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Original Article

Prognostic value of NRF2 in breast cancer patients and its role as a tumor suppressor by directly inhibiting HER2 expression

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Abstract: Whether NF-E2-related factor-2 (NRF2) in breast cancer is oncogenic or tumor-suppressive remains under debate, and little is known about its functions. The aim of this study was to evaluate the pathological and clinical significance of NRF2 in breast cancer patients and its functional roles. We immunolocalized NRF2 in 72 invasive ductal breast cancer patients. Clinicopathological parameters and disease-free survival (DFS) was assessed. Correlation between NRF2 and human epidermal growth factor receptor 2 (ErbB2/HER2) expression was detected in patients and breast cancer cell lines by Real-time PCR, western blotting and proliferation assay. Chromatin-immunoprecipitation (CHIP) assays were used to determine if NRF2 interacts with HER2 promoter. We found that NRF2 was positive in 89% of the cases, and its status was significantly associated with HER2 immunoreactivity. Multivariate analyses revealed that NRF2 status was an independent good prognostic factor for DFS. Inverse correlation between NRF2 and HER2 expression was not only observed in breast cancer patients, but also verified on breast cancer cell lines using NRF2 inducer curcumin. Direct regulation of HER2 expression by NRF2 through its binding to ARE (antioxidant-response element) on the HER2 promoter was confirmed by CHIP analysis, and more NRF2 was enriched on the HER2 promoter when cells were treated with curcumin. Our findings suggest that NRF2 might be a marker of good prognosis in breast cancer patients, and that the NRF2-HER2 interaction might be a promising therapeutic target for treating breast cancer.

Keywords: NRF2, breast cancer, prognostic factor, ARE

Introduction

Based on gene expression profiling, breast cancers can be grouped into four major subtypes: Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2+), and basal like/triple negative breast cancer [1-3]. Subtypes not only differ in gene expression profiles, but respond differently to treatment plans and affect the prognosis of the disease [4]. Luminal A and B patients respond well to endocrine therapy; HER2 enriched breast cancers respond better to trastuzumab (Herceptin) and anthracycline-based chemotherapy; Basal like/Triple negative breast cancer patients do not respond to endocrine therapy or Herceptin but are sensitive to platinum-based chemotherapy and PARP inhibitors [4]. Luminal A and B patients often have better prognosis than HER2+ patients [5-7]. Recently, more anti-HER2 drugs have been approved for HER2+ breast cancers including pertuzumab (Perjeta) [8, 9], the dual EGFR/HER2 TK inhibitor (TKI) lapatinib (Tyverb) [10, 11], and the antibody-drug conjugate trastuzumab emtansine (T-DM1, Kadcyla) [12]. However, a substantial fraction of the HER2+ cancers eventually develop resistance and cancer relapses or progresses, e.g. resistance to single-agent trastuzumab ranges from 66% to 88% [13]. There is a need for a better under-

Table 1. Clinicopathological characteristic of the 72 patients examined in this study

	No. of patients	Percentage	Mean value (minimum-maximum)
Patient age (years)	72	100%	50 (28-75)
Breast cancer molecular subtypes			
Luminal B	24	34%	
Luminal A	6	8%	
HER2 enriched	11	15%	
Basal-like	31	43%	
Menopausal status			
Premenopausal	36	50%	
Postmenopausal	36	50%	
TNM Staging			
I	25	35%	
II	43	60%	
III	4	5%	
Histological type			
Invasive ductal carcinoma	72	100%	
T Classification			
1	27	37%	
2	40	56%	
3	5	7%	
Lymph node metastasis			
Positive	32	44%	
Negative	40	56%	
Pathological type			
1, 2	45	63%	
3	27	38%	
ER status			
Positive	30	42%	
Negative	42	58%	
PR status			
Positive	26	36%	
Negative	46	64%	
ER, PR status			
ER+, PR+	26	36%	
ER-, PR+	0	0	
ER+, PR-	4	6%	
HER2 status			
Positive	35	49%	
Negative	37	51%	
Ki-67 a (%)	72	100%	31 (0-80)
Chemotherapy			
TEC	46	64%	
Other chemotherapy (containing docetaxel)	26	36%	
Endocrine therapy	30	42%	
Follow-up time (months)	72	100%	72 (6-49)
Survival status of patients			
Alive without recurrence	53	72%	
Alive with recurrence	19	27%	
Death from breast cancer	1	1%	
Dead from other cause	4	6%	

[a]Data were evaluated as continuous variables in this study, and their range are summarized as mean (minimum-maximum).

standing of the function of HER2 in breast cancer, the mechanisms responsible for drug resistance in HER2+ patients, and for novel therapeutic strategies.

HER2, initially discovered by Weinberg and associates in 1984 [14], encodes a transmembrane receptor tyrosine kinase (RTK), a member of the ErbB/HER receptor family that consists of four members: EGFR/ErbB1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. Unlike, EGFR and HER3, HER2 has no known ligands. Instead, it forms heterologous dimers with EGFR or HER3 and hence activates the downstream signaling pathways, mainly the phosphatidylinositol 3-kinase (PI3K)/Akt and the Erk1-2/MAPK pathways [15]. HER2 is the preferential dimerization partner for its homologs because it displays a high catalytic activity [16]. HER2 positive breast cancers are often associated with increased aggressiveness, poor prognosis, shorter disease-free survival (DFS), and resistance to chemotherapy and endocrine therapy. Overexpression of HER2-achieved by either transcriptional activation or genome amplification-can lead to cellular transformation and tumorigenesis [17]. Indeed, HER2 is amplified in 25-30% of breast cancers [18, 19]. Whereas there have been numerous studies on HER2 and its association with breast cancer treatment and prognosis, transcriptional regulation of HER2 expression is not well studied. Here, we investigated a transcription factor NFE2-related factor 2 (NRF2) and its regulation of HER2 expression.

