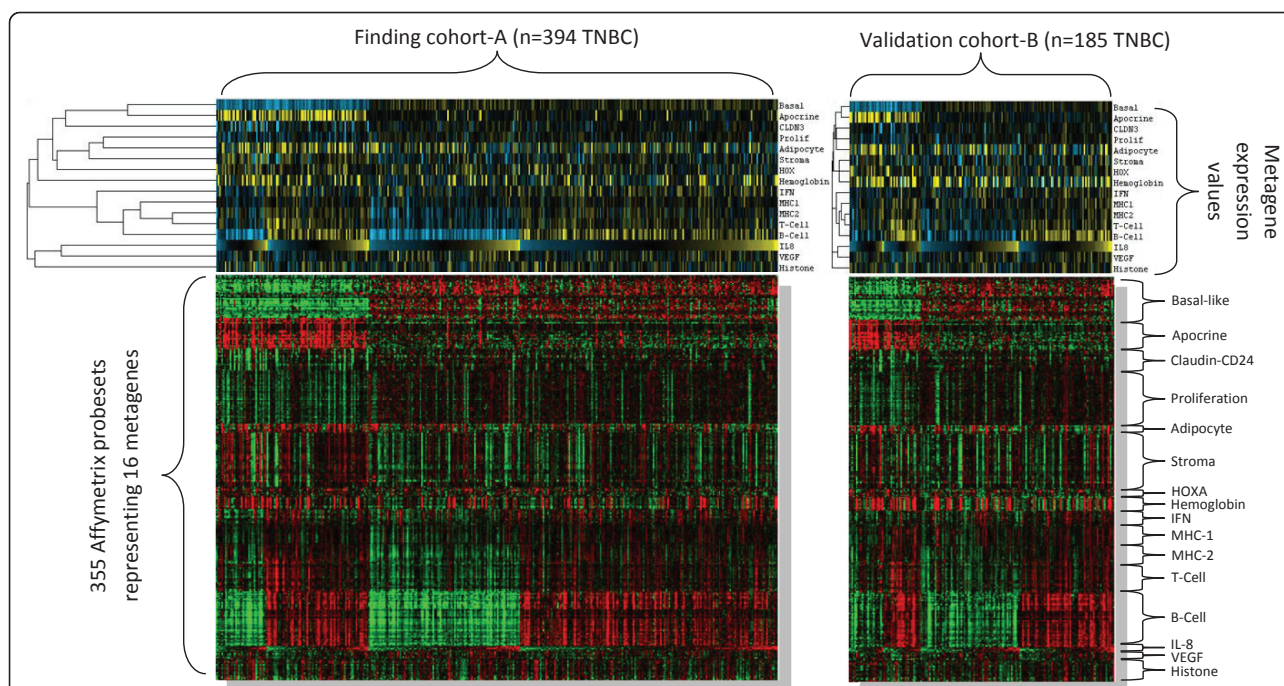


Figure 1 Principal biological phenotypes identified as metagenes among TNBC. Heatmaps of expression values of the 16 metagenes (upper panels) and the 355 individual Affymetrix probe sets (lower panels) are shown for the finding cohort (left panels, $n = 394$) and validation cohort (right panels, $n = 185$). The dendrogram at the left presents the results from hierarchical clustering of the metagenes. Three major clusters were observed representing (i) *basal-like*, *apocrine*, *CLDN-CD24*, *proliferation*, and *adipocyte* metagenes (ii) all five immune cell metagenes, and (iii) the *IL-8* and *VEGF* metagenes, when the hemoglobin and stroma metagenes were left out which display some dataset-bias (see methods). In keeping with these three major phenotypes the samples were sorted according to (1.) Basal-like phenotype, (2.) low vs. high B-Cell metagene, and (3.) the expression value of the *IL-8* metagene. (The 355 individual Affymetrix probesets and the respective metagenes are listed in the Additional file 4, Supplementary Methods).

gene expression data of breast cancer cell lines indicate that carcinoma cells are the main source of the *IL-8* metagene (Figure 2).

Relationship between TNBC and basal-like breast cancer (BLBC)

We observed a clear bimodal distribution of the *basal-like* metagene score among TNBC (Figure 3). This bimodal distribution allows us to derive a cutoff to separate cases into high and low expression groups by fitting two normal distributions to the data (Figure 3). According to this cutoff, 72.8%, 73.0% and 69.7% of TNBC were defined as BLBC in the discovery cohort-A, validation cohort-B, and validation cohort-C, respectively. Table 3 compares the clinical characteristics of BLBC or non-BLBC triple negative cancers the discovery cohort-A. The positive association between high histological grade (G3, $P < 0.001$), younger age ($P = 0.004$) and BLBC were also observed in the validation cohort-C and validation cohort-B, respectively (Additional file 2, Supplementary Table S2).

