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Genetic Polymorphisms and Protein Expression of NRF2 and Sulfiredoxin Predict Survival Outcomes in Breast Cancer

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Abstract

NRF2 activates several protective genes, such as sulfiredoxin (*SRXN1*), as a response to oxidative and xenobiotic stress. Defects in NRF2 pathway may increase cancer susceptibility. In tumor cells, activation of NRF2 may lead to chemo- and radioresistance and thus affect patient outcome. Nine single-nucleotide polymorphisms on *NRF2* gene and eight on *SRXN1* were genotyped in 452 patients with breast cancer and 370 controls. Protein expression of NRF2 and SRXN1 was studied in 373 breast carcinomas by immunohistochemistry. Statistical significance of the associations between genotypes, protein expression, clinicopathologic variables, and survival was assessed. A high level (>25%) of cytoplasmic NRF2 positivity was observed in 237 of 361 (66%) and SRXN1 positivity was observed in 82 of 363 (23%) cases. The *NRF2* rs6721961 genotype *TT* was associated with increased risk of breast cancer [$P = 0.008$; OR, 4.656; confidence interval (CI), 1.350–16.063] and the *T* allele was associated with a low extent of NRF2 protein expression ($P = 0.0003$; OR, 2.420; CI, 1.491–3.926) and negative SRXN1 expression ($P = 0.047$; OR, 1.867; CI = 1.002–3.478). The *NRF2* rs2886162 allele *A* was associated with low NRF2 expression ($P = 0.011$; OR, 1.988; CI, 1.162–3.400) and the *AA* genotype was associated with a worse survival ($P = 0.032$; HR, 1.687; CI, 1.047–2.748). The *NRF2* rs1962142 *T* allele was associated with a low level of cytoplasmic NRF2 expression ($P = 0.036$) and negative sulfiredoxin expression ($P = 0.042$). The *NRF2* rs2706110 *AA* genotype was associated with an increased risk of breast cancer, and the *SRXN1* rs6053666 *C* allele was associated with a decrease in breast cancer risk ($P = 0.011$ and 0.017). *NRF2* and *SRXN1* genetic polymorphisms are associated with breast cancer risk and survival, implicating that mechanisms associated with reactive oxygen species and NRF2 pathway are involved in breast cancer initiation and progression. *Cancer Res; 72(21); 5537–46. ©2012 AACR.*

Introduction

Nuclear factor erythroid 2–related factor 2 (NRF2) is a transcriptional factor, which senses oxidative and xenobiotic stress in the cells (1). When such stress occurs, NRF2 is released from a complex formed with Kelch-like ECH-associated protein 1 (KEAP1), a substrate adaptor of a Cullin 3–based E3 ubiquitin ligase complex, and moves to the nucleus where it associates with maf proteins and then upregulates several stress-related genes such as glutathione *S*-transferases, thioredoxin, thioredoxin reductases, peroxiredoxins, γ-glutamyl cysteine ligase, heme oxygenase 1, NADPH kinone oxidoreductase, and multidrug resistance genes (1, 2). If NRF2 stays in a complex with KEAP1 in cytoplasm, it is degraded through the proteasome pathway (1, 2).

The function of NRF2 is important in the pathogenesis of several diseases. Mice with a nonfunctioning *Nrf2* gene develop early-onset emphysema because of a deficient anti-oxidative response (3). Similarly, mice lacking *Nrf2* develop nutritional steatohepatitis (4). A high amount of unbound NRF2 in cancer cells results in chemoresistance of the tumor cells (5). The importance of NRF2 and KEAP1 in tumorigenesis is underlined by the fact that tumor cells may contain mutations in the respective genes. *NRF2* mutations are present in esophageal, skin, larynx, and lung cancer with a 6% to 13% frequency with the highest prevalence in squamous cell carcinomas (6). A similar mutational frequency has been found for *KEAP1* with an incidence of 15% in lung cancer (7). Loss of KEAP1 function leads to increased NRF2 concentration and chemoresistance in non–small cell lung cancer (8). In addition to somatic mutations, genomic low penetrance DNA variations could have an effect on the function of these genes and their downstream targets.

Peroxiredoxins are enzymes that have the capability of scavenging hydrogen peroxide and other peroxides (9, 10). Peroxiredoxins may undergo reversible oxidation in their cysteine sites to sulfinic acid rendering the molecules to degradation (10, 11). Sulfiredoxin catalyzes the reversal of

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overoxidation of peroxiredoxins, thus salvaging them from inactivation (11). Sulfiredoxin is also involved in deglutathionylation of proteins following nitrosactive or oxidative stress (12). In cell lines, overexpression of sulfiredoxin has been shown to stimulate cell proliferation and apoptosis induced by cisplatin, the effects of which were mediated by phosphorylation of cell-cycle regulators and kinases (13). Sulfiredoxin is induced by NRF2 and AP-1 and protects the lung from tobacco-mediated oxidative damage (14, 15). Increased sulfiredoxin has been linked with oncogenic transformation, and it is overexpressed in various skin cancers (16).

There are many risk factors associated with the development of breast carcinoma, and 1% to 5% of them have a hereditary basis (17). Even though oxidative damage is considered as one mechanism for cancer development, its role in breast cancer has not been extensively studied. Because NRF2 is known to be a sensor of oxidative damage, we studied its expression in different types of breast carcinoma. In addition, we investigated the expression and significance of sulfiredoxin, a known target for NRF2, in breast carcinoma. We also evaluated the effect of *NRF2* and *SRXN1* genetic variation on the protein expression, as well as their role in breast cancer risk and development. The genetic variants and the level of protein staining were also evaluated as predictive factors. This is the first report on *SRXN1* polymorphisms in (breast) cancer.

Materials and Methods

DNA samples

DNA from 452 patients with invasive breast cancer and 370 control subjects from the Kuopio Breast Cancer Project (KBCP) sample set were available for genotyping (Supplementary Table S1). The KBCP sample set consists of 497 prospective breast cancer cases and 458 controls from the province of Northern Savo in Eastern Finland. The KBCP sample material is characterized in more detail by Hartikainen and colleagues (18) and Pellikainen and colleagues (19). Genomic DNA was extracted from peripheral blood lymphocytes of both cases and controls using standard procedures (20). The KBCP has been approved by the ethical committee of University of Eastern Finland and Kuopio University Hospital (Kuopio, Finland).

Tumor material in tissue microarray

The tumor material consisted of 373 cases of invasive breast carcinomas included in the KBCP. The clinical characteristics of the material are shown in Supplementary Table S1. Paraffin-embedded tumor tissue from the primary tumor was obtained from breast cancer surgery. Tissue microarray was constructed as previously described (21).

