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KRAS variation and risk of endometriosis

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Endometriosis is a common gynaecological disease with symptoms of pelvic pain and infertility which affects 7–10% of women in their reproductive years. Activation of an oncogenic allele of Kirsten rat sarcoma viral oncogene homologue (*KRAS*) in the reproductive tract of mice resulted in the development of endometriosis. We hypothesized that variation in *KRAS* may influence risk of endometriosis in humans. Thirty tagSNPs spanning a region of 60.7 kb across the *KRAS* locus were genotyped using iPLEX chemistry on a MALDI-TOF MassARRAY platform in 959 endometriosis cases and 959 unrelated controls, and data were analysed for association with endometriosis. Genotypes were obtained for most individuals with a mean completion rate of 99.1%. We identified six haplotype blocks across the *KRAS* locus in our sample. There were no significant differences between cases and controls in the frequencies of individual single-nucleotide polymorphisms (SNPs) or haplotypes. We also developed a rapid method to screen for 11 common *KRAS* and *BRAF* mutations on the Sequenom MassARRAY system. The assay detected all mutations previously identified by direct sequencing in a panel of positive controls. No germline variants for *KRAS* or *BRAF* were detected. Our results demonstrate that any risk of endometriosis in women because of common variation in *KRAS* must be very small.

Key words: association test/endometriosis/*KRAS*/polymorphism

Introduction

Endometriosis (MIM 131200) is a common gynaecological disease defined as the presence of functional endometrial tissue outside the uterus, most commonly the pelvic peritoneum, the ovaries and the rectovaginal septum (Giudice and Kao, 2004). The disease causes pelvic pain, severe dysmenorrhoea (painful periods) and subfertility. The main pathological processes are peritoneal inflammation, fibrosis and the formation of adhesions and ovarian cysts. Diagnosis of the disease is usually made by visual inspection of the pelvis at laparoscopy, and estimates of the population prevalence are difficult to obtain. The best estimates indicate that endometriosis affects 7–10% of women in their reproductive years (Eskenazi and Warner, 1997; Treloar *et al*., 1999). Endometriosis is generally considered a benign disorder and is not associated with a general increase in the incidence of cancer (Somigliana *et al*., 2006). However, there is good evidence for a modest increase in the frequency of ovarian cancers of endometrioid or clear cell histotypes in women with endometriosis (Somigliana *et al*., 2006). Common risk factors may predispose to both diseases, or endometriotic lesions may undergo somatic mutational events and become precursor lesions.

Three members of the RAS gene family, Harvey rat sarcoma viral oncogene homologue (*HRAS*), Kirsten rat sarcoma viral oncogene homologue (*KRAS*) and neuroblastoma RAS viral (v-ras) oncogene homologue (*NRAS*), are the most common oncogenes associated with human neoplasms (Fabjani *et al*., 2005). Mutations in *KRAS* are found in ovarian, colorectal, pancreatic and lung cancers (Kahn *et al*., 1987; Bos, 1989). Mutations cause constitutive activation of the protein by increasing GDP/GTP exchange or decreasing GTPase activity of the protein, thus leading to increased cell proliferation or differentiation. In an important recent article, Dinulescu *et al*. (2005) demonstrated that specific targeting of the *KRAS* gene in the reproductive tract of mice resulted in the development of endometriosis. When a silenced oncogenic allele of the *KRAS* gene was activated by injection of an adenoviral vector expressing Cre recombinase into the bursal cavity, 47% of mice developed peritoneal endometriosis. In addition, 100% of injected mice also developed benign endometriosis-like lesions at the ovarian surface epithelium (OSE). Peritoneal endometriotic lesions in the mice resembled the histomorphology and biology of human endometriosis. Analysis of epithelial cells within the endometriotic glands isolated by laser capture microdissection demonstrated that the cells had undergone *KRAS* activation. These experiments suggest that activation of *KRAS* may be an important pathway in human endometriosis.

We hypothesized that variation in *KRAS* may influence risk of endometriosis and conducted a case–control study to test for association between common variants in *KRAS* and endometriosis. We also used MALDI-TOF mass spectrometry to screen our cases for evidence of germline mutations commonly associated with ovarian cancer.

