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Breast cancer-derived K172N, D301V mutations abolish Na^+/H^+ exchanger regulatory factor 1 inhibition of platelet-derived growth factor receptor signaling

Shan Cheng [a,e,1], Yang Li [a,1], Ying Yang [b], Duiping Feng [a,2], Longyan Yang [a], Qian Ma [a], Shuai Zheng [a], Ran Meng [a], Shuhui Wang [c], Songlin Wang [a,d], Wen G. Jiang [a,e,f], Junqi He [a,e,*]

[a] Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, China
[b] Proteomic Research Center, Capital Medical University, Beijing 100069, China
[c] Department of Neurology, Beijing Friendship Hospital, Capital Medical University, Beijing 100069, China
[d] Molecular Laboratory for Gene Therapy and Tooth Regeneration, Capital Medical University School of Stomatology, Beijing 100050, China
[e] Capital Medical University-Cardiff University Joint Centre for Biomedical Research, Capital Medical University, Beijing 100069, China
[f] Metastasis and Angiogenesis Research Group, Department of Surgery, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK

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ABSTRACT

Na^+/H^+ exchanger regulatory factor 1 (NHERF1) is a scaffold protein known to interact with a number of cancer-related proteins. *nherf1* Mutations (K172N and D301V) were recently identified in breast cancer cells. To investigate the functional properties of NHERF1, wild-type and cancer-derived *nherf1* mutations were stably expressed in SKMES-1 cells respectively. NHERF1-wt overexpression suppressed the cellular malignant phenotypes, including proliferation, migration, and invasion. *nherf1* Mutations (K172N and D301V) caused complete or partial loss of NHERF1 functions by affecting the PTEN/NHERF1/PDGFRβ complex formation, inactivating NHERF1 inhibition of PDGF-induced AKT and ERK activation, and attenuating the tumor-suppressor effects of NHERF1-wt. These results further demonstrated the functional consequences of breast cancer-derived *nherf1* mutations (K172N and D301V), and suggested the causal role of NHERF1 in tumor development and progression.

Structured summary of protein interactions:
NHERF1 physically interacts with **PDGFRbeta** by pull down
NHERF1 physically interacts with **PTEN** by pull down
PDGFRbeta physically interacts with **PTEN** and **NHERF1** by pull down

1. Introduction

Na^+/H^+ exchanger regulatory factor 1 (NHERF1, also named as EBP50), was first identified as an essential cofactor for cyclic AMP inhibition of Na^+/H^+ exchange in the rabbit renal brush border membrane [1]. It is known to be a scaffold protein highly expressed in the apical membrane of polarized epithelial cells. NHERF1 consists of two tandem PDZ domains followed by an ezrin–radixin–moesin (ERM)-binding region [2,3]. NHERF1 can bind to ion transporters, G protein-coupled receptors, and cytoskeleton-associated ERM proteins via these domains, and has been implicated not only in regulating diverse biological processes for ion transport and second messaging cascades, but also in maintaining cell polarity [2,4].

Recently, increasing evidence has demonstrated that NHERF1 can interact with many cancer-related proteins, such as PTEN [5,6], neurofibromatosis 2 (NF2) [7], spleen tyrosine kinase (SYK) [8] platelet-derived growth factor receptor (PDGFR) [9], epidermal growth factor receptor (EGFR) [10] and β-catenin [11,12], suggesting its possible function in carcinogenesis. Indeed, abnormal expression of NHERF1 has been found in several kinds of tumors, such as hepatocellular carcinoma, breast cancer, and nerve schwannoma [11,13,14]. NHERF1 overexpression in breast tumors is associated with tumor stage, metastatic progression, and poor prognosis [15,16], implying that NHERF1 is a potential oncoprotein. However, contrasting results have recently been reported. Downregulation of endogenous NHERF1 in breast cancer cells promoted proliferation, tumorigenic ability, and delayed the turnover of PDGF-induced AKT phosphorylation [17,18]. In our previous

* Corresponding author. address: Department of Biochemistry and Molecular Biology, Capital Medical University, No. 10 Xitoutiao, You An Men, Beijing 100069, China. Fax: +86 10 8395 0126.
E-mail address: jq_he@ccmu.edu.cn (J. He).
[1] These authors contribute equally in this paper.
[2] Current address: Department of Radiology, First Hospital of Shanxi Medical University, Taiyuan, China.