In unsupervised clustering of the metagenes the *basal-like* metagene clustered next to the *apocrine* metagene

but showed a strong inverse correlation (Figure 1). To quantify the correlation between the *basal-like* metagene and all other metagenes from Table 2 we used quartiles of the respective metagenes. Additional file 2, Supplementary Table S3 presents the six metagenes that displayed significant correlations with the BLBC phenotype in both the discovery and validation cohorts. A positive correlation was found between the BLBC phenotype and the *proliferation* and angiogenesis (*VEGF*) metagenes. A negative correlation was observed for the *apocrine/androgen receptor signaling* and two immune system related metagenes (*MHC-2* and *T-Cell* metagenes), as well as an *adipocyte* related signature.

Since we observed a negative correlation between the *basal-like* metagene and potential markers of normal breast tissue, such as the *adipocyte* metagene, we had to exclude the possibility that we are only distinguishing stroma-rich and stroma-poor samples. As shown in Additional file 1, Supplementary Figure S5, when metagenes for *proliferation*, *adipocytes* and *histones* were compared between BLBC, non-BLBC, and normal breast samples it is clearly demonstrated that the non-BLBC subtype is distinct from normal breast tissues in the

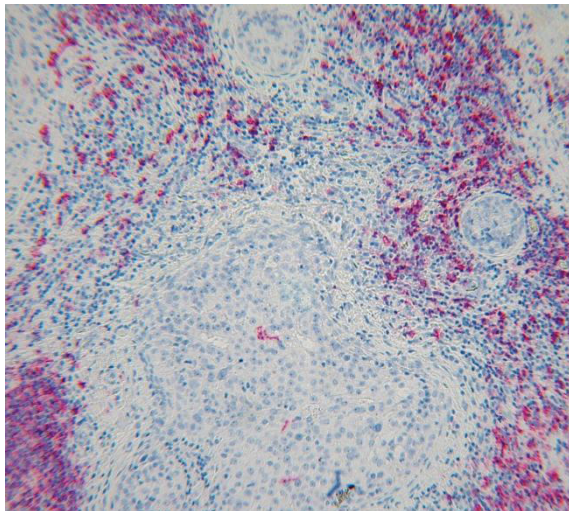
Table 2 Principal biological phenotypes identified as metagenes among TNBC

Biological component	Metagene name	Correlation within metagene cluster	# of probesets in metagene cluster	Key markers	Reference
Basal-like phenotype	Basal-like	0.61	37	KRT-5,-6, -14, -17, SOX10, SFRP1, ELF5, EPHB3, GABRP	[4]
Apocrine/androgen receptor signalling	Apocrine	0.67	27	AR, FOXA1	[18,19]
Immune system:					[4,20,21,23-25]
• B-Cell	B-Cell	0.87	48	IgG	
• T-Cell	T-Cell	0.84	27	TCR, LCK, ITK	
• MHC class II	MHC-2	0.83	14	HLA-DR, -DM, -DP, -DQ	
• MHC class I	MHC-1	0.84	17	HLA-A, -B, -C, -E, -F, -G	
• Interferone response	IFN	0.76	14	OAS1, OAS2, OAS3, MX1	
Stroma*	Stroma	0.83	47	Decorin, Osteonectin, Fibronectin, COL5A1	[26,27]
Claudin-CD24 signature	Claudin-CD24	0.70	19	CLDN3, CLDN4, CD24, ELF3	[28,29]
Proliferation	Proliferation	0.74	47	BUB1, CDC2, STK6, BIRC5, TOP2A,	[35]
Blood *	Hemoglobin *	0.63	17	HBA1, HBA2, HBB	[30]
Adipocytes	Adipocyte	0.74	8	FABP4, PLIN, ADIPOQ, ADH1B	[4]
Angiogenesis	VEGF	0.57	7	VEGF, adrenomedullin, ANGPTL4	[34]
Inflammation	IL-8	0.52	4	IL-8, CXCL1, CXCL2	[31,32]
HOXA gene cluster	HOXA	0.52	8	HOXA-4, -5, -7, -9, -10, -11	[64]
Histone gene cluster	Histone	0.69	19	Histones H2A, H2B	[65]

* The *Stroma* and *Hemoglobin* metagenes displayed a bias between datasets related to different biopsy techniques (see Methods).

