Heparanase gene haplotype (CGC) is associated with stage of disease in patients with ovarian carcinoma

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Heparanase (HSPE-1) and vascular endothelial growth factor (VEGF), proangiogenic growth factors, play important roles in the metastatic biology of ovarian cancer. The aim of the present study was to test for association between single nucleotide polymorphisms (SNPs) in *HSPE-1* and *VEGF* and outcome in ovarian cancer. A mutational analysis was performed on the coding sequence of the *HSPE-1* gene to define high-frequency SNPs. *HSPE-1* polymorphisms, together with two SNPs in the *VEGF* gene, were studied in 136 patients with ovarian cancer. Patients were categorized into two groups, those with FIGO stages 1 and 2 (group 1) and those with stages 3 and 4 (group 2). We identified 10 polymorphisms in the *HSPE-1* gene, those in introns 2, 3 and 5b, and exons 8, 13a and 13b occurring at a minor allele frequency of ≥10%. There was an increase in frequency of those individuals with a genotype that carried at least one copy of the intron 2 (C), exon 8 (G), exon 13a (C) haplotype (CGC) in group 2. Specifically there were 24% with this haplotype in group 2 versus 5% in group 1 (*P* = 0.0184, odds ratio 5.986, 95% confidence interval 1.340–26.752). Most of this association was captured by the intron 2 genotype, where carriage of the C allele was associated with stage (*P* = 0.0148, odds ratio 6.524, 95% confidence interval 1.401–27.921). There was no association between *VEGF* SNPs and stage of disease. The CGC *HSPE-1* haplotype associates with stage in ovarian cancer. This haplotype may affect splicing of the *HSPE-1* gene, as *in silico* it alters the presence of a splicing motif. (*Cancer Sci* 2007; 98: 844–849)

Globally, ovarian cancer accounts for 4% of malignancies in women, with Northern European countries showing the highest incidence across Europe. Recent trends show increasing incidence and mortality in Southern and Eastern Europe.[1] Ovarian cancer remains a threatening disease because of the subtle nature of presenting symptoms and a lack of an effective screening program with the result that patients often present with late stage disease and metastasis.[2] Although early stage 1 disease is detected in only 25% of cases, up to 80% of patients with early stage disease can be cured with surgery and adjuvant chemotherapy.[3]

Angiogenesis is an important contributor to the progression of many tumor types showing a direct correlation between tumor microvessel density (MVD) and poor prognosis.[4] The importance of angiogenesis in the pathophysiology of ovarian tumor progression and metastasis is currently not determined. Conflicting evidence has linked MVD to disease prognosis in some studies but not others.[5,6]

Many factors have been implicated in the angiogenic pathology of ovarian cancer. Two proangiogenic factors with a significant role in ovarian cancer metastasis are heparanase (HSPE-1) and vascular endothelial growth factor (VEGF). Interestingly, estrogen upregulates both *HSPE-1* and *VEGF* gene expression.[7,8]

HSPE-1, an endo-β-D-glucuronidase, degrades heparan sulfate in the extracellular matrix (ECM) and thereby solubilizes matrix-bound growth factors, such as VEGF and basic fibroblast growth factor, required for angiogenesis.[9] In addition, degradation of heparan sulfate glycosaminoglycan loosens the structure of the ECM and basement membrane allowing cell invasion.[10] HSPE-1 is expressed to a higher degree in metastatic ovarian carcinoma cells, compared to cells from primary tumors.[11] HSPE-1 mRNA expression is significantly higher in ovarian cancer associated with massive ascites and high grade.[12] Increased HSPE-1 mRNA and protein expression has been linked with poor prognosis in several other carcinomas including breast, colon and gastric tumors.[13–15]

VEGF, a potent mitogen for endothelial cells and a modulator of vascular permeability, shows increased mRNA expression in primary tumors and metastases.[16] Protein expression is higher in peritoneal metastases than in primary tumors.[17] In early stage disease, VEGF expression is linked to poor clinical outcome.[18] Tumor-derived VEGF seems necessary for ascites formation in ovarian cancer, and serum VEGF correlates with stage of disease and may act as a biomarker for diagnosis.[19]

Genetic mutations capable of inducing an invasive metastatic phenotype have long been sought. Many studies have examined the theory that tumorigenicity and metastasis are caused by a mutation or mutations in somatic cells. Germ line polymorphisms may also play a significant role in the variability in metastatic potential.

Single nucleotide polymorphisms (SNPs) in the *VEGF* gene promoter have been described and are associated with other angiogenic pathologies, such as proliferative retinopathy.[20] Currently there have been no clinical association studies with SNPs in the *HSPE-1* gene. As HSPE-1 and VEGF play an important role in the metastatic biology of ovarian cancer, our hypothesis was that genetic markers of these candidates might be linked to stage or clinical outcome. The aim of the present study therefore was to use high-frequency *HSPE-1* and *VEGF* SNPs to test for association with outcome in patients with ovarian cancer.

