Pharmacogenetic studies in multiple myeloma

Sophie Corthals

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Aan mijn ouders

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Chapter 1

General introduction

Multiple myeloma

Multiple myeloma (MM) is a malignant plasma cell disorder that accounts for approximately 10% of all hematologic cancers.[1-2] MM is characterized by clonal proliferation of malignant plasma cells in the bone marrow, which secrete a homogeneous immunoglobulin product known as monoclonal (M) protein or paraprotein. Typical features of MM include osteolytic bone lesions, renal disease, anemia, hypercalcemia and immunodeficiency.[3]

The pathological development of MM is a multistep process and starts with the emergence of an asymptomatic premalignant stage of clonal plasma cell proliferation known as "monoclonal gammopathy of undetermined significance" (MGUS), occurring in about 3% of individuals above the age of 50. MGUS cells secrete monoclonal immunoglobulin (Ig) which may progress to smouldering MM and ultimately to symptomatic intramedullary and extramedullary multiple myeloma, or plasma cell leukemia; expressing the same Ig. Smouldering MM has a stable intramedullary tumor cell content of >10%, but no osteolytic lesions or other complications of malignant MM. Patients with MGUS have a risk to progress to myeloma or a related malignancy at a rate of 1% a year.[4-5] The prevalence of both MGUS and MM increases markedly with age, and is slightly more common in men than in women. The incidence is about two-fold higher in African Americans than in Caucasians. The median length of survival after diagnosis is approximately 3-5 years.

Diagnosis

Diagnostic criteria for MM require the presence of at least 10% plasma cells in bone marrow and a monoclonal Ig protein (M-protein; usually > 30 g/L) in the serum or urine. In addition, hypercalcemia, renal insufficiency, anemia and lytic bone lesions may be present.

The clinical presentation of MM varies depending on disease stage. The Durie-Salmon (DS) staging system has been the most commonly used staging system for patients with MM since 1975.[6] More recently, the International Myeloma Working Group proposed the International Staging System (ISS),[7-8] which correlates clinical features with survival. This staging system divides patients into three stages, based on serum β_2-microglobulin and albumin levels; Stage 1: β_2-microglobulin concentration < 3.5 μg/mL and albumin concentration ≥ 3.5 g/dL; Stage II: β_2-microglobulin concentration < 3.5 μg/mL and albumin concentration < 3.5 g/dL, or 3.5-5.5 μg/mL; or Stage III: β_2-microglobulin concentration ≥ 5.5 μg/mL. The ISS was validated and demonstrated a median survival of 62 months, 44 months and 29 months for respectively stage I, II, and III.

Molecular genetics of MM

MM is characterized by profound genetic instability, leading to a distinctive combination of gains and losses of whole chromosomes, non-random chromosomal translocations and point mutations. Chromosomal translocations are early events in disease progression and seem to play an important role in the genetic pathogenesis of MM.

Based on cytogenetics, multiple myeloma can be subdivided into two groups; hyperdiploid and non-hyperdiploid.[9] A hyperdiploid karyotype is present in approximately 60% of MM patients and is characterized by trisomies of odd-numbered chromosomes including 3, 5, 7, 9, 11, 15, 19 and 21. Patients with hyperdiploid MM tend to have a better prognosis than those with non-hyperdiploid disease.[10]

Many B cell tumors, including multiple myeloma, are characterized by the presence of chromosomal translocations that are mediated by errors in one of the three B cell DNA-modification mechanisms. These include immunoglobulin heavy chain (IgH) switch recombination, somatic hypermutation and VDJ recombination.[11] These translocations results in dysregulation or increased expression of an oncogene that is positioned near a strong Ig enhancer. The IgH translocations involve several recurrent chromosomal loci, including 11q13 (cyclin D1), 6p21 (cyclin D3), 4p16 (FGFR3 and MMSET), 16q23 (MAF) and 20q11 (MAFB). Primary translocations occur early in pathogenesis, whereas secondary translocations are involved in progression of MM. The oncogene MYC is involved in secondary translocations. These translocations are not mediated by B cell DNA-modification mechanisms, which are not active in healthy or tumor plasma cells. In addition, other IgH translocation partners have been identified in approximately 15% of MM patients.[12-13]

In addition to hyperdiploidy/non-hyperdiploidy and chromosomal translocations; gains and losses of specific chromosomal regions occur in all MM patients. The most common abnormality observed is a deletion of chromosome 13, which is seen in more than 50% of MM cases and is an early event in MM pathogenesis. Such aberrations of chromosome 13 are detectable in all stages of myeloma, and normally involve large segments or the entire long arm, however small interstitial deletions have also been described.[14] Chromosomal gains that recur in more than 30% of MM patients include 1q, 3q, 9q, 11q and 15q.

The bone marrow microenvironment

MM is a bone marrow disorder in which malignant monoclonal B cells differentiate into plasma cells. The pathogenesis of MM is not exclusively determined by the genetic background of the plasma cells. In addition, the interaction of MM plasma cells with the bone marrow microenvironment is also very important. The bone marrow microenvironment consists of the extracellular matrix, and five types of bone marrow stromal cells (BMSCs): fibroblastic stromal cells, osteoblasts, osteoclasts, vascular endothelial cells and lymphocytes. The proliferation, differentiation, and function of MM plasma cells is regulated by reciprocal positive and negative interactions among these cells, which are mediated by a variety of cytokines, receptors and adhesion molecules. BMSCs secrete factors including interleukin 6 (IL-6), insulin-like growth factor 1 (IGF1), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 alpha (SDF1α), and tumor necrosis factor alpha (TNFα). The interaction of MM plasma cells with the extracellular matrix, accessory cells, and secreted cytokines results in the activation of signaling pathways that mediate growth, survival, drug resistance, the migration of MM cells, as well as osteoclastogenesis and angiogenesis.

Multiple myeloma treatment

In spite of major advances in the treatment of MM, the disease inevitably relapses due to the acquisition of drug resistance. The first standard of care in the 1960s was a melphalan/prednisone based regimen as palliative treatment of MM. With this regimen, complete response (CR) rates of approximately 5% were achieved. In the early eighties, high-dose melphalan (HDM), followed by reinfusion of autologous stem cells was introduced and median survival improved to approximately 5 years.[15] The combination of vincristine, doxorubicin (adriamycin), and dexamethasone (VAD) as induction treatment to reduce tumor burden was also widely used, but has now been replaced by novel agents.[16] Dexamethasone alone appeared to be an alternative, since this regimen is probably largely responsible for the effect of VAD.[17] In search for the optimal treatment for MM, several new agents that target myeloma plasma cells and may overcome resistance from conventional agents have been developed including thalidomide,[18] bortezomib,[19] and lenalidomide.[20] These compounds have shown promising activity and tolerability in newly diagnosed myeloma patients, as well as those with relapsed and/or refractory disease.

Thalidomide

Thalidomide was initially introduced in the late fifties as a sedative-hypnotic drug. Despite its withdrawal from the market after its severe teratogenicity was recognized, thalidomide was found to be highly effective in a whole range of dermatological, gastro-intestinal and inflammatory diseases, and reappeared in treatment regimens after its potent anti-myeloma effect was recognized. The drug is tested on many malignant tumors, including leprosy, Behçet syndrome, graft versus host disease and aphthosis in HIV positive patients.

Thalidomide (α-N-phthalimido-glutarimide) is a synthetic derivative of glutamic acid and has been used for cancer treatment because of its anti-angiogenic activity. The introduction of thalidomide in 1999 has revolutionized clinical management of patients with myeloma. Thalidomide treatment has been extensively studied in patients with newly diagnosed, relapsed and/or refractory MM and has achieved response rates of 30% at relapse and even higher rates at presentation.[21]

The exact mechanism of action in MM remains to be elucidated, and is still an active area of research. Thalidomide has the ability to inhibit TNFα production by activated human monocytes.[22]

Apart from this anti-inflammatory property, thalidomide inhibits levels of other cytokines; IL-1β, IL-6,[23] VEGF,[24] beta fibroblast growth factor (βFGF),[25-26] hepatocyte growth factor (HGF),[27] and granulocyte macrophage-colony stimulating factor (GM-CSF), and in addition upregulates the level of intercellular adhesion molecule 1 (ICAM1),[28] vascular cell adhesion molecule 1 (VCAM1), IL-10[29-30] and IL-12.[31]

Survival of myeloma plasma cells is dependent on their interactions with the bone marrow microenvironment. Disruption of these interactions by thalidomide plays a major role in their anti-myeloma activity, which is mediated by the anti-inflammatory, anti-angiogenic,[32] and immunomodulatory properties of the drug including T cell costimulation and activation of NK cells[33] and the ability to modulate the production of cytokines and adhesion molecules.

Thalidomide therapy is however associated with toxic side effects including; constipation, peripheral neuropathy, fatigue, rash and especially venous thromboembolism.

Thalidomide analogues, immunomodulatory drugs (IMiDs), have been synthesized to optimize both anti-TNFα and anti-angiogenic effects, while reducing toxic side effects. Two IMiDs that are currently in advanced-phase clinical trials are lenalidomide (CC-5013; Revlimid) and pomalidomide (CC-4047; Actimid).

Lenalidomide

Lenalidomide (Revlimid; formerly called CC-5013), is a derivative of thalidomide and belongs to the class of immunomodulatory drugs. Despite the similar chemical structures of thalidomide and lenalidomide, the toxic profile is different for both novel agents, and lenalidomide is better tolerated in patients than thalidomide. Moreover, lenalidomide has shown to be significantly more potent in terms of both anti-angiogenic and anti-TNFα activity compared to thalidomide.[20] Lenalidomide has the ability to induce growth arrest or apoptosis in drug-resistant myeloma cell lines, abrogate myeloma cell adhesion to bone marrow stromal cells, and modulate cytokines that promote the growth, survival, and drug resistance of myeloma cells.[34-35]

The most common major adverse event associated with lenalidomide is myelosuppression; mainly neutropenia and thrombocytopenia, which are manageable by dose reduction and growth factor support.[20,36] Lenalidomide; in combination with dexamethasone, is associated with greater risk of venous thromboembolism and anticoagulant prophylaxis is mandatory. Importantly, common adverse events such as sedation, constipation, and neuropathy are limited.

Bortezomib

Bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA, formerly known as PS-341) is a cobalt containing small molecule which specifically inhibits the 26S proteasome. The ubiquitin-proteasome pathway is responsible for the degradation of cellular proteins in a controlled fashion. Inhibition of this pathway leads to disruption of the protein regulation involved in cell cycle control, cell growth, angiogenesis and apoptosis, which eventually results in cell cycle arrest and apoptosis of myeloma cells.[37] As proteasomes are present in all eukaryotic cells, bortezomib has potential as a chemotherapeutic agent in many different tumor types; more specifically it demonstrates a number of MM specific effects. One central mechanism by which bortezomib functions is that it blocks the activation of transcription factor nuclear factor kappa B (NFκB), by inhibiting the breakdown of inhibitory kappa B (IκB), subsequently inactivating multiple downstream pathways known to be important in MM cell signaling.[38] As a result of NFκB inhibition, the adhesion of the myeloma plasma cells to BMSCs is decreased, thus increasing sensitivity to apoptosis and inhibiting paracrine-mediated growth of MM cells.[39] In addition to the inhibition of NFκB, MM specific effects include inhibition of angiogenesis, inhibition of DNA repair by cleaving DNA repair enzymes, and impairment of osteoclast activity.[40] Bortezomib induced apoptosis of MM cells is also associated with caspase 8, 9 and caspase 3 activation.[41]

Bortezomib was introduced in MM treatment after demonstrating striking anti-myeloma activity in a phase I trial.[42] This observation, together with preclinical evidence of anti-myeloma activity, provided the rationale for a phase II trial in relapse and/or refractory MM. This led to the approval of bortezomib by the FDA in 2003. Bortezomib has also been proven effective in the treatment of newly diagnosed MM patients, and has greatly improved the management of MM. Clinical trials with bortezomib in relapsed and/or refractory MM have shown high response rates and a survival advantage.[43-45] Subsequent to this, in newly presenting patients, bortezomib combinations have been shown to be associated with excellent complete response (CR) and very good partial response (VGPR) rates.[46-49] Despite this, patients treated with bortezomib also encounter significant toxicity. The most common adverse events are fatigue, weakness, herpes zoster reactivation, gastrointestinal disturbances (including nausea, vomiting, diarrhea, and constipation), thrombocytopenia, and peripheral neuropathy.[44] In addition to MM, bortezomib is also used in the treatment of non-hematological malignancies.[50]

Complications of multiple myeloma treatment with novel agents

Peripheral neuropathy

Peripheral neuropathy (PN) is defined as degeneration of nerves and occurs frequently in plasma cell disorders (MGUS, MM, Waldenstroms disease, POEMS syndrome and AL-amyloidosis). Although PN can be associated with the underlying disease itself, it can also be related to treatment. PN can involve sensory, motor or autonomic nerve fibers. Assessment of PN is based on clinical and elctrophysiological examination, and is usually graded using the Common Toxicity Criteria of the National Cancer Institute (NCI-CTC version 3.0). Published research has shown the presence of PN in up to 54% of newly diagnosed patients.[51-53] Accurate detection of PN is however influenced by several factors including; the neurotoxicity grading scale used, patient selection, dosing, schedule and duration of treatment, co-medication and co-morbidities.

PN is the most frequent and disabling non-hematological side effect from the use of thalidomide and bortezomib in MM patients. Clinical presentation of thalidomide induced PN (TiPN) and bortezomib induced PN (BiPN) differs substantially.

TiPN has an incidence rate ranging from 25% to 75%. TiPN is a predominantly sensory axonal neuropathy affecting large and small fibers,[54-55] but has also been classified as ganglionopathy.[55-56] Sensory PN is characterized by symmetric hypesthesia (numbness), paresthesias (tingling) or hypesthesia of fingers and toes. Reduction in amplitude or absence of sensory nerve action potentials is the most common electrophysiological alteration that can precede or worsen symptoms after thalidomide withdrawal, and often does not resolve.[57-58] Motor PN occurs less frequently than sensory PN, but can still complicate treatment. The nerve damage caused by thalidomide is irreversible, and mostly occurs within months. The dosing and duration of thalidomide treatment seem to be critical in the development of TiPN. Immediate dose reduction or withdrawal of thalidomide as soon as signs of TiPN develop is recommended.

BiPN is typically sensory and characterized by burning pain, distal paresthesias, hyperesthesia, and hypoesthesia.[59-60] The burning pain is caused by damage to small fibers.[61] Electrophysiological examination reveals low amplitude of sensory action potentials. In the majority of patients, BiPN is reversible and does not seem to be influenced by type or number of previous treatment.[62] BiPN typically occurs after 3 months, reaches a plateau after 5-6 cycles, and does not appear to increase. In relapsed and/or refractory MM, BiPN has been observed in 37%; 22% of which were grade 1-2, 13% were grade 3 and 1 % were grade 4.[19,43-44] In newly diagnosed MM patients, BiPN may affect 47% of patients with up to 16% grade 3-4.[46] Motor PN occurs less frequently after bortezomib treatment, and often follows sensory PN.

Although less frequent and less severe, PN has also been observed by lenalidomide, pomalidomide and later generation proteasome inhibitors, however such data is limited.[63-65]

Risk factors that may predict the occurrence of PN include the presence of PN at baseline, alcohol abuse, diabetes mellitus, vitamin deficiencies and viral infections. No effective treatment of TiPN and BiPN is currently available and therefore prevention of severe PN by close monitoring and dose reduction defines the standard of care.

Venous thromboembolism

Many malignancies, including multiple myeloma, are associated with an increased risk for venous thromboembolism (VTE). VTE includes pulmonary embolism (PE) and deep venous thrombosis (DVT). One of the major contributing factors to the risk of VTE is treatment with immunomodulatory agents; including thalidomide. In addition, disease stage, the type of chemotherapy combination, and the supportive therapy play a role in the development of VTE following thalidomide exposure. Other factors that contribute to VTE risk include mobility and performance status of the patient.

VTE can be associated with MM itself, and there appears to be background rate of 5-10%.[66-68] In patients treated with thalidomide alone, or in combination with other agents such as anthracyclines and dexamethasone,[69-70] VTE rates increased to 10-15%.[67,71-72] In contrast, is has been reported that VTE rates decrease following bortezomib treatment.[48,73-76] VTE occurs early after the initiation of thalidomide treatment.

The mechanism underlying thalidomide related VTE is not known, however, thalidomide regulates the level of cyclooxygenase 2 (COX2), a well described prothrombotic factor. In addition, thalidomide may also influence VTE risk by modulating cytokine levels acting on the endothelial cell. This mechanism depends on the differential apoptotic effects of thalidomide in myeloma plasma cells compared with endothelial cells, which are protected from apoptosis by a decrease in VEGF following thalidomide exposure.[77-78] Another biological effect of thalidomide that may promote VTE is restoration of endothelial cell protease activated receptor 1 (PAR1) expression after damage from doxorubicin, which is often used in combination with thalidomide.[79]

Given the risk of thalidomide associated VTE, patients receiving thalidomide treatment may benefit from thrombosis prophylaxis. Different strategies have been taken ranging from identification of high-risk patients suitable for prophylaxis to prophylactic anticoagulation for all patients.[80]

Pharmacogenetics

The term pharmacogenetics was first introduced in 1959. In pharmacogenetics, the role of a patient's individual genetic variability on the activity, toxicity or kinetics of a particular drug is studied. The genetic profile of a patient is an important cause for this interindividual variation in drug response. Differences in genetic profiles are often due to inherited single nucleotide polymorphisms (SNPs). These SNPs may be located in enzymes involved in drug metabolism, such as the cytochrome P450 enzymes and ATP-binding cassette (ABC) transporters, and genes coding for drug targets. However, not only drug metabolizing enzymes play a role in drug response, genes involved in mechanisms as inflammation, immunity, DNA repair and apoptosis may contribute to drug response. The interindividual differences may be caused by a difference in concentrations of the intended compound at the intended site of action, the pharmacodynamics of a drug. On the other hand, interindividual differences in drug response can be due to differential pharmacokinetics, which includes absorption, distribution, metabolism, and excretion of the drug. Pharmacogenetics will allow adapting a treatment to the genetic profile of a patient.

Genetic variation

Polymorphism (from Greek: poly "many", morph "form") refers to a variation in the DNA sequence among individuals. Polymorphisms which have a population frequency of 1% or more are considered to be common polymorphisms. Genetic polymorphism ranges from single nucleotide changes in the DNA (SNPs), small insertions and deletions of a number of nucleotides, through insertions, deletions, and duplications of large segments of DNA, to translocation of chromosomal segments and even changes in chromosomal number. Although rare genetic variants exist, most of the variation in the human population is attributable to common variants (alleles); SNPs.

Single nucleotide polymorphisms

A SNP is defined as a common DNA sequence variation occurring when a single nucleotide (A, T, C, or G) in the genome differs between individuals (or between paired chromosomes in an individual). SNPs arise due to mutation, normally due to a misincorporation of a nucleotide during replication, or by chemical or physical mutagenesis. Throughout the genome, SNPs occur every 1000-3000 base pairs and have been identified in 93% of all known genes.[81]

Almost all common SNPs have only two variants (alleles). The major allele is the allele found at highest frequency, and the lower frequency allele is referred to as the minor allele. The minor allele frequency (MAF) refers to the frequency at which the less common allele of the SNP occurs in a particular population. The MAF varies with ethnicity and gender due to demographic influences undergone by the population such as migration, genetic drift, and population specific selection. Disease status also affects variability in the frequency of SNPs. It is estimated that there are around 11 million common, > 1% MAF SNPs in the human genome and ~7 million with MAF > 5%.[82]

SNPs can be present in coding, non-coding or intergenic regions. If located in a coding sequence, they can change amino acid sequence (nonsynonymous SNPs; Figure 1A) with correspondent change to protein function to be missense (replacing one amino acid with another), or nonsense (producing an aberrant stop codon). When the amino acid sequence is not changed by a SNP, it is a synonymous or silent variant (Figure 1B). Both nonsynonymous and synonymous SNPs may cause a change in protein structure and function, the level of protein expression, or might influence the assembly of the final messenger RNA template from which the protein is synthesized (alternative splicing). They can also influence promoter activity or the ability of a protein to bind its substrate. Therefore, SNPs may contribute to altered pharmacodynamics and pharmacokinetics, consequently altering the response to therapy and development of adverse reactions. Identification of SNPs that are linked to or contribute to individual drug response variability, may therefore allow the design of personalized regimens based upon genotype.

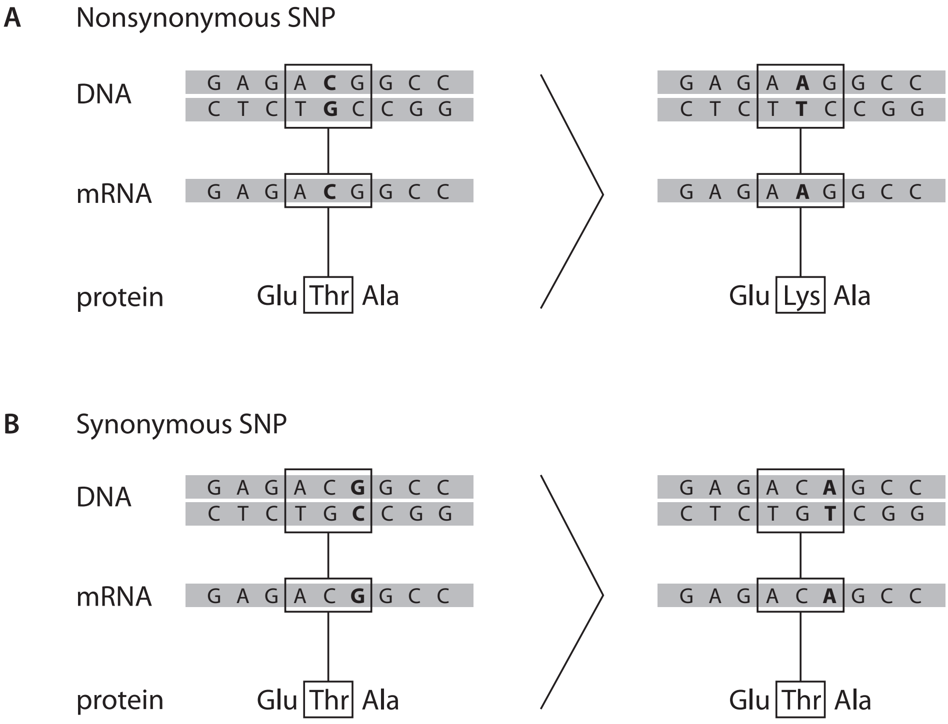

Figure 1. Schematic overview of SNP types. (A) A change of a single nucleotide leads to an amino acid change. The C and A nucleotide are transcribed to mRNA. In the protein, ACG is translated into a Threonine amino acid whereas AAG is translated into a Lysine amino acid. (B) A change of a single nucleotide does not lead to an amino acid change. The G and A nucleotide are transcribed to mRNA. ACG and ACA are both translated into a Threonine amino acid.

Strategies for SNP analysis

To study the association between genetic variation and treatment related toxicity or treatment outcome, different approaches can be used including a candidate gene approach and a genome-wide approach.

The most common approach employed to study associations between a genetic variant and a disease has been to examine one or more candidate genes based on a hypothesis-driven strategy. This strategy requires knowledge of the biological basis of the disease or trait, along with some knowledge of the function of the gene or genes involved.

The genome-wide approach is free of any hypothesis regarding which genes are involved. With the improvement of genotyping technologies and the exponentially growing number of SNPs, genome-wide association analyses have become a useful tool. A genome-wide association analysis makes use of the knowledge that the human genome has a haplotype structure.[83] Haplotypes are particular combinations of SNPs observed together on a chromosome that are inherited together as a unit. These SNPs segregate together more often than expected, and are said to be in linkage disequilibrium (LD). LD is dependent on allele frequencies, as well as recombination. It is therefore only necessary to genotype a small number of SNPs from a particular haplotype, which should provide the information about the other SNPs within this haplotype. In addition, 'tag SNPs' are SNPs in high LD that represent a certain region of the genome, and are therefore useful in genome-wide SNP association studies. Still, several hundred thousand SNPs are analyzed in this approach to gather genome-wide SNP information. One of the advantages of the genome-wide approach is that it enables the detection of the contributions of novel or less obvious genes.

Bank On A Cure SNP panel

The Bank On A Cure (BOAC) SNP panel was designed to examine the association of genetic variations with disease risk and outcome in multiple myeloma.[84] DNA from multiple cooperative groups was banked and genotyped using this custom SNP panel. The panel comprises 3404 SNPs in 964 genes, selected using a candidate gene approach. Pertinent candidate genes were selected by myeloma experts in the International Myeloma Foundation consortium. An initial list was supplemented with referencing established pathway databases,[85-87] generating a candidate gene list spanning 67 molecular pathways important in the biology of myeloma, treatment response, and side effects to conventional and novel agents (Table 1). A literature search[88] was conducted to identify SNPs that had been previously reported as having a functional consequence or relevance in prior etiologic or treatment outcome studies. The following criteria were used to select SNPs; having a MAF greater than 2%, from the candidate gene list, nonsynonymous SNPs present in dbSNP/SNP 500,[89] promoter variants present in homologous regions between human and mouse, in or adjacent to a transcription binding site using the Promolign database,[90] and promoter SNPs identified in the Functional Element SNPs Database (FESD).[91] Tag SNPs in genes considered to be of particular relevance along with population discriminating admixture variants form the X chromosome.[92] Finally, all nonsynonymous SNPs with a MAF greater than 2% in phosphatase, kinase and transferase genes present in the dbSNP database were included.

Table 1. Functional categories on the BOAC custom-built SNP panel.

Functional category	**No. of genes**	**No. of SNPs**
ADME/DMET	130	445
Cancer	406	1558
Carbohydrate Metabolism	69	384
Cell Cycle	230	867
Cell Death	433	1662
Cell Signaling	90	352
Cell-To-Cell Signaling and Interaction	248	880
Cellular Growth and Proliferation	420	1451
Cellular Movement	227	923
DNA Replication, Recombination, and Repair	204	854
Drug Metabolism	20	114
Gene Expression	240	951
Hematological Disease	223	876
Immune Response	247	985
Lipid Metabolism	146	664
Molecular Transport	170	708
Nucleic Acid Metabolism	30	161
Skeletal and Muscular Disorders	64	289
Skeletal and Muscular System Development and Function	77	278
Signaling Kinase, Phosphatase, Transferase	198	885
Inflammation & Immunity	196	813

Table adapted from Van Ness *et al.*[84]

The 3404 pre-selected SNPs can be simultaneously identified using the Affymetrix Targeted Genotyping System, which is based on a molecular inversion probe technology (Figure 2).[93-94]

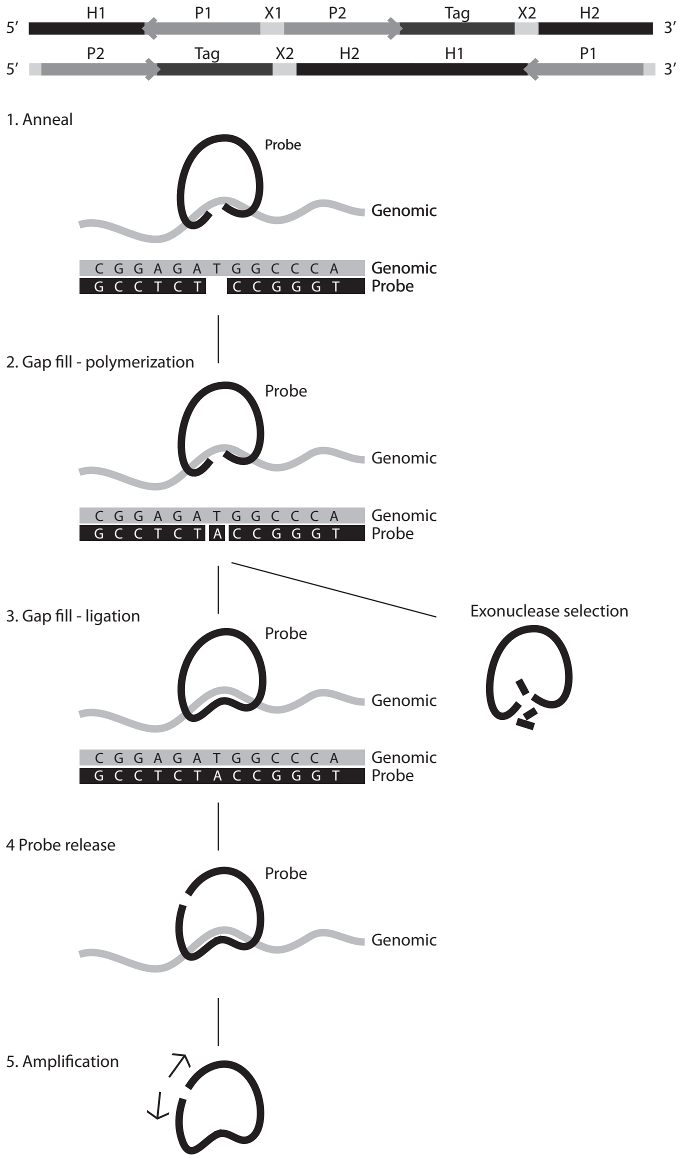

Figure 2. Molecular inversion probe technology. The top panel shows an unreacted probe (top) and an inverted probe (bottom). The probe consists of; 2 homology regions (H1 and H2) unique to each probe, 2 common PCR primer regions (P1 and P2), 1 bar code (Tag) unique for each locus, and 2 common cleavage sites (X1 and X2). The bottom panel shows the enzymatic probe inversion. (1) Genomic DNA, probes, ligase, and polymerase is heat-denatured. Homology regions H1 and H2 hybridize to complementary sites on the genomic DNA. A circular structure with a single nucleotide gap is created. (2) In four separate reactions, unlabeled dATP, dCTP, dGTP or dTTP are added respectively. DNA polymerase adds the nucleotide to the single nucleotide gap when the nucleotide is complementary. (3) DNA ligase closes the gap and a covalently closed circular molecule is formed (left) and exonucleases digest the non-complementary nucleotides and excess linear probes (right). (4) Probes are released from the genomic DNA and cleaved at the abasic site. (5) PCR amplification of probes that were circularized in the gap fill reaction.

MicroRNAs

MicroRNAs (miRNAs) are a class of small non-coding single stranded RNAs of approximately 22 nucleotides in length that are found in both plants and animals.[95] So far, more than 500 human miRNAs have been reported in literature.[96]

MiRNA biogenesis (Figure 3) starts in the nucleus where miRNAs are initially transcribed by RNA Polymerase II (Pol II) to form long primary transcripts known as primary-miRNAs (pri-miRNAs), which are capped and polyadenylated. The pri-miRNAs are processed in the nucleus by the microprocessor complex and associated factors; including the RNase II enzyme Drosha and its co-factor, Pasha (also known as DGCR8) to form a stem-loop hairpin structure, a pre-miRNA of 50-70 nucleotides, in which the miRNA itself is contained.[97-100] The pre-miRNAs are exported into the cytoplasm by the RanGTP-dependent transporter exportin 5.[101-103] Another RNAse III enzyme, Dicer, processes them further into a double stranded RNA of approximately 22 nucleotides in length that contains the mature miRNA and an oligonucleotide of the other arm of the hairpin; referred to as miRNA:miRNA* duplex.[104-107] The functional element of the duplex is determined when it is incorporated into the multiprotein RNA-induced silencing complex (miRISC) complex. Only one strand remains stably associated with miRISC, which becomes the mature miRNA.

MiRNAs primarily function as translational repressors by binding to mRNA in the 3'UTR (un-translated region) with a certain degree of complementation. The mature miRNA incorporated in the miRISC functions as guide, directing the complex to the 3'UTR of their target genes. The degree of complementation can be either perfect or imperfect, resulting in mRNA degradation or protein translation inhibition, respectively (Figure 3). The inhibition mechanism depends on several factors including the miRNA sequence, the target mRNA sequence and the exact composition of the miRISC complex.[108-109]

Because miRNAs are capable of binding their targets with imperfect complementation, each miRNA can possibly interact with a large number of genes; conversely, a single gene can harbor multiple miRNA recognition sites. This fact puts a challenge on the identification of miRNA targets and several algorithms have been developed to predict miRNA targets.[110-112] Critical in the prediction of targets is the 'seed sequence' of the miRNA, which consists of 6–7 nucleotides of the 22 nucleotides comprising a miRNA.[113] However, experimental verification is required before a gene can be considered as a genuine miRNA target.

MiRNAs are involved in critical biological processes including cellular growth and differentiation, development, and apoptosis.[95] Furthermore, miRNAs have been implicated in cancer, which is highlighted by the observation that about 50% of annotated human miRNAs is located at fragile sites across the human genome. These fragile sites are often deleted or amplified at a common breakpoint, and frequently associated with cancer.[114] This observation indicated that miRNAs might play a crucial role in cancer progression.

Large-scale miRNA expression profiling has been explored in many cancer types including hematological malignancies such as chronic lymphocytic leukemia[115] and acute myeloid leukemia.[116-117] However, little is known about the miRNA expression in MM.[118-123]

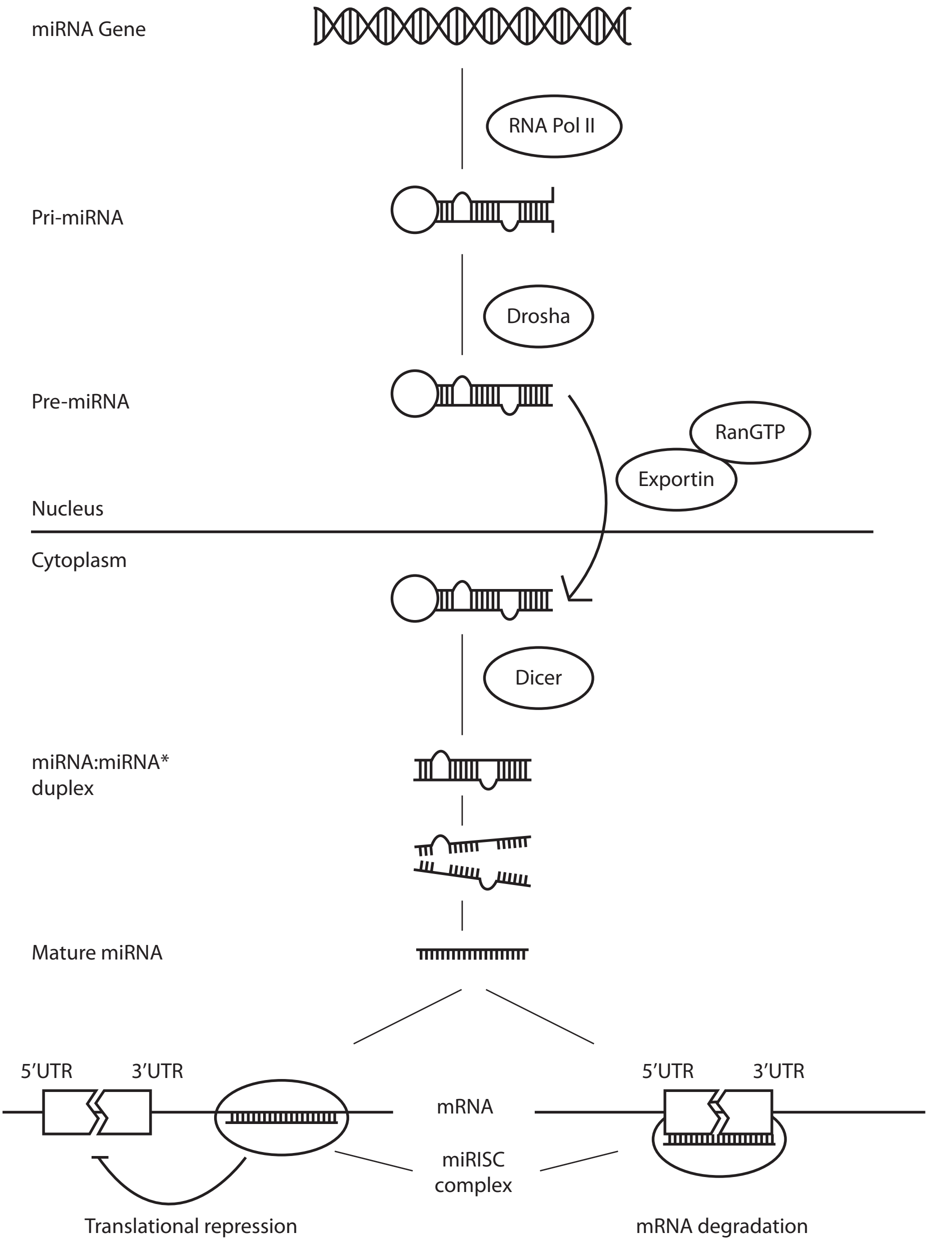

Figure 3. Schematic representation of miRNA biogenesis.

Aims and outline of the thesis

Pharmacogenetics is the study of how genetic variants affect drug response. This genetic variation, often due to SNPs, can affect a patient's response to drugs and the development of toxic side effects. Therefore, gaining better insight into the SNP profile of patients will eventually allow individualized treatment and prediction of side effects.

The thesis presents work divided into two parts. The first part (Chapters 2 through 6) focuses on SNP associations with adverse events of treatment and treatment outcome in MM patients. In **Chapter 2**, the association between inherited genetic variation and the development of MM is discussed. The introduction of novel agents, such as bortezomib and thalidomide, has revolutionized clinical management of patients with MM. However, the therapeutic use of these novel agents is accompanied by various side effects and 30% of the patients have to stop treatment prematurely. SNPs located in the genes involved in detoxification of drugs can lead to alterations in drug-metabolizing enzymes, resulting in altered pharmacokinetics of therapeutic agents, thereby influencing a patient's response to treatment and treatment related toxicity. Thalidomide treatment is associated with the development of VTE. Genetic associations with VTE in MM patients treated with thalidomide are discussed in **Chapter 3**. In Chapters 4, 5, and 6 we have focused on MM treatment with novel agents; bortezomib and thalidomide, which are accompanied by PN. In these chapters the question of whether SNPs can be used to identify patients at risk for PN, and whether SNP association analyses can provide insights in the mechanisms underlying treatment induced PN is explored. Specifically, in **Chapters 4 and 5**, genetic associations with TiPN and BiPN are discussed respectively. **Chapter 6**, discusses the differences between vincristine induced PN (ViPN) and BiPN during induction treatment in MM patients.

The second part of this thesis (Chapters 7 and 8) deals with miRNA expression in MM patients. MiRNA expression profiling is presented in **Chapter 7** as a means to gain more insight in miRNA expression patterns in MM. In **Chapter 8**, we discuss *miRNA-15a* and *miRNA-16* expression in relation to chromosome 13 deletion; a recurrent chromosomal abnormality in MM.

Finally, the results as described in this thesis and their future perspectives are discussed in **Chapter 9**.

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Chapter 2

Inherited genetic variation and the risk of developing multiple myeloma

David C. Johnson, Pieter Sonneveld, Sophie L. Corthals,
Faith E. Davies, Christine Ramos, John D. Shaughnessy Jr,
Brian A. Walker, Walter M. Gregory, Majda Haznadar,
David Gonzalez, André G. Uitterlinden, Henk M. Lokhorst,
Brian G.M. Durie, Bart Barlogie, Brian Van Ness,
Dalsu Baris, Gareth J. Morgan

Submitted

Abstract

We have performed an expanded candidate gene study by combining genotyping data from 2595 presenting myeloma cases with 8974 matched population controls across three cross validating cohorts of European origin from the UK, US and The Netherlands. Strong associations were found with nonsynonymous SNPs in *FCRL5* (rs6679793; odds ratio (OR) = 1.54, $P = 4.2 \times 10^{-14}$); *CYP2C19* (rs3758581; OR = 2.02, $P = 3.08 \times 10^{-9}$); *CAMKK2* (rs1132780; OR = 1.56, $P = 8.58 \times 10^{-8}$) and *SELP* (rs6127; OR = 1.26, $P = 9.5 \times 10^{-7}$). We also see associations with tagging SNPs across potential regulatory regions in *NFATC1* (rs4799055; OR = 0.77, $P = 6.93 \times 10^{-13}$); *CYP1A2* (rs2472304; OR = 1.26, $P = 3.40 \times 10^{-8}$); *IFNGR2*, (rs8131980; OR = 1.24, $P = 4.23 \times 10^{-7}$); *GSTA4* (rs7496; OR = 1.59, $P = 6.33 \times 10^{-6}$); and *TNFRSF17* (rs3743591; OR = 2.92, $P = 1.72 \times 10^{-11}$). These data provide evidence of an underlying genetic susceptibility to multiple myeloma disease.

Introduction

Approaches aimed at identifying the causative factors associated with the risk of developing multiple myeloma (MM), have largely been inconclusive[1-4] and genetic epidemiology approaches offer an important new avenue of research. Evidence supporting a role for genetic variation in the etiology of MM comes from a variety of sources, where a greater risk is seen in first degree relatives of patients with MM[5] and MGUS,[6-7] there are reported familial clusters of MM,[8-11] and an increased risk is seen in African Americans.[12-13] Although studies to date using genetic epidemiological methods have lacked power and have used only a limited numbers of SNPs,[14-18] they have suggested possible associations with genetic variation in a number of pathways, including growth factors, DNA repair, one carbon metabolism, cell cycle progression and apoptosis pathways.[19-24]

In this work, we have taken a hypothesis-driven approach to examine the role of genetic variation associated with MM, using a custom genotyping array to study 2595 presenting MM cases of European origin derived from the UK, US and The Netherlands. The custom array consisted of 3404 SNPs in 964 genes, focusing on SNP variation in molecular pathways involved in the pathogenesis and treatment response of MM[25-27] In order to understand the distribution of these variants within the normal unaffected population, we accessed 8974 population control genotypes generated by the Wellcome Trust Case Controls Consortium (WTCCC) and the Erasmus Rotterdam Health and the Elderly Study Group (ERGO). A comparison of the genotypic distribution between the case and control populations has allowed us to identify genetic variation that associates with MM predisposition.

Materials and methods

A hypothesis-driven custom Bank On A Cure (BOAC) array[26-28] was built by generating a candidate list, comprising of genes with experimental evidence of a functional role linked to the risk and progression of MM from the literature. The candidate gene list was systematically interrogated by SNP databases including dbSNP, SNP500,[29] Promolign[30] focusing on common coding and regulatory SNPs with minor allele frequency (MAF) > 2%. We sought to validate previously published work in myeloma epidemiology and pharmacogenetics in general by including variants with previously described associations or putative functional effects in myeloma. We also had a focus on absorption, distribution, metabolism and excretion (ADME) genes.[31] The final array comprised 3404 SNPs, selected in predicted functional regions within 964 genes spanning 67 molecular pathways.

