KEAP1 Genetic Polymorphisms Associate with Breast Cancer Risk and Survival Outcomes

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Abstract

Purpose: Defective oxidative stress response may increase cancer susceptibility. In tumors, these rescue mechanisms may cause chemotherapeutic resistance, impacting patient outcome. We previously showed that genetic variation in the nuclear factor erythroid 2–related factor 2 (NFE2L2) is associated with breast cancer risk and prognosis. Here we further studied this pathway by investigating Kelch-like ECH-associated protein 1 (KEAP1).

Experimental Design: Five tagging SNPs in the KEAP1 gene were genotyped in 996 breast cancer cases and 880 controls from two Finnish case–control sets. KEAP1 protein expression was studied in 373 invasive breast cancer tumors.

Results: rs34197572 genotype TT was associated with increased risk of breast cancer in the KBPC samples (P = 1.8 × 10−4; OR, 7.314; confidence interval [CI], 2.185–24.478). rs11085735 allele A was associated with lower KEAP1 protein expression (P = 0.040; OR, 3.545) and high nuclear NRF2 expression (P = 0.009; OR, 2.445) and worse survival in all invasive cases (P = 0.023; HR, 1.634). When including treatment data, rs11085735 was associated with recurrence-free survival (RFS; P = 0.020; HR, 1.545) and breast cancer–specific survival (P = 0.016; HR, 1.683) and rs34197572 with overall survival (P = 0.045; HR, 1.304). rs11085735 associated with RFS also among tamoxifen-treated cases (P = 0.003; HR, 3.517). Among radiotherapy-treated cases, overall survival was associated with rs34197572 (P = 0.018; HR, 1.486) and rs8113472 (P = 0.025; HR, 1.455). RFS was associated with rs9676881 (P = 0.024; HR, 1.452) and rs1048290 (P = 0.020; HR, 1.468) among all invasive cases and among estrogen receptor (ER)-positive tamoxifen-treated cases (P = 0.018; HR, 2.407 and P = 0.015; HR, 2.476, respectively).

Conclusions: The present findings suggest that the investigated SNPs have effects related to oxidative stress induced by cancer treatment, supporting involvement of the NRF2/KEAP1 pathway in breast cancer susceptibility and patient outcome. Clin Cancer Res; 21(7): 1591–601. ©2015 AACR.

Introduction

Radiation, inflammation, and various chemicals can lead to oxidative stress, which in turn causes DNA changes and may lead to cancer development (1, 2). Antioxidative enzymes—including superoxide dismutases (SOD), thioredoxins and peroxiredoxins—and nonenzymatic antioxidants contribute to combating oxidative stress (1, 2). The main mediator of cellular adaptation to oxidative and xenobiotic stress is the Nuclear factor erythroid 2–related factor 2 protein (NRF2, encoded by NFE2L2). NRF2 normally resides in the cytoplasm and forms a complex with Kelch-like ECH-associated protein 1 (KEAP1), which directs NRF2 through ubiquitination to proteasomal degradation (3). In the event of oxidative stress, NRF2 is released from the complex and moves to the nucleus. In the nucleus, NRF2 heterodimerizes with small Maf or c-Jun and binds antioxidant response elements (ARE) in downstream genes, thus inducing the expression of several cytoprotective genes—including detoxifying enzymes, drug transporters, antiapoptotic proteins, and proteasomes (3). Once redox balance (basal state) is returned to the cell, KEAP1 moves to the nucleus, binds to NRF2 by its EGE domain, and takes it back to the cytoplasm for degradation (3, 4). Several factors reportedly interrupt the NRF2/KEAP1 interaction, inducing the NRF2 accumulation, and thus interfering with reactive oxygen species (ROS) scavenging (5–11). Recent findings suggest that enhanced ROS detoxification through additional NRF2 functions may be also protumorigenic (12).

In cancer cells, large amounts of unbound NRF2 can result in chemoresistance (13). Tumor cells may contain mutations in the NFE2L2 and KEAP1 genes, underlining the importance of NRF2 and KEAP1 in tumorigenesis. Somatic mutations in KEAP1 predominate in lung cancer (14, 15), whereas NFE2L2 mutations occur with a 6% to 13% frequency in esophageal, skin, larynx, and lung cancer, with the highest prevalence found.

