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ORIGINAL ARTICLE

Polymorphisms in the heparanase gene in multiple myeloma association with bone morbidity and survival

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Abstract

Objectives: In multiple myeloma, heparanase (*HSPE*) is involved in myeloma cell growth, angiogenesis, osteoclastogenesis and shedding of syndecan-1, a key player in myeloma pathophysiology. Different single nucleotide polymorphisms (SNPs) in the *HSPE* gene with effect on gene function have been described, and some are associated with haematological malignancies. *Methods:* In this study, we evaluated four SNPs rs11099592, rs4364254, rs4693608 and rs6535455 in the *HSPE* gene in 348 newly diagnosed multiple myeloma patients with focus on bone morbidity (lytic bone disease and vertebral fractures) and outcome after high-dose chemotherapy with stem cell support (HDT). *Results:* We observed that homozygous carriers of the rs4693608 wild-type A-allele had a higher frequency of vertebral fractures compared to carriers of the variant G-allele, $P = 0.02$. In multivariate analysis, homozygous carriers of the rs6535455 variant T-allele had a longer survival than homo- and heterozygous carriers of the wild-type C-allele, hazard ratio 0.3 (95% CI 0.1–0.7, $P = 0.002$). *Conclusion:* The SNPs rs4693608 and rs6535455 in the *HSPE* gene may influence bone morbidity and outcome in multiple myeloma. Our results are an interesting observation but can be chance findings and need confirmation in studies exploring the functional role of SNPs in the *HSPE* gene in multiple myeloma.

Key words multiple myeloma; heparanase (HSPE); single nucleotide polymorphisms (SNPs); bone disease; survival

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The heparanase (*HSPE)* gene is located on chromosome 4q21.3 and expressed in a variety of normal cells, including endothelial cells, as well as haematopoietic cells (1, 2). Different single nucleotide polymorphisms (SNPs) in the *HSPE* gene have been described, and some are associated with increased mRNA expression (3). Recent studies have examined the potential relation between SNPs in the *HSPE* gene and malignancies. Comparison of genotype frequencies revealed an association between the SNPs rs4364254 in acute leukaemia and rs4693602 in multiple myeloma, while in ovarian cancer, one specific haplotype was associated with disease stage (3, 4).

HSPE is an endoglycosidase that cleaves heparan sulphate proteoglycans into heparan sulphate side chains and core proteoglycans. Expression of HSPE is rare in normal tissue, while in tumours, it becomes evident and increases the angiogenic and metastatic potential of malignant cells (5, 6). HSPE promotes angiogenesis by releasing heparin-binding angiogenic growth factors such as fibroblastic growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) that is trapped in the extracellular matrix (7). HSPE is present in bone marrow plasma from myeloma patients, and high HSPE activity is associated with angiogenesis (8). HSPE enhances osteoclastogenesis through syndecan-1 and may influence bone resorption (9). Bone marrow microenvironment expression of HSPE is described as a negative prognostic factor in myeloma patients (10).

The heparan sulphate proteoglycan syndecan-1 (CD138) is a major regulator of the bone marrow microenvironment that supports myeloma cell growth (11). Cell surface syndecan-1 mediates adhesion of myeloma cells to collagen and other myeloma cells and transmits intracellular signals (12–14). Recent studies on myeloma cell lines have shown that HSPE promotes expression and shedding of syndecan-1 which stimulates endothelial invasion and angiogenesis (10, 15). *In vivo*, high serum levels of syndecan-1 are associated with bone marrow angiogenesis and poor prognosis in patients with multiple myeloma (16, 17).

SNPs in the *HSPE* gene may alter HSPE expression and the influence on syndecan-1 regulation, angiogenesis and osteoclastogenesis. Factors involved in multiple myeloma pathophysiology and associated with prognosis. In this study, we describe four SNPs in the *HSPE* gene in a group of multiple myeloma patients treated with high-dose chemotherapy with stem cell support (HDT) with focus on bone morbidity and outcome.

Material and methods

Patient's clinical data, response criteria, eligibility criteria and treatment have previously been described (18). Briefly, patients diagnosed with multiple myeloma and treated with HDT from August 1994 to August 2004 were recruited from four hospitals in Denmark. A total of 348 patients were included in the study. Of these, 185 patients were included in the high-dose treatment protocols implemented by the Nordic Myeloma Study Group (NMSG no. 5/94, 7/98 and 11/00) (19–21), whereas the remaining 163 patients were treated with similar treatment regimens off protocol.