NRF2, a member of the CNC-bZIP subfamily of basic region/leucine zipper (bZIP) transcription factors, plays a significant role in the adaptive response to oxidative stress by directing various transcriptional programs. NRF2 exists in an inactive cytoplasm-localized state or active nucleus-localized state. When it resides in the cytoplasm, it is kept transcriptionally inactive through binding to its own negative regulator, Kelch-like ECH-associated protein 1 (KEAP1), and forms a complex with KEAP1 and cullin 3 (CUL3) ubiquitin ligase, which directs NRF2 for proteasomal degradation [20]. Upon exposure of cells to electrophiles or oxidative stress-generating agents, NRF2 is transported to the nucleus, binds to response elements on DNA, known as antioxidant response elements (AREs) or electrophile response elements (EpREs), and regulates the expression of genes involved in the response to cellular stress [21]. Whether NRF2 is beneficial or harmful in cancer is the subject of active debate [22]. NRF2 inducing drugs, like curcumin [23], have been widely used for the prevention of cancer. However, recent studies also found that NRF2 mutations and enhanced NRF2 activity might be associated with resistance to chemotherapy and poor survival from cancer treatment [24, 25]. Although NRF2 has oncogenic potential, NRF2 activators are being used already for cancer management and more are under active pharmacological development. It is critical to further dissect the function of NRF2 in breast cancers, especially HER2+ cancers. In this study, we evaluated the association between NRF2 status and clinical patient outcome, the correlation between NRF2 and HER2 expression, and the regulatory effect of NRF2 on HER2 expression in invasive breast cancers.

Materials and methods

Patients and tissue samples

The paraffin-embedded pathological specimens from 72 invasive ductal breast carcinoma patients were obtained from Chinese female patients who underwent surgical treatment from 2010 to 2011 in the Department of Breast Surgery, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu, China. The clinicopathological characteristics of these patients are summarized in **Table 1**. In this clinical trial, the patients did not receive chemotherapy or hormonal therapy before surgery but received chemotherapy containing docetaxel after surgery, and were followed for up to 49 months. In addition, 18 snap-frozen specimens of invasive ductal carcinoma patients including their matching normal tissues, adjacent tissues and breast cancer tissues were used for measuring NRF2 mRNA and protein levels. These tissues were obtained from patients who underwent surgical treatment from 2014 to 2015 from the same hospital. Informed consent was obtained from all the patients before the clinical trials and their surgery. Research protocols followed in this study were approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

Immunohistochemistry

Rabbit polyclonal antibodies for NRF2 (ab137550), p53 (ab131442) and mouse

monoclonal antibody for NQO1 (ab28947), CK5/6 (ab86974) were purchased from Abcam (Cambridge, MA, USA). Mouse MABs for estrogen receptor (ER; ER1D5), progesterone receptor (PR; MAB429), and Ki-67 (MIB1), were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and Dako, respectively. Rabbit MABs for HER2 (ab16662) and LRP (ab92544) were purchased from Abcam.

Immunohistochemistry was performed using paraffin-embedded formaldehyde-fixed tissues sections. Antigen retrieval was enhanced by microwave heating in 10 mM citrate buffer (pH 6.0) for 12 min. Endogenous peroxidase was quenched using 1.5% H_2O_2, followed by blocking with 5% normal goat serum diluted in PBS. The primary antibodies are as previously noted and used at 1:200 in blocking serum. Sections were later treated with biotinylated secondary antibody for 30 min and ABC reagent for 45 min (PK-6101, Vector Labs, Burlingame, CA). Visualization was achieved using the horseradish peroxidase substrate 3,3'-diaminobenzidine (DAB) solution (Solarbio, Beijing, China), and counterstained with hematoxylin.

Scoring of immunoreactivity and statistical analyses

NRF2 and NQO1 immunoreactivities were detected in the nucleus and cytoplasm of the breast carcinoma cells, and cases with more than 25% positive carcinoma cells were considered positive for NRF2, and NQO1 status. NRF2 immunoreactivity was also detected in some epithelial cells of non-neoplastic mammary glands. Cases with more than 25% positive epithelial cells were considered positive for NRF2 in the non-neoplastic glands, the same as for the carcinoma cells. The cutoff for Estrogen receptors (ER) positivity and progesterone receptor (PR) positivity was 1% positive tumor cells with nuclear staining. The Ki-67 labeling index (LI) was expressed as the percentage of positive nuclear staining cells among at least 1000 invasive cells in the area scored, and it was categorized into high and low groups divided by the median value (high, LI ≥ 14%; low, LI < 14%). Positive for HER2 was defined as uniform intense membrane staining of > 10% of invasive tumor cells. The results were scored independently by two pathologists who were blinded with regard to patients' clinical data. Cases with discrepancies were simultaneously re-reviewed by the original two pathologists and a senior pathologist until a consensus was reached.

NRF2 status and clinicopathological factors were evaluated using Student's t-test or a cross-table using the Chi-square 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 were considered statistically significant. *P* values no between 0.05 and 0.10 were considered borderline significant. Parameters considered significant or borderline significant in the univariate analyses were subsequently used in the multivariate analyses in this study. The statistical analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL).

Cell lines

Human breast carcinoma cell lines BT-474, MDA-MB-231, MCF-7 and SKBR3 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 (Sigma-Aldrich), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). MCF-10A (ATCC) was cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 500 ng/ml hydrocortisone, and 100 ng/ml cholera toxin. D407 (retinal pigment epithelial cell line) cells were cultured in DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Cell proliferation assay

Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure the effect of curcumin on BT-474 (ATCC, Manassas, VA) cell proliferation. Cells were plated at a density of 5000 cells per well in 96-well plates with complete medium and incubated with different concentrations of curcumin or mock treated for 12 hr, 24 hr or 48 hr. 10 μl of the cell proliferation reagent WST-8 was added to each well and incubated for 2 h at

