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NHERF1 expression levels in blood and tissue predict breast tumor clinical behaviour

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Abbreviations: NHERF1, Na⁺/H⁺ exchanger regulatory factor 1; NPI, Nottingham Prognostic Index; IHC, immunohistochemistry; ER, estrogen receptor; PR, progesterone receptor; MIB-1, proliferative marker; RPM, revolutions per minute; PBS, phosphate-buffered saline; TBS, tris-buffered saline; SDS, sodium dodecyl sulphate;

Abstract

Aims: Several studies have demonstrated that NHERF1 protein, over-expressed and heterogeneously distributed in different stages of breast cancer, could be used as a tumor marker for prognosis in molecular detection strategies. We observed that tumor infiltrated lymphocytes in the tumour tissue display a high NHERF1 staining in contrast with those present in the contiguous non involved tissue. Hypothesizing that cancer cells elicit a specific T cell response associated with the characteristics of the solid tumor, we evaluated NHERF1 in peripheral lymphocytes from healthy donors and breast cancer patients.

Method and results: NHERF1 levels were analyzed in 55 breast cancer patients and 40 healthy donors and compared these levels with clinical pathological features. NHERF1 was over-expressed in circulatory peripheral lymphocytes from patients compared to those from healthy subjects. Furthermore, in both circulatory lymphocytes and tissues, NHERF1 was positively associated with tumor grade, Nottingham Prognostic Index and estrogen receptor, while there was no association with other clinical parameters in either tissue.

Conclusions: We propose that NHERF1 measurements in circulatory lymphocytes of breast cancer patients may be a valid method to predict breast cancer occurrence and prognosis and may have value in the management of cancer patients.

Introduction

Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1, also known as EBP50) is a 358 residue protein comprised of two tandem PDZ domains and a C-terminal EB region,¹ and functions as a molecular scaffold that coordinates the interaction of many transmembrane proteins and cytosolic second messenger cascades,^{2,3} many of which are involved and related to cancer progression⁴ such as PTEN,⁵ NF2 tumor suppressors,⁶ β -catenin,⁷ PDGFR⁸ and EGFR.⁹ These functions have raised interest in NHERF1 as a potentially attractive target for new clinical approaches in tumor patients. NHERF1 expression in human cancers has been described, in general, to change with progression in both level and in altered intracellular distribution.¹⁰⁻¹⁶ In particular, studies utilizing Western Blot (WB) analysis¹² and immunohistochemistry (IHC)¹³ of a series of tumor and contiguous non-involved breast tissues from the same patient have clearly demonstrated that NHERF1 protein is highly over-expressed in breast tumor tissues and that, furthermore, this over-expression is associated with increasing aggressive clinical characteristics and with poor prognosis. We have further observed a heterogeneous and different distribution of NHERF1 expression in normal breast, *in situ* and invasive tumors, metastatic lymph node and distant metastases.¹⁷ Indeed, cytoplasmic NHERF1 protein expression progressively increased in tumor cells from ductal carcinoma *in situ* to invasive and metastatic tissues and this increased cytoplasmic expression was paralleled by a progressive and significant decrease in membranous NHERF1 expression. Overall, these data have galvanized some to suggest that NHERF1 may be useful as a marker of clinical relevance in breast cancer patients.

Many epidemiologic, preclinical, and clinical studies have demonstrated that the functioning of both the innate and the adaptive immune systems play a role in breast cancer aetiology.¹⁸ Recent advances in genetics and molecular tumor biology¹⁹ underline the impaired immunosurveillance induced by tumor and host dependent mechanisms, involving cytokines and growth factors that primarily mediate the tumor growth and regulate the interaction among tumor cells, tumor stroma and tumor-infiltrating lymphocytes. During tumorigenesis adaptive immune cells, such as lymphocytes, distinguish themselves from innate leucocytes by expression of diverse, somatically generated, antigen-specific receptors exerting multiple effects or functions that are

continually fine-tuned as tissue microenvironments are altered.^{20,21} Moreover, the peripheral blood immune status of patients with primary breast cancer has been analysed in comparison with healthy donors to investigate the expression of immunoregulatory antigens²² and gene-expression²³ in peripheral blood lymphocytes. The different lymphocytic subpopulations identified leads to the hypothesis of systemic immunosuppression, which could open the door for tumor cell dissemination via the blood stream, but also, in line with this view, that peripheral blood can be used to develop a gene/protein expression based test for easy detection of cancer antigens.

Hypothesizing that breast cancer cells elicit a specific T cell response associated with and dependent upon the characteristics of the solid tumor, we quantitatively evaluated NHERF1 expression in circulatory peripheral lymphocytes and matched tissue from patients with diagnosed breast cancer in relation to the main clinicopathological characteristics of the disease to determine its utility as a progression and/or prognostic tumor marker in molecular detection strategies.

Here, we present results comparing the levels of NHERF1 expression with clinical parameters in: the contiguous, non-tumor compartment of the tumor tissue and the tumor compartment, the metastatic lymph node, and the peripheral blood of a group of 55 breast cancer patients. Moreover, in the peripheral blood compartment, we analyzed the differences between these breast cancer patients and a healthy control group.

Materials and methods

PATIENTS

Blood samples were collected from 55 patients with a first diagnosis of primary breast cancer, histologically confirmed at the Pathology Department of our Institute. Among them, 45/55 samples frozen tumour tissue, 24/55 contiguous non tumour frozen tissues and 12/21 frozen lymph nodes were used for NHERF1 protein expression analyses. Moreover, sections from paraffin-embedded specimen were also selected and processed for IHC. Blood samples from a group of 40 healthy donors were also collected. These healthy donors were all female with an age and menopause distribution not significantly different from the patients.

