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Nuclear NHERF1 expression as a prognostic marker in breast cancer

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Our purpose was to investigate whether Na^+/H^+ exchanger regulatory factor 1 (NHERF1) expression could be linked to prognosis in invasive breast carcinomas. NHERF1, an ezrin-radixin-moesin (ERM) binding phosphoprotein 50, is involved in the linkage of integral membrane proteins to the cytoskeleton. It is therefore believed to have an important role in cell signaling associated with changes in cell cytoarchitecture. NHERF1 expression is observed in various types of cancer and is related to tumor aggressiveness. To date the most extensive analyses of the influence of NHERF1 in cancer development have been performed on breast cancer. However, the underlying mechanism and its prognostic significance are still undefined. NHERF1 expression was studied by immunohistochemistry (IHC) in a cohort of 222 breast carcinoma patients. Association of cytoplasmic and nuclear NHERF1 expression with survival was analyzed. Disease-free survival (DFS) and overall survival (OS) were determined based on the Kaplan–Meier method. Cytoplasmic NHERF1 expression was associated with negative progesterone receptor (PgR) ($P = 0.017$) and positive HER2 expression ($P = 0.023$). NHERF1 also showed a nuclear localization and this correlated with small tumor size ($P = 0.026$) and positive estrogen receptor (ER) expression ($P = 0.010$). Multivariate analysis identified large tumor size ($P = 0.011$) and nuclear NHERF1 expression ($P = 0.049$) to be independent prognostic variables for DFS. Moreover, the nuclear NHERF1(−)/ER(−) immunophenotype (27%) was statistically associated with large tumor size ($P = 0.0276$), high histological grade ($P = 0.0411$), PgR-negative tumors ($P < 0.0001$) and high proliferative activity ($P = 0.0027$). These patients had worse DFS compared with patients with nuclear NHERF1(+)/ER(+) tumors (75.4% *versus* 92.6%; $P = 0.010$). These results show that the loss of nuclear NHERF1 expression is associated with reduced survival, and the link between nuclear NHERF1 and ER expression may serve as a prognostic marker for the routine clinical management of breast cancer patients.

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Breast cancer, the most common malignancy in women, is regarded as a heterogeneous group of tumors with different outcomes and responses to treatment.[1] Several biological features that are indicative of clinical aggressiveness and useful for identification of patients at low and high risk have been investigated. [2–4] Recent evidence obtained from our laboratory suggested a fundamental role for Na^+/H^+ exchanger regulatory factor 1 (NHERF1) in human breast and colorectal cancers.[5–8] NHERF1, located on chromosome 17q25.1 (also named SLC9A3R1 and ezrin-radixin-moesin (ERM) binding phosphoprotein 50 EBP50), is a member of a family of scaffold proteins, composed of two tandem PDZ domains and a C-terminal ezrin-binding region.[9] PDZ domains are among the most frequent protein modules involved in protein–protein interactions and directly bind to the carboxyl (C)-terminal PDZ motifs of their ligands.[10] NHERF1 is involved in transmitting signals from the surface into the cell, which could depend on the status of cell–cell adhesion,[11,12] and is also known to have a pivotal role in coordinating the interaction of members of the merlin and ERM (merlin-ezrin-radixin-moesin, MERM) family, transmembrane proteins, and cytosolic second messenger cascades.[11,13] Some studies have shown that NHERF1 is associated with growth factor tyrosine kinase receptors involved in cancer progression.[14–16] Furthermore, we have also found an association of NHERF1 expression with HER2[5] in breast tumors.[17,18] NHERF1 expression is upregulated in response to estrogens and suppressed by antiestrogens in estrogen receptor (ER) positive breast cancer cell lines.[19] The correlation between ER-positive tumors and NHERF1 expression has also been observed in breast carcinoma specimens.[20–22] Importantly, in our recent study we showed that cytoplasmic NHERF1 was overexpressed in ER-negative breast carcinomas.[17] An important role of NHERF1 in regulating carcinogenesis and cancer progression has emerged.

Our group has previously demonstrated that in an '*in vitro*' model of breast cancer cells[23] NHERF1 is able to induce an invasive phenotype. We later showed that NHERF1 protein

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Abbreviations: NHERF1, Na^+/H^+ exchanger regulatory factor 1; ERM, ezrin-radixin-moesin; MERM, merlin-ezrin-radixin-moesin; ER, estrogen receptor; PgR, progesterone receptor; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; DFS, disease-free survival; OS, overall survival; CI, confidence interval; HR, hazard ratio
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expression significantly correlates with aggressive clinical parameters and poor prognosis in both tissues and lymphocytes of breast cancer patients, underlining its possible involvement also in immune response.[7,24] Other studies have demonstrated that NHERF1 is overexpressed in the tumor compared with non tumor counterparts.[20,25,26] We also observed a heterogeneous distribution of cytoplasmic NHERF1 expression in different stages of breast cancer. Observation of the subcellular localization of NHERF1 protein in tumor and contiguous non-involved tissues from the same patient revealed that cytoplasmic NHERF1 expression progressively increased in tumors cells from normal to invasive and metastatic tissues.[5] These data suggest that NHERF1 might become a marker of clinical relevance for breast tumor patients.

