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PRECLINICAL STUDY

Gene expression profiling of luminal B breast cancers reveals NHERF1 as a new marker of endocrine resistance

Thomas Karn · Eugen Ruckhäberle · Lars Hanker · Volkmar Müller · Marcus Schmidt · Christine Solbach · Regine Gätje · Mathias Gehrmann · Uwe Holtrich · Manfred Kaufmann · Achim Rody

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Abstract The luminal B subtype represents a group of high proliferating estrogen receptor positive breast cancers which are associated with a poor prognosis. Genes exclusively expressed in this subtype should help to better understand these tumors. In a finding cohort of 171 breast cancers luminal B specific genes were identified strong expression in highly proliferating *Ki-67 positive/ER positive* tumors but no expression either in *Ki-67 negative/ER positive* or in *Ki-67 positive/ER negative* samples. The clinical relevance of the scaffold protein NHERF1 identified by this strategy was assessed in a total of 3,030 breast cancers. NHERF1 expression was associated with the luminal B subtype both in the finding and validation cohort. A positive correlation of NHERF1 expression with tumor size ($P < 0.001$), grade ($P < 0.001$), and HER2 status ($P = 0.033$) was observed. NHERF1 expression was associated with a worse survival in ER positive breast cancer ($P < 0.001$) and retained its prognostic value in multivariate analysis. For ER positive samples with low NHERF1 expression a benefit of endocrine therapy was detected ($P = 0.007$). In contrast no differences in disease free survival were found for high NHERF1 expressing breast cancers which were either treated with endocrine therapy or no systemic therapy. Our data indicate that NHERF1 expressing breast cancers seem to have a greater risk to develop resistance to endocrine therapy. However, based on previous findings of NHERF1 functioning in PI3K signalling from basic research, these tumors might be appropriate candidates for a targeted therapy of the PI3K/Akt pathway.

Keywords NHERF1 · SLC9A3R1 · EBP50 · Luminal B type breast cancer · Prognosis · Microarray analysis

Abbreviations

ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
NHERF1	Na/H exchanger regulatory factor 1
PDGFR	Platelet-derived growth factor receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PDZ	PSD-95/DlgA/zo-1
MERM	Merlin–ezrin–radixin–moesin
DDR1	Discoidin Domain Receptor 1

Introduction

Proliferation and differentiation are the basic principles of organogenesis and maintenance of tissue integrity. Destabilization of this balance might result in the development of cancer. Breast cancer is a paradigm for the interaction of

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T. Karn (✉) · E. Ruckhäberle · L. Hanker · C. Solbach · R. Gätje · U. Holtrich · M. Kaufmann · A. Rody
Department of Obstetrics and Gynecology, J. W. Goethe-University, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
e-mail: t.karn@em.uni-frankfurt.de

V. Müller
Department of Obstetrics and Gynecology, University Hospital Hamburg-Eppendorf, Hamburg, Germany

M. Schmidt
Department of Obstetrics and Gynecology, Gutenberg-University, Mainz, Germany

M. Gehrmann
Siemens Diagnostics, Leverkusen, Germany

hormonal influences on proliferation and differentiation. In vitro and in vivo data demonstrate that the expression of estrogen receptor (ER) in breast cancer is associated with low proliferation and a favorable prognosis of patients. Recent classification of breast cancer by gene expression profiling revealed distinct molecular subtypes with different disease outcomes [1, 2]. The HER2 negative, ER positive breast cancers can be subdivided in luminal A and luminal B subtypes which can be discriminated by expression of cell cycle genes as well as genomic grade index [1, 3]. These molecular differences between luminal A and B subtypes are associated with an impaired prognosis in breast cancer patients of the luminal B subtype [3, 4]. However, it still remains unclear if such molecular differences are causal for a distinct biology or just represent an epiphenomenon. Differentially expressed genes between luminal A and B subtypes, which are not associated with proliferation, could provide new information on molecular tumor classification as well as prediction of prognosis and treatment response [5, 6].

The adaptor protein NHERF1 (Na^+–H^+ Exchanger Regulatory Factor also named SLC9A3R1 and EBP50) is a member of a family of scaffold proteins, which are characterized by the presence of two PDZ protein interaction domains and a C-terminal domain that binds the cytoskeleton proteins merlin, ezrin, radixin, and moesin (MERM family). NHERF1, located on chromosome 17q25.1, has been shown to be estrogen induced in ER positive breast carcinoma cells [7]. The NHERF1 protein is expressed in the luminal membrane of many epithelia and was elevated in breast tumors compared to adjacent normal tissue [8]. Furthermore its expression correlates closely with the receptor status of breast carcinoma specimens [9]. NHERF1 binds to the cytoplasmic part of different types of transmembrane receptors [10–12] and in addition to members of the MERM family of actin binding proteins. Thereby the protein is involved in transmitting signals from the surface into the cell which could depend on the status of cell–cell adhesion [13, 14].

When comparing gene expression of luminal A and B tumors in a test set of 171 breast cancers we were able to identify 18 genes overexpressed in the luminal B subtype. Among them was the scaffold protein NHERF1 which was previously reported to be induced by estrogen. To analyze the prognostic impact of this gene cluster and its relationship to endocrine therapy we used a large-scale meta-analysis of microarray datasets according to NHERF1 expression.

