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Common variation near *CDKN1A*, *POLD3* and *SHROOM2* influences colorectal cancer risk

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Abstract

We performed a meta-analysis of five genome-wide association studies to identify common variants influencing colorectal cancer (CRC) risk comprising 8,682 cases and 9,649 controls. Replication analysis was performed in case-control sets totalling 21,096 cases and 19,555 controls. We identified three novel CRC risk loci at 6p21 (rs1321311, near *CDKN1A*; $P=1.14\times10^{-10}$), 11q13.4 (rs3824999, intronic to *POLD3*; $P=3.65\times10^{-10}$) and Xp22.2 (rs5934683, near *SHROOM2*; $P=7.30\times10^{-10}$) This brings to 20 the number of independent loci associated with CRC risk, and provides further insight into the genetic architecture of inherited susceptibility to CRC.

Many colorectal cancers (CRCs) develop in genetically susceptible individuals, most of whom are not carriers of germ-line mismatch repair or *APC* mutations[1-3]. Genome-wide association studies (GWASs) have validated the hypothesis that part of the heritable risk of CRC is attributable to common, low-risk variants identifying CRC susceptibility loci at 17 loci[4-10]. The statistical power of individual GWASs is limited by the modest effect sizes of genetic variants and financial constraints on the numbers of variants that can be followed up. Meta-analysis of existing GWAS data offers the opportunity to discover additional disease loci given current projections for the number of independent regions harbouring common variants associated with CRC risk[11]. In this study, we conducted a meta-analysis of GWAS data, followed by validation in multiple independent case-control series, identifying three novel susceptibility loci for CRC.

The discovery phase comprised five GWAS datasets from the UK population, totalling 8,682 cases and 9,649 controls (Supplementary Table 1). The Scotland1 GWAS consisted of genotyping 1,012 early-onset Scottish CRC cases and 1,012 controls using the Illumina HumanHap300 and HumanHap240S arrays (COGS Study). The London phase 1 (UK1) was based on genotyping 940 cases with familial colorectal neoplasia and 965 controls ascertained through the Colorectal Tumour Gene Identification (CoRGI) consortium using Illumina HumanHap550 arrays. Scotland2 was based on an additional 2,057 cases and 2,111 controls (SOCCS Study) and UK2 samples comprised an additional 2,873 CRC cases and 2,871 controls ascertained through the National Study of Colorectal Cancer Genetics (NSCCG). Scotland2 and UK2 samples were genotyped using Illumina Infinium-iSelect and GoldenGate arrays for a common set of 43,140 SNPs: the 14,982 most strongly associated SNPs from UK1; the 14,972 most strongly associated SNPs from Scotland1 and 13,186 SNPs showing the strongest association from a joint analysis of all CRC cases and controls from both phase 1 datasets. The VQ58 GWAS comprised 1,800 CRC cases from the UK-

based VICTOR and QUASAR2 adjuvant chemotherapy clinical trials. The VQ58 cases were

108. North Tyneside General Hospital
109. Northampton General
110. Nottingham City Hospital
111. Peterborough District
112. Poole Hospital, Dorset
113. Portsmouth Oncology Centre, Queen Alexandra Hospital
114. Princess Alexandra Hospital
115. Queen Elizabeth, The Queen Mother Hospital
116. Queen Elizabeth, University Hospital, Birmingham
117. Queen Elizabeth, Woolwich
118. Queens, Burton Upon Trent
119. Raigmore Hospital
120. Royal Berkshire / Berkshire Cancer Centre
121. Royal Bournemouth Hospital
122. Royal Cornwall
123. Royal Derby Hospital
124. Royal Free Hospital
125. Royal Hampshire County Hospital, Winchester
126. Royal Marsden, Fulham Road
127. Royal Marsden, Sutton
128. Royal Preston Hospital
129. Royal Surrey County Hospital, St Lukes
130. Royal Sussex County Hospital, Brighton
131. Salisbury District
132. Scarborough Hospital
133. Scunthorpe General
134. Singleton Hospital
135. South Tyneside
136. Southampton Hospital
137. Southend Hospital
138. Southport & Formby DGH
139. St. Bartholomew's Hospital
140. St. George's Hospital, London
141. St. James' Hospital, Dublin
142. St. James' University Hospital, Leeds (Cookridge)
143. St. Mary's Hospital, Isle Of Wight
144. St. Mary's, London
145. St. Vincent's Hospital, Dublin
146. Staffordshire General Hospital
147. Sunderland Royal Hospital
148. Torbay Hospital
149. University College Hospital London
150. University Hospital of North Staffs NHS Trust
151. Velindre Hospital, Cardiff
152. Wansbeck General Hospital
153. Waterford Regional Hospital, Waterford
154. West Middlesex and Charing Cross
155. West Suffolk Hospital
156. West Wales General
157. Western General, Edinburgh
158. Weston General Hospital, Weston Super Mare
159. Weston Park Hospital, Sheffield
160. Whiston Hospital
161. William Harvey Hospital
162. Withybush General Hospital
163. Worcestershire Royal Hospital
164. Worthing Hospital
165. Yeovil District Hospital
166. Ysbyty Gwynedd
167. Pharmacy Department, Weston Park Hospital, Sheffield
168. Radiotherapy Department, Charing Cross Hospital, London
169. Medical Research Council Clinical Trials Unit, London, UK
170. Institute of Cancer Research, Sutton
171. Barts Mesothelioma Research, 54 New Cavendish Street, London
172. Queen's University, Belfast
173. UCL Hospitals NHS Foundation Trust, London
174. The London Clinic, Consulting Rooms, 116 Harley Street, London
175. Section of clinical trials research, university of leeds
176. Institut Multidisciplinaire d'Oncologie, Clinique de Genolier
177. Christie Hospital, Manchester

