Functional complementation studies identify candidate genes and common genetic variants associated with ovarian cancer survival

Lydia Quaye¹, Dimitra Dafou¹, Susan J. Ramus¹, Honglin Song², Aleksandra Gentry Maharaj¹, Maria Notaridou¹, Estrid Hogdall⁴, Susanne Kruger Kjaer⁴, Lise Christensen⁵, Claus Hogdall⁶, Douglas F. Easton³, Ian Jacobs¹, Usha Menon¹, Paul D.P. Pharoah² and Simon A. Gayther¹,*

¹Gynaecological Oncology Unit, UCL EGA Institute for Women’s Health, University College London, UK, ²CR-UK Department of Oncology, ³Genetic Epidemiology Unit, University of Cambridge, Strangeways Research Laboratory, Cambridge, UK, ⁴Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark, ⁵Department of Pathology, Bispebjerg Hospital, University of Copenhagen, Denmark and ⁶The Juliane Marie Centre, Rigshospitalet, University of Copenhagen, Denmark

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Common germline genetic variation and/or somatic alterations in tumours may be associated with survival in women diagnosed with ovarian cancer. The successful identification of genetic associations relies on a suitable strategy for identifying and testing candidate genes. We used microcell-mediated chromosome transfer approach and expression microarray analysis to identify genes that were associated with neoplastic suppression in ovarian cancer cell lines. Sixty-five tagging single nucleotide polymorphisms (tSNPs) in nine candidate genes were genotyped in ~1700 invasive ovarian cancer cases to look for associations with survival. For two of these genes, loss of heterozygosity (LOH) analysis of tSNPs in 314 ovarian tumours was used to identify associations between somatic gene deletions and survival. We identified significant associations with survival for a tSNP in caspase 5 (CASP5) [hazard ratio (HR) = 1.13 (95% CI: 1.00–1.27), P = 0.042] and two tSNPs in the retinoblastoma binding protein (RBBP8) gene [HR = 0.85 (95% CI: 0.75–0.95), P = 0.007 and HR = 0.83 (95% CI: 0.71–0.95), P = 0.009]. After adjusting for multiple prognostic factors in a multivariate Cox regression analysis, both associations in RBBP8 remained significant (P = 0.028 and 0.036). We then genotyped 314 ovarian tumours for several tSNPs in CASP5 and RBBP8 to identify gene deletions by LOH. For RBBP8, 35% of tumours in 101 informative cases showed somatic allelic deletion; LOH of RBBP8 was associated with a significantly worse prognosis [HR = 2.19 (95% CI: 1.36–3.54), P = 0.001]. In summary, a novel in vitro functional approach in ovarian cancer cells has identified RBBP8 as a gene for which both germline genetic variation and somatic alterations in tumours are associated with survival in ovarian cancer patients.

INTRODUCTION

Epithelial ovarian cancer is the eighth most common cancer in women and the most common cause of cancer death from a gynaecological malignancy. In 2002, there were ~200 000 new diagnoses worldwide and about 125 000 deaths from the disease (1). Despite improvements in the response of patients to adjuvant chemotherapy in recent years, most patients still develop recurrent disease, which may be drug resistant. Consequently, survival rates for the disease have
improved little in more than 30 years with <40% of women with late stage ovarian cancer (stages III/IV) surviving more than 5 years (2,3).

Several studies have shown that the molecular and biological features of a cancer will influence the response to different treatment regimes, and subsequently patient outcome. Some molecular markers serve as good predictors of overall survival after a diagnosis of cancer; the identification of other markers has enabled novel cancer treatments to be developed (4,5). However, there are no reliable prognostic tumour markers for ovarian cancer, and standard treatment consists of non-specific platinum-based chemotherapies.

Evidence has emerged to suggest that germline genetic variation can influence survival in patients with ovarian cancer. Some studies have reported survival differences in patients carrying a BRCA1 or BRCA2 mutation versus non-mutation carriers (6,7). Other studies have investigated the effects of common genetic variation on survival and reported a handful of single nucleotide polymorphisms (SNPs) located in oncogenes, DNA mismatch repair and cell cycle control genes and combinations of SNPs in the vascular endothelial growth factor gene that may be associated with survival (6–11). Finally, there is evidence suggesting that germline genetic variation may also play a role in chemotherapy resistance (12). By identifying genetic polymorphisms that influence survival, it may be possible to predict response to different treatments and ultimately improve the prognosis using individualized treatment strategies.

So far, most studies have used a candidate gene approach to look for genes that affect survival. A limitation of this approach is that candidate gene selection is usually based on a predicted rather than a known function for the gene in ovarian cancer development. Using an approach that focuses on functional evidence for a gene’s involvement in disease aetiology may be more successful at identifying new prognostic markers. A method that we have used to identify putative, functionally relevant candidate genes for ovarian cancer is microcell-mediated chromosome transfer (MMCT). Briefly, ovarian cancer cells act as recipients for fusion with microcells generated from a donor cell line (e.g. murine fibroblasts) containing single copies of human normal chromosomes tagged with a selectable marker (13). The neoplastic phenotype of recipient:donor hybrids is then assayed in vitro and in vivo in order to assess whether chromosome transfer causes a phenotypic change that can be coupled to biological and/or molecular function. This method has been used successfully in the past to identify tumour suppressor ‘like’ genes or gene regions that may have been deleted during tumour progression (14). With the advent of gene expression microarray technologies, this method can also be used to report on the changes that take place throughout the genome in response to chromosome transfer.

