Methylation of *CDKN2B* CpG islands is associated with upregulated telomerase activity in children with acute lymphoblastic leukemia

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Abstract. The aim of the present study was to investigate the association between methylation of cyclin-dependent kinase inhibitor 2B (CDKN2B) CpG islands and telomerase activity in children with acute lymphoblastic leukemia (ALL). A total of 72 children with ALL and 12 children with immune thrombocytopenia (ITP) were subjected to bone marrow aspiration and methylation-specific polymerase chain reaction analysis, and modified telomeric repeat amplification protocol assay analyses, to evaluate *CDKN2B* methylation and telomerase activity, respectively. The results of the present study demonstrated that, of these 72 children with ALL, 31 exhibited *CDKN2B* methylation at diagnosis (43.1%), whereas 41 exhibited no *CDKN2B* methylation (36.9%). However, no *CDKN2B* methylation was detected in the ITP controls. Furthermore, the mean level of telomerase activity was 39.52±39.33 total product generated (TPG) units in children with ALL, which was significantly increased compared with 2.49±2.27 TPG units in the ITP controls (P=0.002). The mean levels of telomerase were 49.09±44.43 and 29.99±32.43 TPG units in children with ALL with and without *CDKN2B* methylation, respectively (P=0.041), therefore children with ALL exhibited significantly increased levels of telomerase. The increased telomerase activity was significantly associated with increased risk of childhood ALL (P=0.023). A total of 22/31 children with ALL with methylated *CDKN2B* (71.0%) and 17/41 children with ALL with unmethylated *CDKN2B* (41.46%) exhibited increased telomerase activity (>15 TPG units). The results of the present study suggest that hypermethylation of *CDKN2B* CpG islands and hyperactivity of telomerase are common events in childhood ALL, and hypermethylation of *CDKN2B* CpG islands was significantly associated with upregulated telomerase activity (P=0.013).

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignancy of white blood cells that exhibits marked occurrence in children (1). Effective treatment options for children with ALL include chemotherapy, radiation, biological therapy and immunotherapy, alone or in combination, with a curable rate of >80%, although <20% patients with ALL remain incurable (1). However, the precise cause of ALL remains unclear, therefore there are difficulties in achieving long-term remission of ALL (2). Tumor cell proliferation is regulated by cell cycle progression and it is well established that cyclin-dependent-kinases (CDKs) serve a key role in cell cycle control in eukaryotes (3). The G_1/S transition, the most restrictive checkpoint for cell cycle progression and cell proliferation, requires the activation of cyclin D-CDK4 and cyclin E-CDK2 complexes (4). However, cyclin-dependent kinase inhibitor 2B (CDKN2B) (p15) and CDKN2A (p16) proteins are able to inhibit the activities of cyclin D-CDK4 and cyclin D-CDK6 complexes to negatively regulate cell cycle progression (5,6). Loss of function of these cyclin-dependent kinase inhibitors may lead to inappropriate cell cycle progression. Therefore, genetic alteration of the *CDKN2B* and *CDKN2A* genes has been investigated, and it has been demonstrated that their genes are localized at chromosomal locus 9p21 (7). Frequent hypermethylation was demonstrated in ALL; however, deletion of *CDKN2B* was rare (8). Hypermethylation of *CDKN2B* CpG islands occurred almost exclusively in hematological malignancies (9,10).

Telomerase is known to be a ribonucleoprotein in human cells that exhibits reverse transcriptase activity (11). Telomerase contains an RNA component to provide a template for the synthesis of repeated telomeric sequences. These repeats are attached to the ends of existing telomeres to maintain telomere lengths in chromosomes (12). Telomerase almost universally provides the molecular basis for cell proliferative capacity (13). Therefore, telomerase activity is present in almost all types

of malignant cell, including hematological malignancies; however, telomerase activity is essentially absent from the majority of wild-type somatic tissues (14). Simon *et al* (15) demonstrated that inactivation of CDKN2A/CDKN2B and pl4ARF-dependent signaling pathways, potentially in conjunction with telomerase activation, may be a critical step for meningioma cells to evade senescence and achieve immortalization. However, adenovirus-mediated overexpression of *CDKN2B* inhibited human glioma cell growth, induced replicative senescence and inhibited telomerase activity (16). Swellam *et al* (17) demonstrated that laryngeal squamous cell carcinoma progression was associated with an increase in telomerase activity; however, deletion of the *CDKN2A* and *CDKN2B* genes may assist in the prognosis and improved classification of patients for treatment. Other previous studies have indicated the importance of *CDKN2B* methylation and upregulated telomerase activity in the development of ALL (18-20). Therefore, in the present study, the association of *CDKN2B* methylation with telomerase activity in childhood ALL, and the association of alterations in *CDKN2B* methylation and telomerase activity with clinicopathological data from children with ALL were investigated. The present study aims to provide a molecular basis for the development of a novel strategy to manage ALL.

Table I. Patient characteristics.

Characteristic	Cases, n (%)
Gender	
Male	42 (58.33)
Female	30 (41.67)
Liver or spleen enlargement	51 (70.83)
CNS involvement	10 (13.89)
Immunotyping	
B-cell ALL	53 (73.61)
T-cell ALL	19 (26.39)
Risk group	
SR	27 (37.11)
IR	39 (54.16)
HR	6 (8.33)

CNS, central nervous system; ALL, acute lymphoblastic leukemia; SR, standard-risk group; IR, intermediate-risk group; HR, high-risk group.