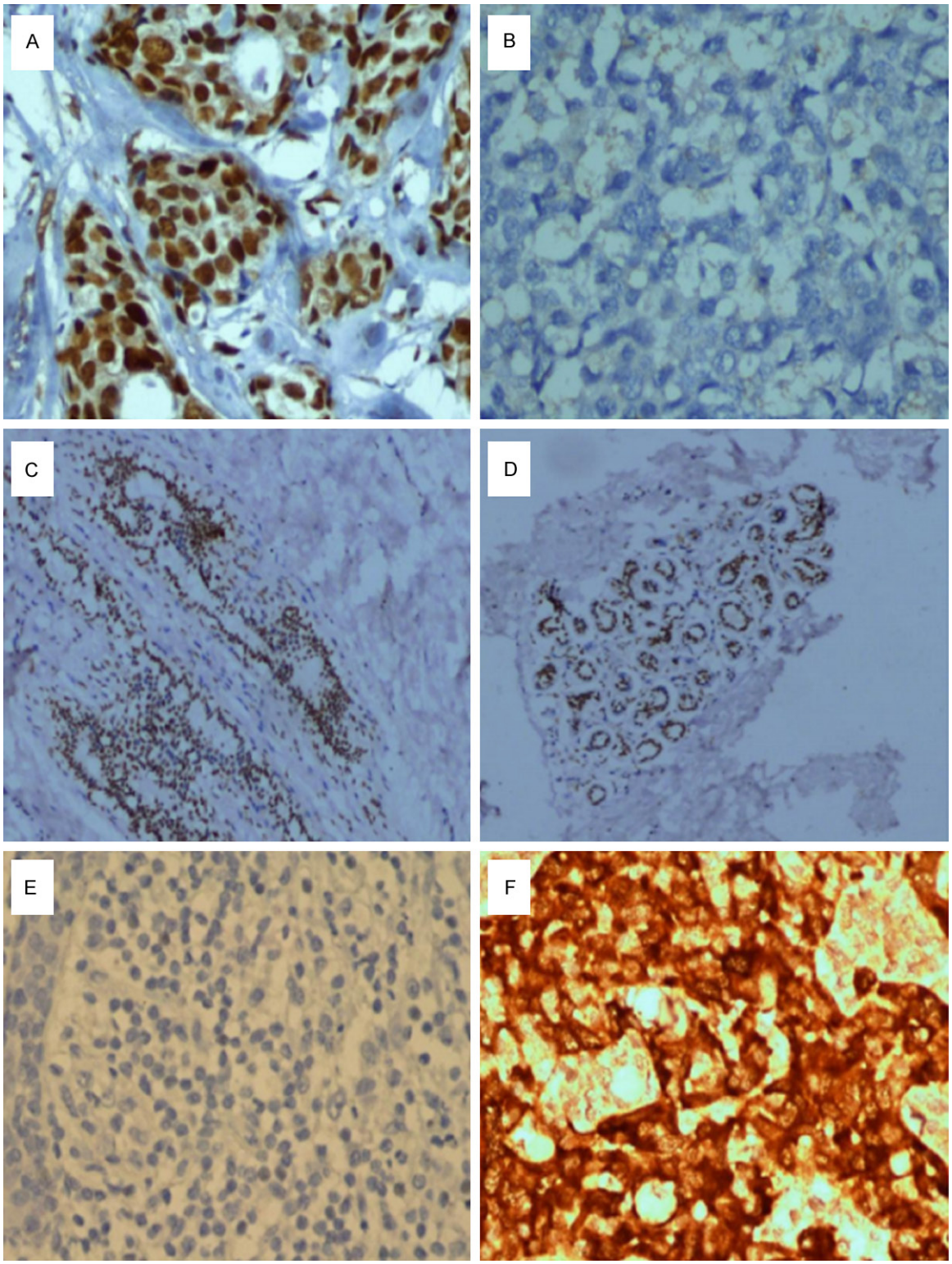

Figure 1. Immunohistochemistry for NRF2 in the breast cancer tissues. A. Positive expression; B. Loss of expression; C. Non-neoplastic mammary glands adjacent to the carcinoma; D. Hyperplasia adjacent to the carcinoma. Immunohistochemistry for NQO1 in the breast cancer tissues; E. Loss of expression; F. High expression. Representative images of samples with different levels of these proteins are shown.

37°C. Viable cell numbers were estimated by measurement of optical density at 450 nm. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells.

Western blotting

Whole cell protein was extracted by RIPA lysis buffer (50 mM HEPES, pH7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA and protease inhibitor cocktail) for 30 min at 4°C. After centrifugation for 20 min at 10,000 rpm, the protein concentrations of the supernatants were measured by BCA Protein Assay Kit (Pierce). Heat denatured protein samples were resolved using 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Sigma). After blocking in 5% non-fat skim milk in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 h, the membranes were incubated sequentially with primary and secondary antibodies. Proteins were then detected by enhanced chemiluminescence detection system (Amersham). Blots shown are representatives of experiments repeated at least three times.

Real-time PCR

Total RNA was extracted from breast carcinoma cell lines or tissue using the TRIzol method (Invitrogen). First-strand cDNAs were synthesized using a Reverse Transcription kit (Invitrogen). Primer sequences used in this study were as follows: NRF2 (NM_004348): Forward 5'-ATAGCTGAGCCCAGTATC-3'; Reverse: 5'-GGTGCCATGGATTTGCCAT-3'; HER2 (NM_001005862.2): Forward: 5'-ATCTGGCGCTTTTGGCACAG-3'; Reverse: 5'-CACCAGCCATCACGTATGCT-3'; ACTB (NM_001101): Forward: 5'-GGCATCGTGATGGACTCCG-3'; Reverse: 5'-GCTGGAAGGTGGACAGCGA-3'.

Chromatin immunoprecipitation (ChIP)

Cells were fixed with 4% formaldehyde, scraped and collected. Protein-DNA complexes were extracted, sonicated, immunoprecipitated and eluted with a Chromatin Immunoprecipitation Assay Kit (Millipore, 17-295) according to the manufacturer's instructions using NRF2 antibodies (Abcam). A nonspecific rabbit IgG (Millipore, PP64) was employed as a negative immunoprecipitation control. Immunoprecipitated genomic DNA was recovered and used in polymerase chain reactions (PCR) with GoTaq DNA polymerase (Promega, M3005). The following primers flanking two putative ARE sites in HER2 promoter were used: HER2-1, Forward: 5'-AGCCCAGTTTCTGCCTTTG-3', Reverse: 5'-AACCACCTTTCCAGCCTCA-3' (83 bp fragment); HER2-2, Forward: 5'-TCTGACCTGGATGCCATAGG-3', Reverse: 5'-ACATGCTCATTTCACGGACC-3' (143 bp fragment). PCR products were analyzed by agarose gel electrophoresis. Real-time PCR using Power SYBR Green reagent (Life Technology, 4367659) was also employed to analyze HER2 promoter DNA immunoprecipitation.