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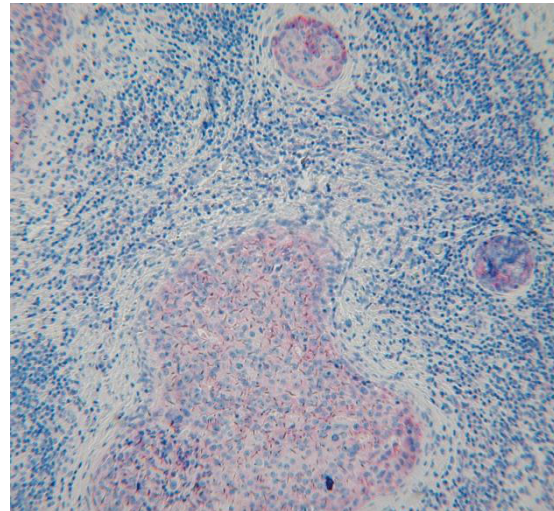


Figure 2 Immunohistochemical analyses of the cellular source of expression of the B-Cell and IL-8 metagenes in TNBC. A) Detection of B-lymphocytes by a CD20 antibody (red staining) in a triple negative breast cancer from the Frankfurt cohort with high expression of B-Cell and IL-8 metagenes. **B)** An adjacent section of the same tumor as in (A) is stained with an IL-8 antibody demonstrating that carcinoma cells are the source of IL-8 expression (red staining). Note the strong IL-8 staining in rod-like structures in the carcinoma cells. Further analyses using antibodies specific for macrophages (CD68) also demonstrated that macrophages are not the cellular source of IL-8 expression in the tumor (Additional file 1, Supplementary Figure S15).

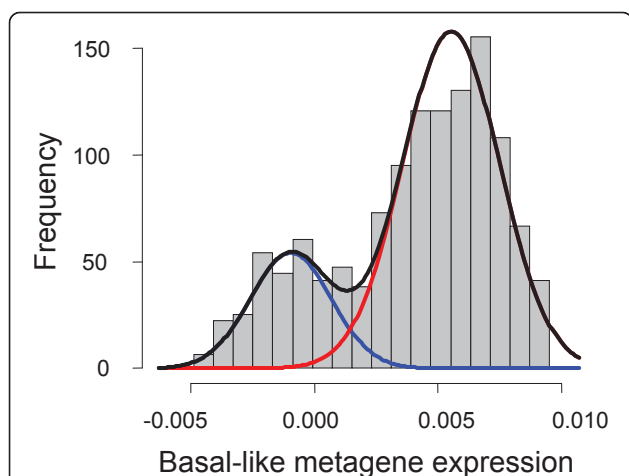


Figure 3 Distribution of the expression of the basal-like metagene among TNBC of cohort-A. The bimodal distribution of the expression of the basal-like metagene among the 394 TNBC samples in the finding cohort-A is shown. A mixture (black line) of two normal gaussian distributions (blue and red lines) was fitted to these data. The interception of the two gaussians was derived as a cutoff (0.0014) for the definition of basal-like tumors. Similar results were obtained for the validation cohorts-B, and -C, as well as from all samples combined.

expression of several metagenes. Proliferation genes have been previously shown to be the most important determinant of cancer vs normal signatures [35]. Furthermore, the strong bimodal distribution of the *basal-like* metagene argues against the possibility that this metagene is inversely describing the degree of contamination with normal tissue which should rather result in a continuous distribution. The non-BLBC tumors in our TNBC dataset mainly represent samples of the “molecular apocrine” type (16.5%), which demonstrates the inverse bimodal distribution as the *basal-like* metagene, and a relatively small group of “claudin-low” tumors (6.3%). The mutual relationship of these three metagenes is shown in Additional file 1, Supplementary Figure S6.