Purpose: Defective oxidative stress response may increase cancer susceptibility. In tumors, these rescue mechanisms may cause chemotherapeutic resistance, impacting patient outcome. We previously showed that genetic variation in the nuclear factor erythroid 2–related factor 2 (NFE2L2) is associated with breast cancer risk and prognosis. Here we further studied this pathway by investigating Kelch-like ECH-associated protein 1 (KEAP1).

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in squamous cell carcinomas (16). The NRF2/KEAP1 pathway has been reported to be somatically significantly altered also in clear cell renal cell carcinoma (17). In breast carcinomas, KEAP1 mutations and putative copy number alterations have rarely been detected (18, 19) and NFE2L2 mutations are even less common (19).

Breast carcinoma—the most common cancer type among women in Western countries—has been associated with many risk factors, some of them with a hereditary basis (20). The link between breast carcinoma development and oxidative damage is supported by the expression of 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (NHE) in breast cancer tissue and the fact that estrogen metabolites induce ROS formation and thus are able to cause oxidative stress (21, 22). We previously reported that germline polymorphisms in NFE2L2 and SRXN1 are associated with breast cancer risk and outcome (23). Another study demonstrated that a combination of risk alleles in iron-related oxidative stress pathway genes (NFE2L2, NQO1, NOS3, and HO-1) are associated with increased risk of postmenopausal breast cancer (24).

KEAP1 polymorphisms have not previously been shown to be associated with breast cancer risk or survival. However, the KEAP1 polymorphism rs1048290 in tumor tissue associated with progression-free survival in endometrioid endometrial adenocarcinoma (25). In addition, in non–small cell lung cancer, low KEAP1 protein expression reported associated with worse overall survival (OS; ref. 26), and loss of KEAP1 function leads to increased NRF2 concentration and chemoresistance (27).

Here, we studied KEAP1 protein expression in 373 cases of invasive breast carcinoma and continued to investigate the NRF2/KEAP1 pathway. We analyzed the influence of 5 tagging SNPs—rs34197572, rs9676881, rs1048290, rs11085735, and rs8113472—in KEAP1 on KEAP1 protein expression, on breast cancer risk, and on patient prognosis. KEAP1 protein expression was also evaluated as a predictive factor and in relation to the immunohistochemical expression of the NRF2 protein. This is the first study to observe the role of KEAP1 polymorphisms as genetic risk factors in breast cancer.

Translational Relevance

Oxidative stress is linked to cancer development. Oxidative stress rescue mechanisms also play a role, as they potentially cause chemoresistance and radioreistance and affect patient outcome. Kelch-like ECH-associated protein 1 (KEAP1) is a regulator of the main mediator of the redox stress response, Nuclear factor erythroid 2–related factor 2 (NRF2). Although oxidative damage is considered a mechanism for cancer development, its role in breast cancer risk and outcome has not been extensively studied. The present study demonstrated that genetic polymorphisms in KEAP1 also affect breast cancer risk and survival. In particular, the investigated polymorphisms modified the effects of radiotherapy and tamoxifen treatment on patient survival. These results contribute to the present knowledge regarding the involvement of NRF2 pathway in breast cancer and in the future may be translated into clinical use and be used in treatment planning and assessing prognosis of patients with breast cancer.

Materials and Methods

DNA samples

This study included 996 patients with breast cancer and 880 healthy control individuals, all of whom were Finnish Caucasian females. Data and DNA samples for genotyping were obtained from 2 population-based sample sets from Finland: the Kuopio Breast Cancer Project (KBCP) from Eastern Finland and the Northern Finnish Oulu Breast Cancer Study (OCBS).

From the KBCP sample set, DNA for genotyping was available from 452 patients with invasive breast cancer (age, 23–91 years) and 370 control subjects (age, 26–77 years; Table 1). The KBCP sample set includes a total of 497 prospective breast cancer cases from the province of Northern Savo in Eastern Finland, diagnosed at the Kuopio University Hospital between 1990 and 1995. It also includes 458 control individuals matched for age and long-term area of residence who were selected from the National Population Register during the same time period. The patient follow-up data extend up to 20 years to February 2011. More details regarding the KBCP have been previously published (e.g., ref. 28). The study participants gave their informed consent and the KBCP has been approved by the ethical committee of the University of Eastern Finland and Kuopio University Hospital.

