NRF2 immunolocalization in human breast cancer patients as a prognostic factor

Yoshiaki Onodera¹, Hozumi Motohashi², Kiyoshi Takagi³, Yasuhiro Miki¹, Yukiko Shibahara¹, Mika Watanabe⁴, Takanori Ishida⁵, Hisashi Hirakawa⁶, Hironobu Sasano^{1,4}, Masayuki Yamamoto⁷ and Takashi Suzuki³

¹Department of Anatomic Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan

²Department of Gene Expression Regulation, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

³Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, 2-1 Seiryō-machi, Aoba-ku, Sendai, Miyagi-ken 980-8575, Japan

⁴Department of Pathology, Tohoku University Hospital, Sendai, Japan

⁵Department of Surgical Oncology, Tohoku University Graduate School of Medicine, Sendai, Japan

⁶Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan

⁷Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan

Correspondence should be addressed to T Suzuki

Email
t-suzuki@patholo2.med.tohoku.ac.jp

Abstract

Nuclear factor erythroid 2-related factor 2 (NRF2 (NFE2L2)) is an important transcriptional activator involved in the cellular defense mechanisms against electrophilic and oxidative stress. Recent studies have demonstrated that the expression of NRF2 protein is upregulated in several human malignancies and is associated with worse prognosis in these patients. However, the pathological and clinical significance of NRF2 has remained largely unknown in breast cancer patients. Therefore, in this study, we immunolocalized NRF2 in 106 breast carcinoma cases. NRF2 immunoreactivity was mainly detected in the nucleus of the breast carcinoma cells and it was positive in 44% of the cases. NRF2 status was significantly associated with histological grade, Ki-67 labeling index, p62 immunoreactivity, and NAD(P) H:quinone oxidoreductase 1 (NQO1) immunoreactivity, and the results of multivariate analyses revealed that NRF2 status was an independent adverse prognostic factor for both recurrence and disease-free survival of the patients. Subsequent *in vitro* studies demonstrated that the expression of NRF2 significantly increased the proliferation activity of MCF7 and SK-BR-3 breast carcinoma cells. These results indicate that nuclear NRF2 protein plays important roles in the proliferation and/or progression of breast carcinoma, and nuclear NRF2 immunoreactivity is therefore considered a potent prognostic factor in breast cancer patients.

Key Words

- ▶ breast
- ▶ carcinoma
- ▶ immunohistochemistry
- ▶ NRF2
- ▶ prognosis
- ▶ proliferation

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Introduction

Breast cancer is one of the most common malignancies in women. Invasive breast cancer is generally regarded as a disease that could metastasize in an early phase (Hüsemann *et al.* 2008), and the clinical outcome of the

patients is markedly influenced predominantly by the proliferative activity of the carcinoma cells (van Diest *et al.* 2004). Nevertheless, breast cancer patients frequently received adjuvant therapy such as endocrine therapy

(tamoxifen, luteinizing hormone-releasing hormone agonists or aromatase inhibitors, etc.) and chemotherapy after surgical treatment, distant recurrence in the patients treated with tamoxifen after surgery has been reported 15% at 10 years (Paik *et al.* 2004) and 25% of the patients who received adjuvant chemotherapy developed distant recurrence (Tevaarwerk *et al.* 2013). Therefore, it is very important to evaluate clinical and biological markers in breast cancer patients to predict its recurrence after surgery and to evaluate indications for additional therapies appropriately.

Nuclear factor erythroid 2-related factor 2 (NRF2 (NFE2L2)) is one of the most important transcriptional activators involved in the cellular defense mechanisms against electrophilic or oxidative stress (Uruno & Motohashi 2011). NRF2 is constantly degraded by KEAP1, a negative regulator of NRF2, via the ubiquitin–proteasome pathway under normal conditions. However, upon exposure to stimuli that inactivate KEAP1, stabilized NRF2 accumulates in the nucleus and induces various cytoprotective genes including NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO1 (HMOX1)) (Surh *et al.* 2008, Baird & Dinkova-Kostova 2011, Taguchi *et al.* 2011). Previous studies have shown that NRF2 activation can protect against many human diseases such as carcinoma, neurodegenerative disease, aging, and cardiovascular disease (Saw & Kong 2011), and then NRF2 has been considered to be a protein that protects humans from genotoxic damage caused by carcinogens (Zhang *et al.* 2012).

However, new emerging evidence has revealed that NRF2 protein is constitutively upregulated in several malignant tumors in contrast to normal cells due to the disruption of KEAP1-mediated degradation of NRF2. Moreover, recent data also indicate that p62/SQSTM1 plays an important role in the accumulation of NRF2 in carcinoma cells (Komatsu *et al.* 2010, Inami *et al.* 2011). An association between NRF2 accumulation and adverse clinical outcome of patients has been reported in lung (Solis *et al.* 2010, Inoue *et al.* 2012), gallbladder (Wang *et al.* 2010), and ovarian (Konstantinopoulos *et al.* 2011) carcinomas. These findings indicate that NRF2 is possibly involved in the growth and/or progression of these carcinomas. NRF2 immunolocalization has been reported in breast carcinoma (Loignon *et al.* 2009, Karihtala *et al.* 2011, Hartikainen *et al.* 2012), but its clinical significance remains largely unclear, including the association with NQO1 and p62 status and whether it can represent a prognostic factor in breast cancer patients. Therefore, in this study, we examined NRF2 in human breast carcinoma

using immunohistochemistry and further carried out *in vitro* studies to explore its clinicopathological significance and biological functions.

