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WWOX tumour suppressor gene polymorphisms and ovarian cancer pathology and prognosis

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ABSTRACT

WWOX is a *bona fide* tumour suppressor, with hypomorphic and knockout mouse models exhibiting increased tumour susceptibility. In ovarian cancer cells WWOX transfection abolishes tumourigenicity, suppresses tumour cell adhesion to extracellular matrix and induces apoptosis in non-adherent cells. One-third of ovarian tumours show loss of WWOX expression, and this loss significantly associates with clear cell and mucinous histology, advanced stage, low progesterone receptor expression and poor survival, suggesting that WWOX status affects ovarian cancer progression and prognosis. Genetic variation in other tumour suppressors (e.g. p53 and XPD) is reported to modify cancer progression/outcome, and single nucleotide polymorphisms (SNPs) within the WWOX gene are reported to associate with prostate cancer risk. We previously identified polymorphic variants within WWOX, some of which have potential to affect its expression. We therefore examined a cancer modifier role for these WWOX variants. Eight SNPs, based upon location, frequency and potential to affect WWOX expression, were genotyped in 554 ovarian cancer patients (CGP samples), and associations with pathological and survival data were examined. The CGP samples demonstrated significant associations after Bonferroni correction between Isnp1 and both tumour grade ($p_{corr} = 0.033$) and histology ($p_{corr} = 0.046$), Isnp8 and tumour grade ($p_{corr} = 0.032$) and T1497G and progression-free survival ($p_{corr} = 0.037$). None of these positive associations were confirmed in an independent ovarian cancer population (Scotroc1 samples, $n = 863$). While these results may suggest that the associations are false positives, differences between the two populations cannot be excluded, and thus highlight the challenges in validation studies.

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1. Introduction

Common fragile sites (CFS) are chromosomal regions that show propensity to exhibit breaks and genomic disruption. There is strong evidence linking CFS and tumourigenesis with homozygous deletions, translocations, viral integrations and carcinogenic damage being observed at CFS in many tumour types.[1] Many of the CFS regions examined possess nearby genes, several spanning the fragile site, that are co-conserved across species, maybe indicating a functional link between the CFS and associated genes. The significance of CFS and their associated genes to tumourigenesis remains an important unanswered question. It is clear, however, that dysregulation of many of these CFS genes is important in the carcinogenic process,[2] and several, such as *WWOX* and FHIT, are tumour suppressors.[3,4]

The *WWOX* (WW-domain containing oxidoreductase) gene on chromosome 16q23 spans the second most commonly observed CFS – FRA16D.[5] *WWOX* is a *bona fide* tumour suppressor gene, and *Wwox* knockout mice show increased lung carcinoma, osteosarcoma and B-cell lymphoma susceptibility.[3] Furthermore, transfection of *WWOX* in cell lines of several tumour types, including ovarian cancer, suppresses *in vivo* tumourigenicity,[5–7] and in some studies can induce apoptosis *in vitro*.[6,8] Somatic point mutations within *WWOX* are extremely rare. Instead, *WWOX* is inactivated by genomic disruption (LOH and homozygous deletion) and epigenetic methylation in ovarian and other tumours.[9–12] *WWOX* is expressed in normal ovarian epithelial cells, but is significantly down-regulated in ovarian tumours.[13,14] Loss of WWOX protein expression is found in 29% of serous and 23% of endometrioid tumours, and is more common in clear cell (42%) and mucinous (70%) cases. Loss of WWOX protein also associates with higher stage tumours and shorter overall survival of patients.[14] These data suggest that *WWOX* status may affect the pathological features and prognostic outcome of ovarian cancer through somatic or constitutive alterations of the gene.