Single-nucleotide polymorphism selection

Tagging single-nucleotide polymorphisms (tagSNP) for *NRF2* and *SRXN1* genes were selected using the HapMap Genome Browser release 2 (Phase 3, NCBI build 36, bdSNP b126) as of February 24 and November 8, 2010 (22). TagSNPs for regions chr2:177799989–177853228 and chr20:573580–583579 were picked out for the CEU population using the Tagger multimarker algorithm with r^2 cutoff at 0.8 and minor allele frequency (MAF) cutoff at 0.05. Two functional polymorphisms on the *NRF2* promoter region were selected on the basis of previous publications (refs. 23, 24; Supplementary Fig. S1A and S1B).

Genotyping of *NRF2* and *SRXN1* SNPs

Genotyping of 6 *NRF2* and 8 *SRXN1* TagSNPs and 2 *NRF2* functional SNPs was done using MassARRAY (Sequenom, Inc.) and iPLEX Gold (Sequenom, Inc.) on 384-well plate format. MassARRAY mass spectrometer (Sequenom, Inc.) was used for spectra acquisitions from the SpetroCHIP. Data analysis and genotype calling were done using TyperAnalyzer Software version 4.0.3.18 (Sequenom, Inc.). Each 384-well plate contained a minimum of 8 nontemplate controls. Duplicate analysis was done for 6.7% of the samples for quality control. All primer sequences and reaction conditions are available upon request. Genotyping of the *NRF2* tagSNP rs2886162 was conducted by 5′-nuclease assay (TaqMan) using the Mx3000P Real-Time PCR System (Stratagene) according to manufacturer's instructions. Primers and probes for rs2886162 were supplied from Applied Biosystems as TaqMan Genotyping Assays. Reactions were carried out in 10-μL volume in 96-well format as previously described (21). Duplicate genotypes were done for 4.2% of samples for quality control. If the duplicate and its pair were discordant, the genotypes for the sample would be discarded.

Immunohistochemistry

Four-micrometer thick tissue sections were cut from the paraffin-embedded blocks. The construction of the microarray blocks and the immunohistochemical staining procedure has been described previously (21, 25). The primary antibodies, rabbit polyclonal anti-human NRF2 (sc-722, Santa Cruz Biotechnology, Inc.), and rabbit polyclonal anti-human sulfiredoxin (14273-1-AP, Proteintech Group) were diluted with 1% bovine serum albumin in PBS to 1:200 and 1:500 working solutions, respectively. The evaluation of NRF2 immunostaining was conducted separately in tumor cell nuclei and cytoplasm. For sulfiredoxin, cytoplasmic immunoreactivity was evaluated. The results for NRF2 were semiquantitated as follows: 0%–5%, negative; >5% to 25%, weak positivity; >25% to 75%, moderate positivity; and >75% to 100%, strong positivity. In the analyses, NRF2 expression was divided in 2 groups: low extent ($\leq$25%) and high extent (>25%) expression. For sulfiredoxin, the presence (>1%) or absence of cytoplasmic expression was recorded.

Statistical analysis

The statistical analyses were conducted with SPSS for Windows software v 14.0 (SPSS). Continuous data were compared using ANOVA. When ANOVA results indicated that groups differed, *post hoc* comparisons were conducted using 2-tailed *t* tests. Categorical data were compared using the Fisher exact test designed for small sample groups. The significance levels for comparisons of the genotype frequencies between cases and controls and for the association between the genotypes and protein expression and clinical variables [tumor grade and size, histologic type, nodal status, estrogen

receptor (ER) status, progesterone receptor (PR) status, HER status] among the cases were also computed using Armitage trend test. The concordance of the genotypes with Hardy–Weinberg equilibrium (HWE) was tested using standard χ^2 test. Survival data were analyzed using the Kaplan–Meier method with the use of the log-rank, Breslow, and Tarone–Ware test in SPSS v 14.0 (SPSS). In multivariate survival analyses, the Cox regression analysis in SPSS v 14.0 (SPSS) was used. *P* values less than 0.05 (2-sided) were considered statistically significant in all tests.

Results

NRF2 rs6721961 and rs2706110 associate with increased risk of breast cancer and *SRXN1* rs6053666 protects against breast cancer

Seven TagSNPs (rs1806649, rs2886162, rs1962142, rs2364722, rs10183914, rs2706110, and rs13035806) and 2 functional SNPs (rs6721961 and rs6706649) were analyzed in the *NRF2* gene region. Eight TagSNPs (rs6085283, rs13043781, rs6076869, rs6053666, rs2008022, rs6116929, rs7269823, and rs6053728) were analyzed in the *SRXN1* gene region (Supplementary Table S2). The SNP genotypes were tested for concordance with the HWE. Among the controls, *NRF2* rs6706649 deviated slightly from HWE with $P = 0.029$. All other genotypes were concordant with the HWE.

Among the invasive breast cancer cases, an association with breast cancer risk was observed with *NRF2* rs6721961 and rs2706110 and *SRXN1* rs6053666 genotypes (Table 1). The rare homozygous genotypes of *NRF2* rs6721961 (*TT*) and rs2706110 (*AA*) associated with increased risk of breast cancer, whereas the common allele was protective (Table 1). The rare allele *C* of *SRXN1* rs6053666 was protective (Table 1). A near-significant association was observed with *NRF2* rs13035806 (Table 1).

NRF2 expression associates with sulfiredoxin expression

High extent (>25%) cytoplasmic NRF2 positivity was seen in 66% (237 of 361) and nuclear (>25%) positivity in 26% (96 of 365) of cases (Fig. 1A–D). High-extent nuclear positivity was observed in 20% (43 of 219) of ductal, 47% (33 of 70) of lobular, and 29% (17 of 59) of other types. Most notably, lobular carcinomas showed significantly more high-extent nuclear NRF2 expression than ductal ones ($P = 0.001$). Twenty-three percent (82 of 363) of the breast tumors displayed positivity for sulfiredoxin (Fig. 1E and F). Twenty-three percent (50 of 219) of

Table 1. Significant associations between the *NRF2* and *SRXN1* genotypes and risk of breast cancer

				Homozygous[b]		Allele positivity[c]	
SNP	***P*** **(χ^2)[a]**	***P*$_{trend}$**	**Associated allele**	***P***	**OR (CI)**	***P***	**OR (CI)**
NRF2							
rs13035806	0.065	0.824	*G*	0.058	0.353 (0.115–1.085)	**0.048**	**0.339** (0.111–1.040)
rs2706110	**0.029**	0.058	*A*	**0.011**	**2.079 (1.175–3.679)**	0.300	1.162 (0.875–1.543)
			G	**0.011**	**0.481 (0.272–0.851)**	**0.010**	**0.487 (0.279–0.851)**
rs10183914	0.474	0.913	ns				
rs1962142	0.335	0.224	ns				
rs1806649	0.850	0.571	ns				
rs2364722	0.276	0.348	ns				
rs6706649	0.141	0.398	ns				
rs6721961	**0.028**	0.113	*T*	**0.008**	**4.656 (1.350–16.063)**	0.377	1.150 (0.843–1.569)
			G	**0.008**	**0.215 (0.062–0.741)**	**0.008**	**0.217 (0.063–0.746)**
rs2886162	0.352	0.449	ns				
SRXN1							
rs6116929	1	0.991	ns				
rs6076869	0.948	0.922	ns				
rs6053666	0.052	**0.028**	*C*	0.079	0.673 (0.432–1.048)	**0.017**	**0.702 (0.524–0.939)**
			T	0.079	1.486 (0.954–2.314)	0.345	1.215 (0.811–1.820)
rs7269823	0.319	0.150	ns				
rs2008022	0.222	0.364	ns				
rs13043781	0.535	0.307	ns				
rs6085283	0.247	0.784	ns				
rs6053728	0.472	0.257	ns				

NOTE: $P_{trend} = P$ value from the Armitage trend test for the overall association with breast cancer risk. Bold text indicates statistical significance.

