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ORIGINAL MANUSCRIPT

Inherited variants in the inner centromere protein (INCENP) gene of the chromosomal passenger complex contribute to the susceptibility of ER-negative breast cancer

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Abstract

The chromosomal passenger complex (CPC) plays a pivotal role in the regulation of cell division. Therefore, inherited CPC variability could influence tumor development. The present candidate gene approach investigates the relationship between single nucleotide polymorphisms (SNPs) in genes encoding key CPC components and breast cancer risk. Fifteen SNPs in four CPC genes (*INCENP*, *AURKB*, *BIRC5* and *CDCA8*) were genotyped in 88 911 European women from 39 case-control studies of the Breast Cancer Association Consortium. Possible associations were investigated in fixed-effects meta-analyses. The synonymous SNP rs1675126 in exon 7 of *INCENP* was associated with overall breast cancer risk [per A allele odds ratio (OR) 0.95, 95% confidence interval (CI) 0.92–0.98, $P = 0.007$] and particularly with estrogen receptor (ER)-negative breast tumors (per A allele OR 0.89, 95% CI 0.83–0.95, $P = 0.0005$). SNPs not directly genotyped were imputed based on 1000 Genomes. The SNPs rs1047739 in the 3′ untranslated region

and rs144045115 downstream of *INCENP* showed the strongest association signals for overall (per T allele OR 1.03, 95% CI 1.00–1.06, *P* = 0.0009) and ER-negative breast cancer risk (per A allele OR 1.06, 95% CI 1.02–1.10, *P* = 0.0002). Two genotyped SNPs in *BIRC5* were associated with familial breast cancer risk (top SNP rs2071214: per G allele OR 1.12, 95% CI 1.04–1.21, *P* = 0.002). The data suggest that *INCENP* in the CPC pathway contributes to ER-negative breast cancer susceptibility in the European population. In spite of a modest contribution of CPC-inherited variants to the total burden of sporadic and familial breast cancer, their potential as novel targets for breast cancer treatment should be further investigated.

Abbreviations

AIC	Akaike's information criterion
AURKB	aurora kinase B
BCAC	Breast Cancer Association Consortium
BIRC5	baculoviral IAP repeat containing 5
CDCA8	cell division cycle associated 8
COGS	Collaborative Oncological Gene-environment Study
CPC	chromosomal passenger complex
ER	estrogen receptor
FRR	familial relative risk
HER2	human epidermal growth factor receptor 2
LD	linkage disequilibrium
MAF	minor allele frequencies
OS	overall survival
PAF	population attributable fraction
RFS	relapse-free survival
SNP	single nucleotide polymorphism
TCGA	The Cancer Genome Atlas.

Introduction

Breast cancer is the most commonly occurring epithelial malignancy among women, with an estimated 1.4 million new cases and >450 000 deaths worldwide (1). Familial aggregation and twin studies have shown the substantial contribution of inherited susceptibility to breast cancer (2,3). Many genetic loci have been identified that contribute to this familial risk (4), including genes with high-penetrance mutations, notably *BRCA1* and *BRCA2*, moderate penetrance genes including *ATM*, *BRIP1*, *CHEK2* and *PALB2* and common lower penetrance alleles, of which >80 have been identified so far. In total, these loci explain ~35% of the familial risk of breast cancer (5) leaving a large portion of the observed familial clustering of the disease unexplained (6).

The chromosomal passenger complex (CPC) is a key regulator of mitosis and is essential for maintenance of genomic stability through its control of multiple processes during both nuclear and cytoplasmic division (cytokinesis) (7,8).

The core CPC is composed of the three non-enzymatic subunits, the microtubule-binding inner centromere protein (INCENP), survivin (baculoviral IAP repeat containing 5, BIRC5) and borealin (cell division cycle associated 8, CDCA8), which regulate the activity, localization and stability of the CPC's catalytic subunit, aurora kinase B (AURKB) (9). INCENP is the platform on which the CPC assembles. The INCENP N-terminus forms a triple-helix bundle with the C-terminus of survivin and N-terminus of borealin (9) that is required for CPC localization to the centromere, anaphase spindle midzone and telophase midbody (9–12). AURKB binds to a conserved region (IN box) at the INCENP C-terminus (13). Strict localization of AURKB by CPC ensures that the kinase, which has >50 substrates (8), phosphorylates the correct targets at the proper steps in cell cycle progression.

Loss of CPC function results in lagging chromosomes during metaphase, leading to segregation errors, and in addition cleavage furrows fail to maintain ingression, resulting in cytokinesis failures (14–18). Moreover, lagging chromosomes can secondarily cause cytokinesis failures during telophase. Furthermore, analyses of point mutations in CPC proteins reveal independent roles of these proteins in the initiation of cytokinesis (19–21). Disturbed CPC function may also be caused by overexpression of CPC subunits and by deregulation of its regulatory kinases and phosphatases. Indeed, high expression levels of INCENP were observed in colorectal cancer cell lines (22), whereas high expression levels of survivin (23) and AURKB (24,25) have been found in various cancers including breast cancer and shown to be associated with poor prognosis (26,27).

Given the key role of the CPC in maintaining genomic stability and the facts that chromosome segregation error (28,29) and overexpression of CPC components are frequently seen in human cancers, we hypothesize that genetic variants in the core CPC genes *INCENP*, *AURKB*, *BIRC5* and *CDCA8* affect breast cancer susceptibility. Thus, the primary aim of this investigation was to assess possible associations between selected tag single nucleotide polymorphisms (SNPs) and potentially functional SNPs in four CPC genes and breast cancer risk. Subsequently, *in silico* analyses of SNP function and gene expression were carried out to provide supportive evidence of the identified risk variants. The secondary aim was to explore genetic associations with the survival of breast cancer patients.

Materials and methods

Study participants

Study subjects were 88 911 women of European ancestry from 39 case-control studies participating in the Breast Cancer Association Consortium (BCAC). All BCAC studies had local ethical approvals and all included individuals gave informed consent (5). Seventeen SNPs were selected for genotyping including 12 tag SNPs for *INCENP*, three potentially functional SNPs in *AURKB*, *BIRC5* and *CDCA8*, one SNP in *AURKB* previously reported to be associated with familial breast cancer risk (30) and one SNP in *BIRC5* previously reported to be correlated with survivin expression (31). SNP genotyping in the BCAC samples was conducted using a custom Illumina Infinium array (iCOGS) in four centers, as part of a multiconsortia collaboration [the Collaborative Oncological Gene-environment Study (COGS)] (5). Genotypes were called using Illumina's proprietary GenCall algorithm. Quality control included checks on call rate, heterozygosity and Hardy–Weinberg equilibrium. Details on BCAC studies and the SNP selection approach can be found in Supplementary Materials and Methods and in Supplementary Tables S1 and S2, available at *Carcinogenesis* Online.