Results

NRF2 immunolocalization in human breast carcinoma

Inactive NRF2 resides in cytoplasm, but translocates to the nucleus upon activation and stimulates expression of its downstream regulated genes. As shown in **Figure 1** and **Table 2**, strong immunoreactivity of NRF2 in the nuclei of breast carcinoma cells (**Figure 1A**) was found in 64 out of 72 patients. Patients from the NRF2 negative group (8/72) either gave no signal or had weak NRF2 staining in the cytoplasm (**Figure 1B**). Furthermore, in another 18 fresh matching samples of breast cancer patients, NRF2 was focally present in the nuclei of epithelial cells in non-neoplastic glands (**Figure 1C**) and hyperplasia (**Figure 1D**) adjacent to the carcinoma. When evaluated using the same cut-off point (25% positive cells), NRF2 immunolocalization of the non-neoplastic glands was found to be positive in all of the normal tissues and tissues adjacent to carcinoma (100% in each 18 samples, respectively), while it was positive in 9 of 18 cases examined (50%) in the matching breast cancer tissues.

The expression of NQO1, a downstream effector of NRF2, significantly correlated with NRF2. Indeed, 50 patients showed positive immunostaining of NQO1 in cytoplasm (**Figure 1F**).

When the mRNA expression of NRF2 and HER2 were examined in fresh tissues of 18 invasive ductal breast cancer patients using real-time PCR, immunohistochemical nuclear status showed significant positive correlation with the mRNA levels of NRF2 ($P = 0.027$; **Figure 2A**). On

Table 2. Association between immunohistochemical nuclear NRF2 status and clinicopathological parameters in 72 breast carcinoma cases

NRF2 status	Positive (n = 64)	Percentage	Negative (n = 8)	Percentage	P
Patient Age * (years)					
≤ 50	35		3		0.463
> 50	29		5		
Menopausal status					
Premenopausal	32	44.44%	4	5.56%	1.000
Postmenopausal	32	44.44%	4	5.56%	
TNM stage					
I	17	23.61%	1	1.39%	0.670
II, III	47	65.28%	7	9.72%	
Pathological tumor factor					
1	26	36.11%	1	1.39%	0.244
2, 3	38	52.78%	7	9.72%	
Lymph node metastasis					
Positive	27	37.50%	5	6.94%	0.543
Negative	37	51.39%	3	4.17%	
Histological grade					
1 (well), 2 (moderate)	39	54.17%	6	8.33%	0.701
3 (poor)	25	34.72%	2	2.78%	
ER					
Positive	27	37.50%	3	4.17%	1.000
Negative	37	51.39%	5	6.94%	
PR					
Positive	25	34.72%	1	1.39%	0.244
Negative	39	54.17%	7	9.72%	
HER2					
Positive	28	38.89%	7	9.72%	0.026
Negative	36	50%	1	1.39%	
Ki-67 * (%)	0.750 ± 3		45.7 ± 12.7		
Ki-67					
High	42	58.33%	6	8.33%	0.710
Low	22	30.56%	2	2.78%	
P53					
Positive	44	61.11%	4	5.56%	0.427
Negative	20	27.78%	4	5.56%	
CK5/6					
Positive	8	11.11%	1	1.39%	0.456
Negative	39	54.17%	2	2.78%	
Chemotherapy					
TEC	43	59.72%	3	4.17%	0.128
Others including T	21	29.17%	5	6.94%	
LRP					
Positive	40	55.56%	2	2.78%	0.060
Negative	24	33.33%	6	8.33%	
NQO1					
Positive	47	62.28%	3	4.17%	0.051
Negative	17	23.61%	5	6.94%	

P < 0.05 was considered as statistically significant. *data are presented as mean ± S.E.M. All other values represent the number of cases and percentage.

the other hand, there was a significant negative correlation of NRF2 status with the mRNA levels of HER2 (P = 0.042; **Figure 2B**).

We further analyzed the association between NRF2 nuclear immunolocalization and other clinicopathological parameters, summarized in **Table 2**. From a panel of proteins analyzed including ER, PR, HER2, Ki-67, P53, and CK5/6, only NRF2 status highly associated with HER2 expression (P = 0.026) and marginally associated with NQO1 status (P = 0.026) and low density lipoprotein receptor-related protein (LRP) status (P = 0.066). Histological grade (P = 0.701), patient age (P = 0.463), classification of Malignant Tumors (TNM stage) (0.670), pathological tumor factor (P = 0.244), lymph node metastasis (P = 0.543) did not show significant association with NRF2 status.

Association between NRF2 status and clinical outcome of breast cancer patients

We first analyzed disease-free survival (DFS) among different breast cancer subtypes. As shown in **Figure 3A**, HER2-enriched (+) patients had substantially worse prognosis than patients with the other three subtypes. The association between NRF2 status and DFS was shown in **Figure 3B** and a strong association between NRF2 status and better clinical outcome was observed. A similar tendency was detected in HER2-enriched (+) breast

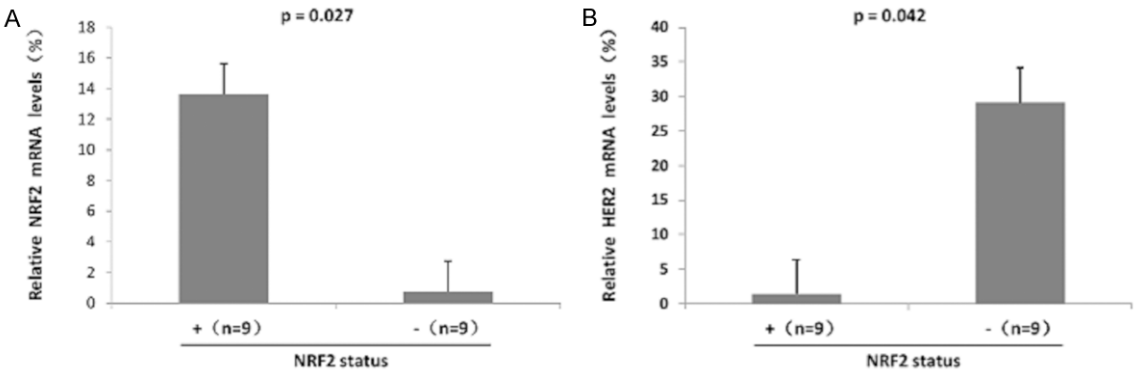

Figure 2. Association between immunohistochemical nuclear NRF2 status and NRF2 (A) and HER2 (B) mRNA levels in 18 breast cancer tissue samples. The statistical analyses were carried out using student's t-test. Inverse correlation between NRF2 and HER2 expression in invasive ductal breast carcinoma. NRF2 (A) and HER2 (B) mRNA levels in 18 breast carcinoma tissue samples.

