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Epigenetic repression of miR-375 is the dominant mechanism for constitutive activation of the PDPK1/RPS6KA3 signalling axis in multiple myeloma

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Summary

Cytogenetic/molecular heterogeneity is the hallmark of multiple myeloma (MM). However, we recently showed that the serine/threonine kinase PDPK1 and its substrate RPS6KA3 (also termed RSK2) are universally active in MM, and play pivotal roles in myeloma pathophysiology. In this study, we assessed involvement of aberrant miR-375 repression in PDPK1 overexpression in MM. An analysis of plasma cells from 30 pre-malignant monoclonal gammopathies of undetermined significance and 73 MM patients showed a significant decrease in miR-375 expression in patient-derived plasma cells regardless of the clinical stage, compared to normal plasma cells. Introduction of miR-375 reduced PDPK1 expression in human myeloma cell lines (HMCLs), indicating that miR-375 is the dominant regulator of PDPK1 expression. In addition, miR-375 introduction also downregulated IGF1R and JAK2 in HMCLs. CpG islands in the *MIR375* promoter were pathologically hypermethylated in all 8 HMCLs examined and in most of 58 patient-derived myeloma cells. Treatment with SGI-110, a hypomethylating agent, and/or trichostatin A, a histone deacetylase inhibitor, increased miR-375 expression, but repressed PDPK1, IGF1R and JAK2 in HMCLs. Collectively, these results show the universal involvement of overlapping epigenetic dysregulation for abnormal miR-375 repression in MM, which is likely to contribute to myelomagenesis and to subsequent myeloma progression by activating oncogenic signalling pathways.

Keywords: multiple myeloma, microRNA, epigenetics, PDPK1, miR-375.

Multiple myeloma (MM) is a clonal B-cell malignancy with high cytogenetic/molecular heterogeneity that is characterized by aberrant expansion of plasma cells within the bone marrow (BM) and occasionally in extramedullary sites in the late clinical phase. MM remains mostly incurable despite major advances in treatment (Rajkumar *et al*, 2014). The complex interplay among cell-intrinsic and -extrinsic molecular mechanisms confers inter-patient diversity and intraclonal heterogeneity in MM (Brioli *et al*, 2014). Thus, treatment outcomes are likely to be improved by identification of a universal and relevant molecular target for therapy.

We have recently identified 3-phosphoinositide-dependent protein kinase 1 (PDPK1), a serine threonine kinase, and its major downstream substrate RPS6KA3 (also termed RSK2), a member of the 90 kDa ribosomal S6 kinase family of serine threonine kinases, as universally active molecules in MM, regardless of the type of cytogenetic abnormality and the mutation status of *RAS*, *RAF* and fibroblast growth factor receptor 3 (*FGFR3*) genes. Importantly, PDPK1/RPS6KA3 signalling was found to play pivotal roles in myeloma pathophysiology by regulating a series of myeloma-promoting molecules, such as MYC, IRF4, D-type cyclins, and PLK1, while inactivation of PDPK1 or the N-terminal domain of RPS6KA3 resulted in induction of apoptosis in myeloma cells, accompanied by activation of BH3-only proteins BCL2L11 (BIM) and BAD (Shimura *et al*, 2012; Chinen *et al*, 2014), while RPS6KA3 activation has been shown to be one of the leading causes of resistance to immunomodulatory

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agents, such as lenalidomide (LEN) (Zhu *et al*, 2015). Autophosphorylation is the dominant mechanism for PDPK1 activation, and therefore, the kinetics of PDPK1 are largely determined by its protein expression level. However, the underlying mechanisms for constitutive PDPK1 overexpression have not been verified in MM, and an understanding of this event may shed further light on the as yet unknown, but potentially universal, mechanism of myelomagenesis.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate many cellular processes, including cell proliferation, differentiation, apoptosis and invasion. miRNAs play crucial roles in a variety of physiological processes, and also contribute to tumour formation and development as oncogenes or tumour suppressors through negative regulation of expression of target genes harbouring complementary sequences to the individual miRNAs. In myeloma, several studies have revealed involvement of aberrant expression of miRNAs and their functions in the pathogenesis and drug resistance of MM (Pichiorri *et al*, 2011; Abdi *et al*, 2016). While most aberrantly expressed miRNAs have been associated with specific cytogenetic/genetic subtypes or clinical manifestations, subtype-independent abnormalities have also been observed for a few miRNAs, including miR-375 and miR-214, in MM (Gutierrez *et al*, 2010; Chi *et al*, 2011). Among these miRNAs, miR-375 has been shown to target PDPK1 and is involved in glucose regulation of insulin gene expression and β-cell growth (El Ouaamari *et al*, 2008). Moreover, involvement of miR-375 repression in PDPK1 overexpression has been shown in several neoplasms, including gastric cancer (Tsukamoto *et al*, 2010) and oesophageal cancer (Li *et al*, 2011); however, the association and functional role of miR-375 in MM have not been determined.

Given this background, the current study was performed to examine involvement of abnormal miR-375 regulation as an underlying mechanism of PDPK1 overexpression. We also investigated the molecular mechanisms of aberrant miR-375 expression to allow clinical translation of this knowledge for diagnosis and treatment of MM targeting the PDPK1/RPS6KA3 signalling axis.

Material and methods

Patient samples

BM samples were obtained from patients with monoclonal gammopathy of undetermined significance (MGUS) ($n = 30$), newly diagnosed multiple myeloma (NDMM) ($n = 34$), and relapsed/refractory multiple myeloma (RRMM) ($n = 39$) between March 2014 and March 2016 at Kyoto Prefectural University of Medicine, Gunma University and Nagoya City University. The diagnosis of MGUS/MM was made according to the International Myeloma Working Group (IMWG) 2014 criteria (Rajkumar *et al*, 2014). This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (Kyoto, Japan; RBMR-G-124-2). Patient samples were collected with informed consent in accordance with the Declaration of Helsinki. BM mononuclear cells were labelled with anti-CD138 MicroBeads and CD138-positive plasma cells were positively isolated using a Mini-MACS separator (Miltenyi Biotec KK, Bergisch Gladbach, Germany). Plasma cells of healthy donors were utilized as control samples.

Cells and reagents

Eleven human myeloma cell lines (HMCLs) were authenticated by mycoplasma detection, DNA fingerprinting, isozyme detection, and cell vitality detection before use in the study. These cell lines were AMO1, NCI-H929, OPM2, and LP-1 (Deutsche Sammlung von Mikroorgranismen und Zellkulturen GmbH, Braunschweig, Germany), RPMI8226 and IM9 (ATCC), KMS-18, KMS-12-BM, KMS-28-PE and KMS-34 (kind gifts from Dr. T. Ohtsuki, Kawasaki Medical School, Okayama, Japan) and AMU-MM1 (a kind gift from Dr. I. Hanamura, Aichi Medical School, Aichi, Japan). Cells were maintained in RPMI-1640 medium containing 10% fetal calf serum, 2 mmol/l L-glutamate and penicillin/streptomycin at 37°C in a fully humidified atmosphere of 5% CO_2 in air. The agents used in the study were LEN (Celgene Corp., Summit, NJ), bortezomib (BTZ; provided by the Screening Committee for Anticancer Drugs (SCAD), Tokyo, Japan), terameprocol (TMP; Sigma-Aldrich, St. Louis, MO), the histone deacetylase (HDAC) inhibitor trichostatin A (TSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and SGI-110, a hypomethylating dinucleotide of decitabine (Selleck Chemicals, Houston, TX).

Assays for growth inhibition

Cells were seeded at $2{\cdot}0 \times 10^5$ cells/ml and treated with various concentrations of BTZ, LEN, or TMP for 48 h, TSA for 24 h or SGI-110 for 72 h. Growth-inhibitory effects were analysed by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). The resultant 50% inhibitory concentration (IC_{50}) values of the agents are provided in the Supporting Information. Cells were also treated with an equal amount of dimethyl sulfoxide (DMSO) as controls.

miR-375 expression analysis by stem-loop reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from isolated plasma cells using a mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA). Mature miR-375 and human RNU6-50P (U6) snRNA were reverse transcribed using Stem–loop RT primer with a TaqMan® MicroRNA RT Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed using TaqMan® Universal PCR Master Mix II with UNG with a

7300 instrument (Applied Biosystems). *MIR375* expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Human RNU6-50P snRNA was examined as a reference for intracellular miR-375 expression analysis.

Mature miRNA introduction and RNA interference

HMCLs were transfected with 20 μmol/l miRNA-375 mimic, negative control mature miRNA (Bioneer Inc., Daejeon, Korea), or small-interfering RNA (siRNA) for *PDPK1* (Takara Bio Inc., Shiga, Japan) using a Haemagglutinating Virus of Japan (HVJ)-envelope vector (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan).

Western blot analysis

Western blot analysis was conducted as described elsewhere (Kobayashi *et al*, 2010). The primary antibodies used are described in the Supporting Information.