Statistical analyses

Single SNP association analysis

The BCAC provided genotype data along with the first 7 genetic principal components to allow adjustment for population stratification (the first 6 components based on ~37 000 uncorrelated polymorphisms plus a seventh specifically derived for the Leuven Multidisciplinary Breast Centre study). Available phenotype data included disease status, hormone receptor status, family history and survival information. The association between genotypes and overall breast cancer risk was investigated in an individual-based fixed-effects model meta-analysis comprising 88 911 study subjects. Per allele odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were estimated by logistic regression in a model that incorporated study and the first 7 principal genetic components as fixed effects. Study heterogeneity

was assessed by the I^2 index. Forest plots were generated and the nearest neighbor method based on the medians of the seven first genetic principal components was used to cluster studies according to genetic similarity.

To assess the familial breast cancer risk, cases with a family history of breast cancer in a first-degree relative were compared with all controls using an additive logistic regression model adjusted for study and the first 7 principal genetic components.

A case-only analysis was carried out to explore whether SNPs in CPC genes are associated with the hormone receptor status of the tumor [estrogen receptor (ER)-positive/negative, progesterone receptor (PR)-positive/negative and human epidermal growth factor receptor 2 (HER2)-positive/negative].

Survival information was available for only ~65% of all cases. The relationship between genotype and overall survival (OS), breast-cancer-specific survival and relapse-free survival (RFS) was investigated in cases, which did not represent with distant metastases at diagnosis. OS was defined as the time between breast cancer diagnosis and death or last follow-up, whichever occurred first. For breast-cancer-specific survival, only deaths from breast cancer according to International Classification of Diseases, 10th Revision code counted as events, whereas deaths from any other cause were censored. RFS was defined as the time between breast cancer diagnosis and locoregional relapse or relapse of distant metastasis after a period of remission, whichever occurred first. Survival times were censored after 15 years (for OS and breast-cancer-specific survival) and 10 years (for RFS). If cases were diagnosed before study entry, survival times were left truncated. Survival analyses were performed by Cox regression models incorporating study and the seven genetic principal components as fixed effects. Per allele hazard ratios and corresponding 95% CIs were reported. In addition Kaplan–Meier estimates of survival were plotted stratified by genotype, and genotype-specific estimated 10 year (for OS and BCCS) and 5 year (for RFS) survival rates were reported.

If an association with the hormone receptor status of the tumor was detected, the subtype-specific disease risk and survival was investigated as well.

Haplotype analysis

Pairwise linkage disequilibrium (LD) between *INCENP* SNPs was measured by r^2 and a LD heat map was generated. Based on different combinations of SNPs and taking the LD block structure into account, we inferred haplotypes using the expectation-maximization algorithm. Haplotype frequencies were calculated. Subsequently, the association between most frequent haplotypes and overall breast cancer risk was analyzed by a logistic regression model adjusted for study and the seven principal components. The model fit was evaluated by Akaike's information criterion (AIC) in order to identify the optimal SNP combination, where the smallest AIC value represents the best model. Haplotype-specific ORs and corresponding 95% CIs were also estimated.

Interaction and pathway analysis

In order to assess the interaction effects of SNPs in different CPC genes on the risk of breast cancer, we carried out a SNP–SNP interaction analysis. The multiplicative interaction index and the interaction contrast ratio were calculated, and deviation from multiplicativity and additivity was tested based on Wilcoxon signed-rank tests. The 95% CIs were computed by bootstrapping with 10 000 simulations. To investigate whether SNPs in genes of the CPC pathway are jointly associated with overall breast cancer risk, *P*-values from single SNP analyses were summarized into one combined *P*-value using Fisher's method for independent tests.

Imputation of genotypes

Multiple imputation of genotypes was performed based on all genotyped *INCENP* SNPs, to detect associations with not directly genotyped but potentially causal SNPs. The European subpopulations from the 1000 Genomes Project phase 1 were used as reference panel (32). Genotypes of only SNPs were imputed in a region centered on the *INCENP* gene. The extent of this region was identified by visual inspection of recombination rates and, spanned at least ±150 kb starting from the first and last reference SNP. A logistic regression model adjusted for study and the seven genetic principal components was applied in subsequent association analyses for imputed genotypes summarized by minus the logarithm of the *P*-value. A gene map of the investigated region was created together with a LD map relying on pairwise r^2 values for imputed and reference SNPs.

Population attributable fraction and familial risk

The population attributable fraction (PAF) was calculated for the SNP showing the strongest association with overall breast cancer risk in order to quantify the proportion of sporadic cases related to the risk variant. Also the familial relative risk (FRR), which reflects the attributable proportion of familial cases, was estimated. The calculation of PAF and FRR relied on the estimated ORs and minor allele frequencies (MAF), together with an assumed prevalence of breast cancer in the general population equal to 7.8% until the age of 74 (33,34). Subsequently, the obtained PAF and FRR of *INCENP* were compared with the PAFs and FRRs of previously identified breast cancer susceptibility variants. Considered susceptibility variants included high-penetrance mutations in *BRCA1* and *BRCA2* (6), moderate penetrance variants in *ATM, BRIP1*, *CHEK2* and *PALB2* (6,35) as well as 80 low-penetrance variants throughout the genome (4).

Expression quantitative trait loci analysis

We examined whether identified risk variants influence gene expression. Information from HapMap, NCBI's Gene Expression Omnibus and The Cancer Genome Atlas (TCGA) was exploited (36,37). For 60 unrelated Utah residents with northern and western European ancestry from the CEPH collection (CEU population), genotype data were available from the International HapMap project. Expression data derived from Epstein-Barr virus-transformed lymphoblastoid cell lines of the same individuals have been made public through Gene Expression Omnibus. The breast cancer study (BRCA) from TCGA provides germline DNA genotypes as well as expression data for tumor and matched normal breast tissue samples. All eQTL analyses involved a two-sided Kruskal–Wallis test. Differences in expression levels between normal and tumor breast tissue samples were analyzed with a two-sided Wilcoxon–Mann–Whitney test.

