Association of NCOA3 Polymorphisms with Breast Cancer Risk

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ABSTRACT

The nuclear receptor coactivator 3 (NCOA3), also known as AIB1, is a coactivator of nuclear receptors like the estrogen receptor. NCOA3 is overexpressed in ~60% of primary human breast tumors, and high levels of NCOA3 expression are associated with tamoxifen resistance and worse survival rate. In contrast, NCOA3 deficiency suppresses v-Ha-ras–induced breast cancer initiation and progression in mice. Here, we analyzed the influence of NCOA3 coding single nucleotide polymorphisms on breast cancer risk by performing a case-control study using a German and a Polish study population and identified an association between NCOA3 polymorphisms and breast cancer. A joint analysis of the German and the Polish study population revealed a significant protective effect for the

1758G>C (Q586H) and 2880A>G (T960T) variants. In addition, haplotype analysis showed a protective effect of the 1758C-2880A and 1758G-2880G haplotypes (odds ratio 0.79; 95% confidence interval, 0.67-0.93; P = 0.004). Because of the impact of NCOA3 in antiestrogen therapy resistance, these polymorphisms might also influence therapy outcome in breast cancer.

INTRODUCTION

Breast cancer is the most frequent cancer among women in developed countries (1). Familial breast cancer constitutes ~10% of total breast cancer (2, 3). Among these, BRCA1 and BRCA2 mutations account for about 20%, and ATM, TP53, and CHEK2 mutations or variants for a minor percentage of breast cancer. Thus, the majority of breast cancer susceptibility genes still remain to be discovered.

Ovarian steroids are strong risk factors for the initiation and progression of breast cancer. Clinical and animal studies have showed that depletion of ovarian steroids or abortion of estrogen receptor (ER) α significantly reduces breast cancer risk (4, 5). Therefore, therapies that inhibit estrogen synthesis or block ER are used for breast cancer treatment (6). ERα is a member of the nuclear receptor family, a group of hormone-inducible transcription factors, which activates gene expression through recruiting multiple coactivators. The nuclear receptor coactivator 3 (NCOA3, also known as AIB1, ACTR, TRAM1, p/CIP, or SRC3) interacts with numerous nuclear hormone receptors like ERα, thyroid hormone receptor, and progesterone receptor (PR) to enhance their transcriptional activation (7–9). NCOA3 is a member of the p160/steroid receptor coactivator family. Like other family members NCOA3 associates with the transcription factor CREB binding protein and possesses histone acetyltransferase activity (9). NCOA3 is overexpressed in ~60% of primary human breast tumors as a result of transcriptional up-regulation or gene amplification (10–13). Overexpression of NCOA3 has been detected in breast tumors positive and negative for ER and PR (14). Similarly, amplification and up-regulation of NCOA3 has been observed in ovarian, pancreatic, and gastric cancers (10, 15, 16). Furthermore, NCOA3 overexpression in invasive breast tumors has been correlated with high levels of human epidermal growth factor receptor 2 (HER2 or ERBB2) and unfavorable antiestrogen therapy outcome (14, 17).

Recent studies have uncovered that the function of NCOA3 is not restricted to nuclear hormone receptors but that NCOA3 also regulates other transcription factors such as TP53 (18), NFκB (19), and ERβ (18, 20, 21). Moreover, NCOA3 deficiency affects the insulin-like growth factor 1 signaling pathway and suppresses v-Ha-ras–induced breast cancer initiation and progression in mice (22). Increased numbers of polyglutamine repeats in the NCOA3 protein have been shown to correlate with higher breast cancer risk in BRCA1 and BRCA2 mutation carriers (23, 24), whereas the repeat length...
does not alter the breast cancer risk among unselected postmenopausal women with breast cancer (25) or breast cancer cases (26).

The present study investigates, for the first time, the influence of *NCOA3* coding single nucleotide polymorphisms (SNP) on breast cancer risk by performing a case-control study on a German and a Polish study population and identifies an association between *NCOA3* single polymorphisms and breast cancer.

**MATERIALS AND METHODS**

**Samples.** A case-control study was done using a German and a Polish study population. The cases in both studies were unrelated, female, *BRCA1/2* mutation-negative individuals with breast cancer. Breast cancer cases were selected according to the criteria that are used for *BRCA1* and *BRCA2* mutation screening. By doing so, we accumulated familial cases and early-onset cases, which are more likely to be due to a genetic cause, in our study population. The controls were chosen from the same geographic area and ethnic background as the breast cancer cases.