Fluorescence in situ *hybridization (FISH)*

FISH was conducted as described previously (Nagoshi *et al*, 2012). Copy number abnormality was assessed by double-colour interphase FISH for *PDPK1* and the centromere of chromosome 16. The probes used are described in the Supporting Information.

DNA extraction and bisulfite conversion

Genomic DNA was extracted from HMCLs and patient-derived plasma cells using an EpiTect LyseAll Lysis Kit (Qiagen, Hilden, Germany). Genomic DNA (200 ng) was modified by treatment with sodium bisulfite using an EpiTect Fast Bisulfite Conversion Kit (Qiagen). Searches for CpG-rich regions (CpG island) and primer design were performed using Meth Primer Tools (http://www.urogene.org/methprimer/).

Methylation-specific PCR (MSP) and bisulfite sequencing

A DNA methylation profiling assay was performed using methylation-specific PCR (MSP) and bisulfite sequencing. EpiTect Control DNA and Control DNA Set (Qiagen) were used as positive (methylated) and negative (unmethylated) controls. Methylated MSP (M-MSP) and unmethylated MSP (U-MSP) were performed as described in the Supporting Information. PCR was performed with AmpliTaq Gold 360 Master Mix (Applied Biosystems). The sensitivities of M-MSP-1 and M-MSP-2 were 10 copies/μl (Fig S1). Bisulfite sequencing PCR was performed with the primers shown in Table SI. The amplicons were cloned with a TOPO® TA Cloning® Kit (Life Technologies) and at least six independent clones for each samples were selected. M13 primers were used to sequence inserted fragments with an Applied Biosystems® 3130 Genetic Analyser. Analysis of bisulfite sequencing and figure design were performed by QUantification tool for Methylation Analysis (QUMA; RIKEN Centre for Developmental Biology, Kobe, Japan; http://quma.cdb.riken.jp/index_j.html).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with a SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling Technologies, Beverly, MA). Antibodies specific for acetylated H3 (06-599, Upstate Biotechnology, Inc. Lake Placid, NY) and H3K4me3 (ab8580, Abcam, Cambridge, UK) were used. DNA (200 ng) was subjected to PCR using Power SYBR Green Master Mix (Applied Biosystems) with specific primers (Table SI). Enrichment was calculated as a percentage of the input. All measurements were performed in triplicate.

RNA extraction from serum, library preparation, and sequencing for miRNA analysis

Circulating small RNA was extracted and purified from serum of patients with MM and healthy donors using a miRCURY™ RNA Isolation Kit Biofluids (Exiqon Inc. Woburn, MA). Extracted RNA samples were converted to cDNA libraries, followed by sequencing using a NEBNext Multiplex Small RNA Library Prep Set (New England BioLabs, Ipswich, MA) and a MiSeq Sequencing Kit (Illumina, San Diego, CA). Expression analysis of miR-375 was performed using CLC Genomics Workbench 8.0 software (Qiagen, Redwood, CA). miR-let7b, miR-let7g and miR-30d were examined as reference miRNAs in circulating miR-375 expression analysis, as their expression levels were constantly high and not significantly different between healthy donors and MM patients in our analyses (data not shown).

Statistics

Expression levels of miR-375 in plasma cells were assessed by one-way analysis of variance after logarithmic conversion using EZR ver.1.21 (Saitama Medical Centre, Jichi Medical University, Saitama, Japan) (Kanda, 2013), which is a graphical user interface for R 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). Analysis of serum miRNA data was performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). All tests were two-sided and $P < 0{\cdot}05$ was considered to be significant.

Results

miR-375 expression is decreased in CD138-positive plasma cells from patients with MGUS, NDMM and RRMM

We first analysed the expression level of miR-375 in CD138-positve plasma cells from 30 MGUS patients, 34 NDMM

patients, 39 RRMM patients and 11 HMCLs by the $2^{-\Delta\Delta Ct}$ method, using cDNA from RPMI8226 cells as a calibrator sample. Because more than 97% of patient-derived CD138-positve plasma cells were morphologically confirmed as myeloma cells, we decided to regard CD138-positve plasma cells as myeloma cells in this study. Compared with plasma cells from 10 healthy controls, the expression levels of miR-375 were significantly lower in patient-derived CD138-positve plasma cells (i.e., myeloma cells) from MGUS ($P < 0{\cdot}05$), NDMM ($P < 0{\cdot}01$) and RRMM ($P < 0{\cdot}01$), and tended to be lower in HMCLs. In contrast, the levels of miR-375 in CD138-positive plasma cells did not differ significantly among MGUS, NDMM, RRMM and HMCLs (Fig 1A). In addition, in patients with available information on chromosomal abnormality, the miR-375 level did not appear to be associated with types of myeloma-specific chromosomal abnormalities, such as *t*(4;14), *t*(11;14), del(17p), or del(13q) (Fig 1B). These findings indicate that miR-375 expression is equivalently repressed in all disease phases from MGUS to RRMM and regardless of cytogenetic subtype.

Introduction of miR-375 reduces the levels of PDPK1 and various myeloma-promoting molecules in HMCLs

Next, we investigated whether abnormal repression of miR-375 causes PDPK1 overexpression in HMCLs. A miR-375 mimic was successfully introduced into RPMI8226 cells using the HVJ envelope vector, and the protein expression level of PDPK1 was decreased 48 h after introduction of miR-375, compared with cells with negative control miRNA (Fig 2A). A similar result was obtained in 6 of the other 7 HMCLs examined, with the exception being KMS28PE cells, although the extent of reduction of PDPK1 differed among cell lines (Fig 2B). In addition, the introduction of miR-375 caused repression of insulin-like growth factor 1 receptor (IGF1R) and Janus kinase 2 (JAK2) in several HMCLs, while expression of SP1, one of the known targets of miR-375, was unchanged by miR-375 introduction in HMCLs (Fig 2C). These results indicate that miR-375 is involved in regulation of PDPK1 expression, as well as expression of IGF1R and JAK2, which are also essential for cell proliferation and survival of myeloma cells (Chiron *et al*, 2013; Zhang *et al*, 2016).

Copy number status of the PDPK1 *gene in HMCLs and patient-derived myeloma cells*

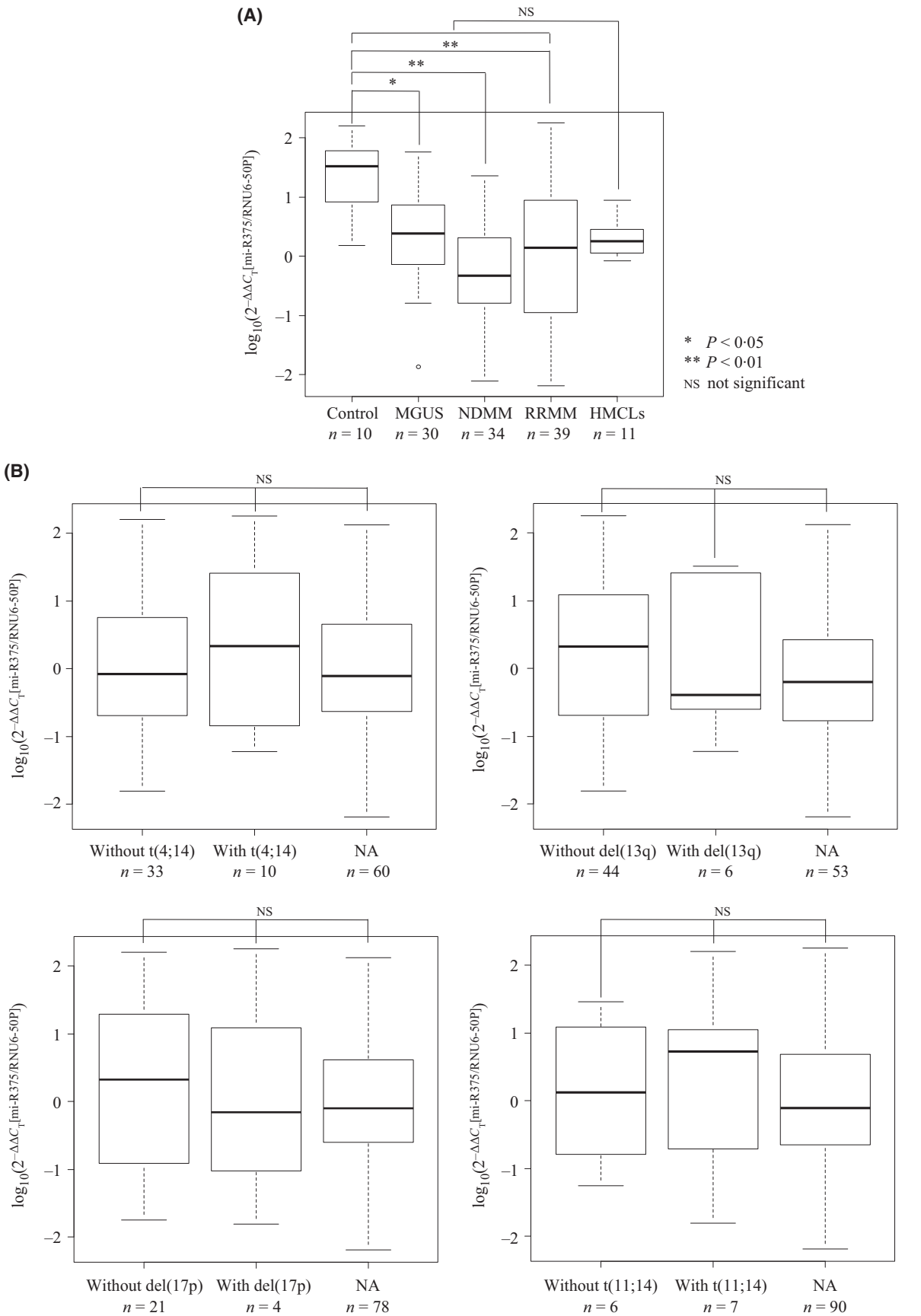
The copy number status was assessed by double colour FISH for *PDPK1* located on the short arm of chromosome 16 and for the centromere of chromosome 16 (CEP16). All 8 HMCLs examined were found to harbour a copy number increase for *PDPK1*. Some cell lines showed equally increased signals for *PDPK1* and CEP16, indicating the presence of chromosome 16 gain (such as trisomy 16), while others showed increased *PDPK1* signals without increased CEP16, suggesting *PDPK1* gene amplification. However, increased *PDPK1* copy number was not found in any patient-derived myeloma cells (Fig S2). Thus, our results indicate that a copy number increase is not the major cause of PDPK1 overexpression in patient-derived myeloma cells, but may be an artificial event that is limited to immortalized HMCLs.

