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Epigenetic silencing of LPP/miR-28 in multiple myeloma

Zhenhai Li,[1] Kwan Yeung Wong,[1] Godfrey Chi-fung Chan,[2] Chor Sang Chim[1]

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[1]Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong, China
[2]Department of Pediatrics and Adolescent Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong, China

Correspondence to
Professor Chor Sang Chim, Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong; jcschim@hku.hk

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ABSTRACT

Aims miR-28-5- is a tumour suppressor microRNA implicated in cancers. As a CpG island is absent in miR-28-5- but present in its host gene, LPP (LIM domain containing preferred translocation partner in lipoma), we hypothesized that miR-28-5p is epigenetically silenced by promoter DNA methylation of its host gene in multiple myeloma.

Methods Methylation-specific PCR, verified by quantitative bisulfite pyrosequencing, was employed to study methylation of LPP/miR-28 in healthy controls (n=10), human myeloma cell lines (HMCLs) (n=15), and primary myeloma marrow samples at diagnosis (n=49) and at relapse (n=18). Quantitative reverse transcription PCR was used to investigate expression of miR-28-5p, LPP and CCND1.

Results LPP/miR-28 was completely unmethylated in all healthy controls and 12 (80%) HMCLs, but partially methylated in three (20%) HMCLs. Methylation of LPP/miR-28 correlated with low expression of miR-285p (p=0.012) and LPP (p=0.037) in HMCLs. In RPMI-8226R cells, in which LPP/miR-28 was partially methylated, 5-AzadC treatment led to demethylation of LPP/miR-28 and re-expression of both miR-28-5p (p=0.0007) and LPP (p=0.0007), whereas continuous culture without 5-AzadC restored LPP/miR-28 methylation and reduced expression of both miR-28-5p (p=0.0013) and LPP (p=0.0025). Moreover, a known miR-28-5p target, CCND1, was expressed at higher levels in HMCLs with LPP/miR-28 methylation than those without, consistent with a tumour suppressor role of miR-28-5p in myeloma. However, in primary samples, LPP/miR-28 was methylated in two (4.1%) at diagnosis, whereas none at relapse.

Conclusions This is the first report of epigenetic regulation of the intronic miR-28-5p expression by promoter DNA methylation of its host gene, hence warrants further study in different cancers.

INTRODUCTION

Multiple myeloma is an incurable haematological malignancy characterized by uncontrolled clonal proliferation of transformed plasma cells in the bone marrow.[1] Clinically, it evolves from a premalignant condition, monoclonal gammopathy of undetermined significance (MGUS),[2] into symptomatic myeloma at a rate of 1% per year,[3] whereby an expanding tumour load of myeloma plasma cells in the bone marrow results in end-organ damages, including lytic bone lesions, anaemia, hypercalcaemia and impaired renal function.[4] Genetically, overexpression of cyclin D1, D2 and/or D3, with either hyperdiploid or non-hyperdiploid karyotypes, is the hallmark of myeloma.[4 5] microRNAs (miRNAs) are endogenous RNAs of ~22 nt that repress expression of target protein-coding genes by binding to the corresponding seed region binding sites in the 3'-UTR, resulting in degradation or translational block of target mRNAs.[6] Altered expression of miRNAs, particularly oncomiRs and tumour suppressor miRNAs, has been shown in human malignancies.[7] Moreover, downregulation of multiple tumour suppressor miRNAs in myeloma has been shown to be mediated by methylation of promoter-associated CpG island.[8 9]

DNA methylation refers to catalytic addition of a methyl (-CH3) group to the carbon 5 position of the cytosine ring in a CpG dinucleotide.[10] Carcinogenesis is characterized by global DNA hypomethylation together with locus-specific hypermethylation at the promoter-associated CpG island of tumour suppressor genes.[11] In cancer cells, methylation of the promoter-associated CpG island of a tumour suppressor protein-coding or non-coding gene is associated with heterochromatin configuration and therefore gene silencing.[12] Previous studies have shown methylation of the promoter-associated CpG island leading to silencing of tumour suppressor protein-coding genes,[13 14] miRNAs[15 16] and long non-coding RNAs.[17 18]

