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SHORT COMMUNICATION

Frequent DAP kinase but not p14 or Apaf-1 hypermethylation in B-cell chronic lymphocytic leukemia

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Abstract Dysregulation of apoptosis, and thus the p14/DAP kinase/HDM2/p53/Apaf-1 pathway, is potentially important in carcinogenesis. Chronic lymphocytic leukemia (CLL), uncommon in the Chinese, is a disease characterized by impaired apoptosis, of the neoplastic lymphocytes. Hypermethylation of *p14, DAP kinase* and *Apaf-1* was studied by methylation-specific polymerase chain reaction (MSP) with primers for methylated (M-MSP) and unmethylated (U-MSP) alleles in 50 diagnostic marrow samples from patients with CLL. Chinese CLL patients had an indolent course similar to Caucasians with median overall survival (OS) of 96 months, which was adversely affected by advanced Rai stage (projected 5-year OS = 72% and 39% for Rai ≤ 2 and Rai > 2; $P = 0.01$). *DAP kinase* was methylated in 18 (36%) patients while *p14* and *Apaf-1* were completely unmethylated in all the primary CLL samples. There was no correlation between *DAP kinase* hypermethylation and age, sex, poor-risk karyotype, lymphocyte count and Rai stage at diagnosis. Projected OS for patients with and without *DAP kinase* hypermethylation were 59 and 57% ($P = 0.91$). *DAP kinase*, but not *p14* and *Apaf-1*, of the DAP kinase/p14/HDM2/p53/Apaf-1 pathway is frequently hypermethylated in CLL, but not of prognostic significance. Moreover Chinese patients with CLL share a similarly indolent clinical course, and this is the first comprehensive study on *p14, DAP kinase* and *Apaf-1* hypermethylation in CLL.

Keywords DAP kinase · P14 · Apaf-1 · Methylation · CLL · Chinese

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Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western hemisphere but not Asia. (Parker et al. 1997) 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 and presents with lymphocytosis, lymphadenopathy and hepatosplenomegaly. (Keating et al. 2003) It runs an indolent clinical course, but may be complicated by development of autoimmune disorders, marrow failure and Richter transformation.

Death associated protein kinase (DAP kinase) is a pro-apoptotic calcium/calmodulin-regulated serine/threonine kinase with a multidomain structure that participates in a wide array of apoptotic systems initiated by IFN-, TNF-, activated Fas, and detachment from extracellular matrix. (Raveh et al. 2001) It counteracts oncogene-induced transformation by activating *p53* in a $p19^{ARF}$-dependent manner, thereby providing an intrinsic p53-dependent apoptotic check-

point that is turned on by oncogenes at the initial stages of transformation. (Raveh et al. 2001)

p14^{ARF} (*p19*^{Arf} in mice) is one of the two genes encoded by the INK4A/ARF locus at chromosome 9p21. (Sherr et al. 2001) Alternative first exons (1α and exon 1β), under the control of different promoters, specify the 5′ ends of p16^{INK4A} and p14^{ARF} respectively. These alternative exons (1α and 1β) are spliced to the same splice acceptor site in exon 2, which is translated in alternative reading frames. p14 physically associates with HDM2, a negative regulator of p53, and protects p53 from HDM2-mediated proteosomal degradation. HDM2 possesses E3 ubiquitin ligase activity, thereby promoting proteasomal degradation of p53. Binding of p14^{ARF} to HDM2 results in localization of HDM2 to the nucleolus, precluding its interaction with p53. (Sherr et al. 2001) Moreover, E3 ubiquitin ligase activity of HDM2 is also inhibited by binding of p14^{ARF}. Therefore, p14^{ARF} is a tumor suppressor by virtue of its ability to stabilize cellular p53 protein.

During the multi-step process of carcinogenesis, the first step is usually activation of a cellular oncogene. However, an intrinsic tumor suppression mechanism is triggered to result in apoptosis of cells carrying activated oncogenes. (Lowe et al. 2004) In particular, *DAP kinase* is upregulated and render apoptosis of cells harboring an activated oncogene in a p14 and p53-dependent manner, thereby conferring intrinsic tumor suppression. (Raveh et al. 2001) Various molecules are involved in the upstream regulation of p53 (DAP kinase, p14 and HDM2), and apoptosis is effected downstream by activation of apaf-1 with formation of apoptosome. (Hengartner et al. 2000) However, while p53 is frequently inactivated in many forms of solid cancers, p53 mutation is infrequent in haematological cancers. (Peller et al. 2003) Therefore, abrogation of this intrinsic tumor suppression mechanism, i.e. DAP kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway is potentially important in CLL.

