IMPACT OF MIRSNPS ON SURVIVAL AND PROGRESSION IN PATIENTS WITH MULTIPLE MYELOMA UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

Running title: MiRSNPs in myeloma

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DISCLOSURE OF CONFLICTS OF INTEREST

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TRANSLATIONAL RELEVANCE:

This is the first study reporting that polymorphisms related to microRNAs (miRSNPs) have prognostic value in multiple myeloma. Two miRSNPs, one in a keratin gene (KRT81), and other in the miRNA biogenesis pathway-related protein exportin-5 (XPO5), can modify the final levels of these proteins and are involved in the prognosis after melphalan-based autologous stem-cell transplantation of patients with multiple myeloma. This information could be helpful in order to incorporate additional measures in the first-line therapy before autologous stem-cell transplantation, according to the genetic background.
ABSTRACT

Purpose: A distinctive new group of polymorphisms is constituted by SNPs in microRNAs (miRNA) processing machinery in miRNA precursor molecules and in miRNA binding sites, known as miRSNPs. The aim of this study was to ascertain the prognostic impact of 6 miRSNPs in multiple myeloma (MM) patients and analyze the functional consequences.

Experimental design: One hundred and thirty seven patients with chemosensitive MM (73M/64F) intensified with ASCT were studied. The median follow-up was 4 years. The genes and SNPs evaluated in genomic DNA by allelic discrimination were KRT81 (rs3660), AFF1 (rs17703261), FAM179b (rs1053667), MIR196A2 (rs11614913), for miRNA target genes and TRBP (rs784567) and XPO5 (rs11077) for miRNA biogenesis pathway.

Results: Overall survival (OS) was significantly longer in patients with KRT81 rs3660 C/C variant (p=0.037). Functional analysis showed that the presence of C variant in KRT81 3'UTR region is related with a reduction of the protein levels. Moreover, the reduction of KRT81 protein levels by siRNA in MM cell lines is related to a decreased proliferation. On the other hand, OS was significantly longer in patients with C/C or A/C variant in XPO5 rs11077 (p=0.012). There was also a significantly longer progression-free survival (PFS) for this SNP (p=0.013). This SNP retained its prognostic impact on PFS and OS in a multivariate regression analysis (p=0.028 and p=0.014, respectively).

Conclusion: This is the first report that relates miRSNPs with prognosis in MM either in a keratin gene (KRT81), target of diverse miRNA MM clusters, or in the miRNA biogenesis pathway related protein exportin-5.
INTRODUCTION

Multiple myeloma (MM) is the prototype of malignant plasma cell disorders. In patients younger than 65 years the treatment is based in an induction therapy followed by high-dose melphalan-based regimens followed by autologous stem cell rescue (ASCT) (1). Even though a number of clinical and biological factors at diagnosis have been associated with survival in the last years, the response to treatment remains the most important prognostic factor (2). The incorporation of novel drugs, particularly thalidomide, bortezomib and lenalidomide in the last decade has resulted in a significant survival prolongation (3). However, MM is still an incurable disease with an important shortening in the expected survival (4).

MicroRNAs (miRNAs) are small non-coding RNAs which can regulate the translation through interactions with target mRNAs as main function. These have been associated with progression and prognosis in several cancers, with a distinctive profile of these molecules (5). MiRNAs are deregulated in myeloma cells, and their pattern of expression in MM seems to be associated with specific genetic abnormalities (6). Their functions can be dual, acting as oncogenic or tumor-suppression genes, according to their targets that can change depending on the cell or tissue involved (7). Recently, it has been described that polymorphisms related to miRNA genes, miRNA binding sites, or in the miRNA processing machinery can affect the final levels and functions of miRNAs. This distinctive and relative new group of polymorphisms is call miRSNPs (8). Although, SNPs in drug metabolic enzymes, DNA repair o MDR1 have been described in association with prevalence, response to treatment, progression-free and overall survival in MM (9,10), no studies have been reported to date with miRSNPs in MM. This novel class of SNPs opens a new area of research in cancer biology and clinical oncology.

