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Genome-wide association analysis identifies three new breast cancer susceptibility loci

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Abstract

Breast cancer is the most common cancer among women. To date, 22 common breast cancer susceptibility loci have been identified accounting for ~ 8% of the heritability of the disease. We followed up 72 promising associations from two independent Genome Wide Association Studies (GWAS) in ~70,000 cases and ~68,000 controls from 41 case-control studies and nine breast cancer GWAS. We identified three new breast cancer risk loci on 12p11 (rs10771399; $P=2.7\times10^{-35}$), 12q24 (rs1292011; $P=4.3\times10^{-19}$) and 21q21 (rs2823093; $P=1.1\times10^{-12}$). SNP rs10771399 was associated with similar relative risks for both estrogen receptor (ER)-negative and ER-positive breast cancer, whereas the other two loci were associated only with ER-positive disease. Two of the loci lie in regions that contain strong plausible candidate genes: *PTHLH* (12p11) plays a crucial role in mammary gland development and the establishment of bone metastasis in breast cancer, while *NRIP1* (21q21) encodes an ER co-factor and has a role in the regulation of breast cancer cell growth.

Breast cancer is one of the most commonly occurring epithelial malignancies in women with an estimated one million new cases and over 400,000 deaths annually worldwide[1]. Familial aggregation and twin studies have demonstrated the substantial contribution of inherited susceptibility to breast cancer [2, 3]. Over the last four years, we and others have conducted several genome-wide association studies (GWAS) and reported breast cancer susceptibility variants at 21 loci [4-14] with an additional locus (*CASP8*) identified through a candidate gene approach 15. These variants are associated with modest risks of the disease (per-allele odds ratios <1.3), and explain ~ 8% of the excess familial risk of breast cancer, while other rarer high and moderate risk loci contribute less than 20%, suggesting that other loci remain to be identified [16].

To identify further breast cancer susceptibility loci, we selected 72 SNPs that were genotyped and found to be significantly associated with breast cancer at $P<0.0001$ in either of two breast cancer GWAS in the UK (UK2 and BBCS) [17, 18]. We attempted to genotype these SNPs in up to 41 case-control studies through the Breast Cancer Association Consortium (BCAC). After quality control (QC) exclusions (see Methods), we analysed data on 54,588 cases of invasive breast cancer, 2401 cases of Ductal Carcinoma *in Situ* (DCIS) and 58,098 controls. In addition, we utilised data from 7 additional breast cancer GWAS from which summary results had been obtained based on imputation to Hapmap 2 CEU. Results from the GWAS and BCAC replication were then combined to derive the overall evidence of association for each SNP based on 69,564 cases and 68,150 controls.

Three SNPs showed strong evidence for association in European women, consistent with the effect seen in the original GWAS (Table 1 and Figure 1). In each case, the genotype-specific odds ratios (ORs) were consistent with an allele dose (log-additive) model (Supplementary Table 1). SNP rs2823093 showed some evidence of heterogeneity in the per-allele ORs among studies in the replication stage ($P=0.002$), with particularly marked associations in two studies (HMBCS, RBCS; Figure 1). The association in the replication stage remained highly significant, however, even after excluding these two studies ($P=7.1\times10^{-7}$). The other two loci showed no evidence of heterogeneity among studies. Two additional SNPs on 17q21, rs2532348 and rs199523 (correlated at $r^2=0.80$ in the UK2 GWAS), gave more limited evidence of replication ($P=0.000078$ and $P=0.0063$) and reached $P=5.8\times10^{-7}$ and $P=2.6\times10^{-6}$ respectively when combined with the GWAS data (Supplementary Table 2). These SNPs were only genotyped in the UK2 GWAS. They could not be imputed using HapMap, and were only successfully genotyped in 12 studies in the BCAC replication. Moreover, for SNP rs2532348 there was evidence of heterogeneity among studies in the per-allele ORs in BCAC ($P=0.001$). Further data will be required to determine whether this SNP is associated with breast cancer risk. Three other SNPs (rs10940235 on 5q11, rs4403040 on

4q21 and rs6027564 on 20q13) showed evidence of replication at P<0.01 but none reached genome-wide levels of statistical significance (Supplementary Table 2).

