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No association of polymorphisms in the cell polarity gene *SCRIB* with breast cancer risk

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Key words

SCRIB, polymorphisms, epithelial cell polarity, breast cancer risk

Abstract

The human homolog of the *Drosophila Scribble* (*SCRIB*) tumor suppressor gene encodes a protein that regulates apical-basolateral polarity in mammalian epithelia and controls cell proliferation. Due to the role of cell polarity proteins in human cancers, we investigated whether genetic variability in *SCRIB* impacts breast carcinogenesis and tumor pathology. Five genetic variants were analyzed for an association with breast cancer risk and histopathological tumor parameters using a single nucleotide polymorphisms (SNP) tagging approach. Genotyping of five tag SNPs was performed by TaqMan® allelic discrimination and RFLP-based PCR using the GENICA population-based breast cancer case-control collection including 1,021 and 1,015 age-matched controls. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by ordinal logistic regression. None of the tag SNPs was associated with breast cancer risk or tumor characteristics. Our findings suggest that genetic variability in the *SCRIB* polarity gene does not contribute to breast cancer development.

Introduction

Breast cancer originates from epithelial cells of the terminal ductal lobular units (TDLU) in the breast [1, 2]. Each TDLU consists of several small units, referred to as acini, which consist of a single polarized layer of luminal epithelial cells surrounding a hollow lumen [2,3]. The establishment and maintenance of polarized architecture is critical for normal function of epithelial cells *in vivo.* During the development of carcinoma, epithelial cells loose their ability to maintain polarized organization, suggesting an important role for molecules that regulate cell polarity in breast cancer.

One of the proteins regulating cell polarity is the tumor suppressor Scribble, recently identified in *Drosophila* [4]. Loss of Scribble is sufficient to induce tumorigenic growth and promote spontaneous invasion and metastasis in combination with activated oncogenes Ras, Raf or Notch [5, 6]. Moreover, loss of Scribble results in loss of polarity and neoplastic tissue overgrowth, demonstrating a direct link between cell polarity regulators and control of proliferation [7].

Recent studies suggest that the human homolog of *Drosophila* Scribble, SCRIB also plays a role in the development of cancer. One study demonstrated that deregulation of SCRIB promotes transformation of mammary epithelial cells *in vitro* and *in vivo* by disrupting cell polarity, three-dimensional morphogenesis and by inhibiting cell death [8]. Moreover, SCRIB is a target protein for the ubiquitin-mediated degradation by human papillomavirus (HPV) E6 oncoprotein, which is considered to have a causal role in the development of cervical cancer [9]. This is consistent with data showing that SCRIB expression is decreased in tumors associated with HPV infection [9, 10]. Additionally, loss of SCRIB expression is frequently observed in colon and lobular breast cancers [11, 12]. Further strong evidence for a role of SCRIB in breast cancer comes from a recent study showing that SCRIB was downregulated in

more than 50% of breast cancers and mislocalized from cell-cell junctions to the cytoplasm in numerous breast cancer cell lines and ductal carcinomas *in situ* [8].

Due to the strong relationship between SCRIB and breast tumorigenesis, we investigated whether genetic variation in *SCRIB* is associated with breast cancer risk and histopathological tumor characteristics using a SNP tagging approach. The dataset includes 1,021 patients from the GENICA population-based collection of incident primary breast cancer cases.

Material and Methods

Study population

The GENICA study participants of the population-based breast cancer case-control study from the Greater Bonn Region, Germany, were recruited between 08/2000 and 9/2004 as previously described [13, 14]. In brief, 1,143 incident breast cancer cases and 1,155 population controls, matched in 5-year classes, participated in the study. Cases and controls were eligible if they were of Caucasian ethnicity, current residents of the study region and below 80 years of age. Among the recruited individuals, DNA samples were available for 1,021 (89%) breast cancer cases and 1,015 (88%) controls. Information on known and potential risk factors was collected for all participants via in-person interviews. The response rate was 88% for cases and 67% for controls. Selected epidemiological characteristics of the patients are shown in Table 1.

