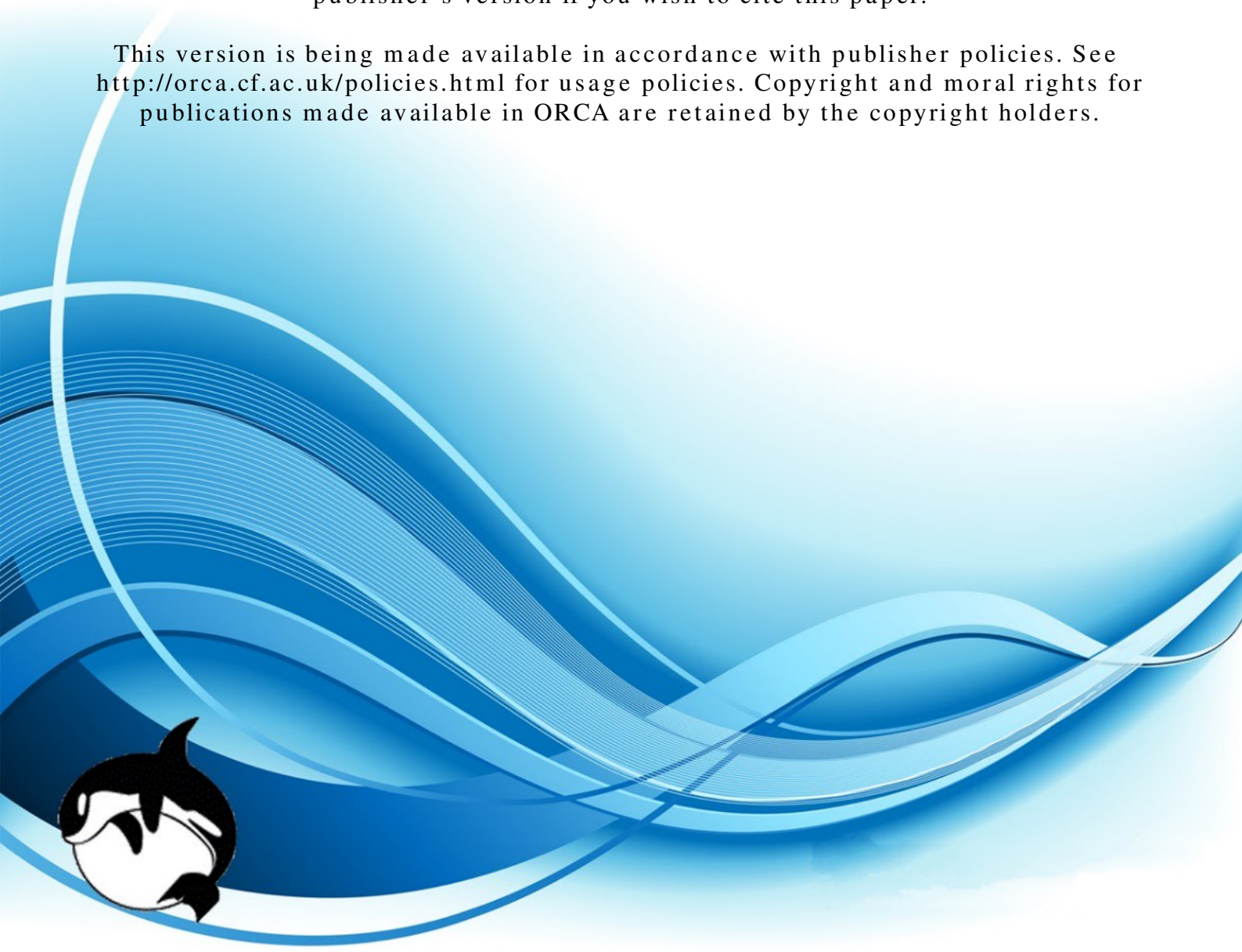

A Novel *NHERF1* Mutation in Human Breast Cancer Inactivates Inhibition by NHERF1 Protein in EGFR Signaling

GUIFANG DU[1,2], CHENGCHENG HAO[1,2], YANAN GU[1,2], ZHIYAN WANG[1,2], WEN G. JIANG[1,2,3], JUNQI HE[1,2] and SHAN CHENG[1,2]

[1]*Department of Biochemistry and Molecular Biology, and* [2]*Beijing Key Laboratory of Cancer & Metastasis Research, Capital Medical University, Beijing, P.R. China;*
[3]*Cardiff China Medical Research Collaborative, Cardiff University School of Medicine, Heath Park, Cardiff, U.K.*

Abstract. *Background: Na^+/H^+ exchanger regulatory factor 1 (NHERF1) has been reported to interact with many cancer-related proteins. We recently identified a novel NHERF1 mutation (E43G) in breast tumours. Materials and Methods: The candidates of NHERF1 mutation were identified in breast cancer tissues by polymerase chain reaction and DNA sequencing. Wild-type NHERF1 and E43G mutation were expressed in NHERF1-knockdown cells (MCF7ΔNHERF1) and low-NHERF1-expressing cells (SKMES-1). The effects of mutated NHERF1 on cell functions were examined using in vitro methods. Glutathione S-transferase pull-down assays and western blotting were performed to study the effects of NHERF1 mutation on its interaction with cancer-related proteins. Results: Compared to wild-type NHERF1, expression of the mutated NHERF1 failed to suppress malignant traits in cancer cells, attenuated interaction of NHERF1 protein with epidermal growth factor receptor (EGFR), and inactivated its inhibition of EGF-induced Akt and extracellular regulated protein kinases (ERK) activation. Conclusion: The results show the causal role of NHERF1 in the regulation of the EGFR pathway and the progression of breast cancer.*

During the past decade, the systemic therapeutic management of breast cancer has undergone a significant transformation. Without targeted therapies, conventional treatment with cytotoxic agents has maximized its potential in terms of patient survival for most types of cancers. Enhanced understanding of the pathogenesis of tumour cell growth and metastasis has led to the identification of growth signalling pathways as targets for these directed therapies (1). Epidermal growth factor receptor (EGFR) is over-expressed or activated in breast cancer (2, 3), and a number of drugs specifically targeting this tyrosine kinase have been developed over the years as anticancer agents (4, 5). Evidence in cells has shown that the scaffolding protein Na^+/H^+ exchanger regulatory factor 1 (NHERF1) can associate with EGFR to regulate its trafficking and expression in breast cancer cells and, modulating its role in progression and metastasis of breast cancer (6).