From the OCBS sample set, DNA for genotyping was available from 544 patients with breast cancer (age, 28–92) and 510 healthy control subjects (age, 18–66 years; ref. 29). The OCBS invasive breast cancer cases were consecutively operated, and data and blood samples collected at the Oulu University Hospital between 2000 and 2007 (Table 1). Patient follow-up data extend up to 12.7 years. The Northern Finnish control subjects were collected from cancer-free Finnish Red Cross blood donors recruited in 2002 and originating from the same geographical region as the studied cancer patient cohort. Control subjects were anonymous. Their genders, ages, and places of birth were known. All control individuals were cancer-free at the time of donation of the blood sample. There was no follow-up on donor health status. Informed consent for participation in the study has been obtained from each patient, and the study has been approved by the Finnish Ministry of Social Affairs and Health, and the ethical committee of Oulu University Hospital.

Genomic DNA was extracted from peripheral blood lymphocytes of both cases and controls using standard procedures (30).

Tumor samples

Tumor material was available from 373 cases of invasive breast carcinomas included in the KBCP. Table 1 shows the clinical characteristics of these cases. Tumor tissue was obtained from the primary tumor during breast cancer surgery. Tissue was paraffin embedded and used to construct a tissue microarray (TMA) as previously described (28).

Immunohistochemistry

Immunohistochemical staining of KEAP1 protein was performed as previously described (23). The primary antibody goat polyclonal anti-human KEAP1 (E-20, sc-15246, Santa Cruz Biotechnology, Inc.) was diluted with 1.5% normal rabbit serum in PBS to make a 1:200 working solution. The antibody dilution was optimized using 3 confirmed KEAP1-positive lung tissue specimens under the pathologist’s supervision. One of these lung tissue samples was used as a positive control when staining the breast cancer TMAs. Negative controls included lung tissue and breast...
KEAP1 Genetic Polymorphism Predicts Outcome in Breast Cancer

Table 1. Clinicopathologic characteristics of the patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>KBCP samples in genotyping</th>
<th>OBCS samples in genotyping</th>
<th>KBCP cases in TMA</th>
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<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
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<tr>
<td>Number of patients</td>
<td>452 (100)</td>
<td>370</td>
<td>537 (100)</td>
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<td>Tumor grade</td>
<td>116 (25.7)</td>
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<td>91 (16.9)</td>
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<td></td>
<td>210 (46.5)</td>
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<td>122 (26.9)</td>
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<td>19 (3.5)</td>
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<td>313 (58.5)</td>
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<td>191 (35.7)</td>
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<td>25 (5.5)</td>
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<td>17 (3.2)</td>
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<td>5 (1.3)</td>
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<td>117 (25.9)</td>
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<td>63 (11.9)</td>
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<td>148 (40%)</td>
<td>155 (28.9)</td>
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<td>162 (30.2)</td>
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<td>86 (19.0)</td>
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<td>116 (25.7)</td>
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<td>256 (48.3)</td>
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<tr>
<td>No</td>
<td>336 (74.3)</td>
<td>—</td>
<td>274 (51.7)</td>
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</table>

Abbreviations: NA, data not available; —, data not applicable.
cancer tissue samples prepared using the same reagents and procedure except that the primary antibody was replaced by the buffer used for dilution. No staining was observed in the negative controls.
The immunohistochemical staining in the microarray for each tumor was done in triplicate. The immunostaining was semi-quantitatively categorized into 4 groups: 0, 0%–5% positive cells; 1, 5%–50% positive cells; 2, 50%–75% positive cells; and 3, over 75% positive cells. The intensity was assessed as: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The quantity and intensity values were summed to create an immunostaining index value: 0, negative (0); 1–3, weak (1); and 4–6, strong (2). The final immunostaining value for each tumor was the arithmetic mean (from values 0–2) of the array triplicates. For statistical analyses, these mean values were divided into 2 groups: ≤1.33 (representing negative/weak staining) and ≥1.67 (representing strong/moderate KEAP1 staining). To analyze the association of KEAP1 protein expression and genotypes with NRF2
Sequencing samples and 9.1% of OBCS samples. If the duplicate genotype control, genotyping was performed in duplicate for 4.2% of KBCP in a 96-well format as previously described (28). For quality Genotyping Assay. Reactions were carried out in a 10-mmol/L volume with an r2 cutoff of 0.8 and a minor allele frequency (MAF) cutoff of 0.05.