In the current study, we investigate for the first time the prognostic significance of NHERF1 subcellular localization in a cohort of 222 well-characterized invasive breast carcinomas with long-term clinical follow-up. We also analyze the association between the clinicopathological characteristics and immunohistochemical expression of NHERF1.

Results

Association between cytoplasmic and nuclear NHERF1 expression and clinicopathological characteristics in invasive breast carcinomas. Both cytoplasmic and nuclear NHERF1 expression were observed in cancer cells. Representative images of NHERF1 staining are shown in Figure 1. NHERF1 immunostaining was predominantly cytoplasmic (Figure 1a). However, in the majority of positive cases for cytoplasmic NHERF1 an intense nuclear staining was also demonstrated (Figure 1b). This was scored separately and its significance was evaluated. In addition, in contiguous non tumor breast tissue, NHERF1 immunoreactivity showed mostly an apical membranous reactivity in epithelia cells (Figure 1c).

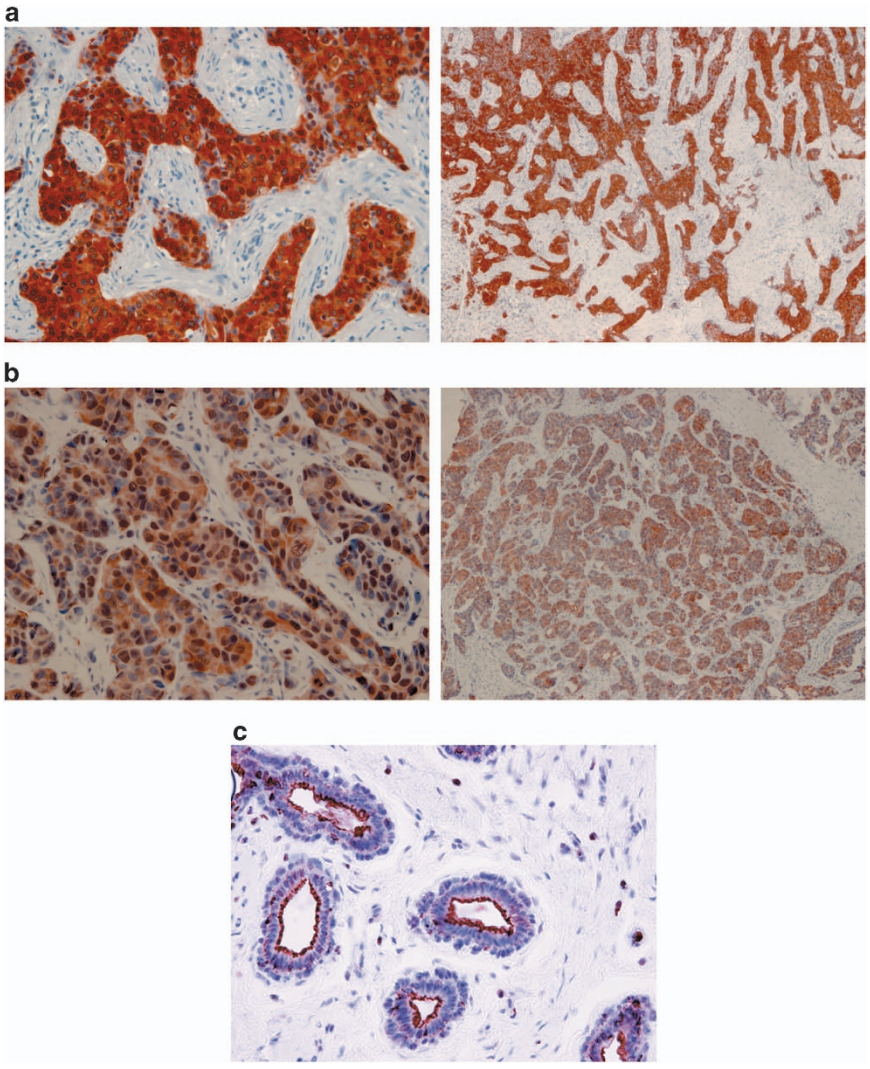

Figure 1 Immunoreactivity and localization of NHERF1 in breast carcinoma. Representative images of immunohistochemical staining: (**a**) positive staining for cytoplasmic NHERF1 in tissue of poorly differentiated IDC (original magnification on the left $\times$ 20) and panoramic view of the tumor (original magnification on the right $\times$ 5); (**b**) NHERF1 antibody stained intensely in the cytoplasm and in the nucleus of the cells of poorly differentiated IDC (original magnification on the left $\times$ 20), and panoramic view of the tumor (original magnification on the right $\times$ 5); (**c**) apical membranous immunoreactivity of NHERF1 in non neoplastic epithelia cells (original magnification $\times$ 20)