In the current study, we have combined MMCT analysis of chromosome 18 with gene expression microarray analysis of two ovarian cancer cell lines to identify novel candidates that may be functionally important in ovarian cancer development. We identified nine differentially expressed genes with a described function suggesting they might play a role in cancer. Data relating to common germline genetic variation were available for these genes from HapMap. This enabled us to evaluate the associations between germline and somatic genetic variation with survival in ~1700 invasive ovarian cancer cases.

RESULTS

Identifying candidate genes using MMCT

Metaphase comparative genomic hybridization (CGH) analysis of 141 primary epithelial ovarian cancers identified several chromosomes (4–6, 13–15 and 18) that show partial or complete deletion in 34–78% of tumours (15). We transferred these chromosomes individually into two epithelial ovarian cancer cell lines (TOV112D and TOV21G) by MMCT. Donor–recipient hybrids were assessed for their neoplastic phenotype in vitro. Chromosome 18 showed strong evidence of neoplastic suppression, evaluated by anchorage-dependent and -independent growth and invasion through a matrigel (Fig. 1). Moreover, this phenotype was not subject to clonal variation (data not shown). Previous evidence has suggested that chromosome 6 is involved in ovarian cancer development following MMCT (16); but the effects for chromosome 18 are novel. Therefore, we chose to investigate TOV112D*18 and TOV21G*18 hybrid cell lines in greater detail by examining their tumourigenic phenotype in vivo following intraperitoneal injection into immunosuppressed mice. TOV112D and TOV21G cell lines both formed undifferentiated ovarian carcinomas with ascites and multiple peritoneal surface implants within 3 weeks. By contrast, TOV112D*18 and TOV21G*18 hybrid cell lines showed very limited tumour growth after 12 weeks (data not shown).

Gene expression microarray analysis was used to compare the transcriptome between two hybrid lines generated from each parental cell line for chromosome 18, and the TOV112D and TOV21G cancer cell lines. Five hundred and forty genes were identified that were either up- or down-regulated in hybrid lines. These were ranked according to a combination of P-value (<0.05) and differences in expression ‘fold-change’ (>|1.5-fold). The top 190 genes were further evaluated according to the known or predicted function, the number of common SNPs and for the gene size (which affects how efficiently the gene is tagged) to identify the nine candidates investigated in this study. All 190 differentially expressed genes are listed in Supplementary Material, Table S1, includes a brief summary of the known function of each gene, the differential expression change in MMCT hybrids versus parental cancer cell lines, and the gene size and number of tagging SNPs identified.

Nine candidate genes were further selected for analysis: AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1 and STAG3. We identified 68 tagging SNPs (tSNPs) in these genes. Of these, three tSNPs (AKTIP rs2271695, AXIN2 rs2240308 and rs4128941) could not be genotyped or efficiently tagged by any other SNP. Therefore, 65 tSNPs were genotyped in 1694 ovarian cancer cases of the MALOVA, SEARCH and UKOPS population-based studies. Details of the cases and populations included in this study are given in Table 1. Brief information about each gene, including the putative function and the number of tSNPs per gene, is given in Table 2.
Evaluating the effects of common genetic variants on ovarian cancer survival

The results of the univariate analyses for all 65 tSNPs are shown in Supplementary Material, Table S2. We identified significant associations between common genetic variation and all-cause mortality for three tSNPs; rs518604 in CASP5 gene, and rs4474794 and rs9304261 in RBBP8 gene (Table 3). The rare allele of rs518604 was associated with

Table 1. Characteristics of study populations used in these analyses

<table>
<thead>
<tr>
<th>Ascertainment</th>
<th>MALOVA</th>
<th>SEARCH</th>
<th>UKOPS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incident cases dx 1994–1999 (municipalities of Copenhagen and Frederiksberg)</td>
<td>Prevalent cases dx 1991–1998; incident cases dx from 1998 (East Anglia, West Midlands and Trent regions of England)</td>
<td>Prevalent and incident cases dx from 2006 from 10 Gynaecological oncology centres in England and Wales</td>
<td></td>
</tr>
<tr>
<td>Participation rate (%)</td>
<td>79</td>
<td>69</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>Denmark</td>
<td>UK</td>
<td>UK</td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>446</td>
<td>847</td>
<td>401</td>
<td></td>
</tr>
<tr>
<td>No. of deaths</td>
<td>301</td>
<td>230</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Time at risk (person years)</td>
<td>1805</td>
<td>3565</td>
<td>515</td>
<td></td>
</tr>
<tr>
<td>Median time at risk (years)</td>
<td>3.26 (0.01–9.72)</td>
<td>4.29 (0.1–8.15)</td>
<td>1.35 (0.01–2.41)</td>
<td></td>
</tr>
<tr>
<td>Annual mortality rate</td>
<td>0.17</td>
<td>0.06</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>13 (3%)</td>
<td>52 (6%)</td>
<td>7 (2%)</td>
<td>72 (4%)</td>
</tr>
<tr>
<td>40–49</td>
<td>71 (16%)</td>
<td>142 (17%)</td>
<td>57 (14%)</td>
<td>270 (16%)</td>
</tr>
<tr>
<td>50–59</td>
<td>130 (29%)</td>
<td>369 (44%)</td>
<td>137 (34%)</td>
<td>636 (38%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>232 (52%)</td>
<td>284 (34%)</td>
<td>200 (50%)</td>
<td>716 (42%)</td>
</tr>
<tr>
<td>Tumour grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>104 (23%)</td>
<td>101 (12%)</td>
<td>45 (11%)</td>
<td>250 (15%)</td>
</tr>
<tr>
<td>2</td>
<td>145 (33%)</td>
<td>161 (19%)</td>
<td>94 (23%)</td>
<td>400 (24%)</td>
</tr>
<tr>
<td>3</td>
<td>168 (38%)</td>
<td>174 (21%)</td>
<td>177 (44%)</td>
<td>519 (31%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>29 (7%)</td>
<td>411 (49%)</td>
<td>85 (21%)</td>
<td>525 (31%)</td>
</tr>
<tr>
<td>Tumour stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>148 (33%)</td>
<td>212 (25%)</td>
<td>132 (33%)</td>
<td>492 (29%)</td>
</tr>
<tr>
<td>Advanced diseasea</td>
<td>298 (67%)</td>
<td>199 (23%)</td>
<td>248 (62%)</td>
<td>745 (44%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0%)</td>
<td>436 (51%)</td>
<td>21 (5%)</td>
<td>457 (27%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>275 (62%)</td>
<td>328 (39%)</td>
<td>193 (48%)</td>
<td>796 (47%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>56 (13%)</td>
<td>138 (16%)</td>
<td>68 (17%)</td>
<td>262 (15%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>43 (10%)</td>
<td>104 (12%)</td>
<td>38 (9%)</td>
<td>185 (11%)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>33 (7%)</td>
<td>83 (10%)</td>
<td>37 (9%)</td>
<td>153 (9%)</td>
</tr>
<tr>
<td>Other</td>
<td>38 (8%)</td>
<td>194 (23%)</td>
<td>65 (16%)</td>
<td>297 (18%)</td>
</tr>
</tbody>
</table>

