A Novel Polymorphism in the Promoter Region of ERBB4 Is Associated with Breast and Colorectal Cancer Risk

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Abstract

Purpose: The receptor tyrosine kinase ERBB4/HER4 plays a role in cell division, migration, differentiation, as well as apoptosis, and is frequently overexpressed in breast and colorectal tumors. To understand the role of genetic variations in the regulation of ERBB4 expression, we identified new polymorphisms and investigated their functional implication and risk association with breast and colorectal cancer.

Experimental Design: We screened colorectal tumors from 92 patients for genetic variants at the ERBB4 ATG -1000 bp 5′ regulatory region by denaturing high-performance liquid chromatography and sequencing. Variants were subjected to DNA-protein interaction analyses (electrophoretic mobility shift assay), reporter gene assays in breast cancer cell lines MDA134 and MDA157, and immunohistochemical analyses of breast tumors. We established genotypic frequencies within a breast cancer case-control collection (1,021 cases, 1,015 population-based controls) and a colorectal cancer case-control collection (459 cases, 569 blood donors) using matrix-assisted laser desorption ionization/time of flight mass spectrometry. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were assessed by multivariate logistic regression.

Results: We identified five new germ line variants -815 A>T, -782 G>T, -638 insTC, -267 C>G, and -219 del10bp. Two variants showed in vitro functional effects. The -782T allele showed lower protein binding affinity and lower promoter activity compared with the -782G allele, however, the -815T allele showed higher protein binding affinity and higher promoter activity. The -782T variant was identified as a risk allele for breast and colorectal cancer (OR, 1.59; 95% CI, 1.06-2.34 and OR, 2.21; 95% CI, 1.22-3.99, respectively).

Conclusion: The ERBB4 -782 G>T polymorphism, by virtue of its in vitro functional implication and incidence, is a risk factor for breast and colorectal cancer.

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Note: Supplementary data for this article is available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/). Requests for reprints: Hiltrud Brauch, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Auerbachstrasse 112, 70376 Stuttgart, Germany. Phone: 49-711-8101-3705; Fax: 49-711-85-9295; E-mail: hiltrud.brauch@ikp-stuttgart.de.

The ERBB4 protein, also known as HER4, is a member of the epidermal growth factor (EGF/ERBB) family of receptor tyrosine kinases, which are key activators of signaling pathways involved in cell division, migration, adhesion, differentiation, and apoptosis (1). ERBB4 participates together with other members EGFR/ERBB1/HER1, ERBB2/HER2, and ERBB3/HER3 in ERBB signaling of which the mitogen-activated protein kinase and phosphatidylinositol-3-kinase – activated AKT pathways are the most prominent targets (1). Aberrant ERBB signaling is a frequent phenomenon of many human cancers with receptor tyrosine kinase overexpression correlating with the oncogenic potential (1). Particularly, EGFR and ERBB2 have well-characterized roles in carcinogenesis and prognosis (2). Both receptor tyrosine kinases are frequently overexpressed in tumor tissues (3), and cancer patients whose tumors have EGFR or ERBB2 alterations tend to have a more aggressive disease associated with variables predicting a poor outcome (2, 4).

A detailed biochemical understanding and availability for extracellular manipulation made them attractive targets for pharmacologic intervention. As of today, a therapeutic monoclonal antibody for the inhibition of ERBB2, i.e., Herceptin...
that variants of the ERBB4 gene expression are available. Because genetic variants in the 5'-regulatory region of EGFR have been shown to alter promoter activity and gene expression in vitro and in vivo (15), there is a possibility that variants of the ERBB4 5'-regulatory region may also exert such a role. We therefore screened the ERBB4 ATG -1000 bp including the 5'-untranslated region with the transcriptional starting site at -98 for the presence of novel genetic variants and studied their functional consequences as well as potential roles in breast and colorectal cancer.