SNPs were genotyped using DNA extracted from the peripheral blood of 2595 presenting MM cases derived from a number of randomized clinical trials from the UK (MRC Myeloma-IX study), US (ECOGA4/A03, ECOG9486, SWOG9321) and The Netherlands (HOVON-50/GMMG-HD3, HOVON-65/GMMG-HD4). The MM cases were genotyped on Affymetrix Targeted arrays using True-Tag protocol version 1.5. Genotype calling involves background subtraction and spectral overlap correction, followed by expectation-maximization (E/M) clustering across all processed arrays. Samples were filtered using a > 90% call rate cut-off and SNPs were filtered for call rates > 95% and for departures from Hardy-Weinberg equilibrium (HWE) $P > 10^{-5}$ across each SNP cluster. To understand and protect the analysis from potential platform effects, 58 DNA samples from Coriell CEPH HapMap individuals were assayed on the custom assay to validate the call performance of the panel. A total of 2606 SNPs were present on both the custom array and HapMap, with a SNP call correlation of 96.1%; SNPs falling below this were removed from the analysis (132 SNPs). The Coriell sample genotype validation was replicated in each of the three labs to ensure there was no differential bias in genotyping scoring between sites.

The control population sets consisted of the WTCCC2 study[32-33] with 3000 individuals from the 1958 British Birth Cohort (58C) and the UK Blood Service collections (UKBS), genotyped on both the Illumina 1.2M Duo (Human1-2M-DuoCustom_v1) and the Affymetrix v6.0 chips; and the 5974 individuals from the Dutch > 55yrs old population controls from the ERGO study[34-35], genotyped on the Illumina 550K array. 2352 autosomal SNPs typed on the targeted array with MAF > 1% were also available by direct genotyping in the publicly accessible WTCCC population datasets. 2164 SNPs were available from the WTCCC2 data genotyped on the Illumina 1.2M Duo array (Human1-2M-DuoCustom_v1). 719 SNPs from the targeted array were available on the Affymetrix v6.0 chip, 601 SNPs of these were not seen on the Illumina array. An additional 230 SNPs was provided by imputation methods. Indirectly population genotypes were generated by imputation with IMPUTE2 as described,[36] utilizing both 1000 genomes and HapMap3 data sets. We also selected a set of proxy SNPs in complete linkage disequilibrium (LD; D′ and $R^2 = 1$) with the target SNP using SNAP.[37] The WTCCC BS dataset were paired with the US cases and the WTCCC 58C were used to provide population controls for the UK cases. In the Dutch series there was 992 SNPs available by direct genotyping on the Illumina 550K array from the ERGO study, a further 255 SNPs were available following imputation and 630 SNPs by identification of proxies. Quality control measures were applied with a > 90% call rate for the controls samples. A call rate of > 95% filter was applied for each SNP assay; departures from HWE with a $P > 10^{-5}$ were also excluded.

Statistical analysis

Analysis was performed in the program PLINK 1.07.[38] Subjects with evidence of cryptic relatedness and non-European background were excluded from the analysis. A genomic inflation factor λ was evaluated based on median chi-squared for each set of analyses and showed little evidence for an inflation of test statistics (Figure 2). A logistic regression based additive model was fitted to each SNP with MM risk as the outcome measure and was adjusted for the covariates age and sex.

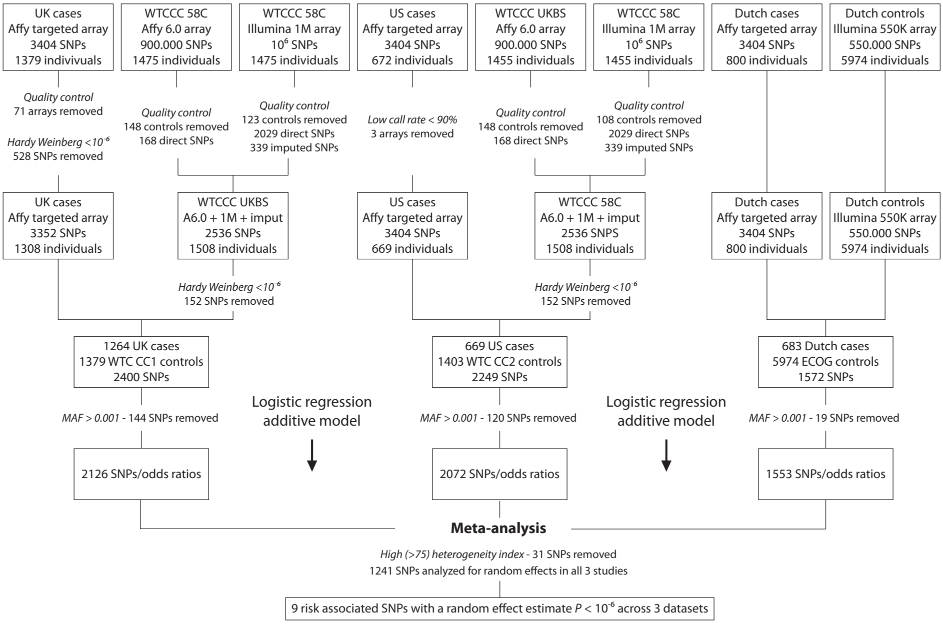


Figure 1. Analysis scheme: quality controls measures were applied across each of the three cases and three control datasets; cases and controls were combined; odds ratios were calculated for single SNPs by logistic regression; meta-analysis of overlapping SNPs.

There were 1240 SNPs in 582 genes fulfilling quality control criteria across all three case-controls sets available for meta-analysis which was carried out to examine associations across the discovery and validation datasets. The PLINK --epistasis option was used for case and control analysis, and the --fast-epistasis option was used for case-only analysis. We performed search of pairwise interactions using epistasis analysis.[39] Combinations were restricted to SNPs more than 5 MB apart or on different chromosomes. To correct for multiple testing in the epistasis analysis, analyses were restricted to SNPs producing nominally significant values of $P < 10^{-4}$.[40]

The relationship of expression with genotype in SNPs associated with risk was examined in a set of 212 UK [41] and 264 Arkansas cases[42-43] with expression data derived from the U133 Plus 2.0 expression array (Affymetrix). Utilizing publicly available data through NCBI GEO datasets and HapMap, we also correlated mRNA expression level with genotype in the same SNPs from 86 Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines in European HapMap individuals using Sentrix Human-6 Expression BeadChips (Illumina).[44] A Wilcoxon-type test for trend[45] was used to compare differences in the distribution of levels of mRNA expression between SNP genotypes in these sets.

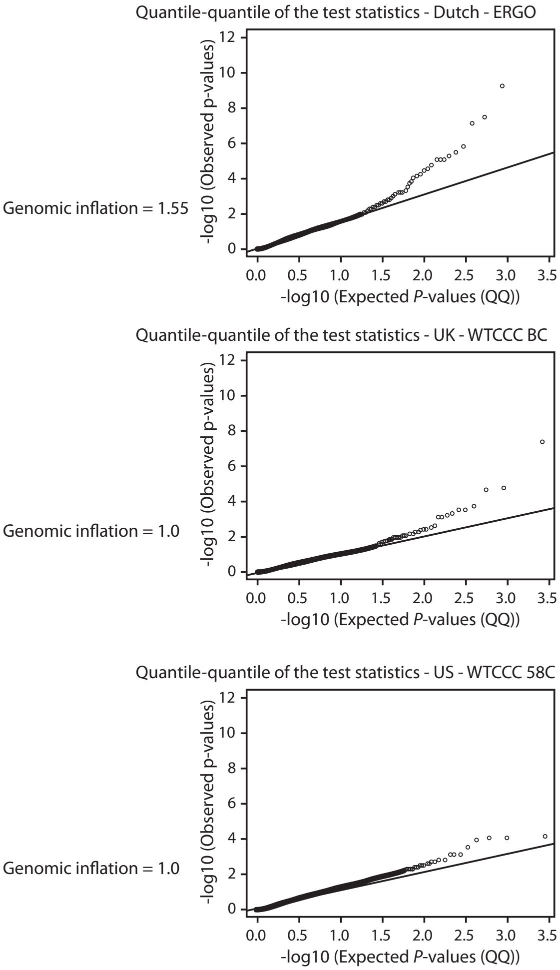


Figure 2. Quantile-quantile plots of test statistics for MM risk across the Dutch, UK and US datasets.

Results

The study design and comparator groups are shown in Figure 1 and the strongest positive associations seen are summarized in Table 1. The nonsynonymous variants significantly associated with myeloma risk across each of the studies are shown as a Forest plot, Figure 3. Further haplotypic structures around the individual SNPs are described in more detail in the supplementary data. The strongest association is seen with the nonsynonymous SNP rs6679793 in the gene

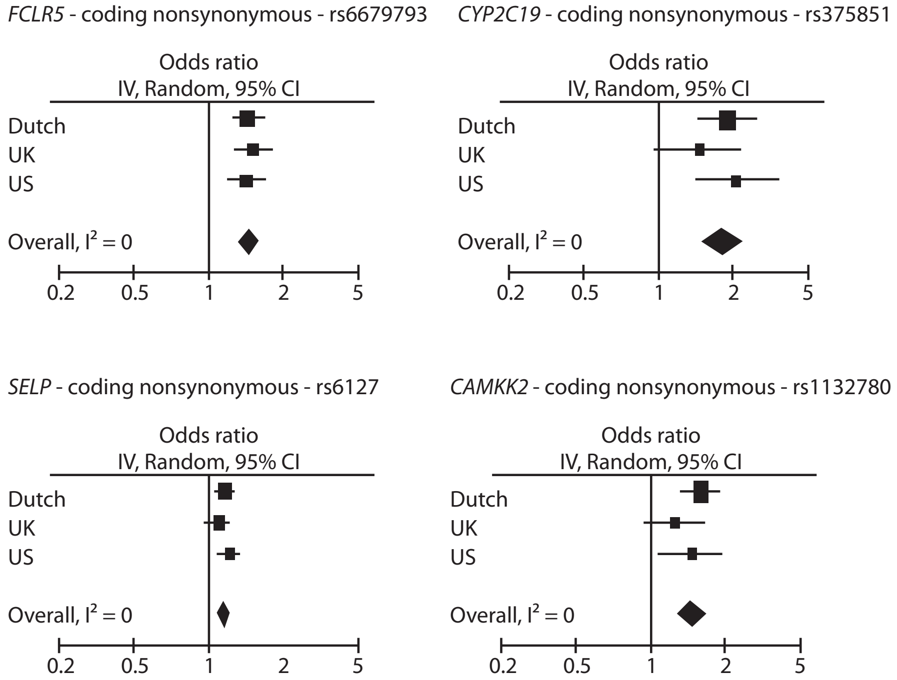
Fc receptor-like 5 (*FCRL5)*. Under a random-effects model, the A allele in the SNP rs6679793 is associated with an increased myeloma risk with a $P = 4.2 \times 10^{-14}$, OR = 1.54, CI 1.38-1.72. An association is seen with an intronic SNP rs4799055 in the gene nuclear factor of activated T-cells, cytoplasmic 1 (*NFATC1*), under a random-effects model the T allele being protective, $P = 6.93 \times 10^{-13}$, OR = 0.77, CI 0.66-0.87. We see an association with a SNP in the untranslated region of the gene tumor necrosis factor receptor superfamily, member 17 (*TNFRSF17*), the G allele of SNP rs3743591 being associated with risk, $P = 1.72 \times 10^{-11}$, OR = 2.92, CI 2.14-3.99, in a random-effect model. An association is seen with a nonsynonymous SNP rs3758581 in cytochrome P450 2C19 (*CYP2C19*), the A allele being associated with increased risk, $P = 3.08 \times 10^{-9}$, OR = 2.02, CI 1.60-2.55 under a random-effect model. We found an association with a nonsynonymous SNP rs1132780 in the gene calcium/calmodulin-dependent protein kinase kinase (*CAMKK2*), the A allele being associated with risk $P = 8.58 \times 10^{-8}$, OR = 1.56, CI 1.32-1.83. An association is found with the intronic SNP rs2472304 in the gene cytochrome P450 1A2 (*CYP1A2*) the G risk allele being associated with an increased risk $P = 3.40 \times 10^{-8}$, OR = 1.26, CI 1.16-1.37. We see an association with the gene interferon gamma receptor 2 (*IFNGR2),* SNP rs8131980, the A allele being associated with increased risk $P = 4.23 \times 10^{-7}$, OR = 1.24, CI 1.14-1.35.

Table 1. Associated myeloma risk variants reaching genomic significance, following meta-analysis for random-effects in Dutch, UK, and US populations.

SNP	**Gene**	**Chr**	**Meta OR (R) - (95% CI)**	**Meta *P* - (R)**	**Dutch OR - (95% CI)**	**Dutch - *P***	**UK OR - (95% CI)**	**UK - *P***	**US OR - (95% CI)**	**US - *P***
rs6679793	*FCRL5*	1	1.54 (1.29-1.82)	4.20×10^{-14}	1.60 (1.29-1.98)	1.46×10^{-6}	1.60 (1.29-1.98)	1.62×10^{-5}	1.50 (1.23-1.84)	8.23×10^{-5}
rs4799055	*NFATC1*	18	0.77 (0.70-0.88)	6.93×10^{-13}	0.75 (0.66-0.86)	1.98×10^{-5}	0.75 (0.66-0.86)	1.62×10^{-5}	0.76 (0.66-0.87)	1.02×10^{-4}
rs3743591	*TNFRSF17*	16	2.92 (2.01-4.49)	1.72×10^{-11}	3.11 (1.70-5.72)	7.36×10^{-8}	3.11 (1.70-5.72)	2.46×10^{-4}	2.24 (0.94-5.36)	6.99×10^{-2}
rs3758581	*CYP2C19*	10	2.02 (1.53-2.94)	3.08×10^{-9}	1.56 (0.96-2.53)	6.29×10^{-6}	1.56 (0.96-2.53)	6.99×10^{-2}	2.32 (1.47-3.67)	3.17×10^{-4}
rs2472304	*CYP1A2*	15	1.26 (1.15-1.48)	3.40×10^{-8}	1.28 (1.10-1.50)	3.83×10^{-5}	1.28 (1.10-1.50)	1.81×10^{-3}	1.19 (1.02-1.39)	3.05×10^{-2}
rs1132780	*CAMKK2*	12	1.55 (1.36-2.12)	8.58×10^{-8}	1.29 (0.93-1.79)	3.38×10^{-6}	1.29 (0.93-1.79)	1.24×10^{-1}	1.55 (1.11-2.16)	1.05×10^{-2}
rs8131980	*IFNGR2*	21	1.24 (1.11-1.43)	4.23×10^{-7}	1.21 (1.04-1.42)	4.77×10^{-4}	1.21 (1.04-1.42)	1.72×10^{-2}	1.25 (1.07-1.47)	5.12×10^{-3}
rs6127	*SELP*	1	1.18 (1.07-1.33)	3.09×10^{-6}	1.10 (0.97-1.25)	1.39×10^{-3}	1.10 (0.97-1.25)	1.25×10^{-1}	1.25 (1.10-1.43)	9.46×10^{-4}
rs7496	*GSTA4*	6	1.59 (1.04-1.97)	6.33×10^{-6}	1.92 (1.32-2.78)	3.02×10^{-2}	1.92 (1.32-2.78)	6.36×10^{-4}	1.53 (1.07-2.20)	1.97×10^{-2}

SNP indicates single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

We see two further associations that did not achieve genome-wide significance, but are close enough to be reported. There is an association with a nonsynonymous SNP rs6127 in the gene selectin P (*SELP*), the C allele being associated with risk, $P = 3.09 \times 10^{-6}$, OR = 1.18, CI 1.09-1.26; and also with a SNP rs7496 in the untranslated region of glutathione S-transferase alpha 4 (*GSTA4*), the T allele being associated with increased risk, $P = 6.33 \times 10^{-6}$, OR = 1.59, CI 1.07-2.20. It is important to note, given that imputation techniques can bias genotype frequencies that SNP rs37458581 was imputed across all three control cohorts, whilst rs6127 was imputed in the



FCLR5 - coding nonsynonymous - rs6679793

CYP2C19 - coding nonsynonymous - rs375851

SELP - coding nonsynonymous - rs6127

CAMKK2 - coding nonsynonymous - rs1132780

Figure 3. Forest plots of an inverse variance (IV) meta-analysis for random-effects.

Dutch cohort only; all other case and controls genotypes across the associated MM risk variants were directly genotyped.

In order to examine potential functionality of significantly associated SNPs present within regulatory regions, we correlated the presence of the risk variant with the expression level of the gene probeset in two separate myeloma datasets and a lymphoblastoid cell line set (HapMap). All associated genes were shown to be expressed highly in myeloma tumor cells and the results of this analysis are given in full in the supplementary data. We see evidence of a significant trend in expression level with the risk associated genotypes in *SELP*, *NFATC1*, *CAMKK2*, *FCRL5*, *GSTA4* in one of the two myeloma expression sets, but no SNP showed a significant correlation with expression across both myeloma sets.

There is increasing evidence that gene-gene interactions (epistasis) could play a role in susceptibility to complex diseases.[46-48] To investigate statistical evidence of epistasis, we tested whether the observed counts for SNP-SNP combinations between associated variants, reflected the expected values within the null hypothesis of no interaction. Examining SNP x SNP interactions in both cases and controls we see that rs6127 (*SELP*) has a significant interaction with a number of SNPs including rs3743591 (*TNFRSF17*) $OR_{interaction} = 0.66$, $P = 1.6 \times 10^{-9}$, rs3758581 (*CYP2C19*) $OR_{interaction} = 1.54$, and rs7496 (*GSTA4*) $P = 6.5 \times 10^{-6}$, $OR_{interaction} = 1.33$, $P = 1.6 \times 10^{-5}$. It is possible to increase the power to detect such interactions by performing a case-only analysis.[49] In the case-only method we see a major interaction between rs6127 (*SELP*) and rs3743591 (*TNFRSF17)*, with seven further interactions exceeding $P > 10^{-5}$ (Table 2).

Table 2. *P*-values generated by SNP x SNP interaction analysis within top SNP associations with MM risk following meta-analysis (cases-only).

SNP1/SNP2	rs6679793	rs4799055	rs3743591	rs3758581	rs2472304	rs1132780	rs8131980	rs6127	rs7496
rs6679793	-	0.39	1.26×10^{-11}	0.02	0.09	0.31	1.33	1.33×10^{-9}	0.02
rs4799055		-	0.60	0.25	0.20	0.05	0.16	0.84	0.57
rs3743591			-	1.85×10^{-5}	0.13	0.40	0.01	5.09×10^{-51}	3.79×10^{-13}
rs3758581				-	0.27	0.85	0.14	1.03×10^{-7}	2.61×10^{-5}
rs2472304					-	0.99	0.49	0.77	0.32
rs1132780						-	0.28	0.15	0.02
rs8131980							-	0.01	0.13
rs6127								-	1.29×10^{-13}
rs7496									-

Discussion

In this study we describe a number of inherited genetic variants that associate with the risk of developing MM that are informative of both the biological and environmental contributions in this respect. It is the largest genetic epidemiological study to date addressing the genetic contribution to the risk of developing MM with the study design laying somewhere between a classical candidate gene study and a whole genome scan. We have assayed 1284 SNPs across 524 genes, in genes that are potentially involved in the pathogenesis of MM and specifically asked how they may modulate the risk of developing MM. The associations seen are strong, across three cohorts of European origin with a number achieving genome wide significance $P < 10^{-7}$. In addition, there is experimental support for the functional role of the variants identified. However, it should be noted, this hypothesis-driven approach cannot detect associations outside the candidate panel and will not, therefore, observe associations potentially detectable by a genome-wide approach.

Micro-environmental interactions and B cell signaling pathways are relevant to the development of myeloma possibly by mediating cell survival following genetic damage. In this context, we find a number of relevant associations including 2 SNPs derived from chromosome 1, a region which is frequently associated with myeloma progression and poor clinical outcome.[42,50-52] The strongest association was seen with the SNP rs6679793 in *FCRL5*, which is situated at bp 157514097 and codes for an amino acid change of Tyrosine to Histidine at position 267. FCRL5, also known as IRTA2, is an immunoglobulin-like cell surface receptor, expressed on the plasma membrane of the majority of MM patients,[53] is involved in B cell differentiation and is thought to play an immunoregulatory role in the marginal zone.[54-55] The second associated SNP from chromosome 1, rs6127, lies at bp 169566313 in the *SELP* gene and results in an amino acid change of Arginine to Asparagine at position 603. SELP is a cell adhesion molecule on the surfaces of activated endothelial cells, with the P-selectin ligand being highly expressed in MM cells compared to normal plasma cells.[56]

We see an association with, the SNP rs8131980 in *IFNGR2* located on chromosome 21 at bp 34810007; IFNGR2 is an integral part of the IFNγ signal transduction pathway and interacts with GAF, JAK1, and/or JAK2 to deliver survival signals. A further associated SNP rs3743591, located in *TNFRSF17* at bp 12059032 on chromosome 16, is a receptor that is preferentially expressed in mature B lymphocytes, and is important for B cell development and autoimmune response. TNFRSF17 can bind to TNFRSF13b also known as BAFF, leading to NFκB and MAPK8/JNK activation. TNFRSF17 also binds to various TRAF family members, and thus may transduce signals for cell survival and proliferation.

The intronic SNP rs4799055, in *NFATC1*, at bp 77182003 on chromosome 18, represents another associated MM risk variant in a gene involved in mediating signaling via cytokine signaling pathways. NFATC1 can influence the expression of cytokine genes, such as IL-2 or IL-4 and may not only regulate B cell activation and proliferation, but also the differentiation and programmed death of T lymphocytes. NFATC1 is also a transcription factor that plays a central role in osteoclast formation. An associated risk variant in a gene mediating similar effects is rs1132780 in *CAMKK2* leading to an amino acid change at position 363 from an Arginine to a Serine, located at bp 121691096 on chromosome 12. CAMKK2 plays a key role in autophagy and cell survival and it is also known that CAMKK-dependent Akt activation inhibits IL-1β-induced NFκB activation through an interference with the coupling of IRAK1 to MyD88.[57] The potential importance of the gene in myeloma was highlighted in an RNAi scan for genes critical in myeloma cell function.[58] Further evidence for variation in immune system related genes impacting on risk comes from the observation of associations with a SNP in the untranslated region of *IL-8RB* (rs1126579), $P < 5.27 \times 10^{-5}$ and *NFκB* intron SNP rs4648133 at $P < 9.32 \times 10^{-7}$. These analyses were only performed across two studies, as the data was not available for the Dutch set.

The associations with absorption, distribution, metabolism, excretion (ADME) genes seen in this study suggest that there are potential environmental exposures and opens the way for further validation in studies designed to investigate gene environment interactions. We see associations with a number of ADME genes including the *CYP2C19* SNP rs3758581 present in poor metabolizer haplotypes *CYP2C19*2* and *CYP2C19*3*, found on chromosome 10 at bp 96602623, resulting in an amino change from Isoleucine to Valine at position 331. CYP2C19 is a key metabolizing gene residing in the endoplasmic reticulum and can be induced to high levels in liver and other tissues by various relevant environmental exposures.[59] The *CYP1A2* SNP rs2472304 is associated with myeloma risk, is located on chromosome 15 at bp 75044238. CYP1A2 is found in the endoplasmic reticulum and is induced by some polycyclic aromatic hydrocarbons (PAHs). We could not demonstrate a relationship between the presence of the risk variant and *CYP1A2* expression levels in the myeloma plasma cells, but this may be due to its impact being mediated at the level of the liver. A further association in an ADME gene with observed with SNP rs7496 in the *GSTA4* gene from chromosome 6 at bp 52842839.

An observation of potential importance from the analysis of the SNP x SNP interactions is the suggestion that the two major pathways outlined above seem to mediate risk in tandem. In the strongest interaction discovered in this analysis we see two risk alleles from the immune response pathway, rs6157 (*SELP*) and rs3743591 (*TNFRSF17*). This interaction was negative, and the risk alleles were seen to be partially exclusive of each other, indicating that may be serving a similar functional role in governing risk. We also then see a number of synergistic interactions between members from the two major pathway, an example of this is interaction between rs6157 (*SELP*) from immune response and rs3758581 (*CYP2C19*) from an ADME pathway.

In addition to describing novel associations we sought to validate previously reported associations with MM risk in our series of patients. While DNA damage and repair has been linked to myeloma risk, we saw no evidence of an association with the DNA repair genes *XRCC5*, *MRE11A*, *BRCA1*, *BRCA2* and *FANCA*. However, we did see a strong association with a variant within *ERCC4*, but we are unsure of the significance of this finding due to the heterogeneity seen between studies. While prior studies have suggested associations with one carbon metabolism, we did not see such an association.[20,60-63] We also did not see an association with genetic variation with growth factor signaling pathways as has been reported previously.[18] Thus while classic epidemiological association studies have identified potential environmental exposures as well as chronic immune stimulation, as being relevant associations with the risk of developing MM and a number of groups have suggested that inherited variation in *IL-6*, *IL-1B*, *TNFα* and *NFκB*,[23,64-72] may play a role in MM risk, none of these associations have been either adequately replicated or reached genome-wide significance.

Our findings provide the first evidence of common genetic variants linked with the risk of developing MM at the level of genome-wide significance. The nine positive associations fall into two broad groups mediating immune response and the response to environmentally encountered carcinogens by ADME genes. We provide evidence that individuals at greatest risk of developing MM carry risk alleles in both the immune response and ADME pathways. Further evaluation of these pathways potentially by sequencing approaches in both MM and MGUS cohorts will provide a greater understanding of the mechanisms driving the transition from a plasma cell to the myeloma tumor cell and potentially enable biomarker discovery to allow anticipation of an individual's MM risk.

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Chapter 3

Genetic associations with thalidomide mediated venous thrombotic events in myeloma identified using targeted genotyping

David C. Johnson, Sophie L. Corthals, Christine Ramos,
Antje Hoering, Kim Cocks, Nicholas J. Dickens, Jeff Haessler,
Hartmut Goldschmidt, J. Anthony Child, Sue E. Bell,
Graham Jackson, Dalsu Baris, S. Vincent Rajkumar,
Faith E. Davies, Brian G.M. Durie, John Crowley,
Pieter Sonneveld, Brian Van Ness, Gareth J. Morgan

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Abstract

A venous thromboembolism (VTE) with the subsequent risk of pulmonary embolism is a major concern in the treatment of multiple myeloma patients with thalidomide. The susceptibility to developing a VTE in response to thalidomide therapy is likely to be influenced by both genetic and environmental factors. To test genetic variation associated with treatment related VTE in patient peripheral blood DNA, we used a custom-built molecular inversion probe (MIP) based single nucleotide polymorphism (SNP) chip containing 3404 SNPs. SNPs on the chip were selected in "functional regions" within 964 genes spanning 67 molecular pathways thought to be involved in the pathogenesis, treatment response and side effects associated with myeloma therapy. Cases and controls were taken from three large clinical trials; MRC Myeloma IX, HOVON-50 and ECOG EA100, which compared conventional treatments with thalidomide in myeloma patients. Our analysis showed that the set of SNPs associated with thalidomide related VTE were enriched in genes and pathways important in drug transport/metabolism, DNA repair and cytokine balance. The effects of the SNPs associated with thalidomide related VTE may be functional at the level of the tumor cell, the tumor related microenvironment, and the endothelium. The clinical trials described in this paper have been registered as follows: MRC Myeloma IX: ISRCTN68454111, HOVON-50: www.clinicaltrials.gov under identifier NCT00028886, and ECOG EA100: www.clinicaltrials.gov under identifier NCT00033332.

Introduction

The introduction of thalidomide and other immunomodulatory drugs has revolutionized clinical management of patients with myeloma. Thalidomide treatment has achieved response rates of 30% at relapse and even higher rates at presentation.[1] Investigation of the specific effects of thalidomide in myeloma remains an active area of research where up regulation of ICAM-1,[2] VCAM-1, IL-10,[3-4] IL-12,[5] and decreased levels of VEGF,[6] βFGF,[7-9] HGF,[10] TNFα,[11] IL-6,[12] sIL-6-R,[13] are thought to play a role in the mechanism of action, which suggests that thalidomide effects the myeloma cell directly as well as its microenvironment.[14]

The therapeutic use of thalidomide has focused attention on venous thrombotic events (VTEs). There appears to be a background rate of 5-10% VTE[15-16] in myeloma possibly due to enhanced expression of tissue factor and VEGF,[17] acquired cytokine mediated activated protein C resistance[18] and downregulation of thrombospondin.[19] In intensively treated patients exposed to thalidomide the rate of VTE increases to 10-15%,[16,20-21] the mechanisms leading to this are uncertain, but it is known that thalidomide regulates the level of COX-2,[22-25] a well described prothrombotic factor. Thalidomide may also modulate the VTE risk by its effects on cytokine levels acting on the endothelial cell, a mechanism dependent on the differential apoptotic effects of thalidomide in myeloma plasma cells compared to endothelial cells, which are protected from apoptosis by decrease of VEGF by thalidomide,[26-28] In this context, it is known that stressed human umbilical vein endothelial cells (HUVECs) upregulate a number of procoagulant factors including PAR-1, P-selectin, E-selectin and tissue factor, with thalidomide protecting these cells from apoptosis potentially enhancing these procoagulant effects, there is some clinical evidence for this mechanism in non-myeloma settings.[29-33]

The risk of developing a VTE following thalidomide exposure depends upon a number of factors including, disease stage, the type of chemotherapy combination and the supportive therapy used. Patient-specific variables also contribute to the excess risk of VTE including immobility, poor performance status, and dehydration. An important clinical observation is that VTEs occur early after the initiation of thalidomide treatment and VTE rates are increased in patients when used in conjunction with anthracycline and dexamethasone[34,35] and can decrease following exposure to bortezomib.[36-40]

The excess risk of thalidomide associated VTE in myeloma has been managed by a number of different strategies, ranging from the identification of high risk patients suitable for prophylaxis to prophylactic anticoagulation for all patients.[41] Aspirin has been suggested to be effective,[42] but its use is controversial because of the lack of a readily applicable mechanism justifying its use. In this work we have examined inherited genetic variation associated with VTE following thalidomide exposure in myeloma patients, using a custom array-based SNP detection tool, in an effort to elucidate the molecular mechanisms contributing to increased risk.

Materials and methods

Clinical samples

Peripheral blood DNA samples were obtained from 544 myeloma cases derived from three randomized clinical trials comparing standard induction treatment for presenting patients with thalidomide containing regimens derived from the Medical Research Council (MRC) Myeloma IX (1966 patients) the Eastern Cooperative Oncology Group (ECOG) EA100 (900) patients and the HOVON-50 study (400 patients; Figure 1). The dose of thalidomide (100-200 mg daily) was comparable between the 3 studies, but the chemotherapy combination used differed. The samples were used as the basis for 2 nested case-control comparisons examining the inherited genetic contribution to the risk of VTE as a consequence of thalidomide exposure. In a discovery set analysis, we compared the genotype results derived from 157 Myeloma IX patients with VTEs, of which 104 were related to thalidomide exposure and 53 unrelated, to a control group of 315 age- and sex-matched myeloma patients also in the trial who did not develop a VTE (198 thalidomide exposed patients and 117 non thalidomide exposed). To validate the frequency distributions, we carried out a second case-control comparison using 23 patients with VTE treated with thalidomide and 49 thalidomide treated controls. To ensure homogeneity of allelic frequencies only patients of European descent were included. This study has been approved by The United Kingdom Multicentre Ethics Committee.

Clinical trials

The Myeloma IX study comprises 2 randomizations: an intensive pathway for younger, fitter patients comparing CVAD (cyclophosphamide 500 mg orally weekly, vincristine 0.4 mg intravenously on days [d] 1-4), doxorubicin 9.0 mg/m^2 on d1-d4, dexamethasone 40 mg on d1-d4 and d12-d15), delivered by a central venous access device with oral CTD (cyclophosphamide, thalidomide, dexamethasone) using the same doses of cyclophosphamide and dexamethasone combined with 200 mg of thalidomide. The second randomization, for older, less-fit patients, compared an attenuated dose of CTD (thalidomide 100-200 mg) to melphalan (7.0 mg/m^2 orally on d1-d4 every 28 days) and prednisolone (MP). All patients at high risk of VTE, defined by clinical criteria, were identified; prophylactic anticoagulation was considered by the treating physician, but it was not specified. The ECOG EA100 study randomized patients to either dexamethasone alone 40 mg daily from d1 to d4 and d12 to d15 or the same dose in combination with thalidomide 200 mg daily. In the study set, from which samples were available, no thromboprophylaxis was used on either arm. The HOVON-50 study randomized patients to either 3 cycles of VAD (vincristine 0.4 mg, intravenous rapid infusion on d1-d4; doxorubicin 9 mg/m^2, intravenous rapid infusion on d1-d4; and dexamethasone 40 mg orally, d1-d4, d9-d12, and d17-d20) or the same regimen but with thalidomide replacing the vincristine (TAD). Thalidomide was given daily at a dose of 200 mg, but could be escalated to 400 mg. All patients in the TAD arm received thromboprophylaxis with low-molecularweight heparin (LMWH). Incident cases of VTE were defined using clinical criteria, and no screening approach was used. The identification of VTE represents current clinical practice with initial clinical identification and subsequent confirmation and definition of the extent of thrombosis using a definitive radiologic investigation. Central venous thrombosis and line-related thrombosis were defined by clinical criteria and subsequently confirmed by ultrasound.

Genotyping, SNP selection, and chip design

DNA was extracted from frozen white blood cell pellets using the Qiagen Flexigene kit (Valencia, CA) and quantified using a Nanodrop spectrophotometer (Wilmington, DE). Genotyping was performed using the Affymetrix targeted genotyping platform (Santa Clara, CA), which is based on a molecular inversion probe technology.[43-45] Patient samples were assayed using a custom-built 3.0K panel comprising 3400 SNPs. SNPs were selected using a hypothesis-driven strategy. Pertinent candidate genes were nominated by myeloma groups in the International Myeloma Foundation - led “Bank On A Cure” (BOAC) consortium. An initial list was supplemented with referencing pathway databases, including BioCarta, Kyoto Encyclopedia of Genes and Genomes (KEGG),[46-47] and Pathway Assist (Ariadne Genomics, Rockville, MD),[48] generating a candidate gene list spanning some 67 molecular pathways important in the biology of myeloma, treatment response, and side effects to conventional and novel agents, which included important genes within the clotting and prothrombotic pathways. Taking the BOAC candidate genes, we completed a literature search[49] to identify SNPs that had been previously reported as having a functional consequence or relevance in prior etiologic or treatment outcome studies. SNPs with a minor allele frequency (MAF) greater than 2% were then systematically selected from the candidate gene list using the following criteria: nonsynonymous SNPs present in dbSNP/SNP 500;[50] promoter variants present in homologous regions between human and mouse, in or adjacent to a transcription binding site utilizing the Promolign database;[51] and promoter SNPs identified in the Functional Element SNPs Database (FESD).[52] We then included Tag SNPs in genes considered to be of particular relevance along with population discriminating admixture variants from the X chromosome.[53] Finally, we included all nonsynonymous SNPs present in the dbSNP database in phosphatase, kinase, and transferase genes with a MAF greater than 2%. The genes and SNPs comprising this panel with allele frequencies are available online.[54]

Statistical analyses

We carried out a Fisher exact Hardy-Weinberg equilibrium (HWE) test at a *P* value less than or equal to 0.001 on all SNPs across the control samples and removed SNPs departing from HWE from the analysis to filter erroneously performing SNPs. We then carried out a “test of missingness” on patient and control status to control for any bias in missing data. We performed a basic Fisher (allelic) association test for disease trait based on a comparison of patients with controls.

We then completed the analysis using 3 genetic models: additive (Cochran-Armitage trend test), dominant, and recessive. To account for multiple testing, we carried out label swapping permutation procedures on each of the SNP assays, with their most significant models used to calculate an empirical *P* value for each SNP. The size of the dataset generated on the BOAC panel is much larger than a typical candidate gene study; we therefore carried out this analysis in the program PLINK,[55] an open-source whole genome association analysis toolset designed for large dataset analysis. The test for epistasis involved testing all pairwise combinations of SNPs. The output consists only of pairwise epistatic results above a *P* level less than 0.001 for each SNP. Combinations were restricted to SNPs more than 1 MB apart or on different chromosomes. This test is only an approximation of the extent of epistasis (SNP-SNP interaction), as it is a naive statistic that does not take linkage disequilibrium (LD) into account. We characterized the haplotypes using Haploview 4.0,[56] and completed haplotype trend regression in Helix-tree (Bozeman, MT). Meta-analysis was performed in SPSS 14.0 (Chicago, IL) using a meta-analysis macro written by Garcia-Granero.[57] Combined odds ratios were calculated using Mantel-Haenszel method for fixed events.

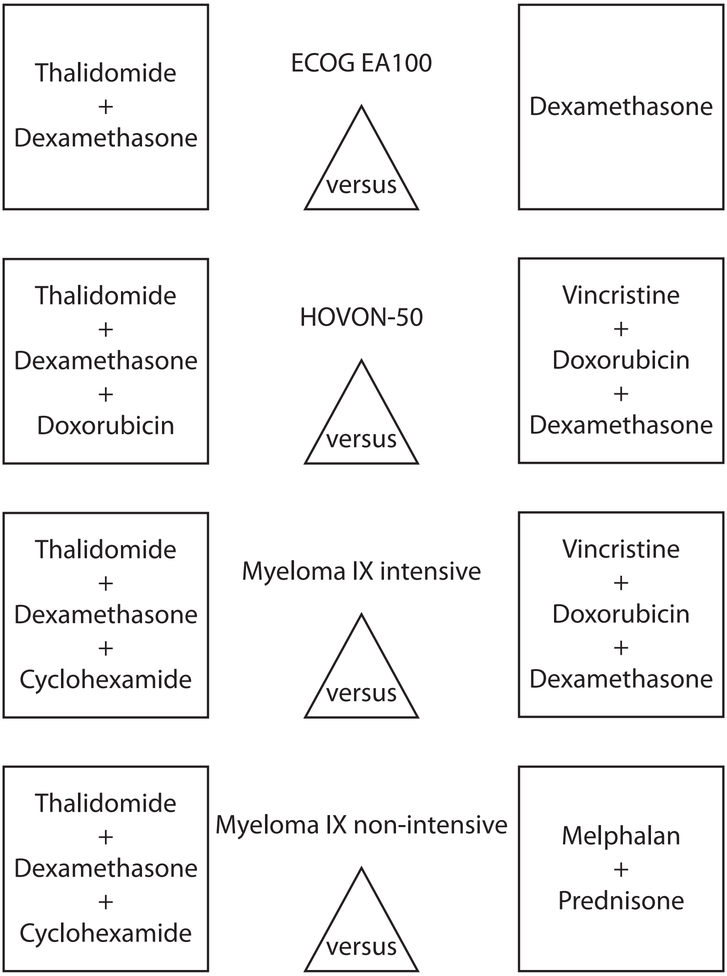


Figure 1. Simplified treatment arms of ECOG EA100, HOVON-50, and Myeloma IX studies.

Biological relevance of the associated SNPs

To examine the possible functionality of the thalidomide related VTE associated SNPs, we used 2 complementary in silico algorithms for prediction of the putative impact of missense variants on protein function, PolyPhen58 (structural) and the SIFT59 (conservation), shown in Table S8. We then used a bioinformatics approach to define the pathways potentially deregulated by the associated and validated genes. We used the functional annotation tool on the DAVID Bioinformatics Resources/Database,[60] to characterize which pathways are most represented in associated gene groups from our single-point analysis. The gene coverage of the BOAC chip was used to form a template/background set, against which associated genes and validated associated SNPs with VTE were tested (Table S9).

Recursive partitioning

To develop a predictive model for the identification of patients at high risk of VTE, we first divided the combined dataset into a training and validation set. We then applied the method of recursive partitioning to the training set.[61] In this approach, a regression tree is built by first finding the SNP which best splits the data into 2 groups (VTE, no VTE). This process is repeated over and over again for the individual subsets until the subgroups reach a minimum size or no improvement can be made. The second stage in recursive partitioning consists of cross validation by trimming back (pruning) the typically complex full tree. The best pruned trees are examined to find which one has the largest classification rate while using the smallest number of SNPs. Sensitivity and specificity are determined for the training and validation set. A receiver operator characteristic (ROC) curve is used to determine the best sensitivity and specificity trade-off.

Results

Clinical results

The Myeloma IX analysis is based on 1966 randomized patients: 984 patients treated with CTD, 557 patients treated with CVAD, and 425 patients treated with MP. In the intensive pathway, the overall rate of VTE was identical in both arms (Table S1). However, there was a qualitative difference between the 2 arms, with deep vein thrombosis (DVT) predominant in the thalidomide treated group and line related thrombosis predominant in the CVAD group. In the nonintensive pathway, very few VTEs were seen in the MP group, whereas in the CTD group there was a 15.0% VTE rate. The median time to VTE in each of the groups was approximately 12 weeks from treatment initiation. The HOVON-50 study had VTE rates of 12.1% and 11.8% in the thalidomide related and standard arms, respectively, with median time to first event of 8.9 weeks. In the ECOGEA100 study, the VTE rates were 17.0% and 3.0% in the thalidomide related and standard arms, respectively.[62]

Panel, sample, and SNP assay validation

Affymetrix constructed and validated the SNP panel reagents. A total of 59 DNA samples from the extensively characterized and genotyped Coriell CEPH HapMap series were assayed to validate the call performance of the BOAC panel. A total of 58 Coriell CEPH HapMap samples were also used in a correlation analysis between the BOAC chip and HapMap study. We did not obtain HapMap data for the remaining Coriell samples and did not perform a correlation analysis. A total of 2606 SNPs were present on both the chip and HapMap. There was a SNP call correlation of 96.1% at 95% confidence levels; SNPs falling below this were removed from the analysis (132 SNPs). The Coriell sample genotype validation was replicated in BOAC labs to ensure there was no differential bias in genotyping scoring between sites. Patients and controls were also genotyped together throughout the experiment to avoid any differential bias in genotype scoring.