*aSpread to regional lymph nodes or distant metastases; dx = diagnosed.

Evaluating the effects of common genetic variants on ovarian cancer survival

The results of the univariate analyses for all 65 tSNPs are shown in Supplementary Material, Table S2. We identified significant associations between common genetic variation and all-cause mortality for three tSNPs; rs518604 in CASP5 gene, and rs4474794 and rs9304261 in RBBP8 gene (Table 3). The rare allele of rs518604 was associated with

Figure 1. In vitro phenotype analysis of TOV112D and TOV21G cancer cell lines and the MMCT hybrids generated from these cell lines. (A) Colony forming efficiency (CFE) in anchorage-independent growth assays for both parental cancer cell lines and five hybrids generated for each of six chromosomes (4, 5, 6, 13, 14, 15 and 18). (B) Invasive ability of both parental cancer cell lines and the same five hybrids generated for each chromosome as described above. Invasiveness in recipient and hybrid cell lines was measured using transwell chambers; 3 × 10⁶ cells were aliquoted into each insert in serum-free culture media and 1% of bovine serum albumin was used as the chemotactant. For both cancer cell lines, the results of invasion assays are generally replicated by anchorage-independent growth assays. Hybrids from TOV21G transfers show evidence of growth suppression for chromosomes 5, 6, 13, 14 and 18; hybrids from TOV112D transfers show evidence of neoplastic suppression for chromosomes 5, 6 and 18. Chromosome 20 was included as a control in these experiments because it rarely shows loss in primary ovarian tumours (Ramus et al. (15)).


**Table 2.** Details of nine candidate genes selected from differential gene expression analysis of TOV21G and TOV112D ovarian cancer cells lines and MMCT-18 hybrids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression: parental versus hybrid lines</th>
<th>Cytoband</th>
<th>Function</th>
<th>Gene size (bp)</th>
<th>No. SNPsa</th>
<th>No. tSNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIFM2</td>
<td>3-fold up in TOV112D and TOV21G hybrids</td>
<td>10q22.1</td>
<td>TP53-induced apoptosis; overexpression induces apoptosis</td>
<td>34,711</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>AKTIP</td>
<td>4-fold up in TOV112D and TOV21G hybrids</td>
<td>16q12.2</td>
<td>Apoptosis; interacts with PKB/Akt</td>
<td>11,978</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>AXIN2</td>
<td>5-fold up in TOV112D hybrids</td>
<td>17q23–q24</td>
<td>Inhibitor of β-catenin in Wnt-signalling pathway; LOH in breast and other cancers</td>
<td>33,084</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>CASP5</td>
<td>7-fold up in TOV21G hybrids</td>
<td>11q22.2–q22.3</td>
<td>Regulation of apoptosis</td>
<td>14,729</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>FILIP1</td>
<td>5-fold up in TOV112D hybrids</td>
<td>3q12.1</td>
<td>Down-regulated in ovarian cancer</td>
<td>281,369</td>
<td>135</td>
<td>8</td>
</tr>
<tr>
<td>RBBP8</td>
<td>7-fold up in TOV112D hybrids</td>
<td>18q11.2</td>
<td>RB1 binding protein; transcriptional regulation of BRCAl; DNA repair; tumour suppressor</td>
<td>93,155</td>
<td>39</td>
<td>4</td>
</tr>
<tr>
<td>RGCO21</td>
<td>3-fold down in TOV112D hybrids</td>
<td>13q14.11</td>
<td>Cell cycle progression regulation; induced by p53 in response to DNA damage</td>
<td>13,323</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>RUVBL1</td>
<td>25-fold down in TOV112D and TOV21G hybrids</td>
<td>3q21</td>
<td>Interacts with MYC; involved in cell growth</td>
<td>42,857</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>STAG3</td>
<td>9-fold up in TOV21G hybrids</td>
<td>7q22.1</td>
<td>Component of cohesin complex; chromosome segregation</td>
<td>43,764</td>
<td>28</td>
<td>3</td>
</tr>
</tbody>
</table>

aCommon variants (minor allele frequency ≥ 0.05).

