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Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer

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Abstract

Common germline genetic variation in the population is associated with susceptibility to epithelial ovarian cancer. Microcell-mediated chromosome transfer and expression microarray analysis identified nine genes associated with functional suppression of tumorogenicity in ovarian cancer cell lines; *AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1* and *STAG3*. Sixty-three tagging single nucleotide polymorphisms (tSNPs) in these genes were genotyped in 1,799 invasive ovarian cancer cases and 3,045 controls to look for associations with disease risk. Two SNPs in *RUVBL1*, rs13063604 and rs7650365, were associated with increased risk of serous ovarian cancer [HetOR = 1.42 (1.15–1.74) and the HomOR = 1.63 (1.10–1.42), *p*-trend = 0.0002] and [HetOR = 0.97 (0.80–1.17), HomOR = 0.74 (0.58–0.93), *p*-trend = 0.009], respectively. We genotyped rs13063604 and rs7650365 in an additional 4,590 cases and 6,031 controls from ten sites from the United States, Europe and Australia; however, neither SNP was significant in Stage 2. We also evaluated the potential role of tSNPs in these nine genes in ovarian cancer development by testing for allele-specific loss of heterozygosity (LOH) in 286 primary ovarian tumours. We found frequent LOH for tSNPs in *AXIN2, AKTIP* and *RGC32* (64, 46 and 34%, respectively) and one SNP, rs1637001, in *STAG3* showed significant allele-specific LOH with loss of the common allele in 94% of informative tumours ($p = 0.015$). Array comparative genomic hybridisation indicated that this nonrandom allelic imbalance was due to amplification of the rare allele. In conclusion, we show evidence for the involvement of a common allele of *STAG3* in the development of epithelial ovarian cancer.

Keywords

risk of ovarian cancer; polymorphism; association studies

BRCA1 and *BRCA2* mutations are responsible for the majority of families containing more than two cases of ovarian cancer.1-3 However, *BRCA1* and *BRCA2* mutation carriers are rare in the population, and these genes are thought to explain <40% of familial ovarian cancer risk.4 It has been proposed that low-penetrance susceptibility alleles that are more common in the population contribute to a significant fraction of the excess familial risk.5

The most widely used study design for identifying common low-penetrance susceptibility alleles for disease is the genetic association study, in which the frequency of single nucleotide polymorphisms (SNPs) is compared between individuals with the disease and unaffected controls. Studies have used either a candidate gene approach, in which SNPs in genes hypothesised to have a functional role in disease development are analysed for their disease association, or a genome wide association study (GWAS) design, which is an empirical approach that evaluates hundreds of thousands of SNPs distributed throughout the genome without any *a priori* functional role in the disease being studied.

During the last 3 years, there have been numerous reports describing common SNPs conferring susceptibility to several common diseases, including several cancers (reviewed in Refs. 6,7). Most published genetic association studies for ovarian cancer have used a candidate gene approach with genes selected from pathways including steroid hormone metabolism, DNA repair and cell cycle control, as well as known oncogenes and tumour

suppressor genes.8-13 Many of the studies reporting statistically significant associations were performed using small sample sizes. Recently, a multicentre international consortium [Ovarian Cancer Association Consortium (OCAC)] has enabled replication analysis of many of these initial findings in samples sizes of up to 9,000 ovarian cancer cases and 11,500 controls. These studies have shown that the majority of genetic associations so far reported are likely to be either weak effects or false-positive associations.14,15

One possible explanation for the failure of candidate gene studies to identify true genetic associations could be that the strategies used for candidate gene selection are inadequate. Often, gene selection is based on predicted rather than a known role for genes in ovarian cancer development; selecting genes for which there is experimentally demonstrable evidence of functional involvement in ovarian cancer may prove a more successful strategy for gene selection. For example, a recently published study in which an *in vitro* model of ovarian cancer suppression was used to identify genes that might be associated with ovarian cancer prognosis, identified common genetic variants in a gene (*RBBP8*) that showed evidence of association with ovarian cancer survival.16

Few genetic association studies have provided a functional rationale for the susceptibility variants that have been identified. There are several possible reasons for this. One reason is that confirmed susceptibility SNPs are rarely nonsynonymous coding variants within genes. The associated SNP may not be the disease-causing variant but instead is probably in linkage disequilibrium with the true causal variant. A substantial proportion of disease associated SNPs are not located within or near known genes suggesting that noncoding DNA variation may impart functional effects; however, our understanding of the biological function of noncoding DNA is rudimentary, making functional analysis of these susceptibility alleles challenging.

In our study, we have evaluated the effects on ovarian cancer risk of common variants in nine candidate susceptibility genes (*AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1* and *STAG3*) identified after performing microcell-mediated chromosome transfer (MMCT) of chromosome 18 into ovarian cancer cell lines16,17 as described below. These genes were first genotyped in 1,799 invasive ovarian cancer cases and 3,045 unaffected controls from three studies; significant associations were genotyped in an additional 4,590 cases and 6,031 controls from ten studies that are part of the OCAC, resulting in a total of 6,389 invasive ovarian cancer cases and 9,076 controls from 13 studies. We also tested the functional effects of SNPs in these candidate genes in primary ovarian tumours by evaluating allele-specific loss of heterozygosity (LOH) for nondisease associated alleles and the frequency of somatic alterations at putative susceptibility loci using array comparative genomic hybridisation (aCGH) analysis.

