

High-density fine-mapping of a chromosome 10q26 linkage peak suggests association between endometriosis and variants close to *CYP2C19*

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Objective: To refine a previously reported linkage peak for endometriosis on chromosome 10q26, and conduct follow-up analyses and a fine-mapping association study across the region to identify new candidate genes for endometriosis.

Design: Case-control study.

Setting: Academic research.

Patient(s): Cases = 3,223 women with surgically confirmed endometriosis; controls = 1,190 women without endometriosis and 7,060 population samples.

Intervention(s): Analysis of 11,984 single nucleotide polymorphisms on chromosome 10.

Main Outcome Measure(s): Allele frequency differences between cases and controls.

Result(s): Linkage analyses on families grouped by endometriosis symptoms (primarily subfertility) provided increased evidence for linkage (logarithm of odds score = 3.62) near a previously reported linkage peak. Three independent association signals were found at 96.59 Mb (rs11592737), 105.63 Mb (rs1253130), and 124.25 Mb (rs2250804). Analyses including only samples from linkage families supported the association at all three regions. However, only rs11592737 in the cytochrome P450 subfamily C (*CYP2C19*) gene was replicated in an independent sample of 2,079 cases and 7,060 population controls.

Conclusion(s): The role of the *CYP2C19* gene in conferring risk for endometriosis warrants further investigation. (Fertil Steril® 2011;95:2236–40. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, linkage, association, subfertility, *CYP2C19*

Endometriosis, a disease affecting 6%–10% of women of reproductive age, is defined as the presence of endometrial-like tissue in sites outside of the uterus, most commonly the pelvic peritoneum, ovaries, and rectovaginal septum (1). Although symptoms vary, affected

women most commonly experience chronic pelvic pain, severe dysmenorrhea, and subfertility. The disease is inherited as a complex genetic trait (1–3), and aggregates within families in humans (4, 5) and nonhuman primates (6). Genetic factors accounted for 52% of

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the variation in liability to endometriosis in an Australian twin study, with a relative recurrence risk of 2.34 for sibs of patients with endometriosis (7).

We previously reported significant genetic linkage (logarithm of odds [LOD] score >3) to endometriosis on chromosome 10q26 in a study of 1,176 families (8). We have now performed further analyses to refine the linkage peak and extensive fine mapping to identify regions associated with risk of developing endometriosis. We used latent class analysis to determine whether stratification by disease stage and/or symptoms was possible, and the relative contribution of these factors to linkage in the region. Subsequently, we genotyped a high-density single nucleotide polymorphism (SNP) panel in 1,144 familial cases and 1,190 controls. The best association signals were detected at three independent loci, although only SNPs at the 96.59-Mb region, harboring the cytochrome P450 family subfamily C (*CYP2C19*) gene, showed evidence of replication in independent case-control samples.

MATERIALS AND METHODS

Refining the Linkage Peak

Latent class analysis The linkage study included 931 affected sister pair families collected by the Queensland Institute of Medical Research (QIMR) and 245 collected by the University of Oxford (8, 9). To refine the published linkage peak we sought to increase the genetic homogeneity of the sample by examining endometriosis subtypes using information about self-rated symptoms of pelvic pain (ever experiencing severe pelvic pain) and subfertility (failure to conceive after trying for 12 months) and physician-diagnosed disease stage (based on the revised American Fertility Society [AFS] classification system) (10). As it can be difficult to stage disease accurately using clinical records alone, a simplified two-stage system was used (9, 10): stage A (revised AFS I-II or some ovarian disease plus a few adhesions) and stage B (revised AFS III-IV). Latent class analysis, a method to find subtypes of related cases from multivariate categorical data, was used to investigate the presence and composition of endometriosis subgroups using the Bayes information criterion (BIC) (11) as the index of model goodness-of-fit. The null hypothesis of a one-class (group) solution (i.e., all individuals belong to the same class or group) is rejected if models with more parameters (groups) provide a smaller BIC value.

Linkage and ordered subset analyses To investigate whether stratifying on subfertility provided a more phenotypically homogenous sample, the approach of Cox et al. (12) was adapted to conduct linkage analyses with families weighted according to reported subfertility (0 = subfertility, 1 = no subfertility). Nonparametric, multipoint, affected-only LOD scores were calculated on the basis of the S-pairs scoring function and an exponential allele-sharing model (exponential LOD [expLOD] scores) using the ALLEGRO analysis package (13). Ordered subset analyses (14) were performed to assess the increased evidence of linkage in subsets of families ordered by their subfertility value relative to the entire sample.

