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PRECLINICAL STUDY

Schlafen-11 expression is associated with immune signatures and basal-like phenotype in breast cancer

Edoardo Isnaldi[1] · Domenico Ferraioli[1,2] · Lorenzo Ferrando[1] · Sylvain Brohée[3] · Fabio Ferrando[1,4] · Piero Fregatti[1,4] · Davide Bedognetti[5] · Alberto Ballestrero[1,4] · Gabriele Zoppoli[1,4]

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Abstract

Purpose Breast cancer (BC) is a heterogeneous disorder, with variable response to systemic chemotherapy. Likewise, BC shows highly complex immune activation patterns, only in part reflecting classical histopathological subtyping. Schlafen-11 (SLFN11) is a nuclear protein we independently described as causal factor of sensitivity to DNA damaging agents (DDA) in cancer cell line models. SLFN11 has been reported as a predictive biomarker for DDA and PARP inhibitors in human neoplasms. SLFN11 has been implicated in several immune processes such as thymocyte maturation and antiviral response through the activation of interferon signaling pathway, suggesting its potential relevance as a link between immunity and cancer. In the present work, we investigated the transcriptional landscape of SLFN11, its potential prognostic value, and the clinico-pathological associations with its variability in BC.

Methods We assessed SLFN11 determinants in a gene expression meta-set of 5061 breast cancer patients annotated with clinical data and multigene signatures.

Results We found that 537 transcripts are highly correlated with SLFN11, identifying "immune response", "lymphocyte activation", and "T cell activation" as top Gene Ontology processes. We established a strong association of SLFN11 with stromal signatures of basal-like phenotype and response to chemotherapy in estrogen receptor negative (ER-) BC. We identified a distinct subgroup of patients, characterized by high SLFN11 levels, ER- status, basal-like phenotype, immune activation, and younger age. Finally, we observed an independent positive predictive role for SLFN11 in BC.

Conclusions Our findings are suggestive of a relevant role for SLFN11 in BC and its immune and molecular variability.

Keywords Schlafen-11 · Immune signatures · Basal-like phenotype · Breast cancer · Biomarker

Edoardo Isnaldi, Domenico Ferraioli, Lorenzo Ferrando, Alberto Ballestrero and Gabriele Zoppoli contributed equally to the present work.

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✉ Gabriele Zoppoli
gabriele.zoppoli@unige.it

[1] Department of Internal Medicine (DiMI), University of Genoa and Ospedale Policlinico San Martino, Viale Benedetto XV, 6, 16132 Genoa, Italy

[2] Comprehensive Cancer Center Leon Berard, Lyon, France

[3] Institut de Pathologie Et de Génétique a.s.b.l, Charleroi, Belgium

[4] Ospedale Policlinico San Martino IRCCS per l'Oncologia, Genoa, Italy

[5] Sidra Medical Center, Doha, Qatar

Abbreviations

BC	Breast cancer
DDA	DNA damaging agents
DFS	Disease-free survival
ER	Estrogen receptor
HT	Hormone treatment
ICR	Immunological constant of rejection
MCA	Multiple correspondence analysis
SLFN11	Schlafen-11
TNBC	Triple-negative breast cancer

Introduction

Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent neoplasm among women [1].

BC is a clinically and molecularly heterogeneous disease and genomic microarray analyses have corroborated the presence of at least four distinct intrinsic molecular subtypes: luminal A, luminal B, basal-like, and HER2 enriched subsets [2, 3]. These subtypes display varying degrees of sensitivity to treatment and highlight the molecular heterogeneity of BC [4].

We and an independent group [5] recently discovered the role of a putative DNA/RNA helicase, Schlafen-11 (SLFN11), for its causal association with sensitivity to DNA damaging agents (DDA), such as platinum salts, topoisomerase I and II inhibitors, and other alkylators in the NCI-60 panel of cancer cell lines [6].

SLFN11 belongs to the Schlafen protein family, which has been implicated in the regulation of important mammalian biological functions, such as control of cell proliferation [7], induction of immune responses [8], and regulation of viral replication [9].