Heterozygous knockout mice exhibit increased tumour susceptibility,[3] but interestingly, tumours arising in these mice were still positive for WWOX expression. This demonstrates that complete loss of expression was not required for tumour development, indicating haploinsufficiency. Thus factors that constitutively modulate WWOX expression may contribute to tumourigenesis. Single nucleotide polymorphisms (SNPs) in many genes, e.g. IL-6 and MDM2, have been shown to modulate their expression,[15,16] and associate with particular clinical features of cancer suggesting a potential cancer modifier role. In addition, SNPs within the WWOX gene have been reported to demonstrate strong linkage with prostate cancer susceptibility.[17,18] We previously identified polymorphic variants in the *WWOX* gene,[10] some of which have potential to affect *WWOX* expression (as discussed below). We therefore investigated whether these naturally occurring variants of the *WWOX* tumour suppressor might associate with clinical characteristics of ovarian cancer. We here examine the genotypes of eight SNPs located within the *WWOX* gene in constitutive DNA of 554 unselected ovarian cancer patients (CGP samples). These SNPs were chosen for this study based upon location, allele frequency and potential to affect *WWOX* expression. They include SNPs within the Kozak translation-start site (C121T), splice consensus sequences (Isnp8 and Isnp13), within (T1497G) or close to (C1442T) predicted microRNA target regions and a non-synonymous coding SNP (A660G). We report associations of *WWOX* variants with tumour histology, advanced grade, earlier age at diagnosis and progression-free survival. We further sought confirmation of these findings in an independent phase III clinical trial population of 863 cancer patients (Scotroc1 samples), which did not reproduce any of the previous associations. This study again highlights the importance of large datasets and independent populations to identify true ovarian cancer modifiers.

2. Patients and methods

2.1. Study populations

Genomic DNA was extracted from blood of 554 women with epithelial ovarian cancer attending various hospitals throughout the UK (CGP samples). All patients were eligible, irrespective of stage and histology, following definitive diagnosis of ovarian carcinoma. The patients gave informed consent for sample collection. Institutional ethical approval was granted by the Lothian University National Health Service Trust, Medicine/Clinical Oncology Research Ethics Subcommittee. The patients were classified according to FIGO stage at presentation. The clinical data are summarised in Table 1. Note that all clear cell tumours, as opposed to other histological types, are regarded as high grade and were considered separately for the analysis of grade. Overall and progression-free survival data were only available for the 235 patients attending the Western General Hospital, Edinburgh, UK, allowing association of *WWOX* variants with survival to be determined in a sub-set of samples. The end-point for overall survival is the patient's date of death, or date last seen by doctor where no event occurred (137 events/235 patients). The end-point for progression-free survival is the date of clinical progression, or date last seen by doctor where no event occurred (162 events/235 patients). The distributions of the clinical factors among these 235 samples are similar to their distributions among all 554 samples (Table 1).

Positive findings in the CGP dataset were then confirmed by testing in a second, independent ovarian cancer population. We chose this approach for two reasons: firstly, we did not wish to split our original CGP dataset into test and validation sets as this would reduce the power of our study; secondly, we had ready access, through the Scottish Gynaecological Cancer Trials Group, to such an independent ovarian cancer population (SCOTROC1 samples). Genotyping was performed on DNA extracted from blood of 863 of the 1077 patients enrolled onto the SCOTROC1 phase III trial. All patients had histologically confirmed epithelial ovarian carcinoma and FIGO stage Ic–IV disease as previously described.[19,20] All patients gave written informed consent, and appropriate ethical review boards approved the study. Clinical data for these patients are summarised in Table 1.

Table 1 – Summary of patient clinical data.

	CGP	CGP survival[a]	Scotroc1
Total number of subjects	554	235	863
Median age at diagnosis, years	59 (22–88)[b]	60 (28–88)[b]	59 (18–84)[b]
Median survival, months	n/d[c]	70 (10–226)[d]	34 (0–41)[d]
Median PFS[e], months	n/d[c]	25 (10–190)[d]	15 (0–41)[d]
FIGO stage			
I	138 (25%)	59 (25%)	61 (7%)
II	64 (12%)	30 (13%)	102 (12%)
III	282 (51%)	119 (51%)	579 (67%)
IV	63 (11%)	26 (11%)	121 (14%)
Unknown	7 (1%)	1 (0%)	0
Tumour grade			
1	70 (13%)	15 (6%)	61 (7%)
2	131 (24%)	51 (22%)	209 (24%)
3	263 (47%)	144 (61%)	466 (54%)
Clear cell cases[f]	40 (7%)	19 (8%)	42 (5%)
Unknown	50 (9%)	6 (3%)	85 (10%)
Histology			
Clear cell	40 (7%)	20 (8%)	42 (5%)
Endometrioid	125 (22%)	63 (27%)	97 (11%)
Mucinous	38 (7%)	13 (5%)	28 (3%)
Serous	298 (54%)	133 (57%)	387 (45%)
Anaplastic	0	0	3 (0%)
Adenocarcinoma	0	0	135 (16%)
Other	0	0	165 (19%)
Unknown	53 (10%)	6 (3%)	6 (1%)

a Patients from the CGP study for whom survival data were available.
b Range of variables shown in parentheses.
c Not determined.
d Minimum and maximum follow-up for patients who have not experienced the event.
e Progression-free survival.
f Clear cell tumours are all regarded as high grade, and were analysed separately.