Genotyping
Four KEAP1 tagSNPs were genotyped using MassARRAY (Sequenom Inc.) and iPLEX Gold (Sequenom Inc.) with a 384-well plate format as previously described (23). For quality control, duplicate analysis was performed for 6.7% of the KBCP samples and 6.5% of the OBCS samples. All primer sequences and reaction conditions are available upon request. The KEAP1 tagSNP rs34197572 was genotyped by 5’ nuclease assay (TaqMan) using the Mx3000P Real-Time PCR System (Stratagene) following the manufacturer’s instructions. Primers and probes for rs34197572 were supplied from Applied Biosystems as Custom TaqMan Genotyping Assay. Reactions were carried out in a 10-mmol/L volume in a 96-well format as previously described (28). For quality control, genotyping was performed in duplicate for 4.2% of KBCP samples and 9.1% of OBCS samples. If the duplicate genotype results were discordant, the sample was discarded.

Sequencing
We detected great deviation from the Hardy–Weinberg equilibrium. Therefore, to confirm the results of the TaqMan analysis, we sequenced all samples with the rs34197572 minor homozygous genotype (29 samples in KBCP and 30 samples in OBCS), 10 samples representing the common homozygous and 5 representing the heterozygous genotypes. The sequencing primers were as follows: forward primer: 5’-ataaaaggtggcaccacagg-3’; reverse primer: 5’-tcacctgctagggttaa-3’. PCR conditions are available on request. Sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and Data Collection version 2.0 software (Applied Biosystems) following the manufacturer’s instructions. Sequences were analyzed using Sequence Scanner v1.0 software (Applied Biosystems). All obtained sequences were in agreement with the TaqMan results.

Statistical analyses
Significance levels for overall comparisons of SNP genotype frequencies between cases and controls were computed using Fisher exact test implemented in IBM SPSS Statistics 19 software (IBM). The Armitage trend test was used to estimate the linear additive trend effect (increasing number of alleles) of the risk allele (32). Logistic regression analysis in IBM SPSS Statistics 19 was performed to calculate genotype-specific ORs and to test the association between genotypes and protein expressions. The deviation from Hardy–Weinberg equilibrium was tested using a standard χ2 test. Survival data were analyzed using the univariate Kaplan–Meier method with the log-rank test and multivariate Cox regression analysis in IBM SPSS Statistics 19. OS and breast cancer-specific survival (BCSS) were calculated as the time from the date of diagnosis to the date of last follow-up or death. Cause of death was coded as either breast cancer or not caused by breast cancer. Relapse-free survival (RFS) was calculated as the time of diagnosis to the time of first relapse (local relapse, contralateral breast cancer, or metastatic disease). A P value of 0.05 (2-sided) or less was considered statistically significant. The initial P values from the breast cancer risk association analyses were corrected for multiple testing using the Benjamini–Hochberg false discovery rate method (33).

Results
Positive KEAP1 protein expression in breast tumors
Cytoplasmic KEAP1 protein expression was negative or weak (0–0.67) in 16.6% of the tumors, moderate (1–1.33) in 48.9%, and high (1.67–2) in 34.6% (Supplementary Fig. S1). KEAP1 staining did not significantly differ between different histologic types of breast carcinoma. Lower KEAP1 protein expression was associated with estrogen receptor (ER)-positive tumors [P = 0.040; OR, 1.711; 95% confidence interval (CI), 1.025–2.856], progesterone receptor (PR)-positive tumors [P = 0.025; OR, 1.683; 95% CI, 1.069–2.652] and HER2-negative tumors [P = 0.034; OR, 1.984; 95% CI, 1.052–3.741; Supplementary Table S1].

KEAP1 protein expression associated with NRF2
High KEAP1 protein expression (≥1.67) was associated with high cytoplasmic NRF2 protein expression [P = 0.003; OR, 2.154; 95% CI, 1.288–3.603; Poverall = 0.003, logistic regression; Supplementary Table S2]. Low cytoplasmic NRF2 expression was more closely associated with lobular tumors than with ductal tumors (with medullary tumors and other histologic types as a reference group; P = 0.007; OR, 2.915; 95% CI, 1.332–6.378, in logistic regression analysis; data not shown). With the analysis limited to tumors with high KEAP1 expression (≥1.67), we observed that tumor histology was associated with cytoplasmic NRF2 expression [P = 0.020; logistic regression]. In particular, lobular histology was associated with negative/low cytoplasmic NRF2 [P = 0.034; OR, 6.250; 95% CI, 1.145–34.123; logistic regression; data not shown]. With the analysis restricted to tumors with high cytoplasmic NRF2 protein expression, KEAP1 protein expression not significantly associated with tumor histology.