The study was performed with the approval of the Ethics Committee of our Institute. Each individual involved in the study signed an informed consent authorizing the Institute to utilize their blood and removed biological tissues for research purpose. Each patient was staged according to the International Union Against Cancer TNM classification.²⁴ Histological grade was performed according to the Elston and Ellis method.²⁵

The Nottingham Prognostic Index (NPI), combining tumor size, lymph node status and histological grade information,²⁶ has been demonstrated to be able to group patients into prognostic categories^{24,25}: a group with a good prognosis (GPG, NPI values <2.5); with a moderate prognosis (MPG, NPI = 2.5–3.5) and with a poor prognosis (PPG, NPI >3.5). The NPI was utilized to score each patient in this study according to their prognostic group.

Estrogen receptor (ER) and progesterone receptor (PR) expression was determined by immunohistochemical assays and categorized as positive or negative cases according to the cut-off value of 10% of positive immunostained cells.²⁷ Tumor proliferative activity (MIB-1 labeling index) was determined as the percentage of tumor cells expressing the growth-related Ki67 antigen by immunohistochemical assay.²⁷

SEPARATION OF PERIPHERAL CIRCULATORY LYMPHOCYTES

To isolate peripheral blood lymphocytes, diluted anti-coagulated whole blood was layered over Ficoll-HypaqueTM and centrifuged 30 min at 1800 revolutions per minute (RPM), over a step gradient consisting of a mixture of the carbohydrate polymer FicollTM and the dense iodine-

containing compound metrizamide. Red blood cells, polymorphonuclear leukocytes and granulocytes centrifuge through the Ficoll-HypaqueTM, while mononuclear cells consisting of lymphocytes, together with some monocytes, band over it and were recovered at the interface. Lymphocytes were then purified by washing the sample with phosphate-buffered saline (PBS) and centrifuging 2 times at 1200 RPM for 10 min.

SAMPLE PREPARATION AND WESTERN BLOTTING

For the analysis of protein expression, frozen tissues and lymphocytes were first homogenized in a homogenization buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 5 mM; ethylene-diamine-tetra-acetic acid 0.5 mM, pH 7.2), in which 2 μ L of protease inhibitor cocktail per ml of buffer was added. An aliquot of 30 μ g of total protein was then heated at 100°C in sodium dodecyl sulfate (SDS) sample buffer (6.25 mM Tris-HCl, pH 6.8, containing 10% glycerol, 3 mM SDS, 1% 2-mercaptoethanol and 0.75 mM of Bromophenol Blue) and separated by 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto Immobilon P (Millipore) for immunoblotting. The membrane was first washed for 30 min with tris-buffered saline (TBS) containing 0.1% Tween 20 and was then blocked with 5% dried fat-free milk in TBS for 1 h followed by washing twice with TBS. The blocked membrane was incubated with the primary antibody overnight at 4°C followed by three washes with TBS. NHERF1 protein expression was analyzed using an over-night incubation with a goat monoclonal antibody against NHERF1 (1:250, BD Biosciences Transduction Laboratories, Lexington, KY). The membrane was incubated with mouse anti-goat IgG (1:2000, Cell Signaling, MA, USA) for 1 h, washed 3 times with TBS and subsequently developed with enhanced luminol-based chemiluminescent substrate (ECLTM Plus, GE Healthcare UK, Buckinghamshire HP7 9NA, UK).

NHERF1 protein expression was quantified by densitometry in which the blot film was scanned with an imaging scanner (Epson, Milan, Italy) and analyzed with ImageJ imaging software (National Institutes of Health Bethesda, MD). After Western analysis, proteins bound to the membrane were stained by Coomassie Blue to confirm that identical amounts of protein had been transferred. To quantify protein levels, the relative NHERF1 optical density of lymphocytes was related by densitometric analysis to that of the breast cancer cell line, MCF7, which was set as 1.

IMMUNOHISTOCHEMISTRY

Immunoreactivity of NHERF1 was performed by IHC on specimens from the same patients, as previously described.¹⁷

Briefly, paraffin embedded tissue sections were incubated with rabbit polyclonal antihuman EBP50 antibody (1:150, clone PA1-090, Affinity Bioreagents, Golden, CO, USA) overnight at 4°C. After incubation, specimens were washed with 1 × PBS, incubated with biotinylated link for 30 min, peroxidase-labelled streptavidin for 30 min, and 3-amino-9-ethylcarbazole substrate-chromogen (Labelled Streptavidin-Biotin2 System-Horseradish Peroxidase; DakoCytomation, Glostrup, Denmark) for 15 min in the dark. After PBS washing, slides were counterstained with haematoxylin and mounted with aqueous mounting medium (DakoCytomation). For negative controls, the primary antibody was omitted and replaced by PBS.

STATISTICAL ANALYSIS

The Kruskal-Wallis non-parametric ANOVA test was applied to analyze NHERF1 expression between different NPI stages while the Mann-Whitney non-parametric test was applied to age, menopausal status, tumor size, nodal status, grade. Correlation analysis of NHERF1 expression with ER, PR, MIB-1 expression was performed with the Spearman-Rank non-parametric test. All comparisons were performed with InStat (GraphPad Software, San Diego, CA).

Results

NHERF1 EXPRESSION IN NON TUMOR AND TUMOR TISSUE COMPARTMENT

Relative NHERF1 protein expression was first measured in Western Blot (WB) in all of the 55 frozen tumours and in contiguous, non involved tissue from 24 of these samples and in frozen metastatic lymph nodes from 12 of the same patients (Figure 1A). We found that with respect to non tumor compartment, NHERF1 protein is highly over-expressed both in primary tumours, as previously reported,¹² and in metastatic lymph nodes, where generally it was even more highly

expressed (Figure 1B).