Material and Methods

Identifying functional candidate genes using MMCT

MMCT of normal human Chromosome 18 was achieved by polyethylene glycol fusion of the epithelial ovarian cancer cell lines TOV112D and TOV21G (American Tissue Culture Collection, LGC Standards, Middlesex, United Kingdom)18 and mouse (A9): human monochromosome hybrid donor cell lines carrying a selectable fusion gene marker, hygromycin phosphotransferase.17 *In vitro* phenotypic analysis was performed by assaying anchorage independent growth in soft agar and invasion through matrigel as described previously.17 For MMCT hybrids displaying significant neoplastic suppression, a combination of cytogenetic analysis, DNA microarray analysis and microsatellite genotyping confirmed the uptake of a complete or partial human chromosome 18 in MMCT hybrids. Expression microarray analysis was performed on the parental cell lines and four

chromosome 18 MMCT hybrids, two generated from each of the parental cell lines as described previously.16 All samples were performed in triplicate. The microarray (Applied Biosystems version 2) contained 32,878 probes for the interrogation of 29,098 genes. An analysis of variance test was used to generate *p* values for statistical differences between groups. The *p* values were adjusted for multiple comparisons.19

Candidate gene selection was based on genes that showed significant differential expression between hybrid and parental cell lines.16 Lists of genes that were up or down regulated in hybrids from TOV21G, TOV112D, or both cancer cell lines were generated. The top 30 ranked genes in each list, based on *p* value and expression fold change, were compiled into a single master list. The functions of these genes were obtained from Gene Cards (http://www.genecards.org) and NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez). Tagged SNPs for each gene were identified from HapMap data release 22/phase II, April 2007, including putative regulatory regions up and down stream of each gene (within 5kb). Common SNPs (minor allele frequency ≥ 0.05) from each gene with a minimum correlation coefficient (r2) of 0.8 were selected and tagged with Haploview and coworkers20 and Tagger21 using the aggressive tagging option. Candidate genes were selected if the function was known or predicted and had a plausible role in cancer; if there was at least a common SNP (MAF ≥ 0.05) for every 2 kb of the gene; and if the number of tSNPs for a gene was between 3 and 20 tSNPs (to be sufficiently tagged and fit into an iplex multiplex). Nine genes *AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1* and *STAG3* were selected for genotyping from which there were 68 tagging SNPs (tSNPs).

Study individuals

In the first stage of our study, we genotyped 1,799 invasive ovarian cancer cases and 3,045 unaffected controls from three populations; MALOVA, Denmark (446 cases; 1,221 controls) and two UK studies; SEARCH (847 cases; 1,229 controls) and UKOPS (506 cases; 595 controls). Of the 1,799 cases, 849 were serous histology; 279 endometrioid; 196 mucinous; 166 clear cell and 309 with mixed/other histology or undifferentiated.

In the second stage, two putative positive associations were followed up in ten additional case control studies as follows: (1) USC, USA (391 cases; 546 controls); (2) DOV, USA (530 cases; 716 controls); (3) HOP, USA (280 case; 603 controls); (4) GEO, USA (327 cases; 429 controls); (5) NCO, USA (622; 747 controls); (6) HAW, USA (70 cases; 158 controls) (7) POC, Poland (456 cases; 460 controls); (8) BAV, Germany (228 cases; 234 controls); (9) GER, Germany (218 cases; 416 controls) and (10) AUS, Australia (768 cases; 1,122 controls). An additional 553 cases and 467 controls from UKOPS and SEARCH called UKO (B) were also included in Stage 2. A total of 4,590 cases and 6,031 controls were genotyped in Stage 2. Only the nonHispanic White individuals from these studies were included in the analysis to avoid heterogeneity due to ethnicity. Details for several of these studies have been published previously15,22 and are summarised in Supporting Information Table 1. Local ethics committee approval was given for the collections and genotyping in all individuals.

SNP genotyping

Of the 68 tSNPs, three SNPs (*AIFM2* rs2271695, *AXIN2* rs4128941 and rs2240308) failed manufacture and could not be efficiently tagged by any other SNP. Therefore, 65 tSNPs were genotyped in Stage 1 using a combination of iPLEX Gold (Sequenom Inc., Hamburg, Germany) and TaqMan ABI 7900HT Sequence Detection System (Applied Biosystems, Cheshire, United Kingdom) as previously described.12 Two iPlex multiplex experiments were designed to analyse 50 SNPs, and the remaining 13 SNPs were analysed by Taqman (Table 1). TaqMan was used to genotype the two SNPs in the Stage 2 studies, apart from the

AUS samples which were genotyped for rs7650365 by iplex. All genotyping included duplicate samples and no DNA template controls for quality control purposes as described previously.15 DNA plates with <90% call rates and studies with <95% call rates were excluded. Studies deviating from Hardy-Weinberg equilibrium (HWE) $p < 0.05$ in controls were excluded. This is reflected in the variable numbers of cases and/or controls that were successfully genotyped for each tSNP. Two SNPs (*RGC32* rs3783197 and *RUVBL1* rs13091198) failed on genotyping quality control criteria.

Loss of heterozygosity analysis

DNA was extracted from 286 formalin-fixed paraffin-embedded (FFPE) tumour tissues from the Danish MALOVA study. Areas with >80% tumour cells were extracted using a Proteinase K DNA extraction method as previously described.23 Tumour DNA and matching germline DNA samples were genotyped for 50 tSNPs by iPlex. Additional quality control criteria were applied to genotypes in tumour tissues; FFPE samples that passed for <80% of the assays were excluded. SNPs with pass rates of <90% for the genomic DNA samples were excluded. After quality control, LOH data were available for 37 tSNPs.