KEAP1 rs34197572 associated with breast cancer risk
We analyzed 5 tagging SNPs in the KEAP1 gene region: rs34197572, rs9676881, rs1048290, rs11085735, and rs8113472 (Supplementary Fig. S2). The controls were in Hardy–Weinberg equilibrium with all studied SNPs (Supplementary Table S3). Within the KBCP sample set, an increased risk of breast cancer was associated with the rs34197572 minor allele T (P = 0.015; OR, 1.74 from the Armitage trend test) and the homozygous genotype TT (P = 0.006; OR, 6.41 compared with the CC genotype). On the other hand, the major allele C was associated with decreased breast cancer risk (P = 0.015; OR, 0.66 from the Armitage trend test; and P = 0.0006; OR, 0.157 for allele positivity; Table 2). The associations were not significant within the OBCS set. However, they were found to be significant in the combined KBCP and OBCS sample set. In the KBCP + OBCS sample set, increased risk of breast cancer was significantly associated with the rs34197572 allele T (P = 0.044; OR, 1.30 from the Armitage trend test) and the homozygous genotype TT (P = 0.003; OR, 2.39 compared with the CC genotype), whereas the common allele C was associated...
rs9676881 and rs1048290 were in tight linkage disequilibrium with decreased breast cancer risk ($P = 0.044$; OR, 0.793 from the Armitage trend test; and $P = 0.003$; OR, 0.421 for allele positivity; Table 2). In addition, in the KBCP + OBCS sample set, the rs11085735 minor allele A was associated with PR-negative tumors ($P = 0.017$; OR, 1.612; 95% CI, 1.089–2.397; log reg; data not shown).

**KEAPI genotypes associated with KEAPI and NRF2 protein expressions**

Within the KBCP sample set, higher KEAPI protein expression was significantly associated with the KEAPI rs9676881 minor allele A ($P = 0.027$; OR, 2.507; 95%CI, 1.109–5.667 for rs9676881 AA) and rs1048290 minor alleles G ($P = 0.024$; OR, 2.545; 95%CI, 1.128–5.746 for rs1048290 GG; Supplementary Table S4). As rs9676881 and rs1048290 were in tight linkage disequilibrium (LD) with an $r^2$ value of 1, they most likely represent the same association. We also found that the KEAPI rs11085735 minor allele A was significantly associated with lower KEAPI protein expression ($P = 0.004$; OR, 2.445; 95% CI, 1.232–4.353; Supplementary Table S4), and with high NRF2 expression ($P = 0.009$; OR, 2.445; 95% CI, 1.232–4.853; Supplementary Table S5).

rs34197572 and rs8113472 associated with OS

To study the effect of the SNPs on the patients' response to breast cancer treatment, we included the treatment data in the survival analyses of the combined KBCP and OBCS cases. Among all invasive cases, the KEAPI rs34197572 minor allele T was associated with poorer OS ($P = 0.045$; HR, 1.304, Cox regression analysis; Fig. 1). When the analysis was limited to all those patients who received radiotherapy (RT), the minor allele T was also associated with OS ($P = 0.018$; HR, 1.486, Cox regression analysis; Fig. 2A), and the difference between genotypes was even more pronounced among the patients who received only RT ($P = 0.037$; HR, 1.948, Cox regression analysis; Fig. 2B). The rs8113472 minor allele A was associated with poorer OS among the patients who received RT ($P = 0.025$; HR, 1.455, Cox regression analysis; Fig. 2C), although this association did not reach statistically significance among all invasive cases ($P = 0.063$, data not shown). Again, the difference between the genotypes (major homozygous vs. minor allele carriers) was more pronounced among the patients receiving only RT ($P = 0.044$; HR, 1.820, Cox regression analysis; Fig. 2D).

