

transplant did not influence NK cell numbers in our cohort and this is most probably due to the cross-sectional nature of the study. Having said this, our observational results indicate a potentially novel role for NK cells in HSCT for non-malignant disease and give an indication for further research in a larger cohort, minimizing heterogeneity.

Disclosure of conflicts of interest

The authors have no conflicts of interest to disclose.

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A peroxisome proliferator-activated receptor gamma (PPARG) polymorphism is associated with zoledronic acid-related osteonecrosis of the jaw in multiple myeloma patients: analysis by DMET microarray profiling

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The aminobisphosphonate zoledronic acid (ZA) is the most important antiresorptive agent for the treatment of multiple myeloma (MM)-related bone disease (BD). Osteonecrosis of the jaw (ONJ) is an important complication of ZA-treated MM patients (Vannucchi *et al*, 2005; Filleul *et al*, 2010). So far, the mechanism of ONJ pathogenesis has not been clearly

elucidated. Recently, a genetic susceptibility to ONJ has been suggested and a polymorphism of the cytochrome P450 *CYP2C8* has been associated with ZA-related ONJ in MM (Sarasquete *et al*, 2008).

To further investigate the genetic bases of ONJ, we genotyped in a case-control study a cohort of 19 MM patients treated with ZA who developed [nine cases, median age 66 years (range: 63–79)] or not [10 matched controls, median age 69 years (range: 63–84)] ONJ. We used the novel Affymetrix DMET™ plus platform (Affymetrix, Santa Clara, CA, USA), which interrogates 1936 genetic variations in 225 genes associated with phase I–II drug metabolism, disposition and transport (Deeken, 2009). The study protocol was approved by our University Hospital Bioethical Committee and informed consent was obtained from each patient. All patients received ZA according to the conventional dose and administration schedule; the ONJ group received 20 ± standard deviation (SD) 5.1 treatment courses and the control group underwent 15.1 ± 4.2 courses. MM patients were homogeneous on clinical and pathological characteristics at diagnosis and on their response to treatment. ONJ was diagnosed by clinical examination and imaging, including radiographs and/or computed tomography or magnetic resonance imaging. Peripheral blood was collected and used for DNA extraction. Genotypes were determined for each single nucleotide polymorphism (SNP) site of the 1931 of all interrogated SNPs and for the five Copy Number Variations (CNVs) included in DMET™ Plus GeneChip. Pharmacogenomic profiles were generated by Affymetrix DMET™ Console software®. Statistical analysis was performed by two-tailed Fisher's exact test. No correction for multiple comparisons was performed. Results are therefore to be interpreted as hypothesis generating.

Eight SNPs were significantly ($P \leq 0.05$) associated with ONJ occurrence. Table I shows these SNPs, the reference and variant allele and the genotype and allele frequencies. All alleles were in Hardy-Weinberg equilibrium. The four genes correlated to the eight statistically relevant SNPs were *PPARG* (peroxisome proliferator-activated receptor gamma), *ABPI* {amiloride binding protein 1 [amine oxidase (copper-containing)]}, *CHST11* [carbohydrate (chondroitin 4) sulfotransferase 11] and *CROT* (carnitine O-octanoyltransferase). The different distribution of SNP alleles and genotypes between ONJ patients and control cases are reported in Table II. The SNP rs1152003, mapping in *PPARG*, showed the strongest association with ONJ. We detected a highly significant ($P = 0.0055$) differential occurrence of the C/C homozygous (HOM) genotype in 77.7% of ONJ cases (7/9) versus only 10% of controls (1/10) (Fig 1A). Moreover, homozygous and heterozygous genotypes for the C variant were differently distributed between ONJ patients and the control group (Table II). The frequency of the C variant allele in the *PPARG* SNP underlines a highly significant association of the C allele with the ONJ group ($P = 0.0064$, Table II). No clinical association has been previously reported for these SNPs.

Table I. SNP polymorphisms associated with ONJ in MM patients.