The ratio of the allele peak heights between the tumour and the germline DNA for heterozygous individuals was used to determine LOH as described previously.23 $L = (at_2 \times an_1)/(at_1 \times an_2)$, where at_1,at_2 and an_1,an_2 are the peak heights of the two alleles of the tumour and the germline DNA, respectively. A value of $L < 0.6$ and $L > 1.67$ was considered LOH.24

Genotyping of 95 of the tumour samples was repeated to confirm that the peak height ratios were reproducible. If a reaction showed LOH in one experiment and then there was a decrease in peak height for the same allele in the other experiment with peak height ratios close to the LOH limit values (<0.8 or >1.4), they were classed as concordant. If the concordance of LOH between the duplicate samples was <85%, the assay was excluded from the analysis. For duplicate samples that were concordant, the log average of the L value was used. If discordant both results were removed.

The calculated frequency of overall LOH for a specific gene was based on the combined analysis of multiple tSNPs within the gene. LOH was recorded if any informative SNP in a gene showed LOH, even if other informative tSNPs did not show LOH. Tumour DNA samples from 12 cases were also analysed by array CGH analysis as previously described.17

Statistical methods

Deviation from HWE was assessed in controls within study populations using standard χ^2 test. Unconditional logistic regression was used to assess the association between each tSNP and risk of ovarian cancer for each study and pooled across studies (stratified by study) with the primary test of association being a test for trend (p-trend) as described previously.12 A log-additive co-dominant model was used, and odds ratios for the heterozygote and rare homozygote relative to the common homozygote were estimated by stratified logistic regression. The programme TagSNPs25 was used to model the relevant multi-marker haplotypes resulting from aggressive SNP tagging. Analysis to test for association with the variants was first performed on all invasive cases and then restricted to the serous histological subtype as this was the most frequent subtype. There were insufficient numbers of the other subtypes for analysis.

We also conducted analysis to determine if haplotype effects were present as described previously.12 Haplotype blocks were defined using the confidence interval option of Haploview and coworkers,20 with minor adjustments to include adjacent SNPs but maintaining the cumulative frequency of the common haplotypes to [mt 90%. *AKTIP,*

RBBP8, RGC32, RUVBL1 and *STAG3* had one haplotype block. The other genes (*AIFM2, AXIN2, CASP5* and *FILIP1L*) had two haplotype blocks. Unconditional logistic regression was used to test the association between each haplotype relative to the most common haplotype.25-27 Haplotypes that occurred with a frequency of 2% or greater in the combined data were considered "common", and those with <2% frequency were pooled as rare haplotypes. To assess the existence of allele-specific LOH, the Fishers exact two tailed test was used to determine if there was a significant deviation from random LOH.

Results

Evaluating ovarian cancer risk associated with common variants in nine candidate genes

We have previously shown that microcell-mediated transfer of normal human chromosome 18 in ovarian cancer cell lines induces functional suppression of the neoplastic phenotype *in vitro* and *in vivo*.17 Subsequently, gene expression microarray analysis was used to compare the transcriptome of parental cancer cell lines with Chromosome 18 cell line hybrids; this identified nine candidate genes (*AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1* and *STAG3*) that were differentially expressed in chromosome 18 hybrids suggesting they are associated with neoplastic suppression in this model (Table 1).

We selected tagging SNPs (tSNPs) with minor allele frequencies (MAF) ≥ 0.05 in each of these genes and evaluated their association with ovarian cancer risk after genotyping up to 1,799 invasive epithelial ovarian cancers and 3,045 healthy controls from three different population-based ovarian cancer case control studies. Sixty-eight tSNPs were identified in these genes but five could not be genotyped because they failed manufacture or quality control, and therefore, data were available for 63 tSNPs. We found evidence of risk association for tSNPs in *AXIN2, CASP5* and *RUVBL1* when the data for all histological subtypes were combined. The rs11079571 in *AXIN2* showed evidence of an increased ovarian cancer risk [heterozygous odds ratio (HetOR) with 95% confidence intervals (CI) 1.23 (1.00–1.51); homozygous OR (HomOR) = 1.73 (0.99–3.01), *p*-trend = 0.038]; the minor alleles of rs518604 in *CASP5* and rs13063604 in *RUVBL1* were also associated with an increased risk [HetOR = 1.39 (1.06–1.81), HomOR = 1.44(1.05–1.97), *p*-trend = 0.0124; and HetOR = 1.14 (0.97–1.34), HomOR = 1.39 [1.02–1.89], *p*-trend = 0.0192, respectively). We found no evidence of association for any tSNPs in the *AIFM2, AKTIP, FILIP1L, RBBP8, RGC32* and *STAG3* genes. These data are summarised in Table 2 and Supporting Information Tables 2 and 3.

When the analysis of these 63 SNPs was restricted to serous ovarian cancer cases, the most common histological subtype, additional ovarian cancer risk associations were identified (Table 2). These included rs13063604 and rs7650365 in *RUVBL1* [HetOR = 1.42 (1.15–1.74) HomOR = 1.63 (1.10–1.42), *p*-trend = 0.0002; and HetOR = 0.97 (0.80–1.17), HomOR = 0.74 (0.58–0.93) *p*-trend = 0.009); rs518604 in *CASP5* [HetOR = 1.36 (0.98–1.88), HomOR = 1.45 (0.99–2.11), *p*-trend = 0.031]; rs4474794 in *RBBP8* [HetOR = 0.83 (0.70–0.98), HomOR = 0.80 (0.63–1.03), *p*-trend = 0.032]; and rs1637001 in *STAG3* [HetOR = 0.84 (0.71–0.99), HomOR = 0.77 (0.56–1.05), *p*-trend = 0.018].