Materials and Methods

Patients. One hundred and thirty-six patients with ovarian cancer attending the Oncology Department at Christie Hospital (Manchester, UK) took part in this study. One hundred and twenty of these patients had undergone surgery for ovarian cancer and had been referred to the Medical Oncology team for further management. These patients' tumors were reviewed by specialist oncological pathologists. Before the patients received chemotherapy, a single 5-mL sample of blood was aspirated into a glass tube containing ethylenediaminetetracetic acid (EDTA) and then frozen at –20°C until processed. Briefly, DNA was isolated using a non-organic isolation method. Blood cells were extracted by washing in 155 mM ammonium chloride, 10 mM potassium hydrogen carbonate and 1 mM EDTA. The cellular pellet was lysed in 25 mM EDTA, 2% sodium dodecylsulfate and 10 M ammonium acetate. Following centrifugation the supernatant was combined with propan-2-ol to precipitate out DNA. The DNA was centrifuged to form a pellet and washed

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Table 1. Patient demographics

	Ovarian cancer patients (n = 136)
FIGO stage	No
1	27
2	13
3	72
4	24
Epithelial histology	
Serous	51
Mucinous	5
Clear cell	20
Endometrioid	30
Serous + endometrioid	15
Mucinous + endometrioid	1
Clear + endometrioid	2
NOS	12
Tumor differentiation	
Well	8
Moderately	30
Poorly	73
NOS	23
NK	2
KP score	
40	2
50	11
60	12
70	23
80	39
90	44
100	5
Median time of follow-up (days)	1225 (133–2396)
Median survival time (days)	1221

KP, Karnofksy performance; NK, not known; NOS, not otherwise specified.

twice in 70% ethanol. DNA was redissolved in dH_2O and quantified by spectrometry at a wavelength of 260 nm. Twenty-five ovarian cancer patients were selected for mutation screening to detect SNP in the *HSPE-1* gene. For patient demographics see Table 1. For the purposes of statistical analysis patients were split into two groups: group 1 comprised those patients with stage 1 and 2 ovarian cancer and group 2 comprised those patients with stage 3 and 4 ovarian cancer. Informed consent was obtained from each participant and the study was approved by South Manchester Local Ethics Committee.

Mutational analysis. SNPs in the *VEGF* gene have been described previously by our group and two high-frequency SNPs, –460 C/T and +405 G/C, have been identified in genetic association studies of disease in which there is a significant angiogenic contribution.[20,21] Currently no SNPs in *HSPE-1* have been reported in clinical studies, so a full coding sequence mutational analysis was undertaken, encompassing all of the exons and intron–exon boundaries in the *HSPE-1* gene (Fig. 1).

All exons of the *HSPE-1* gene were amplified separately by polymerase chain reaction (PCR); primers are listed in Table 2. Exons 13 and 14 of the *HSPE-1* gene were split into overlapping fragments, to obtain amplification products that did not exceed 500 bp. PCR was carried out using either Optimase polymerase (Transgenomic 78990; Transgenomic, Elancourt, France) (exons 7, 8, 9, 10, 12, 13A, 13B, 14A, 14B, 14D and 14E) or Bioline polymerase (Bioline, London, UK) (exons 1, 2, 3, 4, 5, 6, 11 and 14C).

Denaturing high-performance liquid chromatography wave analysis. Denaturing high-performance liquid chromatography (dHPLC) was carried out on WAVE DNA fragment analysis equipment (Transgenomic). PCR products were screened for heteroduplexes by subjecting 25 μL of each PCR sample to a denaturation step (95°C, 5 min) and then a gradual annealing gradient of 1°C/1.5 min down to a final temperature of 4°C. The oven temperature for optimal heteroduplex separation was determined using the WAVEMAKER version 4.1.0 software program (Transgenomic). The temperature giving 70–80% helical fraction of the wild-type DNA was used to screen all samples.

Validation of mutants. Samples showing an alteration in elution profile were selected for sequencing using an ABI377 sequencer (Applied Biosystems, Warrington, UK). Where possible two mutations and two wild types were reamplified using PCR under the same conditions prior to WAVE analysis. Each PCR product was then subjected to cycle sequencing in both the forward and reverse direction using the Big-Dye Terminator kit (Version 1.1, Applied Biosystems).