The aim of this study was to ascertain the prognostic impact of 6 miRSNPs located either in MM related miRNAs target genes or in miRNA biogenesis pathway proteins in MM undergoing ASCT. We tested this hypothesis in a homogenous population of 137 chemosensitive MM patients who had undergone ASCT as part of the first-line therapy in a single institution.
MATERIALS AND METHODS

Patient recruitment and data collection

From March 31st 1994 to November 16th 2010, one hundred ninety two patients with MM underwent ASCT in our institution. 158 (82.3%) of them had chemosensitive disease at the time of ASCT. Of these, 137 patients (74M/63F, median age 55 years; range 26 to 67 years) had a genomic DNA sample available and they were the subject of the present investigation. The induction regimen more frequently used was standard combination chemotherapy (99 patients, 72.3%), mainly alternating polychemotherapy (75%) V[B]CMP (vincristine, [BCNU], melphalan, cyclophosphamide, prednisone) and VBAD[P] (vincristine, BCNU, doxorubicin, dexamethasone [prednisone]). The remaining regimens consisted of thalidomide/dexamethasone (9.5%), and bortezomib-based combinations (18.25%). All patients achieved at least a partial response after one (85%) or two (15%) induction regimens prior to melphalan-based ASCT. Baseline demographics, clinical and laboratory findings and treatment are summarized in Table 1. The median follow-up was 4 years (range 4 months to 16 years). None patient was lost to follow-up. Response, relapse and progression were defined according to European Blood and Marrow Transplantation (EBMT) criteria (11). Moreover, peripheral blood of 50 healthy donors was collected to use as controls for functional analysis. The Ethics Committee of Hospital Clínico of Barcelona provided institutional review board-approval for this study.

DNA extraction and genotyping

Genomic DNA was isolated from bone marrow aspirate slides or peripheral blood using DNeasy Blood & Tissue Kit (Qiagen, Barcelona, Spain) according to the manufacturer’s instructions. All DNA samples were stored at -80°C, suspended in TE buffer. After a selection based in the reported association of the selected miRSNPs with cancer risk (8,12,13), a group of genes and SNPs were selected: KRT81 (rs3660), AFF1 (rs17703261), FAM179b (rs1053667), MIR196A2 (rs11614913), TRBP (rs784567) and XPO5 (rs11077). Inclusion criteria for each SNP included a
minor genotype frequency for the European population \( \geq 0.05 \); and either a known association with a differential susceptibility to cancer development or a differential impact in patients with solid tumors. Previous reports of our group of prognosis association of some of these miRSNPs in lung cancer (14) were also taken into account. SNP analysis was performed by allelic discrimination with TaqMan probes (commercial assays) in an ABI PRISM 7500 Sequence detection system (Applied Biosystems, Foster City, CA) as previously described (15). At least 10% of the samples were studied by PCR in a second time, obtaining the same genotypic results. All samples had a code number at the time of extraction, as guarantee of the blinded analysis.

**mRNA and protein analysis**

Total RNA was extracted from lymphocytes from peripheral blood of healthy donors using Trizol (Invitrogen) as manufacturer's protocol. Total cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan Gene Expression assays (Applied Biosystems) for XPO5 (Hs00382453_m1), KRT81 (Hs00605559_m1) and GUSB (Hs99999908_m1), used as endogenous control.

Total protein was isolated from lymphocytes from peripheral blood of healthy donors using Qiagen Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. XPO5 and KRT81 protein levels were determined using Exportin 5 (XPO5) and keratin 81 (KRT81) ELISA Kit (antibodies-online GmbH, Germany) as per manufacturer's protocol.

**Cell culture and transfection**

The MM cell lines KMM-1 and RPMI-8226 were cultured in RPMI 1640 containing 20% and 10% fetal calf serum (Invitrogen) respectively. These cell lines where obtained from Hematopathology Unit (Dolors Colomer, PhD, Hospital Clínic of Barcelona) just before the experiment. Both cell lines were transfected with 33 nM of Keratin 81 siRNA (sc-95788) and Control si-RNA A (sc-37007A) (Santa Cruz
Biotechnology, Inc) by using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol with minor modifications. Briefly, one hour before transfection both cell lines were counted and 250,000 cells/well were diluted in 50µl of Opti-MEM (Invitrogen) without serum and plated in coated 96-well plates. After, the siRNA and lipofectamine 2000 complexes diluted in 50µl of Opti-MEM were added to the cells and subsequently incubated at 37°C in 5%CO2 for 4 hours without serum. Fifty µl of RPMI 1640 with fetal calf serum were then added for further incubation. All the experiments were performed by triplicate.