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

genotyped using the Illumina Hap300 and Hap370 arrays. The 2,690 controls, typed on the Illumina Human-1.2M-Duo Custom_v1 array, were from the UK population-based 1958 Birth Cohort.

Prior to undertaking the meta-analysis of all GWAS datasets, we searched for potential biases in each case-control series (Supplementary Figure 1). Comparison of the observed and expected distributions showed little evidence for an inflation of the test statistics (Supplementary Figure 2), thereby excluding the possibility of significant hidden population substructure, cryptic relatedness among subjects or differential genotype calling. Principal component analysis showed that the cases and controls were genetically well matched (Supplementary Figure 3; Supplementary Note). Any outliers or related individuals were excluded (Supplementary Methods; Supplementary Figure 1).

We also made use of data on 260 SNPs from 2,183 cases and 2,501 controls which had been genotyped as part of the COINNBS series. These SNPs had been selected as showing some evidence of association with CRC in a previous meta-analysis of the 5 GWAS datasets in which a smaller set of VQ cases had been genotyped[8] (Supplementary Table 1).

Using data from the above six studies, we derived for each SNP joint odds ratios (ORs) and confidence intervals (CIs) under a fixed-effects model, and the associated *P*-values. We identified two SNPs, rs1321311 and rs3824999, showing good evidence of association ($P<5.0\times10^{-5}$) and mapping to distinct loci not previously associated with CRC risk. This threshold did not exclude the possibility that other SNPs represented genuine association signals, but was simply a pragmatic strategy for prioritizing replication.

To validate our findings, we conducted a replication study of rs1321311 and rs3824999, genotyping samples from nine additional case-control series: Colon Cancer Family Registry (CCFR1), UK NSCCG (UK3), UK CORGI (UK4), Edinburgh (Scotland3), Cambridge (Cambridge), Croatian (Croatia), Finnish Colorectal Cancer Predisposition Study (Helsinki), and Swedish (Sweden), together with a Japanese study (Japan) (Supplementary Table 1). In the combined analysis, both rs1321311 ($P=1.14\times10^{-10}$; $P_{het}=0.55$, $I^2=0\%$) and rs3824999 ($P=3.65\times10^{-10}$; $P_{het}=0.05$, $I^2=41\%$) showed evidence for an association with CRC at genome-wide significance (*i.e.*, $P<5.0\times10^{-8}$) (Table 1, Supplementary Table 2).

rs3824999 maps to 11q13.4 at 74,023,198bps, within intron 9 of the *POLD3* gene (polymerase DNA-directed delta 3; MIM 611415; Figure 1). POLD3 is a component of the DNA polymerase-δ complex which comprises proliferating cell nuclear antigen (PCNA), the multisubunit replication factor C and the 4-subunit polymerase complex. As well as being involved in suppression of homologous recombination, the DNA polymerase-δ complex participates in DNA mismatch and base excision repair, key processes shown to be defective in Mendelian CRC susceptibility disorders[12].