Abbreviation: ns, no statistically significant association observed.

[a]*P* from the χ^2 test for overall association with breast cancer risk.

[b]*P*, OR, and CI for the homozygous allele carriers.

[c]*P*, OR, and CI for the homozygous and heterozygous allele carriers.

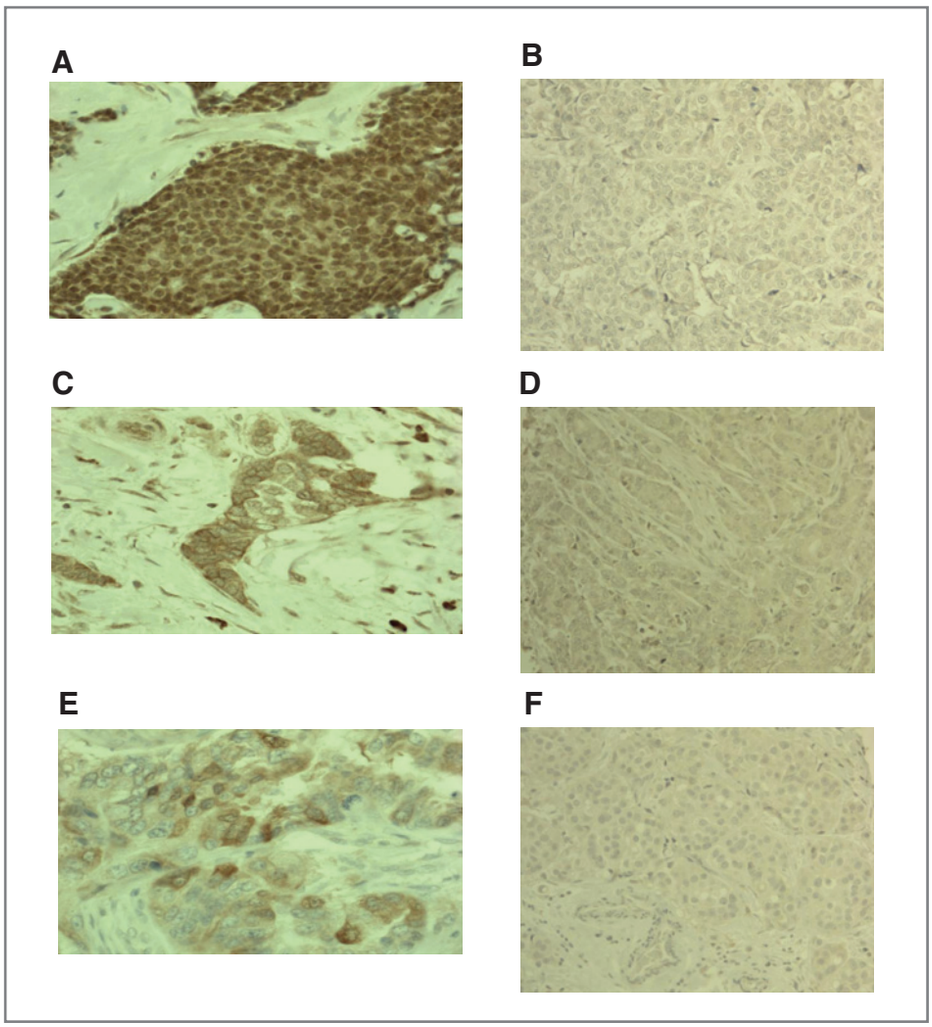

Figure 1. Immunohistochemical staining of NRF2 and sulfiredoxin. Ductal breast carcinoma showing strong nuclear positivity for NRF2 (A), negative nuclear staining for NRF2 (B), strong cytoplasmic positivity for NRF2 (C), negative cytoplasmic staining for NRF2 (D), strong cytoplasmic positivity for sulfiredoxin (E), and negative cytoplasmic staining for sulfiredoxin (F). Magnification, ×250 in A, C, and E and ×110 in B, D, and F.

ductal, 15% (10 of 68) of lobular, and 30% (15 of 50) of other types expressed positivity. Nuclear and cytoplasmic NRF2 expression was associated with sulfiredoxin expression ($P = 0.003$ and $P = 0.008$, respectively; Supplementary Table S3).

NRF2 SNP rare alleles associate with low-extent cytoplasmic NRF2 and sulfiredoxin protein expression

A significant association was observed with cytoplasmic NRF2 protein expression and the *NRF2* rs1962142, rs2886162, and rs6721961 genotypes among the invasive breast cancer cases, the rare alleles associating with low-extent cytoplasmic NRF2 protein expression (Table 2). The rare alleles of *NRF2* rs1962142 and rs6721961 also associated with negative sulfiredoxin protein expression (Table 2). More specifically, *NRF2* rs6721961 rare allele associated with grade 2 tumors [$P_{overall} = 0.041$, $P_{allele\text{-}specific} = 0.012$; OR, 1.975; confidence interval (CI), 1.159–3.365] and *NRF2* rs2886162 rare homozygous genotype *AA* associated with ER-positive breast cancer ($P_{overall} = 0.008$, $P_{allele\text{-}specific} = 0.008$; OR, 2.518; CI, 1.276–4.969). *NRF2* SNP rs1962142 allele *T* also associated with grade 2 tumors (data not shown). This SNP resides in the same haplotype block with rs6721961 and most likely represents the same association as rs6721961.

SRXN1 SNP rs6076869 rare allele associates with cytoplasmic NRF2 protein expression

SRXN1 rs6076869 genotypes associated with cytoplasmic NRF2 protein expression among the invasive breast cancer cases. The rare allele *T* associated with high-extent cytoplasmic NRF2 protein expression (Table 2) and with lobular histology ($P_{overall} = 0.019$, $P_{allele\text{-}specific} = 0.022$; OR, 1.830; CI, 1.092–3.066).