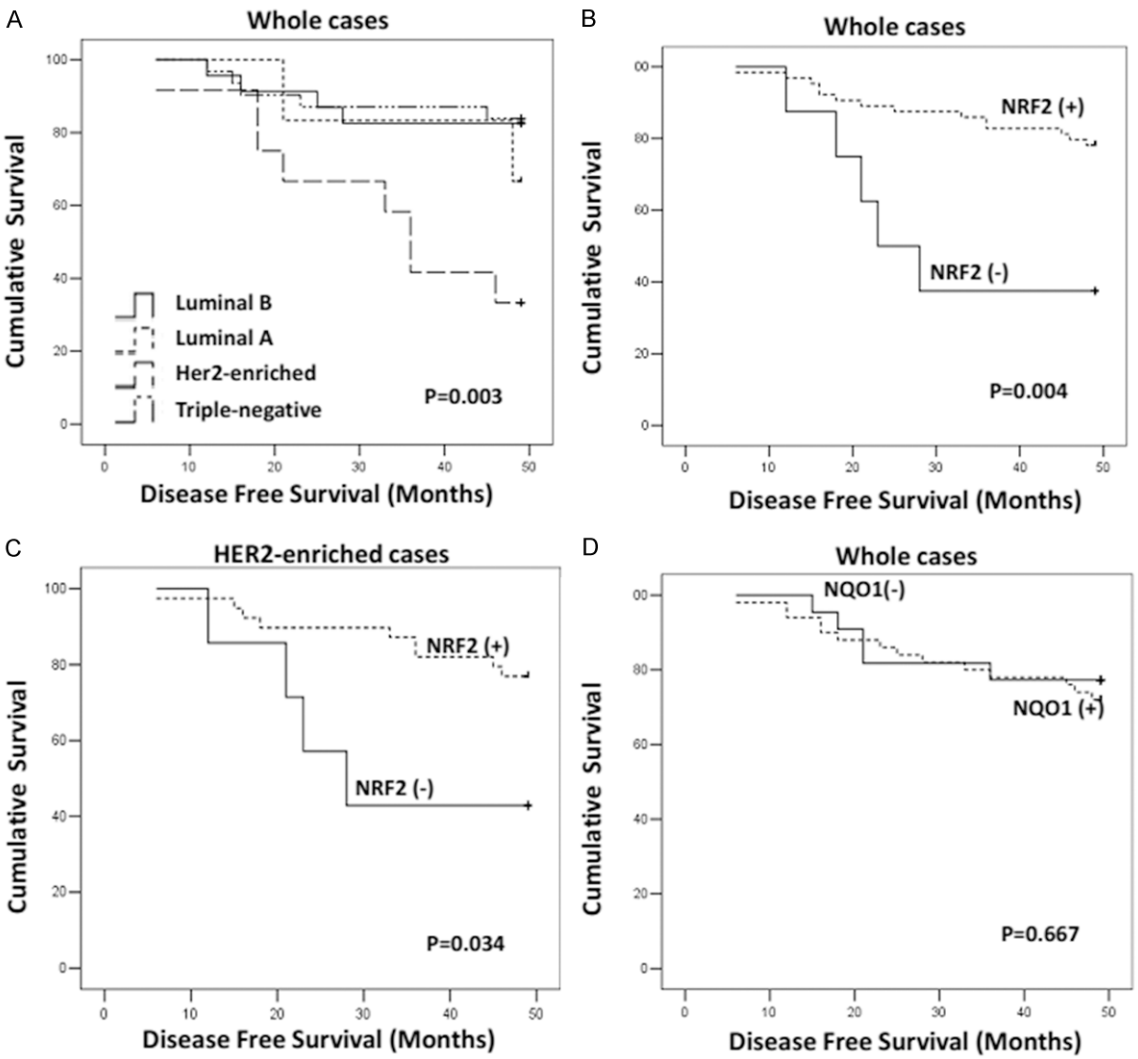

Figure 3. Disease free survival in subgroups of patients. A. According to molecular types; B. According to NRF2 status; C. According to NRF2 status in HER2-enriched(+) cases; D. According to NQO1 status.

Table 3. Univariate analyses of disease-free survival in the 72 breast carcinoma cases

	HR	95.0% CI		P
		Lower	Upper	
NRF2 (+/-)	0.25	0.09	0.7	0.0081
NQO1 (+/-)	1.25	0.45	3.47	0.6692
ER (+/-)	0.8	0.31	2.02	0.632
PR (+/-)	0.8	0.31	2.12	0.6587
HER2 (+/-)	4.17	1.69	10.3	0.002
P53 (+/-)	1.4	0.5	3.88	0.5206
Ki-67 (high/low expression)*	1.11	0.42	2.91	0.8396
CK5/6 (+/-)	2.95	1.03	8.41	0.0431
LRP (+/-)	0.58	0.24	1.43	0.2402
T (T2, 3/T1)	0.86	0.35	2.15	0.7504
TNM stage (II, III/I)	1.37	0.45	4.13	0.5759
Histological grade (3/1, 2)	0.73	0.28	1.92	0.5206
Menopause (pre/post menopause)	0.51	0.2	1.29	0.1549
Age (> 50/≤ 50 years)	0.35	0.12	0.96	0.0425
Lymph node metastasis (+/-)	9.38	2.72	32.31	0.0004

P < 0.05 was considered as statistically significant, described as boldface.
*Data were evaluated as continuous variables and all other data were evaluated as dichotomized variables.