Among patients who did not receive adjuvant therapy ($n = 187$), the rs34197572 and rs8113472 genotypes were not associated with survival. Within both SNPs, OS significantly differed between the treatment-defined strata (RT vs. no adjuvant therapy: $P = 0.024$ for rs34197572 and $P = 0.026$ for rs8113472; log-rank Kaplan–Meier analysis, data not shown). Among RT-treated cases, poorer OS was associated with the rs34197572 allele $T$ ($P = 0.009$ and rs8113472 allele A ($P = 0.015$, log-rank Kaplan–Meier analysis, data not shown). No such associations were found among patients who did not receive adjuvant therapy ($P = 0.818$ for rs34197572 and $P = 0.692$ for rs8113472, log-rank
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KEAP1 rs10482920 and rs9676881 associated with RFS

When the treatment data were included in the analysis of the combined KBCP and OBCS invasive cases, shorter RFS was significantly associated with the KEAP1 minor homozygous genotypes rs10482920 GC (P = 0.020; HR, 1.468) and rs9676881 AA (P = 0.024; HR, 1.452, Cox regression analysis; Fig. 3A and B). These genotypes were also significantly associated with shorter RFS among the ER-positive patients treated with tamoxifen (P = 0.011; HR, 2.139 for rs10482920 and P = 0.063 for rs9676881, Cox regression analysis; Supplementary Table S6). Among patients who did not receive adjuvant therapy (n = 186), the rs10482920 and rs9676881 genotypes were not associated with survival. For both SNPs, RFS significantly differed between the treatment-defined strata (ER-positive tamoxifen-treated vs. cases with no adjuvant therapy): P = 0.029 for rs10482920 and 0.037 for rs9676881 (log-rank Kaplan–Meier analysis, data not shown). The genotypes rs10482920 GC and rs9676881 AA were significantly associated with shorter RFS among tamoxifen-treated patients (P = 0.011 and 0.015, respectively, log-rank Kaplan–Meier analysis, data not shown) but not among patients who did not receive adjuvant therapy (P = 0.522 and 0.569, respectively, log-rank Kaplan–Meier analysis, data not shown).

Figure 1. Association of KEAP1 rs34197572 with OS among all invasive KBCP and OBCS cases. Multivariate analysis stratified by age at diagnosis, stage, ER status, PR status, adjuvant chemotherapy, radiotherapy, and hormone therapy. HRs of death with 95% CI in Cox regression survival analysis.

Kaplan–Meier analysis, data not shown). Multivariate analysis showed that the rs34197572 and rs8113472 genotypes were not associated with survival when analyzed separately in the KBCP and OBCS samples (Supplementary Table S6).

Figure 2. Association of KEAP1 rs34197572 and rs8113472 OS among invasive KBCP and OBCS patients who received radiotherapy. OS with rs34197572 genotypes (A) among patients who received radiotherapy and (B) among patients who received only chemotherapy. OS with rs8113472 genotypes (C) among patients who received radiotherapy and (D) among patients who received only chemotherapy. A and C, HRs of death with 95% CI in Cox regression survival analyses stratified by age at diagnosis, stage, ER status, PR status, adjuvant chemotherapy, and hormone therapy. B and D, HRs of death with 95% CI in Cox regression survival analyses stratified by age at diagnosis, stage, ER status, and PR status.
KEAP1 rs11085735 associated with BCSS

When treatment data were included, Cox multivariate survival analysis revealed that the KEAP1 rs11085735 minor allele A was associated with poorer RFS and BCSS among all invasive cases \( (P = 0.020; \text{HR}, 1.545; 95\% \text{CI}, 1.064–2.027) \) and among tamoxifen-treated cases \( (P = 0.024; \text{HR}, 1.452) \). Among ER-positive cases only in the KBCP cases, this association was also significant \( (P = 0.017; \text{HR}, 2.106) \), Cox regression analysis; Supplementary Table S7). Among the ER-positive combined KBCP and OBCS cases, the rs11085735 minor allele A was also associated with poorer breast cancer survival in univariate analysis \( (P = 0.007, \text{Kaplan–Meier analysis}; \text{Supplementary Fig. S4A and Table S8}) \) but not in the Cox multivariate analysis. Among the ER-positive cases only in the KBCP sample set, this association was also significant in the multivariate analysis \( (P = 0.017; \text{HR}, 2.106, \text{Cox regression analysis}; \text{Supplementary Tables S8 and S9 and Fig. S4B}) \). Among the KBCP cases with lower KEAP1 protein expression \( (<1.33) \), the rs11085735 minor allele A was associated with poorer breast cancer survival in univariate Kaplan–Meier analysis \( (P = 3.6 \times 10^{-5}; \text{Supplementary Table S8 and Fig. S5A}) \) and in multivariate analysis \( (P = 0.001; \text{HR}, 2.733, \text{Cox regression analysis}; \text{Supplementary Table S10 and Fig. S5B}) \). KEAP1 protein expression itself was not significantly associated with survival in Kaplan–Meier analysis, although a trend toward worse survival with lower KEAP1 protein expression was observed (Supplementary Fig. S6).