NHERF1 is a protein with two structural domains named PDZ1 and PDZ2 followed by an ezrin–radixin–moesin-binding region (7, 8). These domains bind to the carboxy-terminal parts of transporters or receptors (9-11). Hence, NHERF1 binds to the C-terminal of EGFR and stimulation with EGF induces interaction of NHERF1 with EGFR to regulate localization, degradation and function of the latter (12). NHERF1 can suppress EGF-induced proliferation of breast cancer cells by inhibiting EGFR phosphorylation and blocking EGFR downstream signalling in breast cancer cells (13).

Loss of heterozygosity (LOH) at the *NHERF1* gene locus (17q25.1) is found in more than 50% of human breast tumours (14), whereas being less frequent in other tumour types, suggesting a pivotal role of NHERF1 during breast carcinogenesis (14). Previous reports have suggested that NHERF1 plays a tumour-suppressor role in breast cancer cell lines. NHERF1 inhibited canonical Wnt signalling and Wnt-dependent cell proliferation in MCF7 and MDA MB-231 cells (15). By interaction with phosphatase and tensin homolog deleted on chromosome 10 (PTEN), NHERF1 counter-balanced phosphatidylinositol 3-kinase/Akt (PI3K/Akt) oncogenic signaling and enhanced cell responses to platelet-derived growth factor receptor (PDGFR) inhibitor in breast cancer cell lines (16). *NHERF1* knockdown accelerated cell-cycle progression, accompanied by increased expression of cyclin E and elevated retinoblastoma phosphorylation level (14). Furthermore, the mammary glands of *NHERF-1-*

Correspondence to: Shan Cheng, Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, China. Tel: +0086 1083950184, e-mail: chengs@ccmu.edu.cn; Junqi He, Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, China. Tel: +0086 1083950125, e-mail: jq_he@ccmu.edu.cn

Key Words: NHERF1 mutation, breast cancer, EGFR.

knockout mice exhibit increased mammary duct density accompanied by increased proliferation and β-catenin activity (17). Our previous studies also showed that over-expression 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 (18). The results of both gain-of-function and loss-of-function studies indicated that NHERF1 acts as a tumour suppressor in tumour cells.

In the present study, a novel *NHERF1* gene mutation (E43G) was identified in a patient with breast cancer. The effects of wild-type (WT) and *NHERF1* mutation E43G on the proliferation, migration, and adhesion of tumour cells were investigated in order to gain insight into NHERF1 function, and to improve our understanding of the role of NHERF1 in tumour development and progression.

Materials and Methods

Cell lines and culture. A human breast cancer cell line, MCF-$7^{\Delta NHERF1}$, with low expression of *NHERF1*, was constructed previously (18), by stably expressing a ribozyme targeted to *NHERF1*. Non-small cell lung cancer cell line SKMES-1 was obtained from the European Collection for Animal Cell Culture (Porton Down, Salisbury, UK). *Cos-7* cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1% penicillin and streptomycin (Gibco BRC, Paisley, UK) in an incubator at 37˚C, with 5% CO_2, and 95% humidity.

Human breast specimens. A total of 20 breast samples were obtained from patients with breast cancer. These tissues were collected immediately after mastectomy, and snap-frozen in liquid nitrogen, with the approvals of the Local Ethical Committee (2013SY15). None of the patients had any underlying medical condition and none were taking any medication.

Mutational analyses of NHERF. The genomic DNA from 20 frozen breast cancer tissues was extracted using the DNeasy kit (Qiagen, Hilden, Germany). All six exons of *NHERF1* gene were amplified by PCR using primers corresponding to the neighbouring intronic sequences, and further analyzed by single-strand conformation polymorphism (SSCP) as previously described (19). The sequences of the intronic primers used for the polymerase chain reaction have already been published (20). The candidates of the mutant gene were then identified by DNA sequencing.

Preparation of expression vectors. Wild-type glutathione S-transferase (GST)–NHERF1 construct was kindly provided by Dr. Jiale Dai (MD Anderson Cancer Center, Houston, TX, USA). *GST–NHERF1* mutation construct was generated from the wild type with the use of Fast MultiSite Mutagenesis System kit (TransGen, Beijing, China). cDNA of *NHERF1-WT* and mutation were cloned into pEASY™-M2 vector using pEASY-Blunt M2 Expression Kit (TransGen) according to the manufacturer's instructions. All constructs were verified by sequencing.

Cell transfection. Subconfluent cells were transfected with *NHERF1* expression constructs using lipofectamine reagent according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA). MCF-$7^{\Delta NHERF1}$ and SKMES-1 cells were selected in culture medium containing 600 μg/ml G418 for 21 days to generate stable *NHERF1*-expressing cell lines. NHERF1 expression was verified by western blotting.

Wound-healing assay. The migratory properties of cells were assessed using a 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 1500 μm. Images of the wound were recorded under a phase-contrast microscope at different time points (0 h, 6 h, 12 h, 24 h). Wound closure/cell migration was evaluated with Image-Pro plus (National Institutes of Health, Maryland, USA).

GST pull-down assays and western blotting. GST pull-down assays and western blotting was performed as described previously (15). Anti-NHERF1was purchased from Becton Dickinson Company (Franklin Lake, New Jersey, USA), anti-extracellular regulated protein kinases(ERK) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho-Akt (Ser473), anti-Akt, anti-EGFR, anti-phospho-EGFR (Tyr1173) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-mitogen-activated protein kinase (MAPK)1/2 (ERK 1/2) was from Millipore (Massachusetts, USA).

Statistical analysis. The results were assessed using non-paired (two-sided) Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant. Each assay was repeated at least three times.

Results

Identification of the NHERF1E43G mutation in breast cancer. The coding region and the intron-exon junctions of the *NHERF1* gene were analyzed by PCR-SSCP (Figure 1A) and confirmed by DNA sequencing (Figure 1B). A previously unreported sequence variant (GAG to GGG), that would result in a switch of codon 43 (Glu-Gly), was identified in the first exon of *NHERF1* in a patient with medullary breast carcinoma. The mutation corresponded to a conserved basic residue in the PDZ1 domain (Figure 1C). The alignment of the NHERF1 protein sequence showed that glutamic acid 43 was highly conserved in the NHERF1 PDZ1 domain throughout evolution and in the PDZ1 domain of the other species in NHERF protein family (Tables I and II), suggesting that it plays an important role in the function of this domain. Furthermore this variant was not found in the 1000 genomes database (http://browser.1000genomes.org).