Subjects and methods

Patients and tissues

A total of 106 specimens of invasive breast carcinoma were obtained from Japanese female patients who underwent surgical treatment from 1991 to 1999 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. The clinicopathological characteristics of these patients are summarized in Table 1. The histological type was invasive ductal carcinoma, not otherwise specified, in all the cases examined, and the patients did not receive chemotherapy or hormonal therapy before the surgery.

Table 1 Clinicopathological characteristics of the 106 patients examined in this study

	No. of patients	Mean value (min.–max.)
Patient age ^a (years)	106 (100%)	57 (31–81)
Menopausal status		
Premenopausal	36 (34%)	
Postmenopausal	70 (66%)	
Stage		
I	33 (31%)	
II	49 (46%)	
III	24 (22%)	
Histological type		
Invasive ductal carcinoma	106 (100%)	
ER status		
Positive	86 (81%)	
Negative	20 (18%)	
PR status		
Positive	77 (73%)	
Negative	29 (27%)	
HER2 status		
Positive	21 (20%)	
Negative	85 (80%)	
Ki-67 LI ^a (%)	106 (100%)	17 (0–73)
Adjuvant therapy received after surgery		
Endocrine therapy	77 (73%)	
Chemotherapy	69 (65%)	
Follow-up time ^a (months)	106 (100%)	103 (3–175)
Survival status of patients		
Alive without recurrence	79 (75%)	
Alive with recurrence	14 (13%)	
Dead due to breast cancer	12 (11%)	
Dead due to other cause	1 (1%)	

All data represent the number of cases and percentage. LI, labeling index (percentage of positive carcinoma cells).

^aData were evaluated as continuous variables in this study, and their ranges (mean (min.–max.)) are also summarized.

Specimens were fixed in 10% formalin and embedded in paraffin wax.

In addition, 20 snap-frozen specimens of invasive ductal carcinoma, not otherwise specified, were obtained from patients who underwent surgical treatment from 2005 to 2006 in the Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan. Specimens fixed in 10% formalin and embedded in paraffin wax were also available in these cases. Informed consent was obtained from all the patients before their surgery. Research protocols followed in this study were approved by the Ethics Committee of the Tohoku University School of Medicine.

Immunohistochemistry

Rabbit polyclonal antibodies for NRF2 (sc-13032X) and HER2 (A0485) were purchased from Santa Cruz Biotechnology and Dako (Carpinteria, CA, USA) respectively. Mouse MABs for estrogen receptor (ER; ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), and p62/SQSTM1 (M162-3) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), Dako, and MBL (Nagoya, Japan) respectively. Goat polyclonal antibody for NQO1 (ab2346) was purchased from Abcam (Cambridge, MA, USA).

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), based on the streptavidin–biotin amplification method, was used in this study according to methods described in a previous report (Suzuki *et al.* 2005). Antigen retrieval was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) for NRF2, ER, PR, HER2, and Ki-67 immunostaining and by heating the slides in a microwave for 20 min in the citric acid buffer for staining with anti-p62 and NQO1 antibodies. Dilutions of primary antibodies used were as follows: 1/1000, NRF2; 1/50, ER; 1/30, PR; 1/200, HER2; 1/50, Ki-67; 1/1000, p62; and 1/1000, NQO1. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H₂O₂) and counterstained with hematoxylin. Human lung carcinoma tissue was used as a positive control for NRF2, p62, and NQO1 immunostaining (Inoue *et al.* 2012). As a negative control, normal rabbit, mouse, or goat IgG was used instead of the primary antibody in this study.

Scoring of immunoreactivity and statistical analyses

NRF2, p62, and NQO1 immunoreactivities were detected in the nucleus, cytoplasm, and cytoplasm of the breast

carcinoma cells respectively, and cases with more than 10% positive carcinoma cells were considered positive for NRF2, p62, and NQO1 status according to the criteria used in a previous report (Inoue *et al.* 2012). NRF2 immunoreactivity was also detected in some epithelial cells of non-neoplastic mammary glands, and cases with more than 10% positive epithelial cells were considered positive for NRF2 in the non-neoplastic glands, the same as for the carcinoma cells. ER, PR, and Ki-67 immunoreactivities were evaluated in more than 1000 carcinoma cells for each case, and their labeling index (LI) was determined subsequently. Cases with ER and PR LI of more than 1% were considered ER- and PR-positive cases (Hammond *et al.* 2011). Ki-67 immunoreactivity was evaluated as a continuous variable (i.e. Ki-67 LI), and it was also dichotomized into high and low groups using the median value (Ki-67 LI=17% in this study) according to the method described in a previous report (Viale *et al.* 2008). HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (Dako), and strongly circumscribed membrane staining of HER2 in more than 10% of carcinoma cells was considered positive.

