No significant association between progesterone receptor exon 4 Val660Leu G/T polymorphism and risk of ovarian cancer

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Epidemiological studies suggest that ovarian cancer is an endocrine-related tumour, and progesterone exposure specifically may decrease the risk of ovarian cancer. To assess whether the progesterone receptor (PR) exon 4 valine to leucine amino acid variant is associated with specific tumour characteristics or with overall risk of ovarian cancer, we examined 551 cases of epithelial ovarian cancer and 298 unaffected controls for the underlying G→T nucleotide substitution polymorphism. Stratification of the ovarian cancer cases according to tumour behaviour (low malignant potential or invasive), histology, grade or stage failed to reveal any heterogeneity with respect to the genotype defined by the PR exon 4 polymorphism. Furthermore, the genotype distribution did not differ significantly between ovarian cancer cases and unaffected controls. Compared with the GG genotype, the age-adjusted odds ratio (95% confidence interval) for risk of ovarian cancer was 0.78 (0.57–1.08) for the GT genotype, and 1.39 (0.47–4.14) for the TT genotype. In conclusion, the PR exon 4 codon 660 leucine variant encoded by the T allele does not appear to be associated with ovarian tumour behaviour, histology, stage or grade. This variant is also not associated with an increased risk of ovarian cancer, and is unlikely to be associated with a large decrease in ovarian cancer risk, although we cannot rule out a moderate inverse association between the GT genotype and ovarian cancer.

Introduction

Ovarian cancer is the sixth most common cancer among women (1), and the lifetime risk of developing ovarian cancer is approximately 1 in 100 in Australian women (2). While mutations in the breast cancer susceptibility genes BRCA1 and BRCA2, or in the mismatch repair genes hMLH1 and hMSH2, are responsible for most ‘hereditary’ ovarian cancers from multiple-case families (3), the vast majority (97%) of Australian patients with ovarian cancer present without a family history of ovarian cancer in a first degree relative (4). However, results from a recent twin study investigating the role of environmental and heritable factors in causation of cancer suggest that genetic factors may account for 22% of the variation in susceptibility to ovarian cancer (5). It is thus likely that ‘low-risk’ genes may account for at least some predisposition to apparently ‘sporadic’ ovarian cancers. In an attempt to identify such low-risk ovarian cancer susceptibility genes, we have chosen the candidate gene approach to compare large samples of women with ovarian cancer to unaffected control subjects.

It has been suggested that increased exposure to progesterone may protect against ovarian cancer (6). This author proposed that: the 10-fold increases in maternal circulating progesterone levels may account for the additional protective aspect of pregnancy beyond that attributable to suppression of ovulation; the progesterone content of the oral contraceptive (OC) pill may explain the large reduction in risk associated with use of OCs (including progestin-only formulations) that cannot be attributed to ovulation suppression alone; and that the decreased progesterone levels associated with higher levels of physical activity in premenopausal women may account for reported increasing trend in risk of ovarian cancer with increasing levels of physical activity (6).

The action of progesterone is mediated via the progesterone receptor (PR). Progesterone binds to PR, and the activated PR protein translocates to the cell nucleus under the control of hormone response elements of PR-responsive genes to regulate their transcription. Thus the cellular responses to progesterone are influenced by both PR protein level and activity in addition to progesterone hormone levels. In support of a protective effect of progesterone, cytosolic PR concentration was observed to be significantly lower in 51 ovarian cancer cases than in 45 benign ovarian tumours and 28 normal ovaries (7), and loss of PR in malignant tumours appeared to be independent of oestrogen receptor loss (7). In addition, exposure to progesterone may affect ovarian tumour characteristics: PR concentration was shown to be decreased in anaplastic tumours compared with more differentiated ones (7); high tumour PR levels have been correlated with longer survival (8,9); loss of heterozygosity in the region of the PR locus has been associated with reduced PR expression and reduced survival (10,11); and retention of heterozygosity in the region of the PR locus has been associated with endometrioid rather than serous or mucinous histology (10,11).