Association Mapping Sample Selection

For the fine-mapping association analyses we genotyped unrelated cases mostly from the families included in the linkage study (8). Subject to DNA availability, cases were chosen to include individuals with the most severe disease (i.e., the highest disease stage or the youngest age at onset if both sisters had the same disease stages). There were 871 such cases from the 931 QIMR families and 231 from Oxford. An additional 40 QIMR cases were chosen from families not included in the original linkage analysis but containing a proband plus at least two affected relatives using the same criteria.

The QIMR controls (N = 952) were chosen from female twin pairs originally recruited for a study of gynecological health (15), including one sample from pairs where neither sister had self-reported endometriosis. Oxford controls (N = 238) were unrelated women recruited in collaborating hospitals who were [1] undergoing laparoscopy for pelvic pain, subfertility, or other

gynecological complaints, hysterectomy, or sterilization; [2] free of endometriosis at surgery, and [3] without a previous surgical diagnosis of endometriosis. All study participants were volunteers, had signed written informed consent, and had provided a blood sample for DNA extraction. Ethics approval was obtained from the QIMR Human Research Ethics Committee, and the United Kingdom Regional Multi-centre and local Research Ethics Committees.

Fine-Mapping

SNP selection The 95% confidence interval for both the published (region 112–129 Mb) and “fertility-related” (region 94–107 Mb) linkage peaks extends more than 36 Mb (National Center for Biotechnology Information build 36; <http://www.ensembl.org>). Assays for 13,589 SNPs were manufactured and the genotyping and initial quality control performed at Illumina Inc. (San Diego, CA) on an Illumina Infinium iSelect custom platform. Across the entire 36 Mb region, gene-based SNPs were included in all exons and 5' and 3' untranslated regions for approximately 250 genes, and under the published and fertility-related linkage peaks SNPs tagged to a minimum pairwise r^2 of 0.97. In an attempt to capture information from rare SNPs (minor allele frequencies [MAFs] <1%) we did not exclude loci with MAFs of 0 in the HapMap (www.hapmap.org), although most variants were common in our dataset; only 6% of SNPs had MAFs <1% (range 0.0002–0.0098) (Table 1).

Quality control and association analyses Additional quality control was performed on genotype data from 2,369 individuals (1,158 cases, 1,211 controls) and 12,537 polymorphic SNPs using PLINK (16). We detected and removed individuals with non-Caucasian ancestry and SNPs with >5% missing genotypes or Hardy-Weinberg P values < 1×10^{-4} in control samples. Thereafter, 1,144 cases (911 QIMR, 233 Oxford), 1,190 controls (952 QIMR, 238 Oxford), and 11,984 SNPs remained in the dataset. Cochran-Mantel-Haenszel tests of association were performed using PLINK including QIMR and Oxford data as different strata to account for any subtle differences between populations in baseline effect (17). Breslow-Day tests were conducted to check that the assumptions of the Cochran-Mantel-Haenszel test (i.e., similar effect size across strata) were true. The significance of the association signals was assessed by permutation (10,000 replicates).

Investigation of Association in a Replication Data Set

We attempted to replicate the results from the “discovery” sample in an independent set of 2,079 cases (1,383 QIMR, 696 Oxford), all surgically confirmed, without a family history, recruited within the QIMR and Oxford studies. Each was genotyped on Illumina Human670Quad Beadarrays for a genome-wide association study (17). There were 7,060 population controls genotyped using [1] Human610Quad (QIMR controls: 1,870 unrelated individuals recruited within the Brisbane Adolescent Twin Study) (18, 19) or [2] Human1M-Duo beadchips (Oxford controls: 5,190 United Kingdom

TABLE 1

Minor allele frequency ranges for 11,984 polymorphic SNPs included in the fine-mapping association analyses.

Minor allele frequencies	SNPs per frequency class
Common MAF > 5%	10,245 (85.5%)
Low frequency MAF 1-5%	1008 (8.4%)
Rare MAF < 1%	731 (6.1%)

Note: MAF = minor allele frequencies; SNP = single nucleotide polymorphism.

Painter. Fine-mapping linkage peaks for endometriosis. Fertil Steril 2011.

unrelated population controls provided by The Wellcome Trust Case Control Consortium 2). Association analysis and meta-analysis of the P values for both datasets were performed using PLINK (16).