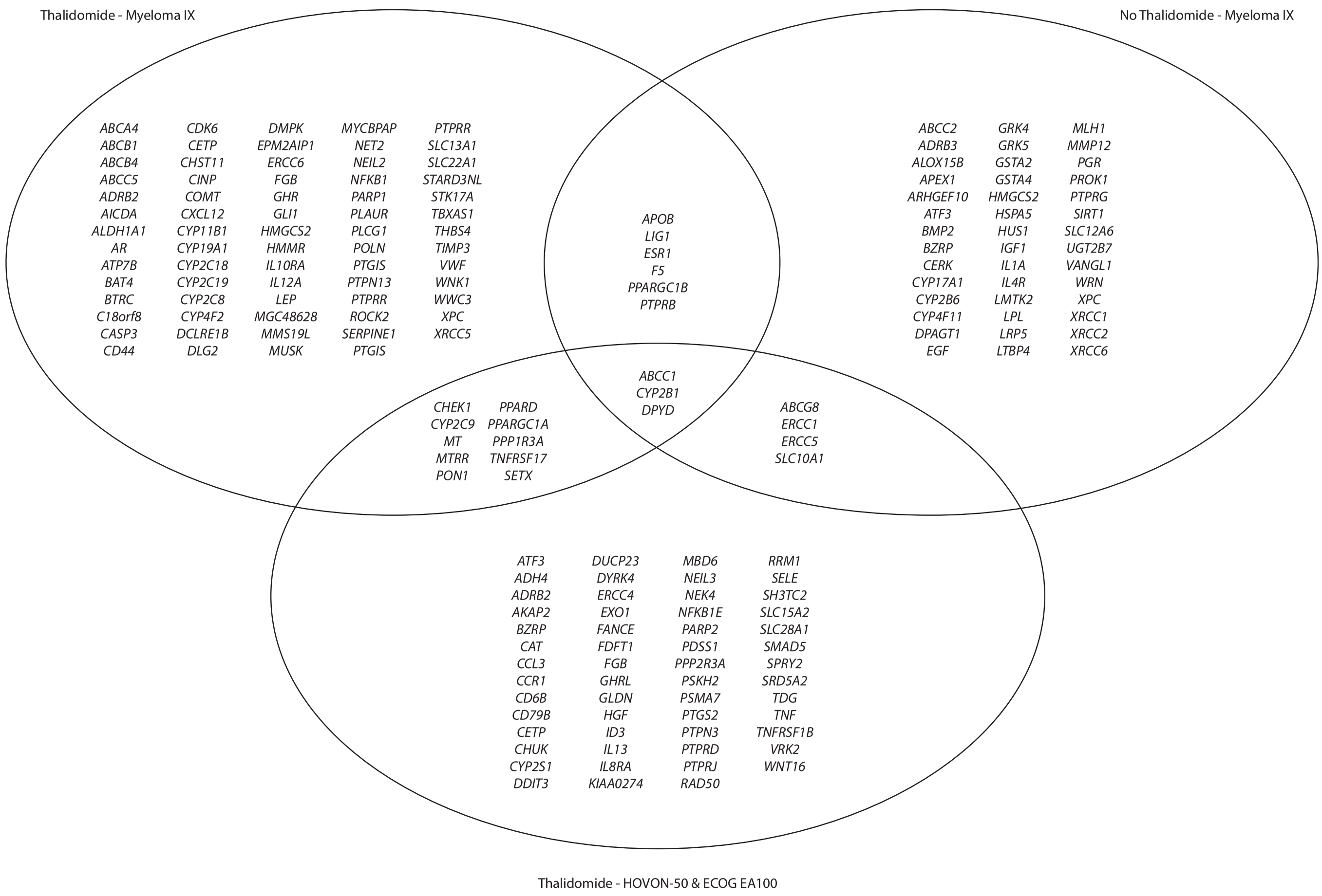


Figure 2. Venn diagram showing overlapping VTE-associated genes between thalidomide Myeloma IX, non-thalidomide Myeloma IX, and thalidomide HOVON-50/ ECOG EA100 analyses.

We observed complete agreement between the known sex and inferred SNP-based sex in all samples. We used Eigenstrat[63] to highlight population stratification and removed 4 population outliers from the analysis. A number of admixture SNPs were included in the SNP panel.[53] Genotype calls for these SNPs demonstrated that patients and controls reflected a sample set drawn from a European population.

Genotyping results and validation

The MRC Myeloma IX study is the largest of the datasets in this study; to capitalize on this, we chose to focus our discovery set on this study and to validate the results on combined data sets from HOVON-50 and ECOG EA100 trials. A set of SNPs for validation was defined by separately determining the distribution of the most significant SNPs in the MRC myeloma IX, HOVON-50, and ECOG EA100 studies. Following testing allelic distributions using the Fisher test to an empirical *P* value less than 0.05 in the discovery set, 120 SNPs were found to be associated with thalidomide associated VTE, involving 71 genes (Table S2). Further genetic model association analysis in Myeloma IX data are listed in Table S3; allelic and genetic model association for non–thalidomide related VTEs are listed in Tables S4 and S5, with allelic and genetic model association for HOVON-50/EA100 trials shown in Tables S6 and S7. With the aim of identifying genes modulating the risk of thalidomide related thrombosis, we compared the distribution of the SNPs identified in this analysis between 3 subgroups: thalidomide associated VTEs from MRC Myeloma IX, non–thalidomide associated VTEs from MRC Myeloma IX, and thalidomide associated VTEs from the combined HOVON-50/ECOG EA100 studies. A Venn diagram depicting the overlapping associated genes in the 3 analyses is shown in Figure 2.

Validation of thalidomide related VTE associated SNPs

To validate the genotyping results in the discovery set, we discarded SNPs with conflicting frequency distributions between studies. This approach may have led to the removal of a number of true positives from the analysis because of the small sizes of the HOVON-50 and ECOG EA100 datasets. SNPs with small effect sizes were also removed from the analysis. As a result of this process we found 24 SNPs (Table 1) associated with VTE in the “discovery set” with consistent distributions in the 2 validation datasets. Haplotype analysis showed that 6 SNPs were in linkage with a stronger proxy SNP and as such were discarded, leaving 18 validated SNPs associated with thalidomide related VTEs. A “forest plot” with odds ratios (ORs) and confidence intervals (CIs) for the combined and individual datasets of the validated SNPs was generated (Figure 3).

Table 1. Allele distributions of cross-trial-validated associated thalidomide related VTE SNPs.

SNP	Chromosome	Gene	Functional class	Minor allele	MAF patients	MAF controls	Major allele	*P*	OR (L95 - U95)	Emperical *P*	Trial	In linkage
rs2302387	7	*ABCB4*	Coding-synonymous	A	0.09	0.14	G	0.042	0.56 (0.31 - 0.99)	0.041	Myeloma IX	
				A	0.38	0.40	G	0.879	0.90 (0.23 - 3.48)	0.857	HOVON-50	
				A	0.00	0.18	G	0.112	NA	0.149	E1A100	
rs1049216	4	*CASP3*	Untranslated	C	0.19	0.29	T	0.009	0.58 (0.38 - 0.88)	0.006	Myeloma IX	
				C	0.28	0.45	T	0.251	0.46 (0.12 - 1.75)	0.345	HOVON-50	
				C	0.08	0.42	T	0.029	0.13 (0.02 - 1.05)	0.087	E1A100	
rs506504	11	*CHEK1*	Coding-nonsynonymous	T	0.07	0.03	C	0.031	2.41 (1.06 - 5.48)	0.044	Myeloma IX	
				T	0.11	0.00	C	0.109	NA	0.146	HOVON-50	
				T	0.25	0.06	C	0.046	5.22 (0.91 - 30.11)	0.060	E1A100	
rs7011	14	*CINP*	Coding-nonsynonymous	T	0.31	0.23	C	0.032	1.52 (1.03 - 2.23)	0.036	Myeloma IX	
				T	0.39	0.25	C	0.358	1.91 (0.48 - 7.64)	0.357	HOVON-50	
				T	0.50	0.22	C	0.051	3.55 (0.95 - 13.2)	0.096	E1A100	
rs4633	22	*COMT*	Coding-synonymous	C	0.42	0.52	T	0.015	0.65 (0.46 - 0.92)	0.016	Myeloma IX	
				C	0.39	0.41	T	0.897	0.92 (0.26 - 3.28)	0.999	HOVON-50	
				C	0.33	0.38	T	0.764	0.82 (0.22 - 3.08)	0.999	E1A100	
rs12022378	1	*DCLRE1B*	Coding-nonsynonymous	T	0.23	0.15	C	0.019	1.67 (1.08 - 2.57)	0.023	Myeloma IX	
				T	0.22	0.09	C	0.247	2.86 (0.46 - 17.8)	0.632	HOVON-50	
				T	0.25	0.22	C	0.823	1.18 (0.27 - 5.13)	0.999	E1A100	
rs4253211	10	*ERCC6*	Coding-nonsynonymous	C	0.16	0.10	G	0.028	1.76 (1.06 - 2.91)	0.035	Myeloma IX	
				C	0.17	0.05	G	0.204	4.20 (0.40 - 44.4)	0.261	HOVON-50	
				C	0.17	0.10	G	0.512	1.80 (0.30 - 10.64)	0.560	E1A100	
rs299295	5	*HMMR*	Coding-nonsynonymous	T	0.28	0.20	C	0.024	1.58 (1.06 - 2.35)	0.041	Myeloma IX	
				T	0.39	0.27	C	0.435	1.70 (0.45 - 6.44)	0.999	HOVON-50	
				T	0.33	0.32	C	0.929	1.06 (0.28 - 4.06)	0.999	E1A100	

NA indicates not applicable; L95, lower 95% CI; and U95, upper 95% CI.

Table 1. continued.

SNP	Chromosome	Gene	Functional class	Minor allele	MAF patients	MAF controls	Major allele	*P*	OR (L95 - U95)	Emperical *P*	Trial	In linkage
rs582537	3	*IL12A*	Intron	A	0.52	0.40	C	0.007	1.60 (1.13 - 2.25)	0.005	Myeloma IX	rs2227314
				A	0.50	0.50	C	0.999	1.00 (0.29 - 3.48)	0.999	HOVON-50	rs2227314
				A	0.58	0.44	C	0.372	1.78 (0.50 - 6.39)	0.591	E1A100	rs2227314
rs10249476	7	*LEP*	Promoter	T	0.41	0.31	G	0.024	1.50 (1.05 - 2.15)	0.026	Myeloma IX	
				T	0.56	0.41	G	0.356	1.81 (0.51 - 6.36)	0.450	HOVON-50	
				T	0.33	0.21	G	0.360	1.90 (0.47 - 7.61)	0.517	E1A100	
rs20579	19	*LIG1*	Untranslated	T	0.07	0.13	C	0.022	0.48 (0.25 - 0.91)	0.017	Myeloma IX	
				T	0.06	0.27	C	0.072	0.16 (0.02 - 1.45)	0.113	HOVON-50	
				T	0.00	0.04	C	0.481	NA	0.999	E1A100	
rs13815	22	*MT*	Coding-nonsynonymous	C	0.26	0.37	G	0.007	0.60 (0.41 - 0.87)	0.010	Myeloma IX	
				C	0.22	0.45	G	0.125	0.34 (0.09 - 1.38)	0.126	HOVON-50	
				C	0.25	0.36	G	0.470	0.59 (0.14 - 2.47)	0.857	E1A100	
rs2410558	8	*NAT2*	Locus, TagSNP	T	0.22	0.30	C	0.024	0.63 (0.42 - 0.94)	0.024	Myeloma IX	
				T	0.22	0.50	C	0.071	0.29 (0.07 - 1.15)	0.226	HOVON-50	
				T	0.25	0.28	C	0.834	0.86 (0.20 - 3.64)	0.999	E1A100	
rs3774968	4	*NFKB1*	Intron	A	0.36	0.46	G	0.017	0.65 (0.46-0.93)	0.017	Myeloma IX	
				A	0.33	0.32	G	0.919	1.07 (0.28-4.05)	0.999	HOVON-50	
				A	0.40	0.46	G	0.728	0.78 (0.20-3.12)	0.800	E1A100	
rs1805414	1	*PARP1*	Coding-synonymous	C	0.25	0.34	T	0.029	0.65 (0.45 - 0.96)	0.033	Myeloma IX	rs1002153, rs2048426,rs2267669
				C	0.22	0.45	T	0.125	0.34 (0.09 - 1.38)	0.328	HOVON-50	rs1002153, rs2048426,rs2267669
				C	0.33	0.36	T	0.862	0.89 (0.23 - 3.37)	0.999	E1A100	rs1002153, rs2048426,rs2267669
rs2267669	6	*PPARD*	Intron	G	0.12	0.21	A	0.006	0.50 (0.30 - 0.83)	0.007	Myeloma IX	
				G	0.00	0.23	A	0.031	NA	0.037	HOVON-50	
				G	0.17	0.18	A	0.903	0.90 (0.16 - 4.93)	0.999	E1A100	
rs2070682	7	*SERPINE1*	Intron	C	0.37	0.46	T	0.034	0.69 (0.48 - 0.97)	0.029	Myeloma IX	

NA indicates not applicable; L95, lower 95% CI; and U95, upper 95% CI.

Table 1. continued.

				C	0.33	0.36	T	0.842	0.88 (0.24 - 3.24)	0.999	HOVON-50	
				C	0.42	0.42	T	0.983	0.99 (0.27 - 3.54)	0.999	E1A100	
rs12922317	16	*TNFRSF17*	Intron,TagSNP	G	0.29	0.40	A	0.011	0.62 (0.43 - 0.90)	0.010	Myeloma IX	rs11862958
				G	0.28	0.40	A	0.428	0.58 (0.15 - 2.26)	0.380	HOVON-50	rs11862958
				G	0.17	0.36	A	0.198	0.36 (0.07 - 1.80)	0.800	E1A100	rs11862958
rs2440	2	*XRCC5*	Untranslated	T	0.43	0.35	C	0.062	1.40 (0.98 - 2.00)	0.047	Myeloma IX	rs207932
				T	0.25	0.18	C	0.611	1.50 (0.31 - 7.19)	0.857	HOVON-50	rs207932
				T	0.42	0.23	C	0.189	2.40 (0.64 - 9.09)	0.226	E1A100	rs207932

NA indicates not applicable; L95, lower 95% CI; and U95, upper 95% CI.

Gene-gene interactions

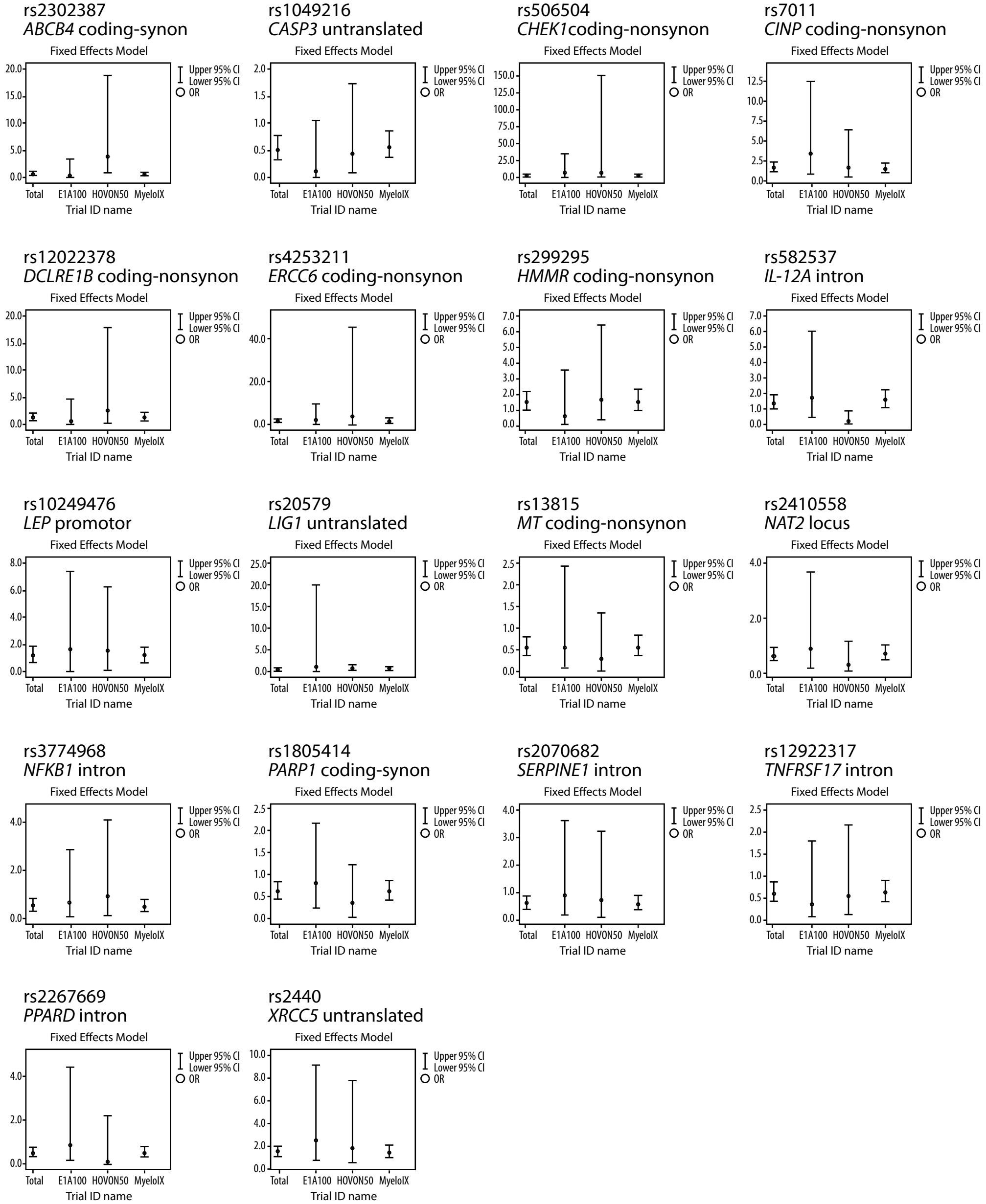
To examine gene-gene interactions, we looked for pairwise combinations mediating risk. The epistatic interactions with a *P* value less than 0.001 are shown in Table S10.

Recursive partioning analysis

To maximize the size of the dataset and thus to maximize the ability to identify relevant SNPs, we combined all the datasets into one and randomly split it into a two-thirds training set and one-third validation set. The data were stratified by trial and VTE patients to ensure that the training and validation sets were comparable. These data included 165 subjects without VTE and 84 subjects with VTE in the training set and 82 subjects without VTE and 42 subjects with VTE in the validation set. The training set was used to identify the top associated genes and SNPs by association at the level of *P* less than 0.05 listed in Table S2. These SNPs were used in a recursive partitioning analysis carried out on the test set with the aim of finding the combination with the best sensitivity and specificity for the identification of VTE. We pruned the tree to find the tree with the highest classification and smallest number of SNPs. The results of this analysis (Figure 4) showed that using 7 SNPs (rs7011 in *CINP*, rs289747 in *CETP*, rs610529 in *ALDH1A1*, rs3829963 in *CDKN1A*, rs2608555 in *GAN*, rs699947 in *VEGF*, and rs168351 in *ALDH1A*) it was possible to identify VTEs correctly in 70% of individuals with a specificity of 59% and sensitivity of 81%. This set of SNPs performed well in the validation set; the set was able to correctly classify VTEs in 61% of individuals with a specificity of 30% and sensitivity of 77% (Tables S12 and S13).

Discussion

This study has analyzed data from 3 large randomized clinical studies comprising 3100 patients, comparing induction treatment for newly presenting patients with myeloma with and without thalidomide. The results of this analysis show that the background rate of VTE in MP treated patients is very low and significantly increases with the addition of thalidomide. In addition we provide further evidence that infusional regimes based on VAD increase VTE rates to around 15%, which is similar to the rates seen with oral thalidomide combinations. The nature of the thrombotic events is qualitatively different between regimes; with all events being either DVT or pulmonary embolism (PE) in the oral thalidomide treated patients, whereas in the intravenous treatment, 50% of the events are central line related. There is a doubling of non-central line related VTE rates in the thalidomide treated patients compared with those receiving infusional induction regimens. The median time to VTE in each of the treatments is approximately 50 to 60 days after the initiation of treatment, a time reflecting the rapid dissolution of the myeloma clone. We have shown previously that response rate is enhanced in thalidomide containing regimes compared with VAD-like regimes, and we postulate that this is important in determining



rs2302387
ABCB4 coding-synon

rs1049216
CASP3 untranslated

rs506504
*CHEK1*coding-nonsynon

rs7011
CINP coding-nonsynon

rs12022378
DCLRE1B coding-nonsynon

rs4253211
ERCC6 coding-nonsynon

rs299295
HMMR coding-nonsynon

rs582537
IL-12A intron

rs10249476
LEP promotor

rs20579
LIG1 untranslated

rs13815
MT coding-nonsynon

rs2410558
NAT2 locus

rs3774968
NFKB1 intron

rs1805414
PARP1 coding-synon

rs2070682
SERPINE1 intron

rs12922317
TNFRSF17 intron

rs2267669
PPARD intron

rs2440
XRCC5 untranslated

Figure 3. Forest plots showing distribution of validated SNPs associated with thalidomide related VTEs across the Myeloma IX, HOVON-50, and ECOG EA100 trials. Error bars indicate upper and lower 95% CIs.

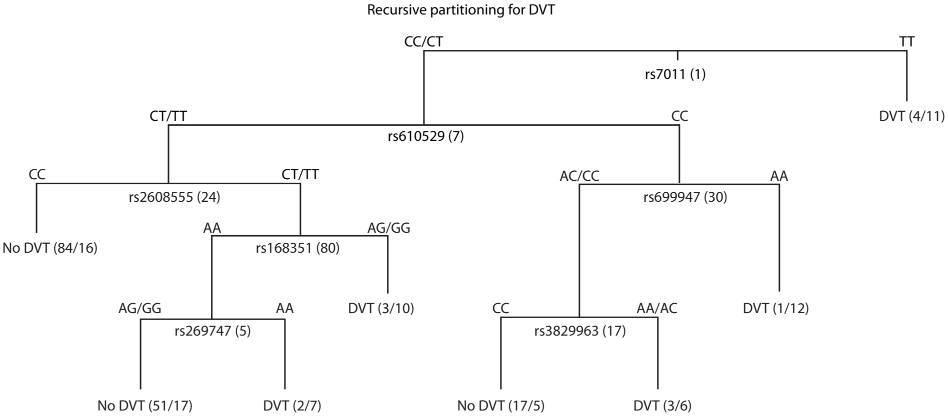
the VTE risk.[64] The mechanistic importance of increased response rates with VTE risk may explain the reduced numbers of VTEs seen in relapsed patients, who are frequently drug resistant and show lower response rates. It is also important not to discount increased VTE risk due to changes in the disease biology related procoagulant profiles of such relapsed patients.

Using a nested case-control design with readily defined exposure and clinical endpoint, this study has given useful information about inherited genetic variants with a moderate effect size affecting the thrombotic response to thalidomide exposure. We chose to use the MRC Myeloma IX study as our initial discovery set because it was the largest and had the most data available with it. Validation in the combined HOVON-50/ECOG study represents a pragmatic decision based on study size, study design, and our desire to identify penetrant variants that can be replicated with relevance to different studies and datasets.

Despite a comprehensive analysis of the genetic variation within the coagulation and prothrombotic pathways, we could not find evidence for a significant association of genetic variation within these pathways with VTE risk following thalidomide exposure. Although we found Factor 5 Leiden (rs6025) to be associated with an increased risk of VTE in this analysis, the thrombotic risk was not increased in patients treated with thalidomide; similar results were seen for polymorphisms in *MTHFR* and *FGB*. We saw no association with thalidomide related VTE in commonly reported VTE risk alleles in *F2*-455G/A (rs3136430) splice variant 20210G/A (rs3136431). We did find weak associations with genes known to mediate the coagulation pathway, including *MTRR*, *PLAUR*, *PPARD*, *PPARGC1A*, *PPARGC1B*, *THBS4*, and *WNK*, but the associated risk was not high. We conclude that we can exclude a major contribution of genetic variation within the coagulation and prothrombotic pathways based on this targeted approach, although smaller contributions to the phenotype may be missed because of the study size and design. Our findings are consistent with previous clinical observations and work by some of the authors, who failed to identify relevant changes in functional assays investigating this pathway.[65-67]

The lack of a strong association with variation in the coagulation cascade suggests that VTE risk is mediated via alternative mechanisms. We identified the 18 SNPs, which validated across the 3 datasets (Figure 3). Using the whole BOAC panel as the background gene set in the DAVID Functional Annotation Clustering tool against the 18 validated genes, generated 3 major enriched annotation clusters. The annotation clusters consisted of 2 “response to stress” groups; a response to DNA damage group, including *CHEK1*, *XRCC5*, *LIG1*, *ERCC6*, *DCLRE1B*, and *PARP1*; a cytokine response group containing *NFKB1*, *TNFRSF17*, *IL-12B*, and *LEP*; and a third related group of “apoptosis” with *CASP3*, *PPARD*, and *NFKB1*. These enrichment groups indicate that genetic variation in response to DNA damage and cytokine mediated apoptosis modulates risk of developing a thalidomide related thrombosis.

High-dose dexamethasone enhances hemostasis, increases platelet activation, and promotes von Willebrand factor (vWF) antigen–dependent thrombosis.[68] Extremely high levels of factor VIII coagulant (FVIII:C) activity and vWF have been found in thalidomide exposed patients.[69] Patients that develop a subsequent VTE had higher vWF antigen (Ag) levels but not FVIII:C levels. High FVIII:C/vWF Ag levels are found in patients with active myeloma; this is probably a reflection of increased bone marrow angiogenesis in myeloma. These prothrombogenic circumstances would contribute to VTE during treatment with a thalidomide-dexamethasone combination.[69] In line with vWF mediating the prothrombotic effects of dexamethasone in thalidomide related VTE, we saw a protective effect of *vWF* nonsynonymous SNP (rs216321) and synonymous SNP (rs216902) in thalidomide treated controls.



rs number	Rank*	Univariate *P*-value	Functional type	Gene	Target Allele
rs7011	1	0.001231074	Coding-nonsynonymous	*CINP*	C/T
rs289747	5	0.007021794	3′ UTR	*CETP*	A/G
rs610529	7	0.008176454	Intron, TagSNP	*ALDH1A1*	C/T
rs3829963	17	0.017841244	Promotor	*CDKN1A*	A/C
rs2608555	24	0.027787883	Coding-nonsynonymous	*GAN*	C/T
rs699947	30	0.031169521	Promotor	*VEGF*	A/C
rs168351	80	0.143913327	Intron, TagSNP	*ALDH1A1*	A/G

*using Fisher's exact test

Figure 4. Predictive tree of thalidomide related thrombosis in myeloma patients following recursive partitioning analysis.

Although there is evidence to suggest thalidomide may damage DNA directly,[70] it is important to note the majority of cases in this analysis were derived from the MRC and HOVON-50 studies, which included either cyclophosphamide or doxorubicin/adriamycin in the treatment regime, which may explain an association with DNA repair genes. Variation in DNA repair capacity could readily affect the response of the myeloma clone to treatment due to the direct relationship between the extent of DNA damage accumulation and the clinical response to alkylating agents.[71] A rapid response and dissolution of myeloma clones with an impaired double-stranded DNA repair pathway would release greater prothrombotic factors that could be either microparticles with surface tissue factor or cytokines and tissue factor. The greater thrombogenesis due to increased dissolution of the myeloma clone may act additively with a dexamethasone-thalidomide interaction on plasma cells,[72] giving rise to an increased number of VTEs in the MRC and HOVON studies.[73-74] An alternative mechanism to explain the increased risk of a VTE associated with DNA repair genes could be based on the observation that thalidomide can protect endothelial cells from doxorubicin induced apoptosis by restoring PAR-1 expression,[75] promoting subendothelial tissue factor exposure, endothelial dysfunction, and platelet activation, and consequently increasing the thrombosis risk.[75-77] Under these conditions, decreased DNA repair capacity could promote clot formation at the endothelium (Figure 5).

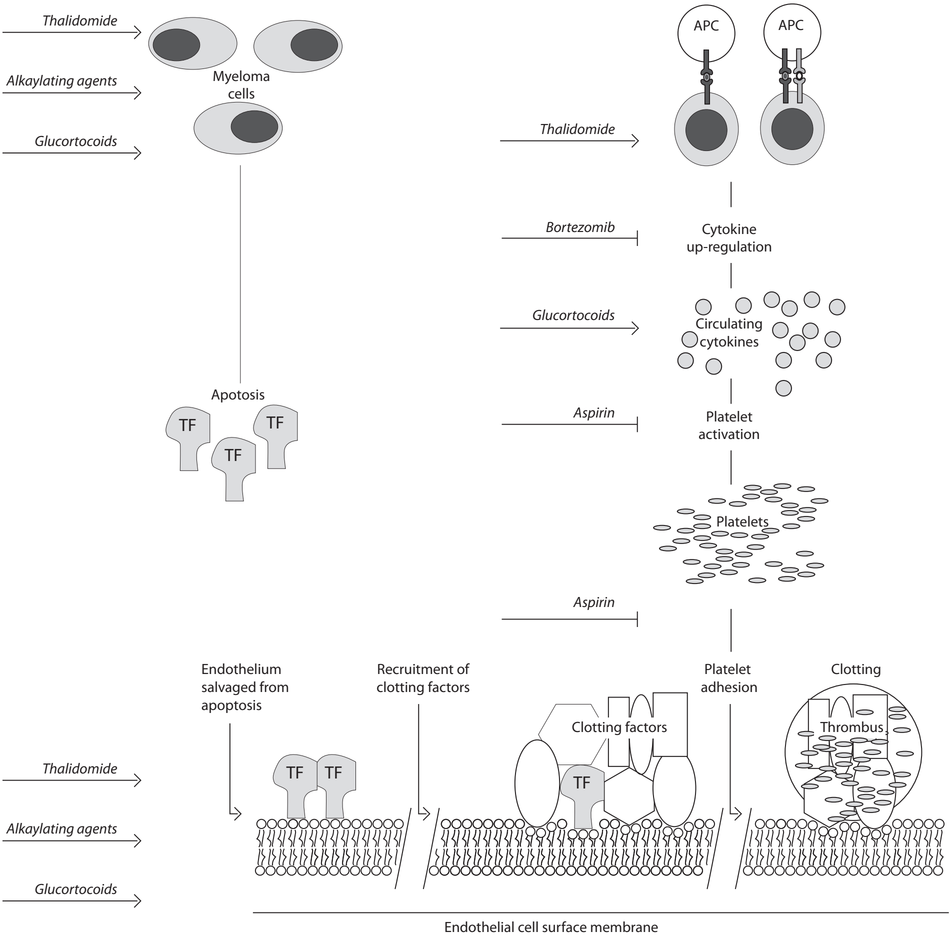


Figure 5. Thalidomide treatment in combination with alkylating agents in myeloma promotes prothrombotic conditions at the endothelium surface via a combination of mechanisms. Mechanisms include rapid apoptosis of myeloma cells leading to circulating tissue factor (TF), exposed TF by endothelium cells salvaged from apoptosis, increased circulating cytokines (e.g., TNFα) with T cell activation by antigen-presenting cells (APCs), and activated platelets in response to increased circulating cytokines.

The enrichment of cytokine mediated apoptosis genes in SNPs associated with thalidomide related thrombosis risk may also give clues to the role bortezomib and aspirin play in VTE management. Low rates of VTE are seen in patients with myeloma treated with bortezomib in thalidomide combinations,[37,40,78] possibly through the prevention of the upregulation of prothrombotic molecules such as thrombomodulin, cytokines, and E-selectin by bortezomib.[79-80] A number of clinical studies have suggested that aspirin[42,81-89] is effective at preventing the excess of VTE seen in thalidomide exposed individuals. Aspirin is classically thought to inhibit platelet COX-2, reducing platelet adhesiveness and modulating risk of arterial thrombosis. Aspirin can also lead to decreased levels of circulating TNFα by inhibiting IKK and therefore NFκB. Higher levels of TNFα and COX-2 lead to an increased risk of apoptosis in endothelial cells, which also become proadhesive to nonactivated platelets.[90] In a thalidomide treatment setting, aspirin may be able to inhibit thalidomide VTE mediated events by lowering circulating TNFα.

Genetic analysis of the multifactorial phenotype that is thalidomide related venous thrombosis is challenging. To minimize experimental artifacts that can be found in many association studies,[91] we have associated a discrete clinical outcome from a homogenous population of similarly treated patients with high-quality genotype data with stringent quality controls. We took a hypothesis-driven candidate gene approach rather than a whole genome scan (WGS)–based approach because it was clear that the number of events to be analyzed would be small, and we were aiming to identify pertinent functional loci variants with moderate to large effect size. We accept that future GWS and sequencing approaches may add relevant variants in unknown pathways. As part of the analysis, we took an exploratory approach to defining whether the SNPs identified could be used to identify patients at high risk of VTE and consequently guide clinical intervention. Guidelines have recently been established to govern clinical indicators for intervention, but these prognostic factors can be difficult to identify and use clinically.[41] The US Food and Drug Administration (FDA) and European Medicines Evaluation Agency (EMEA) have published warnings suggesting the use of thromboprophylaxis with any immunomodulatory derivative of thalidomide (IMiD)–based regimen.[92-94] The results of this recursive partitioning analysis have identified a limited number of SNPs that, when analyzed together, can predict the risk of VTE. Testing for these SNPs has the potential for being clinically useful for identifying high-risk patients for whom therapeutic intervention is required. For clinically defined high-risk patients intervention strategies may not change, but for patients at genetic high risk for whom aspirin was the chosen strategy, intervention with warfarin or LMWH would be more appropriate.

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Chapter 4

Genetic factors underlying the risk of thalidomide related neuropathy in multiple myeloma patients

David C. Johnson, Sophie L. Corthals, Brian A. Walker,
Fiona M. Ross, Walter M. Gregory, Nicholas J. Dickens,
Henk M. Lokhorst, Hartmut Goldschmidt, Faith E. Davies,
Brian G.M. Durie, Brian Van Ness, J. Anthony Child,
Pieter Sonneveld, Gareth J. Morgan

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Abstract

Purpose

To indentify genetic variation that can modulate and predict the risk of developing thalidomide related peripheral neuropathy (TrPN).

Patients and methods

We analyzed DNA from 1512 patients with multiple myeloma. Using a custom-built single nucleotide polymorphism (SNP) array, we tested the association of TrPN with 3404 SNPs. The SNPs were selected in predicted functional regions within 964 genes spanning 67 molecular pathways thought to be involved in the pathogenesis, treatment response and side effects associated with myeloma and its therapy. Cases and controls were derived from two large clinical trials that compared thalidomide with conventional based treatment in myeloma patients; MRC Myeloma-IX and HOVON-50/GMMG-HD3.

Results

We report TrPN associations with SNPs: *ABCA1* (rs363717), *ICAM1* (rs1799969), *PPARD* (rs2076169), *SERPINB2* (rs6103) and *SLC12A6* (rs7164902), where we show cross validation of the associations in both trials. To investigate whether TrPN SNP associations were related to exposure to thalidomide only or general drug related peripheral neuropathy, we performed a second analysis on patients treated with vincristine. We report SNPs associated with vincristine neuropathy, with a seemingly distinct underlying genetic mechanism.

Conclusion

Our results are consistent with the hypothesis that an individual's risk of developing a peripheral neuropathy following thalidomide treatment can be mediated by polymorphisms in genes governing repair mechanisms and inflammation in the peripheral nervous system. These findings will contribute to the development of future neuro-protective strategies with thalidomide therapy and the better use of this important compound.

Introduction

Peripheral neuropathy is a significant adverse event in multiple myeloma patients treated with thalidomide. Thalidomide related peripheral neuropathy (TrPN) typically consists of symmetrical paresthesias, with loss of tactile and pain response as well as numbness and muscle cramps.[1] Rates of neuropathy following thalidomide treatment vary from 15-70%, with the risk of neuropathy being related to the cumulative dose and duration of therapy.[2-6] Factors influencing the risk of neurotoxicity include prior neuropathy, age,[7] previous chemotherapy, and vitamin B12 and/or folate deficicency.[8] The mainstay of TrPN prevention is dose reduction or withdrawal of thalidomide, which can lead to symptom resolution in up to 16 weeks,[9] however in some cases neuropathy is irreversible.

At a pathologic level, TrPN is a length-dependent, predominantly sensory axonal neuropathy affecting large and small fibers,[1,5] but has also been classified as a ganglionopathy.[5,10] Reduction in amplitude or absence of sensory nerve action potentials is the most common electrophysiological alteration that can precede symptoms or worsen after thalidomide withdrawal and often does not resolve.[6,11] Proposed mechanisms to explain TrPN include; anti-angiogenesis, direct toxic effects on the posterior root ganglia and dysregulation of neurotrophin activity through NFκB.

To date there have been only a limited number of small studies investigating the genetic factors that associate with TrPN.[12-13] In this study we have sought to address this by investigating the genetic variation associated with risk of TrPN in two large clinical trials, consisting of patients treated with thalidomide or vincristine and genotyped with a custom targeted panel of 3404 SNPs.

Materials and methods

Clinical samples

Peripheral blood DNA samples were obtained from 1512 presenting multiple myeloma cases derived from two randomized clinical trials; Medical Research Council (MRC) Myeloma-IX study (n=993), and the HOVON-50/GMMG-HD3 (n=519), comparing standard treatment to thalidomide containing regimens. Peripheral neuropathy was assessed using National Cancer Institute (NCI) Common Toxicity Criteria version 2.0. Neuropathy events were only considered following induction therapy and not during maintenance. In a discovery set analysis we compared genotype results from 194 Myeloma-IX patients with neuropathy following exposure to thalidomide, with 416 control patients treated with thalidomide that did not develop neuropathy. Cases and controls were matched for age and sex. As validation we carried out a comparison using 74 thalidomide treated patients with neuropathy from the HOVON-50/GMMG-HD3 trial, with 176 thalidomide treated controls. We then carried out a comparison using cases of neuropathy grade ≥ 2 in the Myeloma-IX dataset; 75 cases with neuropathy were compared with 297 matched controls. The HOVON-50/GMMG-HD3 analysis contained 49 neuropathy ≥ 2 cases with 176 controls. In the non-thalidomide arms, neuropathy events were also observed following vincristine exposure. We carried out a nested case-control comparison using 76 cases of vincristine related neuropathy from the Myeloma-IX trial compared to 307 controls. In the HOVON-50/GMMG-HD3 cohort we compared genotypes from 26 vincristine related neuropathy cases with 226 controls. To ensure homogeneity of allelic frequencies, only patients of European descent were included.

Clinical trials

The Myeloma IX study comprises 2 randomizations: an intensive pathway for younger, fitter patients comparing CVAD (cyclophosphamide 500 mg orally weekly, vincristine 0.4 mg intravenously on days [d] 1-4), doxorubicin 9.0 mg/m^2 on d1-d4, dexamethasone 40 mg on d1-d4 and d12-d15), delivered by a central venous access device with oral CTD (cyclophosphamide, thalidomide, dexamethasone) using the same doses of cyclophosphamide and dexamethasone combined with 200 mg of thalidomide. The second randomization, for older, less-fit patients, compared an attenuated dose of CTD (thalidomide 100-200 mg) to melphalan (7.0 mg/m^2 orally on d1-d4 every 28 days) and prednisolone (MP). The HOVON-50/GMMG-HD3 study randomized patients to either: 3 cycles of VAD (vincristine (0.4 mg, intravenously rapid infusion on d1-d4), doxorubicin (9 mg/m^2, intravenously rapid infusion on d1-d4) and dexamethasone (40 mg orally, d1-d4, d9-d12, and d17-d20)) or the same regimen but with thalidomide (TAD; 200 mg, but could be escalated to 400 mg), replacing the vincristine (Figure 1A).

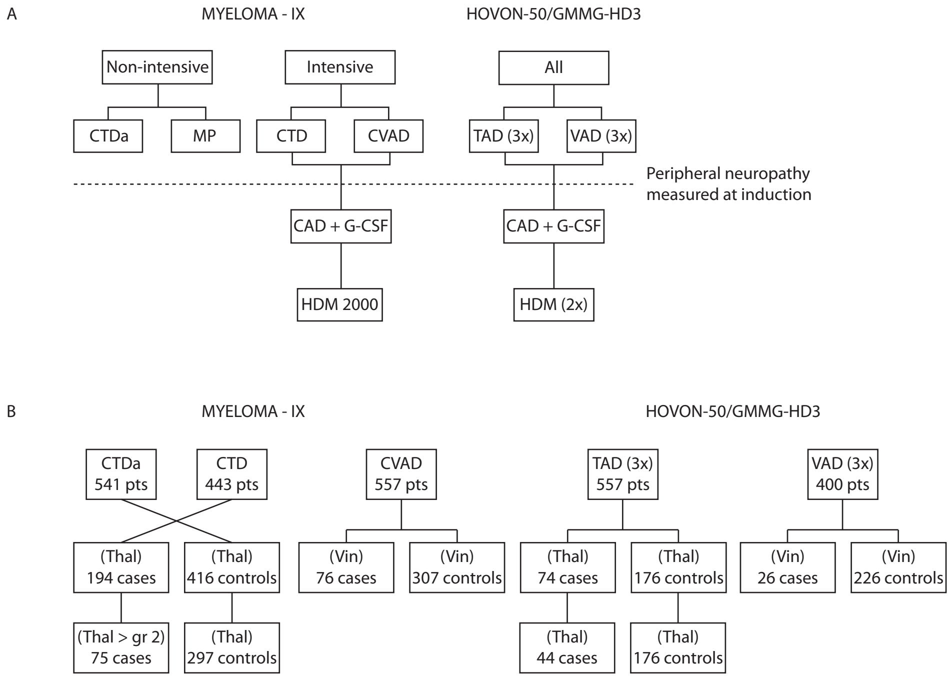

Figure 1. (A) Simplified treatment arms of the Myeloma-IX and HOVON-50/GMMG-HD3 trials. (B) Case and control comparisons used in SNP analysis for patients from the Myeloma-IX and HOVON-50/GMMG-HD3 trials.

Genotyping and SNP selection

DNA was extracted from frozen white blood cell pellets using Flexigene kit (Qiagen, Crawley, UK) and quantified using a Nanodrop® Spectrophotometer (Nanodrop products, Wilmington, USA). Genotyping was performed using an Affymetrix Targeted genotyping True-tag Bank On A Cure (BOAC) array.[14-16] SNPs were selected using a hypothesis-driven strategy, targeting genes and SNPs with previously described associations or putative functional effects. There was no sample ascertainment bias between cases and controls, as genotyping was performed before access to demographic and phenotypic data.

Statistical analyses

Departures from Fisher Exact Hardy-Weinberg equilibrium at a *P* level less than 10^{-5} and bias in missing data were controlled for each SNP. A Cochran-Armitage trend test and a Fishers Exact test were performed to evaluate odds ratios. Genomic inflation factor λ was evaluated based on median chi-squared for each set of analyses. To account for multiple testing we carried out label swapping permutation procedures on each SNP. Proxy association testing was performed where multiple SNPs in a gene were found to be associated, to resolve haplotypes. Association analysis was performed using the program PLINK v1.07.[17] DAVID Bioinformatics Resource[18] was used to characterize pathways enriched in risk associated genes.

The functional impact of regulatory SNPs in association with TrPN, was investigated by ensuring the gene was typically expressed in neurons,[19] and then subsequently examining myeloma tumor expression data in relation to SNP genotype using a Wilcoxon-type test for trend.[20-21]

To generate a predictive strategy based on genetic variation, recursive partitioning analysis was performed using the program Willows.[22] Redundant associated SNPs in linkage disequilibrium (LD) were filtered and recursive partitioning was performed on a training set consisting of two-thirds of the Myeloma-IX dataset to create an initial predictive tree, this tree was "pruned" at level of $P < 0.001$, to a smaller number of classifier SNPs. The final predictive tree was then assessed for its ability to correctly classify the remaining one-third of the Myeloma-IX and HOVON-50/GMMG-HD3 datasets.

