ORIGINAL ARTICLE

Methylation of INK4 and CIP/KIP families of cyclin-dependent kinase inhibitor in chronic lymphocytic leukaemia in Chinese patients

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Background: INK4 (p15, p16, p18 and p19) and CIP/KIP (p21, p27 and p57) are two families of cyclin-dependent kinase inhibitors (CKI) targeting CDK4/6 and CDK2, respectively.
Aim: To study the role of methylation in the inactivation of CKI in chronic lymphocytic leukaemia (CLL).
Materials and methods: Methylation-specific polymerase chain reaction was carried out on DNA obtained from the bone marrow of 56 newly diagnosed patients with CLL.
Results: Similar demographic features and clinical outcome were observed in our patients when compared with Caucasian patients, including an indolent clinical course (10-year overall survival 51%) and advanced Rai stage ($p=0.006$), and a high-risk karyotype such as trisomy 12 and complex aberrations ($p=0.03$). In the INK4 family, methylation in *p15* and *p16* occurred in 20 (35.7%) and 8 (14.3%) patients, respectively. In all, 5 (8.9%) CLL samples harboured concurrent methylation of both *p15* and *p16*. Apart from an association of *p16* methylation with higher presenting leucocyte count (64.5×10^9/l in methylated *p16* and 16.0×10^9/l in unmethylated *p16* patients; $p=0.016$), there was no association between *p15* and *p16* methylation and age, sex and Rai stage. No difference was observed in the overall survival for patients with and without *p15* and *p16* methylation. By contrast, *p18* and *Rb* were unmethylated in all samples. In the CIP/KIP family, apart from infrequent methylation of *p57* in 4 (7.1%) patients, methylation of *p21* and *p27* was uniformly absent.
Conclusion: *p15* and, less frequently, *p16* of the INK4 family of CKI, instead of the CIP or KIP family, were targeted by methylation in CLL. *p16* methylation was associated with a higher lymphocyte count at presentation. This is the first comprehensive study of the epigenetic dysregulation of the INK4 and CIP/KIP families of CKI in Chinese patients with CLL.

Cellular proliferation is mediated by progression through the cell cycle, where two cell cycle checkpoints are located at G_1S and G_2M.[1] Quiescent cells in G_0 phase contain hypophosphorylated retinoblastoma (RB), which sequesters the transcription factor E2F. On activation by mitogens, up regulation of D-type cyclins results in the activation of cyclin-dependent kinases 4 and 6 ($CDK_{4/6}$), leading to phosphorylation of RB.[1] Hyperphosphorylated RB results in release of E2F, which activates transcription of S1-specific genes. At the same time, the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs) will detach from the CDK_2 or cyclin E complex and bind to $CDK_{4/6}$ instead, resulting in de-repression (and thus activation) of CDK_2 and activation of $CDK_{4/6}$, and thus irreversible commitment of the cell to transit the G_1S cell cycle checkpoint.[1] Therefore, the cell cycle is triggered by the binding of cyclin D to and activation of cyclin-dependent kinase 4/6 ($CDK_{4/6}$), and further potentiated by the subsequent activation of CDK_2, but is negatively regulated by the INK4 (p15, p16, p18 and p19) and the CIP/KIP ($p21^{CIP}$, $p27^{KIP1}$and $p57^{KIP2}$) families of CKIs.[2] The INK4 family of CKIs binds to and inhibits $CDK_{4/6}$.[2] In contrast, the CIP/KIP family of CKIs may bind to both CDK_2 and $CDK_{4/6}$, with inhibition of CDK_2 and activation of $CDK_{4/6}$ on binding.[2]

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the Western population, but not in Asians.[3] For example, the age-adjusted incidence of CLL in the US in 2000 was 3.4/100 000. On the other hand, the age-adjusted incidence of CLL in Hong Kong in 2000 was 0.45/100 000, as estimated from the Hong Kong Cancer Registry. Patients are usually elderly people and present with lymphocytosis, lymphadenopathy and hepatospenomegaly.[4] The disease runs an indolent clinical course, but may be complicated by development of autoimmune disorders, marrow failure and Richter transformation.