Figure 2 shows a representative IHC in a paraffin embedded tumor tissue from a breast cancer in which, as we previously demonstrated,¹⁷ the immunolocalization of NHERF1 varies with the patho-physiological characteristics of the tissue. NHERF1 expression was limited to the apical membrane regions in the normal lobules in the organized, non involved part (Figure 2A), while NHERF1 was also expressed in the cytoplasm in tumor region (Figure 2B). Moreover, it is possible to observe the inflammatory component of the tissue with lymphocytes dispersed in both the non tumor and tumor stroma (arrow). Importantly, the majority of the tumor infiltrated lymphocytes (Figure 2B) displayed a very high NHERF1 staining while the rare lymphocytes scattered in the stroma of the contiguous, non involved tissue were not marked for NHERF1. Immunostaining for NHERF1 in a metastatic lymph node (C) confirmed the intense immunoreactivity of the lymphocytes present in the lymph node stroma. Lastly, an intense NHERF1 reactivity was also detected in lymphocytes present in the peritumoral blood vessels (Figure 2D).

These observations confirmed that lymphocytes in tumor areas have a higher NHERF1 expression than those in non-tumor tissues and constitute a 'Proof of Principle' for the study of the association of circulatory lymphocyte NHERF1 expression as a possible diagnostic and prognostic marker.

NHERF1 EXPRESSION IN PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood lymphocytes obtained from the 55 tumor patients and from 40 healthy donors were separated and analyzed for their NHERF1 protein expression in WB using the same anti-NHERF1 antibody. Figure 3A illustrates a typical WB of NHERF1 expression: where it can be seen that the level of NHERF1 expression in peripheral blood lymphocytes from patients is significantly higher than in the healthy group (Figure 3B).

Intriguingly, comparing NHERF1 expression in the various tumor compartments and patients lymphocytes, we observed that the level of NHERF1 expression in the patient circulatory lymphocytes is higher than the non tumor tissue expression (Figure 1B), and significantly lower than the lymph node expression. No significant difference was observed between the NHERF1 level of expression of the tumor and lymphocytes compartments.

CLINICOPATHOLOGICAL ASSOCIATION OF NHERF1 EXPRESSION IN TISSUE AND LYMPHOCYTE

The next question was whether the over-expression of NHERF1 in patient tumor tissue and circulatory lymphocytes are associated with the same clinicopathological characteristics (Table 1). When analyzed in both tumor tissues and lymphocytes according to histological grade: patients with Grade 3 (poorly differentiated) expressed significantly more NHERF1 expression than did patients with Grade 1 (highly differentiated).

We next determined whether the levels of NHERF1 over-expression were associated with the same clinicopathological characteristics in patient tumor tissue and circulatory lymphocytes (Table 1). As can be seen, NHERF1 over-expression in both the tumour tissues and circulatory lymphocytes was significantly associated with both histological grade and with the whole prognosis indicator for operable breast cancer patient, the Nottingham Prognostic Index (NPI).^{24,25} Indeed, as seen in Figure 4, the correlation of NHERF1 expression with respect to NPI significantly increased with increasing NPI in both tissues and lymphocytes ($Y = 0.205x + 2.769$, $r^2 = 0.1826$ vs. $Y = 0.4646x + 2.133$, $r^2 = 0.2538$, in tissues and lymphocytes, respectively). With both measurements, higher NHERF1 expression was associated with more aggressive characteristics.

Moreover, there was a significant correlation ($Y = 8.190x + 53.25$, $r^2 = 0.1915$) between the levels of lymphocyte NHERF1 expression and tissue ER levels, in the ER+ patients (Figure 5). No significant correlation was found in either tissue or lymphocyte NHERF1 expression with either menopausal status, lymph node status or MIB-1 expression. Lastly, while not significant, there were trends of a higher NHERF1 expression being associated with greater tumor size and no PgR expression.

Discussion

Data from a number of laboratories have clearly shown that increased NHERF1 expression in the primary tumor is of clinical significance in that NHERF1 protein expression is increased in tumors compared to the contiguous not involved tissue and that the relative level of increased NHERF1 protein was significantly associated with a more aggressive phenotype and a poor

prognosis.^{12,17} Recent epidemiologic, preclinical, and clinical studies have underlined that the immune profile of tumor-draining lymph nodes is of biologic and clinical importance for patients with breast cancer^{37,38}, suggesting that NHERF1 could also be over-expressed in involved lymph nodes of breast cancer patients. For this reason, here we have further compared the NHERF1 protein expression levels, by WB analysis, of the metastatic lymph node respect to the primary tumor and the contiguous not involved tissue of the same patients. We observed that NHERF1, indeed, is generally even more highly over-expressed in the metastatic lymph node. These data are in line with the very recent study of the immunolocalization of NHERF1 in the tumor and non tumor compartment¹⁷ and these have further confirmed the typical localization of NHERF1, as previously demonstrated,¹² with an heterogeneous distribution of NHERF1 expression in different stages of the disease.

Further, the IHC data (Figure 2) shows that the lymphocytes dispersed in the stroma around tumor lobules and those present in the peritumoral blood vessels were intensely stained for NHERF1, while those dispersed in well organized, normal lobules expressed almost no NHERF1, suggesting that NHERF1 expression could also be important in the immunoresponce to the tumor.