Polymorphism Ref>Var	Effect	Ref. SNP alleles (A//B)	Genotype frequencies			Allele frequencies	
			Ref	HT	Var	A	B
<i>PPARG</i> rs 1152003	NA	C//G	8	9	2	0.658	0.342
<i>ABPI</i> _2357A>G	P545P	G//A	9	9	1	0.289	0.711
<i>ABPI</i> _4064G>A	S630S	G//A	9	9	1	0.711	0.289
<i>ABPI</i> _4107C>G	H645D	C//G	9	9	1	0.711	0.289
<i>CHST11</i> _> (rs2463437)	NA	G//A	7	11	1	0.342	0.658
<i>CHST11</i> _> (rs903247)	NA	C//T	7	10	2	0.368	0.632
<i>CHST11</i> rs2468110	NA	G//A	7	10	2	0.632	0.368
<i>CROT</i> _73879> (rs2097937)	NA	G//A	12	7	0	0.184	0.816

Distribution data for eight SNPs associated with ONJ in MM patients. Polymorphisms are reported as Reference (Ref), Heterozygosity (HT) and Variant (Var). Allele frequencies were calculated in the population included in the study using the Hardy-Weinberg equation.

Direct nucleotide sequencing was carried out on patient specimens to further confirm the presence of genetic variations, using an Applied Biosystems ABI 3100 Genetic Analyser. We found a concordance rate of 100% between DMET genotyping and sequence analysis (Fig 1B).

The rs1152003 SNP maps in the 3'UTR region of *PPARG*, at position 12477055 of chromosome 3 (Genome Build 37.1). Although no clinical correlation has been reported for the rs1152003 variant, polymorphisms in *PPARG* have been associated with increased risk of a variety of diseases (Dallongeville *et al*, 2009). *PPARG* is located in the human chromosome 3, band 3p25. Chromosomal abnormalities, such as 3p deletion, have been identified in several hematologic malignancies. *PPARG* is involved in adipocyte differentiation and in angiogenesis (Rosen & Spiegelman, 2001). Recently, the *PPARG* pathway has been recognized as key mechanism for bone remodelling. It acts on mesenchymal stem cell differentiation by increasing adipogenesis but also inhibiting osteoblast and osteoclast formation. Moreover, *PPARG* polymorphisms correlate with the bone mass density (Ackert-Bicknell *et al*, 2008). However, a recent study on a wide cohort of Korean individuals, with idiopathic, steroid-induced or alcohol-induced osteonecrosis of the femoral head, failed to demonstrate a significant correlation with three common *PPARG* polymorphisms (Kim *et al*, 2007). Interestingly, modulation of *PPARG* activity within the bone marrow microenvironment has been recently shown to interfere with cytokines such as IL6, which is involved with a central role in the pathogenesis of MM (Wang *et al*, 2004), suggesting also that *PPARG* may represent a valuable therapeutic target in MM (Garcia-Bates *et al*, 2008).

The present study also showed that three SNPs identified in *ABPI* were associated with ONJ and were in linkage disequilibrium (data not shown). *ABPI* encodes a membrane

Table II. Allele and genotype frequencies of polymorphisms in MM patients.

SNP and variants	Gene	Allele distribution			Genotype	Genotype distribution		P	Clinical association
		Cases	Controls	P		Cases (%)	Controls (%)		
rs 1152003									
C	<i>PPARG</i>	16/18	9/20	0.0064	CC	7/9 (77.7)	1/10 (10.0)	0.0055	Unknown
G		2/18	11/20		CG	2/9 (22.2)	7/10 (70.0)		
					GG	0/9 (00.0)	2/10 (20.0)		
rs10893									
G	<i>ABP1</i>	2/18	9/20	0.0327	AA	7/9 (77.7)	2/10 (20.0)	0.023	Unknown
A		16/18	11/20		AG	2/9 (22.2)	7/10 (70.0)		
					GG	0/9 (00.0)	1/10 (10.0)		
rs4725373									
G	<i>ABP1</i>	16/18	11/20	0.0327	GG	7/9 (77.7)	2/10 (20.0)	0.023	Unknown
A		2/18	9/20		AG	2/9 (22.2)	7/10 (70.0)		
					AA	0/9 (00.0)	1/10 (10.0)		
rs1049793									
C	<i>ABP1</i>	16/18	11/20	0.0327	CC	7/9 (77.7)	2/10 (20.0)	0.023	Unknown
G		2/18	9/20		CG	2/9 (22.2)	7/10 (70.0)		
					GG	0/9 (00.0)	1/10 (10.0)		
rs2463437									
G	<i>CHST11</i>	15/18	10/20	0.0434	AA	6/9 (66.6)	1/10 (10.0)	0.0198	Unknown
A		3/18	10/20		AG	3/9 (33.3)	8/10 (80.0)		
					GG	0/9 (00.0)	1/10 (10.0)		
rs903247									
C	<i>CHST11</i>	3/18	11/20	0.0205	TT	6/9 (66.6)	1/10 (10.0)	0.0198	Unknown
T		15/18	9/20		CT	3/9 (33.3)	7/10 (70.0)		
					CC	0/9 (00.0)	2/10 (20.0)		
rs2468110									
G	<i>CHST11</i>	14/18	10/20	0.1008	GG	6/9 (66.6)	1/10 (10.0)	0.0198	Unknown
A		4/18	10/20		AG	2/9 (22.2)	8/10 (80.0)		
					AA	1/9 (11.1)	1/10 (10.0)		
rs2097937									
G	<i>CROT</i>	6/18	1/20	0.0381	AG	6/9 (66.6)	1/10 (10.0)	0.0198	Unknown
A		12/18	19/20		AA	3/9 (33.3)	9/10 (90.0)		
					GG	0/9 (00.0)	0/10 (00.0)		