2.2. *Genotyping WWOX in the CGP samples*

Based on previous research[10] eight SNPs were selected spanning the *WWOX* gene and were tested here for a significant effect on tumour pathology, overall survival and progression-free survival in 554 ovarian cancer patients (CGP samples). For SNP typing 200 bp of sequence surrounding each SNP was provided to Applied Biosystems to develop Taqman Assays® using their assay-by-design platform (Foster City, CA). DNA (2 μl at 5 ng/μl) was dispensed into 384-well PCR plates using a Biomek FX robot (Fullerton, CA). Taqman assay-by-design reagent mix (3 μl; ABI, Foster City, CA) was added according to the manufacturers' instructions. The plates were sealed with optical seals (ABI, Foster City, CA) and incubated at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min, before analysis on a 7900HT plate reader (ABI, Foster City, CA).

Where SNPs failed the assay-by-design criteria (C121T, Isnp1, Isnp15 and C1442T), SNAPshot assays were designed as a single multiplex using Primer3 software. DNA (2 μl at 5 ng/μl) was dispensed into 384-well PCR plates using a Biomek FX robot (Fullerton, CA), and amplified on a PTC225 Thermocycler (MJ research, Waltham, MA) using 0.2 μM of each dNTP, 1.5 mM $MgCl_2$, 25 ng each PCR primer and 0.5 U AmpliTaq Gold (ABI, Foster City, CA) under standard cycling conditions (95 °C for 30 s, 60 °C for 15 s and 72 °C for 30 s). Two microliters of PCR product from each of the 4 loci from single individuals was combined in clean 384-well plates and incubated at 37 °C for 15 min and at 80 °C for 15 min with 2 μl ExoSapIt (Amersham Bioscience, Uppsala, Sweden). 1.5 μl of pooled template was combined with 2.5 μl of SNAPshot mix (ABI, Foster City, CA) and 1.25 ng of each SNAPshot primer (Sigma-Genosys, Cambridge, UK) and incubated at 95 °C for 10 s, at 50 °C for 5 s and at 60 °C for 30 s. To each sample 2 U of Shrimp Alkaline Phosphatase was added and incubated at 37 °C for 1 h and at 75 °C for 15 min. The samples were combined with GS120LIZ internal size standard and electrophoresed on a 96-lane capillary automatic sequencer (3700, ABI, Foster City, CA). The data were analysed using Genotyper software (ABI, Foster City, CA) by two independent scorers and any discrepancies re-evaluated.

2.3. *Confirming positive associations in SCOTROC1 samples*

SNPs showing significant associations in the CGP dataset were then genotyped in an independent set of 863 ovarian cancer patients (SCOTROC1 samples) using a Sequenom MALDI-TOF mass spectrometer. PCRs were performed in 384-well plates according to Sequenom protocols, using multiplex reactions (28-plex containing Isnp1 and C1442T, and 22-plex containing C121T, Isnp8 and T1497G), based on output from

Table 2 – WWOX SNP genotype frequencies.