The two most significant of these associations (rs13063604 and rs7650365 in *RUVBL1*) were further investigated after genotyping up to 4,437 additional cases (of which 2,534 were serous) and 5,885 controls from the OCAC. The rs13063604 was genotyped in fewer samples as a second batch of Taqman assay failed manufacture. Neither tSNP was significant in the additional genotyping data alone, and rs7650365 was not significant in a combined analysis (Table 3). However, for rs13063604, the association remained significant in the combined analysis [HetOR = 1.13 (1.00–1.27), HomOR =1.22 (0.96–1.56), *p*-trend = 0.019; Supporting Information Figures 1 and 2]. These results appear to be driven by the

Stage 1 data. In a combined analysis for all histological subtypes, the association for rs13063604 was also marginally significant [HetOR = 1.08 (0.98–1.19), HomOR =1.19 (0.98–1.45), *p*-trend = 0.033].

From the Stage 1 data, we also found evidence of association with ovarian cancer risk for four haplotypes (Supporting Information Table 4). Haplotypes h0011 and h1111 in *AXIN2* Block 2 were associated with decreased and increased risk respectively [OR = 0.57 (0.34–0.95) $p = 0.031$; and OR = 1.21 (1.03–1.42) $p = 0.023$]. This is consistent with the presence or absence of the rare allele of rs11079571 in the second position of the block that was shown to have an increased risk (described above). Haplotypes h000 and h100 in Block 1 of *CASP5* were associated with a decreased risk [OR = 0.72 (0.56–0.94) $p = 0.015$], and increased risk [OR = 1.13 (1.03–1.24) $p = 0.012$], respectively. These haplotypes contain the common and minor allele, respectively, of rs518604 in the first position, which was associated with an increased risk in the analysis described earlier. A global haplotype analysis showed that *CASP5* block 1 and *RUVBL1* were significant ($p = 8.4 \times 10^{-6}$ and $p = 0.0016$, respectively).

Evaluating a functional role for SNPs and candidate genes in primary ovarian tumours

We simultaneously evaluated whether or not the candidate genes and/or tSNPs described above were involved in the somatic genetic development of primary ovarian cancers by studying loss of heterozygosity (LOH) in 286 tumours for which germline genotype data were also available. We hypothesised that low-penetrance susceptibility genes behave like tumour suppressor genes according to Knudson's hypothesis28 and undergo nonrandom loss of the wild-type (nonrisk) allele during tumour development.

Genotyping was performed concurrently in germline and matching tumour DNA for 50 tSNPs from the nine genes performed by iPlex; 37 tSNPs passed the quality control criteria we established for genotyping of FFPE tumour samples (Table 4; Supporting Information Table 5). We observed frequent LOH for tSNPs in the *AXIN2, AKTIP* and *RGC32* (64, 46 and 34% LOH, respectively; Table 4) suggesting a role for these genes (or regions) in ovarian tumour development. We found evidence of allele-specific LOH for one SNP, rs1637001 in *STAG3*; in 16 tumours informative for LOH for this tSNP, the common A allele showed significant preferential loss over the G allele in 94% of cases ($p = 0.015$; Figure 1).

We then used array comparative genomic hydridisation (aCGH) analysis of ovarian tumours to establish whether the allelic imbalances we observed for rs1637001 in *STAG3* were the result of deletion of the common A allele or amplification of the rare G allele. The aCGH was performed for 12 ovarian tumours that showed allelic imbalance for rs1637001. Nine of these tumours showed copy number gain for *STAG3*, and three tumours were copy number neutral (Figure 2); none of the tumours showed deletion of *STAG3*. These data suggest that, for the majority of tumours, there was amplification of the rare G allele rather than deletion of the common A allele. For tumours that were copy number neutral for *STAG3*, it is possible that the somatic alterations that were revealed by LOH analysis were not detected by aCGH due to subsequent amplification of the remaining allele.

Discussion

We have used a functional approach to identify candidate genes from an *in vitro* model of ovarian cancer suppression, and evaluated whether common germline genetic variation for 63 SNPs in these genes is associated with low-penetrance susceptibility to the disease. Of the nine genes identified, we found statistically significant evidence of association with disease risk at the 5% level for three SNPs (in genes *AXIN1, CASP5* and *RUVBL1*) when

all ovarian cancer histological subtypes were analysed, and for an additional three SNPs (in genes *RUVLB1, RBBP8* and *STAG3*) when the analysis was restricted to the serous subtype.

The strongest associations identified were for two SNPs in *RUVLB1*. However, these associations may still be false positives; more stringent significance levels are required to ensure that an identified association is a true positive. It has been proposed that the significance level of candidate gene studies should be $p < 10^{-4}$.29 Therefore, we further investigated the associations for rs13063604 and rs7650365 in *RUVBL1* as part of an international multicentre case control study by the OCAC. This analysis failed to replicate independently the association for either of these SNPs, although the combined analysis of all genotyping remained marginally significant for rs13063604. This variant is located in Intron 9 of the gene and does not appear to be functionally significant *per se*; however, it tags nine other SNPs in the gene, two of which are located in the 3′ untranslated region. Using the bioinformatics tool Pupasuite (http://pupasuite.bioinfo.cipf.es/), none of these SNPs were predicted to be potentially functionally significant. There is evidence to suggest that *RUVBL1* plays an important role in the development of ovarian cancer and other cancer types; *RUVBL1* expression is elevated in ovarian, breast, colon, bladder and lung cancers [Oncomine(Compendia Bioscience, Ann Arbor, MI)].