NRF2 status and clinicopathological factors were evaluated using Student's *t*-test or a cross-table using the χ^2 test. Survival curves were generated according to the Kaplan–Meier method, and statistical significance was calculated using the log-rank test. The results of univariate and multivariate analyses were evaluated using a proportional hazard model (Cox). *P* values <0.05 and 0.05 ≤ *P* values <0.10 were considered significant and borderline significant respectively. Parameters considered significant or borderline significant in the univariate analyses were subsequently used in the multivariate analyses in this study (Suzuki *et al.* 2007a,b). The statistical analyses were carried out using the StatView 5.0J Software (SAS Institute, Cary, NC, USA).

Cell lines

MCF7 and SK-BR-3 human breast carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 (Sigma–Aldrich), containing 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich) and 1% penicillin–streptomycin (Invitrogen).

siRNA transfection

siRNA oligonucleotides for NRF2 used in this study were Stealth RNAi siRNA Duplex Oligoribonucleotides

(Invitrogen). The target sequences of siRNA against *NRF2* were NFE2L2-HSS181505 (si1): 5'-CAAACUGACAGAA-GUUGACAAUUAU-3' (sense) and 5'-AUAAUUGUCAA-CUUCUGUCAGUUUG-3' (anti-sense) and NFE2L2-HSS181506 (si2): 5'-CAAUGAAGCUCAACUUGCAUUAUU-3' (sense) and 5'-AAUUAUGCAAGUUGAGCUUCAUUG-3' (anti-sense). Universal RNAi Negative Control Duplexes (NC; Sigma-Aldrich) were also used as the negative control siRNAs. The siRNAs (10 nM) were transfected using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

Total RNA was extracted from breast carcinoma tissues and cultured cells using the TRIzol Reagent (Invitrogen), and cDNA was synthesized using a RT kit (Superscript II preamplification system) (Gibco-BRL). The LightCycler System (Roche Diagnostics GmbH) was used to semi-quantify the mRNA expression levels by real-time PCR. The primer sequences used in this study were as follows: *NRF2* – NM_004348: forward, 782–800 and reverse, 943–961; *NQO1* – NM_000903: forward, 977–1002 and reverse, 1046–1075; *HO1* – NM_002133: forward, 997–1021 and reverse, 1295–1321; and ribosomal protein L13a (*RPL13A*) – NM_012423: forward, 487–509 and reverse, 588–612 (Suzuki et al. 2007a,b). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 10 s, annealing at 63 °C (*NRF2*, *NQO1*, and *HO1*) or 68 °C (*RPL13A*) for 15 s, and elongation at 72 °C for 15 s. The DNA-binding dye SYBR Green I (Roche Diagnostics GmbH) was used for the detection of PCR products, and the relative mRNA level in each sample was calculated as the ratio of *RPL13A* in this study (Miki et al. 2002).

Immunoblotting

The nuclear and whole-cell proteins from MCF7 and SK-BR-3 cells were extracted using the CellLytic NuCLEAR Extraction Kit (Sigma-Aldrich) and M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA) respectively. The proteins (10 µg) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, the proteins were transferred onto Hybond PVDF membranes (GE Healthcare, Buckinghamshire, UK). Primary antibodies used for immunohistochemistry were anti-NRF2 and anti-NQO1 antibodies and anti-HO1 antibody (ADI-SPA-895, Enzo Life Sciences, Inc., Farmingdale, NY, USA). In addition,

anti-poly (ADP-ribose) polymerase (PARP; 46D11, Abcam) and anti-β-actin (AC-15, Sigma-Aldrich) antibodies were used as internal controls for nuclear proteins (Artus et al. 2006) and whole-cell proteins (Suzuki et al. 2007a,b) respectively. Antibody-protein complexes on the blots were detected using ECL-Plus Western Blotting Detection Reagents (GE Healthcare), and the protein bands were visualized using an LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Cell proliferation and migration assays

One day after transfection of the MCF7 and SK-BR-3 cells with *NRF2* siRNA, medium was changed to phenol-red-free RPMI 1640 medium containing 10% dextran-coated charcoal-FBS. Three days after the transfection, the cell proliferation status was measured by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-²H-tetrazolium, monosodium salt) method using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

The migration properties of MCF7 and SK-BR-3 cells transfected with *NRF2* siRNA were evaluated by wound scratch healing assay (Liang et al. 2007). Briefly, the confluent cell layer was scratched with a sterile plastic P-200 pipette tip after transfection and incubated for 48 h. The migration area was evaluated using the MultiGauge v3.1 Software (Fuji Photo Film), and the relative migration area was then calculated as the ratio of that in the control cells transfected with NC in this study (Iida et al. 2012).