Cytoplasmic and nuclear expression of NHERF1 were analyzed with respect to the main clinicopathological characteristics, and the significant associations are summarized in Table 1. Clinicopathological analysis showed that cytoplasmic NHERF1 overexpression present in 44% of tumor tissues was significantly associated with negative progesterone receptor (PgR) tumors (52%, $P=0.017$) and with HER2 overexpression (56%, $P=0.023$). A statistical trend was observed between positive cytoplasmic expression and ER-negative (51%, $P=0.054$) and Ki67-positive tumors (47%, $P=0.054$), whereas cytoplasmic NHERF1 overexpression was not significantly associated with age at diagnosis, tumor size, lymph node metastasis, or histological grade. In contrast, analysis of the clinicopathological significance of nuclear NHERF1 expression revealed that high nuclear NHERF1 expression present in 46% of tumor tissues was associated with small tumor size (56%, $P=0.026$) and positive ER tumors (54%, $P=0.010$). No statistical significance between nuclear NHERF1 expression and other clinicopathological variables was observed. Moreover, there was no correlation between the expression, either cytoplasmic or nuclear, of NHERF1 and the treatment type. In patients that developed distant metastases (10%), cytoplasmic NHERF1 was overexpressed in 39% (9/23), whereas nuclear NHERF1 was overexpressed in 30% (7/23) of patients. Nuclear NHERF1 was completely absent in 16 patients. Seven of these patients (44%) had a worse survival. In the 51% of patients with positive lymph nodes, cytoplasmic NHERF1 expression was significantly associated with negative PgR (31%, $P=0.001$) and with positive Ki67 tumors (43%, $P=0.018$), whereas

Table 1 Correlation of cytoplasmic and nuclear NHERF1 expression with clinicopathological characteristics of invasive breast cancer patients

Characteristics	No. of pts (%)	Cytoplasmic NHERF1 expression		*P*-value[a]	Nuclear NHERF1 expression		*P*-value[a]
		Negative	Positive		Negative	Positive	
Patients' age							
≤52 Years	120 (54)	68	52	1.000	63	57	0.721
>52 Years	102 (46)	57	45		56	46	
Tumor size (cm)							
≤2	80 (36)	51	29	0.077	35	45	0.026
>2	140 (64)	72	68		83	57	
Lymph node status							
Negative	108 (49)	58	50	0.447	57	51	0.810
Positive	114 (51)	67	47		62	52	
Histological grade[b]							
1	3 (1)	3	0	0.217[c]	2	1	0.814[c]
2	53 (24)	32	21		27	26	
3	165 (75)	89	76		90	75	
Histological type							
IDC	206 (93)	113	93	0.373	110	96	1.000
Other	16 (7)	12	4		9	7	
Receptor status							
ER-negative (≤10%)	96 (43)	47	49	0.054	61	35	
ER-positive (>10%)	126 (57)	78	48		58	68	0.010
PgR-negative (≤10%)	112 (51)	54	58	0.017	66	46	0.095
PgR-positive (>10%)	109 (49)	70	39		52	57	
Ki67 index							
Negative (≤20%)	37 (17)	26	11	0.054	19	18	0.804
Positive (>20%)	181 (83)	96	85		97	84	
HER2							
Negative (0, 1+)	160 (72)	98	62		92	68	0.078
Positive (3+)	61 (28)	27	34	0.023	27	34	
Treatment							
Chemotherapy	101 (45)	50	51	0.062	61	40	0.064
Chemo + hormonotherapy	121 (55)	75	46		58	63	
Distant recurrence							
Absent	199 (90)	111	88	0.665	103	96	0.125
Present	23 (10)	14	9		16	7	

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; NHERF1, Na^+/H^+ exchanger regulatory factor 1; PgR, progesterone receptor; pts, patients
Two patients out 222 had missing values for tumor size; four patients out 222 had missing values for Ki67; one for PgR and one for HER2
[a]*P*-values were calculated with the Pearson χ^2 test
[b]Not performed on one case due to histological type
[c]*P*-values were calculated with the χ^2 test. These were not included in the analyses

nuclear NHERF1 expression was significantly associated with positive PgR tumors (32%, $P=0.038$) (data not shown).