Methods

Study subjects

Cases were drawn from our Australian study of endometriosis (Treloar *et al*., 2002). One sample from women with surgically confirmed endometriosis was selected from 959 families. Where families had multiple cases one sample was chosen from the sister with the more severe stage of disease. Disease severity was assessed retrospectively from medical records by use of the revised American Fertility Society (rAFS) classification system (American Fertility Society, 1985), which assigns patients to one of four stages (I–IV) on the basis of the

extent of the disease and the associated adhesions present. Fifty-nine per cent of cases were classified with moderate to severe stage B (rAFS stages III–IV) endometriosis. Cases with stage B endometriosis are more likely to have ovarian endometriosis. There were 259 cases (27%) that reported having a hysterectomy and 805 cases (86%) that reported having a laparoscopy. Endometriosis in the remaining cases was surgically diagnosed at the time of hysterectomy or in a small number of cases at laparotomy or at other procedures.

We also typed 959 unrelated controls drawn from women who volunteered for a twin study of gynaecological health (Treloar *et al*., 1999); a small number (n = 66) also provided relevant information in a study of twins aged over 50 years (Kirk *et al*., 1999). Control samples were chosen from women considered at low risk of endometriosis including self-report that they had never been diagnosed with endometriosis and information from medical records where available. There were 130 control women who reported having a hysterectomy and 131 women who reported having a laparoscopy. No evidence of endometriosis was reported at any of these procedures. A t-test for equality of means showed that cases were around 10 years younger than controls at the time of reporting ($P < 0.001$); mean age was 35.9 ± 8.9 years in cases compared with 45.6 ± 12.0 years in controls. Age range of cases was 17–64 years (median 35 years) and of controls was 29–90 years (median 43 years).

Ethics approval to obtain medical records and for blood collection and DNA extraction was obtained from the Human Research Ethics Committee of the Queensland Institute of Medical Research (QIMR) and the Australian Twin Registry. DNA was extracted from peripheral blood lymphocytes by the salt precipitation method (Miller *et al*., 1988).

Single-nucleotide polymorphism selection

We selected tagging single-nucleotide polymorphisms (SNPs) across the *KRAS* gene on the basis of data from public databases including the International HapMap Project (http://www.hapmap.org/) and NCBI (http://www.ncbi.nlm.nih.gov/). Thirty-two tagSNPs were selected in the region beginning 10 kb upstream and extending 5 kb downstream of the gene by including SNPs in the coding region with frequency information in HapMap and selecting tagSNPs from phase I and phase II HapMap data so other SNPs within the interval were in strong linkage disequilibrium (r^2 coefficient of ≥0.8) with one of the tagSNPs (Figure 1a).

Genotyping

Assays were designed to type 32 tagSNPs across the *KRAS* locus using the Sequenom MassARRAY Assay Design software (version 3.0). SNPs were typed using iPLEX™ chemistry on a MALDI-TOF Mass Spectrometer (Sequenom Inc., San Diego, CA, USA). PCR reactions were carried out in 2.5 µl in standard 384-well plates. PCR was performed with 10 ng of genomic DNA, 0.5 U of *Taq* polymerase (HotStarTaq, Qiagen, Valencia, CA, USA), 500 µmol of each dNTP and 100 nmol of each PCR primer. PCR thermal cycling was carried out in an ABI-9700 instrument for 15 min at 94°C, followed by 45 cycles of 20 s at 94°C, 30 s at 56°C and 60 s at 72°C. To the completed PCR reaction, 1 µl containing 0.15 U of Shrimp Alkaline Phosphatase was added, and the reaction was incubated for 30 min at 37°C followed by inactivation for 5 min at 85°C. After adjusting the concentrations of extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX assay was performed in a final 5 µl volume extension reaction containing 0.1 µl of termination mix, 0.02 µl of DNA polymerase (Sequenom Inc.) and 600–1200 nM extension primers. A two-step 200 short cycles program was used for the iPLEX reaction: initial denaturation was for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. An additional 40 annealing and extension cycles were then looped back to 5 s at 94°C, 5 s at 52°C and 5 s at 80°C. The final extension was carried out at 72°C for 3 min, and the sample was cooled to 20°C. The iPLEX reaction products were desalted by diluting samples with 15 µl of water and adding 3 µl of resin, then centrifuged to remove resin. The products were spotted on a SpectroChip (Sequenom Inc.), processed and analysed in a Compact Mass Spectrometer by MassARRAY Workstation (version 3.3) software (Sequenom Inc.).

