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BIOLOGICAL AND CLINICAL RELEVANCE OF MIRNA EXPRESSION SIGNATURES IN PRIMARY PLASMA CELL LEUKEMIA

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Summary

Purpose: Plasma cell leukemia (PCL) is a very aggressive and rare hematological malignancy that can be distinguished into primary (pPCL), originating *de novo*, or secondary (sPCL), arising as a leukemic transformation of multiple myeloma (MM). Genomic and clinical differences between pPCL and MM have been demonstrated, mainly based on retrospective studies. This study was aimed at investigating the involvement of miRNAs in pPCL and their possible relationship with higher tumor aggressiveness.

Experimental design: MiRNA expression profiles were analyzed in highly-purified malignant plasma cells from 18 pPCL cases included in a prospective clinical trial. MiRNA expression patterns were evaluated in comparison with a representative series of multiple myeloma (MM) patients, in relation to the most recurrent chromosomal abnormalities (as assessed by fluorescence *in situ* hybridization and single nucleotide polymorphism-array analysis), and in association with clinical outcome. MiRNA expression was also integrated with gene expression profiles and computational prediction of miRNA target genes in pPCL and MM samples in order to identify putative target genes of deregulated miRNAs. The functional role of a few identified miRNAs in plasma cell dyscrasia pathogenesis was explored by transfection of synthetic pre/anti-miRNAs in multiple myeloma cell lines.

Results: We identified a series of deregulated miRNAs in pPCL (42 up- and 41 down-regulated) in comparison with MM. Some of them, based on their reported functions or putative target genes computed by integrative analysis, might have a role in the pathobiology of pPCL, such as *miR-21*, that was found to promote *in vitro* growth of MM cell lines. As regards chromosomal aberrations, the expression of some miRNAs mapped to hot-spot altered regions was associated with DNA copy number of the corresponding genomic *loci*; furthermore, *TP53* deletion, a frequent cytogenetic lesion in our pPCL cohort, was found associated with a trend of down-regulation of *miR-34a*, whose tumor suppressor activity was demonstrated for the first time also in the context of MM. Finally, four miRNAs (*miR-497, miR-106b, miR-181a** and *miR-181b*) were identified having expression levels correlated with treatment response, and four (*miR-92a, miR-330-3p, miR-22,* and *miR-146a*) with clinical outcome.

Conclusions: Overall, this study provides insights into the possible contribution of miRNAs in the pathogenesis of pPCL and suggests targets for future therapeutic investigations.

Introduction

Multistep transformation process from normal to malignant plasma cells

Multiple myeloma (MM) is a genetically complex disease that is becoming more common in today's ageing population. Myeloma belongs to a group of related paraproteinaemias that are characterized by an abnormal clonal plasma cell (PC) infiltration in the bone marrow (BM) (Greipp *et al*., 2005; Kyle *et al*., 2003). Various distinct clinical phases of myeloma can be recognized (Table 1, Figure 1), including monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM; also known as asymptomatic myeloma). Although both of these disease phases lack the clinical features of myeloma, they share some of the genetic features of myelomas that require treatment (Kyle *et al*., 2007). By contrast, symptomatic multiple myeloma is defined by clinical symptoms and evidence of organ damage. A characteristic feature of myeloma cells is the requirement for an intimate relationship with the bone marrow microenvironment, where plasma cells are nurtured in specialized niches that maintain their long-term survival (Mitsiades *et al*., 2006). However, during the progression of the disease, clonal cells develop the ability to proliferate at sites outside of the bone marrow, manifesting as extramedullary myeloma (EMM) and plasma cell leukemia (PCL) (Palumbo and Anderson, 2011). These cells constitute the end stages in the multistep transformation process from normal to malignant plasma cells. Human MM cell lines (HMCLs), which are presumed to include most oncogenic events involved in tumor initiation and progression of the corresponding tumor, have been generated mainly from a subset of extramedullary MM tumors (Kuehl and Bergsagel, 2002).

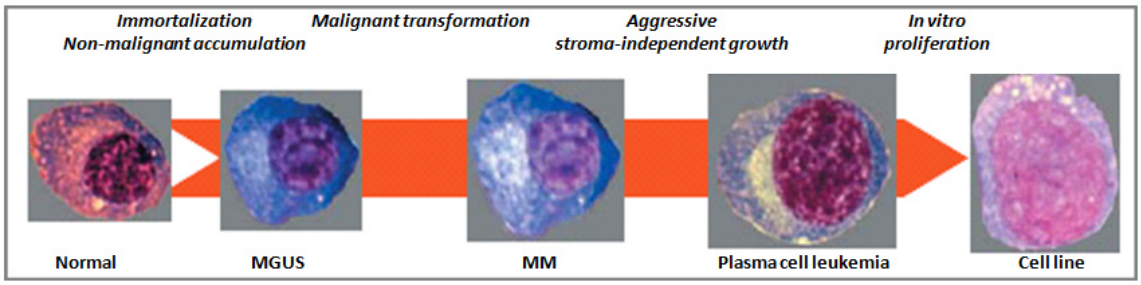


Figure 1 | **Multi-step molecular pathogenesis of MM**. Progression through the different stages of multiple myeloma.

Multiple Myeloma (MM)

Multiple myeloma is characterized by the accumulation of terminally differentiated clonal PCs in the bone marrow, the production of a monoclonal immunoglobulin (Ig) detectable in serum and/or urine and the presence of lytic bone lesions (Gutierrez *et al*., 2007). Osteolytic bone lesions and/or compression fractures detected on routine radiographs, magnetic resonance imaging (MRI), or computed tomographic (CT) scans are the most characteristic markers of end-organ damage in myeloma. Anemia (occurring in 70% of patients), renal failure (50%), and hypercalcemia (25%) are the other established markers of end-organ damage. The presence of one or more of these four markers that is felt to be related to the underlying plasma cell disorder is required for the diagnosis of the disease (Table 1) (Rajkumar, 2005).

Table 1 | Mayo Clinic diagnostic criteria for selected clonal plasma cell disorders.

Disorder	Disease definition
Monoclonal Gammopathy of Undetermined Significance (MGUS)	▪ Serum monoclonal protein < 3 g/dL ▪ Bone-marrow plasma cells < 10% ▪ Absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia, or renal failure
Smoldering Multiple Myeloma (SMM; asymptomatic multiple myeloma)	▪ Serum monoclonal protein (IgG or IgA) ≥ 3 g/dL and/or ▪ Bone-marrow plasma cell ≥ 10% ▪ Absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia, or renal failure
Multiple Myeloma (MM)	▪ Bone marrow plasma cells ≥ 10% ▪ Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) ▪ Evidence of lytic bone lesions, anemia, hypercalcemia, or renal failure

MM accounts for about 10% of all hematological cancers (Kyle and Rajkumar, 2004; Rajkumar and Kyle, 2005), and its age-adjusted annual incidence in the United States is almost 4 per 100,000 (Kyle *et al*., 2004). MM remains an incurable disease, with a median survival being around 3-4 years (ranging from < 1 to > 10 years) and despite some evidence for familial clustering, the effects of genetic background and environment remain to be clarified (Lynch *et al*., 2005).

Cellular origin of myeloma cells

Post-germinal center (GC) B cells that have undergone productive somatic hypermutation, antigen selection, and IgH switching can generate plasmablasts (PBs), which typically migrate to the bone marrow microenvironment that enables differentiation into long-lived plasma cells (Shapiro-Shelef and Calame, 2005). MM

cells are phenotypically similar to PBs/long-lived PCs, including a strong dependence on the BM microenvironment for survival and growth (Kuehl and Bergsagel, 2002). The immunophenotype of MM cells resembles that of normal, terminally differentiated, long-lived BM PC ($CD19^{-}CD20^{-}CD45^{-}CD138^{+}$). In contrast to normal long-lived PCs, MM tumors retain some potential for an extremely low rate of proliferation, usually with no more than a few per cent of cycling cells, until advanced stages of MM (Rajkumar *et al*., 1999).

Genetic architecture and disease progression

At the cytogenetic level MM genome is complex and more reminiscent of epithelial cancers than of more simple leukemias (Morgan *et al*., 2012). Many of the genetic lesions that lead to MM have been defined, and can be categorized as inherited variation, translocations, copy number abnormalities, mutations, methylation and miRNA abnormalities (Table 2).

- **Chromosomal translocations**

The study of chromosomal translocations that are generated by aberrant class switch recombination (CSR) shows that various oncogenes (such as *cyclin D1* (*CCND1*), *CCND3*, *fibroblast growth factor receptor 3* (*FGFR3*), *multiple myeloma SET domain* (*MMSET*; also known as *WHSC1*), *MAF* and *MAFB*) are placed under the control of the strong enhancers of the Ig *loci*, leading to their deregulation (Bergsagel *et al*., 2005; Gonzalez *et al*., 2007) (Table 2). Deregulation of the G1/S transition is a key early molecular abnormality in myeloma, and the consistent deregulation of a D-group cyclin was first noted as a consequence of studying the CSR-driven t(11;14) and t(6;14) translocations, which directly deregulate *CCND1* and *CCND3* respectively (Chesi *et al*., 1996; Shaughnessy, Jr. *et al*., 2001). Non-translocation-based up-regulation of a D-group cyclin can also occur, and in the case of t(14;16) this is modulated through MAF, which up-regulates *CCND2* by binding directly to its promoter (Hurt *et al*., 2004). Cases with t(4;14), which translocates *FGFR3* and *MMSET* at 4p16.3 to the IGH@ enhancers, also up-regulates *CCND2*, but in this case the mechanism is uncertain (Bergsagel *et al*., 2005). The “translocation and cyclin D (TC)” classification is exactly based on spiked expression of genes deregulated by primary IgH translocations and the universal over-expression of *cyclin D* genes by either these translocations or another mechanism (Bergsagel *et al*., 2005; Hideshima *et al*., 2004).

Table 2 | Genetic events underlying the initiation and progression of MM to PCL (Morgan *et al.*, 2012).

Primary genetic events (% of tumors)	Secondary genetic events (% of tumors)
IGH@ translocations and genes affected	**Gains**
• t(4;14): *FGFR3* and *MMSET* (11%)	• 1q: *CKS1B* and *ANP32E* (40%)
• t(6;14): *CCND3* (< 1%)	• 12p: *LTBR*
• t(11;14): *CCND1* (14%)	• 17q: *NIK*
• t(14;16): *MAF* (3%)	**Secondary translocations**
• t(14;20): *MAFB* (1.5%)	• t(8;14): *MYC*
Hyperdiploidy	• Other non-IGH@ translocations
• Trisomies of chr 3, 5, 7, 9, 11, 15, 19 and 21	**Epigenetic events**
	• Global hypomethylation (MGUS to MM)
	• Gene-specific hypermethylation (MM to PCL)
	Molecular hallmarks
	• Immortalization
	• G1/S abnormality (*CDKN2C*, *RB1* (3%), *CCND1* (3%) and *CDKN2A*))
	• Proliferation (*NRAS* (21%), *KRAS* (28%), *BRAF* (5%) and *MYC* (1%))
	• Resistance to apoptosis (*PI3K* and *AKT*)
	• NF-κB pathway (*TRAF3* (3%), *CYLD* (3%) and *I-κB*)
	• Abnormal localization and bone disease (*DKK1*, *FRZB* and *DNAH5* (8%))
	• Abnormal plasma cell differentiation (*XBP1* (3%), *BLIMP1*/*PRDM1* (6%) and *IRF4* (5%))
	• Abnormal DNA repair (*TP53* (6%), *MRE11A* (1%) and *PARP1*)
	• RNA editing (*DIS3* (13%), *FAM46C* (10%) and *LRRK2* (5%))
	• Epigenetic abnormalities (*UTX* (10%), *MLL* (1%), *MMSET* (8%), *HOXA9* and *KDM6B*)
	• Abnormal immune surveillance
	• Abnormal energy metabolism and ADME events (absorption, distribution, metabolism and excretion)
	Deletions
	• 1p: *CDKN2C*, *FAF1* and *FAM46C* (30%)
	• 6q (33%)
	• 8p (25%)
	• 11q: *BIRC2* and *BIRC3* (7%)
	• 13: *RB1* and *DIS3* (45%)
	• 14q: *TRAF3* (38%)
	• 16q: *CYLD* and *WWOX* (35%)
	• 17p: *TP53* (8%)

The resultant classification identifies five groups of tumors: TC1 tumors (18%) express high levels of either *cyclin D1* (11q13) or *cyclin D3* (6p21) as a result of an Ig translocation; TC2 tumors (37%) ectopically express low to moderate levels of *cyclin D1* despite the absence of a t(11;14) translocation; TC3 tumors (22%) are a mixture of tumors that do not fall into one of the other groups, with most expressing *cyclin D2*, but

a few also expressing low levels of *cyclin D1* or *cyclin D3*; TC4 tumors (16%) express high levels of *cyclin D2*, and also *MMSET* (and in most cases *FGFR3*) as a result of a t(4;14) translocation; and TC5 tumors (7%) express the highest levels of cyclin D2, and also high levels of either *MAF* or *MAFB*, consistent with evidence that both MAF transcription factors up-regulate the expression of *cyclin D2*. Other IGH@ translocations are seen in myeloma, but in contrast to the CSR-driven events, CSR-independent translocations tend to occur later in the disease process (Avet-Loiseau *et al*., 2001b; Shou *et al*., 2000). The gene that is typically deregulated by such events is *MYC*, the deregulation of which may lead to a more aggressive disease phase (Nobuyoshi *et al*., 1991).

- **Copy number abnormalities**

Gains and losses of DNA, resulting in copy number alterations, are common events in myeloma (Table 2). The high frequency and recurrent nature of focal loss of copy number and loss of heterozygosity (LOH) suggests that the minimal deleted regions contain tumor suppressor genes that are driver events leading to the development and progression of myeloma (Annunziata *et al*., 2007; Carrasco *et al*., 2006; Walker *et al*., 2010). Most tumor suppressor genes require the inactivation of both alleles and thus have either been identified by the study of homozygous deletions or through the integration of mutational analysis with copy number status (Dickens *et al*., 2010). As regards the loss of negative cell cycle regulators, down-regulation of *CDKN2C* (which encodes INK4C) by loss of chromosome 1p32 is important (Dib *et al*., 2007), as is the inactivation of *CDKN2A* (which encodes INK4A and ARF) by DNA methylation (Gonzalez-Paz *et al*., 2007; Leone *et al*., 2008). Inactivation of *RB1* also affects the G1/S checkpoint and may occur as a result of the loss of chromosome 13, which occurs in 58% of cases; however, homozygous loss and mutational inactivation of *RB1* is infrequent (Walker *et al*., 2010). Other important regions of LOH include 11q (the site of the *BIRC2* and *BIRC3* genes), 16q (the site of *CYLD*) and 14q32 (the site of *TRAF3*) (Annunziata *et al*., 2007; Keats *et al*., 2007). All of these genes are involved in the nuclear factor-κB (NF-κB) pathway, indicating that up-regulation of NF-κB signalling is important in myeloma. The other major class of recurrent genetic abnormality that is seen in myeloma is hyperdiploidy, which is associated with the gain of the odd numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19 and 21. Interstitial copy number gains that are associated with increased gene expression or with activating mutations in oncogenes represent another set of ‘driver’ lesions that can lead to

myeloma progression. A classic example of this is the amplification of chromosome 1q, which harbours numerous potentially relevant oncogenes (for example, *CDC28 protein kinase 1B* (*CKS1B*), *acidic leucine-rich nuclear phosphoprotein 32 family, member E* (*ANP32E*), *BCL-9* and *PDZK1*) (Walker *et al*., 2010; Shaughnessy, 2005). Interstitial copy number gains that may activate the NF-κB pathway are also seen; these include amplification of *NIK* (also known as *MAP3K14*), *TACI* (also known as *TNFRSF13B*) and *lymphotoxin beta receptor* (*LTBR*) (Keats *et al*., 2007).

- **Mutations**

Whole-genome-based and whole-exome-based sequencing strategies have shown that there are approximately 35 non-synonymous mutations per myeloma sample (Chapman *et al*., 2011). There are few recurrently mutated genes in MM, that are, for the most part, known oncogenes. However, a few novel genes have been identified (such as *FAM46C* in 13% of cases and *DIS3* in 11% of cases), and as the numbers of samples analyzed increases, the incidence of recurrent genes will undoubtedly rise. This observation is consistent with a hypothesis whereby it is the deregulation of pathways that is pathogenically important, rather than the deregulation of a specific gene. For example, the high frequency of mutations in the ERK pathway (*NRAS* in 24% of cases, *KRAS* in 27% of cases and *BRAF* in 4% of cases) indicates that the ERK pathway is crucial for myeloma development and points to a treatment strategy that has so far not been harnessed. Deregulation of the PI3K pathway is also important in myeloma, although it is not frequently mutated (Chapman *et al*., 2011).

- **Epigenetic changes**

The most important epigenetic change that is relevant to the pathogenesis of myeloma is global DNA hypomethylation and gene-specific DNA hypermethylation during the transformation of MGUS to myeloma (Walker *et al*., 2011). The most pronounced DNA methylation change is seen in the 15% of patients with the t(4;14) translocation, whose myeloma samples have increased gene-specific DNA hypermethylation compared with myeloma samples of other cytogenetic subgroups. This t(4;14) subgroup over-expresses *MMSET*, which encodes a histone methyltransferase transcriptional repressor (Kim *et al*., 2008; Marango *et al*., 2008; Nimura *et al*., 2009), that mediates histone H3 lysine 36 (H3K36) methylation, and its deregulation leads to global changes in histone modifications that promote cell survival, cell cycle progression and DNA repair (Brito *et al*., 2009; Kuo *et al*., 2011; Marango *et al*., 2008; Martinez-Garcia *et al*., 2011; Pei *et al*., 2011). Other chromatin modifiers are also

deregulated in myeloma, including *lysine-specific demethylase 6A* (*KDM6A*; also known as *UTX*), *KDM6B*, *mixed-lineage leukaemia* (*MLL*) and *homeobox A9* (*HOXA9*), but the full relevance of any resultant chromatin modifications needs further validation (Chapman *et al*., 2011; van Haaften *et al*., 2009).

- Other key genetic events

To date, no consistent mutation of DNA repair genes has been identified in myeloma; however, deletions of chromosome 17p occur in 8% of cases at presentation, and this frequency increases in the later stages of the disease. The key gene at this site is thought to be *TP53*, and mutations of this gene are associated with increased genomic instability and impaired clinical outcomes (Avet-Loiseau *et al*., 2010; Boyd *et al*., 2011; Lode *et al*., 2010).

As regards microRNAs, various studies have produced data that suggest a role for miRNAs in both normal plasma cell development and in the pathogenesis of myeloma (Lionetti *et al*., 2012).

Treatment

The treatment of MM has so far been unrelated to any particular molecular or genetic feature. The cornerstones of conventional anti-myeloma therapy have long been alkylators and corticosteroids, more specifically oral melphalan and prednisone (MP), leading to a median post-treatment overall survival (OS) of 3-4 years. Subsequently, high-dose therapy followed by autologous stem cell transplantation (ASCT) has been introduced into standard practice as it prolongs survival in comparison with conventional chemotherapy (median OS 5–7 years) (Raab *et al*., 2009; Rajkumar, 2009). However, over the last few years, the treatment of MM has changed considerably as a result of drug development (Raab *et al*., 2009): in particular, the immunomodulatory drugs (IMiDs) thalidomide (Singhal *et al*., 1999) and lenalidomide (Rajkumar *et al*., 2005; Richardson *et al*., 2006), and the proteasome inhibitor bortezomib (Richardson *et al*., 2003; Richardson *et al*., 2005), are effective in treating newly diagnosed or relapsed myeloma (Rajkumar, 2009), and have further improved overall and event-free survival. However, there is still a lack of effective therapies targeting the deregulated biological pathways specifically associated with the disease.

Plasma cell leukemia

Definition and epidemiology

PCL is the most aggressive presentation of the PC neoplasms and is characterized by circulating PCs > 2×10^9/L in peripheral blood and a peripheral blood plasmacytosis > 20% (Albarracin and Fonseca, 2011; Kyle *et al*., 1974) (Table 3).

Table 3 | Diagnostic criteria for plasma cell leukemia.

1. Plasma cells > $2x10^9$/L in peripheral blood
2. Plasma cells > 20% of blood leukocytes in peripheral blood
3. Primary plasma cell leukemia (pPCL): presents as *de novo* leukemia
4. Secondary plasma cell leukemia (sPCL): progression from a pre-existing multiple myeloma

International Myeloma Working Group (International Myeloma Working Group, 2003)

Primary PCL (pPCL) is defined as a malignant PC proliferation first diagnosed in the leukemic phase, while secondary PCL (sPCL) corresponds to the leukemic transformation of a previously diagnosed multiple myeloma, probably as a consequence of clonal transformation (Table 3) (International Myeloma Working Group, 2003; Noel and Kyle, 1987). Secondary PCL occurs as a progression of disease in 1 to 4% of all cases of MM (Blade and Kyle, 1999). Altogether 60% of all PCL is pPCL (Jimenez-Zepeda and Dominguez, 2006). In the United States, the incidence is approximately 0.02 to 0.03 cases per 100,000 population accounting for less than 0.2% of all leukemia cases diagnosed from 1997 to 2002 (Yamamoto and Goodman, 2008). As with MM, PCL is more common in African Americans than Caucasians (Yamamoto and Goodman, 2008). There appears to be a 3:2 male to female sex distribution in both primary and secondary PCL (Albarracin and Fonseca, 2011).

Clinical and pathologic features

As compared to MM, pPCL tends to be observed in younger individuals. The median age of diagnosis of pPCL is 55, a decade younger than the average age of MM diagnosis (Tiedemann *et al*., 2008). The median time to leukemic transformation for patients with MM who evolve to sPCL is 21 months (Tiedemann *et al*., 2008). Patients with pPCL present without a MM prodrome, and are characterized by higher prevalence of renal insufficiency and elevated β_2-microglobulin as opposed to newly diagnosed MM. Patients with sPCL are also frequently afflicted with renal insufficiency (Tiedemann *et al*., 2008). In addition to the high number of circulating PCs, extramedullary deposits at other sites are common in PCL. For instance up to 15% of patients will have hepatomegaly, splenomegaly or lymphadenopathy. An interesting

feature of pPCL is that the majority of these individuals do not have clinical evidence of overt bone destruction. Osteolytic lesions are more common in sPCL as they are also more common in pre-existing MM (sPCL 53% *versus* pPCL 18%) (Tiedemann *et al*., 2008). Because of extensive involvement of the bone marrow, patients with PCL have a higher prevalence of anemia and thrombocytopenia (Garcia-Sanz *et al*., 1999; Noel and Kyle, 1987; Tiedemann *et al*., 2008). Likewise other laboratory parameters of disease aggressiveness such as an elevated LDH or β_2-microglobulin are common in PCL cases (Garcia-Sanz *et al*., 1999; Tiedemann *et al*., 2008) (Table 4).

Table 4 | Clinical and biological differences between PCL and MM (Tiedemann *et al*., 2008).

