Epigenetic inactivation of the *miR-34a* in hematological malignancies

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Abstract

miR-34a is a transcriptional target of p53, and implicated in carcinogenesis. We studied the role of *miR-34a* methylation in a panel of hematological malignancies including acute leukemia (AML, ALL), chronic leukemia (CLL and CML), multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL). The methylation status of *miR-34a* promoter was studied in 12 cell lines and 188 diagnostic samples by methylation-specific PCR. *miR-34a* promoter was unmethylated in normal controls but methylated in 75% lymphoma and 37% myeloma cell lines. Hypomethylating treatment led to re-expression of *pri-miR-34a* transcript in lymphoma cells with homozygous *miR-34a* methylation. In primary samples at diagnosis, *miR-34a* methylation was detected in 4% CLL, 5.5% MM samples, and 18.8% of NHL at diagnosis but none of ALL, AML and CML (p=0.011). In MM patients with paired samples, *miR-34a* methylation status remained unchanged at progression. Amongst lymphoid malignancies, *miR-34a* was preferentially methylated in NHL (p=0.018), in particular natural killer/T cell (NK/T cell) lymphoma. In conclusion, amongst hematological malignancies, *miR-34a* methylation is preferentially hypermethylated in NHL, in particular NK/T cell lymphoma, in a tumor-specific manner, therefore the role of *miR-34a* in lymphomagenesis warrants further study.

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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, by catalyzing the cytosine into methylcytosine through DNA methyltransferase.[1,2] Cancer cells are characterized by global DNA hypomethylation but gene-specific hypermethylation of promoter-associated CpG islands of tumor suppressor genes (TSGs), resulting in transcriptional repression, and hence serve as an alternative mechanism of gene inactivation. Based on a pathway-specific approach, multiple TSGs across pathways including cell cycle regulation, JAK/STAT signaling, WNT signaling, and DAP kinase-associated intrinsic tumor suppression have been shown to be inactivated by gene hypermethylation in leukemia, lymphoma and multiple myeloma.[3-11]

MicroRNA (miRNA or miR) is a single-stranded, non-coding RNA molecule of 22-25 nucleotides, which leads to downregulation of target protein expression.[12] miRNAs are involved in carcinogenesis. miRNAs can be either oncogenic (oncomir) when TSGs are targeted, or tumor suppressive (tumor suppressive miRNAs) when oncogenes are targeted.[12] Little is known about the role of hypermethylation of tumor suppressor miRNAs in haemic cancers.

p53 is an important tumor suppressor gene inactivated in the majority of cancers. However, inactivation of *p53* in haemic cancers is relatively uncommon, and present

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in only about 10%-20% of cases.[13] On the other hand, *miR-34a*, localized to 1p36, is a transcriptional target of p53, mediating cellular apoptosis. It was shown to be hypermethylated in breast, colon and lung cancers, and downregulate CDK6 translation by binding on the 3' untranslated region (3' UTR) of the *CDK6* mRNA, thereby demonstrating the tumor suppressor role of *miR-34a*.[14] Therefore, we postulated that while genetic inactivation of *p53* is uncommon in hematological malignancies, epigenetic inactivation of *miR-34a*, a direct transcriptional target of p53, may occur in hematological cancers.

In this study, we aimed to study the role of *miR-34a* methylation in a wide range of hematological malignancies including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL).

Materials and methods

Patient samples

Diagnostic bone marrow or tissue samples were obtained in 20 ALL, 20 AML, 11 CML in chronic phase, 50 CLL, 55 MM and 32 NHL patients. Diagnosis of leukemia and lymphoma were made according to the FAB Classification and WHO Classification of Tumors respectively.[15-18] Patient demographics were listed in Tables

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Deleted: ALL patients comprised 6 common ALL, 1 early B precursor, 10 precursor B ALL and 3 pre-T ALL. Of the 50 CLL patients,[21] there were 23 patients with limited stage (<stage II) and 27 with advanced stage (≥ stage II) disease with a median age of 65.5 years (range: 37-91 years). The median presenting lymphocyte count was $17x10^9$/L (range: 10-$236x10^9$/L). Of the 32 patients with NHL, there were 11 patients with peripheral T cell lymphoma (one anaplastic T-cell [ALCL], four angio-immunoblastic T-cell [AITL], six peripheral T-cell, not otherwise specified [PTCL, NOS]), nine with natural killer –cell (NK-cell) lymphoma (eight extranodal NK/T-cell lymphoma, nasal type, and one blastic NK-cell lymphoma), 12 patients with B-cell lymhoma (eight follicular: grade I to II, two monocytoid B-cell and two mantle cell lymphoma). The median age was 64 years (range: 32 – 86). Seventeen (53.2%) has Ann Arbor stage I/II disease, and 15 (46.9%) had stage III/IV disease. ¶
Of the 55 MM patients, 12 had bone marrow DNA both at diagnosis and progression for methylation study.

1 and 2. The study has been approved by IRB of Queen Mary Hospital with informed consent.