RESULTS

Linkage: Latent Class Analysis

Comparative fits of latent class analysis models determined a two-class solution as the most parsimonious with a minimum BIC of -247.50 (one-class BIC -237.30 ; three-class BIC -220.81). The main phenotypic measure discriminating between the two classes was subfertility. Class 1 families (CL1; 51.7% of the linkage families; 663 QIMR, 138 Oxford) represented an endometriosis type typically without subfertility (91%), a slightly lower proportion with stage B disease (27%), but more common experience of pelvic pain (80.3%). Class 2 families (CL2; 48.3%; 268 QIMR, 107 Oxford) represented a form typically seen with subfertility (89%), a slightly higher proportion with stage B disease (40%), and less common experience of pelvic pain (72.3%).

The results of linkage analyses, performed using the subfertility weighting, did not change substantially by including CL2 families only. Restricting the analysis to CL1 families produced an $\text{expLOD} = 3.62$ at approximately 98 Mb, 27 Mb closer to the centromere than the published significant linkage peak ($\text{expLOD} = 3.08$ at 125 Mb) (Fig. 1). These results were confirmed by the ordered subset analysis, with an increased $\text{expLOD} = 3.58$ when families ranked by fertility scores were successively added to the linkage analysis. The increased evidence for linkage in the fertile subset relative to the entire sample (assessed with 10,000 permutations) was significant ($P=.02$).

Fine-Mapping Association Analyses in the Discovery Sample

Nominal signals of association ($P < 1 \times 10^{-3}$), supported by multiple SNPs, were seen in three regions (Table 2, Fig. 2). The SNPs with the smallest P values were under the fertility-related linkage peak: at approximately 96.59 Mb (top SNP rs11592737, $P=4.9 \times 10^{-4}$, odds ratio [OR] = 1.28) in intron 7 of the *CYP2C19* gene, and at approximately 105.63 Mb (rs12573103, $P=2.5 \times 10^{-4}$, OR = 1.24) upstream of the SH3 and PX domain-containing adaptor

(*SH3PXD2A*) gene. The next smallest P value was for rs2250804 at 124.25 Mb ($P=9.7 \times 10^{-4}$; OR = 1.22) under the published linkage peak, in intron 3 of the HtrA serine peptidase 1 precursor (*HTRAI*) gene. All three signals were independent, with no linkage disequilibrium between the SNPs. However, the permutation analysis showed that none of association signals was significant at a study-wide level ($P < .05$), with corrected empirical P values of .92, .75, and .99 for rs11592737, rs12573103, and rs2250904, respectively.

Exploratory analyses were performed limiting cases to those from the previously published linkage families (1,105 cases) and CL1 families only (755 cases). P values for the SNPs at 96.59 Mb, 105.63 Mb, and 124.25 Mb remained the smallest in these analyses. For rs11592737 at 96.59 Mb, a $P=9.0 \times 10^{-4}$ (OR = 1.26) was obtained including only linkage family cases but was below background levels including only CL1 family cases ($P=5.0 \times 10^{-2}$; OR = 1.18). For rs12573103 at 105.63 Mb, a $P=1.0 \times 10^{-4}$ (OR = 1.25) was obtained including only linkage family cases and $P=5.3 \times 10^{-5}$ (OR = 1.31) for CL1 cases. The signal for rs2250804 at 124.25 Mb was reduced by including only linkage family ($P=1.1 \times 10^{-3}$, OR = 1.23) and CL1 ($P=1.2 \times 10^{-2}$, OR = 1.19) cases.

Replication Analyses in an Independent Case-Control Sample

The SNPs with the smallest P values in the 96.59 Mb, 105.63 Mb, and 124.25 Mb regions were not present on the commercial Illumina arrays typed in the replication samples. However, additional SNPs that were in moderate linkage disequilibrium (LD; pairwise $r^2 > 0.5$) were included in our Illumina iSelect panel, allowing a direct comparison of P values for the discovery and replication datasets. All three SNPs in LD ($r^2 \geq 0.98$) with rs11592737 at 96.59 Mb showed nominal evidence of association ($P=4.0 \times 10^{-2}$ for rs7085745 and 5.0×10^{-2} for rs12243416 and rs11188067; ORs = 1.10) in the replication dataset (Table 2), with corresponding P values between 3.1×10^{-4} and 4.5×10^{-4} (ORs = 1.14) in the meta-analysis of the fine-mapping discovery and replication datasets (Table 2).

There was no replication signal for SNPs at either 105.63 Mb or 124.25 Mb. The SNP in highest LD with rs12573103 at 105.63 Mb (rs1980653, $r^2 = 0.94$) had a $P=.60$ (OR = 1.02) in the replication dataset. The two SNPs in highest LD with rs2250804 at 124.25 Mb (rs2300433 and rs2253755, $r^2 = 0.50$) had P values of .44 and .47 in the replication dataset (Table 2).

