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IMPROVED RISK STRATIFICATION IN MULTIPLE MYELOMA USING A MICRORNA-BASED CLASSIFIER

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Summary

Introduction and purposes.

Multiple myeloma (MM) is a heterogeneous disease. The discovery of a class of small non-coding RNAs (miRNAs) has revealed a new level of biological complexity underlying the regulation of gene expression. It may be possible to use this interesting new biology to improve our ability to risk stratify patients in the clinic.

Methods and experimental design.

We performed global miRNA expression profiling analysis of 163 primary tumors included in the UK Myeloma IX clinical trial. miRNA expression profiling was carried out using Affymetrix GeneChip miRNA 2.0; expression values for 847 hsa-miRNAs were extracted using Affymetrix miRNA QC tool and RMA-normalized. There are also 153 matching samples with gene expression profiles (GEP) and 72 matching cases with genotyping data available for integrative analyses. GEP was generated on Affymetrix HG-U133 Plus 2.0 and the expression values were RMA normalized; genotyping was performed on Affymetrix GeneChip Mapping 500K Array and the copy number values were obtained using GTYPE and dChip and were inferred against normal germ-line counterpart for each sample.

Results.

Firstly we have defined 8 miRNAs linked to 3 Translocation Cyclin D (TC) subtypes of MM with distinct prognoses, including miR-99b/let-7e/miR-125a upregulation and miR-150/miR-155/miR-34a upregulation in unfavourable 4p16 and MAF cases respectively as well as miR-1275 upregulation and miR-138 downregulation in favourable 11q13 cases. The expression levels of the miRNA cluster miR-99b/let-7e/miR-125a at 13q13 have been shown to be associated with shorter progression free survival in our dataset. Interestingly unsupervised hierarchical clustering analysis using these 8 miRNAs identified two subclusters among 11q13 cases, which have differential effect on overall survival (OS). We then evaluated the association of miRNA expression with OS and identified 3 significantly associated miRNAs (miR-17, miR-18 and miR-886-5p) after multiple testing corrections, either per se or in concerted fashion. We went on to develop an "outcome classifier" based on the expression of two miRNAs (miR-17 and miR-886-5p), which is able to stratify patients into three risk groups (median OS 19.4 months vs 40.6 months vs 65.3 months, log-rank test $P = 0.001$). The robustness of the miRNA-based classifier has been validated using 1000 bootstrap replications with an estimated error rate of 1.6%. The miRNA-stratified risk groups are independent from main adverse fluorescence in situ hybridization (FISH) abnormalities (1q gain, 17p deletion and t(4;14)), International Staging System (ISS) and Myeloma IX treatment arm (intensive or non-intensive).

Using the miRNA-based classifier in the context of ISS/FISH risk stratification showed that it can significantly improve the predictive power (likelihood-ratio test $P = 0.0005$) and this classifier is also independent from GEP-derived prognostic signatures including UAMS, IFM and Myeloma IX 6-gene signature ($P < 0.002$). Integrative analyses didn't show enough evidence that the miRNAs comprising the classifier were deregulated via copy number changes; however, our data supported that the mir-17~92 cluster was activated by Myc and E2F3, highlighting the potential importance of Myc/E2F/miR-17~92 negative feedback loop in myeloma pathogenesis. We developed an approach to identify the putative targets of the OS-associated miRNAs and show that they regulate a large number of genes involved in MM biology such as proliferation, apoptosis, angiogenesis and drug resistance.

Conclusion.

In this study we developed a simple miRNA-based classifier to stratify patients into three risk groups, which is independent from current prognostic approaches in MM such as ISS, FISH abnormalities and GEP-derived signatures. The miRNAs comprising the classifier are biologically relevant and have been shown to regulate a large number of genes involved in MM biology. This is the first report to show that miRNAs can be built into molecular diagnostic strategies for risk stratification in MM.

Introduction

Multiple myeloma

Multiple myeloma (MM) is an incurable disease characterized by heterogeneous molecular features, presentation and outcome, and accounts for up to 10% of haematological malignancies, with an incidence in Western countries of about 3-5 per 100,000. The average age of onset is 70 years.[1-3] MM affects antibody-secreting bone marrow (BM) plasma cells (PCs) and shows a wide clinical presentation ranging from the presumed pre-malignant condition of monoclonal gammopathy of undetermined significance (MGUS) to smoldering MM, truly overt and symptomatic MM, and extra-medullary myeloma or plasma cell leukemia (PCL). To date, MM diagnosis is based on the criteria established by the International Myeloma Working Group in 2003,[4] subsequently updated in 2009.[5] MM is defined as symptomatic upon the simultaneous occurrence of clonal plasma cells >10% in BM biopsy, monoclonal serum (or urinary) paraprotein and related organ/tissue impairment. Patients are staged according to Durie and Salmon criteria[6] or, now widely increasingly and more effectively, to the International Staging System (ISS)[7] that consider serum β2-microglobulin and albumin levels. Indeed, ISS could be considered a prognostic index rather than a true staging system, as it is a valid measure of risk of progression and short outcome for patients with MM presentation, and is not strictly a measure of tumor burden or extent unlike staging systems used in other cancers.

MM is characterized by a remarkable genomic instability that encompasses ploidy

and structural rearrangements (Figure 1).[8,9] Such a genomic instability, mediated by the interaction with environmental factors and combined with the normal physiological processes that lead to the generation of antibody diversity, substantially contribute to the immortalization of a myeloma-propagating PCs.

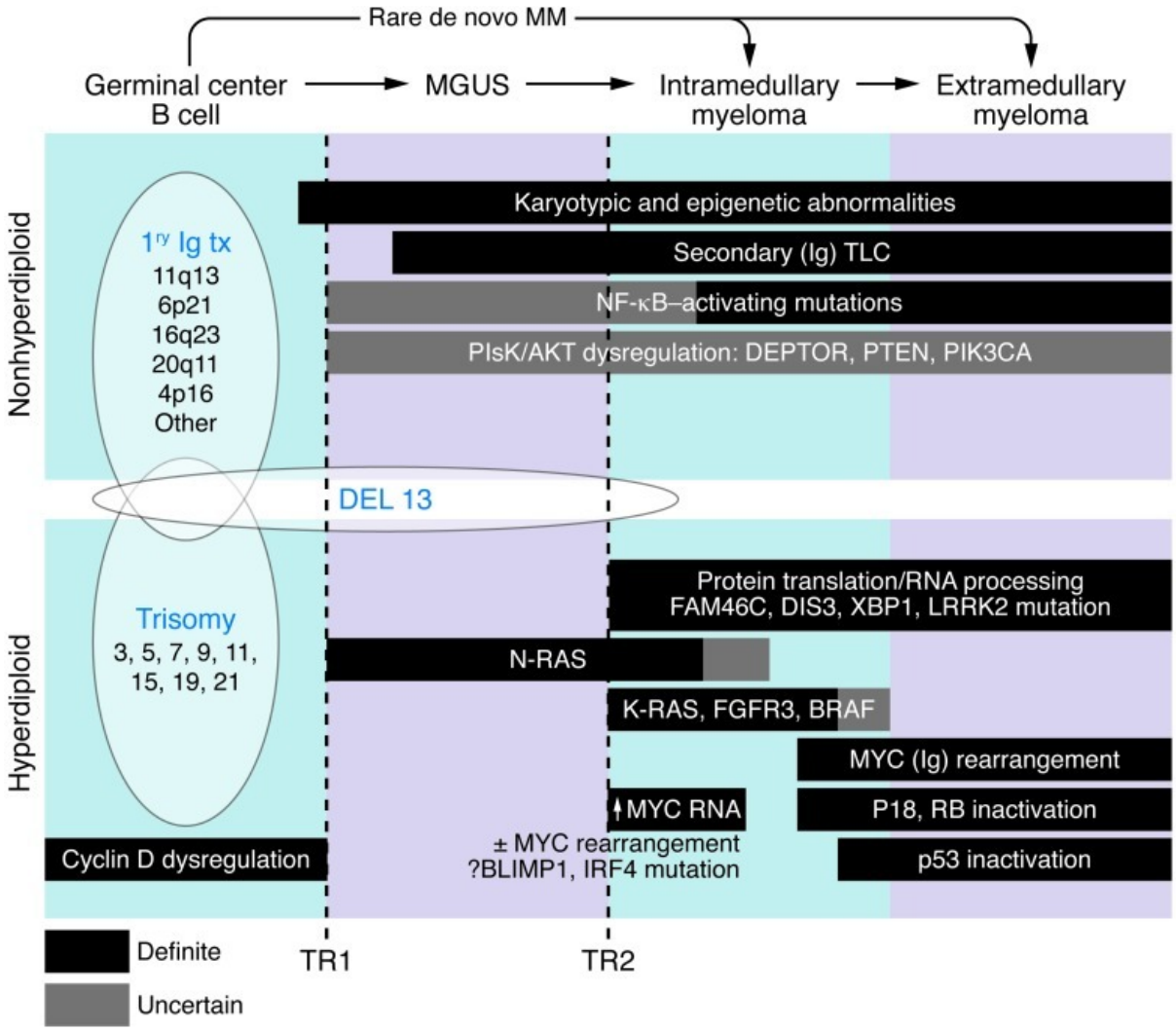

Figure 1. Model for molecular pathogenesis of MGUS and MM (from Kuehl, M and Bergsagel, PL, 2012, *J Clin Invest*).

Based on their large distribution in most clonal cells, the chromosomal translocations generated by aberrant class-switch recombination have been suggested to be initiating events occurring early in the disease process. As a result of these translocations, several genes are aberrrantly placed under the control of the strong

enhancers of the immunoglobulin (*IGH@*) gene loci, leading to their ectopically increased expression. As regards chromosomal numeric imbalances, approximately half of MM tumors are hyperdiploid with non-random trisomies of odd chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, together with a low prevalence of chromosomal translocations involving the *IGH@* on chromosome 14q32.4. The remaining tumors are referred as non-hyperdiploid and are frequently associated with the constitutive activation of *CCND1* (11q13), *CCND3* (6p21), *MAF* (16q23), *MAFB* (20q11), or *FGFR3/MMSET* (4p16.3) genes as a result of *IGH@* translocations.

The mechanisms underlying this dichotomic pattern have not been elucidated but hyperdiploid patients have a generally better prognosis, whereas the t(4;14) and the t(14;16) are associated with a poor prognosis. Other genetic abnormalities (e.g., *N-/K-RAS* and *BRAF* mutations, inactivation of p53 and/or del(17)(p13), Myc deregulation) characterize mainly the non-hyperdiploid fraction of MM cases and are specifically associated with advanced stages of the disease.[8-10] Therefore, the understanding of the molecular bases of the disease represents an important step to dissect the clinical heterogeneity of the disease and the variety of clinical presentation.

Noteworthy, although several promising studies report the relationship between drugs effect and MM subtypes, the treatment of MM has so far been largely independent of any of the main molecular or genetic features, and is related to other factors (e.g. the age of onset, being considered younger patients those with MM presentation before 65 ys); a targeted therapy is a early desirable scenario but currently still not tangible.[11] The conventional long-lasting anti-myeloma therapy (alkylators and corticosteroids, with a median post-treatment overall survival of 3-4 years) have been first implemented by high-dose therapy followed by autologous stem cell transplantation (ASCT, median OS 5–7 years) and then, over the last few years, by the new-generation treatments: the proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide and lenalidomide has been the cornerstone of improving the outcome of patients with myeloma.[12] However, there is still a lack of effective therapies targeting the deregulated biological/molecular pathways specifically associated with subsets of the disease. Only the recent updates in myeloma treatment guidelines have partially introduced risk stratification and molecular features as factors for choosing therapy. For instance, the Mayo Clinic has developed a risk-stratification model termed Mayo Stratification for

Myeloma and Risk-adapted Therapy (mSMART), which dissects patients into high-risk (bortezomib-based treatments) and standard-risk categories (lenalidomide and dexamethasone for patients ineligible for ASCT). Patients with deletion of chromosome 13 or hypodiploidy by conventional cytogenetics, t(4;14), t(14;16) or 17p- by molecular genetic studies, or with a high plasma cell labeling index (3% or more) are considered as high-risk myeloma.[13] The unfavorable prognostic value of t(4;14) and deletion of 17p is in line with other studies that introduced serum β2-microglobulin as discriminant and independent variable in association with genomic alterations.[14]