Schlafen genes were originally identified during screening for growth regulatory genes, and they are differentially expressed during lymphocyte development [10–13]. Later, SLFN11 was described as an early interferon response gene, in association with HIV infection [9]. Furthermore, Murai et al. described molecular mechanisms detailing how SLFN11 is a dominant determinant of sensitivity to DNA-targeted therapies [14]. In particular, SLFN11 inhibits checkpoint maintenance and homologous recombination by removing Replication Protein A from single-stranded DNA [15]. Tang et al. demonstrated that the use of histone deacetylase inhibitors can be used to sensitize SLFN11-inactivated cancers to DDA [16]. Recently, the importance of SLFN11 as a predictor of sensitivity to DDA has been proven in Ewing's sarcomas, ovarian cancer, and colorectal cancer [17–20]. SLFN11 has also been confirmed as a predictive biomarker of PARP inhibitor sensitivity in small cell lung cancer [21].

The aims of our study were to investigate the transcriptional landscape of SLFN11 expression in invasive BC and to identify clinical and pathological parameters that could help explain SLFN11 modulation in BC. In addition, we set up to determine whether SLFN11 expression could be associated with prognosis or response to treatment in this neoplasm.

Materials and methods

Datasets retrieval, pre-processing, and data normalization

Thirty-five gene expression datasets of expression profiles from 7737 tumors were retrieved from public databases or authors' websites [32 sets previously described in [22] and another three: PNC, METABRIC and TCGA [23–25]. Immune phenotypes for TCGA BC cases and leucocyte infiltration were obtained as described in Hendrickx et al. [26].

To ensure comparability of expression values across multiple datasets and microarray platforms (Agilent, Affymetrix or Illumina), we performed 0.95 quantile normalization (using the R/Bioconductor package genefu [27]).

SLFN 11 expression analysis and gene signature enrichment

Whole transcriptome correlation of SLFN11 was performed using Spearman's rank correlation. We selected the top 5th percentile of transcripts that better correlated with SLFN11 expression. Functional annotation of correlators was further performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7 [28] in order to identify significantly enriched pathways [false discovery rate (FDR) < 0.05], particularly Gene Ontology (GO) terms (The Gene Ontology Consortium). DAVID identifies GO categories to which genes belong, determining the statistical significance of non-random representation. To provide an independent assessment of enrichment analysis, we classified patients in molecular subtypes, extracting relative genomic signatures from the *genefu* package [27]. Patients labeled as "normal" PAM50 phenotype were removed, upon concerns of low cancer cellularity and possible ensuing contamination by normal breast tissue [29]. The most significant gene signatures were extracted using a feature selection machine learning approach, called LASSO regression (*glmnet* package).

Multiple correspondence analysis

We investigated the modulation of SLNF11 in breast cancer through the study of the mutual distribution of clinical and pathological categorical data. First, we removed T1a samples, due to their small relative number and size, *T*x and *N*x tumor patients and all those patients with unknown age information, estrogen receptor or HER2 status. For this analysis, SLFN11 expression was subdivided in tertiles of expression (low, intermediate, and high). Exploratory assessment and inter-dependencies relations of data, combined with the extracted gene signatures, were accomplished by multiple correspondence analysis with the *FactoMineR* package.

Survival analysis and time dependency correlation

Survival analyses were performed in order to determine the association of SLFN11 with prognosis in BC. We defined, by univariable statistical analysis, the association between disease-free survival (DFS) and SLFN11 expression ("low"

if in the lower two tertiles and "high" if in the top tertile). The DFS curves were generated using Kaplan–Meier estimators (*survcomp* package), and *p* values were obtained with the log-rank test. For what concerns the analysis of more than one covariates, we employed a stepwise backward-forward Cox proportional hazards regression model. The Akaike Information Criterion allowed the estimation of the best set of clinical and pathological variables described above (*MASS* package).