SNP name	bp from C121T	Polymorphism and location	Genotype frequencies[a] of CGP samples				Genotype frequencies[a] of Scotroc1 samples			
			A/A	A/a	a/a	Unknown	A/A	A/a	a/a	Unknown
C121T	0	C → T in Exon 1	211 (47%)	183 (41%)	52 (12%)	108	370 (44%)	366 (44%)	97 (12%)	30
Isnp1	8538	C → T in Intron I	381 (71%)	146 (27%)	10 (2%)	17	573 (73%)	191 (24%)	24 (3%)	75
Isnp8	64,521	C → T in Intron V	284 (52%)	233 (42%)	32 (6%)	5	448 (52%)	349 (40%)	65 (8%)	1
Isnp13	178,826	A → C in Intron V	143 (26%)	268 (49%)	133 (24%)	10	n/d[b]	n/d	n/d	n/d
A660G	287,104	A → G in Exon 6	220 (40%)	237 (43%)	91 (17%)	6	n/d	n/d	n/d	n/d
Isnp15	325,079	A → G in Intron VI	2 (0%)	67 (13%)	466 (87%)	19	n/d	n/d	n/d	n/d
C1442T	1,112,094	C → T in Exon 9	457 (90%)	46 (9%)	5 (1%)	46	n/d	n/d	n/d	n/d
T1497G	1,112,149	T → G in Exon 9	184 (33%)	273 (50%)	94 (17%)	3	276 (32%)	421 (49%)	158 (19%)	8

a Allele 'A' represents the original nucleotide and allele 'a' the variant nucleotide as indicated in the polymorphism and location column.
b Not determined.

Sequenom assay design software. Forward, reverse and extension reaction primers (5′-ACGTTGGATGTGTCGTCCAG CCCCGCGTA-3′, 5′-ACGTTGGATGAGTTCCTGAGCGAGTGGA C-3′ and 5′-AGGGCCAGGTGCCTCCACAGT-3′ for C121T, 5′-AC G-TTGGATGTAAAGTTGACCCCGTAGCTG-3′, 5′-ACGTTGGAT GTTTCTCTCCCAGATAGCCAG-3′ and 5′-GGCCAGATATAAGA AGGAAGT-3′ for Isnp1, 5′-ACGTTGGATGAGCAGTGTCACGCA TTTTAG-3′, 5′-ACGTTGGATGGAAATCTCCATATGGTTAGC-3′ and 5′-AACAACAACAACAGTCAAGC-3′ for Isnp8, 5′-ACGTTGG ATGCTGTGTTGGAGGGACATTTG-3′, 5′-ACGTTGGATGAAGT GGAGCTCAGAGCGGAT-3′ and 5′-GAGTGCCAGGGCTGGG-3′ for C1442T and 5′-ACGTTGGATGGAATTCCCTGCTTCCCA TTG-3′, 5′-ACGTTGGATGCAGATCCGCAAGAGTAAAGG-3′ and 5′-GAAGAGTAAAGGAAATAAGAGCA-3′ for T1497G) were obtained from Sigma-Genosys. PCR was carried out in 5μl reaction mixture containing 1.25× Qiagen HotStar buffer, 3.5 mM Mg^{2+}, 0.5 mM dNTPs, 100 nM primer mix, 0.5 U Qiagen HotStar Taq polymerase (1 U for 28-plex PCR) and 5 ng DNA.

2.4. Statistics

SNPs were tested for Hardy–Weinberg equilibrium by Fisher's Exact tests. Univariate tests for independence from genotype were performed for clinical covariates histology, grade, stage (χ^2-tests and Fisher's exact tests) and age (1-way ANOVA). When cell counts were too small for the chi-squared distribution to be approximated, genotypes were recategorised as carriage/non-carriage of the rare allele. *P*-values were corrected for multiple testing of the eight SNP variables using the Bonferroni procedure.

Assessment of the *WWOX* SNPs as prognostic factors independent of clinical factors for progression-free and overall survival was performed using a multivariate Cox proportional-hazards regression model. Initially we modelled one SNP variable at a time together with all relevant clinical factors (stage, grade, histology and age at diagnosis). Any SNPs with significant effects after adjusting for multiple testing by the Bonferroni procedure were then combined into a final multivariate prognostic model, again including the relevant clinical factors. Model assumptions were checked using Schoenfeld and Martingale residuals.[21,22] Half of the patients with survival data had missing data for one or more variables (see Tables 1 and 2), which were therefore imputed using a multiple-imputation method,[23,24] prior to running the Cox regression. Running the iterative imputation algorithm for 20 iterations, 10 sets of imputed values for the missing data points were generated.

Chi-squared, Fisher's exact and ANOVA analyses were performed using the WinSTAT excel plug-in or SPSS 15.0 for windows. The survival analysis was carried out using the survival library of the statistical computing package R version 2.4.0 for Windows and missing value imputation was done using the R library MICE. Multiple Cox regression was performed in SPSS 15.0 for windows. Harrell's C index was determined with a .632 bootstrap estimate computed with B = 1000 bootstrap samples using the validate.cph function in Frank Harrell's R library 'Design' (version 2.1-2).