Characteristic		MM	sPCL	pPCL	*P*
Patient number		439	39	41	
Age (years)	median	66	65.7	54.5	< 0.001
Extraosseous plasmacytoma	% cases		6	22	0.21
Osteolytic lesions	% cases	81	53	35	0.19
Splenomegaly	% cases		8	18	0.29
Hepatomegaly	% cases		11	32	0.04
WBC ($x10^9$/L)	median	5.8	15.7	21.5	0.04
PB plasmacytosis (%)	median	0	52	46	0.017
PB plasmacytosis ($x10^9$/L)	median	0	6.3	7.2	0.19
Hemoglobin (g/dL)	median	10.9	9.1	9.4	0.05
Platelets ($x10^9$/L)	median	242	53	98	0.05
Serum creatinine (mg/dL)	median	1.2	1.4	1.9	0.03
Kappa (excl. non-secretors)	% cases	66	58	42	0.24
Lambda (excl. non-secretors)	% cases	34	42	58	0.24
PCLI	median	0.4	3.0	1.4	0.47
BM plasmacytosis	% BM	46	63	78	0.11

WBC: white blood cells; PB: peripheral blood; PCLI: plasma cell labeling index; BM: bone marrow.

Genetics of PCL

- Ploidy

PCL usually has complex hypodiploid or pseudodiploid karyotypes. In two of the largest studies where informative karyotypes were obtained in 34 and 38 patients with PCL, most cases were non-hyperdiploid (NH-MM) consisting mostly of complex hypodiploid or pseudodiploid karyotypes (67 and 63% respectively) (Avet-Loiseau *et al*., 2001a; Tiedemann *et al*., 2008). Very few patients with hyperdiploid MM (H-MM) karyotypes in PCL have been reported, a finding that contrast with those published in MM in which hyperdiploidy is observed in approximately 60% of patients (Blade and Kyle, 1999; Taniwaki *et al*., 1996).

- **IGH translocations**

Chromosome 14q32 translocations are found in a great number of pPCL and sPCL patients (82–87%), as would be expected in NH-MM cases. Based on the study by Tiedemann and colleagues, in pPCL IgH translocations almost exclusively involve 11q13 (*CCND1*), supporting a central etiological role, while in sPCL multiple partner oncogenes are involved, including 11q13, 4p16 (*FGFR3*/ *MMSET*) and 16q23 (*MAF*), recapitulating MM (Tiedemann *et al*., 2008). Avet-Loiseau and colleagues described a higher prevalence of t(11;14) and t(14;16) in PCL as compared with a stage III MM population (33 and 13% *versus* 16 and 1%; $P = 0.025$ and $P = 0.002$, respectively), although in his study the incidence of t(4;14) was identical (12%) (Avet-Loiseau *et al*., 2001a).

Genomic aberrations such as t(4;14), del(13q14), del(17p), del(1p21) and 1q21 gains are adverse risk factors in MM but their significance in PCL is unclear (as they usually indicate more aggressive behavior which is ubiquitous in PCL) (Avet-Loiseau *et al*., 2007; Chang *et al*., 2007; Chang *et al*., 2005; Fonseca *et al*., 2004; Fonseca *et al*., 2006; Shaughnessy, Jr. *et al*., 2007; Chang *et al*., 2006; Hanamura *et al*., 2006). Recently, Chang and colleagues investigated 41 PCL cases aiming to detect chromosome 1q amplifications and 1p deletions, and compared the genetic aberrations with those in 220 MM patients (Chang *et al*., 2009).They report del(17p), del(13q14), del(1p21), 1q21 amplifications and t(4;14) were more frequent in PCL than MM. Deletions of 1p21 were associated with 1q21 amplification ($P = 0.03$) and has a marginal association with del(17p) ($P = 0.06$). They showed patients with 1p21 deletions had a shorter OS than those without such deletions (6.2 *versus* 33.5 months, $P = 0.006$) (Chang *et al*., 2009). Notably patients with t(4;14) had a shorter survival than those without t(4;14) (1.5 *versus* 21.6 months, $P = 0.003$). The presence of del(13q14), del(17p), t(11;14) and 1q21 amplification did not influence survival in this cohort. In a multivariate analysis adjusting for all above genetic risk factors as well as C-reactive protein, calcium and β2-microglobulin levels, only t(4;14) was an independent predictor for a worse OS ($P = 0.008$); 1p21 deletions did not retain the prognostic significance ($P = 0.14$) (Chang *et al*., 2009).

- **Chromosome 13**

In a series of 26 patients Garcia Sanz reported deletion of chromosome 13 in 84% of patients with PCL *versus* 26% for those with MM (Garcia-Sanz *et al*., 1999). In the Mayo study loss of 13q by FISH was found very common in pPCL (85%), more so

than in MM (54%) (P = 0.02); but with no difference in prevalence between pPCL and sPCL (Tiedemann *et al*., 2008). As would be expected in NH-MM Avet-Loiseau reported a high frequency of monosomy 13 (85%) (Avet-Loiseau *et al*., 2001a).

- **Deletions of 17p13 and *TP53* inactivation**

Previous studies reported 17p13.1 deletion in MM as a late event found only in 10% of patients (Fonseca *et al*., 2003), and *TP53* coding mutations in only 3% (Chng *et al*., 2007). In the group of 80 patients reported by Tiedemann there was a prevalence of deletion of 17p13.1, causing allelic loss of *TP53*, in 50% of pPCL and a remarkable 75% of sPCL. Moreover, functionally relevant *TP53* coding mutations were identified in 24% of PCL patients tested, contributing to a substantial prevalence of allelic *TP53* inactivation (by mutation or deletion) of 56% in pPCL and 83% in sPCL. Eleven percent of pPCL and 33% of sPCL tumors showed biallelic inactivation of *TP53* with simultaneous allelic deletion and mutation (Tiedemann *et al*., 2008). Interestingly, monoallelic or biallelic inactivation of *TP53* did not correlate significantly with survival in sPCL, unlike MM, where del(17p13.1) predicts adverse survival (Gertz *et al*., 2005; Lloveras *et al*., 2004). Lack of correlation between *TP53* status and survival may reflect ubiquitous targeting of the TP53 pathway in sPCL.

- ***MYC* abnormalities and *RAS* mutation**

Rearrangement of *MYC* was identified by 3′FISH break apart in 33% of pPCL and sPCL tumors and was complemented by *MYC* amplification or 5′*MYC* translocations in 8 and 17% of patients, respectively (Tiedemann *et al*., 2008). *MYC* rearrangements were associated with a trend toward inferior OS in pPCL (median OS of 8.6 *versus* 27.8 months without rearrangements, P = 0.006). On the other hand, mutations of *K-RAS* or *N-RAS* at codons 12, 13 or 61, previously characterized as functionally activating (Bezieau *et al*., 2001; Ahuja *et al*., 1990; Ortega *et al*., 2006; Liang *et al*., 2006), were found in 27% pPCL and 15% sPCL (Tiedemann *et al*., 2008). Activating mutations of *RAS* were associated with a trend toward poorer outcome in pPCL (P = 0.069). However, the prevalence of *K*- or *N-RAS* mutation in sPCL is comparable to that reported in MM (21%) (Ortega *et al*., 2006), suggesting little, if any, selective pressure for *RAS* activation in secondary leukemic transformation from MM.

Survival

PCL is extremely aggressive, associated with short survival (7 to 11 months), with up to 28% of patients dying within the first month after diagnosis in different studies (Avet-Loiseau *et al*., 2001a; Noel and Kyle, 1987; Tiedemann *et al*., 2008). Survival is

even shorter when PCL occurs in the context of refractory or relapsing MM. In one of the largest series Tiedemann *et al*. reported an overall survival of 1.3 months for sPCL and 11.2 months for pPCL (Tiedemann *et al*., 2008). Accurate prognostication of PCL is hampered by the always low number of cases studied, and the realization that the disease is always associated with an adverse outcome. Most studies confirm that predictable variables of disease progression predict more aggressive course (Garcia-Sanz *et al*., 1999). Garcia-Sanz showed in a very small series that many variables predict outcome, all of them associated with greater tumor bulk and cell proliferation (Garcia-Sanz *et al*., 1999). Tiedemann could not find major prognostic implications for the various genetic factors tested (Tiedemann *et al*., 2008). Avet-Loiseau reported longer survival, but still limited survival for patients with a t (11;14) (12 months, P = 0.001) (Avet-Loiseau *et al*., 2001a), but other series have not reached similar conclusions (Fonseca *et al*., 1998; Lai *et al*., 1998).

Treatment of PCL

Treatment of both pPCL and sPCL, as in MM, is aimed at prolonging survival and maximizing quality of life, as there are no recognized curative regimens (Avet-Loiseau *et al*., 2001a; Hayman and Fonseca, 2001). In general, patients are treated with aggressive induction therapy followed by ASCT in those who are appropriate candidates for this approach. Chemotherapy alone is the principal option for those ineligible for ASCT. The best induction regimen for PCL is not known and there is great variability in clinical practice. Several combinations that include an alkylating agent have been used as sole treatments of PCL or as induction therapy prior to anticipated transplant. Combinations that include melphalan are generally avoided as induction therapy in potential transplant candidates to allow adequate stem cell collection. Most patients need to be treated with the most effective agents currently available (Musto *et al*., 2007; Benson, Jr. and Smith, 2007; Musto *et al*., 2008) and historic data with older agents show poor rates of control. Patients with PCL have had a median overall survival of 4 months when treated with melphalan plus prednisone (Garcia-Sanz *et al*., 1999; Tiedemann *et al*., 2008; Dimopoulos *et al*., 1994). Multi-agent infusional chemotherapy (e.g. vincristine, doxorubicin, plus dexamethasone) results in a superior, yet still poor median overall survival of approximately 15 to 18 months (Garcia-Sanz *et al*., 1999; Tiedemann *et al*., 2008). Another study reported similar results by showing a median OS of 6.8 months for patients treated with VAD

(vincristine, doxorubicin and dexamethasone) *versus* 2 months for those who received MP ($P < 0.05$) (Jimenez-Zepeda and Dominguez, 2006).

MicroRNAs

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 (Ambros, 2004) (Figure 2).

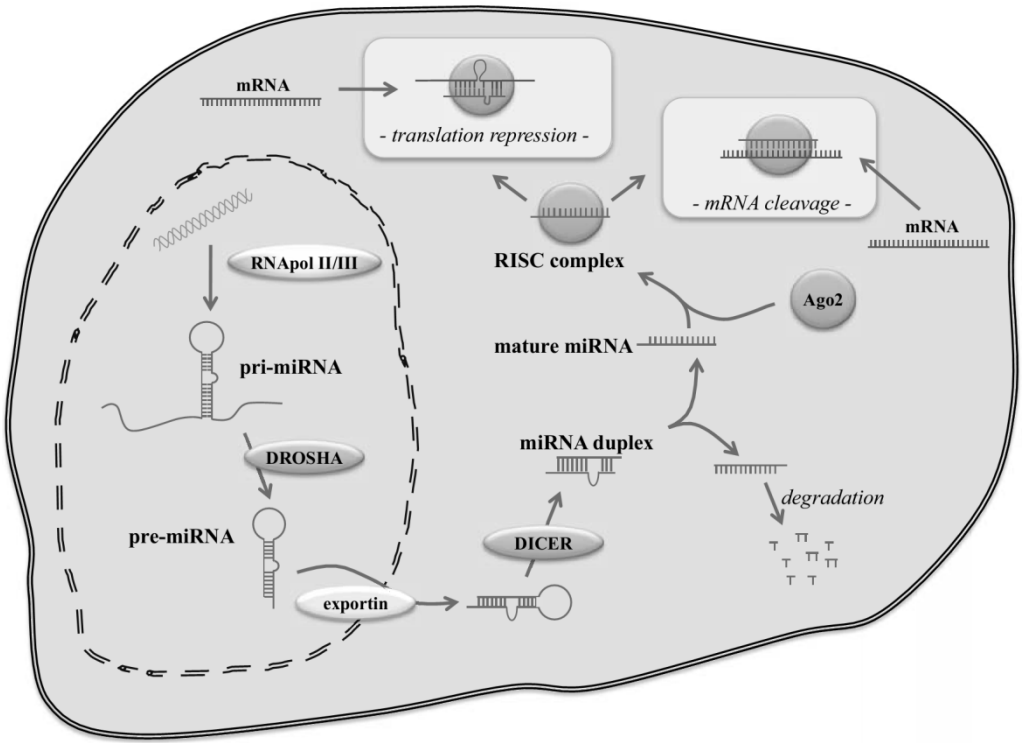


Figure 2 | **MiRNA biogenesis** (adapted from (Lionetti *et al*., 2012)).

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 (Baskerville and Bartel, 2005; Kim and Kim, 2007). 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 (Tsuchiya *et al*., 2006). Two thousand and forty-two human miRNA genes are currently included in the Sanger miRNA registry (miRBase version 19, August 2012), 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 (Sethupathy *et al*., 2006). 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 (Thomas *et al*., 2010). There is strong evidence indicating that miRNAs play important roles in cell processes such as proliferation, development, differentiation and apoptosis (Schickel *et al*., 2008), and it is therefore not surprising that their expression is profoundly deregulated in human cancer (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006). The first direct link between miRNAs and cancer was highlighted by Calin *et al*., who found that the minimal deleted region of chromosome 13q14 in chronic lymphocytic leukemia (CLL) contained the genes encoding *miR-15a* and *miR-16* (Calin *et al*., 2002), 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 (Cimmino *et al*., 2005). 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 (Deng *et al*., 2008). In general 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 (Ventura and Jacks, 2009). 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* (He *et al*., 2005), *miR-155* (Eis *et al*., 2005),

and *miR-21* (Chan *et al*., 2005). Conversely, other miRNAs such as those of *let-7* family have tumor suppressor activity (Johnson *et al*., 2007; Takamizawa *et al*., 2004). 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 (Camps *et al*., 2008; Garzon *et al*., 2008; Lotterman *et al*., 2008; Lu *et al*., 2005; Marcucci *et al*., 2008; Schetter *et al*., 2008; Yu *et al*., 2008).

MicroRNAs in plasma cell dyscrasias

Aberrant miRNA expression in MM has been reported by us and other groups, demonstrating that miRNA deregulation characterizes the progression of the disease, affects important pathways involved in MM cell survival and reflects the different MM genetic subtypes (reviewed in (Lionetti *et al*., 2012)). Furthermore, data obtained more recently *in vitro* and *in vivo* provided a proof-of-principle that miRNA-based treatment has therapeutic activity in preclinical models of MM and support a framework for development of miRNA-based treatment strategies in MM patients (Amodio *et al*., 2012; Di Martino *et al*., 2012). To date, however, no information on miRNA expression in pPCL has been reported.

As stated above, MM is characterized by profound genomic instability involving both numerical and structural chromosomal aberrations. Our first main contribution to the study of the involvement of miRNAs in myeloma was precisely the identification of specific patterns of miRNA expression in different molecular subgroups of the disease. In particular, we have provided evidence that the miRNA transcriptional profiles of 40 MM samples significantly grouped patients according to the proposed TC classification (Hideshima *et al*., 2004), particularly with regard to t(4;14) (TC4) and MAF-translocated (TC5) samples (gray and red branches in Figure 3, respectively) (Lionetti *et al*., 2009b).

In a supervised approach, 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 (Figure 4).

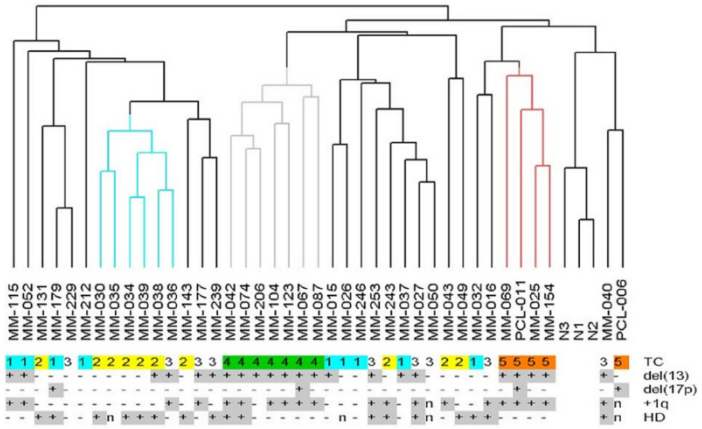


Figure 3 | Unsupervised analysis of miRNA expression profiles in MM samples (Lionetti *et al.*, 2009b). Hierarchical clustering of the samples using the 74 most variable miRNAs (patients in columns, miRNAs in rows). The color scale bar represents the relative miRNA expression changes normalized by the standard deviation. The patients' molecular characteristics are shown above the matrix; n indicates unavailable information. Specific characteristics are enriched in colored sub-branches.

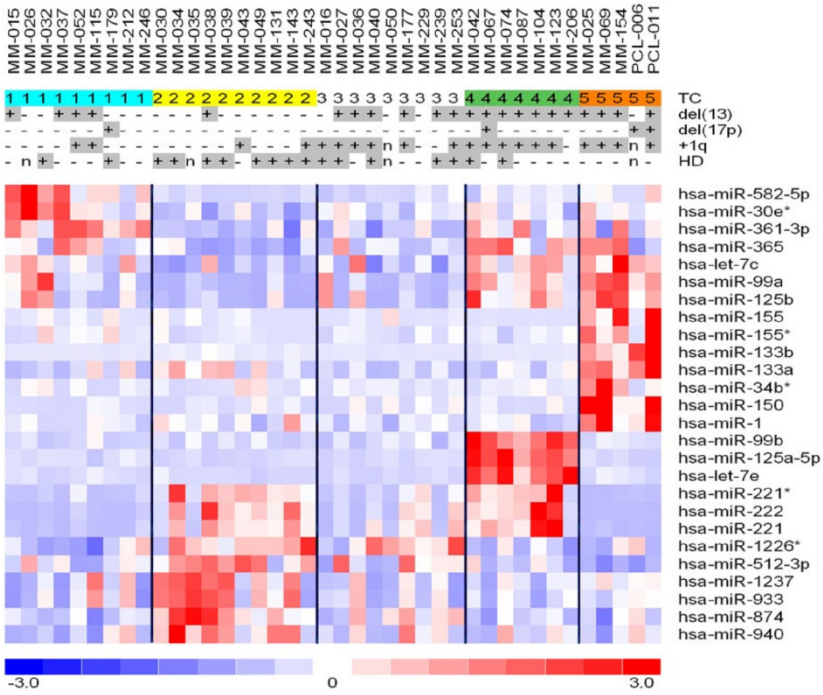


Figure 4 | Identification of miRNA signatures characterizing TC classes (Lionetti *et al.*, 2009b). Heatmap of the differentially expressed miRNAs in MM patients stratified into the 5 TC groups.

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 (Guo *et al.*, 2010a). *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 reported that *miR-125b* promotes B lymphocyte diversification in GCs by inhibiting the premature use of essential transcription factors for PC

differentiation (Gururajan *et al.*, 2010), 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 gene expression in translocation events may modulate miRNA expression. We are currently investigating the role that these miRNAs may have in MM cells, and preliminary results have been also produced for the cluster of *miR-221* and *222*, more expressed in TC4 and TC2 patients (hyperdiploid and with high expression of *CCND2*): these miRNAs modulate the expression of p27 protein in MM cells, thus influencing directly the entry into S phase of the cell cycle (Figure 5).

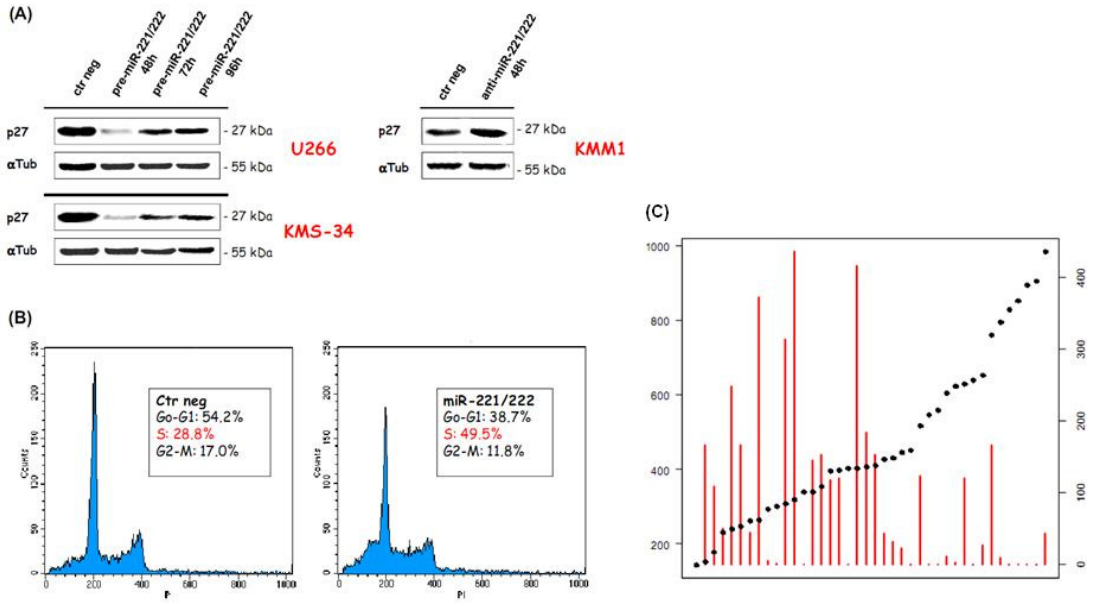


Figure 5 | *MiR-221/222* cluster modulates *p27* in MM, thereby directly affecting entry into S phase (Lionetti *et al.*, 9th Congress of the Italian Society of Experimental Hematology, 2010, Poster#081). (A) Expression of p27 protein in U266, KMS34 and KMM1 cell lines transfected with pre-miRs *221* and *222* (U266 and KMS34), anti-miRs *221* and *222* (KMM1) and relative negative controls. Total cellular extracts collected at the indicated time points after transfection were analyzed by Western blotting using an anti-p27 antibody, and α-tubulin was included as a control for equal protein loading (B) Flow cytometric analysis of U266 cells 72h after transfection with pre-miRs *221* and *222* or relative negative control: the analysis showed an increase in pre-miRs transfected cells with an S DNA content. (C) Significative negative correlation between *P27KIP1* and *miR-222* levels in a dataset of 40 primary MM patients. The red bars represent mature miRNA expression (vertical axis on right side), and the spots represent target gene expression (vertical axis on left side) Horizontal axis: patients samples ordered according to increasing *P27KIP1* expression.

In our series of primary tumors the expression of these two miRNAs is anti-correlated to that of the messenger of *p27*, which is an important gene in MM, considering that:

the induction of its degradation is the mechanism through which progression through the cell cycle is promoted by CKS1B, whose gene is located in the minimal region of amplification in 1q21 and whose over-expression is associated with lower survival in patients at diagnosis (Chang *et al*., 2010); and the stabilization of p27 is one of the mechanisms through which the proteasome inhibitors decrease the proliferation of myeloma cells (Hideshima *et al*., 2003). Also 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) were supposed to target CBFA2T2, a nuclear repressor homologous to ETO that binds to the AML1-ETO complex and may play a role in hematopoietic differentiation (Fracchiolla *et al*., 1998; Lindberg *et al*., 2005), and let-7e putatively targets PTPRE, a positive regulator of osteoclast function and a selective inhibitor of IL-6- and IL-10-induced JAK-STAT signalling (Chiusaroli *et al*., 2004; Tanuma *et al*., 2001) (Figure 6).