NRF2 and *SRXN1* genotypes associate with prognosis/survival

NRF2 rs2886162 rare homozygous genotype *AA* associated with a worse survival compared with the carriers of the common allele *G* [Kaplan–Meier: $P_{log\text{-}rank} = 0.017$ (Supplementary Table S4 and Supplementary Fig. S2)]. This association remained significant in the multivariate analysis [Cox regression: $P = 0.032$; HR, 1.687; CI, 1.047–2.748 (Table 3 and Fig. 2)]. In this multivariate analysis also, the cytoplasmic NRF2

Table 2. Significant associations of the *NRF2* and *SRXN1* genotypes with cytoplasmic NRF2 and sulfiredoxin protein expression

				Allele positivity[c]					
				High-extent NRF2		Low-extent NRF2		Negative sulfiredoxin	
SNP	P_{trend}[a]	P_{trend}[b]	Associated allele	*P*	OR (CI)	*P*	OR (CI)	*P*	OR (CI)
NRF2									
rs1962142	**0.030**	0.022	*T*			**0.036**	**1.742 (1.035–2.933)**	**0.042**	**1.990 (1.015–3.901)**
			C			0.230	0.469 (0.133–1.659)	0.086	0.159 (0.009–2.745)
rs6721961	**0.0008**	0.018	*T*			**0.0003**	**2.420 (1.491–3.926)**	**0.047**	**1.867 (1.002–3.478)**
			G			0.274	0.564 (0.199–1.595)	**0.042**	0.114 (0.007–1.940)
rs2886162	**0.041**		*A*			**0.011**	**1.988 (1.162–3.400)**		
			G			0.428	0.808 (0.476–1.370)		
SRXN1									
rs6076869	**0.012**		*T*	**0.005**	**1.927 (1.217–3.051)**				
			A	0.360	0.712 (0.343–1.478)				

NOTE: Bold text indicates statistical significance.
Abbreviation: ns, no statistically significant association observed.
[a]*P* from the Armitage trend test for the overall association with cytoplasmic NRF2 protein expression.
[b]*P* value from the Armitage trend test for the overall association with sulfiredoxin protein expression.
[c]*P*, OR, and CI for the homozygous and heterozygous allele carriers.

expression was included but it did not associate with survival. However, in the Kaplan–Meier analysis, a difference in the genotype-associated survival was observed between the strata (cytoplasmic NRF2 low vs. high extent, $P = 0.023$, log-rank = 5.163), implying that the genotypes association is most significant among those with low-extent cytoplasmic NRF2 only (Supplementary Table S5 and Supplementary Fig. S3A and S3B). Similar trend was observed with nuclear NRF2 protein expression [$P = 0.019$, log-rank = 5.490 (Supplementary Table S5 and Supplementary Fig. S4A and S4B)].

SRXN1 rs6116929 rare homozygous genotype *GG* and rs2008022 rare allele carriers *CA&AA* had better survival than the common allele [$P_{log\text{-}rank} = 0.063$ and $P_{log\text{-}rank} = 0.012$, respectively (Supplementary Table S4 and Supplementary Fig. S5A and S5B)]. *SRXN1* rs7269823 and rs6085283 rare allele carriers (*AG&GG* and *CT&TT*, respectively) had poorer survival than the common homozygous genotype [$P_{log\text{-}rank} = 0.030$ and $P_{log\text{-}rank} = 0.015$, respectively (Supplementary Table S4 and Supplementary Fig. S5C and S5D). In the Cox regression analysis, including all 4 survival-associated *SRXN1* polymorphisms, only rs2008022 remained significant ($P = 0.012$; HR, 1.645; CI, 1.116–2.425). None of these 4 polymorphisms, however, were independently significant prognostic factors in the multivariate analysis including tumor grade,

Table 3. Variables significantly associated with breast cancer survival in multivariate analysis according to *NRF2* SNP genotypes

Variable	*n*	B (SE)	Wald	HR (95% CI)	*P*
Nodal status					
Negative	168		Ref.		
Positive	122	0.859 (0.235)	13.424	2.362 (1.491–3.740)	0.0002485
HER2 status					
Negative	251		Ref.		
Positive	39	0.932 (0.262)	12.633	2.539 (1.519–4.244)	0.000379
rs2886162					
GG+*GA*	219		Ref.		
AA	71	0.523 (0.243)	4.613	1.687 (1.047–2.718)	0.032

NOTE: Analysis stratified by tumor grade, nodal status, ER status, PR status, histologic type, tumor size, HER2 status, cytoplasmic NRF2 expression, and rs2886162 genotypes. HR (95% CI), HR of breast cancer death and 95% CI from Cox regression survival analysis.
Abbreviations: B (SE), B coefficient with standard error from the Cox regression survival analysis. Ref, reference category.

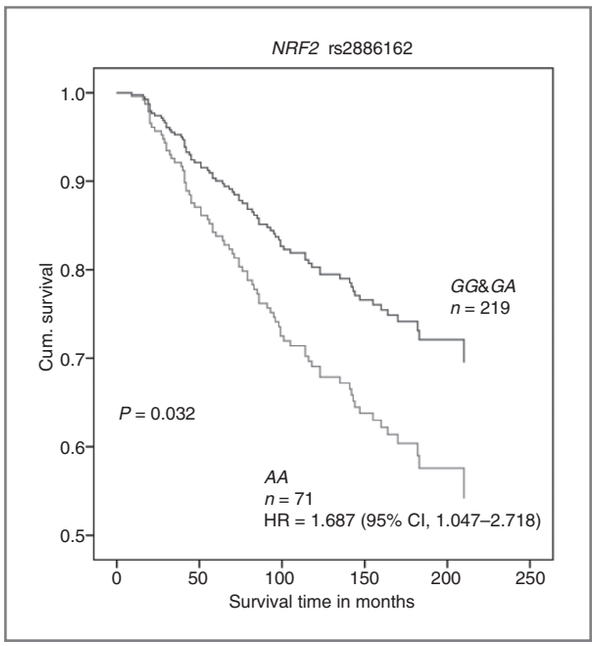

Figure 2. Association of *NRF2* rs2886162 with breast cancer survival in multivariate analysis. Tumor grade, nodal status, ER status, PR status, histologic type, tumor size, HER2 status, cytoplasmic NRF2 expression, and rs2886162 genotypes included in analysis. HR (95% CI), HR of breast cancer death with 95% CI in Cox regression analysis.

nodal status, ER status, PR status, histologic type, tumor size, and HER2 status (Cox regression, data not shown).