Results

NRF2 immunolocalization in human breast carcinoma

NRF2 immunoreactivity was detected in the nuclei of the breast carcinoma cells, and it was also weakly detected in the cytoplasm (Fig. 1A). Of the 106 cases of breast cancer examined in this study, 47 (44%) were NRF2-positive. On the other hand, NRF2 was weakly and focally present in the nuclei of epithelial cells in non-neoplastic glands adjacent to the carcinoma, while it was negative in the stroma (Fig. 1B). When evaluated using the same cut-off point used for carcinoma cells (i.e. 10% positive cells), NRF2 status of the non-neoplastic glands was found to be positive in two of 95 cases examined (2%). When NRF2 was immunolocalized in 24 benign breast disease (BBD) lesions, comprising papilloma, fibroadenoma, sclerosing adenosis, and usual ductal hyperplasia (*n* = 6 respectively), obtained from non-cancerous patients, NRF2 status was

Figure 1

Immunohistochemistry for NRF2, p62, and NQO1 in the breast carcinoma tissues. (A, B and C) NRF2 immunolocalization in breast carcinoma cells (A), non-neoplastic mammary glands adjacent to the carcinoma (B), and fibroadenoma (C). (D and E) p62 immunoreactivity in breast carcinoma cells (D) and non-neoplastic mammary glands (E). (F and G) NQO1

found to be positive in one case (fibroadenoma) examined (4%) (Fig. 1C).

p62 immunoreactivity was detected in the cytoplasm of the carcinoma cells (Fig. 1D) and some epithelial cells of non-neoplastic glands (Fig. 1E). NQO1 was also immunolocalized in the cytoplasm of the carcinoma cells (Fig. 1F) and some epithelial cells of non-neoplastic glands (Fig. 1G).

When the mRNA expression of *NRF2* and *NQO1* was examined in the tissue samples of 20 invasive ductal carcinoma cases using real-time PCR, immunohistochemical nuclear NRF2 status was found to be significantly associated with the mRNA levels of *NRF2* ($P=0.018$; Fig. 1H, left panel) and *NQO1* ($P=0.0048$; Fig. 1H, right panel). The mRNA levels of *NRF2* were also significantly correlated with those of *NQO1* in these cases ($P=0.0061$, $r=0.59$; data not shown).

Associations between immunohistochemical nuclear NRF2 status and various clinicopathological parameters of the patients are summarized in Table 2. NRF2 status was positively associated with histological grade ($P=0.013$), Ki-67 LI ($P=0.011$), p62 status ($P=0.029$), and NQO1 status ($P<0.001$) and marginally associated with pathological tumor factor (pT) ($P=0.082$), lymph node metastasis ($P=0.086$), HER2 status ($P=0.071$), and Ki-67 status ($P=0.052$). No significant association was detected between NRF2 and other factors such as patient

immunoreactivity in breast carcinoma cells (F) and non-neoplastic mammary glands (G). Bar = 100 μ m. Associations between immunohistochemical nuclear NRF2 status and *NRF2* (left panel) and *NQO1* (right panel) mRNA levels in 20 breast carcinoma tissue samples. The statistical analyses were carried out using Student's *t*-test (H).

age, menopausal status, tumor stage, ER status, and PR status in this study.

Association between NRF2 status and clinical outcome of breast cancer patients

As shown in Fig. 2A, NRF2 status was significantly associated with an increased incidence of recurrence ($P=0.0002$ by the log-rank test) among the 106 breast cancer patients examined. The association between NRF2 status and breast cancer-specific survival is shown in Fig. 2B, and a significant association was also detected between NRF2 status and adverse clinical outcome of the patients ($P=0.0004$ by the log-rank test). A similar tendency was detected when NRF2 status was further categorized into four groups (with 5, 10, and 20% positive cells as cut-off points) in these cases (Fig. 2C and D). A similar tendency was also detected in the group that received adjuvant endocrine therapy ($n=77$: $P=0.0083$ for disease-free survival (Fig. 2E) and $P=0.0041$ for breast cancer-specific survival (data not shown)) or adjuvant chemotherapy ($n=69$: $P=0.0010$ for disease-free survival (Fig. 2F) and $P=0.0005$ for breast cancer-specific survival (data not shown)). NRF2 status was positively associated with p62 status as shown in Table 2, but a significant association between NRF2 status and worse prognosis was detected regardless of the p62 status of the cases

Table 2 Association between immunohistochemical nuclear NRF2 status and clinicopathological parameters in 106 breast carcinoma cases

	NRF2 status		P value
	+(n=47)	–(n=59)	
Patient age ^a (years)	56.3 ± 1.6	56.9 ± 1.6	0.80
Menopausal status			
Premenopausal	15 (14%)	21 (20%)	0.69
Postmenopausal	32 (30%)	38 (36%)	
Stage			
I	11 (10%)	22 (21%)	0.17
II	22 (21%)	27 (25%)	
III	14 (13%)	10 (9%)	
Pathological tumor factor (pT)			
1	16 (15%)	30 (28%)	0.082
2–4	31 (29%)	29 (27%)	
Lymph node metastasis			
Positive	27 (25%)	24 (23%)	0.086
Negative	20 (19%)	35 (33%)	
Histological grade			
1 (well)	5 (5%)	15 (14%)	0.013
2 (moderate)	22 (21%)	33 (31%)	
3 (poor)	20 (19%)	11 (10%)	
ER status			
Positive	36 (34%)	50 (47%)	0.29
Negative	11 (10%)	9 (8%)	
PR status			
Positive	32 (30%)	45 (42%)	0.35
Negative	15 (14%)	14 (13%)	
HER2 status			
Positive	13 (12%)	8 (8%)	0.071
Negative	34 (32%)	51 (48%)	
Ki-67 LI ^a (%)	23.8 ± 2.4	15.9 ± 1.9	0.011
Ki-67 status			
High	28 (26%)	24 (23%)	0.052
Low	19 (18%)	35 (33%)	
p62 status			
Positive	36 (34%)	32 (30%)	0.029
Negative	11 (10%)	27 (25%)	
NQO1 status			
Positive	35 (33%)	14 (13%)	<0.0001
Negative	12 (11%)	45 (42%)	