Information on clinical and histopathological tumor characteristics was collected and included histology (ductal, lobular, ductolobular), histological grade (G1, G2, G3), tumor size (T1, T2, T3, T4), lymph node status (N0, N≥1), estrogen receptor (ER) status (positive, negative), progesterone receptor (PR) status (positive, negative), and HER2 status (positive, negative). Tumor grade was determined according to the Nottingham Criteria, which comprises formation of tubuli, nuclear pleomorphism and mitotic rate.

The GENICA study was approved by the Ethic´s Committee of the University of Bonn. All study participants gave written informed consent.

DNA isolation and genotyping

Genomic DNA was extracted from heparinized blood samples (PuregeneTM, Gentra Systems, Inc., Mineapolis, USA) as previously described [15]. Genotyping of *SCRIB* polymorphisms was performed by TaqMan® allelic discrimination and PCR-based restriction-fragment-length-polymorphism analysis. SNP genotyping assays were purchased from Applied Biosystems (Foster City, CA, USA). The pre-designed SNP genotyping assay IDs are C_26008847_10 for rs6558394 and C_30687359_20 for rs11786637 and C_401143_20 for rs7822430; custom assay for rs7844493. PCRs were run in a 5 µl reaction containing 6.25 ng DNA, TaqMan® Genotyping Master Mix (Applied Biosystems) and Assay-Mix. PCR was performed at 50°C for 2 min, 95°C for 10 min and 50 cycles at 92°C for 15 s and 60°C for 1 min. The samples were amplified, read and analyzed using the ABI Prism 7900HT Sequence Detection System and ABI Prism SDS 2.2 software.

SNP rs4875054 was amplified using newly designed primers, a forward primer (5'-AGG AGA GCA GGA TCA GGG GT-3') and a reverse primer (5'-GGC TGA CAT CTT GCC TGT GAG-3'). The reaction was set up in 10 µl containing 12.5 ng genomic DNA, 1x PCR buffer (Qiagen, Hilden, Germany), 2.0 mM MgCl2, 0.1 µM of each primer, 250 µM of each dNTP (Promega, Mannheim, Germany), and 0.4 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). After an initial 15 min step at 95°C, DNA was amplified by 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and followed by a final extension of 10 min at 72°C. PCR products were digested with 2.5 U *MnlI* (New England Bioloabs, Frankfurt, Germany). Sizes of the ethidium-bromide stained fragments were 37 bp and 75 bp for the G allele and 112 bp for the A allele.

Selection of SNPs in *SCRIB* for association studies

To define the set of tag SNPs we used the software Tagger and CEU genotype data from the International HapMap Project [16] spanning the region from position 144945078 to 144972537 of chromosome 8. Common variants with a minor allele frequency (MAF) of ≥0.05 were captured using a correlation coefficient (r^2) between tag and untyped SNPs greater than 0.8. Coding polymorphisms were analyzed for their potential effect on protein function using the web-tools Polyphen [17] and Panther [18, 19].

Haplotype analysis

The linkage disequilibrium (LD) between polymorphisms was determined in the GENICA control group using the software Haploview [20]. The haplotype block structure was defined according to the method of Gabriel *et al*. [21]. Haplotype analysis was performed within the identified haplotype block using the SAS genetics package. Haplotypes were estimated using an expectation-maximization algorithm (proc haplotype).

Statistical analyses

The power calculation was performed using the PGA software [22].

SCRIB genotype frequencies were tested for Hardy-Weinberg equilibrium. Associations between genetic variables and breast cancer risk were analyzed by conditional logistic regression adjusted for six potential epidemiological breast cancer risk factors (menopausal status, family history of breast or ovarian cancer, use of oral contraceptives, use of hormone therapy, body mass index and smoking) and stratified for age. Subsequently, subgroup analysis was performed for these six epidemiological variables. Additionally, the associations between *SCRIB* genotypes and seven clinical and histopathological tumor characteristics (histology, histological grade, tumor size, nodal status, ER status, PR status and HER2 status)

of breast cancer cases were analyzed by conditional logistic regression and chi square test. All tests were two-sided. To correct for multiple testing we divided the significance level of 0.05 by the number of tested variables. In case of epidemiological variables 0.05 was divided by six and thus *P*-values < 0.008 were considered significant. Accordingly for the seven tumor characteristics *P*-values < 0.007 were considered significant. Risk estimates were given as odds ratios (OR) and 95% confidence interval (CI). Statistical analyses were done using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Results