To explore time dependency of SLFN11 modulation, we tested the proportional hazards assumption for a Cox regression model as described previously [30]. We tested a two-sided hypothesis, rejecting the null ones with a $p < 0.05$ and applied multiple corrections of resulting *p* values using the Benjamini–Hochberg method.

Results

SLFN11 expression correlates with BC immune-related transcripts

To investigate the transcriptional landscape of SLFN11 in BC, we conducted a gene expression microarray meta-analysis of 7737 cases from 35 publicly available datasets.

Of 7737 cases, we assessed 5061 patients with SLFN11 expression values. Then, we performed a whole transcriptome correlation analysis with SLFN11 and identified 537 genes in the top 5th percentile of correlation. The list of these 537 transcripts was analyzed for gene ontology (GO) enrichment. Strikingly, immune function processes represented most of the GO terms resulting from such analysis. The overrepresented terms in our sample set are listed in Table 1.

In agreement with such finding, we observed a strong positive association between well-established markers of tumor lymphocytic infiltration with SLFN11 expression such as CD3 and CD8 (Spearman's $\rho = 0.527$, FDR < 0.0001 with the expression of CD3, and $\rho = 0.514$ with the expression of CD8—FDR < 0.0001, see Fig. 1).

Overall, this data purports an association of SLFN11 with immune modulation in BC.

SLFN11 expression correlates with BC immune gene signatures

Next, to validate our observations from an independent perspective, we inferred gene expression signatures from 4740 patients after removing the "normal-like" intrinsic phenotype cases (in light of their low cellularity) and exploited LASSO penalized regression to extract the most relevant signatures associated with SLFN11 expression. In harmony with our previous observations, we observed an independent, strict association with immune-related signatures, in particular with two publicly described signatures '*Immune2*' [31] ($\rho = 0.508$, FDR < 0.0001) and '*Stroma1*' [31] ($\rho = 0.377$, FDR < 0.0001, see Fig. 2).

High expression of SLNF11 is linked with aggressive BC

To better understand the role of SLFN11 in BC modulation, we performed multiple correspondence analysis (MCA) including clinical and pathological parameters, as well as SLFN11 levels ranked by tertiles of expression.

2581 patients from 7 datasets, presenting with all clinical and pathological features including ER and progesterone receptor immunohistochemistry, HER2 status, grade, T, N, intrinsic subtype, and STAT1 signature as a proxy for immune activation [32] were considered for such analysis.

MCA highlighted two clearly separated patient clusters. The "*SLFN11-hot*" cluster is defined by high SLFN11 expression, ER-negative status, high histological grade, basal-like phenotype, immune activation, and younger age at diagnosis (< 50 years old).

Table 1 Top gene ontology (GO) terms associated with SLFN11 expression in breast cancer

Term	Count	Percent	Fold-en.	FDR
Immune response	117	23.2	5.62	5.94 E−53
Positive regulation of immune system process	55	10.9	7.66	3.36 E−29
Cell activation	56	11.1	6.47	8.42 E−26
Leukocyte activation	52	10.3	7.12	8.74 E−26
Regulation of cell activation	44	8.7	8.34	3.08 E−24
Regulation of lymphocyte activation	41	8.1	9.19	4.14 E−24
Lymphocyte activation	45	8.9	7.50	8.28 E−23
Regulation of leukocyte activation	41	8.1	8.19	4.94 E−22
Regulation of T cell activation	35	6.9	9.92	2.69 E−21
Positive regulation of cell activation	34	6.7	10.16	5.84 E−21