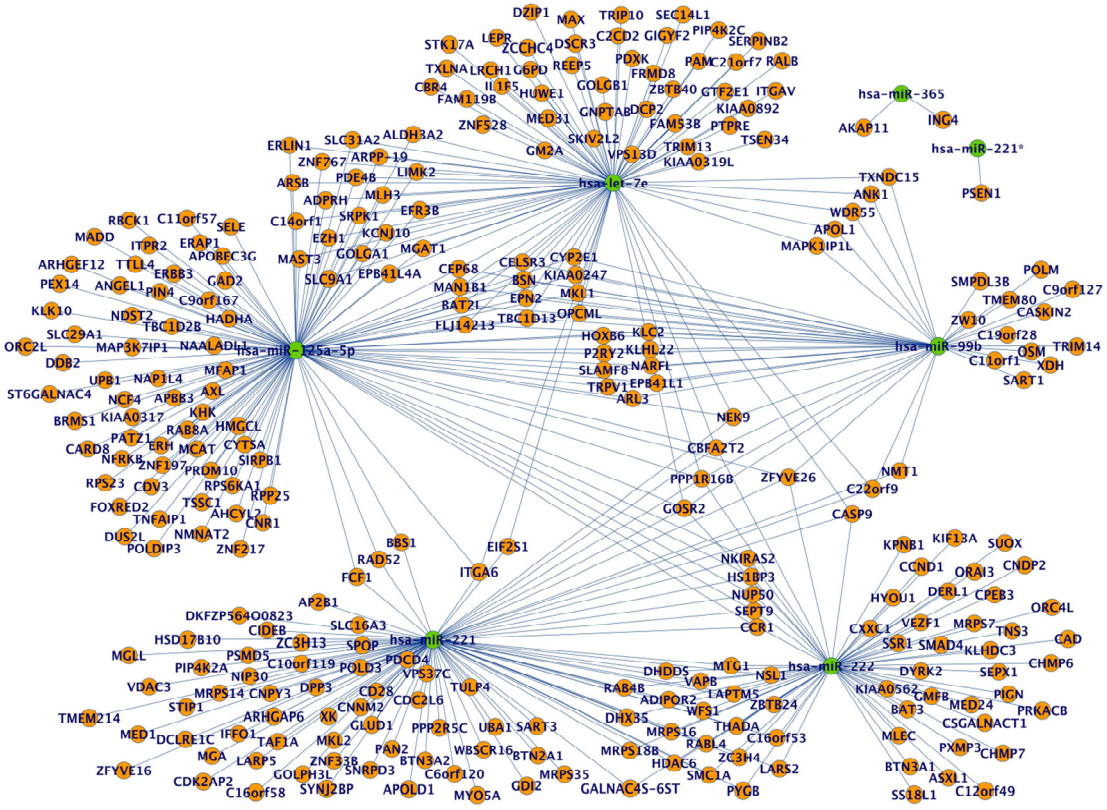


Figure 6 | Predictive functional regulatory relationships (direct edges) between the miRNAs up-regulated in t(4;14) cases (green nodes) and their target genes (orange nodes) (Lionetti *et al*., 2009b). The network-based frame allows the visualization of possible post-transcriptional co-regulated groups of genes and groups of miRNAs sharing one or more target genes.

The data generated in our cohort of patients are partially reflected in other MM series (Lionetti *et al*., unpublished results): Figure 7, for example, shows increased expression of *miR-125a-5p* in almost all MM cases of the dataset of the University of Arkansas over-expressing *MMSET* (A) (Gene Expression Ominibus (GEO) Accession Number GSE17306) (Zhou *et al*., 2010), and in MM patients with t(4; 14) of the dataset of Salamanca's group (B) (GEO Accession Number GSE16558) (Gutierrez *et al*., 2010).

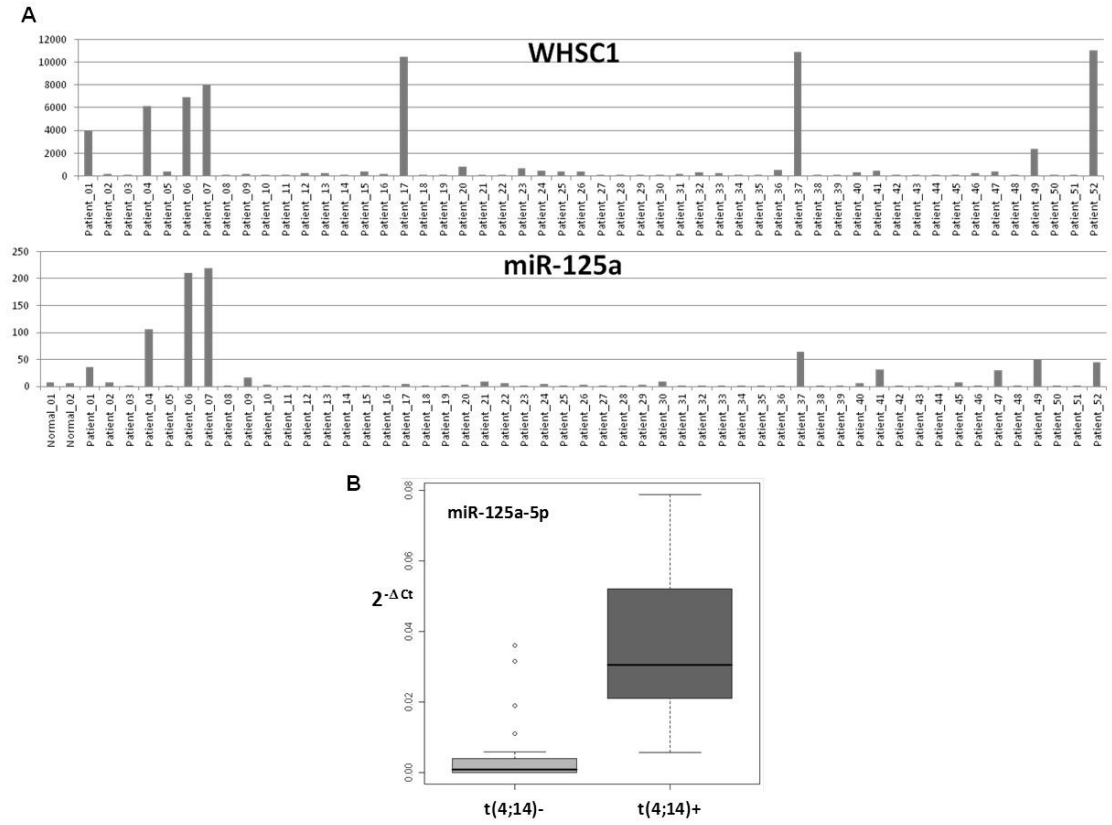


Figure 7 | *MiR-125a-5p* over-expression in TC4 MM samples of independent datasets. (A) *MMSET* and *miR-125a-5p* expression in 52 MM patients profiled on Affymetrix U133 Plus 2.0 GeneChip microarrays and Agilent Human miRNA Microarray Platform V1, respectively (Zhou *et al*., 2010). Normalized gene and miRNA expression data were downloaded from NCBI GEO database (Accession number GSE17306). (B) *MiR-125a-5p* expression in 60 MM samples profiled on TaqMan low-density arrays (Applied Biosystems) (Gutierrez *et al*., 2010) and classified according to the occurrence of t(4;14); miRNA expression data were downloaded from NCBI GEO database (Accession number GSE16558) and expressed as $2^{-\Delta Ct}$ after normalization based on the mean expression of *RNU44* and *RNU48*.

Aim of the study

To investigate the involvement of miRNAs in pPCL and their possible relationship with higher tumor aggressiveness, miRNA expression profiles were analyzed in highly-purified malignant plasma cells from 18 pPCL untreated patients included in a prospective clinical trial. MiRNA expression patterns were evaluated in comparison with a representative series of multiple myeloma (MM) patients, in relation to the most recurrent chromosomal abnormalities (as assessed by fluorescence *in situ* hybridization and single nucleotide polymorphism-array analysis), and in association with clinical outcome. MiRNA expression was also integrated with gene expression profiles in pPCL and MM samples in order to identify putative target genes of deregulated miRNAs.

Materials and Methods

Patient samples

Bone marrow aspirates from pPCL patients were obtained during standard diagnostic procedures. PPCL patients had been diagnosed on the basis of previously described criteria (International Myeloma Working Group, 2003; Albarracin and Fonseca, 2011) and belonged to a national, multicenter pilot clinical trial including 23 patients (12 males, 11 females; median age at diagnosis: 60 ys, range: 44-80 ys). This study was aimed to evaluate safety and anti-tumor activity of lenalidomide and dexamethasone combination in previously untreated pPCL (RV-PCL-PI-350, registered at www.clinicaltrials.gov as NCT01553357) (Musto *et al*., 2011); in particular, enrolled patients received lenalidomide at a dose of 25 mg/d for 21 days and oral dexamethasone at 40 mg on days 1, 8, 15, and 22 for each 28-day cycle. After 4 cycles, responding patients not eligible for ASCT continued until 8 cycles of full-dose LD, if tolerated, followed by a maintenance dose of single agent lenalidomide equal to 10 mg/d on days 1-21 of each 28-day cycle. Patients responding after 4 cycles and eligible for ASCT proceeded according to single Centre transplant policy. Patients not responding after 4 cycles or progressing during this treatment were considered off-study. The primary endpoint was the response rate according to International Uniform Criteria (van de Donk *et al*., 2012; Bird *et al*., 2011); secondary endpoints were safety, progression free survival (PFS) and overall survival (OS): for PFS, subjects were considered treatment failures at the time of progression or death from any cause; OS was defined as time from the date of diagnosis to the date of death or last contact. PFS and OS were measured over a median follow-up of 23 months. The description of safety, efficacy and side effects of the treatment, together with a comprehensive description of the clinical trial, is beyond the scope of this work and will be treated elsewhere (Musto *et al*., manuscript in preparation). All patients gave their informed consent for molecular analyses.

PCs were purified using CD138 immunomagnetic microbeads (MidiMACS, Miltenyi Biotec, Auburn, CA). The purity of the positively selected PCs (≥ 90%) was assessed by means of flow cytometry.

High-density single nucleotide polymorphism (SNP)-array analysis

SNP-array analysis was performed in 17 patients (Table 5 A in the “Results” section).

MiRNA expression profiling

MiRNA expression profiling was performed in 18 out of 23 pPCL patients for whom material was available (molecular and clinical data of the 18 pPCL patients are reported

in Table 5 A in the "Results" section). A series of 39 MM, most of which were included in our previous report, were also profiled for global miRNA expression (Lionetti *et al*., 2009b); patients were selected on the basis of their representativeness with respect to molecular characteristics. Overall, nine MM patients had t(11;14), seven MMs t(4;14), two MMs t(14;16) and one MM t(14;20). The frequencies of the major genetic lesions in MM patients were globally consistent with those reported in literature (Table 5 B); only del(17p) occurred in a slightly lower percentage (Sawyer, 2011). Furthermore, miRNA expression profiling was performed in normal control PCs (NCs) obtained from 4 healthy donors.

Gene expression profiling analysis

Total RNA was available from 21 pPCL (Table 5 A in the "Results" section) and 36 of the 39 MM patients profiled for global miRNA expression.

Fluorescence *in situ* hybridization

Pathological samples from all patients were characterized by fluorescence *in situ* hybridization (FISH) analysis for the presence of the main IGH@ chromosomal translocations and for del(13q14), del(17p13), 1p33 (*CDKN2C*) loss and 1q21.3 (*CKS1B*) gain aberrations, as previously described (Agnelli *et al*., 2009; Fabris *et al*., 2005; Fabris *et al*., 2007). The cut-off levels considered were 10% for fusion or break-apart probes and 20% for numerical abnormalities, as recommended by the European Myeloma Network (EMN) (Ross *et al*., 2012).

TP53 mutation analysis

TP53 mutation analysis was performed on genomic DNA of 17 pPCLs. Oligonucleotide primers used to amplify *TP53* exons 5–9 and polymerase chain reaction (PCR) protocols were downloaded from the IARC TP53 database website (http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf). The amplicons were sequenced directly with sense and anti-sense primers using the Big Dye terminator v1.1 cycle sequencing kit in ABI PRISM 310 automated sequencer (Applied Biosystems, FosterCity, CA).

High-density SNP array analysis

Total genomic DNA was extracted using Wizard genomic purification DNA kit (Promega), then processed and hybridized on Affymetrix GeneChip Human Mapping 250K *NspI* arrays following the manufacturer's protocol (Affymetrix). Each sample

was compared with a set of 48 normal Caucasian HapMap references available on the Affymetrix web site (http://www.affymetrix.com/support/technical/sample_data/500k_data.affx).

Genotyping Console was used to extract raw DNA copy number (CN) probability from CEL files according to Affymetrix guidelines. CN was then estimated using circular binary segmentation and normalized on FISH data using *DNAcopy* and *FBN* packages in R software, respectively, as previously described (Agnelli *et al*., 2009; Mosca *et al*., 2010). Furthermore, DNA CN estimation using CD138 negative bone marrow population as normal counterpart (i.e., CD138+ < 0.5% as assessed by FACS analysis) was performed in 8 patients. The thresholds for the identification of the gained and lost genomic regions resulted as follows: inferred CNs of more than 2.18, 2.52 and 3.41 corresponded to 3, 4 and 5 or more copies, whereas CNs of less than 1.75 and 1.34 corresponded to mono- and biallelic deletion. The genotyping data have been deposited at NCBI Gene Expression Omnibus repository (http://www.ncbi.nlm.mih.gov/geo) and are accessible through GEO Series accession number GSE39383.

MiRNA expression profiling

The total RNA extraction and quality assessment were performed as previously described (Lionetti *et al*., 2009a). Samples were profiled using the one-color technique in accordance with the manufacturer's instructions on the Agilent Human miRNA Microarray V2 (Agilent Technologies, Santa Clara, CA), which consist of 60-mer DNA probes synthesized *in situ* that represent 723 human and 76 human viral miRNAs from the Sanger miRBase (v10.1) (Lionetti *et al*., 2009b). Expression values were extracted according to procedures described in Agilent Feature Extraction Software version 10.1 manual. Non-human probes, miRNAs flagged as "absent" (i.e. expressed below background levels) throughout the whole dataset and miRNAs expired according to Sanger miRBase Release 15 (April 2010) were discarded, and quantile normalization was applied on raw data using the *aroma.light* package for Bioconductor. The data were then converted to obtain positive values throughout the dataset, at a minimum value of 1, and log2 transformed. The raw and normalized miRNA data are available through GEO accession number GSE37053. DNAChip Analyzer (dChip) software (Schadt *et al*., 2001) was used to visualize the selected probe list and to generate hierarchical agglomerative clustering of the samples, performed using Pearson's correlation coefficient and average linkage as distance and linkage metrics, respectively, on those probes whose average change in expression levels varied at least

two-fold from the mean across the dataset; the threshold for significance of sample enrichment was set at $P = 0.0005$. Supervised analyses were carried out using *samr* package for Bioconductor. The threshold for significance (at a q-value of 0) was determined by tuning the Δ parameter on the false discovery rate and controlling the q value of the selected probe list. Enrichment analysis of miRNA categories was performed using TAM (Tool for Annotations of MicroRNAs, V2; http://cmbi.bjmu.edu.cn/tam).

The differentially expressed miRNAs were validated using an independent MM cohort (GEO series GSE17306) (Zhou *et al*., 2010) generated on Agilent Human microRNA Microarray V1, including fifty-two MM patients and two healthy donors. To this aim, a comprehensive dataset was generated including PC specimens from our pPCL cohort and the fifty-two MM samples of the series GSE17306; quantile normalization was applied on the expression values of the 276 miRNAs represented on both platforms and expressed in at least one sample of our series. All samples passed the relative-log expression (RLE) evaluation (namely, the 25^{th} and 75^{th} percentile values required to fall between -1 and 1, respectively), made to prevent biases possibly due to inter-cohort analysis. Supervised analysis was carried out using the same approach (SAM, q-value = 0) applied in the proprietary series.

Gene expression profiling (GEP) analysis

Preparation of DNA single-stranded sense target, hybridization to GeneChip® Gene 1.0 ST arrays (Affymetrix Inc., Santa Clara, CA) and scanning of the arrays (7G Scanner, Affymetrix Inc.) were performed according to manufacturer's protocols. Log2-transformed expression values were extracted from CEL files and normalized using NetAffx Transcript Cluster Annotations, Release 32 (June 2011) and robust multi-array average (RMA) procedure in Expression Console software (Affymetrix Inc.). Non-annotated transcript clusters were discarded. The expression values of transcript cluster ID specific for *loci* representing naturally occurring read-through transcriptions or mapped to more than one chromosomal location were summarized as median value for each sample. The data have been deposited at NCBI Gene Expression Omnibus repository (http://www.ncbi.nlm.mih.gov/geo) and are accessible through GEO Series accession number GSE39925. The differentially expressed genes between pPCL and MM cases were identified using the Significant Analysis of Microarrays software version 4.00 as previously described (Agnelli *et al*., 2007). The functional annotation analysis on the selected lists was performed using the Database for Annotation,

Visualization and Integrated Discovery (DAVID) 6.7 Tool (U.S. National Institutes of Health at http://david.abcc.ncifcrf.gov/).

Quantitative Real-Time PCR (Q-RT-PCR)

MicroRNAs. Selected mature miRNAs underwent Q-RT-PCR using TaqMan® microRNA assays (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's protocol. All of the RNA samples were normalized on the basis of the RNU48 TaqMan® miRNA Assays-Control. The measurement of miRNA expression was performed using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All the samples were run in duplicate. MiRNA expression was relatively quantified using the $2^{-\Delta Ct}$ method (Applied Biosystems User Bulletin No.2). All the samples were run in duplicate. Data were expressed as $2^{-\Delta Ct}$ (Applied User Bulletin No. 2).

Protein-coding genes. Oligo-dT-primed cDNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression levels of selected genes were analyzed in by means of TaqMan Gene Expression Assays and the TaqMan Fast Universal Master Mix were used according to manufacturer's instructions (Applied Biosystems, Foster City, CA). GAPDH-specific pre-developed assay reagent (PDAR) (Applied Biosystems) was used as internal control. The measurement of gene expression was performed using the StepOne Thermocycler (Applied Biosystems). All the samples were run in triplicate. Data were expressed as $2^{-\Delta\Delta Ct}$ (Applied User Bulletin No. 2).

DNA copy number/miRNA expression integrative analysis

To evaluate whether gene dosage effects could be identified for a specific miRNA, the relationship between miRNA expression and the inferred CN of the corresponding miRNA gene (according to NCBI36/hg18) was measured by means of Kendall's tau test. The analyses were performed using appropriate R software packages.

Integrative analysis for miRNA target identification

The integrated analysis of target predictions, miRNA and gene expression data was performed using scripts for R software developed in our laboratory. In particular, we assessed the correlation between the expression data of all possible miRNA-gene pairs in 18 cases of pPCL and 36 cases of MM, and for each significantly anti-correlated pair (using Pearson's correlation coefficient and setting the threshold for Benjamini-

Hochberg adjusted-*P* value at 0.05) we verified the existence of a predicted targeting relationship by 9 computational algorithms. Specifically, queries were submitted to miRecords (http://mirecords.biolead.org) and results were retrieved for the following databases: DIANA-microT, RNA22, NBmiRTar, MirTarget2, PicTar, miRanda, and RNAhybrid; in addition, updated pre-compiled datasets were downloaded from PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and TargetScan (http://www.targetscan.org/). Then, we selected those genes predicted as targets by at least 5 miRNA target prediction programs.

3' UTR Reporter Assay

293T cells were plated at 600,000 cells per well in a 6 well plate. The next day cells were transfected with 30 nM of specific mirVana miRNA mimics (Ambion) or pre-miR negative control (Ambion), 3.0 μg of pMir target firefly luciferase reporter plasmid containing 3' UTR sequences from the putative target gene of interest (Origene) and 0.1 μg of Renilla luciferase expression plasmid pRL-SV40 (Promega) using Lipofectamine 2000 (Invitrogen). At 24 hrs post-transfection cells were analyzed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Each sample was prepared in triplicate and the entire experiment was repeated twice.

Survival analysis

The correlation between the expression levels of each tested miRNA and OS or PFS was tested using the Cox proportional hazards model in the *globaltest* function of R software. Patients were stratified into two groups using sliding thresholds on the expression levels of the most significant miRNAs identified by *globaltest*, and the groups identified by this approach were then tested for association with survival. Survival analysis was conducted with *survcomp* package in R software, using the Kaplan-Meier estimator and log-rank test, and *P* values were calculated according to the standard normal asymptotic distribution. Independence between commonly used prognostic factors and miRNA signatures was tested using multivariate Cox regression procedure of *survival* R package.

Cell lines

NCI-H929, U266, and SKMM1 MM cell lines were available within our research network. RPMI-8226 MM cell line was purchased from Istituto Zooprofilattico Sperimentale (I.Z.S.L.E.R.). OPM1, DOX-6, and LR-5 MM cell lines were kindly

provided by Dr. Eduard Thomson (University of Texas Medical Branch, Galveston, TX, USA), MM1S was purchased by American Type Culture Collection, INA-6 was provided by Dr. Renate Burger (University of Erlangen-Nuernberg, Erlangen, Germany (Burger *et al*., 2001; Tassone *et al*., 2005)). Human BM stromal cells (BMSC) were obtained by long-term culture of BM mononuclear cells (Fulciniti *et al*., 2009; Hamasaki *et al*., 2005; Neri *et al*., 2007). For co-culture, $1x10^5$ CD138+ cells were seeded on $5x10^4$ BMSCs, which had been cultured for 24 to 48 hours in 96-well plates. Cells were grown in RPMI medium containing L-glutamine (Gibco®, Life Technologies, Carlsbad, CA) with the addition of 10% fetal bovine serum (Lonza Group Ltd., Switzerland), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco®, Life Technologies) at 37°C in a 5% CO_2 atmosphere (Tassone *et al*., 2000; Tassone *et al*., 2002). The IL-6 dependent INA-6 cell line was cultured in the presence of 2.5 ng/ml of human recombinant IL-6 (Sigma-Aldrich). Methylcellulose colony assays were performed in RPMI-1640 medium containing 1.1% methylcellulose (Stem Cell Technologies) and 20% FBS. Colony formation was scored at day 14 after plating 2000 cells (SKMM1) or 1000 cells (RPMI-8226) in 18 well plates containing 1 ml of methylcellulose medium.

In vitro transfection of MM cells with synthetic pre-/anti-miRs

Synthetic pre- and anti-miRNAs and corresponding negative controls (miR-NC) were purchased from Ambion (Applied Biosystems). A total of $1x10^6$ cells were electroporated with scrambled (miR-NC) or synthetic pre- or anti-miR at a final concentration of 100nmol/L, using Neon Transfection System (Invitrogen) with 1050 V, 30 ms, 1 pulse. Cell transfection efficiency was evaluated by flow cytometric analysis of FAM-dye-labeled synthetic miRNA inhibitor (Invitrogen) transfection.

Apoptosis analysis

MM cells transfected with pre/anti-miR or scrambled sequence were harvested and treated with Annexin V/7-aminoactinomycin D (7-AAD) solution (BD Pharmingen) at 24, 48, and 72 hours, according to the manufacturer's instructions.