Table 4. Multivariate analyses of disease-free survival in the 72 breast carcinoma cases

		95.0% CI		
	HR	Lower	Upper	P
NRF2	0.21	0.07	0.64	0.0061
HER2	2.66	1.03	6.92	0.0441
Age (> 50/≤ 50)	0.31	0.11	0.88	0.0281
Lymph node metastasis (+/-)	8.23	2.26	29.93	0.0014

cancer (**Figure 3C**) that NRF2 positive patients showed better prognosis. No significant association was found between NQO1 status and DFS (**Figure 3D**).

The univariate analysis of disease-free survival by Cox model (**Table 3**) indicated breast cancer subtypes (P = 0.0036), NRF2 status (P = 0.0081), HER2 status (P = 0.002), CK5/6 status (P = 0.0431), age (with cutoff at 50, P = 0.0425) and lymph node metastasis (P = 0.0004) to be significant prognostic parameters for disease-free survival in the 72 breast carcinoma patients. The multivariate analysis (**Table 4**) revealed that NRF2 status (P = 0.0061, HR = 0.21, CI = 0.07-0.64), HER2 status (P = 0.0441, HR = 2.66, CI = 1.03-6.92), age (P = 0.0281, HR = 0.31, CI = 0.11-0.88) and lymph node metastasis (P = 0.0014, HR = 8.32, CI = 2.26-29.93) were independent prognostic factors. Positive NRF2 showed a strong association with better prognosis. However, Age > 50, positive HER2, and lymph node metastasis all were associated with adverse clinical outcome of the patients.

Inverse correlation between NRF2 and HER2 expression in invasive carcinoma cells

From the analysis performed thus far, NRF2 and HER2 provide positive and negative prognostic factors for breast carcinomas, respectively. To further analyze the association between these two factors, we collected fresh breast cancer tissue from 18 breast carcinoma patients and performed gene expression analysis. Real time PCR data revealed an inverse correlation between NRF2 and HER2 expression in invasive breast carcinomas (**Figure 2A**, **2B**). Similarly, in those breast cancer cell lines (MDA-MB-231 and MCF-7) that have a higher expression of NRF2, HER2 expression was blocked (**Figure 4A**), whereas in BT-474 and SKBR3 cells, NRF2 levels were relatively low and HER2 expression was detected. This inverse correlation between NRF2 and HER2 was only observed in breast cancer cells but not in a non-cancerous breast cell line, MCF-10A or retinal pigment epithelial cells D407. MCF-10A and D407 express low levels of NRF2 but HER2 expression was not detected.

Curcumin has a long history as a compound used in cancer chemoprevention and is known to be a NRF2 activator [26]. Treatment of BT-474 cells with curcumin led to a dramatic increase of NRF2 expression in a time dependent manner (**Figure 4B**). Interestingly, when BT-474 cells were treated with increasing doses of curcumin, HER2 mRNA expression (**Figure 4C**) dropped by 5.4-fold and 50-fold with 35 μM and 50 μM curcumin, respectively, along with a 40% and 2.5-fold increase of NRF2 mRNA levels (**Figure 4D**). The changes of NRF2 and HER2 expression were further confirmed on protein