Proliferation assays
Proliferation in KMM-1 and RPMI-8226 cells transfected with Keratin 81 siRNA or with Control si-RNA A were directly measured at 24 hours after transfection using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) as per the manufacturer's protocol. Cell proliferation was assessed by measuring the absorbance at 490 nm with a VersaMax plate reader (Molecular Devices, Sunnyvale, CA).

Renilla/Luciferase Reporter Assays
Eighth synthetic oligonucleotides, containing from 5’ to 3’: Xhol sticky end (5 bp), a fragment from the 3’UTR of KRT81 gene containing the WT or the SNP sequence (rs3660) (41 bp) or a fragment from the 3’UTR of XPO5 gene containing the WT or the SNP sequence (rs11077) (47 bp), BgIII restriction site (6 bp), and NotI sticky end (2 bp), were cloned in the psiCheck2 vector (Promega) in the 3’ UTR of Renilla luciferase gene. The 2 oligonucleotides for KRT81-WT sense (5’-TCGAGAGTGCTCAGCTACTTTCTCCTCCACTTTGAAAGACCCCTCCCAGATCTGC-3’) and antisense (5’-GGCCCGCAGATCTGGGAGGGGTCTTTCAAAAGTGAGGAGAAGTAGCTGAGCACTC-3’), the two for KRT81-SNP sense (5’-TCGAGAGTGCTCAGCTACTTTCTCCTGCACTTTGAAAGACCCCTCCCAGATCTGC-3’) and antisense (5’-GGCCCGCAGATCTGGGAGGGGTCTTTCAAAAGTGAGGAGAAGTAGCTGAGCACTC-3’) and the 2 oligonucleotides for XPO5-WT sense (5’-TCGAGTACCTCCAAGGACCAGGGGTCTGGGAAGTCTTTAGTGCAACATCCCTA
GATCTGC -3') and antisense (5'-GGCCGCAGATCTAGGGGATGTTAGCACTAAAGACTTCCAGCCTGGTCTCTTG GAGGTAC -3'), the two for XPO5-SNP sense (5'-TCGAGTACCTCCAAGGACCAGGGCTGGGCAGTCTTTAGTGCTAACATCCCCCTA GATCTGC -3') and antisense (5'-GGCCGCAGATCTAGGGGATGTTAGCACTAAAGACTTCCAGCCTGGTCTCTTG GAGGTAC -3') were first annealed with a Tris buffer (100 mM Tris HCl, pH 7.5, 1 M NaCl, 10 mM ethylenediaminetetraacetic acid) in a heating block at 95°C for 5 minutes, followed by a gradual reduction of temperature until room temperature. The psiCheck2 vector was linearized by digestion with NotI and XhoI (New England Biolabs) and purified from an agarose gel. The annealed oligonucleotides were ligated in the linearized psiCheck2 vector into the NotI and XhoI cloning sites located in the downstream of the Renilla luciferase reporter gene with T4 DNA ligase (New England Biolabs). The ligation reaction was transformed in TOP10F' Escherichia coli competent cells (Invitrogen). Positive clones were selected by restriction digestion with BglII and reconfirmed by sequencing.

The 2 cell lines were transfected with 0.5 μg of either the modified psiCheck2 vector containing the insert with the WT sequence (WT-modified psiCheck2) or the modified psiCheck2 vector containing the insert with the SNP (SNP-modified psiCheck2). Then the Renilla luciferase and Firefly luciferase activity was measured at 48 hours after transfection with the Promega Dual luciferase reporter assay system (Promega) in a Veritas microplate luminometer. The transfection efficiency was normalized with the Firefly luciferase gene.