Cell survival and proliferation assays

For cell viability analysis, $1.5x10^5$ MM cells were plated in 6 well plates, electroporated with pre/anti-miR or miR-NC, and then harvested and counted at 24-hour intervals using a Trypan Blue-excluding viable cells assay. Each sample was run

in triplicate. Alternatively, cell survival was evaluated by MTT assay in 96-well plates. In brief, transfected cells were seeded at a density of $1x10^4$ cells per well in 100 µl of culture medium. Every 24 hours, 10 µl of 5 mg/ml MTT (Dimethyl thiazolyl diphenyl tetrazolium, Sigma) reagent were added to each well, and cells were further incubated for 4 h at 37°C. Then medium was removed, and 100 µl of DMSO (dimethyl sulfoxide) were added to each well to dissolve the formazan. The optical density (OD) was evaluated at wave length of 560 nm. Wells without cells (DMSO alone) were used as blank, and experiments were repeated at least three times. Data represent the mean ± SD of 3 independent experiments.

Cell growth was evaluated by BrdU proliferation assay. Electroporated cells were incubated for 4 hours in 6 well plates; after harvesting, they were plated in 96 well plates for BrdU proliferation assay. Cells were counted at 24 hours intervals. BrdU uptake was measured every 24 hours by the DELFIA cell proliferation assay, and luminescence was detected using a Victor 4 plate reader (Perkin Elmer. Waltham, Massachusetts). Each sample was run at least in triplicate.

Western blotting

SDS-PAGE and Western Blotting (WB) were performed according to standard protocols. Briefly, cells were lysed in lysis buffer containing 15mM Tris/HCl pH 7.5, 120mM NaCl, 25mM KCl, 1mM EDTA, 0.5% Triton 100, Halt Protease Inhibitor Single-Use cocktail (100X, Thermo Scientific). Whole cells lysates (50 mg per line) were separated using 4-12% Novex Bis-Tris SDS-acrylamide gels (Invitrogen), electro-transferred on Nitrocellulose membranes (Bio-Rad), and immunoblotted with the mouse anti-CDK6 (Cell Signaling), anti-Bcl-2, anti-γ-tubulin antibodies (Santa Cruz Biotechnology), and the rabbit anti-TP53 antibody (Santa Cruz Biotechnology).

Results

Part I
Chromosomal alterations and miRNA expression profiling in pPCL patients

Major cytogenetic aberrations and *TP53* mutational analysis of pPCL

FISH analyses

The major chromosomal aberrations investigated by FISH in the prospective series of 23 pPCL patients are reported in Table 5 A. Specifically, translocations at 14q32 involving IGH@ *locus* were detected in the large majority of cases (20/23, 87%); t(11;14) was the most frequent (9/23, 39.1%), t(14;16) was identified in 7 cases (30.4%), and t(4;14) was present in 3 patients (13%). The t(14;20) was present in one of 21 investigated cases (4.8%). The t(6;14) was analyzed in 17 cases, all of which tested negative. Deletions of chromosome 13q were found in 17 patients (73.9%), whereas del(17p13) in 8 patients (34.8%). Alterations of chromosome 1 were analyzed in 21 patients: 1p loss was identified in 8 cases (38.1%) and 1q gain in 10 (47.6%); five patients showed the concomitant presence of both aberrations. Overall, a contingency analysis revealed highly significant inverse correlation between the occurrence of 1q gain and of the t(11;14) (adjusted P = 0.006). In agreement with various published retrospective pPCL series, consisting of a heterogeneous number of cases (Avet-Loiseau *et al.*, 2001a; Avet-Loiseau *et al.*, 2012; Chang *et al.*, 2009; Chiecchio *et al.*, 2009; Tiedemann *et al.*, 2008), in our series we found higher frequencies of t(14;16), del(17p), t(11;14) and del(13q) as compared with MM samples, as illustrated in Table 5 B in comparison with the representative MM cohort profiled here for miRNA expression (although statistical significance was not reached for t(11;14) and del(13q).

TP53 mutation analyses

We tested the frequency of *TP53* mutations in 17 cases (Table 5 A). Four patients (4/17, 23.5%) showed a functionally relevant coding mutation in the DNA binding domain critical for TP53 tumor-suppressing activity. Three of these cases were also positive for 17p deletion and showed a missense mutation at exons 6 (I195T in PCL-027) and 8 (R280K in PCL-018 and R273C in PCL-030), respectively. The remaining *TP53*-mutated patient (PCL-037) had an in-frame deletion of 6 nucleotides affecting codons 155-156 in exon 5.

Table 5 | Characteristics of patients. (A) Demographic and molecular characteristics of the 23 pPCL patients included in the study.

Patient	Age	Sex	PP type	del (13q)[a]	del (17p)[a]	1q gain	1p loss[a]	t(4;14)	t(6;14)	t(11;14)	t(14;16)	t(14;20)	c-myc	TP53 mutation	SNP	miRNA	GEP
PCL-010	80	M	IgGλ	-	-	-	-	-	nd	+	-	-	nd	nd	N	N	Y
PCL-014	46	M	BJ-λ	+	+	-	+	-	nd	+	-	-	nd	nd	N	N	Y
PCL-015	78	M	BJ-κ	+	-	+	-	-	-	-	+	-	nd	wt	Y	N	Y
PCL-016	57	F	IgGκ	+	-	+	+	-	-	-	+	-	-	wt	Y[c]	Y	Y
PCL-017	68	F	IgGκ	+	+	+	+	-	-	-	+	-	+	wt	Y[c]	Y	Y
PCL-018	59	F	BJ-κ	+	+	-	-	-	-	+	-	-	-	R280K	Y[c]	Y	Y
PCL-019	67	F	IgMκ	+	+	+	+	-	-	-	+	-	-	wt	Y[c]	Y	Y
PCL-020	79	F	IgGλ	-	-	+	-	-	-	-	-	-	-	wt	Y	Y	Y
PCL-021	48	M	IgGλ	+	-	+	-	+	-	-	-	-	-	wt	Y	Y	Y
PCL-022	50	M	IgGκ	+	+	-	+	-	-	+	-	-	-	nd	N	Y	Y
PCL-023	60	M	IgGκ	+	+	+	+	-	nd	-	+	-	nd	wt	Y[c]	Y	Y
PCL-024	44	M	IgDλ	+	-	nd	nd	-	nd	-	-	nd	nd	nd	N	N	N
PCL-025	64	F	IgGκ	-	-	+	+	-	-	-	-	nd	nd	nd	N	N	N
PCL-026	59	M	IgGκ	+	-	+	-	-	-	-	+	-	+	wt	Y	Y	Y
PCL-027	65	M	BJ-λ	-	+	-	-	-	-	+	-	-	-	I195T	Y	Y	Y
PCL-028	57	F	BJ-κ	+	-	-	-	-	-	+	-	-	-	wt	Y[c]	Y	Y
PCL-029	51	M	IgAλ	-	-	-	-	-	-	-	+	-	-	wt	Y[c]	Y	Y
PCL-030	52	F	BJ-κ	+	+	-	+	-	-	-	-	+	-	R273C	Y[c]	Y	Y
PCL-032	65	F	IgGκ	+	-	+	-	+	-	-	-	-	nd	wt	Y	Y	Y
PCL-034	59	F	IgGλ	+	-	nd	nd	+	nd	-	-	-	nd	nd	N	Y	Y
PCL-035	76	F	BJ-κ	-	-	-	-	-	-	+	-	-	-	wt	Y	Y	Y
PCL-036	71	M	IgGκ	+	-	-	-	-	nd	+	-	-	-	wt	Y	Y	Y
PCL-037	72	M	IgAλ	+	-	-	-	-	-	+	-	-	-	466-471 del6	Y	Y	Y
pos/tested				17/23	8/23	10/21	8/21	3/23	0/17	9/23	7/23	1/21	2/15	4/17			
(%)				73.9%	34.8%	47.6%	38.1%	13.0%	0.0%	39.1%	30.4%	4.8%	13.3%	23.5%			

[a] Only monoallelic deletion. [c] CD138-negative counterpart available.

(B) Characteristics of pPCL patients and MM patients analyzed for miRNA expression (significance of the difference of frequencies between pPCL and MM cases analyzed for miRNA expression assessed by Fisher's exact test; ns: not significant).

Genetic lesion	Frequency in pPCL cases pos/tested (%)	Frequency in the present MM series pos/tested (%)	*P* value	Frequency in MM according to literature data (Sawyer *et al*., Cancer Genetics 2011)
del(13q)	17/23 **(74%)**	22/39 **(56%)**	ns	50%
del(17p)	8/23 **(35%)**	2/39 **(5%)**	0.004	10%
1q gain	10/21 **(48%)**	19/37 **(51%)**	ns	40%
del(1p)	8/21 **(38%)**	na		30%[a]
t(11;14)	9/23 **(39%)**	9/39 **(23%)**	ns	15%
t(4;14)	3/23 **(13%)**	7/39 **(18%)**	ns	15%
t(14;16)	7/23 **(30%)**	2/39 **(5%)**	0.01	6-7%
t(14;20)	1/21 **(5%)**	1/39 **(3%)**	ns	2%

[a]Morgan *et al*., Nature Reviews Cancer 2012

MiRNA expression profiling in pPCL patients

MiRNA expression profiling was performed in 18 of the 23 pPCL patients included in the protocol for which RNA material was available Table 5 A. Two hundred sixty-nine miRNAs were detected as expressed above background levels in at least one sample. The hierarchical clustering of the samples based on the 46 most variable miRNAs across the dataset is reported in Figure 8 A: the groupings appeared unrelated to known molecular characteristics as indicated above the heatmap. The supervised three-class comparison of miRNA expression between pPCL cases carrying different IGH@ chromosomal translocations identified seven differentially expressed miRNAs, of which three were associated with t(4;14) *(let-7e*, *miR-135a* and *miR-148a*), three with MAF translocations (*miR-7*, *miR-7-1** and *miR-454*) and one with t(11;14) (*miR-342-3p*) (Figure 8 B). Notably, some of these miRNAs had already been found in association with specific molecular subgroups in a proprietary dataset of MM patients (Lionetti *et al*., 2009b).

Regarding numerical chromosomal alterations, gains of the long arm of chromosome 1, found in 8 cases, were not associated with the up-regulation of miRNA expression but rather with the down-regulation of 5 miRNAs, i.e. *let-7b*, *miR-152*, *miR-26b*, *miR-342-5p* and *miR-342-3p* (Figure 8 C). *MiR-342-3p* also resulted from the aforementioned multiclass analysis: this is compatible with the fact that t(11;14) negative and positive cases coincided almost exactly with those carrying or not 1q gain, respectively (as indicated by the above-named contingency analysis performed on all pPCL cases included in the protocol). Interestingly, a similar pattern was also found in MM patients (Lionetti *et al*., 2009b). Conversely, comparison of miRNA expression between patients with or without deletion of 13q or 1p did not identify any differentially expressed miRNA at a significant level (data not shown). Patients carrying chromosome 17p deletion over-expressed one miRNA (*miR-654-5p*) at a significant level (Figure 8 D). Thus, on the basis of these supervised approaches, we did not identify any direct influence of chromosomal gain/deletion on the expression of miRNAs involved in each specific alteration. This is in line with what was previously observed in our MM dataset, where we were able to highlight a gene-dosage effect only through a fine and pointwise correlation of the expression of each miRNA gene with the DNA copy number alterations of residing *loci* (Lionetti *et al*., 2009b).

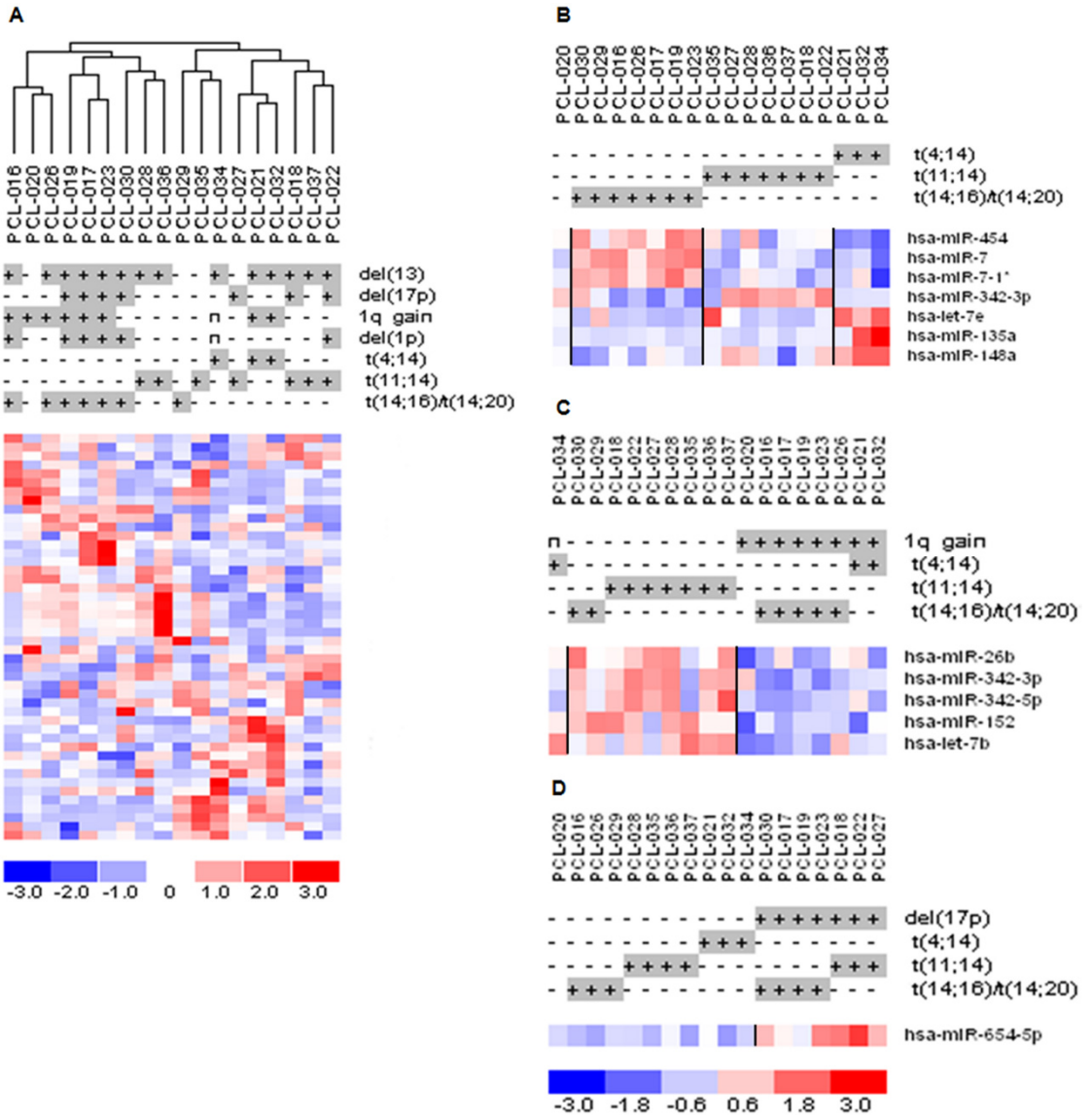


Figure 8 | MiRNA expression profiles in pPCL patients. (A) Hierarchical clustering of the samples using the 46 most variable miRNAs. (B) Heatmap of differentially expressed miRNAs between pPCL patients carrying t(4;14), t(11;14), or t(14;16)/t(14;20). Differentially expressed miRNAs were identified by means of a SAM three-class analysis. MiRNA expression of the pPCL case (PCL-020) not carrying any IGH chromosomal translocation (and therefore not included in the analysis) is also shown. (C) Heatmap of differentially expressed miRNAs between pPCL cases carrying or not 1q gain or (D) del(17p). In all the heatmaps, patients are in columns, transcripts in rows. The color scale bar represents the relative transcript expression changes normalized by the standard deviation, and the color changes in each row represent transcript expression relative to the mean across the samples.

Based on the high frequency (7/18) of 17p deletion in our pPCL panel and the known involvement of *miR-34a* in TP53 pathway, in that TP53 induces *miR-34* family members which in turn target its inhibitor SIRT1, we evaluated the expression of this miRNA in relation to this chromosomal lesion. We found that, although at a less stringent condition in the supervised analysis, 17p deletion was actually associated with

the down-regulation of *miR-34a* (Wilcoxon rank sum test $P = 0.0173$), as also observed by Q-RT-PCR analysis in cases for whom material was available (Pearson correlation coefficient between Q-RT-PCR and microarray expression levels = 0.98) (Figure 9).

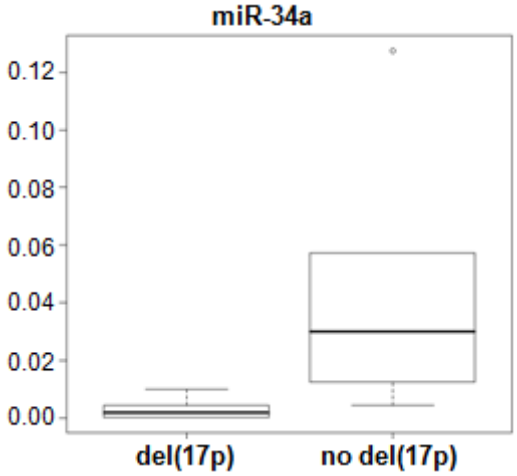


Figure 9 | *MiR-34a* expression in relation to deletion of chromosome 17p in pPCL patients. Box plot of *miR-34a* expression as assessed by quantitative RT-PCR in pPCL patients with or without deletion of chromosome 17p. Expression levels are given as $2^{-\Delta Ct}$.

However, based on available GEP data in pPCL samples, *SIRT1* expression was not significantly inversely correlated with that of *miR-34a*, in all likelihood because the *miR-34a* regulation was demonstrated to act at a translational level. Information on *TP53* mutations was also available for 16 of these patients (Table 5 A): interestingly, almost all of the patients without any chromosomal alteration of the short arm of chromosome 17 did not show any *TP53* mutation, while 3 of 6 cases with 17p deletion also had a *TP53* mutation; a trend of down-regulation of miR-34a expression was observed between samples carrying both 17p deletion and *TP53* mutation and those only carrying 17p deletion, although statistical significance was not reached and the limited number of samples makes it difficult to formulate hypotheses.

Other miRNAs known to be activated by TP53 but not differentially expressed between patients with or without 17p deletion (i.e., other members of *miR-34* family, *miR-145*, *miR-192*, *miR-215*, *miR-107*) mostly had a very low/undetectable expression in pPCL samples, with the exception of *miR-107*. Furthermore, other miRNAs are known to positively or negatively affect the TP53 network, such as, respectively: the aforementioned *miR-192/215* (targeting *MDM2* and cell cycle regulatory genes), *miR-372/373* (that target *LATS2*, involved in a TP53-related positive feedback loop), the liver-specific *miR-122* (that promotes TP53 stability and transcriptional activity by modulating the expression of *CCNG1*), and *miR-29* family members (that up-regulate TP53 levels by directly suppressing the TP53 negative regulators p85α and CDC42); and *miR-125b*, *miR-25*, *miR-30d*, *miR-504* (all directly targeting *TP53* mRNA), *miR-21*

(that can neutralize TP53 activities by targeting multiple critical components of the pathway), and *miR-155* (counteracting the action of TP53 by inhibiting the expression of *TP53INP1*). Interestingly, with the exception of *miR-29* family members, miRNAs cooperating with TP53 were absent/poorly expressed, while *miR-21* was by far the most expressed miRNA, and also *miR-25*, *miR-155*, *miR-125b* and *miR-30d* were among the most highly expressed miRNAs in pPCL patients according to our microarray analysis.

In vitro effects of enforced expression of *miR-34a* in MM cells

Driven by these data and by the observation reported in the literature of the hypermethylation of *miR-34a* promoter in a small fraction of MM patients and cell lines, we decided to investigate the role of this miRNA in MM, in collaboration with the group headed by prof.Tassone of the University of Catanzaro, with which our laboratory is inserted within a "5X1000" AIRC project, aimed at the identification of miRNAs as therapeutic targets in MM and CLL (Di Martino *et al*., 2012).

Expression of *miR-34a* in MM cells

We first evaluated *miR-34a* expression in a series of 11 HMCLs by quantitative RT-PCR. Among these, 2 wild-type *TP53* cell lines (MM1S and NCI-H929) showed significantly higher miR-34a expression as compared with *TP53*-mutated HMCLs (U266, SKMM1, RPMI-8226, INA-6, DOX6, KMS12-PE, OPM1, OPM2, and LR5; Figure 10 A). We next explored if the positive *miR-34a*-TP53 loop was functional in MM cells. Specifically, we treated the *TP53* mutated SKMM1 and RPMI-8226 cells, as well as the *TP53* wild-type NCI-H929 cell line, with nutlin-3, which blocks the TP53-MDM2 inhibitory interaction and thereby induces the expression of TP53-regulated genes (Vassilev *et al*., 2004). As expected, nutlin-3 treatment induced *miR-34a* expression in *TP53* wild-type cells (NCI-H929) but not in *TP53*-mutated SKMM1 and RPMI-8226 cells, as evaluated by q-RT-PCR ($P < 0.05$, Figure 10 B). Nutlin-3-induced up-regulation of *miR-34a* in turn reduced expression of *miR-34a* canonical targets, such as BCL2 and CDK6 proteins. As expected, this effect was not demonstrable in *TP53*-mutated RPMI-8226 cells (Figure 10 C). These findings suggest that *miR-34a* expression is positively modulated by a functional loop in *TP53* wild-type MM cells.

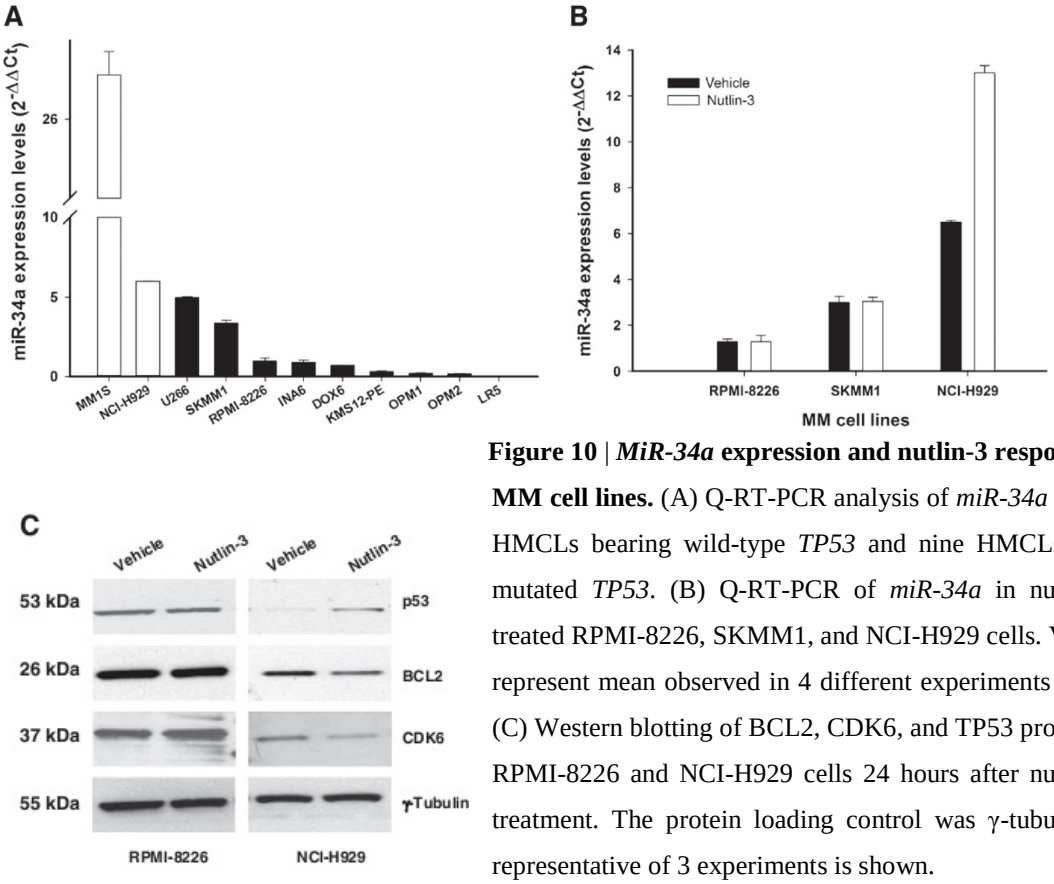


Figure 10 | *MiR-34a* expression and nutlin-3 response in MM cell lines. (A) Q-RT-PCR analysis of *miR-34a* in two HMCLs bearing wild-type *TP53* and nine HMCLs with mutated *TP53*. (B) Q-RT-PCR of *miR-34a* in nutlin-3-treated RPMI-8226, SKMM1, and NCI-H929 cells. Values represent mean observed in 4 different experiments ± SD. (C) Western blotting of BCL2, CDK6, and TP53 protein in RPMI-8226 and NCI-H929 cells 24 hours after nutlin-3-treatment. The protein loading control was γ-tubulin. A representative of 3 experiments is shown.