Materials and methods

All analyses were performed according to the REMARK recommendations for tumor marker studies [15]. A respective diagram of the 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 Supplementary Fig. S3. All analyses were performed using the R software environment (http://www.r-project.org/) and SPSS version 17.0.

Breast cancer samples

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. Patients were selected for this study if they had received adjuvant endocrine therapy or chemotherapy (CMF or EC) and sufficient follow-up data of >2 years were available ($n = 171$). All tissue samples were stored in liquid nitrogen. Samples were characterized according to standard pathology including the ER status by ligand binding assays or immunohistochemistry (IHC). Isolation of RNA and expression profiling using Affymetrix Human Genome U133A microarrays was performed as described elsewhere [5]. Briefly, hybridization intensity data were automatically acquired and processed by Affymetrix Microarray Suite 5.0 software. Arrays were analyzed using MAS5 algorithm. Scans were rejected if the scaling factor exceeded 2 or "chip surface scan" revealed scratches, specks or gradients affecting overall data quality (Refiner, GeneData AG, Basel, Switzerland). MAS5 expression values were log transformed and median centered over arrays.

Identification of a luminal B specific gene cluster

For identification of marker genes specific for the luminal B subtype of breast cancers we used a test set of 171 samples (Frankfurt cohort). Samples were stratified into the following subtypes: HER2 positive, triple-negative and ER positive (luminal). The ER positive subset of samples were further subdivided into low proliferating (luminal A) and high proliferating (luminal B) tumors based on a median split according to Ki-67. Similar results were obtained when was used expression the genomic grade index (GGI) [3] as surrogate marker for proliferation (data not shown). To identify luminal B specific genes which are not just associated with proliferation we defined an artificial *Luminal B Prototype Gene* displaying a binary expression profile with expression ("+1") only in the high proliferating ER positive (luminal B) subset but not ("−1") in the even high proliferating triple-negative tumors or the low proliferating ER positive subset (luminal A). This artificial expression vector consisting of either "+1" or "−1" for

each sample was then included in an unsupervised hierarchical clustering of all probesets from the Affymetrix array to identify genes correlating with the luminal B prototype. Affymetrix probesets were median-centered and hierarchical gene clustering was done by average linkage clustering with Pearson correlation as distance metric Cluster 3.0 software [16, 17].

Centroid method to stratify molecular subtypes of breast cancer

We applied a recently published implementation of different variants of the centroid method to assign breast cancer samples to a molecular subtype [18]. Detailed information and corresponding R-code can be downloaded from the authors of this study at http://rock.icr.ac.uk/collaborations/Mackay/centroid.correlations.Eset/ExpressionSet%20Nearest%20Centroid%20Correlations.pdf. For the results presented in Fig. 2 we performed spearman rank correlations on all probes with centering using the centroids according to Hu et al. [19] downloaded from http://rock.icr.ac.uk/collaborations/Mackay/centroid.correlations.Eset/Hu306.centroids.txt. The analyses were performed independently in seven larger datasets (Frankfurt, Mainz, New York, Stockholm, Transbig, Uppsala, Rotterdam) to assign a total of 1,364 breast cancer samples to a molecular subtype.

Validation microarray dataset

We combined a database of $n = 3{,}030$ Affymetrix HG-U133A and HG-U133Plus2 microarrays from treatment-naïve primary breast cancer samples as we have previously described [20] (Supplementary Table S1). We included 238 of our own samples (datasets Frankfurt, Frankfurt-2, and Frankfurt-3) which have been described previously [5, 6, 21–23] as well as 2,792 samples from 22 different publicly available datasets (Supplementary Table S1): Rotterdam [24–26], Mainz [27], TransBIG [28], Oxford-Untreated [29], London [3], London-2 [30], Oxford-Tamoxifen, Veridex-2 [31], Stockholm [32], Uppsala [33, 34], San Francisco [35], New York [36], MDA133 [37], EORTC [38], Edinburgh [39], ExpO [40], Singapore [41], Genentech [42], Boston [43], Berlin [44], Paris [45], and Tampa [46]. For comparability only the ProbeSets from the Affymetrix HG-U133A microarray were used from seven datasets where HG-U133Plus2.0 microarrays were applied. The clinical characteristics of the patients in the different datasets are given in Supplementary Table S1. Affymetrix expression data were analyzed by using the MAS5.0 [47] algorithm of the *affy* package [48] of the Bioconductor software project [49] (http://www.bioconductor.org/). Subsequently data were $\log_2$ transformed and median centered across arrays. For further normalization the expression values of all the genes on the array were multiplied by a scale factor S so that the magnitude (sum of the squares of the values) equals 1. ER, PgR, and HER2 status were based on genes expression from microarray as we have previously described [20].