NHERF1E43G mutation impaired tumour-suppressive effects on malignant cell phenotypes. Previous studies on the tumour-suppressor functions of NHERF1 were carried out by knockdown or over-expression of *NHERF1* in cancer cell lines (14, 17, 21). Our previous studies also showed that *NHERF1*

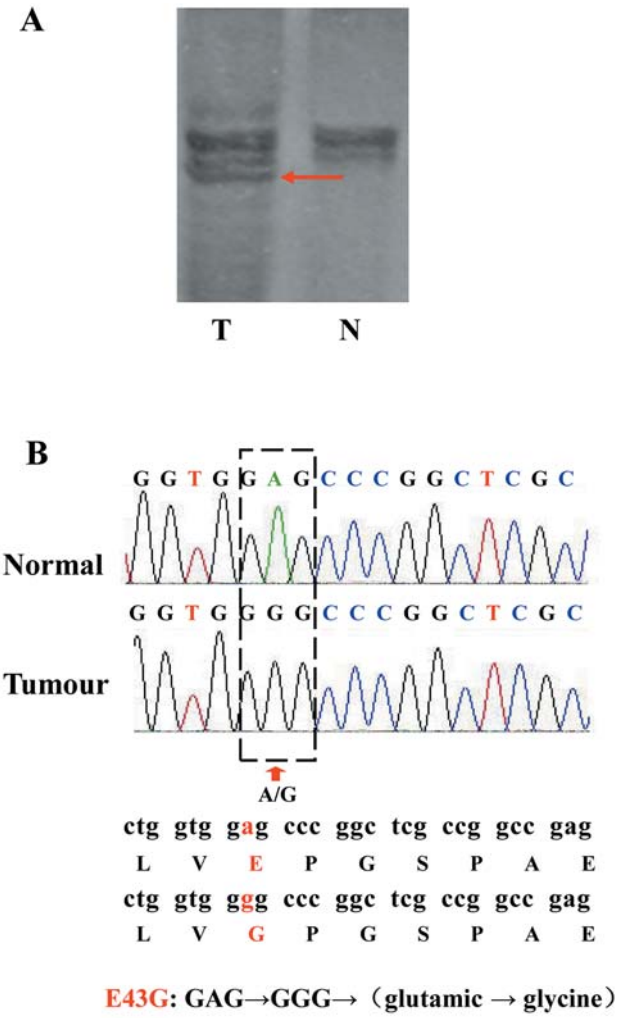

Figure 1. *A novel Na^+/H^+ exchanger regulatory factor 1 (NHERF1) mutation was identified in patients with medullar breast carcinoma. NHERF1 mutation in exon 1 was analysed by single-strand conformation polymor-phism(SSCP) analysis. A: Abnormal migratory SSCP band patterns for the mutation (T) are demonstrated as compared with normal (N). B: A previously unreported sequence variant (GAG to GGG) was identified by sequencing, which would result in a switch of codon 43 (Glu-Gly). C: The mutation corresponded to a conserved basic residue in the PDZ I domain.*

behaves as a tumour suppressor in breast cancer cells (18). To examine the effects of the E43G mutation on the tumour-suppressive function of *NHERF1*, it was transfected into the low-*NHERF1*-expressing cancer cells SKMES-1 and MCF-$7^{\Delta NHERF1}$. Its expression was assessed by western blotting. The expression level of NHERF1 was considerably increased in the stably-transfected cells compared to parental and vector control cells, and in cells with E43G mutation was expressed at similar levels to NHERF1-WT (Figure 2A and B). Major malignant phenotypes, including cell proliferation and migration, were then evaluated. Compared to vector control cells, cell proliferation was suppressed by up to 22% and 46%, respectively, in SKMES-1 (on day 4) and MCF-$7^{\Delta NHERF1}$ (on day 4) cells overexpressing NHERF1-WT (Figure 2C and D), cell migration was also reduced by up to 50% and 30% respectively at 24 h (Figure 2E, F). *NHERF1E43G* mutation was able to rescue the cell proliferation and migration that was suppressed by NHERF1-WT, since there were no significant differences in proliferation and migration rates between control and the mutant-expressing cells (Figure 2C-F). These results indicate that NHERF1-WT acted as a tumour suppressor in SKMES-1 and MCF-$7^{\Delta NHERF1}$ cells, and the breast cancer-derived *NHERF1* mutation E43G abolished its suppressor effects on cell proliferation and migration.

NHERF1E43G mutation altered interaction of NHERF1 protein with EGFR. It has been shown that NHERF1 interacts with EGFR through its PDZ1 domain and inhibits EGFR signalling in breast cancer cells (12, 13, 22). To further investigate if *NHERF1* mutation could affect the interaction between NHERF1 and EGFR, various GST–NHERF1 fusion protein beads were incubated with protein extracts from COS-7 cells, and EGFR was then detected in the pull-down complex. GST–NHERF1-WT associated robustly with endogenous EGFR protein, whereas GST alone did not. Although NHERF1-E43G was able to bind to EGFR, its affinity to EGFR was reduced by almost 40% compared to that of NHERF1-WT (Figure 3). These data indicate that *NHERF1*E43G mutation weakened the interaction of NHERF1 with EGFR.

NHERF1E43G mutation inactivated NHERF1 inhibition of EGF-induced Akt and ERK activation. NHERF1 has been reported to suppress the phosphorylation activation of EGFR, blocking EGFR downstream signaling in breast cancer cells (13), which in turn controls important biological functions such as cell proliferation and migration (23). Since *NHERF1* mutation affected NHERF1–EGFR complex formation, it was reasonable to assume that the mutation could also affect EGFR signaling. To test this hypothesis, we detected phosphorylation of EGFR, Akt and ERK in MCF$7^{\Delta NHERF1}$ cells transfected with different *NHERF1* constructs. EGFR (Figure 4A), Akt (Figure 4B) and ERK (Figure 4C) were all

Table I. *Glutamic acid 43 was highly conserved in the NHERF1 PDZ1 domain throughout different species.*

UniProt Accession Number	Amino acid sequence	Species
O14745	FHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLV	HUMAN
P70441	FHLHGEKGKVGQFIRLVEPGSPAEKSGLLAGDRLV	MOUSE
Q9JJ19	FHLHGEKGKVGQFIRLVEPGSPAEKSGLLAGDRLV	RAT
Q4R6G4	FHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLV	MACAQUE
Q3SZK8	FHLHGEKGKVGQYIRLVEPGSPAEKSGLLAGDRLV	BOVINE
Q28619	FHLHGEKGKVGQYIRLVEPGSPAEKAGLLAGDRLV	RABBIT
Q5ZM14	FHLHGEKGKPGQYIRLVEAGSPAERSGLRAGDRLL	CHICKEN

*All protein sequences were obtained from UniProt.