DNA methylation involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide. (Chim et al. 2002; Herman et al. 2003) In many cancers, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression. Various genes have been shown to be frequently methylated in cancers and leukemias including CLL. (Chim et al. 2002, 2005a, 2006; Esteller et al. 2001) However, as study of isolated genes or random assortment of genes preclude interpretation of the role of methylation in a specific pathway, we conducted a comprehensive study of hypermethylation of the putative tumor suppressors in the DAP kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in CLL. *p53* was not investigated as it does not harbor a CpG island in promoter region (Accession number: J04238).

Materials and method

Patient, diagnosis and treatment

Diagnosis of CLL were made according to standard criteria, (Muller-Hermelink et al. 2001; Wong et al. 1999) which is based on classical morphology, low level of expression of light-chain-restricted surface immunoglobulin, and dual positivity of CD5 and CD23 in the neoplastic lymphocytes by flow cytometry. (Muller-Hermelink et al. 2001; Wong et al. 1999) Patients were staged according to Rai staging system. There were 40 males (80%) and 10 females (20%) with a median age of 65.5 years (range: 37–91 years). There were 11 (22%) stage 0, 12 (24%) stage I, six (12%) stage II, 11 (22%) stage III and 10 (20%) stage IV patients by Rai staging system respectively. The median presenting lymphocyte count was 17×10^9/L (range: 10–236 $\times 10^9$/L) There was variable infiltration of bone marrow by leukemic CLL cells ranging from 37% to 90%, (median : 65%) Patients received treatment if there were B symptoms, symptomatic organomegaly, extreme lymphocytosis, immune cytopenia, or rapid rise in lymphocyte count. Treatment included prednisolone, fludarabine or chlorambucil, or combination chemotherapy such as COPP (cyclophosphamide, vincristine, prednisolone and procarbazine), CVP (cyclophosphamide, vincristine and prednisolone) or FND (fludarabine, mitoxantrone and dexamethasone). (Ma et al. 2004) Cytogenetic data were available in 39 patients. (Wong et al. 1999) Previous studies showed that trisomy 12 in CLL is associated with atypical morphology, progressive disease and poor survival, whereas del(13q) appears to indicate a good prognosis. (Juliusson et al. 1998) Therefore, on this study, poor-risk cytogenetic aberrations was defined as those with trisomy 12 and complex abnormalities, and standard-risk cytogenetic aberration included those with normal karyotype and isolated deletion of 13q14. Poor risk cytogenetic changes (trisomy 12 and complex karyotypes) were found in 16/39 (41.0%). Moreover, we have reported an adverse prognostic impact of poor-risk karyotype (such as trisomy 12 and complex aberrations) on overall survival, OS for patients with poor and standard risk karyotype were 84.5 and 32.2% (P = 0.03). (Chim et al. 2005a)

Methylation-specific polymerase chain reaction (MSP)

High molecular weight genomic DNA was isolated by standard protocols from leukocytes of diagnostic bone marrow aspirates of 50 Chinese CLL patients, eight normal bone marrow donors in addition to DNA from peripheral blood of 12 healthy blood donors to the Hong Kong Red Cross Association.(Chim et al. 2004a) The methylation-specific polymerase chain reaction (MSP) for gene promoter methylation was performed as previously described. (Chim et al. 2004a) The primers for the methylated (M-MSP) and unmethylated (U-MSP) promoters of *p14*, and *DAP kinase* were shown in Table 1. (Estellet et al. 2000; Chan et al. 2002) DNA from normal bone marrow and peripheral blood leukocytes was used as negative control, while methylated control DNA (CpGenome Universal Methylated DNA, Intergen) was used as positive control in all the experiments.

Statistical analysis

Correlation between *DAP kinase* methylation status with continuous (mean age, mean diagnostic haemoglobin, lymphocyte and platelet counts) and categorical variables (sex, Rai staging and poor-risk cytogenetics) were studied by Student *t* test and Chi-square test (or Fisher Exact test) respectively. Overall survival (OS) is measured from the date of diagnosis to the date of last follow-up or death. OS of patients with limited Rai stage (stages 0, I and II) were compared to those with advanced stage (stage III and IV). Survival is plotted by the Kaplan–Meier method and compared by the log-rank test. All *P* values were two-sided.

Results

MSP of positive and negative controls None of the three genes tested were methylated in eight normal bone marrow samples, (Fig. 1) 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).