Effect of combined *NRF2* and *SRXN1* genotypes on prognosis/survival

We further studied the effect of the combined *SRXN1* survival–associated polymorphisms by summing up the number of the risk alleles of the *SRXN1* polymorphisms rs61169295, rs2008022, rs7269823, and rs6085283 for each patient. The highest value possible was 8 and the lowest was 0. The patients were divided in 2 groups: 0–3 risk alleles and 4–8 risk alleles. A trend toward poorer survival was observed with increasing amount of risk alleles in Kaplan–Meier analysis [$P_{\text{log-rank}} = 0.009$ for 0–3 vs. 4–8 risk alleles (Supplementary Table S4 and Supplementary Fig. S6)]. However, the poorest survival was still defined by rs2008022. When the effect of *NRF2* rs2886162 was also considered together with the combined *SRXN1* SNPs, there was a difference in the survival between the strata defined by rs2886162 genotype ($P = 0.014$, log-rank = 6.009). Among the rs2886162 rare allele (*A*) carriers, poorer survival was observed among those with 4–8 SRXN1 risk alleles ($P_{\text{log-rank}} = 0.010$) but no difference among the rs2886162 common homozygotes (*GG*) was observed [$P_{\text{log-rank}} = 0.638$ (Supplementary Fig. S7A and S7B)]. This reflects that the rs2886162 genotype is a stronger prognostic factor than the combined *SRXN1* SNP genotypes; otherwise the effect on survival by *SRXN1* genotypes should also be seen among the rs2886162 common homozygotes. Indeed, in the multivariate analysis including the combined *SRXN1* genotypes, *NRF2* rs2886162 genotype, and other prognostic factors, only rs2886162 genotype, nodal status, and HER2 status remained significant (Supplementary Table S6 and Supplementary Fig. S8A). Similar results were obtained from the multivariate analysis including the clinicopathologic variables, NRF2 protein expression (cytoplasmic and nuclear), sulfiredoxin protein expression, and combined *SRXN1* genotypes and rs2886162 (Supplementary Table S7 and Supplementary Fig. S8B).

NRF2 rs2886162 *AA* genotype independently predicts poorer survival among patients who received chemotherapy or radiotherapy

The effect of *NRF2* rs2886162 rare homozygous genotype *AA* on poor prognosis was also seen separately in the group that had received adjuvant chemotherapy and among those that received postoperative radiotherapy. In the group that had received adjuvant chemotherapy, the rs2886162 genotype *AA* associated with poorer breast cancer survival ($P = 0.019$; HR, 2.43; CI, 1.16–5.08; Fig. 3A) and with poorer recurrence-free survival ($P = 0.003$; HR, 2.83; CI, 1.43–5.61). In the group that received postoperative radiotherapy, the rs2886162 genotype *AA* associated with poorer recurrence-free survival ($P = 0.025$; HR, 1.68; CI, 1.07–2.64; Fig. 3B). Among patients who did not receive any adjuvant therapy ($n = 137$), the rs2886162 genotypes did not associate with survival (data not shown).

SRXN1 genotypes independently predict survival among patients receiving radiation treatment

The effect of the *SRXN1* genotypes on prognosis also holds when radiation treatment is taken into account. *SRXN1* rs6116929 rare homozygous genotype *GG* and rs2008022 rare allele carriers *CA&AA* predicted better prognosis among the patients who received radiotherapy (Supplementary Table S8 and Fig. 4A and B). Also, among the patients treated with radiotherapy, the *SRXN1* rs7269823 and rs6085283 rare allele carriers (*AG&GG* and *CT&TT*, respectively) had poorer survival than the patients carrying the common homozygous genotypes (Supplementary Table S8 and Fig. 4C and D). In addition, among the patients treated with radiotherapy, the *SRXN1* rs6053666 rare homozygous genotype *CC* predicted better prognosis than the common allele carriers (Supplementary Table S8). Interestingly, rs6053666 rare allele also associates with decreased breast cancer risk.

Discussion

NRF2 is a transcription factor, which senses xenobiotic and oxidative stress and activates several anti-oxidative and other protective genes if such stress occurs. Upregulation of NRF2 may thus protect cells from oxidative damage and prevent initiation of carcinogenesis due to mutations caused by such damage. In tumor tissue, on the other hand, activation of NRF2 leads to increased chemo- and radioresistance of the tumor cells, which is reflected by the fact that many tumors display elevated levels of anti-oxidative enzymes compared with normal tissues (26). Recent findings suggest that enhanced detoxification of reactive oxygen species (ROS) with additional NRF2 functions may in fact be also protumorigenic (7).

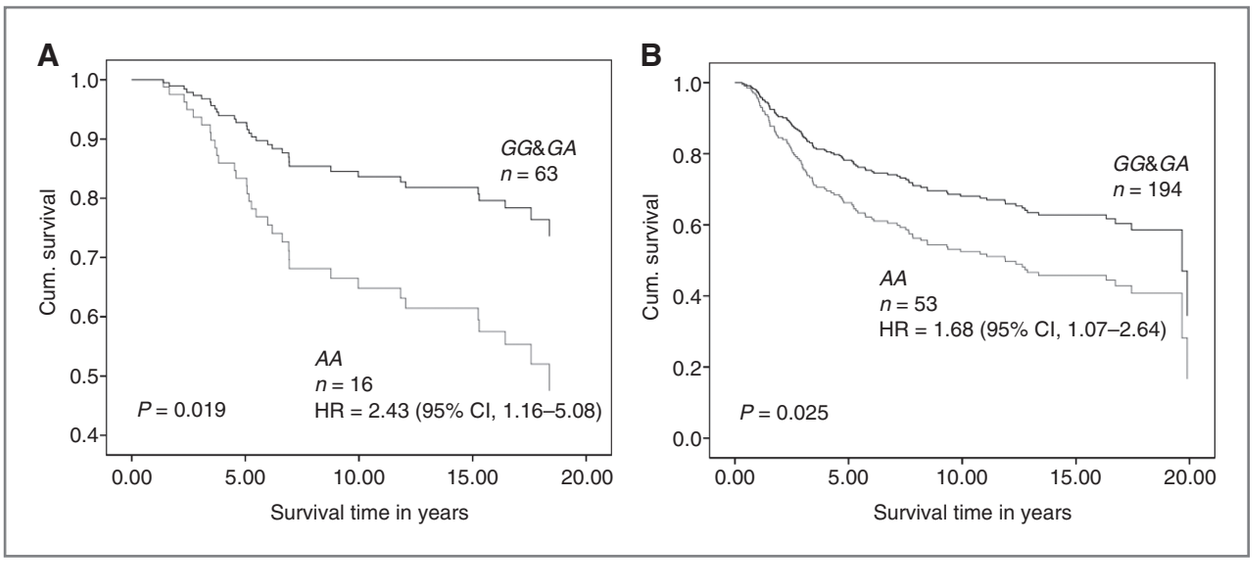

Figure 3. Association of *NRF2* rs2886162 with survival among patients with breast cancer with different therapies. A, breast cancer–specific survival among patients treated with adjuvant chemotherapy. Analysis stratified by age, stage, and radiotherapy. B, recurrence-free survival among patients treated with postoperative radiotherapy. Analysis stratified by age, stage, hormone therapy, and chemotherapy. HR (95% CI), HR of breast cancer death with 95% CI in Cox regression survival analysis.