Genotyping. Polymorphic changes as determined by sequencing were confirmed using Taqman allelic discrimination with the ABI PRISM 7700 sequence detection system (Applied Biosystems). Polymorphisms in introns 2, 3, 5b and exons 8, 13a and 13b all occurred at a minor allele frequency of ≥10% and were selected for genotypic and haplotypic analysis. For all but the intron 13 polymorphisms each allele was identified by either a FAM™ dye 6-carboxyfluorescein (FAM)- or (VIC™)-labeled probe (see Table 3). The intron 13 polymorphisms were identified by sequencing as described in the validation of mutants section. VEGF –460 and +405 variants were genotyped by PCR

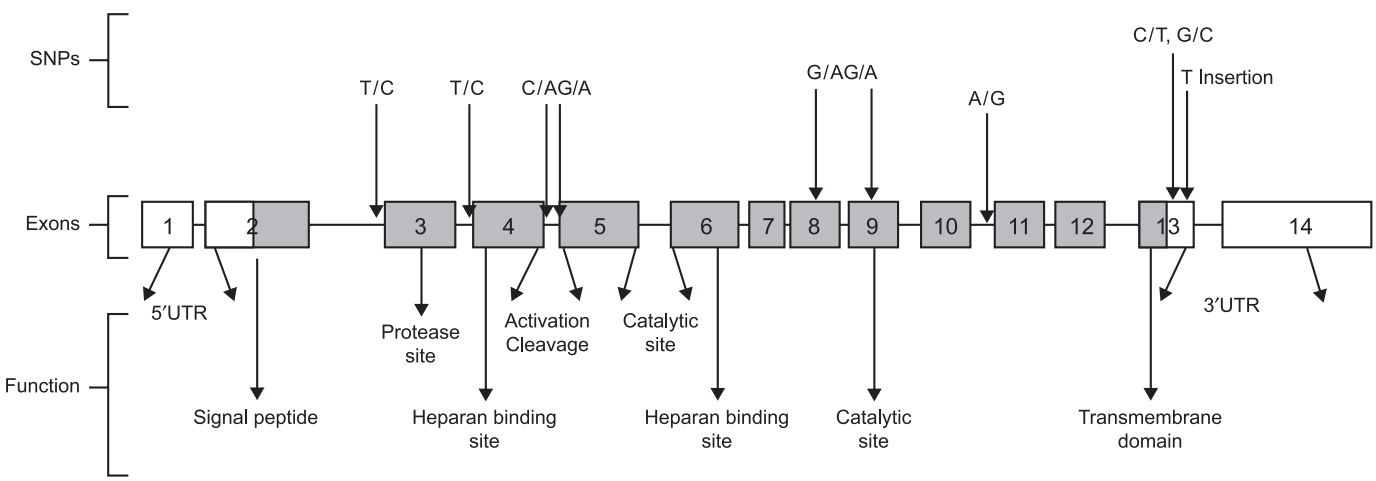

Fig. 1. Heparanase gene map. Coding sequences are highlighted in gray.

Table 2. Polymerase chain reaction primers used to amplify the coding sequence of the *HSPE-1* gene

Exon	Name	Sequence 5′–3′	Length (bp)
1	Hep ex1F	TCAGAATGGGATCTGGGAGT	249
	Hep ex1R	CCGGCTCTCTCCTACTTCCT	
2	Hep ex2F	GCGGGAGGAAGTGCTAGAG	351
	Hep ex2R	GGTGTCAGGAGGACAGGAAA	
3	Hep ex3F	AGGTCCACCAGGAAAGAAGG	294
	Hep ex3R	GCATTCTCATGACGGACTGA	
4	Hep ex4F	TTCAGTCTGCTCACTGTGAAA	246
	Hep ex4R	CCCGAGTCCAACCTATTCAA	
5	Hep ex5F	TTGTCCAATTTTGTTGTCTCAAA	384
	Hep ex5R	TGCTAGTGGGGGATCTCCTT	
6	Hep ex6F	CGCCAGACCTGGTTTTACTT	400
	Hep ex6R	CACTCCAGTCTGGCTGACA	
7	Hep ex7F	AGCAGTAACCAGCCCATTTC	247
	Hep ex7R	TGCAGTCAATTGGCAGTTAAA	
8	Hep ex8F	GGTCAGCAATTAACCTAAAAGCA	298
	Hep ex8R	TGGATAAGTCATAGTGCTTGTTCTC	
9	Hep ex9F	TCAACACCACAGTTGAAACCA	227
	Hep ex9R	AGGATGGAGAAGAGCTGCAC	
10	Hep ex10F	TTGAGCGATTTTGTTTCCAA	232
	Hep ex10R	CACCAGAGGTCCTGAGTTGA	
11	Hep ex11F	GCTTGCCACTGGTTTCATTA	250
	Hep ex11R	CAGGCTTACCCACTAAAACTTGA	
12	Hep ex12F	CATTTGTAATTGAGTTTTTAATTGCTT	300
	Hep ex12R	TTGCTCCCTGTCCAAACTCT	
13	Hep ex13aF	TGACTTTTGTAGCAGCATGG	267
	Hep ex13aR	CTTGAAAAATTCAGTGTCAGGA	
	Hep ex13bF	TGTGATAAGAAATGCCAAAGT	353
	Hep ex13bR	CTGGGTGACAGAGAGAGATTC	
14	Hep ex14aF	TGGGGCAGGAGAATTACTTG	376
	Hep ex14aR	CCGAAAGAAGAAAATCAGCAA	
	Hep ex14bF	TTTTGCTGATTTTCTTCTTTCG	381
	Hep ex14bR	GAAGAACCCAGATCCCTGGT	
	Hep ex14cF	GGCTCCAGCACTTCAGTACG	399
	Hep ex14cR	TCACTGGTAAAAAGCGCAGA	
	Hep ex14dF	TTAGGATAACAGCCCCCAGT	257
	Hep ex14dR	TTCAAATGTGAAAATATAAAAACCAC	
	Hep ex14eF	ATGGAGAGGGTGTTGGTCAG	461
	Hep ex14eR	TGTATTTTGCAGCAACTTGG	