rs1321311 maps to 6p21 at 36,730,878bps within a region of linkage disequilibrium (LD) that encompasses the *CDKN1A* gene (cyclin-dependent kinase inhibitor 1A; MIM 116899; Figure 1). Intriguingly, rs1321311 has been shown to be associated with electrocardiographic QRS duration[13]. *CDKN1A* encodes $p21^{WAF1/Cip1}$ which mediates p53-dependent G1 growth arrest[14]. Moreover, p21 acts as a master effector of multiple tumour

suppressor pathways which are independent of classical p53 tumour suppression. Also, by binding to PCNA, p21 interferes with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating PCNA-dependent DNA repair[14]. Through binding to PCNA, p21 also competes for PCNA binding with DNA polymerase-δ and several other proteins involved in DNA synthesis, thus directly inhibiting DNA synthesis[14]. Similarly, p21 represses MYC-dependent transcription and in turn, MYC disrupts the PCNA–p21 interaction, thus alleviating p21-dependent inhibition of PCNA and DNA synthesis[14]. Decreased p21 expression has been reported to be a feature of dysplastic aberrant crypt foci in colonic mucosa and adenomas. The finding that p21 down-regulation inversely correlates with MSI status in CRC, irrespective of p53 status, again invokes a relationship with defective DNA repair and genomic instability[14].

Including the two newly-discovered SNPs, a total of 19 independent risk SNPs for CRC have been identified, all mapping to autosomal regions of the genome. The risk of sporadic CRC is higher for males in both economically developed and less-developed countries. Furthermore, males are at greater overall CRC risk and have earlier age at onset in Lynch Syndrome[15-17]. It is therefore possible that some of these differences in risk are attributable to sex chromosome genetic variation. To explore this hypothesis, we studied the relationship between SNPs mapping to the sex-specific region of the X-chromosome and CRC risk. Genotypes were analysed using an extension to the standard Cochran-Armitage test for trend[18] (Supplementary Methods).

rs5934683 was the only SNP showing strong evidence of association in the meta-analysis of UK1, UK2, Scotland1, Scotland2, and VQ58. We then genotyped rs5934683 in UK3, Scotland3, UK4, CCFR1, Cambridge, Croatia, Helsinki, Sweden, and Japan (Supplementary Table 1). In the combined analysis, rs5934683 showed evidence for an association with CRC at genome-wide significance (P=7.30×10^{-10}, P_{het}=0.31, I^2=13%; Table 1; Supplementary Table 2).

rs5934683 maps to Xp22.2 within a 43Kb region of LD (9,711,474bps, Figure 1). Two genes map to this region, *GPR143* (G protein-coupled receptor 143; MIM300808) which is expressed by melanocytes and retinal pigment epithelium and *SHROOM2* (shroom family member 2; MIM 300103) a human homolog of the *Xenopus laevis APX* gene. rs5934683 is situated between *GPR143* and *SHROOM2* and appears to be within the distal promoter region of SHROOM2. There is also evidence of longer, less abundant *GPR143* transcripts extending into the SHROOM2 promoter. *SHROOM2* is known to have broad roles in cell morphogenesis, during endothelial and epithelial tissue development[19]. Missense mutations in *SHROOM2* have been detected in large-scale screens for recurring mutations in cancer cell lines[20]. Like *GPR143*, SHROOM2 regulates melanosome biogenesis and localisation in the retinal pigment epithelium[21]. Intriguingly, abnormal retinal pigmentation, similar to the congenital hypertrophy of retinal pigment epithelium (CHRPE) lesions that are a component of the familial adenomatous polyposis syndrome, has been previously been shown to be an extracolonic feature of non-FAP CRC[22,23]. To our knowledge, the relationship between Xp22.2 and CRC risk represents the first evidence for the role of X-chromosome variation in predisposition to a non-sex-specific cancer.

Next we assessed associations between clinico-pathological variables (sex, age at diagnosis, family history of CRC, tumour site, stage or microsatellite instability) and genotype at rs1321311, rs3824999 and rs5934683 through case-only logistic regression (Supplementary Table 3). After adjusting for multiple testing, we did not find any significant association.

To analyse comprehensively the associations at 6p21, 11q13.4 and Xp22.2, we imputed genotypes in GWAS cases and controls using HapMap3 and 1000genomes data for the autosomal regions and HapMap release21 for Xp22.2 (Supplementary Methods; Figure 1). We did not find substantive evidence of stronger associations at the 6p21.2 and Xp22.2 risk loci. However, at the 11q13.4 locus, rs72977282, mapping 3,188bps 5′ to *POLD3*, was more strongly associated with CRC than rs3824999 (Figure 1; Supplementary Table 4). No non-synonymous SNPs showing strong LD (*i.e.* $r^2>0.4/D'>0.8$) with rs1321311, rs3824999 or rs5934683 at 6p21, 11q13.4 and Xp22.2 loci were identified. These data make it likely that the associations between 6p21, 11q13.4 and Xp22.2 and CRC risk are mediated through changes that influence gene expression rather than impacting on protein sequence.