The recent advances, mainly based on next generation sequencing procedure that allows the high-throughput screening of somatic mutations and genetic changes, are contributing to delineate a further complex scenario of the disease (Figure 2). From several studies analyzing the MM genome emerged that mechanisms with a likely role in the biology of myeloma (e.g., NF-kB activation) may be candidate to broad roles by virtue of mutations in multiple members of these pathways.[15-19] The genomic landscape of MM, in particular, pointed to a number of recurrently mutated genes (the *TP53* gene, occurring in almost 10% patients; the *KRAS*, *NRAS* and *BRAF* genes included in the MAPK-pathway, overall occurring in more than half tumors; the functionally uncharacterized *FAM46C* mapped at 1p; the exonuclease encoding *DIS3* gene; the *TRAF3*, *BIRC2*, *BIRC3*, and *CYLD* genes associated with the regulation of the NF-kB signaling pathway), whose overall rate encompasses up to more than 80%, virtually the totality, of MM patients (Figure 3), and that are candidate as causal drivers of the disease. The general notion underlying the progression of myeloma is that multiple mutations in different pathways collaborate to deregulate the intrinsic biology of the PCs; recent data suggest that clonal progression is the key feature of MM evolution into aggressive disease, being the invasive clinically predominant clone typical of MM already present at earlier stages and gradually selected by step-by-step treatments. It is becoming commonly accepted, in fact, that the molecular events acquired during myeloma progression are not acquired in a linear fashion but instead through branching, nonlinear pathways:[9] in line with this, therefore, it is now evident that the genetic complexity of myeloma is based on intraclonal heterogeneity at the level of a myeloma-propagating cell.[18,20-22] Unavoidably, this has (and will have) important effects on the clinical application of both standard and targeted treatment strategies, to which NGS might significantly contribute.[20,23]

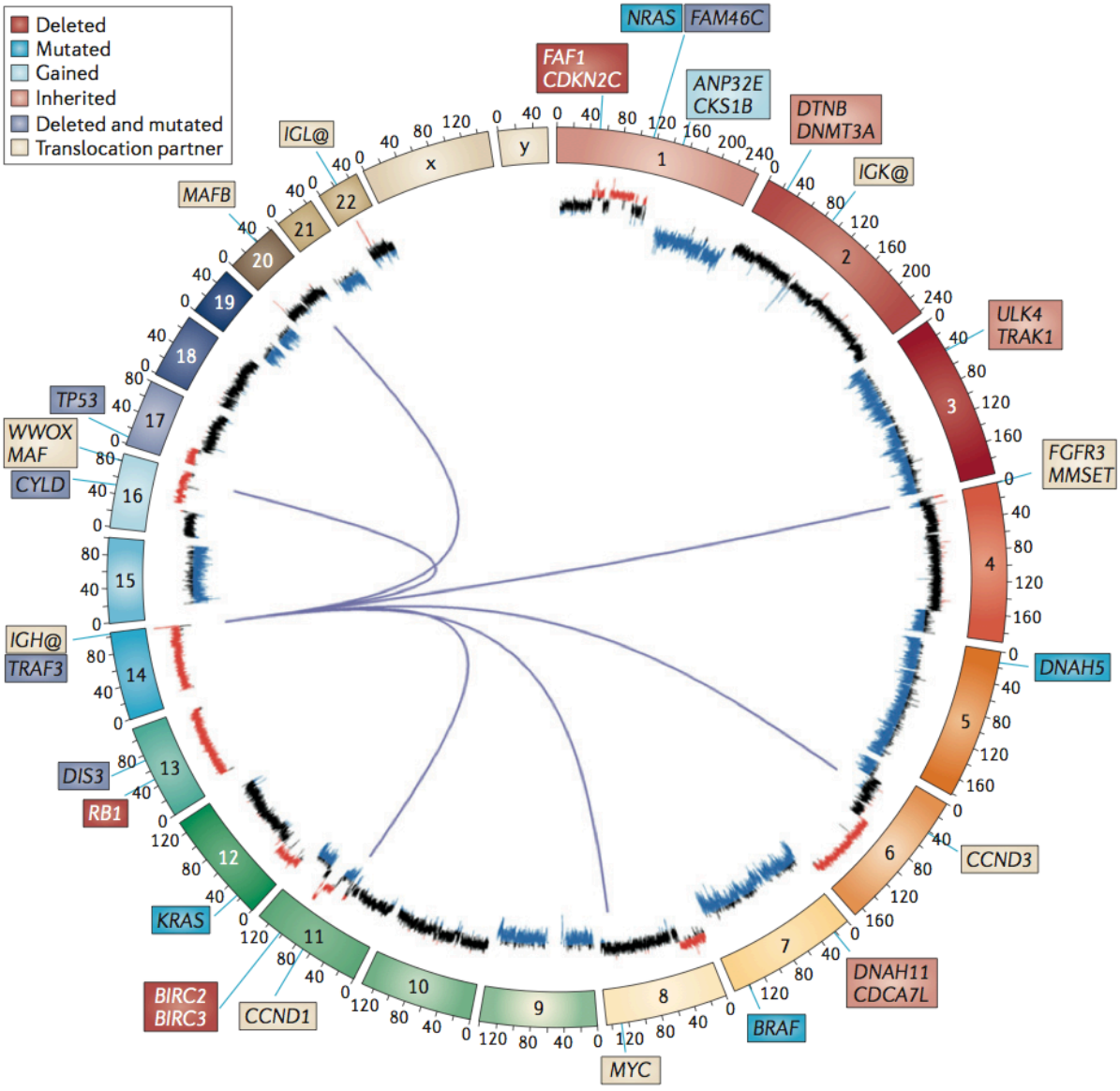

Figure 2. Circos plot showing the key translocations, copy number abnormalities and mutations frequently found in myeloma. The chromosomes are arranged clockwise around the circle. IGH@ translocations are represneted by inner lines emerging from chromosome 14 to the respective partner chromosomes. Copy number data are shown inside the circle: red indicates deletions, blue gains and black normal copy number. Genes targeted by deletions and/or mutations are labelled on the outside of the circle and coloured according to the abnormality as in the top-left legend (from Morgan, GJ, 2013, *Nature Rev*).

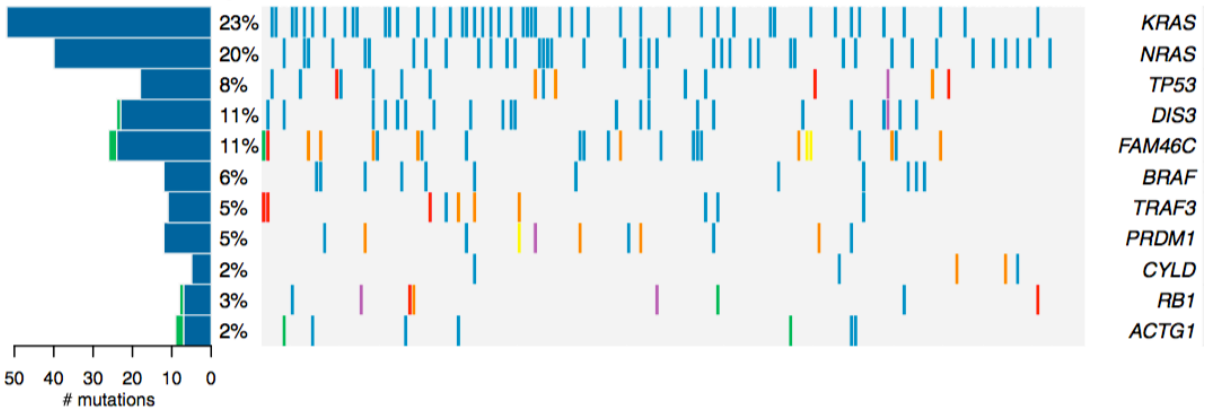

Figure 3. Exemplar heatmap representing individual mutations in a cohort of MM patient samples from Multiple Myeloma Resarch Consortium dataset (light-bliue: missense; red: non-sense; orange: frame-shift; yellow: in frame in-dels; brown: other non synonimous). On the left, the histogram shows the number of mutations in each gene; the percentages represent the fraction of tumors with at least one mutation in the specified gene (modified from Lohr, et al, 2014, Cancer Cell).

Finally, it is also worth mentioning that a characteristic feature of myeloma cells is the requirement for an intimate relationship with the BM microenvironment (Figure 4), where the occurrence of specialized plasma cell *niches* facilitates the growth of the myeloma clone. Several intercellular stimuli have been so far charcterized that sustain PC development in the BM: some of the critical survival and growth factors, such as IL-6, are produced by more than one kind of BM cell. External stimuli, such as hypoxia and internal signals that largerly involve (and result from) MYC dysregulation, stimulate the secretion of factors, e.g. HIF-1α or the pro-angiogenic VEGF, which in turn stimulate endothelial cells to secrete IGF-1. The altered

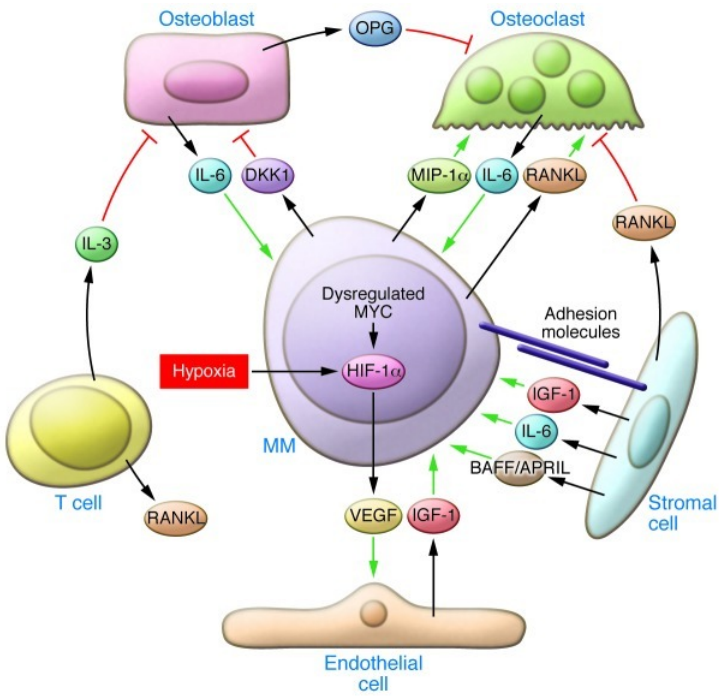

Figure 4. The interactions of MM tumor cells with the BM microenvironment (from Kuehl, M and Bergsagel, PL, 2012).

bone remodeling, hallmark of MM disease, is the effect of an increase in osteoclast activity – mediated by RANKL/RANK interactions, decreased osteoprotegerin (*OPG*), and increased *MIP-1α* – and a decrease in osteoblast activity – mediated by *DKK1* and *IL-3*. The increase in osteoclast activity stimulates the survival and growth of MM cells, at least partially by increased *IL-6*.[10,24,25] Acquiring independence of these interactions is fundamental in the immortalization of a myeloma-propagating cell.

Overall, MM poses a unique challenge for genotyping and expression profiling by virtue of its inherent heterogeneity, with the aim of a fine stratification and early identification of which patients are at risk of progression or relapse, or ultimately to provide the most effective therapeutic regimen to individual patients. Nevertheless, the complexity of the disease represents a serious obstacle to reaching these aims, which is further issued by intrinsic limitations and variability of high-throughput technologies.