Hypermethylation of CpG islands upstream of MIR375 *in HMCLs and patient-derived myeloma (plasma) cells*

Dysregulation of epigenetic mechanisms, such as DNA methylation and histone codes, are important causes of abnormal expression of miRNAs in cancerous diseases, including haematological malignancies (Agirre *et al*, 2012). Therefore, we sought to investigate the possible involvement of epigenetic dysregulation as the underlying mechanism for abnormal miR-375 repression in MM. As previously reported, there are two large CpG islands (CGI) within 3 Kbp of *pre-MIR375*: CGI-1 and CGI-2. CGI-1 is about 720 base pairs (bp) in size and is more distal from *pre-MIR375* compared with CGI-2, which spans about 850 bp and covers the putative *pri-Mir375* transcription start site (Fig 3A) (Avnit-Sagi *et al*, 2009; de Souza Rocha Simonini *et al*, 2010).

We first conducted bisulfite sequencing to investigate the whole methylation status of *MIR375*. This analysis showed that both CGI-1 and CGI-2 were strongly methylated in all three patient-derived myeloma cells in different disease stages (MGUS, NDMM and RRMM) and two HMCLs examined (Fig 3B). MSP analyses further confirmed the constitutive abnormal hypermethylation states of CGI-1 and CGI-2 in all 8 HMCLs examined (Fig 4A). A similar result was found in many, but not all, of the 58 patient-derived myeloma cells from which genomic DNA was available for MSP (Fig 4B). The distal part of CGI-1 was hypermethylated in about 72% of MM patients (MGUS 7/9, NDMM 12/17, RRMM 23/32: total 42/58), while the distal part of CGI-2, including the *MIR375* promoter site, was abnormally hypermethylated in about 57% of MM patients (MGUS 8/9, NDMM 10/17, RRMM 15/32: total 33/58) (Fig 4C). Myeloma cells from 49 of the 58 patients (84·5%) showed hypermethylation in CGI-1 or CGI-2, while abnormal hypermethylation in CGI-1 or

Fig 1. miR-375 expression in multiple myeloma (MM). miR-375 expression levels in CD138-positive plasma cells according to (A) disease phase (monoclonal gammopathy of undetermined significance (MGUS), $n = 30$; newly diagnosed MM (NDMM), $n = 34$; relapsed/refractory MM (RRMM), $n = 39$; and human myeloma-derived cell lines (HMCLs), $n = 11$) and (B) cytogenetic abnormalities. Box-and-whisker plots show expression levels of MIR-375 normalized to RNU6-50P snRNA. Bars represent medians and whiskers are drawn from the 10th to 90th percentile. Levels of MIR-375 were analysed by one-way analysis of variance after logarithmic conversion. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; NS, not significant; NA, not available.


(A)
NS
**
**
*
log10(2−ΔΔCT[mi-R375/RNU6-50P])
2
1
0
−1
−2
Control n = 10
MGUS n = 30
NDMM n = 34
RRMM n = 39
HMCLs n = 11
* P < 0·05
** P < 0·01
NS not significant
(B)
NS
log10(2−ΔΔCT[mi-R375/RNU6-50P])
Without t(4;14) n = 33
With t(4;14) n = 10
NA n = 60
NS
log10(2−ΔΔCT[mi-R375/RNU6-50P])
Without del(13q) n = 44
With del(13q) n = 6
NA n = 53
NS
log10(2−ΔΔCT[mi-R375/RNU6-50P])
Without del(17p) n = 21
With del(17p) n = 4
NA n = 78
NS
log10(2−ΔΔCT[mi-R375/RNU6-50P])
Without t(11;14) n = 6
With t(11;14) n = 7
NA n = 90

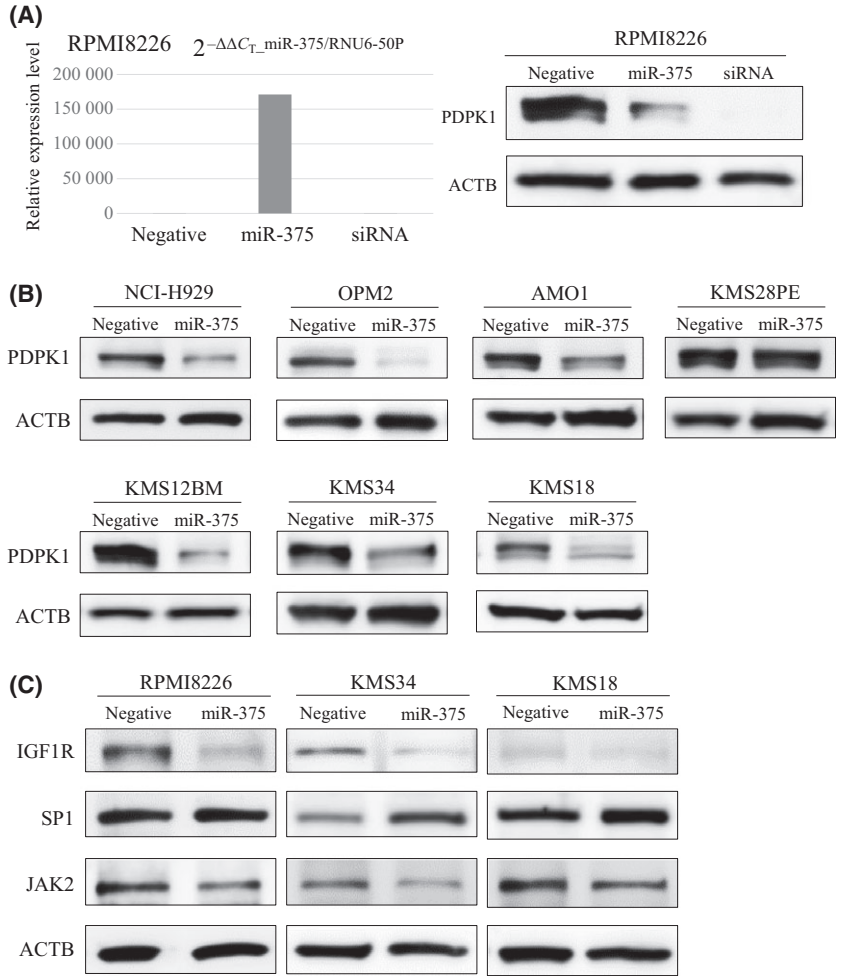


Fig 2. Molecular effects of miR-375 in HMCLs. (A) Introduction of miR-375 mimic (left; results of quantitative RT-PCR analyses of mature miR-375 expression 48 h after transfection) utilizing a haemagglutinating virus of Japan (HVJ)-envelope vector resulted in reduction of PDPK1 in RPMI8226 cells (right; Western blot). Effects of siRNA against *PDPK1* and negative control mature miRNA were evaluated as positive and negative controls, respectively. (B) PDPK1 repression by introduction of miR-375 was confirmed in 7 of 8 HMCLs, in addition to RPMI8226 cells. Results are shown for 7 representative cell lines. (C) Introduction of miR-375 also repressed expression of insulin-like growth factor receptor-1 (IGF1R) and Janus kinase 2 (JAK2), but not SP1, in HMCLs. Results for three representative cell lines are shown.