Functional SNP analysis

In order to explore the functional significance of identified risk variants, the R package *FunciSNP* was used to examine *in silico* annotations with chromatin features available in ENCODE (38,39). The list of examined functional characteristics included five built-in biofeatures [CTCF binding sites, DNaseI hypersensitivity sites, formaldehyde-assisted isolation of regulatory elements signals and known promoter regions] across several cell lines as well as 57 biofeatures (DNaseI hypersensitivity sites, formaldehyde-assisted isolation of regulatory elements signals, transcription factor binding sites, methylation sites, Chromatin State Segmentation by HMM (ChromHMM) and histone modifications by Chip-seq) specifically downloaded for HMEC normal mammary epithelial cells and breast cancer cell lines MCF7 and T47D. Functional SNP analyses were carried out for variants in a 300 kb window centered on the SNP with the strongest association.

Results

Eight tag SNPs (including three surrogates) out of 12 originally selected tag SNPs for *INCENP* were genotyped by the BCAC. Additional genotype data were provided for one upstream and one downstream SNP of *INCENP*. Five SNPs (including one surrogate) out of five originally selected SNPs for *AURKB*, *BIRC5* and *CDCA8* were genotyped. A description of all 15 SNPs genotyped for *INCENP*, *AURKB*, *BIRC5* and *CDCA8* can be found in Supplementary Table S2, available at *Carcinogenesis* Online.

Associations of SNPs in CPC genes with breast cancer risk and survival

A total of 88 911 European women (46 450 cases and 42 461 controls) from 39 BCAC studies were included in association analyses. Reported probability values were not adjusted for multiplicity, they should be interpreted considering that 4 genes and 15 partially linked variants were simultaneously investigated.

Five *INCENP* SNPs (top SNP rs1675126) were associated with a decreased overall breast cancer risk. Women with variant rs1675126 showed the largest breast cancer risk reduction (per minor A allele OR 0.95, 95% CI 0.92–0.98, $P = 0.007$). The two SNPs rs4963459 and rs4963471, located, respectively, upstream and downstream of *INCENP* were associated with overall breast cancer risk as well (rs4963459: per minor A allele OR 1.02, 95% CI 1.00–1.04, $P = 0.021$; rs4963471: per minor G allele OR 1.03, 95% CI 1.01–1.05, $P = 0.003$). Association results for overall breast cancer risk of all SNPs in *INCENP* are shown in Table 1. There was also a weak association of rs2306625 in *CDCA8* with overall breast cancer risk (per minor A allele OR 0.97, 95% CI 0.95–0.99, $P = 0.040$; Table 2).

Figure 1A and B represents the clustering of studies by genetic similarity based on the first genetic principal components. The forest plot on the association with overall breast cancer risk for the top SNP rs1675126 is shown in Figure 1B. The reflection of the geographical study distribution was evident. However, a regional clustering of OR estimates was not obvious. Study heterogeneity was not apparent ($I^2 = 0\%$).

The familial breast cancer risk was increased for women with variants rs2071214 and rs3764384 in *BIRC5* (rs2071214: per minor G allele OR 1.12, 95% CI 1.04–1.21, $P = 0.002$; rs3764384: per minor A allele OR 1.04, 95% CI 1.00–1.08, $P = 0.043$; Supplementary Table S3, available at *Carcinogenesis* Online).

Case-only analysis revealed that four *INCENP* SNPs, which were associated with overall breast cancer risk, showed differential association according to ER (top SNP rs1675126: per minor A allele OR 1.09, 95% CI 1.01–1.16, $P = 0.012$), but not to PR or HER2 tumor status (Table 3). Subsequent analysis of subtype-specific disease risk revealed that five *INCENP* SNPs (top SNP rs1675126) showed stronger associations with risk of ER-negative breast tumors than with overall breast cancer risk. Women with variant rs1675126 showed the largest reduction in risk of developing ER-negative tumors (per minor A allele OR 0.89, 95% CI 0.83–0.95, $P = 0.0005$). This observed association was the strongest among all breast cancer risk analyses and remained statistically significant after correction for multiple testing. The Bonferroni-adjusted *P*-value was $P = 0.04$ (0.0005*75 – considering 15 tests on overall breast cancer risk, 15 tests on familial breast cancer and 15 tests for each of the three hormone receptors). The large sample size of the present association study provided sufficient statistical power to detect small differences between cases and controls in allele frequencies. Table 1 displays association results for all *INCENP* SNPs stratified by ER status. The forest plot on the association with ER-negative breast cancer risk for the top SNP rs1675126 is shown in Figure 1C. The *CDCA8* SNP rs2306625 was associated with HER2 (per minor A OR 0.95, 95% CI 0.91–0.99, $P = 0.033$), but not with ER or PR tumor status (Table 3). No association of rs2306625 with risk of HER2-positive or negative breast tumors was observed (Supplementary Table S4, available at *Carcinogenesis* Online).

No survival association—either with overall, breast cancer specific or relapse-free survival—was observed for the SNPs in *INCENP*, *AURKB* and *BIRC5*. The investigated SNP in *CDCA8* was associated with relapse-free survival. Patients with variant rs2306625 showed an increased risk of relapse (per minor A allele hazard ratio 1.17, 95% CI 1.05–1.31, $P = 0.004$, 89% of the survival times were censored). The 5 year RFS rate was 0.90 (95% CI 0.89–0.91) for patients homozygous for the common allele, 0.89 (95% CI 0.88–0.91) for heterozygotes and 0.88 (95% CI 0.83–0.91) for patients homozygous for the minor allele. The association of rs2306625 with relapse-free survival was stronger when cases with a HER2-positive tumor were compared with all controls (per minor A allele hazard ratio 1.56, 95% CI 1.12–2.17, $P = 0.008$, 84% of the survival times were censored). The results from survival analysis of all SNPs in CPC genes are displayed in Supplementary Tables S5–S8, available at *Carcinogenesis* Online. The relapse-free survival stratified by *CDCA8* rs2306625 genotype is shown in Supplementary Figure S1, available at *Carcinogenesis* Online.

Associations of INCENP haplotypes with overall breast cancer risk

The five *INCENP* SNPs that were singly associated with a decreased overall breast cancer risk were in high LD ($r^2 > 0.8$) and located in two LD blocks comprising a region of ~12 kb (Supplementary Figure S2, available at *Carcinogenesis* Online). Haplotypes were estimated for these SNPs in order to assess their synergistic effect on breast cancer risk. First, the SNPs were ordered according to their *P*-values obtained from overall breast cancer risk analysis. Haplotypes were then inferred for (i) the top two SNPs, (ii) the top three SNPs and (iii) all five SNPs. Among all assessed SNP combinations, the model fit was best for the combination of the top three SNPs (AIC = 238222.1), but did not improve the model fit for rs1675126 alone (AIC = 119150.0). The haplotype frequencies and haplotype-specific estimates for all assessed SNP combinations are displayed in Supplementary Table S9, available at *Carcinogenesis* Online.