3. Results

3.1. Genotyping WWOX SNPs in the CGP samples

Eight SNPs spanning the full length of the *WWOX* gene were chosen for this study based upon their genic location, population frequency and potential to affect *WWOX* expression. Four of these SNPs are exonic – one in Kozak sequence (C121T), one non-synonymous coding (A660G) and two within (T1497G) or close to (C1442T) predicted microRNA target regions in 3′-UTR – and four are in flanking intronic sequence – two (Isnp8 and Isnp13) within splice site consensus sequences.[10]

Constitutive DNA extracted from blood from 554 ovarian cancer patients was genotyped for these *WWOX* SNPs by TaqMan (Isnp8, A660G, Isnp13, and T1497G) or SnapShot (C121T, Isnp1, Isnp15, and C1442T) assay. Genotype frequencies (Table 2) are similar to those we previously reported in a small group of ovarian and colorectal cancer patients ($n = 49$) and individuals without cancer ($n = 54$). Six of the SNPs were in H–W equilibrium as expected, however, two (A660G and C1442T) significantly deviated from the expected frequencies ($p = 0.047$ and $p = 0.013$, respectively). The *p*-value for SNP A660G is only marginally below the $p = 0.05$ cut-off chosen to indicate likely significance, therefore this result may be merely due to chance in our sample population. The *p*-value calculated for C1442T, however, is considerably less than 0.05 and may therefore indicate a true deviation from the expected frequencies rather than a sampling effect. Genotype

frequency for this SNP was similarly deviated from H–W equilibrium in 1358 postmenopausal Danish women without cancer ($p = 0.012$, data not shown). Whilst we cannot exclude genotyping error as an explanation, it should be noted that we screened the Danish samples using a different genotyping assay (Sequenom massarray). Importantly, however, the HapMap SNP database defines nucleotide polymorphisms with an unadjusted H–W statistic $p > 0.001$ as being suitable for mapping and association studies.

3.2. *WWOX SNPs associate with tumour grade and histology in the CGP samples*

We analysed our data for associations between WWOX SNPs and tumour grade, stage, histology or patient age at diagnosis (Table 3). In the cases of four of the SNPs (Isnp1, Isnp8, Isnp15 and C1442T) individuals homozygous for the rarer allele were too few to provide meaningful statistics. Therefore, for these four SNPs the genotype data were reclassified as individuals

Table 3 – Univariate association between WWOX SNP genotypes and clinicopathological data.

	p-values (Bonferroni adjusted p-values)[a]							
	C121T	Isnp1[b]	Isnp8[b]	Isnp13	A660G	Isnp15[b]	C1442T[b]	T1497G
CGP								
Grade (1 versus 2–3)	n/s[c]	0.0041 (0.033)	0.0040 (0.032)	n/s	n/s	n/s	n/s	n/s
Stage (I–II versus III–IV)	n/s	n/s	0.0143 (n/s)	n/s	n/s	n/s	n/s	n/s
Histology	n/s	0.0057 (0.046)	n/s	n/s	0.0275 (n/s)	n/s	n/s	n/s
Age at diagnosis	0.0073 (0.058)	n/s	n/s	n/s	n/s	0.0251 (n/s)	n/s	n/s
Scotroc1								
Grade (1 versus 2–3)	n/d[d]	0.877	0.120	n/d	n/d	n/d	n/d	n/d
Stage (I–II versus III–IV)	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Histology	n/d	0.839	n/d	n/d	n/d	n/d	n/d	n/d
Age at diagnosis	0.575	n/d	n/d	n/d	n/d	n/d	n/d	n/d

a Numbers in parentheses are *p*-values adjusted for the simultaneous multiple testing of eight SNP variables, using Bonferroni method.
b Due to low frequency of individuals homozygous for the rarer allele, these SNP genotypes were analysed as individuals possessing at least one copy of the rarer allele versus individuals homozygous for the common allele.
c Non-significant.
d Not determined.