CGI-2 was not identified in myeloma cells from 9 patients (3 of 17 NDMM, 6 of 32 RRMM: 15·5%). In addition, the frequency of hypermethylation in the CGI-1 lesion did not seem to be influenced by disease stage, while that in CGI-2 was significantly higher in MGUS compared with NDMM or RRMM (Fig 4C). These findings indicate that repression of miR-375 is accompanied by abnormal hypermethylation of upstream CpG islands, including in the promoter site, in most MM patients.

Treatment with epigenetic agents restores miR-375 expression via *histone modifications in HMCLs*

To identify the regulatory mechanism of miR-375 expression in myeloma cells, we next examined the effects of BTZ, LEN, TMP (SP1 inhibitor), TSA, and SGI-110 on expression levels of miR-375 in two HMCLs: RPMI8226 and KMC34 cells. Although BTZ and LEN are central in treatment for MM, neither of these drugs altered miR-375 expression at their IC_{50} level, suggesting that there is no relationship between miR-375 expression and gene regulation by the proteasome pathway or cereblon-associated pathway. In contrast, epigenetic modifiers altered miR-375 expression by varying degrees in RPMI8226 and KMS34 cells. In RPMI8226 cells, IC_{50} levels of TMP, SGI-110 and TSA all increased expression of miR-375, with miR-375 induction being most pronounced by SGI-110. In KMS34 cells, treatment with TSA or SGI-110, but not with TMP, increased expression of miR-375. Interestingly, a combination of TSA and SGI-110 increased miR-375 expression significantly more than with either agent alone (Fig 5).

To further evaluate the effect of SGI-110 alone or SGI-110 plus TSA on histone code modifications, we designed three primer sets to analyse the status of histone H3 acetylation (H3ac) and histone H3 lysine 4 trimethylation (H3K4m3) at approximately 1700 bp upstream of *pre-MIR375*, and performed ChIP-quantitative PCR (qPCR) on cells with or without treatment with SGI-110 or SGI-110 plus TSA. As expected from the results in Fig 5, SGI-110 alone did not cause histone code modification in RPMI8226 or KMS34 cells, but the combination of SGI-110 and TSA increased H3ac and H3K4m3 in all fragments in KMS34 cells, but not in RPMI8226 cells (Fig 6A).

We next examined the effects of SGI-110 and TSA on expression of target proteins, including PDPK1, in RPMI8226 or KMS34 cells. In theory, more induction of miR-375 leads

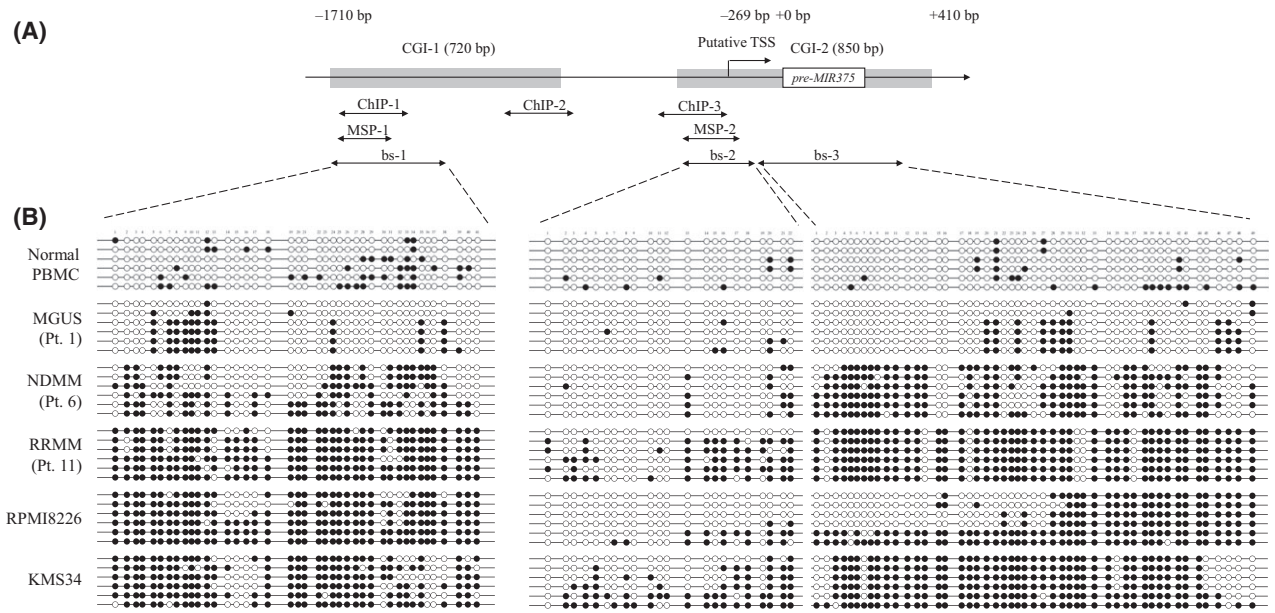


Fig 3. Methylation status of two CpG islands (CGIs) located within 3 Kbp of *pre-MIR375*. (A) Map for CGI-1, CGI-2 (grey boxes) and the putative transcriptional start site (TSS) of *pri-MIR375*. Locations of primer sets for bisulfite sequences (bs-1, bs-2, and bs-3), methylation-specific polymerase chain reaction (PCR) (MSP-1 and MSP-2), and chromatin immunoprecipitation quantitative PCR (ChIP-qPCR; ChIP-1, ChIP-2, ChIP-3) are shown. (B) Results of bisulfite sequencing of two CGIs in normal peripheral blood mononuclear cells (PBMCs), MGUS (Patient 1), NDMM (Patient 6), RRMM (Patient 11), RPMI8226 cells and KMS34 cells (Patient numbers correspond to those in Fig 4). Black and open circles represent methylated and unmethylated CpGs, respectively.

to greater repression of target proteins. Consistent with this theory, only SGI-110 treatment caused reduction of PDPK1 expression in RPIM8226 cells, while both SGI-110 and TSA reduced PDPK1 expression in KMS34 cells. Furthermore, a combination of SGI-110 and TSA markedly repressed expression of PDPK1 in KMS34 cells, while this effect was not prominent in RPMI8226 cells (Fig 6B). These results are all in accord with the results for miR-375 induction by SGI-110 or TSA in the two HMCLs examined. In addition to PDPK1, compared with the treatment with either SGI-110 or TSA, the combination of SGI-110 and TSA showed the greater repression of IGF1R and JAK2 proteins in both RPMI8226 and KMS34 cells. Collectively, these results indicate that overlapping diverse mechanisms for epigenetic dysregulation, i.e. hypermethylation and abnormal histone modifications including acetylation, modify expression levels of miR-375 and its target proteins, such as PDPK1, IGF1R and JAK2. The extent of the effects of hypermethylation and abnormal histone code modification on miR-375 expression varies among cell types, suggesting inter-patient diversity among myeloma clones.

Circulating serum miR-375 levels do not differ between myeloma patients and healthy donors

Finally, we assessed the serum level of circulating miR-375 in MM patients and healthy donors using three different reference miRNAs. The serum miR-375 level did not differ significantly between 15 healthy donors and 61 MM patients (Fig 7), indicating that abnormal repression of miR-375 is an event specific to myeloma cells, and not a systemic event in normal tissues in MM patients.

Discussion

In this study, we focused on the possible involvement of miR-375 repression as a cause for PDPK1 overexpression, which induces PDPK1 activation through autophosphorylation and resultant RPS6KA3 activation. There are several reasons for examining miR-375 in this context. First, *MIR375*, which is located between the *CRYBA2* and *CFAP65* genes on chromosome 2q35, codes for a regulatory miRNA for *PDPK1*, which has been primarily identified to control glucose-induced insulin secretion from human β cells (Jafarian *et al*, 2015; Nathan *et al*, 2015). Second, miR-375 targets several important oncogenes, such as *IGF1R* and *JAK2*, in addition to *PDPK1*, and is a putative tumour suppressor in several cancers, although its role has not been evaluated in myeloma cells (Ding *et al*, 2010; Tsukamoto *et al*, 2010; Kong *et al*, 2012; Yan *et al*, 2014). Third, miR-375 repression has been reported in MM regardless of the types of cytogenetic abnormality (Gutierrez *et al*, 2010), and this independency of the cytogenetic profile seems to be consistent with the cytogenetic-independent universal activation pattern of the PDPK1/RPS6KA3 axis in MM (Chinen *et al*, 2014).