P values <0.05 and 0.05 ≤ P values <0.10 were considered significant and borderline significant and are listed in and italic type respectively. LI, labeling index (percentage of positive carcinoma cells).

^aData are presented as means ± s.e.m. All other values represent the number of cases and percentage.

($P=0.0052$ for disease-free survival in the p62-positive group (Fig. 2G), $P=0.032$ for disease-free survival in the p62-negative group (Fig. 2H), $P=0.032$ for breast cancer-specific survival in the p62-positive group (data not shown), and P value not available for breast cancer-specific survival in the p62-negative group because no patient had died in the NRF2-negative group (data not shown)).

The univariate analysis of disease-free survival by Cox model (Table 3) indicated NRF2 status, lymph node

metastasis, pT, HER2 status, and NQO1 status to be significant prognostic parameters for disease-free survival in the 106 breast carcinoma patients. The multivariate analysis revealed that only NRF2 status ($P=0.0031$) and lymph node metastasis ($P=0.012$) were independent prognostic factors with relative risks over 1.0 (Table 3). In the univariate analysis for breast cancer-specific survival (Table 4), histological grade ($P=0.0065$), NRF2 status ($P=0.0087$), HER2 status ($P=0.0090$), Ki-67 status ($P=0.0043$), ER status ($P=0.022$), and pT ($P=0.031$) were found to be significant prognostic variables and lymph node metastasis ($P=0.064$) exhibited a borderline significance. However, a subsequent multivariate analysis revealed that only NRF2 status was an independent prognostic factor with a relative risk over 1.0 ($P=0.016$).

Survival curves for clinicopathological factors evaluated in the multivariate analysis in Table 3 for NRF2 status are shown in Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article respectively.

Effects of NRF2 expression on the proliferation and migration of breast carcinoma cells

To further evaluate the biological functions of NRF2 in human breast carcinoma cells, we transfected specific siRNA for NRF2 in both ER-positive MCF7 and ER-negative SK-BR-3 breast carcinoma cells. The mRNA expression levels of NRF2 were significantly decreased in cells transfected with specific NRF2 siRNA (si1 or si2) 3 days after transfection compared with those in cells transfected with negative control siRNA (NC) (Fig. 3A, upper panels). The ratio of NRF2 mRNA level to the control level and its P value were as follows: MCF7 – 18% and $P<0.01$ (si1), 16% and $P<0.01$ (si2); SK-BR-3 – 14% and $P<0.001$ (si1), 8% and $P<0.001$ (si2) respectively. The protein levels of nuclear NRF2 were also markedly decreased in cells transfected with NRF2 siRNA compared with those in the control cells grown under the same conditions (Fig. 3A, lower panels). As shown in Fig. 3B, the mRNA levels of the cytoprotective gene NQO1 were significantly lower in both MCF7 and SK-BR-3 cells transfected with si1 or si2 compared with those in cells transfected with NC 3 days after transfection (MCF7, 24% and $P<0.05$ for si1, 22% and $P<0.05$ for si2; SK-BR-3, 32% and $P<0.05$ for si1, 23% and $P<0.05$ for si2). The mRNA levels of the other cytoprotective gene (HO1) were also significantly lower in MCF7 cells grown under the same conditions (25% and $P<0.05$ for si1, 25% and $P<0.05$ for si2). A similar tendency was detected in SK-BR-3 cells, although it did

Figure 2

Disease-free survival (A, C, E, F, G and H) and breast cancer-specific survival (B and D) of breast carcinoma patients according to NRF2 status studied by the Kaplan–Meier method. (A, B, C and D) Total cases ($n=106$), (E, F, G and H) subset groups that received adjuvant endocrine therapy ($n=77$; E) and adjuvant chemotherapy ($n=69$; F), and groups positive ($n=68$; G) or

negative ($n=38$; H) for p62 status. Statistical analysis results were evaluated using the log-rank test. P values <0.05 were considered significant and are shown in bold type. P value was not calculated for data shown in (D), because no patient had died in the '0–4%' group.

not reach significance ($P=0.15$ and 20% for si1, $P=0.11$ and 17% for si2). Immunoblot analysis subsequently confirmed decreased expression of NQO1 and HO1 proteins in MCF7 and SK-BR-3 cells transfected with NRF2 siRNA for 2 days (Fig. 3B, lower panels).