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study, overexpression of NHERF1 in a breast cancer cell line suppressed cell proliferation by promoting apoptosis and inhibiting ERK activation [19], suggestive of a tumor suppressor role for NHERF1. These contradictory reports suggest a complicated role for NHERF1 in breast cancer.

Two point mutations of *nherf1* (K172N and D301V) were recently identified in breast cancer cell lines or primary breast tumors [8]. However, there is no direct evidence to support a causal role for these mutations in cancer development and progression. NHERF1 was reported to form a ternary complex with PDGFR and PTEN in mouse embryonic fibroblasts. NHERF1 depletion enhanced PDGF-induced activation of the PI3K/AKT pathway, cytoskeletal rearrangements, and chemotactic migration [18]. However, the effects of the breast cancer-derived *nherf1* mutations on the interactions of NHERF1 with PDGFR and PTEN have not yet been established.

In this context, we investigated the effects of wild-type (wt) and point mutations of *nherf1* (K172N and D301V) on the proliferation, migration, and invasion of SKMES-1 cells to gain insight into the NHERF1 function, and to improve our understanding of the role of NHERF1 in tumor development and progression.

2. Materials and methods

2.1. Expression vectors

NHERF1 cDNA were cloned into pEF6 vector by using pEF6/V5-His TOPO TA expression kit (Invitrogen, Paisley, Scotland, UK) according to the manufacturer's instructions. NHERF1 cDNA of mutants were excised from GST-NHERF1 mutant constructs K172N and D301V (kindly provided by Dr. Jiale Dai, MD Anderson Cancer Center, Houston, TX) and were subcloned into pEF6 vector. PDGFRβ-CT encoding the last 43 amino acids of human PDGFRβ was amplified via PCR and inserted into pGEX-4T-1 for expression as GST fusion protein.

2.2. Cell lines

Non-small cell lung cancer cell line, SKMES-1, was from the European Collection for Animal Cell Culture (ECACC, Porton Down, Salisbury, UK). HeLa and COS-7 cells were from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in DMEM plus 10% FBS, 1% penicillin and streptomycin (Gibco BRC, Paisley, Scotland, UK).

2.3. Cell transfection

COS-7 cells and SKMES-1 cells were respectively transfected with PDGFRβ and His-NHERF1 by using Hifection II (Applygen, Beijing, China). COS-7 cells were harvested 36 h after transfection. SKMES-1 cells were selected with 1.25 μg/ml blasticidin S in culture medium for further 14 days to generate stable NHERF1-expressing cell lines. NHERF1 expression was verified by Western blotting.

2.4. Knockdown of NHERF1 expression using ribozyme transgenes

Ribozyme transgenes were employed to suppress NHERF1 expression in cells. Briefly, the secondary structure of human NHERF1 was generated using Zucker's RNA mFold software. The ribozyme that specifically targets NHERF1 was generated using touchdown PCR with the appropriate primers (sense: 5′-CTGCAGCCGGGGAGTCTGGGTCCACTGACCGCTGATGAGTCCGTGAGGA-3′; antisense: 5′-ACTAGTCCAAGCCAGGCCAGTTCTTTCGTCCTCACGGACT-3′. The resulting correct inserts were purified and cloned into the pEF6/V5-His-TOPO vector, and then transformed into cells. Knockdown of NHERF1 expression was verified by Western blotting.

2.5. GST pull-down and Western blotting

GST pull-down assays and Western blotting was performed as described previously [20]. Anti-PTEN and anti-pS473AKT antibodies were purchased from Cell Signaling Technology (Danvers, MA), anti-AKT antibody from Sigma (St. Louis, MO), anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ERK, anti-p-ERK and anti-PDGFRβ antibodies from Upstate Biotechnology (Lake Placid, NY). Anti-His antibody was from MBL (Tokyo, Japan). Anti-NHERF1 antibody was obtained from Cell Signaling Technology (Danvers, MA).

2.6. Cell proliferation assay

Cells were seeded in a 96-well plate at 2.5×10^3 cells per well and were stained with 0.5% crystal violet at indicated time points. Viable cells were quantified by measuring absorbance at 540 nm with an Elx800™ spectrophotometer (BioTek, Winooski, VT).