Over the last few years, the high-throughput microarray technologies, particularly global gene expression (GEP) and genome-wide DNA profiling (GWP), have been widely used to investigate the genomic instability underlying the bio-clinical heterogeneity of the disease. Such approach led to promising results, either per se or when analyzed in an integrated fashion. In addition, the recent discovery of microRNA and their involvement in tumor, together with the application of microarray to define their expression profiling is offering an unprecedented perspective of the involvement of non-coding RNAs in MM tumor.

microRNA

MicroRNAs (miRNAs) are small (18-24 nucleotides), evolutionarily conserved non-coding RNAs that bind to the 3'untranslated region (UTR) of target mRNAs and lead to translation repression or mRNA degradation (Figure 5).[26]

They are transcribed by RNA polymerase II from genes located in extra- or intragenic genomic regions into primary precursors (pri-miRNAs) of different lengths. The expression of intronic miRNAs largely coincides with that of their corresponding host genes, thus suggesting that they share the same regulatory sequences.[27,28] Pri-miRNAs are processed into secondary precursors that have a stem-loop structure (pre-miRNAs) of approximately 70 nucleotides that are subsequently translocated to the cytoplasm by

exportins, where they are further processed into 18- to 24-nucleotide duplexes by the RNAase III enzyme DICER1. Only one strand (guide strand) is incorporated as mature miRNA into the RNA-induced silencing complex (RISC) that mediates target RNA cleavage or translational inhibition; the other (passenger) strand is degraded. The RISC leads to mRNA cleavage whenever the complementarity between the mature miRNA and the 3'UTR of the target mRNA is perfect; when the complementarity is partial, it inhibits protein translation. The major component of the RISC is the Argonaute 2 (AGO2) protein, which has RNA cleavage activity and is also involved in miRNA biogenesis.[29]

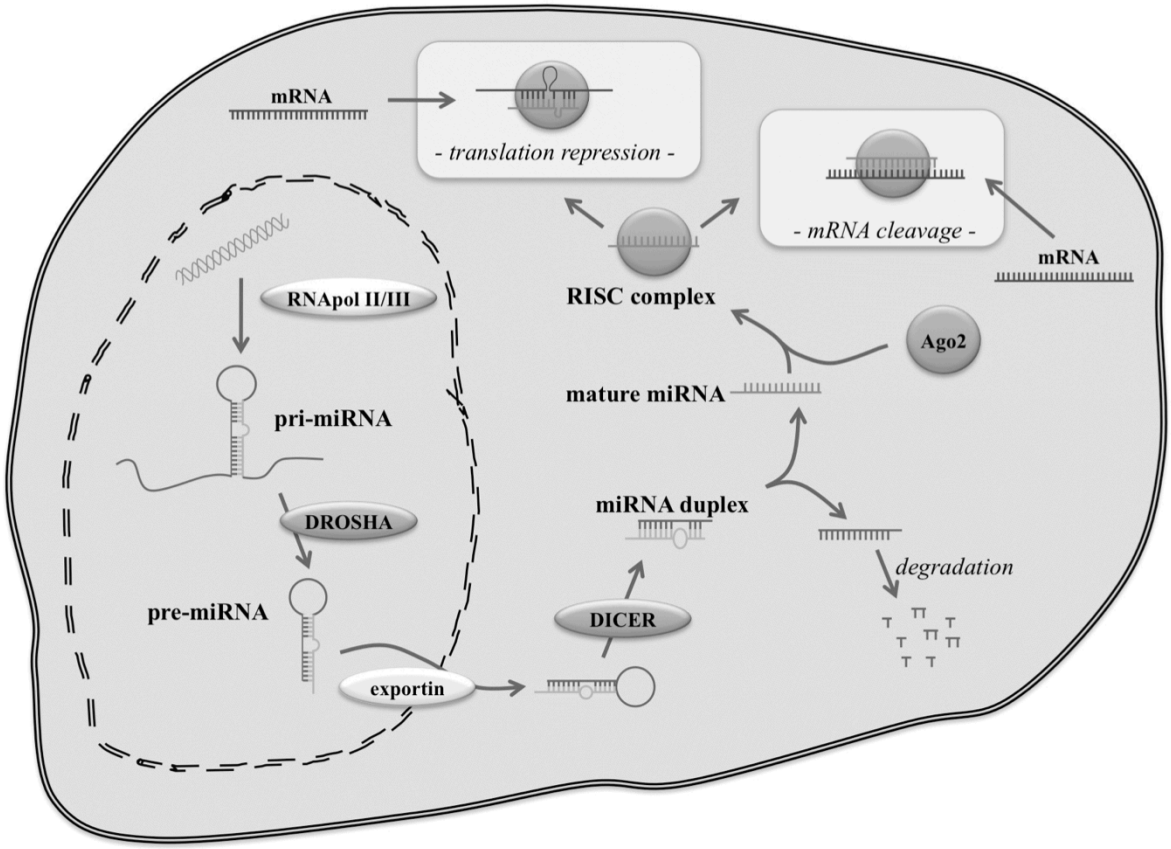

Figure 5. microRNA biogenesis (from Lionetti M, Agnelli L, *et al.* 2012)

One thousand, eight-hundred seventy-two human miRNA genes are currently included in the Sanger miRNA registry (miRBase version 20, June 2013), a database of miRNA sequences and annotations. A single miRNA can control the levels of hundreds of different target genes, and multiple miRNAs can regulate a single mRNA. Various interactions have so far been described, and a number of databases of the target genes of each of the known miRNAs are now available.[30] It has been estimated that more than 60% of human protein-coding genes are subject to miRNA regulation (Friedman *et al.*,

2009). Many of the known miRNAs are clustered in the genome, suggesting that they might work in combination to achieve their biological function. Moreover, a very large number of tools have been implemented as means of predicting targets based on sequence affinity, thus leading to the predicted interactions that represent a strong basis for further in-vitro validation.[31]

There is strong evidence indicating that miRNAs play important roles in cell processes such as proliferation, development, differentiation and apoptosis,[32] and it is therefore not surprising that their expression is profoundly deregulated in human.[33,34] The first direct link between miRNAs and cancer was highlighted by Calin *et al*., that found that the minimal deleted region of chromosome 13q14 in chronic lymphocytic leukemia (CLL) contained the genes encoding miR-15a and miR-16,[35] and that the two miRNAs were down-regulated in over two-thirds of the cases. Later, the same Authors also demonstrated that the miRNAs target BCL2, an anti-apoptotic oncogene that is often over-expressed in CLL.[36] Evidence that miRNAs play a causative role in tumorigenesis have accumulated rapidly; transcriptional deregulations, epigenetic alterations, mutations, DNA copy number abnormalities, and defects in the miRNA biogenesis machinery are all mechanisms contributing to miRNA deregulation in cancer, either alone or in combination.[37] In overall terms, the over-expression of miRNAs targeting one or more tumor suppressor genes (TSG) can inhibit anti-oncogenic pathways, whereas miRNAs defects in repressing one or more oncogenes may lead to a gain in oncogenic potency. Furthermore, mutations in the sequence of mature miRNAs or in the seed-matched sequence of their target mRNAs can reduce or eliminate the repression, and switch it towards other transcripts.[38] The oncogenic role of a number of miRNAs has been experimentally demonstrated in various tumors, including hematological malignancies, as is the case of the pro-tumorigenic cluster miR-17~92,[39] miR-155,[40] and miR-21.[41] Conversely, other miRNAs such as those of let-7 family have tumor suppressor activity.[42,43] Global miRNA expression profiling studies have revealed miRNA dysregulation in virtually all types of cancer and highlighted the usefulness of miRNA profiling in diagnosis and prognosis, and in predicting response to therapy.[44-50] It is not surprising, therefore, that miRNA expression profiles can also be used to classify cancer, often with a greater degree of accuracy than traditional GEP (Lu *et al*., 2005; Calin & Croce, 2006b).

MicroRNA in multiple myeloma biology

The involvement of miRNAs in MM pathogenesis was first described by Pichiorri *et al*., who profiled the miRNA expression of PCs in a restricted series of normal subjects, MGUS and MM samples, and a number of MM-derived cell lines (HMCLs).[51] The analysis led to the identification of different miRNA expression signatures in abnormal PCs that were associated with tumor progression; in particular, miR-181a (which is known to play a role in B and T cell differentiation), the oncogenic cluster miR-106b~25 (in particular miR-93, miR-106b, and miR-25), and miR-21 were all up-regulated in the MGUS and MM samples, whereas miR-32 and the miR-17~92 cluster (particularly miR-19a and miR-19b) were significantly up-regulated only in the MM samples, thus suggesting their possible role in disease progression. The miR-17~92 cluster miRNAs have been recently demonstrated as positively modulated by Myc and associated with poor prognosis (in particular, high expression of miR-17, miR-20 and miR-92 was linked to shorter progression free survival).[52] Functional assays on HMCLs have related the deregulated expression of some of these miRNAs to biological processes that are particularly relevant in MM pathogenesis: miR-181a and miR-181b, the miR-106b~25 cluster and miR-32 interact with the 3'UTR of *PCAF* gene, a regulator of TP53 activity in myeloma cells, whereas miR-19a and miR-19b target SOCS-1, a negative regulator of the *IL-6R/STAT3* pathway that may therefore play a role in the anti-apoptotic signal triggered by IL-6.[51] The relevance of miR-21 to MM was previously suggested by Loffler *et al*., who demonstrated that miR-21 transcription is controlled by IL-6 through a mechanism involving *STAT3*, and that its ectopic expression gives independence of the IL-6-growth stimulus.[53] MiR-21 was then very recently found to be involved in a positive regulatory loop with STAT3 by direct targeting the protein inhibitor of activated STAT3 (*PIAS3*).[54] It has been additionally reported that miR-21 expression was partially driven by NFkB signaling via myeloma cell adhesion to BMSCs and that suppression of miR-21 expression contributed to sensitize MM cells to dexamethasone and doxorubicin and reduce MM cells invasiveness.[55] Recent findings suggest that miRNAs are key players in controlling cell proliferation and differentiation by actively functioning in the TP53 tumor suppressor network.[56] In the context of MM (in which mutation- induced TP53 inactivation is rare), Kumar *et al*. found that the expression of two miRNAs identified as TP53 regulators (miR-25 and miR-30d)

was higher in MM patients than in healthy donors and, interestingly, that the TP53 transcript and miRNA levels were moderately inversely correlated.[57] The inhibition of the two miRNAs in a MM cell line with a wt TP53 gene increased TP53 protein levels and enhanced apoptosis. Pichiorri *et al*. investigated the TP53/MDM2 autoregulatory loop by exploiting a panel of HMCLs and primary tumors with or without inactivating TP53 mutations.[58] Using MDM2 inhibitors, they found that miR-192, miR-194 and miR-215 were only up-regulated in myeloma cells harboring a wt TP53, and demonstrated that they can be considered as TP53 targets. These miRNAs can affect TP53-dependent MM cell growth by inhibiting MDM2 expression; in addition, miR-192 and miR-215 specifically targeted the IGF pathway, thus preventing the enhanced migration of PCs into BM. These findings, together with the evidence of hypermetylation of the promoter region of the mir-194-2/192 cluster probably leading to their down-regulation in MM in comparison with MGUS samples, are of particular interest as TP53 function is retained in the majority of untreated patients and it might be susceptible to successful modulation.[59]

Recent studies have concentrated on the pathogenetic role of miR-15a and miR-16, which are located at 13q14 chromosomal region, that is frequently deleted in CLL, MM and MCL. These two miRNAs are abundantly expressed in multiple human tissues and are involved in anti-proliferative and pro-apoptotic activities, which suggests that they may represent candidate targets of the deletion. Roccaro *et al*. provided the first evidence of the functional role of miR-15a and miR-16 in MM by showing that the two miRNAs regulate the proliferation and growth of HMCLs in vitro and in vivo by inhibiting a number of molecular pathways, such as those involving AKT3, ribosomal protein S6, MAP kinases, and the NF-kB-activator MAP3K7IP3.[60] Furthermore, miR-15a and miR-16 inhibit MM cell-triggered endothelial cell growth and capillary formation in vitro and in vivo, and exert their anti-MM activity of inhibiting migration and growth of MM cells even in the context of the BM milieu, thus overcoming the growth advantage normally conferred by BM stromal cells. A possible explanation of this finding resides in that the suppression of miR-15a/16 expression (possibly mediated by IL-6 secretion) represents one of the mechanisms through which BM stromal cells provide survival support to MM cells. Interestingly, Hao *et al*. reported that melphalan and bortezomib treatment up-regulated miR- 15a/16 expression in MM cells, whereas the interaction of MM cells with BMSCs

inhibited miR-15a/16 expression and suppressed apoptosis of MM cells induced by cytotoxic agents.[61,62] These data strongly suggest the tumor suppressor activity of miR-15a and miR-16, although their role in disease development in primary MM patients and the impact of chromosome 13 deletion on their expression remain controversial. The specific down-regulation of miR-15a and miR-16 (regardless of the status of chromosome 13) was the starting point for Roccaro's study of their functional role in MM,[60] but Pichiorri *et al*. found that miR-15a (as well as other miRNAs mapped on 13q) were up-regulated in MM PCs vs healthy PCs.[51] As discussed later, our group have found a significant correlation between the expression of miR-15a (as well as miR-19a and miR-621) and alterations in the corresponding DNA locus in HMCLs (probably due to miRNA over-expression in aneuploid cell lines with more than two copies of chromosome 13)[63] but not in primary tumors, in which only the expression of miR-17 and miR-20a (13q31) was reduced in cases with 13q deletion.[64] Other Authors have reported varying levels of miR-15a/16[65] as well as of miR-17~92[52] expression in MM patients, regardless of chromosome 13 status. Finally, Gutierrez *et al.* found that, like miR-19a, miR-19b and miR-20a, miR-15a was down-regulated in the patients carrying the *RB* deletion alone in comparison with the normal PC samples or MM patients without this abnormality,[66] and Chi *et al*. reported the down- regulation of both miR-15a/16 and miR-17~92 cluster in del(13) cases.[67] Overall, although there is evidence indicating that miR-15a and miR-16 probably play a role in MM, their relationships with MM biology and the 13q14 deletion remain to be clearly defined.