The effects of NRF2 expression on the proliferation of the breast carcinoma cells are shown in Fig. 3C. The number of MCF7 cells transfected with NRF2 siRNA was significantly lower (82% and $P<0.05$ for si1, 76% and $P<0.05$ for si2) than that of the control cells transfected with NC 3 days after transfection. A similar tendency was also detected in SK-BR-3 cells grown under the same conditions (85% and $P<0.05$ for si1, 72% and

$P<0.01$ for si2). However, relative migration areas in MCF7 and SK-BR-3 cells transfected with siRNA against NRF2 did not change significantly compared with those for the control cells (MCF7, $P=0.95$ for si1 and $P=0.81$ for si2; SK-BR-3, $P=0.90$ for si1 and $P=0.87$ for si2 respectively; Fig. 3D).

In contrast, when exogenous NRF2 was transiently expressed in MCF7 cells, proliferation activity was significantly (114% and $P<0.01$) increased compared with that of the control MCF7 cells transiently expressing mutant NRF2 lacking the activation domain (Mitsuishi et al. 2012) (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

Table 3 Univariate and multivariate analyses of disease-free survival in the 106 breast cancer patients examined

Variable	No. of patients	Univariate	Multivariate	
		P value	P value	Relative risk (95% CI)
NRF2 status (positive/negative)	106 (47/59)	0.0007^A	0.0031	5.36 (1.76–16.34)
Lymph node metastasis (positive/negative)	106 (51/55)	0.0012^A	0.012	3.91 (1.36–11.26)
pT (2–4/1)	106 (60/46)	0.0040^A	0.067	
HER2 status (positive/negative)	106 (21/85)	0.019^A	0.092	
NQO1 status (positive/negative)	106 (49/57)	0.048^A	0.26	
Ki-67 status (high/low) ^a	106 (52/54)	0.16		
p62 status (positive/negative)	106 (68/38)	0.16		
PR status (–/+)	106 (29/77)	0.17		
Histological grade (3/1, 2)	106 (31/75)	0.56		
ER status (–/+)	106 (20/86)	0.60		

Data considered significant ($P<0.05$) are described as boldface. Parameters considered significant or borderline significant in the univariate analyses (A) were used in the multivariate analyses.

^aData were evaluated as continuous variables and all other data were evaluated as dichotomized variables.

Table 4 Univariate and multivariate analyses of breast cancer-specific survival in 106 breast cancer patients

Variable	Number of patients	Univariate	Multivariate	
		P value	P value	Relative risk (95% CI)
Ki-67 LI ^a (73–0%)	106	0.0043*	0.13	
Histological grade (3/1,2)	106 (31/75)	0.0065*	0.82	
NRF2 status (positive/negative)	106 (47/59)	0.0087*	0.016	11.23 (1.41–90.20)
HER2 status (positive/negative)	106 (21/85)	0.0090*	0.15	
ER status (–/+)	106 (20/86)	0.022*	0.67	
pT (pT2–4/pT1)	106 (60/46)	0.031*	0.40	
Lymph node metastasis (positive/negative)	106 (51/55)	0.064*	0.16	
NQO1 status (positive/negative)	106 (49/57)	0.36		
p62 status (positive/negative)	106 (68/38)	0.76		

Data considered significant ($P < 0.05$) were described as boldface. Parameters considered significant or borderline-significant by univariate analyses (*) were used in the multivariate analysis.

^aData were evaluated as continuous variables, and all other data were evaluated as dichotomized variables.

Discussion

This is the first study to demonstrate the association between nuclear NRF2 immunoreactivity and adverse clinical outcome of breast cancer patients, to the best of our knowledge. In the present study, nuclear NRF2 immunoreactivity was detected in 44% of the carcinoma cases, while it was positive in 2% of non-neoplastic breast tissues adjacent to the carcinoma and 4% of BBD tissues. Previous studies have demonstrated that NRF2 immunoreactivity is frequently detected in various human malignancies, such as breast (Loignon *et al.* 2009, Karihtala *et al.* 2011, Hartikainen *et al.* 2012), lung (Inoue *et al.* 2012), gastric (Wang *et al.* 2011), pancreatic (Hong *et al.* 2010), intrahepatic cholangiocellular (Wakai *et al.* 2011), gallbladder (Wang *et al.* 2010), endometrial (Chen *et al.* 2010), and ovarian (Konstantinopoulos *et al.* 2011) carcinomas, and its rate of immunopositivity ranged between 26 and 76% in these studies. The results of the immunohistochemical studies carried out in the present study also indicated a significant association between NRF2 status and immunoreactivity of NQO1, which is known to be an NRF2-induced cytoprotective gene (Lewis *et al.* 2012), and subsequent *in vitro* studies revealed that both MCF7 and SK-BR-3 cells transfected with NRF2 siRNA had decreased NQO1 expression at both mRNA and protein levels. Therefore, nuclear NRF2 immunoreactivity reflects functional NRF2 activity in the breast carcinoma cells, and the relatively wide distribution of NRF2 immunoreactivity indicates the biological importance of the activated NRF2 pathway in human breast carcinoma tissues.