Bone morbidity was assessed from skeletal surveys at diagnosis. The patients were divided into two groups depending on the degree of lytic bone disease: (i) no lytic bone disease and (ii) lytic bone disease. Furthermore, patients were divided into two groups defined by presence of vertebral fractures or not. Due to the retrospective design of the study, skeletal surveys for assessment of bone morbidity were not available for all patients (Table 1).

Time to treatment failure (TTF) and overall survival (OS) were calculated from date of stem cell infusion to date of progression after HDT or death, respectively. Definition of progressive disease has been described previously (18).

The study was approved by the Danish Ethical Committee (01-158/03).

Human tissue samples, DNA purification and detection of single nucleotide polymorphisms

Peripheral blood mononuclear cells (PBMCs) were purified from 292 leukapheresis products by buffy coat preparation. From 56 patients, 10 times 10-μm sections were collected from paraffin-embedded bone marrow samples.

Table 1 Patients' characteristics at the time of diagnosis

	Number of patients (percentage)	Median	Range
Sex			
Male	204 (59%)		
Female	144 (41%)		
Age (yr)		56	(28–69)
<60	247 (71%)		
≥60	101 (29%)		
Beta-2-microglobulin (mg/L)	249 (72%)	3.9	(1.2–57)
Creatinine (μM)	332 (95%)	98	(47–833)
Albumin (g/dL)	297 (85%)	3.5	(0.25–5.3)
Bone morbidity			
No osteolytic lesions	80 (23%)		
Osteolytic lesions	253 (73%)		
No vertebral fractures	146 (42%)		
Vertebral fractures	185 (53%)		
ISS			
I	56 (16%)		
II	94 (27%)		
III	89 (26%)		
NA	109 (31%)		
Durie–Salmon stage			
I	35 (10%)		
II	77 (22%)		
III	228 (66%)		
NA	8 (2%)		
TTF (months)[1]		27.7	(23.4–30.8)
OS (months)[1]		69.8	(60.5–81.6)

ISS, international staging system; TTF, time to treatment failure; OS, overall survival; NA, data not available.
[1]Only censored patients, exclusion of patients with occurrence of secondary malignancies and death without progression.

DNA for analysis was purified from PBMCs by salting out method (22) or from paraffin-embedded tissue by phenol extraction (23).

The SNPs rs11099592, rs4364254, rs4693608 and rs6535455 in the *HSPE* gene were determined on an ABI 7900HT using allelic discrimination and predeveloped assays (Applied Biosystems, Birkerød, Denmark). Reactions of 5 μL contained approximately 50 ng DNA, 2.5 μL mastermix (Applied Biosystems, Birkerød, Denmark) and the predesigned primer and probe mix. DNA from heparin-containing blood samples was treated with heparanase for one hour at 35°C. DNA was precipitated and resuspended in TE. Controls were included in each run, and repeated analysis of 10% subset of samples yielded 100% identical genotypes. Moreover, for 10 patients, DNA from both bone marrow and leukapheresis products was genotyped with identical results.

Statistics

The statistical data were obtained using R statistical software (version 2.9.2, 2009, The R Foundation for Statistical Computing, Vienna, Austria). All tests were two-sided, and *P*-val-

ues < 0.05 were regarded as statistical significant. Fisher's exact test was used to compare categorical variables. The Kaplan–Meier method was used to make survival curves, and the Cox proportional hazards model and log-likelihood statistics to compare differences in TTF and OS between groups.

Results

The characteristics of the patients are summarised in Table 1. The median follow-up of all censored patients was 93.4 months (range 54.6–174.2 months), and the median OS was 69.8 months (range 60.5–81.6 months).

The allele frequencies and genotype distributions of the four SNPs in the *HSPE* gene examined in this study are comparable to the limited published data in previous studies (3, 24). The SNPs rs4364254 and rs6535455 in the *HSPE* gene were determined in all 348 patients, while the genotypes for rs11099592 and rs4693608 were missing for 1 and 3 patients, respectively (Table 2).

Association between *HSPE* genotypes and bone morbidity

At diagnosis, 23% of the patients had no lytic bone disease and 73% had one or several osteolytic bone lesions. Forty-two percentage had no vertebral fractures, and 53% had vertebral fractures (Table 1).

There was no association between the different polymorphisms in the *HSPE* gene and osteolytic bone disease (Table 3).

The SNP rs4693608 was associated with the incidence of vertebral fractures at diagnosis (Table 3). Homozygous carriers of the wild-type A-allele had a higher frequency of vertebral fractures compared to all carriers of the variant G-allele (genotypes AG and GG), $P = 0.02$.

No association was seen between vertebral fractures and the other examined SNPs in the *HSPE* gene.