Results from interaction and pathway analyses are presented in the Supplementary Results, available at *Carcinogenesis* Online.

Genotype imputation of untyped SNPs in the INCENP region

Since several genotyped *INCENP* SNPs were associated with overall and ER-negative breast cancer risk, association mapping was refined by imputing additional variants. The reference panel for imputation comprised 379 individuals of the European subpopulations CEU, TSI (Toscani in Italia), GBR (British from England and Scotland), FIN (Finnish from Finland) and IBS (Iberian populations from Spain) from the 1000 Genomes Project. After visual inspection of recombination rates, an ~465 kb region (from 61 735 132 to 62 201 016 of chromosome 11, NCBI build 37) centered on *INCENP* and comprising 6282 SNPs was selected for genotype imputation. A total of 5078 SNPs fulfilled genotype heterozygosity and were imputed with high accuracy (99.1% median average certainty of best-guess genotypes). Subsequent association analysis revealed that the strongest signal for the association with overall breast cancer risk was obtained for rs1047739 (per minor T allele OR 1.03, 95% CI 1.00–1.06, $P = 0.0009$; Figure 2A). A marginal differential association according to ER tumor status was detected for rs1047739 (per minor T allele OR 1.04, 95% CI 1.00–1.08, $P = 0.005$), but rs144045115 showed the strongest association signal for the association with ER-negative breast cancer risk (per minor A allele OR 1.06, 95% CI 1.02–1.10, $P = 0.0002$; Figure 2B). The two variants are located in the 3′ untranslated region and downstream of *INCENP*. A gene map, recombination rates and LD in the investigated *INCENP* region are represented in Figure 2C, D and E, respectively.

PAF and FRR related to the top INCENP SNP associated with overall breast cancer risk

PAFs and FRRs for *INCENP* SNP rs1047739 compared with previously identified susceptibility variants are displayed in Figure 3. Rs1047739 showed a per allele OR of 1.03 for the association with

Table 1. Association between SNPs in *INCENP* and breast cancer risk

			Overall breast cancer risk			ER-negative breast cancer risk			ER-positive breast cancer risk		
SNP	Genotype	Controls, *N* (%)	Cases, *N* (%)	Per allele OR[a] (95% CI)	*P*-value[b]	Cases, *N* (%)	Per allele OR[a] (95% CI)	*P*-value[b]	Cases, *N* (%)	Per allele OR[a] (95% CI)	*P*-value[b]
rs4963459	GG	13 479 (31.8)	14 552 (31.3)	1.02 (1.00–1.04)	**0.021**	2346 (31.7)	1.02 (0.98–1.06)	0.175	8557 (31.6)	1.01 (0.99–1.03)	0.242
	GA	20 934 (49.3)	22 926 (49.4)			3639 (49.1)			13 322 (49.2)		
	AA	8043 (18.9)	8957 (19.3)			1426 (19.2)			5185 (19.2)		
rs17707648	GG	38 500 (90.7)	41 992 (90.4)	1.00 (0.96–1.05)	0.752	6727 (90.8)	0.98 (0.90–1.07)	0.674	24 464 (90.4)	1.01 (0.96–1.06)	0.566
	GA	3861 (9.1)	4340 (9.3)			666 (9.0)			2541 (9.4)		
	AA	100 (0.2)	118 (0.3)			20 (0.3)			69 (0.3)		
rs1628349	GG	37 684 (88.8)	41 313 (88.9)	0.95 (0.91–0.99)	**0.037**	6646 (89.7)	0.88 (0.81–0.96)	**0.004**	23 951 (88.5)	0.98 (0.93–1.02)	0.401
	GA	4609 (10.9)	4960 (10.7)			743 (10.0)			3008 (11.1)		
	AA	167 (0.4)	176 (0.4)			24 (0.3)			114 (0.4)		
rs1792949	CC	18 911 (44.9)	20 932 (45.4)	0.97 (0.95–0.99)	**0.038**	3401 (46.3)	0.94 (0.90–0.97)	**0.002**	12 024 (44.7)	0.99 (0.97–1.02)	0.799
	CA	18 550 (44.0)	20 081 (43.6)			3190 (43.4)			11 756 (43.7)		
	AA	4699 (11.2)	5100 (11.1)			761 (10.4)			3097 (11.5)		
rs1675063	AA	19 396 (45.7)	21 512 (46.3)	0.97 (0.95–0.99)	**0.029**	3473 (46.9)	0.94 (0.90–0.98)	**0.003**	12 390 (45.8)	0.99 (0.97–1.01)	0.574
	AG	18 594 (43.8)	20 074 (43.2)			3208 (43.3)			11 769 (43.5)		
	GG	4464 (10.5)	4851 (10.5)			729 (9.8)			2908 (10.7)		
rs1675126	GG	34 763 (81.9)	38 102 (82.0)	0.95 (0.92–0.98)	**0.007**	6161 (83.1)	0.89 (0.83–0.95)	**5×10^{-4}**	21 979 (81.2)	0.97 (0.94–1.01)	0.210
	GA	7213 (17.0)	7856 (16.9)			1184 (16.0)			4772 (17.6)		
	AA	482 (1.1)	488 (1.1)			68 (0.9)			319 (1.2)		
rs7129085	AA	16 264 (38.3)	18 062 (38.9)	0.97 (0.95–0.99)	**0.017**	2952 (39.8)	0.93 (0.90–0.97)	**9×10^{-4}**	10 411 (38.5)	0.98 (0.96–1.01)	0.340
	AC	19 943 (47.0)	21 601 (46.5)			3440 (46.4)			12 588 (64.5)		
	CC	6240 (14.7)	6771 (14.6)			1017 (13.7)			4066 (15.0)		
rs3781969	AA	26 025 (61.3)	28 660 (61.7)	0.99 (0.97–1.01)	0.641	4589 (61.9)	0.97 (0.93–1.02)	0.313	16 723 (61.8)	1.00 (0.97–1.02)	0.994
	AC	14 401 (33.9)	15 611 (33.6)			2499 (33.7)			9076 (33.5)		
	CC	2026 (4.8)	2168 (4.7)			324 (4.4)			1266 (4.7)		
rs11230934	AA	22 687 (53.4)	24 937 (53.7)	0.99 (0.97–1.01)	0.514	4031 (54.4)	0.96 (0.92–1.00)	0.074	14 572 (53.8)	1.00 (0.97–1.02)	0.987
	AC	16 638 (39.2)	18 148 (39.1)			2878 (38.8)			10 498 (38.8)		
	CC	3129 (7.4)	3359 (7.2)			503 (6.8)			2000 (7.4)		
rs4963471	AA	23 064 (54.3)	24 865 (53.5)	1.03 (1.01–1.05)	**0.003**	3949 53.3)	1.05 (1.01–1.09)	**0.012**	14 609 (54.0)	1.02 (1.00–1.05)	**0.028**
	AG	16 365 (38.5)	18 265 (39.3)			2915 (39.3)			10 519 (38.9)		
	GG	3032 (7.1)	3314 (7.1)			547 (7.4)			1942 (7.2)		

Probability values <5% are shown in bold.