**Materials and Methods**

**Mutation analyses and identification of germ line variants at the ERBB4 5'-regulatory region.** Tumor DNA from 92 patients with primary colorectal adenocarcinoma was isolated by standard phenol chloroform extraction. PCR was used to amplify the 1 kb ERBB4 5'-regulatory region by three independent reactions. Fragments spanning ATG -1009 to -678 (primers, 5'-CGGCCTTGATGCTGT-3' and 5'-GGGGTAAAGCTGCG-3'; annealing temperature, 55°C) and ATG -701 to -261 (primers, 5'-GACTCGAGGGAAGTCT-3' and 5'-CAGATCCCG-3') were amplified using Qiagen hotstart polymerase (Qiagen). The ATG -313 to +77 fragment (primers, 5'-AGGGAGCGAGCAAGCGAAGG-3' and 5'-GAATGCGTGGGGGCTTCGGCGG-3'; annealing temperature, 64°C) was amplified using FailSafe kit (buffer J; Epicentre). Reactions were carried out according to the manufacturer's instructions. PCR products were subjected to denaturing high-performance liquid chromatography analyses using the Transgenomic WAVE DNA Fragment Analysis System and WaveMaker software (Transgenomic). Chromatograms of each fragment were analyzed and amplifiers with variant elution profiles were subsequently sequenced to confirm putative sequence variations. For this, PCR products were purified with Qiagen PCR purification kit (Qiagen), sequencing reactions were carried out with the ABI PRISM Big Dye terminator cycle sequencing kit 1.1 and sequence analysis was done with an ABI prism 310 automatic sequencer (Applied Biosystems). All ERBB4 mutations identified in tumor tissue were analyzed accordingly in corresponding normal tissue.

**Plasmid constructs.** For the construction of a recombinant plasmid, the ERBB4 5'-regulatory region (-1006 to -5) was amplified from human genomic DNA with forward primer 5'-GAACCGCTTCCG- TAAAGGCCCCCTCC-3' and reverse primer 5'-AGACTCAGGAAAAGTCTCGAATCCCGC-3'. These oligonucleotides introduced a MluI and XhoI restriction site at the 5'- and 3'-ends of the amplified fragment, respectively. The PCR product was digested with XhoI and MluI (GeneCraft) and cloned into appropriately digested luciferase reporter gene vector pGL3-basic (Promega). The identity of the cloned ERBB4 5'-regulatory region was verified by sequencing. Variants of the 5'-regulatory region representing the genetic variants of interest were generated by introducing point mutations using Quickchange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations.

**Transient transfections and reporter gene assays.** Reporter gene assays were carried out for the investigation of differential promoter activities of variant ERBB4 alleles according to Burk et al. (16). Two breast cancer cell lines MDA134 (ER positive) and MDA157 (ER negative) were used for transfection. Statistical significance was determined using one-way ANOVA test with Dunnett's post-test and P < 0.01 was considered statistically significant. The statistical tests were done by GraphPAD Prism version 3.03 software (GraphPad Software, Inc.).

**Electrophoretic mobility shift assays.** DNA-protein interaction was investigated by electrophoretic mobility shift assay (EMSA). The nuclear extract from the MDA134 BC cell line was prepared as described in Schreiber et al. (17). We used the transcription factor binding sites prediction software (TFSEARCH, version 1.3; ref. 18) to predict proteins that might bind to the ERBB4 promoter regions containing the variants under investigation and identified Sp1 as a potential candidate. Therefore, nuclear extract or recombinant Sp1 protein (Promega) were used as protein sources in the EMSA experiments. The sequences of the oligonucleotide probes used are listed in Supplementary Table S1. The sense and antisense oligonucleotides of the respective probes were annealed by incubation at 99°C for 5 min and slow cooling to 25°C. Double-stranded probes were labeled with Cy5.5-dCTP (Amersham Biosciences) by Klenow polymerase (Merck Millipore) for 2 h at 37°C and cleaned by Sephadex G50 (Amersham Biosciences) column purification. Gel retardation assays were carried out as follows: nuclear extract or recombinant Sp1 protein was preincubated with 1 µg of pdIdC in Goodwin binding buffer containing 10 mmol/L of HEPES, 60 mmol/L of KCl, 0.2% NP40, 2.5% Ficoll, 2 mmol/L of DTT for 10 min at room temperature in the absence or presence of specific competitors (unlabeled oligonucleotide probes) or 2 µg of Sp1 antibody (Sp1 mouse monoclonal antibody sc-17824X; Santa Cruz Biotechnology). The labeled probe (1 ng) was added and incubated for 20 min at room temperature. Samples were loaded on a 30-min prerun native 6% polyacrylamide gel and electrophoresis was carried out at room temperature in 0.5x Tris-borate EDTA buffer at 80 V for 80 min. Gel imaging was carried out using Odyssey Infrared Imaging System (LI-COR) at a wavelength of 700 nm.
**Immunohistochemical ERBB4 analysis.** Immunohistochemical stainings were done on 4-µm tissue sections of formalin-fixed paraffin-embedded breast tumors using a 1:50 dilution of monoclonal anti-ERBB4 (HFR-1) mouse monoclonal antibody (Labvision). Immunostaining and scoring was done as described elsewhere (19). Stained tissues were evaluated by an expert pathologist who was unaware of the clinopathologic data and genotype. The differences in mean expression with respect to the genotype were calculated using a nonparametric t test. Statistical tests were done using GraphPAD Prism 3.03 software (GraphPad Software).