Survival analyses. The possible impact of patients, tumor variables, and treatment modalities was investigated by univariate analysis with respect to DFS and OS. At median follow-up (69 months), univariate analysis revealed that large tumor size ($P=0.011$), poor histological grade ($P=0.045$), high Ki67 ($P=0.025$), and PgR-negativity ($P=0.048$) were significantly associated with worse DFS in invasive breast cancer. Improved OS was associated with PgR-positivity and with patients treated with chemo + hormonotherapy in the univariate analysis ($P=0.007$ and $P=0.015$, respectively), (Table 2). Multivariate analysis of the entire cohort, according to a model following the backward process, identified large tumor size (hazard ratio, HR = 2.88, 95% confidence interval, CI 1.28–6.49, $P=0.011$) and nuclear NHERF1 expression (HR = 0.97, 95% CI 0.93–1.00, $P=0.049$) as independent prognostic variables for DFS, whereas only PgR expression was significantly associated with OS (HR = 0.29, 95% CI 0.11–0.71, $P=0.007$), (Table 3). We then investigated the relationship between NHERF1 expression and breast cancer survival. Kaplan–Meier curves revealed that the patients with positive nuclear NHERF1 expression tended toward a higher DFS than the DFS of patients with negative nuclear NHERF1 (5 years, 88% *versus* 80%, $P=0.070$), (Figure 2a). There was no difference in OS between patients with positive and negative nuclear NHERF1 expression (5 years, 93% *versus* 86%, $P=0.234$), (Figure 2b). However, high and low expression of cytoplasmic NHERF1 expression did not correlate with response to DFS and OS. Given the profound effects of estrogen on NHERF1 physiology, we considered

Table 2 Univariate analysis with respect to DFS and OS in 222 patients with invasive breast cancer

Characteristics	No. of pts	No. of events	5-Year% DFS (95% CI)	HR (95% CI)	*P*-value	No. of events	5-Year% OS (95% CI)	HR (95% CI)	*P*-value
Overall	222	42	84 (78–89)	—	—	26	89 (84–93)	—	—
Age (years)									
<52	111	22	85 (77–91)	1.00		12	89 (81–94)	1.00	
≥52	111	20	83 (73–89)	0.93 (0.51–1.70)	0.808	14	89 (81–94)	1.21 (0.56 2.62)	0.622
Histological type									
IDC	206	38	84 (78–89)	1.00		23	90 (85–94)	1.00	
Other	16	4	81 (52–94)	1.31 (0.47–3.68)	0.605	3	79 (47–93)	1.63 (0.49–5.44)	0.424
Lymph node status									
Negative	108	17	87 (78–92)	1.00		10	90 (82–94)	1.00	
Positive	114	25	82 (72–88)	1.39 (0.75–2.58)	0.289	16	89 (80–94)	1.49 (0.68–3.28)	0.322
Tumor size (cm)									
≤2	80	7	90 (82–97)	1.00		5	93 (87–99)	1.00	
>2	140	35	81 (74–88)	2.87 (1.28–6.48)	0.011	21	87 (81–93)	2.42 (0.91–6.43)	0.075
Histological grade									
1 + 2	56	4	92 (71–98)	1.00		2	95 (72–99)	1.00	
3	165	38	81 (74–86)	1.70 (1.01–2.84)	0.045	24	87 (80–91)	1.89 (0.92–3.89)	0.084
Ki67 index									
Negative (≤20%)	37	2	100	1.00		0	100	—	
Positive (>20%)	181	40	80 (73–86)	5.10 (1.23–21.15)	0.025	26	87 (80–91)	—	—
Receptor status									
ER-negative (≤10%)	96	22	82 (72–88)	1.00		15	84 (75–91)	1.00	
ER-positive (>10%)	126	20	86 (78–92)	0.62 (0.34–1.13)	0.117	11	93 (86–97)	0.50 (0.23–1.08)	0.078
PgR-negative (≤10%)	112	27	80 (71–87)	1.00		20	83 (74–89)	1.00	
PgR-positive (>10%)	109	15	88 (79–93)	0.53 (0.28–0.99)	0.048	6	95 (88–98)	0.29 (0.11–0.71)	0.007
HER2									
Negative (0,1 +)	161	27	87 (80–91)	1.00		15	91 (85–95)	1.00	
Positive (3 +)	61	15	77 (62–87)	1.54 (0.82–2.90)	0.177	11	85 (72–92)	2.05 (0.94–4.46)	0.078
Cytoplasmic NHERF1									
Negative	117	20	86 (78–91)	1.00		13	88 (80–93)	1.00	
Positive	105	22	82 (73–89)	1.22 (0.67–2.24)	0.512	13	90 (82–95)	1.10 (0.51–2.37)	0.813
Nuclear NHERF1									
Negative	119	28	80 (71–87)	1.00		17	86 (78–92)	1.00	
Positive	103	14	88 (80–93)	0.55 (0.29–1.05)	0.070	9	93 (85–97)	0.61 (0.27–1.37)	0.234
Treatment									
Chemotherapy	101	25	77 (67–85)	1.00		18	84 (75–90)	1.00	
Chemo + hormonotherapy	121	17	90 (82–94)	0.54 (0.29–1.00)	0.050	8	93 (85–97)	0.36 (0.15–0.82)	0.015