Statistical analyses

All analyses were performed according to the REMARK recommendations for tumor marker studies [15]. We did not apply any optimization of cutoffs to avoid overfitting of the data. Instead, stratification according to NHERF1 (Affymetrix probeset 201349_at) expression in the validation microarray datasets was performed using a median split separately in the ER positive and ER negative subgroups to avoid confounding effects from ER status. However, since the analyzed expression data were assembled from several different datasets still possible confounding effects could have been introduced by systematic technical differences that exist between individual datasets. A systematic bias was not observed when comparing the distributions of NHERF1 expression of samples that were profiled either on U133A or U133Plus2 arrays (Supplementary Fig. S4). Nevertheless, we still validated the survival analysis by performing the median split of NHERF1 expression also on an individual dataset basis which led to identical results (Supplementary Figs. S5 and S6). This stratification on an individual dataset basis was also used when a median split of the mean of all 27 probesets from the luminal B-like cluster was applied in the analyses in Supplementary Fig. S7 to avoid potential bias.

Follow-up data were available for 2,058 of the samples (11 datasets without follow-up, see Supplementary Table S1). Survival intervals were measured from the time of surgery. For nine datasets relapse free survival (RFS) was used as an endpoint ($n = 1{,}180$) while for five datasets only distant metastasis free survival (DMFS) was available ($n = 879$). Thus any local recurrence events are missing from these five datasets. In the conduct of the presented analysis event free survival (EFS) was calculated as preferentially corresponding to the RFS endpoint, but measured with respect to the DMFS endpoint if RFS was not available. We have previously shown [20] that the effect of using these different endpoints was rather small in the overall dataset. However, all results from survival analyses were verified by examining the effect of the different endpoints in stratified analyses. Follow-up data for women in whom the envisaged end point was not reached were censored as of the last follow-up date or at 120 months. We constructed Kaplan–Meier curves and used the log rank test to determine the univariate significance of the variables. A Cox proportional-hazards regression model was used to examine simultaneously the effects of multiple

covariates on survival. The effect of each variable was assessed with the use of the Wald test and described by the hazard ratio, with a 95% confidence interval (95% CI). Subjects with missing values were excluded from the analyses. For the analysis of endocrine systemic therapy we stratified two groups of patients: (i) Untreated patients which did not receive any systemic therapy, (ii) patients with only endocrine treatment which did not receive any type of combined chemotherapy. However, we did not control for differences in length of endocrine therapy. All patient with missing information on treatment or did receive any cytotoxic or combined therapies were excluded from this analysis. All reported *P* values are two sided and *P* values of less than 0.05 were considered to indicate a significant result.

Immunohistochemistry

For immunohistochemical detection of NHERF1 protein expression in breast tissue a monoclonal antibody directed against the human NHERF1 protein (LS-C15004, LifeSpan Biosciences Inc., Seattle, WA) was used. Paraffin sections (2 μm) were mounted on Superfrost Plus slides, dewaxed in xylene, and rehydrated through graduated ethanol to water. Antigens were retrieved by microwaving sections in 1 mM EDTA (pH 8.0) for 20 min at 800 W. Blocking was performed using antibody dilution buffer (DCS Diagnostics, Hamburg, Germany) at room temperature for 15 min. Subsequently, the antibody was diluted 1:100 individually in this buffer. Sections were incubated with antibody for 1 h at room temperature. For negative controls, the primary antibodies were replaced with phosphate-buffered saline. For secondary antibody incubations and detection, the Dako REAL Detection System Alkaline Phosphatase/RED (Dako, Glostrup, Denmark) was used following the protocol of the supplier and sections were counterstained with Mayer's hematoxylin.

Results

Microarray analysis of differentially expressed genes in the luminal B subtype breast cancers

Molecular subtypes of breast cancer according to Sorlie encompass the ER positive luminal A and luminal B subtypes as well as the Her2-like and basal-like subgroups. Luminal B tumors are characterized by high proliferation and a worse prognosis. To gain a more insight into the transcriptional characteristics of luminal B and luminal A tumors we aimed to identify luminal B specific genes which are not related to proliferation. To this end we included a theoretical class vector as a *Luminal B Prototype Gene* in unsupervised gene clustering of Affymetrix microarrays of a test set of 171 samples (Frankfurt cohort). This *Luminal B Prototype Gene* was defined by high expression in the high proliferating ER positive (luminal B) subset but no expression in the even high proliferating triple-negative basal-like subset of tumors or the low proliferating ER positive subset (luminal A). In unsupervised hierarchical clustering this *Luminal B Prototype Gene* clustered together with 27 probesets representing 18 different genes (Supplementary Table S2). As shown in Fig. 1 all of these markers were characterized by increased expression in the luminal B compared to the luminal A subgroup. However, no straight association with the proliferative activity was detectable since in contrast to proliferation markers no expression in the triple-negative subgroup was observed. Most of the 18 genes in the cluster were not known for their contribution to breast cancer. They were located on different chromosomal regions (Supplementary Table S2) suggesting that the expression profile of this cluster did not result from amplification of specific genomic regions in luminal B tumors. Among the genes was DDR1, one of two tyrosine kinase receptors for collagen [50, 51] which have been implicated in cell adhesion in cancer [52]. Another identified marker was the scaffold protein NHERF1 which has been shown to be inducible by estrogen [53] and has been implicated in signal transduction in breast cancer. NHERF has been previously reported to be expressed in ER positive breast carcinoma [9]. Thus we selected NHERF1 as a candidate gene for analysis from the cluster of luminal B specific markers.