ERBB4 promoter polymorphisms and investigation of an associated breast and colorectal cancer risk. A colorectal case-control collection from Slovenia and the German population–based breast cancer case-control collection GENICA were investigated. The colorectal case-control collection consisted of 459 patients with histologically confirmed colorectal carcinoma collected at the Institute of Oncology, Ljubljana between 1996 and 2004, and 569 unrelated blood donors collected at the Blood Transfusion Center of Slovenia (20). Genomic DNA from patients were isolated from snap-frozen normal colonic mucosa using NucPrep chemistry (Applied Biosystems) and of controls from blood mononuclear cells using Puregene kit (Gentra Systems, Inc.). Patient and control characteristics are listed in Table 1. All study participants were of European descent. The GENICA study was approved by the Ethics Committee of the University of Bonn, Germany, and the colorectal cancer study was approved by the National Medical Ethics Committee of the Republic of Slovenia.

Germline variants ERBB4 -815 A>T, -782 G>T, -718 C>T, -638 insTC, -609 G>A, -267 C>G, and -219 del10bp were genotyped to establish allele and genotype frequencies in both case-control collections. Genotyping was carried out with matrix-assisted laser desorption ionization/time of flight mass spectrometry (21, 23) using SpectroCHIP microarray and Bruker Autoflex (Sequenom) as well as MTP Anchor Chip 400/384 TF and Bruker Ultraflex (Bruker Daltonics). All assays were carried out in 384-well plates including four negative controls with no DNA and duplicates of 10% of the samples as a quality control. The position of polymorphisms is reported relative to the initiator ATG (with A as +1) codon. Genotype frequencies and odds ratios (OR) with 95% confidence intervals (CI) were calculated using GraphPad InStat software, version 3.06 (GraphPad Software), and checked for Hardy-Weinberg equilibrium according to Pearson’s χ² test. All OR and P values were calculated from a 2×2 table based on the frequency of each genotype versus the frequency of the major genotype between cases and controls. Multivariate logistic regression analyses were used to obtain adjusted ORs and 95% CIs. Because there was a significant difference with respect to breast cancer risk...
control status for the variables family history of breast cancer and hormone replacement therapy usage (Table 1), we adjusted for these variables in the multivariate analysis. Likewise, there was a significant difference with respect to the colorectal cancer case and control status (Table 1) for the variables age and sex, the reason why we adjusted for these variables in multivariate analysis. Pairwise linkage disequilibrium (LD) and haplotypes were calculated and estimated using Arlequin 2.0 software.

Results

Identification of genetic variants within the ERBB4 5'-regulatory region. Seven genetic variants were identified within the ATG -1000 bp region by mutational analysis of 92 colorectal cancer patients. These included five novel variants, i.e., -815 A>T (2 patients), -782 G>T (8 patients), -638 insTC (1 patient), -267 C>G (5 patients), and -219 del10bp (1 patient), and two known polymorphisms -718 C>T (rs6735626, 61 patients) and -609 G>A (rs6735267, 28 patients; Fig. 1A). To investigate whether the five novel variants represented tumor-specific mutations or germ line variants, we sequenced the patients’ corresponding constitutional DNA and observed that whenever a variant was present in the tumor, it was also present in the germ line.

ERBB4 variants -782T and -815T affect promoter activity in human breast cancer cell lines. We investigated the promoter activity of ERBB4 variants -815T, -782T, -718T, -609A, and -267G by reporter gene assays in MDA134 (ER positive) and MDA157 (ER negative) breast cancer cell lines. Significant changes in promoter activity were observed for the ERBB4 -782T and -815T variants. When compared with the major -782G allele, the promoter activity of the minor -782T allele was significantly reduced to 40% in ER positive (P < 0.01) and to 35% in ER negative cells (P < 0.01), but ERBB4 promoter activity was not completely abolished (Fig. 1B). In contrast, the promoter activity of the -815T variant was significantly increased by 35% in ER positive (P < 0.01) and 55% in ER negative cells (P < 0.01). Other variants did not show any significant changes in promoter activity (Fig. 1B).