Our results show that in the NRF2 pathway, there are genetic polymorphisms that affect both the susceptibility for breast cancer and the outcome of the patients with breast cancer, thus underlining the complex effect of NRF2 in cancer progression. On one hand, the *NRF2* promoter polymorphism rs6721961 associates with breast cancer risk referring to the role of NRF2 in cancer predisposition. The rare allele *T* associates with increased risk of breast cancer and low protein level of both NRF2 and sulfiredoxin. The *T* allele is predicted to destroy a binding site for a transcription factor (intronic enhancer) c-Rel (FastSNP; ref. 27; Supplementary Table S9). Previously, rs6721961 has been shown to be functional (24) and hence it would directly affect the protein expression level of NRF2. Indeed, here we have shown the connection between the *T* allele and decreased NRF2 protein expression in breast cancer tissue, as well as the resulting decrease in sulfiredoxin expression. This is also concordant with the hypothesis that impaired NRF2 function leads to decreased sulfiredoxin function, which in turn affects the function of peroxiredoxins and leads to increased cancer proneness. Decreased NRF2 level presumably might affect also the activation of other NRF2 targets and hence increase cancer susceptibility.

On the other hand, *NRF2* SNP rs2886162 *AA* genotype associates with low NRF2 protein expression level and poorer survival. The effect of the genotype on survival was significant also in the multivariant analysis. In addition, the *NRF2* rs2886162 rare homozygous genotype *AA* independently predicted poorer survival among patients who received adjuvant chemotherapy, and the recurrence-free survival was poorer among radiotherapy–treated *AA* genotype carriers. The rs2886162 association with survival could be through impaired NRF2 (and sulfiredoxin) function as low cytoplasmic NRF2 may result in low sulfiredoxin, even though statistically significant association between rs2886162 and negative sulfiredoxin level was not observed here. (However, a positive overall correlation between NRF2 expression and sulfiredoxin expression was observed). It is also possible that low cytoplasmic NRF2 could be explained by the removal of NRF2 from cytoplasm to the nucleus where it leads to activation of stress response and survival of cancer cells (NRF2 resistency) and thus poorer prognosis. The association with poorer survival in this case could be explained by the fact that *SRXN1* is not the sole target for NRF2 activation. Association of nuclear NRF2 staining with poorer survival has been previously observed in ovarian carcinoma (28). However, in breast cancer, this issue needs further studies, possibly including also KEAP1.

Previous studies on *NRF2* polymorphisms in breast cancer are few. In a cohort of postmenopausal women, specific polymorphisms on *NRF2* (rs1806649), *NQO1*, *NOS3*, and *HO-1* did not have any significance for the risk of breast cancer (29). When the risk polymorphisms of these genes were combined, patients with 3 risk alleles had a 1.5-fold risk and those with a high iron intake had a greater than 2-fold risk (29). In postmenopausal women with oral estrogen replacement therapy, the *NRF2* rs6721961 rare allele seems to modify the risk of thromboembolism (30). *NRF2* polymorphism has, however, been more extensively studied in pulmonary disease (24, 31, 32). While *NRF2* polymorphisms clearly may promote individuals for oxidative damage, no published studies on their significance in lung cancer exist. Lung cancer, as well known, is associated with tobacco smoke, which among other effects provoke development of ROS (33). Polymorphisms rs6721961 and rs6706649 have been studied in gastric carcinogenesis but no overall association with risk was found (34). In gastric cancer, carcinogenesis is predominantly based on *Heliobacter pylori*–induced gastritis leading to gastric atrophy and cancer whereas in breast cancer, hormonal factors play a role (35). It is known that estrogen metabolites induce the formation of ROS

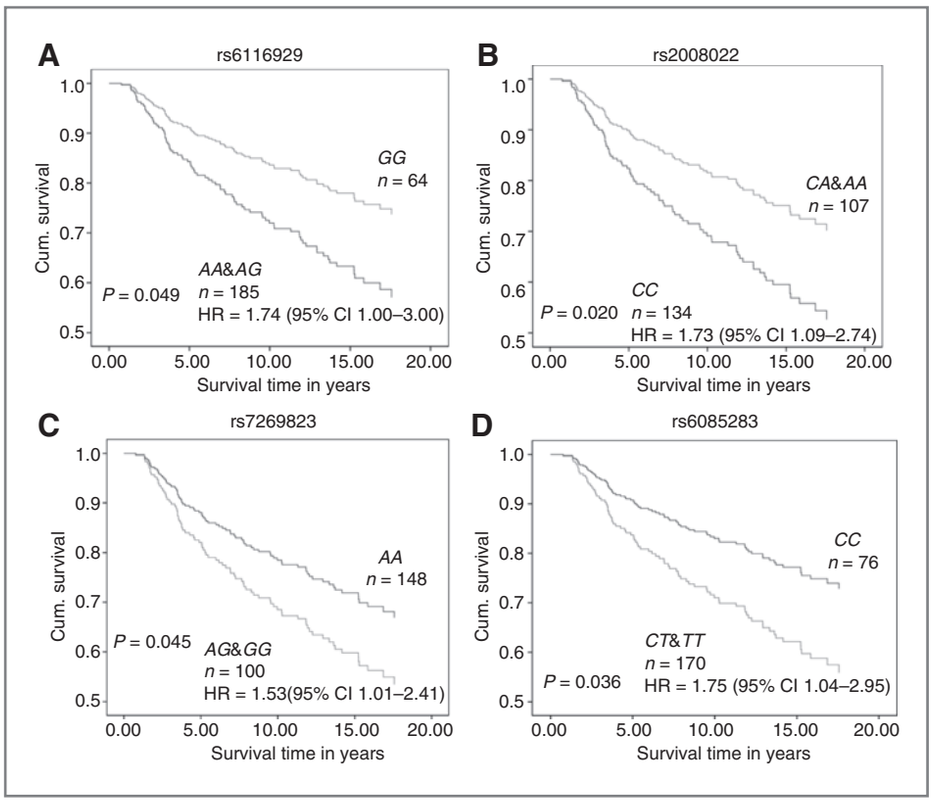

Figure 4. Significant associations of *SRXN1* SNP genotypes with survival among patients with breast cancer receiving radiation treatment. Breast cancer–specific survival according to rs6116929 (A), rs2008022 (B), rs7269823 (C), and rs6085283 (D) genotypes. Analyses stratified by age, stage, hormone therapy, and chemotherapy. HR (95% CI), HR of breast cancer death with 95% CI in Cox regression survival analysis.

(36). In this sense, NRF2 and its dysfunction may be more important in breast carcinogenesis than in gastric cancer.