Fold-en fold enrichment, *FDR* false discovery rate

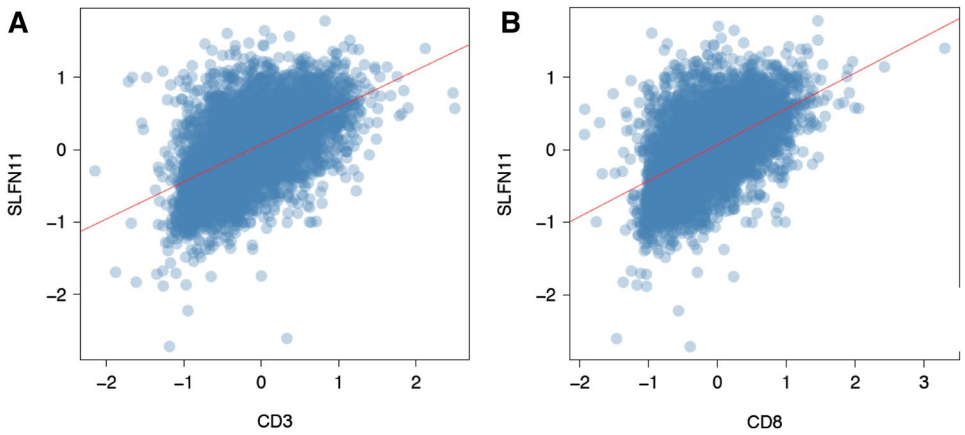

Fig. 1 **a** Correlation between SLFN11 (*y*-axis, *z*-score gene expression values) and CD3 (*x*-axis, *z*-score gene expression values). **b** Correlation between SLFN11 (*y*-axis, *z*-score gene expression values) and CD8 (*x*-axis, *z*-score gene expression values)