Biological effects of *miR-34a* transfection in MM cells

To evaluate the biologic effects of *miR-34a*, we transfected low-*miR-34a* MM cell lines with synthetic *miR-34a* or miR-NC (negative control) by electroporation. The anti-proliferative effect induced by *miR-34a* in MM cells was evaluated by Trypan Blue exclusion assay after synthetic *miR-34a* or miR-NC transfection. We observed a significant growth inhibition in *TP53*-mutated SKMM1 ($P < 0.005$, Figure 11 A), RPMI-8226 ($P < 0.05$, Figure 11 B), and OPM1 ($P < 0.005$, Figure 11 C) MM cells. On the other hand, *TP53*-wild type MM1S and NCI-H929 cells, where a functional TP53-*miR-34a* loop is operative, were not inhibited by *miR-34a*, although transfection of anti-*miR-34a* oligonucleotides indeed produced a growth *stimulus* in these cells (Figure 11 D and E). These data further confirm *miR-34a* role as a negative regulator of MM cell growth in *TP53*-wild type cells, and strengthen the rationale of our experimental strategy of enforced expression in *TP53*-mutated MM cells. We next investigated the induction of apoptosis by Annexin V/7-AAD assay. An increase of apoptotic cell death was observed in *TP53*-mutated cells following transfection with *miR-34a*, but not with miR-NC, after 48 hours (Figure 11 F and G) that was more evident in SKMM1 cells. In contrast, this effect was not observed in *TP53*-wild-type

cells (data not shown). To further explore the anti-MM effects induced by *miR-34a*, we carried out a clonogenic assay to study the colony formation activity of transiently transfected cells. We found a 45% and 20% reduced SKMM1 and RPMI-8226 colony formation, respectively, 14 days after transfection (Figure 11 H). These findings indicate that *miR-34a* inhibits clonogenic properties of MM cells.

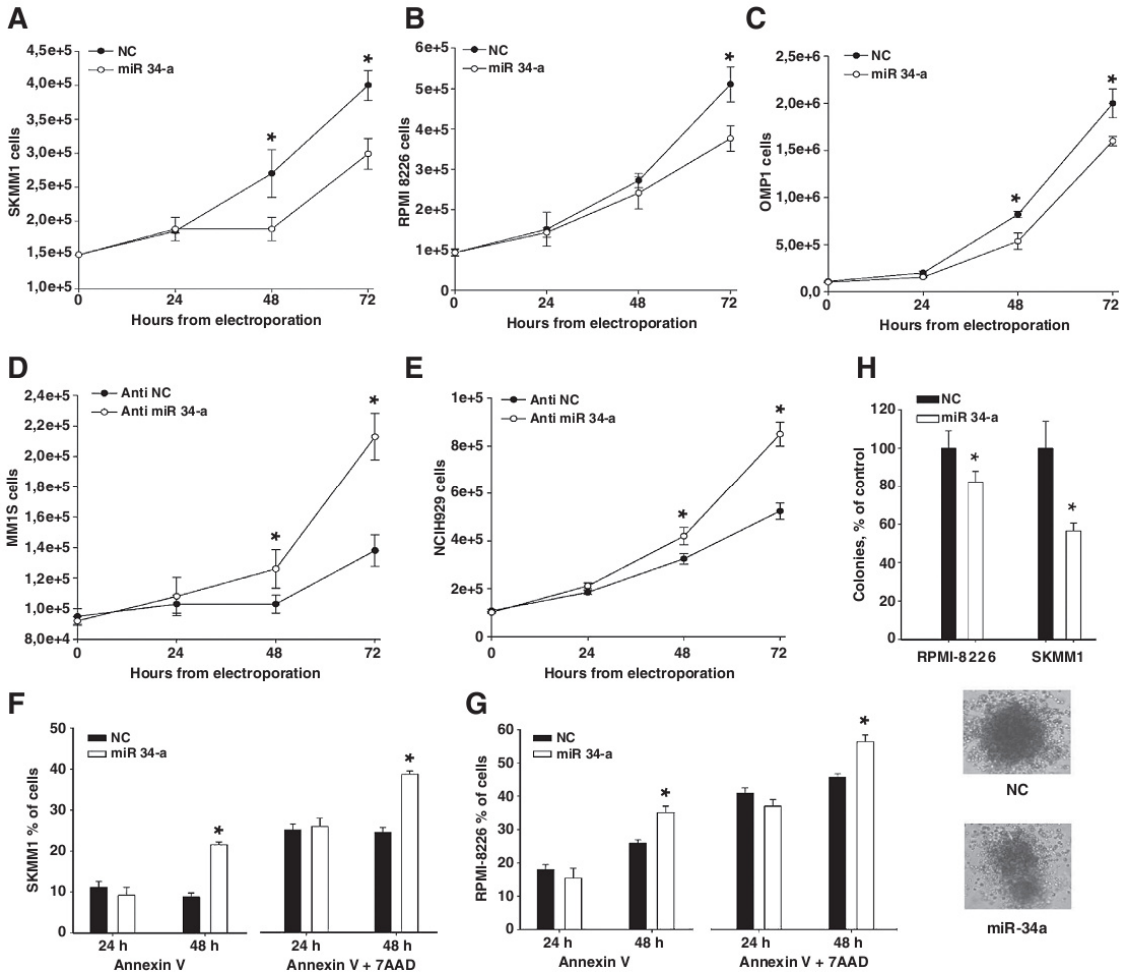


Figure 11 | *MiR-34a* has anti-proliferative activity and induces apoptosis in MM cell lines. Average ± SD values of 3 independent experiments are plotted and *P* values were calculated by 2-tailed Student t-test. Cell growth analysis of SKMM1 (A), RPMI-8226 (B), or OPM1 (C) cells transfected with *miR-34a* or NC. *P* values at 48 and 72 h after transfection are, respectively: 0.01 and 0.005 for SKMM1; 0.001 and 0.05 for RPMI-8226; 0.006 and 0.008 for OPM1. Significant *P* values ($P < 0.05$) are indicated by *. Cell growth analysis of MM1S (D) and NCI-H929 (E) cells transfected with *miR-34a* inhibitor and NC. *P* values: 0.04 and 0.002 for MM1S cells, and 0.04, 0.01, and 0.0008 for NCI-H929 cells at 24, 48, and 72 h after transfection, respectively. Annexin V/7-AAD analysis of SKMM1 (F) and RPMI-8226 (G) cells after transfection with synthetic anti-*miR-34a* or NC. Results are shown as percentage of apoptotic cells. (H) colony formation assay using RPMI-8226 and SKMM1 cells. In all the experiments, *P* was ≤ 0.03 comparing *miR-34a versus* NC. A representative image of *miR-34a* and NC SKMM1 colonies shows homogeneous features of colonies formed by transfected cells with miR-NC, whereas cells transfected with *miR-34a* form irregular heterogeneous colonies.

Downregulation of validated *miR-34a* targets in MM cell lines

To investigate whether genes known to be regulated by *miR-34a* were modulated by exogenous synthetic mimics, we analyzed *BCL2*, *CDK6*, and *NOTCH1* mRNA levels by q-RT-PCR in *miR-34a*-transfected SKMM1 and RPMI-8226 cells. As shown in Figure 12 A and B, we detected a significant down-regulation of *CDK6* and *NOTCH1* mRNA expression 24 hours after cell transfection. This effect occurred together with down-modulation of CDK6 and BCL2 proteins evaluated by Western blot analysis (Figure 12 C and D). Altogether, these results show that synthetic *miR-34a* activity modulates validated targets.

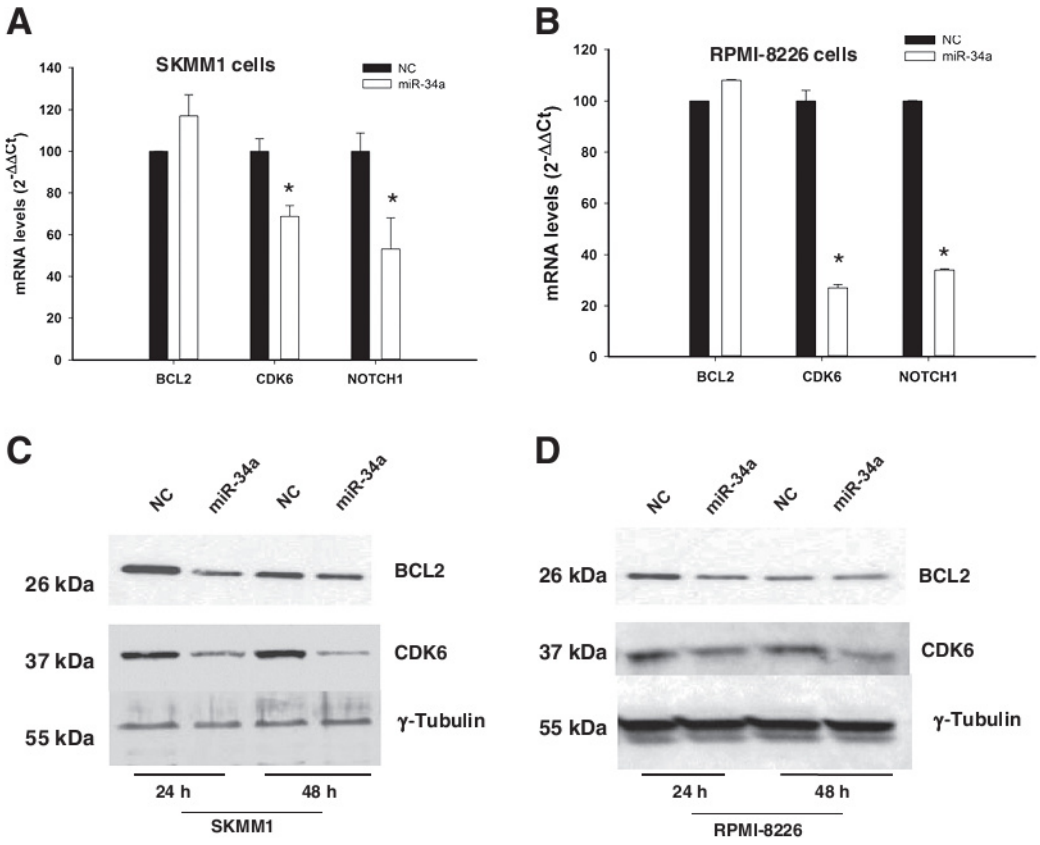


Figure 12 | Molecular effects induced by transient expression of *miR-34a* in MM cells. Q-RT-PCR of *BCL2*, *CDK6*, and *NOTCH1* after transfection with synthetic *miR-34a* or miR-NC in SKMM1 (A) and RPMI-8226 (B) cells. The results are shown as average mRNA expression after normalization with *GAPDH* and ΔΔCt calculations. Data represent the average ± SD of 3 independent experiments. Western blotting of BCL2 and CDK6 protein in SKMM1 (C) and RPMI-8226 (D) cells 24 and 48 hours after transfection with synthetic *miR-34a* or scrambled oligonucleotides (NC). The protein loading control was γ-tubulin. Experiments were conducted in triplicate. *MiR-34a* effects on protein levels reached statistical significance ($P < 0.05$) at all time points.

Influence of chromosomal abnormalities on miRNA expression: evaluation of dosage effects by means of an integrative genomic approach

The influence on miRNA expression of the allelic imbalances detected at DNA genome-wide level was evaluated by performing an integrative analysis of mature miRNA expression and the inferred DNA CN values of the corresponding miRNA gene/s available for 16 of 18 pPCL cases (Table 5 A).

SNP-array

The genome-wide profiles were investigated by SNP-array in the 17 of the 23 pPCL cases for whom DNA material was available (Table 5 A). To confirm their estimation based on public references, CN were also estimated using DNA from CD138 negative BM populations available from eight patients. With the exception of point-wise aberrant signals that were discarded from downstream analyses, all the estimated CN segments were concordant within ± 0.1 with those obtained comparing the eight tumor samples against their normal counterparts (data not shown), suggesting the accuracy of the reported results. The analysis allowed the identification of CN alterations (CNA) in all cases, showing complete concordance with FISH data. A total of 454 aberrations were detected with a median of 20 per sample (range 6-82). Losses were more prevalent (273/454 CNAs; 60.1%), including mono- (229/454, 50.4%) or biallelic deletions (44/ 454, 9.7%). Of the remaining CNAs, 165 (36.3%) were gains (3 or 4 copies), and 16 (3.5%) represented amplifications (5 or more copies). Chromosomes 1, 6, 8, 13, 14, 16, and 17 were the most frequently altered in our series. In particular, deletion of 13q was the most frequent alteration (13/17, 76.5%) detected in our dataset, with most cases (9/13, 69.2%) showing loss of the entire long arm, while deletions involving 17p were found in 6 patients (35%) leading to the definition of two minimally altered regions: the larger (2.6 Mb), spanning the 17p13.2-p13.1 region including the *TP53* gene, and the shorter (0.9 Mb) at 17p12.

DNA copy number/miRNA expression integrated analysis

Considering all miRNA genes except those located on chromosome X, the expression of 23 miRNAs resulted to be significantly correlated with DNA CN on different chromosomes, being chromosome 13 (22%) and the short arm of chromosome 1 the most involved (22%) (Table 6 and Figure 13 for a representative example).

Table 6 | MiRNAs whose expression in pPCL is significantly positively associated with the DNA copy number (CN) of the genomic *locus* harboring the corresponding gene (Kendall's τ correlation, one-sided $P < 0.05$).

miRNA	Chromosomal location[a]	CN = 1, n	CN = 2, n	CN = 3, n	CN = 4, n	CN = 5, n
hsa-miR-197	1p13.3	4	11	1	/	/
hsa-miR-186	1p31.1	3	13	/	/	/
hsa-miR-30e	1p34.2	3	12	/	1	/
hsa-miR-30e*	1p34.2	3	12	/	1	/
hsa-miR-30c	1p34.2	3	12	/	1	/
hsa-miR-29c*	1q32.2	/	9	6	/	1
hsa-miR-28-5p	3q28	1	13	2	/	/
hsa-miR-103	5q35.1	1	13	1	1	/
hsa-miR-148a	7p15.2	1	13	1	1	/
hsa-miR-210	11p15.5	/	14	1	1	/
hsa-miR-483-5p	11p15.5	/	14	1	1	/
hsa-miR-331-3p	12q22	2	13	/	1	/
hsa-miR-15a	13q14.3	12	3	1	/	/
hsa-miR-92a	13q31.3	9	6	1	/	/
hsa-miR-19a	13q31.3	9	6	1	/	/
hsa-miR-20a	13q31.3	9	6	1	/	/
hsa-miR-20a*	13q31.3	9	6	1	/	/
hsa-miR-342-3p	14q32.2	3	12	/	1	/
hsa-miR-630	15q24.1	1	14	/	1	/
hsa-miR-1225-5p	16p13.3	2	13	/	1	/
hsa-miR-140-5p	16q22.1	6	8	/	1	1
hsa-miR-22	17p13.3	5	11	/	/	/
hsa-miR-181d	19p13.12	/	13	2	1	/

[a]For miRNAs produced from distinct genomic *loci*, the expression of the mature miRNA was correlated with the DNA CN of each of these genomic locations. The chromosomal location of the genomic *locus* resulted to be associated with mature miRNA expression is indicated, along with the number of pPCL cases having the reported inferred DNA copy number of that *locus*.

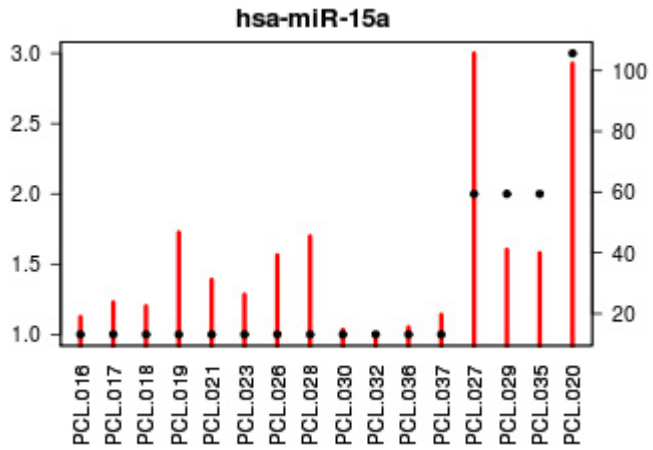


Figure 13 | Representative significant correlation between inferred DNA CN values of 13q14.3 chromosomal *locus* and *miR-15a* expression levels in pPCL. The bars represent mature miRNA expression (right vertical axis), and the spots DNA CN values (left vertical axis). Horizontal axis: pPCLs by increasing CN of 13q14.3.

Specifically, we identified *miR-15a* at 13q14.3 and 4 members of the cluster *miR-17~92* (i.e. *miR-19a*, *miR-20a*, *miR-20a** and *miR-92a*) at 13q31.3 on chromosome 13 (12 and 9 cases showed one copy of the aforementioned genomic *loci*, respectively), and five mature miRNAs (*miR-30e*, *miR-30e**, *miR-30c*, *miR-186* and *miR-197*) encoded by genes mapped to 1p present in single copy in 3 or 4 patients respectively. Two miRNAs (*miR-210* and *miR-483-5p*) were encoded by genes mapped to the cytoband 11p15.5 which was gained in one patient and present in four copies in a near-tetraploid case showing the highest expression. Furthermore, CN of chromosome 16 was correlated with the expression of two miRNAs, *miR-140-5p* and *miR-1225-5p*, encoded respectively by genes mapped to 16q22.1 (monoallelically deleted in 6 patients) and 16p13.3 (present in one copy in two samples). We also highlighted *mir-22*, mapped to 17p13.3. Interestingly, specific CN/miRNA expression correlations found in the present series of pPCLs were previously identified by us in MM patients and/or HMCLs: specifically, *miR-342-3p* at 14q32.2 (whose gene was present in single copy in 2 patients and in 4 copies in one patient), *miR-20a*, *miR-20a**, *miR-210* and *miR-140-5p* were previously found to have a DNA CN-dependent expression in our series of 40 MM samples; *miR-15a*, *miR-19a* and *miR-22* were identified by applying the same approach in a panel of 16 MM cell lines, and the DNA CN of the *mir-30e* gene affected the expression of both mature products (*miR-30e* and *miR-30e**) also in cell lines and primary MM cases (Lionetti *et al*., 2009a; Lionetti *et al*., 2009b).

Part II

MiRNA expression profiling of pPCL and MM patients

To determine whether the natural grouping of miRNA expression profiles might distinguish PCs from NCs, MM and pPCL, we performed an unsupervised analysis of our dataset including 4 NCs, 39 MMs, 18 pPCLs. Based on the 76 most variable miRNAs across the dataset, healthy donors represented a clearly distinct transcriptional entity, and all pPCL samples were grouped together, along with 8 MM samples (Figure 14 A); interestingly, all the MM cases carrying t(14;16) or t(14;20) were included in the pPCL cluster, whereas all but two of the t(4;14) and two of the t(11;14) MM cases were grouped in the MM main cluster. Prompted by these observations, we evaluated in pPCL cases the expression of miRNAs previously identified as distinctive of MM TC (translocation/cyclin D) groups (Lionetti *et al*., 2009b); notably, the overall pattern of miRNA expression in pPCL was globally similar to that of the TC5 MM molecular subgroup, regardless of the presence of MAF translocations (Figure 14 B), with the only exception of those miRNAs that strongly discriminated MM TC4 cases (*miR-125a-5p, miR-99b* and *let-7e*, which were found over-expressed also in t(4;14) pPCLs).

The direct comparison of miRNA expression profiles between pPCL and MM samples by supervised analysis identified 42 up-regulated and 41 down-regulated miRNAs in the pPCL group (Table 7 and Figure 15 A).

The up-regulation of *miR-155*, *miR-21*, *miR-142-3p*, *miR-142-5p* and *miR-103* in pPCL *versus* MM patients was confirmed by performing Q-RT-PCR in all samples for which RNA was available (11 pPCL and 30 MM patients) (Pearson correlation coefficients between Q-RT-PCR and microarray-based expression levels = 0.97, 0.68, 0.65, 0.66 and 0.68, respectively).

The TAM enrichment analysis of miRNA categories of the list of 42 up-regulated miRNAs identified as most significant (FDR < 15%) the over-representations of four classes of miRNAs, namely miRNAs defined as onco-miRNAs or involved in immune response, immune system or hematopoiesis (Figure 15 B). No enriched functional category was found in the list of the 41 down-regulated miRNAs.