Effect of ERBB4 variants on DNA-protein interaction. We performed EMSAs using nuclear proteins from MDA134 cells (Figs. 2 and 3). The DNA-protein complex with the major ERBB4 -782G allele probe showed a more intense band when compared with the complex obtained with the minor T allele probe (Fig. 2A, lanes 2 and 6). In competition assays with labeled G allele probes and an excess of unlabeled competitor oligonucleotides, we observed that the protein-DNA complex 1 labeled G allele probes and an excess of unlabeled competitor antibody (Fig. 2B, lane 2). The same binding pattern was observed with -782T allele probe with nuclear extract led to the formation of a Sp1 complex and a supershifted Sp1 immunocomplex from MDA134 nuclear extract suggests that Sp1 protein is present in this nuclear extract (Fig. 2B, lanes 3 and 4). The observed differences in the electrophoretic mobility of the DNA-protein complex 1 and Sp1 complex, together with the absence of a supershifted immunocomplex, however, suggest that in the MDA134 nuclear extract, a protein(s) other than Sp1 binds to the ERBB4 -782T-containing promoter region.

In the case of the ERBB4 -815 A>T variant, we observed strong Sp1 binding (Fig. 3B). Incubation of the -815A allele probe with MDA134 nuclear extract or recombinant Sp1 protein led to the formation of a Sp1 complex (Fig. 3B, lanes 1 and 2), which was confirmed by supershift with Sp1 antibody in both cases (Fig. 3B, lanes 2 and 6). In addition, we observed a faster migrating complex (complex 2), which was also present when using the Sp1 consensus probe (Fig. 3B, lanes 1 and 3). However, we infer that this complex may not contain Sp1 protein due to a lack of supershift (Fig. 3B, lanes 2 and 4). The same binding pattern was observed with -815T probe (Supplementary Fig. S1, lanes 5-8). Both protein-DNA complexes (Sp1 complex and complex 2) were more pronounced whenever a variant was present in the tumor, it was also present in the germ line.

Identification of genetic variants within the ERBB4 5’-regulatory region. Seven genetic variants were identified within the ATG -1000 bp region by mutational analysis of 92 colorectal cancer patients. These included five novel variants, i.e., -815 A>T (2 patients), -782 G>T (8 patients), -638 insTC (1 patient), -267 C>G (5 patients), and -219 del10bp (1 patient), and two known polymorphisms -718 C>T (rs6735626, 61 patients) and -609 G>A (rs6735267, 28 patients; Fig. 1A). To investigate whether the five novel variants represented tumor-specific mutations or germ line variants, we sequenced the patients’ corresponding constitutional DNA and observed that whenever a variant was present in the tumor, it was also present in the germ line.

ERBB4 variants -782T and -815T affect promoter activity in human breast cancer cell lines. We investigated the promoter activity of ERBB4 variants -815T, -782T, -718T, -609A, and -267G by reporter gene assays in MDA134 (ER positive) and MDA157 (ER negative) breast cancer cell lines. Significant changes in promoter activity were observed for the ERBB4 -782T and -815T variants. When compared with the major -782G allele, the promoter activity of the minor -782T allele was significantly reduced to 40% in ER positive (P < 0.01) and to 35% in ER negative cells (P < 0.01), but ERBB4 promoter activity was not completely abolished (Fig. 1B). In contrast, the promoter activity of the -815T variant was significantly increased by 35% in ER positive (P < 0.01) and 55% in ER negative cells (P < 0.01). Other variants did not show any significant changes in promoter activity (Fig. 1B).

Effect of ERBB4 variants on DNA-protein interaction. We performed EMSAs using nuclear proteins from MDA134 cells (Figs. 2 and 3). The DNA-protein complex with the major ERBB4 -782G allele probe showed a more intense band when compared with the complex obtained with the minor T allele probe (Fig. 2A, lanes 2 and 6). In competition assays with labeled G allele probes and an excess of unlabeled competitor oligonucleotides, we observed that the protein-DNA complex 1 required higher concentrations of T allele competitor than G allele competitor to diminish the band intensity (Fig. 2C; paired t test, P = 0.0274). These data show that the binding affinity of nuclear proteins to the minor T allele was significantly lower compared with the major G allele.