2.7. Wound-healing assay

The migratory properties of cells were assessed by wound-healing assay. A variety of cells were seeded at a density of 2×10^5 cells/well into a 24-well plate and allowed to reach confluence. The layer of cells was then scraped with a fine gauge needle to create a wound of approximately 200 μm. The movement of cells to heal the wound was recorded and analyzed using a time-lapsed video system. Migration of the cells was monitored and recorded on a video for 90 min. Wound closure/cell migration was evaluated with motion analysis and line morphometry software (Optimus 6).

2.8. Cell invasion assay

Transwell chambers, equipped with a 6.5 mm diameter polycarbonate filter insert (pore size 8 μm) (Becton Dickinson, Labware, Oxford, UK), were pre-coated with 50 μg/insert of Matrigel (BD Biosciences, Oxford, UK). Cells were seeded at a density of 3×10^4 cells/insert. 3 days later, the cells that invade through the Matrigel were fixed, stained, and quantified as described before [21].

2.9. Statistical analysis

The 2-sample *t* test was used for normally distributed data. *P* Value of less than 0.05 is considered to be statistically significant.

3. Results

3.1. NHERF1 overexpression reversed malignant phenotypes of SKMES-1 cells

Previous studies on the tumor-suppressor functions of NHERF1 were carried out by knockdown of NHERF1 expression in either normal or high-NHERF1-expressing cancer cell lines [17,18]. However, suppression of the malignant phenotype by overexpression of NHERF1 has not yet been extensively studied. It is therefore necessary to determine its biological roles by using a low-NHERF1-expressing cancer cell model. NHERF1 transcript level was evaluated and compared among different cancer cell lines to screen the low-NHERF1- expressing cancer cell lines. The lowest level of NHERF1 transcripts was observed in the SKMES-1 cells (Fig. S1).

NHERF1 was then stably transfected into SKMES-1 cells and its expression was assessed by Western blotting. The expression level

of His-NHERF1 was considerably increased in transfected cells, compared with parental or vector control cells (Fig. 1A). Major malignant phenotypes, including cell proliferation, invasion, and migration, were then evaluated. Compared with parental or vector control cells, cell proliferation was suppressed by up to 60% in cells overexpressing NHERF1-wt on day 5 (Fig. 1B). Cell migration and invasion were also reduced by up to 30% and 25% at 90 min and 72 h, respectively (Fig. 1C–D).

3.2. Breast cancer-derived nherf1 mutations impair tumor-suppressor function of NHERF1

Two missense mutations of *nherf1* (K172N and D301V) were previously found in a breast tumor and in the SUM149PT breast cancer cell lines, respectively. To further examine if these mutations could inactivate the tumor-suppressor function of NHERF1, they were introduced into SKMES-1 cells respectively. As demonstrated by Western blotting, these mutants were expressed at similar levels to NHERF1-wt (Fig. 1E). We then explored their effects on the malignant phenotype of cancer cells, such as proliferation, migration and invasion. As shown in Fig. 1F, each of the *nherf1* mutations significantly inactivated the cell proliferation suppressed by NHERF1-wt, to a similar level that shown in vehicle control cells. In wound healing assay, both of the *nherf1* mutations abolished the inhibition of NHERF1-wt-mediated cell migration, since there were no significant differences in migration rates between control and both of the two mutants-expressing cells (K172N and D301V) (Fig. 1G), which is consistent with the results