Analysis of NHERF1 expression in a combined dataset of 3,030 breast cancer samples

To validate the high expression of NHERF1 in luminal B breast cancer two different methods for molecular subtype definition were applied. First, we used the simple definition of Hugh et al. [54] which is based on single markers (ER, PgR, HER2) to stratify the triple-negative, HER2 positive and luminal groups. The luminal subtype is further stratified into luminal A and luminal B based on the expression of the proliferation marker Ki-67. Furthermore ER positive HER2 positive tumors are also characterized as luminal B according to this method [54]. For this strategy we used previously determined cutoff values for Affymetrix gene expression measurements which we have previously shown to result in very high accuracy when compared to IHC/biochemical assay [20]. Similar results where obtained when we used the immunohistochemically determined parameters (data not shown). As shown in Fig. 2a highest expression of NHERF1 is observed in the luminal B subtype when this method was applied to the Frankfurt dataset of 171 samples. Second, we also used the centroid method

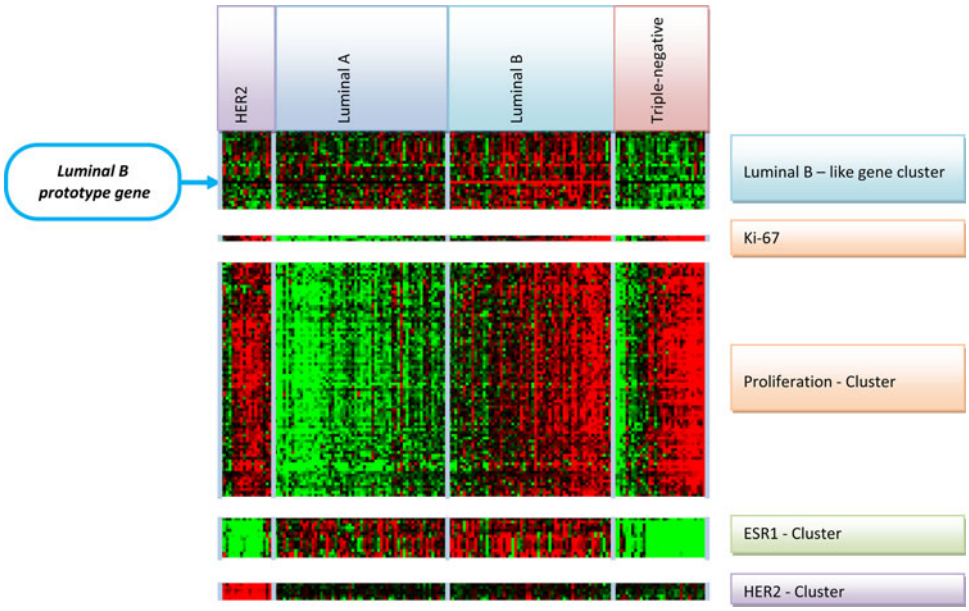

Fig. 1 Identification of a *luminal B-like* gene cluster. Heatmaps of selected gene clusters from a hierarchical clustering of Affymetrix expression data from 171 breast cancers are shown. Samples are ordered according to the molecular subtype definitions. An artificial *Luminal B Prototype Gene* displaying a binary expression profile with expression only in the high proliferating ER positive (luminal B) subset was included in the cluster process and allowed the identification of the upper *Luminal B-like gene cluster*. The 18 different genes in this cluster display an increased expression in the luminal B subtype. Heatmaps of clusters of proliferation markers, ER associated genes, and HER2 associated genes are shown below to demonstrate their difference to the *Luminal B-like gene cluster*

to define molecular subtypes from gene expression data using the intrinsic gene set according to Hu et al. [19]. As shown in Fig. 2b the same result was obtained with highest expression of NHERF1 in the luminal B subtype. Next, to validate these results in a larger sample cohort we used a combined dataset of 3,030 breast cancer samples with available Affymetrix microarray data that we have previously described [20, 55]. Again we applied both methods for molecular subtype definition while leaving out the 171 samples from our finding cohort. Figure 2c demonstrates the high expression of NHERF1 in the luminal subtype when the method of Hugh et al. [54] was used for subtype definition of the 2,859 validation samples. The centroid method was applied independently to the six largest datasets (Mainz, New York, Stockholm, Transbig, Uppsala, Rotterdam). Figure 2d displays the expression of NHERF1 among the 1,193 samples from these datasets stratified into molecular subtypes according to the centroid method. Again, highest NHERF1 expression was observed in the luminal B subtype. In summary, irrespectively of the applied method or dataset NHERF1 demonstrated highest expression in the luminal B subtype as compared to any other subgroup ($P < 0.001$, Mann–Whitney U test).