Discussion

Oxidative stress is linked with cancer development, and defensive oxidative stress rescue mechanisms can also play a role in increasing cancer susceptibility, potentially causing chemoresistance and affecting treatment outcome. KEAP1 is a regulator of NRF2, which is the main mediator of the redox stress response.

Figure 3.
Association of KEAP1 rs1048290 and rs9676881 with RFS in KBCP and OBCS cases. A, rs1048290 among all invasive cases. B, rs9676881 among all invasive cases. C, rs1048290 among ER-positive tamoxifen-treated patients with breast cancer. D, rs9676881 among ER-positive tamoxifen-treated patients with breast cancer. A and B, HRs of breast cancer recurrence with 95% CI in Cox regression survival analysis stratified by age at diagnosis, stage, ER status, PR status, adjuvant chemotherapy, radiotherapy, and hormone therapy. C and D, HRs of breast cancer recurrence with 95% CI in Cox regression survival analyses stratified by age at diagnosis, stage, PR status, and radiotherapy.
response. We previously reported that polymorphisms in the oxidative stress response pathway affect breast cancer risk and the survival outcome of patients with breast cancer (23). The present results show that genetic polymorphisms in KEAP1 also seem to affect the breast cancer risk and survival, further supporting the involvement of the NRF2 pathway in breast cancer. In particular, the investigated polymorphisms seemed to modify the effects of radiotherapy and tamoxifen treatment on patient survival. Implications of the modifying role of lower KEAP1 protein expression were also observed.

In 2 populations, we examined 5 tagging SNPs covering the KEAP1 gene region, along with KEAP1 protein expression by immunohistochemistry. We found that the minor alleles of 2 SNPs in KEAP1—rs9676881 and rs1048290—were associated with higher KEAP1 protein expression and shorter RFS. The association with RFS was observed among all invasive cases, and among ER-positive tamoxifen-treated cases. The SNP rs9676881 is located only 16 bp downstream from the 3'-untranslated region (UTR) of KEAP1 in a possible transcription factor–binding and enhancer region (34, 35). SNP rs1048290 is a synonymous variant in the KEAP1 coding region, which is located in the sequence encoding the beta strand B of blade IV in the Kelch-1 domain, residing 33 bp (11 codons) from the codon for the amino acid whose side chain contacts the NRF2-derived peptide in the crystal structure (36).

The rs1048290 and rs9676881 polymorphisms are in tight LD with each other ($r^2 = 1$, Ensembl, 1000Genomes data, Finnish population; refs. 35, 37) and could also be in LD with another functional variant that affects KEAP1 protein expression or function, such as a miRNA-binding site in the 3'UTR of KEAP1. Loss of miR-200a is reportedly correlated with KEAP1 upregulation and NRF2 downregulation in breast cancer cell lines (38). The KEAP1 3’UTR contains a binding site for miR-200a (TargetScan; ref. 39) that is 410 bp upstream from rs9676881, and rs9676881 and rs1048290 could be in LD with a variant that affects miR-200a binding, resulting in a higher KEAP1 level. Five other miRNA-binding sites are predicted in the KEAP1 3’UTR (miRNA.org; ref. 40). These SNPs appear to have the potential to affect KEAP1 protein level, although the effects on patient survival are probably very complex.

Antiestrogen agents have been reported to stimulate NAD(P)H:dehydrogenase, quinone 1 (NQO1) transcription and to protect against estrogen-induced DNA damage in estrogen-dependent breast cancer cells (41, 42). Tamoxifen acts as a selective estrogen receptor modulator that binds the NQO1 promoter and activates NQO1 gene transcription in breast cancer cells (41, 42). Thus, tamoxifen has been proposed as a possible chemopreventive agent for use against breast cancer. However, after tumor development and the use of tamoxifen, cancer cell survival could actually be promoted, as tamoxifen induces NQO1, which would protect the cancer cells against estrogen-induced DNA damage. Tamoxifen can also induce oxidative stress (43–45), which would then activate the NRF2 pathway. Estrogen also reportedly acts on the ERα ligand-binding domain and inhibits NQO1 promoter activity; therefore, inhibition of estrogen signaling leads to activation of NRF2-dependent NQO1 transcription (46). Breast cancer cells transfected with ERα shRNA exhibit reduced nuclear NRF2.
localization as well as cytoplasmic KEAP1 accumulation, which would bind any remaining NRF2, preventing antioxidant response and allowing ROS generation (47). Among patients with endometrioid endometrial adenocarcinoma who received adjuvant (CT and/or RT) treatment, heterozygous rs1048290 minor allele carriers (analyzed from tumor tissue) have previously been found to show shorter progression-free survival compared with CC homozygotes (25). Furthermore, in ovarian cancer, KEAP1 downregulation by miR-141 reportedly induces cisplatin resistance, whereas KEAP1 overexpression significantly enhances cisplatin sensitivity (48). However, this did not lead to activation of NRF2 target genes but rather to activation of the NF-κB pathway.