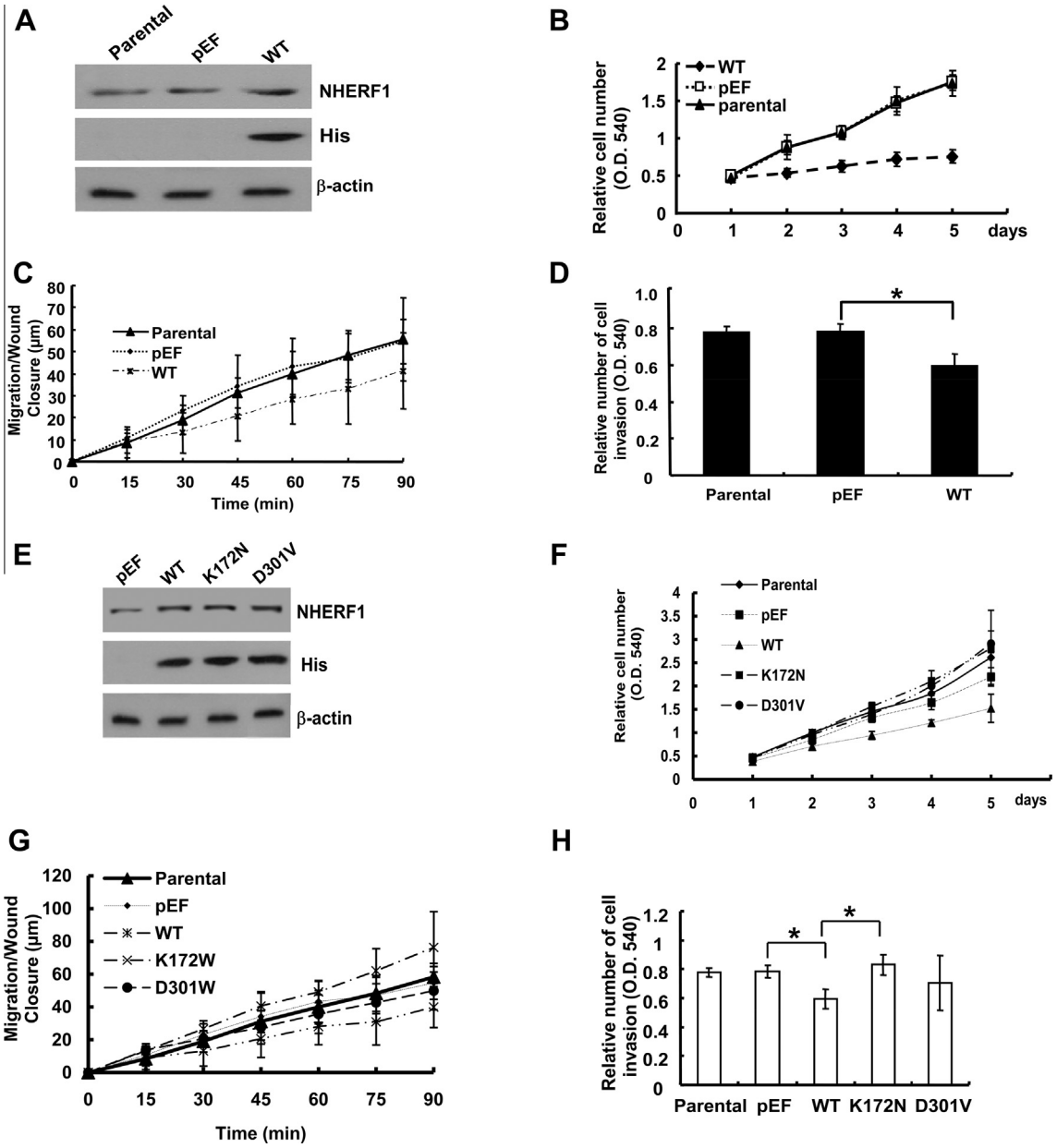

Fig. 1. Breast cancer-derived *nherf1* mutations (K172N and D301V) abolished the antitumor effects of NHERF1 in SKMES-1 cells. Exogenous NHERF1 wild type (A) or mutants (E) was stably expressed in SKMES-1 cells, and detected with anti-NHERF1 or anti-His antibodies. NHERF1-wt overexpression suppressed cell proliferation (B), migration (C), and invasion (D) of SKMES-1 cells. *nherf1* Mutations (K172N and D301V) inactivated the cell proliferation (F), migration (G) and invasion (H) suppressed by NHERF1-wt. In panels B–D and F–H, data represent mean values ± standard deviation (SD) of six (B, C, F, G) or triplicate (D, H) samples. The results shown are representative of three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

obtained from cytodex-2 bead motility assay (data not shown). Similarly, cell invasion rates of both mutants had no statistically difference compared with vehicle control cells (Fig. 1H). These results indicated that NHERF1-wt acted as a tumor suppressor in SKMES-1 cells, and the breast cancer-derived *nherf1* mutations (K172N and D301V) abolished or reduced its function of tumor-suppressor effects.