Methylation-specific polymerase chain reaction (MSP)

DNA was extracted from bone marrow samples of ALL, AML, CML, CLL and MM at diagnosis, diagnostic tissues (either lymph node or nasal biopsy in nasal NK-cell lymphoma) in patients with NHL, lymphoma cell lines (SU-DHL-1 (ALK+), SUP-M2 (ALK+), SUP-T1, and KARPAS-299 (ALK+)), and myeloma cell lines (KMS-12-PE, LP-1, MOLP-8, NCI-H929, OPM-2, RPMI-8226, U-266, and WL-2) by standard method. MSP for aberrant gene promoter methylation was performed as previously described.[3,8] Treatment of DNA with bisulphite for conversion of unmethylated cytosine to uracil (but unaffecting methylated cytosine) was performed with a commercially available kit (EpiTect Bisulphite Kit, Qiagen, Germany). Primers used for the methylated MSP (M-MSP) and unmethylated MSP (U-MSP) are shown in supplementary Table. MSP primers for *miR-34a* promoter was designed within a region of established promoter activity upstream the characterized complete sequence of primary *miR-34a* transcript.[19,20] DNA from normal bone marrow donors (N=5) was used as negative control, while enzymatically methylated control DNA (CpGenome Universal Methylated DNA, Chemicon) was used as positive control in all the experiments. MSP was performed in a thermal cycler (9700, Applied Biosystems,

Foster City, CA, USA) with the following cycling conditions: 95^{o}C for 5 minutes, 35 cycles of 95^{o}C for 30 seconds, specific annealing temperature for 30 seconds (supplementary Table), 72^{o}C for 30 seconds, and a final extension of 10 minutes at 72^{o}C. The MSP mixture contained 50 ng of bisulphite-treated DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 pmol of each primer, 1 X PCR buffer, and 2.5 units of AmpliTaq Gold DNA Polymerase (ABI, Foster City, CA, USA) in a final volume of 50 µl. Ten microliters of PCR products were loaded onto 6% non-denaturing polyacrylamide gels, electrophoresed, and visualized under ultraviolet light after staining with ethidium bromide.

Cell lines and culture

LP-1 and RPMI-8226 were kind gifts from Dr Orlowski (Department of Hematology/Oncology, MD Anderson Cancer Center, USA). WL-2 was kindly provided by Dr Andrew Zannettino (Myeloma and Mesenchymal Research Laboratory, Division of Haematology, Institute of Medical and Veterinary Science, Adelaide, Australia). Other lymphoma (SU-DHL-1, SUP-M2, SUP-T1, and KARPAS-299) and multiple myeloma (KMS-12-PE, MOLP-8, OPM-2, and U-266) cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) (Braunschweig, Germany). NCI-H929 was purchased from American Type Culture Collection (ATCC). Cell cultures were maintained in RPMI

media 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37°C.

Hypomethylating treatment of cell lines with homozygous *miR-34a* methylation

Lymphoma (KARPAS-299) cells were seeded in six-well plates at a density of $1x10^6$ cells/ml, and cultured with 0.5-1 μM of 5-Aza-2'-deoxycytidine (5-AzadC) (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. Cells on day 0 and day 3 of cell treatment were harvested.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated and reverse transcribed using *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) and SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) respectively, according to the manufacturers' instructions. RT-PCR primers for the *pri-miR-34a* and *p53* transcript, and PCR conditions were summarized in supplementary Table.

Statistical analysis

Correlation between *miR-34a* methylation status with type of haemic malignancy was computed by the Chi-square test (or Fisher Exact test). All p-values were two-sided.

Results

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ChIP-PCR. Chromatin immunoprecipitation (ChIP) assays were carried out following the manufacturer's manual (Upstate Biotechnology, Cat# 17-610). Briefly, $2x10^6$ cells were used per ChIP. Cells were treated with 1% formaldehyde for 10 min. After washing by cold PBS, cells were resuspended in lysis buffer and sonicated on ice using 431A cup horn (Misonix, Farmingdale, NY, USA). Chromatin was sheared into fragments ranging between 200 and 800 bp in size. After removing 1% as 'Input', immunoprecipitation was performed by adding anti-H3K4me3 (Upstate, Cat# 04-745), anti-H3K9me3 (Upstate, 17-625), anti-H3K9ac (Upstate, 17-658), anti-H3K27me3 (Upstate, 17-622) and normal rabbit IgG respectively and incubating at 4°C overnight. To collect the immunoprecipitated complexes, magnetic protein A beads were added. After washing, the beads were treated with proteinase K and crosslinks were reversed by heating. Then DNA was extracted and used in confirmation PCR with the oligonucleotide primers

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MSP

Controls. Sequencing of the M-MSP products from the positive control showed the expected nucleotide changes after bisulphite treatment, therefore confirming complete bisulphite conversion and specificity of MSP (Figure 1A). None of the five normal control marrows showed aberrant methylation of *miR-34a* (Figure 1B). The positive and negative controls showed the expected MSP results (normal DNA: U-MSP positive / M-MSP negative; methylated DNA: U-MSP negative / M-MSP positive).