restriction fragment length polymorphism, as in our previously described method.[21]

Haplotyping. All *HSPE-1* polymorphisms were tested for deviation from Hardy–Weinberg Equilibrium (HWE) in the ovarian cancer patient group as a whole and using the HWE test for use in small samples in the stage 1 and 2 and 3 and 4 groups.[22] EHPlus was used to measure marker–marker association and thus the degree of linkage disequilibrium (LD) between SNPs on the *HSPE-1* gene.[23] A utility program called PMPlus was used to prepare data files for EHPlus. Briefly, these programs tested for marker–marker association by comparing the likelihoods of the data under the assumptions of no association and of association. This method is described in detail at http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software/pm.htm. This analysis reported χ^2 values, with appropriate degrees of freedom (*df*), which were converted into *P*-values. These values acted as a measure of association between SNPs and thus an indication of linkage disequilibrium (LD) on the *HSPE-1* gene. The SNPHAP program was used to estimate haplotype frequency and infer haplotypes for all patients. In brief, this program implements a fairly standard method for estimating haplotype frequencies using data from unrelated individuals. It uses an expectation maximization algorithm to calculate estimates of haplotype frequencies given genotype measurements that do not specify phase. The algorithm also allows for some genotype measurements to be missing (due, for example, to PCR failure). It also allows multiple imputation of individual haplotypes. This technique is fully described at http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt.

Statistical analysis. Pearson's χ^2-test was used to compare differences in qualitative variables, where appropriate a continuity correction was applied. When the expected frequency was <5% a Fisher's exact test was applied. Mann–Whitney *U*-test was used for the comparison of quantitative variables. For all hypothesis tests, a two-tailed significance level $P < 0.05$ was considered statistically significant. Data analysis was carried out using SPSS software 11.5.

Results

We analyzed the DNA from 136 patients with epithelial ovarian cancer. The patient accrual period was 2002–2004. Mean age at

Table 3. Taqman primers and probes

Polymorphism	Sequence 5′–3′	Allele detected
Intron 2		
Forward primer	CCACCAGGAAAGAAGGAAGTAAGAAT	
Reverse primer	GCTTTGGAGAACTGTTAGGAAGACA	
FAM probe	AAAGTATAACTGTTTTTTC	C
VIC probe	AAGAAAGTATAACTATTTTTTC	T
Intron 3		
Forward primer	AAATATATGCGATTTCAGTCTGCTCACT	
Reverse primer	CCGTAACTTCTCCTCCACATCAG	
FAM probe	TTTAGAAGGTCTGTTTTCA	T
VIC probe	TTTAGAAGGTCTATTTTCA	C
Intron 5		
Forward primer	TGGTTTTACTTTTCTTGACTTTGAATTACAAGTTTT	
Reverse primer	TGAAAGACAGCAATTCTCAAAATATATATCGAGA	
FAM probe	CTTTTAAATACTGCTATATGT	A
VIC probe	CTTTTAAATACTGCTGTATGTT	G
Exon 8		
Forward primer	TGTGTAATTTAGCTACTATTTGAATGGACGG	
Reverse primer	TTTGCACAGATGAAATAAAAATGTCCAATACA	
FAM probe	ACTGCTACCAGGGAAG	G
VIC probe	CTGCTACCAAGGAAG	A