Genes involved in progesterone metabolism and function are thus putative low-risk ovarian cancer predisposition genes, and common functional allelic variants affecting gene expression or protein function in this pathway are candidates for molecular epidemiological case–control studies examining association with ovarian cancer risk. The progesterone receptor gene contains several polymorphisms. Exonic amino acid substitution sequence variants reported in the sequence database (GenBank accession number P06401) include the N-terminal
region Ser226Gly, Cys256Val and Thr344Ser variants, and the hinge region exon 4 Val660Leu variant. Other variants include an exon 5 C→T nucleotide polymorphism resulting in a synonymous His770His substitution, and an intronic 306 bp Alu insertion polymorphism (12). Linkage disequilibrium is reported to exist between the exon 4 Leu variant, the exon 5 T variant and the Alu insertion (13,14), and this complex of three variants has been termed PROGINS (13,14). Although there is no formal published data indicating functional significance for the PROGINS complex, data from transient co-transfection assays published in abstract form report that the insertion allele exhibits higher mRNA stability, increased amount of protein and higher transcriptional activity than the wild-type allele (13,14), with highest transcriptional activity for the heterodimeric receptor (13,14). This functional data would suggest that the PROGINS complex, especially in heterozygote form, might be associated with a decreased risk of ovarian cancer.

The Alu insertion variant, either alone or as part of the PROGINS complex, has been investigated for its association with ovarian cancer. The initial study of 67 cases and 184 controls from Ireland and Germany (15) reported a difference in distribution of the Alu insertion genotype between cases and controls (P = 0.025) and suggested that the insertion was over-represented in ovarian cancer cases compared with controls, although the raw data indicate that the difference in allele frequency was driven by results from the 26 cases and 101 controls from Germany. The same research group has reported in abstract form (13) that the frequency of the PROGINS complex correlates with the incidence of ovarian cancer in five different ethnic groups, but specific details of results were not reported. Subsequent independent studies of British subjects (231 ovarian cancer cases and 220 controls) (16) and north American subjects (96 cases and 101 controls) (17) found no differences in distribution of the Alu insertion genotype.

We have undertaken a large case–control comparison to assess whether the PR exon 4 G→T Val660Leu amino acid substitution variant is associated with overall risk of ovarian cancer in the Australian population. This particular variant was chosen for analysis since it is the only amino acid substitution polymorphism reported to be in linkage disequilibrium with the Alu insertion as part of the PROGINS complex, and may account directly for the functional differences reported to be associated with the PROGINS complex. Since there is evidence to suggest that the mutational pathway differs between invasive and low malignant potential (LMP) ovarian tumours (18,19), and that the aetiology of ovarian tumours differs according to histological classification (20–22), we first examined whether the T variant was associated with specific ovarian tumour characteristics such as tumour behaviour (LMP versus invasive), histology, stage or grade, and then examined if it was associated with an increased risk of ovarian cancer.

### Materials and methods

#### Subjects

Selected characteristics of cases and controls are displayed in Table I.

<table>
<thead>
<tr>
<th>Description of ovarian cancer cases and controls</th>
<th>Cases</th>
<th>Controls</th>
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<tr>
<td>Age (years)</td>
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</tr>
<tr>
<td>&lt;40</td>
<td>56</td>
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<tr>
<td>40–49</td>
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<tr>
<td>70+</td>
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</tr>
<tr>
<td>Range</td>
<td>19–95</td>
<td>30–90</td>
</tr>
<tr>
<td>Mean</td>
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<td>50.9</td>
</tr>
<tr>
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<td>13.9</td>
</tr>
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<td>Epidemiological information</td>
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<tr>
<td>Parity</td>
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<tr>
<td>Nulliparous</td>
<td>82</td>
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</tr>
<tr>
<td>1–3 live births</td>
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<tr>
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</tr>
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</tr>
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</tr>
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<td>13</td>
</tr>
<tr>
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<td>317</td>
<td>87</td>
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</table>

*Data not available (–) on oral contraceptive use, tubal ligation and hysterectomy for controls.

Brisbane Hospital, Queensland (n = 190). Histopathological information available for these cases included tumour behaviour [low malignant potential (LMP) or invasive], histology, stage and grade. The series comprised 97 (17%) LMP and 459 (83%) invasive tumours. LMP tumours were characterized by cellular proliferation with stratification and pleomorphism but no stromal invasion, whereas invasive tumours were characterized by invasion of the ovarian stroma (23). There were 329 (59%) serous, 69 (12%) mucinous, 64 (12%) endometrioid, 32 (6%) clear cell carcinoma, 12 (2%) mixed Mullerian, 25 (5%) mixed and 17 (3%) undifferentiated tumours, as well as 8 (1%) of unknown histology. For analysis with respect to histology, the mixed Mullerian and mixed histologies were treated as a single mixed group. Patients were staged at laparotomy in accordance with the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO) (24). Of the 426 invasive tumours of known stage, there were 77 (18%), 48 (11%), 259 (61%) and 42 (10%) at FIGO stages 1, 2, 3 and 4, respectively. Grade definitions were drawn directly from pathology reports for 393 of the invasive tumours, and comprised 52 (13%) grade 1, 8 (2%) grade 1/2, 107 (27%) grade 2, 56 (14%) grade 2/3, 163 (42%) grade 3, 1 (≤1%) grade 3/4 and 5 (1%) grade 4 tumours. The grades 1/2, 2/3 and 3/4 reflected grades considered indistinguishable by the pathologists. For analysis of trends in grade with genotype frequency, the eight tumours of grade 1/2 were excluded, while grades 3, 3/4 and 4 were treated as a single group of grade 3–4. Detailed demographic, medical, reproductive and contraceptive information was collected in a face-to-face interview as part of the population-based case–control study (4), and was therefore available for 366 (66%) case subjects. These population-based cases did not differ from population-based cases unavailable for molecular analysis with regard to age, family history of breast or ovarian cancer, parity, oral contraceptive use, tubal ligation and hysterectomy, or with respect to tumour behaviour (P > 0.4).