In an alternate approach, a "risk score classifier" was generated based on the summation of the associated "at risk" and "protective" SNPs. Using a training set of two-thirds of the Myeloma-IX dataset, the $\log_{10}$(odds ratios) of the associated SNPs for each patient was summed. This scoring system was assessed for its ability to correctly classify patients "at risk" in the remaining one-third of the Myeloma-IX and HOVON-50/GMMG-HD3 datasets.

Table 1. Clinical demographics of patients within the Myeloma-IX study.

		Cases with neuropathy	%	Controls without neuropathy	%
Thalidomide	All neuropathy	192	31.8	416	68.2
	> grade 2 neuropathy	76	12.5	416	68.2
	Sensory neuropathy	91	69.1	NA	NA
	Sensory and motor neuropathy	28	14.4	NA	NA
	Motor neuropathy	27	16.5	NA	NA
	Sex	135 males	69.6 (males)	225 males	54 (males)
		59 females	31.4 (females)	191 females	46 (females)
	Median age	62 years	NA	65 years	NA
	Median cycles of thalidomide	5	NA	5	NA
	Median time to neuropathy (weeks)	8	NA	NA	NA
ISS	1	34	23.6	84	27.2
	2	54	37.5	121	39.2
	3	56	38.9	104	33.7
Paraprotein type	IgG	107	55.7	243	58.4
	IgA	46	24	82	19.7
	IgM	2	1	2	0.4
	IgD	4	2	9	0.8
	Light chain only	17	8.9	56	13.4
	Missing data	16	8.3	24	5.7
FISH status	IgH translocation	52/115	45.2	116/241	48.1
	Hyperdiploidy	60/114	52.6	122/229	53.2
	t(4;14)	12/115	10.4	31/237	13
	t(11;14)	14/115	12.2	33/237	14
	Deletion 1p32.1	12/92	13	26/202	12.9
	Gain 1q21	33/99	33.3	72/205	35.1
Vincristine	All neuropathy	76	19.8	307	NA
	> Grade 2 neuropathy	31	40.7	307	NA
	Median time to neuropathy (weeks)	8	NA	NA	NA
No thalidomide	All neuropathy	19	7.6	231	NA
or vincristine	> Grade 2 neuropathy	7	2.8	231	NA
	Median time to neuropathy (weeks)	6	NA	NA	NA

NA indicates not applicable.

Results

Clinical results

The clinical demographics of the Myeloma-IX trial patients across the whole trial (Table 1) were comparable to the samples derived from it in the case-control comparison (Table S1), and included 970 cases treated with CTD and 550 with CVAD (Figure 1B). In this analysis 31.8% of patients developed neuropathy, with grade ≥ 1, 11% grade ≥ 2, and 3.6% grade ≥ 3, following exposure to thalidomide. The median time to neuropathy was eight weeks. A higher frequency of grade ≥ 3 was seen in older patients, at 5%. In patients exposed to vincristine, 33.6% developed neuropathy in a median of eight weeks. In contrast, 6.4% patients not exposed to thalidomide or vincristine, developed grade 1 neuropathy in a median time of six weeks (Table S2). We did not see significant differences between patients with and without neuropathy for previously described risk factors such as immunoglobulin type, and age.[7] Assessing the rates of TrPN based on the ISS stage, we show that tumor burden is not related to risk. Similarly, as fluorescence in situ hybridization (FISH) variants are distributed evenly between patients with and without neuropathy, the pathogenic subtype does not seem to contribute to risk. Interestingly, we identified increased rates in male cases compared to females, the cause for which is uncertain. In order to address the hypothesis that patients who respond best to thalidomide are more sensitive to TrPN, we examined the relationship of neuropathy to response during induction in the Myeloma-IX study. While we saw a greater percentage of complete responses (CR) and very good partial responses (VGPR) in patients lacking neuropathy, the difference did not reach statistical significance (Pearson Chi-square test, $P = 0.55$; Figure 2A). Additionally, we did not see a significant relationship between response and time to neuropathy (Figure 2B). These clinical data show the variability of risk between patients and the lack of any simple clinical variants that can predict risk.

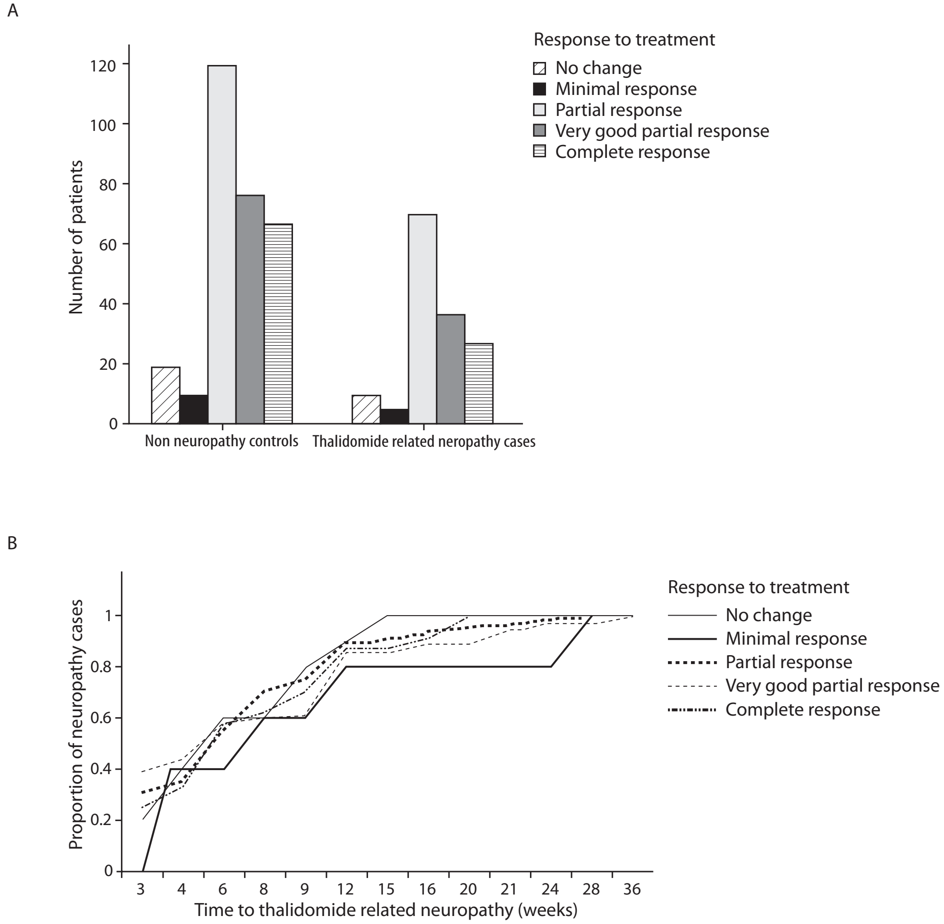

Figure 2. (A) Response status versus neuropathy during induction therapy in the Myeloma-IX study. (B) Time to neuropathy in relation to response during induction therapy with thalidomide in the Myeloma-IX study.

Genotyping results

To address the hypothesis that genetic variation mediates the risk of TrPN we compared the genetic contribution to cases that developed neuropathy with those who did not. The initial analysis included all TrPN cases and a secondary analysis was carried out using only neuropathy of grade ≥ 2. SNPs significantly associated with TrPN ($P < 0.01$) in the Myeloma-IX dataset are shown in Table 2. The associated SNPs were similar in grade ≥ 1 and grade ≥ 2 analyses, indicating a common mechanism for thalidomide neuropathy across the groups.

Table 2. SNP associations with thalidomide related neuropathy (grade ≥ 2) in the Myeloma-IX study, using a Trend test for association, permutated *P* < 0.01. The genomic inflation factor λ is 1.0.

SNP	Chr	Alleles	Odds ratios Fishers Exact (95% CI)	*P* for Trend	Gene	SNP type
rs246220	16	G > C	0.49 (0.33-0.73)	< 0.001	*ABCC1*	Intron,TagSNP:*ABCC1*
rs7164902	15	A > G	0.60 (0.44-0.80)	0.001	*SLC12A6*	Coding-synonymous
rs1805386	13	G > A	0.56 (0.39-0.79)	0.001	*LIG4*	Coding-synonymous
rs3740066	10	T > C	1.51 (1.18-1.93)	0.001	*ABCC2*	Coding-synonymous
rs4752904	11	G > C	0.69 (0.54-0.88)	0.002	*PTPRJ*	Coding-nonsynonymous
rs8192341	8	A > C	1.48 (1.14-1.93)	0.003	*SFTPC*	Coding-nonsynonymous
rs2292334	6	A > G	0.71 (0.55-0.91)	0.004	*SLC22A3*	Coding-synonymous
rs246	11	A > G	1.54 (1.15-2.07)	0.005	*CYP2C9*	Intron
rs4839469	1	A > G	1.56 (1.13-2.15)	0.005	*VANGL1*	Coding-nonsynonymous
rs3735481	7	A > C	1.50 (1.12-2.00)	0.006	*PPIA*	Intron
rs2272037	15	T > C	1.40 (1.10-1.78)	0.007	*IGF1R*	Intron
rs231775	2	G > A	1.38 (1.08-1.77)	0.007	*CTLA4*	Coding-nonsynonymous
rs914959	1	T > C	0.70 (0.54-0.90)	0.007	*DPYD*	Intron,TagSNP:*DPYD*
rs3136794	8	G > A	1.62 (1.15-2.29)	0.009	*POLB*	Intron

SNP indicates single nucleotide polymorphism; Chr, chromosome; CI, confidence interval.

Findings from the Myeloma-IX study were validated in a second comparable trial; HOVON-50/GMMG-HD3, and significantly associated SNPs in this study are shown in Table S2 and S4. In order to cross validate the findings between the trials, we investigated whether a significantly associated SNP in either trial alone (permutated *P* ≤ 0.05) was associated with risk in the second trial. This included 103 SNPs from the Myeloma IX and 82 SNPs from HOVON-50/GMMG-HD3. Using this approach on TrPN grade ≥ 2, we found five cross validating SNPs in different genes (Table 3). It should be noted however, that the size of the validation set may be under powered to validate all associations in the discovery set. Associated regulatory region SNPs were analyzed for a relationship with gene expression (Figure S1).

Table 3. SNP associations with thalidomide related neuropathy (grade ≥ 2) associated in both the Myeloma-IX and HOVON-50/GMMG-HD3 trials, using a Trend test for association, permutated *P* < 0.05.

			(UK- Myeloma IX)		(Dutch- HOVON-50)			
SNP	Chr	Alleles	Odds ratios Fishers Exact (95% CI)	Permutated *P* for Trend	Odds ratios Fishers Exact (95%CI)	Permutated *P* for Trend	Gene	SNP Type
rs7164902	15	A > G	0.60 (0.44-0.80)	< 0.001	0.47 (0.25-0.87)	0.023	*SLC12A6*	Coding-synonymous
rs6103	18	G > C	0.70 (0.52-0.95)	0.018	0.56 (0.30-1.07)	0.054	*SERPINB2*	Coding-nonsynonymous
rs2076169	6	G > A	0.60 (0.38-0.95)	0.026	0.27 (0.08-0.90)	0.025	*PPARD*	Intron
rs363717	9	C > T	0.71 (0.52-0.98)	0.041	0.46 (0.22-0.97)	0.045	*ABCA1*	Untranslated
rs1799969	19	A > G	0.67 (0.44-1.03)	0.050	0.40 (0.15-1.04)	0.046	*ICAM1*	Coding-nonsynonymous

SNP indicates single nucleotide polymorphism; Chr, chromosome; CI, confidence interval.

Haplotype analysis of *ABCC1* variants with TrPN, reveal that the main effect can be attributed to a two SNP haplotype involving SNPs rs246217 and rs246218 (Figure S2). Analysis of the 12 genotyped SNPs in the *ABCC2* gene reveal a four SNP haplotype, with three SNPs additively contributing to risk of TrPN (Figure S3).

To investigate whether the observed associations with thalidomide were drug specific, we sought to contrast findings with vincristine treated patients. SNPs significantly associated with vincristine related neuropathy in the Myeloma-IX dataset are shown in (Table 4, S3, and S4).

Table 4. SNP associations with vincristine related neuropathy (grade ≥ 2) associated in both the Myeloma-IX and HOVON-50/GMMG-HD3 trials, using a Trend test for association, permutated $P < 0.05$.

			(UK- Myeloma IX)		(Dutch- HOVON-50)			
SNP	**Chr**	**Alleles**	**Odds ratios Fishers Exact (95% CI)**	**Permutated *P* for Trend**	**Odds ratios Fishers Exact (95%CI)**	**Permutated *P* for Trend**	**Gene**	**SNP type**
rs7242	7	G > T	1.88 (1.17-3.01)	0.006	2.15 (1.15-4.00)	0.015	*SERPINE1*	Untranslated
rs2082382	5	G > A	0.50 (0.30-0.83)	0.009	0.52 (0.27-1.02)	0.051	*ADRB2*	Promoter
rs1555026	1	C > T	2.73 (1.29-5.76)	0.009	2.45 (0.95-6.30)	0.049	*ID3*	Locus
rs1042714	5	G > C	0.51 (0.31-0.85)	0.010	0.52 (0.27-1.01)	0.047	*ADRB2*	Coding-nonsynonymous
rs1934951	10	T > C	1.90 (1.14-3.16)	0.012	2.36 (1.20-4.65)	0.016	*CYP2C9*	Intron
rs7214723	17	C > T	1.70 (1.06-2.71)	0.020	1.90 (1.03-3.51)	0.038	*CAMKK1*	Coding-nonsynonymous
rs1058932	10	A > G	1.74 (1.04-2.89)	0.027	2.18 (1.11-4.30)	0.024	*CYP2C8*	Untranslated
rs2301157	13	A > G	0.61 (0.38-1.00)	0.039	0.52 (0.27-0.98)	0.035	*SLC10A2*	Untranslated
rs228832	20	T > C	0.51 (0.27-0.96)	0.040	0.39 (0.16-0.94)	0.030	*NFATC2*	Intron

SNP indicates single nucleotide polymorphism; Chr, chromosome; CI, confidence interval.

Using inherited genetic variation as a clinical means of risk stratification to adjust thalidomide dose is an important clinical goal. In an effort to build a predictive model based on a limited number of predictive SNPs, we examined two classification methods. In the first method, we utilized recursive partitioning to create a predictive tree. This tree predicted cases and controls with a sensitivity of 38% and a specificity of 31% in the Myeloma-IX validation set, and a sensitivity of 30.7% and a specificity of 81.6% in HOVON-50/GMMG-HD3 dataset (Figure S4). The second method; cumulative risk score, predicted cases of neuropathy with a sensitivity of 100% and a specificity of 77.8% in the Myeloma IX validation, and sensitivity of 60% and a specificity of 70.5% in HOVON-50/GMMG-HD3 dataset (Figure S5).

Discussion

Using a hypothesis-driven candidate gene approach we show for the first time that there is a significant genetic contribution to the risk of TrPN. Thalidomide is used in the treatment of a number of diseases where the increased production of TNFα is thought to be pathogenically important, but the benefits of thalidomide can be limited by the onset of TrPN. This study, the largest of its kind to date, has allowed us to identify genetic variants that contribute to TrPN risk. These findings provide important insights into the therapeutic management of these patients and will stimulate further research into protective strategies for patients treated with thalidomide. While we considered taking a genome-wide approach to identify such genetic variation, this was not applicable because of issues with statistical power. In addition, the candidate gene approach allowed us to examine our candidate regions in greater detail than could be achieved with mapping arrays such as the Affymetrix 500K or Illumina 550K.

The doses of thalidomide used in the study were moderate at 100-200 mg, and the neuropathy occurred early, after a median of 3 cycles of treatment. This group of cases is distinct from the group who develop neuropathy associated with prolonged exposure, which is dependent on the total dose of thalidomide received and was not studied here. We did not identify a clinical parameter which was associated with early onset neuropathy and patients were not unduly sensitive to thalidomide, with response rates being similar in cases and controls. We cannot be certain about associations with other co-morbid conditions such as diabetes, diet and alcohol consumption as the data were not systematically collected. Cases with pre-existing neuropathy were excluded.

A number of ATP-Binding Cassette (*ABC*) transporters genes were linked with TrPN. Both *ABCC1* SNPs rs246217 and rs246218 are intronic, but lie within transcription binding domains and may mediate levels of expression of *ABCC1*. Two SNPs in *ABCC2* ((rs3740066) and (-24) C > T (rs717620)) are also associated with neuropathy risk. rs717620 has been associated with decreased ABCC2 function *in vitro*[23-25] and toxicity in other systems,[26] whereas rs3740066 may modulate substrate specificity via codon usage therefore influencing the translation rate.[27] Also, in *ABCA1* rs363717 is a cross validating SNP and is found in the binding domain of the microRNA, hsa-miRNA-299. Weaker associations were also seen with *ABCB1* and its role in mediating peripheral neuropathy in response to taxane exposure has been previously reported.[28-29] Interestingly, thalidomide can modulate the function of ABCC2 and ABCB1,[30] both of which are active in neuronal function.

We saw a number of associations with other absorption, distribution, metabolism, excretion (*ADME*) genes, including a promoter SNP in *FMO6* (rs1736565) and the ion channel gene *SLC12A6* (rs7614902), previously associated with peripheral neuropathy.[31] It has been suggested that CYP2C19 plays a role in thalidomide metabolism,[32-34] however, it is now known that thalidomide breakdown is dependent on spontaneous non-enzymatic degradation.[35] Consistent with this we found no association of neuropathy risk with functional variants in *CYP2C19*, or other genes in the *CYP2C* subfamily. No consistent association was seen with common N-acetyltransferase 2 (*NAT2*) variants, as previously reported**.**[13]

Pathway analysis of the TrPN associated SNPs highlights the association with genes involved in the development of the central and peripheral nervous system. A conserved promoter SNP (rs1611753), which affects the expression of the gene *SPRR1A*, was significantly associated with

TrPN in HOVON-50/GMMG-HD3 samples. The gene *SPRR1A* is expressed by axotomized neurons and promotes axonal outgrowth.[36] Variation in neurological genes may dictate the ability of a damaged neuron to undergo repair and may mediate risk of neuropathy.

We also describe associations with SNPs in genes mediating neuro-inflammation, with some 35% of the TrPN associated SNPs having an inflammatory role. We see associations with *SERPINB2* (rs6103), a gene induced in injured neurons[37] and *PPARD* (rs2076169) which may also indicate a role of neuro-inflammation in the pathology mechanism.

We found 20% of the TrPN associated genes in this study to have some transmembrane transporter activity, consistent with a hypothesis where variants in transmembrane transporter genes mediate their effects at the level of the peripheral nerve.[38] At this site, they negatively impact the ability of the dorsal root ganglions to repair itself following exposure to the toxic effects of thalidomide and/or promote neuro-inflammatory change. Prophylactic pharmacological therapies aimed at modulating ion channel activity may prove useful in reducing neuro-toxicity caused by axonal membrane ion channel variation.[39-40]

The genetic mechanisms underlying thalidomide and vincristine neuropathy appear distinct. SNP associations with vincristine related neuropathy include rs2082382 and rs1042714 in *ADRB2.* Association with vincristine related neuropathy was observed in a highly conserved promoter SNP (rs7214723) in *CAMKK1*, which is expressed at higher levels in neurons resistant to oxidative stress. We also see an association with nonsynonymous SNPs in *CYP2C9* (rs1934951) and *NFATC2* (rs228832), conserved intronic SNPs in *ID3* (rs1555026) and *SLC10A2* (rs2301157), and in *CYP2C8* (rs1058932) which is located in the binding site for the microRNA hsa-miRNA-96. Interestingly, we see little overlap of the genes associated with TrPN in comparison with vincristine related neuropathy. We propose that this is indicative of a fundamentally different pathological mechanism between these neuropathies. In contrast, similar TrPN associated genes have been observed in studies investigating peripheral neuropathy in response to paclitaxel, bortezomib and docetaxel which result in sensory peripheral neuropathies as opposed to the sensorimotor neuropathy associated with exposure to the vinca alkaloids and taxol.

We show that simple clinical factors do not allow the identification of patients at greater risk of neuropathy following thalidomide exposure, for which dose adjustment or cessation of therapy would be appropriate. Consequently, we have attempted to define a limited number of SNPs that can identify high risk patients suitable for more intensive clinical monitoring. In this approach, we have used two risk classification methods both of which can identify patients at increased risk, but the predictive value of these is not adequate to totally avoid thalidomide usage. However, this approach can be used to identify patients requiring greater clinical vigilance and suitable counseling.

The poor risk prediction in our classifiers is due to a number of limitations, which include; a) naive assumptions in modeling methods, the risk score method assumes interactions to be solely additive, whilst recursive partitioning method interactions are assumed to be uni-directional,

b) hypothesis-driven approaches do not consider all variation contributing to an outcome, c) population specific effects, and d) the impact of rare variation. Despite these drawbacks, the limited number of SNPs identified in this study can be used as a simple and useful method for identifying patients at high risk of TrPN, who in turn may benefit from greater clinical vigilance.

Acknowledgements

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Chapter 5

Genetic factors underlying the risk of bortezomib induced peripheral neuropathy in multiple myeloma patients

Sophie L. Corthals, Pieter Sonneveld, David C. Johnson,
Joost L.M. Jongen, Yvonne de Knegt, Hartmut Goldschmidt,
Henk M. Lokhorst, Stéphane Minvielle, Florence Magrangeas,
Roman Hajek, Orhan Sezer, Jean-Luc Harousseau,
Bronno van der Holt, Rowan Kuiper, Brian G.M. Durie,
Brian van Ness, Gareth J. Morgan, Hervé Avet-Loiseau

Submitted

Abstract

Bortezomib induced peripheral neuropathy (BiPN) is a major concern in the treatment of multiple myeloma, however little is known about the mechanism underlying this dose-limiting side effect. To identify genetic risk factors that are associated with development of BiPN in multiple myeloma patients, a pharmacogenetic association study was performed in a discovery set of 238 multiple myeloma patients who were treated with bortezomib from the randomized clinical trial; IFM 2005-01. Validation was performed using 231 multiple myeloma patients from the randomized clinical trial HOVON-65/GMMG-HD4 and a Czech Republic dataset. A total of 3404 single nucleotide polymorphisms (SNPs) in 964 genes spanning 67 molecular pathways related to the biology of multiple myeloma, treatment response and toxicity, were analyzed for a possible association with BiPN. Chi-square association analysis revealed a significant association between development of BiPN and SNPs in proinflammatory genes *TNFα* (rs2857605, rs2228088), prothrombin (rs3136516) and *PARP1* (rs2280712, rs1805408, rs1805407, rs1805405, rs1002153). Additional associations were observed in genes with neurological functionality *NGFR* (rs11466155), *NEFL* (rs2976437, rs2976436), and *IKBKAP* (rs10979601, rs10759326). This study shows that SNPs in genes involved in inflammatory disease or neurological disorders are associated with BiPN. The results contribute to a better understanding of the pathogenic mechanisms underlying the development of BiPN.

Introduction

Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) is a cobalt containing small molecule which specifically inhibits the 26S proteasome. The ubiquitin-proteasome pathway is responsible for the degradation of cellular proteins. Inhibition of this pathway leads to disruption of the protein regulation involved in cell cycle control, cell growth, angiogenesis and apoptosis, which eventually results in cell cycle arrest and apoptosis of myeloma cells.[1] Bortezomib blocks the activation of transcription factor nuclear factor-kappa B (NFκB), by inhibiting the breakdown of inhibitory kappa B (IκB), subsequently inactivating multiple downstream pathways known to be important in MM cell signaling.[2] The introduction of bortezomib has greatly improved the management of multiple myeloma (MM). Clinical trials with bortezomib in relapsed and/or refractory MM have shown high response rates and a survival advantage.[3-5] Subsequent to this, in newly presenting patients, bortezomib combinations have been shown to be associated with excellent complete response (CR) and very good partial response (VGPR) rates.[6-8]

Bortezomib is generally well tolerated in patients of all ages; however the dose-limiting toxicity is peripheral neuropathy, which frequently requires a dose reduction or treatment discontinuation.[3-4,9-10] Peripheral neuropathy is present in 10 percent of MM patients at diagnosis, and has a clinical and electrophysiological phenotype which is different from bortezomib induced peripheral neuropathy (BiPN). BiPN is often related to cumulative dosing and predominantly sensory resulting from axonal degeneration.[11-13] It is characterized by burning pain, distal paresthesias, numbness and neuropathic pain. In the majority of patients, BiPN is reversible and does not seem to be influenced by number or type of previous treatments,[14] although preexisting neuropathy in MM patients may contribute to BiPN.[9]

Little is known about the mechanism underlying BiPN and to date two animal models of BiPN have been described.[12-13] Some of the mechanisms that may contribute to the development of BiPN[15] include decreased neurotrophin-p75NTR signaling through inhibition of NFκB,[16-17] damage to mitochondria and the endoplasmic reticulum, together with autoimmune factors and inflammation.[13,18] The nature of BiPN and its clinical features are highly suggestive of a genetically mediated process, as some patients develop BiPN during the first cycle, whereas other patients may tolerate up to 10 cycles without any sign of BiPN. In this study, we identify genetic risk factors associated with the development of BiPN in newly diagnosed patients with MM who were treated with bortezomib. A custom-built SNP array was used that contains 3404 SNPs located in genes that have a role in the pathogenesis and clinical characteristics of MM, as well as metabolic and drug transport genes.

Patients and methods

Patients

The study was performed in patients who had been included in two randomized clinical trials, i.e. the HOVON-65/GMMG-HD4[19] (ISRCTN64455289) trial for newly diagnosed patients with MM (n=833), and the IFM 2005-01 trial (NCT00200681; n=493), both of which compared standard induction treatment (vincristine, adriamycin and dexamethasone; VAD) with a bortezomib combination prior to high-dose therapy (HDT) and stem cell transplantation. In addition, a prospectively collected set of samples (n=56) from the Babak Research Institute in the Czech Republic was included as part of the cooperative program of the International Myeloma Foundation and International Myeloma Working Group. This set contained patients who were treated uniformly with bortezomib and dexamethasone at relapse. All patients gave written informed consent for this genetic study. Patients with amyloidosis or monoclonal gammopathy of unknown significance were excluded. Adverse events (AEs) were prospectively assessed using standard National Cancer Institute Common Toxicity Criteria for Adverse Events, version 3.0 (CTCAE 3.0).[20] All patients treated with bortezomib from whom diagnostic blood samples were obtained were included in the analysis. To ensure homogeneity of allelic frequencies, 15 patients of non-European descent were excluded from the study. In total 238/246 patients from IFM 2005-01, 183/412 patients from HOVON-65/GMMG-HD4 and 48/56 from the Czech Republic who were randomized for treatment with bortezomib were included in the analysis. Samples were divided into a discovery and validation set. In discovery set, comprising 238 IFM 2005-01 patients, 99 bortezomib treated patients who developed BiPN were analyzed against 139 patients that did not develop BiPN (Figure 1B). As validation, an association analysis was performed using the 183 HOVON-65/GMMG-HD4 and 48 Czech Republic patients; 131 patients who developed BiPN were compared to 100 patients that did not develop BiPN (Figure 1B).

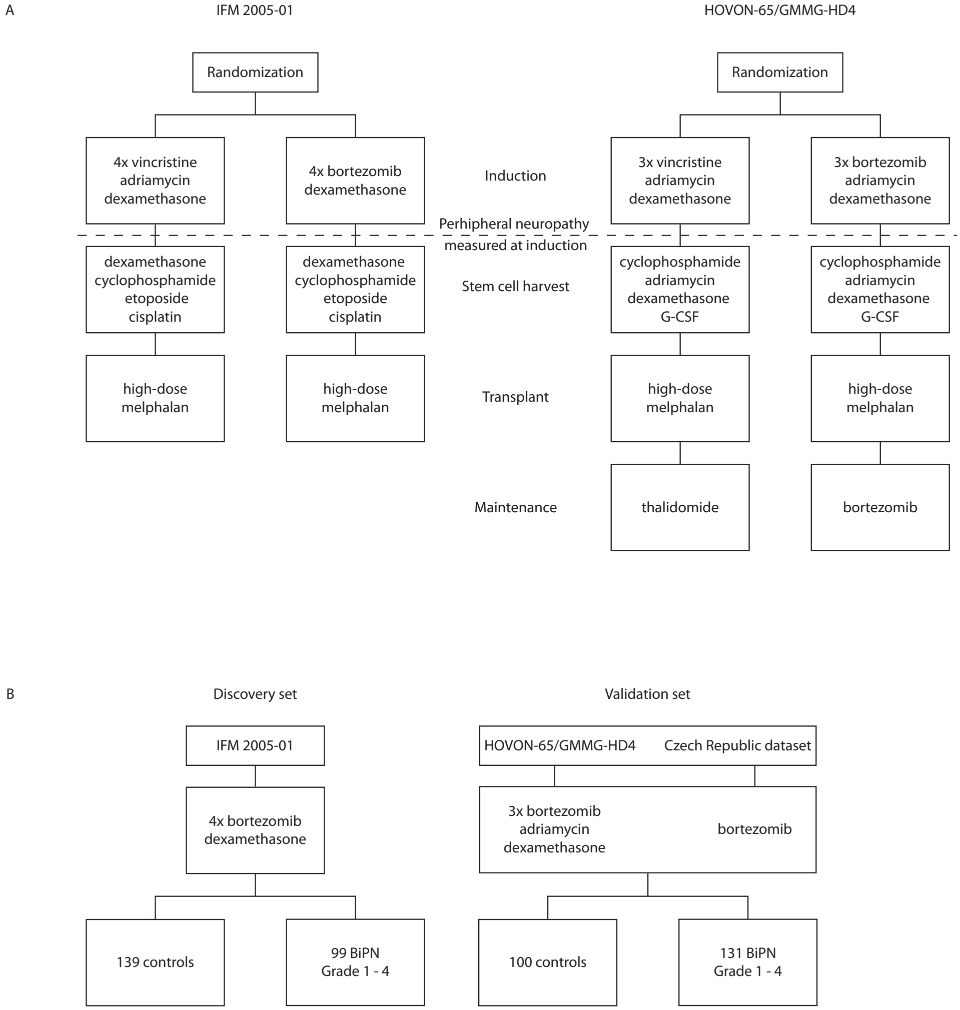

Figure 1. Schematic representation of MM patient treatment and numbers. (A) Schematic representation of IFM 2005-01 and HOVON-65/GMMG-HD4 treatment. (B) BiPN case and control comparisons used in SNP analysis for discovery and validation set. G-CSF indicates granulocyte colony-stimulating factor; BiPN, bortezomib induced peripheral neuropathy.

Clinical trials

In the IFM 2005-01 trial, patients aged 18-65 years were randomized to receive 4 cycles of VAD or bortezomib 1.3 mg/m^2 days 1, 4, 8, 11 combined with dexamethasone (40 mg orally, days 1-4, 9-12, 17-20) before transplantation (Figure 1A). In the HOVON-65/GMMG-HD4 trial, patients aged 18-65 years were randomized to receive 3 cycles of PAD (bortezomib 1.3 mg/m^2 days 1, 4, 8, 11, doxorubicin 9 mg/m^2, days 1-4 and dexamethasone 40 mg orally, days 1-4, 9-12, 17-20) or standard VAD at 28 day intervals as induction regimen prior to high-dose therapy (Figure 1A).

Genotyping

DNA was extracted from peripheral blood nucleated cells or CD138 negative bone marrow cells and quantified using a Nanodrop Spectrophotometer (Nanodrop products, Wilmington, USA). Genotyping was performed using an Affymetrix Targeted genotyping custom-built panel, comprising 3404 SNPs, selected using a hypothesis driven strategy, targeting genes and SNPs with previously described associations or putative functional effects.[21-22] There was no sample ascertainment bias between cases and controls, as genotyping was performed before access to demographic and phenotypic data.

Statistical analysis

The clinical characteristics, ISS stages and other prognostic variables of the patients from the HOVON-65/GMMG-HD4 trial who were included in the analysis (n=183) were compared with those not included (n=229). Chi-square and Fisher's exact tests were used to compare nominal variables and continuous variables were analyzed using the Mann-Whitney U-test (Table 1).

SNPs departing from Hardy-Weinberg equilibrium ($P < 0.00001$) were excluded from further analysis. To evaluate SNP associations with BiPN and calculation of odds ratios (ORs) a chi-square and a Fishers exact test were performed using the discovery set. Genomic inflation factor λ was evaluated based on median chi-squared. Analysis was performed in the program PLINK v1.07.[23] To account for multiple testing, 10.000 permutation tests were performed using the max(T) permutation procedure implemented in PLINK. In this procedure, two sets of empirical significance values were calculated: pointwise estimates of an individual SNP's significance (empirical pointwise *P*-values) and corrected values for multiple testing (corrected empirical *P*-values). The pointwise *P*-values have been used to indicate the significance level of association. The associated gene sets were subjected to Ingenuity Pathway Analysis (Ingenuity System Inc., USA). In order to evaluate the effect of nonsynonymous SNPs associated with BiPN, these SNPs were characterized using the prediction program Sorting Intolerant From Tolerant (SIFT).[24]

As validation, a Cochran Mantel-Haenszel stratified association test was performed in an independent dataset comprised of patients from the HOVON-65/GMMG-HD4 trial and patients from the Czech Republic to evaluate cross validating SNP associations and ORs. Specifically, ORs from significant SNPs (pointwise $P < 0.05$) in the discovery set were selected for validation. A one-sided test for OR > 1 (OR < 1) was performed to test whether ORs in the discovery and validation set cross validated.

Table 1. Demographic and clinical characteristics of MM cases and controls restricted to bortezomib treated patients in the IFM 2005-01 trial.

	Discovery set		**Validation set**						
	IFM 2005-01		**HOVON-65/GMMG-HD4**					**Czech Republic dataset**	
	Included in SNP analysis (n = 238)		**Not included in SNP analysis (n = 229)**		**Included in SNP analysis (n = 183)**		***P*-value**	**Included in SNP analysis (n = 48)**	
Age									
Median (years)	57		57		57		0.62	nd	
Range (years)	35-68		31-65		32-65			nd	
Sex	no.	percentage	no.	percentage	no.	percentage		no.	percentage
M	132	61%	144	63%	109	60%	0.54	27	56%
F	85	39%	85	37%	74	40%		17	35%
nd								4	8%
ISS									
I	nd		90	39%	82	45%	0.25	22	46%
II	nd		73	32%	46	25%		7	15%
III	nd		48	21%	32	17%		7	15%
nd	nd		18	8%	23	13%		12	25%
M protein heavy chain									
IgA	59	25%	54	24%	35	19%	0.45	12	25%
IgG	129	54%	130	57%	119	65%		29	60%
IgD	6	3%	4	2%	2	1%		0	0%
LCD	0	0%	36	16%	24	13%		3	6%
nd	44	18%	5	2%	3	2%		4	8%
M protein light chain									
Kappa	148	62%	148	65%	122	67%	0.78	27	56%
Lambda	71	30%	76	33%	59	32%		17	35%
nd	19	8%	5	2%	2	1%		4	8%
BiPN grade									
0	139	58%	122	53%	80	44%	0.36	20	42%
1	27	11%	54	24%	52	28%		10	21%
2	57	24%	29	13%	26	14%		13	27%
3	11	5%	16	7%	19	10%		5	10%

LCD indicates light chain disease; ISS, International Staging System; BiPN, bortezomib induced peripheral neuropathy; nd, not determined.

Results

For the association analysis, 469 MM patients who had been treated with bortezomib were assessable. The demographics and clinical characteristics of patients with BiPN and controls were not different from the whole patient group in the HOVON-65/GMMG-HD4 trial (Table 1). In the discovery set, 27 patients developed BiPN grade 1, 57 grade 2, 11 grade 3, and 4 grade 4. The BiPN rates and clinical characteristics of both the discovery set and the validation set are shown in Table 1. Figure 2 shows the time to BiPN for each grade separately in patients from the HOVON-65/GMMG-HD4 trial, which are included in the validation set. The median time to BiPN grade 1 was 6 weeks, and 7 weeks to grade 2, 3 or 4. The peripheral neuropathy rates In the VAD treatment arm of the HOVON-65/GMMG-HD4 trial, which will not further be discussed, are shown in Table S1. The clinical characteristics age, immunoglobulin type and ISS stage, did not contribute to risk of BiPN.

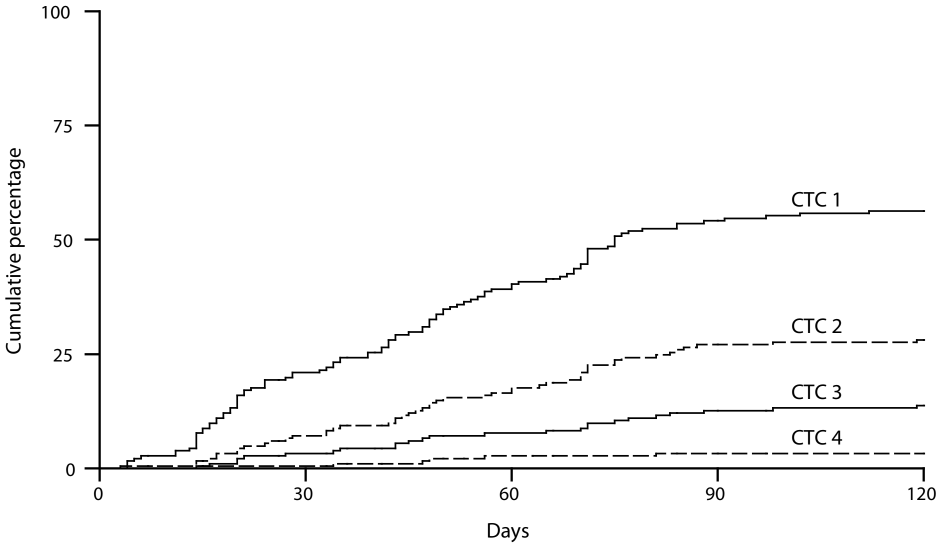

Figure 2. BiPN rates by time to BiPN in the HOVON-65/GMMG-HD4 trial. Cumulative percentage of MM patients from the HOVON-65/GMMG-HD4 (n=412) who developed grade 1 (26%), grade 2 (13%), grade 3 (9%) or grade 4 (3%) BiPN shown in time (days). CTC indicates common toxicity criteria.

After imputation and removing monomorphic SNPs, a panel containing 2534 SNPs was analyzed for association by conducting a chi-square association analysis. No evidence of deviation from Hardy-Weinberg equilibrium at $P = 0.00001$ was observed for the 2534 SNPs in either case or control samples. The results for all SNPs associated with BiPN at pointwise *P*-level less than 0.01 are presented in Table 2 and Table S2 shows associated SNPs at pointwise *P*-level less than 0.05.

Table 2. SNPs associated with BiPN (pointwise $P < 0.01$) using a chi-square association analysis. The genomic inflation factor λ is 1.0201.

SNP	Chr	Alleles	OR	95% CI	Pointwise *P* for Chi-square	Gene	SNP type
rs3759217	12	C > T	2.76	1.58 - 4.84	< 0.001	*CDKN1B*	Locus
rs11466155	17	C > T	1.87	1.25 - 2.80	0.004	*NGFR*	Coding-synonymous
rs6033	1	A > G	2.53	1.30 - 4.94	0.006	*F5*	Coding-nonsynonymous
rs2228088	6	G > T	0.56	0.36 - 0.87	0.006	*TNF*	Coding-synonymousymous,TagSNP:*TNF*
rs2686184	8	G > A	1.72	1.19 - 2.49	0.006	*FDFT1*	3′ UTR
rs12721516	1	C > T	0.55	0.34 - 0.90	0.007	*CSF1*	Coding-nonsynonymous
rs6945306	7	G > C	1.71	1.17 - 2.52	0.007	*STK31*	Coding-nonsynonymous
rs228851	20	G > T	0.59	0.41 - 0.86	0.008	*NFATC2*	Intron
rs3136516	11	A > G	0.61	0.42 - 0.88	0.008	*F2*	Intron
rs6018	1	T > G	2.42	1.23 - 4.74	0.009	*F5*	Coding-nonsynonymous
rs584589	17	A > G	2.01	1.16 - 3.47	0.009	*NGFR*	Promoter
rs4148949	10	C > T	0.60	0.41 - 0.88	0.010	*CHST3*	Untranslated

SNP indicates single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

The observed significant SNPs were similar in patients with BiPN grade ≥ 2, ≥ 3 or grade 4, indicating that the mechanisms underlying BiPN may be common across the groups. In this study we did not analyze the early onset versus late onset BiPN. The results of the analysis performed in the discovery set (IFM 2005-01 trial) were validated using an independent dataset from the Czech Republic who were treated uniformly with bortezomib and dexamethasone at relapse combined with the HOVON-65/GMMG-HD4 trial. The validation set included 131 randomly assigned patients who developed BiPN and 100 patients who did not develop BiPN. A Cochran Mantel-Haenszel stratified association test was performed. Significantly associated SNPs (pointwise $P < 0.05$) in this validation set are shown in Table S3. To investigate whether significant SNPs (pointwise $P < 0.05$; n=70) in the discovery set cross validated with this external reference set a one-sided test for ORs was performed. This resulted in one significantly cross validating SNP; rs619824 in CYP17A1 (Table 3). Because of the limited power of the validation set, not all associations in the discovery set (pointwise $P < 0.05$) could be validated, and therefore, cross validating SNPs at *P*-level < 0.2 are shown in Table 3.

To evaluate the mechanisms mediating the development of BiPN, a pathway analysis of the associated genes (pointwise $P < 0.05$) was performed. Top related functions were “Cancer” and “Immunological disease”.

Table 3. Cross validation of significantly associated SNPs in the IFM 2005-01 discovery set**.** A Cochran chi-square test and a Mantel-Haenszel stratified association test were used to analyze SNP associations in the discovery set (IFM 2005-01) and the validation set (HOVON-65/GMMG-HD4 and Czech Republic samples) respectively. A one-sided test for ORs was performed to investigate cross validation of significant SNPs (pointwise $P < 0.05$) in the discovery set.