The data for rs13063604 and rs7650365 in *RUVBL1* were stronger in serous ovarian cancer cases than in all histological subtypes combined. If different germline genetic variants confer susceptibility to different disease subtypes, then disease heterogeneity may be one of the reasons for a lack of success in identifying genetic susceptibility alleles for ovarian cancer. There is increasing evidence from high-penetrance gene studies that the underlying genetic basis of ovarian cancer can contribute to disease heterogeneity. For example, *BRCA1* and *BRCA2* appear to confer susceptibility mainly to the development of the serous subtype.30 Some studies have also suggested that disease heterogeneity can be influenced by common alleles of low penetrance. Studies by the OCAC that are sufficiently large to enable stratification by subtype have identified genetic variants that may be associated with specific subtypes of the disease.22,31

From the data presented, we cannot rule out the possibility that risk associations exist for other germline genetic variants in these genes (*AIFM2, AKTIP, AXIN2, CASP5, FILIP1L, RBBP8, RGC32, RUVBL1* and *STAG3*). The combined sample size from the three studies provides 98% power at the 5% significance level to detect a co-dominant allele with a frequency of 0.3 that confers a relative risk of 1.2, and 95% power to detect a dominant allele with a frequency of 0.1 that confers a relative risk of 1.3. Of the 303 common variants, 288 were tagged with r2 ≥ 0.8 and 290 with r2 ≥ 0.5; however, some unidentified common as yet SNPs may not have been tagged efficiently, and we did not evaluate rarer variants with MAFs <0.05. Risk associations for these genes may also exist for some of the rarer histological subtypes of ovarian cancer, but we did not have sufficient statistical power to detect such associations.

During the course of this study, a GWAS for ovarian cancer was completed and published. 22 This GWAS study analysed a partly overlapping set of patients as used in this study, with cases from UKOPS and SEARCH as well as other UK studies. With access to the data from this GWAS (which analysed 1,819 cases and 2,343 controls for 507,094 genotyped SNPs and ~2 million imputed SNPs) we were able to reevaluate all tSNPs identified in these nine genes including the five SNPs that were not analysed; we found no evidence of association with disease risk in the GWAS data for all histologies combined (Supporting Information Table 6). Three SNPs were significant in the serous only analysis; two in *CASP5* rs518604

and rs523104 and one in *FILIP1L* rs12494994. *CASP5* rs518604 is the only SNP significant in both studies, and it is currently being investigated further in additional cases and controls.

One of the features of our study is that candidate genes were selected on the basis of having a possible functional role in an *in vitro* model of ovarian cancer suppression. To our knowledge, none of the nine candidate genes investigated have been previously assessed for their association with ovarian cancer risk. For some of the genes, we identified, there are previously reported evidence of a role in ovarian cancer development, in addition to that described for *RUVBL1*: For example, we have previously shown that germline variants in the *BRCA1* interacting gene *RBBP8* may be associated with survival after a diagnosis of ovarian cancer,16 and another study has shown downregulation of *FILIP1L* in primary ovarian tumours suggesting it may be an ovarian cancer tumour suppressor gene [Oncomine (Compendia Bioscience, Ann Arbor, MI)].

Although numerous common genetic variants have now been shown to be associated with the risks of several cancer types, rarely has any functional rationale been established for their risk association. Therefore, we investigated whether SNPs within all nine genes were somatically altered in primary ovarian cancers. The SNP rs1637001 in the *STAG3* gene shows significant nonrandom allelic imbalance by LOH analysis in tumours. This was shown by array CGH to be the likely result of amplification of the minor G allele; amplification rather than deletion of an allele has previously been suggested as a potential mechanism to explain detected LOH at a SNP.32 The same SNP in this gene also showed evidence of association with disease risk in serous ovarian cancer cases, although this was not confirmed in the imputed data from the GWAS. The potential synergy between a putative risk association for a germline genetic variant and the preferential somatic amplification of one of the alleles during tumour development is intriguing, and further investigation is worthwhile.

Although there are several examples in the published literature of allele-specific imbalance of polymorphic markers in primary tumours (reviewed in Refs. 33-36), synergy between genetic risk alleles and somatic alterations has not been reported before. For example, in one study, analysis of the *DAL1* gene in breast cancer found that 94% of tumours showing LOH retained the C allele of the C2166T SNP; in another study, 73% of lung tumours with LOH involving the *P34* gene retained the G allele of the A106G SNP; and a third study reported 83% of breast tumours with loss of the pro allele of Arg72Pro in the *P53* gene. What is unclear for the rs1637001 variant located in the 3′UTR of *STAG3* is whether or not the preferential allelic amplification targets rs1637001 or *STAG3* specifically. The amplified region detected by aCGH in tumours extended across several genes including *STAG3*; neither is it clear that the amplification is functionally relevant to ovarian cancer development. More detailed *in vitro* cell biology studies will be needed to address this. *STAG3* is a component of the meiosis specific cohesion complex.37,38 It is not expressed in embryonic stem cells that form follicle like ovarian structures, and this is thought to contribute to the inability of those cells to progress through meiosis.39 The gene is activated in lymphoma cells after mutant p53 has been induced by irradiation.40 Other studies have implicated *STAG3* mutations with chromosomal instability in colorectal cancer,41 and the gene is associated with chromosome segregation and downregulation in testicular cancer.42