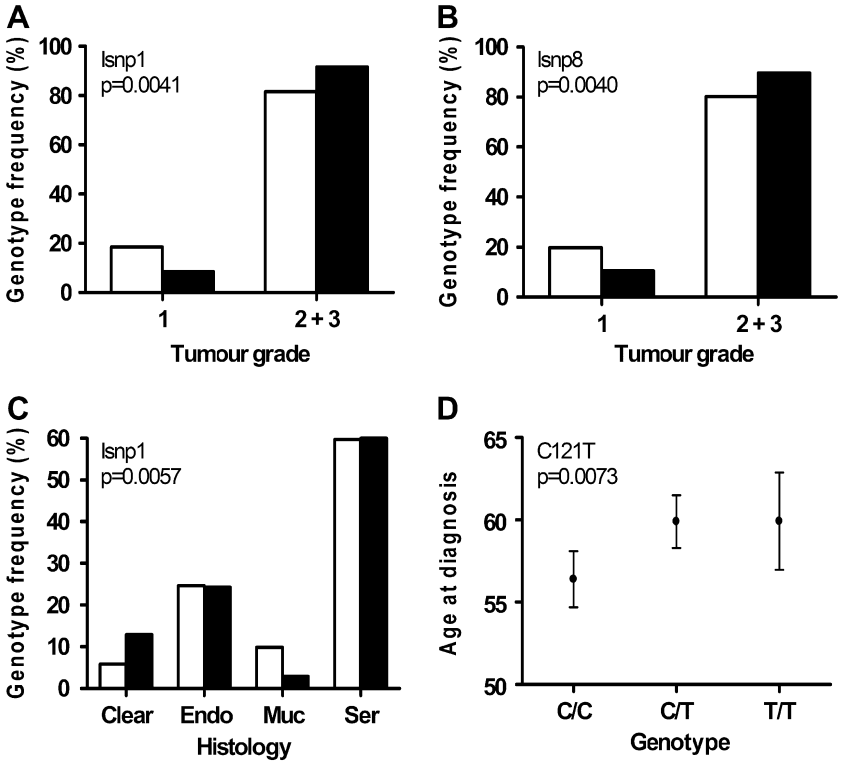

Fig. 1 – Relative frequencies in CGP samples of (A) Isnp1 and (B) Isnp8 genotypes stratified by grade, (C) Isnp1 genotypes stratified by histology. White bars, C/C; black bars, C/T + T/T. Clear, clear cell; Endo, endometrioid; Muc, mucinous; and Ser, serous. (D) Mean age at diagnosis ±95% confidence intervals stratified by C121T genotype.

Table 4 – Association between each *WWOX* SNP and survival data in the CGP dataset ($n = 235$), independent of clinical factors.

SNP	p-values (Bonferroni-adjusted p-values)[a]	
	Progression-free survival	Overall survival
C121T	n/s[b]	n/s
Isnp1	n/s	n/s
Isnp8	n/s	n/s
Isnp13	n/s	n/s
A660G	n/s	n/s
Isnp15	n/s	n/s
C1442T	0.0443 (n/s)	n/s
T1497G	0.0046 (0.0368)	0.0284 (n/s)

a Numbers in parentheses are p-values adjusted for the simultaneous multiple testing of eight SNP variables, using Bonferroni method.
b Non-significant.

possessing at least one copy of the rarer allele versus individuals homozygous for the common allele. In all other cases, the three genotypes were treated separately for analysis.

Two SNPs (Isnp1 and Isnp8) showed associations with tumour grade ($p = 0.0041$ and $p = 0.0040$, respectively), which remained significant after correcting for multiple testing of eight SNP variables using the Bonferroni method (corrected p-values shown in parentheses in Table 3). In both cases, presence of at least one copy of the rarer allele (Isnp1 T allele or Isnp8 T allele) positively correlated with higher grade (Fig. 1A and B).

Isnp1 also showed association with clear cell histology ($p = 0.0057$), as did the A660G SNP ($p = 0.0275$), and the association with Isnp1 remained significant after Bonferroni correction (Table 3). The presence of at least one copy of the rarer Isnp1 T allele positively correlated with clear cell histology, while the C/C genotype positively correlated with mucinous tumours (Fig. 1C).