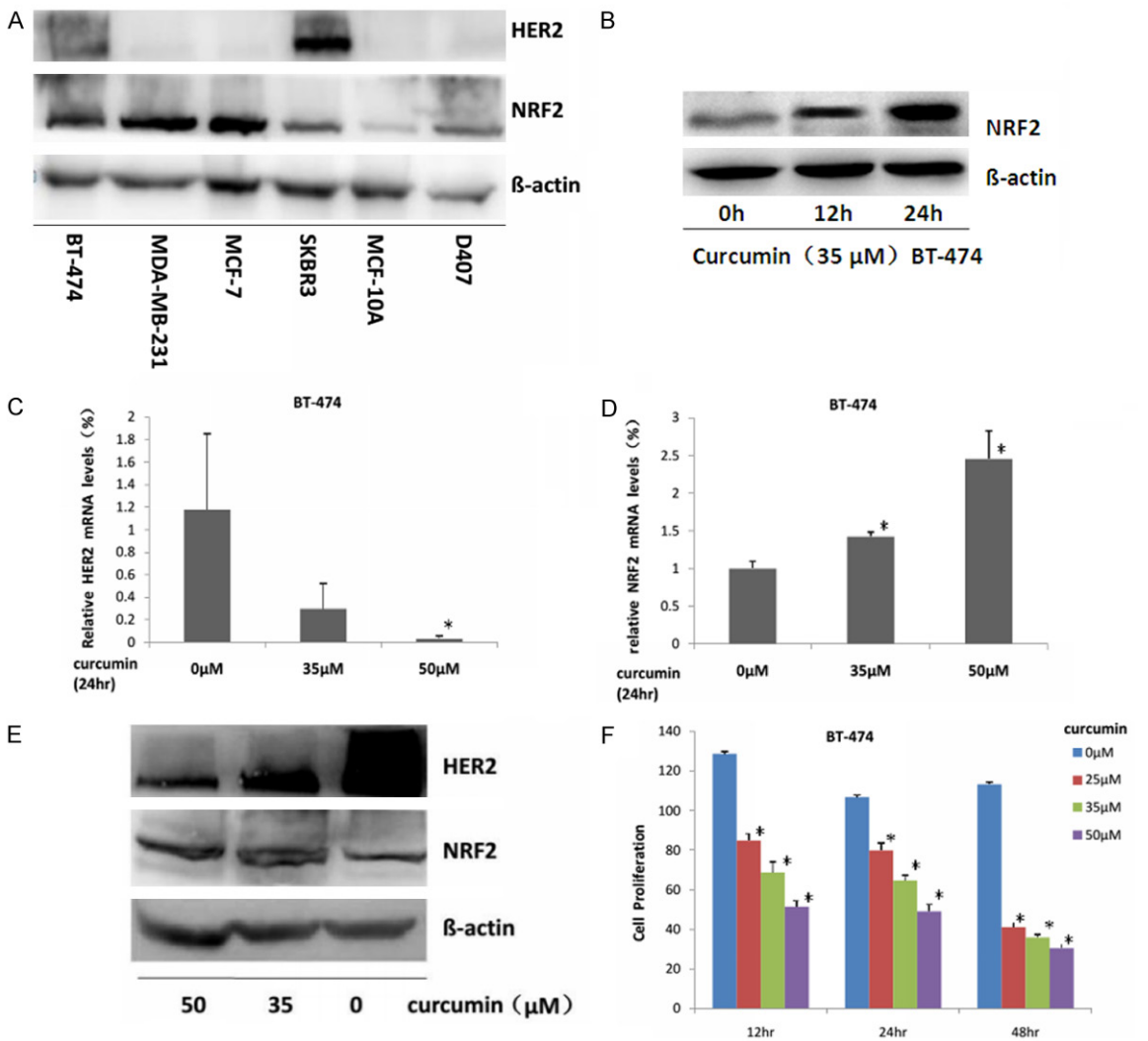

Figure 4. (A) Expression of NRF2 and HER2 in 4 breast cancer cell lines. Expression levels in MCF-10A and D407 were included as controls. (B) Time course of NRF2 induction from BT-474 cells by 35 μM curcumin. B-Actin served as loading control. Samples were collected at different time points and subjected to SDS-PAGE western blot analysis. (C and D) HER2 and NRF2 mRNA levels in BT-474 cells after incubation with different doses of curcumin for 24 hr. Data are depicted as relative expression normalized to mRNA levels from mock treated cells. (E) Protein levels of NRF2 and HER2 were also analyzed by western blot from cells same treated as in (C) and (D). (F) Effect of NRF2 on BT-474 cell proliferation. Incubation of BT-474 cells with curcumin resulted in marked reduction of cell proliferation in a time and dose dependent manner.

levels by western blotting as shown in **Figure 4E**.

NRF2 expression reduced the proliferation of breast carcinoma cells

As HER2 has an established role in promoting breast cancer growth through activation of its downstream signaling pathways, such as MAPK and PI3K/AKT. Here in our study, NRF2 exhibited a negative regulation of HER2 expression. We next sought to ask whether NRF2's regulation on HER2 affects breast cancer proliferation. To examine this, we manipulated NRF2 expression by using NRF2 activator curcumin. As shown in **Figure 4F**, treatment of BT-474 cells with curcumin reduced cell proliferation markedly, in a time and curcumin dose dependent manner.

NRF2 binds to the promoter regions of HER2 and inhibits its transcription

Based on the DNA sequence analysis of the HER2 promoter region, two putative ARE sites were first detected. To determine whether

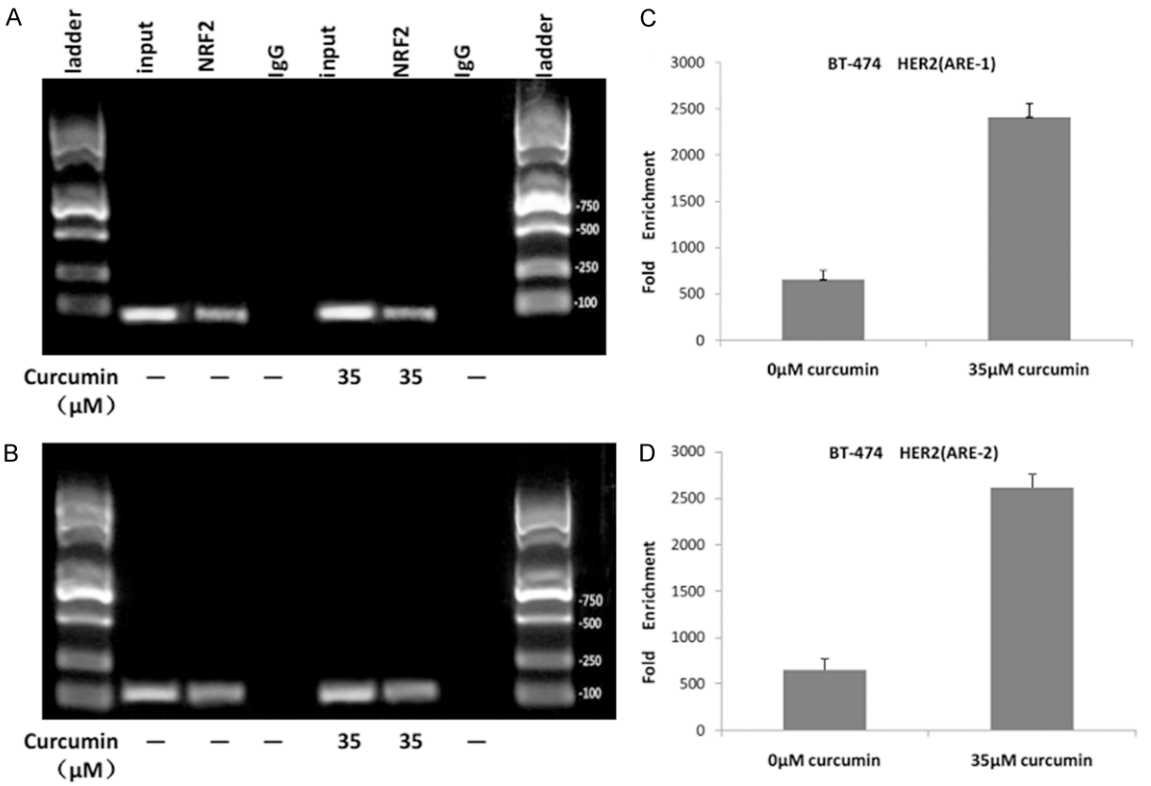

Figure 5. NRF2 binds to the promoter regions of HER2. A, C. CHIP assay showed NRF2 binds to HER2 promoter ARE-1 area in BT-474 cells. B, D. CHIP assay showed NRF2 binds to HER2 promoter ARE-2 area in BT-474 cells.

NRF2 interacts with the promoter of HER2, we performed chromatin immunoprecipitation (ChIP) assay in cultured cells using an antibody to NRF2. These experiments confirmed that NRF2 binds to the promoter region of HER2 (**Figure 5A**, **5B**). As a positive control, NRF2 was also enriched on NQO1 promoter, a downstream effector of NRF2. NRF2 did not show significant binding to the promoter region of α-Tubulin. Real time PCR was also employed to analyze HER2 promoter DNA immunoprecipitated with anti-NRF2 antibody. As shown in **Figure 5C**, **5D**, more HER2 promoter ARE regions immunoprecipitated with anti-NRF2 antibody, suggesting more binding of NRF2 to HER2 promoter after curcumin treatment. Taken together, these data demonstrated that NRF2 binds to the promoter region of HER2 and functions as a novel transcriptional suppressor of HER2 gene expression.