Lymphoma cell lines. The profile of methylation of *miR-34a* in lymphoma cell lines was shown in Figure 1C. SUP-T1 and KARPAS-299 (ALK+) were homozygously methylated for *miR-34a*; whereas SU-DHL-1 (ALK+) was hemizygously methylated for *miR-34a*; and only SUP-M2 (ALK+) was completely unmethylated for *miR-34a*.

Myeloma cell lines. The profile of methylation of *miR-34a* in myeloma cell lines was shown in Figure 1D. KMS-12-PE, OPM-2, and WL-2 were homozygously methylated for *miR-34a*; whereas LP-1, MOLP-8, NCI-H929, RPMI-8226, and U-266 were completely unmethylated for *miR-34a*.

Primary samples. *miR-34a* was hypermethylated in none of CML, ALL and AML in addition to 2 (4%) CLL, 3 (5.5%) MM and 6 (18.8%) NHL samples. (Figure 2) Amongst the lymphoid malignancies, there was significantly more frequent *miR-34a* methylation in NHL than MM, CLL or ALL (p=0.027). In lymphoma samples,

miR-34a was more frequently methylated in NK/T cell (N=4; 44.4%) than B-cell (N=2; 16.7%) or PTCL, NOS (N=0) (p=0.039). (Table 2) In the 12 MM patients with bone marrow samples at both diagnosis and progression including two with *miR-34a* methylation at diagnosis, the methylation status of *miR-34a* did not change at disease progression (data not shown).

5-AzadC treatment of KARPAS-299 cells

Untreated KARPAS-299 cells were completely methylated for *miR-34a*. Upon 5-AzadC demethylation treatment, *miR-34a* U-MSP signal emerged on day 3 (Figure 3A), with re-expression of *pri-miR-34a* as shown by semi-quantitative RT-PCR (Figure 3B). *p53*, the transcriptional activator of *pri-miR-34a*, was constitutively expressed before and after 5-AzadC treatment. (Figure 3B)

Discussion

Methylation studies require precise identification of gene promoter region and CpG island for proper MSP primer design. The complete sequence of primary *miR-34a* transcript has previously been described by other groups using rapid amplification of cDNA ends (RACE),[19,20] and published to GenBank (accession: EF592573).[19] The promoter region and transcription start site (TSS) of *miR-34a* is therefore mapped to > 30 kb upstream of mature *miR-34a*. The promoter activity of which under the control of p53, the transcriptional activator of *miR-34a*, has been

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5-AzadC and VPA treatment of KMS-12-PE cells ¶

Untreated KMS-12-PE cells were completely methylated for *miR-34a*. Upon 5-AzadC demethylation treatment, *miR-34a* U-MSP signal emerged on day 3. (Figure 3A) However, *pri-miR-34a* transcript was not reexpressed by 5-AzadC alone or a combination of 5-AzadC and VPA. (Figure 3B) NCI-H929, which was unmethylated for *miR-34a* with constitutive expression of *pri-miR-34a*, served as positive control for *pri-miR-34a* expression. *p53*, constitutively expressed in NCI-H929, was also expressed in KMS cells both before and after treatment of 5-AzadC +/- VPA. (Figure 3B) Moreover, PCR on DNA fragment pulled down from chromatin immunoprecipitation (i.e. ChIP-PCR) in untreated KMS-12-PE cells showed relative abundance of trimethyl H3K9 and trimethyl H3K27 in *Alu* repeat element and the homozygously methylated *miR-34a* promoter but not *GAPDH* promoter. (Figure 4B, rows 1-3) Upon 5-AzadC treatment, high level of trimethyl H3K9 and trimethyl H3K27 persisted in addition to persistent absence of trimethyl H3K4 and acetylated H3K9

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confirmed by luciferase reporter assay.[19] CpG island has been found in the above region. Hence, our MSP primer sets designed for this study were selected according to the above region, where > 30 kb upstream of mature *miR-34a*, with TSS, p53 binding site, proven promoter activity, and CpG island.

In this study, there are several interesting observations. First, we showed that *miR-34a* is not methylated in normal blood cells but is hypermethylated in myeloma and lymphoma cell lines, and hence it is tumor-specific and consistent with its tumor suppressor role.[19] By contrast, miRs such as *miR-127* and *miR-373* are hypermethylated in both normal and tumor cells, which will hence represent tissue-specific but not tumor-specific miR methylation.[21] Moreover, in KARPAS-299 cells, *miR-34a* hypermethylation was associated with gene silencing, which was reversed by hypomethylating treatment.