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Table 4. *HSPE-1* polymorphisms

dbSNP designation	Base pair numbering	Genome accession no.	Sequence	Allele frequency
Intron 2	**12 387**	**rs4328905**	**TGGAAAAAA(T/C)AGTTATACT**	**T 0.90**
Intron 3	**15 282**	**rs12501123**	**CTGTGAAAA(T/C)AGACCTTCT**	**T 0.76**
Intron 5a	21 677		TGGTGAGTA(C/A)CCCAGGGAA	
Intron 5b	**23 832**	**rs6535455**	**AATACTGCT(G/A)TATGTTTGC**	**G 0.77**
Exon 8	**25 317**	**rs11099592**	**CTGCTACCA(G/A)GGAAGATTT**	**G 0.76**
Exon 9	25 903		AGGCGGAGC(G/A)CCCTTGCTA	
Intron 10	32 510		ACCACCACA(A/G)CTAGGATTA	
Exon 13a	**39 566**	**rs6855404**	**ACTGGGACA(C/T)T(G/C)TCAGTGCTA**	**C 0.80**
Exon 13b	**39 568**	**rs6856901**		**G 0.80**
Intron 13 T ins	39 729		ATTTATTTT(T ins)GAGACAGAA	

Those highlighted in bold occurred at a minor allele frequency of ≥10% and were selected for analysis in the whole cohort. Base pair numbering is from translation start site. Allele frequency was determined in the whole patient group.

Table 5. Pairwise linkage disequilibrium for the common single nucleotide polymorphisms of the *HSPE-1* gene

	Intron 3	Intron 5b	Exon 8	Exon 13a	Exon 13b
Intron 2	0.1577	0.2132	0.1447	0.9979	0.9979
Intron 3		<0.0001	<0.0001	0.3043	0.6985
Intron 5b			<0.0001	0.6962	0.6962
Exon 8				0.6731	0.6731
Exon 13a					<0.0001

Statistical significance (*P*-value) determined by EHPlus. EHPlus was used to measure marker–marker association and thus the degree of linakge disequilibrium (LD) between SNPs on the *HSPE-1* gene.[23] This method is described in detail at http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software/pm.htm.

Table 6. Heparanase haplotype frequency

Haplotype	Stage 1 & 2	Stage 3 & 4	
CGC	2.5% (2)	12.5% (24)	
TGC	65.7% (54)	53.6% (103)	
TGT	10.0% (8)	11.5% (22)	
TAC	12.5% (10)	13.5% (26)	
TAT	7.5% (6)	7.8% (15)	
CGT	0.0% (0)	1.0% (2)	
			P-value
CGC	2.5% (2)	12.5% (24)	0.011
Not CGC	97.5% (78)	87.5% (168)	

The most striking change in frequency is that of the CGC haplotype. Thus carriage of the CGC haplotypes versus no carriage, was compared statistically. This analysis is represented in the lower section of the table

Table 7. Heparanase 3 loci genotype frequency

Genotype	Stage 1 and 2	Stage 3 and 4	
TGC/**CGC**	2.5% (1)	12.5% (12)	
TAC/**CGC**	0.0% (0)	4.2% (4)	
TAT/**CGC**	0.0% (0)	2.1% (2)	
CGC/CGC	0.0% (0)	1.0% (1)	
CGC/CGT	0.0% (0)	1.0% (1)	
TGT/**CGC**	2.5% (1)	3.1% (3)	
TGC/TGC	47.5% (19)	27.1% (26)	
TGC/TGT	12.5% (5)	13.5% (13)	
TGC/TAC	15.0% (6)	18.8% (18)	
TGC/TAT	10.0% (4)	8.3% (8)	
TGT/TGT	2.5% (1)	1.0% (1)	
TGT/TAT	0.0% (0)	3.1% (3)	
TGT/CGT	0.0% (0)	1.0% (1)	
TAC/TAC	2.5% (1)	1.0% (1)	
TAC/TAT	5.0% (2)	2.1% (2)	
			P-value
Carries CGC	5.0% (2)	24.0% (23)	0.0184
Does not Carry CGC	95.0% (38)	76.0% (73)	

Genotypes with a least one copy of the CGC haplotype are highlighted in bold. These genotypes were compared to those which did not carry this haplotype, in the lower section of the table.

diagnosis was 60 years (range 23–84 years). Staging was carried out according to the current Federation of Gynecology and Obstetrics (FIGO) classification. Histologically, there were 51 serous adenocarcinomas, five mucinous adenocarcinomas, 30 endometrioid adenocarcinomas, 20 clear cell carcinomas and 18 of mixed histology (15 of serous + endometrioid, one of mucinous + endometrioid and two of clear cell + endometrioid) and 12 not classifiable (pathologists were unable to identify a subtype, e.g. endometrioid or serous). Median duration of follow-up was 1225 days (range 133–2396 days) (Table 1).

Polymorphic variants were identified in introns 2 (T/C), 3 (T/C), 5a (C/A), 5b (G/A), 10 (A/G) and 13 (T insertion) and exons 8 (G/A), 9 (G/A) and 13 (C/T and G/C) (Fig. 1; Table 4). The polymorphism in exon 8 produces an amino acid substitution (codon arginine 307 lysine), and exon 9 a synonymous change of an alanine residue (codon 352). The two polymorphisms in exon 13 occur in an untranslated region of the gene.

