Brief report
Epigenetic inactivation of the MIR34B/C in multiple myeloma

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We postulated that MIR34B/C, a direct transcriptional target of TP53, might be inactivated by promoter hypermethylation in multiple myeloma (MM). MIR34B/C promoter methylation was studied in 8 normal marrow controls, 8 MM cell lines, 95 diagnostic, and 23 relapsed/progressed MM samples by methylation-specific PCR. MIR34B/C was methylated in 6 (75.0%) MM cell lines but not normal controls.

5-Aza-2’-deoxycytidine led to MIR34B/C promoter demethylation and MIR34B/C re-expression. Moreover, restoration of MIR34B/C led to reduced cellular proliferation and enhanced apoptosis of myeloma cells. In primary samples, methylation of MIR34B/C occurred in 5.3% at diagnosis and 52.2% at relapse/disease progression (P < .001). In 12 MM patients with paired samples at diagnosis and relapse/progression, MIR34B/C methylation was acquired in 6 at relapse/progression. In conclusion, MIR34B/C is a tumor suppressor in myeloma. Hypermethylation of MIR34B/C is tumor-specific. Frequent MIR34B/C hypermethylation during relapse/progression but not at diagnosis implicated a role of MIR34B/C hypermethylation in myeloma relapse/progression.

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Introduction
DNA methylation involves the addition of a methyl group to the number 5 carbon of the cytosine ring in the CpG dinucleotide. Global DNA hypomethylation, but transcriptional silencing of tumor suppressor genes by hypermethylation of promoter-associated CpG islands, is a hallmark of cancers. In multiple myeloma (MM), the pathogenic role of DNA hypermethylation has been illustrated in multiple tumor suppressor genes. MicroRNA (miRNA) is a class of short, single-stranded, noncoding RNAs, which inhibits the expression of targeted protein. In carcinogenesis, tumor suppressor (TS) miRNAs can be inactivated by promoter hypermethylation.

TP53 mutation is pivotal in epithelial cancer carcinogenesis but present in only 5% in MM. With such rare TP53 mutation, failure of TP53-mediated apoptosis in MM might be mediated by loss of function of genes that are upstream or downstream to TP53. Along this theme, study of DAPK1, CDKN2A, and APAF1 methylation showed frequent DAPK1 methylation in MM at diagnosis. Alternatively, inactivation of TP53 direct transcriptional targets, the MIR34 family miRNAs, might lead to failure of TP53-mediated apoptosis. In this connection, MIR34A methylation has been studied in a panel of hematologic malignancies, which showed infrequent MIR34A hypermethylation in MM. As MIR34B/C cluster is also a direct transcriptional target of TP53, MIR34B/C methylation might be important in MM.

Herein, we study MIR34B/C methylation in cell lines and primary bone marrow samples at diagnosis and relapse/progression of MM. Moreover, the TS function of MIR34B/C is demonstrated.

Methods
Patients and samples
Bone marrow samples were obtained from 95 myeloma patients at diagnosis and 23 patients at relapse/progression. Of the 23 relapsed (N = 5)/progressed (N = 18) myeloma patients, 12 had paired samples at both diagnosis and relapse/progression. The diagnosis of MM was based on standard criteria. Patient demographics are described in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Of the 23 relapsed/progressed myeloma patients, 5 relapsed from complete remission, and 18 progressed from plateau phase/stable disease. Definitions of relapse and disease progression followed the criteria of the European Group for Blood and Marrow Transplantation Registry. Briefly, “relapse” from complete remission was defined as the reappearance of the same paraprotein detected by serum/urine protein electrophoresis, appearance of new bone lesion or extramedul- lary plasmacytoma, or unexplained hypercalcemia. The definition of “disease progression” from plateau phase/stable disease was the same as the definition of relapse except that “> 25% increase in paraprotein level” replaced “reappearance of the same paraprotein.” The study has been approved by the Institutional Review Board of Queen Mary Hospital with informed consent in accordance with the Declaration of Helsinki.

MSP
DNA extraction from marrow buffy coat and bisulfite conversion were performed using QIAamp DNA Blood Mini Kit and EpiTect Bisulfite Kit (QIAGEN), respectively, as described. Methylation-specific PCR (MSP) for aberrant miRNA methylation was performed as described. Primers and conditions are as follows: Methylated-MSP forward, 5'-ATTCGTTTCGTTTCGCGTTCGTTTC-3'; reverse, 5'-TTTTTATTTGTTTTGTTTTGTGTTTGTTTT-3'; Unmethylated-MSP forward, 5'-TTTTATTTTGGTTTTGTTTTTGTTTGTGGT-3'; and reverse, 5'-CAACTACAATCCAAACAAATCC-3' (2mM/60°C/38×). Sensitivity of the M-MSP was 10⁻³ (supplemental Figure 1).