MiRNA expression in different molecular types of MM

As stated above, MM is characterized by profound genomic instability involving both numerical and structural chromosomal aberrations. A number of global gene expression profiling (GEP) studies have clearly shown that distinctive gene expression signatures are associated with the major molecular subtypes.[68-78] Very recently, the potential impact of MM genetic lesions on miRNA expression has also been investigated using the same global analyses on the basis of evidence showing that genomic alterations frequently deregulate miRNA expression in human cancer.[79-82]

Applying an integrative genomic approach to a representative panel of 16 HMCLs

and in 19 primary MM tumors, Lionetti *et al.*[64] found that a large number of miRNA genes were located in genomic areas affected by allelic imbalances that may lead to significantly altered expression levels: these include mir-17 and mir-20a, which belong to a cluster at 13q31 deleted in almost 40% of patients; miR-140-3p at 16q22.1, a region that is recurrently involved in LOH and has prognostic and possible pathogenetic importance in myeloma;[83] and a number of miRNAs located in odd-numbered chromosomes involved in hyperdiploidy.

Information concerning the association between miRNA expression and the major genetic lesions in myeloma (particularly chromosomal translocations) is still limited and partly controversial. Lionetti *et al.* have provided evidence that the miRNA transcriptional profiles of 40 MM samples significantly grouped the patients according to the proposed TC (translocation and cyclin D) classification, particularly with regard to t(4;14) (TC4) and MAF-translocated (TC5) samples (Figure 6). In line with the consistency of these signatures within groups, it is worth noting that a leave-one-out cross-validation approach to the dataset showed that 13 miRNAs could classify the large majority of samples (up to 80%).[64] and unpublished results The most striking finding was the highly specific expression of three clustered miRNAs (miR-99b, let-7e, and miR-125a-5p) mapping to 19q13.33 in the t(4;14) cases. It has very recently been demonstrated that these miRNAs are particularly abundant in hematopoietic stem cells (HSCs), and that miR-125a mediates HSC expansion when expressed in primary cells. MiR-99a, let-7c and miR-125b, which are moderately over-expressed in the TC5 cases, belong to a paralogous cluster at 21q21.1, and it has been

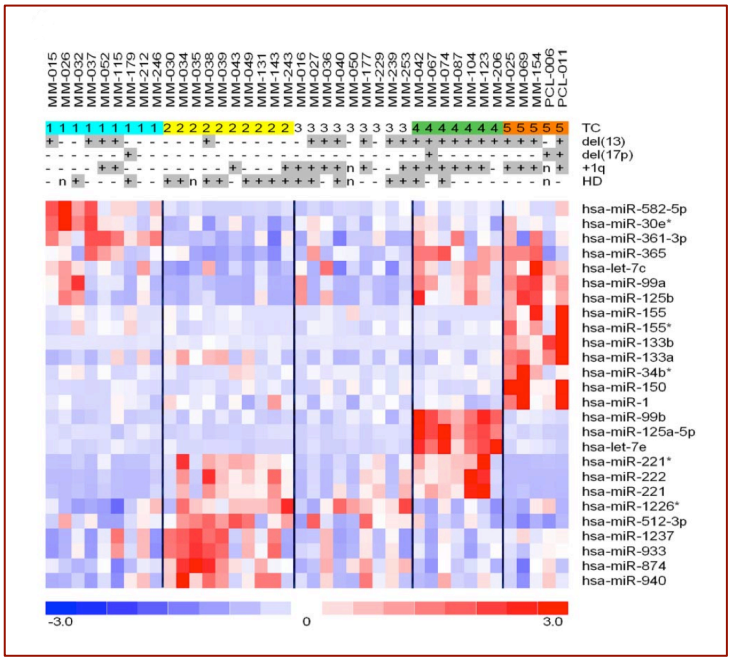

Figure 6. Identification of miRNA signatures characterizing TC classes (Lionetti et al., 2009b). Heatmap of the differentially expressed miRNAs in MM patients stratified into the 5 TC groups.

reported that miR-125b promotes B lymphocyte diversification in germinal centers by inhibiting the premature use of essential transcription factors for PC differentiation,[84] thus suggesting a pathogenetic role for miR-125b associated with impaired expression. Interestingly, none of the miRNAs significantly discriminating the TC groups associated with IGH translocations was localized in the chromosomal regions specific for the corresponding cytogenetic abnormality, which suggests that mechanisms other than those directly deregulating mRNA expression in translocation events may modulate miRNA expression. The integrative analysis defining a network of putative functional miRNA-target regulatory relations, which was based on computational target prediction and supported by miRNA/mRNA expression data, reinforced the suggestion that some of the miRNAs differentially expressed in IGH translocated cases may play important roles in the biology of MM PCs. As many as five of the seven miRNAs associated with t(4;14) target CBFA2T2, a nuclear repressor homologous to ETO that binds to the AML1-ETO complex and may play a role in hematopoietic differentiation,[85,86] and let-7e targets PTPRE, a positive regulator of osteoclast function and a selective inhibitor of IL-6- and IL-10-induced JAK-STAT signalling[87,88].

Using different technical and analytical approaches to a similarly representative panel of patients, Gutierrez *et al*. identified a number of miRNAs that were differentially expressed in myeloma cells from t(4;14), t(14;16), t(11;14), RB-deleted (as a single abnormality) or cytogenetically normal patients, as compared with healthy controls.[66] Only two (miR-214 and miR-375) were commonly deregulated in the myeloma samples regardless of cytogenetic characteristics. Confirming previous data,[64] miR-1 and miR-133a were over-expressed in patients with the t(14;16) chromosomal translocation, a finding that strengthens the hypothesis of a link between the deregulation of *MAF* genes and the expression of this miRNA cluster. By combining the miRNA and mRNA expression data from the same samples, Gutierrez *et al*. also identified putative target genes with important roles in cancer or the control of MM cell biology, such as *CCND2*, which was up-regulated in MM subtypes t(4;14), t(14;16) and monosomy 13, and has target sites for a number of the miRNAs that are significantly deregulated in these cytogenetic subtypes. This suggests that the underexpression of various miRNAs found in many MM cases may be responsible for the increased CCND2 expression.

Finally, two recent works did not confirm extensively previous data concerning

specific patterns of miRNA expression associated with known molecular characteristics in MM patients, most likely because of the relatively small number of patients, or subgroup of, included in the series investigated by the Authors.[67,89] In particular, clustering analysis in the paper by Cothals *et al*. showed that miRNA expression classified MM patients into subgroups which could not be distinguished on the basis of other known characteristics, whereas Chi *et al*. described miRNA transcripts which may be deregulated in the transition from normal to MGUS or MM presentation. In both of these papers the Authors described miRNAs associated with clinical outcome, although in the paper by Corthals *et al*. the associations did not reach significance after multiple test corrections. Chi *et al*. found that the expression of miR-153, miR-490, miR-455, miR-642, miR-500, miR-596, miR-548d, miR-373, miR-554 and miR-888 was related to event-free survival.

MiRNA expression in the prognostic stratification of myeloma.

Zhou *et al*. analyzed the miRNA expression profiling in two healthy donors and 52 newly diagnosed MM patients, and concluded that higher total miRNA expression levels (calculated as the mean expression levels of expressed miRNAs) might be associated with the onset of MM.[90] Among the pathological samples, unlike those of other types of cancer,[91-94] higher total miRNA expression seemed to be associated with a worse outcome as it was positively associated with the same Authors' GEP-defined high-risk score (RS) and proliferation index (PI).[78] In line with this, the RS and PI were associated with the expression of a number of miRNAs, four of which (miR-106a, miR-106b, miR- 17-5p and miR-20b) target the tumor suppressor gene *p21CIP1* in HMCLs. The putative role of miRNA over-expression in myeloma progression was suggested by the silencing of *EIF2C2*/*AGO2* and *DICER1* in HMCLs, which led to decreased cell viability, cell cycle arrest and the induction of apoptosis. The Authors also found that, although associated with a bad prognosis in MM, *AGO2* expression did not correlate with total miRNA expression in the primary tumor samples, suggesting that *AGO2* may not be the only biogenetic factor affecting the global expression of miRNAs in myeloma cells. A more recent study of the expression of *DICER1* and *DROSHA* genes in patients with MGUS, smoldering and symptomatic MM found that *DICER1* was less expressed in the symptomatic MM cases

than in healthy donors or MGUS patients;[95] furthermore, among the symptomatic patients, median progression-free survival was significantly longer in those with higher *DICER1* expression levels. As recent evidence suggests that alterations in *DICER1* may lead to altered miRNA expression levels,[96] an apparent disagreement emerged: the first study directly correlated higher total miRNA expression with poorer outcomes, whereas the second found that lower *DICER1* expression levels were a marker of shorter progression-free survival. However, the miRNA biogenesis should be considered as a multi-step process and an alteration in only one of its components is probably insufficient to alter miRNA expression as a whole: further studies are warranted to clarify the contribution of each of the components involved in miRNA biogenesis, and elucidate how other genetic or epigenetic factors can lead to the selectively altered expression of particular miRNAs. Another very recent evidence links the miRNA processing machinery to MM prognosis: a single nucleotide polymorphism (SNP) in exportin-5 (*XPO5*), rs11077, has been in fact associated with significant longer PFS and OS in MM patients after ASCT:[97] this SNP affected protein translation in HMCLs, perhaps through the introduction of a binding site for a miRNA. In the same paper, the impact of miRNAs on MM prognosis was also inferred by the observation that *KRT81* rs3660 C/C variant was associated with significantly longer OS: this polymorphism in the 3'UTR region of the gene, facilitating the binding of some miRNAs reported to be up-regulated in MM, produced a reduction in protein levels that correlated with lower proliferation in HMCLs.

Aim fo the study

In the present work, based on the findings of microRNA deregulation in abnormal PCs and the raising evidence of their association with outcome, we test the hypothesis that some patterns of miRNA expression in myeloma could correlate with survival, aimed at providing new molecular biomarkers of outcome.

To this aim, we analyzed the miRNA expression profiles of a large, prospective and representative panel of 163 primary tumors included in the phase 3 multicenter factorial design Myeloma Research Council (MRC) Myeloma IX clinical trial, and correlated the expression with overall and progression free survival. Furthermore, we evaluated whether a miRNA-based classifier might be proficiently used to stratify high risk myeloma patients independent of and integrating other prognostic risk stratifications (based on International Staging System, cytogenetics and gene expression).

Finally, we took advantage of the corresponding gene expression and genome-wide profiles available for the patients included in the dataset to evaluate the putative relationships with the transcriptional and genomic *milieu*.

Materials and methods

Patient samples

Bone marrow aspirates were obtained from newly diagnosed myeloma patients in the MRC Myeloma IX study during standard diagnostic procedures following informed consent. The study was approved by the National Research Ethics Service (MREC 02/8/95) and registered under ISRCTN68454111. The design, patient evaluation and end points of this trial have been extensively reported previously:[98] in summary, the trial recruited 1960 patients, who were allocated to two main treatment pathways (intensive or non-intensive) at the discretion of the treating physician taking account of the age and performance status. The median follow-up of this trial at the time of the present study (november, 2011) was 5.9 years. 153 patients with GEP have been stratified according to the TC classification described previously.[73]

miRNA profiling

Plasma cells were selected to a purity of >90% as determined by both microscopy and flow cytometry, from bone marrow aspirate samples using CD138 magnetic bead sorting, subsequently small RNA was extracted and enriched using a modified protocol for Qiagen Allprep kit or Trizol Invitrogen kit. miRNA expression profiling was then carried out in 185

cases according to Affymetrix recommended protocol. Briefly, the enriched small RNA was processed using the FlashTag labeling kit, which uses a tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The labelled RNA was then hybridized to Affymetrix GeneChip® microRNA arrays v1.0 and scanned using a GeneChip® scanner 3000 7G. Expression values for 847 human miRNAs were extracted from CEL files using Affymetrix miRNA QC tool software (RMA normalized and log2-transformed). After quality control using R package affyPLM, 163 samples were included based on the metrics resulting from NUSE (Normalized Unscaled Standard Error) analysis, namely showing values less than 1.05. The microarray data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE41276. The patients included in the miRNA expression analysis were representative of patients entered into the overall trial (Table 1).

Table 1. Demographic, serological and molecular features of the patients included in the miRNA expression analysis compared with those who entered into the overall trial. No significant differences at $P < 0.05$ were evidenced.