Recently, miR-28–5 p, embedded in the sixth intron of LPP (LIM domain containing preferred translocation partner in lipoma), was found to be a tumour suppressor downregulated in large B-cell lymphomas,[19] natural killer/T-cell lymphoma,[20] hepatocellular carcinoma[21] and colorectal cancer.[22] For example, overexpression of miR-28–5 p in colorectal cancer cells reduced cellular proliferation, migration and invasion in vitro and in vivo, which was accompanied by direct targeting of HOXB3 and inhibition of CCND1, which is important in transition from G1 to S phase in the cell cycle.[22] Interestingly, CCND1 is frequently overexpressed in myeloma.[23] Moreover, a promoter-associated CpG island is present at miR-28–5 p host gene, LPP. Herein, as intronic miRNAs can be coregulated by promoter of the host gene,[24 25] we hypothesized that miR-28–5 p may be a tumour suppressor miRNA silenced by promoter methylation through its host gene, LPP, in myeloma. Moreover, the expression of miR-28–5 p might inversely correlate to that of its target gene CCND1. To verify this hypothesis, we studied the methylation of LPP/miR-28 in healthy normal controls, myeloma cell lines and primary myeloma marrow samples at diagnosis and at relapse. The expression

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of CCND1 was also examined and correlated to miR-28–5 p in myeloma cell lines.

METHODS

Patient samples

Bone marrow samples were obtained from 49 patients with myeloma at diagnosis and 18 at relapse/progression. The diagnosis of myeloma was based on standard criteria of the International Myeloma Working Group.[26] Complete staging work-up included bone marrow examination, skeletal survey, serum and urine protein electrophoresis, and serum immunoglobulin levels. Among the 49 newly diagnosed myeloma cases, there were 21 women and 28 men, with a median age of 63 (33–88) years. Plasma cell percentage ranges from 10% to 95%, with mean and median of 44.24% and 42.0%, respectively. Apart from four patients with insufficient clinical data of International Staging System,[27] there were eight stage I, 21 stage II and 16 stage III cases. There were 15 IgA, 27 IgG, 2 IgD, 3 light chain and 2 non-secretary myeloma. According to the criteria of the European Group for Blood and Marrow Transplantation Myeloma Registry,[28] 'relapse' from complete remission (CR) was defined as the reappearance of the same paraprotein detected by serum/urine protein electrophoresis, appearance of new bone lesion or extramedullary plasmacytoma, or unexplained hypercalcaemia. The study has been approved by the Institutional Review Board of Queen Mary Hospital (UW 05–269 T/932).

Cell culture

Human myeloma cell lines (HMCLs) LP-1 and RPMI-8226 were kindly provided by Professor Robert Orlowski (Department of Lymphoma/Myeloma, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA). JJN-3, OCI-MY5 and RPMI-8226R were kindly provided by Professor Wee Joo Chng (Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore). WL-2 was kindly provided by Professor Andrew Zannettino (Myeloma Research Programme, The University of Adelaide, Australia). KMS-11/BTZ and OPM-2/BTZ were obtained from Kyowa Hakko Kirin (Tokyo, Japan). NCI-H929 was purchased from American Type Culture Collection (Manassas, Virginia, USA). KMS-12-PE, MOLP-8, OPM-2 and U-266 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). MMLAL[29] and MMKKF (unpublished) were established from the myelomatous pleural effusion of patients with myeloma. Cell cultures were maintained in RPMI-1640 medium (IMDM for LP-1, DMEM + IMDM for MMLAL), supplemented with 10% or 20% fetal bovine serum, 50 U/mL of penicillin and 50 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C. All cell culture reagents were purchased from Invitrogen (Carlsbad, California, USA).

Methylation-specific PCR (MSP)

Genomic DNA was isolated from peripheral buffy coat of 10 healthy donors, primary myeloma marrow samples at diagnosis or at relapse/progression, and 15 myeloma cell lines using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was treated with bisulfite for conversion of all unmethylated cytosines into uracils using EpiTect Bisulfite Kit (Qiagen), and served as templates for methylated-MSP (M-MSP) and unmethylated-MSP (U-MSP). MSP primer sequences and conditions are listed in table 1. Enzymatically methylated DNA (Chemicon, Temecula, California, USA) was used as positive control for M-MSP and negative control for U-MSP after bisulfite treatment.