3.3. NHERF1 facilitates the formation of PTEN/NHERF1/PDGFRβ complex

It has been proven that NHERF1 inhibits PDGF signaling in breast cancer cells, and NHERF1 has been reported to interact with PTEN and PDGFRβ respectively [17]. However, there is no direct evidence that NHERF1 can bridge the PTEN/NHERF1/PDGFRβ complex. To confirm the role of NHERF1 in the formation of the PTEN/NHERF1/PDGFRβ complex, GST-PDGFRβ-CT fusion protein beads were incubated with the protein extracts from COS-7 cells, and endogenous PTEN were then detected by Western blotting. As shown in Fig. 2A, NHERF1 overexpression robustly enhanced the amount of PTEN in the pull-down complexes. Accordingly, knockdown of NHERF1 expression decreased the amount of PTEN in the pull-down complexes (Fig. 2B). These data indicated that NHERF1 could facilitate the PTEN/NHERF1/PDGFRβ complex formation.

3.4. nherf1 Mutations alter its interaction with PTEN and PDGFRβ

To further investigate if *nherf1* mutations could affect the formation of PTEN/NHERF1/PDGFRβ complex, various GST-NHERF1 fusion protein beads were incubated with protein extracts from COS-7 cells, and PTEN were then detected in the pull-down complex. GST-NHERF1-wt associated robustly with endogenous PTEN protein, whereas GST alone did not. Although both NHERF1-K172N and NHERF1-D301V were able to bind to PTEN, their affinities with PTEN were reduced by almost 20%, compared with that of NHERF1-wt (Fig. 2C). Furthermore, the effect of the mutations on the NHERF1-wt/PDGFRβ interaction were also analyzed in COS-7 cells transfected with PDGFRβ. GST-NHERF1-wt robustly associated with exogenous PDGFRβ protein, and this interaction was unaffected by NHERF1-K172N mutation; however, NHERF1-D301V promoted up to a 2-fold increase in NHERF1-PDGFRβ inter-