In silico analysis identified the Sp1 protein as a potential binding partner of the ERBB4 -782 and -815-containing region, a reason why we did additional EMSA experiments with recombinant Sp1 protein. In the case of the -782G allele, we observed a Sp1-containing complex (Sp1 complex) with reduced electrophoretic mobility compared with that of complex 1, which was confirmed by a supershift with the Sp1 antibody (Fig. 2B, lanes 5 and 6). Incubation of the ERBB4 -782G allele probe with nuclear extract led to the formation of complex 1 (Fig. 2B, lane 1), which was not supershifted by Sp1

antibody (Fig. 2B, lane 2). The same binding pattern was observed with the -782T probe (Supplementary Fig. S1, lanes 1-4). Lanes 3, 4, 7, and 8 of Fig. 2B show control reactions with a Sp1 consensus probe. The formation of a Sp1 complex and a supershifted Sp1 immunocomplex from the MDA134 nuclear extract suggests that Sp1 protein is present in this nuclear extract (Fig. 2B, lanes 3 and 4). The observed differences in the electrophoretic mobility of the DNA-protein complex 1 and Sp1 complex, together with the absence of a supershifted immunocomplex, however, suggest that in the MDA134 nuclear extract, a protein(s) other than Sp1 binds to the ERBB4 -782T-containing promoter region.

In the case of the ERBB4 -815 A>T variant, we observed strong Sp1 binding (Fig. 3B). Incubation of the -815A allele probe with MDA134 nuclear extract or recombinant Sp1 protein led to the formation of a Sp1 complex (Fig. 3B, lanes 1 and 2), which was confirmed by supershift with Sp1 antibody in both cases (Fig. 3B, lanes 2 and 6). In addition, we observed a faster migrating complex (complex 2), which was also present when using the Sp1 consensus probe (Fig. 3B, lanes 1 and 3). However, we infer that this complex may not contain Sp1 protein due to a lack of supershift (Fig. 3B, lanes 2 and 4). The same binding pattern was observed with the -815T probe (Supplementary Fig. S1, lanes 5-8). Both protein-DNA complexes (Sp1 complex and complex 2) were more pronounced whenever a variant was present in the tumor, it was also present in the germ line.
when using the minor T allele probe in comparison with the major A allele probe (Fig. 3A, lanes 6 and 2, respectively). Competition assays with labeled T allele probe and excess of unlabeled competitor oligonucleotide showed that complex 2 was competed out at lower amounts of T allele competitor (Fig. 3C) compared with the same amounts of A allele competitor, a difference that was close to significance (paired t test, \( P = 0.078 \)). There was no difference in competition of Sp1 complex with respect to the two competitor oligonucleotides (SupplementaryFig. S2; paired t test, \( P = 0.14 \)). These data indicate that the binding affinity of some nuclear proteins to the minor T allele might be higher when compared with the major A allele. No DNA-protein interactions were observed for the other analyzed ERBB4 promoter variants.

Expression of ERBB4 protein in breast tumors does not differ between carriers of the -782G/G and the G/T genotype. We analyzed the expression of ERBB4 protein in breast tumor tissues from 36 patients carrying the major -782G/G genotype and 21 patients carrying the heterozygous G/T genotype. There was no difference in expression levels between the two groups with respect to membranous, cytoplasmic, or nuclear staining (data not shown).

The ERBB4 -782T allele is associated with an increased risk for breast and colorectal cancer. To perform cancer risk association studies, we first needed to investigate whether the newly identified variants were single nucleotide polymorphisms or rare disease–associated germ line mutations. Therefore, we established allele and genotype frequencies in populations from Slovenia and Germany. The variants ERBB4 -638 insTC and -219 del10bp were rare, with allele frequencies of <0.5%, and were not further analyzed. The minor allele frequencies of -815 A>T, -782 G>T, and -267 C>G ranged between 0.8% and 3%. These variants, together with the known polymorphisms, -718 C>T (minor allele frequency 43% and 45%, respectively) and -609 G>A (minor allele frequency 27% and 29%, respectively) met Hardy-Weinberg equilibrium in cases and controls.
controls and were further analyzed in subsequent association studies (Table 2). Based on genotype frequencies in breast cancer and colorectal cancer case-control collections, we calculated ORs to obtain genotype-associated disease risk estimations (Table 2). The heterozygous genotype ERBB4-782G/T was significantly associated with an increased risk of developing breast cancer (OR, 1.59; 95% CI, 1.06-2.34) and colorectal cancer (OR, 2.21; 95% CI, 1.22-3.99). No other cancer risk associations were observed.