Secondly, in primary samples, *miR-34*a was preferentially methylated in lymphoma samples, in particular, extranodal NK/T cell lymphoma, nasal type. NK-cell lymphoma is an Epstein-Barr virus–associated, aggressive extranodal lymphoma more frequently encountered in Asia, and Central and South America.[22] Various tumor suppressor genes have been shown to be frequently hypermethylated in NK-cell lymphoma including *p73, CDKN2A, CDKN2B, hMLH1 and RARβ*,[23] but hypermethylation of *miR-34a* is likely the first report of methylation of miR in

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NK-cell lymphoma. On the other hand, *miR-34a* methylation is infrequent in other hematological malignancies, in contrast to solid cancer such as prostatic carcinoma, in which *miR-34a* methylation was detected in 79% of primary solid cancer samples.[14]

Thirdly, *p53* is an important independent prognostic factor in CLL, with a much inferior survival in patients with *p53* inactivation by either del(17p) or *p53* mutation.[24] However, unlike solid cancers, in which *p53* inactivation may occur in up to 60% of cases,[25] del(17p) and *p53* mutation only occur in ≤10% of CLL patients at diagnosis. However, other components of the *p53*-associated tumor suppression pathways have been shown to be inactivated by mutation or hypermethylation. For example, ATM, involved in the ATM-CHK2-P53 pathway, is inactivated by del(11q23) in about 20% of patients is another adverse prognostic factor for survival.[26] Moreover, death-associated protein kinase (DAPK), involved in the DAPK/p14/HDM2/p53/Apaf-1 apoptosis pathway, has been shown to be frequently hypermethylated in CLL.[27] Furthermore, recently, *miR-34b* and *miR-34c*, with similar function to *miR-34a*, has been shown to be hypermethylated in colon cancer.[28] These will be another interesting target of methylation to study.

In addition to hypermethylated promoter, *miR-34a* is localized to 1p36, which is frequent for loss of heterozygosity (LOH) in different kinds of cancers. LOH serves as one of the mechanisms for TSG inactivation in fulfilling the Kundson's two-hit

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hypothesis.[29] Loss of 1p heterozygosity is common in solid cancers such as breast, colon, lung, and neuroblastoma.[30-31] In hematological cancers, loss of 1p36 heterozygosity and the associated tumor suppressor genes is also common.[32-34] In particular, high frequency of promoter hypermethylation for *p73* or LOH of *p73* at 1p36 was found in NHL patients.[34] Hence, *miR-34a* inactivation might potentially result from promoter hypermethylation together with LOH at 1p36 in the samples, thereby fulfilling the Kundson's two-hit hypothesis for TSG inactivation.

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In summary, amongst hematological malignancies, *miR-34a* is preferentially hypermethylated in NHL, in particular NK-cell lymphoma, in a tumor-specific manner. *miR-34a* methylation is associated with reversible gene silencing, and the role of *miR-34a* in lymphomagenesis warrants further study.

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References

1. Chim CS, Liang R, Kwong YL. Hypermethylation of gene promoters in hematological neoplasia. Hematological Oncology. 2002;20(4):167-176.
2. Herman JG, Baylin SB. Gene Silencing in Cancer in Association with Promoter Hypermethylation. 2003. p. 2042-2054.
3. Chim CS, Liang R, Tam CYY, Kwong YL. Methylation of p15 and p16 Genes in Acute Promyelocytic Leukemia: Potential Diagnostic and Prognostic Significance. Journal of Clinical Oncology, 2001. p. 2033-2040.
4. Chim CS, Wong SY, Kwong YL. Aberrant gene promoter methylation in acute promyelocytic leukaemia: profile and prognostic significance. Br J Haematol. 2003; 122(4): 571-8.
5. Chim CS, Kwong YL, Liang R. Gene hypermethylation in multiple myeloma: lessons from a cancer pathway approach. Clinical lymphoma & myeloma. 2008 Dec;8(6):331-339.
6. Chim CS, Pang R, Fung TK, Choi CL, Liang R. Epigenetic dysregulation of Wnt signaling pathway in multiple myeloma. Leukemia. 2007;21(12):2527-2536.
7. Chim CS, Liang R, Fung TK, Choi CL, Kwong YL. Epigenetic dysregulation of the death-associated protein kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in multiple myeloma. J Clin Pathol. 2007 June 1, 2007;60(6):664-669.
8. Chim CS, Fung TK, Cheung WC, Liang R, Kwong YL. SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway. Blood. 2004 June 15, 2004;103(12):4630-4635.
9. Chim CS, Wong ASY, Kwong YL. Epigenetic dysregulation of the Jak/STAT pathway by frequent aberrant methylation of SHP1 but not SOCS1 in acute leukaemias. Annals of Hematology. 2004;83(8):527-532.
10. Chim CS, Fung TK, Liang R. Disruption of INK4//CDK//Rb cell cycle pathway by gene hypermethylation in multiple myeloma and MGUS. Leukemia. 2003;17(12):2533-2535.
11. Chim CS, Liang R, Leung MH, Yip SF, Kwong YL. Aberrant gene promoter methylation marking disease progression in multiple myeloma. Leukemia. 2006;20(6):1190-1192.
12. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857-866.
13. Peller S, Rotter V. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. Hum Mutat. 2003; 21(3): 277-84.
14. Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Körner H, Knyazev P, Diebold J, Hermeking H. Inactivation of miR-34a by aberrant CpG

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Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000; 403(6765): 41-5.¶

Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet. 2009; 10(5): 295-304.

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methylation in multiple types of cancer. Cell Cycle. 2008 Aug 15;7(16):2591-600.

15. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 1976; 33: 451-458.