Quantitative bisulfite pyrosequencing

Bisulfite-treated DNA was amplified with a pair of methylation-unbiased primers with a specific PCR product overlapping the MSP amplicon. Forward primer: 5'- TAT GGG GGA GGG GGA TTT A - 3'; reverse primer: 5' - biotin - CCT TCT CTC TAA ACC TCA ACT AC - 3'; condition: 2 mM $MgCl_2$/58°C/50X. The PCR product was purified and a stretch of seven consecutive CpG dinucleotides was pyrosequenced with sequencing primer: 5'- GGG GTT GGA GTT TTG - 3'.

Quantification of LPP, miR-28–5 p and CCND1

Total RNA was isolated using mirVana miRNA Isolation Kit (Ambion, Austin, Texas, USA). For LPP and CCND1, reverse transcription was performed using QuantiTect Reverse Transcription Kit (Qiagen), followed by quantitative reverse transcription PCR (qRT-PCR), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH)as endogenous control. qRT-PCR primer sequences and conditions of LPP and GAPDH are listed in table 1. TaqMan assay of CCND1 (Hs00765553_m1) and GAPDH (Hs02758991_g1) was also employed. For miR-28–5 p, reverse transcription was performed using TaqMan MicroRNA RT Kit (ABI), followed by qRT-PCR using TaqMan MicroRNA Assay Kit (ABI), with RNU48 as endogenous control. Expression levels of LPP, miR-28–5 p and CCND1 were calculated by ΔCT method. Correlation between LPP/miR-28 methylation and miR-28–5 p or LPP expression was analyzed by Student's t-test.

Table 1 Primer sequences and PCR reaction conditions

Primer set	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	$MgCl_2$/Tm/cycles	Reference
(1) Methylation-specific PCR (MSP)					
LPP/miR-28					
M-MSP	CGC GGT TTA ATT ACG GGG TGT AC	CCA AAA AAA TCC GAA CAA AAA ACG	119	1.5 mM/56°C/35X	—
U-MSP	TTG TGG TTT AAT TAT GGG GTG TAT	CAA AAA AAT CCA AAC AAA AAA CA	119	2.0 mM/58°C/38X	—
(2) Quantitative reverse transcription PCR					
LPP	GTG CAA TGT GTG TTC CAA GC	TGG CAT AAT AGG CTC CTT GC	211		40
GAPDH	ACC ACA GTC CAT GCC ATC ACT	TCC ACC ACC CTG TTG CTG TA	452		—

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSP, methylation-specific PCR; M-MSP, MSP for the methylated allele; Tm, annealing temperature; U-MSP, MSP for the unmethylated allele.