Distribution data for eight SNPs associated with ONJ in MM patients. Allele and genotype distribution between case and control groups. Polymorphisms are reported as rs number used in the human SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). The *P* value was calculated by two-tailed Fisher's exact test.

glycoprotein that is expressed in many epithelial and haematopoietic tissues. Moreover, a further three ONJ-associated SNPs map to *CHST11*, which was recently described as a factor required for proper chondroitin sulfation and cartilage morphogenesis. Expression of the chondroitin sulfotransferase genes is crucial for the correct mammalian bone morphogenesis. Finally, the ONJ-associated rs2097937 maps to *CROT*, whose protein is involved in the trans-esterification of acyl-CoA molecules.

Our findings indicate that genetic polymorphisms are involved in the pathogenesis of ONJ in MM patients. The highly significant association of ONJ with the rs1152003 SNP polymorphism in *PPARG* strongly suggests this genetic variant as candidate biomarker for the identification of MM patients at risk of ONJ if treated with ZA. In fact, the C/C genotype demonstrated an odds ratio of 31.5 (95% confidence interval,

2.35–422.32) for developing ONJ following ZA treatment. Differently from the recent report (Sarasquete *et al*, 2008), where the study was based on the 500K Affymetrix high density array, we used the DMET platform that interrogates only highly selective SNPs associated with drug toxicity and has the advantage of avoiding an extremely high number of comparisons, which requires statistical corrections and large patient cohorts. We propose the rs1152003 C/C genotype as a candidate genetic biomarker for ONJ, which warrants validation in larger series.

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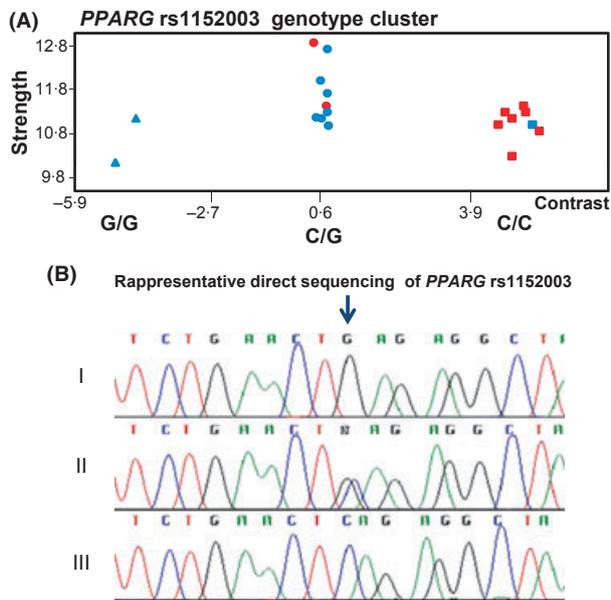


Fig 1. SNP rs1152003 genotype clustering of MM patients. (A) The red colour symbols are MM patients with ONJ. Blue colour symbols are matched MM control patients. Genotypes are identified as homozygote reference allele (▲), heterozygote (●) and homozygote variant allele (■). (B) Representative electropherogram of direct sequencing analysis of a homozygote reference allele (I), heterozygote (II) and homozygote variant allele (III). The variant rs1152003 in PPARG gene was analyzed using the following PCR primers: Forward: 5'-TCCTTCTGCAAGGCAGT'TTT-3' and Reverse: 5'-CACGCCTTCAGGGAAGCTTAG-3'. The black arrow indicates the G, C or G/C sequences.