Abbreviations: CI, confidence interval; DFS, disease-free survival; ER, estrogen receptor; HR, hazard ratio; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; NHERF1, Na^+/H^+ exchanger regulatory factor 1; OS, overall survival; PgR, progesterone receptor

NHERF1 expression in association with ER expression. Interestingly, we found that the nuclear NHERF1(−)/ER(−) immunophenotype (27%, $n=61$) was mainly associated with negative prognostic factors such as large tumor size ($P=0.0276$), high histological grade ($P=0.0411$), PgR-negativity ($P<0.0001$), high Ki67 ($P=0.0027$) tumors, and chemotherapy ($P<0.0001$), (data not shown). Furthermore, distant metastases were more frequently found in patients with nuclear NHERF1(−)/ER(−) tumors (13/61, 21%) than those with nuclear NHERF1(+)/ER(+) (2/68, 3%) tumors. When nuclear NHERF1 and ER expressions were categorized as one variable, Kaplan–Meier curves showed that patients with the nuclear NHERF1(−)/ER(−) immunophenotype had worse DFS compared with patients with the nuclear NHERF1(+)/ER(+) immunophenotype (75.4% *versus* 92.6%; log rank $\chi^2=6,583$; $P=0.010$), (Figure 2c). The difference between the two immunophenotype tumors did not appear significant in OS (83.6% *versus* 94.1%; log rank $\chi^2=3,274$; $P=0.070$), (Figure 2d).

Table 3 Multivariate analysis with respect to DFS and OS in invasive breast cancers

Characteristics	DFS		OS	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Model following backward process				
Tumor size	2.88 (1.28–6.49)	0.011	—	—
Nuclear NHERF1	0.97 (0.93–1.00)	0.049	—	—
PgR	—	—	0.29 (0.11–0.71)	0.007

Abbreviations: CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; NHERF1, Na^+/H^+ exchanger regulatory factor 1; OS, overall survival; PgR, progesterone receptor

Discussion

In this study, we examined the expression of NHERF1 by immunohistochemistry (IHC) in invasive breast carcinomas. To our knowledge, this is the first study to investigate the cytoplasmic and nuclear expressions of this protein and their association to survival.

In breast cancer, the subcellular localization of NHERF1 is deeply altered, moving from less to more aggressive tumors with a predominant cytoplasmic expression.[5] Our data showed that the NHERF1 protein is distributed in the cytoplasm of invasive breast cancer cells, and its overexpression is associated with some aggressive clinical parameters as demonstrated by the association with negative