Recent advances in genetics and molecular tumor biology underline the impaired immunosurveillance induced by tumour. Host dependent mechanisms, such as growth factor and cytokine production, primarily mediate the tumor growth and regulate the interaction among tumor cells, tumor stroma, and tumor-infiltrating lymphocytes,¹⁹ and that both the innate and the adaptive immune system may have a causal role in breast cancer etiology.^{18, 28-30}

In this line, it has been suggested that circulating leukocytes can be viewed as scouts, continuously maintaining a vigilant and comprehensive surveillance of the body for signs of infection or other threats,³¹⁻³⁵ including cancer.³⁶ Indeed, it has been suggested that tumor-infiltrating leukocytes are useful to predict response to neoadjuvant chemotherapy in patients with breast carcinoma.²⁹

Based on these observations and on a recent evidence that underlines a specific immunological response detected in the peripheral blood of breast cancer patients,³⁵ we analyzed NHERF1 protein expression levels in extracted peripheral blood lymphocytes: and we observed that there is a response in these cells to the levels of NHERF1 expression in the tumour. Indeed,

lymphocyte NHERF1 levels were significantly higher in patients with breast cancer compared with the NHERF1 levels in the control group, strongly suggesting the opportunity to validate NHERF1 expression in circulatory lymphocytes as a diagnostic marker in breast cancer patients.

Further, the lymphocyte NHERF1 expression directly reflects the NHERF1 expression levels observed in tumor tissues from the same patient as the level of NHERF1 expression in circulatory lymphocytes is higher than non tumor tissue expression; while no significant difference was observed between the NHERF1 level of expression of the tumor and lymphocytes compartments.

These results confirm the evidence for the implication of NHERF1 protein in immunologic events associated with neoplastic diseases. The most interesting observation is that this is the first report of a protein identically expressed in peripheral blood lymphocytes and tumor tissue of patients, and which underlines the association between NHERF1 expression in lymphocytes and clinicopathological factors, such as histological grade, in tumor tissues. These observations may provide important clues regarding the pathogenesis of this disease.

Various studies have analyzed the importance of histological grade as a prognostic factor in carcinoma of the breast.^{26,39,40} Accurate grading of invasive breast carcinomas is extremely important; in our study we have also considered another prognostic tool widely adopted in breast cancer⁴¹, the NPI. It was constructed for patients with primary operable breast cancer, based on three factors (tumor size, tumor grade and nodal status) and this index is useful in accurately predicting prognosis and for use in adjuvant therapies to improve the survival rates of breast cancer patients.⁴² Using NPI index we observed that tissue and lymphocyte NHERF1 protein expression was significantly higher in PPG tumors compared to GPG tumors.

Moreover, in line with previous reports,^{11,13,12,36} we also observed a strong association of lymphocyte NHERF1 expression with ER positive tumors. The presence of high levels of ER in benign breast epithelium signifies an increased risk of breast cancer, suggesting a role for ER in breast cancer initiation, promotion and progression.⁴³ In breast epithelial cells, ER activation promotes cell division and contributes significantly to breast cancer progression, by inducing proliferation and invasion.^{44,45,46} Interestingly, the NHERF1 gene has been found to be transcriptionally regulated by estrogen:^{36,47} the ER, as a nuclear transcription factor, interacts directly with the Estrogen Responsive Element in order to initiate NHERF1 transcription.³⁶ Based

on these observations, as the ER is currently being used, NHERF1 expression levels in lymphocytes might be useful as a predictive marker in ER+ breast cancer treatments.

In conclusion, our results confirm the implication of NHERF1 protein in immunologic events associated with neoplastic diseases, and underline the relationship between NHERF1 expression in lymphocytes and clinicopathological factors such as histological grade, NPI and ER in tumor tissues from the same patients. The measurement of NHERF1 protein levels in circulatory lymphocytes has the possibility to extend the uses of NHERF1 as tumor biomarker in breast cancer, supporting our previous data.¹⁷