Author contributions

M.T.D., P.S.T. and P.F.T. designed the study and performed data interpretation; M.T.D. wrote the manuscript; M.T.D and M.A. generated datasets. M.T.D. provided statistical analysis and generated figures; P.H.G., P.V. and M.C. performed data

mining; E.P., T.P., I.C., T.C. and M.R. provided blood samples and clinical data; E.L. and F.B. performed sequence analysis; P.S.T. and P.F.T. reviewed the manuscript.

Disclosures

The authors declare no conflicts of interest to disclose.

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Phase II randomized trial of bevacizumab *versus* bevacizumab and thalidomide for relapsed/refractory multiple myeloma: a California Cancer Consortium trial

Vascular endothelial growth factor (VEGF) is upregulated in multiple myeloma (MM), and circulating VEGF levels may correlate with response to therapy (Hideshima *et al*, 2005; Pittini *et al*, 2002). Thalidomide has been part of the standard treatment for MM and is thought to inhibit VEGF-associated angiogenesis (Du *et al*, 2004). Bevacizumab, a monoclonal antibody directed against VEGF-A, inhibits VEGF (Jenab-Wolcott & Giantonio, 2009). Accordingly, we set out to test the efficacy and safety of bevacizumab alone and in combination with thalidomide in MM patients.

A phase II prospective randomized/stratified trial led by the California Cancer Consortium, and including the University of Chicago, was approved by the Cancer Therapy Evaluation Program/National Cancer Institute of the National Institutes of Health (N01-CM-62209). Patients with prior thalidomide exposure received bevacizumab alone (Arm A). Thalidomide-naïve patients were randomized to either arm B (bevacizumab alone) or C (combination therapy). The study was closed early due to poor accrual, attributable to competing trials providing access to lenalidomide and bortezomib (Knight, 2005; Lu *et al*, 2009; Moschetta *et al*, 2010).

The primary objectives were response rate, event-free survival, and toxicity. The secondary objective was to measure markers of angiogenesis and assess any correlation with outcome. Immunohistochemical (IHC) staining of VEGF (VG-1; Neomarkers, Fremont, CA, USA) and its two receptors, VEGFR1/Flt-1 (AB-1; Neomarkers) and VEGFR2/KDR (AB-1; Neomarkers) was carried out on bone marrow clots or cores obtained at baseline.

The study was conducted between October 2001 and November 2004. Patients aged 18 years or older, with relapsed/progressive MM and a Karnofsky performance status (KPS) >60% were enrolled. All patients signed a voluntary informed consent form, approved by the institutional review boards of the participating institutions.

Bevacizumab was given at 10 mg/kg intravenously over a 90-min period every 14 d. Thalidomide was escalated from

100 mg/d by 100 mg/week, up to 400 mg/d. Treatment cycle length was 56 d. Treatment was discontinued due to disease progression, development of grade 3 or 4 toxicities that did not resolve to grade 1 or less (maximum 3 weeks' delay was allowed), non-compliance, or patient request or physician discretion. The National Cancer Institute's Common Toxicity Criteria version 2.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf) was used for toxicity and adverse event reporting.

Complete response was defined as disappearance of the paraprotein in the serum and/or urine by immunofixation and <5% plasma cells on bone marrow evaluation. A partial remission was defined as a $\geq 50\%$ reduction but still detectable level of paraprotein, and if present, a $\geq 50\%$ reduction in urine M-component. Stable disease was defined as <50% reduction in paraprotein, or if the patient had light-chain disease only, a >50% reduction in the urine M-component (Bence-Jones protein). Progressive disease was defined as a 25% increase in paraprotein from the lowest level observed, measured on at least two separate occasions 2 weeks apart. We defined event-free survival (EFS) as synonymous with time to treatment failure (TTF) to avoid reporting artificially long progression-free survival in patients who declined further protocol therapy prior to progression. TTF was therefore defined as the time from the first day of treatment to the first observation of disease progression, death, or treatment cessation due to toxicity or patient refusal.

Fourteen patients consented; one withdrew prior to initiation of treatment, and another became ineligible due to a drop in KPS. Twelve evaluable patients, eight female, four male, (median age: 58 years, range: 50–75) were enrolled; six received bevacizumab alone (Arms A or B); six received combination therapy (Arm C). Eight of the patients were enrolled with stage III disease (Durie & Salmon, 1975) and two each with stages I and II. The median β_2 microglobulin was 2.7 mg/l (range 1.0–9.9 mg/l) with nine cases of IgG κ , two patients with IgG λ , and one case of non-secretory myeloma. Previous treatments