16. Second MIC Cooperative Study Group..Morphologic, immunologic and cytogenetic (MIC) working classification of the acute myeloid leukaemias. Br J Haematol 1988; 68: 487-94.

17. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating MJ, Montserrat E, Rai KR, Kipps TJ; International Workshop on Chronic Lymphocytic Leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008;111(12):5446-56.

18. Swerdlow SH, Campo Eharris NL, Jaffe ES, Pileri SA, Stein H, Thile J, Vardiman JW. WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues. 4th Edition, International Agency for Research on Cancer, Lyon 2008.

19. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Molecular Cell 2007; 26(5): 745-752.

20. Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., *et al.* (2007). Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle*, *6*(13), 1586-1593.

21. Lujambio A, Esteller M. CpG Hypermethylation of Tumor Suppressor microRNAs in Human Cancer. Cell Cycle. 2007 June 15, 2007;6(12):1455-1459.

22. Chim CS, Ma SY, Au WY, Choy C, Lie AK, Liang R, Yau CC, Kwong YL. Primary nasal natural killer cell lymphoma: long-term treatment outcome and relationship with the International Prognostic Index. Blood. 2004; 103(1): 216-21.

23. Siu LL, Chan JK, Wong KF, Choy C, Kwong YL. Aberrant promoter CpG methylation as a molecular marker for disease monitoring in natural killer cell lymphomas. Br J Haematol. 2003 Jul;122(1):70-7.

24. Zenz T, Benner A, Döhner H, Stilgenbauer S. Chronic lymphocytic leukemia and treatment resistance in cancer: the role of the p53 pathway. Cell Cycle. 2008; 7(24): 3810-4.

Deleted: <#>Chim CS, Fung TK, Wong KF, Lau JS, Liang R. Frequent DAP kinase but not p14 or Apaf-1 hypermethylation in B-cell chronic lymphocytic leukemia. J Hum Genet. 2006;51(9):832-8.

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Kawamoto K, Hirata H, Kikuno N, Tanaka Y, Nakagawa M, Dahiya R. DNA methylation and histone modifications cause silencing of Wnt antagonist gene in human renal cell carcinoma cell lines. Int J Cancer. 2008 Aug 1;123(3):535-42.¶
Kondo Y, Issa JP. Enrichment for histone H3 lysine 9 methylation at Alu repeats in human cells. J Biol Chem. 2003 Jul 25;278(30):27658-62.¶

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<#>McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB. Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. Cancer Res. 2006; 66(7): 3541-9.¶

25. Soussi T, Dehouche K, Béroud C. p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. Hum Mutat. 2000;15(1):105-13.

26. Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, Döhner K, Bentz M, Lichter P. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000; 343(26):1910-6.

27. Chim CS, Fung TK, Wong KF, Lau JS, Liang R. Frequent DAP kinase but not p14 or Apaf-1 hypermethylation in B-cell chronic lymphocytic leukemia. J Hum Genet. 2006;51(9):832-8.

28. Toyota, M., Suzuki, H., Sasaki, Y., Maruyama, R., Imai, K., Shinomura, Y., *et al.* (2008). Epigenetic Silencing of MicroRNA-34b/c and B-Cell Translocation Gene 4 Is Associated with CpG Island Methylation in Colorectal Cancer. *Cancer Res, 68*(11), 4123-4132.

29. Knudson, A. G. (1997). Karnofsky Memorial Lecture. Hereditary cancer: theme and variations. *J Clin Oncol, 15*(10), 3280-3287.

30. Ragnarsson, G., Eiriksdottir, G., Johannsdottir, J. T., Jonasson, J. G., Egilsson, V., & Ingvarsson, S. (1999). Loss of heterozygosity at chromosome 1p in different solid human tumours: association with survival. Br J Cancer, 79(9-10), 1468-1474.

31. Caren, H., Fransson, S., Ejeskar, K., Kogner, P., & Martinsson, T. (2007). Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumours. Br J Cancer, 97(10), 1416-1424.

32. Mori, N., Morosetti, R., Spira, S., Lee, S., Ben-Yehuda, D., Schiller, G., et al. (1998). Chromosome Band 1p36 Contains a Putative Tumor Suppressor Gene Important in the Evolution of Chronic Myelocytic Leukemia. Blood, 92(9), 3405-3409.

33. Naoki, M., Roberta, M., Hideaki, M., & Koeffler, H. P. (2003). Progression of myelodysplastic syndrome: allelic loss on chromosomal arm 1p. British Journal of Haematology, 122(2), 226-230.

34. Beatriz, M.-D., Barbara, M., Marta, C., Maria Jose, G., Josep, N., Carmen, R., et al. (2002). Frequent inactivation of the <I>p73</I> gene by abnormal methylation or LOH in non-Hodgkin's lymphomas. International Journal of Cancer, 102(1), 15-19.

35. Candeias MM, Powell DJ, Roubalova E, Apcher S, Bourougaa K, Vojtesek B, et al. (2006). Expression of p53 and p53//47 are controlled by alternative mechanisms of messenger RNA translation initiation. Oncogene, *25*(52), 6936-6947.