For women of Asian ancestry, SNP rs10771399 (12p11) was also associated with breast cancer risk, with the estimated OR being similar to that in women of European ancestry (Supplementary Table 3). There was no significant evidence of association for either SNPs rs1292011 (12q24) or rs2823093 (21q21) in women of Asian ancestry. For rs2823093, the estimated OR was in the opposite direction than that in women of European ancestry, but the estimates did not differ significantly (Supplementary Table 3).

SNP rs10771399 showed strong evidence of association with both estrogen receptor (ER)-positive and ER-negative breast cancer, with the estimated per-allele ORs being similar (based on 24,775 ER-positive and 7,122 ER-negative cases; Supplementary Table 4a). In contrast, for SNPs rs1292011 and rs2823093, the association was confined to ER-positive breast cancer, with no evidence of association for ER-negative disease (Supplementary Table 4a). These latter results conform to the general pattern of a preponderance of common susceptibility loci for ER-positive disease identified through GWAS based on cases unselected for disease subtype [19, 20]. In terms of per-allele OR, SNP rs10771399 has one of the strongest effects identified to date for ER-negative breast cancer (OR 0.85, 95%CI 0.80-0.90). For all three SNPs, the per-allele OR for DCIS was similar to that for invasive disease (based on up to 2,148 DCIS cases; Supplementary Table 4b). For SNP rs10771399, the estimated OR was higher for 10 studies in which cases were selected for a positive family history and/or bilaterality, as would be expected under a polygenic model [21] (*P*=0.027, Supplementary Table 5); however, exclusion of data from these studies made little difference to the estimated OR. There was no evidence for difference in the per-allele OR by age at diagnosis for any SNP (Supplementary Table 4c).

SNP rs10771399 lies in a ~300kb linkage disequilibrium (LD) block on 12p11 that contains one known gene, *PTHLH* (Parathyroid Hormone like Hormone isoform 1), also called *PTHrP* (Parathyroid hormone–related protein; Figure 2a). PTHrP is expressed in a wide variety of tissues and in many malignancies, including 60% of breast tumors and is required for normal mammary gland and bone development [22-25]. During lactation it is released by the mammary gland to regulate the transfer of calcium from the skeleton to the milk [26, 27]. Tumor secreted PTHrP mimics the action of parathyroid hormone (PTH) by binding to its receptor PTH1R [28] promoting humoral hypercalcemia as well as metastasis of breast cancer cells to the bone [23, 29-31]. It has been suggested that PTHrP enhances tumorigenesis through its pro-proliferative and anti-apoptotic activity by promoting survival in cells subjected to apoptosis [32, 33]. However, conflicting data regarding the correlation of PTHrP expression level and breast cancer survival have been found [24, 34-36]. Moreover, a recent study reported that loss of PTHrP accelerates tumor incidence in DCIS and is associated with monocyte infiltration [37].

SNP rs1292011 on 12q24 lies in a ~ 100 kb LD block that contains no known genes (Figure 2b). SNPs in this region have been found to be associated with squamous esophageal carcinoma, renal cell carcinoma, liver adenoma, heart disease and type 1 diabetes as well as blood pressure and PSA levels [38-47]. Two plausible cancer candidate genes, *MAPKAPK5* (mitogen-activated protein kinase-activated protein kinase 5, also called *MK5/PRAK*) and *TBX3* (T-box3), lie within 2 Mb of rs1292011. MAPKAPK5 is a member of the serine/threonine kinase family and is directly activated by *Myc* [48]. TBX3 plays a role in mammary gland development[49] and its haplo-insufficiency is associated with Ulnar-Mammary disorder[50]. *TBX3* was found to be amplified and over-expressed in several cancers including breast cancer [51-54] and at high levels in plasma from breast and ovarian cancer patients [52]. Recently, it has been shown that estrogen regulates the expansion of breast cancer stem cells

through the FGF/FGFR/TBX3 pathway [52, 55] and that *TBX3* is a direct downstream target of the Wnt/beta-catenin pathway [56]. The expression of *TBX3* was found to be significantly higher (P<0.0001) in ER-positive than in ER-negative breast cancer tumors in two independent datasets containing 781 tumors (with HGU-133A Affymetrix expression data) [57] and 244 tumors (with 44k Agilent expression data) [58]. These data suggest that the association of rs1292011 with ER-positive breast cancer could be mediated through its effect on *TBX3*.