**Table 3.** A summary of Cox regression analysis for tSNPs significantly associated with survival (stratified by study) of invasive ovarian cancer cases

<table>
<thead>
<tr>
<th>Gene</th>
<th>Case No. tSNP</th>
<th>All subtypes</th>
<th>Serous subtype</th>
<th>Univariate HR (95% CI), P-trend</th>
<th>Multivariate HR (95% CI), P-trend</th>
<th>Univariate HR (95% CI), P-trend</th>
<th>Multivariate HR (95% CI), P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKTIP</td>
<td>rs7189819</td>
<td>1624</td>
<td>1.12 (1.00–1.27), P = 0.056</td>
<td>1.17 (1.01–1.36), P = 0.036</td>
<td>1.08 (0.90–1.31), P = 0.403</td>
<td>0.86 (0.69–1.09), P = 0.235</td>
<td>0.70 (0.54–0.92), P = 0.056</td>
</tr>
<tr>
<td>AXIN2</td>
<td>rs451111</td>
<td>1618</td>
<td>1.05 (0.94–1.17), P = 0.423</td>
<td>1.16 (1.00–1.34), P = 0.043</td>
<td>1.08 (0.90–1.29), P = 0.386</td>
<td>0.91 (0.80–1.04), P = 0.468</td>
<td>0.85 (0.73–0.99), P = 0.036</td>
</tr>
<tr>
<td>CASP5</td>
<td>rs518604</td>
<td>1645</td>
<td>1.13 (1.00–1.27), P = 0.042</td>
<td>1.16 (1.00–1.34), P = 0.047</td>
<td>1.15 (0.95–1.38), P = 0.151</td>
<td>0.85 (0.73–0.99), P = 0.036</td>
<td>0.85 (0.73–0.99), P = 0.049</td>
</tr>
<tr>
<td>RBBP8</td>
<td>rs447794</td>
<td>1640</td>
<td>0.85 (0.75–0.95), P = 0.007</td>
<td>0.88 (0.75–0.92), P = 0.098</td>
<td>0.89 (0.73–1.06), P = 0.191</td>
<td>0.83 (0.69–0.99), P = 0.036</td>
<td>0.83 (0.71–0.95), P = 0.009</td>
</tr>
<tr>
<td>RGCO21</td>
<td>rs904261</td>
<td>1602</td>
<td>0.83 (0.71–0.95), P = 0.009</td>
<td>0.87 (0.72–0.95), P = 0.143</td>
<td>0.87 (0.68–1.00), P = 0.233</td>
<td>0.83 (0.69–0.99), P = 0.036</td>
<td>0.83 (0.71–0.95), P = 0.009</td>
</tr>
<tr>
<td>STAG3</td>
<td>rs224671</td>
<td>1617</td>
<td>1.07 (0.95–1.19), P = 0.268</td>
<td>1.17 (1.02–1.35), P = 0.03</td>
<td>1.11 (0.93–1.32), P = 0.235</td>
<td>1.11 (0.93–1.32), P = 0.05</td>
<td>1.11 (0.93–1.32), P = 0.235</td>
</tr>
<tr>
<td>RUVBL1</td>
<td>rs1637001</td>
<td>1659</td>
<td>1.11 (0.97–1.26), P = 0.119</td>
<td>1.20 (1.02–1.42), P = 0.029</td>
<td>1.18 (0.97–1.44), P = 0.101</td>
<td>1.11 (0.97–1.26), P = 0.119</td>
<td>1.18 (0.97–1.44), P = 0.101</td>
</tr>
</tbody>
</table>

The data for all tSNPs are given in Supplementary Material, Table S1. HR, hazard ratio; CI, confidence interval; Multivariate: results adjusted for >60 years, tumour stage and grade.

Worse survival [hazard ratio (HR) = 1.13 (95% CI: 1.00–1.27), P = 0.042]. The rare alleles of rs447794 and rs9304261 were associated with a better prognosis [HR = 0.85 (95% CI: 0.75–0.95), P = 0.007 and HR = 0.83 (95% CI: 0.71–0.95), P = 0.009, respectively]. These data are illustrated in Figure 2. The genotype distributions for all SNPs are given in Supplementary Material, Table S3. We looked for evidence of a combined effect for the two significant SNPs in RBBP8 in association with overall survival. Using Cox regression analysis, we found that the interaction between rs447794 and rs9304261 was significantly associated with better prognosis [HR = 0.94 (95% CI: 0.91–0.98), P = 0.007].

We also evaluated the associations between different haplotypes and survival (Table 3). Three haplotypes in CASP5 showed evidence of association: a haplotype in block 1 (h0000), which was associated with a decreased hazard [HR = 0.86 (95% CI: 0.77–0.97), P = 0.014]; the combined rare haplotypes of block 1 were associated with an increased hazard [HR = 4.56 (95% CI: 1.71–12.12), P = 0.002] and a haplotype in block 2 (h100011) was associated with a decreased hazard [HR = 0.5 (95% CI: 0.26–0.98), P = 0.042] (Table 4). Three haplotypes in RBBP8 were also associated with survival: h0000 was associated with worse survival [HR = 1.16 (95% CI: 1.03–1.31), P = 0.015], and h0011 and h0111 were both associated with better survival [HR = 0.60 (95% CI: 0.39–0.93), P = 0.022 and HR = 0.81 (95% CI: 0.71–0.94), P = 0.005, respectively] (Table 3).