LD and haplotype prediction. To better understand the contribution of polymorphisms of the ERBB4 5′-regulatory region to cancer risk, we performed haplotype analyses. Pairwise LD across the 1 kb region is shown in Table 3A. The highest LD was observed between -718 C>T and -609 G>A (\(r^2 = 0.47\)), whereas other polymorphisms showed low levels of LD. Six haplotypes with frequencies >0.5% in populations of European descent, as well as corresponding frequencies in breast and colorectal cancer populations, are listed in Table 3B. Haplotype 5 (ERBB4-815A, -782T, -718T, -609G, and -267C) was associated with an increased risk for breast cancer (OR, 1.73; \(P = 0.020\)) and colorectal cancer (OR, 2.10; \(P = 0.013\)). It is noteworthy that this is the sole haplotype including the -782T variant, which has been identified as the at-risk allele in the preceding single locus analysis.

Discussion

According to a current hypothesis, cancer susceptibility is largely polygenic, which infers that many genetic polymorphisms may cooperate in the carcinogenic process; each conferring a small effect on the overall cancer risk (24). Based on clinical observations (5, 11, 12) and biological plausibility (3, 5, 8), we attempted a candidate gene approach for breast and colorectal cancer susceptibility by scrutinizing the ERBB4 promoter region for its cancer risk potential. On the gene structural and functional level, we identified five novel variants and showed their effects on promoter activity. On the population level, we established allele and genotype frequencies in two

![Fig. 3. EMSA analyses of the -815 A-T containing ERBB4 5′-regulatory region using nuclear protein extract of MDA134 breast cancer cells or recombinant Sp1 protein.](image-url)
European populations and did association tests in large population-based case-control collections to understand whether any of these variants might be associated with the risk of developing breast or colorectal cancer.

In search of ERBB4 promoter variants, we first analyzed DNA from colorectal tumors for the presence or absence of mutations in the ATG -1000 bp 5'-regulatory region. We chose these tumors because ERBB4 is similarly expressed in colorectal and breast tumors; however, the latter have not been available to us. We observed seven genetic variants which, upon analyses of the breast tumors, however, the latter have not been available to us. Interestingly, the protein binding affinity of the minor T allele. Accordingly, the minor T allele may be responsible for the up to 1.5-fold observed increase of promoter activity. Altogether, our results underscore that both polymorphisms show effects on promoter activity. Moreover, they suggest a more versatile role for the ERBB4 -782 G>T polymorphism due to its putative multiple protein binding partners and capacity to obliterate transcriptional activity to a substantial degree.

Table 2. Frequency of ERBB4 genetic variants in German and Slovenian populations as well as corresponding breast and colorectal cancer populations

