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NFIB is a potential target for estrogen receptor-negative breast cancers

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ABSTRACT

Background: The association between nuclear factor I/B (NFIB) gene and triple negative breast cancer has been previously suggested.

Methods: We investigated the relationship between NFIB mRNA and protein expression and molecular subtypes of breast cancer as well as the effect of NFIB silencing on the proliferation and apoptosis of breast cancer cells. Also, the clinical importance of NFIB expression was investigated in 163 breast cancer patients.

Results: By using 20 frozen human breast cancer tissues and various breast cancer cell lines, we observed a significant high level of NFIB mRNA level in triple negative breast cancer. NFIB protein was upregulated in ER negative breast cancer tissues but the expression level was similar between HER2 subtype and triple negative subtype. The clinical significance of NFIB was further examined in a tissue microarray from 163 invasive breast cancer patients, and the immunohistochemistry results showed a significant association between NFIB expression and nuclear grade, ER, and HER2 expression status. NFIB positive tumors were more likely to have high nuclear grade, ER negativity and HER2 over-expression. HCC1954 cells transfected with siRNA against NFIB showed a significant reduction in cell proliferation and increase in apoptotic signaling pathway.

Conclusions: Our results show a potential role of NFIB as a novel target in ER negative breast cancers.

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1. Background

Breast cancer is the most common solid malignancy and is the leading cause of cancer-related deaths worldwide regardless of the degree of development (Jemal et al., 2011). Annually more than 450 thousand deaths are attributable to breast cancer worldwide. Recently, the global trend for breast cancer mortality has begun to show a significant decline especially for developed countries (Karim-Kos et al., 2008), and this trend can be explained by the adoption of screening mammography and development of

effective adjuvant treatments (Berry et al., 2005). For example, adjuvant tamoxifen treatment has reduced the risk of breast cancer death by 31% in women with estrogen receptor (ER) positive tumors (EBCTBG, 2005). However, we still do not have a valid target to develop novel therapeutics in ER negative tumors including the triple negative breast cancer. These tumors are often treated with non-specific cytotoxic chemotherapeutic agents which result in significant morbidity and decrement in quality of life. Therefore, identifying a novel target for ER negative breast cancer is an active topic of research these days.

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Previously, we have reported a specific gene copy number alteration for triple negative breast cancer patients using array CGH (Han et al., 2008). Despite the small number of samples, we also observed a significant increase in *nfib* DNA in triple negative breast cancer tissues shown by RT-PCR. *Nfib* is a member of human nuclear factor I gene, and is known as an important regulator in lung and brain development (Grunder et al., 2002; Qian et al., 1995; Steele-Perkins et al., 2005). Recent studies have also indicated that *nfib* gene rearrangement is also involved in the development of various types of tumors (Geurts et al., 1998; Nilsson et al., 2005; Persson et al., 2009).

In this study, we investigated the expression frequency of NFIB protein in human breast cancer tissues and its clinical implications. Also, by silencing *nfib* gene, we assessed the role of NFIB in breast cancer cell proliferation and survival.

2. Materials and methods

2.1. Reagents and cell lines

Insulin, hydrocortisone and cholera toxin were purchased from Sigma (St. Louis, MO); EGF was from Upstate Biotechnology (Lake Placid, NY). All of the other media, buffer, and ingredients for cell culture were purchased from Invitrogen Life Sciences (Carlsbad, CA). The MCF10A (breast epithelial cell line), ER positive breast cancer cell lines (T47D, MCF7, ZR75-1), and ER negative cell lines (SKBR3, HCC1954, MDA-MB436, MDA-MB468, Hs578 T, MDA-MB231) were obtained from ATCC (Manassas, VA, USA). NFIB antibody was purchased from Upstate Biotechnology (Waltham, MA). β -actin antibody was purchased from Sigma. Antibodies against CDK2, CDK6, cyclin D3, cyclin B1, phospho-Rb, Rb, p21, Bcl2, Bad, phospho-Bad, Bid and Bax are purchased from Sigma, Cell signaling or Upstate company.

2.2. Cell cultures

All cell lines except MCF10A were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin. MCF10A cell line was grown and was cultured in Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 supplemented with 5% horse serum, 10 mmol/L HEPES buffer, 10 ng/mL insulin, 20 ng/mL EGF, 100 ng/mL cholera toxin, and 0.5 mg/mL hydrocortisone at 37 °C in a 5% CO₂ incubator. All cell lines were fed twice a week and passaged on a weekly basis. Spheres were generated from single cells of HCC1954 seeded at 10³ cells in 10 mm low attachment plates (Falcon) and cultured in serum-free DMEM. Cells were grown under these conditions as nonadherent spherical clusters.