Table II. *Glutamic acid 43 was highly conserved in the PDZ1 domain of NHERF1 and NHERF2 proteins.*

UniProt Accession Number	Amino acid sequence	Protein & Species
O14745	FHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLV	NHERF1 HUMAN
Q15599	FHLHGEKGRRGQFIRRVEPGSPAEAAALRAGDRLV	NHERF2 HUMAN
Q9JHL1	FHLHGEKGRRGQFIRRVEPGSPAEAAALRAGDRLV	NHERF2 MOUSE
Q920G2	FHLHGEKGRRGQFIRRVEPGSPAEAAALRAGDRLV	NHERF2 RAT
Q8SQG9	FHLHGEKGRRGQFIRRVEPGSPAEAAALRAGDRLV	NHERF2 RABBIT

* All protein sequences were obtained from UniProt.

dramatically phosphorylated in both MCF7$^{\Delta NHERF1}$ parental and vector control cells following 5 min stimulation with 100 ng/ml EGF. NHERF1-WT over-expression significantly inhibited Akt and ERK activation and blocked EGF-induced EGFR phosphorylation. However, the NHERF1 mutation E43G abolished NHERF1-WT inhibition of EGFR, Akt and ERK activation (Figure 4), Meanwhile the base levels of Akt and ERK activation were not detectably different in cells transfected with different *NHERF1* constructs (data not shown). Taken together, these data suggest that cancer-derived *NHERF1* mutation (E43G) abolished the inhibition of the phosphorylational activation of EGFR and its downstream signaling through Akt and ERK signaling pathways induced by EGF.

NHERF1E43G mutation resulted in loss of EGF-induced tumour-suppressor effects of NHERF1-WT. Since the mutation of *NHERF1* affects EGFR activation in breast cancer cells, *NHERF1* mutation could potentially mediate changes in cell migration of these breast cancer cells. EGF-induced cell migration was investigated in cells stably transfected with *NHERF1-WT* and mutation in order to explore which potential signaling transduction proteins were modulated by *NHERF1*. After pre-culture in serum-free medium for 36 h, cells were treated with 100 ng/ml EGF. NHERF1-WT significantly inhibited the proliferation and migration rate of MCF-7$^{\Delta NHERF1}$ cells induced by EGF compared to parental and vector control cells. In contrast, *NHERF1* E43G mutation removed the inhibitory effect of NHERF1 in the EGF-induced malignant cell phenotypes (Figure 5). Taken together, these results suggest that NHERF1 inhibited EGF-induced cell malignant phenotypes in breast cancer cells, but the E43G mutation resulted in complete loss of the tumour-suppressor effects of NHERF1-WT.

Discussion

Breast cancer is regarded as a heterogeneous group of tumours with different outcomes and responses to treatment. Recent reports suggest a fundamental role for *NHERF1* in human breast cancer. Down-regulation of endogenous NHERF1 in breast cancer cells promoted proliferation, tumourigenic ability, and delayed the turnover of PDGF-induced Akt phosphorylation (24). In our previous study, over-expression of *NHERF1* in a breast cancer cell line suppressed cell proliferation by promoting apoptosis and inhibiting ERK activation (18), suggestive of a tumour-suppressive role for *NHERF1*. However, contrasting results have recently been reported. NHERF1 over-expression in breast tumours is associated with tumour stage, metastatic progression, and poor prognosis (25), implying that NHERF1 is a potential oncoprotein. These contradictory reports suggest a complicated role for NHERF1 in breast cancer.