MSP in primary CLL marrow samples *DAP kinase* was methylated in 18 (36%) patients while *p14* and *Apaf-1* were completely unmethylated in all the primary marrow samples.(Fig. 1)

Statistical analysis

There was no association between *DAP kinase* methylation and age ($P = 0.11$), sex ($P = 0.46$), Rai stage at diagnosis ($P = 0.52$), poor-risk karyotype (0.73) and lymphocyte count at diagnosis ($P = 0.53$). (Table 2) Median OS for the whole group was 96 months. Projected 5-year OS in patients with limited (Rai stage ≤ 2) and advanced (Rai stage > 2) disease were 72 and 39% ($P = 0.01$). Patients with and without *DAP kinase* methylation were 59 and 57% ($P = 0.91$)(Fig. 2).

Table 1 Methylation-specific polymerase chain reaction (MSP): primer sequences and reaction conditions

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Tm/cycles	Reference
DAP kinase				
M-MSP	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	63°C/35	Chan et al. 2002
U-MSP	GGAGGATAGTTGGATTGAG TTAATGTT	CAAATCCCTCCCAAACACCAA	–	–
P14				
M-MSP	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	65°C/35	Esteller et al. 2000
U-MSP	TTTTTGGTGTTAAAGGGT GGTGTAGT	CACAAAAACCCTCACTCACAACAA	–	–
Apaf-1				
M-MSP*	TATTGCGATATTGTTTTAAATTCGA	GAAACGTAACTAAACCTCAAAAACG	64°C/35	Genebank AB070829
U-MSP**	TATTGTGATATTGTTTTAAATTTGA	CAAAACATAACTAAACCTC AAAAACAC	–	

Tm annealing temperature, *M-MSP* methylation-specific polymerase chain reaction for the methylated allele, *U-MSP* MSP for the unmethylated allele, Primers for *Apaf-1*: *M-MSP** nucleotides 785–809, *U-MSP*** nucleotides 938–963 in Genbank accession number AB070829