When comparing samples only based on ER status as shown in Supplementary Fig. S1 highest NHERF1 transcript levels were observed in ER positive breast cancers whereas ER negative tumors rarely express the gene. This low expression of NHERF1 in the ER negative tumors is in line with previous studies by others in breast cancer cell lines that have indicated that NHERF1 is an ER-regulated gene [53]. Similar results were obtained when we performed immunohistochemical analyses. In ten randomly selected luminal B tumor samples a strong cytoplasmic staining with an antibody raised against NHERF1 was observed while no or weak expression was detectable in all ER negative tumors (Supplementary Fig. S3). It should be noted, however, that a few tumor cells in some of the ER negative samples also express NHERF1 indicating additional ER-independent modes of NHERF1 induction.

Since NHERF1 has been shown to be regulated by estrogen the elevated expression of NHERF1 observed in luminal B subtype tumors in comparison to luminal A tumors might point to an altered endocrine responsiveness. To address this point we next analyzed the prognostic and predictive relevance of NHERF1 expression regarding endocrine therapy in the combined larger cohort of breast cancers.

Correlation of NHERF1 expression with clinical parameters in ER positive and ER negative breast cancer

We first analyzed the clinical parameters associated with NHERF1 expression. We performed separate analyses of

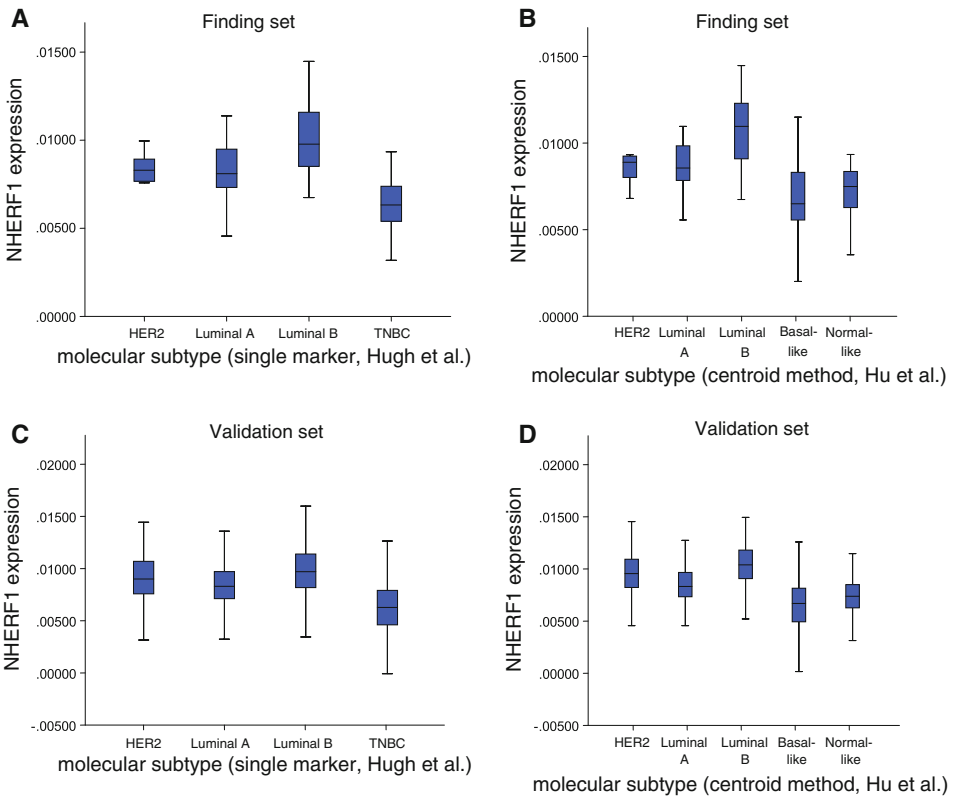

Fig. 2 High expression of NHERF1 in the luminal B subtype of breast cancer. *Box plots* of the normalized NHERF1 expression values from Affymetrix microarrays in the molecular subtypes of breast cancer are shown for 171 samples from the finding dataset *Frankfurt* (**a**, **b**) as well as 2,859 (**c**, **d**) samples from the validation datasets. The molecular subtypes were either defined by expression of single markers (ER, PgR, HER2, and Ki-67) according to the method of Hugh et al. [54] (**a**, **c**) or using the centroid method and the intrinsic gene set according to Hu et al. [19] (**b**, **d**). In (**d**) only the six largest datasets encompassing a total of 1,193 samples were used

ER positive and ER negative tumors to avoid confounding effects of the above-demonstrated association of NHERF1 expression with the ER status of the tumor. The clinical parameters of ER negative and ER positive breast cancers stratified according to high and low NHERF1 expression are presented in Table 1. In ER positive cancers high NHERF1 expression is associated with larger tumor size (χ^2-test, $P < 0.001$), node positive disease ($P = 0.024$), and poor histological grading ($P < 0.001$). Moreover, as shown in Table 1 a positive correlation of NHERF1 and HER2 expression was observed for ER negative and positive tumors. 70.1% of the ER positive HER2 positive samples and 80.4% of the ER negative HER2 positive samples were found in the group with high NHERF1 expression, respectively. In ER negative a positive association of NHERF1 expression with lymph node status and higher age.