The German breast cancer cases were classified into six categories based on family history: (A1) families with two or more cases of breast cancer including at least two cases with onset under the age of 50 years (134 cases); (A2) families with at least one male breast cancer case (5 cases); (B) families with one or more cases of breast cancer and at least one ovarian cancer (54 cases); (C) families with two or more cases of breast cancer including one case diagnosed before the age of 50 years (16 cases); (D) families with one or more cases of breast cancer diagnosed after the age of 50 years (133 cases); and (E) a single case of breast cancer with diagnosis before the age of 35 years (16 cases; ref. 27).

Inclusion criteria for the Polish breast cancer cases were (a) at least two first-degree relatives with breast and/or ovarian cancer regardless of age, (b) breast cancer diagnosed below the age of 35 years without family history, (c) bilateral breast cancer regardless of family history, (d) breast and ovarian cancer diagnosed in one patient regardless of family history (category 1-4, 297 cases), (e) breast cancer diagnosed below the age of 50 years regardless of family history (133 cases; ref. 28).

The analysis was done by using genomic DNA of 357 German breast cancer cases and 1,195 German controls as well as genomic DNA of 434 Polish breast cancer cases and 449 Polish controls, resulting in a total of 791 cases and 1,644 controls analyzed. The study was approved by the ethics committee of the University of Heidelberg, Heidelberg, Germany.

**Single Nucleotide Polymorphism Verification.** For the verification of annotated SNPs from the dbSNP database (National Center for Biotechnology Information), PCR products of the respective regions using genomic DNA of 22 to 23 randomly chosen breast cancer cases were generated and sequenced. Primer sequences were designed based on sequences NT_011362 and NM_006534 and are available upon request.

**Genotyping.** The polymorphisms Q586H (G>C) and T960T (A>G) were investigated using TaqMan allelic discrimination. Primer and TaqMan MGB probes were purchased from Applied Biosystems (Foster City, CA): SNP Q586H: forward 5'-CTGGGCTTTATGCGACCAA-3', reverse 5'-GCTCTCCTTACCTTCTGGTACCTGA-3'; TaqMan probes: forward 5'-TTCAATGTCATCTCAAAAT-3' VIC, reverse 5' -CAATGTCATCTCAAAAT-FAM; SNP T960T: forward 5'- CCTGCAGTGTTGCT-3', reverse 5'-CTCGCACCCTGG-TATGCTATTAGAC-3'; TaqMan probes: forward 5'-CTATTCCTCACATTGCCCT-3' VIC, reverse 5'-TTCCCACTTGCCTC-3' FAM. Five nanograms genomic DNA were used per assay. PCR was done at 50°C for 2 minutes, 95°C for 10 minutes, and 40 to 45× (92°C, 15 seconds, and 60°C, 60 seconds). Samples were analyzed with the ABI Prism 7900HT detection system using SDS 1.2 software (Applied Biosystems). At least 8% of all genotyping results including all rare homozygous genotypes were confirmed by sequencing as described above.

**Statistical Analysis.** Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for genotype and haplotype frequencies between breast cancer cases and controls using logistic regression adjustment for country. The logistic regression analysis was done using SAS Version 8.2. Haplotypes for the Q586H and T960T polymorphisms were determined using the PHASE 2 software created by Stephens et al. (http://archmedes.well.ox.ac.uk/pise/PHASE-simple.html). Power calculation was carried out using power and sample size calculation software PS version 2.1.31 (http://www.mc.vanderbilt.edu/prevmid/ps/). With the present sample size and a carrier frequency of 0.2 in the control population, we had a power of 90% to detect an OR of about 0.65. Calculations for Hardy-Weinberg equilibrium were carried out using the Hardy-Weinberg equilibrium tool offered by the Institute of Human Genetics, Technische Universität, Munich, Germany (http://ihg.gsfc.nasa.gov/cgi-bin/hw/hwa1.pl).