Mutation screening

MALDI-TOF mass spectrometry also offers a rapid method to screen for common mutations within genes (James *et al*., 2006). Seven *KRAS*-activating mutations in codons 12 and 13 were selected on the basis of published reports of mutations identified in tumours (*KRAS* mutations G12A, G12C, G12D, G12R, G12S, G12V and G13D). We included the common *BRAF* mutations V600D, V600E, V600K and V600R in a single multiplex set because of parallel studies in colon cancer. The variants were assayed by a modified genotyping assay using primer extension. A 2.5 µl PCR was performed using 10 ng of genomic DNA, 0.1 U of HotStar Taq polymerase (Qiagen), 100 µM of dNTPs and 100 nmol of PCR primers in 1× standard buffer provided with the enzyme supplemented to 3 mM $MgCl_2$. Thermal cycling was carried out for 15 min at 95°C, followed by 45 cycles of 20 s at 94°C, 30 s at 56°C and 60 s at 72°C. Shrimp Alkaline Phosphatase was used to complete the PCR reaction as per standard procedures. Post-PCR was performed in a final 5 µl extension reaction containing 1200 nM each forward and reverse extension primers as summarized in Table I, 10 µM each of ddATP, dGTP, dATP and dCTP, and 0.08 U/µl ThermoSequenase (Sequenom Inc.) in 0.22× PCR buffer. The reactions were heated in an ABI-9700 thermocycler for 30 s at 94°C then cycled 99 times for 5 s at 94°C, 15 s at 52°C and 5 s at 72°C. Samples were then desalted and spotted as described in the *Genotyping* section. Samples from tumours or endometriotic lesions will often be mixtures of cell types. To address this question, we diluted *BRAF* V600K mutation DNA from a melanoma cell line with different proportions of wild-type germline DNA. The mutant allele could be detected in a mixture, when the proportion of mutant alleles was >10% (data not shown).

Statistical analysis

Marker genotypes were inspected and results tested for departures from Hardy–Weinberg equilibrium (HWE) separately for cases and controls using the PEDSTATS program (http://www.sph.umich.edu/csg/abecasis/PedStats/index.html). Genotypes for all but two markers were consistent with HWE. No obvious genotyping errors were apparent for the two markers (rs11047882 and rs2970531), and data for these markers were excluded from further analysis. The Haplo.stat (Schaid *et al*., 2002) and UNPHASED/COCAPHASE (Dudbridge, 2003) programs were used to test for association between endometriosis and individual markers or combinations of markers (haplotypes) (Dudbridge, 2003). In addition to obtaining nominal P-values, 10 000 permutation tests were performed to obtain a region-wide empirical P-value for each marker. This maintained the individual genotype as a whole, whereas the individual's status was shuffled. This method preserves the correlation between SNPs (linkage disequilibrium) while breaking the relation between status and the genotypes. For each replicate or permutation, each SNP was tested for association and the most significant P-value was stored. The global significance level was derived from these permutation tests. Haplotype blocks were determined by Haploview (Barrett *et al*., 2005) using the default method of Gabriel *et al*. (2002).

We performed power calculations for our case–control study assuming a disease (endometriosis) prevalence of 10% using the Genetic Power Calculator (Purcell *et al*., 2003). Power calculations for our total sample assumed loci with dominant, recessive and multiplicative modes of inheritance (MOI) and were based on 959 unrelated cases and 959 unrelated controls. All power calculations used a significance threshold (α) of P = 0.001 assuming correction for up to 50 independent SNPs per gene.