Analysis of the prognostic and predictive value of NHERF1 in ER positive and ER negative breast cancers

Kaplan–Meier analyses of EFS according to NHERF1 expression were performed separately for the subgroups of ER positive and ER negative breast cancers. As shown in Fig. 3 a poor survival of patients with tumors displaying high NHERF1 expression was observed in the ER positive subgroup (5 years EFS $73.6 \pm 1.7\%$ vs. $81.3 \pm 1.4\%$, $P < 0.001$; Fig. 3a) while no significant difference among ER negative breast cancers was detected (Fig. 3b). Similar results were obtained when the analysis was repeated on a dataset by dataset basis to control for potential bias between individual datasets (Supplementary Fig. S5). Since the patients received different types of adjuvant therapy the poor survival in the ER positive subgroup could represent a combined measure of prognosis and endocrine response prediction. To analyze a potential predictive effect of NHERF1 expression for the response to endocrine therapy we selected the subset of 1,285 ER positive patients which were either treated with endocrine therapy only or did not receive any adjuvant systemic treatment. We then compared these two groups in separate Kaplan–Meier analyses for either tumors with low NHERF1 expression (Fig. 4a) or high NHERF1 expression (Fig. 4b). A difference in EFS as benefit from endocrine therapy was observed among the group of tumors with low NHERF1 expression (5 years EFS $85.8 \pm 2.0\%$ vs. $78.6 \pm 2.2\%$, $P < 0.009$; Fig. 4a) but not in the group with high NHERF1 expression ($P = 0.28$; Fig. 4b). Again, the corresponding analysis on a dataset by dataset basis controlling for potential bias between individual datasets led to similar results (Supplementary Fig. S6).

Table 1 Correlation of NHERF1 expression with clinical parameters in ER positive and ER negative breast cancer

Parameter (total $n = 3,030$)	ER positive ($n = 2,158$)			ER negative ($n = 872$)		
	Low NHERF1	High NHERF1	*P*-value	Low NHERF1	High NHERF1	*P*-value
Lymph node status ($n = 2,284$)						
LNN	631 (53.6%)	547 (46.4%)	0.024	252 (58.7%)	177 (62.3%)	0.001
LNP	231 (47.4%)	256 (52.6%)		83 (43.7%)	107 (56.3%)	
Age ($n = 1,672$)						
>50 years	415 (49.5%)	424 (50.5%)	n.s.	114 (45.6%)	136 (54.4%)	0.006
≤50 years	196 (52.5%)	177 (47.5%)		123 (58.6%)	87 (41.4%)	
Tumor size ($n = 1,834$)						
≤2 cm	310 (57.7%)	327 (42.3%)	<0.001	70 (51.1%)	67 (48.9%)	n.s.
>2 cm	362 (44.9%)	445 (55.1%)		185 (52.4%)	168 (47.6%)	
Histological grading ($n = 1,837$)						
G3	123 (36.4%)	215 (63.6%)	<0.001	188 (53.6%)	163 (46.4%)	n.s.
G1–2	532 (52.9%)	473 (47.1%)		66 (46.2%)	77 (53.8%)	
PgR ($n = 3,030$)						
Positive	781 (51.0%)	749 (49.0%)	n.s.	92 (45.1%)	112 (54.9%)	n.s.
Negative	298 (47.5%)	330 (52.5%)		343 (51.3%)	325 (48.7%)	
HER2 ($n = 3,030$)						
Positive	46 (29.9%)	108 (70.1%)	<0.001	49 (19.5%)	202 (80.5%)	<0.001
Negative	1,033 (51.5%)	971 (48.5%)		386 (62.2%)	235 (37.8%)	

Separate median splits were performed among ER positive and ER negative tumors to avoid confounding effects of the association of NHERF1 expression with ER status

Multivariate Cox regression analysis

In univariate analysis NHERF1 displayed a hazard ratio (HR) of 1.49 (95% CI 1.23–1.80, $P < 0.001$) for disease recurrence in the ER positive sample cohort. To compare the prognostic value of NHERF1 with standard parameters (tumor size, nodal status, grading, age, and HER2 expression) a multivariate Cox regression analysis was performed using $n = 786$ patients with ER positive tumors for which all the parameters were available. The result of this analysis is presented in Table 2. NHERF1 emerged as the strongest prognostic marker for disease free survival (HR 1.37, 95% CI 1.05–1.79, $P = 0.02$) beside tumor size (HR 0.55, 95% CI 0.41–0.74, $P < 0.001$). Further addition of Ki-67 expression, however, led to loss of significance of NHERF1 in the multivariate Cox analysis among ER positive tumors ($P = 0.15$, Supplementary Table S5). This result was not unexpected since the cluster of luminal B-like genes is per definition strongly correlated to Ki-67 expression among ER positive tumors. Nevertheless when the mean of all 27 probesets from the luminal B-like gene cluster was used as a metric to replace the single measurement of NHERF1 it retained significance in the corresponding analysis ($P = 0.015$, Supplementary Table S6).