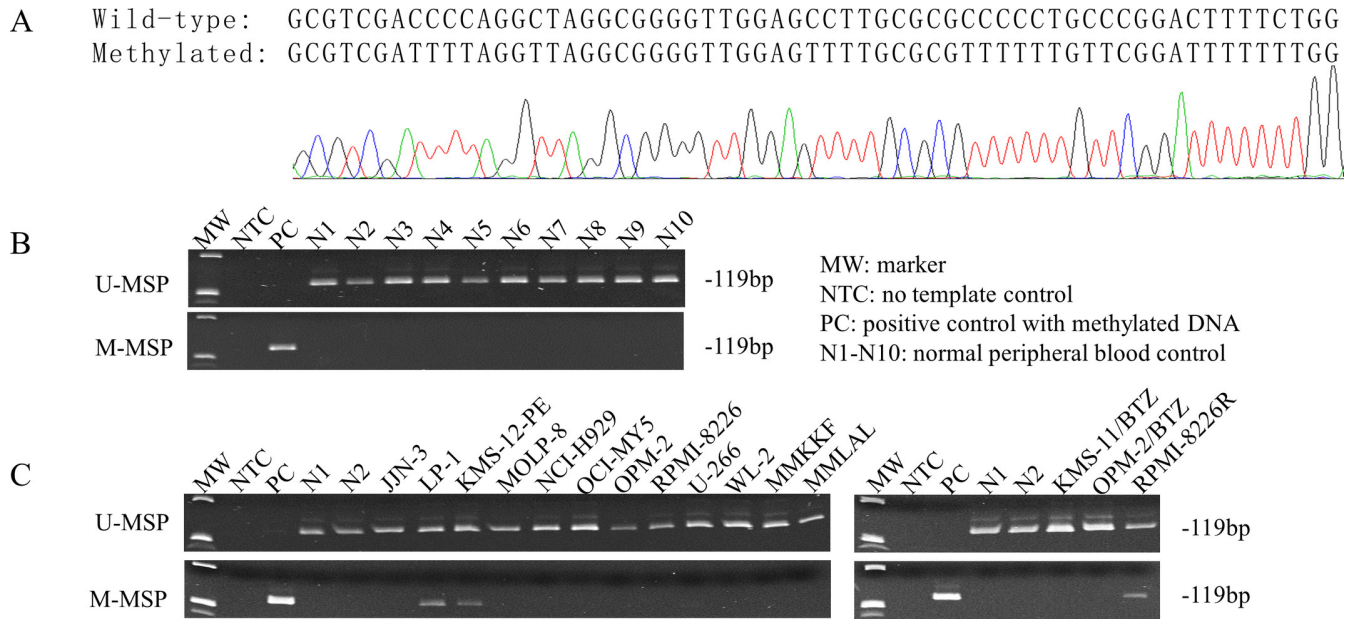

Figure 1 Methylation of LPP/miR-28 in healthy controls and human myeloma cell lines. (A) Sequencing analysis of M-MSP products from a positive control of methylated DNA showed the conversion of all unmethylated cytosines into uracils (turned into thymidines after PCR) but unchanged of all methylated cytosines, indicating complete bisulfite conversion and specificity of MSP. (B) M-MSP and U-MSP showed LPP/miR-28 was completely unmethylated in healthy controls, whereas completely methylated in positive control of methylated DNA. (C) M-MSP and U-MSP showed LPP/miR-28 was partially methylated (MU) in myeloma cell lines KMS-12-PE, LP-1 and RPMI-8226R, whereas completely unmethylated (UU) in JJN-3, MOLP-8, NCI-H929, OCI-MY5, OPM-2, RPMI-8226, U-266, WL-2, MMKKF, MMLAL, KMS-11/BTZ and OPM-2/BTZ. MSP, methylation-specific PCR; M-MSP, methylated-MSP; U-MSP, unmethylated-MSP.

p Values were two-sided and p<0.05 was defined as significant difference.

5-Aza-2′-deoxycytidine (5-AzadC) treatment

The partially methylated myeloma cell line, RPMI-8226R, was cultured in 25 cm^2 flasks at a density of 1×10^6 cells/mL. Cells were treated with fresh medium with 1 μmol/L 5-AzadC (Sigma-Aldrich, St Louis, Missouri, USA) in every 24 hours for 3 days and subsequently grew fresh medium without 5-AzadC for further 6 days. Cells were harvested for DNA and RNA extraction on days 3 and 9. Relative expression level of LPP and miR-28–5 p in 5-AzadC treated groupas compared to the corresponding untreated group were calculated by $2^{-\Delta\Delta CT}$ method. p Values were two-sided and p<0.05 was defined as significant difference.

RESULTS

Methylation of the putative LPP/miR-28 promoter in healthy controls andHMCLs

Methylation status of the putative LPP/miR-28 promoter was studied by MSP in healthy normal controls (n=10) and HMCLs (n=15). Complete bisulfite conversion and specificity of the MSP were confirmed by direct sequencing of the M-MSP products from a positive control of methylated DNA, which showed the conversion of all unmethylated cytosines into uracils (turned into thymidines after PCR), whereas all methylated cytosines remained as cytosines (figure 1A). None of the healthy controls showed methylation of LPP/miR-28 (figure 1B). Conversely, in myeloma cell lines, LPP/miR-28 was partially methylated (MU) in KMS-12-PE, LP-1 and RPMI-8226R, as evidenced by positive PCR amplification in both M-MSP and U-MSP. By contrast, JJN-3, MOLP-8, NCI-H929, OCI-MY5, OPM-2, RPMI-8226, U-266, WL-2, MMKKF, MMLAL, KMS-11/BTZ and OPM-2/BTZ were completely unmethylated (UU) (figure 1C). Furthermore, methylation status of the cell lines shown by MSP was verified by quantitative bisulfite pyrosequencing, which showed a methylation level ranging from 13.3% to 48.4% in partially methylated cell lines, and <10% in completely unmethylated cell lines (online supplementary figure 1). Together, LPP/miR-28 was methylated in a myeloma-specific manner.