Statistical methods

Statistical analyses were performed with PAWS statistics software 18.0 for Windows® and GraphPad 5. Progression-free survival (PFS) was defined as survival from ASCT until relapse or dead from any cause. Overall survival (OS) was calculated from the time of ASCT to last follow-up or death. Survival probabilities were estimated using the Kaplan-Meir method and analyzed by means of the log-rank test. Cox proportional hazards model was used to estimate the risk ratio of
events (relative risk, RR) with the respective confidence interval (CI), and to multivariate analysis. T-Test was used to compare differential expression between genotypic groups. The genotypes were combined for statistical reasons when different biological plausible combinations were analysed, and two of them showed an overlapped trend. Statistical significance was defined as $p \leq 0.05$. 
RESULTS

miRSNP and prognosis

In the overall population, the median PFS from ASCT was 2.8 years (CI 95% 2 to 3.7 years), with a median OS of 9.7 years (CI 95% 5.2 to 14.2 years). Two miRSNPs had prognostic impact after ASCT. The first one, rs3660 in KRT81 gene, was associated with a significantly longer OS in patients with CC (21) vs. CG or GG genotype (111) (median not reached vs. 7 years; p=0.037, RR 3.26 [CI 95% 1.1 to 10.5]) (Figure 1A). Only three late deaths were observed in the favourable group with 21 patients at risk. When only patients who underwent into ASCT after first line treatment were considered, this significance was retained (p=0.046). The same variants in this miRSNP showed a longer PFS (median 4.3 vs. 2.4 years) but not reaching statistical significance (p=0.24).

The second miRSNP was rs11077 in exportin-5 gene (XPO5). Patients with CC/AC genotype (86) had a significantly longer OS when compared to patients with AA genotype (31) (median not reached years vs. 7.2 years; p=0.012, RR 2.5 [IC 95% 1.2 to 5]) (Figure 1B). Interestingly, and in the same line, patients with CC/AC genotype showed a higher PFS (median 4.3 vs. 2 years; p=0.013, RR 1.97 [IC 95% 1.12 to 3.4]) (Figure 2). The most unfavourable variant (AA) showed also a significantly shorter PFS when compared to the two other genotypes (AC, p=0.032; CC, p=0.023). The complete response rate was not significantly different among the different genotype groups. When only patients who received ASCT after first line treatment were considered, the effect over OS was even deeper (p=0.005; RR 2.9 [IC 95% 1.3 to 6.4]). Furthermore, the effect on OS was also significant in the subset of patients achieving CR after ASCT (p=0.03).

The miRSNP rs784567 in TRBP gene showed a trend towards a shorter OS (p=0.103) and PFS (p=0.083) in the same sense. All the patients were heterozygote to AFF1 (rs17703261), so no further analysis was performed on this SNP. No associations with prognosis or toxicities were observed with the other SNPs studied.
The main baseline clinical and laboratory characteristics of the patients were not significantly different between these genotypic groups. The incorporation of new drugs (thalidomide and bortezomib) into the induction regimens prior to ASCT according to the genotypes with clinical difference was no different neither in KRT81 rs3660 (26.8% vs. 28.7%, p=884) nor XPO5 rs11077 (22.1% vs. 32.3%, p=0.332). There was no significant difference in pre-transplant response between the two genotypes with clinical difference neither in KRT81 rs3660 (p=0.409) nor XPO5 rs11077 (p=0.915). In fact, CR rate in both groups were quite similar (19% vs. 14.4% and 15.1 vs. 12.9%) respectively. A Cox multivariate regression analysis including age, international staging system immunoglobulin isotype, and XPO5, KRT81 and TRBP miRSNPs showed that the SNP in XPO5 was the only parameter to retain its prognostic impact on PFS and OS (p=0.028 and p=0.014, respectively). For historical reasons, there was only cytogenetic information in 41 patients. The distribution of poor prognostic alterations (t(4;14), t(14;16) and del17p) was not significantly different between the genotypic groups.

The miRSNP in KRT81 3’ UTR region affects protein translation in MM cell lines. The analysis of the 3’UTR region of KRT81 showed that the miRSNP is located in the miRNA binding site for miR-17, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, and miR-519d (as predicted by TargetScan 5.1). The nucleotide change produces the lost of a nucleotide binding in the seed sequence region of these miRNAs as showed in Figure 3A. In order to analyze the functional effect of this nucleotide change in the expression of KRT81, we constructed a vector containing the SNP or the WT genotype in the 3’UTR region of Renilla luciferase gene (WT/SNP-modified psicheck2 vectors) and we transfected both vectors in two myeloma cell lines. A reduction by 9% and 25% in the the Renilla luciferase activity analysis 48 hours after transfection was observed in KMM-1 (p=0.14) and RPMI-8226 (p=0.01) respectively with the SNP-modified psicheck2 vector (Figure 3B). These results indicate that the presence of the polymorphisms enhances the binding of miRNAs to this sequence resulting in a reduction of protein levels.
The miRSNP in KRT81 3'UTR region alters KRT81 levels in healthy lymphocytes
In order to verify the observed effect in protein reduction by SNP presence in the in vitro assays, we analyzed the mRNA and protein levels of KRT81 in lymphocytes from 50 healthy controls. No differences in mRNA levels were observed.