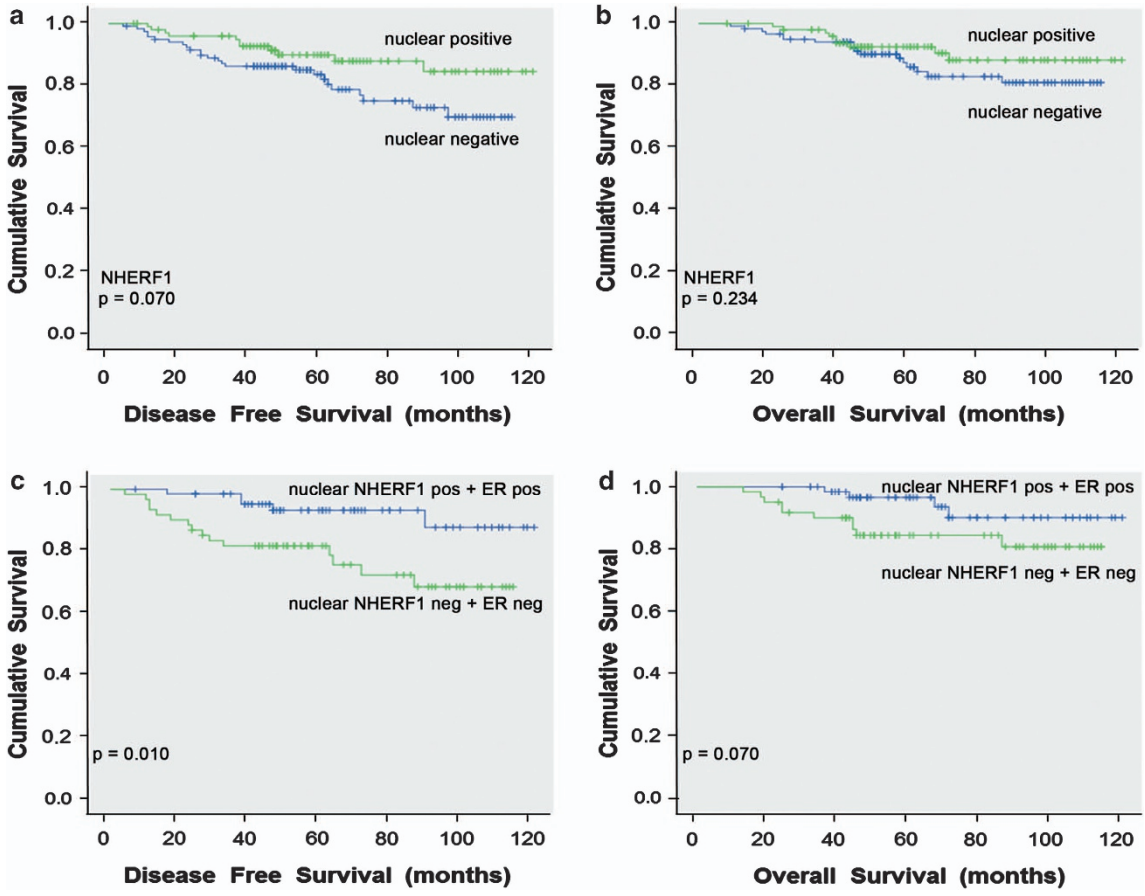

Figure 2 Association between nuclear NHERF1 expression and survival. (**a**) Patients with positive nuclear NHERF1 expression tended toward a higher DFS than the DFS of patients with negative nuclear NHERF1 (**b**) No difference in OS between patients with positive and negative nuclear NHERF1 expression. Association between nuclear NHERF1 expression and survival in NHERF1/ER immunophenotype. (**c**) The patients with nuclear NHERF1(−)/ER(−) immunophenotype had worse DFS compared with patients with nuclear NHERF1(+)/ER(+) immunophenotype. (**d**) OS in patients with nuclear NHERF1(−)/ER(−) immunophenotype was not significantly shorter

hormonal status, high proliferative activity, and unfavorable prognosis. Furthermore, we found cytoplasmic NHERF1 significantly increased in HER2-positive tumors. As a scaffolding protein, NHERF1 recruits membrane proteins into functional complexes through its PDZ domains and is associated with a number of G protein-coupled receptors, ion channels, and growth factor tyrosine kinase receptors.[11,15] In a previous study, we demonstrated that NHERF1 is strongly colocalized with HER2 in '*in situ*' breast carcinoma overexpressing HER2, in invasive tumors, and in distant metastases[5]. A positive association between NHERF1 expression and HER2 in breast cancer has been subsequently described by Karn *et al.*[22] Cytoplasmic NHERF1 strongly related to HER2 could be able to create new signaling pathways that drive the subverted cellular functions exhibited by tumor cells. It was hypothesized that NHERF1 may behave either as a tumor suppressor, when it is localized in the plasma membrane, or as an oncogenic protein, when it is shifted to the cytoplasm.[12] Interestingly, we found NHERF1 also present in the nucleus of invasive breast tumor tissues. The role of NHERF1 in the nucleus of breast tumors was not examined. Of the clinicopathological variables tested in this study, nuclear NHERF1 expression showed an association with small tumor size and positive ER expression, differently from the cytoplasmic cellular localization. We hypothesize that, differently from colon cancer behavior,[16,27] subcellular distribution of NHERF1 into the nucleus of breast tumors is the result of a translocation necessary for a function, which leads to a better clinical outcome. In contrast, we observed that loss of nuclear NHERF1 protein expression was associated with reduced survival. The data regarding survival are only exploratory, however, and the results must therefore be confirmed. These differences in cellular location of the scaffold protein NHERF1 could be of high clinical importance. Moreover, multivariate survival analyses identified nuclear NHERF1 as an independent prognostic variable for DFS in this cohort of invasive breast cancer patients.