To examine if any directly typed or imputed SNPs lie within or very close to a putative transcription factor binding/enhancer element, we conducted a bioinformatic search using Transfac[24], ENCODE CHIP-Seq and ENCODE UW DNAaseI Hypersensitivity data. These analyses did not provide evidence that rs1321311, rs3824999 and rs5934683or any closely correlated SNP maps to a known or predicted region of transcriptional regulation (Supplementary Table 4).

To explore whether the rs1321311, rs3824999 and rs5934683 associations (or SNP proxies) reflect *cis*-acting regulatory effects on *POLD3*, *CDKN1A*, *GPR143* or *SHROOM2*, we conducted expression studies using Illumina HT-12 arrays using RNA extracted from 42 samples of normal colonic epithelium (Supplementary Table 5). We also analyzed publicly-available mRNA expression data from fibroblasts, lymphoblastoid cell lines (LCL), T-cells, adipose tissue and CRC[25,26] (Supplementary Table 5). *In silico* analysis revealed a statistically significant relationship between rs1321311 genotype and expression of *CDKN1A.* However, this was observed only in the LCLs and T-cell data, with no evidence of an effect in colon (Supplementary Table 5). We also found that the risk allele at rs5934683 was associated with a striking reduction in *SHROOM2* expression in both normal colonic-epithelium and CRC tissue (Supplementary Figure 4). The relationship between *SHROOM2* expression in normal colonic epithelium and rs5934683 genotype was very strong ($P=1.3\times10^{-7}$) and was significant after accounting for all genes tested on the HT-12 array ($P=9.0\times10^{-4}$). Indeed, rs5934683 genotype accounted for 55% of the variation in *SHROOM2* expression. Exploring the relationship between *SHROOM2* expression, rs5934683 risk genotype and CRC causation will be of considerable interest, not least because of the observations of an association between excess pigmented lesions in the retinal pigment epithelium and CRC[22,23]. There was no significant difference in the observed MAF of rs5934683 between female and male cases raising the possibility that skewed X-inactivation might underscore the associated CRC risk. Favored X-inactivation producing a normal phenotype has been documented in X-linked dominant disease[27] and skewed X-inactivation has been implicated as a risk factor for breast cancer[28]. The expression data were consistent with full dosage compensation but, due to sample and effect

sizes, we are currently unable to confirm or refute a dosage effect on risk. There was no detectable relationship between rs3824999 and *POLD3* expression from any of the expression studies. It should be noted that these exploratory analyses could only detect >5% difference in RNA expression by genotype with 80% power at a single time point and hence we could not exclude any subtle effects of genotype on target tissues relevant to CRC.

By pooling GWAS data and conducting extensive replication analyses, we have identified three new loci influencing CRC susceptibility. The loci are of modest effect size, which is unsurprising given that common alleles with a larger impact on CRC were likely to have been discovered in previous studies. While additional analyses are required to determine the functional consequences that lead to CRC, our findings highlight the importance of variation in genes encoding components of the $p21^{WAF1/Cip1}$ signalling pathway in CRC. This pathway, elucidated through the extended interaction network of *CDKN1A*, incorporates not only *POLD3* discovered as a CRC locus here, but also *MYC* and other genes (including *SMADs* and other *TGF*-β pathway genes) that we have previously identified as risk factors for CRC.

URLs

The R suite can be found at http://www.r-project.org

Detailed information on the tag SNP panel can be found at http://www.illumina.com

dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP

HapMap: http://www.hapmap.org

1000Genomes: http://www.1000genomes.org

SNAP http://www.broadinstitute.org/mpg/snap

IMPUTE: https://mathgen.stats.ox.ac.uk/impute/impute.html

SNPTEST: http://www.stats.ox.ac.uk/~marchini/software/gwas/snptest.html

Transfac Matrix Database: http://www.biobase-international.com/pages/index.php?id=transfac

Wellcome Trust Case Control Consortium: www.wtccc.org.uk

Mendelian Inheritance In Man: http://www.ncbi.nlm.nih.gov/omim

SIFT: http://sift.jcvi.org/

PolyPhen: http://genetics.bwh.harvard.edu/pph

Globocan: http://globocan.iarc.fr

Cancer Genome Atlas project: http://cancergenome.nih.gov

The ENCODE Project: ENCyclopedia Of DNA Elements: http://www.genome.gov

Genevar (GENe Expression VARiation): http://www.sanger.ac.uk/resources

Catalogue Of Somatic Mutations In Cancer: http://www.sanger.ac.uk/genetics/CGP/cosmic

METHODS

Ethics statement

Collection of blood samples and clinico-pathological information from subjects was undertaken with informed consent and ethical review board approval at all sites in accordance with the tenets of the Declaration of Helsinki.

