Genetic polymorphisms and protein expression of NRF2 and sulfiredoxin predict survival outcomes in breast cancer

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Précis: Potentially seminal findings identify biomarkers in a core ROS stress pathway that may be generally impactful in determining the survival outcomes of patients with breast cancer.
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 (SNPs) on NRF2 gene and eight on SRXN1 were genotyped in 452 breast cancer patients 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, clinicopathological variables and survival was assessed. A high level (>25%) of cytoplasmic NRF2 positivity was observed in 237/361 (66%) and SRXN1 positivity was observed in 82/363 (23%) of the cases. The NRF2 rs6721961 genotype TT was associated with increased risk of breast cancer ($P=0.008$, $OR=4.656$, $CI=1.350-16.063$) 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 ($P$ values 0.011 and 0.017). NRF2 and SRXN1 genetic polymorphisms are associated with breast cancer risk and survival, implicating that mechanisms associated with ROS and NRF2 pathway are involved in breast cancer initiation and progression.
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 KEAP1 (Kelch-like ECH-associated protein 1), 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, gamma 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 non-functioning Nrf2 gene develop early onset emphysema because of a deficient antioxidative 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 oesophageal, skin, larynx and lung cancer with a 6-13 % 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 to the function of these genes and their downstream targets.
Peroxiredoxins are enzymes, which have capability in 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 catalyses the reversal of 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 1-5% 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. Since NRF2 is known to be a sensor of oxidative damage, we studied its expression in different types of breast carcinoma. Additionally 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 (Supplemental 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 et al. 2005 (18) and Pellikainen et al. 2003 (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.

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 Supplemental 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 (SNP) selection

Tagging single nucleotide polymorphism (tagSNPs) for NRF2 and SRXN1 genes were selected using the HapMap Genome Browser release 2 (Phase 3, NCBI build 36, bdSNP
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 MAF (minor allele frequency) cutoff at 0.05. Two functional polymorphisms on the NRF2 promoter region were selected on the basis of previous publications (23, 24). (Supplemental Figure S1A and B)

**Genotyping of NRF2 and SRXN1 SNPs**

Genotyping of six NRF2 and eight SRXN1 tagging single nucleotide polymorphisms (TagSNPs) and two NRF2 functional SNPs was done using MassARRAY® (Sequenom Inc., San Diego, CA, USA) 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 non template 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 performed by 5’ nuclease assay (TaqMan) using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) according to manufacturer’s instructions. Primers and probes for rs2886162 were supplied from Applied Biosystems (Foster City, CA, USA) 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-μm-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., Santa Cruz, CA, USA) and rabbit polyclonal anti-human sulfiredoxin (14273-1-AP, Protein Tech Group, Chicago, IL, USA) were diluted with 1 % bovine serum albumin in PBS to 1:200 and 1:500 working solutions, respectively. The evaluation of NRF2 immunostaining was performed separately in tumor cell nuclei and cytoplasm. For sulfiredoxin cytoplasmic immunoreactivity was evaluated. The results for NRF2 were semiquantitated as follows; 0-5 %=negative, over 5 to 25 %= weak positivity, over 25 to 75 %=moderate positivity, over 75 to 100 %= strong positivity. In the analyses, NRF2 expression was divided in two groups, low extent (< 25 %) and high extent (> 25 %) expression. For sulfiredoxin the presence (> 1 %) or absence of cytoplasmic expression was recorded.