For Peer Review

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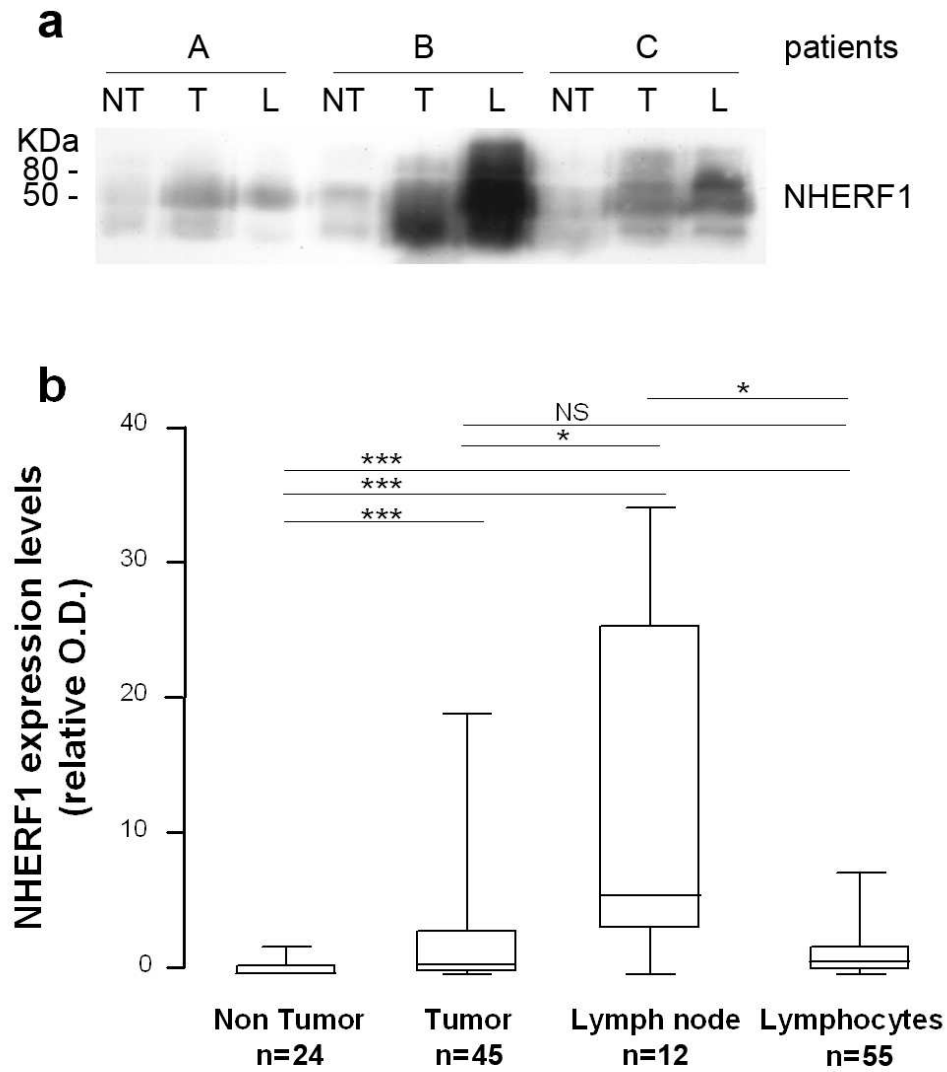
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Table 1. Clinicopathological associations of NHERF1 protein expression levels in tumor tissue and lymphocytes.

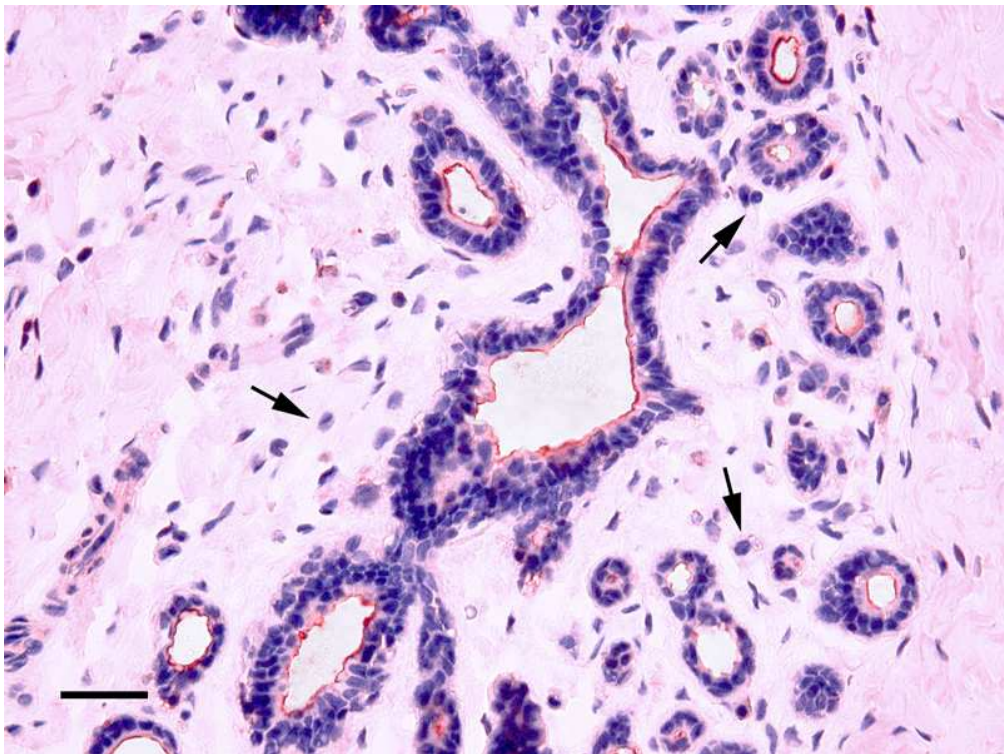
Clinicopathological characteristics		Tumor tissues			Lymphocytes		
		<i>n</i>	NHERF1 expression	<i>P</i> -value	<i>n</i>	NHERF1 expression	<i>P</i> -value
Age (years)	≤55	17	0.74	NS	31	0.58	NS
	>55	26	0.58		24	0.97	
Menopausal status	pre	11	0.62	NS	23	0.52	NS
	post	32	0.73		32	1.03	
Lymph node status	negative	22	0.93	NS	24	0.89	NS
	positive	21	0.86		31	0.76	
Tumor size (cm)	≤2	13	0.51	NS	25	1.06	NS
	>2	30	0.81		30	0.74	
Histological Grade	1	9	0.23	0.0001	20	0.58	0.0017
	2	17	0.69		24	1.00	
	3	17	3.14		11	2.5	
Hormonal receptor status	ER-	16	0.74	NS	22	0.87	NS
	ER+	27	0.71		33	0.92	
	PgR-	24	1.01	NS	28	0.91	NS
	PgR+	19	0.59		27	0.82	
Proliferation Index	MIB1-	20	0.72	NS	24	0.64	NS
	MIB1+	23	0.71		31	0.98	
NPI	GPG	7	0.41	0.0206	13	0.85	0.0005
	MPG	13	0.75		17	0.95	
	PPG	23	1.74		25	1.7	

Data are presented as median. All other values represent number of patients. The *P*-value was calculated using the Kruskal-Wallis non-parametric ANOVA test and Mann-Whitney non-parametric test. Differences with *P* < 0.05 were considered to be significant. NS, not significant.