SNPs in the *HSPE* gene, response, time to treatment failure and overall survival after high-dose treatment

The SNP rs6535455 was associated with overall survival. Homozygous carriers of the variant T-allele lived longer than carriers of the wild-type C-allele. The median survival for the genotypes TT, CT and CC was 117.7, 65.9 and 69.6 months, respectively (Table 2). In univariate analysis, there was a borderline significant difference in overall survival between the genotype TT and the genotypes CT and CC, $P = 0.07$ (Fig. 1). In a multivariate analysis adjusting for beta-2-microglobulin, creatinine and Durie–Salmon stage, well-known prognostic factors in multiple myeloma, homozygous carriers

Table 2 Uni- and multivariate analyses of the association between different genotypes in the *HSPE* gene, time to treatment failure (TTF) after high-dose treatment and overall survival (OS). Heterozygous and homozygous carriers of the variant allele are compared with homozygous carriers of the wild-type allele either alone or together

		TTF (months)			OS (months)		
	Number (%)	Median	HR (95% CI)	*P*-value	Median	HR (95% CI)	*P*-value
rs11099592							
CC	204 (59%)	26.2	1		64.6	1	
CT	126 (36%)	28.7	0.7 (0.7–1.2)	0.60	75.1	0.9 (0.6–1.1) *0.9 (0.6–1.3)*	0.30 *0.68*
TT	17 (5%)	17.7	1.5 (0.9–2.6)	0.11	65.7	1.2 (0.7–2.1) *1.0 (0.4–2.1)*	0.58 *0.91*
CT+TT	143 (41%)	28.6	1.0 (0.8–1.5)	0.91	70.1	9.9 (0.7–1.2) *0.9 (0.7–1.3)*	0.42 *0.69*
rs4364254							
TT	130 (37%)	27.7	1		70.1	1	
CT	153 (44%)	26.2	1.0 (0.7–1.3)	0.83	65.6	1.0 (0.7–1.3) *1.0 (0.6–1.6)*	0.83 *0.97*
CC	65 (19%)	28.6	0.9 (0.7–1.3)	0.61	69.8	1.0 (0.6–1.4) *1.2 (0.8–2.0)*	0.79 *0.36*
CT+CC	218 (63%)	27.6	1.0 (0.7–1.2)	0.71	69.8	1.0 (0.7–1.3) *0.8 (0.6–1.2)*	0.79 *0.24*
rs4693608							
AA	95 (28%)	29.9	1		70.1	1	
AG	135 (39%)	29.9	1.0 (0.7–1.3)	0.92	75.1	0.9 (0.7–1.3) *0.8 (0.5–1.3)*	0.63 *0.39*
GG	115 (33%)	23.3	1.1 (0.8–1.3)	0.41	65.7	1.1 (0.8–1.5) *1.0 (0.7–1.6)*	0.63 *0.85*
AG+GG	250 (72%)	26.2	1.1 (0.8–1.4)	0.71	69.7	1.0 (0.7–1.4) *0.9 (0.6–1.4)*	0.98 *0.71*
rs6535455							
CC	175 (50%)	27.8	1		69.5	1	
CT	146 (42%)	25.1	1.0 (0.8–1.2)	0.80	65.9	1.1 (0.8–1.4) *1.2 (0.9–1.7)*	0.73 *0.28*
TT	27 (8%)	44.6	0.7 (0.4–1.1)	0.15	117.7	0.6 (0.3–1.1) *0.4 (0.1–0.8)*	0.10 ***0.02***
CT+TT	173 (50%)	27.6	0.9 (0.7–1.2)	0.48	69.8	1.0 (0.7–1.3) 1.0 (0.7–1.4)	0.81 *0.96*

HR, hazard ratio; CI, confidence interval. In the multivariate analysis (italics) adjusted for beta-2-microglobulin, creatinine and Durie–Salmon stage. Bold value indicates $P < 0.05$.