Characteristic	miRNA Expression (n=163)	Myeloma IX (n=1960)
Age, years		
Mean	64.7	64.6
SD	10.0	10.2
Serum β2 microglobulin (β2M), mg/l		
Mean	5.6	6.1
SD	4.0	5.9
Total patients	116	1789
Serum albumin (Alb), g/l		
Mean	34.7	34.7
SD	6.96	7.0
Total patients	163	1858
Haemoglobin (Hb), g/dl		
Mean	10.7	10.8
SD	1.82	4.3
Total patients	163	1880
Deletion 13q		
%	40.8	45.3
No. patients	60	473
Total patients	147	1043
Gain 1q21		
%	40.2	38.9
No. patients	58	351
Total patients	144	902
t(4;14)		
%	14.5	11.4
No. patients	22	120

Characteristic	miRNA Expression (n=163)	Myeloma IX (n=1960)
Total patients	152	1052
Deletion 17p		
%	7.5	8.4
No. patients	11	85
Total patients	147	1015
t(11;14)		
%	16.5	13.9
No. patients	25	146
Total patients	151	1047
Hyperdiploidy		
%	58.4	57.7
No. patients	87	578
Total patients	149	1002
Treatment pathway, % of patients		
Intensive	57.1	56.7
Non-intensive	42.9	43.3

Gene Expression and Copy Number Analysis

Gene expression profiling (GEP) of 261 samples were generated on Affymetrix HG-U133 Plus 2.0 arrays,[99] and the expression values were RMA normalized and log2-transformed. Affymetrix GeneChip Mapping 500K Array sets were performed as described in Dickens *et al.*.[99] Briefly, loss-of-hetozigosity (LOH) and copy number data were extracted from the raw feature intensities using the Affymetrix GCOS software (version 1.4.0). The tumor copy number values were then inferred against normal germ-line counterpart, for each sample, using Affymetrix GTYPE software and dChipSNP.[100]

Finally, 153 of GEP samples and 72 genotyping samples have miRNA profiling data available for integrative analysis. The associated microarray datasets have been deposited into GEO under accession number GSE15695.

Quantitative RT-PCR

The expression of miR-886-5p, miR-17 and miR-18a was analyzed in purified CD138+ cells by means of real-time quantitative polymerase chain reaction (Q-RT-PCR) using the TaqMan® microRNA assays (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's protocol. The measurement of transcript expression was performed

using the Applied Biosystems StepONE Real-Time PCR System. All of the RNA samples were run in duplicate and normalized on the basis of the expression of miR-103.[101] The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. All signals with Ct≥ 35 were manually set to undetermined. Data were expressed as 2-ΔCt (Applied User Bulletin No. 2).

Statistical analysis

All analyses were performed in R 2.12.2 and Bioconductor 2.7. Multivariate Cox regression analyses were conducted to investigate the association of miRNA expression with progression-free (PFS) and overall survival (OS), where the expression level of each miRNA were used as continuous variable and treatment pathways (intensive or non-intensive) as covariate. miRNAs with a p-value less than 0.05 were selected as being associated with survival irrespective of treatment pathway. For robustness, only the miRNAs that remained significant after being corrected for multiple testing (Benjamin and Hochberg's method, $P < 0.05$) were used to construct an outcome classifier to divide patients into different risk groups. The distribution of OS between risk groups of patients was estimated using the Kaplan-Meier method (log-rank test). Internal cross-validation was performed via bootstrapping on the final prognostic model (1000 replications). The independence of the risk groups defined by miRNA expression from other important risk predicting factors was tested by using multivariate Cox regression. Performance of predictive models was compared by likelihood-ratio test (R package *anova.coxph*). The association of trend between the ISS/FISH risk groups and the miRNA-derived risk groups was investigated using linear by linear association test (R package *coin*).

Differentially expressed miRNAs between a particular TC subgroup of interest and the other subgroups were selected using significance analysis of microarray (SAM) (Bioconductor package *samr*), with a 1000-permutation adjustment and 5% false discovery rate (FDR).

As stated above, among the 163 samples there were also 72 cases with SNP-based mapping array data and 152 cases with fluorescence in situ hybridization (FISH) results.

Integrative analyses were carried out to explore the mechanisms of miRNA deregulation. Either Wilcoxon or Kruskal-Wallis test was used to look at the associations between miRNA expression levels and corresponding copy number values, as well as FISH lesions.

miRNA target prediction and correlation with gene expression

In order to be identified as putative targets of a particular miRNA, genes have to fulfil the following criteria: (1) the targets are predicted by at least 3 of the 11 programs in miRecords,[102] a resource for animal miRNA-target interactions which integrate the following target prediction tools: DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan; (2) the targets are statistically associated with OS ($P < 0.05$). The association between GEP and OS was tested following the same workflow as described above, to produce two lists of genes associated with shorter OS (n=1569) and longer OS (n=1311) respectively; and (3), a significant inverse Pearson correlation needs to be identified between the expression of a miRNA and its targets ($P < 0.05$). Correlation analyses between gene expression and miRNA expression was carried out among the 153 patients where GEP was available; only those interactions with negative correlation coefficients (r) were selected.

Results

Identification of miRNA expression profiles associated with the prognostic groups based on TC classification

In an initial analysis of the dataset, we assessed the miRNA expression patterns in TC classification groups with prognostic relevance (4p16, MAF and 11q13 groups). In the 153 cases with both GEP and miRNA profiling data, there were 26 cases of 4p16, 7 cases of MAF, 26 cases of 11q13, 42 cases of D1, 31 cases of D2, 12 cases of D1+D2, 1 case of D3 and 8 cases of unknown classification; subsequently, the single D3 case and the unclassified cases were excluded from further analyses. We performed a one-to-one comparison between the test groups with the other major subtypes using SAM in 144 cases. The resulting lists were examined for intersections to find the miRNAs consistently being upregulated (or downregulated) in the subgroup of interest (Table 2).

The 144 samples were grouped into 4p16, 11q13, MAF and others (comprising D1, D2 and D1+D2) based on TC classification, and the expression characteristics of the 4 subgroups were visualized using a heatmap (Figure 7). A distinct upregulation of the miRNA cluster 99b/let-7e/125a on 19q was identified in TC 4p16 cases, as well as miR-150/miR-155/miR-34a upregulation in MAF subgroup, largely confirming what has been seen previously by us in a smaller series. [64] In addition, upregulation of miR-1275 and downregulation of miR-138 were observed in 11q13 cases.

Table 2. The specific deregulation of miRNAs in (A) 4p16, (B) MAF and (C) 11q13 subtypes compared to other subtypes (SAM, FDR < 0.05 based on 1000 permutations). The reported values represent the miRNA's fold changes in the subgroup of interest compared to 5 other major subgroups individually (>1 for upregulation, <1 for down regulation).

(A)

miRNA	4p16 vs. 11q13	4p16 vs. D1	4p16 vs. D2	4p16 vs. D1+D2	4p16 vs. MAF	Cytoband
hsa-let-7e	3.806016	4.511331	4.771102	4.179846	5.073125	19q13.41
hsa-miR-125a-5p	7.616727	7.868159	7.854732	6.590528	9.236523	19q13.41
hsa-miR-99b	2.736672	3.085145	3.022427	2.920532	3.194268	19q13.41

(B)

miRNA	MAF vs. 11q13	MAF vs. D1	MAF vs. D2	MAF vs. D1+D2	MAF vs. 4p16	Cytoband
hsa-miR-155	6.817351	13.68937	20.93044	17.78436	21.58904	21q21.3
hsa-miR-34a	5.794281	7.291856	5.715695	5.865755	3.979102	1p36.22
hsa-miR-150	12.42424	10.69551	8.258373	5.252736	5.208516	19q13.33

(C)

miRNA	11q13 vs. D1	11q13 vs. D2	11q13 vs. D1+D2	11q13 vs. 4p16	11q13 vs. MAF	Cytoband
hsa-miR-1275	1.84345	2.729543	2.90606	1.955498	2.529239	6p21.31
hsa-miR-138	0.081537	0.11135	0.098762	0.212333	0.173282	16q13/3p21.32

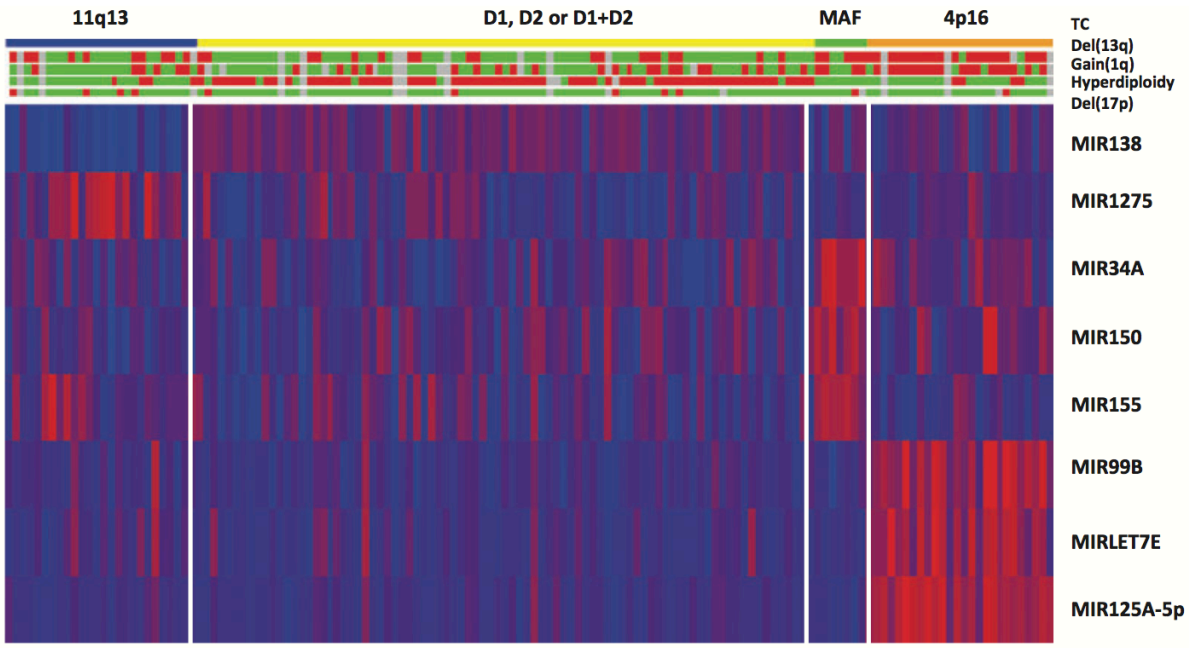

Figure 7. MicroRNA signatures for TC classification: the heatmap shows the distinct expression characteristics of the favourable 11q13 group, unfavourable 4p16 and MAF groups in contrast to the rest of cases (D1, D2, D1+D2), according to the 8 differentially expressed miRNAs. The TC subgroups and FISH abnormalities (green, no chromosomal abnormality; red, chromosomal abnormality; grey, not known) are shown in colours above the heatmap. The colour scale bar in the heatmap represents the relative miRNA expression with red representing upregulation and blue representing downregulation.

miRNA expression associated with OS

After removal of those miRNAs with a percentage detection call (defined by Affymetrix QC Tool) of less than 2% across the samples, the expression values of 38 miRNAs were identified as being associated with OS as continuous variables according to Cox regression analyses ($P < 0.05$, **Table 3**). Three clusters of miRNAs located at cytobands 13q31.1 (mir-17~92: miR-17, miR-18a, miR-20a, miR-19b-1, miR-92a-1), Xq26.2 (mir-106a~363: miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2) and Xq26.3 (miR-503, miR-542-5p, miR-424-star) were identified as being associated with survival, comprising 13 of the 38 potentially deregulated miRNAs. Clusters mir-17~92 and mir-106a~363 are of particular interest, as the association between members of these clusters and OS remained significant or borderline significant ($P < 0.06$) after being corrected by multiple testing (Benjamin and Hochberg's method).