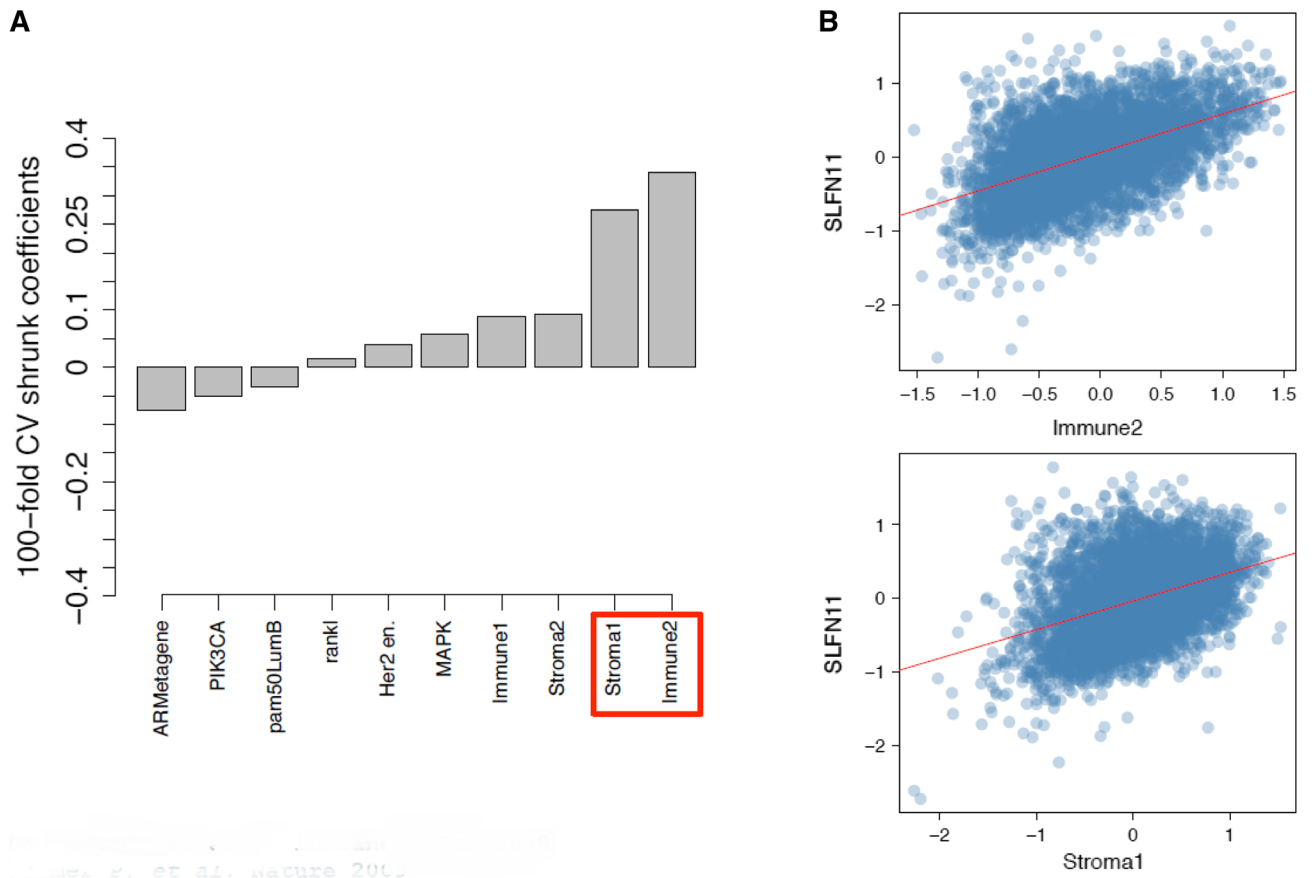

Fig. 2 **a** Bar plot shows the LASSO regression coefficient weights related to the gene signatures of interest: the highest weighted signatures are highlighted in a red contoured box. **b** The upper and lower scatterplots show the correlation between SLNF11 and the most relevant gene signatures resulting from previous variable selection analysis

The *"SLFN11-cold"* cluster is characterized by low/intermediate SLFN11 expression, ER-positive status, lack of HER2 amplification, older age at diagnosis (> 50 years old), and low/intermediate STAT1 expression (see Fig. 3).

In summary, high SLFN11 expression correlates with aggressive tumors with signs of immune activation (basal-like phenotype, higher histological grade, younger age), whereas lower SLFN11 expression can be observed in luminal, less aggressive neoplasms characterized by low immune activation.

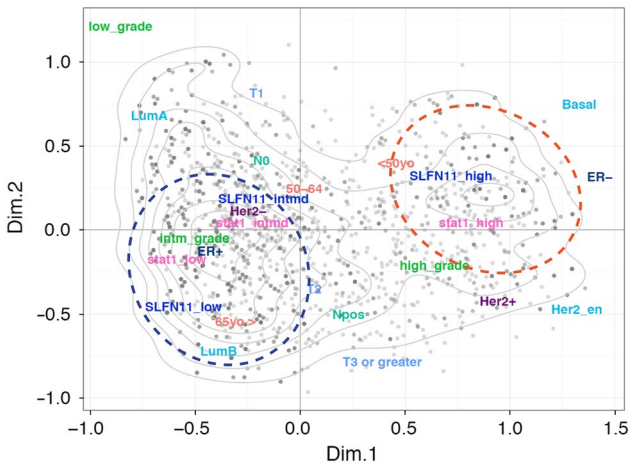

Fig. 3 MCA showing the relationship patterns between clinico-pathological variables and SLFN11 expression in breast cancer. x- and y-axes represent the first and second dimension (Dim.1 and Dim.2) of the MCA analysis performed on clinical and pathological data, as well as SLFN11 expression, divided in tertiles, from 2581 BC patients. Patients are represented by small grey dots and categorical variables are colored. In particular, patients with high-grade tumors also show high SLFN11 expression levels (highlighted by the red dashed circle), whereas the cluster of patients with SLFN11 low and intermediate expression tumors is also characterized by low and intermediate (Low/Intm) STAT1 expression, HER2-, ER+ cancers (steel-blue dashed circle)

SLFN11 overexpression is independently associated with better prognosis

To evaluate whether SLFN11 expression could be associated with prognosis or response to treatment in BC, we evaluated 2093 patients from 3 different datasets with complete information concerning DFS and type of treatment.

By univariable analysis, SLFN11 was not associated with prognosis (HR = 1.09 for SLFN11-high vs. low expression, 95% CI 0.88–1.36, $p = 0.37$).