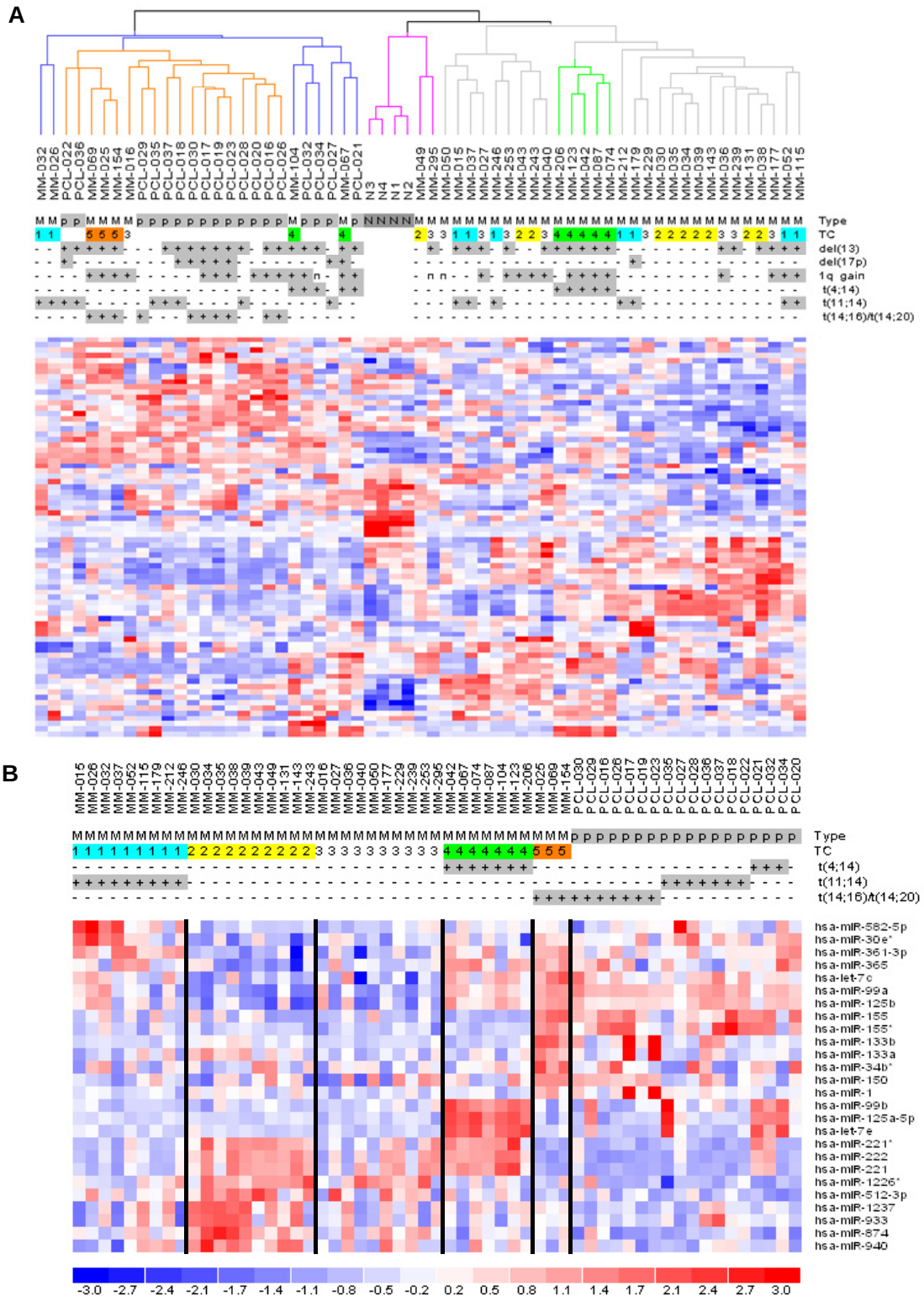


Figure 14 | MiRNA expression profiles in NCs, pPCL and MM patients. (A) Dendrogram of the 4 NCs, 18 pPCL and 39 MM samples clustered according to the expression profiles of the 76 most variable miRNAs. Specific characteristics are enriched in colored sub-branches: pink, NC; grey, MM; blue, PCL; orange, t(14;16) or t(14;20) samples; green, t(4;14) samples. (B) Heatmap of the 26 miRNAs previously identified as distinctive of MM TC groups (Lionetti *et al.*, 2009b) in MM and pPCL patients. MM patients are grouped on the basis of TC classification; pPCL samples are ordered according to the IGH translocation type.

Table 7 | Differentially expressed miRNAs in pPCL compared with MM samples.

miRNA[a, b]	SAM score[c]	miRNA[a, b]	SAM score[c]
hsa-miR-21	7.34	**hsa-miR-513b**	-5.75
hsa-miR-301a	5.13	***hsa-miR-513a-5p***	-5.35
hsa-miR-374a	4.98	***hsa-miR-494***	-4.61
hsa-miR-330-3p	4.79	**hsa-miR-513c**	-4.47
hsa-miR-454	4.71	***hsa-miR-638***	-4.06
hsa-miR-142-3p	4.68	**hsa-miR-193b***	-4
hsa-miR-155*	4.38	**hsa-miR-1224-5p**	-3.61
hsa-miR-301b	4.29	**hsa-miR-193a-5p**	-3.57
hsa-miR-140-5p	4.09	***hsa-miR-222***	-3.5
hsa-miR-18a	4.03	***hsa-miR-145***	-3.49
hsa-miR-99a	3.95	**hsa-miR-23a***	-3.46
hsa-miR-590-5p	3.95	***hsa-miR-221***	-3.4
hsa-miR-362-3p	3.94	**hsa-miR-139-3p**	-3.37
hsa-miR-155	3.91	**hsa-miR-423-5p**	-3.13
hsa-miR-7-1*	3.9	**hsa-miR-1226***	-3.08
hsa-miR-21*	3.47	hsa-miR-126	-3.05
hsa-miR-628-5p	3.32	**hsa-miR-1225-5p**	-3
hsa-miR-20a*	3.22	**hsa-let-7a**	-2.95
hsa-miR-18b	3.17	***hsa-miR-572***	-2.91
hsa-miR-19a	3.15	***hsa-miR-671-5p***	-2.91
hsa-miR-29b-1*	3.1	***hsa-miR-663***	-2.89
hsa-miR-660	3.07	***hsa-miR-765***	-2.86
hsa-miR-424	2.93	hsa-miR-874	-2.84
hsa-miR-142-5p	2.81	***hsa-miR-188-5p***	-2.8
hsa-miR-100	2.76	***hsa-miR-370***	-2.8
hsa-miR-103	2.59	hsa-miR-636	-2.77
hsa-miR-532-5p	2.56	**hsa-miR-135a***	-2.71
hsa-miR-181d	2.56	***hsa-miR-345***	-2.68
hsa-miR-7	2.54	**hsa-miR-34c-3p**	-2.55
hsa-miR-26b	2.53	hsa-miR-1234	-2.52
hsa-miR-125b	2.53	hsa-miR-193b	-2.47
hsa-miR-340	2.52	hsa-miR-221*	-2.46
hsa-miR-374b	2.52	hsa-miR-1229	-2.46
hsa-miR-362-5p	2.49	hsa-miR-877*	-2.38
hsa-miR-20b	2.48	hsa-miR-625*	-2.34
hsa-miR-98	2.46	*hsa-miR-324-3p*	-2.3
hsa-miR-551b	2.45	hsa-miR-96	-2.21
*hsa-miR-181a**	2.33	*hsa-miR-148a*	-2.19
hsa-miR-505*	2.3	**hsa-miR-483-5p**	-2.17
hsa-miR-542-3p	2.27	hsa-miR-1228	-2.16
hsa-miR-210	2.18	hsa-miR-223	-2.14
hsa-miR-500*	2.15		

[a] MiRNAs are in bold whose trend of expression from MM to pPCL was significantly maintained (Jonckheere-Terpstra test, $P < 0.005$) when considering the healthy controls. [b] MiRNAs are in italic if differentially expressed between pPCL from proprietary cohort and MM cases from GEO series GSE17306. [c] Up- and down-regulated miRNAs are ordered according to SAM scores.

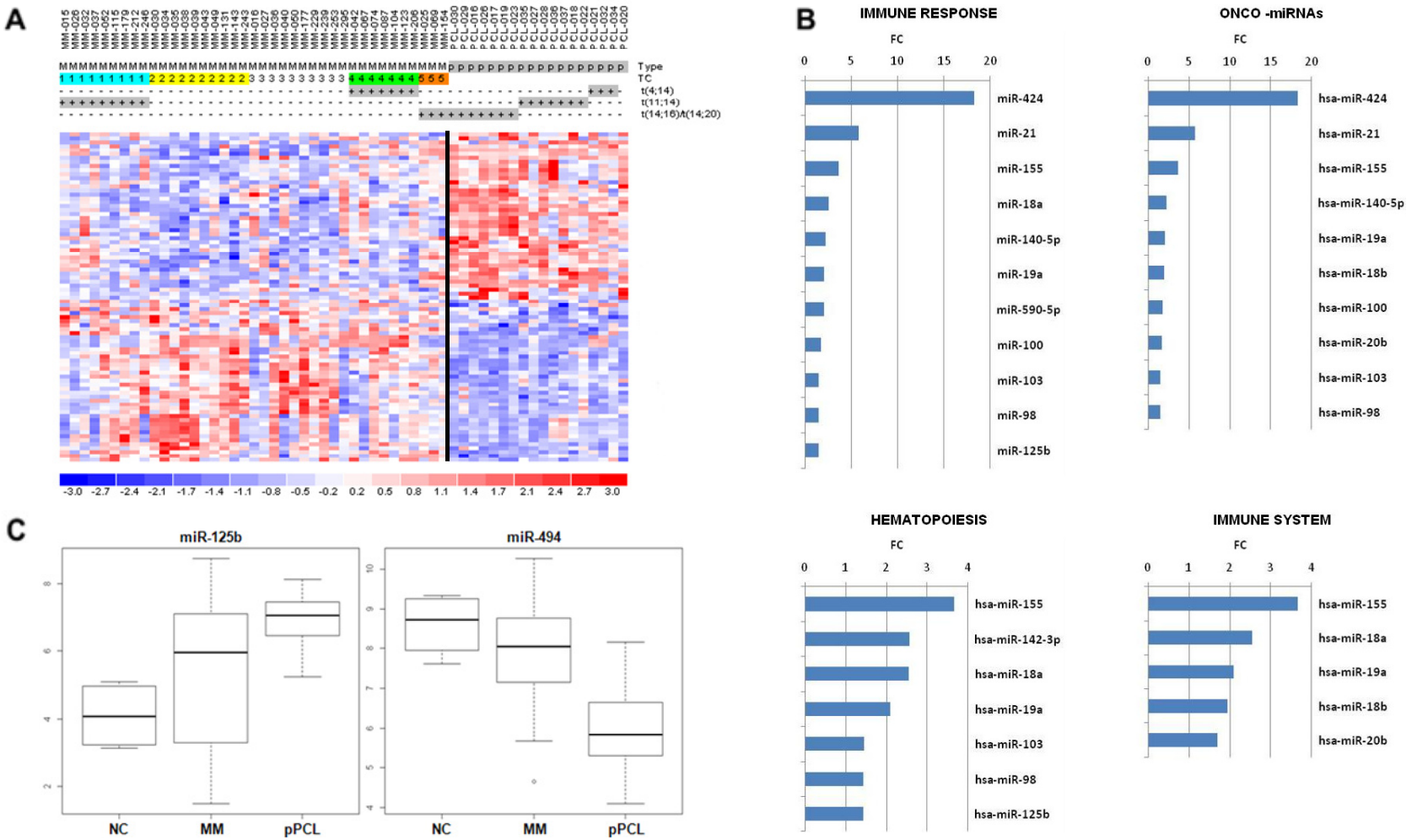


Figure 15 | Differential miRNA expression between pPCL and MM patients ((A) Heatmap of the miRNAs identified as differentially expressed between pPCL and MM patients. (B) Enriched functions in up-regulated miRNAs in pPCL. The average fold change (FC) in expression level of pPCL *versus* MM samples is shown for each miRNA. MiRNAs are ranked according to their FC. (C) Boxplots of two representative differentially expressed miRNAs maintaining a statistically significant trend of differential expression (Jonckheere-Terpstra test, $P < 0.005$) when considering the healthy controls (NC→MM→pPCL) (*miR-125b* resulted up-regulated and *miR-494* down-regulated in pPCL *versus* MM samples).

Validation of miRNA differential expression between pPCL and MM in an independent dataset

The differentially expressed miRNAs between pPCL and MM patients were validated using an independent MM cohort (GEO series GSE17306) (Zhou *et al*., 2010) generated on Agilent Human microRNA Microarray V1, including fifty-two MM patients and two healthy donors. To this aim, we generated a comprehensive dataset including PC specimens from our pPCL cohort and the fifty-two MM samples of the series GSE17306; after verifying the absence of biases possibly due to inter-cohort analysis, a supervised analysis was carried out using the same approach (SAM, q-value = 0) applied in the proprietary series. Notably, when comparing our pPCL cases with MM cases of this independent dataset, we found that 41 (80%) of the 51 miRNAs belonging to our pPCL miRNA signature and represented on Agilent microRNA Microarray V1 showed the same trend of differential expression between pPCL and MM (Table 7).

Modulation of miRNAs included in MM-pPCL signature throughout progression from normal plasma cells to PCL

In addition, based on the hypothesis that the deregulation of these miRNAs might be compatible with the "strength" of neoplastic transformation, we investigated whether among the 83 differentially expressed miRNAs between MM and PCL a trend might be identified in relationship with normal donors. Interestingly, for 56/83 (approximately 70%) miRNAs differentially expressed between pPCL and MM, the trend of expression (increasing or decreasing) from healthy controls, through MM, to pPCL was significantly maintained (Jonckheere-Terpstra test, $P < 0.005$, (Table 7 and Figure 15 C).

Oncogenic role of *miR-21* in malignant plasma cells

The putative oncogenic role of some miRNAs over-expressed in pPCL, suggested by the functional enrichment analysis and by consistent trends of expression from normal plasma cells to pPCL, was directly demonstrated for miR-21 by *in vitro* experiments performed in collaboration with the group headed by Prof.Tassone at the University of Catanzaro. First of all, we evaluated *miR-21* expression in HMCLs, and found variable levels of expression; notably, we found > 3 fold increase in *miR-21* expression when the IL-6-dependent INA-6 cells were cultured adherent to hBMSCs.

To study the anti-MM activity of *miR-21* inhibition, we transfected MM cell lines with *miR-21* inhibitors. By trypan blue exclusion cell count and BrdU proliferation assay, we found significantly decreased cell growth in MM cell lines expressing high *miR-21* levels (KMS26, U266 and OPM2) (Figure 16 A and B). In these cells, we also observed reduction of cell survival by MTT assay (Figure 16 C). Conversely, *miR-21* inhibitors did not affect cell proliferation or survival of MM cell lines with low *miR-21* expression (NCI-H929, MM1S, RPMI-8226) (data not shown).

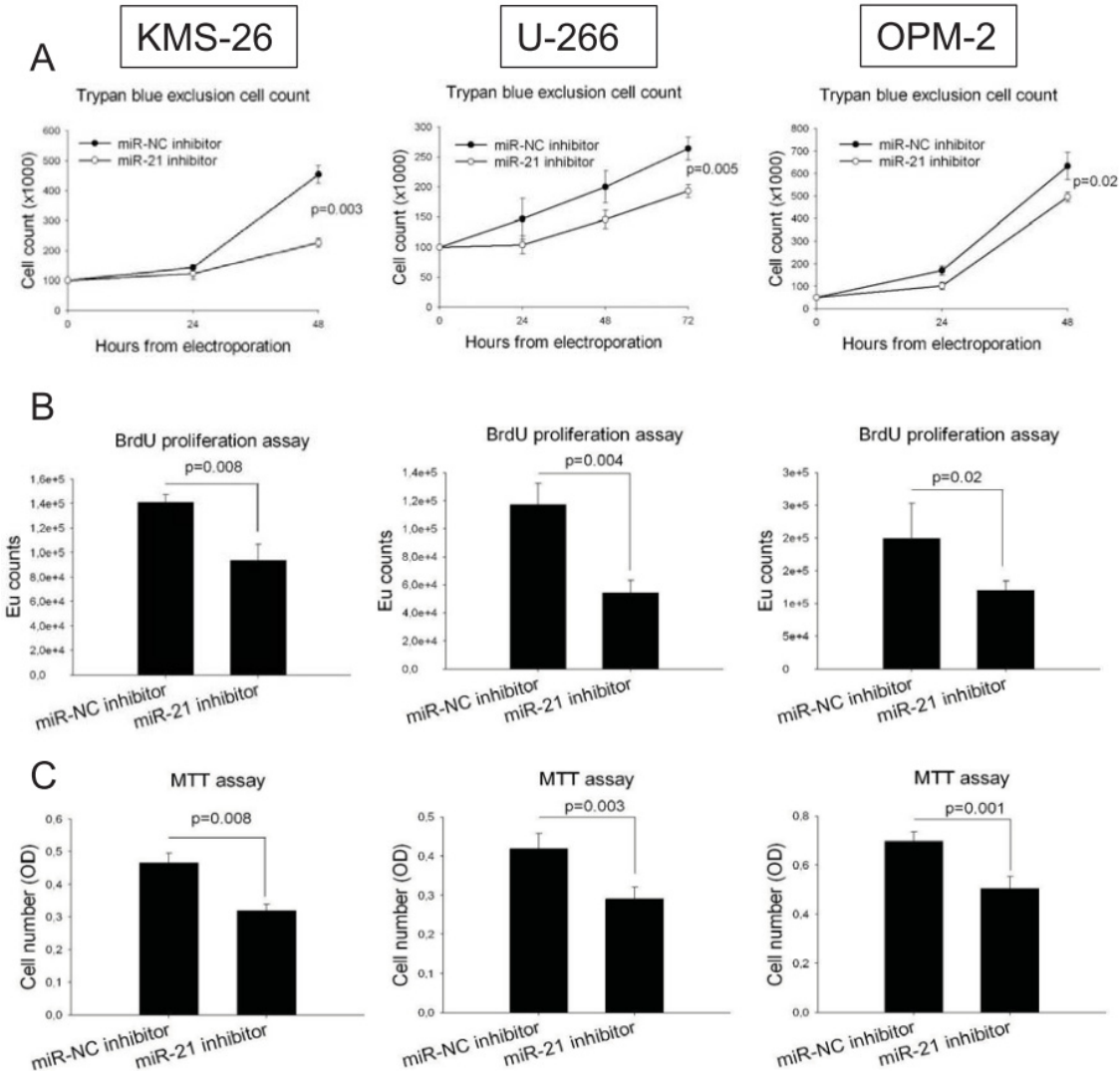


Figure 16 | Effects of ectopic expression of *miR-21* inhibitors in MM cell lines. (A) Trypan blue exclusion growth curves were generated from KMS26, U266 and OPM2 cells transfected with *miR-21* inhibitors or scrambled controls (miR-NC inhibitors). (B) BrdU proliferation assay was performed in KMS26, U266 and OPM2 cells 48h after transfection with *miR-21* inhibitors or scrambled controls. (C) MTT survival assay was performed in KMS26, U266 and OPM2 cells 48h after transfection with *miR-21* inhibitors or scrambled controls. Averaged values ± SD of three independent experiments are plotted. *P* values were obtained using two-tailed t test.

BMSCs in the human BM *milieu* strongly support survival and proliferation of MM cells. Since *miR-21* expression in INA-6 cell line was significantly enhanced by adherence of cells to hBMSCs, we next evaluated whether *miR-21* inhibition could overcome the supportive effects of the BM microenvironment. To this aim, we evaluated the anti-tumor activity of *miR-21* inhibition in a context closely resembling the intramedullary stage of MM. Specifically, we cultured IL-6-dependent MM cell line INA-6 adherent to hBMSCs and enforced the expression of *miR-21* inhibitors in both cell types. As shown in Figure 17 A, *miR-21* inhibition affected viability of MM cells to a similar extent as does hBMSCs deprivation. *MiR-21* inhibition was also observed in INA-6 cells cultured in an IL-6-enriched culture medium (Figure 17 A). We wondered whether this effect was due to *miR-21* inhibition directly in INA-6 cells, in hBMSCs, or in both. Therefore, we investigated the effects of *miR-21* inhibition in INA-6 cells co-cultured with non transfected hBMSCs, and found that the anti-tumor effect was similar to that obtained when *miR-21* inhibition was performed in both cell types (Figure 17 B). In contrast, no effects were observed when *miR-21* was inhibited only in hBMSCs adhering to INA-6 cells (Figure 17 B). From these findings, we conclude that *miR-21* inhibitors abrogate the supporting activity of hBMSC on MM cell lines.

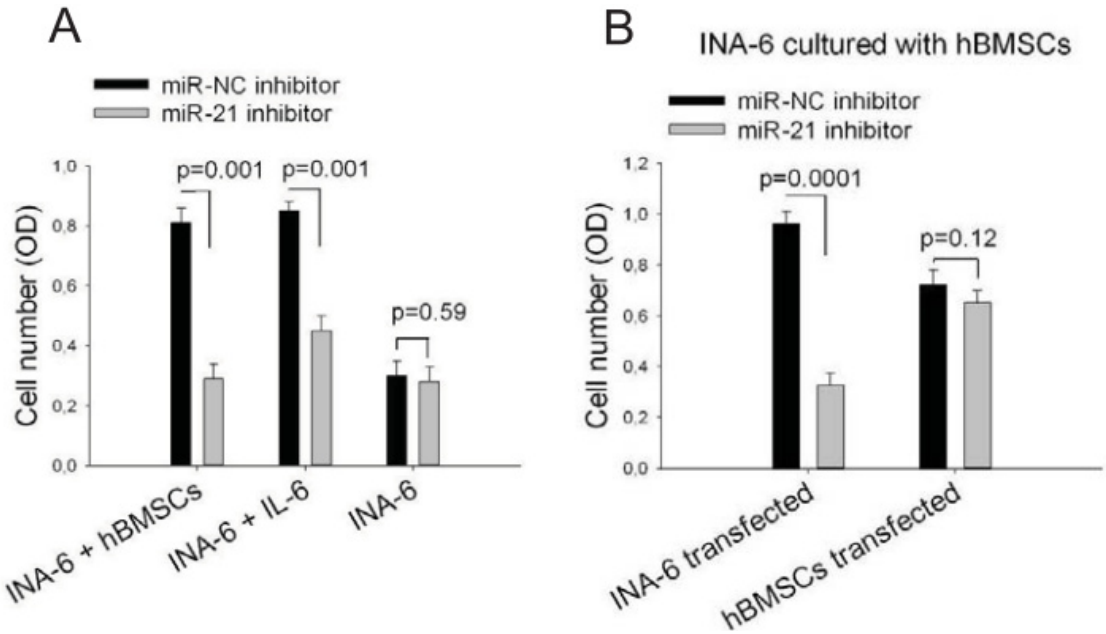


Figure 17 | *MiR-21* inhibition antagonizes pro-survival effect of BM *milieu*. (A) MTT assay of INA-6 cells cultured adherent to hBMSCs, in IL-6-enriched culture medium, or in IL-6-free culture medium. The assay was performed 48 hours after transfection of co-cultured cells with *miR-21* inhibitors or scrambled controls (miR-NC inhibitors). (B) MTT assay of INA-6 cells cultured with hBMSCs was performed 48 hours after either INA-6 cells or hBMSCs were independently transfected with *miR-21* inhibitors or scrambled controls.

Part III

Integrative miRNA/GEP analysis for miRNA target identification

To gain further insights into the function of differentially expressed miRNAs between pPCL and MM cases, we took advantage of the availability of gene expression data for all 18 pPCL cases and 36 of the 39 MM patients analyzed for miRNA expression for performing an integrative analysis of miRNA and gene expression data combined with miRNA target prediction. This approach can only identify putative target genes whose regulation involves mRNA degradation rather than translation repression. In particular, we assessed the correlation between the expression data of all possible miRNA-gene pairs in 18 pPCL and 36 MM cases, and for each significantly anti-correlated pair we verified the existence of a predicted targeting relationship by 9 computational algorithms, then selecting those genes predicted as targets by at least 5 miRNA target prediction programs. In this way, seventy-four statistically significant negative correlations involving differentially expressed miRNAs and putative target genes were computed (Table 8). These targeting relationships were exerted by 24 distinct miRNAs (11 down- and 13 up-regulated) on 69 different genes, and in 5 cases one gene was anti-correlated with more than one miRNA. All the 74 predicted interactions are reported in Figure 18. Several anti-correlated genes were involved in biological functions related to the regulation of cellular component organization according to the DAVID functional annotation tool. Interestingly, most of these anti-correlated genes were differentially expressed between these pPCL and MM samples (as highlighted in Table 8).