5-azadC treatment
Each of KMS-12-PE and LP-1 was cultured at 1 x 10⁶ cells/mL with 5-aza-2’-deoxycytidine (5-azadC, 1.5µM) for 7 days.
Quantification of MIR34B

Total RNA isolation, RT, and quantitative real-time PCR of MIR34B were respectively performed using mirVana miRNA Isolation Kit, TaqMan MicroRNA RT Kit, and TaqMan MicroRNA Assay Kit (ABI) as described.15 SNORD48 was selected as reference using 2⁻¹⁸H11002/H9004/H9004 Ct method.18

Overexpression studies of MIR34B

Precursor MIR34B (100nM; Ambion) was transfected into 1/H11003 10⁶ KMS-12-PE cells using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics), as described.15 Nontargeting miRNA precursor was used negative control. Cells were harvested at 48 hours for MTT, trypan blue, and cell cycle analyses (supplemental Methods).

Statistical analysis

Frequencies of MIR34B/C methylation in primary samples at diagnosis and at relapse were compared by χ² test. Experimental results of continuous variables after MIR34B transfection were compared by Student t test. All P values were 2-sided.

Results and discussion

MSP showed completely unmethylated MIR34B/C amplification in the 8 normal marrow controls but solely methylated amplification in the methylated positive control, testifying that MIR34B/C hypermethylation was tumor-specific (Figure 1A). On the 8 myeloma cell lines (Figure 1B), 6 (75%) carried MIR34B/C methylation, with KMS-12-PE, LP-1, and NCI-H929 being homozygously methylated, MOLP-8, OPM-2, and WL-2 hemizygously methylated, and RPMI-8226 and U-266 completely unmethylated.

Furthermore, in these cell lines, MIR34B/C methylation had a trend of lower level of MIR34B expression (supplemental Figure 2). Moreover, 5-azadC treatment of KMS-12-PE and LP-1 cells, both being homozygously methylated for MIR34B/C, led to demethylation of MIR34B/C promoter as evidenced by the emergence of the U-MSP signal, with reexpression of mature MIR34B as shown by TaqMan stem-loop quantitative RT-PCR (Figure 2A). Therefore, MIR34B/C methylation is frequent in myeloma cell lines leading to reversible miRNA silencing.

Moreover, if MIR34B/C is a TS in MM, restoration of MIR34B/C expression in myeloma cells is expected to inhibit cellular proliferation and/or induce cellular apoptosis. On transfection of precursor MIR34B into KMS-12-PE, in which MIR34B was homozygously methylated, restoration of mature MIR34B expression, as indicated by TaqMan stem-loop quantitative RT-PCR (Figure 2Bi), was associated with a 15% decrease of cellular proliferation by MTT assay (P = .05, Figure 2Bii), a 12.5% increase of dead cells by trypan blue (P = .02, Figure 2Biii), and a 10% increase of cells in sub-G₁ phase by propidium iodide staining (P = .02, Figure 2Biv), compared with transfection of negative control. Therefore, the results showed that MIR34B reexpression led to inhibition of cellular proliferation and enhancement of apoptosis in myeloma cells, thereby confirming MIR34B as a TS in myeloma.

In primary myeloma samples, methylation of MIR34B/C was found in only 5 of 95 (5.3%) MM samples at diagnosis but in 12 of 23 (52.2%) MM samples at relapse/progression (P < .001). Of these 23 relapse/progression samples, 12 patients had paired samples at both diagnosis and relapse/progression, with 1 patient showing MIR34B/C methylation at both diagnosis and relapse. Interestingly, in the remaining 11 patients with unmethylated...
MIR34B/C at diagnosis, 6 (54.5%) acquired MIR34B/C methylation at the time of relapse/progression. Moreover, direct sequencing of the M-MSP products from a methylated primary sample at relapse showed the expected nucleotide changes after bisulfite treatment, thereby confirming complete bisulfite conversion and authenticity of methylation (Figure 1E). Therefore, despite rare methylation in myeloma at diagnosis, MIR34B/C methylation is frequently acquired at relapse/progression and, hence, a useful marker of relapse/disease progression. This is further illustrated by the much more frequent MIR34B/C methylation at relapse/progression in the paired marrow samples.

Furthermore, using “miR-34b” and “methylated” as key words in a PubMed search, although MIR34B/C methylation has been shown to be associated with advanced stage of cancer or higher risk of recurrence,7,19,20 our finding of acquisition of MIR34B/C methylation at relapse/progression of myeloma is probably the first report supporting MIR34B/C methylation as a marker of relapse/disease progression of MM. This is further illustrated by the much more frequent MIR34B/C methylation at relapse/progression in the paired marrow samples.

In conclusion, MIR34B/C is a TS in myeloma. Hypermethylation of MIR34B/C is tumor-specific with reversible miRNA silencing. Moreover, frequent MIR34B/C hypermethylation in MM patients at relapse but not diagnosis implicated a role of MIR34B/C hypermethylation in disease progression of MM.

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Authorship
Contribution: C.S.C. designed the study; K.Y.W. and R.L.H.Y. conducted the experiments; C.S.C. and C.C.S. collected samples and retrieved clinical data; C.S.C., K.Y.W. and R.L.H.Y. analyzed the data; D.-Y.J. and R.L. supervised conduction of experiments; and all authors were involved in the writing and final approval of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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