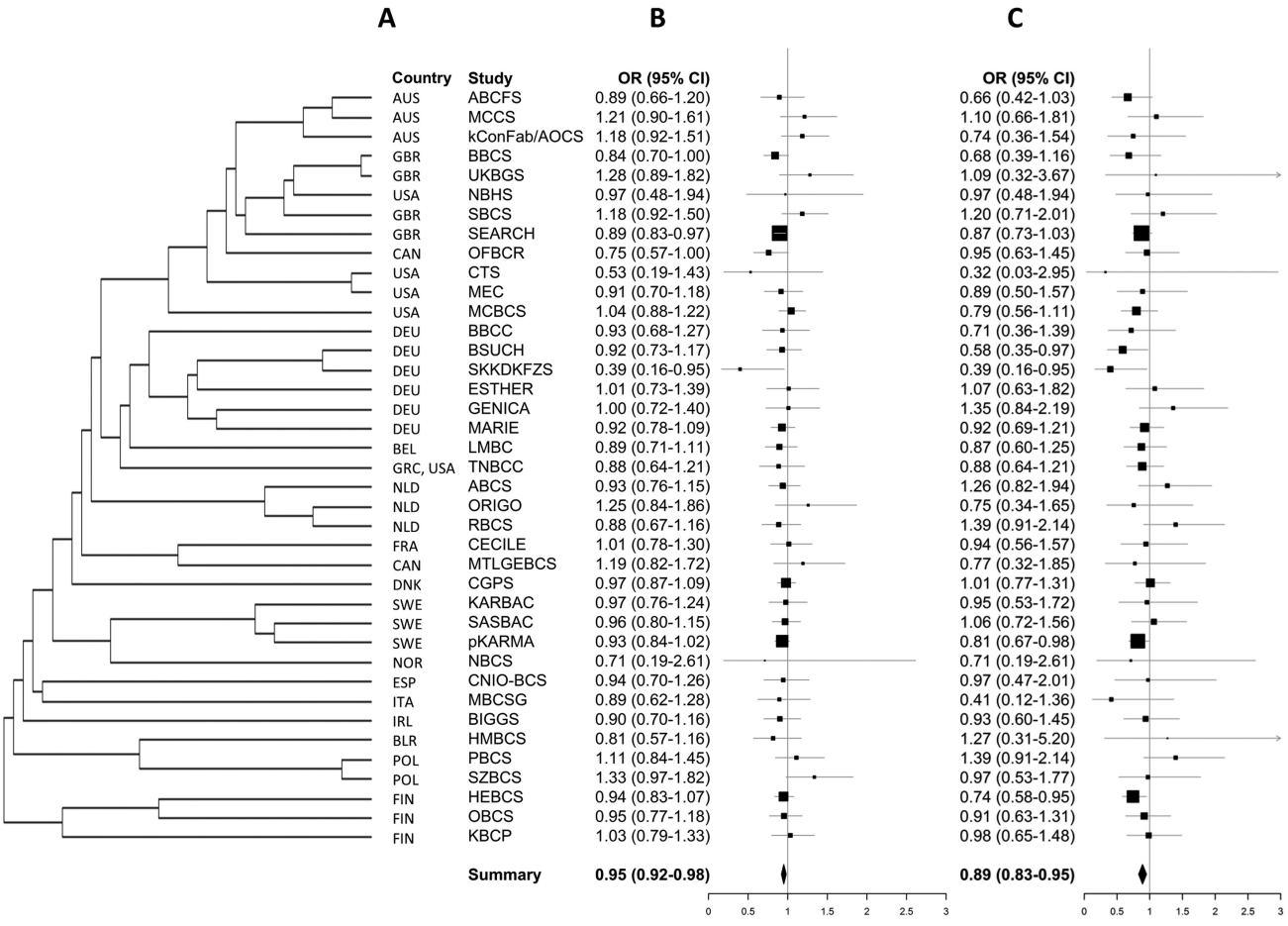
[a]OR adjusted for a fixed study effect and the first 7 principal components.

[b]Probability value based on logistic regression and an additive model.

Table 2. Association between SNPs in *AURKB*, *BIRC5* and *CDCA8* and breast cancer risk

				Overall breast cancer risk		
Gene	SNP	Genotype	Controls, *N* (%)	Cases, *N* (%)	Per allele OR[a] (95% CI)	*P*-value[b]
AURKB	rs1059476	GG	33 953 (80.0)	37 051 (79.8)	0.94 (0.97–1.00)	0.154
		AG	7966 (18.7)	8818 (19.0)		
		AA	523 (1.2)	568 (1.2)		
	rs2241909	AA	18 882 (44.5)	20 753 (44.7)	0.98 (0.96–1.00)	0.196
		AG	18 844 (44.4)	20 508 (44.2)		
		GG	4687 (11.1)	5135 (11.1)		
BIRC5	rs2071214	AA	37 965 (89.4)	41 512 (89.4)	1.02 (0.98–1.06)	0.293
		AG	4356 (10.3)	4792 (10.3)		
		GG	139 (0.3)	145 (0.3)		
	rs3764384	GG	19 315 (45.5)	20 958 (45.1)	1.00 (0.98–1.02)	0.602
		AG	18 690 (44.0)	20 492 (44.1)		
		AA	4449 (10.5)	4991 (10.8)		
CDCA8	rs2306625	GG	28 220 (66.5)	31 003 (66.8)	0.97 (0.95–0.99)	**0.040**
		AG	12 698 (29.9)	13 821 (29.8)		
		AA	1526 (3.6)	1590 (3.4)		

Probability values <5% are shown in bold.
aOR adjusted for a fixed study effect and the first 7 principal components.
bProbability value based on logistic regression and an additive model.