**RESULTS**

To evaluate public database SNPs in the coding region of the *NCOA3* gene we sequenced 22 to 23 randomly chosen breast cancer samples (44-46 alleles). Out of the 12 polymorphisms listed, 5 could be detected in our sample set: rs6094740 R218C (3/46), rs6018604 intron 9 (2/46), rs609475S P355L (1/46), rs6094756 L369F (0/46), rs6125065 P382L (0/46), rs6125057 A416A (0/46), rs1052765 G460R (0/46), rs2230781 P559S (0/46), rs2230782 Q586H (6/44), rs2664573 G904G (0/44), rs2076547 A927A (1/44), and rs2076546 T960T (6/44). The polymorphisms Q586H (1758G>C) and T960T (2880A>G) were confirmed by sequencing as described above. The rare variants of these two SNPs were not linked to each other. The genotype distributions of both polymorphisms were consistent with Hardy-Weinberg equilibrium in both populations analyzed. Furthermore, the results of 8% resequenced samples including 100% of the rare homozygous calls were in complete agreement with the genotyping results.

**NCOA3 Variants and Risk of Breast Cancer.** Regarding the Q586H (1758G>C) variant, the rare genotypes CC and GG were more frequent in controls compared with cases (Table 1). In the German population, the difference in genotype frequency was significant (OR, 0.70; 95% CI, 0.51-0.97; P = 0.034) and
In silico Analysis. A WU-Blastp search indicates that Gln586 is highly conserved not only among orthologous but also among homologous genes, with one surprising exception (Table 2). The rat NCOA3 protein exhibits proline at this position, whereas the homologous proteins NCOA1 and NCOA2 in rat also exhibit glutamine. Gln586 is located 35 amino acids upstream of the first LXXLL motif that has been shown to be essential for nuclear receptor binding.

Investigating a putative functional relevance of the T960T (ACA->ACG) showed that this variant does not seem to affect sequence motifs like exonic splice site enhancers, exonic splice site silencers, and splice site donor or acceptor motifs. RNA stability modeling, using MFOLD (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html), revealed no significant difference in RNA stability between the polymorphic variants either. However, comparing codon usage preference for threonine in NCOA3 (NM_006534, determined using DNASTAR Lasergene Software) versus the codon usage preference in the Homo sapiens Codon Usage Database (hs_CUD, ftp://ftp.kazusa.or.jp/pub/codon/current/species/Homo_sapiens.pri, including 41,507 human coding DNA sequences) showed a distinct shift from ACG (0% usage in NCOA3 versus 12% in hs_CUD) toward ACA (40% usage in NCOA3 versus 28% in hs_CUD; Table 3). Interestingly, ACAG is the only codon that is never used in NCOA3 wild-type. Moreover, we analyzed all 61 codons encoding the 23 different amino acids in humans by the same trend was detected in the Polish population. Logistic regression analysis of the two populations confirmed the protective effect of 1758G>C (OR, 0.79; 95% CI, 0.67-0.93; \( P = 0.035 \); Table 1). An even stronger effect of the rare 1758C variant could be detected in German and Polish populations when comparing the rare homozygous frequency CC versus the genotype frequencies GG and GC in cases and controls (German population: OR, 0.40; Polish population: OR, 0.38). Logistic regression analysis of both populations results in an OR of 0.39 (95% CI, 0.14-1.05). However, because the number of rare homozygous carriers was very low, this effect is at the borderline of significance (\( P = 0.061 \)). Likewise, the rare variant of the T960T (2880A>G) polymorphism was more frequent in controls compared with cases in both populations. Regression analysis of both populations results in an OR of 0.78 (95% CI, 0.63-0.99, \( P = 0.045 \)). The rare variants of both SNPs analyzed were not linked to each other. Instead, the rare variant 2880G was observed to be linked to the common variant of the 1758G>C polymorphism. Thus, the theoretically most preventive haplotype 1758C-2880G (C-G) did not exist. Comparison of the haplotype 1758G-2880A (G-A) versus all other protective haplotypes [1758G-2880G (G-G) and 1758C-2880A (C-A)] showed that protective haplotypes [1758G-2880G (G-G) and 1758C-2880A (C-A)] were more frequent in controls compared with cases. A joint analysis of both populations resulted in an OR of 0.79 (95% CI, 0.67-0.93; \( P = 0.004 \); Table 1).
the frequencies in the hs_CUD. Surprisingly, all codons—except AUG, because it is the only codon for methionine and UGG, as it is the only codon for tryptophan—show a codon usage shift in favor of A/T instead of G/C at the third codon position in NCOA3 compared with the average use in the human Coding Sequence Database (data not shown). Thus, the ACA>ACG polymorphism provokes a shift from the preferred codon ACA in NCOA3 to the less preferred codon ACG as well as a shift from the preferred A/T at the third position in NCOA3 to the less preferred G/C. Some unproven hypothesis suggests that aberrations from the preferred codon usage influence translational and/or transcriptional efficiency as well as the translational accuracy (29–31).