Selection of SNPs in *SCRIB* for association studies

Based on HapMap data, eight common SNPs (MAF ≥0.05) located in exons and introns of *SCRIB* were tagged by five genotyped variants, rs6558394, rs7844493, rs11786637, rs7822430 and rs4875054 (Figure 1). Functional implications of *SCRIB* polymorphisms have not been reported so far. However, the tag SNP rs6558394 resulting in an amino acid substitution (L422P) may impact the function of the protein as suggested by *in silico* analyses.

Associations of SNPs in *SCRIB* with overall breast cancer risk and histopathological tumor parameters

We analyzed the five tag SNPs in *SCRIB* within the GENICA study population. Call rates were greater than 99.3% and repeated analysis of 5% of samples showed 99% concordance. Genotype frequencies of cases and controls met Hardy-Weinberg equilibrium. No association was observed with breast cancer risk in general (Table 2) or in subgroup analysis with respect to menopausal status, family history of breast or ovarian cancer, use of oral contraceptives, use of hormone therapy, body mass index and smoking (data not shown). Frequencies of

SCRIB haplotypes encompassing SNPs in strong linkage disequilibrium (rs7822430, rs11786637) did not differ significantly between cases and controls (data not shown).

Further, none of the SNPs was associated with histopathological tumor parameters considering histological grade, tumor histology, tumor size, lymph node status, ER, PR and HER2 status (data not shown).

Discussion

In the present study we tested the hypothesis whether genetic variability of *SCRIB* impacts breast cancer risk and/or tumor pathology. Such a relationship seemed plausible because it recently has been shown that *SCRIB* inhibits breast cancer formation and that deregulation of *SCRIB* by down regulation or mislocalization promotes dysplastic and neoplastic growth by disrupting morphogenesis and cell polarity and by inhibiting cell death [8]. To the best of our knowledge, the present investigation of five *SCRIB* SNPs in the GENICA population-based case-control study is the first one to address this possible relationship.

None of the five tag SNPs in the *SCRIB* gene was associated with breast cancer risk in general or any of the subgroup risks addressed by menopausal status, family history of breast or ovarian cancer, use of oral contraceptives, use of hormone therapy, body mass index and smoking. There was also no association with histopathological tumor parameters including histology, histological grade, tumor size, lymph node status, ER, PR and HER2 status.

Our study had a 90% power to detect minimum ORs of 1.34-1.40 (p=0.05, two sided test, dominant model). The range reflects the variation of minor allele frequencies of the five polymorphisms in cases, which were at 10-38%.

The results of this study of no link of *SCRIB* genetic variation with breast cancer risk and tumor parameters are relevant due to the important role of this polarity gene in breast cancer suppression. However, due to the lack of functional studies it is unknown if the

polymorphisms analyzed do influence functional aspects of SCRIB such as protein activity, localization or stability.

At least three evolutionary conserved groups of proteins play a central role in the establishment and maintenance of apical-basolateral polarisation of epithelial cells: the Crumbs-Pals1(Stardust)-Patj and the Par3(Bazooka)-Par6-aPKC protein complexes that localize to the apical membrane domain and promote apical-membrane-domain identity [23, 24], the function of which are antagonized by the basolaterally localized Lethal giant larvae (Lgl), Scribble (Scrib) and Discs large (Dlg) proteins, which together promote basolateral membrane identity [7]. Thus, it may be possible that genetic variability in other genes linked with basolateral polarisation or in genes promoting apical polarisation are associated with breast cancer risk and/or tumor parameters.

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Competing interests The authors declare that they have no competing interests.

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Figure legend

Figure 1 - Genomic structure of the human *SCRIB* gene. The region 3,000 bp upstream of exon 1 is indicated by a grey box. Exons are given in black and introns in white. The entire region contains eight SNPs with a minor allele frequency of ≥0.05, which were analyzed by a SNP tagging approach. The five tag SNPs are shown in bold. The gene structure corresponds to the Ensembl transcript ID ENST00000356994.