LD was observed across all six SNP ($P < 0.0001$). Pairwise LD existed between the intron 3, 5b and exon 8 SNP, and between the exon 13a and 13b variants, where there was complete LD (Table 5). LD was not present between the intron 2 SNP and all other polymorphisms, nor was it present between the exon 13 variants and other SNP.

Of the 64 theoretical possible haplotypes, a total of eight six-locus haplotypes were present in the ovarian cancer group. Five of these haplotypes were present at a frequency of >2%, accounting for 98.6% of the total haplotypes. Examination of these haplotype patterns revealed that these five haplotypes could be uniquely identified using the intron 2, exon 8 and exon 13a loci. Thus five three-locus haplotypes captured 99.2% of the eight six-locus haplotypes. For this reason, analysis was limited to these three polymorphisms.

The intron 2, exon 8 and exon 13a haplotypes were statistically examined at a haplotypic and genotypic level (Tables 6,7). Due to the large number of possible haplotypes and genotypes, analysis was broken down to represent the most striking difference in frequency (i.e. that containing the CGC haplotype, or genotypes that contained the CGC haplotype). First, haplotype frequency was compared. There was an increased frequency of

Table 8. Frequency of *HSPE-1* polymorphisms in ovarian cancer patients

HSPE-1 polymorphism	Stage 1 and 2	Stage 3 and 4	*P*-value
Intron 2			
TT	94.9% (37)	74.7% (71)	0.027
TC	5.1% (2)	23.2% (22)	
CC	0.0% (0)	2.1% (2)	
Exon 8			
GG	64.1% (25)	55.4% (51)	0.248
GA	28.2% (11)	41.3% (38)	
AA	7.7% (3)	3.3% (3)	
Exon 13a			
CC	66.7% (26)	61.4% (54)	0.699
CT	30.8% (12)	33.0% (29)	
TT	2.5% (1)	5.6% (5)	

the CGC haplotype in patients in the stage 3 and 4 group ($P = 0.011$, odds ratio [OR] 5.571, 95% confidence interval [CI] 1.285–24.166) (Table 6). Second, genotype frequency was compared. There was an increased frequency of those individuals with a genotype that contained at least one copy of the CGC haplotype in the stage 3 and 4 group compared to the stage 1 and 2 group, 24 versus 5%, respectively ($P = 0.0184$, OR 5.986, 95% CI 1.340–26.752) (Table 6). Only one individual was homozygous for this haplotype; this individual was in the stage 3 and 4 group. Further analysis revealed that the intron 2 genotype was significantly linked with stage of disease, 5.1% CT (stage 1 and 2) versus 23.2% (stage 3 and 4) ($P = 0.027$; Table 8). Thus the majority of this association was captured by the intron 2 genotype, where carriage of the C allele was associated with stage ($P = 0.0148$, OR 6.524, 95% CI 1.401–27.921). There was no association between the exon 8 and 13a genotypes and stage (Table 8). Therefore, we suggest that the CGC haplotype association with stage of disease is predominantly explained by the presence of the intron 2 genotype.

Prediction of the presence of a splicing motif was carried out using the RESCUE-ESE exonic splicing enhancer (ESE) prediction software program.[24] The presence of the C allele resulted in the presence of an ESE (AAACAG) that was not present in the T allele variant.

There were no differences in *HSPE-1* haplotype or SNP frequency compared to histological type of ovarian cancer, bulk of residual disease after surgery or response to therapy. There was no association between *VEGF* genotype of stage of ovarian cancer (data not shown).

Discussion

Without angiogenesis, tumors are limited in size to 1–2 mm, being the distance that oxygen can diffuse from the nearest capillary.[25] Vascularization of tumors through angiogenesis allows the tumor to expand in size and provides a portal for access of tumor cells to the systemic circulation, enabling metastatic spread. Two important angiogenic factors, VEGF and HSPE-1, are implicated in the pathophysiology of ovarian and other carcinomas. Whilst it is clear that both these factors are expressed pathophysiologically in increased amounts at the mRNA and protein levels, there has been no study that assesses the contribution of the patient's genetic background to the outcome of disease.

This study provides evidence of a significant association of an intronic change in the *HSPE-1* gene with stage of disease. Currently, we speculate that two potential mechanisms might account for this association: first, that this SNP exerts a functional role, or second, that this polymorphism acts as a marker that is in tight LD with other SNP with a functional role in the *HSPE-1* promoter.