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Figure 1. Methylation of *miR-34a*. (A) Schematic diagram showing the locations of p53 binding site (solid green box), and distribution of CpG dinucleotides (solid vertical lines) over transcription start site (TSS) of *pri-miR-34a* and precursor *miR-34a* (solid black box). Sequence analysis of the M-MSP product from bisulphite-treated positive control DNA showed that the cytosine [C] residues of CpG dinucleotides were methylated and remained unchanged, whereas all the other C residues were unmethylated and were converted to thymidine [T], confirming complete bisulphate conversion and specificity of MSP. (B) U-MSP showed that the methylated control [P] was totally methylated, and all five normal controls [N1-N5] were unmethylated. In the M-MSP, the methylated control was methylated but all normal controls were unmethylated. (C) For the lymphoma cell lines, SUP-T1 and KARPAS-299 were completely methylated for *miR-34a*. (D) For the myeloma cell lines, KMS-12-PE, OPM-2, and WL-2 were completely methylated for *miR-34a*; whereas LP-1, MOLP-8, NCI-H929, RPMI-8226, and U-266 were completely unmethylated for *miR-34a*.

Figure 2. Methylation of *miR-34a* in primary samples (B: reagent blank; S: primary sample; N: normal control; P: positive control with methylated DNA).

Figure 3. Effect of 5-Aza-2'-deoxycytidine (5-AzadC) treatment on KARPAS-299 lymphoma cells. (A) M-/U-MSP analysis of *miR-34a* promoter methylation status (P: positive control; N: normal bone marrow; D0: day 0; D3: day 3; B: reagent blank). (B) semi-quantitative RT-PCR analysis of the *p53* status and *pri-miR-34a* expression (MW: marker; B: reagent blank; D0: day 0; D3: day 3; NoRT: negative control without reverse transcriptase). Sequencing analysis of the semi-quantitative RT-PCR products for the detection of *p53* and *pri-miR-34a* expression was shown in the upper panel. 5-AzadC treatment resulted in progressive demethylation of *miR-34a* promoter, and re-expression of the *pri-miR-34a* in KARPAS-299 cells.

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Figure 4. Chromatin Immunoprecipitation (ChIP) assay of KMS-12-PE myeloma cells with 5-Aza-2'-deoxycytidine (5-AzadC) with or without valproic acid (VPA). A) Schematic diagram showing the locations of the ChIP primer sets (R1 and R2) used, p53 binding site (solid green box), and the distribution of CpG dinucleotides (solid vertical lines) over the transcription start site (TSS) of *pri-miR-34a* and the mature *miR-34a* (solid black box). B) Chromatin DNA was

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Table 1. Patient demographics

Type	
CLL (N=50)	
Gender (male/female)	40/10
Age (years)	
median	65.5 years
range	37-91 years
Rai stage	
≤ 2	23 (46%)
>2	27 (54%)
Lymphocyte count (range; median)	10-236 x 10^9/L (median: 17)
MM (N=55)	
Gender (male/female)	35/20
Age (years)	
median	57 years
range	25-87 years
Durie Salmon stage (I/II/III)	
I	10 (18.2%)
II	14 (25.5%)
III	31 (56.4%)
Ig type	
G	32 (58.2%)
A	13 (23.6%)
LC	10 (18.2%)
AML (N=20)	N=20
Gender (M/F)	9/11
Age (years)	
median	41.5
range	20-72
FAB type	
M1/M2/M4/M5	3/14/2/1
ALL (N=20)	N=20
Gender (M/F)	11/9
Age (years)	
median	35
range	13-62
MIC type	
C/PB/EPB/T	6/10/1/3
CML (N=11)	
Chronic phase	11
Gender (M/F)	7/4
Age (years)	
median	41
range	22-87

G: IgG type; A: IgA type; LC: light chain myeloma; M1: FAB M1; M2: FAB M2; M4: FAB M4; M5: FAB M5; MIC: morphologic-immunophenotypic-classification; C: common ALL; PB: precursor B ALL; EBP: early B precursor; T: pre-T ALL

Table 2. Non-Hodgkin's lymphoma patient demographics and *miR-34a* methylation result

Lineage	Type	N	M : F	Age (years) range (median)	Nodal/extranodal	*miR-34* methylated	*miR-34a* unmethylated
Mature T	AITL	4	3 : 1	56-74 (68)	all nodal	0	4
	ALCL	1	1 : 0	49 (49)	Nodal	0	1
	PTCL, NOS	6	5 : 1	48-75 (68)	3 nodal/3 extranodal (muscle/tonsil/maxillary mass)	0	6
	NK/T	9	5 : 4	48-74 (68)	3 nasal, 6 extranasal (3 soft tissue; 2 LN; 1 NK-cell leukemia)	4	5
Mature B	FL	8	6 : 2	50-77 (64)	all nodal	1	7
	MCL	2	2 : 0	56-77	Both nodal + BM	0	2
	NMZL	2	1 : 1	56-73 (64)	Both nodal + BM	1	1