Statistical analysis

The statistical analyses were performed with SPSS for Windows software v 14.0 (SPSS, Chicago, IL, USA). Continuous data were compared using analysis of variance (ANOVA). When ANOVA results indicated that groups differed, post hoc comparisons were performed using two-tailed t-tests. Categorical data were compared using Fisher’s 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, histological type, nodal status, estrogen receptor status, progesterone receptor status, HER status)
among the cases were also computed using Armitage’s trend test. The concordance of the genotypes with Hardy-Weinberg equilibrium was tested using standard chi-squared 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 Inc.). In multivariate survival analyses the Cox regression analysis in SPSS v 14.0 (SPSS Inc.) was used. P-values less than 0.05 (two-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 tagging SNPs (rs1806649, rs2886162, rs1962142, rs2364722, rs10183914, rs2706110 and rs13035806) and two functional SNPs (rs6721961 and rs6706649) were analysed in the *NRF2* gene region. Eight tagging SNPs (rs6085283, rs13043781, rs6076869, rs6053666, rs2008022, rs6116929, rs7269823 and rs6053728) were analyzed in the *SRXN1* gene region (Supplemental Table S2). The SNP genotypes were tested for concordance with the Hardy-Weinberg equilibrium (HWE). Among the controls *NRF2* rs6706649 deviated slightly from HWE with a *P* value of 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 % (237/361) and nuclear (> 25 %) positivity in 26 % (96/365) of cases (Figure 1A-D). High extent nuclear positivity was observed in 20 % (43/219) of ductal, 47 % (33/70) of lobular and 29 % (17/59) of other...
types. Most notably, lobular carcinomas showed significantly more high extent nuclear NRF2 expression than ductal ones ($P=0.001$). Twenty-three percent (82/363) of the breast tumors displayed positivity for sulfiredoxin (Figure 1E and F). Twenty-three percent (50/219) of ductal, 15 % (10/68) of lobular and 30 % (15/50) of other types expressed positivity. Nuclear and cytoplasmic NRF2 expression was associated with sulfiredoxin expression ($P=0.003$ and $P=0.008$, respectively, Supplemental 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 ($P_{(\text{Overall})}=0.041$, $P_{(\text{Allele specific})}=0.012$, OR=1.975, CI=1.159-3.365) and $NRF2$ rs2886162 rare homozygous genotype AA associated with ER positive breast cancer ($P_{(\text{Overall})}=0.008$, $P_{(\text{Allele specific})}=0.008$, 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 ($P_{\text{Overall}}=0.019$, $P_{\text{Allele specific}}=0.022$, 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 to the carriers of the common allele G (Kaplan-Meier $P_{\text{log rank}}=0.017$, Supplemental Table S4, Supplemental Figure S2). This association remained significant in the multivariate analysis (Cox regression $P=0.032$, HR=1.687, CI=1.047-2.748, Table 3, Figure 2). In this multivariate analysis also the cytoplasmic NRF2 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 extent 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 (Supplemental Table S5, Supplemental Figure S3A and B). Similar trend was observed with nuclear NRF2 protein expression ($P=0.019$, log rank 5.490) (Supplemental Table S5, Supplemental Figure S4A and B).

SRXN1 rs6116929 rare homozygous genotype GG and rs2008022 rare allele carriers CA&AA had better survival compared to the common allele ($P_{\text{log rank}}=0.063$ and $P_{\text{log}}$
SRXN1 rs7269823 and rs6085283 rare allele carriers (AG&GG and CT&TT, respectively) had poorer survival compared to the common homozygous genotype ($P_{\text{log rank}}=0.030$ and $P_{\text{log rank}}=0.015$, respectively, Supplemental Table S4, Supplemental Figure S5C and D). In the Cox regression analysis including all four survival-associated SRXN1 polymorphisms only rs2008022 remained significant ($P=0.012$, HR=1.645, CI=1.116-2.425). None of these four polymorphisms however, were independently significant prognostic factors in the multivariate analysis including tumor grade, nodal status, ER status, PR status, histological 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 zero. The patients were divided in two groups; 0-3 risk alleles and 4-8 risk alleles. A trend towards 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, Supplemental Table S4 Supplemental Figure 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 ($P_{\text{log rank}}=0.638$) (Supplemental Figure S7A and B). 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 (Supplemental Table S6, Supplemental Figure S8A). Similar results were obtained from the multivariate analysis including the clinicopathological variables, NRF2 protein expression (cytoplasmic and nuclear), sulfiredoxin protein expression, combined SRXN1 genotypes and rs2886162 (Supplemental Table S7, Supplemental Figure S8B).

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

The effect of NRF2 rs2886162 rare homozygous genotype AA on poor prognosis was also seen separately in the group that had received adjuvant chemotherapy (CT) and among those that received postoperative radiation therapy (RT). In the group that had received adjuvant CT the rs2886162 genotype AA associated with poorer breast cancer survival ($P=0.019$, $HR=2.43$, CI=1.16-5.08) (Figure 3A) and with poorer recurrence-free survival ($P=0.003$, $HR=2.83$, CI=1.43-5.61). In the group that received postoperative RT the rs2886162 genotype AA associated with poorer recurrence-free survival ($P=0.025$, $HR=1.68$, CI=1.07-2.64) (Figure 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 RT (Supplemental Table S8, Figure 4A and B). Also, among the patients treated with RT, the SRXN1 rs7269823 and rs6085283 rare allele carriers (AG&GG and CT&TT, respectively) had poorer survival compared to the patients carrying the common homozygous genotypes (Supplemental Table S8, Figure 4C and D). In addition, among the patients treated with RT the SRXN1 rs6053666 rare homozygous genotype CC predicted better prognosis compared to the common allele carriers (Supplemental 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 antioxidative 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 antioxidative enzymes compared to normal tissues (26). Recent findings suggest that enhanced detoxification of reactive oxygen species with additional NRF2 functions may in fact be also protumorigenic (27).