In conclusion, we have used a functional model of ovarian cancer suppression to identify and test candidate susceptibility genes for ovarian cancer. We have identified functional evidence suggesting allele-specific imbalance for somatic genetic alterations in primary ovarian tumours for a SNP in the *STAG3* gene. These studies highlight the importance of international consortia like the OCAC to validate putative genetic risk associations; but they also emphasise some of the limitations and challenges that face the scientific community in

trying to elucidate the functional rationale underlying the numerous genetic associations that have been identified for multiple complex disease traits.

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Abbreviations:

GWAS	genome wide association study
HetOR	heterozygous odds ratio
HomOR	homozygous odds ratio
HWE	Hardy-Weinberg equilibrium
LOH	loss of heterozygosity
MMCT	microcell-mediated chromosome transfer
SNP	single nucleotide polymorphisms
tSNP	tagging single nucleotide polymorphisms

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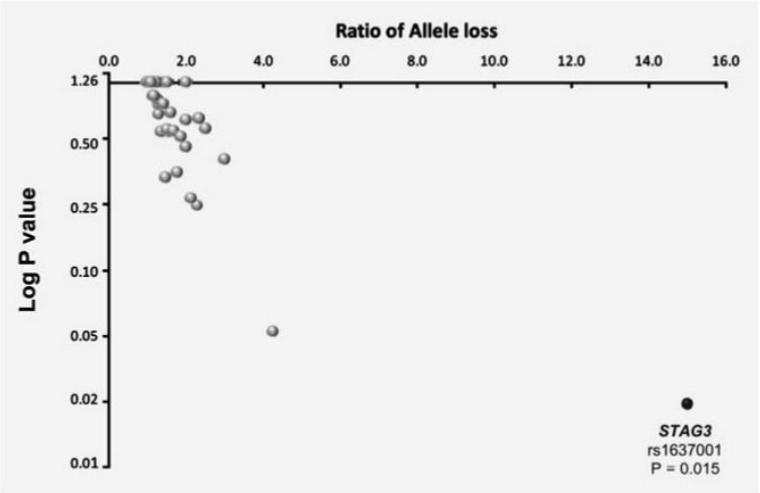

Figure 1.

Allele-specific LOH for 37 tSNPs. For each of the 37 SNPs the log of the *p*-value of difference from random loss of alleles is plotted against the ratio of allele loss. The grey spots indicate SNPs showing random LOH; the black spot is rs1637001 in *STAG3*, which shows significant deviation from random LOH ($p = 0.015$).

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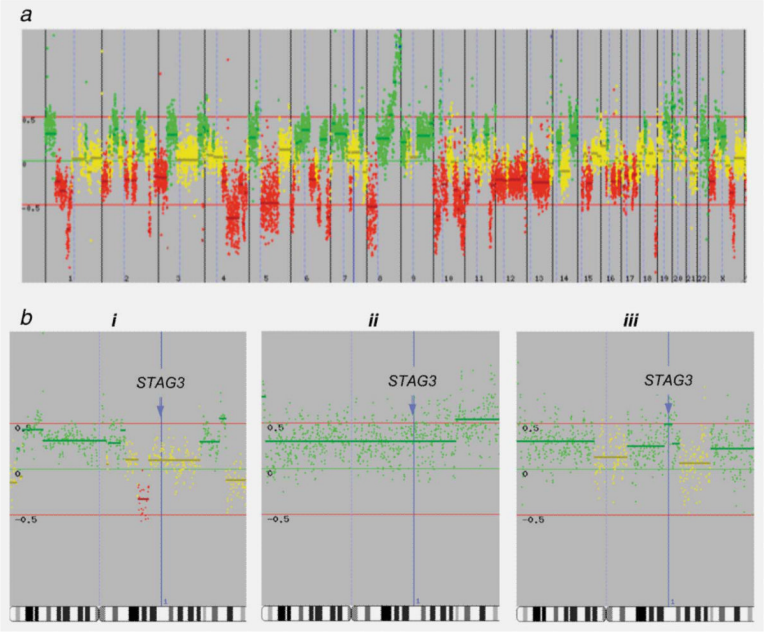

Figure 2.

The aCGH profiles of tumours showing copy number variation for rs1637001 in *STAG3*. (*a*) Array CGH profiles are presented in linear chromosome order, from the telomere of the p-arm to the telomere of the q-arm for each chromosome. Yellow spots indicate no change in copy number between normal and tumour DNA; green spots indicate an increase in DNA copy number in the tumour; red spots indicates loss of DNA copy number. (*b*) aCGH profiles for Chromosome 7 only in three tumours. The position of STAG3 is indicated by the blue line and arrow. (*i*) A tumour showing normal copy number at *STAG3* and LOH with loss of allele A; thus the predicted genotype is GG. (*ii*) A tumour showing three copies of *STAG3* and LOH with loss of allele A; thus the predicted genotype is AGG. (*iii*) A tumour with LOH and loss of allele G, with four copies of *STAG3*; thus, the predicted genotype is AAAG.