<table>
<thead>
<tr>
<th>Single nucleotide polymorphism</th>
<th>Genotype/ minor allele</th>
<th>GENICA controls (%)</th>
<th>GENICA breast cancer cases (%)</th>
<th>Adjusted OR (95% CI)</th>
<th>Slovenian controls (%)</th>
<th>Slovenian colorectal cancer cases (%)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-815 A&gt;T</td>
<td>A/A</td>
<td>972 (98.48)</td>
<td>977 (98.99)</td>
<td>1</td>
<td>520 (98.48)</td>
<td>422 (99.06)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A/T</td>
<td>15 (1.52)</td>
<td>10 (1.01)</td>
<td>0.68 (0.30–1.52)</td>
<td>8 (1.52)</td>
<td>4 (0.94)</td>
<td>0.44 (0.10–1.88)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-782 G&gt;T</td>
<td>G/G</td>
<td>949 (96.74)</td>
<td>939 (94.66)</td>
<td>1</td>
<td>511 (96.60)</td>
<td>412 (92.79)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>G/T</td>
<td>32 (3.26)</td>
<td>53 (5.34)</td>
<td>1.59 (1.06–2.34)</td>
<td>18 (3.40)</td>
<td>32 (7.21)</td>
<td>2.21 (1.22–3.99)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-718 C&gt;T</td>
<td>C/C</td>
<td>325 (31.40)</td>
<td>336 (33.50)</td>
<td>1</td>
<td>164 (29.23)</td>
<td>135 (29.67)</td>
<td>1</td>
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<tr>
<td></td>
<td>C/T</td>
<td>525 (50.72)</td>
<td>475 (47.36)</td>
<td>0.87 (0.71–1.07)</td>
<td>287 (51.16)</td>
<td>231 (50.77)</td>
<td>1.035 (0.69–1.56)</td>
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<tr>
<td></td>
<td>T/T</td>
<td>185 (17.87)</td>
<td>192 (19.14)</td>
<td>1.02 (0.79–1.33)</td>
<td>110 (19.61)</td>
<td>89 (19.56)</td>
<td>0.97 (0.75–1.25)</td>
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<tr>
<td>-609 G&gt;A</td>
<td>G/G</td>
<td>514 (53.54)</td>
<td>515 (53.09)</td>
<td>1</td>
<td>233 (51.32)</td>
<td>238 (57.49)</td>
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<td></td>
<td>G/A</td>
<td>375 (39.06)</td>
<td>375 (38.66)</td>
<td>1.01 (0.83–1.22)</td>
<td>182 (40.09)</td>
<td>150 (36.23)</td>
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<td></td>
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<td>71 (7.40)</td>
<td>80 (8.25)</td>
<td>1.19 (0.84–1.69)</td>
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<td>26 (6.28)</td>
<td>0.56 (0.22–1.09)</td>
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<td></td>
<td>A/T</td>
<td>0.27</td>
<td>0.29</td>
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<tr>
<td>-267 C&gt;G</td>
<td>C/C</td>
<td>928 (93.55)</td>
<td>935 (91.76)</td>
<td>1</td>
<td>548 (97.34)</td>
<td>439 (96.06)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>C/G</td>
<td>52 (5.24)</td>
<td>64 (6.28)</td>
<td>1.17 (0.82–1.68)</td>
<td>15 (2.66)</td>
<td>18 (3.94)</td>
<td>1.43 (0.49–4.11)</td>
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<td></td>
<td>G/G</td>
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<td>1 (0.10)</td>
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<tr>
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<td>G frequency</td>
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</table>

*The positions of the genetic variants were counted from the ATG codon. OR and 95% CIs were calculated based on the frequency of each genotype versus the major genotype between cases and controls.

†Adjusted for family history of breast cancer and hormone replacement therapy usage.

‡Adjusted for age and sex.
These interpretations are in line with our patient-based results from breast and colorectal cancer risk association studies from Germany and Slovenia. From the five ERBB4 promoter polymorphisms included in the analyses, the -782 G>T polymorphism was significantly associated with higher cancer risks. In detail, the frequency of the heterozygote genotype G/T was 3.3% in the GENICA population-based controls and 3.4% in the Slovenian blood donor controls. This frequency was significantly increased in the corresponding cancer groups, which translates into a 1.6-fold increased risk for breast cancer and a 2.2-fold increased risk for colorectal cancer. The -782 G>T polymorphism is less frequent than the known promoter polymorphisms rs673626 and rs6735267. Yet, our findings draw substance from the large sizes of case-control comparisons (i.e., >2,000 study subjects from the GENICA breast cancer collection and >1,000 study subjects from the Slovenian colorectal cancer collection), appropriate study design, and high genotyping call rates of >97%. Despite the lower size of the colorectal cancer collection, we had an 85% power to detect a risk allele with a frequency of 2%, which confers a relative risk of 2.2 at the 0.05 level of significance. In the colorectal cancer study, cases and controls did not match with respect to age and sex; however, genotype frequencies matched those of the larger GENICA control group and were therefore regarded relevant. Haplotype analysis did not add more information about the influential single nucleotide polymorphism’s accuracy. The only haplotype associated with an increased risk for breast and colorectal cancer was the haplotype that included the -782T variant, which has been identified as the at-risk allele in the single locus analysis. At this point, we are aware of the possibility that some of our observed associations may vanish upon stringent correction for multiple testing. Due to the explorative nature of our study, we purposely did not perform multiple testing corrections in order to not eliminate potentially important results from this first investigation. To understand the relevance of our data, however, it will be important to replicate these findings in independent data sets.

Our mutational, in vitro functional, and population-based cancer studies suggest a role for the ERBB4 -782 G>T polymorphism in breast and colorectal cancer risk. Whether this polymorphism will be useful to identify individuals at increased cancer risk should be further pursued in large international population-based studies. This is encouraged by recent developments in which CASP8 (rs1045485), originally identified as a putative breast cancer susceptibility gene in two British populations (26), was successfully confirmed within a comprehensive international effort of the Breast Cancer Association Consortium on the basis of >30,000 breast cancer cases and controls (27).