In breast epithelial cells, ER activation significantly contributes to breast cancer progression by inducing proliferation and invasion.[28] Fouassier *et al.*[29] indicated that both the expression and distribution of NHERF1 are regulated by estrogens and contribute to proliferative response in epithelial cells. Tumor NHERF1 protein expression levels were reported as being directly related with increasing ER levels in ER-positive tumors. It is noteworthy that cytoplasmic NHERF1 is strongly associated with ER expression in ER-positive breast tumors,[20,21,24] and more recently Karn *et al.*[22] observed that high NHERF1 expression was associated with poor survival in ER-positive patients. Importantly, in a previous study we found an inverse link between cytoplasmic NHERF1 overexpression and ER status in ER-negative patients, suggesting an ER-independent alternative pathway for NHERF1 in ER-negative breast cancers.[17] The correlation between expression of NHERF1 and ER status continues to be intriguing for its potential clinical application. On the other hand, on investigation of the relationship between NHERF1 and clinical outcome of the patients we found that patients with high nuclear NHERF1 expression showed increased DFS, particularly the ER-positive patients. If the relationship between nuclear NHERF1 expression evaluated as a single marker and survival was not significant, it implies that other factors may interact with NHERF1 to influence clinical outcome, for example, the presence of NHERF1 in the cytoplasmic cell compartment. Rather interestingly, in this study we observed that the nuclear NHERF1(−)/ER(−) immunophenotype was associated with aggressive clinicopathological parameters and unfavorable prognosis. Kaplan–Meier analysis in the patients with ER-negative tumors showed a strong association between nuclear negative NHERF1 expression and worse outcome.

In conclusion, we found that the loss of nuclear NHERF1 is associated with reduced survival, and for the first time we report an interesting link between nuclear NHERF1 and ER status as a prognostic marker for the routine clinical management of breast cancer. We found that nuclear NHERF1 expression identifies more than a quarter of patients with ER-negative tumors who may benefit from more aggressive therapeutic management, and more than a third of patients with both positive parameters, for whom less intervention may be warranted. Thus, we hope that in the near future our observation could be validated by other analyses in this field on a larger series of patients.

Materials and Methods

Patient characteristics. This study involved 222 patients with a diagnosis of invasive breast cancer. All patients underwent surgery at the NCRC Istituto Tumori 'Giovanni Paolo II' of Bari between 1998 and 2004 and were enrolled into a prospective multicenter clinical study.[30] Patients were eligible if: they were females $\leq$70 years of age; had histological diagnosis of invasive breast carcinomas of any size with one- to three-positive axillary nodes or node-negative tumors $>$1 cm; had radical tumor resection; had no evidence of metastatic disease. Patients were excluded if they had a previous history of invasive breast cancer, or other previous or concomitant malignancies or concomitant diseases. The study was approved by the institutional review boards of each participating center. The patients were required to be accessible for follow-up, and their informed consent was obtained before assignment to treatment. The clinicopathological characteristics considered in this study are summarized in Table 1. The median age of the patients was 52 years (range 36–70 years) and median follow-up was 69 months (range 0–122 months). The majority of the tumors had tumor size $>$2 cm (64%), and 51% had nodal involvement. Seventy-five percent of patients had poorly differentiated tumors, according to the Scarf-Bloom-Richardson grade system,[31] and 93% were invasive ductal carcinomas (IDCs). Information regarding ER, PgR, Ki67 index and HER2 expression was collected from the Pathology Department of our Institute. Fifty-seven percent and 49% of patients were ER- and PgR-positive, respectively. Ki67 index was positive in 83% and HER2 was positive in 28% of the patients. Overexpression of cytoplasmic NHERF1 was examined in 44% (97/222) of tumor tissues. In 46% (103/222) of tumor tissues, NHERF1 showed also a nuclear localization in addition to cytoplasmic NHERF1 immunoreactivity. All patients received adjuvant chemotherapy and 96% (121/126) of patients with ER-positive tumors received adjuvant tamoxifen for 5 years after the end of chemotherapy. For five (4%) patients the treatment data were not available.

Ethics statement. This study was approved by the Institutional Review Board of our Institute. Before undergoing routine surgery, all patients signed an informed consent form authorizing the Institute to utilize their removed biological tissue for research purposes according to ethical standards.