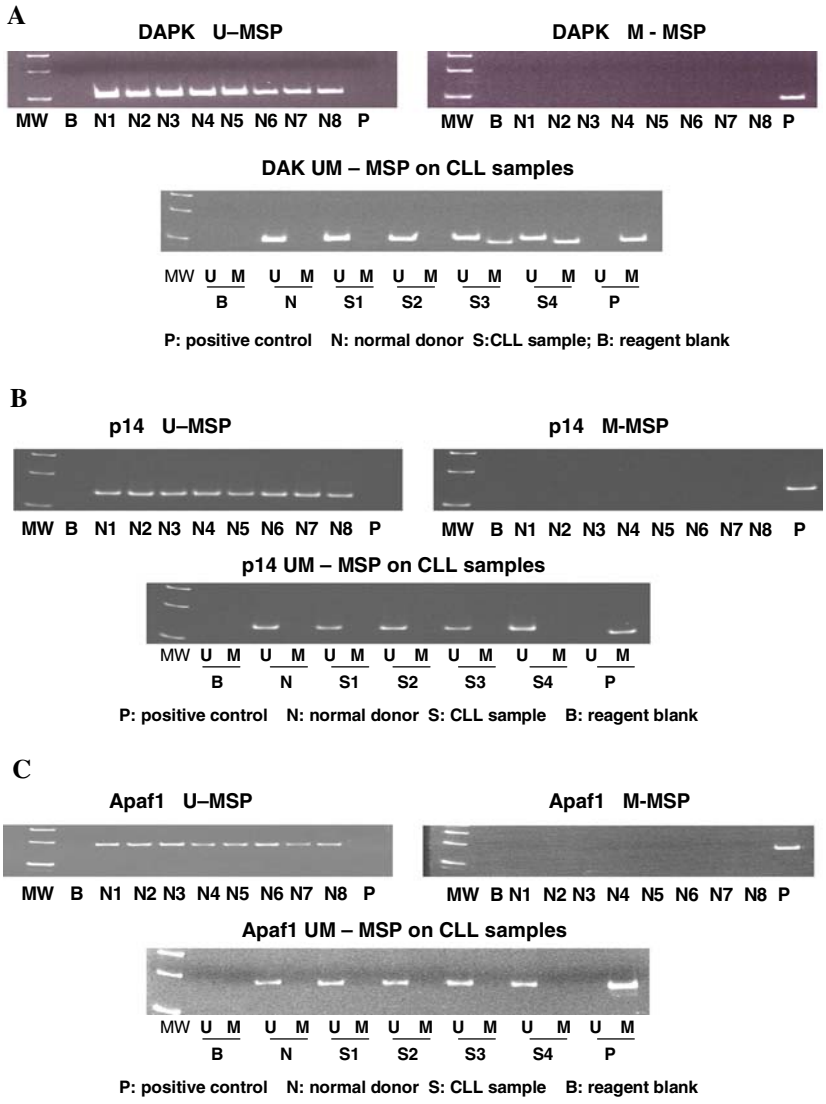

Fig. 1 Hypermethylation of *DAP kinase*. **a** *DAP kinase* methylation. U-MSP showed that the methylated control (M) was totally methylated. All 8 normal controls (N1–N8) showed an amplifiable band. In the M-MSP, the methylated control showed an amplifiable band, but none of the normal controls showed amplification. For the CLL samples, S3 and S4 showed *DAP kinase* hypermethylation. *B* reagent blank, *P* methylated positive control, *MW* molecular weight control, *N1–N8* normal marrow DNA, *S1–S4* primary CLL samples. **b** *p14* hypermethylation U-MSP showed that the methylated control (M) was totally methylated. All 8 normal controls (N1–N8) showed an amplifiable band. In the M-MSP, the methylated control showed an amplifiable band, but none of the normal controls showed amplification. For the CLL samples, none showed *p14* hypermethylation. *B* reagent blank, *P* methylated positive control, *MW* molecular weight control, *N1–N8* normal marrow DNA, *S1–S4* primary CLL samples. **c** *Apaf-1* hypermethylation. U-MSP showed that the methylated control (M) was totally methylated. All 8 normal controls (N1–N8) showed an amplifiable band. In the M-MSP, the methylated control showed an amplifiable band, but none of the normal controls showed amplification. For the CLL samples, none showed *Apaf-1* hypermethylation. *B* reagent blank, *P* methylated positive control, *MW* molecular weight control, *N1–N8* normal marrow DNA, *S1–S4* primary CLL samples

Discussion

Despite a lower incidence of CLL in Orientals, (Wong et al. 1999; Kwong et al. 1994) including an elderly median presenting age and an indolent clinical course (median OS: 96 months). (Keating et al. 2003) Moreover, similar to Caucasian patients, advanced Rai stage disease conferred an inferior OS. Therefore, our series represented CLL patients similar to Caucasian patients.

Ideally, MSP status of the neoplastic lymphocytes is best studied if marrow cells have been sorted for CD5

Table 2 Association of *DAP kinase* hypermethylation with patients' characteristics

DAP kinase	Methylated	Unmethylated	*P* value
Number	18 (36%)	32 (64%)	–
Median age	68	62	0.11
Sex			
Male	13	27	0.46
Female	5	5	–
Median lymphocyte count	51.1	39.1	0.52
Rai stage			
≤II	9	20	0.53
>II	9	12	–
Poor-risk karyotype*			
No	6	16	0.73
Yes	5	10	–

*Karyotype and *DAP kinase* hypermethylation data available in 37/50 patients

and CD23 dually positive cells. Here, MSP of the genes has been validated in normal control DNA by demonstration of the lack of methylation in normal controls, and thus M-MSP amplification is specific to tumor cells. Given that methylation detected by MSP is a positive signal with a high sensitivity (as evidenced by serial dilution of positive methylated control DNA: 1 in 10^3 for *RARα*, (Chim et al. 2005b) 1 in 10^4 for *p16* and *p73*, (Chim et al. 2001a, 2004b) and 1 in 10^5 for *p15* gene, (Chim et al. 2001b) our results are still valid without sorting of the marrow for lymphocytes, given that the marrow in these patients had leukemic infiltration ranging from 37 to 90% (median: 65%).(Materials and method) On the other hand, the presence of U-MSP amplification was likely due to the invariable contamination of the marrow by unmethylated normal cells. Moreover, the lack of tumor cell selection or enrichment precludes assessment of important information such as the level of *DAP kinase* methylation and expression of DAP kinase in primary samples. If there is no intratumoral heterogeneity, the level of methylation such as mono- or bi-allelic methylation might have been studied by quantitative MSP, and the level of DAP expression by RT-PCR or Western hybridization).