The polymorphism rs34197572 is downstream of the KEAP1 3'UTR and was also associated with breast cancer risk. SNP rs34197572 was not associated with KEAP1 protein level but can be connected to breast cancer susceptibility via the ROS scavenging/NRF2 pathway, as impaired ROS response increases cancer risk. SNP rs34197572 was also associated with OS among all cases of invasive breast cancer, as well as among those treated with RT and those treated with only RT. Review of the previously published data revealed no direct functional consequences for this variation. Approximately 700 bp upstream of rs34197572, there is a possible transcription factor-binding site (Encode; ref. 49). It is possible that rs34197572 is in LD with another functional variant, which could explain a potential connection with breast cancer risk and survival. This SNP could also impact survival via another mechanism, independent of oxidative stress.

The SNP rs8113472 was associated with OS among all invasive cases, including those treated with RT and those treated with only RT. This SNP was not associated with KEAP1 protein expression. The SNP rs8113472 is located in intron 1 of KEAP1. It has not been reported to impact any regulatory regions or having functional consequences. Therefore, the functional variant explaining this association with survival remains to be elucidated.

For the final studied SNP rs11085735, the minor allele A was associated with lower KEAP1 protein expression and increased nuclear NRF2 expression. rs11085735 resides in intron 2, which intervenes with the exon sequence encoding the Kelch-1 domain (between the B and C beta strands of blade III). As the SNP is located near a coding region of a functional element of the protein, it may be in LD with a variant that affects the Kelch-1 domain structure and thus affects the interaction between KEAP1 and NRF2. Impairment of the affinity of KEAP1 for NRF2 could potentially enhance NRF2 accumulation in the nucleus. Increased nuclear NRF2 expression implies that NRF2 is activating genes for an ROS response and therefore facilitating the cancer cell survival and resulting in poorer patient survival. Indeed, the rs11085735 minor allele was associated with poorer BCSS and DFS among all invasive cases, poorer OS among RT-treated patients, and shorter DFS among tamoxifen-treated patients. Cases with lower KEAP1 protein expression and the rs11085735 A allele also showed poorer breast cancer survival. One intriguing possibility is that impaired interaction of KEAP1 and NRF2 in patients with the rs11085735 minor allele A shows an enhanced effect of ROS scavenging. This would result in lower KEAP1 protein expression and higher nuclear NRF2, which were both observed to be associated with this allele, and would lead to poorer patient survival.

Overall, here we report that in certain treatment groups, these presently studied SNPs seem to worsen the prognosis of patients with breast cancer, thus supporting the possible involvement of the NRF2/KEAP1 pathway. RT, estrogen, and tamoxifen each increase oxidative stress, which activates NRF2 function (13, 22, 43–45, 50). Enhanced ROS scavenging in tumors could lead to cancer recurrence or poor survival. None of the investigated SNPs showed significant effects within the group of patients who did not receive any adjuvant therapy, implying that these SNPs specifically modified the effects of radiotherapy and tamoxifen treatment on patient survival.

In conclusion, here we report that genetic variants in the KEAP1 gene were associated with the outcome of patients with breast cancer. One of these variants, rs34197572, was also associated with risk of breast cancer. These SNPs may cause defects in the oxidative stress rescue mechanisms. Impaired response to oxidative stress causes prolonged exposure of the tissues to damaging agents, thus increasing cancer susceptibility. On the other hand, if the stress response system is functioning in the cancer tissue in response to damage caused by therapy, it could also help the cancer cells survive. The present findings support the hypothesis that the KEAP1/NRF2 pathway plays a role in breast cancer. Further studies are needed to identify the functional variants affecting KEAP1 protein expression and interaction with NRF2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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