Methylation and expression of LPP/miR-28 in myeloma cell lines

In myeloma cell lines, methylation status of LPP/miR-28 was correlated with expression of LPP and miR-28–5 p, as detected by qRT-PCR. Of the 15 myeloma cell lines, LPP/miR-28 methylated cell lines (n=3) had significantly lower expression of both LPP (p=0.037; figure 2A,B; online supplementary figure 3A) and its intronic miR-28–5 p (p=0.012; figure 2C; onlinesupplementary figure 3B) than the unmethylated cell lines (n=12).

Moreover, RPMI-8226R cells, which were partially methylated for LPP/miR-28, were treated with a demethylation agent, 5-AzadC, followed by pyrosequencing and qRT-PCR. Results showed that treatment with 5-AzadC led to demethylation and re-expression of LPP/miR-28 on day 3, as indicated by decrease of the mean methylation percentage of a stretch of seven consecutive CpG dinucleotides from 46.7% to 26.7% after 5-AzadC treatment (online supplementary figure 2), and concomitant re-expression of both LPP and miR-28–5 p by 6.8-fold and 6.0-fold, respectively, as compared with the untreated control (figure 2D). On the other hand, when 5-AzadC-treated cells were further cultured in fresh medium without 5-AzadC, LPP/miR-28 was remethylated with a mean methylation level of 41.9%, and LPP and miR-28–5 p were repressed to 2-fold and 1.6-fold, respectively, compared with the untreated group (LPP:

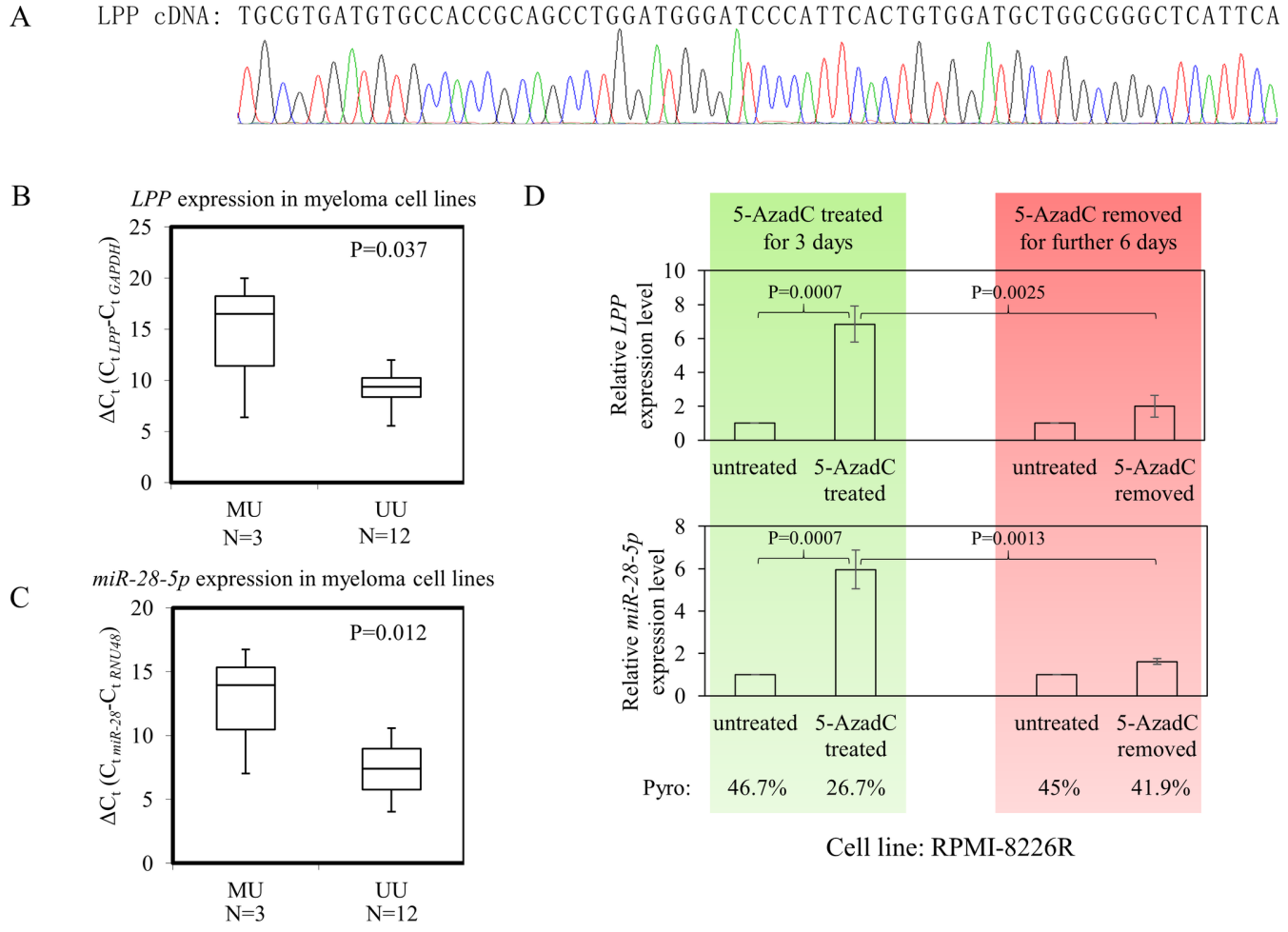

Figure 2 Methylation and expression of LPP/miR-28 in myeloma cell lines. (A) Sequencing analysis of quantitative reverse transcription PCR (qRT-PCR) product confirmed the specificity of LPP. (B,**C**) qRT-PCR analysis showed the expression of LPP (B, p=0.037) and miR-28–5 p (C, p=0.012) was significantly lower, as indicated by larger ΔCt, in methylated myeloma cell lines than the unmethylated cell lines. (D) The partially methylated myeloma cell line, RPMI-8226R, was treated with 1 µmol/L of demethylation agent, 5-AzadC for 3 days, after which 5-AzadC-treated cells were further cultured continuously in fresh medium without 5-AzadC for additional 6 days. On day 3, on treatment of 5-AzadC, quantitative bisulfite pyrosequencing showed decreased LPP/miR-28 methylation percentage with concomitant LPP and miR-28–5 p re-expression measured by qRT-PCR. On day 9, on removal of 5-AzadC for 6 days, methylation and reduced expression of LPP/miR-28 were restored.

p=0.0025, miR-28–5 p: p=0.0013) on day 9 (figure 2D). Therefore, these data suggested that the expression of LPP/miR-28 was negatively regulated by promoter DNA methylation.

Expression of LPP/miR-28 and CCND1 in myeloma cell lines

As the tumour suppressor function of miR-28–5 p was demonstrated by downregulation of CCND1,[22] which is commonly overexpressed in myeloma, the expression of CCND1 was also examined and correlated with methylation and expression of LPP/miR-28 in myeloma cell lines. By qRT-PCR, the expression of CCND1 was found higher in myeloma cell lines associated with methylated and low expression of miR-28–5 p than those associated with unmethylated and high expression of miR-28–5 p, indicative of an inverse relationship between the expression of miR-28–5 p and CCND1 in myeloma cell lines (figure 3; online supplementary figure 4).

Methylation of LPP/miR-28 in primary myeloma samples at diagnosis and at relapse

Methylation of LPP/miR-28 was studied in both primary myeloma samples at diagnosis (n=49) and at relapse (n=18) by MSP. Results showed that there were only two (4.1%) cases at diagnosis that had methylation of LPP/miR-28 and none at relapse (figure 4; online supplementary figure 5).

DISCUSSION

There were several observations in this study. First, our data demonstrated methylation-mediated silencing of LPP/miR-28 in myeloma cells, but not in healthy controls, and hence tumour-specific. By contrast, some genes have been shown to be hypermethylated in both tumour and normal controls, and hence tissue-specific but not tumour-specific methylation (eg, miR-517c,[30] miR-373[31] and miR-9–2[32]). Moreover, the methylation-mediated silencing of LPP/miR-28 was reversible as evidenced by the restoration of LPP/miR-28 expression on 5-AzadC demethylating treatment, and conversely LPP/miR-28 repression with promoter remethylation on removal of 5-AzadC. Hence, these data were consistent with previous studies showing that tumour-suppressive miRNAs may be silenced by promoter DNA methylation in a tumour-specific and reversible manners.[15 16 33 34]