					One-sided test for ORs		**IFM 2005-01 discovery set**			**HOVON-65/GMMG-HD4 and Czech Republic validation set**		
SNP	**Chr**	**Alleles**	**Gene**	**SNP type**	**Unadjusted *P*-value discovery set**	**Unadjusted *P*-value validation set**	**OR**	**95% CI**	**Pointwise *P* for Chi-square**	**OR**	**95% CI**	**Pointwise *P* for Cochran Mantel-Haenszel**
rs619824	10	G > T	*CYP17A1*	3'UTR	0.018	0.029	0.64	0.44 - 0.93	0.010	0.70	0.48 - 1.01	0.045
rs2857605	6	A > G	*TNF*	Intron,TagSNP:*TNF*	0.021	0.046	0.57	0.36 - 0.92	0.034	0.69	0.45 - 1.06	0.082
rs6945306	7	G > C	*STK31*	Coding-nonsynonymous	0.006	0.082	1.71	1.17 - 2.52	0.007	1.33	0.89 - 1.97	0.204
rs10979601	9	C > T	*IKBKAP*	Coding-nonsynonymous	0.054	0.098	1.57	0.99 - 2.48	0.048	1.38	0.85 - 2.26	0.194
rs10759326	9	T > G	*IKBKAP*	Coding-nonsynonymous	0.054	0.128	1.57	0.99 - 2.48	0.048	1.32	0.82 - 2.12	0.259
rs2472299	15	G > A	*CYP1A1*	Promoter	0.017	0.133	0.62	0.42 - 0.92	0.022	0.80	0.54 - 1.19	0.291
rs3136516	11	A > G	*F2*	Intron	0.009	0.141	0.61	0.42 - 0.88	0.008	0.82	0.56 - 1.18	0.284
rs2074351	7	G > A	*PON1*	Intron,TagSNP:*PON1*	0.042	0.176	1.51	1.01 - 2.26	0.050	1.22	0.80 - 1.86	0.356
rs762551	15	A > C	*CYP1A2*	Intron,TagSNP:*CYP1A*	0.021	0.182	0.63	0.42 - 0.93	0.027	0.83	0.56 - 1.23	0.393
rs1149901	10	C > T	*GATA3*	Locus,Untranslated	0.023	0.204	1.62	1.07 - 2.45	0.015	1.20	0.78 - 1.83	0.447
rs2124459	21	T > C	*CBS*	Intron	0.031	0.205	0.66	0.45 - 0.96	0.047	0.85	0.58 - 1.25	0.400
rs1052637	2	G > C	*DDX18*	Coding-nonsynonymous	0.034	0.207	0.66	0.45 - 0.97	0.031	0.85	0.58 - 1.25	0.411
rs854556	7	C > T	*PON1*	Intron,TagSNP:*PON1*	0.025	0.213	0.64	0.44 - 0.95	0.031	0.85	0.58 - 1.26	0.418
rs2227956	6	T > C	*HSPA1L*	Coding-nonsynonymous	0.015	0.221	0.52	0.30 - 0.88	0.018	0.84	0.53 - 1.32	0.464
rs3733890	5	G > A	*BHMT*	Coding-nonsynonymous	0.038	0.233	0.66	0.45 - 0.98	0.037	0.86	0.57 - 1.30	0.443
rs1296028	8	A > G	*FDFT1*	3'UTR	0.042	0.235	0.64	0.42 - 0.99	0.045	0.86	0.56 - 1.31	0.501
rs2228233	14	C > T	*NFATC4*	Coding-synonymous	0.040	0.306	0.65	0.43 - 0.98	0.048	0.90	0.60 - 1.35	0.626
rs3758581	10	G > A	*CYP2C19*	Coding-nonsynonymous	0.021	0.309	2.35	1.12 - 4.97	0.047	1.19	0.60 - 2.35	0.698
rs854555	7	C > A	*PON1*	Intron,TagSNP:*PON1*	0.027	0.315	1.53	1.05 - 2.24	0.032	1.10	0.74 - 1.65	0.629
rs9640663	7	A > G	*PTPN12*	Coding-nonsynonymous	0.036	0.318	0.67	0.46 - 0.98	0.042	0.91	0.63 - 1.33	0.652

SNP indicates single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

Table 3. continued.

rs121	7	A > G	*OSBPL3*	Intron	0.011	0.343	1.61	1.11 - 2.32	0.011	1.08	0.74 - 1.57	0.682
rs2239330	16	C > T	*ABCC1*	Coding-synonymous	0.013	0.348	0.59	0.39 - 0.90	0.012	0.92	0.61 - 1.40	0.723
rs1049434	1	A > T	*SLC16A1*	Coding-nonsynonymous	0.008	0.354	1.65	1.14 - 2.39	0.012	1.08	0.74 - 1.57	0.760
rs878201	1	G > A	*Admixture*	Admixture	0.031	0.366	1.54	1.04 - 2.29	0.015	1.07	0.72 - 1.61	0.746
rs2007231	1	T > C	*NRAS*	Intron	0.034	0.372	0.66	0.44 - 0.97	0.044	0.94	0.63 - 1.39	0.747
rs440454	6	C > T	*RDBP*	Locus,Intron	0.037	0.410	0.62	0.39 - 0.97	0.032	0.95	0.64 -1.43	0.826
rs3212254	14	C > A	*RIPK3*	Coding-nonsynonymous	0.034	0.459	2.12	1.04 - 4.31	0.042	1.04	0.54 - 1.99	1.000
rs2686184	8	G > A	*FDFT1*	3' UTR	0.004	0.475	1.72	1.19 - 2.49	0.006	1.01	0.70 - 1.47	1.000
rs6768093	3	T > A	*ATR*	Locus	0.031	0.488	0.66	0.45 - 0.96	0.036	0.99	0.68 - 1.45	0.984
rs7169	1	T > C	*SLC16A1*	Untranslated	0.008	0.500	1.65	1.14 - 2.39	0.012	1.00	0.68 - 1.46	1.000
rs584589	17	A > G	*NGFR*	Promoter	0.011	0.507	2.01	1.16 - 3.47	0.009	1.00	0.59 - 1.68	1.000
rs3776432	5	G > A	*NSUN2*	Intron	0.032	0.510	1.52	1.04 - 2.24	0.028	0.99	0.66 - 1.50	1.000
rs1641536	17	G > A	*SHBG*	3' UTR	0.014	0.517	0.43	0.22 - 0.86	0.021	1.01	0.56 - 1.82	0.965
rs2227930	3	A > T	*ATR*	Coding-synonymous	0.041	0.539	0.67	0.46 - 0.98	0.046	1.02	0.70 - 1.48	0.918
rs2033178	12	C > T	*IGF1*	Intron	0.008	0.578	2.42	1.23 - 4.74	0.014	0.93	0.45 - 1.93	0.855
rs2227928	3	C > T	*ATR*	Coding-nonsynonymous	0.041	0.605	0.67	0.46 - 0.98	0.046	1.05	0.72 - 1.53	0.806
rs4148949	10	C > T	*CHST3*	Untranslated	0.009	0.606	0.60	0.41 - 0.88	0.010	1.05	0.72 - 1.53	0.798
rs2280712	1	G > A	*PARP1*	Intron	0.036	0.611	0.57	0.34 - 0.97	0.041	1.07	0.66 - 1.75	0.797
rs1805408	1	A > G	*PARP1*	Intron	0.029	0.646	0.56	0.33 - 0.95	0.030	1.10	0.67 - 1.80	0.720
rs1002153	1	T > C	*PARP1*	Intron	0.029	0.654	0.56	0.33 - 0.95	0.030	1.10	0.68 - 1.81	0.709
rs1805405	1	C > A	*PARP1*	Intron	0.029	0.664	0.56	0.33 - 0.95	0.030	1.11	0.68 - 1.82	0.709
rs2231142	4	C > A	*ABCG2*	Coding-nonsynonymous	0.034	0.705	2.12	1.04 - 4.31	0.042	0.83	0.43 - 1.62	0.624
rs11466155	17	C > T	*NGFR*	Coding-synonymous	0.002	0.709	1.87	1.25 - 2.80	0.004	0.89	0.60 - 1.34	0.597
rs212090	16	T > A	*ABCC1*	Untranslated,TagSNP:*ABCC1*	0.015	0.724	0.63	0.44 - 0.92	0.013	1.12	0.77 - 1.63	0.570
rs4148946	10	T > C	*CHST3*	Untranslated	0.012	0.728	0.62	0.42 - 0.90	0.013	1.12	0.77 - 1.63	0.532
rs4799055	18	G > T	*NFATC1*	Intron	0.015	0.773	1.61	1.10 - 2.35	0.030	0.87	0.60 - 1.26	0.499
rs228851	20	G > T	*NFATC2*	Intron	0.005	0.788	0.59	0.41 - 0.86	0.008	1.16	0.80 - 1.68	0.451

SNP indicates single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

Table 3. continued.

					One-sided test for ORs		IFM 2005-01 discovery set			HOVON-65/GMMG-HD4 and Czech Republic validation set		
SNP	**Chr**	**Alleles**	**Gene**	**SNP type**	**Unadjusted *P*-value discovery set**	**Unadjusted *P*-value validation set**	**OR**	**95% CI**	**Pointwise *P* for Chi-square**	**OR**	**95% CI**	**Pointwise *P* for Cochran Mantel-Haenszel**
rs212087	16	C > T	*ABCC1*	Intron,TagSNP:*ABCC1*	0.028	0.820	0.66	0.46 - 0.96	0.025	1.19	0.82 - 1.73	0.402
rs9885672	6	T > C	*KIAA0274*	Coding-nonsynonymous	0.024	0.820	1.86	1.08 - 3.21	0.024	0.78	0.46 - 1.33	0.357
rs1801105	2	C > T	*HNMT*	Coding-nonsynonymous	0.031	0.848	1.95	1.06 - 3.60	0.041	0.74	0.42 - 1.31	0.295
rs2228088	6	G > T	*TNF*	Coding-synonymousymous,TagSNP:*TNF*	0.010	0.869	0.56	0.36 - 0.87	0.006	1.43	0.77 - 2.67	0.314
rs3817074	19	C > T	*BAX*	Intron	0.031	0.895	1.95	1.06 - 3.60	0.029	0.67	0.36 - 1.25	0.258
rs1405655	19	T > C	*NR1H2*	Intron	0.028	0.915	1.56	1.05 - 2.32	0.036	0.76	0.52 - 1.12	0.163
rs6018	1	T > G	*F5*	Coding-nonsynonymous	0.008	0.932	2.42	1.23 - 4.74	0.009	0.53	0.23 - 1.22	0.155
rs2973015	5	A > G	*GHR*	Intron	0.015	0.935	0.63	0.44 - 0.91	0.017	1.34	0.92 - 1.94	0.151
rs504122	13	C > T	*SPRY2*	Coding-nonsynonymous	0.021	0.935	0.63	0.43 - 0.93	0.018	1.34	0.92 - 1.97	0.076
rs163078	2	C > T	*CYP1B1*	Intron,TagSNP:*CYP1B1*	0.017	0.944	0.63	0.43 - 0.92	0.026	1.38	0.93 - 2.05	0.113
rs6027	1	A > G	*F5*	Coding-nonsynonymous	0.014	0.944	2.30	1.17 - 4.54	0.015	0.50	0.21 - 1.18	0.117
rs2976437	8	A > G	*NEFL*	Promoter	0.009	0.985	1.63	1.13 - 2.37	0.013	0.66	0.45 - 0.96	0.030
rs6033	1	A > G	*F5*	Coding-nonsynonymous	0.005	0.986	2.53	1.30 - 4.94	0.006	0.42	0.20 - 0.91	0.044
rs2976436	8	C > T	*NEFL*	Promoter	0.009	0.992	1.63	1.13 - 2.37	0.013	0.62	0.43 - 0.92	0.017

SNP indicates single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

Proinflammatory genes are associated with BiPN

Several SNPs in genes that regulate the inflammatory response were associated with BiPN. One of the most significant SNPs, rs3136516 ($P = 0.008$) was an intronic SNP located in prothrombin (coagulation factor II; *F2*), a gene that acts as a mediator in the neurotoxic cascade leading to neurodegenerative diseases.[25] Two SNPs that lie within or in close proximity of the *TNFα* gene (rs2857605 and rs2228088; Figure S1) were associated with BiPN.

The PARP pathway regulates the expression of genes involved in promoting inflammatory reactions and neuronal dysfunction.[26] A group of five intronic SNPs in *PARP1* (rs1805405, rs1805407, rs1805408, rs1002153, and rs2280712) were significantly associated with BiPN.

Associations with neuropathy susceptibility genes

The NFκB pathway is central to the immune response and two of the most significant SNPs are located in the *IKBKAP* gene. Both SNPs, rs10979601 and rs10759326, are conservative coding nonsynonymous SNPs and potentially functional in the regulation of splicing. This is a particularly relevant association because hereditary sensory and autonomic neuropathy type III, or familial dysautonomia (FD), can be caused by mutations in the *IKBKAP* gene, leading to poor development, reduced survival, and progressive degeneration of the sensory and autonomic nervous system.[27] Importantly, the FD causing mutation, which acts by altering the splicing of *IKBKAP* in a tissue-specific manner,[28] is located within a haplotype that includes the associated SNP rs2230792.

Mutations in neurofilament, light polypeptide (*NEFL*) cause Charcot-Marie-Tooth Neuropathy Type 2E/1F, the most common inherited peripheral neuropathy.[29-30] Two promoter SNPs (rs2976437 and rs2976436) in *NEFL* were significantly associated with BiPN.

Two SNPs were located in the nerve growth factor receptor (*NGFR;* rs11466155 and rs584589), a gene particularly important in respect to neurological functions. The NFGR signals via NFκB activation and binds neutrophin precursors that stimulate neuronal cells survival and differentiation.

Associations with absorption, distribution, metabolism, and excretion (ADME) genes

Previous studies showed that cytochrome P450 isoforms CYP3A4, CYP2C19, CYP1A2, CYP2D6, and CYP2C9 are the major enzymes involved in bortezomib metabolism.[31] The hypothesis that the development of BiPN depends on exposure to active bortezomib or metabolites assumes an association with SNPs that lie within genes of the cytochrome P450 family. Our results indicate that out of 122 investigated SNPs located in the five cytochrome P450 isoforms listed above, only one SNP in *CYP2C19* (rs3758581) and one SNP in *CYP1A2* (rs762551) were associated with BiPN risk.

Other associations with ADME genes observed in this analysis included three SNPs (rs2239330, rs212090, rs212087), located in the ATP-binding cassette transporter *ABCC1*, and a nonsynonymous SNP (rs2231142) in the *ABCG2* gene.

Discussion

The pathogenic mechanisms underlying BiPN have not been established yet and this is the first study exploring the association of genetic markers with development of BiPN. Our results suggest that the mechanisms underlying the development of BiPN are multifactorial. They strongly support that SNPs in important genes of the inflammatory response may play an essential role in the pathogenesis of BiPN. SNPs that lie within proinflammatory cytokines, such as *TNFα*, *PARP1* and *prothrombin*, are significantly associated with BiPN. TNFα has been implicated in the pathogenesis of several neurodegenerative diseases, including multiple sclerosis, Alzheimer disease, and human immunodeficiency virus-related encephalopathy.[32-34] Additionally, the TNFα system is activated in diabetic polyneuropathy, which leads to increased microvascular permeability, hypercoagulability and even direct nerve damage. Improvement of diabetic polyneuropathy following suppression of TNFα has been shown in several animal models.[35-36] Since bortezomib's target NFκB is intricately related to the TNF receptor system in the nervous system, an association of *TNF*-SNPs with BiPN may not be surprising.[37] Furthermore, neuropathic pain, one of the determinants of the CTCAE-neuropathy score and thus of BiPN severity, is mediated through TNF-mediated induction of stress-activated kinases like p38 MAPK.[38-39] PARP1 plays an important role in cell death and inflammation, most likely through its interaction with several transcription factors among which NFκB. It is thought that neuronal cell death is mediated by PARP1 activation.[26] The association of *TNFα* gene SNPs with BiPN may therefore hint to a possible application of the TNFα inhibitors or monoclonal antibodies for the treatment of BiPN. While there is no effective treatment available for BiPN apart from dose modification or treatment discontinuation, hence the recognition of the inflammation system being important in the pathogenesis of BiPN may provide new therapeutic targets to be explored. Promising results have been reported for TNFα suppression using infliximab in diabetic polyneuropathy in animal models.[40-42]

A further unifying mechanism underlying BiPN is the inability to repair neurological damage. While prothrombin plays an important role in the coagulation cascade, emerging evidence indicates that prothrombin can significantly contribute to pathological conditions in the central nervous system. Prothrombin is expressed in neuronal cells as well as in astrocytes, which indicates a role of prothrombin in the brain.[43-46] Furthermore, thrombin can have either cytoprotective or cytotoxic effects on neurons, modify the morphology of neurons and astrocytes, and induce glial cell proliferation.[47] Prothrombin synthesis increases after brain injury, which results in neuron protection through activation of protease-activated receptors (PARs).[48-49] Finally, reduced levels of prothrombin were detected in patients with traumatic brain injury.[50] Together, these studies emphasize the possibility that genes regulating neuronal proliferation and damage repair may be directly involved.

Another important finding is the association of SNPs with BiPN that lie within essential neuropathy susceptibility genes. We observed that the risk of developing BiPN is based on genetic variation acting at the level of the neuron. Consistent with this, we show that although bortezomib is known to be metabolized by cytochrome P450 enzymes, only a small number of genetic polymorphisms lying within these genes were found to be associated with BiPN., However, these associations included one SNP in *CYP1A2* and one in *CYP2C19*, which are both known to be involved in bortezomib metabolism.[31]

Because in some patients there is a rapid onset of neuropathy that cannot be predicted on clinical grounds, there is a need to be able to predict the susceptibility of a patient to develop BiPN. The ultimate objective would be to identify genetic markers that predict BiPN development and combine these with clinical risk factors. Our results contribute to reaching this aim; however, further research to the different strategies and methods aimed at building a predictive model is needed.

In conclusion, we evaluated genetic risk factors associated with BiPN in MM patients. Although further research will be necessary to confirm the involvement of the inflammation system in BiPN, our study provides important evidence that proinflammatory cytokines are involved in BiPN development.

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Chapter 6

Mechanisms of peripheral neuropathy associated with bortezomib and vincristine in patients with newly diagnosed multiple myeloma: a prospective analysis of data from the HOVON-65/GMMG-HD4 trial

Annemiek Broyl*, Sophie L. Corthals*, Joost L. M. Jongen,
Bronno van der Holt, Rowan Kuiper, Yvonne de Knegt,
Mark van Duin, Laila el Jarari, Uta Bertsch,
Henk M. Lokhorst, Brian G.M. Durie,
Hartmut Goldschmidt, Pieter Sonneveld

*These authors contributed equally to this work

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Summary

Background

Bortezomib induced peripheral neuropathy is a dose-limiting toxicity in patients with multiple myeloma, often requiring adjustment of treatment and affecting quality of life. We investigated the molecular profiles of early onset (within one treatment cycle) versus late onset (after two or three treatment cycles) bortezomib induced peripheral neuropathy and compared them with those of vincristine induced peripheral neuropathy during the induction phase of a prospective phase 3 trial.

Methods

In the induction phase of the HOVON-65/GMMG-HD4 trial, patients (aged 18–65 years) with newly diagnosed Salmon and Durie stage 2 or 3 multiple myeloma were randomly assigned to three cycles of bortezomib-based or vincristine-based induction treatment. We analyzed the gene expression profiles and single nucleotide polymorphisms (SNPs) of pretreatment samples of myeloma plasma cells and peripheral blood, respectively. This study is registered, number ISRCTN64455289.

Findings

We analyzed gene expression profiles of myeloma plasma cells from 329 (39%) of 833 patients at diagnosis, and SNPs in DNA samples from 369 (44%) patients. Early onset bortezomib induced peripheral neuropathy was noted in 20 (8%) patients, and 63 (25%) developed the late onset type. Early onset and late onset vincristine induced peripheral neuropathy was noted in 11 (4%) and 17 (7%) patients, respectively. Significant genes in myeloma plasma cells from patients that were associated with early onset bortezomib induced peripheral neuropathy were the enzyme coding genes *RHOBTB2* (upregulated by 1.59 times; $P = 4.5 \times 10^{-5}$), involved in drug-induced apoptosis, *CPT1C* (1.44 times; $P = 2.9 \times 10^{-7}$), involved in mitochondrial dysfunction, and *SOX8* (1.68 times; $P = 4.28 \times 10^{-13}$), involved in development of peripheral nervous system. Significant SNPs in the same patients included those located in the apoptosis gene *caspase 9* (odds ratio [OR] 3.59, 95% CI 1.59–8.14; $P = 2.9 \times 10^{-3}$), *ALOX12* (3.50, 1.47–8.32; $P = 3.8 \times 10^{-3}$), and *IGF1R* (0.22, 0.07–0.77; $P = 8.3 \times 10^{-3}$). In late onset bortezomib induced peripheral neuropathy, the significant genes were *SOD2* (upregulated by 1.18 times; $P = 9.6 \times 10^{-3}$) and *MYO5A* (1.93 times; $P = 3.2 \times 10^{-2}$), involved in development and function of the nervous system. Significant SNPs were noted in inflammatory genes *MBL2* (OR 0.49, 95% CI 0.26–0.94; $P = 3.0 \times 10^{-2}$) and *PPARD* (0.35, 0.15–0.83; $P = 9.1 \times 10^{-3}$), and DNA repair genes *ERCC4* (2.74, 1.56–4.84; $P = 1.0 \times 10^{-3}$) and *ERCC3* (1.26, 0.75–2.12; $P = 3.3 \times 10^{-3}$). By contrast, early onset vincristine induced peripheral neuropathy was characterized by upregulation of genes involved in cell cycle and proliferation, including *AURKA* (3.31 times; $P = 1.04 \times 10^{-2}$) and *MKI67* (3.66 times; $P = 1.82 \times 10^{-3}$), and the presence of SNPs in genes involved in these processes - e.g., *GLI1* (rs2228224 [0.13, 0.02–0.97, P = 1.18 x 10^{-2}] and rs2242578 [0.14, 0.02–1.12, P = 3.00 x 10^{-2}]). Late onset vincristine induced peripheral neuropathy was associated with the presence of SNPs in genes involved in absorption, distribution, metabolism, and excretion - e.g., rs1413239 in *DPYD* (3.29, 1.47–7.37, 5.40 x 10^{-3}) and rs3887412 in *ABCC1* (3.36, 1.47–7.67, P = 5.70 x 10^{-3}).

Interpretation

Our results strongly suggest an interaction between myeloma related factors and the patient's genetic background in the development of treatment induced peripheral neuropathy, with different molecular pathways being implicated in bortezomib induced and vincristine induced peripheral neuropathy.

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Introduction

Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) is a boronic acid dipeptide, which specifically inhibits the chymotryptic site of the 26S proteasome. In patients with newly diagnosed myeloma, bortezomib in combination with conventional drugs resulted in high rates of complete response and very good partial response.[1–4] This drug is generally well tolerated; however, one of its most frequent and potentially disabling side effects is the development of a painful, sensory peripheral neuropathy,[5–7] often requiring dose modification or discontinuation of bortezomib, which negatively affects clinical endpoints and quality of life.[8] Grade 1 and 2 bortezomib induced peripheral neuropathy can arise in 27–75% of patients with recurrent multiple myeloma and in 25–33% of those with newly diagnosed multiple myeloma, whereas grade 3 and 4 peripheral neuropathy might affect 0–30% of patients with recurrent disease and 0–18% of those with newly diagnosed disease.[9] In most patients, this side effect is reversible and does not seem to be affected by the number or type of previous treatments.[7] Bortezomib induced peripheral neuropathy results from axonal degeneration,[10,11] often occurring within the first cycles of treatment, and does not seem to increase after the fifth cycle of bortezomib.[7]

Little is known about the mechanism of bortezomib induced peripheral neuropathy, but a multifactorial pathogenesis seems likely. Damage to mitochondria and endoplasmic reticulum through activation of apoptosis has been seen in dorsal root ganglia of mice given bortezomib.[11] Additionally, mechanisms such as dysregulation of mitochondrial calcium homoeostasis,[12] autoimmune factors and inflammation,[13] and blockade of nerve-growth-factor-mediated neuronal survival through inhibition of the activation of nuclear factor kappa B (NFκB)[6] could contribute to bortezomib induced peripheral neuropathy. Evidence that multiple myeloma is also implicated in peripheral neuropathy was described by Ropper and Gorson[14] in 1998. Baseline neuropathy is present in 15–20% of patients with newly diagnosed myeloma,[15,16] which might be of both axonal and demyelinating subtypes.[14] The role of myeloma related factors in peripheral neuropathy related to treatment is not clear. Bortezomib induced peripheral neuropathy was noted at higher frequencies in patients with multiple myeloma than in those with solid tumors.[17] Richardson and colleagues[16] characterized the possible role of myeloma related factors in bortezomib induced peripheral neuropathy using plasma cells from patients with multiple myeloma. Additionally, we have noted that inherited single nucleotide polymorphisms (SNPs) are associated with a higher probability of developing thalidomide induced or bortezomib induced peripheral neuropathy (Corthals SL, unpublished data). We therefore analyzed myeloma related gene expression and inherited patient variations as indicators of the potential risk of developing treatment related peripheral neuropathy. We investigated whether particular molecular profiles were specific for early onset versus late onset bortezomib induced peripheral neuropathy and compared these with genetic profiles associated with early onset versus late onset vincristine induced peripheral neuropathy to elucidate molecular differences associated with the development of peripheral neuropathy after the different treatments.

Methods

Patients

833 patients (aged 18–65 years) with newly diagnosed Salmon and Durie stage 2–3 multiple myeloma were enrolled in a prospective, randomized phase 3 trial (HOVON-65/GMMG-HD4; EudraCTnr2004-000944-26) in 75 centers in The Netherlands, Germany, and Belgium.[3] Patients were excluded if they had amyloidosis or monoclonal gammopathy of unknown significance, and baseline peripheral neuropathy of grade 2 or more.

The trial was done in accordance with the Declaration of Helsinki, and was approved by a medical ethics review committee. We obtained written informed consent from the patients for treatment and sample procurement.

Procedures

Patients were randomly assigned to three cycles of induction treatment with vincristine 0.4 mg intravenously on days 1–4, doxorubicin 9 mg/m^2 intravenously on days 1–4, and dexamethasone 40 mg orally on days 1–4, 9–12, and 17–20 or bortezomib 1.3 mg/m^2 intravenously on days 1, 4, 8, and 11, doxorubicin 9 mg/m^2 intravenously on days 1–4, and dexamethasone 40 mg orally on days 1–4, 9–12, and 17–20. Stem cells were mobilized by use of cyclophosphamide 1000 mg/m^2 intravenously on day 1, doxorubicin 15 mg/m^2 intravenously on days 1–4, dexamethasone 40 mg orally on days 1–4, and granulocyte colony-stimulating factor (filgrastim) 10 µg/kg per day subcutaneously, divided in two doses per day, from day 5 until last stem cell collection. After induction therapy, patients received one or two cycles of high-dose melphalan (200 mg/m^2 intravenously) with autologous stem cell rescue followed by maintenance treatment with thalidomide (50 mg per day orally; group assigned to vincristine-based induction treatment) or bortezomib (1.3 mg/m^2 intravenously once every 2 weeks; group assigned to bortezomib-based induction treatment) for 2 years. Treatment was not masked for physicians and patients.

Severity of neuropathy was graded at baseline and after each treatment cycle by use of the National Cancer Institute's Common Toxicity Criteria for Adverse Events criteria (version 3.0).[18] All data were analyzed centrally. No neurological assessment was undertaken to objectify peripheral neuropathy. Since grade 1 peripheral neuropathy could easily be missed or misinterpreted, and because it does not include pain or interfere with the activities of daily life, we decided that grade 1 peripheral neuropathy was not clinically significant enough for the molecular analysis and therefore cases of this grade were excluded. Furthermore, the dose-modification guidelines established during the SUMMIT,[6] CREST,[5] and APEX[19] trials did not recommend discontinuation of bortezomib or dose modifications when grade 1 bortezomib induced peripheral neuropathy occurred. We did not routinely assess data for diabetes and vascular disease. Development of peripheral neuropathy after the first cycle of induction treatment is described as early onset, and after two to three cycles of induction treatment as late onset. Vincristine induced peripheral neuropathy was used as a reference when we assessed the incidence and severity of bortezomib induced peripheral neuropathy.

RNA isolation and microarray processing was done as previously described.[20] Microarray data presented in this report have been stored in the Gene Expression Omnibus database (National Center for Biotechnology Information, Bethesda MD, USA), accession number GSE19784. Gene expression arrays were done with RNA extracted from myeloma plasma cells that were purified ($\geq$ 80%) from the extra bone marrow aspiration taken at diagnosis and met the criteria for quality.[20]

DNA was extracted from peripheral blood nucleated cells or CD138-negative bone marrow cells and quantified by use of the Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE, USA). Samples were genotyped by use of the Affymetrix Targeted Genotyping (Affymetrix, Santa Clara, CA, USA) custom-built panel, with 3404 SNPs, selected with a hypothesis-driven strategy, targeting genes and SNPs for which associations or putative functional effects have been noted (Corthals SL, unpublished data).

Statistical analysis

For differences in incidence of baseline and grade 2–4 peripheral neuropathy after one cycle and after two to three cycles of bortezomib-based and vincristine-based treatment, χ^2 analysis was done with a two-sided *P* value of 0.05. For gene expression data, class comparison of groups of arrays was done with one-way ANOVA in Partek Genomics Suite (version 6.4), followed by multiple test correction with a false discovery rate of less than 0.05.

For SNP genotyping data, deviations from Fisher's exact *t* test for Hardy-Weinberg equilibrium at $P < 0.00001$ and bias in missing data were controlled for each SNP. SNPs with a minor allele frequency of less than 5% and a call rate of less than 80% were removed from further analysis. To assess SNP associations with treatment related peripheral neuropathy and calculation of odds ratios (ORs), a Cochran-Armitage trend test and a Fisher's *t* exact test were done. We assessed the genomic inflation factor λ based on the median χ^2 for each analysis with PLINK (version 1.07).[21] To account for multiple testing, 10.000 permutation tests were done with the max(T) permutation procedure with PLINK. To assess the effect of nonsynonymous SNPs associated with bortezomib induced peripheral neuropathy and vincristine induced peripheral neuropathy, SNPs were characterized by use of the prediction program Sorting Intolerant From Tolerant (version 4.0.3).

Analysis of the gene and SNP sets for peripheral neuropathy associated with bortezomib and vincristine was done by use of Ingenuity Pathway Analysis software (version 8.7).

This study is registered as an International Standard Randomized Controlled Trial, number ISRCTN64455289.

Role of the funding source

The sponsors had no role in the design, gathering, analysis, and interpretation of the data, or the writing of the report. The corresponding author had full access to all the data and the final responsibility to submit for publication.

Results

We did gene expression arrays for 329 (39%; 170 treated with bortezomib, 159 treated with vincristine) of 833 patients included in the trial, and SNP profiles for samples taken from 369 (44%; 186 treated with bortezomib, 183 treated with vincristine) patients. Simultaneous gene expression and SNP data were obtained for 185 patients; only SNP data were available for 184 patients, and only gene expression data were available for 144 patients. The baseline clinical characteristics of 513 patients included in this study were not different from the whole patient group included in the trial (Table S1).

Table 1. Incidence of baseline, bortezomib induced, and vincristine induced peripheral neuropathy.

	Bortezomib-based induction treatment (n = 250)	Vincristine-based induction treatment (n = 250)	*P*-value
Baseline peripheral neuropathy	8 (3%)	13 (5%)	0.37
Peripheral neuropathy after one cycle			
PNP grade 2 - 4	20 (8%)	11 (4%)	0.27
PNP grade 2	10 (50%)	9 (82%)	
PNP grade 3	7 (35%)	1 (9%)	0.18*
PNP grade 4	3 (15%)	1 (9%)	
Peripheral neuropathy after two or three cycles			
PNP grade 2 - 4	66 (26%)	17 (7%)	< 0.0001
PNP grade 2	31 (47%)	11 (65%)	
PNP grade 3	24 (36%)	6 (35%)	0.72*
PNP grade 4	8 (12%)	0 (0%)	

Data are number (%), unless otherwise indicated. The denominator for calculation of the percentages of patients with grades 2, 3, and 4 drug related peripheral neuropathy was the total number of patients presenting with drug related peripheral neuropathy after one or two to three cycles of treatment, respectively. *For difference in percentage of patients with grade 3 and 4 peripheral neuropathy associated with bortezomib and vincristine among the total number of patients presenting with grade 2–4 bortezomib induced and vincristine induced peripheral neuropathy, respectively.

Table 1 shows the incidence of peripheral neuropathy at baseline and after treatment with bortezomib-based and vincristine-based induction treatments in 500 of 513 patients who were fully assessable and had a minimum follow-up of 40 months. The median time to development of bortezomib induced peripheral neuropathy was 42 days (range 0–137). Cumulative dose of bortezomib given before development of peripheral neuropathy was 13 mg/m^2. 52 patients (21%) developed grade 1 bortezomib induced peripheral neuropathy, and 34 (14%) developed grade 1 peripheral neuropathy before progressing to a higher grade. When patients developed peripheral neuropathy, the dose of bortezomib was adjusted according to the established guidelines for dose modification.[5–7,19] Median time to development of vincristine induced peripheral neuropathy was 37 days (range 0–171). Cumulative dose of vincristine given before development of peripheral neuropathy was 4 mg. 60 (24%) patients developed vincristine induced peripheral neuropathy, and 18 (7%) developed grade 1 peripheral neuropathy before progressing to a higher grade. When patients developed vincristine induced peripheral neuropathy, vincristine was discontinued and supportive treatments such as pregabalin were used. Overall, baseline peripheral neuropathy was noted in only a small number of patients (Table 1). The proportion of patients developing late onset bortezomib induced peripheral neuropathy was significantly higher than that of patients with late onset vincristine induced peripheral neuropathy (Table 1).

Table 2. Differentially expressed genes in early onset and late onset bortezomib induced and vincristine induced peripheral neuropathy.

Probeset ID	Gene name	Gene description	Factor difference in expression	*P*-value
Grade 2-4 peripheral neuropathy (n=15) versus no peripheral neuropathy (n=134) after one cycle of bortezomib				
225189_s_at	*RAPH1*	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	2.24	3.04 x 10^{-2}
235014_at	*LOC147727*	Hypothetical LOC147727	2.15	1.91 x 10-^{2}
1569872_a_at	*LOC650392*	Hypothetical protein LOC650392	1.98	9.65 x 10^{-4}
213056_at	*FRMD4B*	FERM domain containing 4B	1.74	8.42 x 10^{-3}
227984_at	*LOC650392*	Hypothetical protein LOC650392	1.71	1.19 x 10^{-3}
225478_at	*MFHAS1*	Malignant fibrous histiocytoma amplified sequence 1	1.68	5.34 x 10^{-9}
226913_s_at	*SOX8*	SRY (sex determining region Y)-box 8	1.68	4.28 x 10^{-13}
204810_s_at	*CKM*	Creatine kinase, muscle	1.67	1.11 x 10^{-30}
1569871_at	*LOC650392*	Hypothetical protein LOC650392	1.65	1.77 x 10^{-19}
228057_at	*DDIT4L*	DNA-damage-inducible transcript 4-like	1.59	5.59 x 10^{-20}
Grade 2-4 peripheral neuropathy (n=44) versus no peripheral neuropathy (n=78) after two or three cycles of bortezomib				
205590_at	*RASGRP1*	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	2.97	2.14 x 10^{-2}
204527_at	*MYO5A*	Myosin VA (heavy chain 12, myoxin)	1.93	3.21 x 10^{-2}
235065_at	...	...	1.57	3.19 x 10^{-2}
205422_s_at	*ITGBL1*	Integrin, beta-like 1 (with EGF-like repeat domains)	1.44	1.35 x 10^{-3}
228113_at	*RAB37*	RAB37, member RAS oncogene family	1.41	3.69 x 10^{-3}
210321_at	*GZMH*	Granzyme H (cathepsin G-like 2, protein h-CCPX)	1.37	3.19 x 10^{-2}
226969_at	*MTR*	5-methyltetrahydrofolate-homocysteine methyltransferase	1.34	4.26 x 10^{-2}
204072_s_at	*FRY*	Furry homolog (Drosophila)	1.31	4.94 x 10^{-2}
236442_at	*DPF3*	D4, zinc and double PHD fingers, family 3	1.30	3.38 x 10^{-3}
243329_at	...	...	1.30	4.26 x 10^{-2}
Grade 2-4 peripheral neuropathy (n=9) versus no peripheral neuropathy (n=129) after one cycle of vincristine				
208235_x_at	*GAGE7*	G antigen 7	11.55	3.21 x 10^{-3}
206640_x_at	*GAGE12I*	G antigen 12I	11.46	4.29 x 10^{-3}
207739_s_at	*GAGE2C*	G antigen 2C	7.76	1.62 x 10^{-3}
208155_x_at	*GAGE6*	G antigen 6	6.88	1.06 x 10^{-5}
206897_at	*PAGE1*	P antigen family, member 1 (prostate associated)	6.76	4.29 x 10^{-3}
216063_at	*HBBP1*	Hemoglobin, beta pseudogene 1	6.24	4.04 x 10^{-2}
207086_x_at	*GAGE4*	G antigen 4	6.16	3.29 x 10^{-5}
206626_x_at	*SSX1*	Synovial sarcoma, X breakpoint 1	5.93	2.61 x 10^{-2}
207912_s_at	*DAZ1*	Deleted in azoospermia 1	5.86	1.06 x 10^{-4}
214957_at	*ACTL8*	Actin-like 8	4.93	1.32 x 10^{-10}
Grade 2-4 peripheral neuropathy (n=10) versus no peripheral neuropathy (n=103) after two or three cycles of vincristine				
210632_s_at	*SGCA*	Sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)	4.08	3.35 x 10^{-2}
210992_x_at	*FCGR2C*	Fc fragment of IgG, low affinity IIc, receptor for (CD32)	2.49	3.57 x 10^{-2}
241991_at	...	...	1.80	3.35 x 10^{-2}
206771_at	*UPK3A*	Uroplakin 3A	1.59	2.94 x 10^{-2}
241365_at	...	...	1.57	3.35 x 10^{-2}
236266_at	*RORA*	RAR-related orphan receptor A	1.53	2.94 x 10^{-2}
214059_at	*IFI44*	Interferon-induced protein 44	1.51	4.92 x 10^{-6}
230477_at	...	...	1.48	2.94 x 10^{-2}
237322_at	*MIAT*	Myocardial infarction associated transcript (non-protein coding)	1.45	2.94 x 10^{-2}
239239_at	...	...	1.33	2.94 x 10^{-2}

First column is the probe set identification number. Genes were ranked from highest to lowest change; the first ten genes with the highest changes are shown.

Gene expression arrays for 15 patients developing early onset grade 2–4 bortezomib induced peripheral neuropathy were compared with arrays of patients who did not develop bortezomib induced peripheral neuropathy (Table 2). Grade 2–4 early onset bortezomib induced peripheral neuropathy was characterized by 19 differentially expressed genes (false discovery rate < 0.05). The genes showing the highest changes in the gene expression arrays are shown in Table 2, and the complete number of differentially expressed probe sets are shown in Table S2. The genes showing the highest change in expression included *RAPH1* (involved in signal transduction), *FRMD4B*, *MFHAS1* (possibly an oncogene regulated by NFκB or tumor necrosis factor), and *DDIT4L* (a DNA-damage inducible transcript; Table 2). Genes that might play a direct part in bortezomib induced peripheral neuropathy are transcription regulator *SOX8* (involved in development of peripheral nervous system), *CPT1C* and *RHOBTB2* (Table S2). Ingenuity pathway analyses of gene and SNP sets showed enrichment of genes implicated in the canonical pathway of signaling mediated by AMP-activated protein kinase (AMPK), including *CPT1C*, *CKM*, and *PIK3CG* (three of 156 genes involved in AMPK signaling were upregulated, $P = 7.33 \times 10^{-5}$).

Gene expression arrays for 44 patients with grade 2–4 late onset bortezomib induced peripheral neuropathy were characterized by 27 differentially expressed genes, using the same false discovery rate as for early onset (Table S2), and showed a different pattern of gene expression to that in early onset bortezomib induced peripheral neuropathy, without overlap (Table 2). *RASGRP1* showed the highest change in patients with late onset bortezomib induced peripheral neuropathy compared with patients without this side effect (Table 2). Furthermore, we noted upregulation of genes involved in transcription regulation, including *TRERF1*, *TRPS1*, and *MDM2*. We noted enrichment of genes involved in the development and function of the nervous system, including *SOD2* and *MYO5A*.

All significant SNPs (permuted $P < 0.01$) associated with grade 2–4 early onset bortezomib induced peripheral neuropathy are shown in Table 3 (values of permuted $P < 0.05$ are shown in Table S3). Several SNPs associated with early onset bortezomib induced peripheral neuropathy were located in *caspase 9* (rs4646091, rs2020895, rs2020903, rs4646032, and rs4646034). Other highly associated SNPs were located in genes *RDM1*, *ALOX12*, *IGF1R*, and *LSM1* (Table 3). Pathway analysis of these associated genes showed enrichment of genes involved in cell death (14 genes, $P = 5.25 \times 10^{-3}$–4.93×10^{-2}), DNA repair (14 genes, $P = 5.25 \times 10^{-3}$–4.93×10^{-2}), and development and function of the nervous system (four genes, $P = 2.01 \times 10^{-3}$).

The SNPs that were characteristic of late onset bortezomib induced peripheral neuropathy were mainly located in DNA repair genes, such as *ERCC3*, *ERCC4*, *ATM*, *BRCA1*, *EXO1*, and *MRE11A* (Table 3, and S3). Pathway analysis showed enrichment of associated SNPs located in genes involved in the development and function of the nervous system (three genes, $P = 3.35 \times 10^{-3}$–1.69×10^{-2}) and in inflammatory disease (26 genes, $P = 2.09 \times 10^{-3}$–4.95×10^{-3}).

The genetic profile of myeloma plasma cells from nine patients who developed grade 2–4 early onset vincristine induced peripheral neuropathy showed overexpression of the genes for testis cancer antigens, of which the *GAGE* genes were mainly upregulated (Table 2).

Table 3. Single nucleotide polymorphisms associated with bortezomib induced and vincristine induced peripheral neuropathy.