In addition, the question arises whether the effects of the polymorphisms may also influence tumor biology and behavior. We observed cytoplasmic, nuclear, and membranous ERBB4 protein expression in breast tumors; however, there was no difference with respect to the underlying major or minor -782 genotypes. This may not be surprising because breast cancer risk may rather be reflected by features of the normal mammary gland, yet ERBB4 expression in normal breast is too low to be quantitatively analyzed (9). Therefore, our ERBB4 expression findings do not contradict the observed genotype-associated risk associations, but rather point to other regulatory mechanisms of ERBB4 expression in tumors. We infer that the role of ERBB4 genotypes may be limited to normal tissue.

Recently, it has been shown that ERBB4 functions as a proapoptotic protein suppressing the growth of malignant cells, and when activated, induces cell killing of breast tumor cells and enhances apoptosis (28). From this, we may infer that

| Table 3. Pairwise LD of five ERBB4 genetic variants and major haplotypes with their frequencies in German and Slovenian control as well as cancer populations |

(A) Pairwise LD

<table>
<thead>
<tr>
<th>ID</th>
<th>Major haplotypes</th>
<th>GENICA controls (%)</th>
<th>GENICA breast cancer cases (%)</th>
<th>OR</th>
<th>P</th>
<th>Slovenian controls (%)</th>
<th>Slovenian colorectal cancer cases (%)</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGGC</td>
<td>53.56</td>
<td>52.72</td>
<td>1</td>
<td>52.69</td>
<td>52.21</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AGGC</td>
<td>26.70</td>
<td>27.53</td>
<td>1.05</td>
<td>0.810</td>
<td>28.93</td>
<td>28.93</td>
<td>23.82</td>
<td>0.83</td>
</tr>
<tr>
<td>3</td>
<td>AGGC</td>
<td>15.17</td>
<td>13.34</td>
<td>0.89</td>
<td>0.218</td>
<td>14.09</td>
<td>14.09</td>
<td>17.66</td>
<td>1.27</td>
</tr>
<tr>
<td>4</td>
<td>AGGC</td>
<td>2.65</td>
<td>3.23</td>
<td>1.24</td>
<td>0.304</td>
<td>1.41</td>
<td>1.41</td>
<td>1.97</td>
<td>1.42</td>
</tr>
<tr>
<td>5</td>
<td>AGGC</td>
<td>1.52</td>
<td>2.59</td>
<td>1.73</td>
<td>0.020</td>
<td>1.71</td>
<td>1.71</td>
<td>3.55</td>
<td>2.10</td>
</tr>
<tr>
<td>6</td>
<td>AGGC</td>
<td>0.72</td>
<td>0.49</td>
<td>0.70</td>
<td>0.495</td>
<td>0.71</td>
<td>0.71</td>
<td>0.46</td>
<td>0.66</td>
</tr>
</tbody>
</table>

NOTE: In A, D’ abs (bottom left half) and r2 (top right half).
*The order of genetic variants from left to right are: -815 A>T, -782 G>T, -718 C>T, -609 G>A and -267 C>G with the minor allele being highlighted in black.

*Odds ratios and P values were calculated based on the frequency of each haplotype versus haplotype 1 between cases and controls.
high levels of ERBB4 may have a protective cellular effect, thus explaining why a low constitutional expression genotype may confer breast and colorectal cancer risk. This interpretation is in line with the reported improved breast cancer survival times of patients with high ERBB4 expression in their tumors (11, 12). Moreover, there has been mention that the nuclear localization of an ERBB4 isoform is associated with worse outcome compared with membrane localization (9). Whether the ERBB4 -782 G>T polymorphism plays a role in the aberrant expression of this or any other deleterious ERBB4 isoform remains to be determined. Finally, it will be important to understand the role of this polymorphism on the ERBB effector pathways mitogen-activated protein and phosphatidylinositol-3 kinase–activated AKT, which may eventually stimulate progress into the development of targeted drug treatment for breast and colorectal cancer.

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References

A Novel Polymorphism in the Promoter Region of ERBB4 Is Associated with Breast and Colorectal Cancer Risk

Matjaz Rokavec, Christina Justenhoven, Werner Schroth, et al.


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