AUS, Australia; BEL, Belgium; BLR, Belarus; CAN, Canada; DEU, Germany; DNK, Denmark; ESP, Spain; FRA, France; FIN, Finland; GBR, United Kingdom; GRC, Greece; IRL, Ireland; ITA, Italy; NLD, Netherlands; NOR, Norway; POL, Poland; SWE, Sweden; USA, United States

Figure 1. (**A**) Clustering of studies based on the first genetic principal components. The distance between merged studies reflects their genetic similarity. (**B**) Forest plot for the association between rs1675126 and overall breast cancer risk. (**C**) Forest plot for the association between rs1675126 and ER-negative breast cancer risk.

Table 3. Association between SNPs in CPC genes and hormone receptor status

Gene	SNP	Genotype	Cases, *N* (%)	ER status		PR status		HER2 status	
				Per allele OR[a] (95% CI)	*P*-value[b]	Per allele OR[a] (95% CI)	*P*-value[b]	Per allele OR[a] (95% CI)	*P*-value[b]
INCENP	rs4963459	GG	14552 (31.3)	0.99 (0.95–1.03)	0.656	1.00 (0.96–1.04)	0.831	0.95 (0.89–1.01)	0.127
		GA	22926 (49.4)						
		AA	8957 (19.3)						
	rs17707648	GG	41992 (90.4)	1.05 (0.96–1.15)	0.234	1.02 (0.94–1.11)	0.558	0.98 (0.85–1.12)	0.797
		GA	4340 (9.3)						
		AA	118 (0.3)						
	rs1628349	GG	41313 (88.9)	1.09 (1.00–1.18)	**0.046**	1.04 (0.97–1.13)	0.215	1.07 (0.94–1.21)	0.281
		GA	4960 (10.7)						
		AA	176 (0.4)						
	rs1792949	CC	20932 (45.4)	1.04 (1.00–1.09)	**0.025**	1.03 (0.99–1.07)	0.111	1.01 (0.95–1.08)	0.607
		CA	20081 (43.6)						
		AA	5100 (11.1)						
	rs1675063	AA	21512 (46.3)	1.03 (0.99–1.08)	0.074	1.02 (0.98–1.06)	0.229	1.03 (0.96–1.09)	0.362
		AG	20074 (43.2)						
		GG	4851 (10.5)						
	rs1675126	GG	38102 (82.0)	1.09 (1.01–1.16)	**0.012**	1.05 (0.99–1.12)	0.064	1.03 (0.93–1.14)	0.569
		GA	7856 (16.9)						
		AA	488 (1.1)						
	rs7129085	AA	18062 (38.9)	1.04 (1.00–1.08)	**0.028**	1.02 (0.98–1.06)	0.243	1.01 (0.95–1.08)	0.594
		AC	21601 (46.5)						
		CC	6771 (14.6)						
	rs3781969	AA	28660 (61.7)	1.00 (0.96–1.05)	0.706	1.00 (0.96–1.05)	0.706	1.00 (0.93–1.08)	0.827
		AC	15611 (33.6)						
		CC	2168 (4.7)						
	rs11230934	AA	24937 (53.7)	1.02 (0.98–1.07)	0.218	1.01 (0.97–1.05)	0.469	1.06 (0.81–1.38)	0.654
		AC	18148 (39.1)						
		CC	3359 (7.2)						
	rs4963471	AA	24865 (53.5)	0.97 (0.93–1.02)	0.273	0.98 (0.94–1.02)	0.528	0.99 (0.92–1.06)	0.775
		AG	18265 (39.3)						
		GG	3314 (7.1)						
AURKB	rs1059476	GG	34056 (80.0)	1.04 (1.00–1.09)	0.052	1.03 (0.99–1.08)	0.071	0.99 (0.94–1.05)	0.873
		AG	7999 (18.8)						
		AA	526 (1.2)						
	rs2241909	AA	18934 (44.5)	1.00 (0.97–1.03)	0.721	0.98 (0.96–1.01)	0.423	0.98 (0.94–1.01)	0.306
		AG	18907 (44.4)						
		GG	4711 (11.1)						
BIRC5	rs2071214	AA	38090 (89.4)	1.03 (0.97–1.09)	0.316	1.04 (0.98–1.10)	0.115	0.98 (0.91–1.05)	0.593
		AG	4370 (10.3)						
		GG	139 (0.3)						
	rs3764384	GG	19384 (45.5)	0.97 (0.95–1.00)	0.110	0.97 (0.94–1.00)	0.067	0.98 (0.95–1.01)	0.374
		AG	18748 (44.0)						
		AA	4461 (10.5)						
CDCA8	rs2306625	GG	28308 (66.5)	1.00 (0.96–1.03)	0.845	0.98 (0.95–1.02)	0.508	0.95 (0.91–0.99)	**0.033**
		AG	12740 (29.9)						
		AA	1535 (3.6)						

Probability values <5% are shown in bold.

[a]OR adjusted for a fixed study effect and the first 7 principal components.

bProbability value based on logistic regression and an additive model.