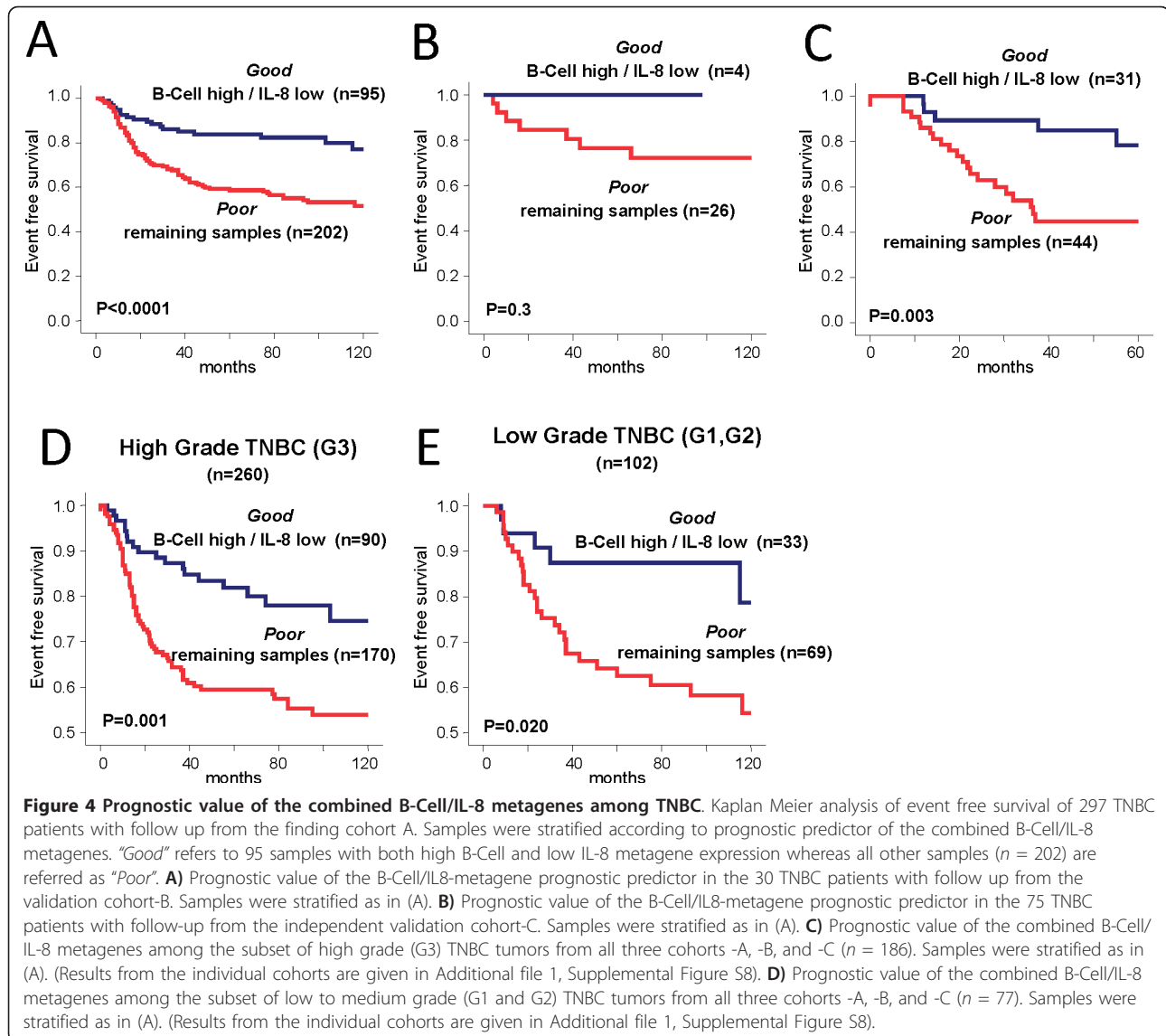
Prognostic value of the different biological phenotypes in TNBC

To assess the prognostic value of the metagenes, we analyzed the event free survival of patients as a function of metagene expression. The *basal-like* metagene had no significant effect on survival (Additional file 1, Supplementary Figure S7). In contrast, five other metagenes including the *IL-8*, *Histone*, *VEGF*, *B-Cell*, and *T-Cell* metagenes showed significant prognostic values when considered as continuous variables in univariate analysis (Additional file 2, Supplementary Table S4). In a step-wise multivariate Cox regression analysis only three of these, the *IL-8*, *Histone*, and the *B-Cell* metagenes, remained significant (Additional file 2, Supplementary Table S5). The *IL-8* and *Histone* metagenes were positively correlated with one another in all data sets (see Figure 1). The *B-cell* and *IL-8* metagenes were associated with prognosis but with an opposing direction. Based on these observations, we derived a *B-Cell* /*IL-8* metagene ratio as a prognostic index for TNBC. Figure 4A demonstrates that patients with a high expression of the *B-Cell* and low expression of the *IL8* metagene have significantly better prognosis than other TNBC patients (HR 0.37, 95% CI 0.22 to 0.61; $P < 0.001$). The five-year event-free survival was $84 \pm 4\%$ for the good prognosis group ($n = 95$) compared to $59 \pm 4\%$ for the rest of the patients. In validation cohort B ($n = 30$), there was a non-significant trend for better survival for patients with high B-cell low IL8 metagene expression ($P = 0.3$, Figure 4B). Since this cohort has limited power due to the small sample size, we also tested the prognostic value on a separate and larger ($n = 75$) validation cohort of TNBC samples [16]. The B-cell/IL8 metagene ratio had significant prognostic value in this second validation cohort C, the hazard ratio (HR) was 0.26, (95% CI 0.10 to 0.68) and the five-year DFS was $78 \pm 9\%$ vs. $45 \pm 8\%$, ($P = 0.003$) (Figure 4C). The prognostic value was independent of histological grade; Figure 4D, E shows pooled data from all three cohorts to increase sample size, (see also Additional file 1, Supplementary Figure S8