On the other hand, when taking into account clinical and pathological parameters as well as type of treatment and intrinsic subtypes, SLFN11 high expression was independently associated with better prognosis (HR = 0.61, 95% CI 0.41–0.91, $p = 0.0153$). Moreover, we could define an interaction between SLFN11 expression and hormone treatment (HT), with high-SLFN11 patients undergoing HT being characterized by worse outcome (HR: 1.81, 95% CI 1.11–2.96, p value for interaction = 0.0175, Fig. 4, panel a).

To better understand this not obvious observation, we investigated SLFN11 expression and HT in relation with possible time dependencies violating the Cox proportional hazards assumption. Indeed, in our analysis high SLFN11 levels subtended a worse prognosis in the first 2 years after diagnosis only in patients undergoing HT (Fig. 4, panel b).

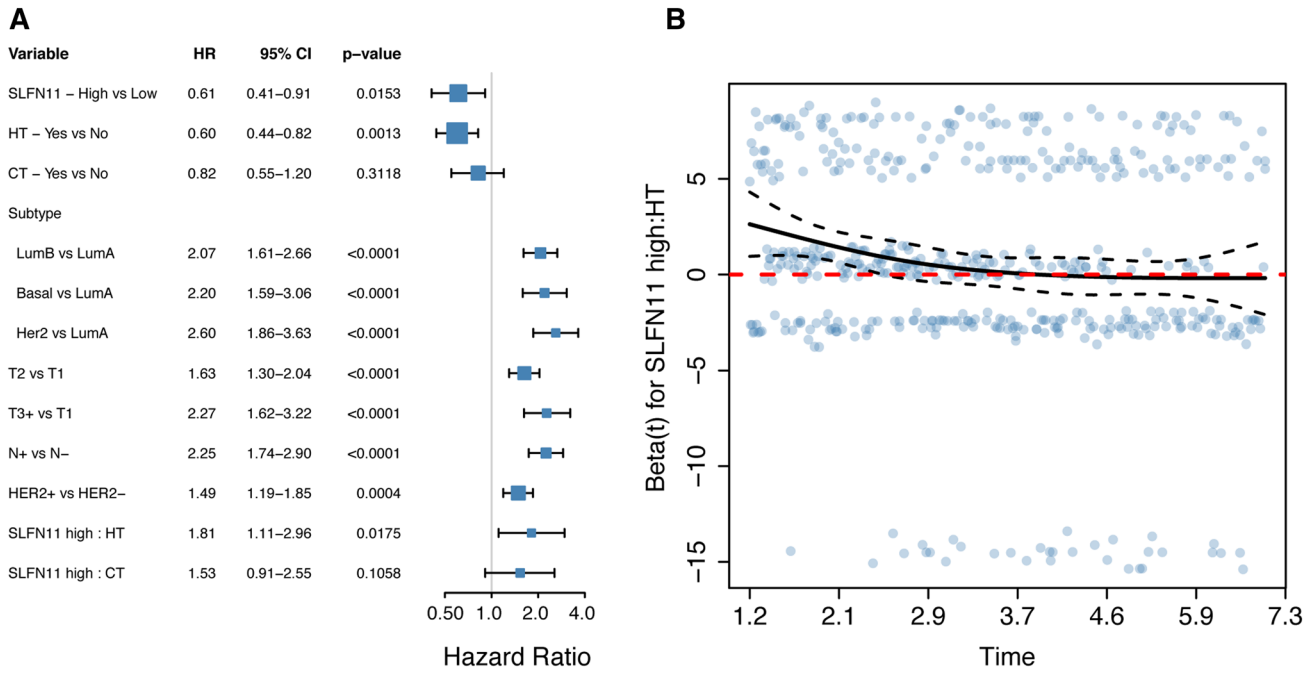

Fig. 4 **a** Forest plot of Cox regression model for DFS in 2093 BC patients with complete anatomopathological and clinical follow-up data. **b** Plot of scaled Schoenfeld residuals. Red dashed and blue dotted lines represent, respectively, the null effect (null log hazard ratio) and a ±2-standard-error band around the fit. On the *x*-axis, time is expressed in years