Discussion

NRF2's role in breast cancer is controversial. NRF2 has long been considered a tumor suppressor, with many NRF2 activators (e.g. sulphoraphane [27, 28], oleanane triterpenoids [29-31] and curcumin [32, 33]) extensively used for their chemopreventive activity against cancer. Recently, gain-of-function mutations in NRF2 [34, 35] were discovered and reports about the oncogenic features of the gene presented [36-39], which have raised increasing concerns about the safety of long-term augmentation of NRF2 activity. The debate on this topic is vigorous: is it a "good" or "bad" gene?

In this study, we aimed to dissect out the function of NRF2 in breast cancer and its association with patient outcomes, and using a specific cell line, focused in vitro efforts primarily on the Luminal B subtype. Our analysis of 72 invasive ductal breast carcinoma patients demonstrated a strong negative association between HER2 status and patient disease free survival (DFS). In contrast, a positive NRF2 suggested a better patient outcome. Our data therefore conflict with an earlier published study that suggested that the presence of NRF2 in tumors implied worse prognosis. In the report by

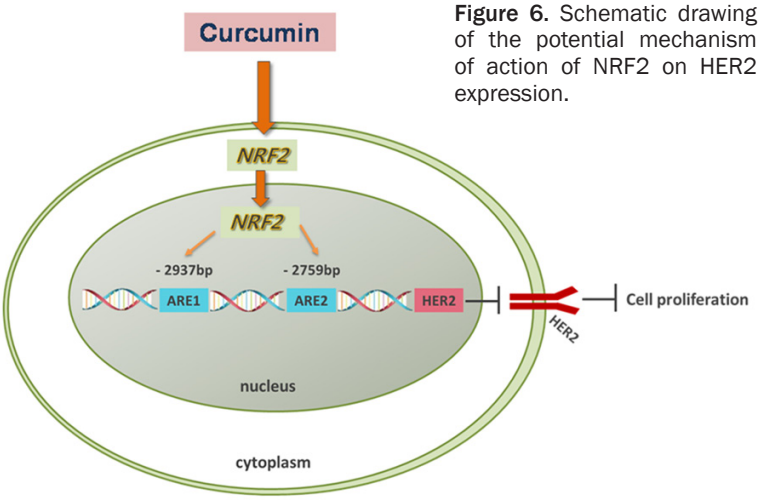

Figure 6. Schematic drawing of the potential mechanism of action of NRF2 on HER2 expression.

expression in a time and dose dependent manner. And again, HER2 expression declined markedly when NRF2 expression was induced with curcumin, suggesting a tight control of HER2 expression by curcumin through NRF2 activation. Consistent with the positive association of NRF2 status and patient outcome, BT-474 cell proliferation decreased dramatically upon treatment with the NRF2 activator curcumin, with dose and time dependence clearly evident (**Figure 4**).

Onodera et al. [40], the association between NRF2 status and patient outcome was studied in 106 breast cancer cases with all subtypes. Although our sample size was smaller, we strictly followed the inclusion and exclusion criteria for screening and then used the same chemotherapy for treatment in all subjects; therefore treatment-effect bias was small. Breast cancer is highly heterogeneous and different sampling decisions will lead to different results. As can be seen from TNM staging data (**Table 1**), 95% of patients had early stage breast cancer. By contrast, in Onodera's study, 22% patients were considered as having advanced breast cancer. Furthermore, our study and Onodera's are the only two reports regarding NRF2 and prognosis of breast cancer patients.

From a comprehensive multivariate analysis, we revealed four independent risk factors for DFS in early invasive ductal breast cancers, namely NRF2 status, HER2 status, age and lymph node metastasis. Gene expression studies revealed that NRF2 expression inversely correlated with HER2 in 18 freshly prepared patient samples. This inverse correlation between NRF2 and HER2 was further validated in breast cancer cell lines, where high NRF2 expression cell lines exhibited much less or absent HER2 expression (**Figure 4**). Taken altogether, a tight regulation of expression between NRF2 and HER2 is suggested.

A Luminal B subtype cancer cell line, BT-474 was selected for manipulation of NRF2 expression to study the effect on HER2 expression and cell behavior. Curcumin induced NRF2

We sought the mechanism that might be responsible for NRF2 regulation of HER2 expression. As a transcription factor, NRF2 binds to the antioxidant response element (ARE, 5'-TGACnnnGC-3') to regulate the transcription of many target genes, including those involved in the response to oxidative stress [41]. Through an analysis of the HER2 promoter region, two putative ARE-like sites were located at -2759 bp and -2937 bp upstream of the start codon: ARE-1, AAATGGGGATAATGACCCAGCCAC, ARE-2, CAAGCCACAGGGTGGGACTTGACTGGGCAGTGGG.

Chromatin immunoprecipitation analysis demonstrated the binding of NRF2 protein to both ARE-like sites (**Figure 5**). Treatment with curcumin led to enhanced enrichment of NRF2 on the HER2 promoter. Binding of NRF2 to ARE usually activates target gene expression. In this case, inhibition of HER2 expression occurred after NRF2 binding to the ARE-1 and ARE-2 sites on the promoter. This is not the first inhibitory effect of NRF2 reported, as it has also been reported that binding of NRF2 to the promoter of RON resulted in gene expression inhibition as well [41]. Our work provides another case identifying NRF2 as a repressor of gene expression, in this case HER2, which plays an important role in breast cancer.

In conclusion, NRF2 appears to be an independent positive prognostic factor of for disease-free survival. In vitro studies depicted a possible mechanism for NRF2, functioning as a tumor suppressor in breast cancer, especially Luminal B subtype, by directly inhibiting expres-

sion of HER2 (**Figure 6**). Such regulation of HER2 expression by NRF2 can be enhanced by curcumin. Our findings demonstrated that NRF2-HER2 might be a promising therapeutic target for treating breast cancer.

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Disclosure of conflict of interest

None.

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