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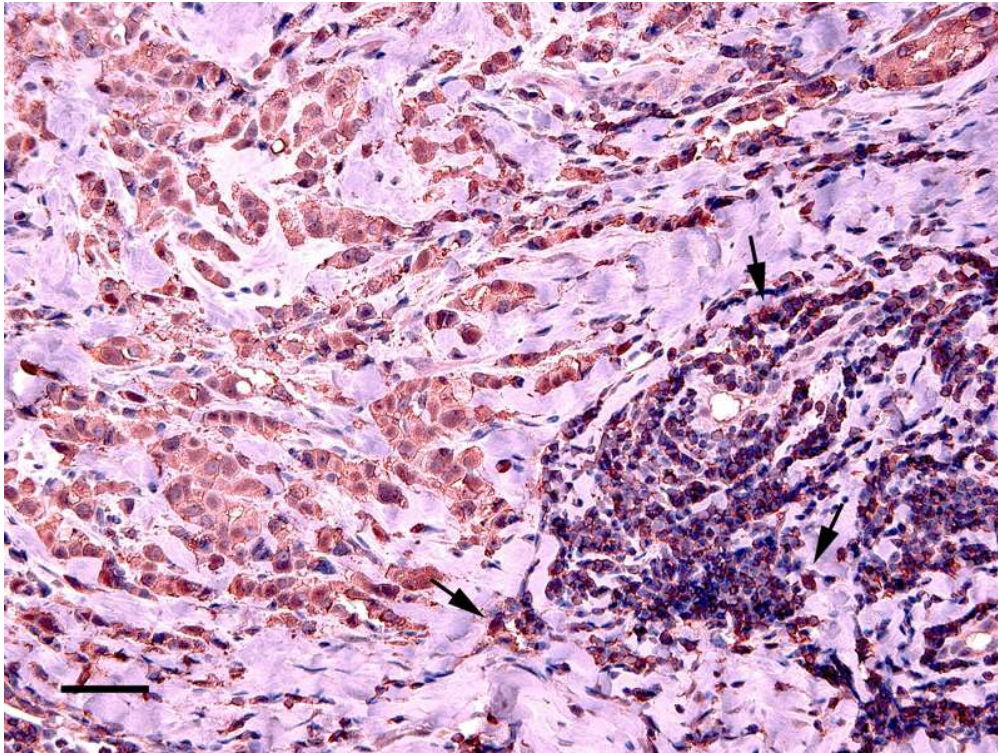