Table 3 Clinical parameters of TNBC with basal-like breast cancer (BLBC) or non-BLBC phenotype

Parameter	Information available*		Non-BLBC	BLBC	Total (n = 394)	P-value
			(n = 107, 27.2%)	(n = 287, 72.8%)		
lymph node status	n = 308	LNN	50 (64.9%)	190 (82.3%)	240	0.002
		N1	27 (35.1%)	41 (17.7%)	68	
Age 50 yrs	n = 309	≤ 50 yr	27 (34.6%)	124 (53.7%)	151	0.004
		> 50 yr	51 (65.4%)	107 (46.3%)	158	
Tumor size	n = 309	≤ 2 cm	16 (20.5%)	69 (29.9%)	85	0.14
		> 2 cm	62 (79.5%)	162 (70.1%)	224	
Histological grade	n = 309	G3	45 (57.0%)	182 (79.1%)	227	< 0.001
		G1&2	34 (43.0%)	48 (20.9%)	82	

* Number of cases with available information on the specific parameter in the finding cohort-A



for the individual cohorts). Moreover, the prognostic value of the B-cell/IL8 metagene ratio was observed both in BLBC and non-BLBC TNBCs ($P = 0.001$ and $P = 0.006$, respectively; Additional file 1, Supplementary Figure S9). The proportion of BLBC cases was similar in the Good and Poor prognosis groups defined by the B-cell/IL8 metagene ratio (75.2% and 71.8%, respectively; $P = 0.54$).

To assess a potential predictive value for sensitivity to systemic adjuvant chemotherapy, the patients were stratified by adjuvant treatment. In the discovery cohort, 186 patients received no adjuvant systemic treatment and 81 patients received chemotherapy (mostly Cyclophosphamide Methotrexate Fluorouracil; CMF). Better prognosis was observed for the high B-cell/low IL8 group in both untreated ($P = 0.001$) as well as chemotherapy treated patients ($P = 0.05$; not shown). A

potential predictive value of the B-cell and IL8 metagenes was also analyzed in 191 patients with TNBC who received neoadjuvant chemotherapy. We assembled this cohort of samples with information on pathologically complete response (pCR) from seven datasets. As shown in Additional file 1, Supplementary Figure S10 the B-cell metagene had a modest predictive value with an area under the curve (AUC) of 0.606 consistent with our previous results [22]. The predictive value for the IL8 metagene was smaller (AUC -0.552). Combining both metagenes increased the AUC to 0.612 (95% CI 0.519 to 0.704; $P = 0.018$).

In multivariate Cox regression analysis, including lymph node status, age, tumor size, and histological grade, only the combined B-Cell/IL8-metagene score showed strong independent prognostic value in both the

discovery cohort (HR 0.38, 95% CI 0.22 to 0.67, $P = 0.001$) and in the second, larger validation cohort-C, (HR 0.21, 95% CI 0.07 to 0.62, $P = 0.005$). The only other variable with borderline statistical significance (HR 0.40; 95% CI 0.17 to 0.99, $P = 0.046$) was lymph node status in validation cohort-C (Table 4). However, even in univariate analyses the remaining clinical variables did not show a significant prognostic value in the analyzed cohorts. This might be attributed to the fact that most TNBC are usually highly proliferating and grading is not as important for prognosis in this subtype as it is in ER positive disease; in addition, the power of our analysis may be limited to detecting the modest effect of age and tumor size on prognosis within this sample set. The inclusion of a term for chemotherapeutic treatment in the multivariate analysis further reduced the sample size to 213 patients in cohort-A (no treatment information was available for patients from validation cohort-B). Of these 213 patients only 37 were treated with chemotherapy. The combined B-Cell/IL8-metagene score remained significant ($P = 0.001$) in the corresponding multivariate analysis (Additional file 2, Supplementary Table S9A). Unexpectedly, chemotherapy treatment was associated with a worse prognosis probably due to chance or some form of selection bias to include higher risk patients in these public data sets (Additional file 2, Supplementary Table S9A). This selection bias is consistent with a significant higher portion of node positive patients in the chemotherapy group ($P = 0.001$) and a trend for a higher histological grade ($P = 0.074$; Additional file 2, Supplementary Table S9B).