Table 3. miRNAs associated with OS ($P < 0.05$) (N=38). 24 of them were upregulated in shorter survivors (HR > 1.0) and 14 were downregulated (HR < 1.0). Those labelled * remained significant after correction for multiple testing.

miRNAs	P value	P value after multiple testing correction	Hazard Ratio (HR)	Cytoband
*** hsa-miR-886-5p**	.0002	.0385	1.74	5q31.1
*** hsa-miR-18a**	.0002	.0385	1.39	13q31.1
*** hsa-miR-17**	.0003	.0385	1.54	13q31.1
hsa-miR-501-3p	.0005	.0506	2.21	Xp11.23
hsa-miR-1260	.0006	.0506	0.31	14q24.3
hsa-miR-18b	.0007	.0506	2.24	Xq26.2
hsa-miR-106a	.0008	.0527	1.52	Xq26.2
hsa-miR-17-star	.0031	.1762	1.45	13q31.1
hsa-miR-339-3p	.0048	.2090	0.75	7p22.3
hsa-miR-503	.0050	.2090	1.57	Xq26.3
hsa-miR-92a	.0056	.2090	1.28	13q31.1/Xq26.2
hsa-miR-20a	.0056	.2090	1.35	13q31.1
hsa-miR-20b	.0061	.2115	1.38	Xq26.2
hsa-miR-129-3p	.0111	.3477	2.49	7q32.1/11p11.2
hsa-miR-19b	.0116	.3477	1.30	13q31.1/Xq26.2
hsa-miR-494	.0124	.3482	1.64	14q32.31
hsa-miR-575	.0137	.3617	1.43	4q21.22
hsa-miR-615-3p	.0153	.3833	0.40	12q13.13
hsa-miR-31	.0179	.4156	1.78	9p21.3
hsa-miR-1308	.0188	.4156	1.20	p22.11
hsa-miR-629	.0201	.4156	1.62	15q23
hsa-miR-542-5p	.0203	.4156	2.32	Xq26.3
hsa-miR-424-star	.0217	.4239	1.60	Xq26.3
hsa-miR-891b	.0247	.4627	0.43	Xq27.3
hsa-miR-152	.0258	.4651	0.85	17q21.32
hsa-miR-155	.0300	.5108	0.91	21q21.3
hsa-miR-890	.0306	.5108	1.65	Xq27.3
hsa-miR-1271	.0353	.5387	2.07	5q35
hsa-miR-650	.0361	.5387	1.21	22q11.22
hsa-miR-886-3p	.0374	.5387	1.28	5q31.1
hsa-miR-16-1-star	.0376	.5387	0.40	13q14.2
hsa-miR-541	.0383	.5387	0.49	14q32.31
hsa-miR-1324	.0418	.5698	0.49	3p12.3
hsa-let-7g-star	.0449	.5757	0.54	3p21.1
hsa-miR-491-5p	.0455	.5757	0.63	9p21.3
hsa-miR-216b	.0468	.5757	0.54	2p16.1
hsa-miR-92a-2-star	.0481	.5757	0.56	Xq26.2
hsa-miR-570	.0486	.5757	0.67	3q29

Some miRNAs located within these two clusters are homologous in sequence and, therefore, are classified as members of the same family (Figure 8). The miRNAs within these clusters were co-expressed in our data, suggesting that they are subject to common regulatory mechanisms. An exception is miR-363 located within the mir-

106a~363 cluster, which might be due to the presence of an alternative/additional regulatory mechanism. The expression levels of miR-17 and miR-106a, despite belonging to separate clusters located on different chromosomes, are highly correlated (r = 0.94). The higher expression of miR-886-5p and miR-886-3p, originating from opposite arms of the same pre-miRNA, are both associated with shorter OS.

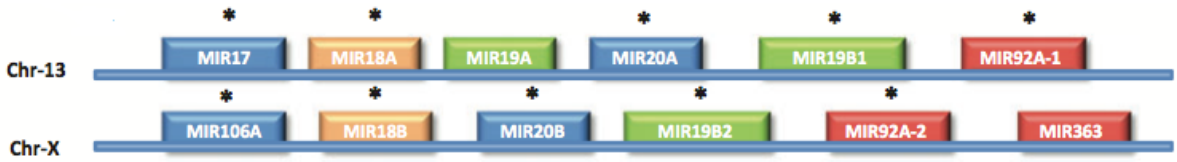

Figure 8. *MIR17HG* and *MIR106~363* clusters and their roles in myeloma pathogenesis: the scheme depicts the genomic structure of the miRNA clusters located on chromosomes 13 and X. The colours indicate sequence homology between the individual miRNAs. miRNAs labelled with * are upregulated in short survivors (*P* < 005).

miRNA expression associated with PFS

Using the same work-flow as for OS, 35 miRNA were identified being associated with PFS. Although after correction for multiple testing none of these miRNAs remained significant, it is worth mentioning that the members of the cluster at 19q13 (let-7e, miR-125a-5p and miR-99b) being identified strongly associated with TC 4p16 were among the top 6 most differentially expressed miRNAs associated with PFS, adding further evidence to the global prognostic importance of the t(4;14) in myeloma (Table 4).

Table 4. miRNAs associated with PFS (unadjusted $P < 0.05$).

miRNA	P value	P value after multiple testing correction	HR	Cytoband
hsa-miR-339-3p	0.00017	0.07534	0.701153	7p22.3
hsa-miR-99b	0.001165	0.220564	1.461024	19q13.41
hsa-miR-125a-5p	0.00161	0.220564	1.249882	19q13.41
hsa-miR-9-star	0.001992	0.220564	3.360287	5q14.3/15q26.1/1q22
hsa-miR-650	0.003322	0.294368	1.27696	22q11.22
hsa-let-7e	0.004263	0.314777	1.246946	19q13.41
hsa-miR-1285	0.005716	0.326173	2.286495	2p13.3/7q21-q22
hsa-miR-551b-star	0.00589	0.326173	2.934288	3q26.2
hsa-miR-409-3p	0.00753	0.367169	0.409305	14q32.31
hsa-miR-491-5p	0.008526	0.367169	0.578563	9p21.3
hsa-miR-1202	0.009117	0.367169	1.523442	6q25.3
hsa-miR-512-5p	0.011773	0.434629	2.364608	19q13.42
hsa-miR-25-star	0.01562	0.480018	1.642398	7q22.1
hsa-miR-338-3p	0.016025	0.480018	0.493484	17q25.3
hsa-miR-194	0.016442	0.480018	1.406287	11q13.1/1q41
hsa-miR-200c	0.01788	0.480018	1.303194	12p13.31
hsa-miR-548p	0.018421	0.480018	2.79399	5q21.1
hsa-miR-151-3p	0.023515	0.564778	1.856842	8q24.3
hsa-miR-135a-star	0.024496	0.564778	2.357257	3p21.1/12q23.1
hsa-miR-548i	0.025498	0.564778	0.472353	8p23.1/3q21.2/4p16.1/Xq21.1
hsa-miR-339-5p	0.02788	0.588126	0.768534	7p22.3
hsa-miR-626	0.029375	0.591516	0.436553	15q15.1
hsa-miR-664	0.033687	0.599703	0.458207	1q41
hsa-let-7a-star	0.033988	0.599703	0.476679	9q22.32/22q13.31/11q24.1
hsa-miR-1295	0.034652	0.599703	2.315558	1q24.3
hsa-miR-150-star	0.036524	0.599703	1.45902	19q13.33
hsa-miR-744	0.038362	0.599703	1.299559	17p12
hsa-miR-503	0.039966	0.599703	1.33183	Xq26.3
hsa-miR-139-5p	0.040541	0.599703	1.88743	11q13.4
hsa-miR-483-5p	0.040612	0.599703	1.7496	11p15.5
hsa-miR-125b	0.044886	0.618771	1.108454	21q21.1/11q24.1
hsa-miR-16-1-star	0.04525	0.618771	0.453842	13q14.2
hsa-miR-589	0.047881	0.618771	0.53818	7p22.1
hsa-miR-606	0.048302	0.618771	0.619731	10q22.2
hsa-miR-18b-star	0.048887	0.618771	1.918677	Xq26.2

Construction of a miRNA-based classifier for OS

Three upregulated miRNAs (miR-886-5p, miR-17 and miR-18a) were significantly associated with OS after correction for multiple testing (Benjamin and Hochberg's method, $P < 0.05$). The expression of these miRNAs was validated by means of Q-RT-PCR in a fraction of samples (58 cases). A very good concordance with microarray data was found for all transcripts (Pearson correlation coefficients of the expression of each

miRNA as determined by microarray or Q-RT-PCR were 0.86, 0.72 and 0.75 respectively). Unsupervised K-means clustering was applied to each miRNA across 163 samples to define a threshold splitting samples with higher expression from those with lower expression (Figure 9).

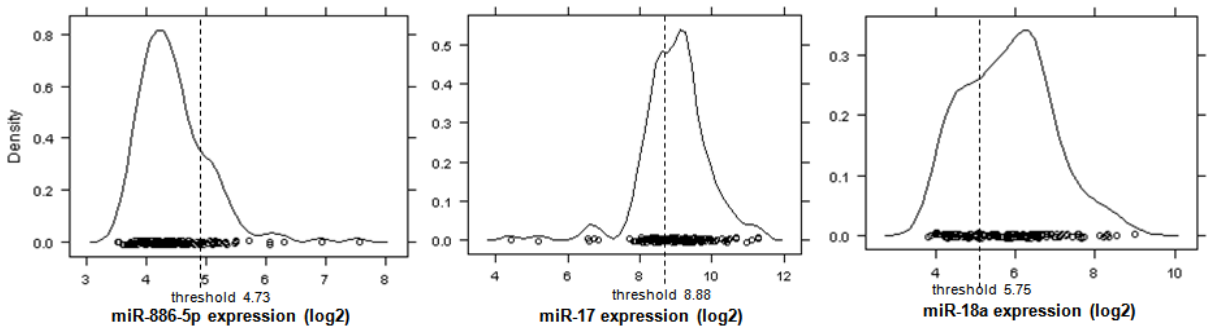

Figure 9. Density plots showing expression pattern of miR-17, miR-18a and miR-886-5p across 163 samples and the thresholds for high and low expression.

After stepwise selection in a multivariate Cox regression model, using treatment pathway as a covariate, miR-886-5p and miR-17 were shown to have the strongest discriminative power for OS (Figure 10); consequently these two miRNAs were used to construct an outcome classifier.

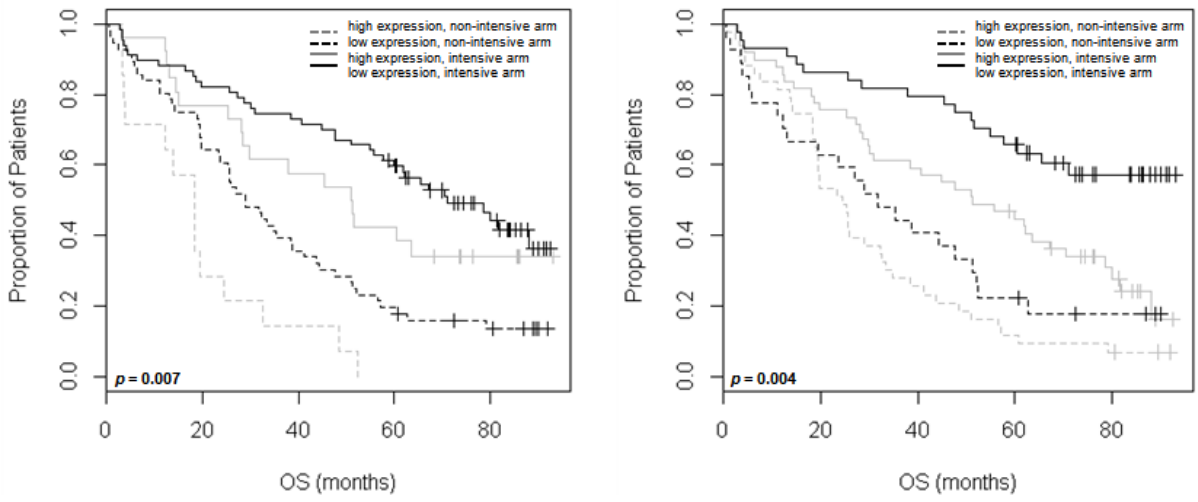

Figure 10. Kaplan-Meier estimated curves of the groups defined by high/low expression levels of miR-886-5p (left panel) and miR-17 (right panel), which is consistent across both treatment pathways.

The proportion of samples defined as having higher expression of miR-886-5p and miR-17 were 24.5% and 56.4% respectively. Based on the expression levels of these two miRNAs, 163 patients were divided into three groups: a high risk group (both expression levels high) comprising 13.5% of the patients, a median risk group (either high) comprising 54% cases and a low risk group (both low) comprising 32.5% cases (Figure 11A). These three groups have significantly differential OS (log-rank test *P* = 0.001, median OS 19.4 months vs. 40.6 months vs. 65.3 months). The median risk group and high risk group have a hazard ratio (HR) of 1.79 (95% Confidence Intervals [CI]: 1.15-2.78) and 2.89 (95% CI 1.60-5.20), respectively, relative to the low risk group. The stability of the miRNA-based classifier was assessed using bootstrap resampling. Based on 1000 replicates, the mean significance was 0.004 with a standard error of 0.02, and the majority (98.4%) of the *P* values were less than 0.05. It is not surprising that the OS classifier based on the expression of two miRNAs was not associated with PFS in this dataset, since neither miR-17 nor miR-886-5p expression was associated with PFS; although the pathogenic role of mir-17~92 in myeloma has been well demonstrated.[51,103] To exclude the possibility that the association of miRNA classifier with OS was due to non-myeloma-related mortality, 22 cases who died from reasons other than progressive myeloma (mostly other cancers, heart disease, stroke and infection) were censored at the time of death. The results showed that the three risk groups still had significantly differential effect on myeloma-specific survival (log-rank test *P* = 0.002, median survival 28.2 months vs. 51.5 months vs. not reached). Further analysis on post-relapse survival for 141 cases who relapsed before or at the time of death showed a strikingly differential effect among the three risk groups (log-rank test *P* = 2.4x10-7, median survival 6.1 months vs. 18.1 months vs. 35.1 months, Figure 11B), which largely accounted for its impact on OS while lacking the significance on PFS.