2.3. Patients and tumor specimens

Western blot and RT-PCR against NFIB were done by using frozen breast cancer tissues. Fresh frozen tissues used in this study were obtained from the frozen tissue archives at the Cancer Research Institute, Seoul National University, and used with the informed consent of all study participants

(SNUH IRB H-0512-502-163). Immunohistochemical staining against NFIB using paraffin-embedded tissue and the review of medical record were also approved (SNUH IRB H-1103-086-355). All tumors were excised between February 2006 and January 2008 and were histopathologically confirmed as invasive ductal carcinoma. Patients with recurrent or stage IV breast cancer, or patients who underwent neoadjuvant chemotherapy were excluded. Tissue samples obtained during surgery were collected in the operating room within 20 min of devascularization, frozen in liquid nitrogen and stored at –80 °C.

For immunohistochemical staining against NFIB protein, we used tissue microarray consisted of 180 breast cancer patients' tissues.

2.4. Immunohistochemical staining against NFIB protein

Our protocol for immunohistochemistry has been described previously (Moon et al., 2010). Briefly, we have constructed a tissue microarray using the breast cancer tissues from the Institution's pathology archives. We randomly selected 180 breast cancer patients who underwent an operation at Seoul National University Hospital from January 1999 to December 2006. Only the patients with primary invasive carcinoma were selected, and the patients with in situ tumors or recurrent tumors were excluded. The tissues were stained with diluted mouse monoclonal antibody against NFIB (1: 200, Abnova). Antigen retrieval was facilitated with microwaving the tissue for 21 min, and the rest of the staining procedure followed the standard avidin–biotinylated peroxidase complex method. Any NFIB positivity in epithelial carcinoma cells were classified as NFIB positive cases.

2.5. siRNA transfection

siRNA of the human NFIB gene and non-specific scrambled siRNA were purchased from Invitrogen. The siRNAs were transfected into HCC1954 cells using LipofectaminTM RNAi-MAX (Invitrogen). In the 6-well plates, NFIB specific siRNA oligomer (10 pmol/well) were diluted 250 μ l Opti-MEM I (Invitrogen) reduced serum medium and mixed with 5 μ l of LipofectaminTM RNAi-MAX. After 20 min incubation at room temperature, the complexes were added to the cells with antibiotics-free medium in a total volume of 2.0 ml. Silencing of NFIB gene was confirmed by immunoblot and RT-PCR.

2.6. RT-PCR (reverse transcription-polymerase chain reaction)

RNA from the breast cancer tissues and their paired normal tissues was treated with DNase I and reverse transcribed using a commercial kit: SuperScript III First-Strand Synthesis System (Invitrogen). PCR was performed in iCycler (BioRad, Hercules, CA) using a commercial SYBR green reaction mix (Takara) and the following primers: AAACCCAGCACTTTGTGTCC and TCTTGGGGAAGAATCCTGTG for NFIB (NM_005596.3), GGAATGTGGGCTTTGTGTTC and TGCCCTGGAGATTTTGTAGT for RPLP0 (NM_053275). The NFIB mRNA level in each sample was corrected by corresponding level of RPLP0. In order to validate

NFIB silencing efficiency, RNA from the transfected HCC1954 cells and control HCC1954 cells was reverse transcribed to cDNA. PCR was performed using following primers: GCCAAGT-GACGCTGACATTA and GGAGCTCGTAGAGGATGTGC for NFIA (NP_005595.4), ACCTGGCATAACGACCTGAAC and GGGCTGTT-GAATGGTGACTT for NFIC (NM_005597.2), GAGATGGA-GAGCCCTGTTGA and GAGAGGGCACTGCAGAAGTC for NFIX (NM_002501.2), CACTGTGTTGGCGTACAGGT and TCATCAC-CATTGGCAATGAG for β -actin (NM_167053.1). The NFIB, NFIA, NFIC or NFIX mRNA level in each sample was corrected by the corresponding level of β -actin.