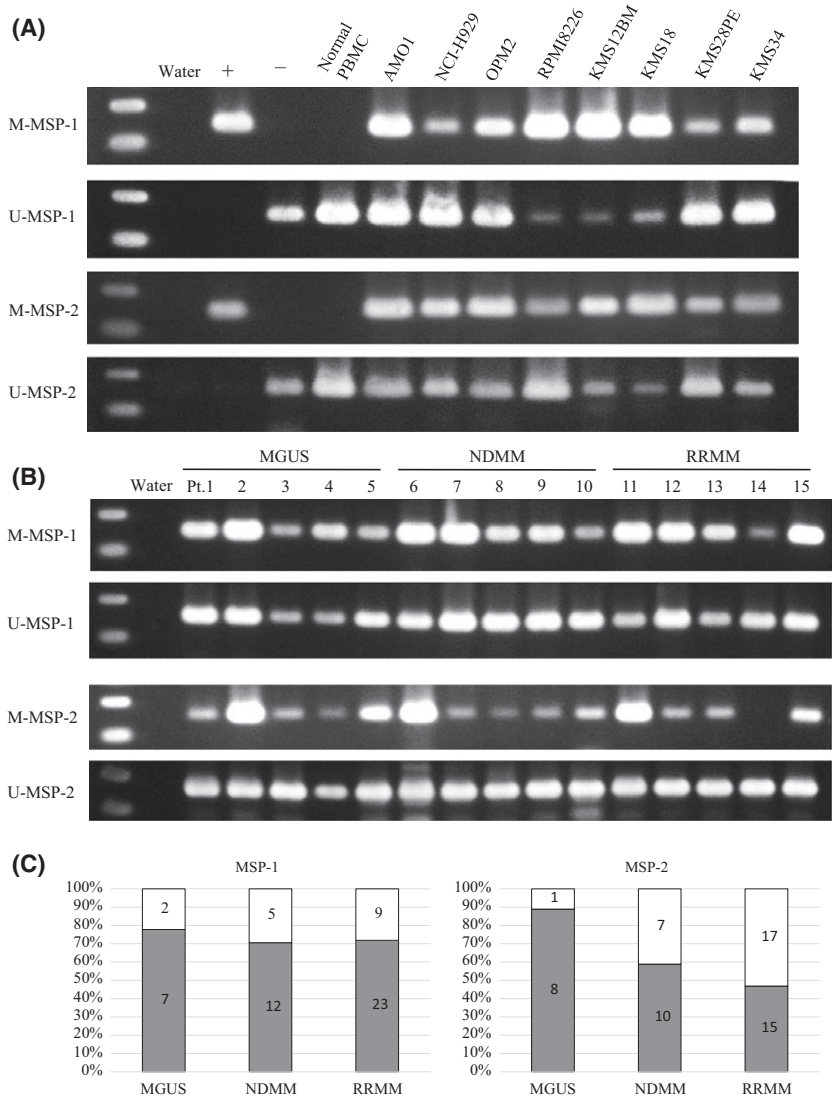


Fig 4. *MIR375* hypermethylation in patient-derived CD138-positive myeloma cells and HMCLs analysed by MSP. (A) MSP-1 and MSP-2 showed methylation in all eight HMCLs, but not in normal PBMCs. Methylated and unmethylated controls are shown in the (+) and (−) lanes, respectively. (B) methylation-specific PCR (MSP) analyses of 15 patient-derived myeloma cells. Each clinical stage included 5 samples. All patient-derived myeloma cells were methylated in MSP-1 and MSP-2, except for MSP-2 in RRMM Patient 14. (C) Frequencies of patients with hypermethylation at MSP-1 and MSP-2 in 58 patient-derived myeloma cells (MGUS, $N = 9$, NDMM, $N = 17$; RRMM, $N = 32$): grey, methylated; white, unmethylated.

As expected, we found repression of miR-375 in most MM patient-derived myeloma cells from all clinical stages (MGUS, NDMM and RRMM), regardless of the cytogenetic profile. More importantly, introduction of the *MIR375* gene decreased expression of PDPK1 (IGF1R and JAK2 as well) in most HMCLs, suggesting functional involvement of miR-375 in PDPK1 expression in MM. Since it has been reported that an increased gene copy number accounts for *PDPK1* overexpression in breast cancer cells (Maurer *et al*, 2009), we additionally examined whether a copy number increase of *PDPK1* was also present in MM. However, this was not found in patient-derived myeloma cells. Collectively, these findings suggest that repression of miR-375 is the dominant mechanisms for PDPK1 overexpression in myeloma from an early clinical stage (MGUS) to an advanced disease stage (RRMM).

Dysregulation of miRNA expression is induced by various mechanisms, including deletion, amplification, mutation, epigenetic alteration or dysregulation of upstream transcription factors that regulate target miRNAs (Croce, 2009). Among these, dysregulated epigenetic processes have been associated with miR-375 repression in various cancers. For instance, hypermethylation at a CGI upstream of *MIR375* has been associated with miR-375 repression in hepatocellular carcinoma, melanoma and oesophageal cancer (Furuta *et al*, 2010; Li *et al*, 2011; Mazar *et al*, 2011), and abnormal histone acetylation has been linked to dysregulated miR-375 expression in oesophageal cancer and myeloproliferative neoplasms (Isozaki *et al*, 2012; Yin *et al*, 2015). In our study, CGI-1 and CGI-2, which are within 3 Kbp of *pre-MIR375*, were hypermethylated in all 8 HMCLs examined and the majority of 58 patient-derived myeloma cells. Although we were unable to evaluate the relationship between miR-375 expression levels and the degree of methylation at the two CGIs in patient-derived plasma cells (data not shown), our results indicate that repression of miR-375 is caused by abnormal hypermethylation of at least one of these CGIs, including in the promoter region of *MIR375*. Indeed,

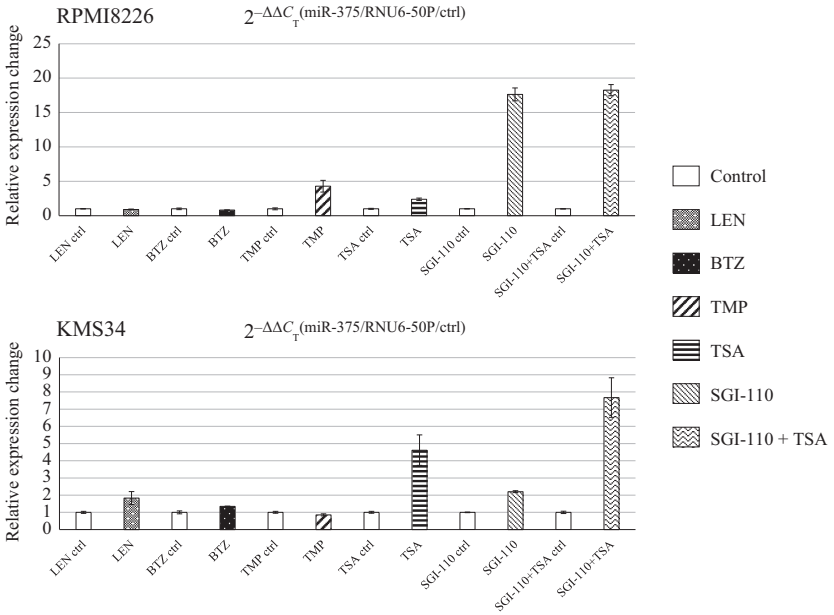


Fig 5. Changes in miR-375 expression with various agents. RPMI8226 (upper) and KMS34 (lower) cells were treated with each agent at their IC_{50} level. Expression levels of miR-375 determined by quantitative RT-PCR and normalized to control RNU6-50P snRNA are shown. The miR-375 level in control cells treated with an equivalent amount of DMSO was considered to be 1·0. The diagram shows the mean ± SD of three independent experiments. ctrl, control; LEN, lenalidomide; BTZ, bortezomib; TMP, terameprocol; TSA, trichostatin A.

treatment with SGI-110 or TSA restored the expression level of miR-375 in a cell-type dependent manner, and a combination of SGI-110 and TSA further increased the level of miR-375, especially in KMS34 cells. Presumably, TSA promoted conversion of silenced chromatin into an open chromatin structure by increasing H3ac and H3K4m3, and thereby made regions demethylated by SGI-110 more susceptible to unknown transcriptional factors in KMS34 cells. These findings support the idea that repression of miR-375 is caused by overlapping epigenetic dysregulations, while the impact of CGI hypermethylation and histone code changes on miR-375 repression most likely varies among cell types in MM.

Although somewhat paradoxical, both global hypomethylation and gene-specific methylation can promote transformation of MGUS to MM with diverse methylation profiles according to cytogenetic subgroups (Salhia *et al*, 2010; Walker *et al*, 2011). In contrast, the methylation status of miR-375 was not influenced by disease stage; the frequencies of patients with *MIR375* methylation were mostly equivalent among MGUS, NDMM and RRMM, suggesting that *MIR375* methylation occurs as an early event in myelomagenesis in most patients. ChIP-qPCR for analysis of histone modifications was performed only in HMCLs due to the lack of sufficient amounts of samples from patient-derived cells; therefore, we were unable to evaluate involvement of histones in miR-375 expression in patient-derived myeloma cells. However, abnormal histone modification is frequently associated with disease progression and is commonly considered to be an acquired molecular event in carcinogenesis in various cancers (Barlesi *et al*, 2007; Elsheikh *et al*, 2009; Mosashvilli *et al*, 2010). This is consistent with our finding of functional involvement of histone acetylation in miR-375 repression in HMCLs derived from patients with aggressive MM in an advanced clinical phase.