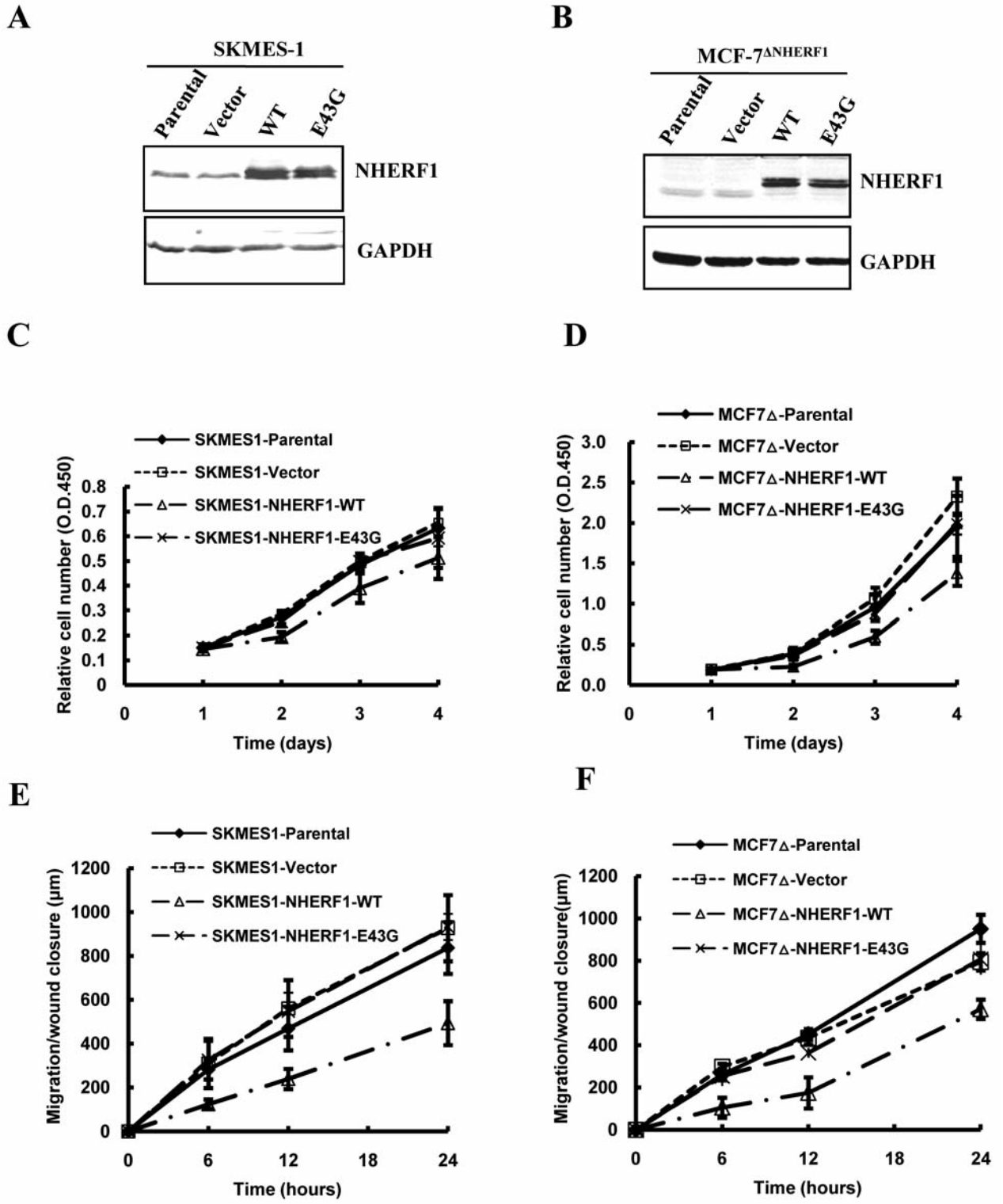

Figure 2. *Breast cancer-derived Na^+/H^+ exchanger regulatory factor 1(NHERF1) E43G mutation abolished the antitumor effects of NHERF1 on cell migration in SKMES1 and MCF-7$^{\Delta NHERF1}$ cells. The wild-type NHERF1 (WT) and the E43G mutation were stably transfected into SKMES1 and MCF-7$^{\Delta NHERF1}$ cells and detected with anti-NHERF (A, B). E43G mutation impaired tumor-suppressive function of NHERF1, including cell proliferation (C, D) and migration (E, F). The results represent mean values±SD of three independent experiments. *p<0.05, **p<0.01. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.*

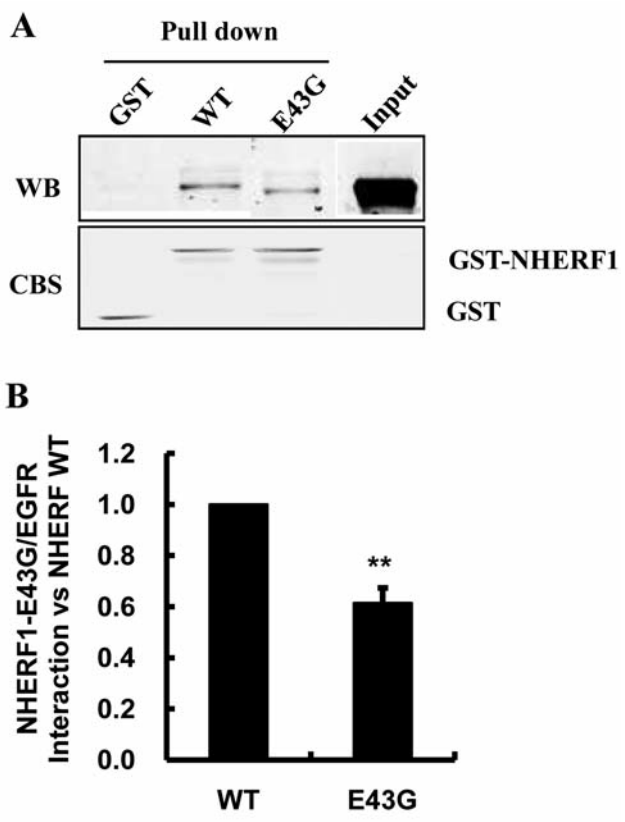

Figure 3. *Na^+/H^+ exchanger regulatory factor 1 (NHERF1) E43G mutation altered its interaction with epidermal growth factor receptor (EGFR). Glutathione S-transferases (GST)–NHERF1 beads were incubated with COS-7 cell lysates, and then the pull-down complexes were subjected to EGFR immunoblotting. A: NHERF1 mutation (E43G) weakened NHERF1 interaction with EGFR by GST pull-down assay. The Coomassie blue-stained gel was used to visualize the same amount of input of fusion proteins. B: The signals were quantified by densitometric analysis. The results represent mean values±SD of three independent experiments. **p<0.01. WT: Wild-type.*

In this study, the coding region and the intron-exon junctions of the *NHERF1* gene derived from 20 breast cancer tissues were analyzed. A novel *NHERF1* sequence variant (GAG to GGG) resulting in a switch of codon 43 (Glu-Gly) was firstly identified (Figure 1B). The mutation corresponded to a conserved basic residue in the PDZ1 domain (Figure 1C). The sequence is highly conserved according to the alignment of the sequence of PDZ1 of NHERF1 protein in various animals (Tables I and II). These data suggest that a variation in PDZ1 domain could greatly impact the cell phenotype or even cause cell death, making it hard for cells to survive and to identify cells carrying the sequence variation in the PDZ domain. Results of the cell function assays showed that the E43G mutation significantly inactivated the NHERF1 suppression of proliferation and migration of $MCF7^{\Delta NHERF1}$ and SKMES-1 cells. These findings further support the tumour-suppressive function of NHERF1 in tumour cells, and suggest that the *NHERF1* mutation may play a causal role in tumour development and progression. Here, we demonstrated that NHERF1 interacted with EGFR (Figure 3) and suppressed EGF-induced proliferation and migration of $MCF\text{-}7^{\Delta NHERF1}$ cells (Figure 5) by inhibiting EGFR phosphorylation (Figure 4A) and blocking EGFR downstream signaling in breast cancer cells (Figure 4B and C). While *NHERF1* E43G mutation attenuated the interaction of NHERF1 protein with EGFR (Figure 3) and markedly inactivated NHERF1-suppressed activation of Akt and ERK upon EGF stimulation. Hence, loss of suppression of ERK and Akt activities in the cells carrying *NHERF1* mutations might explain their increased malignant cell phenotypes.