Table 1 Demographic data of the patients

	n	%
Patients	56	100
Sex		
Men	44	78.6
Women	12	21.4
Median age (years)	37–91 (64.4)	
Presenting lymphocyte count ($\times10^9$/l)	2–236 (17.5)	
Rai stage		
>2	22	40.7
⩽2	32	59.3
Unknown	2	
Karyotypic abnormalities	39	
del 13q14	3	7.7
Trisomy 12	5	12.8
Complex	11	28.2
Normal	20	51.3

Abbreviations: $CDK_{4/6}$, cyclin-dependent kinases 4 and 6; CKI, cyclin-dependent kinase inhibitors; CLL, chronic lymphocytic leukaemia; MSP, methylation-specific polymerase chain reaction; M-MSP, methylated MSP; U-MSP, unmethylated MSP; PCR, polymerase chain reaction

DNA methylation, catalysed by DNA methyltransferase, includes the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide, converting it to methylcytosine.[5 6] In many cancers and haematological malignancies, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression. This may serve as an alternative mechanism of gene inactivation.[5 6] Indeed, epigenetic dysregulation of cell cycle control has been shown in various haematological malignancies.[7–10] In CLL, the importance of cell cycle dysregulation has been suggested by the effective induction of apoptosis of B-CLL cells by CDI.[11] Despite the frequency of the disease, data on methylation in CLL, especially with regard to a defined cellular pathway, are surprisingly scanty. Therefore, we hypothesised that epigenetic inactivation of the INK_4 and CIP/KIP families of CKI may be associated with CLL. *p19* of the INK4 family was not included in the analysis, as tumour suppressor activity has not been shown in transgenic mice deficient in *p19*.[12] This is the first comprehensive study of the epigenetic dysregulation of the INK4 and CIP/KIP families of CKI in Chinese patients with CLL.

PATIENTS AND METHODS

Patients and treatment

The diagnosis of CLL was made according to standard criteria,[13] and staged according to the Rai staging system.[13] In all, there were 44 men and 12 women, with a median age of 64.5 (range: 37–91) years. The median lymphocyte count was 17×10^9/l. Apart from two patients with an uncertain Rai stage at diagnosis, there were 22 (40.7%) and 32 (59.3%) patients with Rai stage >2 and $\leqslant2$, respectively (table 1). Treatment would be given for B symptoms, symptomatic organomegaly, extreme lymphocytosis, immune cytopenia or a rapid rise in lymphocyte count. The treatment included prednisolone, fludarabine or chlorambucil, or combination chemotherapy such as COPP (cyclophosphamide, vincristine, prednisolone and procabazine), CVP (cyclophosphamide, vincristine and prednisolone) or FND (fludarabine, mitoxantrone and dexamethasone).[14]

High-molecular-weight genomic DNA was isolated by standard protocols from diagnostic bone marrow aspirates of 56 patients with CLL and 12 normal bone marrow donors in addition to DNA from the peripheral blood of 12 healthy blood donors from the Hong Kong Red Cross Association.[15] Cytogenetic data were available in 39 patients.[16] Previous studies showed that trisomy 12 in CLL is associated with atypical morphology, progressive disease and poor survival, whereas del(13q) seems to indicate a good prognosis.[17] Therefore, in this study, patients with poor-risk cytogenetic aberrations were those with trisomy 12 and complex abnormalities, and patients with standard-risk cytogenetic aberration included those with normal karyotype and isolated deletion of 13q14.