We identified associations between serous ovarian cancer and survival for tSNPs in the AKTIP, AXIN2, CASP5 and STAG3 genes. Notably, rs518604 in CASP5 was still associated with worse survival in serous cases and remained marginally significant [HR = 1.16 (95% CI: 1.00–1.34), P = 0.047]. In addition, the rare allele of rs523104 in CASP5 was associated with improved survival in serous cases [HR = 0.85 (95% CI: 0.73–1.00), P = 0.049] (Table 3). We also identified several haplotypes in the CASP5, AKTIP, AXIN2 and STAG3 genes that were associated with survival only in serous cases (Table 4 and Supplementary Material, Table S4).
For the significant associations ($P < 0.05$), we adjusted for prognostic factors, which included age $>$ 60, clinical stage and histopathological grade. After adjustment, rs518604 in $CASP5$ and the haplotype block h010 were no longer significant; but associations for rs4474794 and rs9304261 of $RBBP8$ with better survival remained significant; $[HR = 0.85$ (95% CI: 0.69–0.99), $P = 0.036]$. 

**Table 4.** Cox regression analysis of haplotypes (stratified by study) that are significantly associated with ovarian cancer survival

<table>
<thead>
<tr>
<th>Gene</th>
<th>Haplotype</th>
<th>Frequency</th>
<th>All subtypes</th>
<th>Serous subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Univariate HR (95% CI), $P$-trend</td>
<td>Multivariate HR (95% CI), $P$-trend</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Univariate HR (95% CI), P-trend</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Multivariate HR (95% CI), $P$-trend</td>
</tr>
<tr>
<td>$CASP5$ (block 1)</td>
<td>h010</td>
<td>0.45</td>
<td>0.86 (0.77–0.97), $P = 0.014$</td>
<td>0.90 (0.78–1.03), $P = 0.128$</td>
</tr>
<tr>
<td></td>
<td>h100</td>
<td>0.42</td>
<td>1.12 (1.00–1.26), $P = 0.05$</td>
<td>1.08 (0.94–1.23), $P = 0.295$</td>
</tr>
<tr>
<td></td>
<td>Rare</td>
<td>0.004</td>
<td>4.56 (1.71–12.12), $P &lt; 0.005$</td>
<td>11.72 (4.13–33.23), $P = 0.016$</td>
</tr>
<tr>
<td>$CASP5$ (block 2)</td>
<td>h10001</td>
<td>0.03</td>
<td>0.5 (0.26–0.98), $P = 0.042$</td>
<td>0.85 (0.55–1.32), $P = 0.479$</td>
</tr>
<tr>
<td></td>
<td>h0000</td>
<td>0.62</td>
<td>1.16 (1.03–1.31), $P = 0.015$</td>
<td>1.17 (1.02–1.35), $P = 0.006$</td>
</tr>
<tr>
<td>$RBBP8$</td>
<td>h0010</td>
<td>0.03</td>
<td>0.60 (0.39–0.93), $P = 0.022$</td>
<td>0.69 (0.41–1.16), $P = 0.016$</td>
</tr>
<tr>
<td></td>
<td>h0011</td>
<td>0.23</td>
<td>0.81 (0.71–0.94), $P = 0.005$</td>
<td>0.82 (0.69–0.98), $P = 0.026$</td>
</tr>
<tr>
<td>$STAG3$</td>
<td>h011</td>
<td>0.27</td>
<td>1.11 (0.98–1.26), $P = 0.1$</td>
<td>1.11 (0.96–1.29), $P = 0.152$</td>
</tr>
</tbody>
</table>

The complete data for all haplotypes are given in Supplementary Material, table S3.

HR, hazard ratio; CI, confidence interval; Multivariate: results adjusted for $>60$ years, tumour stage and grade; SNP order in haplotypes, 5’ to 3’ of the genes $CASP5$ (block 1): rs518604, rs523104 and rs318128; $CASP5$ (block 2): rs17446518, rs9651713, rs3181175, rs3181174, rs2282657 and rs507879; $RBBP8$: rs7239066, rs11082221, rs4474794 and rs9304261, $STAG3$: rs11762932, rs2246713 and rs1637001.

‘0’ represents common allele and ‘1’ represents rare allele.
RBBP8
tSNPs and haplotypes in the
in ovarian cancer
than 7-fold increases in gene expression in TOV112D+
ovarian cancer. Both
fore, we looked for further evidence for their involvement in
the strongest evidence of an association with survival. There-
h0011 rs4474794 and rs9304261, and the haplotypes h0000 and
P = 0.74–0.98), respectively. The interaction we found between
rs4474794 and rs9304261, and the haplotypes h0000 and
h0011 RBBP8 also remained significant after adjustment.

Additional evidence of a role for CASP5 and RBBP8 in ovarian cancer
tSNPs and haplotypes in the RBBP8 and CASP5 genes showed
the strongest evidence of an association with survival. There-
fore, we looked for further evidence for their involvement in
ovarian cancer. Both RBBP8 and CASP5 showed greater
than 7-fold increases in gene expression in TOV112D18
and TOV21G18 hybrid cell lines, respectively, compared with
parental cancer cell lines, suggesting they behave like
tumour suppressor genes.