Our results demonstrate that in the NRF2 pathway there are genetic polymorphisms that affect both the susceptibility for breast cancer and the outcome of the breast cancer patients, 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 (intrinsic enhancer) c-Rel (FastSNP, ref. 28) (Supplemental 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 demonstrated 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, \textit{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 \textit{NRF2} rs2886162 rare homozygous genotype AA independently predicted poorer survival among patients who received adjuvant chemotherapy, and the recurrence-free survival was poorer among RT-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 \textit{SRXN1} is not the sole target for NRF2 activation. Association of nuclear NRF2 staining with poorer survival has been previously observed in ovarian carcinoma (29). However, in breast cancer this issue needs further studies, possibly including also \textit{KEAP1}.

Previous studies on \textit{NRF2} polymorphisms in breast cancer are few. In a cohort of postmenopausal women, specific polymorphisms on \textit{NRF2} (rs1806649), \textit{NQO1}, \textit{NOS3}
and HO-1 did not have any significance for the risk of breast cancer (30). When the risk polymorphisms of these genes were combined, patients with three risk alleles had a 1.5 fold risk and those with a high iron intake had a greater than 2 fold risk (30). In postmenopausal women with oral estrogen replacement therapy the NRF2 rs6721961 rare allele seems to modify the risk of thromboembolism (31). NRF2 polymorphism has, however, been more extensively studied in pulmonary disease (24, 32, 33). 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 provokes development of ROS (34). Polymorphisms rs6721961 and rs6706649 have been studied in gastric carcinogenesis but no overall association with risk was found (35). In gastric cancer, carcinogenesis is predominantly based on H. pylori induced gastritis leading to gastric atrophy and cancer, while in breast cancer hormonal factors play a role (36). It is known that estrogen metabolites induce the formation of reactive oxygen species (37). In this sense NRF2 and its dysfunction may be more important in breast carcinogenesis than 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 RT 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. 38). rs6116929 locates in downstream region and the rare allele G (which associated with better survival) is
predicted to destroy binding sites for CdxA, cap and deltaE (F-SNP, ref. 39). rs6116929 is also only 84 base pairs from rs6076869 (D' 0.99), the rare allele of which associated with increased NRF2 protein. rs6076869 rare allele T is predicted to destroy a GATA-X binding site (FastSNP) and to create cap and AP-4 TF binding sites (F-SNP). rs6085283 resides in intron 1 and the rare allele T (which associated with poorer survival) creates a binding site for Oct-1 transcription factor (FastSNP). Also, the rs2008022 (in intron 1) rare allele A (which associated with better survival) destroys a GATA-2 transcription binding site, and is 1079 base pairs 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 LD with rs2008022 (D' -1) and rs6053666 (D' 0.7). (Supplemental 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 (40). Moreover, peroxiredoxin III promotes breast cancer cell proliferation and peroxiredoxins I, II and III protect cells from oxidative damage induced apoptosis (41, 42). 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 RT. 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. rs6053666 resides on the 3'UTR.
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) (Supplemental 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 histological subgroups, lobular invasive carcinomas showed a stronger expression of nuclear positivity than ductal ones. Lobular carcinoma is a tumor type showing low or non-existent expression of E-cadherin. Interestingly, NRF2 activation by sulphoraphane was reported to cause down regulation of EMT type changes in rat kidney tubular epithelial cells, including E-cadherin (43). 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 co-workers (2009) found that NRF2 protein expression was decreased in seven of the ten breast cancer cell lines they studied (44). They also detected lower levels of NRF2 in seven of the ten studied breast cancer tumor samples compared to normal breast tissue (45). Unfortunately the authors did not specify subcellular localization of the staining or the histological subgroups of the tumors. High gene expression of NRF2 has been reported to associate with poor prognosis among ER positive breast cancer (46).
To conclude, we have observed that \textit{NRF2} and \textit{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 antioxidative mechanisms may thus pave the way for new treatment modalities based on inhibition of such mechanisms in breast cancer cells.

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REFERENCES

1. Itoh K, Mimura J, Yamamoto M. Discovery of the Negative Regulator of Nrf2,


Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in

factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis

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


7. Hayes JD, McMahon M. NRF2 and KEAP1 mutations: permanent activation of an

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.

9. Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in


38. www.ensembl.org
Table 1. Significant associations between the NRF2 and SRXN1 genotypes and risk of breast cancer.