Two SNPs (C121T and Isnp15) associated with patient age at diagnosis ($p = 0.0073$ and $p = 0.0251$, respectively), although neither association remained significant after Bonferroni correction, with the corrected p-value for C121T ($p = 0.0584$) just above the cut-off for significance (Table 3). Individuals with the C/C genotype at C121T showed an earlier age at diagnosis compared to those with the other two genotypes (mean 56 years versus 60 years, Fig. 1D).

3.3. *WWOX* SNP T1497G is an independent prognostic indicator in CGP samples

Follow-up data were available for 235 patients from the CGP study who were treated at the Western General Hospital, Edinburgh, UK (median follow-up 8.2 years). Any missing data for these patients were imputed as described in the methods, and the effect of the WWOX SNPs on progression-free survival (PFS) and overall survival was determined separately for each SNP, as described in Section 2.

T1497G ($p = 0.0045$) and C1442T ($p = 0.0443$) showed association with PFS, and T1497G showed association with overall survival ($p = 0.0284$). However, only the association of T1497G with PFS remained significant after Bonferroni correction (Table 4).

The final prognostic model for PFS (as described in Section 2) including T1497G and important clinical covariates is given in Table 5. T1497G was found to be an independent prognostic indicator of PFS (HR = 2.1, 95% CI = 1.28–3.40, $p = 0.0030$ for G/G. Table 5). The patients carrying the T/T

Table 5 – Prognostic model (T1497G and clinical covariates) for progression-free survival in multivariate Cox PH regression.

	CGP ($n = 235$)			Scotroc1 ($n = 863$)		
	HR[a]	95% CI[b]	p[c]	HR[a]	95% CI[b]	p[c]
T1497G						
T/G	1.6	1.13–2.32	0.0092	0.9	0.71–1.04	0.123
G/G	2.1	1.28–3.40	0.0030	0.9	0.68–1.10	0.223
Age at diagnosis	1.0	1.01–1.04	0.0003	1.0	0.99–1.01	0.938
Stage						
III–IV	3.4	2.21–5.23	0.0000	3.4	2.55–4.65	0.000
Grade						
2	3.7	1.19–11.65	0.0239	1.7	1.10–2.56	0.016
3	5.4	1.76–16.60	0.0033	1.5	0.97–2.20	0.071
Histology						
Clear cell	3.0	0.84–10.43	0.0929	1.8	1.19–2.74	0.006
Endometrioid	0.6	0.41–0.91	0.0166	0.7	0.49–0.94	0.019
Mucinous	0.4	0.09–1.75	0.2167	1.0	0.58–1.76	0.960
Anaplastic	n/d[d]	n/d	n/d	1.0	0.24–3.94	0.972
Adenocarcinoma	n/d	n/d	n/d	1.1	0.86–1.40	0.441
Other	n/d	n/d	n/d	1.0	0.81–1.27	0.895

a Hazard ratio.
b 95% confidence interval.
c P-value.
d Not determined since no cases of these were present in CGP samples.

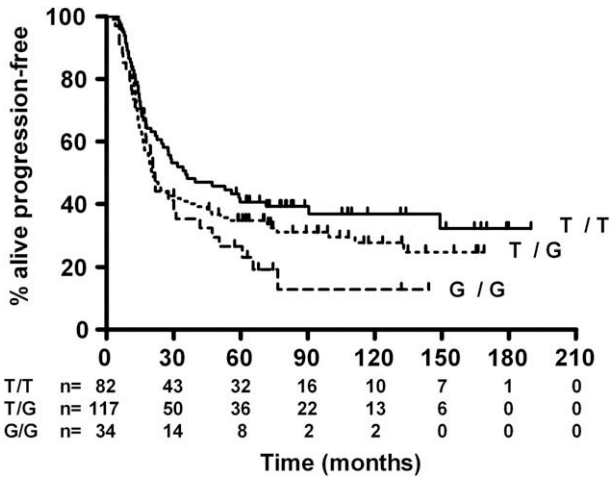

Fig. 2 – The *WWOX* T1497G SNP is an independent prognostic indicator for progression-free survival (PFS) in the CGP samples. Kaplan–Meier curve of PFS stratified by T1497G genotype. Patients at risk numbers are shown below x-axis for each genotype. T/T genotype (continuous line), T/G (dotted line) and G/G genotype (dashed line).

genotype ($n = 82$, median PFS = 34.8 months) exhibited better prognosis than those with either the T/G or G/G genotype ($n = 151$, median PFS = 20.7 months, Fig. 2). The discriminatory capacity of the model was assessed using Harrell c-statistic, as described in methods, and the c-index was calculated to be 0.709, indicating a 70% concordance between the predicted and observed progression-free survival.