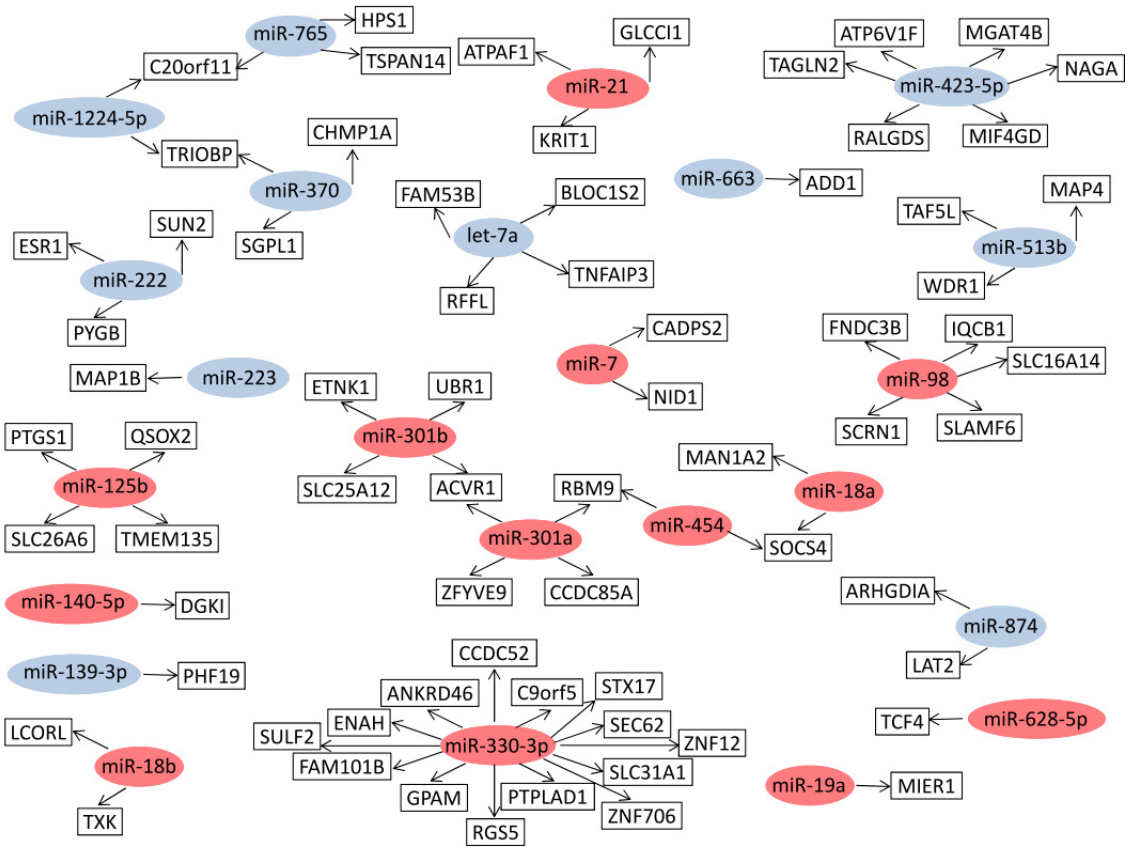


Figure 18 | MiRNA-putative target regulatory relations supported by expression data. Up-regulated miRNAs in pPCL are indicated in red, down-regulated in light blue.

Table 8 | Negative correlations involving differentially expressed miRNAs between pPCL and MM patients and putative target transcripts.

microRNA[a]	RefSeq ID	Gene symbol[b]	No. of algorithms predicting the target gene	Correlation coefficient
hsa-let-7a	NM_001001342	*BLOC1S2**	5	-0.52
	NM_173809	*BLOC1S2**	5	-0.52
	NM_014661	*FAM53B**	5	-0.53
	NM_001017368	*RFFL**	5	-0.57
	NM_057178	*RFFL**	5	-0.57
	NM_006290	*TNFAIP3**	5	-0.54
hsa-miR-1224-5p	NM_017896	*C20orf11*	5	-0.53
	NM_007032	*TRIOBP*	5	-0.56
hsa-miR-125b	NM_000962	*PTGS1*	5	-0.54
	NM_080591	*PTGS1*	5	-0.54
	NM_181701	*QSOX2*	5	-0.57
	NM_001040454	*SLC26A6*	5	-0.57
	NM_022911	*SLC26A6*	5	-0.57
	NM_022918	*TMEM135**	6	-0.52
hsa-miR-139-3p	NM_015651	*PHF19**	5	-0.53
hsa-miR-140-5p	NM_004717	*DGKI**	5	-0.52
hsa-miR-18a	NM_006699	*MAN1A2**	6	-0.52
	NM_004232	*SOCS4**	5	-0.58
hsa-miR-18b	NM_153686	*LCORL*	5	-0.59
	NM_003328	*TXK*	5	-0.53
hsa-miR-19a	NM_001077701	*MIER1*	5	-0.52
	NM_001077703	*MIER1*	5	-0.52
	NM_020948	*MIER1*	6	-0.52
hsa-miR-21	NM_001042546	*ATPAF1**	5	-0.52
	NM_022745	*ATPAF1**	5	-0.52
	NM_138426	*GLCCI1**	5	-0.59
	NM_001013406	*KRIT1**	5	-0.54
	NM_004912	*KRIT1**	7	-0.54
	NM_194454	*KRIT1**	7	-0.54
	NM_194455	*KRIT1**	6	-0.54
	NM_194456	*KRIT1**	7	-0.54
hsa-miR-222	NM_000125	*ESR1*	6	-0.52
	NM_002862	*PYGB*	5	-0.53
	NM_015374	*SUN2**	5	-0.56
hsa-miR-223	NM_005909	*MAP1B*	5	-0.55
hsa-miR-301a	NM_001105	*ACVR1**	7	-0.52
	NM_001080433	*CCDC85A*	6	-0.52
	NM_001031695	*RBM9**	5	-0.53
	NM_001082576	*RBM9**	5	-0.53
	NM_001082578	*RBM9**	5	-0.53
	NM_014309	*RBM9**	5	-0.53
	NM_004799	*ZFYVE9**	5	-0.52
hsa-miR-301b	NM_001105	*ACVR1**	6	-0.52
	NM_018638	*ETNK1**	5	-0.54
	NM_003705	*SLC25A12*	5	-0.52
	NM_174916	*UBR1**	5	-0.52
hsa-miR-330-3p	NM_198401	*ANKRD46**	5	-0.62
	NM_032012	*C9orf5**	5	-0.60
	NM_144718	*CCDC52**	5	-0.54
	NM_001008493	*ENAH*	6	-0.58
	NM_018212	*ENAH*	6	-0.58
	NM_182705	*FAM101B**	5	-0.55
hsa-miR-330-3p	NM_020918	*GPAM**	7	-0.58
	NM_016395	*PTPLAD1**	5	-0.62
	NM_003617	*RGS5**	6	-0.54
	NM_003262	*SEC62**	5	-0.54
	NM_001859	*SLC31A1**	5	-0.59
	NM_017919	*STX17**	5	-0.54
	NM_018837	*SULF2**	5	-0.62
	NM_198596	*SULF2**	5	-0.62
	NM_006956	*ZNF12*	5	-0.53
	NM_016265	*ZNF12*	5	-0.53
	NM_001042510	*ZNF706**	6	-0.55
	NM_001042511	*ZNF706**	6	-0.55
	NM_016096	*ZNF706**	6	-0.55
hsa-miR-370	NM_001083314	*CHMP1A**	5	-0.59
	NM_002768	*CHMP1A**	5	-0.59
	NM_003901	*SGPL1*	5	-0.52
	NM_007032	*TRIOBP*	5	-0.57
hsa-miR-423-5p	NM_004231	*ATP6V1F**	5	-0.69
	NM_014275	*MGAT4B**	5	-0.58
	NM_054013	*MGAT4B**	5	-0.58
	NM_020679	*MIF4GD**	5	-0.53
	NM_000262	*NAGA*	5	-0.52
	NM_001042368	*RALGDS**	5	-0.56
	NM_006266	*RALGDS**	5	-0.56
	NM_003564	*TAGLN2**	5	-0.53
hsa-miR-454	NM_001031695	*RBM9**	5	-0.54
	NM_001082576	*RBM9**	5	-0.54
	NM_001082577	*RBM9**	5	-0.54
	NM_001082578	*RBM9**	5	-0.54
	NM_004232	*SOCS4**	5	-0.52
hsa-miR-513b	NM_030884	*MAP4**	5	-0.52
	NM_001025247	*TAF5L**	5	-0.55
	NM_005112	*WDR1**	5	-0.52
	NM_017491	*WDR1**	5	-0.52
hsa-miR-628-5p	NM_001083962	*TCF4**	5	-0.61
hsa-miR-663	NM_001119	*ADD1*	5	-0.52
	NM_014189	*ADD1*	5	-0.52
	NM_014190	*ADD1*	5	-0.52
hsa-miR-7	NM_001009571	*CADPS2*	5	-0.56
	NM_002508	*NID1*	5	-0.58
hsa-miR-765	NM_017896	*C20orf11*	5	-0.55
	NM_000195	*HPS1**	5	-0.52
	NM_030927	*TSPAN14**	6	-0.53
hsa-miR-874	NM_004309	*ARHGDIA**	5	-0.54
	NM_014146	*LAT2*	5	-0.53
	NM_032463	*LAT2*	5	-0.53
hsa-miR-98	NM_022763	*FNDC3B*	6	-0.53
	NM_001023570	*IQCB1**	5	-0.54
	NM_001023571	*IQCB1**	5	-0.54
	NM_014766	*SCRN1*	5	-0.56
	NM_052931	*SLAMF6**	6	-0.52
	NM_152527	*SLC16A14*	6	-0.57

[a]Up-regulated miRNAs in pPCL are in bold, down-regulated miRNAs in pPCL are in italic. [b]Differentially expressed genes between the same pPCL and MM samples (SAM, q-value=0) are marked with an asterisk.

Target validation assays

We then performed a target validation assay for some of the anti-correlations found in our analysis. To this aim, we focused on genes that, based on previous literature data, might have potential involvement in plasma cell dyscrasia, such as *ACVR1*/*miR-301a* and *miR-301b* (Figure 19 A and B), *SULF2*/*miR-330-3p* and *TNFAIP3*/*let-7a*. Interestingly, luciferase expression from the 3'UTR sequence of *ACVR1* was suppressed by almost 20% (one-sided Student's t-test, $P = 0.002$) in 293T cells co-transfected with pACVR1 3'UTR, *miR-301a* and *miR-301b* (Figure 19 C), while the same approach did not confirm the putative *SULF2*/*miR-330-3p* and *TNFAIP3*/*let-7a* anti-correlations (data not shown).

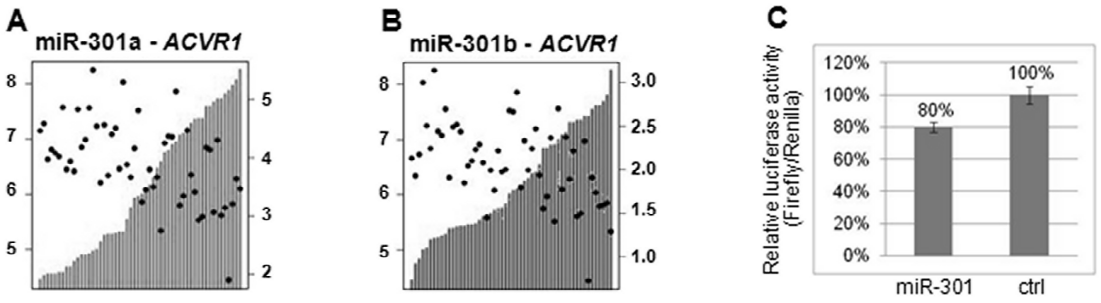


Figure 19 | MiRNA/target anticorrelation in MM. Significant negative correlation of *ACVR1* mRNA with (A) *miR-301a* and (B) *miR-301b* levels. The spots represent mature miRNA expression (scale on right-sided vertical axis), and the grey bars represent target gene expression (scale on left-sided vertical axis) in the dataset of 36 MM and 18 pPCL patients. Horizontal axis: patient samples ordered according to increasing miRNA expression. (C) Luciferase assay showing decreased luciferase activity in cells transfected with pre-*miR-301a* and pre-*miR-301b* compared to cells transfected with pre-miR negative control. All experiments were performed in triplicate. Results were plotted as relative luciferase activity mean ± standard deviation.

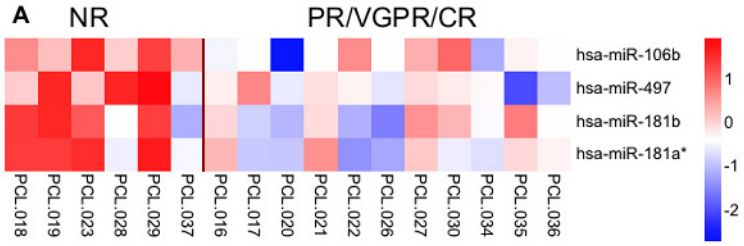
ACVR1 codes for the receptor of bone morphogenetic protein 4 (BMP4), and was recently reported to be increased in MM samples compared with normal controls and correlated with the level of plasma cell infiltration (Grcevic *et al*., 2010). These data provide an important basis for further validation experiments, necessary to determine the actual occurrence of putative targeting relationships and thus the final impact of miRNA deregulation on gene expression.

Part IV

Clinical relevance of miRNA profiles in pPCL

Finally, we evaluated miRNA expression in the context of clinical outcome of the prospective and homogeneously treated series of pPCL patients, including all cases except one, lost at follow-up. Specifically, we aimed to assess whether the occurrence of a miRNA signature at diagnosis might be associated with response to treatment, PFS, or OS. Considering the primary end-point of our study, we investigated the dataset using stringent supervised analysis seeking differentially expressed miRNAs in patients who failed to respond to frontline, planned 4-cycle therapy of lenalidomide/dexamethasone (NR). The analysis led to the identification of four mature miRNAs (*miR-106b*, *miR-497*, *miR-181b*, and *miR-181a**) up-regulated in non-responder patients compared to responder ones, namely those with complete response (CR), very good partial response (VGPR) and partial response (PR) (Figure 20). No specific differentially expressed miRNA could be evidenced when PR, VGPR and CR were considered as different classes of response. This finding will helpfully integrate results on efficacy and side effects of the first-line treatment of lenalidomide/dexamethasone in pPCL (Musto *et al*., manuscript in preparation).

A



B

Gene	Score(d)	Numerator(r)	Denominator(s+s0)	Fold Change	*q*-value (%)
hsa-miR-181a*	-2.279093613	-1.237107431	0.542806765	-2.357254344	0
hsa-miR-181b	-1.75381027	-1.339388372	0.763701978	-2.530440185	0
hsa-miR-497	-1.746650119	-0.654268587	0.374584801	-1.573817862	0
hsa-miR-106b	-1.654248764	-0.802261123	0.48497006	-1.743832078	0

Figure 20 | MiRNAs associated with response to treatment in pPCL. (A) Heatmap of the four differentially expressed miRNAs identified by supervised analysis of pPCL patients stratified according to response rate (NR *versus* PR/VGPR/CR). (B) List of the four differentially expressed miRNAs identified by stringent supervised analysis between non-responder and responder patients (SAM, q-value = 0). MiRNAs are ordered according to the SAM score (d); the numerator and denominator of the relative formula are also shown, together with the fold-change and q-value.

Furthermore, we assessed the relationship between each of the most variable miRNAs across the dataset (i.e. those whose average ratio of the expression values on the mean

was higher than 1.5) and either PFS or OS, representing the secondary end-points of the prospective protocol. Of the 114 most variable miRNAs, two reached a significant correlation ($P < 0.01$) with PFS (*miR-22* and *miR-146a*, Figure 21 A and B), allowing the division of samples into two groups with different outcome.

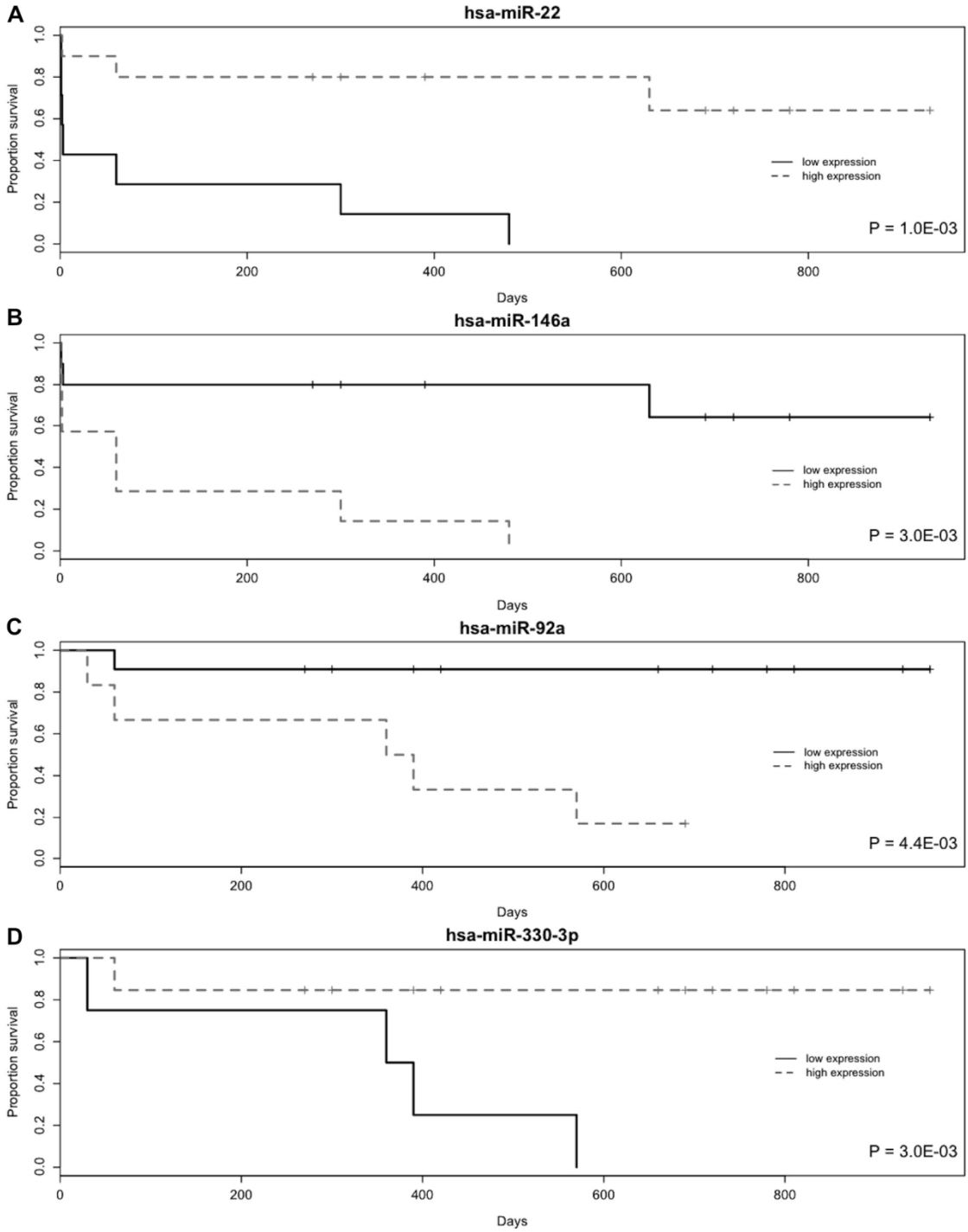


Figure 21 | MiRNAs associated with PFS and OS in pPCL. Kaplan–Meier estimated progression-free survival curves for *miR-22* (A) and *miR-146a* (B), and overall survival curves for *miR-92a* (C) and *miR-330-3p* (D) in pPCL cohorts. The samples have been stratified into two groups (dashed line: high expression; solid line: low expression) according to the thresholds on sorted expression profiles that led to the highest hazard ratio.

The expression of the two miRNAs retained independency from all the molecular characteristics available, as well as from age, sex, LDH levels, renal function and hematologic parameters (Table 9 A and B). As regards OS, two miRNAs reached a significant correlation with the clinical endpoint (*miR-92a* and *miR-330-3p*) (Figure 21 C and D). In multivariate analyses, none of the two miRNAs were independent of patients being subjected to ASCT, indicating that this therapeutic approach points definitively towards a more favorable outcome (Table 9 C and D). It is worth mentioning that none of the cytogenetic aberrations was associated *per se* with PFS and OS (Mosca *et al*., 2013). In particular, we evaluated whether the prevalence of t(4;14), t(11;14), MAF-related translocations, 1p loss, 1q gain, del(13q14), and del(17p13) correlated with the secondary endpoints of the clinical trial, namely OS and PFS. However, none of the considered cytogenetic aberrations was significantly associated neither with OS nor with PFS (data not shown).

Table 9. Multivariate analysis comparing each of the four miRNA-models (A: *miR-22*, B: *miR-146a*, C: *miR-92a*, D: *miR-330-3p*) with biological and molecular variables in pPCL series. The model was available for all covariates with the exception of the cases ("nd") in which the occurrence of covariate was radically inversely correlated with being part of the high-risk group (namely, for ASCT in OS analysis) or whenever the configuration of survival data *versus* covariates value in any group (i.e. the distribution of events) prevented the calculation of hazard ratio model.