SNP rs2823093 lies in a ~ 130 Kb LD block containing no known genes. The nearest gene, ~900 Kb downstream, is *NRIP1* (Nuclear Receptor interacting protein 1) (Figure 2 c) or also called *RIP140 (*Receptor-interacting protein 140). RIP140 acts as a strong transcriptional repressor for nuclear receptors [59, 60]. It interacts with estrogen receptor α (ERα), represses the ER signalling and inhibits its mitogenic effects [61]. This repression is mediated through interaction with FHL1, a protein involved in suppressing cancer cell growth and migration [62]. Several lines of evidence suggest that RIP140 plays an important role in the regulation of breast cancer cell growth. Knockdown of *RIP140* was found to induce growth promotion in an ER-positive breast cancer cell line [61]. This protein was also highly induced following the treatment of human breast cancer cells with retinoids, known for their breast cancer growth suppression and their anti-estrogenic effects [63-66]. A Spanish case-control study, which genotyped SNPs in 91 breast cancer candidate genes in ~700 cases and ~700 controls, identified a relatively rare SNP at this locus (rs926184 - MAF~2%), located 175 Kb upstream of rs2823093, which showed a modest association with breast cancer [67]. These two SNPs are, however, not correlated (r^2=0 in HapMap CEU). The expression of *NRIP1* has been shown to be significantly higher in ER-positive than ER-negative tumors (p<0.0001) [57, 58] suggesting that the association of rs28323093 with ER-positive breast cancer could be mediated through its effect on *NRIP1* expression [57, 58].

The three novel susceptibility variants identified in this study are relatively common (MAF 0.11-0.41) and together explain ~0.7% of the familial risk of breast cancer, and bring the total contribution of common low-penetrance breast cancer susceptibility loci to ~9%. The relative risks associated with these variants are modest, with the per-allele ORs for the risk allele ranging from 1.07 to 1.22 fold, but the causal variants underlying some of these loci might confer more substantial risks. The present work highlights the importance of combining GWAS and large-scale replication studies with tumor subtyping in the identification and characterisation of breast cancer susceptibility loci.

The genes in these regions (if proven to be the causal genes) underscore that diverse mechanisms are likely to be relevant to breast cancer pathogenesis. Re-sequencing of these loci, combined with fine-scale mapping and functional analyses will provide more insights into the genetic architecture of breast cancer and the pathogenesis of the disease.

Methods

GWAS analysis

Primary genotype data were obtained for nine breast cancer GWAS in populations of European ancestry (Supplementary Table 6). Standard QC was performed on all scans, as follows. We excluded all individuals with low call rate (<95%), extreme high or low heterozygosity ($P<10^{-5}$), and all individuals evaluated to be of non-European ancestry (>15% non-European component, by multidimensional scaling using the three Hapmap2 populations as a reference). We excluded SNPs with: call rate <95%; call rate <99% and MAF<5%, all SNPs with MAF<1%, and SNPs whose genotype frequencies departed from Hardy-Weinberg equilibrium at $P<10^{-6}$ in controls or $P<10^{-12}$ in cases. For highly

significant SNPs the genotype intensity cluster plots were examined manually to judge reliability, either centrally or by contacting the original investigators.

Data were imputed for all scans for ~2.6M SNPs using HapMap version 2 CEU as a reference, using the program Mach v1.0. Estimated per-allele ORs and standard errors were generated from the imputed genotypes using Probabel[69]. For two studies (UK2 and HEBCS), estimates were adjusted by the first three principal components, since this was found to materially reduce the inflation. Residual inflation was then adjusted for by multiplying the variance by a genomic control adjustment factor, based on the ratio of the median chi-squared test statistic to its expected value. BBCS and UK2 used the same control data (WTCCC2) but different genotyping platforms. These studies were imputed separately. For the combined analysis, the control set was divided randomly between the two studies, in proportion to the size of case series, to provide disjoint strata. For a limited subset of SNPs that could not be imputed (including rs2532348 and rs199523 on 17q21), genotype data from the original scan(s) were used in the analysis.