In addition to *NRF2* polymorphisms, we observed that polymorphisms on *SRXN1* also are associated with breast cancer risk and survival. Four *SRXN1* SNPs associated with breast cancer survival in Kaplan–Meier analysis (rs6116929, rs2008022, rs7269823, and rs6085283). Among patients who received radiotherapy, these SNPs also associated independently with survival in the multivariate analysis. Interestingly, there are regulatory features in the regions where rs6116929 and rs6085283 reside (Ensembl; ref. 37). The rs6116929 is located in downstream region and the rare allele *G* (which is associated with better survival) is predicted to destroy binding sites for CdxA, cap, and deltaE (F-SNP; ref. 38). The rs6116929 is also only 84 bp from rs6076869 ($D' = 0.99$), the rare allele of which is associated with increased NRF2 protein. The rs6076869 rare allele *T* is predicted to destroy a GATA-X binding site (FastSNP) and to create cap and AP-4 transcription factor–binding sites (F-SNP). The rs6085283 resides in intron 1 and the rare allele *T* (which is associated with poorer survival) creates a binding site for Oct-1 transcription factor (FastSNP). Also, the rs2008022 (in intron 1) rare allele *A* (which is associated with better survival) destroys a GATA-2 transcription factor–binding site and is 1,079 bp from rs13043781 ($D' = -0.98$), the rare allele of which is predicted to destroy v-Myb binding site (FastSNP). There were no predicted or detected functional effects for rs7269823, which also resides in intron 1. However, it is in linkage disequilibrium with rs2008022 ($D' = -1$) and rs6053666 ($D' = 0.7$; Supplementary Table S9). It is possible that these polymorphisms affect the level or function of sulfiredoxin, and the cancer cells exhibiting low sulfiredoxin expression have lower tolerance for oxidative damage and the response for oxidative damage is poor, which promotes/enhances the death of the cancer cell and a better response to treatment and thus leads to better outcome. The effect of sulfiredoxin in breast carcinoma could be connected to its role in converting peroxiredoxins to a functional, reduced state. Some peroxiredoxins have been associated with progression of breast cancer. Overexpression of peroxiredoxin VI in breast carcinoma cell lines leads to a more invasive phenotype with a higher proliferative activity (39). Moreover, peroxiredoxin III promotes breast cancer cell proliferation, and peroxiredoxins I, II, and III protect cells from oxidative damage–induced apoptosis (40, 41). We also found that *SRXN1* rs6053666 rare allele *C* lowered the risk of breast cancer and the *CC* genotype associated with better prognosis among the patients who received radiotherapy. Such influences may be ascribed to the known function of sulfiredoxin on the oxidative state of peroxiredoxins regulating the redox state and metabolism of hydrogen peroxide in cells. The rs6053666 resides on the 3′ untranslated region of the *SRXN1* gene and is predicted to participate in splicing regulation (alternative splicing). Three exonic splicing enhancer (ESE)–binding sites are predicted for allele *C* (SF2/ASF, SC35, and SRp55), and none for allele *T* (FastSNP, F-SNP; Supplementary Table S9).

The protein expression of NRF2 has not previously been studied in large clinical materials of breast cancer. Our results show that NRF2 is strongly expressed in the cytoplasm of

breast carcinoma cells, showing a high frequency expression in 66% of the cases. High-extent nuclear expression, indicating increased functional activity of the protein, was present in 26% of the cases. In the histologic subgroups, lobular invasive carcinomas showed a stronger expression of nuclear positivity than ductal ones. Lobular carcinoma is a tumor type showing low or nonexistent expression of E-cadherin. Interestingly, NRF2 activation by sulphoraphane was reported to cause downregulation of epithelial–mesenchymal transition (EMT)-type changes in rat kidney tubular epithelial cells, including E-cadherin (42). Thus, NRF2 activation might be one additional factor influencing the loss of E-cadherin expression in lobular breast carcinoma. On the other hand, lobular carcinoma cells could be more sensitive in their reaction to oxidative stress, leading to a more abundant nuclear expression of NRF2. Previously, Loignon and colleagues found that NRF2 protein expression was decreased in 7 of the 10 breast cancer cell lines they studied (43). They also detected lower levels of NRF2 in 7 of the 10 studied breast cancer tumor samples than in normal breast tissue (44). Unfortunately, the authors did not specify subcellular localization of the staining or the histologic subgroups of the tumors. High gene expression of *NRF2* has been reported to associate with poor prognosis among ER-positive breast cancer (45).

To conclude, we have observed that *NRF2* and *SRXN1* polymorphisms influence breast cancer susceptibility and survival, and the protein expression has an effect on breast cancer survival. All in all, ROS-associated mechanisms appear to play a role in the behavior and treatment of breast cancer to the extent of being reflected in the survival of the patients. Future studies would be needed for the confirmation of the functional SNPs and their effect on the NRF2 and sulfiredoxin protein expression, as well as studies on peroxiredoxins and AP-1. Studying anti-oxidative mechanisms may thus pave the way for new treatment modalities based on inhibition of such mechanisms in breast cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: A. Mannermaa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Hartikainen, A. Mannermaa, Y. Soini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Hartikainen, M. Tengström, A. Mannermaa, Y. Soini
Writing, review, and/or revision of the manuscript: J.M. Hartikainen, M. Tengström, V.-M. Kosma, V.L. Kinnula, A. Mannermaa, Y. Soini
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.-M. Kosma, A. Mannermaa, Y. Soini
Study supervision: A. Mannermaa, Y. Soini

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References

1. Itoh K, Mimura J, Yamamoto M. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxid Redox Signal 2010;13:1665–78.
2. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacol Res 2008;58:262–70.
3. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. J Clin Invest 2004;114:1248–59.
4. Sugimoto H, Okada K, Shoda J, Warabi E, Ishige K, Ueda T, et al. Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice. Am J Physiol Gastrointest Liver Physiol 2010;298:G283–94.
5. Hu L, Miao W, Loignon M, Kandouz M, Batist G. Putative chemopreventive molecules can increase Nrf2-regulated cell defense in some human cancer cell lines, resulting in resistance to common cytotoxic therapies. Cancer Chemother Pharmacol 2010;66:467–74.
6. Kim YR, Oh JE, Kim MS, Kang MR, Park SW, Han JY, et al. Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. J Pathol 2010;220:446–51.
7. Hayes JD, McMahon M. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. Trends Biochem Sci 2009;34:176–88.
8. Singh A, Boldin-Adamsky S, Thimmulappa RK, Rath SK, Ashush H, Coulter J, et al. RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. Cancer Res 2008;68:7975–84.
9. Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in antioxidant defense and redox signaling. Biochem J 2009;425:313–25.
10. Rhee SG, Yang KS, Kang SW, Woo HA, Chang TS. Controlled elimination of intracellular H(2)O(2): regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. Antioxid Redox Signal 2005;7:619–26.
11. Woo HA, Jeong W, Chang TS, Park KJ, Park SJ, Yang JS, et al. Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-cys peroxiredoxins. J Biol Chem 2005;280:3125–8.
12. Findlay VJ, Tapiero H, Townsend DM. Sulfiredoxin: a potential therapeutic agent? Biomed Pharmacother 2005;59:374–9.
13. Lei K, Townsend DM, Tew KD. Protein cysteine sulfinic acid reductase (sulfiredoxin) as a regulator of cell proliferation and drug response. Oncogene 2008;27:4877–87.
14. Soriano FX, Baxter P, Murray LM, Sporn MB, Gillingwater TH, Hardingham GE. Transcriptional regulation of the AP-1 and Nrf2 target gene sulfiredoxin. Mol Cells 2009;27:279–82.
15. Singh A, Ling G, Suhasini AN, Zhang P, Yamamoto M, Navas-Acien A, et al. Nrf2-dependent sulfiredoxin-1 expression protects against cigarette smoke-induced oxidative stress in lungs. Free Radic Biol Med 2009;46:376–86.
16. Wei Q, Jiang H, Matthews CP, Colburn NH. Sulfiredoxin is an AP-1 target gene that is required for transformation and shows elevated expression in human skin malignancies. Proc Natl Acad Sci USA 2008;105:19738–43.