Results

In total, thirty SNPs were genotyped spanning a region of 60.7 kb across the *KRAS* locus (an average spacing of one SNP every 2.02 kb; Figure 1a). Genotypes were obtained for most individuals with a mean completion rate of 99.1%. The minor allele frequencies of the SNPs ranged from 0.005 to 0.473 (Table I). One SNP was typed twice on 2784 DNA samples at different times using the Sequenom hME or iPlex methods, and the data were used to estimate genotyping technical error rate. There were two discordant genotypes, and the estimated genotyping error frequency was 0.036%.

The *KRAS* locus spans over 60 kb, and there is little evidence of linkage disequilibrium between markers at different ends of the gene. Analysis of variation across the *KRAS* locus in control samples (Figure 1b and c) identified six haplotype blocks (combinations of SNPs defining common chromosomal segments segregating in the

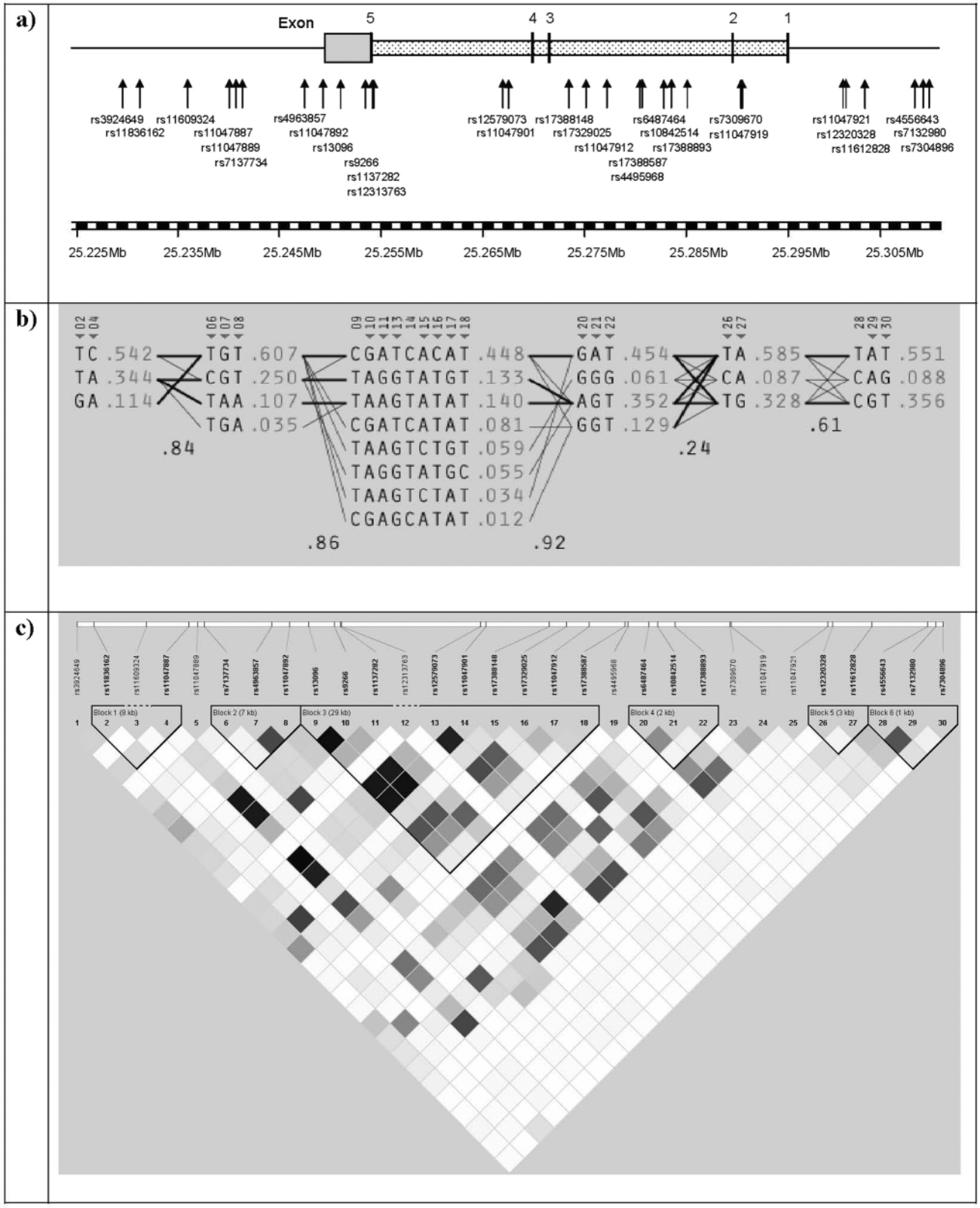

Figure 1. Variation in the human *KRAS* gene. (**a**) The genomic structure of the *KRAS* gene showing the location of the thirty tagSNPs genotyped. (**b**) Common haplotype blocks where dashed lines indicate locations of transition from one common haplotype to the next. (**c**) Linkage disequilibrium plot of single-nucleotide polymorphism (SNP) estimated as r^2 using Haploview.

population). Cases showed similar patterns of variation (data not shown). We chose SNPs across the *KRAS* locus using a SNP tagging strategy where representative SNPs were genotyped that had a high correlation ($r^2 > 0.8$) with other known SNPs in the gene. Common variants increasing risk of endometriosis would be expected to show evidence of association with one or more of the tagging SNPs genotyped.

Table I. Single-nucleotide polymorphisms (SNPs) genotyped across the *KRAS* locus

Number	dbSNP ID	Position	Role	Alleles	Frequency[a]	Association[b] (X_1^2)	*P*-value
1	rs3924649	25229541	3′-flanking	C/T	0.316	3.272	0.071
2	rs11836162	25231138	3′-flanking	G/T	0.108	1.372	0.241
3	rs11609324	25235982	3′-flanking	C/G	0.015	0.271	0.603
4	rs11047887	25239939	3′ UTR	A/C	0.447	1.685	0.194
5	rs11047889	25240755	3′ UTR	A/G	0.016	0.79	0.374