SNP ID	Chromosome	Gene	Single nucleotide polymorphism type	Odds ratio (95% CI)	*P*-value	Permuted *P*-value
Grade 2–4 peripheral neuropathy (n=13) versus no peripheral neuropathy (n=147) after one cycle of bortezomib						
rs2251660	17	*RDM1*	Coding-nonsynonymous	3.65 (1.55-8.57)	$9.06\,x10^{-4}$	$2.40\,x10^{-3}$
rs4646091	1	*CASP9*	Intron	3.56 (1.59-8.14)	$1.43\,x10^{-3}$	$2.90\,x10^{-3}$
rs1126667	17	*ALOX12*	Coding-nonsynonymous	3.50 (1.47-8.32)	$2.95\,x10^{-3}$	$3.80\,x10^{-3}$
rs434473	17	*ALOX12*	Coding-nonsynonymous	3.50 (1.47-8.32)	$2.95\,x10^{-3}$	$4.10\,x10^{-3}$
rs7823144	8	*LSM1*	Intron	4.11 (1.48-11.39)	$2.30\,x10^{-3}$	$7.60\,x10^{-3}$
rs1879612	15	*IGF1R*	Intron	0.22 (0.07-0.77)	$9.42\,x10^{-3}$	$8.30\,x10^{-3}$
rs1029871	3	*NEK4*	Coding-nonsynonymous	0.30 (0.11-0.81)	$8.31\,x10^{-3}$	$9.30\,x10^{-3}$
Grade 2–4 peripheral neuropathy versus (n=49) no peripheral neuropathy (n=80) after two or three cycles of bortezomib						
rs1799800	16	*ERCC4*	Intron	2.74 (1.56-4.84)	$5.16\,x10^{-4}$	$1.00\,x10^{-3}$
rs1799801	16	*ERCC4*	Coding-synonymous	2.48 (1.43-4.28)	$8.85\,x10^{-4}$	$1.10\,x10^{-3}$
rs2300697	2	*SRD5A2*	Intron	0.63 (0.37-1.05)	$4.80\,x10^{-2}$	$2.90\,x10^{-3}$
rs1059293	21	*IFNGR2*	Untranslated,Intron	2.30 (1.37-3.87)	$8.97\,x10^{-4}$	$3.20\,x10^{-3}$
rs2276583	2	*ERCC3*	Locus	1.26 (0.75-2.12)	$3.87\,x10^{-1}$	$3.30\,x10^{-3}$
rs189037	11	*ATM*	Locus,Untranslated	0.53 (0.32-0.89)	$2.32\,x10^{-2}$	$3.60\,x10^{-3}$
rs10501815	11	*MRE11A*	Intron,TagSNP:*MRE11A*	3.27 (1.39-7.74)	$4.41\,x10^{-3}$	$4.20\,x10^{-3}$
rs664677	11	*ATM*	Intron	0.57 (0.34-0.96)	$4.36\,x10^{-2}$	$5.90\,x10^{-3}$
rs664982	11	*ATM*	Intron	0.51 (0.30-0.85)	$1.72\,x10^{-2}$	$6.20\,x10^{-3}$
rs6131	1	*SELP*	Coding-nonsynonymous	0.43 (0.23-0.83)	$6.69\,x10^{-3}$	$6.30\,x10^{-3}$
rs1130499	7	*PTPRN2*	Coding-nonsynonymous	0.43 (0.23-0.79)	$6.23\,x10^{-3}$	$6.60\,x10^{-3}$
rs4722266	7	*STK31*	Coding-nonsynonymous	0.29 (0.12-0.74)	$5.66\,x10^{-3}$	$8.30\,x10^{-3}$
rs2267668	6	*PPARD*	Intron	0.35 (0.15-0.83)	$9.30\,x10^{-3}$	$9.10\,x10^{-3}$
Grade 2–4 peripheral neuropathy versus (n=7) no peripheral neuropathy (n=151) after one cycle of vincristine						
rs7739752	6	*PPARD*	Intron	13.43 (3.90-46.22)	$6.34\,x10^{-7}$	$8.00\,x10^{-4}$
rs2288087	9	*ALDH1A1*	Intron,TagSNP:*ALDH1A1*	7.62 (1.68-34.65)	$1.40\,x10^{-3}$	$1.50\,x10^{-3}$
rs1494961	4	*HEL308*	Coding-nonsynonymous	6.67 (1.47-30.32)	$2.30\,x10^{-3}$	$2.60\,x10^{-3}$
rs6901410	6	*PPARD*	Intron	9.67 (2.65-35.30)	$7.75\,x10^{-5}$	$6.00\,x10^{-3}$
rs6902123	6	*PPARD*	Intron	9.67 (2.65-35.30)	$7.75\,x10^{-5}$	$6.00\,x10^{-3}$
rs2274407	13	*ABCC4*	Coding-nonsynonymous	7.15 (2.02-25.31)	$2.94\,x10^{-4}$	$6.10\,x10^{-3}$
rs909253	6	*LTA*	Intron	4.67 (1.52-14.34)	$3.09\,x10^{-3}$	$6.60\,x10^{-3}$
rs6457816	6	*PPARD*	Intron	8.89 (2.46-32.17)	$1.40\,x10^{-4}$	$7.30\,x10^{-3}$
rs1041981	6	*LTA*	Coding-nonsynonymous	4.52 (1.47-13.88)	$3.58\,x10^{-3}$	$7.40\,x10^{-3}$
rs3803258	13	*SLC10A2*	Untranslated	4.30 (1.45-12.74)	$3.51\,x10^{-3}$	$7.40\,x10^{-3}$
rs3749442	3	*ABCC5*	Coding-synonymous	4.64 (1.53-14.05)	$2.72\,x10^{-3}$	$9.60\,x10^{-3}$
Grade 2–3 peripheral neuropathy (n=14) versus no peripheral neuropathy (n=104) after two or three cycles of vincristine						
rs10515114	5	*CART*	Locus	4.62 (1.68-12.72)	$7.92\,x10^{-4}$	$2.90\,x10^{-3}$
rs6873545	5	*GHR*	Intron	0.09 (0.01-0.67)	$3.44\,x10^{-3}$	$3.60\,x10^{-3}$
rs3734354	6	*SIM1*	Coding-nonsynonymous	3.30 (1.39-7.82)	$2.31\,x10^{-3}$	$5.10\,x10^{-3}$
rs11688	1	*JUN*	Coding-synonymous	5.00 (1.8-13.91)	$9.10\,x10^{-4}$	$5.20\,x10^{-3}$
rs4129472	5	*GHR*	Intron	0.11 (0.01-0.80)	$6.46\,x10^{-3}$	$5.20\,x10^{-3}$
rs1413239	1	*DPYD*	Intron,TagSNP:*DPYD*	3.29 (1.47-7.37)	$3.03\,x10^{-3}$	$5.40\,x10^{-3}$
rs1045020	5	*SLC22A5*	Untranslated	4.80 (1.83-12.61)	$1.48\,x10^{-3}$	$5.40\,x10^{-3}$
rs9885672	6	*KIAA0274*	Coding-nonsynonymous	3.89 (1.62-9.33)	$2.05\,x10^{-3}$	$5.60\,x10^{-3}$
rs3887412	16	*ABCC1*	Intron,TagSNP:*ABCC1*	3.36 (1.47-7.67)	$3.31\,x10^{-3}$	$5.70\,x10^{-3}$
rs6886047	5	*GHR*	Intron	0.10 (0.01-0.72)	$3.97\,x10^{-3}$	$6.10\,x10^{-3}$
rs1236913	9	*PTGS1*	Coding-nonsynonymous	5.40 (1.79-16.28)	$1.43\,x10^{-3}$	$6.30\,x10^{-3}$
rs2644983	16	*ABCC1*	Intron,TagSNP:*ABCC1*	4.22 (1.69-10.50)	$2.27\,x10^{-3}$	$6.60\,x10^{-3}$
rs1042713	5	*ADRB2*	Coding-nonsynonymous	0.23 (0.08-0.69)	$5.30\,x10^{-3}$	$7.20\,x10^{-3}$
rs1966265	5	*FGFR4*	Coding-nonsynonymous	3.47 (1.51-7.94)	$3.40\,x10^{-3}$	$7.30\,x10^{-3}$
rs2308327	10	*MGMT*	Coding-nonsynonymous	3.38 (1.33-8.58)	$3.69\,x10^{-3}$	$7.30\,x10^{-3}$
rs5759197	22	*BZRP*	Intron	2.93 (1.31-6.53)	$6.32\,x10^{-3}$	$7.60\,x10^{-3}$
rs1005658	22	*BZRP*	Locus	3.14 (1.39-7.08)	$6.04\,x10^{-3}$	$8.50\,x10^{-3}$
rs7441774	4	*UGT2B7*	Intron	3.60 (1.40-9.23)	$6.61\,x10^{-3}$	$9.60\,x10^{-3}$

The gene profiles of ten patients who developed grade 2 or 3 late onset vincristine induced peripheral neuropathy showed only ten differentially expressed genes, including *RORA* and *IFI44* (Table 2).

Table 3 shows SNPs significantly associated with early onset vincristine induced peripheral neuropathy. Four of the most highly associated SNPs (rs7739752, rs6901410, rs6902123, and rs6457816) were located in the transcription factor *PPARD*. Additionally, an intronic (rs909253) and a coding nonsynonymous SNP (rs1041981) in *LTA* were significantly associated with early onset vincristine induced peripheral neuropathy. Other significant SNPs were located in genes for transporter enzymes *ABCC4*, *ABCC5*, and *SLC10A2*, oxidizing enzyme *ALDH1A1*, and *GLI1* (Table 3 and S3). Pathway analysis showed enrichment of associated SNPs located in genes involved in cellular growth and proliferation (four genes, $P = 1.14 \times 10^{-2}–4.95 \times 10^{-2}$).

Some intronic SNPs in the dihydropyrimidine dehydrogenase gene *DPYD* and some in the ABC transporter gene *ABCC1* were associated with late onset vincristine induced peripheral neuropathy (Table 3). Pathway analysis showed that most significant SNPs (permuted $P < 0.05$) were located in genes for absorption, distribution, metabolism, and excretion (six genes, $P = 2.06 \times 10^{-2}–4.18 \times 10^{-2}$).

Discussion

The genetic profiles of patients with early onset bortezomib induced peripheral neuropathy suggest the involvement of genes involved in transcription, apoptosis, and AMPK-mediated signaling. The possible role of AMPK-mediated signaling is of particular interest because this enzyme functions by stimulating the signaling pathways that replenish cellular ATP supplies in response to low glucose, hypoxia, ischaemia, or heat shock, which might be triggered in myeloma cells in response to bortezomib. *CPT1C* codes for an enzyme found in neuron mitochondria that is involved in transport of hydrophobic fatty acid chains into mitochondria, and plays a part in mitochondrial dysfunction. It might also have an important role in bortezomib induced peripheral neuropathy, since damage to mitochondria and endoplasmic reticulum through activation of a mitochondrial-based apoptotic pathway by bortezomib was noted in dorsal root ganglia of mice given bortezomib.[11] *RHOBTB2*, encodes another enzyme implicated here, has been shown to be upregulated during drug induced apoptosis, being mainly dependent on E2F1.[22] Knockout of *RHOBTB2* with small interfering RNAs has been shown to delay the onset of drug induced apoptosis.[22] *RASGRP1* is involved in many processes, including apoptosis and calcium-ion binding, which are potentially interesting for its role in bortezomib induced peripheral neuropathy. The presence of polymorphisms in the apoptosis gene *caspase 9*, which plays an important part in bortezomib induced apoptosis, suggests the possible contribution of this enzyme to early onset peripheral neuropathy.[23,24] One of the most significant SNPs (rs1029871) might have a role in the splicing regulation of *NEK4*, which is involved in the regulation of cell cycle and cell division. Furthermore, SNPs in enriched pathways like DNA repair and nervous system development and function were associated with early onset bortezomib induced peripheral neuropathy.

Late onset bortezomib induced peripheral neuropathy was associated with genes involved in the development and function of the nervous system. We noted upregulation of the superoxide dismutase gene *SOD2* in myeloma plasma cells; *SOD2* is regulated by tumor necrosis factor α and NFκB, and is known to have a role in the survival of neurons. Patients with diabetes and a polymorphism in the *SOD2* gene, leading to reduced *SOD2* activity, have been shown to be at increased risk of developing diabetic peripheral neuropathy.[25] The protective effect of *SOD2* might be eliminated with bortezomib induced apoptosis, which might trigger a susceptibility to oxidative stress in treated patients. Three SNPs associated with late onset bortezomib induced peripheral neuropathy were located in *SERPINB2* (plasminogen activator inhibitor-2). *SERPINB2*, with *SERPIN-1* (plasminogen activator inhibitor-1), tissue-type plasminogen activator, and urokinase-type plasminogen activator, has been shown to be induced in dorsal root ganglion neurons after peripheral axotomy in mice.[26] These serpins might also act as autocrine or paracrine regulators of plasminogen-activator-mediated nerve regeneration processes.[26] The associated SNPs might affect *SERPINB2* expression through their effect on splicing regulation. Besides genes involved in development of the nervous system, proinflammatory genes might play an important part in the pathogenesis of late onset bortezomib induced peripheral neuropathy, based on the presence of intronic SNPs in *MBL2* and *PPARD* (Corthals SL, unpublished data), and of about 30% of SNPs with reported inflammatory roles. The hypothesis that the DNA repair pathway is involved in bortezomib induced peripheral neuropathy, and that this side effect might be caused by the inability to repair neuronal damage (Corthals SL, unpublished data), could be substantiated by the presence of SNPs in *BRCA1* (rs16941 and rs799917). These nonsynonymous SNPs might have an effect on the phosphorylation state of a protein, which has been shown to abolish the P871L phosphorylation site in *BRCA1*.[27] Therefore, early onset and late onset bortezomib induced peripheral neuropathies were both associated with a myeloma genetic profile that was characterized by genes involved in the development of the nervous system; however, apoptosis was also a characteristic for the development of early onset bortezomib induced peripheral neuropathy. Genetic polymorphisms in genes involved in nervous system development and DNA repair play a part in both the early and late onset of this side effect.

A comparison of the molecular profiles of bortezomib induced peripheral neuropathy and vincristine induced peripheral neuropathy showed no overlap in associated genes or SNPs. Genes involved in cell cycle and proliferation were mainly associated with early onset vincristine induced peripheral neuropathy, both in the analyses of genetic pathways and SNPs. Additionally, involvement of proinflammatory genes in early onset vincristine induced peripheral neuropathy was substantiated by the finding of SNPs in *PARP1* and *LTA*, and two SNPs in *GLI1* (rs2228224 and rs2228226), which both encode an amino acid change; rs2228226 has been shown to affect *GLI1* activity, thereby affecting the inflammatory response.[28]

Genes implicated in drug absorption, distribution, metabolism, and excretion have been shown to be involved in chemotherapy induced peripheral neuropathy.[29] In accordance with this finding, an association was noted for late onset vincristine induced peripheral neuropathy with nine intronic SNPs in *ABCC1*; vincristine is known to be a substrate of the protein coded for by this gene.

In conclusion, this study provides the first large dataset in which the contribution of both the inherited genetic constitution of the host (patient) and the tumor (myeloma) to the development of bortezomib induced peripheral neuropathy has been reported (panel). We identified molecular factors that are associated with bortezomib induced peripheral neuropathy in patients with newly diagnosed multiple myeloma. Genes for apoptosis contribute to early onset bortezomib induced peripheral neuropathy, whereas genes that have a role in inflammatory pathways and DNA repair contribute to the development of late onset peripheral neuropathy, indicating that distinct genetic factors are involved in the development of early onset and late onset forms of this side effect. Bortezomib induced and vincristine induced peripheral neuropathy arise through different molecular mechanisms. Our findings strongly suggest an interaction between myeloma related factors and the patient's genetic background in the development of bortezomib induced peripheral neuropathy. Profiles of genetic risk might be used in future to identify patients with an increased risk of bortezomib induced peripheral neuropathy.

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Chapter 7

MicroRNA profiling in multiple myeloma

Sophie L. Corthals, Su Ming Sun, Rowan Kuiper,
Yvonne de Knegt, Annemiek Broyl, Bronno van der Holt,
H. Berna Beverloo, Justine K. Peeters, Laila el Jarari,
Henk M. Lokhorst, Sonja Zweegman,
Mojca Jongen-Lavrencic, Pieter Sonneveld

Submitted

Abstract

MicroRNAs (miRNAs) play a role in critical biological processes including cellular growth and differentiation in cancer. We set out to analyze the role of miRNAs in multiple myeloma (MM).

MiRNA and gene expression profiles were determined in 45 newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial. Unsupervised hierarchical clustering revealed distinctive miRNAs signatures, consisting of 4 MM clusters and 1 normal bone marrow cluster. The MM clusters were characterized by up- and downregulation of distinctive miRNAs. One of the cluster signatures was dominated by the miRNA clusters miRNA-17~92 and miRNA-106~25. Our data suggest that the miRNA signatures do not correlate with cytogenetic subgroups, i.e. deletion 13q14, t(4;14), t(11;14), and t(14;16). Upregulation of *let-7f*, *miRNA-194* and *miRNA-296* expression was borderline associated with better overall survival ($P = 0.06$). We identified a significant inverse correlation between *miRNA-21* expression and gene expression of two of its validated targets, *PDCD4* ($P = 1.6 \times 10^{-4}$) and *RECK* ($P = 7.7 \times 10^{-4}$).

MiRNA profiling defined distinctive miRNA signatures in MM patients. In addition, MM patients with common translocations showed specific expression of miRNAs. Our results showed a trend of an association between overall survival and miRNA expression. These results suggest a role of *miRNA-21* in the pathogenesis of MM.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy, characterized by the accumulation of malignant plasma cells in the bone marrow (BM). MM is characterized by profound genetic instability, leading to chromosomal abnormalities. Chromosomal translocations are early events in disease progression, and seem to play an important role in the genetic pathogenesis of MM. Myeloma can be divided into hyperdiploid and non-hyperdiploid disease. Hyperdiploidy; which confers a better prognosis, is present in 60% of myeloma patients, and is characterized by trisomies of chromosomes 3, 5, 9, 11, 15, 19 and 21.[1] Non-hyperdiploid myeloma is associated with recurrent translocations of the immunoglobulin heavy chain (IgH) involving several chromosomal loci, including 11q13 (cyclin D1), 6p21 (cyclin D3), 4p16 (FGFR3 and MMSET), 16q23 (MAF) and 20q11 (MAFB).

In recent years, gene expression profiling has contributed to a better understanding of the genetics of MM and it has been used for MM classification.[2-5] Two major genetic classification systems have been developed; the Translocation and cyclin D (TC) classification and the University of Arkansas for Medical Science (UAMS) molecular classification of myeloma. The UAMS classification was recently confirmed by our own group, and three new clusters were identified.[3]

MicroRNAs (miRNAs) are a class of small non-coding single stranded RNAs of approximately 22 nucleotides in length that regulate protein levels by binding to either partially or complete complementary sites in messenger RNAs (mRNAs), leading to translational repression or transcript degradation respectively.[6] MiRNAs play a role in critical biological processes including cellular growth and differentiation.[6] Studies in acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) revealed that miRNAs play an important role in the pathogenesis of the disease and that miRNA signatures are associated with different cytogenetic subtypes.[7-10] This suggests a possible function of miRNAs in the pathogenesis of MM. Until now, information about miRNA expression in MM is limited.[11-16] Previously, unsupervised analyses of miRNA expression in MM identified unique clusters, which were not associated with chromosomal abnormalities, while supervised analysis showed a specific miRNA expression pattern for these MM subgroups.[11-12]

In line with these studies, we have investigated the miRNA expression level of 365 miRNAs in 45 newly diagnosed MM patients enrolled in the HOVON-65/GMMG-HD4 trial.[17] We addressed the question whether MM patients could be classified based on miRNA expression patterns, and if there is an association with known chromosomal abnormalities in MM. We correlated miRNA expression data with gene expression data in order to gain more insight into candidate miRNA target genes. Finally, a possible association of miRNA expression with response to therapy; progression free survival (PFS) and overall survival (OS) was evaluated.

Materials and methods

Patients

BM plasma cell samples were obtained from 45 newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial (EudraCT nr 2004-000944-26, ISRCTN64455289).[17] Informed consent for treatment protocols and sample procurement was obtained from all patients included in this study, in accordance with the Declaration of Helsinki.

Four healthy BM samples were obtained from subjects undergoing BM harvest for allogeneic transplantation donorship. BM aspirates were obtained from the posterior iliac crest, and BM cells were freshly separated by density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). Myeloma plasma cells were then purified using CD138 magnetic microbeads (MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched aliquots were assessed for purity, and samples with CD138 positive plasma cell purity > 80% were included in this study. A total of 45 samples were analyzed for miRNA expressions profiling. Of 45 samples, 39 were also assessed for gene expression profiling.

Cytogenetic analysis

At least 200 interphase nuclei per sample were analyzed for recurrent chromosomal abnormalities using epi-fluorescence microscopy and image analysis software. In several cases, a preceding analysis of selected myeloma cells based on light chain counterstaining or morphology was performed. Hybridization efficiency was validated on plasma cells obtained from BM of a healthy donor and thresholds for gains, deletions and translocations were set at 10%.

Interphase fluorescence in situ hybridization (FISH) analysis was performed on purified MM plasma cells as previously described.[18-19] Detection of translocations t(11;14)(q13;q32), t(4;14)(p16;q32) and (14;16)(q32;q23) were determined using probes LSI IgH/CCND1, LSI IgH/FGFR3 and LSI IgH/MAF, respectively (Vysis, Abbott Molecular, Abbott Park, Illinois, USA) or for the detection of t(11;14)(q13;q32), t(4;14)(p16;q32) also Poseidon Probes (Kreatech, Amsterdam, The Netherlands) were used. LSI D13S319 (13q14.3) SpectrumOrange Probe was used to detect 13q14 deletions in combination with CEP 9 SpectrumGreen Probe to detect additional copies of chromosome 9 indicative for the presence of a hyperdiploid clone (Vysis, Abbott Molecular, Abbott Park, Illinois, USA). In addition, LSI 13 (RB1) 13q14 SpectrumOrange Probe was used to determine the deletion status of the RB gene.

RNA extraction

Total RNA was extracted from CD138 magnetic cell selected (MACS) MM plasma cells, with a minimum purity of > 80%, using miRVana miRNA Isolation Kit (Ambion, Austin, TX), according to manufacturer's protocol. RNA levels and quality were assessed using the Nanodrop ND100

(Nanodrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Assessments

Response was evaluated on an intention-to-treat basis according to the European Group for Blood and Marrow Transplant[20] criteria to which a very good partial response (VGPR) was added; defined as a 90% or greater reduction in the serum M-protein plus urinary M-protein level less than 100 mg/24 hours.[21] In addition, near complete response (nCR) was added; defined as CR but without negative immunofixation (i.e. either positive or not done).

Definition of end points

The primary endpoint of the HOVON-65/GMMG-HD4 trial was progression free survival. PFS was calculated from randomization until progression, relapse or death from any cause. OS was measured from randomization until death from any cause. Patients still alive were censored at the date of last contact.

MiRNA expression profiling

All 45 MM samples were investigated for miRNA expression using the TaqMan Human MicroRNA Array v1.0 (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) containing 365 different human miRNAs in addition to three small nuclear RNA controls (*RNU24*, *RNU48* and *RNU6B*) that are used for data normalization. MicroRNA hairpin reverse transcriptase (RT) primers for the 365 mature miRNAs were divided into 8 multiplex pools, and used for RT reactions with 100 ng total RNA as template per multiplex reaction. Reversed transcriptase and real-time PCR were done according to manufacturer's protocol. Briefly, RT reactions of 10 µl contained the following: 2 µl of 50 ng/µl of total RNA, 1.0 µl 10x RT buffer, 0.2 µl dNTPs (100 mM total), 2.0 µl Multiscribe Reverse Transcriptase (50 U/µl), 0.125 µl AB RNase Inhibitor (20 U/µl), 1 µl multiplex RT primers and 3.675 µl H_2O. Reactions were incubated in a Primus HT Thermocycler (MWY Biotech, High Point, NC) at 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min and then held at 4 °C. Eight independent RT reactions were carried out for each pool of miRNA primers. Diluted RT reaction products were mixed with TaqMan Universal PCR Mastermix no AmpErase uracil-*N*-glycosylase (Applied Biosystems) and loaded on TaqMan Low Density Arrays (Applied Biosystems, part number 4384792). Real-time quantitative RT-PCR reactions were incubated at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 sec and 60 °C for 1 min by using the ABI 7900 HT Sequence Detection System (SDS; Applied Biosystems).

The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. MiRNAs were considered as present when Ct-values were lower than 35. SDS 2.3 software (Applied Biosystems) was used to analyze real-time RT-PCR data. *RNU48* was used as endogenous control, since this was the most consistently expressed in our patient dataset. The relative quantification method, $2^{-\Delta Ct}$, was used to calculate the expression.[22] A log2 transformation was performed to obtain a normal linear distribution.

Hierarchical clustering with the centered correlation metric with average linkage was performed for unsupervised analysis using BRB Array tools version 3.8.0. Robustness indices (BRB Array tools) were calculated to give an indication about reproducibility of the clusters. Translocation and clinical data were projected next to the dendogram. A supervised approach was used to determine the miRNA expression profiles driving the clusters; a Mann-Whitney U test was applied for each cluster using the Partek Genomics Suite 6.4 software (Partek Inc., St Louis, MO), comparing each cluster to the remaining clusters. A false discovery rate (FDR) was calculated using a step-up approach (Benjamini and Hochberg[23]) with a 0.05 significance value to compensate for multiple testing errors.

Supervised analysis for deletion 13q14, t(4;14), t(11;14), and t(14;16) and response to therapy was performed. Specifically, differentially expressed miRNAs were identified by comparing patients with a chromosomal abnormality and patients without this abnormality. For response to therapy, good responders (nCR/CR) were compared to patients who responded less well (VGPR or less).

Log-rank tests for univariate association with PFS and OS were performed for each of the 365 miRNAs. MiRNA expression was divided in 4 quartiles. Log-rank tests were performed for quartile 1 (Q1) versus Q2 through Q4 and Q4 versus Q1 through Q3 in order to identify up- and down-regulated miRNAs respectively. An FDR was calculated using a step-up approach (Benjamini and Hochberg[23]) for each log-rank *P*-value and considered statistically significant when < 0.05. Distributions of OS for miRNAs identified by the log-rank tests were evaluated using the Kaplan-Meier method with PASW statistics v18 (SPSS Inc., Chicago, IL, USA).

Functional pathway analysis of miRNAs was performed using Ingenuity Pathway Analysis Software (Ingenuity Systems, http://www.ingenuity.com).

Gene expression profiling

RNA target labeling and hybridization to gene expression arrays was performed as previously described.[3] Briefly, the Two-Cycle Eukaryotic Target Labeling Assay (Affymetrix) was used to obtain sufficient amounts of cRNA in order to hybridize 10 μg of fragmented, biotin labeled cRNA to Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix Inc, Santa Clara, CA) in accordance with Affymetrix protocols. Arrays were washed, stained, and finally scanned in a GeneChip Scanner 3000 station (Affymetrix). Quality controls of arrays using GeneChip Operating Software (GCOS) included scaling factor (SF), and percentage of genes present (GP). Arrays with SF difference < 3 and GP > 20% were further analyzed. Raw data from selected gene expression arrays (CEL-files) were pre-processed using GCRMA in Partek Genomics Suite, version 6.4. Final quality control of arrays included relative log expression (RLE) and normalized unscaled standard errors (NUSE) from the affyPLM package (www.bioconductor.com). Arrays showing a NUSE value > 1.05 and aberrant RLE plots were excluded from analysis. Microarray data are available at the NIH Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE19784).

Using the median expression values of *Dicer*, *Drosha* and *Ago* as a cut-off, patients were divided in high and low expression level groups. The relationship of *Dicer*, *Drosha* and *Ago* with PFS and/or OS was analyzed using univariate log-rank tests.

MiRNA-mRNA correlation and target prediction

To identify putative miRNA targets, an integrative analysis of miRNA and mRNA expression data was performed. A Spearman's rank correlation test was performed to identify significant ($P <$ 0.001) inversely correlated miRNA-mRNA pairs. Next, miRNA target prediction for significant miRNA-mRNA pairs was carried out applying 4 different prediction algorithms; TargetScan 5.1,[24] PicTar,[25] miRDB,[26] and MicroCosm.[27] Only those miRNA-mRNA pairs that were predicted by three or more target prediction programs were selected to reduce the possibility of false positive predictions.

Results

Patients

MiRNA expression arrays of purified myeloma plasma cells were obtained from 45 MM patients at diagnosis. The baseline characteristics, cytogenetic abnormality status, response to therapy, and survival data for these patients are shown in Table S1. Chromosome 13q14 deletion was present in 13 (29%) patients, t(4;14) in 6 (13%), t(11;14) in 2 (4%), and 1 (2%) patient presented with t(14;16) at baseline. Concerning response; 6 patients showed no change (NC), 9 patients achieved a partial response (PR), 10 patients a VGPR, 9 patients a nCR and 9 patients achieved a CR. Median PFS was 19.2 months (range 0.5-38.6) and median OS was 26.9 months (range 0.5-40.2).

Unsupervised analysis of miRNA expression

Bone marrow samples of 45 MM patients and 4 normal BM donors were analyzed using unsupervised hierarchical sample clustering based on the miRNA expression. Figure 1 shows a dendogram and heatmap of 1 normal BM cluster and 4 MM clusters with distinctive miRNA expression profiles. An additional cluster of 1 sample was not defined. The robustness indices per cluster are shown in Table S2. Despite the incompleteness of the cytogenetic data, combining this with the hierarchical clustering suggested that the miRNA expression patterns were not associated with recurrent cytogenetic translocations or 13q14 deletion. Four normal BM samples clustered together (Figure 1). To determine the miRNA expression signatures of the 4 MM and 1 normal

BM cluster each cluster was compared to the remaining 4 clusters using the Mann-Whitney U test with a FDR < 0.05 (Partek Genomics Suite 6.4 software). The 4 MM clusters and the normal BM cluster were characterized by up- and downregulation of distinctive miRNAs. The top 10 significant miRNAs for each cluster in comparison to the others are shown in Table 1. The complete list of differentially expressed miRNAs (FDR < 0.05) characterizing each cluster is shown in Table S3. MM cluster 1 (n=8) was characterized by upregulation of 17 miRNAs including *miRNA-130a*, *miRNA -424*, and *miRNA-622*; MM cluster 2 (n=9) by upregulation of *miRNA-576* and *miRNA-106b*; MM cluster 3 (n=4) by upregulation of 23 miRNAs among which *miRNA-372* and *miRNA-200a* and downregulation of *miRNA-146b*; MM cluster 4 (n=23) was characterized by downregulation of 60 miRNAs including miRNAs of clusters miRNA-17~92 (*miRNA-19a*, *-19b*, and *miRNA-92*) and miRNA-106~25. Our data suggest that there is no association of the clusters based on miRNA expression patterns and known recurrent cytogenetic translocations or 13q14 deletion.

Supervised analysis of miRNA expression

We investigated the expression patterns of miRNAs among cytogenetic subgroups of MM. A supervised analysis using a Mann-Whitney U test with a FDR < 0.05 for each cytogenetic subgroup (deletion 13q14, t(4;14), t(11;14), t(14;16) was performed and discriminating miRNAs identified are presented in Table 2. Comparing each chromosomal abnormality to the remaining samples, we identified 5 miRNAs differentially expressed in MM patients with a t(11;14) and 1 miRNA in t(14;16). The 5 miRNAs deregulated in MM patients with t(11;14) (n=2), were all upregulated compared with the remaining MM patients. These miRNAs were not clustered together on the same chromosomal locus, but were located on different chromosomes, which did not include chromosome 11 or 14. *MiRNA-600*, located at chromosome 9, was upregulated in the MM patient harboring a t(14;16) compared with the remaining MM patients.

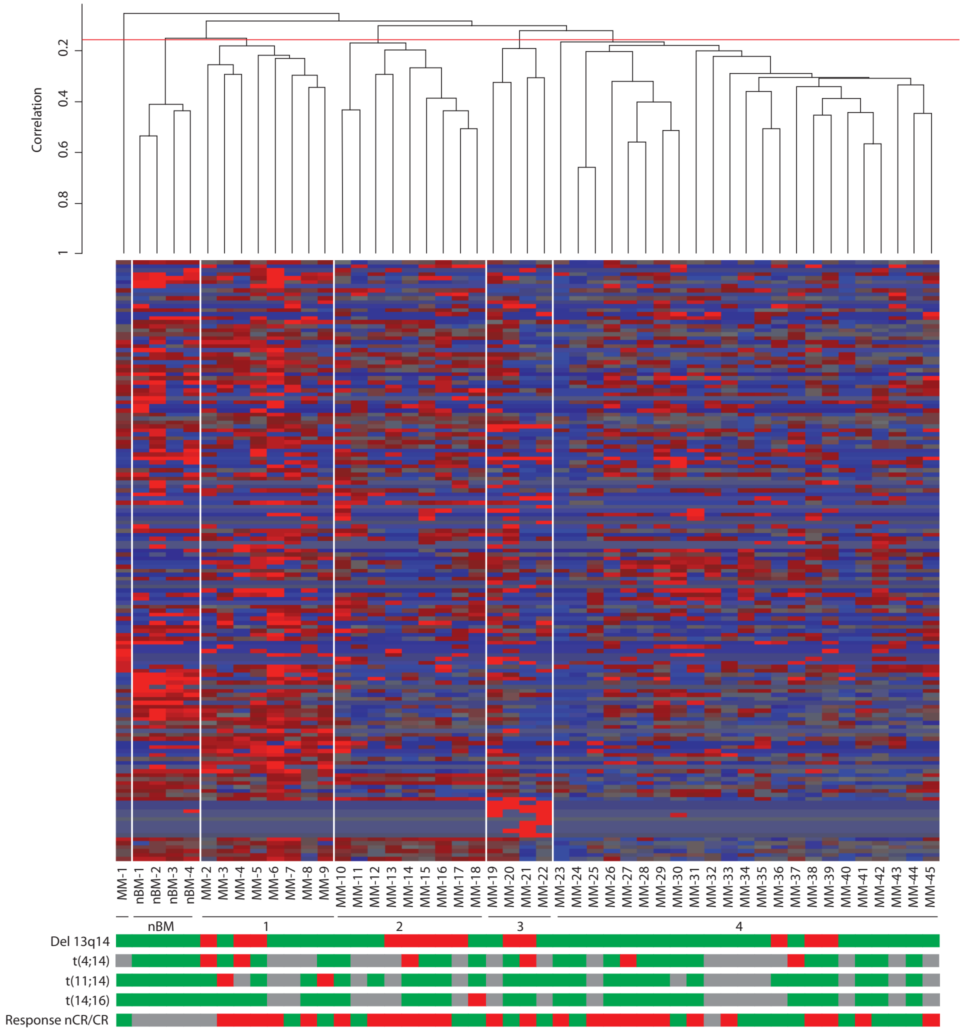

Figure 1. Unsupervised analysis of miRNA expression. Hierarchical clustering of average linkage with the centered correlation metric was performed for unsupervised analysis using BRB Array tools version 3.8.0. Samples are depicted in columns, miRNAs in rows. The color scale bar represents the relative miRNA expression normalized using the relative quantification method, $2^{-\Delta Ct}$, with red representing positive expression (upregulation) and blue representing negative expression (downregulation) of a miRNA. Four MM clusters and 1 normal bone marrow cluster with distinctive miRNA expression profiles were identified. An additional cluster of 1 sample was not defined (MM-1). Cytogenetic and clinical data are shown under the dendogram and heatmap. Green indicates no chromosomal abnormality present; red, chromosomal abnormality and gray, not determined. Concerning response; green indicates nCR/CR; red, ≤ VGPR and gray, not determined.

Table 1. Top differentially expressed miRNAs per cluster. To determine the miRNA expression signature of clusters, a Mann-Whitney U test was applied for each cluster using the Partek Genomics Suite 6.4 software. A FDR was calculated using a step-up approach with a 0.05 significance value to compensate for multiple testing errors. For each cluster the top 10 (or less) up- and/or downregulated miRNAs in comparison to the remaining clusters are shown.

Cluster	No. of samples	miRNA	Chromosome	*P*-value	Up/downregulated
nBM	4	*hsa-miR-28*	3	0.031	up
		hsa-miR-30c	1/6	0.031	up
		hsa-miR-30d	8	0.031	up
		hsa-miR-199a#	1/19	0.031	up
		hsa-miR-214	1	0.031	up
		hsa-miR-135b	1	0.031	up
		hsa-miR-31	9	0.031	up
		hsa-miR-203	14	0.031	up
		hsa-miR-367	4	0.031	up
		hsa-miR-485-5p	14	0.031	up
MM 1	8	*hsa-miR-130a*	11	0.009	up
		hsa-miR-424	X	0.009	up
		hsa-miR-622	13	0.009	up
		hsa-miR-206	6	0.009	up
		hsa-miR-320	8	0.014	up
		hsa-miR-601	9	0.015	up
		hsa-miR-433	14	0.016	up
		hsa-miR-22	17	0.024	up
		hsa-miR-650	22	0.029	up
		hsa-miR-219	6/9	0.035	up
MM 2	9	*hsa-miR-576*	4	0.015	up
		hsa-miR-106b	7	0.041	up
MM 3	4	*hsa-miR-372*	19	< 0.001	up
		hsa-miR-200a	1	< 0.001	up
		hsa-miR-504	X	< 0.001	up
		hsa-miR-518c#	19	< 0.001	up
		hsa-miR-520g	19	< 0.001	up
		hsa-miR-609	10	< 0.001	up
		hsa-miR-96	7	< 0.001	up
		hsa-miR-488	1	0.004	up
		hsa-miR-516-5p	19	0.004	up
		hsa-miR-448	X	0.014	up
MM 4	23	*hsa-let-7c*	21	< 0.001	down
		hsa-miR-125b	11/21	< 0.001	down
		hsa-miR-100	11	< 0.001	down
		hsa-miR-142-5p	17	< 0.001	down
		hsa-miR-99a	21	< 0.001	down
		hsa-miR-32	9	< 0.001	down
		hsa-miR-106b	7	0.001	down
		hsa-miR-30a-5p	6	0.001	down
		hsa-miR-148b	12	0.001	down
		hsa-let-7g	3	0.001	down

nBM indicates normal bone marrow; MM, multiple myeloma.

Table 2. Differentially expressed miRNAs per cytogenetic abnormality. Supervised analysis for deletion 13q14, t(4;14), t(11;14), and t(14;16) was performed using a Mann-Whitney U test implemented in Partek Genomics Suite 6.4 software. A FDR was calculated using a step-up approach with a 0.05 significance value to compensate for multiple testing errors. Differentially expressed miRNAs were identified by comparing patients with a chromosomal abnormality and patients without this abnormality.

Cytogentic abnormality	No. of cases	miRNA	Chromosome	*P*-value	Up/downregulated
13q14	12	-	-	-	-
t(4;14)	6	-	-	-	-
t(11;14)	2	*hsa-miR-122a*	18	0.010	up
		hsa-miR-33	22	0.010	up
		hsa-miR-489	7	0.010	up
		hsa-miR-519e	19	0.010	up
		hsa-miR-555	1	0.010	up
t(14;16)	1	*hsa-miR-600*	9	< 0.001	up

We investigated whether there was a relation between the miRNA expression levels and response to therapy or survival. We could not identify a specific miRNA expression pattern for good responders (nCR/CR) or PFS. Log-rank tests revealed 3 upregulated miRNAs that were associated with a better OS at borderline significance level, i.e. *let-7f* (*P* = 0.06), *miRNA-194* (*P* = 0.06), and *miRNA-296* (*P* = 0.06) (Figure 2).

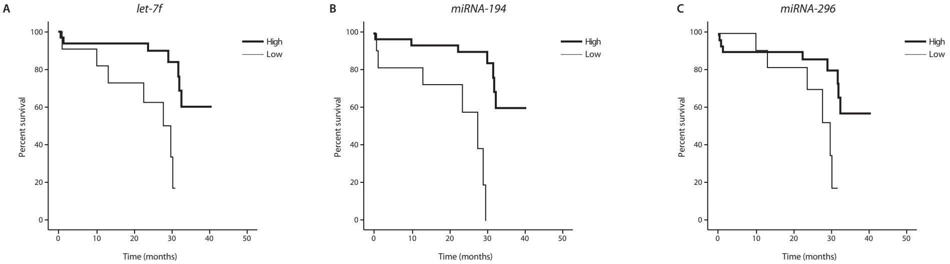

Figure 2. Overall survival of MM patients according to miRNA expression. Log-rank tests for univariate association with OS were performed for each of the 365 miRNAs. Log-rank tests were performed for quartile 1 (Q1) versus Q2 through Q4 and Q4 versus Q1 through Q3 in order to identify up- and downregulated miRNAs respectively. A FDR was calculated for each log-rank *P*-value. MiRNA expression values were divided in two categories; low (25% lower expression values) and high (75% upper expression values). Distributions of OS for miRNAs identified using the log-rank tests were evaluated using the Kaplan-Meier method. (A) OS according to *let-7f* expression (*P* = 0.06). (B) OS according to *miRNA-194* expression (*P* = 0.06). (C) OS according to *miRNA-296* expression (*P* = 0.06).

Finally, we investigated the effect of *Dicer*, *Drosha* and *Ago*, three genes required for miRNA biogenesis, on survival in MM patients. MM patients were divided in high and low expression groups for all three genes and log-rank tests were performed. A better PFS was identified at borderline significance level (*P* = 0.07) in patients having a low *Dicer* expression level (Figure 3).

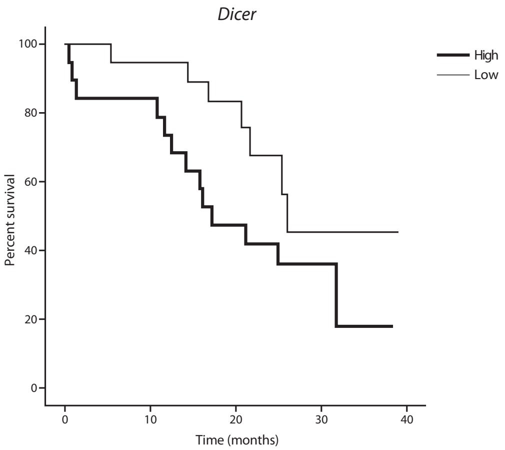

Figure 3. Progression free survival of MM patients according to *Dicer* expression. A log-rank test for univariate association with PFS was performed for *Dicer* gene expression. MM patients were divided in a high and low expression level group. Log-rank test *P*-value = 0.071.

MiRNA and mRNA expression profiles

MiRNAs function primarily as translational repressors by binding to mRNA, which results in mRNA degradation or protein translation inhibition. We therefore determined the mRNA expression profile of 39/45 MM patients for whom sufficient RNA was available using the Human Genome U133 Plus 2.0 Array. Gene expression analysis provides indirect evidence since the direct interaction of an individual miRNA to a specific mRNA is not detected. However, it does detect changes in mRNA level due to mRNA cleavage and degradation possibly caused by miRNA-mRNA interactions.[28] Therefore, analysis of mRNA and miRNA levels is sufficient to identify miRNA-target interactions that result in mRNA degradation and have been shown to contribute to the detection of miRNA targets.[29] Our approach assumes an inverse correlation if a functional interaction between miRNA and mRNA is present. Spearman's rank correlation test identified 130 inverse correlations with $P < 0.001$. Prediction scores were calculated using four of the currently available target prediction programs, including TargetScan 5.1,[24] PicTar,[25] miRDB,[26] and miRBase,[27] and only those miRNA-mRNA pairs that were identified by at least three prediction programs were selected (Table 3). This analysis identified 18 miRNA-mRNA inverse correlations; including 11 miRNAs and 15 genes. Two of these miRNA-mRNA interactions have been validated previously; *miRNA-21* has been shown to target programmed cell death 4 (*PDCD4*),[30-31] as well as reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*), and *CDC34* has been identified as a target of *let-7*.[32] The miRNA-mRNA interactions concerning *miRNA-21* have been validated by quantitative RT-PCR.