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Table 1

Candidate genes selected from differential gene expression between parental cancer cells lines and MMCT-18 hybrids

Gene	Expression: parental *vs* hybrid lines	Cytoband	Function	Gene size (bp)	SNPs with MAF > 0.05	No. tSNPs	tSNPs analysed	SNPs captured $r^2 \geq 0.8$	SNPs captured $r^2 \geq 0.8$ (%)
AIFM2	3.4 fold increase in TOV112D and 1.8 fold increase in TOV21G hybrids	10q22.1	TP53-induced apoptosis; over expression induces apoptosis	34,711	17	13	**12**	13/17	76
AKTIP	5.7 fold increase in TOV112D and 3 fold increase in TOV21G hybrids	16q12.2	Apoptosis; interacts with PKB/Akt;	11,978	7	4	**4**	7/7	100
AXIN2	5 fold increase in TOV112D hybrids	17q23-q24	Inhibitor of β-catenin in Wnt signalling pathway; LOH in breast and other cancers.	33,084	14	12	**10**	12/14	86
CASP5	7 fold increase in TOV21G hybrids	11q22.2-q22.3	Regulation of apoptosis.	14,729	17	9	**9**	17/17	100
FILIP1L	5 fold increase in TOV112D hybrids	3q12.1	Down regulated in ovarian cancer.	281,369	135	8	**8**	135/135	100
RBBP8	7 fold increase in TOV112D hybrids	18q11.2	RB1 binding protein; transcriptional regulation of *BRCA1*; DNA repair; tumour suppressor	93,155	39	4	**4**	39/39	100
RGC32	3 fold decrease in TOV112D hybrids	13q14.11	Cell cycle progression regulation; induced by p53 in response to DNA damage.	13,323	17	8	**7**	15/17	88
RUVBL1	47 fold decrease in TOV112D and 2 fold decrease in TOV21G hybrids	3q21	Interacts with *MYC*; involved in cell growth	42,857	29	7	**6**	22/29	76
STAG3	9 fold increase in TOV21G hybrids	7q22.1	Component of cohesin complex; chromosome segregation	43,764	28	3	**3**	28/28	100
Total					303	68	**63**	288/303	95

Captured SNPs refer to the number and proportion of criteria SNPs covered by the HapMap Data Release 22.

Abbreviations: MAF: minor allele frequencies; MMCT: microcell-mediated chromosome transfer; SNPs: single nucleotide polymorphisms.

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Table 2

Genotype specific risks from stage 1 (SEA, UKO and MAL)

					All subtypes			Serous		
Gene	**tSNP**	**Controls[1]**	**Cases[1]**	**Serous cases**	**HetOR[2] (95% CI)**	**HomOR[2] (95% CI)**	***p*-trend**	**HetOR[2] (95% CI)**	**HomOR[2] (95% CI)**	***p*-trend**
AXIN2	rs11079571	1,206	839	326	1.23 (1.00–1.51)	1.73 (0.99–3.01)	0.038	1.22 (0.92–1.63)	1.74 (0.84–3.63)	0.11
CASP5	rs518604	1,195	438	270	**1.39 (1.06–1.81)**	**1.44 (1.05–1.97)**	**0.012**	1.36 (0.98–1.88)	1.45 (0.99–2.11)	**0.031**
RBBP8	rs4474794	2,895	1,764	829	0.94 (0.82–1.07)	0.88 (0.72–1.06)	0.21	**0.83 (0.70–0.98)**	0.80 (0.63–1.03)	**0.032**
RUVBL1	rs13063604	1,724	1,266	537	1.14 (0.97–1.34)	**1.39 (1.02–1.89)**	**0.019**	**1.42 (1.15–1.74)**	**1.63 (1.10–2.42)**	**0.0002**
RUVBL1	rs7650365	2,672	1,645	769	1.08 (0.93–1.26)	0.86 (0.72–1.03)	0.11	0.97 (0.80–1.17)	**0.74 (0.58–0.93)**	**0.009**
STAG3	rs1637001	2,967	1,784	843	**0.86 (0.76–0.98)**	0.92 (0.73–1.16)	0.07	**0.84 (0.71–0.99)**	0.77 (0.56—1.05)	**0.018**

Bold indicates significant p values <0.05.

Abbreviations: OR: odds ratio; CI: confidence interval.

[1]The differences in the numbers of cases and controls between tSNPs reflect the exclusion of data from some studies that did not pass genotyping quality control criteria.

[2]OR and CI compared to common homozygote.

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Table 3

Genotype specific risks for Stage 2 and all samples combined for the two most significant serous *RUVBL1* tSNPs