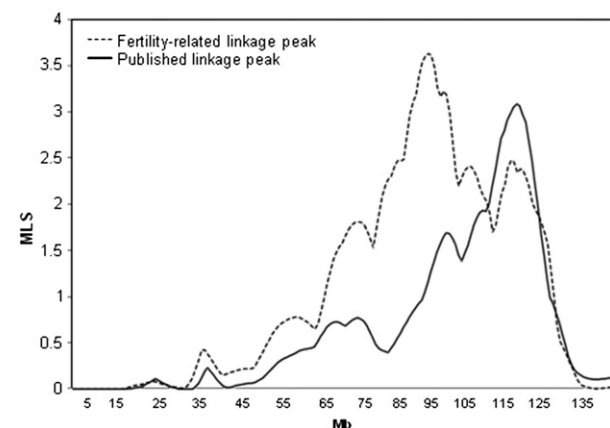
DISCUSSION

Analysis of endometriosis stage, pelvic pain, and subfertility symptoms identified two classes of families, distinguished primarily by the presence or absence of subfertility. Separate linkage analyses with the two classes produced significantly increased evidence for linkage among families without subfertility, shifting the linkage peak approximately 27 Mb. We genotyped SNPs at high density across the region covered by both the published and fertility-related linkage peaks and found evidence of association at three independent loci. Although this was not significant at a study-wide level, there was evidence for replication of the signal(s) at 96.59 Mb within the *CYP2C19* gene in the independent set of endometriosis cases.

The most significantly associated SNP at 96.59 Mb is in intron 7 of *CYP2C19*, which participates in the metabolism of drugs and estrogen (E) including conversion of E_2 to estrone (E_1), and the production of E_1 and E_2 2 α - and 16 α -hydroxylation metabolites (20, 21). The key SNP rs11592737 is in complete LD with rs12248560 (the second

FIGURE 1

Chromosome 10 linkage peaks for endometriosis.



Painter. Fine-mapping linkage peaks for endometriosis. *Fertil Steril* 2011.

TABLE 2

Association signal in the 96.5-Mb and 105.6-Mb regions for the fine-mapping (discovery), replication, and combined datasets.

Linkage peak SNP	Position	<i>r</i> ^{2a}	RAF	Fine-mapping		Replication		Meta-analysis	
				<i>P</i> value	OR ^b	<i>P</i> value	OR	<i>P</i> value	OR
96.59 Mb									
rs11592737	96593404		0.79 (A)	4.9×10^{-4}	1.28 (1.11–1.47)	—	—	—	—
rs12243416	96444146	0.99	0.79 (G)	7.6×10^{-4}	1.28 (1.11–1.47)	5.0×10^{-2}	1.10 (1.00–1.20)	8.4×10^{-4}	1.14 (1.05–1.22)
rs11188067	96483699	0.99	0.79 (A)	6.7×10^{-4}	1.28 (1.11–1.47)	5.0×10^{-2}	1.10 (1.00–1.20)	8.4×10^{-4}	1.14 (1.05–1.22)
rs7085745	96672504	0.98	0.79 (T)	1.0×10^{-3}	1.28 (1.11–1.47)	4.0×10^{-2}	1.10 (1.00–1.20)	7.6×10^{-4}	1.14 (1.05–1.22)
105.63 Mb									
rs12573103	105629231		0.48 (A)	2.5×10^{-4}	1.24 (1.10–1.39)	—	—	—	—
rs10748858	105629504	0.68	0.41 (G)	3.2×10^{-4}	1.24 (1.10–1.39)	.60	1.02 (0.95–1.10)	6.0×10^{-3}	1.09 (1.02–1.15)
rs1980653	105644154	0.94	0.49 (T)	1.8×10^{-3}	1.20 (1.07–1.34)	.11	1.06 (0.99–1.14)	1.3×10^{-3}	1.01 (1.04–1.17)
rs11191865	105662832	0.93	0.49 (G)	1.5×10^{-3}	1.20 (1.07–1.35)	.13	1.06 (0.98–1.14)	1.7×10^{-3}	1.01 (1.04–1.17)
124.25 Mb									
rs2250804	124254868		0.33 (C)	9.7×10^{-4}	1.23 (1.09–1.39)	—	—	—	—
rs2300433	124233447	0.50	0.31 (G)	4.8×10^{-2}	1.13 (1.00–1.28)	.44	0.96 (0.89–1.05)	.53	1.04 (0.96–1.14)
rs2253755	124231547	0.50	0.31 (G)	4.5×10^{-2}	1.13 (1.00–1.28)	.47	0.97 (0.89–1.05)	.50	1.02 (0.96–1.09)

Note: Results are shown for the most significant single nucleotide polymorphism (SNP) in each region, and for the replication and meta-analysis for SNPs in moderate linkage disequilibrium (*r*² > 0.5) for which the replication samples had been genotyped. RAF = risk allele frequency.