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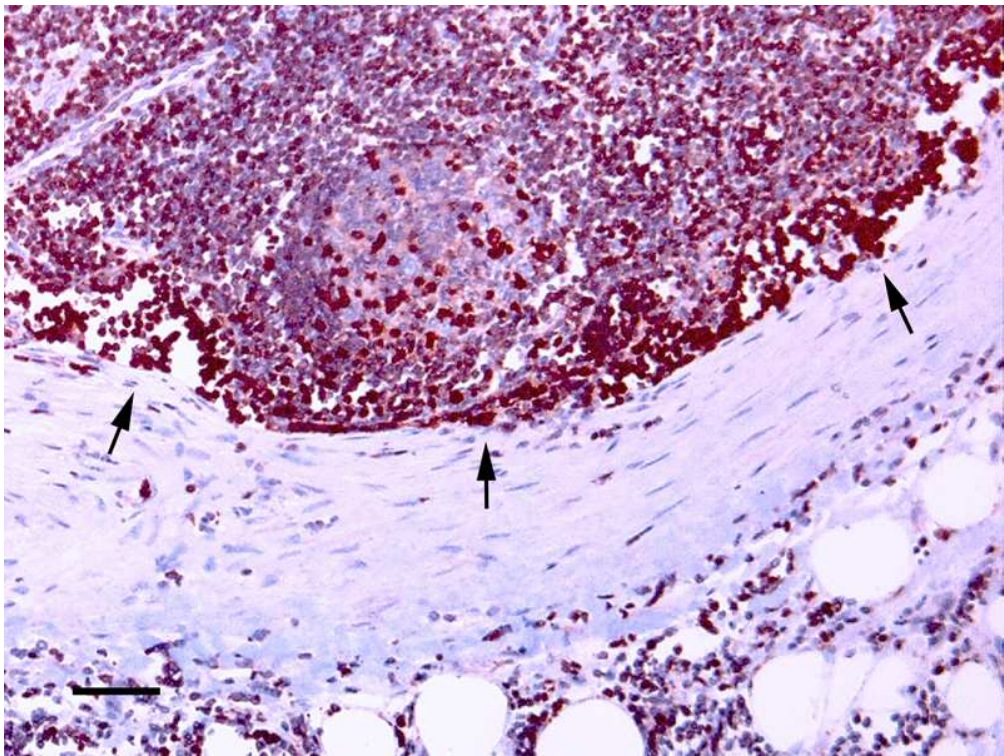
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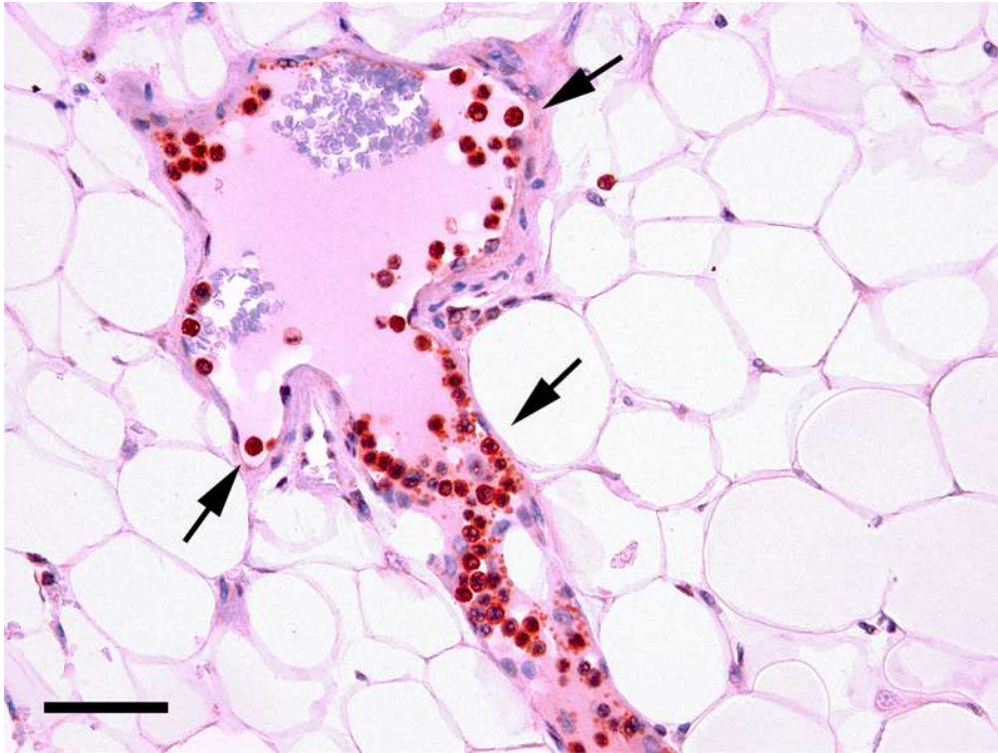
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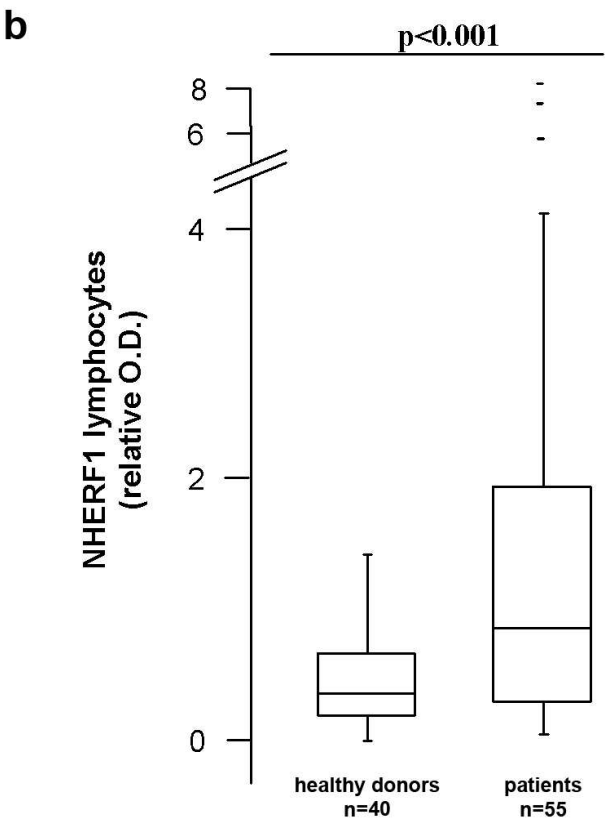
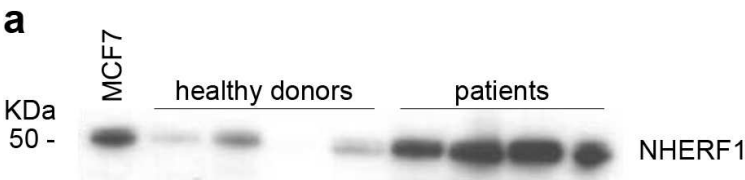
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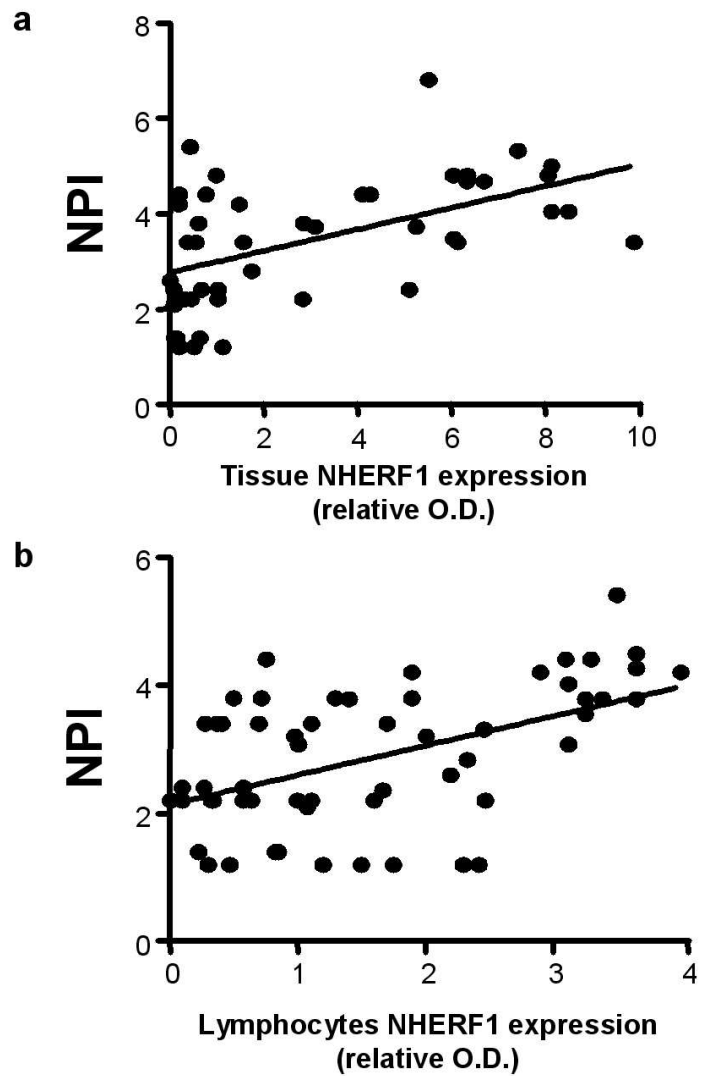