SGI-110 is a second-generation hypomethylating prodrug of which decitabine is the well-characterized active metabolite (Griffiths *et al*, 2013). SGI-110 resensitizes platinum-resistant ovarian cancer cells to cisplatin by restoring expression of tumour suppressor genes and differentiation-associated genes, and suppressing putative drivers of cisplatin resistance (Fang *et al*, 2014). SGI-110 has also been tested in clinical trials, mainly in haematological malignancy, and the US Food and Drug Administration has granted an orphan drug status for SGI-110 for treatment of acute myeloid leukaemia. HDAC inhibitors, such as panobinostat, have been utilized as therapy for MM (San-Miguel *et al*, 2014), whereas the therapeutic effects of currently available hypomethylators, such as azacitidine or decitabine, have been questionable in clinical settings. Our results suggest that SGI-110, a newer agent with higher bioavailability, may have therapeutic potency for MM, especially in combination with HDAC inhibitors, through modulation of expression of tumour suppressor genes, including *MIR375*, that regulate not only the PDPK1/RPS6KA3 signalling axis but also cell proliferation pathways of IGF1R or JAK2.

Circulating miRNAs are stable in the circulation in a cell-free form (Mitchell *et al*, 2008) and their expression patterns are altered in MM. For instance, lower levels of circulating miR-744 and miR-let7e have been associated with poor survival of MM patients (Kubiczkova *et al*, 2014). This association between low circulating miR-375 and a poor prognosis has also been reported in non-small-cell lung cancer and distal gastric adenocarcinoma (Zhang *et al*, 2012; Yu *et al*, 2013); however, this was not the case in MM in the current study. The mechanism for decreased circulating miR-375 has been unclear in previous reports. We speculate that expression of circulating miR-375 might be influenced by the systematic condition of patients with solid cancers, such as high

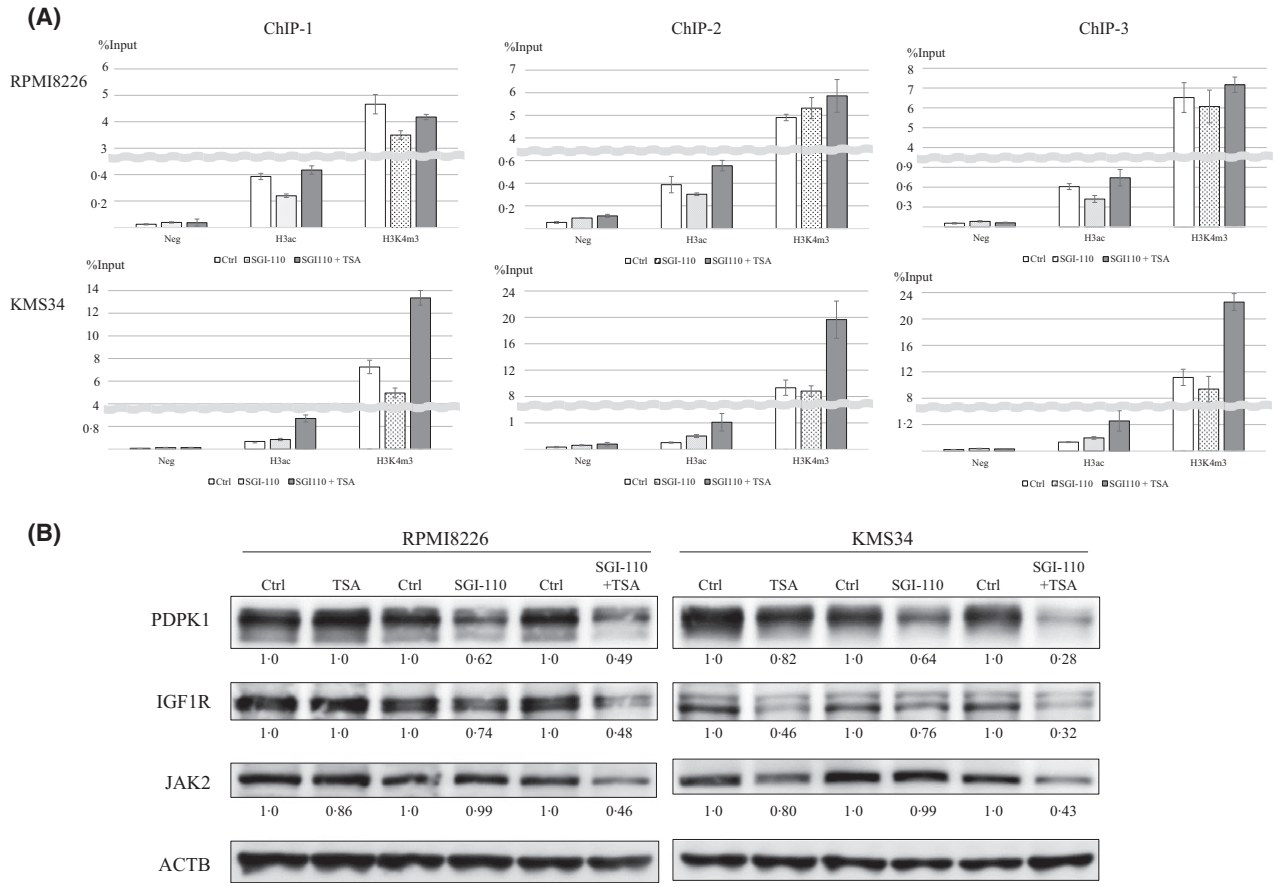


Fig 6. Changes in chromatin modifications and repression of PDPK1 by epigenetic agents. (A) Chromatin immunoprecipitation quantitative reverse transcription polymerase chain reaction (ChIP-qPCR) analyses showed changes in chromatin modification by treatment with SGI-110 or SGI-110 with TSA in RPMI8226 (upper) and KMS34 (lower) cells. Cross-linked chromatin was immunoprecipitated with antibodies specific for histone H3 acetylation (H3ac) and histone H3 lysine 4 trimethylation (H3K4m3). qPCR was performed for analysis of purified DNA with three different ChIP primer sets (ChIP-1, ChIP-2 and ChIP-3 (see Figure 3a). Results are shown as percentages of the input (normalized against input). The diagram shows the mean of independent triplicate experiments ± SD. (B) Epigenetic agents repressed PDPK1, IGF1R and JAK2 in RPMI8226 and KMS34 cells. Expression levels of those proteins relative to untreated cells (ctrl) were calculated with ImageJ software (imagej.nih.gov/ij/) and are described below each band.

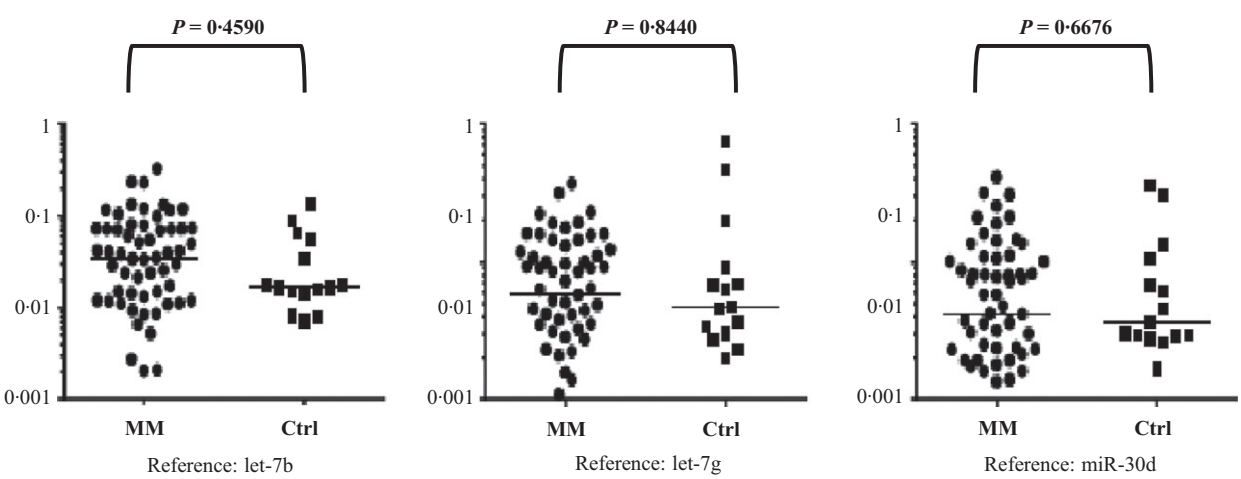


Fig 7. Expression levels of circulating serum miR-375 in MM patients and healthy controls (Ctrl). Levels of circulating serum miR-375 were analyzed using three references, miR-let7b, miR-let7g, and miR-30d, in 61 MM patients and 15 healthy donors. Bars represent the median. Differences between groups were not significant.

interleukin-10 production by tumour-associated macrophages that negatively regulate miR-375 expression (Komohara *et al*, 2014; Garikipati *et al*, 2015). A further study is needed to examine the different patterns of circulating miR-375 between solid cancers and MM. Nevertheless, our study showed that repression of miR-375 is a tumour cell-specific event, and not a systemic event, in MM.