RBBP8 is of particular interest because it is located on
chromosome 18 (18q11.2–q12), which was the chromosome
transferred by MMCT. Fluorescence in situ hybridization
analysis suggested that only a partial copy of human chromo-
some 18 had been transferred into TOV112D cells (Fig. 3).
This was confirmed using a combination of microarray com-
parative genomic hybridization (aCGH) and microsatellite
analysis. These data indicate that the partial chromosome
transfer comprised a 10 Mb region spanning 18p11.21–
18q11.2. Analysis of several databases indicates that this
region contains 26 coding genes, one of which is RBBP8.
We compared the expression of RBBP8 in TOV112D18
hybrids with the expression of 13 other genes in this region
for which data were available. RBBP8 was substantially over-
expressed in TOV112D18 hybrids relative to any other gene
in the region. For 10 genes, we saw an increase in expression
in hybrid lines ranging from 0.63- to 2.14-fold, which is equiv-
alent to an increased expression resulting from a single copy
transfer. For two other genes, RIOK3 and CAYBR, we saw a
4.86- and 3.32-fold increase in expression, respectively.
Together, these data suggest that RIOK3, CAYBR and
RBBP8 are activated, but that RBBP8 is the primary target.

We looked for evidence that CASP5 and RBBP8 are
involved in the development of primary ovarian cancers. We
genotyped three SNPs located in RBBP8 and seven SNPs in
CASP5 in germline DNA and tumour tissues from 314 inva-
sive ovarian cancers, to look for gene deletions measured as
loss of heterozygosity (LOH). For RBBP8, 101 cases were
informative for at least one SNP. The frequency of LOH at
the RBBP8 locus was 34%. For CASP5, 238 cases were
informative and LOH was detected in 134 (56%) tumours. We
evaluated survival in cases showing deletions of CASP5 or
RBBP8 in their primary tumour. Patients with deletions at
the CASP5 or RBBP8 loci had significantly worse survival
than cases without deletions [HR = 1.41 (95% CI: 1.02–
1.96), P = 0.040 and HR = 2.19 (95% CI: 1.36–3.54), P =
0.001, respectively] (Fig. 2).

DISCUSSION
We have used a functional approach to identify candidate
genes from an in vitro model of ovarian cancer suppression
that may be involved in the development of epithelial
ovarian cancers, and evaluated whether common germline
genetic variation within these genes is associated with survival
after diagnosis of the disease. The goal was to use a more reliable strategy for gene identification compared with previous studies, which have mainly focussed on specific pathways and/or genes for which ‘true’ evidence of function in ovarian cancer is limited (6–9).

Of the nine genes identified, we found statistically significant evidence of an association with survival at the 5% level for three tSNPs in two genes, CASP5 and RBBP8. Neither of these genes has previously been implicated directly in ovarian cancer development. The association for rs518604 in CASP5 was marginal and was no longer significant after adjusting for multiple prognostic factors, and so this may be a false-positive finding. The associations for rs4474794 and rs9304261 in RBBP8 were stronger and remained significant after adjusting for age, stage and grade. However, the results need to be taken with caution as the survival results were not corrected for multiple testing.

Both rs4474794 and rs9304261 are located in intronic and flanking regions of RBBP8 and are tagged with an $r^2 = 0.56$. The known or predicted functions of these tSNPs and additional SNPs they tag were assessed with Pupasuite (17, 18). A SNP, rs930910, which is tagged by rs9304261 ($r^2 = 0.89$), is upstream of RBBP8 and is predicted to be a transcription factor binding site. We also found an additional seven SNPs tagged by rs4474794 and five SNPs tagged by rs9304261 that are conserved in mice, perhaps suggesting a level of functional importance. However, with the available information, we were unable to find compelling reasons why any of these SNPs should influence survival. Re-sequencing and fine-mapping of this region is probably required to identify the true causal variant.

No associations were found between survival and tSNPs in the remaining seven genes: AIFM2, AKTIP, AXIN2, FILIP1L, RGC32, RUVBL1 and STAG3. However, we cannot rule out that one or more common variants in these genes is associated with patient survival; even though the tSNPs we studied were selected to adequately tag the common variation in each gene, it remains possible that unidentified variants were not efficiently tagged. In addition, some common variants would have been poorly tagged because some tSNPs failed assay design and were not genotyped. Finally, these genes might harbour rare variants that influence survival; but these would not have been identified or genotyped as part of this study.

The design of this study has its limitations. To date, this is the largest study investigating germline genetic variation and survival in ovarian cancer; however, likely effect sizes for common genetic variants are small—most cancer susceptibility alleles so far identified confer relative risks <1.2 and power is limited to detect such alleles at the highly stringent levels of significance needed where the prior probability of association is low. This study was not large enough to identify associations with stringent levels of statistical significance and neither SNP was significant at the $P < 0.00001$ level, which has been suggested as the threshold for candidate gene studies (18). In addition, neither SNP is likely to be significant at a nominal $P < 0.05$ using simple corrections for multiple testing (e.g. Bonferroni); but it is unclear that such adjustments are appropriate because they do not take into account the correlation that exists between SNPs that tag the genetic variation across gene regions. Unfortunately, we were unable to evaluate associations between germline genetic variants and other clinical features of disease, such as chemotherapeutic response and resistance and disease recurrence. These were epidemiological collections and not clinical trial cohorts, and so the data for additional clinical variables were incomplete; thus, we had insufficient statistical power to identify associations with other clinical variables. The use of prevalent cases may be seen as a weakness; but as long as the left truncation is taken into account in the analysis (as we did here) their inclusion does not result in a bias of the hazard ratio estimates provided the proportional hazards assumption holds. Even if the proportional assumption is violated, their test of association remains valid. For a given sample size and mean duration of follow-up, inclusion of prevalent cases reduces power, as the number of events will be smaller. However, in this analysis, inclusion of the prevalent cases increases sample size and the number of events, and so provides greater power than would be achieved if the analysis was restricted to incident cases only. Disease heterogeneity may also be important and many genetic variants associated with prognosis may only be important in specific subtypes. Unfortunately, the power to detect subgroup-specific effects is even lower and some variants are likely to have general effects that are subgroup independent. Another issue is the fact that we analysed all-cause mortality, rather than only mortality from ovarian cancer. Some women will die from causes other than ovarian cancer, but this misclassification is likely to be small as most of the women in the age group will die from their cancer.