Interestingly, we observed a genotype significant dose-dependent reduction in the KRT81 levels, where the WT genotype showed the higher levels and the SNP the lower levels (average reduction 37%), with an intermediate value for heterozygous patients (average reduction 15.48%) (ANOVA p=0.033).

KRT81 reduction in myeloma cell lines are related to lower proliferation
Since we have observed that the presence of the SNP is related to lower protein production and moreover the patients harboring the homozygote SNP genotype had better prognosis, we wanted to study whether or not the reduction of KRT81 gene is affecting the proliferative rate in myeloma cell lines. Then we transfected both myeloma cell lines with a KRT81-siRNA or with a control-siRNA and analyzed the proliferation using MTS at 24 hours of transfection. A reduction in the proliferation rate by 27.31% (p=0.1) and 61.11% (p=0.045) for KMM-1 and RPMI-8226 respectively was observed in the KRT81-siRNA transfected cells. (Figure 4)

The miRSNP in XPO5 3’ UTR region affects protein translation in MM cell lines
The analysis of the 3’UTR region of XPO5 no showed any miRNA binding site in the miRSNP region which can be affected by the nucleotide change (TargetScan 5.1). However, the nucleotide change produces the apparition of a binding site for miRNA hsa-miR-4763-5p (http://www.mirbase.org/search.shtml) as showed in Figure 5A. In order to analyze the functional effect of this nucleotide change in the expression of XPO5, we have constructed a vector containing the SNP or the WT genotype in the 3’UTR region of Renilla luciferase gene (XPO5-WT/SNP-modified psicheck2 vectors) and we have transfected both vectors in two myeloma cell lines. A reduction by 18% and 25% in the the Renilla luciferase activity analysis 24 hours after transfection was observed in KMM-1 and RPMI-8226 respectively with the SNP-modified psicheck2 vector (Figure 5B). This result showed that the presence of the polymorphisms
allows the binding of new miRNAs to this sequence producing a significant reduction of XPO5 protein levels.

The miRSNP in XPO5 3'UTR region alters XPO5 levels in healthy lymphocytes

In order to analyze if the miRSNP in XPO5 is playing a role in prognosis by affecting the XPO5 levels we analyzed the mRNA and protein levels of XPO5 in 50 healthy controls. Although no differences in mRNA levels were observed, the C/C genotype was associated with a 17.4% reduction of the related protein, XPO5 (p=NS), on peripheral blood lymphocytes.
DISCUSSION

In the present study we report for first time that polymorphisms related to microRNAs (miRSNPs) have a prognostic impact in multiple myeloma. Two miRSNPs, one in a keratin gene (KRT81), and other in the miRNA biogenesis pathway related protein exportin-5 (XPO5), are involved in the prognosis after melphalan-based autologous stem-cell transplantation of patients with multiple myeloma.