AITL: angioimmunoblastic T-cell lymphoma; ALCL: anaplastic large-cell lymphoma; PTCL: peripheral T-cell lymphoma, not otherwise specified; FL: follicular lymphoma; MCL: mantle cell lymphoma; NMZL: Nodal marginal zone lymphoma; NK: Extranodal NK/T-cell lymphoma (nasal or extranasal type); BM: bone marrow; LN: lymph node

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ChIP-PCR. Chromatin immunoprecipitation (ChIP) assays were carried out following the manufacturer's manual (Upstate Biotechnology, Cat# 17-610). Briefly, $2x10^6$ cells were used per ChIP. Cells were treated with 1% formaldehyde for 10 min. After washing by cold PBS, cells were resuspended in lysis buffer and sonicated on ice using 431A cup horn (Misonix, Farmingdale, NY, USA). Chromatin was sheared into fragments ranging between 200 and 800 bp in size. After removing 1% as 'Input', immunoprecipitation was performed by adding anti-H3K4me3 (Upstate, Cat# 04-745), anti-H3K9me3 (Upstate, 17-625), anti-H3K9ac (Upstate, 17-658), anti-H3K27me3 (Upstate, 17-622) and normal rabbit IgG respectively and incubating at 4°C overnight. To collect the immunoprecipitated complexes, magnetic protein A beads were added. After washing, the beads were treated with proteinase K and crosslinks were reversed by heating. Then DNA was extracted and used in confirmation PCR with the oligonucleotide primers that were picked from *GAPDH* promoter[22] and *Alu* repeats.[23] (supplementary Table) For *mir-34a*, two sets of ChIP-PCR primers (supplementary Table) embedded in the promoter-associated CpG island were used, one is 540 bp upstream of *miR-34a* transcription start site (TSS), and the other overlap the p53 binding site which is downstream of *miR-34a* TSS.[24] (Figure 4) The PCR products were visualized by 6% polyacrylamide gel electrophoresis and the assays were done in duplicate.

5-AzadC and VPA treatment of KMS-12-PE cells

Untreated KMS-12-PE cells were completely methylated for *miR-34a*. Upon 5-AzadC demethylation treatment, *miR-34a* U-MSP signal emerged on day 3. (Figure 3A) However, *pri-miR-34a* transcript was not reexpressed by 5-AzadC alone or a combination of 5-AzadC and VPA. (Figure 3B) NCI-H929, which was unmethylated for *miR-34a* with constitutive expression of *pri-miR-34a*, served as positive control for *pri-miR-34a* expression. *p53*, constitutively expressed in NCI-H929, was also expressed in KMS cells both before and after treatment of 5-AzadC +/- VPA. (Figure 3B) Moreover, PCR on DNA fragment pulled down from chromatin immunoprecipitation (i.e. ChIP-PCR) in untreated KMS-12-PE cells showed relative abundance of trimethyl H3K9 and trimethyl H3K27 in *Alu* repeat element and the homozygously methylated *miR-34a* promoter but not *GAPDH* promoter. (Figure 4B, rows 1-3) Upon 5-AzadC treatment, high level of trimethyl H3K9 and trimethyl H3K27 persisted in addition to persistent absence of trimethyl H3K4 and acetylated H3K9 at the *miR-34a* promoter. (Figure 4B, row 4) Treatment of KMS-12-PE cells with a combination of 5-AzadC and VPA led to appearance of trimethyl H3K4 together with a low level of acetylated H3K9. (Figure 4B, row 5) Similar histone modification was observed by the use of ChIP-PCR primers amplifying over the p53 binding site. (Figure 4B, rows 6-8)

Primary samples. *miR-34a* was hypermethylated in none of CML, ALL and AML in addition to 2 (4%) CLL, 3 (5.5%) MM and 6 (18.8%) NHL samples. (Figure 5) Amongst the lymphoid malignancies, there was significantly more frequent *miR-34a* methylation in NHL than MM, CLL or ALL (p=0.027). In lymphoma samples, *miR-34a* was more frequently methylated in NK (N=4; 44.4%) than B-cell (N=2; 16.7%) or PTCL, NOS (N=0) (p=0.039). (Table 1) In the 12 MM patients with bone marrow samples at both

diagnosis and progression including two with *miR-34a* methylation at diagnosis, the methylation status of *miR-34a* did not change at disease progression (data not shown).

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Similarly, in the myeloma line KMS-12-PE, homozygous *miR-34a* methylation was associated with gene silencing. However, hypomethylating treatment of KMS-12-PE cells did not result in *miR-34a* reexpression despite successful demethylation. Therefore, the histone profile at the *miR-34a* promoter was studied to see if failure of miR reexpression was due to unsuccessful modification of histone code. ChIP study of KMS-PE-12 cells showed that, as expected, a euchromatin histone code (i.e. trimethyl H3K4 and acetyl H3K9) was associated with *GAPDH* promoter, and a silencing heterochromatin histone code (i.e. trimethyl H3K9 and trimethyl H3K27) with the *Alu* repeat elements, which is normally hypermethylated.[12] Therefore, in addition to the technical controls evidenced by the positive amplification in the input DNA, and absence of amplification in the IgG control, *GAPDH* and *Alu* repeats served as biological controls for open and closed chromatin configurations. However, despite restoring transcription-ready, open histone code at the *miR-34a* promoter in KMS-12-PE cells, i.e. emergence of trimethyl H3K4 and acetyl H3K9, upon treatment of cells with a combination of 5-AzadC and VPA, *miR-34a* expression was still not demonstrated. This might be explained by the fact that p53, and hence its transcriptional target, *miR-34a*, is inducible upon DNA damage, successful demethylation may render a transcription-ready state but *miR-34a* reexpression would require presence of p53, and hence additional trigger of DNA damage.[26] However, RT-PCR for *p53* showed that *p53* transcript was indeed present, and hence absence of p53 could not account for non-expression of *miR-34a*. Another possibility was that the histone code as revealed by ChIP-PCR did not