Replication stage

SNPs for replication were genotyped in 46 studies, of which 4 were case-only studies that did not contribute to the current analysis (Supplementary Table 7). Data from BBCS were excluded as the same cases were included in the GWAS. Seven studies (HABCS, HMBCS, HUBCS, KARBAC, RBCS, SEARCH and SEBCS) were analysed by Fluidigm for 72 SNPs (Supplementary Table 2). We selected 63 SNPs selected from UK2: one replaced by a better surrogate, and one failed, so only data were available for 61 SNPs. Ten SNPs were selected from BBCS and one SNP was selected from both scans (The original SNP, rs1975930, also referred to as rs56003999, did not work by Fluidigm and in some iPlex analyses and was replaced by a surrogate rs10771399, r^2=0.95, which was typed in all studies). Samples from 27 studies were genotyped by iPlex for 29 SNPs that showed the strongest associations. Seven additional studies (ABCFS, CGPS, MCCS, NC-BCFR, OFBCR, PBCS, UKBGS) were genotyped by Taqman for up to 4 SNPs that showed association after the Fluidigm and iPlex genotyping, including all three 3 SNPs discussed in detail here. We restricted the analysis to individuals of European or East Asian ancestry, since the sample size for other ethnicities was too small to give meaningful results.

All studies complied with BCAC genotyping QC standards by including at least 2% of samples in duplicate and a common set of 93 CEPH DNAs used by the HapMap Consortium (HAPMAPPT01, Coriell Institute for Medical Research, Cambden, NJ). Genotype data were excluded for: any sample that consistently failed genotyping for >20% of the SNPs typed; all samples on any one plate that had a SNP call rate <90%; all genotype data for any SNP where overall call rate was <95%; and all genotype data for any SNP where duplicate concordance was <98% (based on 2% of samples genotyped in duplicate). In addition, for any SNP for which the *P*-value for departure from Hardy-Weinberg equilibrium for controls was <0.005, clustering of the intensity plots was reviewed manually and the data excluded if clustering was judged to be poor. After QC exclusions we analysed data on 54,588 cases of invasive breast cancer, 2,401 cases of DCIS and 58,098 controls.

Per-allele and genotype-specific odds ratios for the replication stage were estimated using logistic regression, adjusted for study. Women of European and Asian ancestry were analysed separately. NC-BCFR contributed cases and controls to both European and Asian analyses; for the remaining studies the subjects were either predominantly European or predominantly Asian, and subjects from other minority ethnicities were excluded.

Statistical significance levels from the GWAS and BCAC replication phases were obtained by combining the logOR estimates and standard errors as in a fixed effect meta-analysis.