SLFN11 is independent from BC immune activation status in prognosis prediction

Finally, we derived leucocyte infiltration and immune phenotypes in the most extensively analyzed set available to us, TCGA, as previously described [26]. We could indeed confirm that SLFN11 expression is associated with leucocyte infiltration (Spearman's $\rho = 0.61$, $p < 0.0001$, see Supplementary Figure S1). However, in a survival model taking into account the interaction of SLFN11 and the recently described BC "low" and "high" immunological constant of rejection (ICR) phenotypes ($N = 318$) [26], we could not find a significant interaction in determining prognosis between the two variables. Surprisingly, however, our model suggested that high SLFN11/high ICR cases may have a short-term worse prognosis than other cases (adjusted HR = 2.68, 95% CI 0.28–25.56, $p = 0.1483$, with a p value for violating the proportional hazards assumption = 0.1114).

Discussion

In the present article, we investigated for the first time how SLFN11 is modulated in BC, analyzing more of 7000 BC cases available from 35 public datasets. Our findings demonstrated a strong correlation of SLNF11 expression with immune system transcriptomic markers, in particular with transcripts involved in immune system processes such as "*prolymphocyte activation*", "*immune response*", and "*T cell activation*". Our findings document the relationship between SLFN11 and immunity in BC, initially suggested by previous works in other settings [9, 33, 34]. In analogy with our findings, Stewart et al. recently published that SLFN11 high expression in small cell lung cancer is positively correlated with immune regulatory pathways, particularly with Type 1 interferon pathway genes [33]. Therefore, SLFN11 appears to have a significant role not only in innate immunity processes such as defense response to virus [9] or DNA damage repair [6], but also in adaptive immune response to cancer.

SLFN11 in addition to its known expression by cancer cells [6] could indeed be expressed by immune cells during anti-tumoral response, potentially behaving as a marker of T cell infiltration in BC as well as in other tumor types. The consistent association of SLNF11 with immunity is exemplified by its strong correlation with tumor infiltrating lymphocyte markers (CD3 and CD8 in our analyses).

Of note, we identified a strong independent correlation of SLFN11 with two immune gene signatures, namely *stroma1* and *immune2*. In the last few years, several prognostic and/or predictive gene expression signatures have been published in BC [35–37]. Desmedt et al. in their comprehensive meta-analysis showed as several prognostic gene signatures differ in prognostic abilities according to the BC subtype and as only immune response modules seem to predict prognosis in ER-negative/HER2 negative BC patients [32]. On the other hand, we previously pointed out the prognostic and predictive value of immune gene signatures in primary TNBC underlining the activation of Th1/effector immune response [35]. Our findings show both high expression of SFLN11 in a subgroup of patients with TNBC-like features and a strong correlation with immune signatures, in particular *immune2*, supporting an involvement of SLFN11 during the effector immune response in BC. In parallel, stroma signatures have also been developed in BC in order to predict clinical outcome and treatment response [38–40]. Particularly, Finak et al. developed a 26 gene stroma-derived prognostic predictor in which a good-outcome cluster overexpresses a distinct set of immune-related genes, including T cell and NK cell markers indicative of a Th1-type immune response (GZMA, CD52, CD247, CD8A) [41]. Winslow et al. showed that a specific immune gene signature (C1Q), represented by genes such as DZMH, GZMA, GZMK, CD3D, CD3G, CD247, CD8A, coding for proteins involved in cytotoxic immune response in TNBC, is associated with low risk of recurrence. Finally, their results support that the molecular profile of a Th-1/immune response ($CD4^+$ T cells) is an important prognostic marker in BC [39] as also hypothesized by Gu-Trantien et al. in her work [42]. In good agreement with such independent observations, in our study SFLN11 is highly associated with stromal signatures, in particular *stroma1*, and expression of T cell markers, supporting the idea of a role of SLFN11 in Th-1/effector immune response in BC.