A

covariates	HR	lo95%CI	up95%CI	*P*
miR-22 model	0.091	0.017	0.483	0.005
del(13q)	2.340	0.438	12.503	0.320
miR-22 model	0.112	0.022	0.561	0.008
del(17p)	1.079	0.295	3.941	0.909
miR-22 model	0.097	0.018	0.520	0.007
gain(1q)	0.513	0.131	2.007	0.338
miR-22 model	0.113	0.023	0.570	0.008
del(1p)	0.714	0.191	2.668	0.616
miR-22 model	0.067	0.010	0.453	0.006
t(11;14)	2.470	0.457	13.356	0.294
miR-22 model	0.093	0.016	0.561	0.010
t(4;14)	1.820	0.165	20.121	0.625
miR-22 model	0.108	0.021	0.559	0.008
MAF translocations	0.940	0.247	3.576	0.928
miR-22 model	0.122	0.019	0.779	0.026
ASCT	0.845	0.166	4.290	0.839
miR-22 model	0.148	0.030	0.724	0.018
Hb	nd	nd	nd	nd
miR-22 model	0.125	0.024	0.643	0.013
LDH	1.668	0.402	6.927	0.481
miR-22 model	0.117	0.022	0.626	0.012
creatinine	1.173	0.295	4.658	0.821

B

covariates	HR	lo95%CI	up95%CI	*P*
miR-146a model	8.469	1.633	43.909	0.011
del(13q)	1.719	0.322	9.162	0.526
miR-146a model	8.342	1.606	43.327	0.012
del(17p)	0.828	0.215	3.188	0.784
miR-146a model	7.988	1.524	41.868	0.014
gain(1q)	0.637	0.168	2.423	0.509
miR-146a model	7.258	1.453	36.260	0.016
del(1p)	0.837	0.228	3.063	0.788
miR-146a model	8.121	1.545	42.681	0.013
t(11;14)	1.111	0.267	4.627	0.885
miR-146a model	9.248	1.524	56.113	0.016
t(4;14)	1.757	0.159	19.444	0.646
miR-146a model	7.570	1.503	38.114	0.014
MAF translocations	1.295	0.361	4.647	0.692
miR-146a model	6.277	1.064	37.016	0.042
ASCT	0.648	0.136	3.100	0.587
miR-146a model	5.771	1.179	28.244	0.031
Hb	nd	nd	nd	nd
miR-146a model	6.902	1.341	35.522	0.021
LDH	1.793	0.441	7.288	0.414
miR-146a model	6.934	1.361	35.326	0.020
creatinine	1.597	0.415	6.146	0.496

C

covariates	HR	lo95%CI	up95%CI	*P*
miR-92a model	28.410	2.214	364.543	0.010
del(13q)	8.326	0.587	118.094	0.117
miR-92a model	13.060	1.452	117.499	0.022
del(17p)	0.663	0.127	3.453	0.626
miR-92a model	9.896	1.101	88.905	0.041
gain(1q)	1.348	0.236	7.693	0.737
miR-92a model	11.080	1.260	97.456	0.030
del(1p)	1.773	0.328	9.576	0.506
miR-92a model	11.647	1.354	100.165	0.025
t(11;14)	0.899	0.162	4.991	0.903
miR-92a model	9.273	1.078	79.757	0.043
t(4;14)	nd	nd	nd	nd
miR-92a model	12.400	1.410	109.053	0.023
MAF translocations	1.724	0.315	9.437	0.530
miR-92a model	1.372	0.124	15.169	0.796
ASCT	nd	nd	nd	nd
miR-92a model	8.886	1.036	76.225	0.046
Hb	nd	nd	nd	nd
miR-92a model	12.020	1.380	104.724	0.024
LDH	1.378	0.247	7.685	0.715
miR-92a model	11.349	1.309	98.406	0.028
creatinine	1.249	0.221	7.062	0.802

D

covariates	HR	lo95%CI	up95%CI	*P*
miR-330-3p model	0.087	0.014	0.563	0.010
del(13q)	3.366	0.328	34.513	0.307
miR-330-3p model	0.117	0.021	0.658	0.015
del(17p)	0.944	0.179	4.978	0.946
miR-330-3p model	nd	nd	nd	nd
gain(1q)	nd	nd	nd	nd
miR-330-3p model	0.129	0.022	0.743	0.022
del(1p)	1.053	0.193	5.735	0.952
miR-330-3p model	nd	nd	nd	nd
t(11;14)	nd	nd	nd	nd
miR-330-3p model	0.144	0.026	0.797	0.027
t(4;14)	nd	nd	nd	nd
miR-330-3p model	0.118	0.021	0.666	0.015
MAF translocations	0.989	0.182	5.360	0.990
miR-330-3p model	1.823	0.165	20.199	0.625
ASCT	nd	nd	nd	nd
miR-330-3p model	0.149	0.027	0.820	0.029
Hb	nd	nd	nd	nd
miR-330-3p model	0.118	0.021	0.657	0.015
LDH	1.022	0.192	5.448	0.979
miR-330-3p model	0.109	0.018	0.677	0.017
creatinine	0.786	0.122	5.051	0.800

Discussion

In this study we reported a miRNA expression profiling analysis of a series of pPCL patients included in a prospective clinical trial aimed at exploring efficacy and safety of lenalidomide and dexamethasone combination as first line therapy in previously untreated patients. At the same time, pPCL samples included in the trial and for whom material was available were subjected to FISH, genotyping and GEP analyses. MiRNA expression patterns were evaluated in relation to the most recurrent chromosomal abnormalities and in comparison with a representative series of MM patients, identifying miRNAs that may contribute to explain the aggressive phenotype of pPCL, based on their known or inferred functions from integrative analysis of miRNA and GEP data or on *in vitro* functional analyses in HMCLs. Finally, we identified miRNAs putatively involved in pPCL clinical outcome.

Chromosomal alterations and miRNA expression profiling in pPCL patients

Existing studies have so far demonstrated that pPCL shows the main molecular characteristics of other plasma cell dyscrasias (e.g., IGH@ translocations or 13q and 17p deletions). As regards chromosomal translocations, those involving the 14q32 *locus* and the *CCND1*, *FGFR3*/*MMSET*, or *MAF* partner genes have been reported occurring in the majority of primary, as well as in secondary PCLs. Herein, we identified the occurrence of IGH@ translocations in ~87% of pPCL cases; such an incidence overlaps with those reported by several authors (Avet-Loiseau *et al*., 2001a; Avet-Loiseau *et al*., 2012; Chang *et al*., 2009; Chiecchio *et al*., 2009; Tiedemann *et al*., 2008), confirming the widely accepted notion that IGH@ translocations are almost an ubiquitous event in PCL. However, the frequency of the different types of partner *loci* still remains controversial and this is probably related to the heterogeneous series investigated or geographical factors, as already suggested by other Authors (Tiedemann *et al*., 2008). Specifically, in our study the frequency of the t(11;14) was approximately 40%, quite similar to that reported by some authors (Avet-Loiseau *et al*., 2001a; Chiecchio *et al*., 2009), but lower than that reported by others (Chang *et al*., 2009; Tiedemann *et al*., 2008). As regards IGH@ translocations involving *MAF* gene, we found higher frequency (30.5%) than those reported in previous works, where incidence in pPCL was approximately 10–17% (Avet-Loiseau *et al*., 2001a; Avet-Loiseau *et al*., 2012) if not absent (Tiedemann *et al*., 2008), and almost identical to that reported by Chiecchio *et al*. (Chiecchio *et al*., 2009). Finally, we found a 13%

incidence of t(4;14), quite similar to an earlier report (Avet-Loiseau *et al*., 2001a) but different from other studies in which it was found at higher frequency (21–25%) (Avet-Loiseau *et al*., 2012; Chang *et al*., 2009) or completely absent (Chiecchio *et al*., 2009; Tiedemann *et al*., 2008). Concerning the impact of chromosomal alterations on miRNA expression, IGH@ translocations seemed to be less associated with distinct miRNAs profiles compared to what we observed in MM patients, although some miRNAs deregulated in a similar manner have emerged: apart from *let-7e*, over-expressed in t(4;14) pPCL cases, other miRNAs were found up-regulated (although at lower levels of statistical significance) in this molecular group of pPCL in agreement with what found in MM cases, i.e. *miR-125a-5p*, *miR-221*, *miR-221**, and *miR-222*; likewise, also *miR-150* was up-regulated in *MAF*-translocated pPCL cases (FDR = 10%). An interesting similarity was highlihted between miRNA expression profiles of pPCL and MM TC5 patients, suggesting further investigations on whether pPCL might share some clinico-biological features with *MAF*-translocated MM.

Regarding numerical chromosomal alterations, we confirmed the remarkable frequency of del(13q) in pPCL which is higher than that reported in MM (Walker *et al*., 2010). We found this lesion in 17/23 (74%) patients; based on SNP-array data we confirmed the loss of the entire chromosome in the majority of cases, with the exception of three pPCLs showing relatively large interstitial deletions encompassing the 13q14 region. Interestingly, several deregulated miRNAs in association with alterations involving the corresponding genomic *loci* were mapped to chromosome 13. In particular, the down-regulation of *miR-15a* in patients carrying chromosome 13 deletion is of particular importance given the frequency of this genomic lesion in pPCL and the experimental evidence that it may acts as a tumor suppressor miR in malignant plasma cells (Hao *et al*., 2011a; Roccaro *et al*., 2009). Roccaro *et al*. indeed 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-κB-activator MAP3K7IP3 (Roccaro *et al*., 2009). 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 (Hao *et al*., 2011a; Hao *et al*., 2011b). 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 (Table 10): in fact, 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 (Roccaro *et al*., 2009), but Pichiorri *et al*. found that *miR-15a* (as well as other miRNAs mapped on 13q) was up-regulated in MM PCs *versus* healthy PCs (Pichiorri *et al*., 2008).

Table 10 | *MiR-15a* and *miR-16* expression in MM (adapted from Lionetti *et al*., Current Cancer Drug Targets 2012).

Reference	Platform	Result
Pichiorri *et al*., PNAS 2008	Academic miRNA microarray	miR-15a up-regulated in MM *versus* healthy PCs.
Roccaro *et al*., Blood 2009	Liquid phase luminex microbead miRNA profiling	miR-15a/16 down-regulated in relapsed/refractory MM *versus* healthy PCs (absent in MM with del(13) and down-regulated in those without deletion).
Lionetti *et al*., Genes Chromosomes Cancer 2009	Agilent miRNA microarray V1	miR-15a expression correlates with DNA copy number in HMCLs.
Lionetti *et al*., Blood 2009	Agilent miRNA microarray V2	miR-15a/16 expression unaffected by chromosome 13 status in MM.
Gutierrez *et al*., Leukemia 2010	Applied Biosystems TaqMan low-density arrays	miR-15a down-regulated in MM with RB deletion *versus* other MM or healthy PCs.
Corthals *et al*., Leuk. Res 2010	Applied Biosystems TaqMan miRNA assays	miR-15a/16 up-regulated in MM than healthy PCs independently of chromosome 13 status.
Zhou *et al*., PNAS 2010	Agilent miRNA microarray V1	miR-15a/16 marginally up-regulated in MM than healthy PCs.
Chi *et al*., Biology Direct 2011	µRNA microarray	down-regulation of both miR-15a/16 in del(13) cases.

Our group found a significant correlation between the expression of *miR-15a* and alterations of the corresponding DNA *locus* in HMCLs (probably due to miRNA over-expression in aneuploid cell lines with more than two copies of chromosome 13) (Lionetti *et al*., 2009a) but not in primary MM tumors, in which only the expression of *miR-17* and *miR-20a* (13q31) was reduced in cases with 13q deletion (Lionetti *et al*.,

2009b). Other Authors have reported varying levels of *miR-15a*/*16* (Corthals *et al*., 2010) as well as of *miR-17~92* (Chen *et al*., 2011) 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 samples of patients carrying the *RB* deletion alone in comparison with the normal PC samples or the samples of MM patients without this abnormality (Gutierrez *et al*., 2010), and Chi *et al*. reported the down-regulation of both *miR-15a*/*16* and *miR-17~92* cluster in del(13) cases (Chi *et al*., 2011).

As regards *miR-17~92*, copy number of chromosome 13 (although correlated with the expression of some of the miRNAs belonging to the cluster, i.e. *miR-19a*, *miR-20a*, *miR-20a** and *miR-92a*) was unlikely the only factor affecting its expression in our pPCL cohort; in fact we found that some miRNAs belonging to the cluster resulted significantly over-expressed in pPCL compared to MM cases independently of their CN status. This appears to be in line with the reported evidence of the oncogenic potential of this miRNA cluster, as also specifically demonstrated in the context of myeloma cells (Chen *et al*., 2011; Pichiorri *et al*., 2008).

Our study confirmed the increased frequency of *TP53* deletion in comparison with that reported in MM. Furthermore, we identified four *TP53*-mutated cases, three of which associated with a gene deletion. Therefore, ~13% of cases in our series showed biallelic inactivation of *TP53*, similarly to that reported by other Authors (Chiecchio *et al*., 2009; Tiedemann *et al*., 2008). Interestingly, our data represent the first evidence in the context of plasma cell dyscrasias of a decreased expression of *miR-34a* in association with *TP53* abnormalities, as already found in other tumors, such as CLL (Mraz *et al*., 2009): importantly, enforced expression of *miR-34a* in MM cell lines indicated the this miRNA exerts anti-myeloma activity *in vitro*. The high sensitivity of *TP53*-mutated MM cells is of interest, as *TP53* inactivation is a rare event at diagnosis, but it becomes common when intramedullary MM progresses to a drug-resistant and more aggressive phenotype, as well as in pPCL and sPCL, suggesting that the "biologically end-stage" disease might benefit from therapies restoring the TP53 function through *miR-34a* enforcement. Furthermore, SNP-array data showed the loss of a large part of 17p in all but one cases tested positive del(17p); this finding is reminiscent of what was reported in CLL by our previous study (Fabris *et al*., 2008) suggesting that other important tumor suppressor genes/miRNAs located in this region may contribute to the tumor development and expansion in pPCL. In this regard, one of the several down-regulated

miRNAs in pPCL in association with the loss of the corresponding genomic *loci* that have already been linked to cancer as having a tumor suppressor role was *miR-22* (Takata *et al*., 2011; Xiong, 2012), precisely mapped at chromosome 17p13.3 and whose down-regulation was found associated with shorter progression free survival in our pPCL cohort, as will be discussed in more detail later. Other down-regulated putative tumor suppressor miRNAs identified were *miR-331-3p* (12q22) (Guo *et al*., 2010b), *miR-342-3p* (14q32.2) (Wang *et al*., 2011a) and *miR-30e* and *miR-30c* (Wu *et al*., 2009), these latter localized at chromosome 1p34.2, another hot-spot altered region as emerged from the DNA genome-wide analysis; overall, indeed, the incidence of 1p loss and 1q gain was ~40 and 50%, respectively, which is only slightly higher than that reported in MM by us and several other groups (Agnelli *et al*., 2009; Fabris *et al*., 2007; Hanamura *et al*., 2006; Sawyer, 2011).

MiRNA expression profiling of pPCL and MM patients

The clustering of samples based on the expression of the 76 most variable miRNAs indicated a significant grouping of pPCL and MM cases in two main branches, respectively. The differences in miRNA expression profiles of MM and pPCL highlighted by the unsupervised analysis were confirmed by direct comparison of these clinical entities: pPCL and MM patients were found to differentially express 83 miRNAs, roughly half of which over-expressed in one group with respect to the other. It is worth noting that the expression of approximately 70% of these miRNAs maintained a statistically significant trend when considering normal donors, reinforcing the hypothesis that the deregulation of these miRNAs might be compatible with the "strength" of neoplastic transformation. Furthermore, up-regulated miRNAs in pPCL were enriched in "onco-miRNAs", such as *miR-21*, *miR-155* (Faraoni *et al*., 2009; Tili *et al*., 2009) and miRNAs belonging to the paralogous clusters *miR-17~92* and *miR-106a~363* (*miR-18a, miR-19a, miR-18b* and *miR-20b*). In particular, *miR-21* was found over-expressed in the majority of cancer types analyzed, acting as a cancer biomarker. Its relevance in MM was first 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 from the IL-6-growth *stimulus* (Loffler *et al*., 2007). More recently, *miR-21* inhibition was shown to have a synergistic anti-MM effect with dexamethasone or doxorubucin (Wang *et al*., 2011b), and our preliminary

data indicated that targeting *miR-21* inhibits *in vitro* MM cell growth. Importantly, *miR-21* expression in MM cells was significantly enhanced by adherence of cells to hBMSCs, and anti-MM activity of *miR-21* inhibitors was exerted also in the context of bone marrow *milieu*, antagonizing the BMSCs protective role on MM cells. Globally, based on these findings, the increased expression of *miR-21* in pPCL compared with MM patients seems to be closely related to the independence of the leukemic cells from the bone marrow microenvironment. *MiR-17~92* functions pleiotropically during both normal development and malignant transformation to promote proliferation, inhibit differentiation, augment angiogenesis and sustain cell survival, and its over-expression has been observed in multiple tumor types (Olive *et al*., 2010). In addition to the initial evidence of its important role in MM (Pichiorri *et al*., 2008), a recent study demonstrated that MYC may inhibit MM cell apoptosis by activating the *miR-17~92* cluster, leading to the down-modulation of the pro-apoptotic protein BIM. Interestingly, MM patients with higher expression of *miR-17*, *miR-20* or *miR-92* had shorter PFS (Chen *et al*., 2011). On the contrary, several miRNAs with lower expression in pPCL compared with MM are thought to play a tumor suppressor role in various tumor types, such as *miR-663* (Pan *et al*., 2010; Tili *et al*., 2010), *miR-193b* (Xu *et al*., 2010; Chen *et al*., 2010; Li *et al*., 2009a), *miR-126* (Meister and Schmidt, 2010) and *let-7a* (Boyerinas *et al*., 2010). Interestingly, *let-7a* has been reported to be repressed by, and in turn repress, MYC (Boyerinas *et al*., 2010), and to be involved in the IL-6 pathway (Meng *et al*., 2007; Iliopoulos *et al*., 2009). Notably, high expression levels of *miR-142-5p*, *miR-21*, *miR-125b*, *miR-103*, *miR-99a*, *miR-26b* or members of the cluster *miR-17~92*, up-regulated in pPCL, were found to be associated with GEP-defined high risk score in MM patients (Zhou *et al*., 2010).

Several over-expressed miRNAs in pPCL also seem to be related to immune response, particularly the above mentioned *miR-17~92* cluster (Olive *et al*., 2010), *miR-155* (Faraoni *et al*., 2009; Tili *et al*., 2009) and *miR-21* (Iliopoulos *et al*., 2010; Sheedy *et al*., 2010). Specifically, *miR-155* and *miR-21*, known to share an important role in tumorigenesis, may represent important links between cancer and inflammation, likely through their relationship with the NF-κB pathway, known to promote their transcription (Ma *et al*., 2011). Furthermore, *miR-301a*, another up-regulated miRNA in pPCL, has been demonstrated to be activated by NF-κB (Ma *et al*., 2011). Both *miR-21*, *miR-155* and *miR-301a* in turn affect the NF-κB pathway, leading to its activation

or inhibition, in part dependent on the cellular context (Li *et al*., 2009b; Ma *et al*., 2011) (Figure 22).

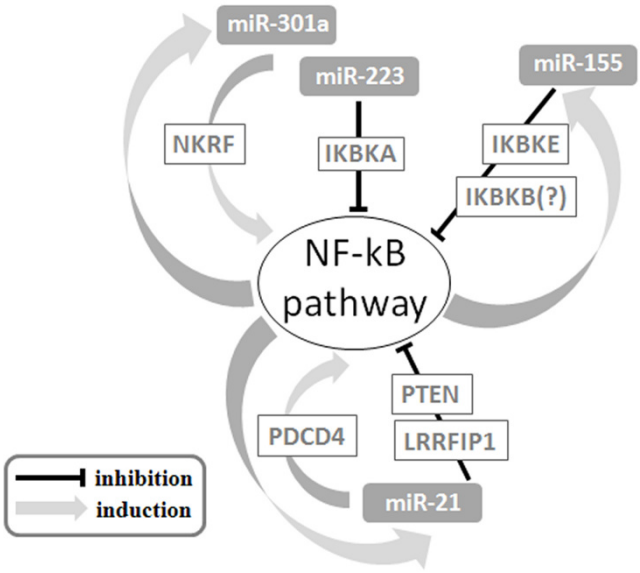


Figure 22 | Involvement in NF-κB pathway of some deregulated miRNAs in pPCL. *MiR-301a*, *miR-155* and *miR-21*, up-regulated in pPCL, are transcriptionally activated by NF-κB pathway and in turn affect it. By controlling the expression of *IKK-epsilon* and possibly *IKK-beta*, *miR-155* limits NF-κB activation, constituting a negative feed-back loop (Ma *et al*., 2011); on the contrary, *miR-301a* was reported in solid tumors to down-regulate *NF-κB-repressing factor* (*Nkrf*), thus creating a positive feed-back loop for persistent NF-κB activation (Ma *et al*., 2011). Experimental evidence in different cellular contexts supports either *miR-21* activating or inhibitory effect on NF-κB pathway (Li *et al*., 2009b; Ma *et al*., 2011). *MiR-223*, significantly down-regulated in pPCL, was shown in macrophages to negatively affect the non-canonical NF-κB pathway through the targeting of *IKK-alpha* (Ma *et al*., 2011).

Notably, gene expression profiling analysis of a dataset of pPCL and MM samples including most of the patients analyzed for miRNA expression profiling identified several differentially expressed NF-κB pathway associated genes between MM and pPCL (Todoerti. *et al*., submitted). The repression of a member of NF-κB pathway, *IKK-alpha*, has been reported also for *miR-223* (Ma *et al*., 2011), a hematopoietic specific miRNA involved in several types of leukemia and solid tumors. *Mir-223* was down-regulated in our pPCL series and nonetheless its absence has recently been observed in extramedullary plasmacytoma (Yu *et al*., 2011); it was also found to suppress cell proliferation by targeting IGF-1R (Jia *et al*., 2011), involved in motility and invasiveness control in MM cells. Overall, these findings strongly suggest an important role for deregulated miRNAs involved in NF-κB pathway in pPCL and may

have implications for future therapeutic approaches, given, for example, the efficacy of bortezomib, a drug which interferes with NF-κB pathway, in pPCL patients.

Clinical relevance of miRNA profiles in pPCL

We investigated whether specific miRNA signatures could be associated with the response rate after four cycles of therapy, which represented the primary endpoint of the study. Interestingly, a four-miRNA signature (*miR-106b*, *miR-181a**, *miR-181b* and *miR-497*) was found significantly up-regulated in non-responding cases. Of note, both *miR-106b* and *miR-181b* were reported to be up-regulated in MM cells compared to healthy PCs (Pichiorri *et al*., 2008; Roccaro *et al*., 2009) and associated with increased MM GEP-defined risk score (Zhou *et al*., 2010) and, interestingly, *miR-181a** was one of up-regulated miRNAs in pPCL *versus* MM cases; this finding makes this miRNA a potential therapeutic target in pPCL.

Moreover, a further contribution to clinical prognostication was represented by identification of four additional miRNAs, the expression of which was associated with pPCL cases with a worse outcome: in particular, the down-regulation of *miR-22* and the up-regulation *miR-146a* implied shorter PFS, while down-regulation of *miR-330-3p* and up-regulation of *miR-92a* were accompanied by lower OS. Interestingly, *miR-146a* deregulation has been associated with the pathogenesis of several human diseases, including solid tumors and hematopoietic malignancies, and this miRNA is thought to play an important role in the regulation of innate immune and inflammatory responses through a negative feedback pathway involving NF-κB (Labbaye and Testa, 2012; Ma *et al*., 2011). This finding, together with previously described interactions between deregulated miRNAs and NF-κB pathway, warrants further investigations to clarify whether the miRNAs/NF-κB network may have a role in pPCL outcome. Notably, in our series *miR-22* was found significantly down-regulated in accordance with allelic loss of its residing *locus* at 17p13.3; however, *TP53* deletion *per se* was not associated with PFS (data not shown), thus suggesting that factors other than deletion of 17p may affect *miR-22* expression. *Mir-22* has already been linked to cancer through its putative tumor suppressor role; it is activated by TP53, suppresses NF-κB activity and is thought to repress the MYC binding proteins MAX and MYCBP and to be inhibited by MYC itself (Takata *et al*., 2011; Xiong, 2012). As regards *miR-92a*, it is a member of the above mentioned cluster *miR-17~92*, and its up-regulation in MM has been reported

in comparison with normal PCs (Pichiorri *et al*., 2008), in relation to increased MM GEP-defined risk score (Zhou *et al*., 2010), and in association with shorter PFS (Chen *et al*., 2011). Considering that pPCL represents a high-risk clinical entity *per se*, biological information at diagnosis could be helpful to guide clinicians in therapeutic decisions; therefore, larger prospective series of patients would be required to better elucidate the clinical relevance of miRNAs in pPCLs.

Overall, our study represents the first attempt to investigate the involvement of miRNAs in pPCL, the most aggressive form of plasma cell dyscrasia, and our data may contribute towards the development of functional approaches to analyze the activity of deregulated miRNAs and their possible role as novel therapeutic targets.

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