6	rs7137734	25241389	3′ UTR	C/T	0.26	1.788	0.181
7	rs4963857	25247667	3′ UTR	A/G	0.1	2.031	0.154
8	rs11047892	25249300	3′ UTR	A/T	0.134	2.514	0.113
9	rs13096	25251108	3′ UTR	A/G	0.43	1.323	0.250
10	rs9266	25253484	3′ UTR	C/T	0.43	1.282	0.258
11	rs1137282	25254044	3′ UTR	C/T	0.206	0.094	0.759
12	rs12313763	25254121	3′ UTR	C/T	0.005	0.061	0.806
13	rs12579073	25267066	Intron	A/C	0.445	1.129	0.288
14	rs11047901	25267596	Intron	A/G	0.44	1.207	0.272
15	rs17388148	25273453	Intron	G/T	0.088	0.868	0.352
16	rs17329025	25275080	Intron	A/G	0.473	2.455	0.117
17	rs11047912	25277177	Intron	C/T	0.266	0.137	0.711
18	rs17388587	25280487	Intron	A/G	0.065	1.692	0.193
19	rs4495968	25280774	Intron	A/G	0.014	0.311	0.577
20	rs6487464	25282723	Intron	C/T	0.352	0.236	0.627
21	rs10842514	25283549	Intron	C/T	0.473	2.884	0.090
22	rs17388893	25285132	Intron	A/C	0.059	0.089	0.766
23	rs7309670	25290240	Intron	A/G	0.204	0.288	0.591
24	rs11047919	25290388	Intron	A/C	0.406	3.526	0.060
25	rs11047921	25299314	Promoter	A/C	0.069	0	0.984
26	rs12320328	25299731	Promoter	A/G	0.09	0.401	0.527
27	rs11612828	25303396	Promoter	C/T	0.34	2.379	0.123
28	rs4556643	25308561		C/T	0.439	0.762	0.383
29	rs7132980	25309344		A/G	0.358	0.017	0.896
30	rs7304896	25309979		G/T	0.082	1.501	0.221

[a]Minor allele frequency in 959 control samples.
[b]Controls compared with 959 endometriosis cases.

We found no evidence for association between endometriosis and individual tagging SNPs in *KRAS* for either the allelic or the genotypic association tests. Tests of association between endometriosis and haplotypes or combinations of SNPs also showed no evidence for *KRAS* variation contributing to risk of endometriosis. There was no evidence for association when the analysis was restricted to more severe cases of endometriosis (data not shown). A small number of controls (131 individuals) had a previous record of laparoscopy where endometriosis was excluded. When we restricted the control sample to this group, there was no significant difference between cases and this subset of controls, although restricting controls to the subset with a record of laparoscopy would decrease power to detect association.