Discussion

Proliferation is a major hallmark of all cancers and is generally associated with poor prognosis. But not all clinical observations might be explained by proliferation alone. It has been suggested that high proliferating ER positive luminal B breast cancers have a worse outcome than ER negative tumors especially in the long run. The observation that expression of markers associated with proliferation (e.g., Ki-67 or the genomic grade index GGI) is even higher in ER negative cancers suggests that proliferation alone might not be the sole reason for this poor prognosis. In our study a comparison of luminal B tumors to other subgroups revealed a cluster of 18 genes which were specifically expressed in this subgroup of breast cancers. Previous studies reported that amplification of genes could be more frequent in luminal B tumors [56]. However, while some of the genes in this cluster have been previously shown to be amplified in breast cancer, most of the genes were located on different chromosomal regions (Supplementary Table S2) suggesting that the expression profile of this cluster did not result from amplification. The expression of the genes in this cluster is strongly correlated. When a combined expression metric from all 27 probesets of the cluster was used we obtained similar results as we

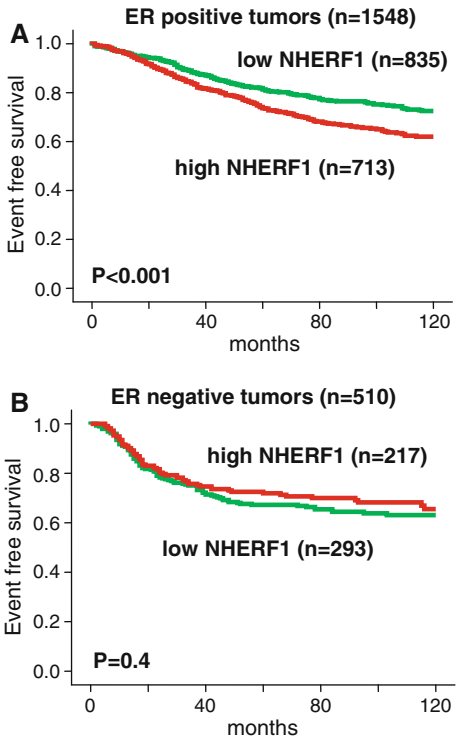

Fig. 3 Survival analysis in ER positive and ER negative breast cancers according to NHERF1 expression. Kaplan–Meier analyses of event free survival according to the expression of NHERF1 were performed separately in the ER positive (**a**) and ER negative (**b**) subgroups of breast cancers. A prognostic value of NHERF1 expression was observed among ER positive tumors only ($P < 0.001$)

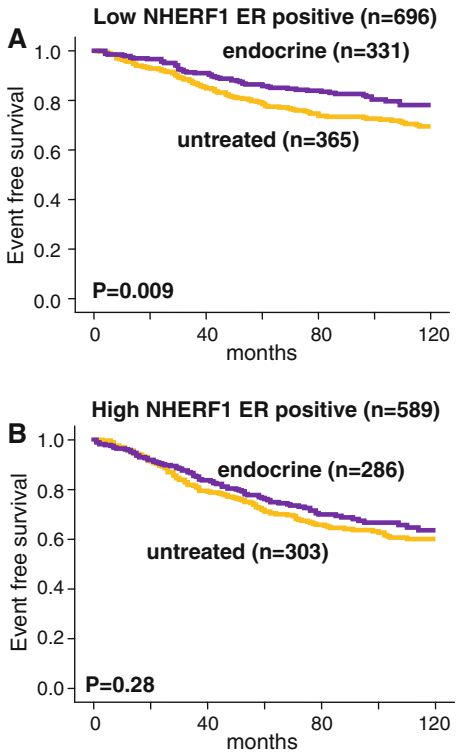

Fig. 4 Predictive value of NHERF1 for response to endocrine treatment in ER positive breast cancer. ER positive breast cancer patients were analyzed for EFS according to treatment with endocrine therapy. Kaplan–Meier analyses were performed separately in the *low NHERF1* (**a**) and *high NHERF1* (**b**) subgroups of tumors. A difference in EFS between endocrine treated patients and patients without systemic treatment ($P = 0.009$) was observed only in the subgroup with low NHERF1 expression

had observed for NHERF1 (Supplementary Fig. S7). Such a metric could even improve prognostic power as also would an optimization of cutoff values. However, the aim of our study was not to derive a complex prognostic signature but to get hints on differences between luminal A and luminal B tumors beyond their obvious differences in proliferative activity. Since it is well known that proliferation is one of the most important prognostic factors it is also not surprising that adding Ki-67 to the multivariate analysis of NHERF1 led to the loss of significance of NHERF1 expression (Supplementary Table S5; while the complete luminal B-like cluster still remained significant, Supplementary Table S6). However, in contrast to NHERF1 and the full cluster, Ki-67 expression as proliferation marker had no significant predictive value for the response to endocrine treatment in Supplementary Fig. S7 (panels F and G).