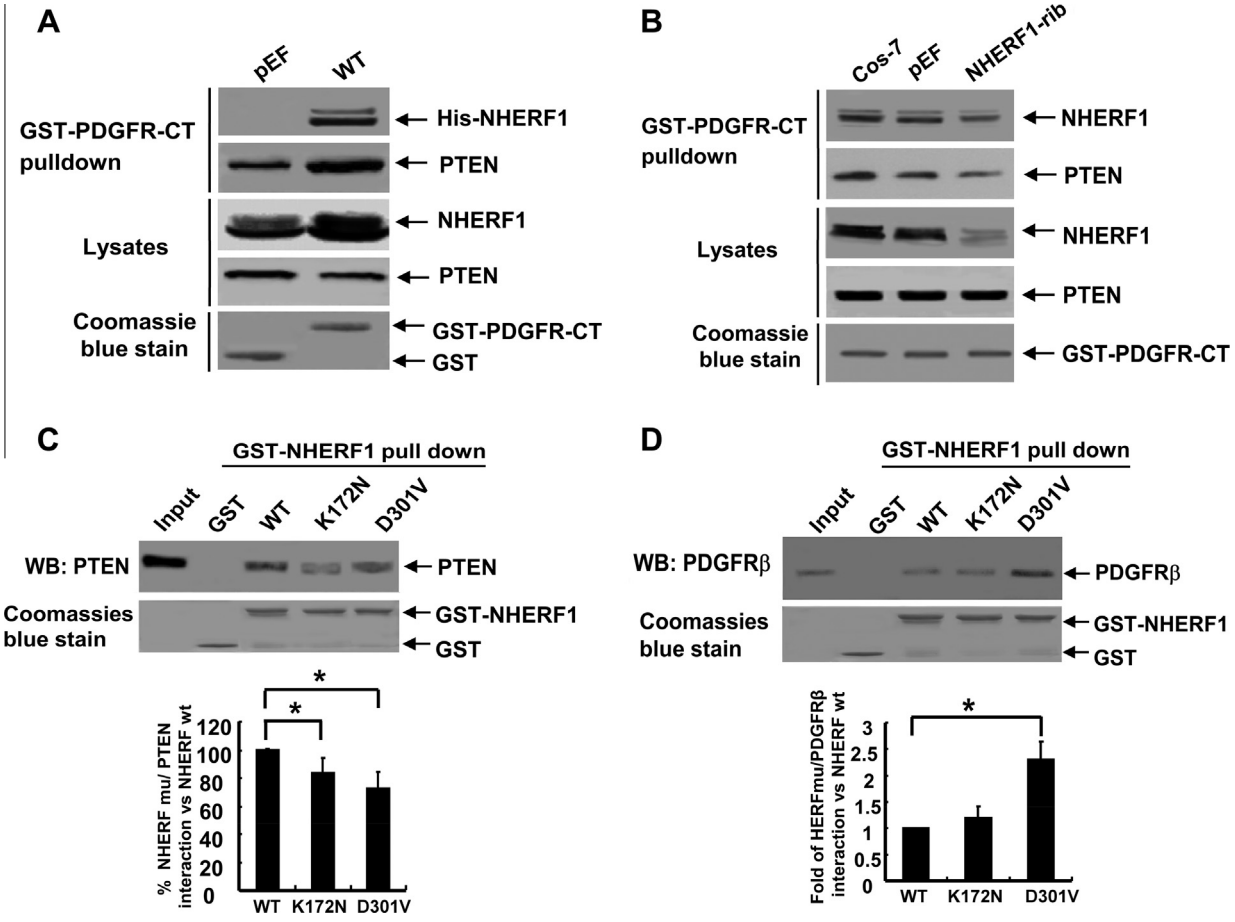

Fig. 2. *nherf1* Mutations (K172N and D301V) reduced the affinity of NHERF1 with PTEN or PDGFRβ. (A) Overexpressing NHERF1-wt enhanced the PTEN/NHERF1/PDGFRβ complex formation in COS-7 cells. GST-PDGFRβ-CT beads were incubated with lysates of COS-7 cells transfected with control vector or pEF-NHERF1-wt. Pull-down complexes were then subjected to NHERF1 and PTEN immunoblotting respectively. (B) Knocking down NHERF1 expression attenuated the PTEN/NHERF1/PDGFRβ complex formation. COS-7 cells were transfected with vector control or NHERF1 ribozyme and then harvested in lysis buffer 48 h post-transfection. GST-PDGFRβ-CT beads were incubated with the cell lysates. The bound proteins were subjected to PTEN and NHERF1 immunoblotting. (C) *nherf1* Mutations (K172N and D301V) altered NHERF1 interaction with PTEN. GST-NHERF1 beads were incubated with COS-7 cell lysates, and then the pull-down complexes were subjected to PTEN immunoblotting. (D) *nherf1* Mutations (K172N and D301V) affected NHERF1 interaction with PDGFRβ by GST pull-down assay. GST-NHERF1 beads were incubated with lysates of COS-7 cells transfected with PDGFRβ. The bound proteins were subjected to PDGFRβ immunoblotting. In A and B, the cell lysates were normalized by the same amount of PTEN. In A–D, the Coomassie-blue stained gel to visualize the same amount of input fusion protein. The signals were quantified by densitometry analysis. The results represent mean values ± SD of three independent experiments.

action (Fig. 2D). These results suggested that NHERF1-K172N and NHERF1-D301V could affect the formation of PTEN/NHERF1/PDGFRβ complex.

3.5. *nherf1* Mutations inactivate NHERF1 inhibition of AKT and ERK activation induced by PDGF

The PTEN/NHERF1/PDGFRβ complex has been reported to be involved in the regulation of AKT and ERK signaling pathways, which in turn control important biological functions such as cell proliferation and migration [5,17,18]. Since *nherf1* mutations affected the PTEN/NHERF1/PDGFRβ complex formation, it was reasonable to assume that these mutations could also affect PI3K/AKT or ERK signaling. To test this hypothesis, we detected phosphorylation of AKT and ERK in SKMES-1 cells transfected with various NHERF1 constructs. Both AKT (Fig. 3A) and ERK (Fig. 3B) were dramatically phosphorylated in either SKMES-1 parental or vector control cells following 15-min stimulation with PDGF. NHERF1-wt overexpression significantly inhibited both AKT and ERK activation. However, the NHERF1 mutations, especially D301V, totally abolished the NHERF1-wt inhibition of AKT and ERK activation (Fig. 3), Meanwhile the base levels of AKT and ERK activation have no detectable difference in cells transfected with a variety of NHERF1 constructs (Fig. S2). Taken together, these data suggested that cancer-derived *nherf1* mutations (K172N and D301V) abolished the inhibition of PI3K/AKT and/or ERK signaling pathways activation induced by PDGF.