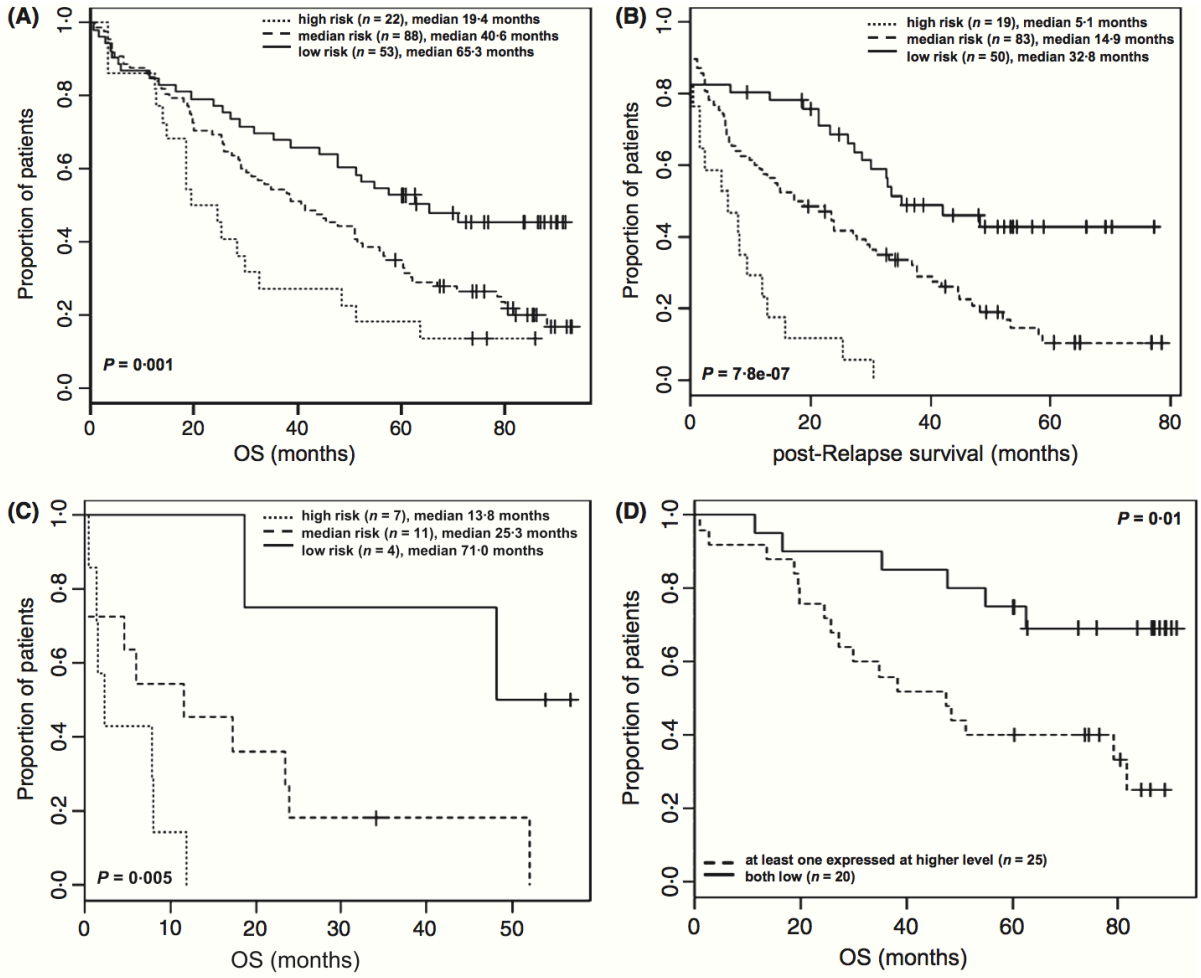

Figure 11. Survival of patients was stratified according to the miRNA-based classifier. **(A)** Patients (N = 163) were divided into three groups: high risk (both miRNA expression high), median risk (either high) and low risk (both low) based on expression levels of miR-17 and miR-886-5p. **(B)** Further analysis on post-relapse survival for 141 relapsed cases showed a remarkable differential effect among the three risk groups. **(C)** The miRNA-based classifier is also able to identify subgroups within 22 t(4;14) cases, which had differential OS (median 13.8, 25.3 and 71.0 months respectively, *P* = 0.005). **(D)** In 45 patients classified as being at low risk by ISS plus FISH abnormalities those with high expression of at least one of the two miRNAs (miR-17 and miR-886-5p) have shorter OS compared to the rest of the cases (median 47.6 months vs not reached, *P* = 0.01).

The miRNA-based classifier improves the ISS/FISH based risk stratification and is independent of GEP signatures

International Staging System (ISS) and FISH abnormalities including adverse IgH translocations [t(4;14), t(14;16) or t(14;20)], gain(1q) and del(17p) have been previously identified as independent prognostic factors,[104] as was the treatment pathway (log-rank

test $P < 0.001$) in the MRC Myeloma IX dataset examined here. Therefore, multivariate Cox regression analysis was carried out to test the independence of the miRNA-based risk groups from these important predictive factors. The results of this analysis confirmed the independent prognostic value of the miRNA-based classifier ($P = 0.0004$, Table 5). This model shows a significant improvement of predictive capability compared to that without the miRNA-based classifier (likelihood-ratio test $P = 0.0004$). Furthermore the miRNA-based classifier is able to identify subgroups within t(4;14) cases with different outcome (median OS 13.8, 25.3 and 71.0 months respectively, $P = 0.005$) (Figure 11C), while no other FISH lesions could identify prognostically significant subgroups within these t(4;14) cases ($p > 0.05$, data not shown).

Table 5. Multivariate Cox regression analysis showing the independence of the miRNA-defined risk groups from other important predictive factors in 97 patients with all variables available.

	HR	HR-95%CI	P-value	
miR-groups	2.11	1.40-3.20	0.0004	***
ISS	1.37	0.97 -1.95	0.08	
Adverse_translocations	2.25	1.18 -4.28	0.01	*
Gain(1q)	0.98	0.57-1.68	0.94	
Del(17p)	3.14	1.16 -8.52	0.02	*
Path (non-intensive)	2.67	1.53-4.64	0.0005	***

***** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$**

Recently, a prognostic model has been developed by us based on the co-segregation of adverse prognostic FISH lesions and the ISS.[104] We investigated whether the miRNA classifier could be usefully incorporated into this type of risk stratification approach. We found a positive association between the ISS/FISH risk groups and the risk groups defined by the miRNA-based classifier (linear by linear association test $P = 0.005$) (Table 6). Importantly, despite this association, the ISS/FISH low-risk patients who showed high expression levels of at least one of these two miRNAs, had shorter OS (median 47.6 months) compared to the remaining patients, 70% of whom remained alive after 7 years ($P = 0.01$) (Figure 11D).

Table 6. Matrix depicting a positive correlation between the risk groups based on FISH abnormalities & ISS and the risk groups defined by miRNA-based classifier (linear by linear association test *P* = 0.005).

	Risk groups defined by miRNA-based classifier			
Risk groups based on FISH abnormalities and ISS	**Low (%)**	**Median (%)**	**High (%)**	**Grand Total (%)**
Low	20 (44.4)	19 (42.2)	6 (13.3)	45 (100)
Intermediate	10 (27.8)	22 (61.1)	4 (11.1)	36 (100)
High	0	12 (75)	4 (25)	16 (100)
Grand Total	30	53	14	97

The expression levels of some individual miRNAs belonging to the mir-17~92 and mir-106a~363 clusters have previously been reported as being associated with the UAMS' GEP-defined risk score.[90] By using the published method[78] we applied this 70-gene signature to our series of 153 samples with matching GEP data to stratify them into high- and low-risk groups based on the gene-risk model. Then, we constructed a multivariate model including both 70-gene-defined risk groups and miRNA-defined risk groups, showing that miRNA-based classifier retained independent prognostic significance from UAMS' gene-risk model (*P* = 0.002). In a similar fashion, the prognostic value of the 2-miRNA model was also confirmed to be independent from both IFM-signature[105] and Myeloma IX 6-gene signature[99] with even more significant effect (*P* < 0.001).

Putative targets of OS-associated miRNAs

Since miRNAs have been shown to exert the functional effects via cleavage of the mRNAs of their target genes,[106,107] we looked at the putative targets of the OS-associated miRNAs to gain insights into potential mechanistic associations. In this context it is known that the members of mir-17~92 and mir-106a~363 clusters share sequence homology and therefore could potentially target the same genes. Validated targets of these miRNAs include *CDKN1A*, *SOCS1* and *BCL2L11* in myeloma cell lines,[51,90] together with the pro-apoptotic genes *PTEN*, *E2F1* and the anti-angiogenic genes *CTGF*, *THBS1* in other cell types.[108-111] We assessed the correlation of expression of these miRNAs with their

potential target genes (*PTEN*, *E2F1*, *CTGF* and *THBS1*) in 153 patient samples for whom both miRNA and gene expression profiling data were available. The results showed that there were significant inverse correlations between *E2F1* expression and at least one of the cluster members ($P < 0.05$). Trends have also been observed for the expression levels of *CTGF*, *THBS1* and *PTEN* being inversely correlated with members of these two clusters. This observation suggests that the expression levels of these genes may be pathologically relevant to the adverse prognosis associated with the expression of these miRNAs. Using the selection criteria described in the methods section, the putative targets of miR-886-5p, the other miRNA forming the classifier, were identified (Table 7). Among these, lower expression of the top candidate target *NR3C1* was associated with shorter OS (data not shown).

Table 7. Putative targets of miR-886-5p identified using the selection criteria described in methods (ranked by p values for correlation between miRNA and mRNA expression).
#: no. of predictions by miRecord Databases

Gene Symbol	Gene Title	Chromosomal Location	#	P Value for Correlation
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	chr5q31.3	3	0.0017
TEX261	testis expressed 261	chr2p13.3	3	0.0049
LDLR	low density lipoprotein receptor	chr19p13.3	3	0.0054
MXRA7	matrix-remodelling associated 7	chr17q25.1	3	0.0107
RAB11FIP4	RAB11 family interacting protein 4 (class II)	chr17q11.2	3	0.0115
IQSEC1	IQ motif and Sec7 domain 1	chr3p25.2	3	0.0134
SH2B1	SH2B adaptor protein 1	chr16p11.2	3	0.0156
PLA2G2D	phospholipase A2, group IID	chr1p36.12	3	0.0164
MED22	mediator complex subunit 22	chr9q34.2	3	0.0175
CLN6	ceroid-lipofuscinosis, neuronal 6, late infantile, variant	chr15q23	3	0.0190
FADS2	fatty acid desaturase 2	chr11q12.2	3	0.0213
PLEKHA1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	chr10q26.13	3	0.0276
ICOSLG	inducible T-cell co-stimulator ligand	chr21q22.3	3	0.0329
GNG7	guanine nucleotide binding protein (G protein), gamma 7	chr19p13.3	3	0.0369
FAM109A	family with sequence similarity 109, member A	chr12q24.12	3	0.0478

The correlation of OS-associated miRNAs with cytogenetic abnormalities, copy numbers and transcriptional regulation

We examined the association between miR-17 and miR-886-5p expression levels and FISH abnormalities including del(13q), del(17p), t(4;14), del(1p) and gain(1q). The results of this analysis showed that miR-17 expression was significantly associated with del(1p) and gain(1q) ($P < 0.05$). However, although miR-17 is located on 13q, which is frequently deleted in myeloma patients, we did not find any correlation between 13q deletion and the expression level of this miRNA. The expression of miR-886-5p was associated with t(4;14) and del(13q) ($P < 0.05$). This is not surprising as these abnormalities are very tightly linked. In order to explore further how these two miRNAs are deregulated in myeloma, we investigated the correlation between their expression and the tumour acquired DNA-based copy number at their chromosomal locations. Our data indicated that neither miR-17 nor miR-886-5p expression levels were copy number sensitive (Figure 12), suggesting that other mechanisms could be responsible.

Finally, as the mir-17~92 and mir-106a~363 clusters have previously been shown to be activated by Myc and E2F3 (O'Donnell *et al.*, 2005; Woods *et al.*, 2007), we evaluated the correlations between the gene expression and the expression of the miRNAs within these two clusters, and significant positive correlations were identified for both genes ($P < 0.05$).