The CASP5 and RBBP8 genes were both substantially over-expressed in MMCT-18 hybrids compared with parental ovarian cancer cell lines. This suggests that activation of these genes is associated with suppression of the neoplastic phenotype, which in turn indicates that they behave like tumour suppressor genes. The data for RBBP8 were particularly compelling. The gene was one of only a handful of genes in a 10 Mb region that were introduced into an ovarian cancer cell line by MMCT; and RBBP8 was more highly expressed than any other gene in this region. LOH analysis in 314 ovarian tumours provides support for this. Allelic deletion is one of the common mechanisms by which tumour suppressor genes are inactivated. CASP5 and RBBP8 were deleted in 56 and 35% of invasive tumours, respectively, both of which represent common somatic alterations in ovarian cancer (reviewed in 19).

Deletions of the CASP5 gene, and particularly the RBBP8 gene, were associated with a worse survival in ovarian cancer cases. These data need to be treated with some caution; the result for CASP5 was marginal and the sample size in both cases was too small to enable adjustment for other prognostic factors. There may also be other mechanisms of gene inactivation that we have not screened for (e.g. methylation), which might influence these findings. To the best of our knowledge, this study represents the first report suggesting the involvement of either gene in the somatic development of ovarian cancers or associations between somatic alterations in these genes and survival.

The known function of RBBP8 lends support to the hypothesis that the gene behaves as a tumour suppressor and can influence patient response to therapy and therefore survival. RBBP8 (alternative nomenclature CTIP) was initially identified...
because of its interaction with the retinoblastoma protein (20). RBBP8/CTIP has also been shown to interact in vivo with the BRCA1 protein C-terminal region (BRCT) repeat domains of the BRCA1 gene; this interaction is ablated by truncating BRCA1 mutations (21). Li et al. (22) have shown that RBBP8/CTIP and BRCA1 dissociate from each other following exposure to ionizing radiation and that this is mediated through the action of the ataxia telangiectasia (ATM) protein that phosphorylates RBBP8/CTIP to regulate DNA damage response. More recently, Wu et al. have shown that RBBP8/CTIP is overexpressed in most oestrogen receptor alpha (ER) positive but not ER negative breast cancer cell lines and that expression status is associated with patient response to neoadjuvant endocrine therapy in primary breast cancers. Wu et al. (23) also found that silencing RBBP8/CTIP conferred tamoxifen resistance in breast cancer cells.

In summary, we have identified a gene RBBP8 that is activated in a functional model of neoplastic suppression in ovarian cancer cell lines. Common germline genetic variants in this gene and somatic gene deletions in primary tumours were independently associated with survival after a diagnosis of ovarian cancer. Previous studies in breast cancer suggest that the RBBP8 protein interacts with the BRCA1 and retinoblastoma proteins, and is associated with Tamoxifen resistance, suggesting a plausible link between its function and survival in ovarian cancer.

MATERIALS AND METHODS

Identifying candidate genes using MMCT

MMCT and phenotype analysis. MMCT of normal human chromosomes was achieved after polyethylene glycol fusion between the epithelial ovarian cancer cell lines TOV112D and TOV21G (24), and mouse (A9): human monochromosome hybrid donor cell lines carrying a selectable fusion gene marker, hygromycin phosphotransferase, as previously described (13). MMCT hybrid lines were tested for their in vitro, tumourigenic phenotype by assaying anchorage-dependent and -independent growth and invasion through a matrigel as previously described (13,26). More detailed methodological information relating to MMCT and the in vitro phenotype analyses are provided in supplementary information.

In vivo tumourigenicity for TOV21G and TOV112D cell lines and two MMCT-18 hybrids generated from each line was evaluated after intraperitoneal injection into immunocompromised mice xenografts (nu/nu). Two animals were sacrificed at 3-, 6-, 9- and 12-week time points, and detailed post-mortem examinations carried out to evaluate tumour burden (25).

Differential gene expression analysis. Expression microarray analysis was performed for RNA isolated from each parental ovarian cancer cell line and two MMCT chromosome 18 hybrids generated from each line. The microarray (Applied Biosystems version 2) contained 32,878 probes for the interrogation of 29,098 genes. All samples were assayed in triplicate. Primary analysis was performed using ArrayExpress software (Applied Biosystems). Microarray data were analysed using Spotfire DecisionSite™ for functional genomics (Spotfire AB, Goteborg, Sweden) and R version 1.9.1. Arrays were normalized, and genes were excluded if the signal-to-noise ratio was <3. An ANOVA test was used to generate P-values for statistical differences between groups. P-values were adjusted for multiple comparisons using the technique described by Benjamini and Yekutieli (27). Genes were considered statistically different between groups if they had an adjusted P < 0.01 and an average fold-change difference of greater than 1.6. Gene ontology analysis was performed with the Panther classification system (http://www.pantherdb.org).