Gene	tSNP	Controls[1]	Cases[1]	Serous cases	All subtypes			Serous		
					HetOR[2] (95% CI)	HomOR[2] (95% CI)	*p*-trend	HetOR[2] (95% CI)	HomOR[2] (95% CI)	*p*-trend
rs13063604	POC	427	432	203	0.99 (0.75,1.32)	1.6 (0.88,2.91)	0.38	1.01 (0.71,1.43)	1.59 (0.77,3.29)	0.46
	DOV	706	526	301	0.95 (0.75,1.21)	0.74 (0.45,1.22)	0.34	0.96 (0.72,1.27)	0.67 (0.36,1.25)	0.35
	HOP	559	263	151	0.94 (0.69,1.28)	1.44 (0.78,2.66)	0.67	0.93 (0.63,1.37)	1.45 (0.69,3.03)	0.76
	STA	418	315	213	1.25 (0.92,1.70)	1.21 (0.60,2.47)	0.20	1.19 (0.84,1.68)	1.29 (0.59,2.83)	0.32
	USC	529	379	237	1.15 (0.86,1.52)	0.97 (0.58,1.65)	0.65	0.96 (0.69,1.33)	0.9 (0.48,1.66)	0.74
	All	2,639	1,915	1218	1.04 (0.92,1.18)	1.09 (0.85,1.41)	0.40	1 (0.86,1.16)	1.05 (0.77,1.42)	0.83
Summary	Stage 1	1,724	1,266	537	1.14 (0.97–1.34)	**1.39 (1.02–1.89)**	**0.019**	**1.42 (1.15–1.74)**	**1.63 (1.10–2.42)**	**0.0002**
	Stage 2	2,639	1,915	1,218	1.04 (0.92–1.18)	1.09 (0.85–1.41)	0.40	1.00 (0.86–1.16)	1.05 (0.77–1.42)	0.83
	Combined	4,363	3,181	1,755	1.08 (0.98–1.19)	1.19 (0.98–1.45)	**0.033**	**1.13 (1.00–1.27)**	**1.22 (0.96–1.56)**	**0.019**
rs7650365	POC	562	543	343	0.87 (0.66,1.15)	0.8 (0.58,1.12)	0.20	0.89 (0.65,1.21)	0.8 (0.56,1.14)	0.23
	DOV	706	524	300	1 (0.76,1.30)	1.05 (0.77,1.45)	0.79	0.97 (0.70,1.33)	0.93 (0.64,1.36)	0.75
	HOP	579	266	151	0.69 (0.49,0.97)	0.88 (0.59,1.31)	0.43	0.65 (0.43,0.98)	0.75 (0.46,1.24)	0.21
	STA	405	313	208	0.86 (0.61,1.21)	0.81 (0.53,1.24)	0.33	0.83 (0.56,1.24)	1.02 (0.64,1.62)	0.97
	USC	533	382	237	0.97 (0.72,1.32)	1.03 (0.71,1.49)	0.96	1 (0.69,1.43)	1.22 (0.79,1.87)	0.43
	AUS	1,121	766	462	1 (0.80,1.25)	1.23 (0.94,1.59)	0.15	0.96 (0.74,1.25)	1.27 (0.94,1.72)	0.14
	BAV	229	215	124	1.03 (0.66,1.59)	1.25 (0.74,2.12)	0.46	0.89 (0.53,1.49)	1.17 (0.64,2.14)	0.72
	GER	410	215	112	1.38 (0.93,2.05)	0.98 (0.60,1.59)	0.98	1.19 (0.73,1.95)	0.89 (0.49,1.64)	0.86
	HAW	157	70	36	0.82 (0.42,1.59)	0.64 (0.30,1.36)	0.29	1.03 (0.43,2.47)	0.82 (0.30,2.22)	0.79
	NCO	725	600	349	1 (0.77,1.29)	0.92 (0.68,1.24)	0.63	0.9 (0.67,1.22)	0.84 (0.59,1.19)	0.34
	UKO (B)	458	543	212	1.1 (0.82,1.48)	0.91 (0.64,1.29)	0.68	1.22 (0.82,1.82)	1.22 (0.77,1.92)	0.43
	All	5,885	4,437	2534	0.97 (0.88,1.06)	0.97 (0.87,1.09)	0.62	0.94 (0.84,1.05)	0.99 (0.87,1.13)	0.86
Summary	Stage 1	2,672	1,645	769	1.08 (0.93–1.26)	0.86 (0.72–1.03)	0.11	0.97 (0.80–1.17)	**0.74 (0.58–0.93)**	**0.009**
	Stage 2	6,010	4,437	2,534	0.97 (0.88–1.06)	0.97 (0.87–1.09)	0.62	0.94 (0.84–1.05)	0.99 (0.87–1.13)	0.86
	Combined	8,682	6,129	3,303	1.02 (0.94–1.1)	0.96 (0.87–1.05)	0.40	0.94 (0.86–1.04)	0.92 (0.82–1.03)	0.142

Bold indicates significant p values <0.05.

The *p* value heterogeneity between studies for Stage 2 for all subtypes was 0.482 and 0.547 and for serous only was 0.624 and 0.579 for rs13063604 and rs7650365, respectively. Not all studies could be genotyped for rs13063604 as a second batch of Taqman assay failed manufacture.

Abbreviations: OR: odds ratio; CI: confidence interval.

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[1] The difference in the numbers of cases and controls between tSNPs reflects the exclusion of data from some studies that did not pass genotyping quality control criteria.

[2] OR and CI compared to common homozygote.

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Table 4

Frequency of LOH in tumours for each candidate gene

Gene	Chromosomal location	No. tSNPs per gene	No. tumours heterozygous	No. tumours with LOH	Percentage of LOH
AIFM2	10q22.1	7	202	55	27
AKTIP	16q12.2	4	137	63	46
AXIN2	17q23-q24	5	235	150	64
CASP5	11q22.2-q22.3	5	214	70	33
FILIP1L	3q12.1	5	196	63	32
RBBP8	18q11.2	3	148	34	23
RGC32	13q14.11	3	185	63	34
RUVBL1	3q21	3	167	51	31
STAG3	7q22.1	2	162	30	19

A tumour was considered to be heterozygous if heterozygous at any tSNP within the gene and to have LOH if LOH in at least one of the tSNPs. Abbreviations: LOH: loss of heterozygosity; tSNPs: tagging single nucleotide polymorphisms.