The results of this study also demonstrated that the status of nuclear NRF2 immunoreactivity was significantly associated with Ki-67 LI and histological grade in breast

carcinoma and marginally associated with pT. In addition, the results of *in vitro* studies indicated that in both MCF7 and SK-BR-3 cells transfected with siRNA against NRF2, cell proliferation was significantly decreased. Ki-67 antibody recognizes cells in all phases of the cell cycle except in the G0 (resting) phase, and Ki-67 LI is known to be closely correlated with the proliferative activity of breast cancer cells (de Azambuja *et al.* 2007), while histological grade is evaluated by the mitotic rate, nuclear atypia, and tubule formation of breast carcinoma cells. Previously, Singh *et al.* (2008) had reported that RNAi-mediated reduction of NRF2 expression suppressed the anchorage-independent growth and tumor growth of lung carcinoma cells, and Lister *et al.* (2011) had reported that RNAi depletion of NRF2 resulted in a decrease in the proliferation of pancreatic carcinoma cells, which are in good agreement with the results of the present study. In addition, Kim *et al.* (2011) reported that NRF2 blockade suppressed colon tumor angiogenesis by inhibiting hypoxia-inducible factor 1 α (HIF1 α), and Mitsuishi *et al.* (2012) have recently demonstrated that NRF2 redirected glucose and glutamine into anabolic pathways and supported proliferation in addition to enhancing cytoprotection. These results as well as the results of the present study indicate that NRF2 plays an important role in the increased proliferative activity of breast carcinoma cells. On the other hand, previous reports had also indicated the protective role of NRF2 against DNA damage and mammary carcinogenesis in non-neoplastic breast epithelial cells (Singh & Bhat 2012, Singh *et al.* 2013), and Becks *et al.* (2010) reported that *Nrf2*-knockout mice were more susceptible to mammary carcinogenesis. Therefore, NRF2 may have dual roles in breast carcinoma, i.e. protection against carcinogenesis and promotion of carcinoma progression

Figure 3

Effects of NRF2 expression on the cytoprotective gene expression and cell proliferation and migration properties in breast carcinoma cells. (A) Expression of *NRF2* mRNA evaluated by real-time PCR in MCF7 (left upper panel; white bars) and SK-BR-3 (right upper panel; gray bars) cells transfected with *NRF2*-specific siRNA (si1 and si2) or negative control siRNA (NC). Lower panels show nuclear NRF2 immunoreactivity in MCF7 (left lower panel) and SK-BR-3 (right lower panel) cells transfected with si1, si2, or NC. In each lane, 10 µg of nuclear protein were loaded. PARP immunoreactivity is also shown as an internal control of nuclear protein. (B) *NQO1* and *HO1* mRNA levels in MCF7 (left upper and middle panels; white bars) and SK-BR-3 (right upper and middle panels; gray bars) cells transfected with *NRF2* siRNA evaluated by real-time PCR. Lower panels

show immunoblotting for *NQO1* and *HO1* in MCF7 (left lower panel) and SK-BR-3 (right lower panel) cells transfected with si1, si2, or NC. In each lane, 10 µg of whole protein were loaded. β-actin immunoreactivity is shown as an internal control. (C) Proliferation assays in MCF7 and SK-BR-3 cells. The relative cell proliferation was evaluated as a ratio (%) compared with the control cells (left bar of each chart). (D) Migration assays in MCF7 and SK-BR-3 cells. The relative migration area was evaluated as a ratio (%) compared with the control cells (left bar of each chart). Data are presented as the means ± s.d. ($n=3$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs control cells (left bar). The statistical analyses were carried out using one-way ANOVA and Fisher's protected least significant difference.

(Lau *et al.* 2008), by preventing normal cells from transforming into carcinoma cells and promoting the survival of carcinoma cells under a deleterious environment. Considering that we examined the functions of NRF2 in already transformed breast carcinoma cells in this study, further examinations are required to address the conflicting roles of NRF2, including NRF2 activation in the process of breast carcinogenesis.

The results of the present study demonstrated that NRF2 status was significantly associated with the recurrence and adverse clinical outcome of breast cancer patients, and a similar tendency was also detected in patients who received adjuvant therapy. Moreover, the results of multivariate analyses revealed that NRF2 status was indeed an independent prognostic factor for both recurrence and breast cancer-specific survival.