We developed a rapid method to screen for 11 common *KRAS* and *BRAF* mutations on the Sequenom MassARRAY system by using a ddATP and dGTP, dCTP, dTTP terminator that gave extensions of 1–7 nucleotides depending on the sequence (James *et al*., 2006). A panel of 66 melanoma cell lines and 13 ovarian cancer cell lines/colorectal polyps was used for mutation-positive controls, and the combined results correctly detect all 11 known mutants. We detected all mutations that had been previously identified by direct sequencing in this panel. No germline variants for *KRAS* or *BRAF* were found amongst 959 endometriosis cases and 959 controls tested.

Discussion

The Ras/Raf/mitogen-activated protein kinase (MAPK) pathway is a critical molecular signalling cascade through which extracellular signals can be transmitted into the nucleus to regulate cell proliferation or differentiation through altered gene expression. Activation of an oncogenic allele of *KRAS* in the reproductive tract of mice results in the development of endometriosis, suggesting that activation of *KRAS* is an important pathway in the initiation and/or progression of this disease (Dinulescu *et al*., 2005). We tested whether common variation in *KRAS* influences risk of endometriosis by genotyping 30 SNPs spanning the *KRAS* locus in 959 endometriosis cases and 959 controls. We found no evidence for differences in the frequencies of individual SNPs or combinations of SNPs (SNP haplotypes) in endometriosis cases compared with controls.

We estimated power for our case–control study (Figure 2). For our total sample of 959 cases and 959 controls, there is >80% power to detect dominant disease-predisposing alleles of frequencies 0.05, 0.25 and 0.5 contributing genotype relative risks (GRRs) of 1.7, 1.5 and 1.8, respectively. For recessive alleles, there is >80% power to detect alleles of frequencies 0.25 and 0.5, with GRRs of 2.3 and 1.6, respectively. For a multiplicative MOI, there is >80% power to detect alleles of frequencies 0.05, 0.25 and 0.5 contributing GRRs of 1.7, 1.4 and 1.3, respectively. These calculations demonstrate that our sample has high power to detect novel gene associations of small to moderate effect. However, because our cases are highly selected in terms of family history, compared with a standard case–control association study, our sample will have considerably more power to detect gene associations. Indeed, Antoniou and Easton (2003) showed that for multiplicative and dominant models, cases selected in terms of family history can provide approximately twice the power to detect association compared with unselected cases. For recessive models, cases with two affected siblings and cases with an affected parent and sibling, respectively, provide approximately 100–150% and 18–43% more power to detect association compared with unselected cases. It is therefore unlikely that common genetic variation in the *KRAS* gene is responsible for initiation and/or maintenance of endometriosis in women.

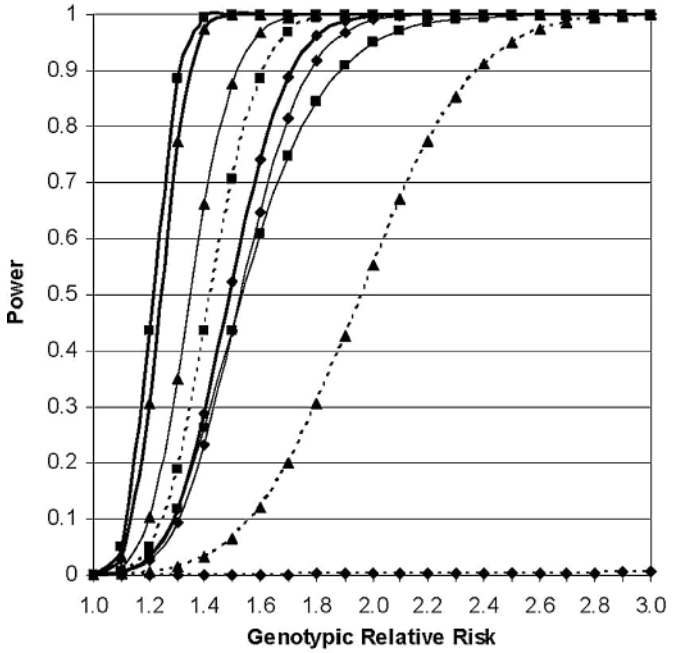

Figure 2. Power curves by genotypic relative risk of association tests in 959 endometriosis cases and 959 unaffected controls for dominant (unbroken line), recessive (broken line) and multiplicative (thick unbroken line) disease-predisposing alleles of frequencies of 0.05 (◆), 0.25 (▲) and 0.5 (■).