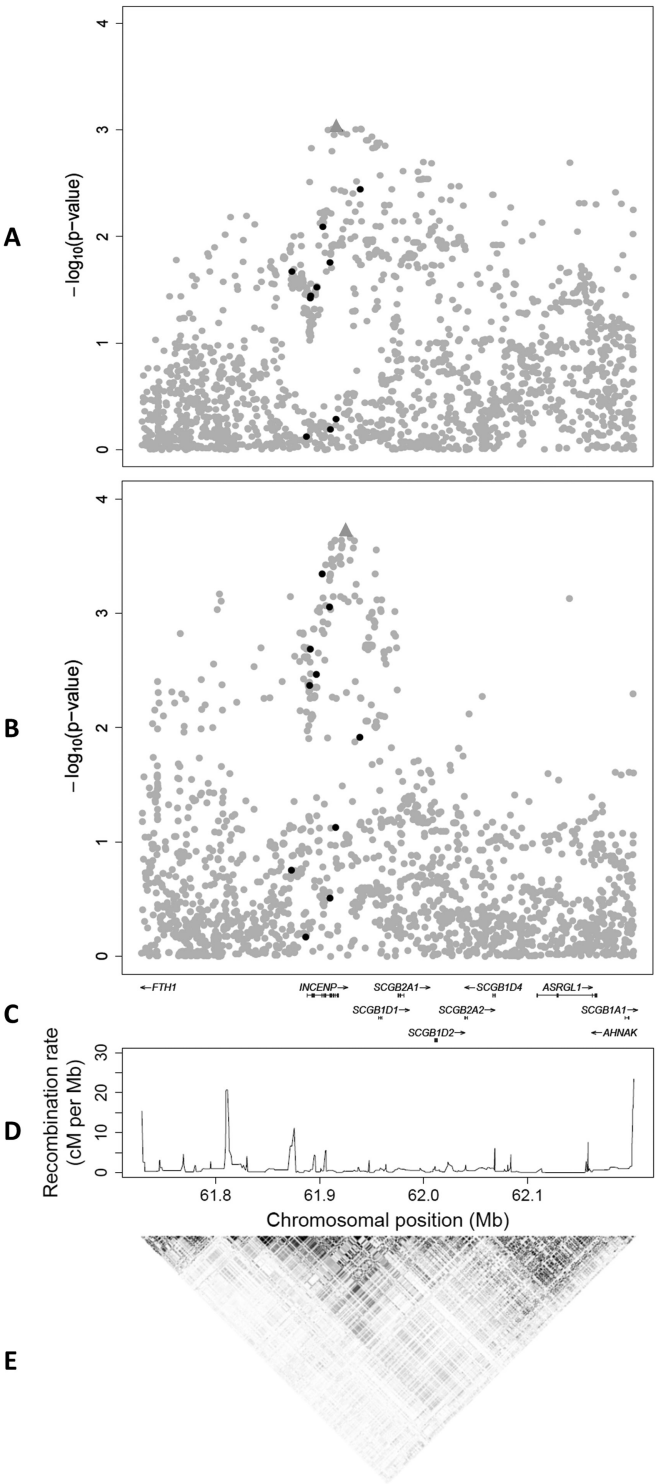


Figure 2. (**A**) Association between overall breast cancer risk and genotyped (black) and imputed (gray) SNPs in the greater *INCENP* region. (**B**) Association between ER-negative breast cancer risk and genotyped (black) and imputed (gray) SNPs in the greater *INCENP* region. Both plots show the $-\log_{10}$ *P*-values based on logistic regression adjusted for study and seven principal components. Only imputed SNPs with MAF > 0.01 are depicted. The imputed SNPs with the smallest *P*-value (rs1047739 and rs144045115) are shown as gray triangles. (**C**) Gene map including all genes in the investigated region. (**D**) Recombination rates in the investigated region. Chromosomal positions refer to NCBI build 37. (**E**) LD heatmap based on genotype data retrieved from the European subpopulations from HapMap phase 3 showing pairwise r^2 values [from 0 (white) to 1 (black)].

overall breast cancer risk and a MAF of 0.24. Assuming a cumulative risk of breast cancer in the European Union of 7.8% until the age of 74, rs1047739 results in a PAF of 1.4% and a FRR of 1.0. In comparison with other susceptibility variants, the *INCENP* SNP rs1047739 contributed to a higher PAF than any rare variant in *BRCA1*, *BRCA2*, *ATM*, *BRIP1*, *CHEK2* or *PALB2*. Most recently identified common susceptibility variants showed larger PAFs and FRRs than rs1047739, where FGFR2 rs2981579 showed the second highest PAF

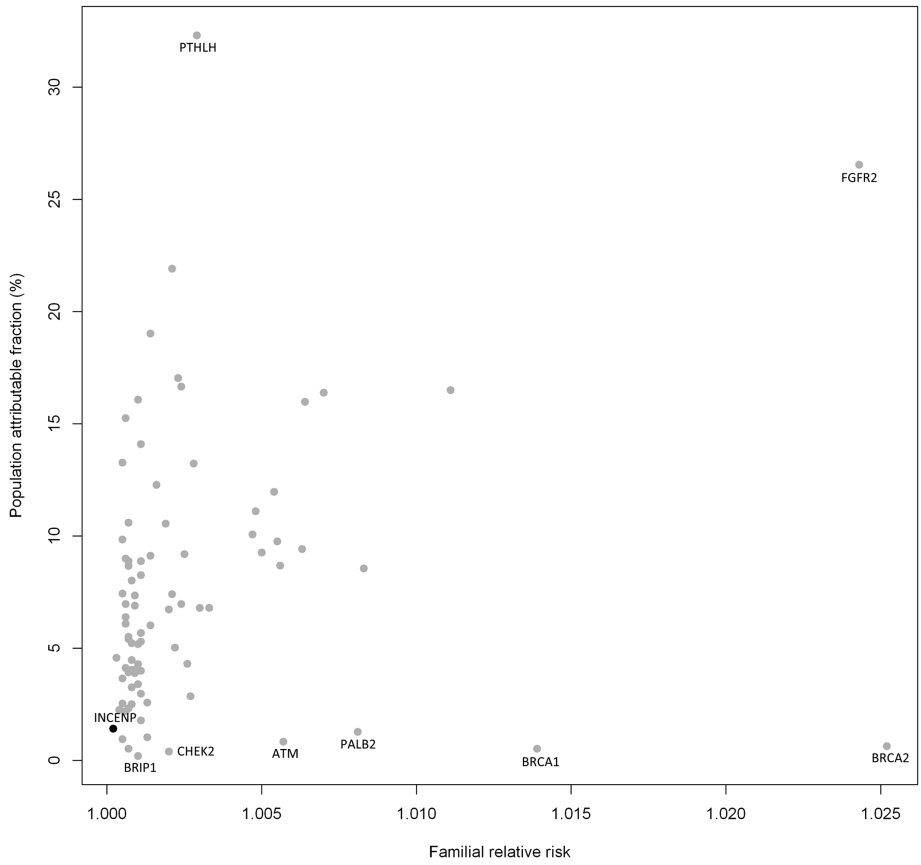


Figure 3. PAFs versus FRRs for all breast cancer susceptibility variants of low, moderate and high penetrance (gray dots). The top imputed *INCENP* SNP rs1047739 is shown as a black dot.

after PTHLH rs10771399 and the highest FRR among all common variants. The updated list of breast cancer susceptibility variants along with the corresponding ORs, MAFs, PAFs and FRRs are listed in Supplementary Table S10, available at *Carcinogenesis* Online.

Associations of INCENP SNPs with gene expression

All variants located between the upstream SNP rs4963459 and the downstream SNP rs4963471 of *INCENP*, including 103 SNPs available for 60 HapMap individuals (expression in lymphoblastoid cells) and 34 SNPs available for 447 TCGA individuals (expression in 60 normal breast tissue samples and 387 tumor breast tissue samples), were examined regarding their impact on gene expression. The mean expression level was −2.84 (±0.54) in the complete set of normal breast tissue samples and −1.88 (±0.80) in the complete set of tumor breast tissue samples ($P < 0.0001$). Two SNPs [expected five (103 × 0.05)] were associated with gene expression in lymphoblastoid cells and one SNP [expected two (34 × 0.05)] was associated with gene expression in normal breast tissue. All of these three SNPs were also associated with overall and ER-negative breast cancer risk. Nine SNPs [expected two (34 × 0.05)] were associated with gene expression in tumor breast tissue. However, none of these were associated with risk of breast cancer. Distribution of INCENP expression levels per SNP genotypes is displayed in Supplementary Figure S3, available at *Carcinogenesis* Online.