Relationship of the identified metagenes to known prognostic signatures

The correlation of several published prognostic gene signatures to the metagenes discovered within the pure

TNBC cohort was analyzed by hierarchical clustering using the gene expression data from cohort-A (Additional file 4, Supplementary Methods Section 13). As shown in Additional file 1, Supplementary Figure S11, the “recurrence score” [36], “genomic grading index” (GGI) [37], and the “wound response signature” [38] display high correlation to the proliferation metagene. On the other hand the “7-gene immune response (IR) signature” [39], the “stroma derived prognostic predictor” (SDPP) [40], and the “368 gene medullary breast cancer signature” [16] were all highly correlated to immune cell metagenes. The magnitude of the correlation ($R^2 = 0.4$ to approximately 0.7) between the different immune metagenes and the related signatures is at the same high level as the correlation between genes within other metagene clusters ($R^2 = 0.5$ to approximately 0.7; Table 2). We demonstrated previously [22] that even if the different immune metagenes can discriminate between distinct types of immune cells, the actual infiltration of tumors generally represents a mixture of these different immune cells. In most cases, the differences in the proportions in this mixture are smaller than the global differences in lymphocyte infiltration between individual tumors. Therefore, different immune signatures often carry redundant prognostic information and can replace each other. In contrast to the immune cell metagenes no correlation between the IL8 metagene and other signatures were observed.

Discussion

It has been suggested that TNBC represent a group of several molecularly [3] and clinically [41,42] distinct disease subtypes. We used gene expression data of a cohort of 394 TNBC to identify molecular subsets within this tumor type. The definition of TNBC was based on gene

Table 4 Multivariate analysis of EFS according to standard parameters and the combined B-Cell/IL8-metagene in TNBC

Variable		Finding cohort A*				Validation cohort C*			
		No. of patients†	Hazard ratio	95% CI	P-value‡	No. of patients§	Hazard ratio	95% CI	P-value‡
Lymph node status	LNN vs N1	210 vs 27	0.59	0.31 to 1.12	0.10	43 vs 29	0.40	0.17 to 0.99	0.046
Age	> 50 vs ≤ 50	113 vs 124	0.75	0.48 to 1.17	0.21	48 vs 24	1.68	0.65 to 4.38	0.29
Tumor size	≤ 2 cm vs > 2 cm	71 vs 166	0.73	0.44 to 1.21	0.22	11 vs 61	0.99	0.28 to 3.42	0.98
Histological grading	G3 vs G1 and 2	166 vs 71	1.11	0.68 to 1.81	0.68	59 vs 13	0.53	0.22 to 1.29	0.16
B-Cell/IL8-Signature	<i>Good vs Poor</i>	78 vs 159	0.38	0.22 to 0.67	0.001	29 vs 43	0.21	0.07 to 0.62	0.005

* Results from multivariate Cox analysis of event free survival in the TNBC finding cohort A and validation cohort C are presented.

† information on all parameters was available for 237 of the 297 TNBC samples with follow up data from the finding cohort A.

‡ Significant P-values are given in bold

§ information on all parameters was available for 72 of the 76 TNBC samples with follow up data from the validation cohort C.

|| “Good” refers to high B-Cell metagene together with low IL8 metagene expression compared to all the remaining samples referred as “Poor”.

expression data which is not the standard definition used in the clinic. This might be a caveat but holds the promise that samples erroneously characterized as receptor-negative by immunohistochemistry do not introduce noise into our analysis. We identified 16 metagenes associated with several distinct biological processes that showed variable expression across TNBC (Table 2). Some of the metagenes seem to point to the distinct origins of these cancers [43,44]. These include the basal-like [4], the apocrine [18,19], and the claudin-low [28,29] subtypes of TNBC. Other metagenes were related to non-neoplastic cellular constituents of the tumor microenvironment including stroma [26,27], blood cell [30] and adipocytes [4], as well as signatures for angiogenesis [23,34] and inflammation [31-33]. Five metagenes appear to reflect the variable presence of immune cells and may contribute to the clinical behavior of the cancer [4,20-25,27,45] (Table 2).