DISCUSSION

To investigate the influence of NCOA3 coding single nucleotide polymorphisms on breast cancer risk, we did a case-control study using a German and a Polish study population. NCOA3 was selected as a candidate gene because its high impact in breast cancer has been shown in several studies. It is overexpressed in ~60% of primary human breast tumors (10–13) and high levels of NCOA3 expression are associated with tamoxifen resistance and worse survival rate (17). In contrast, NCOA3 deficiency suppresses v-Ha-ras–induced breast cancer initiation and progression in mice (22).

We accumulate familial cases for our study because it has been shown that the use of familial cases in case-control studies significantly increases the power to detect rare alleles contributing to risk or protective effects in breast cancer (32, 33). A substantially lower sample size is required for a study using familial breast cancer cases to achieve the same power as compared with a study using unselected cases. For instance, a familial breast cancer cases to achieve the same power as comparing the codon usage frequencies in NCOA3 versus the frequencies in the hs_CUD. Surprisingly, all codons—except AUG, because it is the only codon for methionine and UGG, as it is the only codon for tryptophan—show a codon usage shift in favor of A/T instead of G/C at the third codon position in NCOA3 compared with the average use in the human Coding Sequence Database (data not shown). Thus, the ACA>ACG polymorphism provokes a shift from the preferred codon ACA in NCOA3 to the less preferred codon ACG as well as a shift from the preferred A/T at the third position in NCOA3 to the less preferred G/C. Some unproven hypothesis suggests that aberrations from the preferred codon usage influence translational and/or transcriptional efficiency as well as the translational accuracy (29–31).

The polymorphism Q586H (1758G>C) that results in a nonconservative amino acid exchange was chosen for analysis because of its putative functional effect. A WU-Blastp alignment search indicates that Gln586 shows a high degree of conservation in orthologous and homologous genes (Table 2). Gln586 is located 35 amino acids upstream of the first LXXLL motif that has been shown to be essential for nuclear receptor binding. As it had been shown that silent SNPs also have the capability to be functionally relevant (35) and to perform haplotype analysis, the T960T (2880A>G) polymorphism was additionally chosen for analysis. This polymorphism causes an aberration from the preferred codon usage for threonine in NCOA3 (Table 3). The rare variants of both polymorphisms were not linked to each other. A joint analysis of both study populations revealed a significantly decreased breast cancer risk for both polymorphisms analyzed. Moreover, haplotype analysis showed a protective effect of the 1758C-2880A and 1758G-2880G haplotypes (OR, 0.79; 95% CI, 0.67-0.93; P = 0.004).

We hypothesize that Q586H may alter the structure of NCOA3 and that T960T (ACA>ACG)—due to an alteration in codon usage—may decrease NCOA3 transcription and/or translation; thus, both polymorphisms are suggested to reduce NCOA3 function. However, functional studies are needed to clarify the particular functional effects of these variants. The protective effects of both polymorphisms regarding breast cancer that we have found in the present study are biologically motivated because a depressed ER function would be expected to be associated with a decreased breast cancer risk. These data are in agreement with the observations in mice in which NCOA3 deficiency affects insulin-like growth factor 1 signaling pathway and suppresses v-Ha-ras–induced breast cancer initiation and progression in mice (22).