Table 2 MSP primers and PCR conditions

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Tm and cycles	Size in bp	Locus	Reference
p15						
M-MSP	TGAGGATTTCG CGACGCGTTC	CGTACAATAA CCGAACGAC CGATCG	63°C/35	162	9p21	15
U-MSP	TGAGGATTTTG TGATGTGTTT	CATACAATAA CCAAACAAC CAATCA		162		
p16						
M-MSP	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA	65°C/35	150	9p21	15
U-MSP	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A		151		
p18						
M-MSP	TTATCGAATTG TTATTTTCGTT CG	CGTCTCGCCG AAAAAATAA TC	64°C/35	93	1p32	15
U-MSP	GGGTTATTGAA TTGTTATTTTT GTTTG	CATCCATCTC ACCAAAAAAA TAATC		95		
RB						
M-MSP	GGGAGTTTCGC GGACGTGAC	ACGTCGAAAC ACGCCCCG	66°C/35	152	13q14	15
U-MSP	GGGAGTTTTGT GGATGTGAT	ACATCAAAAC ACACCCCA		152		
p21						
M-MSP	TTG GGC GCG GAT TCG TC	CTA AAC CGC CGA CCC GA	62°C/35	100	6p21	25
U-MSP	TTA GTT TTT TGT GGA GTT G	CTC AAC TCT AAA CCA CAA		120		
p27						
M-MSP	AAG AGG CGA GTT AGC GT	AAA ACG CCG CCG AAC GA	66°C/35	195	12p13	25
U-MSP	ATG GAA GAG GTG AGT TAG T	AAA ACC CCA ATT AAA ACA		212		
p57						
M-MSP	TCG GTT AGG TTT GAG CGA GC	TAC GTA TAC GAA AAA CGC GAC GAC	62°C/35	137	11p15	26
U-MSP	TTG GTT AGG TTT GAG TGA GTG A	TCT ACA TAT ACA AAA AAC ACA ACA A		139		—

PCR, polymerase chain reaction; M, methylated; U, unmethylated; MSP, methylation-specific PCR.

Methylation-specific polymerase chain reaction

The methylation-specific polymerase chain reaction (MSP) for gene promoter methylation was carried out as described in detail previously.[7 17] Briefly, treatment of DNA with bisulphite for conversion of unmethylated, but not methylated, cytosine to uracil was carried out with a commercially available kit (CpGenome DNA modification kit, Intergen, New York, New York, USA) according to the manufacturer's instructions. Table 2 shows the primers for the methylated (M-MSP) and unmethylated (U-MSP) gene promoter regions for *p15, p16, p18, Rb, p21, p27* and *p57*.[18] DNA from eight normal donors was used as negative control, whereas methylated-control DNA (CpGenome Universal Methylated DNA, Intergen) was used as positive control in all the experiments. MSP was carried out in a thermal cycler (9700, PE Biosystems, Foster City, California, USA). The polymerase chain reaction (PCR) mixture contained 50 ng of bisulphite-treated DNA, 0.2 mM deoxynucleoside triphosphates, 2 mM magnesium chloride, 10 pmol of each primer, 1 × PCR buffer II and 2.5 units of AmpliTaq Gold (Perkin-Elmer Biosystems, Wellesley, Massachusetts, USA) in a final volume of 50 µl.

DNA sequencing

The identity of the methylated and unmethylated sequences was confirmed by automated DNA sequencing. PCR products were gel purified, sequenced bi-directionally (DYEnamic ET Terminator Cycle Sequencing kit, Amersham Biosciences, Piscataway, New Jersey, USA), and analysed on an automated DNA sequence analyser (ABI Prism 3700 DNA analyser, Applied Biosystem, Foster City, California, USA). M-MSP of methylated positive control and selected patients were sequenced.

Statistics

Correlations between *p15* and *p16* methylation and continuous variables (median age, median lymphocyte counts) and categorical variables (sex and Rai staging) were studied by Mann–Whitney test and χ^2 test, respectively. Overall survival is defined as the time from diagnosis to the time of death or last follow-up. Overall survivals of patients with limited Rai stage (stages 0, I and II) were compared with those with advanced stage (stage III and IV). The effect of karyotype was studied by comparing overall survival in patients with standard-risk and poor-risk cytogenetic changes. Survival curves are plotted by the Kaplan–Meier method, and compared by the log rank test. All p values were two-sided.