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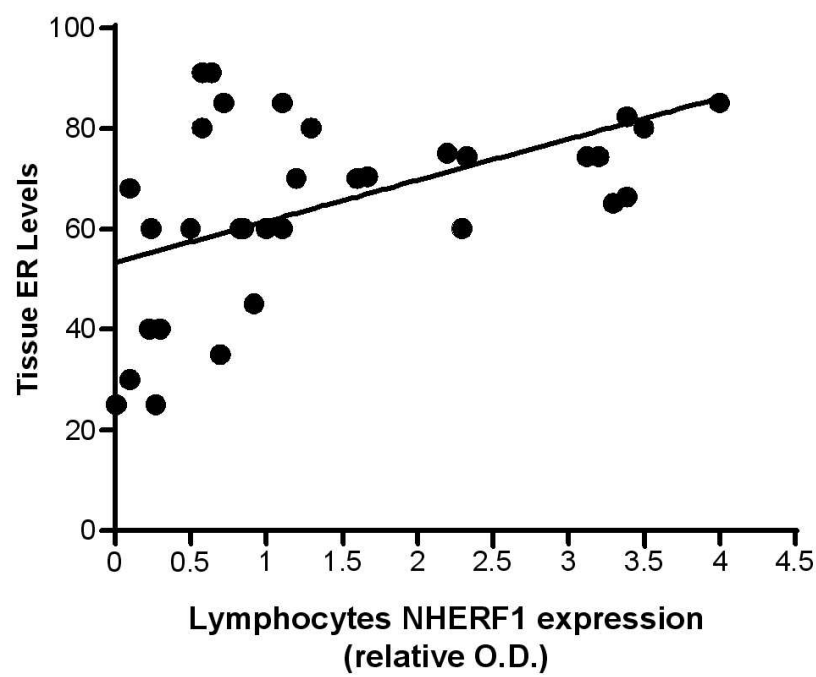
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Figure legends

Figure 1. NHERF1 protein levels are increased in tumor compartments. (a) Representative WB of NHERF1 protein expression in lysates from three patients: breast tumor (T), its contiguous non tumor breast tissue (NT) and metastatic lymph node (L). (b) Complessively, the data indicate an overall significant increase in levels of NHERF1 protein in primary tumors and lymph node with respect to the non tumor tissue. NHERF1 lymph node expression also significantly increased with respect to the tumor. Lymphocyte NHERF1 expression significantly increased with respect to the NHERF1 expression in non tumor tissue and significantly lower with respect to the lymph node compartment. Samples were extracted for total protein and NHERF1 expression analyzed by WB as described in Materials and Methods.

Figure 2. NHERF1 immunolocalization in well organized and invaded lobules of the mammary gland and lymph node. In the non tumor compartment (a) of the tissue, NHERF1 localized only apically in the epithelial cells lining the lobules and there are some lymphocytes are dispersed in the stroma which are not stained with NHERF1 (arrow). The invaded tissue (b) shows NHERF1 immunolocalization in the metastatic spreading cells and in the lymphocytes located in adjacent regions. Lymphocytes in metastatic lymph node (c) and peritumour blood vessels (d) also show an intense immunoreactivity. Bar = 32 μ m.

Figure 3. NHERF1 protein levels are increased in patient's lymphocytes. Lymphocytes were extracted for total protein and NHERF1 expression analyzed by WB as described in the Materials and Methods. (a) Representative WB of NHERF1 protein expression in four healthy donors and four patients. (b) Grouped data indicate a significant increase in median value of patients NHERF1 expression in peripheral lymphocytes with respect to the median value found in the healthy donors.

Figure 4. NHERF1 expression in both patient tissue and lymphocytes increases with poor prognosis. Sperman-Rank correlation between tissue (a) and lymphocyte (b) NHERF1 protein expression and NPI, based on tumor size, grade and node status of the patients ($Y = 0.205x + 2.769$, $r^2 = 0.1826$ vs. $Y = 0.4646x + 2.133$, $r^2 = 0.2538$, in tissues and lymphocytes, respectively). Data indicate that there is a significant linear correlation between the overall tissue NHERF1 expression and the NPI index

Figure 5. Lymphocyte NHERF1 expression correlates with ER levels, in ER+ patients. Sperman-Rank correlation between lymphocyte NHERF1 protein expression and ER levels; data indicate that there is a linear correlation ($Y = 8.190x + 53.25$, $r^2 = 0.1915$) between the ER levels in the tumor tissue and the NHERF1 expression levels found in the corresponding lymphocytes of the patients.

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Acknowledgements

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