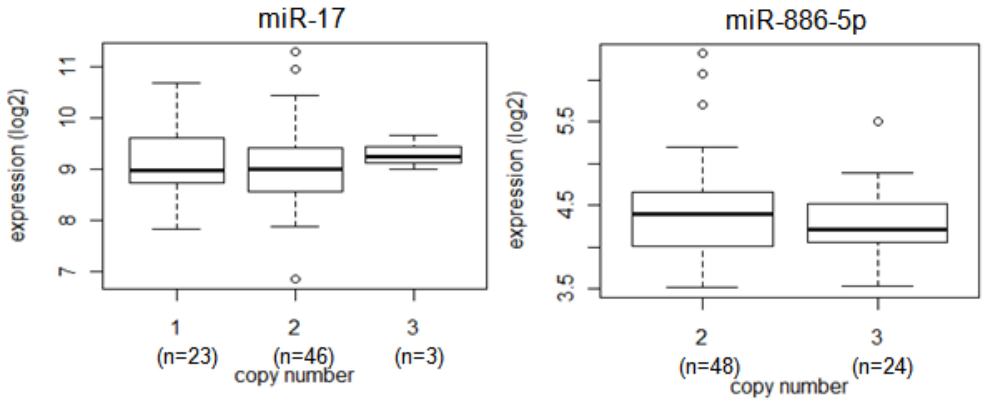

Figure 12. The correlation analyses of miRNA expression and their copy numbers show that the expression levels of miR-886-5p and miR-17 are not copy number sensitive.

Discussion

In the present study, we have comprehensively analyzed the miRNA expression profiling in a prospective cohort including 163 cases from Myeloma IX I trial, and correlated the miRNAs expression pattern with outcome in order to outline their possible role in MM prognostication. In myeloma, the TC classification has been used to define subgroups with distinct prognoses with the 11q13 translocated group being linked to favourable outcome and 4p16 and MAF translocated groups being linked to unfavourable outcome. In our study, 8 miRNAs were identified as being deregulated distinctly in these three subgroups, suggesting that these miRNAs could play an important role in the pathogenesis of these distinct molecular subgroups.

Among the differentially expressed miRNAs, miR-125a, let-7e, miR-150, miR-34a (positively associated with either TC 4p16 or MAF) and miR-138 (negatively associated with TC 11q13) have also been shown to be upregulated in myeloma cells in comparison to their normal counterparts.[51,67,90] The cluster of miRNAs that was strongly correlated with TC 4p16, including miR-125a, let-7e and miR-99b, was shown to be associated with shorter PFS in our dataset. Interestingly miR-125a has been demonstrated to have a role in hematopoietic stem cells, increasing their number both in vivo and in vitro,[112,113] suggesting possible relevance to myeloma stem cell biology. In addition, transgenic mice ectopically expressing the MAF-associated miRNAs (miR-150 or miR-155) either show dramatic impairment of B cell differentiation[114] or develop high-grade B cell lymphoma.[115] *CCND1* has been previously identified as direct target of miR-138,[116] which was significantly downregulated in TC 11q13.

We found three clusters of miRNAs associated with adverse OS outcome in myeloma patients: mir-503~424 (on Xq26.3), mir-17~92 (on 13q31.1) and mir-106a~363 (on Xq26.2). The expression level of mir-503~424 has previously been found to be upregulated in malignant tissues and has been associated with impaired survival in a number of cancers,[117-119] supporting its potential relevance. Previous studies have shown that members of the clusters mir-17~92 and mir-106a~363 are downregulated during the normal germinal center B-cell to plasma cell transition,[120] suggesting that upregulation of these variants in plasma cells may adversely affect their biological behaviour. Indeed, previous work on miRNA expression in myeloma has shown that the upregulation of these miRNAs is associated with either the transformation from MGUS to myeloma or with an mRNA-based risk score.[58,90] In this study, for the first time, we have shown that a high level of expression of these two clusters is associated with an adverse clinical outcome in a series of well characterized clinical samples.

Currently, ISS plus the FISH–based abnormalities are used to define prognosis; however, this approach does not capture all the clinical variability and there is potential for it to be improved. By combining the expression level of miR-17 and miR-886-5p, we classified patients into three subgroups associated with significant differences in OS, which were retained in multivariate analyses when taking into account ISS and FISH–based model. Notably, when the miRNA-based classifier was removed from this model, the predictive power was significantly reduced (P = 0.0004). The robustness of the miRNA-based classifier has been validated using one-thousand bootstrap replications with an estimated error rate of 1.6%. Importantly, the definition of the aggressiveness of the clinical behaviour of newly presenting cases can be improved by the incorporation of this miRNA-based classifier into currently used strategies. In this context, we have shown that within the group classified as being at low risk using ISS/FISH approach the expression of these miRNAs can define a further group (comprising half of the cases) with a significantly worse clinical outcome, which really should belong to intermediate risk group. The miRNA-based risk classifier is also able to identify prognostically important subgroups within t(4;14) cases; notably the t(4;14) cases with low expression level of both these two miRNAs show prolonged median OS of 71 months. These findings are supported by a large body of literature showing that miRNA-based classifiers predict

survival in various types of cancers, which are independent from currently known clinicopathological features.[50,121-125] Although there is no independent validation dataset available, one of the two miRNAs comprising the classifier (miR-17) was associated with GEP risk score;[90] the classifier, therefore, could be considered as partially validated. The other miRNA, miR-886-5p, is not present on their array. The classifier identified in this study is not significantly associated with PFS, and we have confirmed that its differential effect on OS is largely due to the impact on post-relapse survival. OS and PFS are known to be different endpoints; therefore, the strongest predictors for each of them are not necessarily the same. Furthermore OS remains the gold standard for demonstrating clinical benefit in myeloma patients. Notably the oncogenic role of the clusters mir-17~92 and mir-106a~363 in myeloma has been well demonstrated,[58,90] although none of the members is associated with PFS. To this regard, it could be conceivable that the deregulation of specific genes/miRNAs might have long-term effects on myeloma cells and/or their interactions with other environmental components, which might not be reflected by PFS. Indeed, miRNAs have recently been recognized as key regulators in the neoplastic microenvironment.[126]

An important question is whether these miRNA clusters are biologically relevant and actually mediate the biological changes associated with the poor prognosis in MM. Recently, two studies provide functional evidence that miRNAs within mir-17~92 and mir-106a~363 clusters target critical genes including *BCL2L11*, *SOCS1* and *CDKN1A*, which are known to be involved in both myeloma cell proliferation and apoptosis.[51,90] In one of these studies the oncogenic role of miR-19a/b was also confirmed in a nude mice model in which regression of transplanted tumours after treatment with an antagonist was achieved.[51] *BCL2L11* (BIM) and *CDKN1A* (p21) are also two main downstream effectors of TGFβ signalling, the inactivation of which is a major step in the development of a variety of human tumors.[127] The proapoptotic genes *PTEN*, *E2F1*, and anti-angiogenic genes *CTGF*, *THBS1* have also been previously shown to be targets of these two clusters of miRNAs.[108-111] In our study we observed the inverse correlations of the expression level between these genes and their regulating miRNAs, suggesting that an important interaction could also exist in myeloma.

The association of mir-17~92 expression and copy number of chromosome 13q in MM is currently a controversial issue. In keeping with another report,[52] our analysis did

not identify an association between expression of mir-17~92 and del(13q); however, a few other studies observed an at least partial correlation.[64,66,67] This discordance may be due to the variability of the clinical samples. *MYC* deregulation is important in myeloma,[128,129] and recent evidence suggested that *MYC* not only regulates expression of protein-coding genes directly, but also controls the expression of a large set of miRNAs.[110,130] In particular, *MYC* upregulation has been shown to directly activate the miR-17~92 and miR-106a~363 clusters,[110] suggesting that it may play an important role in miRNA deregulation in MM. Indeed in this study we identified a significant positive correlation between *MYC* expression and the expression levels of individual miRNAs within these two clusters. Despite being on different chromosomes, the high correlation between miR-17 and miR-106a may support them being co-regulated. However, as the correlations between the expression of *MYC* and members of the two clusters are modest (R value up to 0.3), other genes may also be important in their deregulation. mir-17~92 cluster has previously been shown to be activated by the *E2F* family member *E2F3*,[131] and *E2F* and *MYC* are known to transactivate each other, suggesting a possible complex regulatory signal for miR-17~92 expression (Figure 13). These observations also highlight the potential importance of Myc/E2F/miR-17~92 negative feedback loop in cancer.

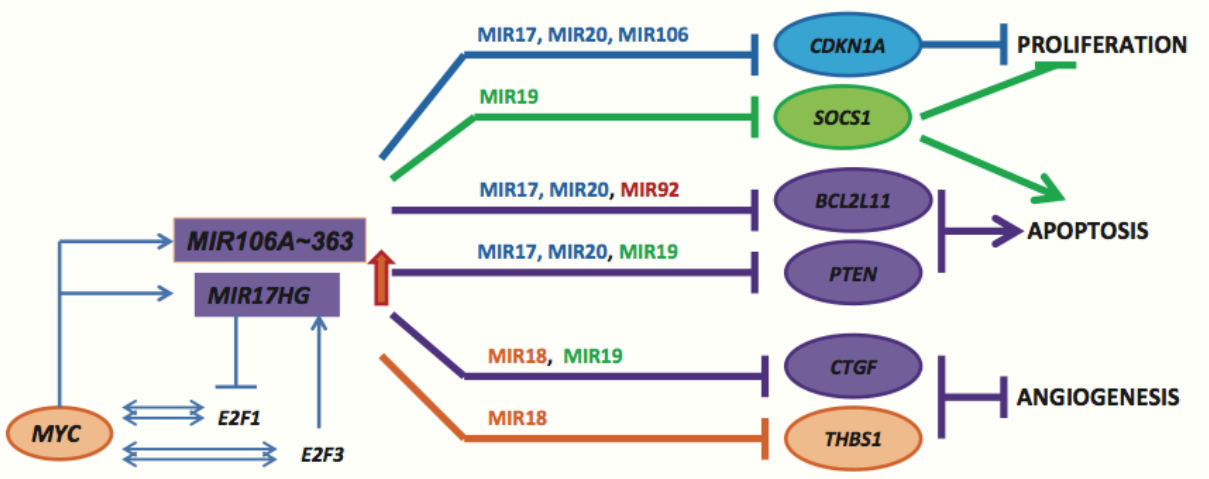

Figure 13. A proposed model of miRNA/MYC/E2F interaction and downstream targets in MM (O'Donnell et al, 2005; Dews et al, 2006; Novotny et al, 2007; Pichiorri et al, 2008; Xiao et al, 2008; Zhou et al, 2010).

The deregulated genes downstream of the deregulated miRNAs could mediate the prognostic effect of these miRNAs; therefore, we developed an approach to identify the

putative targets of miR-886-5p, the other miRNA comprising the classifier. The pathogenic role of miR-886-5p has not been previously reported in myeloma; however, it was shown to be upregulated in Burkitt Lymphoma compared to some other lymphoma types.[132,133] MiR-886-5p inhibits apoptosis of cervical cancer cells by down-regulating the production of Bax.[134] Increased miR-886-3p, which originates from the same pre-miRNA, has recently been associated with chemo-resistance in bladder cancer, which was translated to impaired overall survival.[135] As the top target for miR-886-5p, *NR3C1* is the glucocorticoid receptor gene and its downregulation has been associated with glucocorticoid resistance and inferior prognosis in MM;[136,137] the association of the expression of miR-886-5p with prognosis was confirmed in our dataset. One of the other potential targets *ICOSLG* (inducible co-stimulator ligand) is expressed on tumour cells and has been reported to have an important role in tumor immunity; it also induces B-cell differentiation into plasma cells. It has been demonstrated that cytotoxic T cells play a critical role in myeloma cell elimination;[138,139] therefore, it is not surprising that *ICOSLG* expression has an effect on OS of myeloma patients. The precursor of miR-886-5p and miR-886-3p, previously proposed to be a vault RNA, a component of the vault complex implicated in cancer drug resistance, was recently shown neither to be a genuine pre-miRNA nor a vault RNA;[140] miR-886 binds directly to PKR (Protein Kinase RNA-activated) and silencing of miR-886 activates PKR and its downstream pathways, eIF2α phosphorylation and the NF-κB pathway, leading to impaired cell proliferation.[140] The association of miR-886-5p expression level with OS warrants validation and additional studies to investigate its potential roles in MM pathogenesis.

MM is a genetically complex disease with a well described heterogeneity in clinical outcome. Recent research highlights the contribution of a new class of non-coding genes, miRNA, in myeloma pathogenesis. In this work, we have developed a 2-miRNA-based classifier able to stratify MM patients into three risk groups. The classifier significantly improves the predictive power of an outcome predictor comprising ISS and FISH-based abnormalities; therefore, it may represent a complementary prognostic tool in clinical practice after being validated using independent dataset. The miRNAs related to the classifier are biologically relevant, and integrative analyses indicate that they are putative candidates regulating a large number of genes involved in MM biology such as

proliferation, apoptosis, angiogenesis and drug resistance. In this context, miRNAs can be built into molecular diagnostic strategies for risk stratification as well as being used as treatment targets in MM.

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