3.4. *Examining findings in Scotroc1 samples*

To exclude false positive associations we attempted to confirm our findings using Sequenom massarrays in an independent population of 863 ovarian cancer patients from the Soctroc1 clinical trial. Genotype frequencies (Table 2) showed no significant differences from the frequencies in the CGP samples. All four SNPs were in H–W equilibrium.

We first examined in the Scotroc1 data our previous positive associations with clinicopathological features of ovarian cancer (Isnp1 and Isnp8 versus tumour grade, Isnp1 versus histology and C121T versus age at diagnosis). None of these were confirmed as significant ($p < 0.05$) in the Scotroc1 samples (Table 3). We next examined the previous association of T1497G with PFS in a multivariate Cox regression model. Again, analysis in the Scotroc1 samples did not confirm a significant association ($p = 0.223$, Table 5).

4. Discussion

Our data initially demonstrated significant associations of *WWOX* SNPs (C121T, Isnp1, Isnp8 and T1497G) with higher grade, clear cell histology, earlier age at diagnosis and shorter PFS in the CGP samples, but none of these were subsequently reproduced in the SCOTROC1 sample set. These initially exciting associations in a fairly large dataset were not inconsistent with the published data for *WWOX* in ovarian cancer, where loss of expression has been associated with clear cell and mucinous histologies, and with poor survival.[14] Although loss of WWOX expression was not found to associate with tumour grade in ovarian cancer, an association with grade has also been reported in lung cancer.[25]

Furthermore, three of these four SNPs alter sequences that could potentially regulate *WWOX* expression; C121T in the Kozak translation initiation site, Isnp8 in the splice donor site of intron V and T1497G in a predicted binding site for the microRNA miR-134. Published studies have demonstrated a link between other SNPs within the *WWOX* gene and *WWOX* gene expression,[26] and have identified potential enhancer elements within *WWOX* introns.[27] Associations of *WWOX* SNPs with prostate cancer incidence[17,18] and HDL-cholesterol levels[28] have also been recently reported, demonstrating that natural variants of *WWOX* may indeed have functional effects.

However, the fact that our initial associations did not reproduce in the Scotroc1 samples suggests that these are either false positives, or are specific to characteristics of the initial study population that were different in the SCOTROC1 population. The CGP samples were collected from unselected patients with confirmed diagnosis of ovarian cancer, and therefore represent a general ovarian cancer population. Since we did not possess a second unselected ovarian cancer population, and we did not wish to lose the power of our study by splitting our original population into test and validation sets, we attempted to confirm our original findings in samples from the Scotroc1 phase III clinical trial. As a result there are some differences between the two datasets. In particular, the CGP samples have a higher proportion of stage I and grade 1 tumours compared to those of the SCOTROC1 study (for which stages Ia and Ib tumours were excluded),[19,20] and hence exhibit longer median survival. If we remove the stage I cases from the CGP dataset we still detect the significant associations with tumour grade (Isnp1 $p = 0.0156$, Isnp8 $p = 0.0098$) and PFS (T1497G $p = 0.0088$), but these are no longer resistant to Bonferroni correction. Therefore, the lack of validation in the Scotroc1 samples could in part reflect the paucity of early stage tumours as compared to the CGP samples.

Although disappointing, the absence of confirmation in independent populations is a common observation. Several recent studies have attempted to re-examine the previously reported genetic associations with ovarian cancer, using large independent cohorts.[19,29,30] Of 37 SNPs examined, only 3 were confirmed, and even these exhibited only marginal significance or gave positive findings only in a specific subset of samples.[29,30]

In summary, while our data, generated from 554 CGP ovarian cancer patients, does support a role for WWOX as a modifier of cancer progression, this was not validated in an independent population of 863 Scotroc1 patients. While this may be indicative of false positive associations, the differences in patient populations, particularly of early stage tumours, could explain this lack of validation.

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The sponsors had no involvement in any aspect of the study.

Conflict of interest statement

None declared.

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