Table 3 SNPs in the *HSPE* gene and bone morbidity in patients with multiple myeloma. The differences between the three genotypes and between homozygous carriers of the wild-type allele and hetero- and homozygous carriers of the variant allele are tested

	Osteolytic bone disease			Vertebral fractures		
	No Number (percentage)	Yes Number (percentage)	*P*-value	No Number (percentage)	Yes Number (percentage)	*P*-value
rs11099592						
CC	52 (27%)	142 (73%)	0.32	81 (42%)	114 (58%)	0.55
CT	26 (21%)	96 (79%)		56 (47%)	63 (53%)	
TT	2 (12%)	14 (88%)		8 (50%)	8 (50%)	
CT+TT	28 (20%)	110 (80%)	0.19	64 (47%)	71 (53%)	0.31
rs4364254						
TT	31 (25%)	93 (75%)	0.78	48 (39%)	76 (61%)	0.27
CT	33 (22%)	115 (78%)		71 (49%)	75 (51%)	
CC	16 (26%)	45 (74%)		27 (44%)	34 (56%)	
CT+CC	49 (23%)	160 (77%)	0.79	98 (47%)	109 (53%)	0.14
rs4693608						
AA	20 (22%)	70 (78%)	0.57	30 (34%)	59 (66%)	0.05
AG	36 (27%)	95 (73%)		66 (50%)	65 (50%)	
GG	24 (22%)	85 (78%)		49 (45%)	59 (55%)	
AG+GG	60 (25%)	180 (75%)	0.67	115 (48%)	124 (52%)	**0.02**
rs6535455						
CC	41 (25%)	126 (75%)	0.26	72 (43%)	96 (57%)	0.89
CT	30 (21%)	111 (79%)		63 (46%)	75 (54%)	
TT	9 (36%)	16 (64%)		11 (44%)	14 (56%)	
CT+TT	39 (23%)	127 (77%)	0.90	74 (45%)	89 (55%)	0.66

Bold value indicates $P < 0.05$.

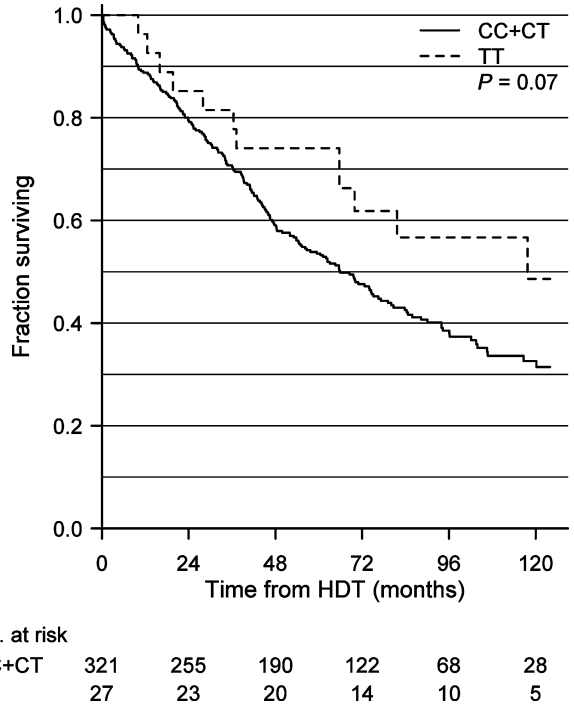

Figure 1 Overall survival for multiple myeloma patients treated with high-dose chemotherapy and stem cell support (HDT) divided into homozygous carriers of the variant T-allele and hetero- and homozygous carriers of the wild-type C-allele for the SNP rs6535455 in the *HSPE* gene.

of the variant T-allele had a significant longer overall survival than homozygous carriers of the wild-type C-allele [hazard ratio 0.4 (95% CI 0.1–0.8), $P = 0.02$ (Table 2)]. Similar result was seen comparing homozygous carriers of the variant T-allele with all carriers of the wild-type C-allele [hazard ratio 0.3 (95% CI 0.1–0.7), $P = 0.002$].

In accordance with this finding, homozygous carriers of the variant T-allele seemed to have a longer TTF after HDT than carriers of the wild-type C-allele 44.6 vs. 26.6 months, respectively. The hazard ratio was 0.7 (95% CI 0.5–1.1, $P = 0.13$). Furthermore, the percentage of patients achieving complete response after HDT seemed to be higher for homozygous carriers of the variant T-allele than carriers of the wild-type C-allele 57% vs. 39%, $P = 0.12$.

None of the three other examined SNPs in the *HSPE* gene were associated with response, time to treatment failure or overall survival after HDT.

Discussion

In multiple myeloma, HSPE is involved in myeloma cell growth, angiogenesis and osteoclastogenesis through release of angiogenic cytokines as well as shedding of syndecan-1. In this study, we have described four different SNPs in the *HSPE* gene, and one of our observations was that homozygous carriers of the variant A-allele in the SNP rs4693608 had a higher frequency of vertebral fractures at diagnosis. Moreover, we observed that homozygous carriers of the variant T-allele in the SNP rs6535455 lived longer than hetero- and homozygous carriers of the wild-type C-allele.