RESULTS

Patient outcome

Overall survival for the whole group was 64.4% at 5 years and 51.2% at 10 years. Five-year overall survival in patients with advanced (>2) and limited (⩽2) Rai stage was 44.4% and 76.7% (p = 0.006), respectively. Poor-risk cytogenetic changes (trisomy 12 and complex karyotypes) were found in 16 of 39

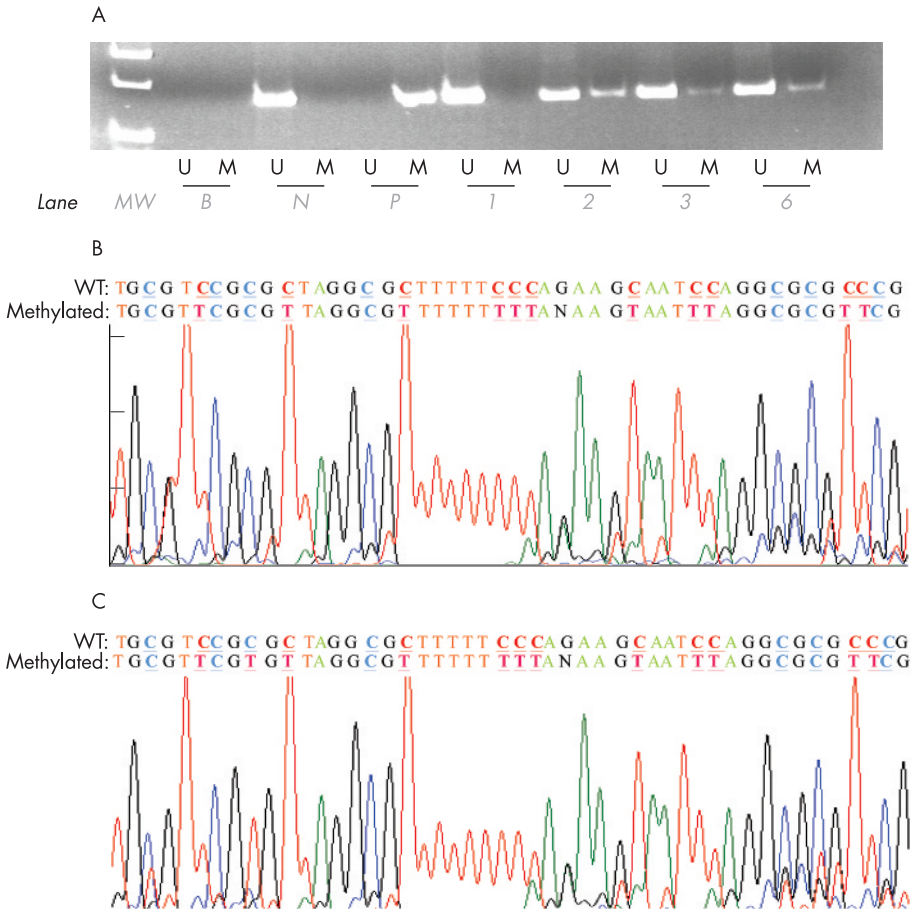

Figure 1 (A) Methylated methylation-specific polymerase chain reaction (M-MSP) and unmethylated methylation-specific poylmerase chain reaction (U-MSP) of *p15* in primary chronic lymphocytic leukaemia (CLL) samples. B, reagent blank; N, normal marrow DNA controls; P, methylated positive control; patients 1, 2, 3 and 6, primary CLL marrow samples. (B) Sequence of positive control M-MSP product and (C) sequence of M-MSP of the primary CLL marrow sample. Wildtype (WT) cytosine (C) residues that remained unchanged in methylated CpG are coloured blue and underlined, whereas those that were changed to thymidine (T) are coloured red and underlined. U-MSP shows that the methylated control (M) was totally methylated.