Finally, epigenetic dysregulations of miR-375 were found to be the cause of PDPK1 overexpression in MM, but other mechanisms regulating miR-375 repression and PDPK1 overexpression may also exist, such as abnormal transcriptional factor regulation or CTCF binding status (Avnit-Sagi *et al*, 2009; de Souza Rocha Simonini *et al*, 2010). Indeed, the two CGIs we assessed in this study include various transcription factor binding sites, and thus transcription factors, including CTCF, may regulate miR-375 expression. Further studies are needed to examine this possibility.

In conclusion, this study revealed that expression of miR-375 is pathologically and universally decreased in clonal plasma cells from MGUS to RRMM patients, and that repression of miR-375 is caused by overlapping epigenetic dysregulations. Dysregulation of miR-375 might occur during early myelomagenesis and may be a critical molecular event in promoting constitutive activation of several signalling pathways for cell proliferation and survival, including PDPK1/RPS6KA3, IGF1R and JAK2 pathways. These results may be translatable into development of novel therapeutic and diagnostic strategies targeting miR-375 in MGUS and MM.

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Authorship statement

S. Tatekawa, Y. Chinen and J. Kuroda designed the study; S. Tatekawa, Y. Chinen, Y. Matsumura-Kimoto, T. Narita, M. Ri and T. Taki performed the research; Y. Shimura, H. Nagoshi, T. Kobayashi, E. Kawata, N. Uoshima and H. Handa contributed to collection of patients samples and clinical data; S. Tatekawa and J. Kuroda wrote the manuscript; and M. Taniwaki, H. Handa and S. Iida edited the manuscript and provided vital conceptual insights. J. Kuroda supervised the whole process.

Conflict of interest

The authors have nothing to disclose. There are no conflicts of interest to report.

Financial disclosure statement

The authors have nothing to disclose. There are no conflicts of interest to report.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. The primers for each PCR assessments.

Figure S1. The sensitivities of M-MSP-1 and M-MSP-2.

Figure S2. The analyses of dual colour interphase FISH.

References

Abdi, J., Jian, H. & Chang, H. (2016) Role of micro-RNAs in drug resistance of multiple myeloma. *Oncotarget*, **7**, 60723–60735.

Agirre, X., Martinez-Climent, J.A., Odero, M.D. & Prosper, F. (2012) Epigenetic regulation of miRNA genes in acute leukemia. *Leukemia*, **26**, 395–403.

Avnit-Sagi, T., Kantorovich, L., Kredo-Russo, S., Hornstein, E. & Walker, M.D. (2009) The promoter of the pri-miR-375 gene directs expression selectively to the endocrine pancreas. *PLoS ONE*, **4**, e5033.

Barlesi, F., Giaccone, G., Gallegos-Ruiz, M.I., Loundou, A., Span, S.W., Lefesvre, P., Kruyt, F.A. & Rodriguez, J.A. (2007) Global histone modifications predict prognosis of resected non small-cell lung cancer. *Journal of Clinical Oncology*, **25**, 4358–4364.

Brioli, A., Melchor, L., Cavo, M. & Morgan, G.J. (2014) The impact of intra-clonal heterogeneity on the treatment of multiple myeloma. *British Journal of Haematology*, **165**, 441–454.

Chi, J., Ballabio, E., Chen, X.H., Kusec, R., Taylor, S., Hay, D., Tramonti, D., Saunders, N.J., Littlewood, T., Pezzella, F., Boultwood, J., Wainscoat, J.S., Hatton, C.S. & Lawrie, C.H. (2011) MicroRNA expression in multiple myeloma is associated with genetic subtype, isotype and survival. *Biology Direct*, **6**, 23.

Chinen, Y., Kuroda, J., Shimura, Y., Nagoshi, H., Kiyota, M., Yamamoto-Sugitani, M., Mizutani, S., Sakamoto, N., Ri, M., Kawata, E., Kobayashi, T., Matsumoto, Y., Horiike, S., Iida, S. & Taniwaki, M. (2014) Phosphoinositide protein kinase PDPK1 is a crucial cell signaling mediator in multiple myeloma. *Cancer Research*, **74**, 7418–7429.

Chiron, D., Maiga, S., Surget, S., Descamps, G., Gomez-Bougie, P., Traore, S., Robillard, N., Moreau, P., Le Gouill, S., Bataille, R., Amiot, M. & Pellat-Deceunynck, C. (2013) Autocrine insulin-like growth factor 1 and stem cell factor but not interleukin 6 support self-renewal of human myeloma cells. *Blood Cancer Journal*, **3**, e120.

Croce, C.M. (2009) Causes and consequences of microRNA dysregulation in cancer. *Nature Reviews Genetics*, **10**, 704–714.

Ding, L., Xu, Y., Zhang, W., Deng, Y., Si, M., Du, Y., Yao, H., Liu, X., Ke, Y., Si, J. & Zhou, T. (2010) MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. *Cell Research*, **20**, 784–793.

El Ouaamari, A., Baroukh, N., Martens, G.A., Lebrun, P., Pipeleers, D. & van Obberghen, E. (2008) miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic b-cells. *Diabetes*, **57**, 2708–2717.

Elsheikh, S.E., Green, A.R., Rakha, E.A., Powe, D.G., Ahmed, R.A., Collins, H.M., Soria, D., Garibaldi, J.M., Paish, C.E., Ammar, A.A., Grainge, M.J., Ball, G.R., Abdelghany, M.K., Martinez-Pomares, L., Heery, D.M. & Ellis, I.O. (2009) Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Research*, **69**, 3802–3809.

Fang, F., Munck, J., Tang, J., Taverna, P., Wang, Y., Miller, D.F., Pilrose, J., Choy, G., Azab, M., Pawelczak, K.S., VanderVere-Carozza, P., Wagner, M., Lyons, J., Matei, D., Turchi, J.J. & Nephew, K.P. (2014) The novel, small-molecule DNA methylation inhibitor SGI-110 as an ovarian cancer chemosensitizer. *Clinical Cancer Research*, **20**, 6504–6516.

Furuta, M., Kozaki, K.I., Tanaka, S., Arii, S., Imoto, I. & Inazawa, J. (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis*, **31**, 766–776.

Garikipati, V.N., Krishnamurthy, P., Verma, S.K., Khan, M., Abramova, T., Mackie, A.R., Qin, G., Benedict, C., Nickoloff, E., Johnson, J., Gao, E., Losordo, D.W., Houser, S.R., Koch, W.J. & Kishore, R. (2015) Negative regulation of miR-375 by Interleukin-10 enhances bone marrow-derived progenitor cell-mediated myocardial repair and function after myocardial infarction. *Stem Cells*, **33**, 3519–3529.

Griffiths, E.A., Choy, G., Redkar, S., Taverna, P., Azab, M. & Karpf, A.R. (2013) SGI-110: DNA methyltransferase inhibitor oncolytic. *Drugs Future*, **38**, 535–543.

Gutierrez, N.C., Sarasquete, M.E., Misiewicz-Krzeminska, I., Delgado, M., De Las Rivas, J., Ticona, F.V., Ferminan, E., Martin-Jimenez, P., Chillon, C., Risueno, A., Hernandez, J.M., Garcia-Sanz, R., Gonzalez, M. & San Miguel, J.F. (2010) Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. *Leukemia*, **24**, 629–637.

Isozaki, Y., Hoshino, I., Nohata, N., Kinoshita, T., Akutsu, Y., Hanari, N., Mori, M., Yoneyama, Y., Akanuma, N., Takeshita, N., Maruyama, T., Seki, N., Nishino, N., Yoshida, M. & Matsubara, H. (2012) Identification of novel molecular targets regulated by tumor suppressive miR-375 induced by histone acetylation in esophageal squamous cell carcinoma. *International Journal of Oncology*, **41**, 985–994.

Jafarian, A., Taghikani, M., Abroun, S., Allahverdi, A., Lamei, M., Lakpour, N. & Soleimani, M. (2015) The generation of insulin producing cells from human mesenchymal stem cells by MiR-375 and Anti-MiR-9. *PLoS ONE*, **10**, e0128650.

Kanda, Y. (2013) Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplantation*, **48**, 452–458.

Kobayashi, T., Kuroda, J., Ashihara, E., Oomizu, S., Terui, Y., Taniyama, A., Adachi, S., Takagi, T., Yamamoto, M., Sasaki, N., Horiike, S., Hatake, K., Yamauchi, A., Hirashima, M. & Taniwaki, M. (2010) Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. *Leukemia*, **24**, 843–850.

Komohara, Y., Jinushi, M. & Takeya, M. (2014) Clinical significance of macrophage heterogeneity in human malignant tumors. *Cancer Science*, **105**, 1–8.