Effects of common genetic variants in candidate genes and survival in ovarian cancer

Study subjects. The 1694 individuals included in this study were confirmed cases of invasive epithelial ovarian cancer from three different case–control studies from Denmark and the UK. These studies were: (i) The Danish MALOVA study (446 cases); (ii) The UK SEARCH study (847 cases) and (iii) The UK ovarian cancer population study (UKOPS) (401 cases). All women were of white ethnic origin. Epidemiological and clinical data for MALOVA and SEARCH cases have been published previously (8) and are summarized in Table 1. The UKOPS study contains incident cases aged between 35 and 86 years from 10 gynaecological oncology National Health Service centres throughout the UK. A small subset of the UKOPS samples were from the UKCTOCS screening trial. The most current follow-up for these samples occurred in June 2008 for the UKCTOCS samples, and in August 2008 for the remaining samples. All study participants were followed up by a ‘flagging study’ for cancer registrations and deaths by the NHS Information System for Health and Social Care (formerly the Office of National Statistics—ONS) for England and Wales. Women who had not consented to their details being sent to ONS were not flagged. Local Ethics Committee approval was given for the collections and genotyping in all individuals.

Gene and tagging SNP selection. Candidate genes were selected because they were differentially expressed between MMCT-18 hybrids and the parental ovarian cancer cell lines. Genes were ranked according to P-value (P ≤ 0.05) and also expression fold-change. The top 30 ranking genes were selected for further analysis. SNP tagging was performed as previously described (8) using HapMap data release 22/phase II, April 2007, on NCBI build 36 assembly, dbSNP b126 (www.hapmap.org). Candidates were chosen for further analysis if: (i) the function indicated a putative role in cancer; (ii) there was at least one common SNP for every 2 kb (adequate tagging of the gene) and (iii) there were at least 3 but no more than 20 common tagging SNPs (tSNPs) (scale of the multiplex). The TagSNPs programme
was used to estimate haplotype dosages as previously described (8).

**Genotyping.** A combination of iPLEX® Gold (Sequenom Inc.) and TaqMan® ABI® 7900HT Sequence Detection System (Applied Biosystems) was used to genotype the samples as previously described (28,29). If we were unable to genotype a tSNP, a different tSNP in LD with \( r^2 > 0.8 \) was genotyped instead. Sample plates with poor call rates (<90%) were excluded from analysis. For quality control, duplicate samples and no template controls were included in genotyping studies. For LOH analysis, DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumour tissues from the Danish MALOVA study was genotyped with iPLEX® Gold. The regions that were microdissected from the tumour tissues contained >80% neoplastic cells (30). To establish the presence of LOH, the peak heights of the alleles in germline and tumour DNA were compared and a ratio of \(<0.6\) or \(>1.67\) was used to indicate LOH (30). Genotyping was performed in two stages: (i) a test set of 181 invasive ovarian tumours and (ii) a validation set of 228 tumours, which included 95 tumours from stage (i). We applied a staged design to test the reproducibility of LOH analysis performed in FFPE tissues, rather than a validation of the LOH frequencies from the first stage. Where we identified LOH in the first set of tumour tissues, we observed 98% LOH concordance in the same 95 tumours that were included in stage (ii). This indicates that LOH genotyping was very accurate.

**Statistical methods.** Cox regression analysis, stratified by study, was used to assess the effect of each tSNP on all-cause mortality from invasive epithelial ovarian cancer. Follow-up was censored at the date of death from any cause, the date a participant was last known to be alive or at 10 years after diagnosis if the participant was still alive. There was a variable time between diagnosis and patient recruitment, thus left truncation was allowed for in the analysis. Appropriate allowance for left truncation provides an unbiased estimate of the relative hazard, provided that the proportional hazard assumption is not violated. Log–log survival curves were used to ensure that the assumptions of proportional hazards were met. The primary tests of association were likelihood ratio tests for trend (1 degree of freedom), based on the number of rare alleles carried. We used Cox regression analysis, stratified by study, to estimate the HR per rare allele carried. HRs were adjusted for the prognostic factors: age at diagnosis (year), tumour stage [Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stages I, II, III and IV], tumour grade (high, moderate and low) and histological subtype (serous, mucinous, endometrioid and clear cell) for each tSNP that was significantly associated with survival \( (P < 0.05) \). Samples with missing clinical data were excluded from this multivariate analysis. Disease heterogeneity may influence our findings. Therefore, we stratified cases by histological subtype and repeated the analyses for serous ovarian cancers only; sample sizes were too small to perform this analysis for other subtypes. Although tSNPs may not be associated with survival, a haplotype combining two or more tSNPs may show an association. The haplotype definitions, dosage estimations and analyses are as described in (8).

There are two haplotype blocks in **AIFM2**, **AXIN2**, **CASP5** and **FILIP1L**; the remaining genes had one haplotype block each. Cox regression analysis was used to establish whether there were associations between subject-specific expected haplotype indicators and ovarian cancer mortality. Cox regression analysis was also used to evaluate associations between the effect of LOH in **CASP5** and **RBBP8** in 314 tumour tissues from the MALOVA study and survival. STATA version 8.2 was used for all statistic analyses and to produce Kaplan–Meier plots of survival estimates. All reported \( P \)-values are two-sided.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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