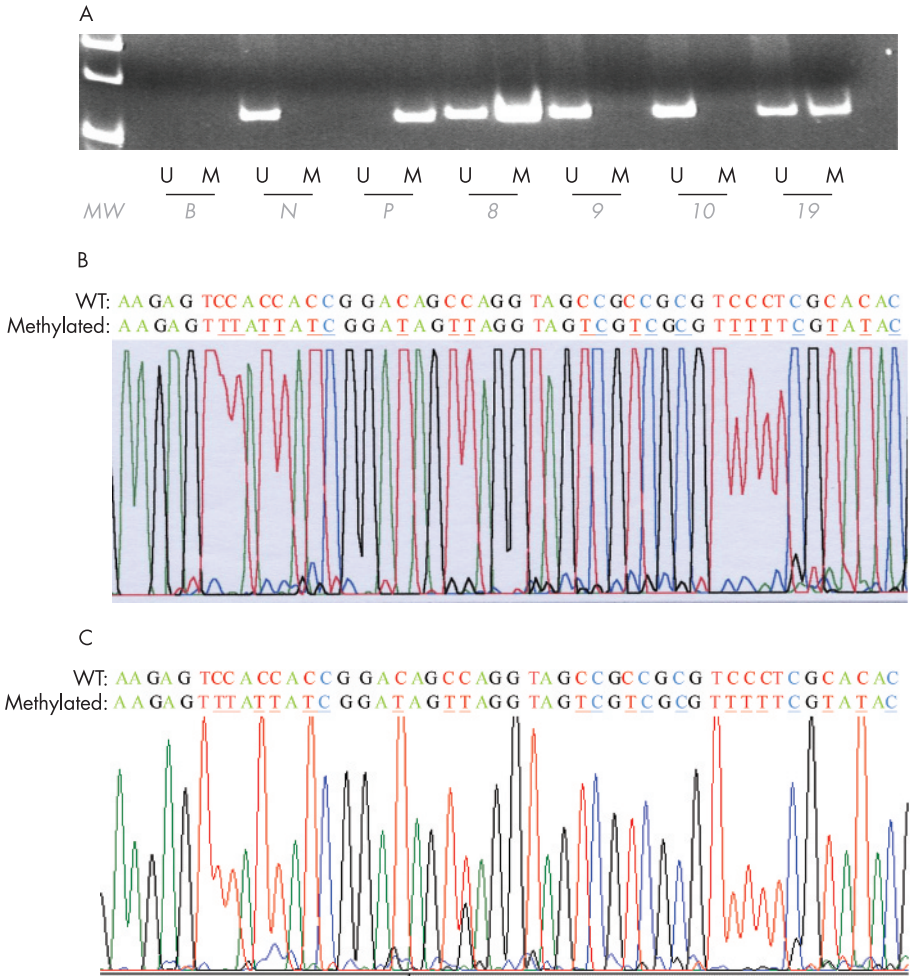

Figure 2 (A) Methylated methylation-specific polymerase chain reaction (M-MSP) and unmethylated methylation-specific polymerase chain reaction (U-MSP) of *p57* in primary chronic lymphocytic leukaemia (CLL) samples. B, reagent blank; N, normal marrow DNA controls; P, methylated positive control; patients 8, 9, 10 and 19, primary CLL marrow samples. (B) Sequence of positive control M-MSP amplification. (C) M-MSP sequence of primary CLL marrow sample. Wildtype (WT) cytosine and (C) residues that remained unchanged in methylated CpG were coloured blue and underlined, whereas those that were changed to thymidine (T) are coloured red and underlined. U-MSP shows that the methylated control (M) was totally methylated.

patients (41.0%; table 1). Overall survival for patients with poor-risk and standard-risk karyotype was 84.5% and 32.2%, respectively ($p = 0.03$).

Controls

None of the seven genes tested were methylated in 12 normal bone marrow and 12 normal peripheral blood samples. 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; figs 1A and 2A). Authenticity of MSP was confirmed by sequencing of samples from the M-MSP products from methylated positive control (figs 1B and 2B).

MSP in primary CLL marrow samples

In the INK4 family, methylation of *p15* and *p16* occurred in 20 (35.7%) and 8 (14.3%) patients, respectively (table 3). Of them, 5 (8.9%) patients with CLL harboured concurrent methylation of both *p15* and *p16*. In contrast, *p18* and *Rb* are unmethylated. In the CIP or KIP family, apart from infrequent methylation of *p57* in 4 (7.1%) patients, methylation of *p21* and *p27* are absent. Authenticity of MSP was confirmed by sequencing samples from the M-MSP products from methylated samples (figs 1C and 2C).