Tissue samples for DNA extraction were not collected from the controls included in the original case–control study (4), and it was thus not possible to use these population-based controls for genotype analysis. Control individuals from another source were utilized for genotype analysis, as described previously (25). Briefly, 300 unrelated adult female monozygotic twins (only one per pair) were selected from a sample of 3348 twins recruited through the volunteer National Australian Twin Registry for the Semi Structured Assessment for the Genetics of Alcoholism research study (26). Like cases, controls were almost exclusively of European descent, and were recruited from major cities in the eastern states of Australia (26). Controls were selected to match as closely as possible the date of birth distribution observed for
ovarian cancer patients, namely one-third from each of 1900–1925, 1926–1938 and 1939–1970. Limited epidemiological information, collected by self-completed questionnaire, was available from controls to adjust for confounding.

Ethical clearance for collection of subject information and blood from cases and controls was given by the Queensland Institute of Medical Research Ethics committee.

Genotype detection
Germline DNA was extracted from peripheral blood by a salt precipitation method (27) for controls, and for cases collected through the Royal Brisbane Hospital. DNA was extracted from archival paraffin blocks using the method of Levi et al. (28) for cases ascertained through the population-based case-control study. Tissue blocks were from sites distant from the tumour, and displayed no evidence of cancer cells. The PR exon 4 Val660Leu G/T polymorphism (GenBank accession number P06001) was detected using the PE ABI Prism 7700 Sequence Detection System (SDS) for multi-colour real-time or end-point fluorogenic PCR detection (PE Applied Biosystems, Foster City, CA). A 68 bp PCR product was amplified using the forward and reverse primers 5′-AGA GCA CTG GAT GCT GTT GCT-3′ and 5′-TGG CTT AGG GCT TGG CTT T-3′, respectively. TaqRI enzyme digestion and high resolution agarose gel electrophoresis was used to identify GG and TT homozygote DNA controls as required standards for the SDS allelic discrimination assay. Using the standard protocol for SDS allelic discrimination assay, fluorescently-labelled probes 5′-6-carboxyfluorescein (FAM)-CAC AGC CAG TGG GCG TTC-CA-6-carboxytetramethylrhodamine (TAMRA)-3′ and 5′-6-carboxy-4,7,2′,7′-tetrachlorofluorescein (TET)-CAC AGC CAT TGG GCG TTC-CA-TAMRA-3′ were used to detect the G and T alleles, respectively. The final concentration of reagents in the PCR mix (reaction volume 25 μl) was 1× TaqMan Universal PCR Master Mix (PE Applied Biosystems), 900 nM primers, 100 nM FAM-G probe and 150 nM TET-T probe. Reaction mix was added to 30 ng genomic sample DNA that had been pre-dried in pre-dried 96-well plates. PCR reactions were incubated in the ABI 7700 SDS PCR machine for 2 min at 50°C, 10 min at 95°C, followed by 45 two-step cycles of 15 s at 95°C and 1 min at 62°C. Genotype analysis was performed on amplified samples using the ABI PRISM 7700 software, following standard procedures. Repeatability of the ABI PRISM 7700 SDS genotyping was assessed by re-analysis of a sub-sample of 121 DNA samples, selected on the basis of DNA availability. Successful re-amplification of samples generated confirmatory genotype results in all instances. Genotype results were obtained for 551 cases and 298 controls, constituting a PCR success rate of 99%.