17. Tavassoli FA, Devilee P. Tumours of the breast and female genital organs. World Health Organization classification of tumours. Lyon, France: IARC Press; 2003.
18. Hartikainen JM, Tuhkanen H, Kataja V, Dunning AM, Antoniou A, Smith P, et al. An autosome-wide scan for linkage disequilibrium–based association in sporadic breast cancer cases in Eastern Finland: three candidate regions found. Cancer Epidemiol Biomarkers Prev 2005;14: 75–80.
19. Pellikainen MJ, Pekola TT, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen M, et al. p21WAF1 expression in invasive breast cancer and its association with p53, AP-2, cell proliferation, and prognosis. J Clin Pathol 2003;56:214–20.
20. Vandenplas S, Wiid J, Grobler-Rabie A, Brebner K, Ricketts M, Wallis G, et al. Blot hybridization of genomic DNA. J Med Genet 1984;21: 164–72.
21. Kauppinen JM, Kosma V-M, Soini Y, Sironen R, Nissinen M, Nykopp TK, et al. ST14 gene variant and decreased matriptase protein expression predict poor breast cancer survival. Cancer Epidemiology Biomarkers Prev 2010;19:2133–42.
22. Available from: http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r2_B36/. [cited 2010 Feb 24 and 2010 Nov 8.]
23. Yamamoto T, Yoh K, Kobayashi A, Ishii Y, Kure S, Koyama A, et al. Identification of polymorphisms in the promoter region of the human NRF2 gene. Biochem Biophys Res Commun 2004;321:72–9.
24. Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lanken PN, et al. Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. FASEB J 2007;21: 2237–46.
25. Voutilainen K, Anttila M, Sillanpää S, Tammi R, Tammi M, Saarikoski S, et al. Versican in epithelial ovarian cancer: relation to hyalyronan, clinicopathological factors and prognosis. Int J Cancer 2003;107: 359–64.
26. Karihtala P, Soini Y. Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. APMIS 2007;115:81–103.
27. Yuan H-Y, Chiou J-J, Tseng W-H, Liu C-H, Liu C-K, Lin Y-J, et al. FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. Nucleic Acids Res 2006;34:W635–41.
28. Konstantinopoulos PA, Spentzos D, Fountzilas E, Francoeur N, Sanisetty A, Grammatikos AP, et al. Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer. Cancer Res 2011;71:5081–9.
29. Hong CC, Ambrosone CB, Ahn J, Choi JY, McCullough ML, Stevens VL, et al. Genetic variability in iron-related oxidative stress pathways (Nrf2, NQ01, NOS3, and HO-1), iron intake, and risk of postmenopausal breast cancer. Cancer Epidemiol Biomarkers Prev 2007;16: 1784–94.
30. Bouligand J, Cabaret O, Canonico M, Verstuyft C, Dubert L, Becquemont L, et al. Estrogen and Thromboembolism Risk (ESTHER) Study Group. Effect of NFE2L2 genetic polymorphism on the association between oral estrogen therapy and the risk of venous thromboembolism in postmenopausal women. Clin Pharmacol Ther 2011;89:60–4.
31. Masuko H, Sakamoto T, Kaneko Y, Iijima H, Naito T, Noguchi E, et al. An interaction between Nrf2 polymorphisms and smoking status affects annual decline in FEV1: a longitudinal retrospective cohort study. BMC Med Genet 2011;12:97.
32. Cho HY, Jedlicka AE, Reddy SP, Zhang LY, Kensler TW, Kleeberger SR. Linkage analysis of susceptibility to hyperoxia. Nrf2 is a candidate gene. Am J Respir Cell Mol Biol 2002;26:42–51.
33. Valavanidis A, Vlachogianni T, fiotakis K. Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. Int J Environ Res Public Health 2009;6:445–62.
34. Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, et al. Nrf2 gene polymorphism and gastric carcinogenesis. Hepatogastroenterology 2008;55:750–4.
35. Sepulveda AR, Graham DY. Role of Helicobacter pylori in gastric carcinogenesis. Gastroenterol Clin North Am 2002;31:517–35.
36. Okoh V, Deoraj A, Roy D. Estrogen-induced reactive oxygen species-mediated signaling contribute to breast cancer. Biochim Biophys Acta 2011;1815:115–33.
37. Available from: www.ensembl.org. [cited 2012 Jul 25.]
38. Lee PH, Shatkay H. F-SNP: computationally predicted functional SNPs for disease association studies. Nucleic Acids Res 2008;36: D820–4.
39. Chang XZ, Li DQ, Hou YF, Wu J, Lu JS, Di GH, et al. Identification of the functional role of peroxiredoxin 6 in the progression of breast cancer. Breast Cancer Res 2007;9:R76.
40. Chua PJ, Lee EH, Yu Y, Yip GW, Tan PH, Bay BH. Silencing the Peroxiredoxin III gene inhibits cell proliferation in breast cancer. Int J Oncol 2010;36:359–64.
41. Bae JY, Ahn SJ, Han W, Noh DY. Peroxiredoxin I and II inhibit H2O2-induced cell death in MCF-7 cell lines. J Cell Biochem 2007;101: 1038–45.
42. Noh YH, Baek JY, Jeong W, Rhee SG, Chang TS. Sulfiredoxin translocation into mitochondria plays a crucial role in reducing hyperoxidized Peroxiredoxin III. J Biol Chem 2009;284:8470–7.
43. Shin DH, Park HM, Jung KA, Choi HG, Kim JA, Kim DD, et al. The NRF2-heme oxygenase-1 system modulates cyclosporine A-induced epithelial-mesenchymal transition and renal fibrosis. Free Radic Biol Med 2010;48:1051–63.
44. Loignon M, Miao W, Hu L, Bier A, Bismar TA, Scrivens PJ, et al. Cul3 overexpression depletes Nrf2 in breast cancer and is associated with sensitivity to carcinogens, to oxidative stress, and to chemotherapy. Mol Cancer Ther 2009;8:2432–40.
45. Aritake N, Tamaki Y, Masuda N, Nakano Y, Monden T, Noguchi S, et al. High expression of two genes selected by iAFLP: a new prognostic factor of estrogen receptor-positive breast cancer. Oncol Rep 2004; 12:381–7.