The choice of control samples for genetic studies in endometriosis is problematic (Zondervan *et al*., 2002, #253). Diagnosis and hence exclusion of disease require surgery, and this is not possible for population-based samples or the volunteer twin sample used in the present study. Alternatively, control samples drawn from patients who undergo laparoscopy for other conditions and where endometriosis is excluded may represent distinct genetic subsets of other gynaecological phenotypes that could confound comparisons with endometriosis patients. We chose to compare our sample of cases with samples from volunteer twins recruited into other studies with self-report data that they had never been diagnosed with endometriosis and were therefore at low risk of endometriosis. There was no significant association between KRAS variation and endometriosis when we restricted the control sample to individuals with a record of laparoscopy and no evidence of endometriosis.

Endometriosis shares some characteristics with ovarian tumours including neoangiogenesis, cellular proliferation and invasion. The majority of malignant ovarian tumours in adult women are epithelial ovarian cancers, and endometriosis is associated with an increase in the frequency of ovarian cancers of endometrioid or clear cell histotypes (Somigliana *et al*., 2006). Mutations in codons 12 and 13 of *KRAS* are reported in ovarian, colorectal, pancreatic and lung cancers (Kahn *et al*., 1987; Bos, 1989). *KRAS* mutations can be detected by several methods, the most common being real-time PCR or direct sequencing. However, these methods are expensive and have relatively low throughput for screening large sample sets. We developed a rapid method for screening 11 common mutations in *KRAS* and *BRAF* in a single PCR reaction using MALDI-TOF mass spectrometry. We tested the method by typing a panel of 66 melanoma cell lines and 13 ovarian cancer cell lines/colorectal polyps with known mutations. All mutations previously identified by direct sequencing in this panel were detected. We typed the 11 common mutations in our endometriosis cases and controls and found no germline mutations in *KRAS* or *BRAF* in our samples. Germline mutations in *KRAS* and *BRAF* have been reported (Niihori *et al*., 2006; Schubbert *et al*., 2006), but these are associated with developmental conditions with overlapping features including Noonan syndrome (MIM 163950) and cardio-facio-cutaneous syndrome (MIM 115150). It is therefore unlikely that functional mutations in *KRAS* exist in endometriosis patients without other associated phenotypes.

Our results demonstrate that the risk of endometriosis in women is not influenced by common variation in *KRAS*. We recently reported the first genome-wide scan for endometriosis and identified a region of significant linkage on chromosome 10q26 (maximum LOD score 3.09) (Treloar *et al*., 2005). *KRAS* maps to chromosome 12p12.1 and does not lie under peaks of significant or suggestive linkage in the genome scan, and the present study extends this result by demonstrating no evidence for association with common variants in *KRAS*. However, targeting the *KRAS* gene in the reproductive tract of mice resulted in the development of endometriosis (Dinulescu *et al*., 2005). Therefore, *KRAS* may play a role in the development or progression of endometriosis in women by cells in the reproductive tract or endometriotic lesions acquiring somatic mutations in *KRAS*.

Areas of transformation of endometrial tissue have been observed in surgical specimens from patients with ovarian cancer (Ballouk *et al*., 1994), and some studies suggest that tissues in ovarian endometrial cysts may be monoclonal in origin (Jimbo *et al*., 1997). If *KRAS* does play a role in the aetiology of endometriosis in women, it may be through somatic mutations in endometriotic lesions. The MALDI-TOF assay described here represents a high-throughput screening assay that could be used to screen for common somatic mutations in *KRAS* in appropriate tissue from endometriotic lesions.

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