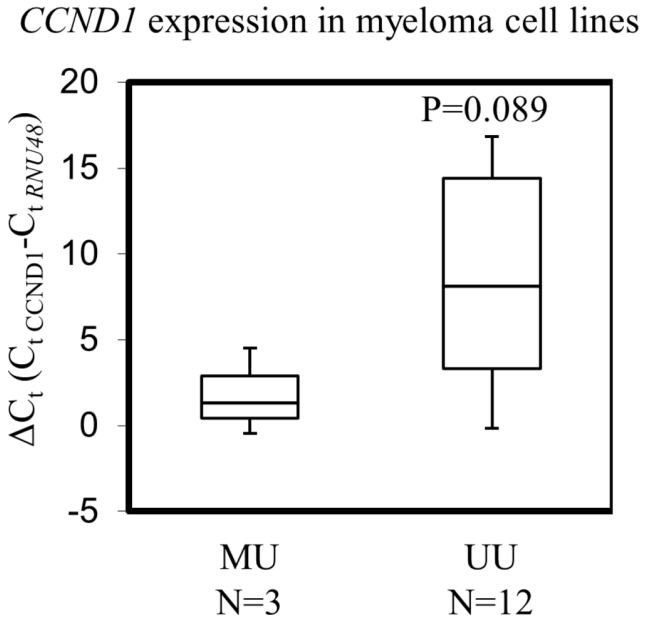

Figure 3 Expression of CCND1 in myeloma cell lines. Quantitative reverse transcription PCR analysis showed the expression of CCND1 was found relatively higher (p=0.089) in myeloma cell lines associated with methylated and lower expression of miR-28–5 p than those associated with unmethylated and higher expression of miR-28–5 p. MU, partially methylated; UU, completely unmethylated.

Second, the current study reported for the first time that expression of both LPP and its intronic miR-28–5 p was regulated by DNA methylation of LPP, consistent with previous reports of regulation of intronic miRNAs by promoter DNA methylation of its protein-coding host gene. For example, intronic miR-126 was found epigenetically silenced by promoter DNA methylation of its host gene EGFL7 (EGF-like domain, multiple 7) in malignant pleural mesothelioma.[35] While miR-28–5 p has also been reported to be coexpressed with its host gene, LPP, in B-cell lymphomas[36] and myeloproliferative neoplasms,[37] our study revealed for the first time that coexpression of both miR-28–5 p and LPP is under epigenetic regulation via promoter DNA methylation of LPP. Interestingly, the expression of miR-28 in myeloma as compared with normal plasma cells remains controversial. For instance, by miRNA profiling in CD138-sorted plasma cells of normal, MGUS and myeloma at diagnosis, Chi *et al*[38] demonstrated that the expression of miR-28 was upregulated in myeloma as compared with normal plasma cells. By contrast, based on a similar miRNA profiling by Pichiorri *et al*, miR-28 was not found to be differentially expressed between myeloma and normal plasma cells.[39] Therefore, the role of LPP/miR-28 methylation and expression in disease progression of myeloma warrants further study in an expanded cohort with RNA extracted from CD138-sorted plasma cells.

Finally, bioinformatic analysis indicated CCND1 as a potential target of miR-28–5 p based on complementarity of the 3' UTR nucleotide sequence of CCND1 with seed region sequence of miR-28–5 p, which was indeed confirmed by previous luciferase assay.[22] Therefore, it is possible that the frequent overexpression of CCND1 in myeloma might be mediated by DNA methylation and hence silencing of miR-28–5 p. Therefore, we checked if CCND1 expression in HMCLs correlated with LPP/miR-28 methylation. Indeed, in myeloma cell lines, an inverse correlation of CCND1 expression with LPP/miR-28 methylation was shown, consistent with the notion that LPP methylation leads