The EGFR is one of four members of the HER family of receptor tyrosine kinases. Triggering of EGFR with its potential ligands (EGF, ransforming growth factor-α or amphiregulin) lead to homo- and heterodimerization of the ErbB receptors, and subsequent autophosphorylation of the tyrosine domain leads to downstream signalling of Ras/MAPK (26), PI(3)K/Akt (27), phospholipase C- protein kinase C (28), and janus kinase/signal transducers and activators of transcription (29) pathways. This in turn resultsin cell differentiation and migration. NHERF1, as a signaling adaptor, can associate with EGFR to regulate the latter's functional expression and activation in breast cancer cells and, modulating its role in progression and response to treatment.

The *C*-terminus of EGFR interacts with NHERF1 through its PDZ1 domain (30), where the E43G mutation of *NHERF1* was identified and located in. It could be imagined that the change in the hydrophobic property of the amino acid residues, [e.g. the acidic amino acid residue (Glu) substituted by the non-polar aliphatic amino acid residue (Gly)], could lead to a change of the spatial structure of the protein, resulting in the different biological activities of NHERF1 in cells. In fact, *NHERF1* E43G mutation altered the binding affinity of NHERF1 to EGFR (Figure 3), and led to the loss of its suppression in the EGFR pathway (Figure 4). However, the mutation could be a useful tool for studying the effects of *NHERF1* on the regulation of the EGFR pathway in cells and the progression and metastasis of breast cancer through the EGFR pathway. Moreover, the one-point mutation in the scaffold protein NHERF1 caused the observed changes in the abilities of proliferation and migration of cells, suggesting that when studying the regulation of the EGFR pathway in breast cancer, some scaffold proteins involved in the pathway should be considered.

In conclusion, a novel *NHERF1* gene mutation (E43G) was isolated and identified in breast tumours. Compared to wild-type NHERF1, expression of the mutated NHERF1 failed to suppress malignant properties of human cancer cells. The E43G mutation attenuated the interaction between

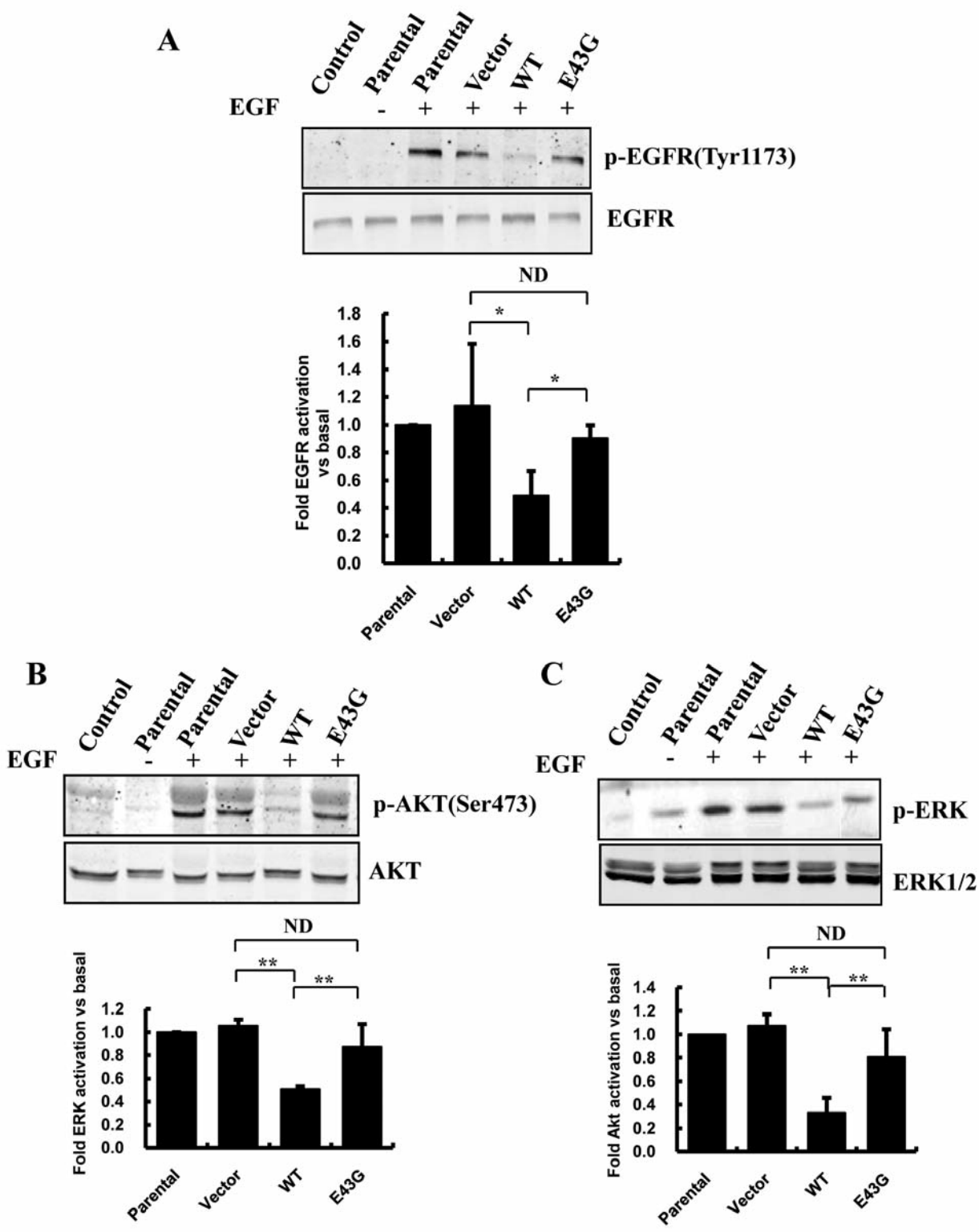

Figure 4. *Na^+/H^+ exchanger regulatory factor 1(NHERF1) E43G mutation caused loss-of-function of NHERF1 inhibition of Akt and extracellular regulated protein kinases(ERK) activation induced by epidermal growth factor(EGF). MCF-$7^{\Delta NHERF1}$ cells stably transfected with various NHERF1 constructs were serum starved for 36 h and then stimulated with or without EGF for 5 min. Immunoblot was detected with anti-pY1173EGFR (A), anti-EGFR (A), anti-pS473Akt (B), anti-Akt (B), anti-p-ERK (C) and anti-ERK (C). The signals were quantified by densitometric analysis. Data are given as the fold that of stimulated parental cells. The results represent mean values±SD of three independent experiments. *p<0.05, **p<0.01. WT: Wild-type.*