Datasets, sample preparation and genotyping

Full details of each dataset are provided in the Supplementary Note.

DNA was extracted from samples using conventional methods and quantified using PicoGreen (Invitrogen). The VQ, UK1, and Scotland1 GWA cohorts were genotyped using Illumina Hap300, Hap240S, Hap370, or Hap550 arrays. 1958BC and NBS genotyping was performed as part of the WTCCC2 study on Hap1.2M-Duo Custom arrays. The CCFR1 samples were genotyped using Illumina Hap1M or Hap1M-Duo arrays. In UK2 and Scotland2, genotyping was conducted using custom Illumina Infinium arrays according to the manufacturer's protocols. Some COIN SNPs were typed on custom Illumina Goldengate arrays. To ensure quality of genotyping, a series of duplicate samples was genotyped, resulting in 99.9% concordant calls in all cases. Other genotyping was conducted using competitive allele-specific PCR KASPar chemistry (KBiosciences Ltd, Hertfordshire, UK), Taqman (Life Sciences, Carlsbad, California) or MassARRAY (Sequenom Inc., San Diego, USA). All primers, probes and conditions used are available on request. Genotyping quality control was tested using duplicate DNA samples within studies and SNP assays, together with direct sequencing of subsets of samples to confirm genotyping accuracy. For all SNPs, >99% concordant results were obtained.

Quality control and sample exclusion

We excluded SNPs from analysis if they failed one or more of the following thresholds: GenCall scores <0.25; overall call rates <95%; MAF<0.01; departure from Hardy-Weinberg equilibrium (HWE) in controls at $P<10^{-4}$ or in cases at $P<10^{-6}$; outlying in terms of signal intensity or X:Y ratio; discordance between duplicate samples; and, for SNPs with evidence of association, poor clustering on inspection of X:Y plots. We excluded individuals from analysis if they failed one or more of the following thresholds: duplication or cryptic relatedness to estimated identity by descent (IBD) >6.25%; overall successfully genotyped SNPs<95%; mismatch between predicted and reported gender; outliers in a plot of heterozygosity *versus* missingness; and evidence of non-white European ancestry by PCA-based analysis in comparison with HapMap samples (http://hapmap.ncbi.nlm.nih.gov). Details of all sample exclusions are provided in Supplementary Figure 1.

To identify individuals who might have non-northern European ancestry, we merged our case and control data from all sample sets with the 60 European (CEU), 60 Nigerian (YRI), and 90 Japanese (JPT) and 90 Han Chinese (CHB) individuals from the International HapMap Project. For each pair of individuals, we calculated genome-wide identity-by-state distances based on markers shared between HapMap2 and our SNP panel, and used these as dissimilarity measures upon which to perform principal components analysis. Principal components analysis was performed in R using CEU, YRI and HCB HapMap samples as reference. The first two principal components for each individual were plotted and any individual not present in the main CEU cluster (that is, >5% of the PC distance from HapMap CEU cluster centroid) was excluded from subsequent analyses (Supplementary Figure 3).

We had previously shown the adequacy of the case-control matching and possibility of differential genotyping of cases and controls using Q-Q plots of test statistics. The inflation factor λ_{GC} was calculated by dividing the mean of the lower 90% of the test statistics by the mean of the lower 90% of the expected values from a χ^2 distribution with 1 d.f. Deviation of the genotype frequencies in the controls from those expected under HWE was assessed by χ^2 test (1 d.f.), or Fisher's exact test where an expected cell count was <5.

Statistical and bioinformatic analysis

Main analyses were undertaken using R (v2.6), Stata v.11 (College Station, Texas, US) and PLINK (v1.06) software[29]. The association between each SNP and risk of CRC was assessed by the Cochran-Armitage trend test. Odds ratios (ORs) and associated 95% confidence intervals (CIs) were calculated by unconditional logistic regression. Meta-analysis was conducted using standard methods[30]. Cochran's Q statistic to test for heterogeneity[30] and the I^2 statistic to quantify the proportion of the total variation due to heterogeneity were calculated[31]. I^2 values ≥75% are considered characteristic of large heterogeneity[31,32]. Associations by sex, age and clinic-pathological phenotypes were examined by logistic regression in case-only analyses.