Kreike *et al.* [9] detected similar metagenes among 97 TNBC analysed with a different microarray platform. That study suggested that the TNBC clinical phenotype can be equated to the BLBC molecular class determined by the centroid method [46] since 95% of the TNBCs were assigned *basal-like* molecular class [47]. However, the centroid method is highly susceptible to the composition of the dataset that is used to define the reference centroids [48] and variants of the method can lead to different results [49]. Bertucci *et al.* [50] identified only 71% of their 172 TNBC cases as *basal-like* when using a slightly different version of the centroid method for molecular classification. When we applied different versions of the centroid method to 1,364 breast cancers, 65% to 90% of the TNBC samples ($n = 172$) were assigned to the basal-like class depending on the method used (Additional file 2, Supplementary Table S6). In this paper we took a different approach and first identified metagenes and used these metagenes to define molecular subsets among TNBC. One of our metagenes corresponded closely to the gene signatures that are used to define BLBC in the centroid based methods. Our results indicate that BLBC defined based on the *basal-like* metagene expression represent around 73% of TNBC (Table 3 and Additional file 2, Supplementary Table S2).

The proportion of BLBC among TNBC in our study is similar to results from an immunohistochemical study by Rakha *et al.* [7] that defined BLBC by the expression of CK5/6, CK14, CK17 or EGFR. These authors observed a worse survival of the 165 patients with BLBC compared to the remaining 67 TNBC cases, which expressed none of these markers. However, we did not detect differences in the prognosis of BLBC and non-BLBC type triple negative cancers (Additional file 1, Supplementary Figure S7). In the study by Rakha *et al.*

the prognostic effect was mainly confined to 103 untreated patients. Still, even when we analyzed untreated patients ($n = 186$) separately, we detected no prognostic value of the BLBC phenotype (not shown). Our results are also contrary to the immunohistochemical study of Cheang *et al.* [51], which used CK5/6 and EGFR antibodies for TNBC stratification. They also observed a worse prognosis of 336 BLBC TNBC compared to 303 non-BLBC TNBC. However, our study is not directly comparable to these prior reports because our definition of BLBC is fundamentally different from the IHC-based methods. Our results are in line with several other genomic profiling studies that reported limited prognostic value for the BLBC molecular class among clinically triple negative cancers [18,19,50].

We observed strong prognostic value for several of the other metagenes (Additional file 2, Supplementary Table S4). An improved prognosis was observed for patients with tumors displaying high expression of immune system related metagenes which supports recent reports [20,23-25,27,39,40,52,53]. An association with decreased survival was observed for high expression of inflammation (IL-8), an angiogenesis/hypoxia signature (VEGF) [34], and histone-related metagenes (Additional file 2, Supplementary Table S4 and Figure 1). A simple combination of high B-Cell and low IL8 metagene expression identifies a subset of TNBC patients (32% of all) with a favorable prognosis and a five-year event-free survival of 84%. In multivariate analysis, only this metagene ratio and lymph node status were significant predictors of TNBC in our cohort of patients (Table 4 and Figure 4D, E). Other known prognostic factors in breast cancer, such as age, tumor size and histological grade, were not significant in our cohorts, even in univariate analysis. Most TNBC are high grade and, therefore, grade is not as important for prognosis in this subtype as it is in ER positive disease. TNBCs are also often associated with younger age but the impact of age and tumor size for prognosis within this subtype is not yet fully clear. Still it cannot be excluded that a bias in our cohort is the reason for the lack of the significance of these factors. Our analyses of neoadjuvant treated TNBC samples suggest modest predictive value of the B-cell/IL8 metagene ratio for currently used chemotherapies [22,54] (Additional file 1, Supplementary Figure S10). We also observed a pure prognostic value in untreated patients of finding the cohort in line with other reports on B-cell metagene [24,27]. Treatment information on the samples from the validation cohort was not available.

Our observation is important since every currently available genomic prognostic signature, (for example, the 70-gene profile [55], Recurrence Score [36], Genomic Grading Index [37]), assigns poor prognostic risk status to all TNBC samples despite their variable

outcome [56-58]. One of these signatures, the Rotterdam-76-gene prognostic signature [59], was developed in a way to allow prognostic stratification of ER-negative cancers. However, similar to other reports [9] we were not able to demonstrate a prognostic value for this signature (Additional file 1, Supplementary Figure S12).