Potential functional *INCENP* SNPs

The *INCENP* SNP rs1047739 was annotated with three histone modifications by H3K27me3, H3K36me3 and H4K20me1, indicating an actively transcribed and accessible chromatin region that marks RNA polymerase II elongation and a silenced promoter. Moreover, rs1047739 overlapped with the chromatin state of transcriptional elongation. Altogether, the 15 variants tightly linked ($r^2 \geq 0.8$) to SNP rs1047739 showed features consistent to open chromatin, promoter silencing, blocked enhancer activity and repressed gene expression. Detailed information is presented in Supplementary Table S11, available at *Carcinogenesis* Online.

Discussion

This is the first study that investigates whether genetic variability in genes of the core CPC including *INCENP*, *AURKB*, *BIRC5* and *CDCA8* may affect primarily the overall, familial and subtype-specific breast cancer risk and secondarily the survival.

The INCENP protein of the CPC is a scaffold protein that comprises two functional subunits: The N-terminus binds to BIRC5 and CDCA8, which is required for the localization of the complex to the centromeres of chromosomes, whereas the conserved C-terminus binds AURKB partly activating the kinase. This allows AURKB to phosphorylate a C-terminal Thr-Ser-Ser motif in INCENP and a Thr in the T-loop of its kinase domain, resulting in full AURKB activation (40,41). INCENP is phosphorylated not only by AURKB but also by Cdk1, which is involved in Polo-like kinase 1 recruitment to the kinetochores and also in the progression from metaphase to anaphase (42). Yet, the molecular mechanisms by which SNPs in *INCENP* and other CPC genes influence breast cancer risk are unknown.

We found that several genotyped and imputed SNPs within and downstream of *INCENP* were associated with overall

and particularly with ER-negative breast cancer risk. The SNP rs1675126 showed the strongest association signal with overall and ER-negative breast cancer risk among all genotyped *INCENP* SNPs. The association with ER-negative breast cancer risk is of particular interest. Only 20–25% of all breast tumors are ER-negative. ER-negative breast cancer is often diagnosed at an earlier age and has a worse prognosis than ER-positive breast cancer. So far, seven loci specifically associated to ER-negative breast cancer susceptibility have been identified (43). The imputed SNP that showed the strongest association signal in the overall breast cancer risk analysis was rs1047739 located in the 3′ untranslated region. Even though rs1047739 showed also a differentiated association regarding ER tumor status, imputed rs144045115 downstream of *INCENP* showed the strongest association with ER-negative breast cancer risk. *In silico* analyses indicated that rs1047739 is located in an accessible chromatin region actively transcribed and that three miRNAs (has-miR-346, has-miR-632 and has-miR-654-3P predicted by Targetscan and MicroCosm Targets 5) bind to the rs1047739 containing region, suggesting that it may be the causal variant. The exact molecular mechanisms of how rs1047739 influences *INCENP* transcription should be further investigated *in vitro*. It has been previously reported that expression levels of INCENP are increased in tumor cells (22). This is in line with our finding that INCENP expression was increased in tumor breast tissue samples compared with normal breast tissue samples based on data from TCGA.

In a previous publication, rs2241909 in *AURKB* was associated with familial breast cancer risk in a German study population (30). We could not replicate this association in our present large data set from several European study populations. Instead we observed that the two SNPs rs3764384 and rs2071214 in *BIRC5* were associated with familial breast cancer risk. It was also observed that rs2306625 in *CDCA8* was particularly associated with relapse-free survival. Therefore, rs2306625 may eventually influence both the risk of disease onset and in case of tumor development the pathological characteristics of the tumor and/or its response to treatment. The per G allele increased risk and better-prognosis finding would contrast with *BRCA1/2* mutations in breast cancer (44), but would mimic on the other hand mutations of mismatch repair genes in colorectal cancer (45).

Low recombination rates downstream of *INCENP* allowed for genotype imputation in the region of six secretoglobin genes (*SCGBs*) taking 10 genotyped *INCENP* SNPs as reference. *SCGBs* are members of a supergene family and most of them are localized in a dense cluster on chromosome 11q13 including *SCGB1D1*, *SCGB1D2*, *SCGB2A1*, *SCGB2A2*, *SCGB1D4* and *SCGB1A1* (46). The *SCGBs* encode small secretory proteins and seem to play a role in the modulation of inflammation, tissue repair and tumorigenesis. Some *SCBGs* are overexpressed in breast cancer (47–49) and are more frequently associated with ER-positive tumors (50,51). Interestingly, imputation results indicated variants in the region of *SCGB1D1* throughout *SCGB1A1*, which were associated with overall breast cancer risk [top SNP rs3781965 (located in intron 2 of *SCGB1D1*): per minor T allele OR 1.02, 95% CI 1.00–1.04, $P = 0.001$], whereas associations with ER-negative tumors were detected only for the upstream and gene region of *SCGB1D1* [top SNP rs2232935 (located upstream of *SCGB1D1*): per minor T allele OR 1.05, 95% CI 1.02–1.09, $P = 0.0003$]. Even though the biological functions of *SCGB* products are still poorly understood and variants in the 3′ region of *INCENP* showed stronger association signals in breast cancer risk analysis, initial results on the possible association between inherited variation in *SCGBs* and breast cancer susceptibility should be explored based on directly typed variants in future consortial work.

In conclusion, taking advantage of BCAC and COGS efforts that translated into a homogeneous, high-quality genotyping of 88 911 women from 39 European studies, we were able to identify potential novel variants in the *INCENP* gene, which associated with a 3% per allele increased risk of breast cancer, and with a 6% per allele increased risk of ER-negative breast tumors. This study demonstrates the benefit of scientific collaborations leading to large sample collections in order to identify low-penetrance variants, in particular for disease subtypes. It is likely that next generation sequencing in combination with the integration of information on additional layers of genetic variability will refine marker association signals and unravel increasing proportions of sporadic and familial cases of disease. In parallel, the identification of new susceptibility variants may point to novel drug targets. Due to the established involvement in the regulation of cell division, this is probably the most relevant aspect of the identified associations between CPC variants and breast cancer.

Supplementary material

Supplementary Materials and methods, Results, Tables S1–S11 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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