For SNPs on the non-pseudoautosomal region of X chromosome, males carry only one copy and in females most loci are subject to X inactivation[33]. To test for X chromosome associations we used an extension to the standard, 1df Cochran-Armitage test for trend, proposed by Clayton (2008)[18] whereby males can be regarded as homozygous females. This 1df trend test adjusts for the different variances for males and females.

Prediction of the untyped SNPs was carried out using IMPUTEv2, based on HapMap Phase III haplotypes release 2 (HapMap Data Release 27/phase III Feb 2009 on NCBI B36 assembly, dbSNP26) and 1000genomes. Imputation of the X chromosome loci was only possible using IMPUTEv1 with HapMap Data Release 21 on NCBI Build 35. Imputed data were analysed using SNPTEST v2 to account for uncertainties in SNP prediction. An imputation info score of 0.95 was used to remove SNPs with poor imputation quality. LD metrics between HapMap SNPs were based on Data Release 27/phase III (Feb 2009) on NCBI B36 assembly, dbSNP26, viewed using Haploview software (v4.2) and plotted using SNAP. LD blocks were defined on the basis of HapMap recombination rate (cM/Mb) as defined using the Oxford recombination hotspots[34] and on the basis of distribution of

confidence intervals defined by Gabriel *et al* [35]. To annotate potential regulatory sequences within disease loci we implemented *in silico* searches using Transfac Matrix Database v7.29 [24], and PReMod10[36] [36]software. We used the *in silico* algorithms SIFT and PolyPhen to predict the impact of amino acid substitutions.

Relationship between SNP genotype and mRNA expression

Expression studies in colonic epithelium—To examine for a relationship between SNP genotype and mRNA expression in colonic epithelium, 42 samples were collected fresh immediately after surgical resection of specimens for colorectal cancer (n=34), solitary adenoma (n=5) or benign conditions (not inflammatory bowel disease) (n=3). For 2 of the 42 subjects, 3 samples of mucosa were harvested from different locations of the fresh resected bowel. Normal epithelium was dissected from muscularis propria, and samples snap frozen and placed in RNAlater (Applied Biosystems) and kept at 4°C overnight before storage at −80°C. Tissue was disrupted and homogenised using TissueLyser LT (Qiagen) and RNA extracted using Ribopure kit (Applied Biosystems). RNA integrity and concentration were assessed on an Agilent Bioanalyzer, RNA purity (A260/A280 and A260/A230) on Nanodrop. RT-PCR products were analysed on HumanHT-12 Expression BeadChip which were scanned using the Illumina HiScan. Array data processing and analysis was performed using Illumina GenomeStudio software (version 2011.1). Microarray data were exported from Illumina Beadstudio software, processed and normalized using the R, Bioconductor beadarray and limma packages[37,38]. Prior to normalization probes that were not detected (detection *P*-value>0.01) on the microarrays were removed. Microarrays were Quantile normalized to remove technical variation. Three mucosa samples were available for 2 of the 42 subjects and in which we used the average signal of the replicates in the analysis. The limma package was used to find differential expressed genes, using the functions lmFit, eBayes and topTable. To test all associations between SNPs and expression, a linear model was fitted to the expression level of each probe, using this genotype value as effect. For SNPs associations with gene expression on the X chromosome, gender was added to the model. Significant associations were considered as < 0.05 using P-values adjusted for multiple testing using the Benjamini, Hochberg method from R's p.adjust function

In silico analysis of publicly available expression data—We analysed expression data generated from: (1) Fibroblast, lymphoblastoid cell lines (LCL) and T-cells derived from the umbilical cords of 75 Geneva GenCord individuals[25]; (2) 166 adipose, 156 LCL and 160 skin samples derived from a subset of healthy female twins of the MuTHER resource[26] using Sentrix Human-6 Expression BeadChips (Illumina, San Diego, USA)[39,40] (3) AgilentG4502A_07_3 custom gene expression data on 154 CRCs as part of the Cancer Genome Atlas project: http://cancergenome.nih.gov. Power of assays to establish a relationship between genotype and expression we made using STATA software.