Immunohistochemistry. NHERF1 expression pattern was examined in 222 tumor samples from invasive breast cancer patients. Sections of 4-μm-thickness were cut from formalin-fixed and paraffin-embedded histological blocks, and these were immunohistochemically stained for NHERF1 using standard immunoperoxidase techniques as previously described[5]. Briefly, sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and pretreated with 0.01 M sodium citrate buffer at pH 6.0 in a water bath. After endogenous peroxidase activity blocking with 0.3% H_2O_2 buffer solution, sections were incubated with a

rabbit polyclonal EBP50 antibody for NHERF1 (clone PA1-090; Affinity Bioreagents, Golden, CO, USA; 1:150 dilution in PBS/BSA 1%) overnight at 4 °C. The bound antibody was visualized with 3-amino-9-ethylcarbazole substrate-chromogen (DakoCytomation, Glostrup, Denmark) in the dark and counterstained with Mayer's haematoxylin. As a positive internal control, we used paraffin-embedded cell pellets from MCF-7 cell lines, expressing high levels of NHERF1. For negative control, the primary antibody was omitted and replaced by PBS pH 7.6. ER, PgR, Ki67 index, and HER2 expression were assessed by IHC at the Pathology Department of our Institute.

Immunohistochemical assessment. For NHERF1, cytoplasmic and nuclear localization were examined. All stained samples were scored in a blind manner by two independent investigators who had no prior knowledge of the clinicopathological data.[5] Protein expression was quantified by counting the positive cells in three representative areas of tumor for each section at $\times 20$ magnification and expressed as a percentage of positive cells/section. According to the median value, the cases were classified positive when cytoplasmic NHERF1 immunoreactivity was present in $\geq 65\%$ of tumor cells (median value 65) and when nuclear NHERF1 expression was detected in $>0\%$ of tumor cells examined (median value 0). ER, PgR and Ki67 immunostaining were confined to the nucleus. The cutoff value for ER and PgR was 10%. Tumors with ER or PgR expression were scored as positive when nuclear staining was present in $>10\%$ and scored negative when $\leq 10\%$ of the tumor cells had nuclear staining. For the Ki67 index, we adopted the cutoff value of 20%, and the tumors with a Ki67 $>20\%$ were considered highly proliferating. The Ki67 cutoff represents the median value of the scores relative to all breast tumor samples analyzed during the last 5 years within our Institute. HER2 was scored as 0, 1+, 2+ or 3+ using a monoclonal antibody (MoAb clone CB11, Novocastra Laboratories Ltd, Newcastle, UK), in accordance with the Herceptest scoring system (Food and Drug Administration accepted): 0 = no membranous immunoreactivity or $<10\%$ of cells reactive; 1+ = incomplete membranous reactivity in $>10\%$ of cells; 2+ $\geq 10\%$ of cells with weak to moderate complete membranous reactivity; and 3+ = strong and complete membranous reactivity in $>10\%$ of cells. Cytoplasmic immunoreactivity was ignored. Cases scoring 0 and 1+ were classified as negative. HER2 was considered to be positive if immunostaining was 3+ or if a 2+ result showed gene amplification by fluorescence *in situ* hybridization (FISH). In FISH analyses, each copy of the *HER2* gene and its centromere 17 (*CEP17*) reference were counted. The interpretation followed the criteria of the ASCO/CAP guidelines for HER2 IHC interpretation for breast cancer;[32] positive if the *HER2/CEP17* ratio was higher than 2.2.

Follow-up and statistical analyses. Pearson's χ^2 test and Fisher's exact test were used for analysis of associations between NHERF1 and age, tumor size, lymph node status, histological type, histological grade, receptor status, Ki67 and HER2.

The results from the immunohistochemical analyses of NHERF1 were assessed in relation to disease-free survival (DFS) and overall survival (OS). DFS (in months) was defined as the time from diagnosis to the date of locoregional or distant recurrence, second invasive breast carcinoma, second primary cancer and/or death without evidence of breast cancer or to the date of last contact. OS (in months) was defined as the time from diagnosis to the date of last contact or of death from any cause. Forty-two breast cancer relapses and 26 deaths were observed in DFS and OS, respectively. Twenty-three of these 42 were distant metastases; in 7 patients these occurred in the lung, in 2 patients in the pleura, in 3 patients in the liver, in 3 patients in the bone, in 2 patients in the central nervous system, in 2 patients in the peritoneum and in 3 patients in the lymph nodes. In one patient there was recurrence without location information. DSF and OS probabilities and 95% CI were computed by the Kaplan–Meier product-limit method and compared by the log rank test. Cox regression analysis was performed to assess prognostic factors, including the variables that were statistically significant in univariate analysis, and also ER status, HER2, nuclear NHERF1 and lymph node status. The model was optimized using a backward stepwise regression. All statistical differences were considered significant at the level of $P<0.05$. Statistical analyses were performed using SPSS 14.0 statistical software (SPSS Inc., IL, Chicago, USA).

Conflict of Interest

The authors declare no conflict of interest.

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