<table>
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<td>0.058</td>
<td>0.353 (0.115-1.085)</td>
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<tr>
<td>rs2706110</td>
<td>0.029</td>
<td>0.058</td>
<td>A</td>
<td>0.011</td>
<td>2.079 (1.175-3.679)</td>
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<td>0.011</td>
<td>0.481 (0.272-0.851)</td>
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<tr>
<td>rs10183914</td>
<td>0.474</td>
<td>0.913</td>
<td>ns</td>
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<tr>
<td>rs1962142</td>
<td>0.335</td>
<td>0.224</td>
<td>ns</td>
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<td></td>
</tr>
<tr>
<td>rs1806649</td>
<td>0.850</td>
<td>0.571</td>
<td>ns</td>
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</tr>
<tr>
<td>rs2364722</td>
<td>0.276</td>
<td>0.348</td>
<td>ns</td>
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<tr>
<td>rs6706649</td>
<td>0.141</td>
<td>0.398</td>
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<td>rs6721961</td>
<td>0.028</td>
<td>0.113</td>
<td>T</td>
<td>0.008</td>
<td>4.656 (1.350-16.063)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>0.008</td>
<td>0.215 (0.062-0.741)</td>
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<tr>
<td>rs2886162</td>
<td>0.352</td>
<td>0.449</td>
<td>ns</td>
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<td></td>
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<td>SRXN1</td>
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<td>rs6116929</td>
<td>1</td>
<td>0.991</td>
<td>ns</td>
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<tr>
<td>rs6076869</td>
<td>0.948</td>
<td>0.922</td>
<td>ns</td>
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<tr>
<td>rs6053666</td>
<td>0.052</td>
<td>0.028</td>
<td>C</td>
<td>0.079</td>
<td>0.673 (0.432-1.048)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>T</td>
<td>0.079</td>
<td>1.486 (0.954-2.314)</td>
</tr>
<tr>
<td>rs7269823</td>
<td>0.319</td>
<td>0.150</td>
<td>ns</td>
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<tr>
<td>rs2008022</td>
<td>0.222</td>
<td>0.364</td>
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<tr>
<td>rs13043781</td>
<td>0.535</td>
<td>0.307</td>
<td>ns</td>
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<td>rs6085283</td>
<td>0.247</td>
<td>0.784</td>
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<tr>
<td>rs6053728</td>
<td>0.472</td>
<td>0.257</td>
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</table>

a P from the chi-squared 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.
<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Associated High extent NRF2</th>
<th>OR (CI)</th>
<th>Associated Low extent NRF2</th>
<th>OR (CI)</th>
<th>Associated Negative sulfiredoxin protein expression</th>
<th>OR (CI)</th>
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<tbody>
<tr>
<td>NRF2</td>
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<td>rs1962142</td>
<td>T</td>
<td>0.030</td>
<td>1.742 (1.035-2.933)</td>
<td>0.042</td>
<td>1.990 (1.015-3.901)</td>
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<tr>
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<td>C</td>
<td>0.230</td>
<td>0.469 (0.133-1.659)</td>
<td>0.086</td>
<td>0.159 (0.009-2.745)</td>
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<tr>
<td>rs6721961</td>
<td>T</td>
<td>0.0008</td>
<td>2.420 (1.491-3.926)</td>
<td>0.047</td>
<td>1.867 (1.002-3.478)</td>
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<tr>
<td></td>
<td>G</td>
<td>0.274</td>
<td>0.564 (0.199-1.595)</td>
<td>0.042</td>
<td>0.114 (0.007-1.940)</td>
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<tr>
<td>rs2886162</td>
<td>A</td>
<td>0.011</td>
<td>1.988 (1.162-3.400)</td>
<td>0.042</td>
<td>1.990 (1.015-3.901)</td>
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<tr>
<td></td>
<td>G</td>
<td>0.428</td>
<td>0.808 (0.476-1.370)</td>
<td>0.042</td>
<td>0.114 (0.007-1.940)</td>
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<tr>
<td>SRXN1</td>
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</tr>
<tr>
<td>rs6076869</td>
<td>T</td>
<td>0.012</td>
<td>1.927 (1.217-3.051)</td>
<td>0.005</td>
<td>1.712 (0.343-1.470)</td>
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<tr>
<td></td>
<td>G</td>
<td>0.360</td>
<td>0.712 (0.137-1.598)</td>
<td>0.270</td>
<td>0.612 (0.269-1.414)</td>
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</tr>
</tbody>
</table>

*P* from the Armitage trend test for the overall association with cytoplasmic NRF2 protein expression. 