Kong, K.L., Kwong, D.L., Chan, T.H., Law, S.Y., Chen, L., Li, Y., Qin, Y.R. & Guan, X.Y. (2012) MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. *Gut*, **61**, 33–42.

Kubiczkova, L., Kryukov, F., Slaby, O., Dementyeva, E., Jarkovsky, J., Nekvindova, J., Radova, L., Greslikova, H., Kuglik, P., Vetesnikova, E., Pour, L., Adam, Z., Sevcikova, S. & Hajek, R. (2014) Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. *Haematologica*, **99**, 511–518.

Li, X., Lin, R. & Li, J. (2011) Epigenetic silencing of microRNA-375 regulates PDK1 expression in esophageal cancer. *Digestive Diseases and Sciences*, **56**, 2849–2856.

Maurer, M., Su, T., Saal, L.H., Koujak, S., Hopkins, B.D., Barkley, C.R., Wu, J., Nandula, S., Dutta, B., Xie, Y., Chin, Y.R., Kim, D.I., Ferris, J.S., Gruvberger-Saal, S.K., Laakso, M., Wang, X., Memeo, L., Rojtman, A., Matos, T., Yu, J.S., Cordon-Cardo, C., Isola, J., Terry, M.B., Toker, A., Mills, G.B., Zhao, J.J., Murty, V.V., Hibshoosh, H. & Parsons, R. (2009) 3-Phosphoinositide-dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma. *Cancer Research*, **69**, 6299–6306.

Mazar, J., DeBlasio, D., Govindarajan, S.S., Zhang, S. & Perera, R.J. (2011) Epigenetic regulation of microRNA-375 and its role in melanoma development in humans. *FEBS Letters*, **585**, 2467–2476.

Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R., Wyman, S.K., Pogosova-Agadjanyan, E.L., Peterson, A., Noteboom, J., O'Briant, K.C., Allen, A., Lin, D.W., Urban, N., Drescher, C.W., Knudsen, B.S., Stirewalt, D.L., Gentleman, R., Vessella, R.L., Nelson, P.S., Martin, D.B. & Tewari, M. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciencesof United States of America*, **105**, 10513–10518.

Mosashvilli, D., Kahl, P., Mertens, C., Holzapfel, S., Rogenhofer, S., Hauser, S., Buttner, R., Von Ruecker, A., Muller, S.C. & Ellinger, J. (2010) Global histone acetylation levels: prognostic relevance in patients with renal cell carcinoma. *Cancer Science*, **101**, 2664–2669.

Nagoshi, H., Taki, T., Hanamura, I., Nitta, M., Otsuki, T., Nishida, K., Okuda, K., Sakamoto, N., Kobayashi, S., Yamamoto-Sugitani, M., Tsutsumi, Y., Kobayashi, T., Matsumoto, Y., Horiike, S., Kuroda, J. & Taniwaki, M. (2012) Frequent PVT1 rearrangement and novel chimeric genes PVT1-NBEA and PVT1-WWOX occur in multiple myeloma with 8q24 abnormality. *Cancer Research*, **72**, 4954–4962.

Nathan, G., Kredo-Russo, S., Geiger, T., Lenz, A., Kaspi, H., Hornstein, E. & Efrat, S. (2015) MiR-375 promotes redifferentiation of adult human beta cells expanded *in vitro*. *PLoS ONE*, **10**, e0122108.

Pichiorri, F., De Luca, L. & Aqeilan, R.I. (2011) MicroRNAs: new players in multiple myeloma. *Frontiers in Genetics*, **2**, 22.

Rajkumar, S.V., Dimopoulos, M.A., Palumbo, A., Blade, J., Merlini, G., Mateos, M.V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P., Landgren, O., Paiva, B., Dispenzieri, A., Weiss, B., LeLeu, X., Zweegman, S., Lonial, S., Rosinol, L., Zamagni, E., Jagannath, S., Sezer, O., Kristinsson, S.Y., Caers, J., Usmani, S.Z., Lahuerta, J.J., Johnsen, H.E., Beksac, M., Cavo, M., Goldschmidt, H., Terpos, E., Kyle, R.A., Anderson, K.C., Durie, B.G. & Miguel, J.F. (2014) International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *The Lancet Oncology*, **15**, e538–e548.

Salhia, B., Baker, A., Ahmann, G., Auclair, D., Fonseca, R. & Carpten, J. (2010) DNA methylation analysis determines the high frequency of genic hypomethylation and low frequency of hypermethylation events in plasma cell tumors. *Cancer Research*, **70**, 6934–6944.

San-Miguel, J.F., Hungria, V.T., Yoon, S.S., Beksac, M., Dimopoulos, M.A., Elghandour, A., Jedrzejczak, W.W., Gunther, A., Nakorn, T.N., Siritanaratkul, N., Corradini, P., Chuncharunee, S., Lee, J.J., Schlossman, R.L., Shelekhova, T., Yong, K., Tan, D., Numbenjapon, T., Cavenagh, J.D., Hou, J., LeBlanc, R., Nahi, H., Qiu, L., Salwender, H., Pulini, S., Moreau, P., Warzocha, K., White, D., Blade, J., Chen, W., de la Rubia, J., Gimsing, P., Lonial, S., Kaufman, J.L., Ocio, E.M., Veskovski, L., Sohn, S.K., Wang, M.C., Lee, J.H., Einsele, H., Sopala, M., Corrado, C., Bengoudifa, B.R., Binlich, F. & Richardson, P.G. (2014) Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, doble-blind phase 3 trial. *The Lancet Oncology*, **15**, 1195–1206.

Shimura, Y., Kuroda, J., Ri, M., Nagoshi, H., Yamamoto-Sugitani, M., Kobayashi, T., Kiyota, M., Nakayama, R., Mizutani, S., Chinen, Y., Sakamoto, N., Matsumoto, Y., Horiike, S., Shiotsu, Y., Iida, S. & Taniwaki, M. (2012) RSK2(Ser227) at N-terminal kinase domain is a potential therapeutic target for multiple

myeloma. *Molecular Cancer Therapeutics*, **11**, 2600–2609.

de Souza Rocha Simonini, P., Breiling, A., Gupta, N., Malekpour, M., Youns, M., Omranipour, R., Malekpour, F., Volinia, S., Croce, C.M., Najmabadi, H., Diederichs, S., Sahin, O., Mayer, D., Lyko, F., Hoheisel, J.D. & Riazalhosseini, Y. (2010) Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. *Cancer Research*, **70**, 9175–9184.

Tsukamoto, Y., Nakada, C., Noguchi, T., Tanigawa, M., Nguyen, L.T., Uchida, T., Hijiya, N., Matsuura, K., Fujioka, T., Seto, M. & Moriyama, M. (2010) MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. *Cancer Research*, **70**, 2339–2349.

Walker, B.A., Wardell, C.P., Chiecchio, L., Smith, E.M., Boyd, K.D., Neri, A., Davies, F.E., Ross, F.M. & Morgan, G.J. (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*, **117**, 553–562.

Yan, J.W., Lin, J.S. & He, X.X. (2014) The emerging role of miR-375 in cancer. *International Journal of Cancer*, **135**, 1011–1018.

Yin, L.H., Zheng, X.Q., Li, H.Y., Bi, L.X., Shi, Y.F., Ye, A.F., Wu, J.B. & Gao, S.M. (2015) Epigenetic deregulated miR-375 contributes to the constitutive activation of JAK2/STAT signaling in myeloproliferative neoplasm. *Leukemia Research*, **39**, 471–478.

Yu, H., Jiang, L., Sun, C., Guo, L., Lin, M., Huang, J. & Zhu, L. (2013) Decreased circulating miR-375: a potential biomarker for patients with non-small-cell lung cancer. *Gene*, **534**, 60–65.

Zhang, W.H., Gui, J.H., Wang, C.Z., Chang, Q., Xu, S.P., Cai, C.H., Li, Y.N., Tian, Y.P., Yan, L. & Wu, B. (2012) The identification of miR-375 as a potential biomarker in distal gastric adenocarcinoma. *Oncology Research*, **20**, 139–147.

Zhang, Z., Mao, H., Du, X., Zhu, J., Xu, Y., Wang, S., Xu, X., Ji, P., Yu, Y., Cao, B., Han, K., Hou, T., Xu, Z., Kong, Y., Jiang, G., Tang, X., Qiao, C. & Mao, X. (2016) A novel small molecule agent displays potent anti-myeloma activity by inhibiting the JAK2-STAT3 signaling pathway. *Oncotarget*, **7**, 9296–9308.

Zhu, Y.X., Yin, H., Bruins, L.A., Shi, C.X., Jedlowski, P., Aziz, M., Sereduk, C., Kortuem, K.M., Schmidt, J.E., Champion, M., Braggio, E. & Keith Stewart, A. (2015) RNA interference screening identifies lenalidomide sensitizers in multiple myeloma, including RSK2. *Blood*, **125**, 483–491.