First, the intron 2 SNP might exhibit a functional role. A significant conceptual shift has occurred over recent years to the view that even translationally silent sequence variations or intronic sequences should raise suspicion and be seriously considered as being responsible for the pathology observed in the patient.[26] Although the intron 2 polymorphism does not directly change the protein sequence of the *HSPE-1* gene, it could potentially affect intron–exon splicing. Indeed the presence of the C allele predicts the presence of an ESE (AAACAG) that was not present in the T allele variant. Thus this C allele as part of the ESE may affect protein binding to this region and modulate exon splicing. Aberrant mRNA splicing could result in mRNA including intron 2, truncated mRNA including part of intron 2 but not exons 3 and 4, or mRNA in which exon 2 was linked to exon 4 with the elimination of exon 3. Exon 2 is important on a functional level as it affects signal peptide cleavage and exon 4 is concerned with activation cleavage.

Interestingly, there is evidence that not only are members of the heparanase gene family found as alternatively spliced species but also that intron 2 is involved. Human heparanase 2 (*HPA2*), which has significant homology to *HSPE-1*, yields three different mRNAs, encoding putative proteins of 480, 534 and 592 amino acids.[27] In addition, *HSPE-1* derived from the blind subterranean mole rat *Spalax ehrenbergi*, which is 85% homologous to the human enzyme, also produces a splice variant lacking 16 amino acids, encoded by exon 7.[28]

The second speculation as to the link between the intron 2 SNP and stage of disease relies on this SNP acting as a genetic marker, possibly being in tight linkage disequilibrium with another SNP upstream in the *HSPE-1* promoter region. The concept of tagged SNP defining large blocks of DNA in LD is commonly accepted following the Haplotype Map (HAPMAP, http://www.hapmap.org) study. In the present study we have identified significant blocks of DNA across the *HSPE-1* gene in tight LD. If similar areas of LD are present upstream of intron 2, the speculation that functional promoter SNP potentially controlling the level of HSPE-1 mRNA expression in response to stimuli may be associated with intron 2 SNP is not unreasonable. The potential roles of this SNP remain to be fully elucidated in future studies.

The lack of association between *VEGF* promoter polymorphisms and outcome in ovarian cancer could imply that the magnitude of the physiological stimuli (e.g. hypoxia) for gene expression in cancer might override any contribution from genetic modulation. Interestingly, HSPE-1 has recently been shown to be a controller of VEGF expression mediated by activation of Src signal transduction.[29] Therefore, the differential association of *HSPE-1* and not *VEGF* genetic markers with stage of disease could reflect the relative controlling influence that these genes have over the metastatic process. In addition to controlling *VEGF* gene expression and release of VEGF and other proangiogenic growth factors from the ECM reservoir, HSPE-1 directly endows the migrating cell with invasive properties.[30]

The present study is the first to document the common SNP in the *HSPE-1* gene in patients with ovarian cancer. It provides the first evidence associating a genetic variant of HSPE-1, intron 2 SNP, with the FIGO stage of ovarian cancer and highlights the need for further studies of the variant. At present the findings are important in that they represent patients attending one of the largest cancer centers in the UK. Ultimately, however, the frequency of these SNP will need to be determined in a large multicenter study. It will also be important to study the effects of this polymorphic variant in splicing *in vitro*, to fully elucidate the functional role of this SNP. Understanding the significance of this C allele substitution in the *HSPE-1* gene may focus attention on the important functional aspects of the enzyme in cancer angiogenesis and invasion.