*P* from the Armitage trend test for the overall association with sulfiredoxin protein expression. 

ns; no significant association observed. 

P, OR and CI for the homozygous and heterozygous allele carriers.
Table 3. Variables significantly associated with breast cancer survival in multivariate analysis according to NRF2 SNP genotypes.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>B (SE)</th>
<th>Wald</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal status</td>
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<tr>
<td>Negative</td>
<td>168</td>
<td>Ref.</td>
<td>0.859 (0.235)</td>
<td>13.424</td>
<td>2.362 (1.491-3.740)</td>
</tr>
<tr>
<td>Positive</td>
<td>122</td>
<td>0.859 (0.235)</td>
<td>13.424</td>
<td>2.362 (1.491-3.740)</td>
<td>0.0002485</td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>251</td>
<td>Ref.</td>
<td>0.932 (0.262)</td>
<td>12.633</td>
<td>2.539 (1.519-4.244)</td>
</tr>
<tr>
<td>Positive</td>
<td>39</td>
<td>0.932 (0.262)</td>
<td>12.633</td>
<td>2.539 (1.519-4.244)</td>
<td>0.000379</td>
</tr>
<tr>
<td>rs2886162</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>GG+GA</td>
<td>219</td>
<td>Ref.</td>
<td>0.523 (0.243)</td>
<td>4.613</td>
<td>1.687 (1.047-2.718)</td>
</tr>
<tr>
<td>AA</td>
<td>71</td>
<td>0.523 (0.243)</td>
<td>4.613</td>
<td>1.687 (1.047-2.718)</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Analysis stratified by tumor grade, nodal status, ER status, PR status, histological type, tumor size, HER2 status, cytoplasmic NRF2 expression and rs2886162 genotypes.

HR (95% CI); Hazard ratio of breast cancer death and 95% confidence interval from Cox regression survival analysis.

Ref.: Reference category
FIGURE LEGENDS

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

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

Figure 3. Association of NRF2 rs2886162 with survival among breast cancer patients with different therapies. A) Breast cancer specific survival among patients treated with adjuvant chemotherapy. Analysis stratified by age, stage and radiation therapy. B) Recurrence-free survival among patients treated with postoperative radiation therapy. Analysis stratified by age, stage, hormone therapy and chemotherapy. HR (95% CI)= Hazard ratio of breast cancer death with 95% confidence interval in Cox regression survival analysis.

Figure 4. Significant associations of SRXN1 SNP genotypes with survival among breast cancer patients receiving radiation treatment. Breast cancer specific survival according to
A) rs6116929, B) rs2008022, C) rs7269823, and D) rs6085283 genotypes. Analyses stratified by age, stage, hormone therapy and chemotherapy. HR (95% CI)= Hazard ratio of breast cancer death with 95% confidence interval in Cox regression survival analysis.
Figure 1.
Figure 2.

*Graph showing survival time in months for different genotypes of NRF2 rs2886162.*

- **GG & GA**
  - n = 219
  - P = 0.032
  - HR = 1.687 (95% CI, 1.047-2.718)

- **AA**
  - n = 71
  - Survival time in months
Figure 3.

A

GG&GA
n=63

AA
n=16
HR= 2.43 (95% CI, 1.16-5.08)

P=0.019

Survival time in years

Cum Survival

B

GG&GA
n=194

AA
n=53
HR= 1.68 (95% CI, 1.07-2.64)

P=0.025

Survival time in years

Cum Survival
Figure 4.

**A**

rs6116929

- **GG**
  - n = 64
- **AA & AG**
  - n = 185
  - HR = 1.74 (95% CI 1.00-3.00)

- **P = 0.049**

Survival time in years

**B**

rs2008022

- **CA & AA**
  - n = 107
- **CC**
  - n = 134
  - HR = 1.73 (95% CI 1.09-2.74)

- **P = 0.020**

Survival time in years

**C**

rs7269823

- **AA**
  - n = 148
- **AG & GG**
  - n = 100
  - HR = 1.53 (95% CI 1.01-2.41)

- **P = 0.045**

Survival time in years

**D**

rs6085283

- **CC**
  - n = 76
- **CT & TT**
  - n = 170
  - HR = 1.75 (95% CI 1.04-2.95)

- **P = 0.036**

Survival time in years
Genetic polymorphisms and protein expression of NRF2 and sulfiredoxin predict survival outcomes in breast cancer

Jaana M. Hartikainen, Maria Tengström, Veli-Matti Kosma, et al.

Cancer Res  Published OnlineFirst September 10, 2012.