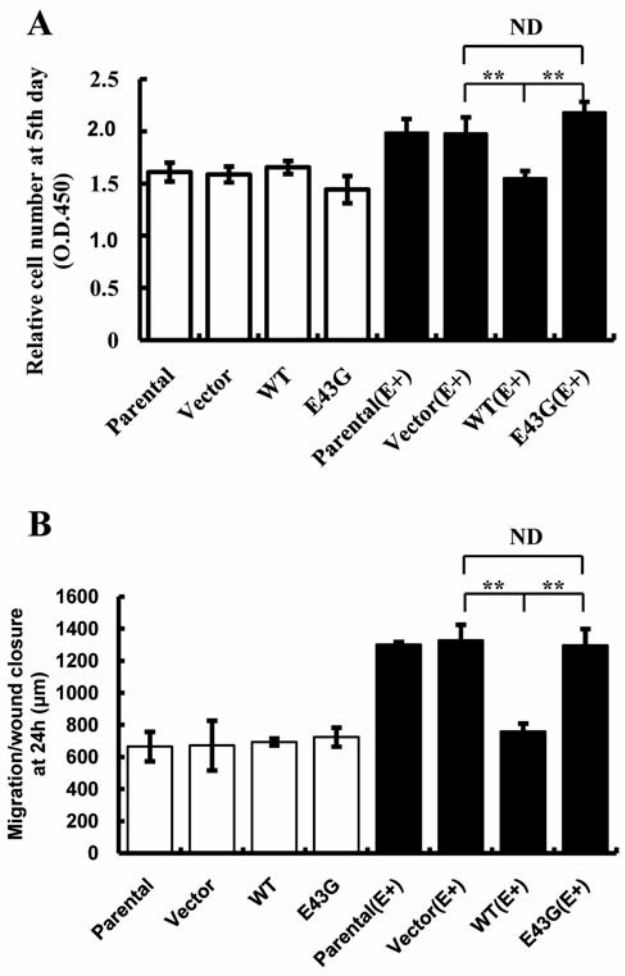

Figure 5. *Na^+/H^+ exchanger regulatory factor 1 (NHERF1) E43G mutation resulted in loss of the epidermal growth factor (EGF)-induced tumour-suppressive effects of wild-type NHERF1-WT in MCF-7$^{\Delta NHERF1}$ cells. The EGF induced cell migration was investigated in cells stably transfected with NHERF-WT and mutation. After pre-culture in serum-free medium for 36 h, cells were treated by EGF (E+). NHERF1 significantly inhibited proliferation (A) and migration (B) rate of MCF-7$^{\Delta NHERF1}$ cells compared to parental and vector control cells, while NHERF1 E43G mutation removed the inhibition of NHERF1in EGF-induced cell proliferation (A) and migration (B). The signals were quantified by densitometric analysis. The results represent mean values±SD of three independent experiments. *p<0.05, **p<0.01. ND: No differences.*

NHERF1 and EGFR proteins, which inactivated NHERF1 inhibition of EGF-induced Akt and ERK activation, resulting in the total loss of suppression of NHERF1 in EGF-induced malignant cell phenotypes. These results further demonstrate the functional consequences of breast cancer-derived *NHERF1* mutation (E43G), and suggest the causal role of *NHERF1* in the regulation of the EGFR signaling pathway and the progression of breast tumours.

Acknowledgements

This work was supported by National Natural Science Foundation of the People's Republic of China (No. 81572704) and the Foundation of Beijing Educational Committee (No. KM201310025003). We also wish to thank the Beijing Municipal Science & Technology Commission for their support (No. Z151100001615039).

References

1 Cosmai L, Gallieni M and Porta C: Renal toxicity of anticancer agents targeting HER2 and EGFR. J Nephrol *28(6)*: 647-57, 2015.

2 Reis-Filho JS, Milanezi F, Carvalho S, Simpson PT, Steele D, Savage K, Lambros MB, Pereira EM, Nesland JM, Lakhani SR and Schmitt FC: Metaplastic breast carcinomas exhibit egfr, but not her2, gene amplification and overexpression: Immunohistochemical and chromogenic *in situ* hybridization analysis. Breast Cancer Res *7(6)*: R1028-1035, 2005.

3 Koumangoye RB, Nangami GN, Thompson PD, Agboto VK, Ochieng J and Sakwe AM: Reduced annexin a6 expression promotes the degradation of activated epidermal growth factor receptor and sensitizes invasive breast cancer cells to EGFR-targeted tyrosine kinase inhibitors. Mol Cancer *12(1)*: 167, 2013.

4 Bourdeanu L and Luu T: Targeted therapies in breast cancer: Implications for advanced oncology practice. J Adv Pract Oncol *5(4)*: 246-260, 2014.

5 Vivanco I, Robins HI, Rohle D, Campos C, Grommes C, Nghiemphu PL, Kubek S, Oldrini B, Chheda MG, Yannuzzi N, Tao H, Zhu S, Iwanami A, Kuga D, Dang J, Pedraza A, Brennan CW, Heguy A, Liau LM, Lieberman F, Yung WK, Gilbert MR, Reardon DA, Drappatz J, Wen PY, Lamborn KR, Chang SM, Prados MD, Fine HA, Horvath S, Wu N, Lassman AB, DeAngelis LM, Yong WH, Kuhn JG, Mischel PS, Mehta MP, Cloughesy TF and Mellinghoff IK: Differential sensitivity of glioma- *versus* lung cancer-specific *EGFR* mutations to EGFR kinase inhibitors. Cancer Discov *2(5)*: 458-471, 2012.

6 Bellizzi A, Greco MR, Rubino R, Paradiso A, Forciniti S, Zeeberg K, Cardone RA and Reshkin SJ: The scaffolding protein NHERF1 sensitizes EGFR-dependent tumor growth, motility and invadopodia function to gefitinib treatment in breast cancer cells. Int J Oncol *46(3)*: 1214-1224, 2015.

7 Saponaro C, Malfettone A, Dell'Endice TS, Brunetti AE, Achimas-Cadariu P, Paradiso A and Mangia A: The prognostic value of the Na (+)/H(+) exchanger regulatory factor 1 (NHERF1) protein in cancer. Cancer Biomark *14(2-3)*: 177-184, 2014.