2.7. Immunoblotting

Cells were washed twice with PBS, total cell lysates were prepared in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM PMSF). Protein concentration was measured by the Bradford assay using a Bio-Rad Protein Assay kit (BioRad Laboratories, Hercules, CA) according to the manufacturer's directions. Equal amounts of cell lysates were separated by 10% SDS-PAGE and the proteins were electrotransferred to Hybond-ECL nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). Blots were blocked with blocking buffer (5% nonfat dry milk in TBS containing 0.05% Tween 20) for 1 h and then incubated overnight at 4 °C with anti-NFIB, anti-CDK2, anti-CDK6, anti-cyclin

D3, anti-cyclin B1, anti-phosphor-Rb, anti-Rb or anti-p21 antibody for cell cycle analysis and anti-Bcl2, anti-Bid, anti-phosphor-Bad, anti-Bad or anti-Bax for anoikis stress. Blots were washed three times in TBS containing 0.05% Tween 20 and then incubated with peroxidase-conjugated affinity-purified rabbit anti mouse IgG (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA) or peroxidase-conjugated affinity-purified mouse anti rabbit or mouse IgG for 1 h at room temperature. Labeled proteins were detected with an ECL-enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK). Each membrane was reprobed with β -actin antibody (Sigma) to assess protein loading.

2.8. Assessment of cell proliferation and chemotherapy sensitivity

To assess proliferation assay of adherent or sphere cultured cells, NFIB silencing cells or control cells were seeded at various number of cells per well (96-well Ultra Low plates; Falcon) in 100 μ L spheres condition medium or control medium. After 3 days, cell viability was determined by using CellTiter-Glo[®] Luminescent Cell Viability Assay Kit (Promega). These experiments were performed in triplicate.

2.9. Cell cycle analysis

NFIB silencing cells or control cells were harvested, fixed with 70% ethanol for 1 h, washed with PBS, and treated for 1 h at

Figure 1 – NFIB mRNA expression level according to breast cancer phenotype in human breast cancer tissues. NFIB expression levels are shown according to the ER expression status (a), HER2 over amplification status (b), and molecular subtypes of breast cancer (c) determined by RT-PCR. The association between NFIB protein levels and molecular subtypes were also examined by western blot densitometry (d). The levels of NFIB protein regulation according to the ER status were examined in human breast cancer tissues (e) and various breast cancer cell lines (f).

Table 1 – Demographic and clinical information of the studied patients.

Variable		Value/frequency
Mean age (years $\pm$ SD)		48.4 $\pm$ 11.7
Mean tumor size (cm $\pm$ SD)		3.66 $\pm$ 1.2
Mean number of metastatic lymph nodes (range)		4.9 $\pm$ 8.3 (0–44)
Mean number of harvested lymph nodes (range)		16.7 $\pm$ 7.2 (2–44)
NFIB expression in epithelial carcinoma cells	NFIB negative	85 (52.1%)
	NFIB positive	78 (47.9%)
T stage	T1	23 (14.1%)
	T2	99 (60.7%)
	T3	25 (15.3%)
	T4	13 (8.0%)
	T unknown	3 (1.8%)
N stage	N0	67 (41.1%)
	N1	44 (27.0%)
	N2	21 (12.9%)
	N3	28 (17.2%)
	N unknown	3 (1.8%)
Histologic grade	Low (Grade 1–2)	55 (33.7%)
	High (Grade 3)	90 (55.2%)
	Unknown	18 (11.0%)
Nuclear grade	Low (Grade 1–2)	84 (51.5%)
	High (Grade 3)	69 (42.3%)
	Unknown	7 (4.3%)
Estrogen receptor status	Negative	97 (59.5%)
	Positive	59 (36.2%)
	Unknown	7 (4.3%)
Progesterone receptor status	Negative	98 (60.1%)
	Positive	58 (35.6%)
	Unknown	7 (4.3%)
HER2 over-expression	Negative	88 (54.3%)
	Positive	67 (41.4%)
	Unknown	7 (4.3%)

37C with 100 g/ml RNase A (Sigma, St. Louis, MO), followed by 25 g/ml propidium iodide (Sigma). Flow cytometry was performed using a FACSCalibur. These experiments were performed in triplicate.

3. Result

3.1. The level of NFIB expression in human breast cancer tissues and various breast cancer cell lines

First, we investigated the expression level of NFIB in human breast cancer tissues with various phenotypes. Frozen breast cancer tissues from 20 breast cancer patients were used to investigate the association between NFIB mRNA expression and breast cancer subtypes. The mean NFIB mRNA expression level was significantly higher in ER negative breast cancer tissues when compared to ER positive cancers ($p = 0.016$, Figure 1a). HER2 amplified tumors showed a trend of low NFIB mRNA expression, but did not show a statistical significance (Figure 1b). When the tumors were classified into molecular subtypes based on the immunohistochemical profiles

of ER, PR, and HER2 (Carey et al., 2007), triple negative subtype showed significantly higher NFIB mRNA expression compared to luminal subtypes while triple negative had no significant difference when compared to HER2 subtype (Figure 1c). NFIB protein level was determined by western blotting using 14 invasive breast cancer tissues. ER positive tumors showed low expression of NFIB (Figure 1e) and the level of NFIB protein was significantly higher in triple negative subtype ($p = 0.02$, Figure 1d). However, the level of NFIB protein in HER2 subtype was similar to that of triple negative subtype, a finding which differed from mRNA expression profiles. The NFIB protein expression level was also determined in various breast cancer cell lines. ER negative breast cancer cell lines showed marked upregulation of NFIB expression compared to ER positive cell lines (Figure 1f).