Table 3. MiRNA-mRNA inverse correlations. To identify putative miRNA targets, an integrative analysis of miRNA and mRNA expression data was performed. A Spearman's rank correlation test was performed to identify significant ($P < 0.001$) inversely correlated miRNA-mRNA pairs. Next, miRNA target prediction for significant miRNA-mRNA pairs was carried out applying 4 different prediction algorithms; TargetScan 5.1,[24] PicTar,[25] miRDB[26] and MicroCosm.[27] To reduce the possibility of false positive predictions, only those miRNA-mRNA pairs that were predicted by three or more target prediction programs are shown.

miRNA	Gene	Spearman's correlation coefficient	*P*-value	TargetScan	MicroCosm	Pictar	miRDB	Validated target
hsa-miR-125b	*MAN1B1*	-0.61	< 0.001	-0.30	17.82	3.22	-	no
hsa-miR-21	*PDCD4*	-0.58	< 0.001	-0.43	15.35	1.3	62	yes
hsa-let-7c	*CDC34*	-0.57	< 0.001	-0.05	18.36	8.88	70	yes
hsa-miR-148b	*RAB34*	-0.57	< 0.001	-0.41	16.16	3.79	66	no
hsa-miR-26a	*CAMSAP1*	-0.55	< 0.001	-0.27	15.51	1.84	71	no
hsa-miR-218	*SEC61A1*	-0.55	< 0.001	-0.27	-	6.13	72	no
hsa-miR-125b	*MAN1B1*	-0.54	< 0.001	-0.30	17.82	3.22	-	no
hsa-miR-30a-5p	*KCTD5*	-0.54	< 0.001	-0.21	15.95	2.19	-	no
hsa-miR-218	*ARAF*	-0.54	< 0.001	-0.17	15.52		50	no
hsa-miR-148b	*RAB34*	-0.54	< 0.001	-0.41	16.16	3.79	66	no
hsa-miR-30d	*UBAC1*	-0.53	0.001	-0.31	16.71	-	55	no
hsa-miR-125b	*ESRRA*	-0.52	0.001	-0.30	15.40	6.48	-	no
hsa-miR-21	*RECK*	-0.52	0.001	-0.47	15.49	1.69	62	yes
hsa-miR-30d	*MAN1B1*	-0.52	0.001	-0.20	16.03	5.16	-	no
hsa-miR-23a	*NIN*	-0.52	0.001	-0.38	-	10.85	94	no
hsa-let-7f	*ZBTB5*	-0.51	0.001	-0.25	-	2.08	57	no
hsa-let-7c	*SLC35D2*	-0.51	0.001	-0.43	16.85	3.53	57	no
hsa-miR-130b	*MLLT10*	-0.51	0.001	-0.20	-	3.76	69	no

Discussion

We analyzed the miRNA expression profiles of 45 newly diagnosed patients with MM.[17] Unsupervised hierarchical clustering revealed a subdivision in 4 MM clusters and 1 normal BM cluster characterized by up- and downregulation of a number of miRNAs. Cytogenetic and clinical data were projected on this clustering. Despite missing cytogenetic data and small sizes of the cytogenetic subgroups t(11;14) and t(14;16) our data suggest that miRNA clusters may not be defined according to chromosomal abnormalities. The 4 normal BM samples clustered together, and myeloma plasma cells had a distinctive miRNA expression pattern when compared to these normal bone marrow samples. We were able to confirm upregulation of 6 miRNAs (*miRNA-214*, *-135b*, *-196a*, *-155*, *-203*, and *-486*) in normal BM, which has been recently described by Gutiérrez *et al.*[11]

It has been shown by Gutiérrez *et al.*[11] that when using a unsupervised approach, a subtle distinction in miRNA expression pattern between MM cytogenetic subgroups may not be detectable, whereas supervised analysis may identify them. Using a supervised analysis, a miRNA signature of patients with t(11;14) (n=2) was determined including upregulation of *miRNA-122a*, *miRNA-33*, *miRNA-489*, *miRNA-519e*, and *miRNA-555*. Upregulation of *miRNA-600* characterized a t(14;16) (n=1). However, cytogenetic subgroups were small and validation of our findings in an independent dataset is necessary.

An important question that remains is what drives this miRNA clustering in MM patients. Our data suggest that miRNAs may have an independent role in the pathogenesis of MM, not related to the cytogenetic abnormalities. Therefore, the functional studies on the miRNAs that define the clusters may reveal the answer to this question in the future.

The differential miRNA expression in distinct clusters is a novel finding in MM. An interesting observation is the identification of a miRNA signature of MM cluster 4 comprised of upregulated miRNAs including *miRNA-21*, *-19a*, and *-19b*, which have all been shown to be involved in the signal transducer and activator of transcription 3 (*STAT3*) and interleukin 6 (*IL-6*) anti-apoptotic pathway, representing a putative mechanism for the malignant growth of myeloma plasma cells.[13,33] In addition, miRNA clusters miRNA-17~92 (*miRNA-19a*, *-19b*, and *miRNA-92*) and miRNA-106~25 (*miRNA-106b*, *-93*, and *miRNA-25*) were highly represented in the miRNA signature of MM cluster 4. Pichiorri *et al.*[13] recently described a miRNA signature in MM, which is characterized by upregulation of the miRNA-17~92 and miRNA-106~25 clusters, having nearly identical sequences. The miRNA-17~92 cluster has an oncogenic role by regulating expression of its pro-apoptotic targets phosphatase and tensin homolog (*PTEN*), E2F transcription factor 1 (*E2F1*), and BCL2-like 11 (*BCL2L11*).[34] Consequently, this cluster plays an essential role in B cell development. The miRNA-106~25 cluster may have a similar function since *E2F1* and *BCL2L11* have also been described as targets of this miRNA cluster.[35]

It is well known that the identification of miRNA targets is difficult, due to the capability of miRNAs to bind their targets with imperfect complementarity. Several algorithms have been developed to predict miRNA targets, however, a large number of these predicted targets may be false positive, and experimental verification is required before a gene can be considered as a genuine miRNA target. In this study we chose to investigate the inverse correlations between miRNA and gene expression profiles. This analysis revealed a number of significant inverse correlations where mRNAs were based on the algorithms also predicted as potential target. One example is a highly significant inverse correlation between *miRNA-21* and two of its previously validated targets, *PDCD4*[30-31] and *RECK*. *MiRNA-21* is upregulated in a wide variety of cancers and has been described as an oncogenic miRNA exhibiting an anti-apoptotic role.[33,36] In myeloma cells, *miRNA-21* has been shown to be upregulated as well.[13,33] The tumor suppressor gene *PDCD4* is downregulated or lost in several tumor types.[37-38] Therefore, *PDCD4* has been opted as molecular target in cancer treatment.[39] In addition, downregulation of *PDCD4* is essential for maximal induction of activating protein 1 (AP-1) upon RAS oncogenes. RECK has been shown to be involved in angiogenesis, through inhibition of MMP2 and MMP9.[40-41] Furthermore, *miRNA-21* has been identified as a *STAT3* target gene, therefore contributing to its anti-apoptotic role.[33] STAT3 is an important mediator of IL-6 signaling,[42] which is essential for the growth and survival of myeloma plasma cells. The confirmation of an inverse correlation of *miRNA-21* with *PDCD4* and *RECK* in this study indicates that this gene possibly blocks apoptosis of myeloma plasma cells, and that it may provide a promising therapeutic target.

The inverse correlation between *let-7* and *CDC34* has been shown recently in primary fibroblast.[32] In this study *let-7* expression leads to downregulation of CDC34 and G_2/M cell cycle arrest via the cyclin dependent kinase CDC2 and Wee 1.

Using log-rank tests we identified 3 borderline significant miRNAs ($P = 0.06$), *let-7f*, *miRNA-194* and *miRNA-296*, whose upregulation was associated with a better OS. One of these miRNAs, *miRNA-296*, has a significant adverse impact on survival in esophageal squamous cell carcinoma.[43] Interestingly, a higher expression of *miRNA-296* predicted a better survival in our study. *MiRNA-296* is involved in drug resistance in part through regulation of apoptosis and regulation of the ATP-binding cassette (ABC) transporter *ABCB1* (*MDR1*).[43] This regulatory miRNA is furthermore involved in angiogenesis in tumors by modulating growth factor receptors.[44] MiRNA *let-7f* also promotes angiogenesis by targeting anti-angiogenic genes.[45] In lung cancers, it has been shown that low levels of *let-7* correlated with shorter survival after resection,[46] which corresponds with our finding that a low level of *let-7f* is associated with shorter OS. In addition, our finding that upregulation of *miRNA-194* is associated with a better OS, is in line with a recent study by Pichiorri *et al.*[47] in which they show that *miRNA-194* expression inhibits cell growth and enhances apoptosis in MM cell lines in a p53 dependent manner. They furthermore show that *miRNA-194* targets murine double minute 2 (*MDM2*) and that a combination of this miRNA with *miRNA-192*, *miRNA-215* and a p53 activator could be a successful therapy for MM. In addition, the identified prognostic miRNAs may be used as predictive markers. Their role in the pathogenesis of MM as well as possible therapeutic targets remains to be elucidated.

Finally, it has previously been shown that high expression levels of *Dicer*, an essential gene for miRNA biogenesis, were associated with metastatic disease in prostate adenocarcinoma.[48] In line with these results, our study revealed a shorter PFS in patients with a high *Dicer* expression level at borderline significance level. However, in other tumor types, the relationship between *Dicer* and survival was found to be reversed.[49-50] These conflicting results indicate that the influence of Dicer on clinical outcome may be dependent on the tumor type.

In conclusion, the study presented here demonstrates that miRNA expression in MM is deregulated compared to normal plasma cells. Unsupervised analysis showed that MM patients can be classified according to their miRNA expression pattern, and that these miRNA profiles may not be characterized by cytogenetic subgroups. Despite the small size of cytogenetic subgroups, supervised analysis revealed distinctive miRNA expression in MM patients with a t(11;14) or t(14;16). A trend towards better OS was observed for patients with high expression of *let-7f*, *miRNA-194* and *miRNA-296*. Furthermore, integration of miRNA and mRNA data showed the putative interaction between *miRNA-21* and two of its validated targets; *PDCD4* and *RECK*, suggesting a functional relationship between miRNA expression and gene expression. These data contribute to a better understanding of the role of miRNAs in the pathogenesis of MM.

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Chapter 8

MicroRNA-15a and *microRNA-16* expression and chromosome 13 deletion in multiple myeloma

Sophie L. Corthals, Mojca Jongen-Lavrencic,
Yvonne de Knegt, Justine K. Peeters,
H. Berna Beverloo, Henk M. Lokhorst, Pieter Sonneveld

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Abstract

Deletion of chromosome 13, observed in more than 50% of multiple myeloma (MM) patients, is associated with poor prognosis; however, a tumor suppressor gene has not yet been identified in the region. We have investigated whether miRNA-15a and miRNA-16-1, located on chromosome 13q14, are downregulated or deleted in 26 MM patients.

Deletions of chromosome 13 were observed in 12/26 (46.2%) MM patients when analyzed by FISH. Copy number variation (CNV) analysis with SNP mapping arrays have defined 8 additional patients with a chromosome 13 aberration at the *miRNA-15a* and *miRNA-16-1* location. Overall, chromosome 13q14.3 deletions were present in 20/26 (76.9%) of MM patients. Both miRNAs displayed a wide range of expression, while no difference in *miRNA-15a* or *miRNA-16* expression between patients with or without a chromosome 13 deletion was found.

MiRNA-15a and *miRNA-16* display a range of expression patterns in MM patients, independent of the chromosome 13 status. These findings suggest that genes other than *miRNA-15a* and *miRNA-16* may be involved in the mechanism underlying the prognostic significance of chromosome 13q deletions. Furthermore, the results show that CNV analysis using SNP mapping arrays is a more comprehensive and accurate method to determine chromosome 13 aberrations than standard FISH.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy, characterized by the accumulation of malignant plasma cells in the bone marrow (BM). MM represents approximately 15% of all hematological malignancies.[1] In spite of conventional and high-dose chemotherapy treatment followed by stem cell transplantation, all patients eventually relapse and their median survival is 3-5 years.[2]

MM is characterized by profound genetic instability, leading to chromosomal abnormalities. Chromosomal translocations are early events in disease progression and seem to play an important role in the genetic pathogenesis of MM. The most common abnormality observed is a deletion of chromosome 13, which is seen in more than 50% of MM cases and is an early event in MM pathogenesis. Such aberrations of chromosome 13, are detectable in all stages of myeloma and normally involve large segments or the entire long arm, however small interstitial deletions have also been described.[3] The most commonly deleted marker in the 13q14 region is D13S319,[4] located distal to the retinoblastoma-1 (*RB1*) gene. Since deletions at chromosome 13 have been associated with poor prognosis,[5-11] it is now common practice to determine the presence of chromosome 13 deletions by fluorescence in situ hybridization (FISH) in patients with newly diagnosed MM. Because of the adverse prognostic role of these deletions, the presence of MM tumor suppressor gene(s) on chromosome 13q has been suggested, but so far not identified.

MicroRNAs (miRNAs) are a class of small non-coding single stranded RNAs of approximately 22 nucleotides in length. So far, more than 500 human miRNAs have been reported in literature.[12] MiRNAs negatively regulate gene expression by binding to partially complementary sites in messenger RNAs (mRNAs). As a result, the mRNA is degraded or remains untranslated, leading to decreased levels of mRNA and protein respectively.[13]

A large number of miRNAs are located at fragile sites across the human genome. These sites are often deleted or amplified at a common breakpoint, and they are frequently associated with cancer.[14] Therefore, miRNAs have been suggested to function as tumor suppressor and oncogenes and play a role in the pathogenesis of MM.[15] In chronic lymphocytic leukemia (CLL), *miRNA-15a* and *miRNA-16-1*; located on chromosome 13q14, are frequently downregulated or deleted and therefore a tumor suppressor activity and pathogenic role has been hypothesized.[16-17]

The aim of this study is to evaluate the expression of *miRNA-15a* and *miRNA-16* in MM and to study the potential association between miRNA expression levels and chromosome 13 deletions in myeloma plasma cells.

Materials and methods

Patient samples and cell lines

We obtained bone marrow samples from newly diagnosed patients with MM who were included in a prospective randomized clinical trial. Bone marrow aspirates were obtained from the posterior iliac crest and bone marrow cells were freshly separated by density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). Myeloma plasma cells were then purified using CD138 magnetic microbeads (MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched aliquots were assessed for purity and samples with CD138 positive plasma cell purity > 80% were included in this study.

RNA isolation

Small RNAs; transcripts less than 200 nucleotides in length including miRNAs, were isolated from CD138 magnetic cell selected (MACS) MM plasma cells using miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA), according to manufacturer´s protocol.

RNA levels and quality were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies).

PCR analysis

Mature *miRNA-15a* and *miRNA-16* expression levels in MM plasma cells were determined by real-time PCR using TaqMan miRNA assay; which uses stem-loop reverse transcription (RT) followed by real-time PCR (Applied Biosystems, P/N: 4373123 and 4373121).[18] Briefly, 10 ng of small RNA was used in each 15 µl reaction, and reverse transcribed to cDNA. RT reactions were carried out at 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min and then held at 4 °C. All RT reactions including no-template controls were run in duplicate.

Next, 1.33 µl of this RT product was used for a 20 µl real-time PCR reaction, containing 10 µl TaqMan 2x Universal PCR Master Mix, No AmpErase uracil-*N*-glycosylase (Applied Biosystems), 1 µl TaqMan MicroRNA assay 20x, and 7.67 µl nuclease free water. The reaction mixture was incubated at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 sec and 60 °C for 1 min by using the ABI 7900 HT Sequence Detection System (SDS; Applied Biosystems). The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. All experiments were performed in duplicate and miRNAs were considered as present when Ct-values were lower than 35.

SNP mapping array analysis

Genomic DNA was isolated from peripheral blood by salting out precipitation. Tumor DNA was extracted from CD138 MACS MM plasma cells using Qiagen RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Genome-wide single nucleotide polymorphism (SNP) genotyping was performed using the Illumina Infinium HumanHap550 Genotyping BeadChip according to manufacturer's instructions (Illumina, San Diego, CA, USA) containing over 550.000 unique tag SNP markers.

Genotypes for all arrays were calculated using BeadStudio's genotyping module (v2.0, Illumina). Data was imported into Partek Genomics Suite 6.4 software (Partek Inc., St Louis, MO) for further analysis; allele intensities were calculated for 25 genomic DNA MM samples and 26 tumor DNA MM samples, of which 20 had paired normal samples. For these 20 samples, paired analysis was performed. For the remaining 6 samples, a group of 25 genomic blood DNA MM samples was set as a baseline for calculating genotypes and DNA copy number (CN). The CN at each SNP was estimated from the allele intensity data by normalizing each sample to a reference (paired or baseline). In addition, allele specific copy number variation (AsCN) was calculated by estimating the number of copies for each allele, rather than total copies of each chromosome. Significant regions of aberration for each sample were found by the segmentation algorithm according to the following criteria: 1) neighboring regions have statistically significantly different mean intensities ($P < 0.0001$), 2) minimum signal to noise ratio for each transition of 0.3, 3) breakpoints (region boundaries) were chosen to give optimal statistical significance ($P < 0.0001$), 4) detected regions must contain a minimum of 10 markers. Finally, loss of heterozygosity (LOH) was estimated from the imported sample genotypes (Beadstudio) by a Hidden Markov Model, using the same baseline as in the segmentation algorithm (25 genomic blood DNA MM samples). CN, AsCN, LOH and allelic ratios were integrated and visualized in a genomic browser within Partek Genomics Suite.

Cytogenetic analysis and FISH

Chromosome analysis was performed on fresh bone marrow samples taken at diagnosis. These samples were cultured in RPMI medium with 10% serum and in Iscove's medium containing interleukin 4 and interleukin 6, and harvested after 96 hours according to standard cytogenetic techniques. Metaphase cells were analyzed using both QFQ- and RBA-banding. The resulting karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[19]

Fluorescence in situ hybridization (FISH) was performed using standard protocols.[20] LSI D13S319 (13q14.3) SpectrumOrange Probe was used to detect 13q14 deletions in combination with CEP 9 SpectrumGreen Probe to detect additional copies of chromosome 9 indicative for the presence of a hyperdiploid clone (Vysis, Abbott Molecular, Abbott Park, Illinois, USA). In addition, LSI 13 (*RB1*) 13q14 SpectrumOrange Probe was used to determine the deletion status of the *RB* gene.

At least 200 interphase nuclei per sample were analyzed using an epi-fluorescence microscope (Zeiss, Axio-Imager Z1, Sliedrecht, The Netherlands) and Isis Software (Metasystems, Altlussheim, Germany), with a subsequent analysis of selected myeloma cells based on morphology.

Data analysis

SDS 2.3 software (Applied Biosystems) was used to analyze real-time RT-PCR data.

Accurate normalization of this data is essential for quantification of miRNA levels. In this study, the stability of five candidate reference small nuclear RNAs was examined using the validation program GeNorm.[21] The two most stable reference genes, *RNU24* (Applied Biosystems, P/N: 4373379) and *RNU48* (Applied Biosystems, P/N: 4373383), were selected and used for internal normalization. The relative expression levels of *miRNA-15a* and *miRNA-16* compared to CD138 sorted plasma cells from normal bone marrow were determined using the $2^{-\Delta\Delta Ct}$ method.[22]

The Mann-Whitney U test was applied to determine if the presence of a chromosome 13 aberration resulted in a significant difference of the *miRNA-15a* or *miRNA-16* gene expression levels.

These statistical analyses were performed with SPSS Statistical software version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). All analyses were two-tailed and differences were considered statistically significant when *P*-values were less than 0.05.

Results

Chromosome 13 deletions

We determined the chromosome 13 status of 26 MM patients by two different methods. Firstly, FISH analysis was performed showing chromosome 13 deletions in 11/26 patients (Table 1 and S1). However, with FISH analysis only the status of both probes RB1 and D13S319 located at

Table 1. Chromosome 13 aberrations of the region flanking *miRNA-15a* and *miRNA-16-1* identified by FISH and segmentation analysis using SNP mapping arrays.

Patient	FISH	Segmentation	Analysis Type	Start	End	Cytoband	Length (bps)	Copy Number	# Markers	Genes
MM 1	Δ13	Del	Paired	42503650	57487878	13q14.11 - 13q21.1	14984228	0.6930	2789	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 2	Del	Del	Paired	27313597	57610331	13q12.2 - 13q21.2	30296734	1.0028	6342	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 3	Del	Del	Paired	47862787	114121253	13q14.2 - 13q34	66258466	0.9633	13801	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 4	Del	Del	Paired	45560358	52963785	13q14.12 - 13q21.1	7403427	0.8485	1488	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 5	Del	Del	Paired	48984705	53079298	13q14.3 - 13q21.1	4094593	0.7389	828	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 6	Del	Del	Paired	43781771	70193498	13q14.11 - 13q21.33	26411727	0.6852	4764	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 7	Del	Del	Paired	40498617	70309383	13q14.11 - 13q21.33	29810766	0.5797	5616	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 8	Del	Del	Paired	47786017	104600044	13q14.2 - 13q33.2	56814027	1.2421	11446	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 9	Del	Del	Paired	39203461	66142848	13q13.3 - 13q21.32	26939387	0.9434	5040	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 10	Del	Del	Paired	40531599	53088969	13q14.11 - 13q21.1	12557370	0.9634	2661	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 11	Del	Del	Unpaired	45324420	52963785	13q14.12 - 13q21.1	7639365	0.9586	1529	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 12	Del	Del	Unpaired	22372135	78494745	13q12.12 - 13q31.1	56122610	1.0474	11979	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 13	N	Del	Paired	49431816	49631113	13q14.3	199297	1.0060	19	*KCNRG, TRIM13*
MM 14	N	Del	Paired	49024071	52210918	13q14.3 - 13q21.1	3186847	1.2947	572	*ARL11, C13orf1, EBPL, KCNRG, KPNA3, RCBTB1, TRIM13*
MM 15	N	Del	Paired	26462920	104775995	13q12.13 - 13q33.2	78313075	0.8103	16439	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 16	N	Del	Paired	34866084	97736842	13q13.3 - 13q32.2	62870758	1.4238	12446	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 17	N	Del	Paired	49358246	49631113	13q14.3	272867	0.5809	23	*C13orf1, KCNRG, TRIM13*
MM 18	N	Del	Unpaired	37338941	52972100	13q13.3 - 13q21.1	15633159	1.0293	3373	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 19	N	Del	Unpaired	48984705	52958855	13q14.3 - 13q21.1	3974150	1.3794	796	*ARL11, C13orf1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, TRIM13*
MM 20	N	Del	Unpaired	48951859	68490076	13q14.3 - 13q21.33	19538217	1.5462	3374	*ARL11, C13orf1, DLEU1, EBPL, KCNRG, KPNA3, PHF11, RCBTB1, SETDB2, TRIM13*
MM 21	N	N	Paired							
MM 22	N	N	Unpaired							
MM 23	N	N	Paired							
MM 24	N	N	Paired							
MM 25	N	N	Paired							
MM 26	N	N	Paired							

N indicates normal; Del, deletion; Δ13, deletion.

chromosome 13q14 are determined, while the exact locations of *miRNA-15a* and *miRNA-16-1* cannot be visualized. The presence of both probes does not exclude that *miRNA-15a* and *miRNA-16-1* are absent by partial deletion. Using karyotyping, 1 additional patient (patient MM 1) had a chromosome 13 deletion, resulting in 12/26 patients (46.2%) having a chromosome 13 aberration detected. Secondly, we have performed copy number variation (CNV) analysis using SNP mapping arrays (Illumina Infinium HumanHap550 Genotyping BeadChip) to determine the exact deleted regions at chromosome 13. Paired analysis of purified MM cells with genomic DNA could be performed on 20/26 samples to determine CNV, while unpaired analysis was performed on 6/26 samples using the total group of genomic DNA samples as a baseline reference. The median genotype call rate in the 26 tumor samples was 93.7% (range: 83.6% - 99.3%) and in the 25 control samples 99.6% (range: 94.7% - 99.9%).

Applying a segmentation algorithm on copy numbers, a minimally deleted region on chromosome 13 (49431816 bp – 49631113 bp), in which *miRNA-15a* and *miRNA-16-1* are located, was observed in 20/26 patients, 15 of which were from the paired analysis ($P < 0.00001$; Table 1, 2, S1, S2, Figure 1, and S1). Segmentation analysis revealed 8 additional patients with a chromosome 13 deletion compared to FISH analysis. Deleted regions were determined with a minimum of 10 significant markers, however with 1 marker, negative samples remained negative. AsCN analysis further revealed the absence of homozygous deletions in these patients. Uniparental disomy (UPD; copy number-neutral LOH), was detected in one patient (MM 17) in small regions across chromosome 13, however at the exact location of *miRNA-15a* and *miRNA-16-1* no UPD was detected in any patients (Figure 1 and Figure S1).

We next examined the expression of transcripts other than *miRNA-15a* and *miRNA-16-1*, which are located within this deleted region. Known genes (NCBI) such as; *ARL11*, *C13orf1*, *DLEU1*, *EBPL*, *KCNRG*, *KNAP3*, *PHF11*, *RCBTB1*, *SETDB2* and *TRIM13*, were located in this region, however, none of these genes are thought to be involved in the pathogenesis of MM (Table 1 and S1). In accordance with previous research, we did not find any changes in the expression of these genes for patients with a chromosome 13 deletion.

A highly correlated cluster of miRNAs; *miRNA-15b* and *miRNA-16-2*, is located on chromosome 3. *MiRNA-15a* and *miRNA-15b* are derived from the same seed sequence, however they differ in their mature sequence. *MiRNA-16-1* and *miRNA-16-2* on the other hand do not differ in their mature sequence, and therefore cannot be distinguished. For this reason, we are not able to separate *miRNA-16-1* and *miRNA-16-2* expression using the Real-Time PCR assay, since it is the mature miRNA sequence primes the assay. As these highly correlated miRNAs are located on chromosome 3, we have also examined this region. Analysis of this chromosome was performed as described for chromosome 13, using the segmentation algorithm to determine the amplified and deleted regions (*miRNA-15b* and *miRNA-16-2* location 161.605.070 bp – 161.605.307 bp). Deletion of this region on chromosome 3 was found in 2 MM patients samples. In contrast, amplification was found in 6 MM patient samples (Table 2 and S2).

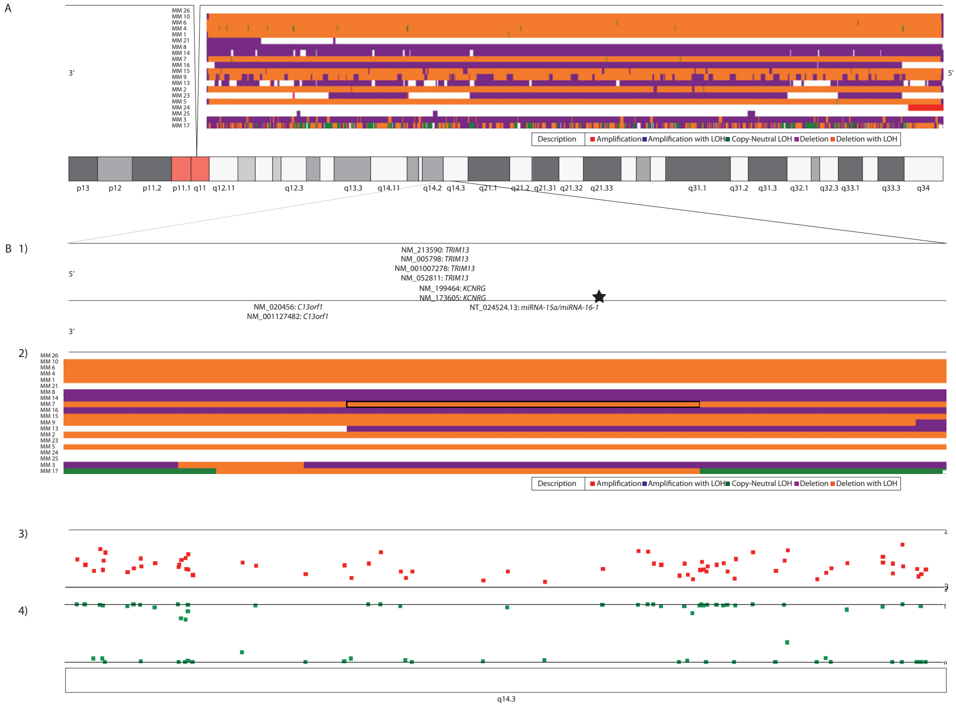

Figure 1. Copy number alterations of chromosome 13 as determined by paired segmentation analysis. SNP mapping arrays (Illumina Infinium HumanHap550 Genotyping BeadChip) were used to determine the exact deleted regions at chromosome 13 MM cases. (A) The results of the paired segmentation analysis of chromosome 13 (20/26 MM patients). (B) A region on chromosome 13; 13q14.3, including the *miRNA-15a* and *miRNA-16-1* locus, is depicted at larger magnification. In panel 1; the *miRNA-15a* and *miRNA-16-1* locus (star) is shown within the genome browser. Panel 2 shows the results of the paired segmentation analysis in more detail. A deletion (orange and purple) was detected in 15/20 MM patients. Uniparental disomy (UPD; green) was detected in patient MM 17, in the regions flanking *miRNA-15a* and *miRNA-16-1*. CN values for SNPs (red dots) are plotted in panel 3. CN values of 2 represent diploid copy number, whereas values of 1 and 0 represent heterozygous and homozygous deletion respectively. A representative patient (MM 7) shows values of 1 or ≤ 1, indicating deletion. In panel 4; the B allele frequency is displayed. Each point (green dot) represents the B allele frequency of one SNP. B allele frequencies of 0 and 1 represent homozygous signals (AA or BB), while an allele frequency of ~0.5 represents an equal contribution from both alleles (AB). A representative MM patient sample (MM 7) shows a deletion with LOH (black box in segmentation panel) which produces a characteristic pattern that lacks heterozygote signal.
For visualization purposes the order of the samples is MM 26, MM 10, MM 4, MM 1, MM 21, MM 8, MM 14, MM 7, MM 16, MM 15, MM 9, MM 13, MM 2, MM 23, MM 5, MM 24, MM 25, MM 3, MM 17.

An overview of the results of FISH and CNV analysis for chromosome 13 and 3 is shown in Table 1 and 2, respectively. The combined analysis of FISH and CNV revealed 20 (76.9%) of 26 MM patients with a chromosome 13 aberration at 13q14.3.

Table 2. Chromosome 3 aberrations of the region flanking *miRNA-15b* and *miRNA-16-2* identified by segmentation analysis using SNP mapping arrays.

Patient	FISH	Segmentation Chr 3	Segmentation Chr 13	Analysis Type	Start	End	Cytoband	Length (bps)	Copy Number	# Markers	Genes
MM 1	ND	N	Del	Paired							
MM 2	ND	Amp	Del	Paired	149248389	175337487	3q24 - 3q26.31	26089098	2.55458	4583	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 3	ND	Amp	Del	Paired	125085329	187093089	3q21.1 - 3q27.2	62007760	3.10386	11110	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 4	ND	N	Del	Paired							
MM 5	ND	Del	Del	Paired	153671566	170804202	3q25.2 - 3q26.2	17132636	1.50403	2794	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 6	ND	Amp	Del	Paired	123068741	173268549	3q13.33 - 3q26.31	50199808	3.13116	8971	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 7	ND	N	Del	Paired							
MM 8	ND	N	Del	Paired							
MM 9	ND	N	Del	Paired							
MM 10	ND	N	Del	Paired							
MM 11	ND	N	Del	Unpaired							
MM 12	ND	N	Del	Unpaired							
MM 13	ND	N	Del	Paired							
MM 14	ND	N	Del	Paired							
MM 15	ND	Amp	Del	Paired	143029158	172793368	3q23 - 3q26.31	29764210	3.19039	5168	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 16	ND	Amp	Del	Paired	127261906	173217727	3q21.2 - 3q26.31	45955821	2.76892	8108	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 17	ND	Del	Del	Paired	161470498	161817497	3q26.1	346999	0.71038	24	*IFT80, KPNA4, SMC4, TRIM59*
MM 18	ND	N	Del	Unpaired							
MM 19	ND	N	Del	Unpaired							
MM 20	ND	N	Del	Unpaired							
MM 21	ND	Amp	N	Paired	153560983	165316823	3q25.2 - 3q26.1	11755840	2.40647	1970	*ARL14, B3GALNT1, IFT80, IL12A, KPNA4, NMD3, PPM1L, SCHIP1, SMC4, TRIM59*
MM 22	ND	N	N	Unpaired							
MM 23	ND	N	N	Paired							
MM 24	ND	N	N	Paired							
MM 25	ND	N	N	Paired							
MM 26	ND	N	N	Paired							

N indicates normal; Del, deletion; Amp, amplification; ND, not determined.

MiRNA-15a and *miRNA-16* gene expression in MM patients

A TaqMan miRNA assay was used to determine the relative *miRNA-15a* and *miRNA-16* expression levels in myeloma cells of 26 MM patients. Both *miRNA-15a* and *miRNA-16* were expressed in all 26 MM samples, although the level of expression varied across the samples. The median expression value of *miRNA-15a* and *miRNA-16* was 0.84 and 1.16, respectively (both values in log2 scale, relative to the geometric mean). Figure 2 shows that no significant association was found between the chromosome 13 deletion status and the *miRNA-15a* expression levels using FISH ($P = 0.38$) and CNV ($P = 0.25$). In addition, statistical analysis showed no significant association between *miRNA-16* expression and chromosome 13 status determined by FISH ($P = 0.40$) and CNV ($P = 0.27$). Since we are not able to separate *miRNA-16-1* and *miRNA-16-2* expression, results are only shown for *miRNA-15a*. We also evaluated the expression levels of *miRNA-15a* and *miRNA-16* in CD138 sorted plasma cells from normal individuals. *MiRNA-16* was found to be expressed at 1.4 fold lower levels in normal plasma cells when compared with myeloma plasma cells, suggesting an increased expression of this miRNA in MM.

These findings demonstrate that *miRNA-15a* and *miRNA-16* are displayed at a range of expression levels in MM patients which are higher than in normal plasma cell counterparts. The expression of these miRNAs varies independent of the chromosome 13 status.

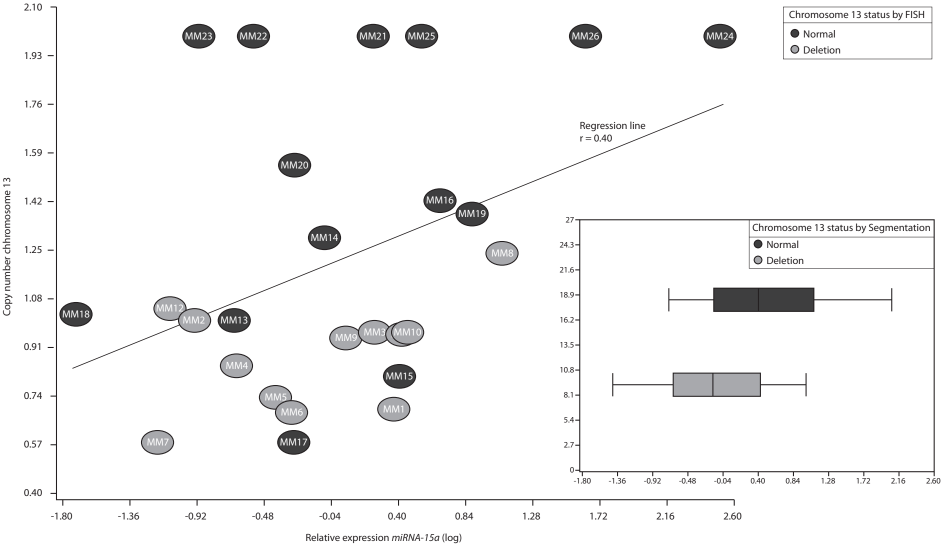

Figure 2. Copy number of chromosome 13 in correlation with *miRNA-15a* gene expression in multiple myeloma (MM) patient samples. A TaqMan miRNA assay was used to determine the relative *miRNA-15a* expression levels in myeloma plasma cells of 26 MM patients. The normalized relative expression of *miRNA-15a* in 26 MM patient samples is displayed on the X-axis. *MiRNA-15a* is expressed in all 26 MM samples, although the level of expression varies across the samples. SNP mapping arrays (Illumina Infinium HumanHap550 Genotyping BeadChip) were used to determine copy number values of chromosome 13, which are shown on the Y-axis. Copy number values of 2 were detected in 6/26 MM patients, indicating diploid copy number; values less than 2 represent heterozygous and homozygous deletions, which were found in 20/26 MM patients. Patients are colored by chromosome 13 status as determined by FISH; being either normal (dark grey) or deletion (light grey). Segmentation analysis has shown 8 additional patients with a chromosome 13 aberration in the region containing *miRNA-15a* and *miRNA-16-1*. A linear regression line reveals there is no correlation between the *miRNA-15a* expression and the chromosome 13 status as determined by segmentation analysis.

The picture insert shows a box plot representation of the normalized relative expression of *miRNA-15a* in 26 MM patients samples (X-axis), in correlation with the chromosome 13 status determined by segmentation (Y-axis). The box plot shows no significant difference in *miRNA-15a* gene expression ($P = 0.38$ and $P = 0.25$ respectively, Mann-Whitney U test) between patients with a deletion (light grey, n= 6) and normal chromosome 13 status (dark grey, n=20).

Discussion

In recent years, the prognostic and biological significance of chromosome 13 deletions has been extensively studied in MM. Deletion of chromosome 13 is detected in approximately 50% of patients by FISH and in 10–20% of patients using conventional karyotyping.[11,23-25] It has been suggested that patients with a whole chromosome 13 deletion detected by cytogenetics, have a worse prognosis in contrast to chromosome 13q deletions detected by FISH.[26-29] Nevertheless, the presence of 13q deletions and their impact on prognosis suggest that one or more tumor suppressor genes are located on chromosome 13q and may be involved in the pathogenesis of MM. The identification of a tumor suppressor gene remains a difficult task, and currently, no other genes flanking the *miRNA-15a* and *miRNA-16-1* region appear to have a role in pathogenesis of MM. Calin *et al.,*[16] reported that in CLL patients; *miRNA-15a* and *miRNA-16-1*; located on chromosome 13q14, are often downregulated or deleted and therefore could play a role in the pathogenesis of CLL. Loss of chromosome 13 occurs, like in MM, in approximately 50% of CLL patients.[30] However, there are essential differences between MM and CLL concerning chromosome 13q deletions. First of all, chromosome 13q deletions have an adverse prognostic role in MM, whereas CLL patients with 13q deletions as a sole abnormality have the longest estimated survival times.[30] Thus, the role of *miRNA-15a* and *miRNA-16-1* in the mechanism behind the prognostic significance of chromosome 13q deletions in CLL and MM may differ. Furthermore, homozygous deletions seem to be rare in MM but are observed in approximately 10% of CLL patients. This was demonstrated in the present study, whereby all patients presenting with a deletion were heterozygous. Finally, deletions of chromosome 13 in CLL patients are of varying length, but almost always involve the 13q14.3 region, whereas in MM, the deletions often involve loss of the entire chromosome.[31]

Downregulation of *miRNA-15a* and *miRNA-16-1* has been reported in cases of MM, CLL and diffuse B Cell Lymphomas.[16-17,32] In this study, the two miRNAs were expressed in all 26 MM patients examined, even when chromosome 13 was deleted. Since these two miRNAs may play a role in the pathogenesis of MM, other regulatory mechanisms must exist.[15,17] One could argue that the two miRNA genes are still present due to a partial deletion. Applying the segmentation algorithm, we could accurately determine the region of deletion according to the presence of a nearby SNP and to detect regions of allelic imbalance due to copy number loss or gain, or copy neutral LOH.[33-34] The segmentation method can be applied to both paired and unpaired tumor samples, showing an increased sensitivity and high specificity for detecting allelic imbalances in heterogeneous samples.[33-34] The CNV analysis demonstrated that the region where *miRNA-15a* and *miRNA-16-1* are located was deleted in 20 MM patient samples. It is therefore highly unlikely that chromosome 13 deletions that are not detected by CNV analysis are partial deletions. Compensation by the non-deleted allele could explain the expression level of *miRNA-16* since all chromosome 13 deletions in this study are heterozygous.

In addition, a highly correlated cluster of miRNAs, *miRNA-15b* and *miRNA-16-2*, are located on chromosome 3. The observed *miRNA-15a* and *miRNA-16* expression levels could be due to persistent expression of these miRNAs. With the Real-Time PCR assay used in this study, we are not able to make a distinction between *miRNA-16-1* and *miRNA-16-2* expression, since the mature miRNA sequence is identical for both.

Currently, FISH analysis is the standard method to determine chromosome 13 deletion status. Large genomic aberrations can be detected by FISH; nevertheless, the resolution of this method is limited. As this study shows, FISH might provide false-negative outcomes. SNP mapping arrays increase the resolution enormously and provide a more robust and sensitive determination of chromosomal aberrations than FISH as we have shown. Indeed, 8 additional patients were found to have a chromosome 13 deletion at the *miRNA-15a* and *miRNA-16-1* location not detected by FISH. CNV analysis using SNP mapping arrays seems to be a highly specific method to determine chromosomal regions of deletion or amplification status. In addition, the high resolution of SNP mapping arrays may facilitate the identification of tumor suppressor genes and oncogenes involved in the pathology of MM. Although this study clearly shows the discrepancy between FISH and CNV analysis and the efficacy of SNP mapping arrays in detecting chromosomal aberration, there are some issues regarding the accuracy of the method that have to be taken into account. First, substantial CNV has been reported in germline DNA. For that reason it is most desirable to use matched tumor and control samples for paired analysis. It is important, especially for smaller aberrations, that a direct comparison between tumor DNA and matched germline DNA is performed.[35] Because paired CN analysis was carried out for 20/26 MM samples, this makes the data highly reliable. Furthermore, CNV calculations are based on the allele intensity data. The quality of the allele intensity, which is indirectly measured by SNP call rate, should be sufficient and SNP arrays with low call rates should therefore be removed from the analysis.

BCL2, an anti-apoptotic gene, is often expressed in many types of cancer, including leukemia's and lymphomas which imply a role for *BCL2* in the pathogenesis of these malignancies.[36] A recent study suggests that both *miRNA-15a* and *miRNA-16*-a negatively regulate *BCL2*-mRNA levels in CLL.[37] Downregulation or deletion of *miRNA-15a* and *miRNA-16-1* may therefore result in increased expression of *BCL2* and inhibition of apoptosis. In MM we did not observe a correlation between the *miRNA-15a* and *miRNA-16* expression levels and the BCL2 protein and gene expression (data not shown).