^a *r*² values between the best SNPs in each region (in bold) to those included both in the fine-mapping dataset and on the Illumina 610 K chips, calculated using the fine-mapping data.

^b Odds ratios (OR) were calculated for the risk allele, indicated in parentheses.

Painter. Fine-mapping linkage peaks for endometriosis. *Fertil Steril* 2011.

best SNP in the region), a functional variant located in the *CYP2C19* promoter. The rs12248560 “T” allele (*CYP2C19**17; <http://www.cypalleles.ki.se/cyp2c19.htm>) increases the rate of *CYP2C19* transcription and was initially thought to produce an “ultra-rapid” metabolizer form of the *CYP2C19* protein (22), although a recent review found drug metabolic rates within the ranges seen for wild-type homozygotes (23).

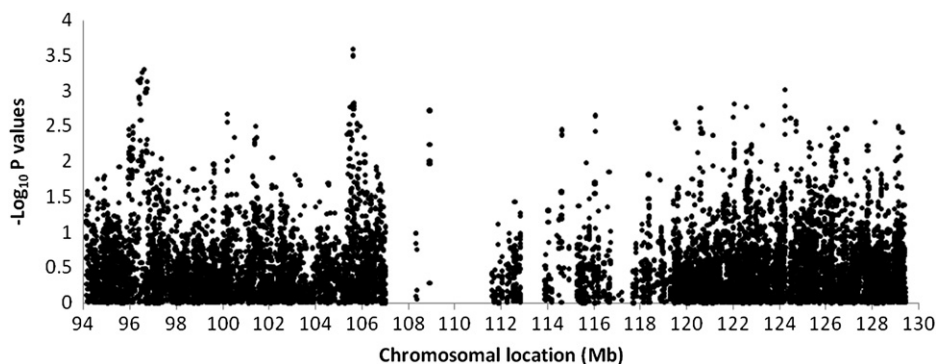
Further evidence for a role for *CYP2C19* in diseases influenced by E comes from a recent study showing a decreased risk of breast cancer in rs12248560 carriers, possibly through increased catabolism resulting in lower overall E levels (24). *CYP2C19* has also been associated with endometriosis in a small study of 50 cases and 50 controls suggesting that affected women were significantly more likely (*P* = .023; OR = 3.12) to be heterozygous carriers of rs4244285, a splice site defect that abrogates gene expression (25). However, these findings

were not replicated in another small study of 46 cases and 39 controls (26). Although both studies were clearly underpowered, the initial study adds further support to *CYP2C19* as a plausible endometriosis candidate gene. The *P* value for rs4244285 in our discovery sample was only nominally significant (*P* = .011; OR = 1.23), indicating that this SNP is not driving our association signal in the region. Several lines of evidence now point to a role for *CYP2C19* either through the effect on transcription of the rs12248560 variant or additional rare and low frequency alleles in LD with this SNP. The association result does not account for the linkage signal. Linkage to this region could result from a combination of a common variant like the one described in the present study in *CYP2C19* and rare variants of larger effect that might be observed in only a few families.

There was no evidence for replication of the 105.63 Mb or 124.25 Mb signals. The directions of the effects were the same for both the

FIGURE 2

Fine-mapping association analysis results across the 36-Mb region under the published and fertility-related linkage peaks for endometriosis.



Painter. Fine-mapping linkage peaks for endometriosis. *Fertil Steril* 2011.

fine-mapping and replication datasets, and both regions harbor plausible candidate genes for endometriosis: *SH3PXD2A* is an interaction partner of ADAM metallopeptidase domain 12 (*ADAM12*), which has a role in uterine decidualization in mice (27, 28). *HTRA1* is up-regulated in human decidual cells, suggesting a role in preparing the endometrium for embryo implantation (29). It is likely that these associations represent false-positive signals, although we were unable to test the key SNPs directly in the replication study. In addition, the discovery sample used familial cases but these were not available for the replication sample, and nonfamilial cases may have different underlying disease etiology.

We detected evidence of genetic association in a region of significant linkage to endometriosis on chromosome 10. This signal does not fully account for the previously reported or fertility-related linkage peaks. However, the finding of suggestive association, and the presence of an extremely plausible candidate gene in the region of association, suggest that further investigation is warranted. Future studies should include replication in other samples, a search for rare and novel genetic variants, and gene expression studies.

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