Table 1 - Characteristics of the GENICA study population

Characteristic		Cases n (%)	Controls n (%)
Age (years)	20-44	124 (12.1)	129 (12.7)
	45-49	99 (9.7)	97 (9.6)
	50-54	155 (15.2)	149 (14.7)
	55-59	140 (13.7)	141 (13.9)
	60-64	202 (19.8)	193 (19.0)
	65-69	140 (13.7)	138 (13.6)
	≥70	161 (15.8)	168 (16.6)
Menopausal status	Premenopausal	249 (24.7)	235 (23.5)
	Postmenopausal	758 (75.3)	764 (76.5)
Breast or ovarian cancer in first-degree relatives	No	860 (84.2)	927 (91.3)
	Yes	161 (15.8)	88 (8.7)
OC use (years)	Never	372 (36.5)	368 (36.3)
	>0 to <5	180 (17.7)	185 (18.3)
	5 to <10	134 (13.2)	120 (11.8)
	≥10	333 (32.7)	340 (33.6)
HT use (years)	Never	506 (49.8)	509 (50.3)
	>0 to <10	245 (24.1)	290 (28.6)
	≥10	266 (26.2)	214 (21.1)
BMI (kg/m^2)	<20	90 (8.8)	72 (7.1)
	20 to <25	469 (45.9)	471 (46.4)
	25 to <30	305 (29.9)	324 (31.9)
	≥30	157 (15.4)	148 (14.6)
Smoking	Never	586 (57.5)	555 (54.7)
	Former	192 (18.8)	215 (21.2)
	Current	242 (23.7)	245 (24.1)

OC: oral contraceptive, HT: hormone therapy, BMI: body mass index

Table 2 - *SCRIB* polymorphisms and breast cancer risk

SNP	Genotype [Allele]	Cases n (%)	Controls n (%)	OR_{adj}[a] (95% CI)	Global *P* value[b]
rs6558394 A>G	AA	396 (39.8)	387 (38.9)	1.00 (reference)	0.79
	AG	464 (46.7)	474 (47.7)	0.94 (0.77-1.14)	
	GG	134 (13.5)	133 (13.4)	0.98 (0.74-1.30)	
	[G]	732 (36.8)	740 (37.2)		
rs7844493 A>G	AA	757 (76.0)	764 (76.9)	1.00 (reference)	0.91
	AG	225 (22.6)	217 (21.8)	1.05 (0.85-1.30)	
	GG	14 (1.4)	13 (1.3)	1.03 (0.48-2.24)	
	[G]	253 (12.7)	243 (12.2)		
rs11786637 T>C	TT	805 (80.7)	806 (81.3)	1.00 (reference)	0.94
	TC	187 (18.7)	179 (18.1)	1.04 (0.83-1.31)	
	CC	6 (0.6)	6 (0.6)	0.97 (0.31-3.06)	
	[C]	199 (10.0)	191 (9.6)		
rs7822430 C>A	CC	365 (36.8)	401 (40.4)	1.00 (reference)	0.08
	CA	493 (49.8)	452 (45.5)	1.24 (1.02-1.50)	
	AA	133 (13.4)	140 (14.1)	1.03 (0.78-1.36)	
	[A]	759 (38.3)	732 (36.9)		
rs4875054 G>A	GG	458 (45.9)	465 (46.7)	1.00 (reference)	0.95
	GA	443 (44.3)	431 (43.3)	1.03 (0.85-1.24)	
	AA	98 (9.8)	100 (10.0)	1.00 (0.73-1.36)	
	[A]	639 (32.0)	631 (31.7)		

[a] Odds ratio conditional on age in 5-year groups adjusted for menopausal status, family history of breast or ovarian cancer, use of oral contraceptives, use of hormone therapy, body mass index and smoking

[b] Global *P* value of testing the null hypothesis of no association between SNP and breast cancer risk based on conditional logistic regression

Figure

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