match the chromatin configuration at the transcription start site (TSS) as the TSS of the primary *miR-34a* was localised at >30Kb upstream of the mature *miR-34a*. (Figure 4A) However, our ChIP-PCR primer was indeed selected from the *miR-34a* TSS DNA sequence, and hence represented an accurate histone status at the TSS. On the other hand, the R1 region is more than 500bp upstream of *miR-34a* TSS, whereas the p53 binding site was about 230 base pairs downstream of *miR-34a* TSS. To ensure restoration of a similar euchromatin status at both the p53 binding site and *miR-34a* TSS, we designed another set of ChIP-PCR primers at the p53 binding site, which also showed emergence of trimethyl H3K4 and acetyl H3K9 data after hypomethylating therapy. Therefore, non-expression of *miR-34a* could not be acccounted by a discordant chromatin configuration at the p53 binding site and the *miR-34a* TSS. Finally, in this ChIP study, sustained high levels of trimethyl H3K9 and H3K27 was demonstrated despite successful promoter DNA demethylation. The persistence of this heterochromatin histone code has also been reported in colorectal cancer cells SW480, in which trimethyl H3K9 and trimetyl H3K27 persisted or even increased, when the hypermethylated soluble Wnt inhibitors SFRP2 and SFRP5 were successfully demethylated and reexpressed upon hypomethylation treatment.[27] Therefore, it appeared that the emergence of trimethyl H3K4 and possibly acetyl H3K9 accounts for the miR reexpression, and persistent H3K9 and H3K27 trimethylation does not impede gene reexpression.

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Figure 3. Treatment of KMS-12-PE myeloma cells with 5-Aza-2'-deoxycytidine (5-AzadC) with or without valproic acid (VPA). (A) M-/U-MSP analysis of *miR-34a* promoter methylation status. 5-AzadC treatment resulted in progressive demethylation of *miR-34a* promoter in KMS-12-PE cells. (B) Semi-quantitative RT-PCR analysis of the

p53 and *pri-miR-34a* expression. No expression of the *pri-miR-34a* transcript upon 5-AzadC alone or combined 5-AzadC + VPA treatment in KMS-12-PE cells. NCI-H929 myeloma cell line, which was completely unmethylated for *miR-34a* and expressed *pri-miR-34a* was used as positive control. RT-PCR of *GAPDH* served as an internal control (B: reagent blank; NoRT, negative control without reverse transcriptase).

Figure 4. Chromatin Immunoprecipitation (ChIP) assay of KMS-12-PE myeloma cells with 5-Aza-2'-deoxycytidine (5-AzadC) with or without valproic acid (VPA). A) Schematic diagram showing the locations of the ChIP primer sets (R1 and R2) used, p53 binding site (solid green box), and the distribution of CpG dinucleotides (solid vertical lines) over the transcription start site (TSS) of *pri-miR-34a* and the mature *miR-34a* (solid black box). B) Chromatin DNA was immunoprecipitated with antibodies for trimethyl H3K4, trimethyl H3K9, acetyl H3K9, trimethyl H3K27 and normal rabbit IgG. PCR amplification of the *GAPDH* promoter region, *Alu* repetitive sequence and *miR-34a* promoter in chromatin immunoprecipitated DNA fragments. In untreated KMS-12-PE cells, there was a high level of trimethyl H3K4 and modest amount of acetyl H3K9 but absence of trimethyl H3K9 at *GAPDH* promoter. (first row) Conversely, there was a high level of trimethyl H3K27 and modest amount of trimethyl H3K9, but absence of trimethyl H3K4 and acetyl H3K9 at the *Alu* repeat sequence (second row) and *miR-34a* promoter (third row). After 5-AzadC treatment, there was persistently high level of trimethyl H3K9, trimethyl H3K27, and absence of trimethyl H3K4 and acetyl H3K9, at the *miR-34a* promoter. (fourth row) However, combined treatment with 5-AzadC and VPA led to appearance of a substantial level of trimethyl H3K4 and a modest amount of acetyl H3K9. (fifth row) Histone modification status observed was consistent between

ChIP primer sets designed prior the TSS of *pri-miR-34a* and over the p53 binding site. (sixth to eighth rows)

Figure 5. Methylation of *miR-34a* in primary samples (B: reagent blank; S: primary sample; N: normal control; P: positive control with methylated DNA).