8 Morales FC, Takahashi Y, Kreimann EL and Georgescu MM: Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 organizes ERM proteins at the apical membrane of polarized epithelia. Proc Natl Acad Sci USA *101(51)*: 17705-17710, 2004.

9 Loureiro CA, Matos AM, Dias-Alves A, Pereira JF, Uliyakina I, Barros P, Amaral MD and Matos P: A molecular switch in the scaffold NHERF1 enables misfolded CFTR to evade the peripheral quality control checkpoint. Sci Signal *8(377)*: ra48, 2015.

10 Courbebaisse M, Leroy C, Bakouh N, Salaun C, Beck L, Grandchamp B, Planelles G, Hall RA, Friedlander G and Prie D: A new human *NHERF1* mutation decreases renal phosphate transporter NPT2A expression by aPTH-independent mechanism. PLoS One *7(4)*: e34764, 2012.

11 Georgescu MM, Morales FC, Molina JR and Hayashi Y: Roles of NHERF1/EBP50 in cancer. Curr Mol Med *8(6)*: 459-468, 2008.
12 Claperon A, Guedj N, Mergey M, Vignjevic D, Desbois-Mouthon C, Boissan M, Saubamea B, Paradis V, Housset C and Fouassier L: Loss ofEBP50stimulates EGFRactivity to induce EMTphenotypic features in biliary cancer cells. Oncogene *31(11)*: 1376-1388, 2012.
13 Yao W, Feng D, Bian W, Yang L, Li Y, Yang Z, Xiong Y, Zheng J, Zhai R and He J: EBP50 inhibits EGF-induced breast cancer cell proliferation by blockingEGFR phosphorylation. Amino Acids *43(5)*: 2027-2035, 2012.
14 Pan Y, Wang L and Dai JL: Suppression of breast cancer cell growth by Na^+/H^+ exchanger regulatory factor 1 (NHERF1). Breast Cancer Res *8(6)*: R63, 2006.
15 Cheng S, Zhang J, Zhu P, Ma Y, Xiong Y, Sun L, Xu J, Zhang H and He J: The PDZdomain protein CALinteracts with MGLUR5Aand modulates receptor expression. J Neurochem *112(3)*: 588-598, 2010.
16 Cao L, Duanmu W, Yin Y, Zhou Z, Ge H, Chen T, Tan L, Yu A, Hu R, Fei L and Feng H: Dihydroartemisinin exhibits anti-glioma stem cell activity through inhibiting p-Aktand activating caspase-3. Pharmazie *69(10)*: 752-758, 2014.
17 Troncoso M, Cuello Carrion FD, Guinazu E, Fanelli MA, Montt-Guevara M, Cabrini RL, Caron RW and Kreimann EL: Expression of NHERF1 in colonic tumors induced by 1,2-dimethylhydrazine in rats is independent of plasma ovarian steroids. Horm Cancer *2(4)*: 214-223, 2011.
18 Cheng S, Li Y, Yang Y, Feng D, Yang L, Ma Q, Zheng S, Meng R, Wang S, Wang S, Jiang WG and He J: Breast cancer-derived K172N, D301V mutations abolish Na^+/H^+ exchanger regulatory factor 1 inhibition of platelet-derived growth factor receptor signaling. FEBS Lett *587(20)*: 3289-3295, 2013.
19 Guo CY, Xu XF, Wu JY and Liu SF: Pcr-sscp-DNA sequencing method in detectingPTEN gene mutation and its significance in human gastric cancer. World J Gastroenterol *14(24)*: 3804-3811, 2008.
20 Dai JL, Wang L, Sahin AA, Broemeling LD, Schutte M and Pan Y: *NHERF* (Na^+/H^+ exchanger regulatory factor) gene mutations in human breast cancer. Oncogene *23(53)*: 8681-8687, 2004.
21 Malfettone A, Silvestris N, Paradiso A, Mattioli E, Simone G and Mangia A: Overexpression of nuclear NHERF1 in advanced colorectal cancer: Association with hypoxic microenvironment and tumor invasive phenotype. Exp Mol Pathol *92(3)*: 296-303, 2012.
22 Lazar CS, Cresson CM, Lauffenburger DA and Gill GN: The Na^+/H^+ exchanger regulatory factor stabilizes epidermal growth factor receptors at the cell surface. Mol Biol Cell *15(12)*: 5470-5480, 2004.
23 Golding SE, Morgan RN, Adams BR, Hawkins AJ, Povirk LF and Valerie K: Pro-survival Aktand ERK signaling from EGFRand mutant EGFRVIII enhances DNA double-strand break repair in human glioma cells. Cancer Biol Ther *8(8)*: 730-738, 2009.
24 Oh HY, Lee EJ, Yoon S, Chung BH, Cho KS and Hong SJ: Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERKsignal transduction. Prostate *67(10)*: 1061-1069, 2007.
25 Song J, Bai J, Yang W, Gabrielson EW, Chan DW and Zhang Z: Expression and clinicopathological significance of oestrogen-responsive ezrin-radixin-moesin-binding phosphoprotein 50 in breast cancer. Histopathology *51(1)*: 40-53, 2007.
26 Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB and Dent P: Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: Inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. Mol Biol Cell *12(9)*: 2629-2645, 2001.
27 Tsai PC, Hsieh CY, Chiu CC, Wang CK, Chang LS and Lin SR: Cardiotoxin iii suppresses MDA-MB-231 cell metastasis through the inhibition of EGF/EGFR-mediated signaling pathway. Toxicon *60(5)*: 734-743, 2012.
28 Zhou Q, Li G, Deng XY, He XB, Chen LJ, Wu C, Shi Y, Wu KP, Mei LJ, Lu JX and Zhou NM: Activated human hydroxy-carboxylic acid receptor-3 signals to MAP kinase cascades *via* the PLC-dependent PKC andMMP-mediated EGFRpathways. Br J Pharmacol *166(6)*: 1756-1773, 2012.
29 Xu N, Wang SQ, Tan D, Gao Y, Lin G and Xi R: EGFR, wingless and JAK/STATsignaling cooperatively maintain Drosophila intestinal stem cells. Dev Biol *354(1)*: 31-43, 2011.
30 Li H, Zhang B, Liu Y, and Yin C: EBP50 inhibits the migration and invasion of human breast cancer cells *via* LIMK/cofilin and the PI3K/Akt/mTOR/MMP signaling pathway. Med Oncol *31*: 162, 2014.

Received January 18, 2016
Revised February 19, 2016
Accepted February 22, 2016