Keratins have been recognized as regulators of cellular functions, including polarization, protein synthesis, membrane traffic and signalling (16). To date, the only keratin for which a specific DNA variant has been associated with cancer predisposition is KRT5 in basal cell carcinoma (17). Notwithstanding the former, their expression levels have been described as prognostic markers in several epithelial tumors (16). SNP rs3660 is located in the 3’UTR of KRT81, a predicted binding site for several miRNAs such miR-17 and miR-93, among others (13), which has been previously observed up-regulated in MM (18). Their functions include down regulation of expression of SOCS-1, a gene that plays a critical role as inhibitor of IL-6 growth signalling (18). In the present study we have observed in two myeloma cell lines, through a Renilla luciferasesa experiment, that the presence of the SNP can affect the final protein levels, where the presence of the C allele in the 3’UTR region facilitates the binding of the miRNAs producing a reduction in the protein levels. This observation is related to the lost of a G≡C binding by the presence of the polymorphism. This alters hybridization process, affecting the minimum free energy (mfe). Using as example KRT81-miR-17 binding, we can observe that the presence of the SNP reduces the mfe necessary for the hybridization from WT-mfe=-8.8 Kcal/mol to SNP-mfe=-10.7 kcal/mol (as predicted by RNAHybrid: http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). When more negative the free energy of a structure, the more likely is formation of that structure. Then the presence of the SNP facilitates the RNA-miRNA binding. Interestingly, this result is confirmed by protein analysis of KRT81 levels in lymphocytes from healthy controls where we found significant differences in KRT81 levels according the genotype. To further investigate the functional consequences of alteration of KRT81 levels in multiple myeloma, we analyzed proliferation levels after KRT81 degradation by siRNA, observing that the presence of the SNP was correlated to lower
proliferation. Both lines were affected in a similar way, but with a different degree, by the silent mRNA for *KRT81* and with the transfection with a vector to SNP or the WT genotype in the *Renilla* luciferase gene. Baseline proliferation, more prominent in RPMI 8226 cells, could be a theoretical reason. We measured the mRNA level for *KRT81* by RT-PCR in both cell lines. KRT81 was 10.2% less expressed in KMM-1 than in RPMI 8226 (*p* = NS). Thus, differential expression of this gene could be also involved in these findings. All these results allow us to speculate that the observed better OS in patients with MM harbouring the SNP, could be explained by the reduction in the levels of KRT81 in this group of patients which are related with a lower proliferative phenotype of tumor cells. Moreover, recently in the whole genome sequencing of malignant plasma cells, mutations in some keratins (i.e. KRT31) have been described (19). These recent findings support the importance of keratin proteins in MM. Validation in larger series is warranted, particularly when some association such as OS with *KRT81* rs3360 would perhaps not be statistically significant if corrected *P* values for multiple comparisons would have been carried out.

Exportin-5 is found in the nuclear membrane and mediates the transport of proteins and other cargo between the nucleic and cytoplasmic compartments. One of these functions involves miRNA biosynthesis (20). The first precursor of miRNA, the primary miRNA, goes through nuclear maturation by RNAase DROSHA system, forming the pre-miRNA. Exportin-5 is then in charged to relocate this molecule in the cytoplasm, where the RNAase DICER complex finished the miRNA maturation process. In some tumor cells, particularly with microsatellite instability, a mutated and inactive XPO5 traps pre-miRNAs in the nucleus, resulting in a reduced miRNA processing, as well as a decreased miRNA-target inhibition (21). Moreover, the restoration of XPO5 functions reverses the impaired export of pre-miRNAs and has tumor-suppressor features. rs11077 is a polymorphism found in the 3’-UTR region of the gene. It was first correlated with oesophageal cancer risk (5). Of interest, this SNP has recently been associated with a trend towards a higher disease-control rate in metastatic colon cancer (22). Our group has recently reported a correlation with time to progression in non-small-cell lung cancer (14). However, the molecular mechanism by which these polymorphisms lead to improving the prognosis after
ASCT is not yet understood. It could be possible that SNPs in the downstream region of XPO5 could be associated with altered expression levels of this protein and, consequently, with quantitative variations in mature miRNA levels in the cytoplasm (8). In order to prove this hypothesis, we have have analyzed if the expression of Renilla luciferase is affected by the genotype. Interestingly we observed a reduction in the Renilla levels in the vector containing the SNP variant. One possible explanation for this protein levels reduction with the SNP variant is the fact that the presence of the SNP allows the binding of miRNAs that has not previously predicted by bioinformatic methods (miRbase, TargetScan). Moreover, we observed a trend to a lower protein expression in healthy donor lymphocytes harbouring the SNP genotype. Sample size and probably many involved heterogeneous factors could condition these levels and limit the statistical conclusions in this regard. In spite of a lack of difference between the response degrees after ASCT, potential variable plasma cell sensitivity to melphalan exposure can not be excluded and further investigation in this regard is warranted. Interestingly, other members of exportin family have been observed altered in cancer. Thus, a recurrent mutation in other exportin (XPO1) has been described in chronic lymphatic leukemia patients with unmutated inmunoglobulins, a finding that support the implications of this carries in haematological malignancies (23).