3.2. The clinical implication of NFIB expression in 163 breast cancer patients

The clinical implication of NFIB expression was determined by immunohistochemistry using the tissue microarray made of 180 breast cancer tissues. NFIB staining results were available in 163 patients who were included in the further analysis. The clinicopathologic information of the included patients are shown in the Table 1.

Human breast cancer tissues showed various intracellular NFIB staining patterns (Figure 2), and among the 163 studied patients, 78 (47.9%) showed positive NFIB expression. The association between various clinicopathologic features and NFIB expression was investigated and shown in the Table 2. NFIB expression showed significant association with ER, PR, HER2 expression status, and nuclear grade. ER/PR negative, and HER2 positive breast cancer cells were more likely to express NFIB protein. Among the 163 patients, disease recurrence, including distant and local failure, developed in 49 patients. NFIB expression status did not show significant relationship with tumor recurrence ($p = 0.632$, log-rank test).

3.3. Effect of NFIB silencing on the proliferation and survival of HCC1954 cells

HCC1954 cells showed high levels of NFIB protein expression (Figure 1f). We knocked down NFIB in HCC1954 cells with specific NFIB siRNA and tested the proliferative capacity of transfected cells (Figure 3a). siRNA transfection resulted in a significant reduction in cell proliferation rate in HCC1954 cells when compared to control (Figure 3b). Interestingly, the anti-proliferative effect of NFIB knockdown was more evident when the cells were cultured in the anchorage-independent conditions (Figure 3c). Flow cytometry of siRNA-transfected cells showed a significant change in cell cycle distribution showing increased arrest of cells in G2/M phase (Figure 3d). Western blotting of cell cycle-regulatory proteins showed that NFIB knockdown resulted in downregulation of CDK2 and upregulation of p21. Also, the phosphor-Rb and Rb protein levels were upregulated while cell cycle analysis did not show G0/G1 arrest. Also various Bcl2-family molecules showed differential regulation according to the NFIB expression status, suggesting a potential role NFIB in breast cancer cell apoptosis (Figure 3e).

Figure 2 — Representative pictures of NFIB staining in human breast cancer tissues. Immunohistochemistry using the anti-NFIB antibody on a paraffin-embedded tissue section of human breast cancer tissue shows various NFIB expression patterns, varying from negative expression (a) to strong expression in cytoplasmic or nuclear area (b and c, respectively).

4. Discussion

Identifying a novel target for cancer is important not only because it provides a novel carcinogenesis mechanism but also it can lead to a development of an effective therapeutic agent. In breast cancer, targeted agents, such as tamoxifen and trastuzumab, have achieved a lower recurrence rate and breast cancer-related deaths (EBCTBG, 2005; Piccart-Gebhart et al., 2005). However, treatment options for patients with triple negative breast cancer which lack a clinically-useful target are still limited other than non-specific cytotoxic chemotherapeutic agents. Furthermore, most patients treated with targeted agents eventually develop treatment resistance (Nahta et al., 2006). These limitations of current therapeutic targets in breast cancer are the main reasons for the need of novel target discovery in breast cancer research.

In our previous study, we have suggested *nfib* gene as a potential target for triple negative breast cancer by using array CGH approach (Han et al., 2008). In that study, DNA level, assessed by real-time PCR, showed NFIB gene amplification was specific to triple negative breast cancer tissues among 32 breast cancer patients. In the current study, we have also observed the increased expression level of NFIB mRNA in

ER negative and HER2-negative breast cancer tissues (Figure 1a–c). However, further analysis including protein expression evaluation revealed that NFIB protein was also commonly expressed in HER2 over-expressing tumors as well as triple negative tumors (Figure 1d and Table 2). The underlying mechanism for this discrepancy between mRNA level and protein level was not investigated in our present study. Further research is needed to address the issues of transcription regulation, post-translational modifications and the potential presence of unknown mechanism which prevent the degradation of NFIB protein in the HER2 subtype of breast cancer. Notably, Fujita et al. (Fujita et al., 2008) have reported that NFIB transcription is regulated by miR-21, a key microRNA that regulates oncogenic processes (Selcuklu et al., 2009). They have shown that miR-21 inhibits translation of NFIB mRNA and accelerates the NFIB clearance in premyelocytic HL-60 cells. The miR-21 which targets NFIB has also been identified as a differentially expressed microRNA during the early carcinogenic process of breast cancer (Hannafon et al., 2011), suggesting a potential regulatory mechanism for NFIB transcription by microRNA system.