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References

1 Bray F, Loos AH, Tognazzo S, La Vecchia C. Ovarian cancer in Europe: cross-sectional trends in incidence and mortality in 28 countries, 1953–2000. *Int J Cancer* 2005; **113**: 977–90.
2 Jacobs IJ, Skates SJ, MacDonald N *et al*. Screening for ovarian cancer: a pilot randomised controlled trial. *Lancet* 1999; **353**: 1207–10.
3 Trimbos JB, Vergote I, Bolis G *et al*. Impact of adjuvant chemotherapy and surgical staging in early-stage ovarian carcinoma: European Organisation for Research and Treatment of Cancer-Adjuvant ChemoTherapy in Ovarian Neoplasm Trial. *J Natl Cancer Inst* 2003; **95**: 113–25.
4 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249–57.
5 Gasparini G, Bonoldi E, Viale G *et al*. Prognostic and predictive value of tumour angiogenesis in ovarian carcinomas. *Int J Cancer* 1996; **69**: 205–11.
6 Ogawa S, Kaku T, Kobayashi H *et al*. Prognostic significance of microvessel density, vascular cuffing and vascular endothelial growth factor expression in ovarian carcinoma: a special review for clear cell adenocarcinoma. *Cancer Lett* 2002; **176**: 111–18.
7 Elkin M, Cohen I, Zcharia E *et al*. Regulation of heparanase gene expression by estrogen in breast cancer. *Cancer Res* 2003; **63**: 8821–6.
8 Sengupta K, Banerjee S, Saxena N, Banerjee SK. Estradiol-induced vascular endothelial growth factor-A expression in breast tumor cells is biphasic and regulated by estrogen receptor-a dependent pathway. *Int J Oncol* 2003; **22**: 609–14.
9 Vlodavsky I, Friedmann Y, Elkin M *et al*. Mammalian heparanase: Gene cloning, expression and function in tumor progression and metastasis. *Nat Med* 1999; **5**: 793–802.
10 Parish CR, Freeman C, Hulett MD. Heparanase: a key enzyme involved in cell invasion. *Biochim Biophys Acta-Rev Cancer* 2001; **1471**: M99–108.
11 Ginath S, Menczer J, Friedmann Y *et al*. Expression of heparanase, Mdm2, and erbB2 in ovarian cancer. *Int J Oncol* 2001; **18**: 1133–44.
12 Kodama J, Shinyo Y, Hashen G, Hongo A, Yoshinouchi M, Hiramatsu Y. Heparanase messenger RNA expression in epithelial ovarian tumor. *Int J Mol Med* 2003; **12**: 961–4.
13 Cohen I, Pappo O, Elkin M *et al*. Heparanase promotes growth, angiogenesis and survival of primary breast tumors. *Int J Cancer* 2006; **118**: 1609–17.
14 Nobuhisa T, Naomoto Y, Ohkawa T *et al*. Heparanase expression correlates with malignant potential in human colon cancer. *J Cancer Res Clin Oncol* 2005; **131**: 229–37.
15 Takaoka M, Naomoto Y, Ohkawa T *et al*. Heparanase expression correlates with invasion and poor prognosis in gastric cancers. *Lab Invest* 2003; **83**: 613–22.
16 Boocock CA, Charnockjones DS, Sharkey AM *et al*. Expression of vascular endothelial growth-factor and its receptors flt and Kdr in ovarian-carcinoma. *J Natl Cancer Inst* 1995; **87**: 506–16.
17 Gadducci A, Viacava P, Cosio S *et al*. Vascular endothelial growth factor (VEGF) expression in primary tumors and peritoneal metastases from patients with advanced ovarian carcinoma. *Anticancer Res* 2003; **23**: 3001–8.
18 Shen GH, Ghazizadeh M, Kawanami O *et al*. Prognostic significance of vascular endothelial growth factor expression in human ovarian carcinoma. *Br J Cancer* 2000; **83**: 196–203.
19 Tanir HM, Ozalp S, Yalcin OT, Colak O, Akcay A, Senses T. Preoperative serum vascular endothelial growth factor (VEGF) in ovarian masses. *Eur J Gynaecol Oncol* 2003; **24**: 271–4.
20 Ray D, Mishra M, Ralph S, Read I, Davies R, Brenchley P. Association of the VEGF gene with proliferative diabetic retinopathy but not proteinuria in diabetes. *Diabetes* 2004; **53**: 861–4.
21 Watson CJ, Webb NJA, Bottomley MJ, Brenchley PEC. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine* 2000; **12**: 1232–5.
22 Elston RC, Forthofer R. Testing for Hardy–Weinberg equilibrium in small samples. *Biometrics* 1977; **33**: 536–42.
23 Zhao HY, Zhang SL, Merikangas KR *et al*. Transmission/disequilibrium tests using multiple tightly linked markers. *Am J Hum Genet* 2000; **67**: 936–46.
24 Fairbrother WG, Yeo GW, Yeh R *et al*. RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucl Acids Res* 2004; **32**: W187–90.
25 Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002; **94**: 883–93.
26 Baralle D, Baralle M. Splicing in action: assessing disease causing sequence changes. *J Med Genet* 2005; **42**: 737–48.
27 McKenzie E, Tyson K, Stamps A *et al*. Cloning and expression profiling of Hpa2, a novel mammalian heparanase family member. *Biochem Biophys Res Com* 2000; **276**: 1170–7.
28 Nasser NJ, Nevo E, Shafat I, Ilan N, Vlodavsky I, Avivi A. Adaptive evolution of heparanase in hypoxia-tolerant spalax: gene cloning and identification of a unique splice variant. *Proc Natl Acad Sci USA* 2005; **102**: 15 161–6.
29 Zetser A, Bashenko Y, Edovitsky E, Levy-Adam F, Vlodavsky I, Ilan N. Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation. *Cancer Res* 2006; **66**: 1455–63.
30 He XT, Brenchley PEC, Jayson GC, Hampson L, Davies J, Hampson IN. Hypoxia increases heparanase-dependent tumor cell invasion, which can be inhibited by antiheparanase antibodies. *Cancer Res* 2004; **64**: 3928–33.