We used an unsupervised class discovery approach to first identify the main molecular subtypes within the data and then assess the prognostic differences between the molecular subsets. Interestingly, when we performed an independent supervised analysis that compared TNBC cases with or without recurrence, we also identified IL-8 as the top ranked gene associated with poor prognosis (Additional file 1, Supplementary Figure S13 and Additional file 2, Supplementary Table S8). However, gene signatures obtained through supervised analysis were not superior to the molecular structure based prognostic predictions in validation (Additional file 1, Supplementary Figure S14). In addition, the biological interpretation of the empirically derived prognostic signature is more difficult than the interpretation of metagenes. In summary, we performed the largest unsupervised analysis of pooled gene expression data from TNBC. We describe a new prognostic signature for these cancers that identify about one-third of TNBC as relatively low risk for recurrence. These cancers are characterized by high B-cell and low IL-8 metagene expression and have about 84% recurrence-free survival at five-years. Whereas, this may not be sufficiently high to forego adjuvant chemotherapy, these observations pave the way to develop a clinically useful multivariate prognostic model for TNBC. A combined, prognostic score, including clinical variables, such as nodal status and perhaps tumor size, and molecular variables, such as optimized B-cell and IL-8 metagenes (measured by an RT-PCR or array-based method), may identify patients with very low risk of recurrence even with ER-, PgR- and HER2-negative breast cancer. Equally important, the prognostic importance of B-cells and the negative impact of IL-8 suggest potential novel therapeutic strategies for TNBC that can be tested in the clinic [31,32]. It could allow the selection of those patients who could profit most from novel immune stimulating drugs like anti-CTLA-4 antibodies that have shown promise in melanoma [60,61]. IL8 could also directly increase the survival of breast cancer stem cells after chemotherapy [62], which can be blocked with IL8 directed drugs [63]. Such an effect might explain the triple negative paradox with high relapse rates despite a good initial response to chemotherapy.

Conclusions

In the largest and most comprehensive analysis of all available gene expression data in TNBC, we first

identified structures in the molecular data without considering any clinical outcome. Subsequently, these molecular phenotypes were correlated with survival in multivariate analysis, including routine clinical and pathological variables. Our most important observation is that a high B-cell presence and low IL-8 activity identifies a good prognosis group, even in the absence of systemic therapy, among TNBC. These observations directly point to therapeutic interventions, such as the inhibition of the IL-8 pathway and activation of the immune system in the tumor microenvironment that could benefit patients with this disease.

Additional material

Additional file 1: Supplementary Figures S1 to S15. An Adobe file containing 15 supplementary figures (S1 to S15).

Additional file 2: Supplementary Tables S1 to S7. An Adobe file containing seven supplementary tables (S1 to S7).

Additional file 3: Supplementary Tables S8. An Excel file containing a supplementary table (S8) containing lists of probesets and corresponding information from the supervised analysis by SAM.

Additional file 4: Supplementary Methods. An Adobe file containing supplementary information on methodology and six additional supplementary figures (S16 to S21), which are referred to within this supplementary methods.

Additional file 5: Supplementary R files. A zipped package containing an R script file of the analysis with respective links to the complete dataset files in GEO and a text file of the metagene probesets used in the R analysis.

Abbreviations

AUC: area under the curve; BLBC: basal-like breast cancer; CK: cytokeratine; DMFS: distant metastasis free survival; EFS: event free survival; EGFR: epidermal growth factor receptor; ER: estrogen receptor; FNA: fine needle aspiration; GGI: genomic grading index; HER2: human epidermal growth factor receptor 2; HR: hazard ratio; IL: interleukine; IR: immune response; MHC: major histocompatibility complex; PgR: progesterone receptor; REMARK: recommendations for prognostic and tumor marker studies; RFS: Relapse free survival; SDPP: stroma derived prognostic predictor; TNBC: triple negative breast cancer; VEGF: vascular endothelial growth factor.

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Authors' contributions

AR, TK and UH conceived the study, carried out the analyses and wrote the manuscript. CL and LP added experimental data, participated in the interpretation of the data and in writing the manuscript. ER, LH, RG, CS AA, MS and VM provided patients and samples, obtained follow-up data and helped to draft the manuscript. DM and TK performed the statistical analysis. MK initiated the study and participated in the design and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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