Heterogeneity in the OR association with each SNP by ER status was evaluated using a case-only analysis, by logistic regression. Heterogeneity by age was evaluated by fitting a linear age × genotype interaction term.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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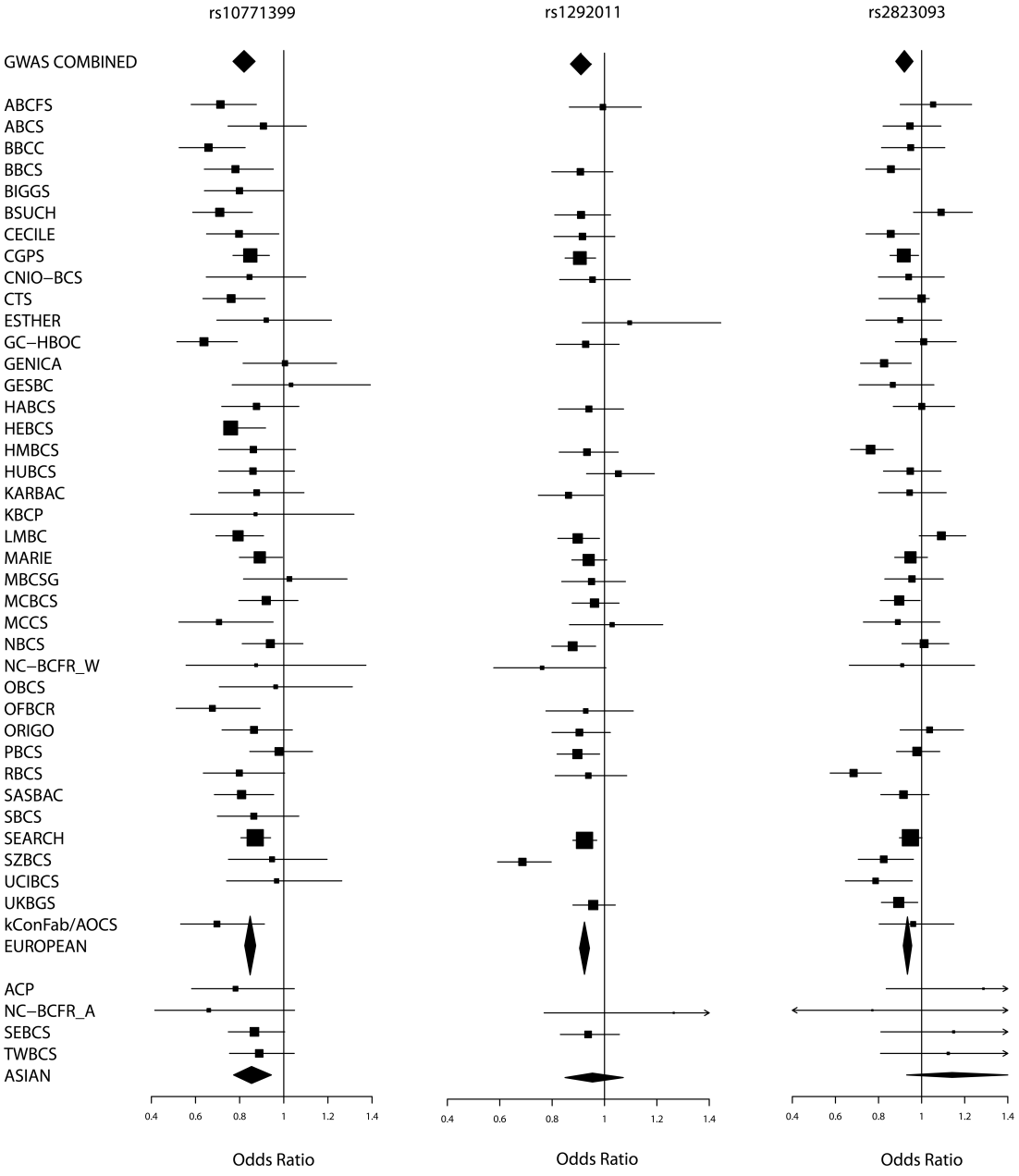

Figure 1.

Forest plots for the 3 SNPs showing evidence of association with breast cancer. Squares represent the estimated per-allele odds ratio (OR) for individual studies. The area of square is inversely proportional to the precise of the estimate. Diamonds represent the summary OR estimates for the subgroups indicated. Horizontal lines represent 95% confidence limits.