In summary, this is the first report concerning miRSNPs involved in the miRNA network in haematological malignancies. A polymorphism in the binding site for diverse miRNA clusters in keratin 81 gene (KRT81), a relevant gene in the structural cytoplasm framework, was associated with prognosis in MM. Moreover, a SNP in XPO5 was associated with a significantly longer PFS and OS in MM patients after ASCT. MiRSNPs emerged as new promising markers for disease progression in cancer, and specifically in multiple myeloma.
AUTHORSHIP CONTRIBUTORS

C.F.L. and A.N. performed the assays, collected and analyzed data, performed statistical analysis, wrote and reviewed the paper; J.B. designed the research, collected and analyzed data, performed statistical analysis, wrote and reviewed the paper; M.T.C., N.T., L.R. and M.R. treated the patients, collected data and reviewed the paper. T.D., R.T. and G.F. performed the assays and reviewed the paper. M.M. reviewed and approved the paper.
REFERENCES


Table 1. Patient characteristics (n=137)

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*Available in 127 patients.
Figure legends.

Figure 1. Overall survival of multiple myeloma patients after autologous stem cell transplantation (ASCT) according to the presence of polymorphisms (A) rs3660 in KRT81 (C/C = SNPs, C/G = heterozygous SNP, G/G = wild type) and (B) rs11077 in XPO5 (C/C = SNPs, A/C = heterozygous SNP, A/A = wild type).

Figure 2. Progression-free survival after ASCT according to the presence of polymorphism rs11077 in XPO5.

Figure 3. A. Schematic representation of how the miRSNP rs3660 located in the 3'UTR region of KTR81 gene is affecting the miRNA binding.

B. Renilla/luciferase assay at 48 hours with the KRT81 WT or SNP-modified psicheck2 vector transfected in KMM-1 and RPMI-8226 myeloma cell lines.

Figure 4. Proliferation rate analyzed by MTS at 24 hours of transfection of both myeloma cell lines with a KRT81-siRNA or with a control-siRNA.

Figure 5. A. Schematic representation of the apparition of a binding site for miRNA hsa-miR-4763-5p when miRSNP rs11077 in XPO5 appears.

B. Renilla/luciferase assay at 48 hours with the XPO5 WT or SNP-modified psicheck2 vector transfected in KMM-1 and RPMI-8226 myeloma cell lines.
Figure 1.

A

C/C
rs3660
p=0.037

C/C or A/C

Median survival: NR vs. 7 years

G/G or C/G

B

rs11077
p=0.012

C/C or A/C

Median survival: NR vs. 7.2 years

A/A

Time from autologous stem cell transplantation (years)

Cum Survival

0.0 0.2 0.4 0.6 0.8 1.0

0 5 10 15 20
Figure 2.

![Graph showing progression-free survival](image)

- **rs11077**
- C/C: Events-free survival
- A/A: Events-free survival

- C/C vs A/A: p = 0.032
- A/C vs A/A: p = 0.023
Figure 3

A

rs3660 WT

KRT81

...UCAGCUACUUUCUCCUCGCACUUUG...

miR-17

GAUGGACGUGACAUU -- CGUGAAAC

rs3660 SNP

KRT81

...UCAGCUACUUUCUCCUCCACUUUG...

miR-17

GAUGGACGUGACAUU -- CGUGAAAC

B

Renilla / Luciferase

WT KMM1

SNP KMM1

WT RPMI

SNP RPMI

p=0.14

p=0.01

-8.89%

-25.29%
Figure 4

KMMI

RPMI

Control

siRNA

p=0.1

p=0.0451

OD

OD
Figure 5

A

rs11077 WT

XPO5

...AGGACCAGGGCUGGG...AAGUC...

No matches were found

rs11077 SNP

XPO5

...AGGACCAGGGGCUGGG...CAGUC...

miR-4763-5P

UCGUCCUCCC GAC CCGUC CGC

B

Renilla / Luciferase

WT KM11

SNP KM11

WT RPMI

SNP RPMI

p=0.0023

p=0.001

-18.19%

-23.5%
IMPACT OF MIRSNPS ON SURVIVAL AND PROGRESSION IN PATIENTS WITH MULTIPLE MYELOMA UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

Carlos Fernandez de Larrea, Alfons Navarro, Rut Tejero, et al.

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