Assignment of microsatellite instability (MSI) in colorectal cancers

Tumour MSI status in CRCs was determined using the mononucleotide microsatellite loci BAT25 and BAT26, which are highly sensitive MSI markers. Briefly, 10 mm sections were cut from formalin-fixed paraffin-embedded CRC tumours, lightly stained with toluidine blue

and regions containing at least 60% tumour microdissected. Tumour DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and genotyped for the BAT25 and BAT26 loci using either ^{32}P–labelled or fluorescently-labelled oligonucleotide primers (UK2/3 and COINNBS studies respectively). Samples showing more than or equal to five novel alleles, when compared with normal DNA, at either or both markers were assigned as MSI-H (corresponding to MSI-high)[41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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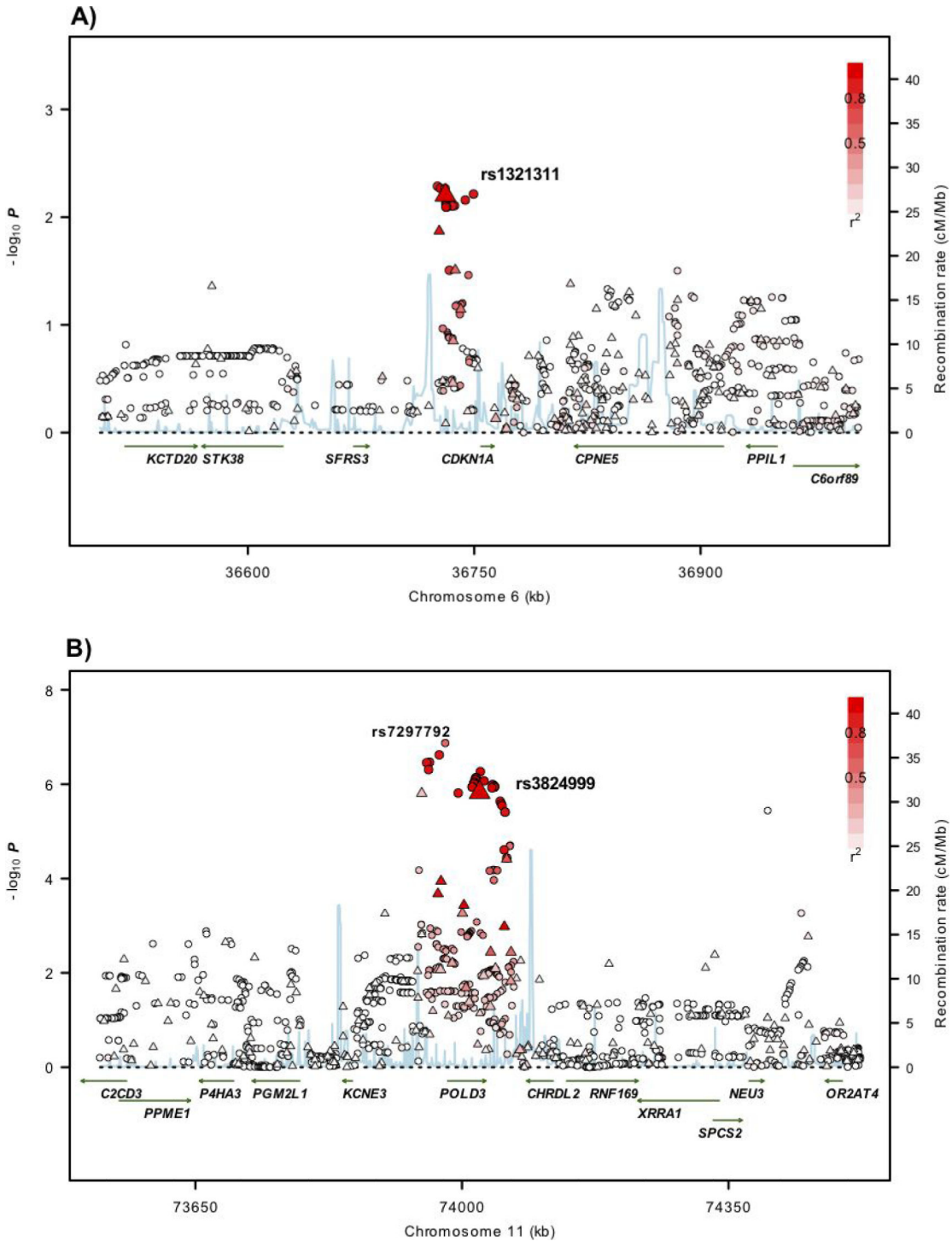

