KRAS variation and risk of endometriosis

Zhen Zhen Zhao, Dale R. Nyholt, Lien Le, Nicholas G. Martin, Michael R. James, Susan A. Treloar and Grant W. Montgomery

1Molecular Epidemiology Laboratory and 2Genetic Epidemiology Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia
3To whom correspondence should be addressed at: Molecular Epidemiology Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland 4029, Australia. E-mail: grant.montgomery@qimr.edu.au

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 (HRA5), Kirsten rat sarcoma viral oncogene homologue (KRAS) and neuroblastoma RAS viral (v-ras) oncogene homologue (NRA5), are the most common oncogenes associated with human neoplasms (Fabyani 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 (Trelour 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 r-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 (r² 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 (HotStar Taq, 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. 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 MgCl₂. 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 1, 10 μM each of ddATP, ddGTP, dATP and dCTP, and 0.08 U/μl Thermo Sequenase (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 (Scheid 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 1). 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
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.
single-nucleotide polymorphisms (SNPs) genotyped across the KRAS locus.

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 (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.

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 0.5, respectively. For multiplicative MOI, 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 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 associations.

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 associations.
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 control sample to individuals with a record of laparoscopy and no evidence 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. KRAS variation and risk of endometriosis

Acknowledgements

The authors thank Barbara Haddon for co-ordination of family recruitment, Dr Dan O’Connor for reviewing medical records, Anjali Henders and Megan Campbell for managing sample processing and DNA preparation, Renee Mayne for genotyping and Kevin Spring, Vicki Whitehall and Jeremy Arnold for providing positive control samples for mutation detection. This study was supported by the Australian Government’s Cooperative Research Centre’s Program and National Health and Medical Research Council of Australia (339430).

References


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 (■).


Purcell S, Cherny SS and Sham PC (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19,149–150.