Association of gene methylation with demographic data and overall survival

No association was found between *p15* methylation and age ($p = 0.19$) and diagnostic Rai stage ($p = 0.77$), presence of poor-risk karyotype ($p = 0.74$) and median lymphocyte count at presentation. No correlation was observed between *p16* methylation and sex ($p = 0.99$), Rai stage ($p = 0.99$), poor-risk karyotype ($p = 0.63$) and age ($p = 0.72$), but *p16* methylation was associated with a high median lymphocyte count at diagnosis ($p = 0.016$). Similarly, no correlation was identified between *p57* methylation and sex ($p = 0.99$), Rai stage ($p = 0.99$), poor-risk karyotype ($p = 0.50$), age ($p = 0.47$) and presenting lymphocyte count ($p = 0.17$). Projected 5-year overall survival for patients with and without *p15* methylation was 66% and 59%, respectively ($p = 0.75$; fig 3).

DISCUSSION

Ideally, MSP status of the neoplastic lymphocytes will only be elucidated if marrow cells have been sorted for CD5 and CD23 dually positive cells. Here, MSP of the genes has been first validated in normal control DNA (both normal bone marrow and normal peripheral blood DNA) by showing a lack of methylation and further verified by sequencing. This

Table 3 Association between gene methylation and demographic characteristics

	p15			*p16*			*p57*		
	Unmethylated	Methylated	p	Unmethylated	Methylated	p	Unmethylated	Methylated	p
Median age	63.5	68.5	0.19	64.5	65.5	0.72	64.5	67.5	0.47
Median lymphocyte count	17.0	21.5	0.25	16.0	64.5	0.016	16.0	31.5	0.17
Sex									
Men	30	14	0.31	38	6	0.99	41	3	0.99
Women	6	6		10	2		11	1	
Rai stage									
⩽2	21	10	0.77	27	5	0.99	30	2	0.99
>2	13	9		19	3		20	2	
Poor-risk karyotype									
Yes	15	8	0.74	21	2	0.63	21	2	0.50
No	9	7		13	3		16	0	

Association between gene methylation and age and lymphocyte count was analysed by the Mann–Whitney test, and association with sex, Rai stage and presence of poor-risk karyotype was analysed by the χ^2 or Fisher's exact test.

confirms that gene methylation is an aberration that does not exist in normal cells, be it marrow or peripheral blood cells. Given that methylation detected by MSP is a positive signal with a high sensitivity (up to 1×10^5 for *p15* gene),[7] our results are still valid without sorting for lymphocytes. The presence of amplification in U-MSP in some methylated primary samples was due to the presence of the unmethylated gene in normal marrow cells.

Although global hypomethylation has been first shown in CLL,[19] a recent genome-wide methylation study showed that 2.5–8% of the CpG islands are aberrantly methylated in a non-random manner in CLL.[20] Moreover, although CLL has conventionally been described to be a disease with impaired apoptosis, the role for cell cycle dysregulation may be less important. However, a subpopulation of cycling B cells,[21] especially those in the pseudofollicles, may be important in disease progression.

Although there are recent reports of *p53* and *ZAP-70* gene methylation in CLL, with the inherent effect on prognosis,[22 23] data on methylation of *p15* and *p16* in CLL are surprisingly scanty. Two studies reporting on the frequency of *p15* and *p16* methylation in Caucasian patients were available. One study showed infrequent *p15* (5% of patients) and *p16* (9%) methylation, whereas another showed frequent methylation of *p15* (50%) and less frequently of *p16* (17%), in Caucasian patients with CLL.[24 25] Our study on Chinese patients for methylation of *p15* and p16 showed a rate of 37.5% and 14.3%, respectively, which is comparable to that in the Caucasians. It is noteworthy that deletion or mutation associated with *p15* and *p16* is extremely rare in CLL.[26] Therefore, methylation of *p15* and *p16* may be the major mechanism of *p15* and *p16* gene inactivation in CLL. Compared with the high frequency (>70%) of *p15* methylation in acute leukaemia,[7 8] the lower frequency of *p15* methylation in CLL suggested a less important role for cell cycle dysregulation in CLL. Interestingly, a similar rate of *p15* methylation has also been reported in multiple myeloma, a disease also characterised by a mature B cell phenotype and impaired apoptosis instead of cell proliferation.[9] Our data also showed that *p16* methylation was associated with a higher leucocyte count at presentation (64.5×10^9/l in patients with methylated *p16* and 16.0×10^9/l in patients with unmethylated *p16*; $p = 0.016$), suggesting the activation of the cell cycle.