Through our unbiased analysis of SLFN11 expression in relation with clinico-pathological BC variables, we discovered two distinct BC patient subgroups. In the "*SLFN11-hot*" cluster, we observed a high expression of the signature of STAT1, a key mediator of type I and type II interferon response. Among its many functions, STAT1 promotes Th1 immune response and TCD8+ cell recruitment [43]. This type of immune activation is predominant in TNBC, a subgroup of BC that is considered highly immunogenic. TNBC typically presents a worse prognosis than other BC subtypes, with—however—a very heterogeneous response to current systemic chemotherapies and absence of actionable molecular targets. To overcome this issue, current clinical trials testing a combination of immunotherapy and chemotherapy in TNBC are ongoing [44].

In our analysis, we demonstrated that SLFN11 expression is strictly related to BC-immunity, in particular in TNBC. The "*SLFN11-hot*" cluster encompasses a distinct BC subgroup with TNBC-like features, strong immune activation, better prognosis, and better response to systemic treatments compared to other BC subtypes. On the other hand, the "*SLFN11-cold*" cluster might represent a different subgroup of scarcely immunogenic BC with minor response to systemic treatment. Therefore, SLFN11

as immune-related biomarker is an intriguing venue for further translational research.

In our time dependency analysis, we identified a subgroup of high-SLFN11 BC patients treated with HT presenting with worse outcome in the first 2 years of follow-up. This behavior shows similarities with TNBC and suggests that the phenomenon that we observed might be actually due to a subset of hormone receptor-poor patients with a biological behavior analogous to that of TNBC. This is, however, just a hypothesis since we did not have the availability of ER expression level by immunohistochemistry in the evaluated dataset for a precise quantitation of ER by standard methods. Our observation is in agreement with recent literature, since several papers confirmed the analogies between TNBC and Luminal-B BC concerning survival rates [45], response to neoadjuvant chemotherapy [46], high mutational burden, and immunogenic profile characterized by higher expression of TIL [47]. Finally, Luminal-B BC are poorly responsive to HT [48] and could be stratified by immune profile analysis into different prognostic groups [49], so that in future studies on BC, we believe SLFN11 expression should be assessed together with other established parameters for prognostic and predictive purposes.

Our lack of identifying a clear association between SLFN11 levels and immune activation in BC in determining prognosis is somehow puzzling. We may speculate that SLFN11 levels in cancer cells play an independent role in response to DDAs when considered together with immune status in BC. As a consequence, we strongly advocate for future studies to morphologically deconvolute SLFN11 expression in cancer cells and in immune infiltrate in selected BC cohorts to reach a causal understanding of the role of this protein. On the other hand, our inconclusive results in assessing the relation of SLFN11 and immune activation in BC may be due to both the relatively low number of events ($N = 42$) and the insufficient length of the follow-up time (median 2.5 years) of publicly available BC TCGA data. Moreover, the suggestion of a worse short-term prognosis again favors the idea of high SLFN11 being a characteristic of BC cancer with such behavior, as TNBC is. The negative prognostic effect of SLFN11 in the high ICR BC cases is puzzling, and we should be careful in overinterpreting substantially indecisive results. Our analyses have several limitations. Among them the heterogeneity concerning the origin of data, chip design, and clinical annotation are unavoidable. Moreover, we did not perform preclinical experiments for our findings, which are of associative nature so far—albeit suggestive—, and SLFN11 location in BC is yet to be determined, since the contribution from infiltrating lymphocytes may be determinant in this regard.

Conclusion

In summary, a consistent and evident pattern emerges, highlighting the strong correlation of SLFN11 with the immune system in BC, as well as its meaningful associations with clearly distinct clinico-pathological BC phenotypes and clinical outcome. Further studies will have to focus on biological, well-annotated, and homogeneous specimens from clinical BC cohorts to further unravel SLFN11 role in BC.

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Compliance with ethical standards

Availability of data and material All raw data used for the generation of the expression set we analyzed are available in GEO under their respective publication IDs. Normalized expression data are available upon request to the Corresponding Author.

Conflict of interest The authors declare no conflict of interest.

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