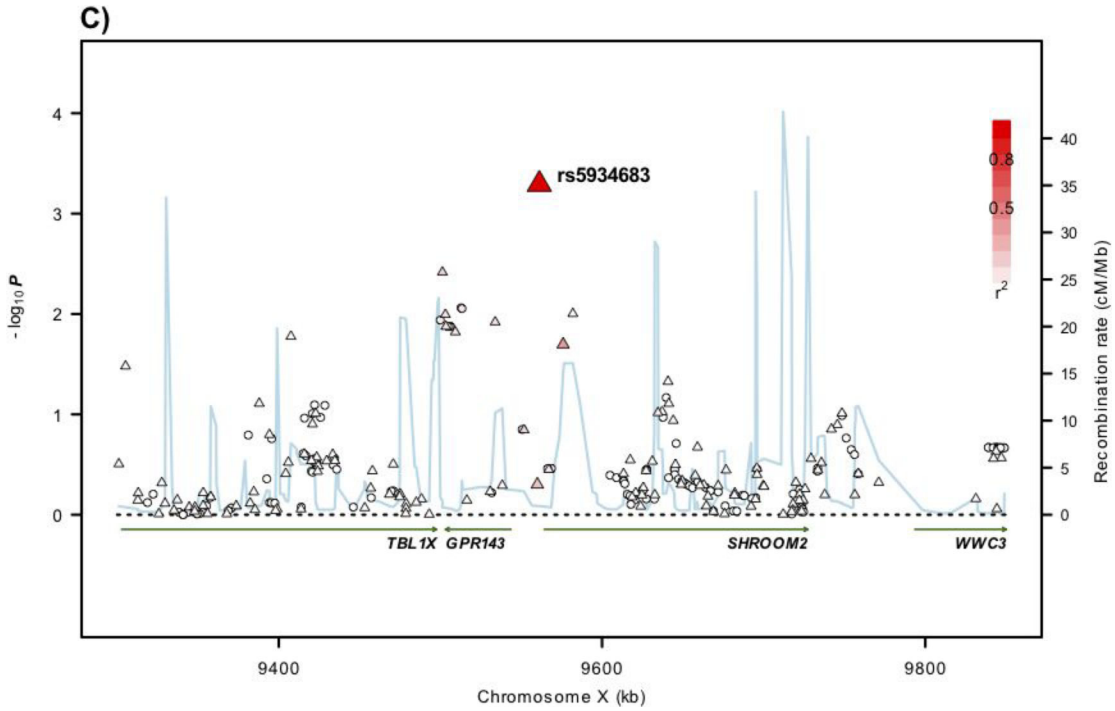

Figure 1. Regional plots of association results and recombination rates for the 6p21, 11q13.4, Xp22.2 susceptibility loci

(a-d) Association results of both genotyped (triangles) and imputed (circles) SNPs in the GWAS samples and recombination rates within the loci: (a) 6p21, (b), 11q13.4, (c) Xp22.2. For each plot, $-\log_{10} P$ values (*y* axis) of the SNPs are shown according to their chromosomal positions (*x* axis). The top genotyped SNP in each combined analysis is a large triangle and is labelled by its rsID. The colour intensity of each symbol reflects the extent of LD with the top genotyped SNP: white (r^2=0) through to dark red (r^2=1.0). Genetic recombination rates (cM/Mb), estimated using HapMap CEU samples, are shown with a light blue line. Physical positions are based on NCBI build 36 of the human genome. Also shown are the relative positions of genes and transcripts mapping to each region of association. Genes have been redrawn to show the relative positions; therefore, maps are not to physical scale.

Table 1

Summary results for the SNPS: rs1321311 (6p21), rs3824999 (11q13.4) and rs5934683 (Xp22.2) associated with CRC risk.

SNP	STUDY	OR[a]	95% CI[b]	*P*-value
rs1321311	Discovery	1.09	1.05-1.14	4.79×10^{-5}
	Replication	1.09	1.05-1.14	5.74×10^{-6}
	Japan	1.18	1.03-1.36	1.71×10^{-2}
	Combined	**1.10**	**1.07-1.13**	**1.14×10^{-10}** (P_{het} = 0.55, I^2 =0%)
rs3824999	Discovery	1.08	1.05-1.13	1.77×10^{-5}
	Replication	1.07	1.04-1.11	2.06×10^{-5}
	Japan	1.09	0.99-1.19	8.46×10^{-2}
	Combined	**1.08**	**1.05-1.10**	**3.65×10^{-10}** (P_{het} = 0.05, I^2 = 41%)
rs5934683	Discovery	1.08	1.04-1.12	8.19×10^{-5}
	Replication	1.07	1.04-1.10	2.16×10^{-6}
	Japan	1.04	0.93-1.16	5.38×10^{-1}
	Combined	**1.07**	**1.04-1.10**	**7.30×10^{-10}** (P_{het} = 0.31, I^2 = 13%)

[a] Odds ratio.

[b] 95% Confidence Interval.