NFIB gene is located at 9p24.1 and is a transcription factor which plays an important role in viral replication and fetal organ development (Gronostajski, 2000; Grunder et al., 2002; Nagata et al., 1982; Qian et al., 1995; Steele-Perkins et al.,

Table 2 — Association between various clinicopathologic parameters and NFIB expression.

Variables		NFIB negative	NFIB positive	p
Tumor size		3.7 ± 2.1	3.6 ± 1.8	0.564
No of metastatic lymph nodes		5.0 ± 8.3	4.7 ± 8.3	0.803
Axillary LN metastasis	Node negative	30 (37%)	37 (47%)	0.2
	Node positive	52 (63%)	41 (53%)	
Histologic grade	Low grade (1–2)	31 (44%)	24 (32%)	0.175
	High grade (3)	40 (56%)	50 (68%)	
Nuclear grade	Low grade (1–2)	55 (71%)	29 (39%)	<0.001
	High grade (3)	23 (29%)	46 (61%)	
ER	Negative	38 (48%)	59 (77%)	<0.001
	Positive	41 (52%)	18 (23%)	
PR	Negative	37 (47%)	61 (79%)	<0.001
	Positive	42 (53%)	16 (21%)	
HER2	Negative	59 (76%)	29 (38%)	<0.001
	Positive	19 (24%)	48 (62%)

Figure 3 – Changes in cell proliferation and cell cycle arrest after silencing NFIB in HCC1954 cells. Cells were either transfected with scramble siRNA or NFIB siRNA(a), and the proportion of surviving cells were calculated in adherent culture and floating culture conditions (b and c, respectively). Each data point represents mean $\pm$ SD, and data were obtained from 5 independent experiments. The cell cycle distribution of NFIB known down cells and control cells analyzed by flow cytometry (d) and various cell cycle- or apoptosis-related protein levels were tested using cell lysates (e). Relative expression levels of proteins in HCC1954 cells treated with NFIB siRNA measured by densitometry are as follows; 0.67 for CDK2, 1.27 for CDK6, 1.05 for cyclin D3, 1.41 for cyclin B1, 4.85 for phosphor-Rb, 1.71 for Rb, 1.22 for p21, 0.22 for Bcl2, 1.99 for phosphor-Bad, 1.04 for Bad, 2.29 for Bid, 0.79 for Bax. All expression values are adjusted to the β -actin expression.

2005), and there are four NFI genes in mammals (*nfia*, *nfib*, *nfic*, and *nfix*). Recently, the role for NFIB in the development and progression of various types of neoplasm has been demonstrated. In lipoma and salivary gland tumors, NFIB has been shown to form a fusion gene with either HMGA2 or MYC (Geurts et al., 1998; Italiano et al., 2008; Mitani et al., 2010; Nilsson et al., 2005; Persson et al., 2009; Pierron et al., 2009). The evidence supporting the relationship between the genetic instability of NFIB and triple negative breast cancer phenotype is now accumulating. The NFIB gene rearrangement and MYB-NFIB fusion has been reported in adenoid cystic carcinoma of breast (Persson et al., 2009). Adenoid cystic carcinoma of breast is a unique subtype of breast carcinoma which can be classified into triple negative breast cancer (Kreike et al., 2007). Another recent study has suggested that acquired uniparental disomy, a common genetic variation in cancer development which has been recognized recently, at chromosome 9 p (MTAP, NFIB) was a significant predictor of triple negative breast cancer phenotype from the analysis of public genomic datasets (Tuna et al., 2010). These studies indicate a potential role of NFIB in the development and progression in triple negative breast cancer as well as the value of NFIB.

5. Conclusions

To summarize our findings, we validated our previous finding in an independent group of patients and tested the clinical implication of NFIB protein expression in a cohort of 163 breast

cancer patients. NFIB protein expression had significant association with nuclear grade, estrogen receptor status, and HER2 over-expression status. Unlike the DNA and mRNA expression, NFIB protein level was similar between HER2 subtype and triple negative subtype. Furthermore, silencing NFIB gene resulted in reduced proliferation and an increase in the apoptotic signaling pathway, suggesting a potential value of NFIB as a novel target in ER negative breast cancer.

Conflict of interest

None declared.

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