4. Discussion

Previous reports have been suggested that NHERF1 plays a tumor suppressor role in breast cancer cell lines. NHERF1 inhibited canonical Wnt signaling and Wnt-dependent cell proliferation in MCF7 and MDA MB-231 cells [22]; By interaction with PTEN, NHERF1 counterbalanced PI3K/Akt oncogenic signaling and enhanced cells respond to PDGFR inhibitor in breast cancer cell lines [9,18,23]; NHERF1 knockdown accelerated cell cycle progression accompanied by increased expression of cyclin E and elevated Rb phosphorylation level [17]. Furthermore, the mammary glands of NHERF1-knockout mice exhibit increased mammary duct density accompanied by increased proliferation and β-catenin activity [22]. The present study showed that overexpression of NHERF1 reduced cell proliferation, motility, and invasion of low-NHERF1-expressing SKMES-1 cells, and knockdown of NHERF1 enhanced the migratory and invasive ability of MCF-7 cells (Fig. S3). Two point mutations of *nherf1* (K172N and D301V) has recently been identified in breast cancer cells and breast tumor [8], but it has not yet been characterized if these mutations cause NHERF1 loss-of-function. The current studies showed that the K172N and D301V mutants significantly inactivated the NHERF1 suppression of SKMES-1 cells proliferation, migration, and invasion. The results of both gain-of-function and loss-of-function studies indicated that NHERF1 acted as a tumor suppressor in tumor cells.

In clinical perspectives, NHERF1 overexpression was observed in breast cancer tissues, which is associated with increasing tumor grade, aggressive clinical behavior, and poor prognosis [24–26]. This was contradictory with NHERF1 to be a cancer suppressor. However, it has been proven that compensatory expression mechanisms exist for many anti-oncogenes [27–29]. The p73, as the first discovered anti-oncogene of p53 family member, is a stress-response gene and a downstream effector in the p53 pathway [27]. The p73 protein is expressed at very low levels in normal tissues and differentially expressed in a number of tumors [28,29]. $P16^{INK4A}$ is recognized as a tumor suppressor mainly because of the prevalence of genetic inactivation of the $p16^{INK4A}$ gene in virtually all types of human cancers. However, it has also been shown that an elevated level of expression (upregulation) of $P16^{INK4A}$ is involved in cellular senescence, and cancer progression [30,31]. Thus, upregulation of NHERF1 expression during breast carcinogenesis might also involve compensatory mechanism.

NHERF1, as a signaling adaptor, can regulate multiple cellular processes via interaction with its different binding partners. NHERF1 mutations may alter the binding affinity of NHERF1 to some of its signaling partners, and lead to the loss of its anticancer functions. Here, we found that NHERF1-K172N and NHERF1-D301V mutants attenuated its interaction with PTEN; NHERF1-D301V mutant increased its interaction with PDGFRβ. Furthermore, we previously identified Ser302 as a phosphorylation site by cdc2, and this phosphorylation has been shown to regulate NHERF1 homodimerization [32]. D301V mutation is adjunct to Ser302 residue. Thus, it will be interesting to investigate if