One prominent marker within the gene cluster previously reported for its expression in ER positive breast cancer was NHERF1 (sodium-hydrogen exchanger regulatory factor 1) located on chromosome 17q25.1. This adapter protein is involved in the anchoring and regulation of ion channels and different types of receptors to the actin cytoskeleton through binding to ERM (ezrin/radixin/moesin) proteins. It assembles macromolecular complexes at the apical membrane of epithelial cells in many epithelial tissues, including the mammary gland. NHERF1 has been shown to control the localization and regulation GPCRs [10] and tyrosine kinase receptors as PDGFR and EGFR [11] and it couples them with other signalling molecules such as PTEN [12]. Recent evidence obtained from different laboratories demonstrated that NHERF1 is an important player in cancer progression [14].

The positive association of NHERF1 mRNA expression with lymph node involvement and poor histological grading that we have observed is in line with results from western blot analysis of NHERF1 protein expression [8]. In addition an immunohistochemical study of 149 breast cancer cases have demonstrated that NHERF1 mRNA expression correlates with protein expression and an elevated cytoplasmic accumulation of NHERF1 was associated with tumor stage, as well as lymph node and ER status [57]. Some studies have suggested that NHERF1 expression promotes an invasive phenotype in breast cancer cells [8]. Moreover, in glioblastoma multiforme (GBM) tumors increased expression was specific for highly invasive cells

Table 2 Multivariate Cox regression analysis of NHERF1 expression and standard parameters among ER positive tumors

Parameter	$n = 786$	HR	95% CI	P-value
NHERF1 (high vs. low)	361 vs. 425	1.37	1.05–1.79	**0.020**
Lymph node status (LNN vs. N1)	505 vs. 281	0.82	0.63–1.07	0.15
Patient age (>50 years vs. ≤50 years)	532 vs. 254	0.84	0.64–1.11	0.22
Histological grading (G3 vs. G1–2)	186 vs. 600	1.30	0.97–1.73	0.075
Tumor size (≤2 cm vs. >2 cm)	340 vs. 446	0.55	0.41–0.74	**<0.001**
HER2 status (pos. vs. neg.)	47 vs. 739	1.48	0.93–2.35	0.097
PgR status (pos. vs. neg.)	597 vs. 189	0.86	0.64–1.14	0.30

Significant P-values are given in bold

that reside in the rim of tumors and depletion of NHERF1 arrested migration of glioblastoma cells [58]. In contrast, earlier studies suggested that NHERF1 is a tumor suppressor gene in breast cancer [59]. It appears that, depending on its subcellular distribution, NHERF1 may behave either as a tumor suppressor, when it is localized at the plasma membrane, or as an oncogenic protein, when it is shifted to the cytoplasm [14]. Recent data from Fouassier et al. indicate that both the expression and distribution of NHERF1 are regulated by estrogens and contribute to the proliferative response in epithelial cells [60]. Mangia et al. demonstrated that in the membrane NHERF1 was colocalized with overexpressed HER2. Interestingly we also detected a positive association between NHERF1 expression and HER2 status in our study (Table 1). This result is in line with the concept of others to include ER positive HER2 positive tumors in the definition of luminal B cancers [54]. Breast cancerogenesis was characterized by increased cytoplasmic expression of NHERF1 as the tumor progresses. In metastatic lymph nodes the cellular distribution of NHERF1, however, was exclusively cytoplasmic [61]. They concluded that the switch from apical membranous to cytoplasmic expression is compatible with a dual role for NHERF1 as a tumor suppressor or tumor promoter dependent on its subcellular localization. Our exemplary immunohistochemical analyses of NHERF1 also revealed a cytoplasmatic localization in the tumor cells while in normal breast tissue an apical localization was detected (Supplementary Fig. S3).

These results would be in accordance with a model where either loss of NHERF1 as a scaffold at the membrane or titration of other components by overexpression of NHERF1 in the cytoplasm could disrupt inhibitory complexes which under normal circumstances are present at the cell membrane. These differences in localized NHERF1 expression between tumors could be of high clinical importance in light of findings that NHERF1 expression confers susceptibility to PDGFR pharmacological inhibition by STI-571 (gleevec) depending on the presence of PTEN [62, 63]. These findings were in line with previous studies demonstrating that normal NHERF1 at the membrane inhibits PI3K signalling by forming a bridge between PDGFR and PTEN. According to the proposed model mentioned above one would suggest that either loss or overexpression of NHERF1 would result in an activated Akt pathway. In a very recent article Creighton et al. [64] have identified a gene signature for activation of the PI3K/Akt pathway from proteomic as well as gene expression analysis. When we used the corresponding gene signature to score the activity of the PI3K/Akt pathway we found higher values in ER positive tumors with high NHERF1 expression. The same result was obtained when luminal B tumors were compared to luminal A samples (Supplementary Fig. S2).

In conclusion we have identified a cluster of genes which was exclusively expressed in the luminal B subtype of breast tumors. Even though detailed information on systemic treatment is available only for a subset of patients our results suggest that overexpression of NHERF1, an adaptor protein found in this cluster, defines a subgroup of ER positive tumors which seem to have a greater risk to develop resistance to endocrine therapy. Data from basic research on this protein might even allow speculations if those tumors could be the appropriate candidates for a targeted therapy of the Akt pathway, e.g., by PI3K inhibitors such as BEZ-235.

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Conflict of interest There are no conflicts of interest to declare by the authors.

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