Statistical analysis
The Student’s t-test was used to compare the mean age of cases and controls. The Hardy–Weinberg equilibrium (HWE) assumption was assessed for case and control groups by comparing the observed numbers of different genotypes with those expected under HWE for the estimated allele frequency, and comparing the Pearson goodness-of-fit statistic with a χ² distribution with one degree of freedom. Differences in the distribution of PR exon 4 genotype amongst cases stratified according to tumour behaviour, histology, grade and stage were used by the χ² test. Since the χ² test is less powerful when the expected cell frequency is less than 5, but more powerful when it is greater than 5, cells with expected frequencies less than 5 were combined to increase expected frequency to 5 or greater, as per Breslow-Day and the rule of 5. When the χ² test was used, the χ² test (tumour behaviour, histology, and stage) was used. Similarly, differences in genotype distribution were assessed with respect to age and parity (0.5 for all comparisons).

Discussion
Previous studies have suggested that progesterone exposure mediated via the PR may affect ovarian tumour characteristics (7–11), and that increased progesterone exposure may protect against the overall risk of ovarian cancer (6,7). The PROGINS complex, including the PR exon 4 codon 660 T variant, has been reported to be associated with increased transcriptional activation relative to the wild-type (13,14). Thus, one might expect the PR exon 4 variant to be associated with specific tumour characteristics, and specifically with a decreased risk of ovarian cancer. Furthermore, since the PROGINS–wild-type heterodimer was reported to be associated with the greatest transcriptional activity relative to the wild-type (13), a protective effect for the PR exon 4 T allele might be greatest for individuals who are heterozygous for this variant. However, the data presented in this study provide no evidence that the genotype defined by the PR exon 4 G→T codon 660 polymorphism is associated with tumour characteristics such as tumour behaviour, histology, grade or stage. The
TT genotype occurred at a very low frequency, and thus we were unable to evaluate the effect of this genotype on ovarian cancer risk. There was no evidence that the PR exon 4 GT genotype is associated with an increased risk of ovarian cancer. Interestingly, the OR for the GT genotype fell below unity (0.78), which is not inconsistent with the hypothesis that the heterozygote genotype of purported greatest activity (13) would be associated with increased progesterone exposure, leading to a reduction in risk of ovarian cancer. However, this decreased risk was not statistically significant (95% CI = 0.57–1.08; P = 0.1). This study of 551 cases and 298 controls had 80% power at the 5% significance level to detect an OR of 0.63 (37% decrease in risk) associated with the GT genotype.

There was no association between genotype and the ovarian cancer risk factors age and parity in either cases or controls, indicating that confounding due to these factors was improbable. Confounding due to other factors such as OC use, tubal ligation or hysterectomy cannot be excluded, but the absence of an association between genotype and these risk factors in cases suggests at least that interaction between PR genotype and OC use is unlikely, and provides some reassurance that confounding is unlikely. Confounding due to differences in ethnicity was unlikely, since controls were almost exclusively of European descent (26), and the same was true for cases given that ovarian cancer is rare in other population groups and that the majority of Australians are Anglo–Celtic in origin. In addition, comparison of the control group of the present study to a series of 832 younger controls collected as part of an Australian breast cancer case–control study showed no difference in genotype frequency (P = 0.4; unpublished data), suggesting that control group genotype frequencies are representative of the population.

Since the PR exon 4 T variant is reported to be in linkage disequilibrium with the Alu insertion (13,14), we also compared our findings to published data on the Alu insertion polymorphism (15–17). The homozygote Alu insertion (purportedly equivalent to the TT genotype) was rare (average 3 and 2.5% in cases and controls, respectively), and similar in frequency to that observed for the TT genotype in this study (2% for cases and controls). The frequency of the heterozygote Alu insertion (purportedly equivalent to the GT genotype) was 22% (range 16–34%) in cases, and 21% (range 18–25%) in controls (15–17), compared with 26% in cases and 30% in controls in our dataset. For the pooled sample of published data on the Alu insertion (15–17), there was no difference between cases and controls in the genotype frequency of the heterozygote Alu insertion compared with the wild-type (P = 0.9), and thus no evidence that the Alu insertion is associated with either an increased or decreased risk of ovarian cancer.

In conclusion, the PR exon 4 codon 660 leucine variant encoded by the T allele does not appear to be associated with ovarian tumour behaviour, histology, stage or grade. There is no evidence to suggest that this variant, reported to be in linkage disequilibrium with the PR intronic Alu insertion (13,14), increases the risk of ovarian cancer. The GT genotype is unlikely to be associated with a large decrease in ovarian cancer risk, but we cannot exclude a moderate inverse association between the GT genotype and ovarian cancer, and a larger study would be necessary to establish whether such an association exists.

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References


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