Previous studies have demonstrated that patients with NRF2 expression in their carcinoma cells generally had worse prognosis in lung (Solis *et al.* 2010, Inoue *et al.* 2012), gallbladder (Wang *et al.* 2010), and ovarian (Konstantinopoulos *et al.* 2011) carcinomas, which is consistent with the results of the present study. NRF2 activation conferred resistance to chemotherapeutic drugs, including 5-fluorouracil, cisplatin, and paclitaxel, which are frequently used in breast carcinoma, in several carcinoma cells (Shibata *et al.* 2008, 2011, Zhang *et al.* 2010, Lister *et al.* 2011), and Loignon *et al.* (2009) reported that Cullin 3 ubiquitin E3 ligase (*CUL3*)-silenced MCF7 cells had increased NRF2 protein levels and exhibited high resistance to both doxorubicin and paclitaxel. Little information is available about the association between NRF2 and resistance to endocrine therapy, but Kim *et al.* (2008)

reported that the expression of NRF2-dependent antioxidant proteins was increased in tamoxifen-resistant breast carcinoma cells and that tamoxifen resistance was partially reversed by NRF2 siRNA. Therefore, residual carcinoma cells following surgical treatment in NRF2-positive breast carcinoma may still have the potential to grow and/or metastasize more rapidly, despite adjuvant therapy, thereby resulting in an increased recurrence and a poorer prognosis in breast carcinoma patients compared with NRF2-negative cases. The clinical patient material examined was of limited quantity in this study, and replication studies with a larger sample set are needed to confirm the clinical significance of NRF2 in breast carcinoma.

In this study, a significantly positive association was detected between NRF2 and p62 immunoreactivities. p62 is a multi-domain protein that serves as a scaffold in the signaling cascades resulting in the activation of nuclear factor- κ B (NF- κ B; Wooten *et al.* 2005, Nakamura *et al.* 2010). p62 also acts as a cargo receptor for autophagic degradation of ubiquitinated targets (Dikic *et al.* 2010). The results of recent studies revealed that p62 contributes to the accumulation of NRF2 by disrupting the association between NRF2 and KEAP1, which is a negative regulator of NRF2 (Komatsu *et al.* 2010), and that the pathway controlled by NRF2 is activated in p62-positive cases of hepatocellular carcinoma (Inami *et al.* 2011). Therefore, p62 is considered to play an important role in NRF2 stabilization in breast carcinoma. However, we also detected nuclear NRF2 immunoreactivity in 11 out of 38 (29%) p62-negative breast carcinoma cases. These results indicate that other mechanisms, such as somatic mutations of *KEAP1* or *NRF2* genes and promoter methylation of *KEAP1* (Taguchi *et al.* 2011), may be responsible for NRF2 stabilization in these cases. Another possible reason for NRF2 accumulation is the transcriptional upregulation of *NRF2* by oncogene-dependent signaling (DeNicola *et al.* 2011). In our immunohistochemical analysis of breast carcinoma, NRF2 status was marginally associated with HER2 status, implying that a HER2-mediated signaling pathway increases the expression of *NRF2*.

p62 has been demonstrated to be involved in the promotion of survival signals, including the proliferation and induction of anti-apoptotic genes (Ciani *et al.* 2003, Seibenheuer *et al.* 2004), and increased p62 protein levels are actually correlated with the aggressive phenotypes of breast carcinoma (Rolland *et al.* 2007, Choi *et al.* 2013). In the present study, a significant association between NRF2 status and worse prognosis was detected regardless of the p62 status of breast carcinoma cells, which was similar to

the result reported by Inoue *et al.* (2012) that simultaneous accumulation of p62 and NRF2 results in a worse prognosis than their individual accumulation in lung carcinoma cells. Considering that p62 alone has not been established as an independent prognostic factor of breast carcinoma in the present study and previous reports, an NRF2-dependent pathway is suggested to play an important role in the exacerbation of a poor prognosis in breast cancer patients, rather than p62 functions, and it may have important therapeutic potential.

In this study, NQO1 status was found to be significantly associated with an increased incidence of recurrence, but not with breast-cancer-specific survival of the patients. Recently, Jamshidi *et al.* (2012) have immunolocalized NQO1 in breast carcinoma, but they did not detect a significant association between NQO1 immunoreactivity and overall survival of the patients. NQO1 has been viewed as a 'good' protein that protects humans from genotoxic damage (Siegel & Ross 2000, Cheng *et al.* 2010). However, recent studies have indicated that NQO1 is expressed at high levels in many solid tumors, and Cheng *et al.* (2010) have shown that NQO1 is involved in the initiation stage of melanoma development and induction of melanoma cell proliferation. In addition, it has been reported that the overexpression of NQO1 increases the levels of antioxidant enzymes, providing tumors with increased protection against cytotoxic agents allowing for rapid carcinoma progression (Lau *et al.* 2008). Therefore, NQO1 may have dual roles in carcinomas similar to NRF2, and further investigations are required to clarify the biological functions of NQO1 in breast carcinoma.

In summary, nuclear NRF2 immunoreactivity was detected in 44% of the breast carcinoma cases. NRF2 status was positively associated with NQO1 immunoreactivity and Ki-67 LI and turned out to be an independent prognostic factor of a worse outcome for both recurrence and disease-free survival of the patients following multivariate analyses. Subsequent *in vitro* studies revealed that NRF2 significantly induced the expression of *NQO1* mRNA and increased the proliferation activity of both MCF7 and SK-BR-3 breast carcinoma cells. These results indicate that nuclear NRF2 protein plays important roles in the proliferation and/or progression of breast carcinoma. Nuclear NRF2 immunoreactivity is therefore considered a potent prognostic factor in breast cancer patients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0234>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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