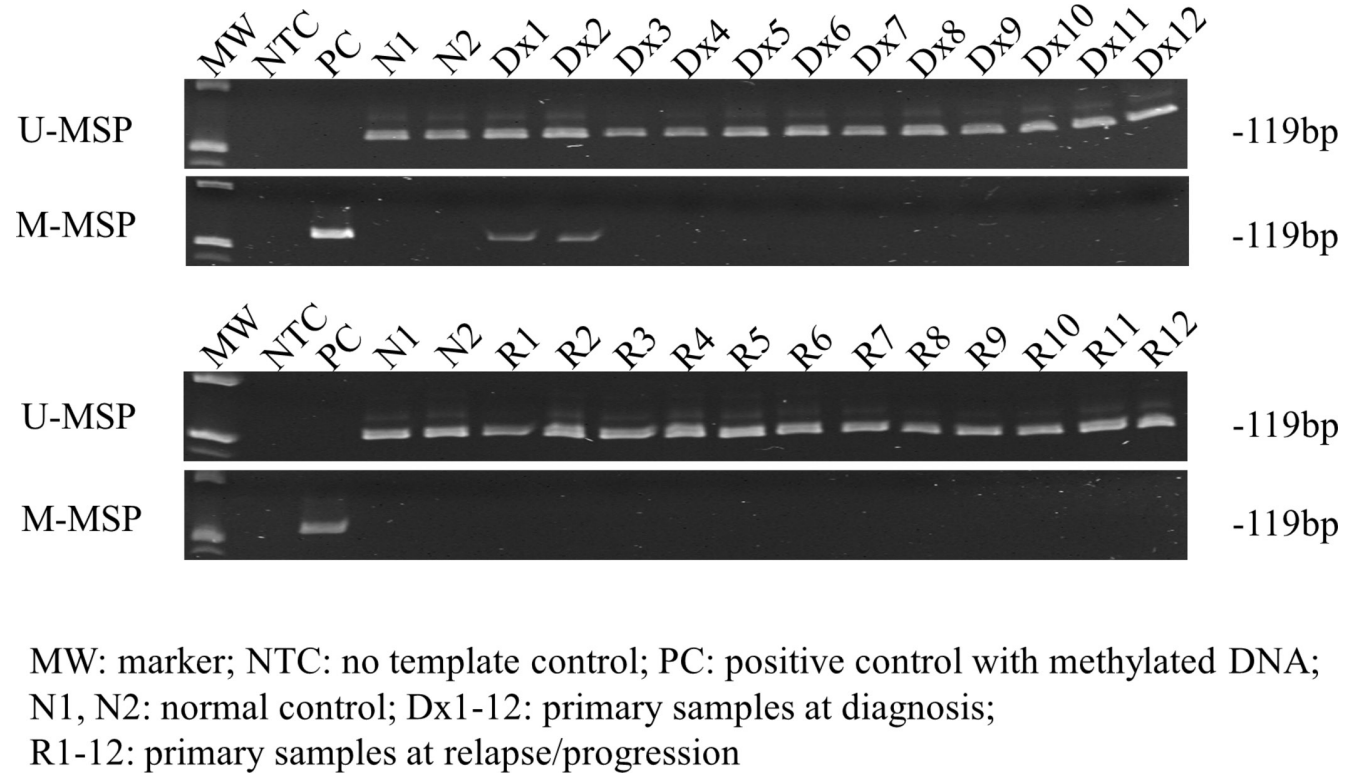

Figure 4 Methylation of LPP/miR-28 in myeloma bone marrow samples at diagnosis (Dx) and at relapse/progression (R). MSP showed LPP/miR-28 was methylated in two myeloma cases at diagnosis and none at relapse. MSP, methylation-specific PCR; M-MSP, methylated-MSP; U-MSP, unmethylated-MSP.

to miR-28–5 p silencing and consequently CCND1 overexpression. Therefore, methylation of miR-28–5 p might be one of the mechanisms contributing to CCND1 overexpression.

In conclusion, methylation of LPP/miR-28 was tumour-specific, reversible and inversely correlated with expression of miR-28–5 p and its host gene, LPP, in myeloma, testifying silencing of an intronic miRNA by DNA methylation of its host gene. Moreover, methylation-mediated silencing of miR-28–5 p was associated with upregulation of its target protein-coding gene, CCND1, in myeloma cell lines, hence likely a tumour suppressor miRNA. As this is the first report of regulation of miR-28–5 p expression by promoter DNA methylation of the host gene, further study in different cancers is warranted.

Take home messages

- Methylation-mediated silencing of LPP/miR-28 in myeloma cells was tumour-specific.
- Intronic miR-28–5 p was epigenetically silenced by promoter DNA methylation of its host gene, LPP, in multiple myeloma.
- Methylation-mediated silencing of miR-28–5 p was associated with upregulation of its target protein-coding gene, CCND1, in myeloma cell lines, hence likely a tumour suppressor miRNA.

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Contributors ZL carried out the experiments. KYW, ZL and CSC drafted the manuscript. KYW and CSC conceived of the study. All authors participated in the design of the study. All authors read and approved the final manuscript.

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Ethics approval Institutional Review Board of Queen Mary Hospital.

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