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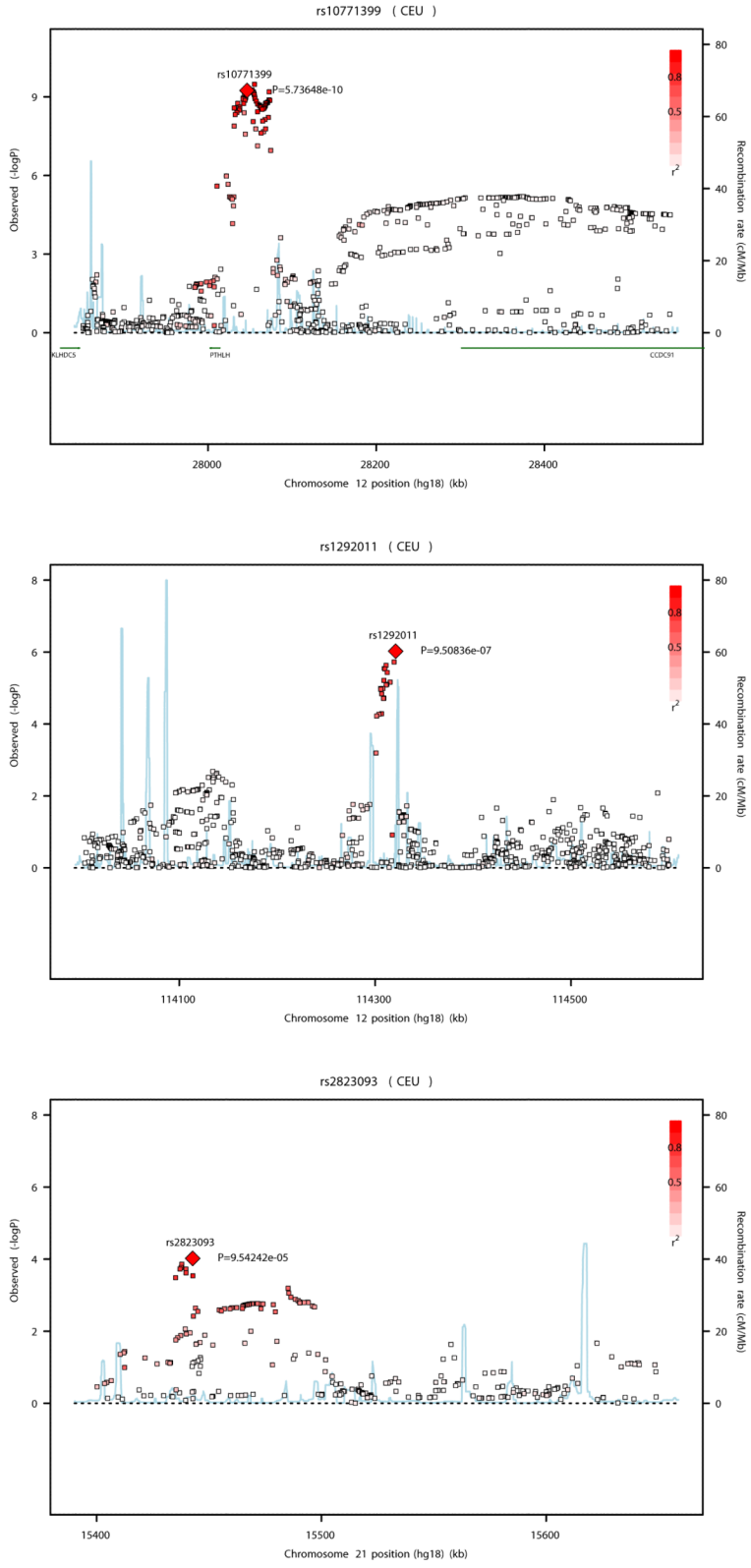

Figures 2a, b and c.

Association plots for the three new breast cancer susceptibility loci at (a) 12p11 (b) 12q24 and (c) 21q21 drawn using the SNAP software35 [68]. Genotyped and imputed SNPs are plotted based on their chromosomal position in build 36 on the X axis and their overall *P* values (as −log10 values) from the UK2 and BBCS GWAS on the Y axis. For each region, the most strongly associated SNP is represented by a diamond. The intensity of the red shading reflects the strength of correlation (r^2) between the best SNP and the other SNPs in the region. Genes present in the region (if any) are indicated in green.

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Table 1

SNP	Chromosome Position[1]	Alleles[2]	MAF	Stage	Per-allele OR (95%CI)[3]	P	Combined P
rs10771399	12p11 28046347	AG	0.12	UK2	0.79 (0.71-0.87)	3.1×10^{-6}	
			0.11	BBCS	0.84 (0.74-0.96)	.008	
			0.10	Other GWAS	0.83 (0.75-0.91)	5.7×10^{-5}	
			0.12	BCAC replication	0.85 (0.83-0.88)	3.3×10^{-27}	2.7×10^{-35}
rs1292011	12q24 114320905	AG	0.41	UK2	0.88 (0.83-0.94)	5.8×10^{-5}	
			0.42	BBCS	0.95 (0.88-1.03)	0.23	
			0.40	Other GWAS	0.91 (0.86-0.96)	.0008	
			0.41	BCAC replication	0.92 (0.91-0.94)	6.2×10^{-14}	4.3×10^{-19}
rs2823093	21q21 15442703	GA	0.26	UK2	0.96 (0.89-1.03)	0.21	
			0.26	BBCS	0.88 (0.76-0.92)	.00013	
			0.26	Other GWAS	0.91 (0.85-0.97)	.0032	
			0.27	BCAC replication	0.94 (0.92-0.96)	1.7×10^{-9}	1.1×10^{-12}

[1] Build 36

[2] Minor allele listed second

[3] Per copy of the minor allele