### Table 2. WU-Blastp analysis of the human NCOA3 protein sequence

<table>
<thead>
<tr>
<th>NCOA3</th>
<th>Species</th>
<th>Accession</th>
<th>Description</th>
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<td>Q9Y6Q9</td>
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<tr>
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<td>O57539</td>
<td>EIQQVSSVCQQSGREHLEKDVKE</td>
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<td>Q15596</td>
<td>E—PSEGTTGQAESSCHPEKEKND</td>
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<tr>
<td>NCOA2</td>
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<td>Q61026</td>
<td>E—PSEGTTGQAESSCHPEEIQGKD</td>
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<td>E—PSEGTTGQAASSCHPEIQGKD</td>
</tr>
<tr>
<td>NCOA2</td>
<td>Xenopus</td>
<td>Q9W705</td>
<td>EEQP-ESATGGGEGCHSNEQNL</td>
</tr>
<tr>
<td>NCOA1</td>
<td>Human</td>
<td>O43792</td>
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</tr>
<tr>
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<td>NCOA1</td>
<td>jap. Quail</td>
<td>Q8UVH3</td>
<td>EIASILNEMIQSDGG—AGDGKPLD</td>
</tr>
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**NOTE.** WU-Blastp analysis of the human NCOA3 protein sequence using the European Molecular Biology Laboratory-EBI server (http://www.ebi.ac.uk/blast2; MVIEW). Gene isoforms, species, uniprot numbers (the model protein XP_233944 for rat NCOA1 is shown as no other sequence is available yet), and protein sequences next to Q586H are shown, illustrating the high conservation of Q586.

### Table 3. Codon usage frequencies for threonine in NCOA3

<table>
<thead>
<tr>
<th>NCOA3</th>
<th>Hs codon usage database</th>
</tr>
</thead>
<tbody>
<tr>
<td>22× ACU</td>
<td>0.35 0.24</td>
</tr>
<tr>
<td>16× ACC</td>
<td>0.35 0.36</td>
</tr>
<tr>
<td>25× ACA</td>
<td>0.40 0.28</td>
</tr>
<tr>
<td>0× ACG</td>
<td>0.00 0.12</td>
</tr>
<tr>
<td>63× Threonine</td>
<td></td>
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</table>

**NOTE.** Codon usage frequencies for threonine in NCOA3 are shown compared with the Homo sapiens Codon Usage Database (accessed April 10, 2002 from ftp://ftp.ncbi.nih.gov/pub/codon/current/species/Homo_sapiens.pr) including 41,507 human coding DNA sequences. The ACA>ACG polymorphism provokes a shift from the preferred codon ACA in NCOA3 to the less preferred codon ACG.
It would be valuable to investigate if these NCOA3 polymorphisms affect the risk of other cancers like ovarian cancer and also others, such as pancreatic, gastric, or hepatocellular cancer, in which NCOA3 has been shown to be amplified or overexpressed, and in which steroid hormones such as estrogens are unlikely to play a critical role (15, 16, 36). In these cancers the coactivator function of NCOA3 on transcription factors different from steroid hormone receptors like TP53, NfκB, or E2F1 are expected to be most important (18, 19, 37).

Therapeutic agents that reduce the estrogen level or compete with ER, such as tamoxifen, have contributed at least in part to the markedly decrease in deaths from breast cancer over the past decade (38). Nevertheless, many breast cancers either fail to respond or become resistant to these therapies. NCOA3 plays a major role in the development of resistance. In tamoxifen-treated patients, high levels of NCOA3 expression are associated with tamoxifen resistance and worse survival rate. Patients with high levels of both NCOA3 and epidermal growth factor receptor 2 (HER2 or ERBB2) exhibit the worst responses to tamoxifen therapy (17). Potentially, NCOA3 associates with ER independently of estrogen, which is mediated by epidermal growth factor—mediated phosphorylation of ER and/or NCOA3 (39). Recently, lines of evidence have been found that NCOA3 also functions as a coactivator of one of the key cell cycle regulators, E2F1, and thus might contribute—a independent of ER—to therapy resistance (37). As a result, it will be helpful to investigate whether the NCOA3 polymorphisms analyzed in this study influence antiestrogen therapy outcome as well.

ACKNOWLEDGMENTS

We thank K. Wagner and S. Wilkeme for sample preparation.

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3. Hemminki K, Granstrom C, Czene K. Attributable risks for familial breast cancer and also others, such as pancreatic, gastric, or hepatocellular cancer. In which NCOA3 has been shown to be amplified or overexpressed, and in which steroid hormones such as estrogens are unlikely to play a critical role (15, 16, 36). In these cancers the coactivator function of NCOA3 on transcription factors different from steroid hormone receptors like TP53, NfκB, or E2F1 are expected to be most important (18, 19, 37).
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