We showed frequent hypermethylation of *DAP kinase* but not *p14* and *Apaf1* in CLL, consistent with the pathogenesis involving resistance to apoptosis in CLL cells. Moreover, frequent *DAP kinase* methylation has also been demonstrated in B-cell lymphoma but not myeloid or T-cell malignancies, *and* thus might be important in carcinogenesis of B-cell malignancies. (Katzenellenbogen et al. 1999)

p14 hypermethylation has been extensively studied in solid cancers, and has been shown to be modestly

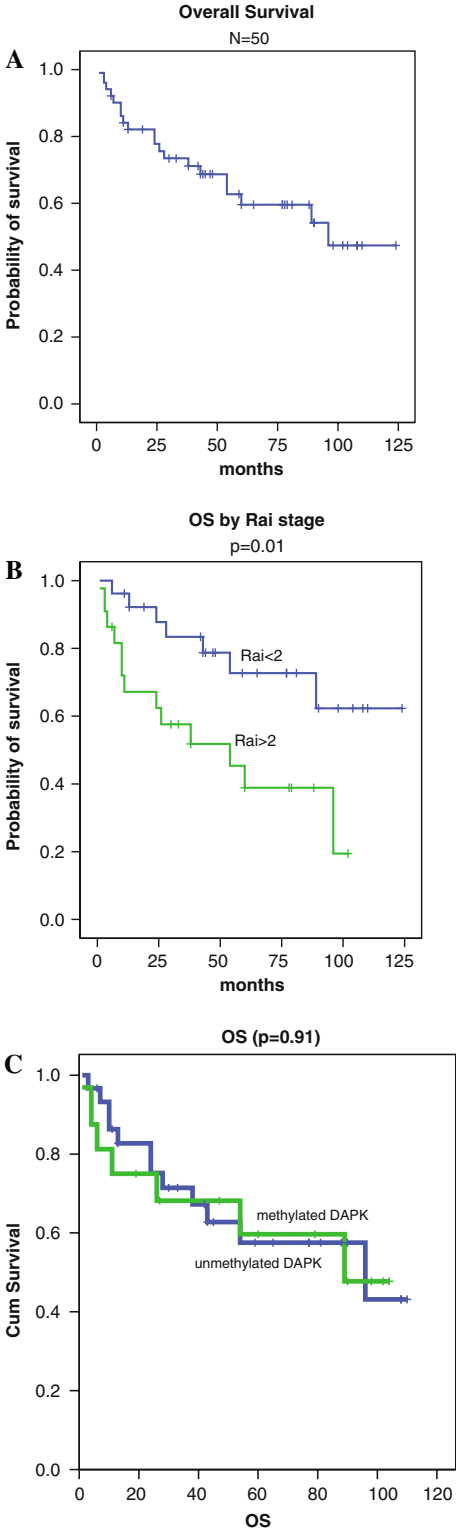

Fig. 2 Overall survival. **a** OS of all CLL patients. Median OS was 96 months. **b** Advanced Rai stage was associated with an inferior OS. Projected 5-year OS of CLL patients with limited and advanced Rai stage disease were 72 and 39%, $P = 0.01$. **c** There was no difference in the OS of patients with and without *DAP kinase* hypermethylation

methylated (10–30%) in renal, gastric and colorectal cancers. (Estellet et al. 2001) In haematological cancers, apart from accelerated phase of CML, where 40% of patients carried *p14* methylation, (Nagy et al. 2003) *p14* hypermethylation is uncommon, and has been reported in only 8% of ALL, (Romen-Gomez et al. 2004) but not t-MDS/AML ($N = 81$). (Christiansen et al. 2003) We demonstrated the absence of *p14* hypermethylation in CLL, consistent with detection of p14 expression in the tumor cells of CLL patients. (Taniguchi et al. 1999) Previous studies have reported the absence of *p14/p16* gene deletion in CLL, (Drexler et al. 1998) together with our data of absence of *p14* methylation of *p14* in CLL, *p14* does not appear to be targeted in the dysregulation of the DAP kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway.

Hypermethylation of *Apaf-1* has been detected in melanoma, (Soengas et al. 2001) and recently in acute leukaemia. (Roman-Gomez et al. 2004) As yet, there is no data on *Apaf-1* methylation in CLL, and our study is the first report of the absence of *Apaf-1* hypermethylation in CLL, consistent with the finding of Apaf-1 protein expression in CLL. (Winkler et al. 2005)

In summary, *DAP kinase* but not *p14* or *Apaf-1* is frequently targeted by methylation, suggesting an important role of DAP kinase dysregulation in the DAP kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway. The clinical behavior of CLL in Orientals is similar to Caucasian CLL patients.

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