A tumour suppressor property of *p18* gene has been demonstrated in mice.[27] However, *p18* methylation was absent in our patients in this and other studies.[25 26] A literature review indicates that *p18* methylation is probably not targeted in cell cycle dysregulation in CLL.[28 29] Although 13q deletions are common in CLL,[30] *Rb* is not associated with the minimally deleted region, and thus genes other than *Rb* are targeted.[30] Our results showed that Rb was not methylated in CLL.

We have also shown the infrequent *p57*, and absence of *p21* and *p27*, methylation in CLL. $p21^{CIP1/WAF1/CDKN1A}$ is a tumour suppressor gene inducible by wildtype p53 in the presence of DNA damage, leading to cell cycle arrest. This is the first report of the absence of *p21* methylation in CLL. *p21* methylation has been shown to be rare in other lymphoid malignancies.[31] Infrequent $p27^{KIP1/CDKN1B}$ methylation has

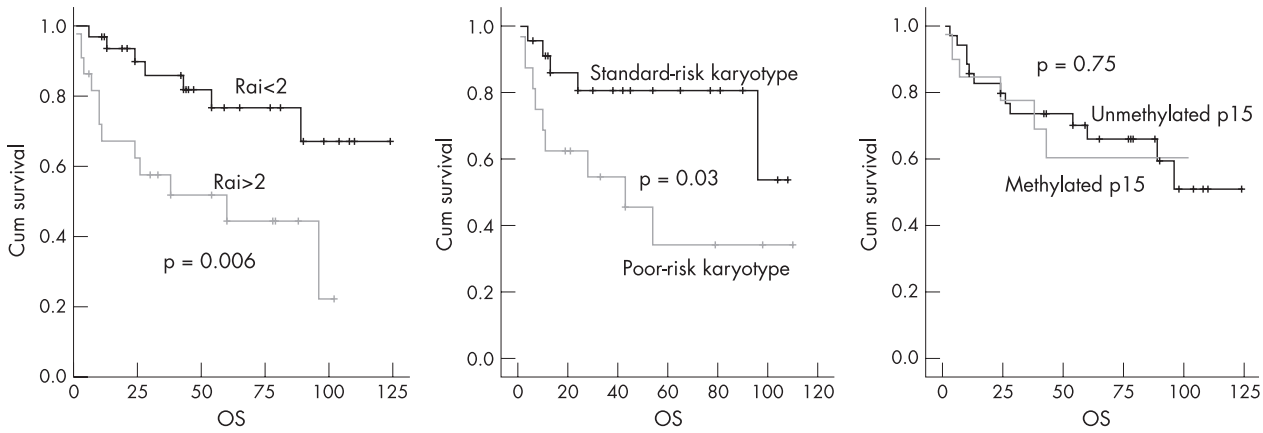

Figure 3 Overall survival (OS) for patients (A) with and without advanced Rai stage disease, (B) poor-risk karyotype and (C) *p15* methylation.

been reported to occur in solid cancers,[32] but has not been studied in haematological cancers. However, recent studies showed that cellular p27 level may be down regulated by post-translational ubiquitination and proteasomal degradation.[33] Moreover, functional inactivation of p27 may also be mediated by the phosphorylation of the nuclear localisation domain, resulting in the cytoplasmic mislocalisation of p27, thus precluding p27 from its negative regulation of CDK_2.[33] Therefore, although *p27* methylation may not have an important role in its inactivation, post-translational modification by ubiquitination or phosphorylation may still be important in oncogenesis. Therefore, our data suggest that epigenetic inactivation of the cell cycle control primarily targets the CDK4/INK4 complex and does not require additional inactivation of the CDK2/CIP/KIP. Indeed, a recent study showed that CDK2 (target of inhibition by KIP proteins), but not CDK4, is dispensable for cell proliferation,[34] underscoring the importance of CDK4 in cell proliferation and thus the potential importance of INK4 targeting by methylation.

In summary, *p15* and, less frequently, *p16* of the INK4 family, instead of the CIP or KIP families, are targets of methylation in CLL, and thus might be important for its pathogenesis, posing potentially important targets for 5-azacytidine demethylation.

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