In conclusion, the current study has shown that CD138 MACS plasma cells of MM patients express both *miRNA-15a* and *miRNA-16* heterogeneously. The levels of *miRNA-15a* and *miRNA-16* expression vary independent from the presence of chromosome 13q deletions. In addition, these high resolution, genome-wide SNP mapping arrays may provide an excellent tool to identify partial chromosomal aberrations and genes.

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Chapter 9

General discussion

Single nucleotide polymorphism association studies in multiple myeloma

Although much progress has been made in the treatment of multiple myeloma (MM) during the last decade, many patients die from treatment failure. In addition, a large number of MM patients experience serious adverse events from the drugs they receive. There is a critical need to discover new drugs, and in addition, it is important to optimize existing drug regimens to minimize adverse events and to optimize drug efficacy.

There are several reasons why a patient may experience excessive toxic side effects including dosing errors, drug–drug interactions, drug allergies and medication errors. However, in the majority of patients with severe adverse drug reactions no explanation can be found. Since the completion of the Human Genome Project[1] and the International HapMap Project,[2] there has been increasing interest in the patient's individual genetic disposition as a risk factor for the development of adverse drug events, and as a potential variable for drug efficacy. After the discovery of the first single nucleotide polymorphism (SNP), it became clear that SNPs could have an impact on individual drug response. The human genome is estimated to have about 12 million common SNPs. Their potential to contribute to inter-individual differences in drug responsiveness, or the frequency of toxic side effects is well recognized. An important aim of SNP association studies is to identify SNPs that contribute to the activity, toxicity or kinetics of a particular drug, and the variability across a patient population; the field of pharmacogenetics.[3] In pharmacogenetics, the aim is to assess a patient's individual genetic variability in relation to drug effects. A pending question of clinical importance in cancer is whether pharmacogenetics can be used to predict adverse events and the clinical outcome following treatment, which would lead to the development of genetic tests for the identification of the most suitable drug for a specific patient. So far, the translation of pharmacogenetic research into clinical practice has not yet been made. Ultimately, the utility and clinical application of pharmacogenetics to improve safety, drug efficacy and quality of life of the patient must be supported by prospective analyses in comprehensive clinical trials.

The main focus of part I was to study the pharmacogenetics in patients with multiple myeloma; a fatal bone marrow cancer. We studied the inherited genetic variations in newly diagnosed MM patients, with the aim of identifying genetic variability that may predict treatment effects. Patients who were included in 3 large prospective clinical trials were genetically characterized, and their pharmacogenetics was compared with the clinical data. We took a hypothesis-driven candidate gene approach because the number of events to be analyzed would be small, and we were aiming to identify pertinent functional loci variants with moderate to large effect size. High quality genotype data with stringent quality controls of a homogenous population of similarly treated patients were studied, in order to minimize experimental artifact that can be found in many association studies.[3]

A pending question of clinical importance is whether genetic factors influence the susceptibility to development of MM. Until now, studies using genetic epidemiological methods have lacked power, due to the limited number of SNPs and samples. The study presented in chapter 2 is the largest genetic epidemiology study that addresses this question in MM to date, and its design lays somewhere between a classical candidate gene study and a whole genome scan. We identified a number of SNPs showing causative effect with risk of developing MM. Classic epidemiological association studies have identified chronic immune stimulation; mediated via a number of environmental factors, as being a relevant association with the risk of developing MM.[4] Herein, we described associations with genetic variation within genes mediating the biology of B lymphocytes that could mediate such immune effects, and within which minimal biological variation could affect the risk of transformation to myeloma. We saw association with 2 SNPs in *FCLR5* and *SELP*, derived from chromosome 1; a region which is frequently associated with MM progression and poor clinical outcome.[5-7] In addition, we found associations in genes involved in micro environmental interactions and B cell signaling pathways, which are relevant to the development of MM; possibly by mediating cell survival following genetic damage. The potential role of environmental exposures in MM risk has been previously suggested, but replication has proven difficult. In this context, we identified associations with absorption, distribution, metabolism, excretion (ADME) genes including *CYP19A1* and *CYP1A2*, opening the way for further validation in studies designed to investigate gene environment interactions. Finally, we have shown an association with genetic variation in genes involved in growth factor signaling pathways as suggested previously, the most investigated being *IL-6*, *IL-1B*, *TNFα*, and *NFκB*. Although the associations seen are strong, and are informative about both the biological and environmental contributions to MM risk, this hypothesis-driven approach cannot detect associations outside the candidate panel, and will not observe associations potentially detectable by a genome-wide approach.

Another application of pharmacogenetics is the identification of inherited genetic variations that play a role in the development of adverse events following treatment. In chapter 3, we studied the association of SNPs with thalidomide induced venous thromboembolism (VTE). Patients with MM have an increased risk (5-10%) to develop VTE because of the underlying disease, concomitant thrombophilic factors, and the administration of chemotherapy.[8] In patients treated with thalidomide alone, or in combination with other agents such as anthracyclins and dexamethasone,[9-10] VTE rates increase to 10-15%.[11-13] In contrast, is has been reported that VTE rates decrease following bortezomib treatment.[14-18] Guidelines have recently been established to govern clinical indicators for intervention, but these prognostic factors can be difficult to identify and use clinically.[19] Given that genetic tests to identify patients at risk are not clinically available yet, it is of great importance that the management of adverse events is optimal. To come to a decision about which prophylaxis strategy is most suitable, causative factors need to be identified. Thus, the application of pharmacogenetics for the identification of the mechanisms underlying the development of adverse events is of great importance. The main challenge in this is to understand the biological context in which these genetic variants act, and how they determine an adverse event such as VTE. The results of chapter 3 contributed considerably to reaching this aim. Interestingly, despite a comprehensive analysis of the genetic variation within the coagulation and prothrombotic pathway, we could not find evidence for a significant association of genetic variation within these pathways with VTE risk following thalidomide exposure, suggesting other pathways to be involved in the development of VTE. Indeed, the set of 18 SNPs associated with thalidomide induced VTE could be divided into three groups; a response to DNA damage group including *CHEK1*, *XRCC5*, *LIG1*, *ERCC6*, *DCLRRE1B*, and *PARP1*; a cytokine response group containing *NFKB1*, *TNFRSF17*, *IL-12B*, and *LEP*; and a third related group of apoptosis with *CASP3*, *PPARD*, and *NFKB1*. These enrichment groups indicate that genetic variation in response to DNA damage and cytokine-mediated apoptosis modulates risk of developing a thalidomide related thrombosis. Variation in DNA repair capacity could readily affect the response of the myeloma clone to treatment, due to the direct relationship between the extent of DNA damage accumulation and the clinical response to alkylating agents.[20] A rapid response and dissolution of myeloma clones with an impaired double stranded DNA repair pathway, would release greater pro-thrombotic factors that could be either micro-particles with surface tissue factor or cytokines and tissue factor. The greater thrombogenesis due to increased dissolution of the myeloma clone, may act additively with a dexamethasone-thalidomide interaction on plasma cells,[21] giving rise to an increased number of VTEs.[22-23] An alternative mechanism to explain the increased risk of a VTE associated with DNA repair genes, could be based on the observation that thalidomide can protect endothelial cells from doxorubicin induced apoptosis by restoring *PAR-1* expression;[24] promoting sub-endothelial tissues factor exposure, endothelial dysfunction, platelet activation and consequently increase the thrombosis risk.[24-26] Under these conditions, decreased DNA repair capacity could promote clot formation at the endothelium.

As part of the analysis in chapter 3, recursive partitioning was used to identify a limited number of SNPs that, when analyzed together, can predict the risk of VTE. A set of 7 SNPs was identified that could correctly predict VTEs in 70% of the patients. Testing for these SNPs has the potential for being clinically useful for identifying high risk patients for whom therapeutic intervention is required. The intervention strategies for patients may change according to the genetically defined risk.

Among the variety of adverse events that are associated with MM treatment, peripheral neuropathy (PN) has been one of the principal non-hematological, dose-limiting adverse events of thalidomide and bortezomib treatment. In chapters 4, 5, and 6, we addressed questions related to PN in newly diagnosed MM patients who were treated with thalidomide or bortezomib. PN, induced by thalidomide (TiPN) or bortezomib (BiPN), is one of the most frequent and potentially disabling adverse events,[27-28] frequently requiring dose modification or discontinuation which negatively affects clinical endpoints and quality of life.[29] Baseline myeloma associated PN is observed in up to 54% of newly diagnosed MM patients.[30-32] Grade 1-2 BiPN can occur in up to 27-75% and 25-33% of patients with recurrent or newly diagnosed MM, respectively, while grade 3-4 BiPN may affect 0-30% of patients with recurrent disease and 0-18% with newly diagnosed myeloma.[17] The overall TiPN incidence ranges from 12-44%, or grade 1-2 of 22%, and grade 3-4 of 6%.[33]

Chapter 4 provides new insights into the mechanisms underlying TiPN. Our results were consistent with the hypothesis that an individual's risk of developing TiPN can be mediated by SNPs in genes governing repair mechanisms, and inflammation in the peripheral nervous system. In addition, a number of ATP-Binding Cassette (ABC) transporters genes were linked with TiPN including: *ABCC1*, *ABCC2*, *ABCA1*, and *ABCB1*. Interestingly, thalidomide can modulate the function of ABCC2 and ABCB1,[34] both of which are active in neuronal function. Pathway analysis of the TiPN associated SNPs, highlights the association with genes involved in the development of the central and peripheral nervous system. A SNP found within a conserved promoter region, affecting the expression of the gene *SPRR1A*, was significantly associated with TiPN. The gene *SPRR1A* is expressed by axotomized neurons and promotes axonal outgrowth.[35] Variation in neurological genes may dictate the ability of a damaged neuron to undergo repair, and may mediate risk of neuropathy.

We have shown that simple clinical factors do not allow the identification of patients at greater risk of neuropathy following thalidomide exposure, for which dose adjustment, or cessation of therapy would be appropriate. Consequently, we have attempted to define a limited number of SNPs that can identify high risk patients suitable for more intensive clinical monitoring. In this approach, we have used two different risk classification methods: the risk score method and recursive partitioning, both of which can identify patients at increased risk, although the predictive value of these methods is not adequate to totally avoid thalidomide usage. However, this approach can be used to identify patients requiring greater clinical vigilance and suitable counseling. The poor risk prediction in our classifiers is due to a number of limitations, which include: a) naive assumptions in modeling methods; the risk score method assumes interactions to be solely additive, whilst recursive partitioning method interactions are assumed to be uni-directional, b) hypothesis-driven approaches do not consider all variation contributing to an outcome, c) population specific effects, and d) the impact of rare variation. Despite these drawbacks, the limited number of SNPs identified in this study can be used as a simple and useful method for identifying patients at high risk of TiPN, who in turn may benefit from greater clinical vigilance.

In chapters 5 and 6, SNP association studies were performed to gain more insight into the mechanisms involved in the development of BiPN. In the future, it will become increasingly important to combine and integrate different microarray technologies in order to come to a detailed understanding of a disease. Conclusions that have clinical consequences will need to be based on the results of different platforms. The integration of gene expression and genomic data, could lead to a better understanding of the variation in genomic structure that has an effect on gene regulation, and how this contributes to disease. In chapter 6, we performed an integrative analysis by combining gene expression and SNP data of newly diagnosed MM patients, who did or did not develop BiPN or vincristine induced PN (ViPN).

The results discussed in chapters 5 and 6 indicate that the mechanisms underlying the development of BiPN are multifactorial, with different molecular pathways being implicated in early onset (after 1 cycle of bortezomib induction treatment) and late onset (after 2 or 3 cycles of bortezomib induction treatment) BiPN. Our findings strongly suggest an interaction between myeloma related factors, and the patient's genetic background in the development of BiPN. A pathway of major relevance involved in the development of late onset BiPN, is the inflammatory pathway. SNPs that lie within proinflammatory cytokines such as *TNFα*, *PARP1*, and *MBL2* are associated with late onset BiPN. Particularly interesting is the association with *TNFα*, since *TNFα* has been implicated in several neurodegenerative diseases, including multiple sclerosis, Alzheimer disease, human immunodeficiency virus-related encephalopathy, and diabetic neuropathy.[36-40] Bortezomib's target; NFκB, is intricately related to the TNF receptor system in the nervous system, and interacts with PARP1, which is involved in neuronal cell death.[41] While there is no effective treatment available for BiPN apart from dose modification or treatment discontinuation, the recognition of the inflammation system being involved in the pathogenesis of BiPN, may provide new therapeutic targets to be explored. Promising results have been reported for TNFα suppression using infliximab in diabetic polyneuropathy in animal models,[42-44] which supports the possible application of TNFα inhibitors or monoclonal antibodies for the treatment of BiPN. This aspect will clearly need to be studied further.

Our results also suggest a possible direct involvement of neuropathy susceptibility genes and genes that regulate neuronal proliferation, and damage repair in late onset BiPN. We noted upregulation of the superoxide dismutase gene *SOD2* in myeloma plasma cells; *SOD2* is regulated by TNFα and NFκB, and is known to have a role in the survival of neurons. Patients with diabetes and a polymorphism in the *SOD2* gene, leading to reduced SOD2 activity, have been shown to be at increased risk of developing diabetic peripheral neuropathy.[45] The protective effect of SOD2 might be eliminated with bortezomib induced apoptosis, which may trigger a susceptibility to oxidative stress in treated patients. While the involvement of these genes may be expected, we are the first to describe the involvement of the inflammation system in the pathogenesis of BiPN, which may provide new therapeutic targets to be explored.

Finally, the genetic profiles of patients with early onset BiPN suggest the involvement of genes involved in AMPK-mediated signaling. The possible role of AMPK-mediated signaling is of particular interest, because this enzyme functions by stimulating the signaling pathways that replenish cellular ATP supplies in response to low glucose, hypoxia, ischemia, or heat shock, which might be triggered in myeloma cells in response to bortezomib. Other genes associated with early onset BiPN are involved in apoptosis (*RHOBTB2* and *RASGRP1*) and transcription.

A comparison of the molecular DNA profiles of TiPN and ViPN in chapter 4, and BiPN and ViPN in chapter 6, showed no overlap in associated genes or SNPs. Genes involved in cell cycle and proliferation were mainly associated with early onset ViPN, both in the analyses of genetic pathways and SNPs. An interesting observation is the involvement of inflammatory genes in all three types. Involvement of proinflammatory genes in early onset ViPN, was substantiated by the finding of SNPs in *PARP1*, *LTA*, and *GLI1*. Despite this overlap, we propose that the involvement of distinct molecular pathways is indicative of a fundamentally different pathological mechanism between these three types of neuropathy. In contrast, similar TiPN associated genes have been seen in studies investigating peripheral neuropathy in response to paclitaxel, and docetaxel, which result in sensory peripheral neuropathies, as opposed to the sensorimotor neuropathy associated with exposure to the vinca alkaloids and taxol.

Substantial challenges still remain before these discoveries find widespread application in clinical practice. First, it is necessary to replicate the results in order to minimize the number of false positive and false negative classifications.[46-48] The strongest evidence that a true genetic variant is associated with adverse events, and thus may be causal, is to replicate the result in a separate independent cohort.[49] Additionally, one of the shortcomings of genetic association studies is that the identified association between a single genetic variant and a disease outcome may have been caused by linkage disequilibrium with another genetic variant. In using a candidate gene approach, it is therefore important to consider gene haplotypes. It is presumed that the identification of a few alleles within a haplotype block, can unambiguously identify all other polymorphic sites in its region. Furthermore, large sample sizes are needed to provide enough statistical power in order to detect the effect of a genetic variant on disease etiology, clinical outcome, or treatment side effects.

The candidate gene studies discussed in part I of this thesis, focused on the identification of SNPs that are most important for treatment related toxicity. Our results show that a candidate gene approach is successful for investigating drug toxicity. Our candidate gene studies have provided initial insights into genetic factors affecting susceptibility to the adverse events VTE and PN. Our results indicate that the possibility to develop simple genetic tests (based on associated SNPs) to determine the most suitable drug for an individual MM patient may become possible in the near future. The knowledge presented within this thesis, may therefore contribute to the development of more personalized approaches of MM management.

Undoubtedly, future research using either a candidate gene or a genome-wide approach, will contribute to further insights in the role of SNPs in MM. Integration of the different genomic techniques, including gene expression, miRNA expression and genome-wide SNP genotyping arrays, will lead to a more detailed understanding of MM, and the adverse events following treatment.

MicroRNA expression in multiple myeloma

MicroRNAs (miRNAs) have been shown to have an important role in various cellular processes, such as apoptosis, differentiation and development. There is considerable potential to target miRNAs as a novel approach in the treatment of MM. It is therefore of great importance to elucidate the miRNA expression pattern in MM and determine the role of miRNAs in MM pathogenesis. Although we are at an early stage of understanding the roles of miRNAs in MM, the importance of these molecules is clear. It has been previously shown that gene expression profiling can be used for the classification of MM.[50-52] In addition to this, it has become clear that the possibility exists to differentiate between normal plasma cells and myeloma plasma cells based on unique miRNA expression signatures.[53-55] In chapter 8, we performed a miRNA expression profiling study which confirms this finding. This study demonstrated that the expression of various miRNAs in MM is deregulated compared to normal plasma cells. Unsupervised analysis showed that MM patients can be classified according to their miRNA expression pattern and that these miRNA profiles were not characterized by cytogenetic subgroups, as previously demonstrated with gene expression. Our results suggest that miRNA profiling could make an important contribution to the classification of MM.

To understand the role of miRNAs in the pathogenesis of MM, it is necessary to determine the targets of significant miRNAs. It is well known that the identification of miRNA targets is difficult, due to the capability of miRNAs to bind their targets with imperfect complimentarity.[56] To date, only a small number of specific targets have been experimentally validated. It is therefore interesting to see that an inverse correlation between *miRNA-21* expression levels and two of its validated target genes; *PDCD4* and *RECK*, could be identified. Analysis of the mRNA and miRNA levels as described in chapter 8 may identify miRNA-target interactions that result in mRNA degradation, and could lead to the identification of disease related miRNA targets.[57-58]

In other malignancies, a correlation has been shown between certain miRNAs and clinical outcome, which indicates the potential of miRNAs to be used in determining a specific course of treatment.[59-60] Indeed, in chapter 8 we identified a borderline significant association between the expression of three miRNAs and overall survival. Further studies in the near future, must confirm and validate our findings and thereby the conclusions regarding the ability of these miRNAs to predict clinical outcome in MM.

A large number of miRNAs are located at fragile sites across the human genome. These sites are often deleted or amplified at a common breakpoint, and they are frequently associated with cancer.[61] Therefore, miRNAs have been suggested to function as tumor suppressor and oncogenes, and play a role in the pathogenesis of MM.[55] In chronic lymphocytic leukemia (CLL), *miRNA-15a* and *miRNA-16-1*; located on chromosome 13q14, are frequently downregulated or deleted, and therefore a tumor suppressor activity and pathogenic role has been suggested.[62-63] A similar function has been hypothesized for *miRNA-15a* and *miRNA-16-1* in MM. The results of chapter 9 suggest otherwise, and provided new insights into the prognostic implications of chromosome 13q deletions. We investigated the expression of *miRNA-15a* and *miRNA-16* in MM, and studied the potential association between miRNA expression levels and chromosome 13q deletions in myeloma plasma cells. We demonstrated that the levels of *miRNA-15a* and *miRNA-16* expression vary, independent from the presence of chromosome 13q deletions. Since chromosome 13q deletions have an impact on prognosis,[64-67] it will be important to assess whether our findings can be reproduced in independent data series.

The results presented in part II of this thesis may contribute to a better understanding of the complex role of miRNAs in the pathogenesis of MM. It has become clear that the identification of altered miRNA expression, as well as their targets, may provide new opportunities for therapeutic strategies.

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Chapter 10

Summary/Samenvatting



Summary

Although considerable progress has been made during the past decade in the treatment of multiple myeloma (MM), many patients die from treatment failure. In addition, a large number of MM patients experience serious adverse side effects to the drugs they receive. Although the introduction of drugs such as thalidomide and bortezomib have enhanced the ability to move toward personalized treatment, there still remains a critical need for novel treatment approaches. In addition, it is important to optimize existing drug efficacy and drug regimens in order to minimize adverse events.

Chapter 1 presents a general introduction to multiple myeloma with emphasis on single nucleotide polymorphisms (SNPs), and microRNAs (miRNAs). In addition, an outline of the subsequent chapters is provided.
Following the first chapter, the thesis is separated into two parts: Part I and Part II.

Part I on SNP association studies in MM comprises chapters 2, 3, 4, 5, and 6.
Pharmacogenetics; the field that studies the role of a patient's individual genetic variability on the activity, toxicity or kinetics of a particular drug, will allow the adaption of a treatment to the genetic profile of a patient. Variation in the genetic profile of a patient is often due to SNPs, which can affect a patient's response to drugs, and the development of toxic adverse events. Therefore, gaining better insight into the SNP profile of patients will eventually allow personalized treatment and prediction of adverse events.

In **chapter 2**, a hypothesis-driven approach is taken to examine the role of inherited genetic variation within MM, using a custom genotyping array to study 2595 presenting MM cases of European origin; derived from the UK, US, and The Netherlands. The custom SNP Bank On A Cure (BOAC) array consists of 3404 SNPs in 964 genes, focusing on SNP variation in molecular pathways involved in the pathogenesis and treatment response of MM. In order to understand the distribution of these variants within the normal population, we access 8974 population control genotypes from publicly available datasets. A comparison of the genotypic distribution between the case and control populations allows us to identify genetic variation that associates with MM predisposition. These genetic variations include nonsynonymous SNPs in immunity genes *FCRL5* and *SELP*; both derived from chromosome 1, a region frequently associated with MM progression and poor clinical outcome. A nonsynonymous SNP in *CAMKK2*, mediating signaling via cytokine signaling pathways and critical for myeloma cell function is also associated with MM. The potential role of environmental exposures in MM risk is supported by the association with a SNP in *CYP2C19*; an absorption, distribution, metabolism, excretion (ADME) gene.

Chapter 3 describes the influence of SNPs on the susceptibility to developing a venous thromboembolism (VTE). VTE, with the subsequent risk of pulmonary embolism, is a major concern in

the treatment of MM patients with thalidomide. Our analysis shows that a set of SNPs associating with thalidomide induced VTE is enriched in genes and pathways important in drug transport and metabolism, DNA repair, and cytokine balance. In addition, recursive partitioning analysis defines a set of SNPs that could be used to identify patients at high risk for VTE.

In **chapter 4**, the association between SNPs and thalidomide induced peripheral neuropathy (TiPN) is assessed using the BOAC SNP array. Peripheral neuropathy (PN) is a serious adverse event in MM patients. Rates of TiPN vary from 15-70%, with the risk of neuropathy being related to the cumulative dose and duration of therapy. The repose to TiPN is dose reduction or withdrawal of thalidomide, which can lead to symptom resolution in up to 16 weeks, however in some cases TiPN is irreversible.

TiPN associations are identified with SNPs in *ABCA1*, *ICAM1*, *PPARD*, *SERPINB2*, and *SLC12A6*, and these results are cross validated in two independent prospective clinical trials. These findings are consistent with the hypothesis that an individual's risk of developing TiPN can be mediated by SNPs in genes governing repair mechanisms and inflammation in the peripheral nervous system. It is also investigated whether TiPN SNP associations are related to exposure to thalidomide only or general drug related PN, and a second analysis is performed on patients treated with vincristine. This identifies SNPs associated with vincristine induced peripheral neuropathy (ViPN), with a seemingly distinct underlying genetic mechanism.

Peripheral neuropathy is also the dose-limiting toxicity of bortezomib treatment, which frequently requires dose reduction or treatment discontinuation. The mechanisms underlying bortezomib induced peripheral neuropathy (BiPN) are largely unknown. Therefore, in **chapter 5**, a pharmacogenetic association study is performed in two prospective clinical trials using the BOAC SNP array. Stratified association analysis reveals a significant association between development of BiPN and proinflammatory genes. The results strongly support the idea that SNPs located in important genes involved in inflammatory response such as *TNFa*, *prothrombin*, and *PARP1*, may play an important role in the pathogenesis of BiPN. A further unifying mechanism underlying BiPN is the inability to repair neurological damage. Another important finding in this study, is the association of SNPs with BiPN that lie within the essential neuropathy susceptibility genes *IKBKAP*, *SERPINB2,* and *DPYD*.

In **chapter 6,** the molecular factors associated with chemotherapy induced PN using gene expression profiles and SNP genotyping data are assessed. A comparison between ViPN and BiPN shows that different genetic factors are involved in the development of PN after vincristine or thalidomide treatment, suggesting a distinct molecular mechanism. Specifically, myeloma derived genetic profiles and patient SNP data shows an association of genes involved in apoptosis and response to oxidative stress with early onset BiPN (after one treatment cycle). Inflammation and nervous system development dominate the genetic and SNP profile of late onset BiPN (developed later during induction treatment). Cell cycle and proliferation genes characterize early onset ViPN, while inflammation factors and SNPs in ADME genes dominate the profile of late onset ViPN.

Part II on miRNA expression in MM comprises chapters 7 and 8.
Part II of this thesis focuses on the role of miRNA expression in the pathogenesis of MM. MiRNAs have been shown to have an important role in various cellular processes, such as apoptosis, differentiation and development. There is considerable potential to target miRNAs as a novel approach in the treatment of MM. It is therefore of great importance to elucidate the miRNA expression pattern in MM and determine the role of miRNAs in MM pathogenesis.

In **chapter 7** we investigate miRNA expression profiles in myeloma plasma cells from 45 newly diagnosed MM patients. In the same series, gene expression profiles are determined in MM cells. This study demonstrates that miRNA expression in MM is deregulated compared to normal plasma cells. Unsupervised analysis shows that MM patients can be classified according to their miRNA expression pattern, and that these miRNA profiles are not characterized by cytogenetic subgroups. A trend towards better overall survival is observed for patients with high expression of *let-7f*, *miRNA-194* and *miRNA-296*. Furthermore, integration of miRNA and mRNA data shows the putative interaction between *miRNA-21* and two of its validated targets; *PDCD4* and *RECK*, suggesting a functional relationship between miRNA expression and gene expression.

Chapter 8 assesses the question whether *miRNA-15a* and *miRNA-16-1*, located on chromosome 13q14, are downregulated or deleted in 26 MM patients. Deletion of chromosome 13, observed in more than 50% of MM patients, is associated with poor prognosis; however, a tumor suppressor gene has not yet been identified in the region. Chromosome 13q deletions are determined by copy number variation (CNV) using genome-wide SNP arrays and fluorescence in situ hybridization (FISH). CNV analysis reveals a number of additional patients with a chromosome 13q deletion. This study shows that MM plasma cells express both *miRNA-15a* and *miRNA-16* heterogeneously. The levels of *miRNA-15a* and *miRNA-16* expression vary independent of the presence of chromosome 13q deletions.

Chapter 9 as final chapter represents a general discussion in which the described results of the pharmacogenetic studies are discussed in more depth and placed in a broader context.

Samenvatting

Ondanks de aanzienlijke vooruitgang van de behandeling van multipel myeloom (MM) in het afgelopen decennium, sterft een groot deel van de patiënten door het falen van de behandeling. Tevens is er een groot aantal MM patiënten met ernstige bijwerkingen van de medicijnen. Hoewel de introductie van nieuwe medicijnen zoals thalidomide en bortezomib hebben bijgedragen aan de weg naar een gepersonaliseerde behandeling, blijft het noodzakelijk om nieuwe behandelmethoden te ontwikkelen. Daarnaast is het van groot belang om bestaande behandelmethoden te optimaliseren ter voorkoming van ernstige bijwerkingen.

Hoofdstuk 1 is een algemene introductie over multipel myeloom, waarin de nadruk ligt op single nucleotide polymorfismen (SNPs) en microRNAs (miRNAs). Ook wordt er een kort overzicht van de hierop volgende hoofdstukken gegeven.
Na hoofdstuk 1 bestaat dit proefschrift uit twee afzonderlijke delen: Deel I en Deel II.

Deel I beschrijft SNP associatie studies in MM en bestaat uit de hoofdstukken 2, 3, 4, 5 en 6.
Farmacogenetica; het veld dat de invloed van de individuele genetische variabiliteit op de activiteit, toxiciteit en kinetiek van een bepaald geneesmiddel onderzoekt, draagt bij aan de ontwikkeling van de aanpassing van een behandeling van een patiënt aan de hand van zijn of haar genetisch profiel. Variatie in het genetisch profiel van een patiënt is vaak te wijten aan SNPs, welke de respons op een geneesmiddel en de ontwikkeling van toxische bijwerkingen kunnen beïnvloeden.

In **hoofdstuk 2** wordt met behulp van een hypothese gestuurde aanpak de rol van erfelijke genetische variatie binnen MM bestudeerd, waarbij gebruik gemaakt wordt van een aangepaste genotyperings array om 2595 MM patiënten van Europese afkomst bij aanvang te onderzoeken; afkomstig uit de Verenigde Staten, Groot-Brittannië en Nederland. De aangepaste Bank On A Cure (BOAC) array bestaat uit 3404 SNPs in 964 genen, gericht op SNP variatie in moleculaire signaalroutes die betrokken zijn bij de pathogenese en de respons op de behandeling van MM. Met het doel de distributie van deze varianten binnen de normale populatie te begrijpen, hebben we toegang gehad tot 8974 controle genotypen uit publiek toegankelijke datasets. Door een vergelijking te maken tussen de distributie in patiënten en controle populaties is het mogelijk geweest polymorfismen te identificeren, die geassocieerd zijn met het risico op MM. Onder deze polymorfismen vallen nonsynonymous SNPs in de immuun genen *FCRL5* en *SELP*; beide afkomstig van chromosoom 1, een regio die vaak geassocieerd is met MM progressie en een slecht klinisch resultaat. Een nonsynonymous SNP in *CAMKK2*, die de signalering via de cytokine signaalroutes beïnvloedt en cruciaal is voor het functioneren van myeloomcellen, wordt ook geassocieerd bevonden met MM. De mogelijke rol van omgevingsfactoren in het risico op

MM wordt ondersteund door de associatie met een SNP in *CYP2C19*; een absorptie, distributie, metabolisme, en excretie (ADME) gen.

Hoofdstuk 3 beschrijft de invloed van SNPs op de gevoeligheid voor het ontwikkelen van een veneuze trombo-embolie (VTE). VTE, met het daaruit voortvloeiende risico op een longembolie, is een groot probleem bij de behandeling van MM patiënten met thalidomide. De analyse toont aan dat een set van SNPs, geassocieerd met thalidomide geïnduceerde VTE, verrijkt is met genen en signaalroutes die een belangrijke rol spelen in het transport en de distributie van geneesmiddelen, DNA herstel en cytokine evenwicht. Bovendien wordt met behulp van een recursieve partitionerings analyse een SNP set gedefinieerd, die gebruikt zou kunnen worden bij de identificatie van patiënten met een verhoogd risico op VTE.

In **hoofdstuk 4** wordt de associatie tussen SNPs en thalidomide geïnduceerde perifere neuropathie (TiPN) nader onderzocht met behulp van de BOAC SNP array. Perifere neuropathie (PN) is een ernstige bijwerking bij MM patiënten. Percentages TiPN variëren van 15-70%, waarbij het risico op neuropathie gerelateerd is aan de cumulatieve dosis en de duur van de therapie. De behandeling van TiPN omvat een verlaging van de thalidomide dosis of het stopzetten van de behandeling, wat binnen 16 weken tot een afname van de symptomen kan leiden. In sommige gevallen is TiPN echter onomkeerbaar.

TiPN associaties worden geïdentificeerd met SNPs in *ABCA1*, *ICAM1*, *PPARD*, *SERPINB2*, en *SLC12A6*. Deze resultaten kunnen worden gevalideerd in twee onafhankelijke prospectieve klinische studies. De bevindingen zijn consistent met de hypothese dat het risico van een individu op het ontwikkelen van TiPN gestuurd kan worden door SNPs in genen belangrijk in herstel- en ontstekingsmechanismen in het perifere zenuwstelsel. Er wordt ook onderzocht of TiPN SNP associaties gerelateerd zijn aan de blootstelling aan thalidomide alleen of geneesmiddelen gerelateerde TiPN in het algemeen, waarvoor een tweede analyse wordt uitgevoerd met vincristine behandelde patiënten. Hieruit komen SNPs naar voren die geassocieerd zijn met vincristine geïnduceerde perifere neuropathie (ViPN), met een duidelijk verschillend onderliggend genetisch mechanisme.

Perifere neuropathie is eveneens een dosis-limiterende toxiciteit bij behandeling met bortezomib, waardoor het vaak nodig is de dosis te verlagen of de behandeling te staken. De mechanismen welke ten grondslag liggen aan bortezomib geïnduceerde perifere neuropathie (BiPN) zijn grotendeels onbekend. Met dit doel is er in **hoofdstuk 5** een farmacogenetische associatie analyse uitgevoerd binnen twee prospectieve klinische studies met behulp van de BOAC SNP array.

Gestratificeerde associatie analyse toont een significante associatie aan tussen de ontwikkeling van BiPN en pre- inflammatoire genen. De resultaten ondersteunen sterk het idee dat SNPs, gelegen in belangrijke genen betrokken bij de inflammatoire respons, zoals *TNFα*, *prothrombine* en *PARP1*, mogelijk een essentiële rol spelen in de pathogenese van BiPN. Een ander onderliggend mechanisme is het onvermogen om neurologische schade te herstellen. Een belangrijk resultaat van deze analyse is de associatie van BiPN met SNPs die in de essentiële neuropathie susceptibiliteitsgenen *IKBKAP*, *SERPINB2* en *DPYD* liggen.

In **hoofdstuk 6** wordt onderzoek gedaan naar de moleculaire factoren die geassocieerd zijn met chemotherapie geïnduceerde PN, waarbij gebruik wordt gemaakt van genexpressie profielen en SNP genotypering data. Uit een vergelijking tussen ViPN en BiPN blijkt dat verschillende genetische factoren betrokken zijn bij de ontwikkeling van PN als gevolg van een behandeling met vincristine of thalidomide, waarmee gesuggereerd wordt dat de moleculaire mechanismen verschillend zijn. De myeloom afgeleide genetische profielen en SNP data van de patiënten laat met name een associatie zien tussen genen die betrokken zijn bij apoptose en de reactie op oxidatieve stress met de aanvang van BiPN in een vroeg stadium (na 1 behandelingscyclus). Ontstekings- en zenuwstelsel ontwikkeling domineren het genetisch- en SNP profiel van de in een laat stadium ontwikkelde BiPN (later tijdens de inductiebehandeling). Celcyclus en proliferatie genen zijn karakteristiek voor ViPN in een vroeg stadium, terwijl ontstekingsfactoren en SNPs in ADME genen het profiel van de in een laat stadium ontwikkelde ViPN domineren.

Deel II beschrijft miRNA expressie in MM en omvat hoofdstuk 7 en 8.

Deel II van dit proefschrift richt zich op de rol van miRNA expressie in de pathogenese van MM. Het is inmiddels duidelijk geworden dat miRNAs een belangrijke rol spelen in verscheidene cellulaire processen zoals apoptose, differentiatie en ontwikkeling. De nieuwe benadering in de behandeling van MM om miRNAs als target te gebruiken heeft aanzienlijk veel potentie. Het is daarom van groot belang om de miRNA expressieprofielen binnen MM te ontrafelen en de rol van miRNAs in de pathogenese van MM vast te stellen.

In **hoofdstuk 7** is er onderzoek gedaan naar de miRNA expressieprofielen van myeloom plasmacellen van nieuw gediagnosticeerde MM patiënten. In dezelfde serie patiënten worden genexpressie profielen van MM cellen bepaald. In dit onderzoek komt naar voren dat miRNA expressie ontregeld is in vergelijking met normale plasmacellen. De analyse laat zien dat MM patiënten geclassificeerd kunnen worden op basis van het miRNA expressieprofiel, en dat deze miRNA profielen niet gekarakteriseerd worden door cytogenetische subgroepen. Patiënten met een hoge expressie van *let-7f*, *miRNA-194*, *miRNA-296* neigen naar een betere algehele overleving. Daarnaast blijkt na het integreren van de miRNA en mRNA expressie data, dat er een mogelijke interactie tussen *miRNA-21* en twee van zijn gevalideerde targets; *PDCD4* en *RECK* bestaat, wat een functionele relatie tussen miRNA expressie en genexpressie suggereert.

Hoofdstuk 8 onderzoekt de vraag of *miRNA-15a* en *miRNA-16-1*, gelegen op chromosoom 13q14, een verlaagde expressie vertonen of gedeleteerd zijn in 26 MM patiënten. Deletie van chromosoom 13 komt in meer dan 50% van MM patiënten voor en is geassocieerd met een slechte prognose; een tumor suppressor gen is echter nog niet geïdentificeerd in deze regio. Chromosoom 13q deleties worden vastgesteld met behulp van copy nummer variatie (CNV), waarbij gebruik wordt gemaakt van genoom-wijde SNP array en fluorescent in situ hybridisatie

(FISH). CNV analyse doet een aantal extra patiënten met een chromosoom 13q deletie naar voren komen. Dit onderzoek laat zien dat MM plasma cellen zowel *miRNA-15a* als *miRNA-16* heterogeen tot expressie brengen. De *miRNA-15a* en *miRNA-16* expressie niveaus variëren onafhankelijk van de aanwezigheid van een chromosoom 13 deletie.

Hoofdstuk 9 bevat tenslotte een algemene discussie waarin de beschreven resultaten van de farmacogenetische studies worden bediscussieerd en in een breder perspectief worden geplaatst.

Abbreviations

ADME	Absorption, distribution, metabolism, excretion
AML	Acute myeloid leukemia
ANOVA	Analysis of variances
AsCN	Allele specific copy number variation
BiPN	Bortezomib induced peripheral neuropathy
BM(SC)	Bone marrow (stromal cell)
BOAC	Bank On A Cure
bp	Base pair
cDNA	Complementary deoxyriboncleic acid
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CN(V)	Copy number (variation)
CR	Complete response
Ct	Threshold cycle
CTD	Cyclophosphamide, thalidomide, dexamethasone
CVAD	Cyclophosphamide, vincristine, adriamycin, dexamethasone
DNA	Deoxyribonucleic acid
DS	Durie-Salmon staging system
DVT	Deep vein thrombosis
FDA	US Food and Drug Administration
FDR	False discovery rate
FISH	Fluorescence in situ hybridization
GM-CSF	Granulocyte macrophage-colony stimulating factor
HapMap	Haplotype map
HDM	High-dose melphalan
HDT	High-dose therapy
HOVON	Stichting Hemato-Oncologie voor Volwassenen Nederland
HWE	Hardy-Weinberg Equilibrium
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IκB	Inhibitory kappa B
IL	Interleukin
IMiD	Immunomodulatory drug
ISS	International staging system
LD	Linkage disequilibrium
LOH	Loss of heterozygosity

MAF	Minor allele frequency
MB	Megabases
MGUS	Monoclonal gammopathy of undetermined significance
MIP	Molecular inversion probe
miRNA	MicroRNA
MM	Multiple myeloma
mRNA	Messenger ribonucleic acid
NC	No change
NCI-CTC	National Cancer Institute-Common Toxicity Criteria
nCR	Near complete response
NFκB	Nuclear factor kappa B
NK cells	Natural killer cells
OR	Odds ratio
OS	Overall survival
PAD	Bortezomib, adriamycin, dexamethasone
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PFS	Progression free survival
PN	Peripheral neuropathy
PR	Partial response
RB1	Retinoblastoma-1
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
TAD	Thalidomide, adriamycin, dexamethasone
TiPN	Thalidomide induced peripheral neuropathy
TNFα	Tumor necrosis factor alpha
TrPN	Thalidomide related peripheral neuropathy
UPD	Uniparental disomy
VAD	Vincristine, adriamycin, dexametasone
VGPR	Very good partial response
ViPN	Vincristine induced peripheral neuropathy
VTE	Venous thromboembolism

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Sophie

Curriculum vitae

Sophie Corthals werd geboren op 16 maart 1981 te Amersfoort. In 1999 behaalde zij haar Gymnasium diploma aan Het Nieuwe Eemland College in Amersfoort. Aansluitend ging zij Biologie studeren aan de Universiteit Utrecht. Na het behalen van haar bachelor Biologie in 2003 stapte zij over naar de Vrije Universiteit in Amsterdam waar zij in 2006 haar master Oncology behaalde. Tijdens haar studie heeft ze onderzoek gedaan naar 'De rol van tyrosine kinases in de ontwikkeling en prognose van acute myeloide leukemie (AML) bij kinderen' onder supervisie van Dr. C. Michel Zwaan en Dr. Jacqueline Cloos op de afdeling Kinderoncologie/Hematologie van het Vrije Universiteit Medisch Centrum in Amsterdam. Ook deed zij onderzoek naar 'Precursor-B acute lymphoblastische leukemie (ALL) cellen stimulatie door Toll-like receptoren en de anti -ALL T cel response' onder begeleiding van Dr. Kirk Schultz op de afdeling Kinderoncologie/Hematologie van het Child & Family Research Institute in Vancouver (Canada). Na het behalen van haar master begon ze in juni 2006 als promovendus in de onderzoeksgroep van Prof.dr. Pieter Sonneveld op de afdeling Hematologie van het Erasmus MC (promotoren Prof.dr. Pieter Sonneveld en Dr. Mojca Jongen-Lavrencic). Aldaar vond het onderzoek beschreven in dit proefschrift plaats.

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Name PhD student: Sophie Leontien Corthals
Erasmus MC Department: Hematology
Research School: Molecular Medicine
PhD period: 2006 - 2010
Promotors and Supervisors: Prof.dr. P. Sonneveld, Dr. M. Jongen-Lavrencic

	Year	ECTS
1. PhD Training		
Courses		
Partek Training Course (MolMed)	2008	0.5
Bioinformatic Analysis, Tools and Services (MolMed)	2008	0.5
SNPs and Human Diseases (MolMed)	2007	2
Workshops		
Browsing Genes and Genomes with Ensembl (MolMed)	2007	0.5
Applied Bioinformatics (MolMed)	2007	0.5
Presentations		
8 Hematology Presentations	2006-2010	5
3 Journal Club Presentations	2006-2010	2
(Inter)national conferences		
11th Molecular Medicine Day, Erasmus MC, Rotterdam (Poster)		
XIth International Myeloma Workshop, Kos, Greece (Poster 2x)	2007	2
XIIth International Myeloma Workshop, Washington, USA	2008	2
12th Molecular Medicine Day, Erasmus MC, Rotterdam (Poster)	2008	0.5
European Hematology Association, Copenhagen, Denmark (Poster)	2008	2
European Myeloma Network meeting, Rotterdam	2009	2
American Society of Hematology, New Orleans, USA (Poster 3x)	2009	2
European Hematology Association, Barcelona, Spain (Oral)	2010	5
American Society of Hematology, Orlando, USA (Oral)	2010	5
2. Other		
Writing Application René Vogels Stipendium	2009	1
Research visit, Department of Haemato-Oncology, The Institute of Cancer Research, Sutton, Surrey, UK.	2006	2
Total		34.5