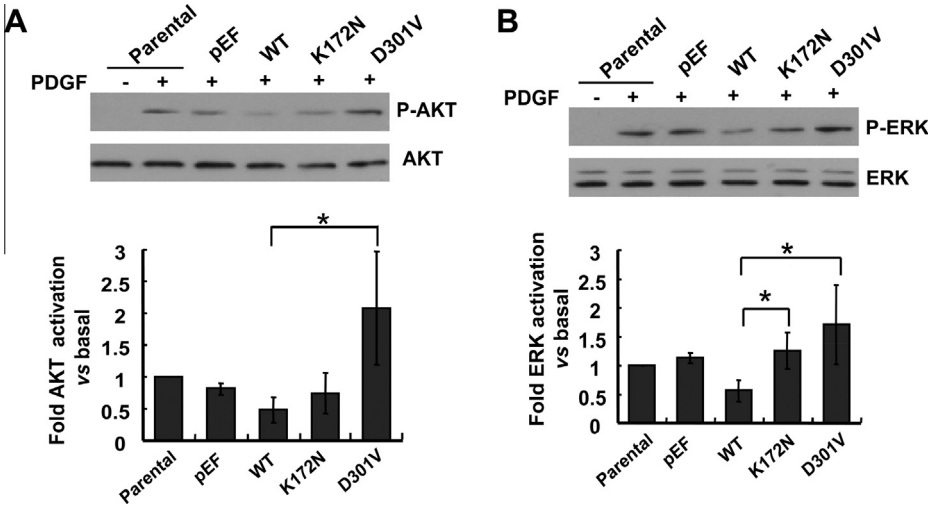

Fig. 3. *nherf1* Mutations (K172N and D301V) cause loss-of-function of NHERF1 inhibition of AKT and ERK activation induced by PDGF. SKMES-1 cells stably transfected with various of NHERF1 mutants were serum starved overnight, and then stimulated with or without PDGF for 15 min. Immunoblot was detected with anti-pS473AKT (A) or anti-p-ERK antibody (B) (upper panels). Membranes were stripped and reprobed with anti-AKT (A) or anti-ERK (B) respectively in middle panels. The signals were quantified by densitometry analysis (lower panels). Data is given as fold of stimulated parental cells. The results represent mean values ± SD of three independent experiments. $^{*}P < 0.05$ relative to the cells transfected with NHERF1-wt.

D301V mutation can affect NHERF1 phosphorylation at Ser302, and further modify NHERF1 intramolecular interaction.

Except K172N and D301V, other point mutations in *nherf1* including R180W have also been identified in breast cancer cells by Dai's team [8] and our unpublished data. R180W mutation also abolish the tumor-suppressor effects of NHERF1, even though the R180W mutation has less effect of NHERF1 interaction with PTEN or PDGFRβ (data not shown), and it may influence its interaction with other tumor suppressors such as SYK and Merlin [8]. Different point mutation may result in the different changes in spatial structure which could lead to the different biologic activities in cells. These findings further support the tumor-suppressor function of NHERF1 in tumor cells, and suggest that these NHERF1 mutations may play a causal role in tumor development and progression.

It was reported that knockdown of NHERF1 expression leads to defective PTEN recruitment to PDGFR, suggesting NHERF1 is required for the PDGFRβ/PTEN complex assembly [5]. However, there is no direct evidence that NHERF1 bridges the PTEN/NHERF1/PDGFRβ complex. Here, to confirm the role of NHERF1 in the formation of the PTEN/NHERF1/PDGFRβ complex, we conducted a pull-down experiment (Fig. 2A and B) using GST-PDGFRβ-CT to pull down the COS-7 lysates with both NHERF1 overexpression (Fig. 2A) or knockdown (Fig. 2B). Either overexprssion or knockdown of NHERF1 significantly influenced the PTEN/PDGFRβ interaction, which directly demonstrated that NHERF1 bridged the formation of PTEN/NHERF1/PDGFRβ complex. Disruption of this complex was reported to lead to an increase of PDGF-induced AKT signaling and cell migration [18]. In this study, we showed that *nherf1* K172N and D301V mutations markedly inactivated NHERF1-suppressed activation of AKT and ERK upon PDGF stimulation. Hence, loss of the suppression of ERK and AKT activities in the cells carrying *nherf1* mutations might explain their increased cell proliferative phenotype.

The *nherf1* mutations were initially identified from breast cancer cells and tissues. However, NHERF1 was highly expressed in all of the breast cancer cell lines which we screened, and the only cell line suitable for this study was SKMES-1, a lung cancer cell line (Fig. S1). The data obtained here therefore have implications on breast cancer as well as on lung cancer. Our preclinical study showed that NHERF1 expression deregualted in human lung cancer, and associated with lung cancer metastasis. Also, NHERF1 expression levels were negatively correlated with aggressive stage of lung cancer (our unpublished data). It has also been shown that NHERF1 plays an important role in the respiratory condition in cystic fibrosis [33,34]. In human lung cancer, however, the candidate binding partners have been demonstrated to be aberrant and are linked to disease progression. Targeting of these partners proteins have been demonstrated to be beneficial to the patients. For examples, PDGFR inhibitors showed potential benefit in reducing growth of lung tumor [35,36]. The same have been reported for Akt, PI3K and EGFR [37,38].

Overall, our study demonstrated that NHERF1-wt overexpression reversed the malignant phenotypes of SKMES-1 cells, including cell proliferation, migration, and invasion. Breast cancer-derived *nherf1* mutations (K172N and D301V) affected the formation of PTEN/NHERF1/PDGFRβ complex, inactivated the NHERF1 inhibition of PDGF-induced AKT and ERK activation, and resulted in complete or partial loss of the tumor-suppressor effects of NHERF1-wt. These results support a role for *nherf1* mutation in tumor development and progression, and provide an evidence of the functional consequences and signaling properties of breast cancer-derived *nherf1* mutations, thus gain more insight into the pathogenesis of breast cancer and lung cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.026.

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