Osteolytic bone disease and vertebral fractures are one of the important manifestations of multiple myeloma. Animal models

have shown that enhanced *HSPE* expression by myeloma cells upregulates their spreading to bone and stimulates systemic osteoclastogenesis and osteolysis, thus mimicking the osteolytic bone disease seen in multiple myeloma. The osteolytic process is partly driven by HSPE-induced expression of receptor activator of NF-κB ligand (RANKL), while no change was seen in expression of the RANKL inhibitor osteoprotegerin (OPG) (25, 26). In bone marrow biopsies from multiple myeloma patients, a positive correlation has been observed between myeloma cells expression of HSPE and RANKL (26). Recently, a study has shown that HSPE may have an inhibitory effect on osteoblast differentiation and mineralization. HSPE shifts the differentiation from osteoblastogenesis to adipogenesis partly through increased secretion of Dickkopf1 (DKK1) by osteoblast progenitors and myeloma cells (27).

In this study, we observed that homozygous carriers of the variant A-allele in the *HSPE* gene SNP rs4693608 had a higher frequency of vertebral fractures, while no difference was observed for the degree of lytic bone disease. Previous studies have found that healthy individuals carrying the SNP rs4639608 genotype AA had a higher mRNA expression of *HSPE* than individuals with the genotype GG (3). An increased expression of HSPE in carriers of the rs4639608 genotype AA may stimulate osteoclastogenesis and osteoclast activity through RANKL activation and inhibit osteoblastogenesis thereby augment the risk of vertebral fractures as seen in this study. Even though HSPE may affect the differentiation of osteoblasts and osteoclasts, several other factors have influence in the complex process that leads to the characteristic lytic bone lesions in multiple myeloma. This may explain why no association between the rs4639608 genotype AA and the degree of lytic bone disease was seen in this study. Indeed, osteopenia and osteopenic vertebral fractures may be seen in myeloma patients without lytic bone lesions (28).

In myeloma cell line studies, high *HSPE* expression is associated with increased level of VEGF, syndecan-1, RANKL and hepatocyte growth factor (HGF), and all factors are reported to be involved in myeloma pathophysiology (15, 26, 29). Intercellular communication through exosomes is stimulated when expression of HSPE in a human myeloma cell line is enhanced or tumour cells are exposed to exogenous HSPE (30). Furthermore, through increased shedding of syndecan-1 and expression of proteases, that is, matrix metalloproteinase 9 (MMP-9), it seems as HSPE facilitates a more aggressive tumour phenotype in multiple myeloma (31). *In vivo*, a recent study has demonstrated that HSPE involvement in multiple myeloma progression may be due to downregulation of the chemokine CXCL10 (32).

In multiple myeloma, *HSPE* is primarily expressed by cells in the bone marrow microenvironment, that is, T cells, monocytes and osteoclasts and rarely by myeloma cells (10). High bone marrow HSPE expression is associated with shorter event free and overall survival after HDT, and *HSPE* mRNA expression level is higher among multiple myeloma patients than healthy controls (3, 8).

Our observation that homozygous carriers of the variant T-allele of the *HSPE* gene SNP rs6535455 had an OS nearly twice as long as carriers of the wild-type C-allele may support the hypothesis that homozygous carriers of the variant T-allele have a less aggressive or more chemo-sensitive myeloma disease due to a lower HSPE expression. Unfortunately, there is limited published data concerning the SNP rs6535455 and no information about the possible influence on HSPE expression.

More knowledge about the functionality of HSPE and the influence of SNPs on HSPE expression in multiple myeloma and other malignancies is warranted, especially, as clinical phase I studies now test the modified heparin SST0001 in patients with advanced multiple myeloma and antiheparanase compounds such as PI-88, M402 and PG545 in pancreatic cancer and hepatocellular carcinoma (33, 34).

In conclusion, our observations indicate that specific SNPs in the *HSPE* gene may affect bone morbidity and survival in multiple myeloma patients. As our results may be chance findings, our study can only indicate a possible association and support the hypothesis that SNPs in the *HSPE* gene are involved in myeloma pathophysiology. It is necessary with further experimental studies including immunohistochemistry, gene expression profiling and angiogenesis examination to explore the functional effect of SNPs in the *HSPE* gene in multiple myeloma.

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Authorship

NFA, UV and AJV designed the study. NFA, PG, HG, NA and AJV collected the data. NFA, UV, TBW and AJV were responsible for analysis and interpretation of data. NFA wrote the paper. UV, TBW, PG, HG, NA and AJV revised the manuscript critically. All authors approved the submitted and final version of the paper.

Conflict of interest

The authors disclose no conflict of interest.

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