Materials and methods

Patients and controls. The present study included 72 newly diagnosed children with ALL who received treatment at the Division of Hematology and Oncology, Shenzhen Children's Hospital (Shenzhen, China) between 1st October 2009 and 30th May 2013. The patients were participating in the ongoing Multicenter Trial of GD-2008 ALL protocol (21). All patients were diagnosed with ALL, and their age ranged between 2 and 12 years. The patients included 42 males and 30 females, 53 of which were B-lineage ALL and 19 T-lineage ALL. These patients were stratified into three risk groups according to the following criteria: i) Standard-risk group (SR), prednisone-good responders (PGR), age at diagnosis between 1 and 6 years, initial white blood cell count (WBC) of <$20x10^9$ cells/l, M1 marrow (<5% blasts) or M2 marrow (between 5 and 25% blasts) on day 15, and M1 marrow on day 33; ii) intermediate-risk group (IR), PGR, age at diagnosis <1 or ≥6 years and/or WBC ≥$20x10^9$ cells/l, M1 or M2 marrow on day 15, and M1 marrow on day 33, or fulfilling SR criteria, but with M3 marrow (>25% blasts) on day 15 and M1 marrow on day 33; iii) high-risk group (HR), prednisone-poor responders (PPR), and/or M2 or M3 marrow on day 33, or fulfilling IR criteria, but with M3 marrow on day 15. Therefore, there were 27/72 (37.11%) SR patients, 39/72 (54.6%) IR patients and 6/72 (8.3%) HR patients (Table I).

Bone marrow samples were obtained at the time of initial diagnosis prior to chemotherapy. Control bone marrow samples were collected from 12 children with immune thrombocytopenia (ITP) who did not exhibit any hematological malignancies or any other tumorous disease. The ITP patients were received treatment at the Division of Hematology and Oncology, Shenzhen Children's Hospital (Shenzhen, China) between 1st October 2009 and 30th May 2013, and their age ranged between 2 and 10 years. The patients included 4 males and 8 females. The control bone marrow samples were aspirated and stored in heparinized tubes, mononuclear cells were separated using Ficoll-Isopaque (Axis-Shield Diagnostics, Ltd., Oslo, Norway) density centrifugation (22), and stored at -80˚C until use.

Written informed consent was obtained from the parents of the patients, and the present study was approved by the Shenzhen Children's Hospital and was conducted according to institutional guidelines in accordance with The Declaration of Helsinki.

Methylation-specific polymerase chain reaction (MSP). Genomic DNA was extracted from the mononuclear cells using a Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) and treated with sodium bisulfite as described previously (23) using the reagents from the EpiTect Bisulfite kit (Qiagen, Inc., Valencia, CA, USA). Bisulfite treatment induced deamination of unmethylated cytosines, converting unmethylated CpG sites into uracil guanine pairs (UpG), while not altering the methylated sites. Therefore, methylated and unmethylated DNA sequences were able to be distinguished using MSP. 1 μg of the genomic DNA samples were amplified using the polymerase chain reaction (PCR) from the EpiTect MSP Kit (Qiagen, Inc., Valencia, CA, USA) with two pairs of gene promoter-specific primers that recognize the methylated and the unmethylated CpG sites. The primers used to detect the methylated *CDKN2B* promoter were 5'-GCGTTCGTATTTTGCGGTT-3' (forward) and 5'-CGT ACAATAACCGAACGACCGA-3' (reverse), whereas primers to detect the unmethylated *CDKN2B* promoter were 5'-TGT GATGTGTTTGTATTTTGTGGTT-3' (forward) and 5'-CCA TACAATAACCAAACAACCAA-3' (reverse) as described in a previous study (24). The PCR amplification conditions were as follows: Initial denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec; and a final extension at 72˚C for 10 min. PCR products were then

separated using 1.5% agarose gels and post-stained with 1% ethidium bromide. 50 bp DNA Ladder (Takara Bio, Inc., Otsu, Japan) was used as marker and images were captured using ultraviolet illumination.

Assessment of telomerase levels. Telomerase levels was detected by the TRAPeze® Telomerase Detection Kit (Millipore-Chemicon, Billerica, MA, USA). A total of $1x10^6$ bone marrow mononuclear cells were incubated in 200 μl ice-cold 1X CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-Mercaptoethanol, 0.5% CHAPS, 10% Glycerol) for 30 min and centrifuged at 12,000 x *g* for 30 min at 4˚C. Cell lysates were resuspended in 50 μl reaction mixture (5.0 μl 10X TRAP buffer, 1.0 μl 50X dNTPs mix, 2.0 μl Ts primer, 1.0 μl TRAP Primer mix, 2 unit *Taq* enzyme, 2 μl cell lysates) and subjected to 33 PCR cycles of 94˚C for 30 sec and 60˚C for 30 sec, and a final extension at 72˚C for 10 min using a thermocycler (Eppendorf, Hamburg, Germany). The PCR primers used to detect telomerase were 5'-AATCCGTCGAGCAGA GTT-3' (forward) and 5'-CCCTTACCCTTACCCTTACCC TAA-3' (reverse). The PCR products were separated using gel electrophoresis in a 10% acrylamide gel and 0.2% Ag-stained (GE Healthcare Life Sciences, Shanghai, China), and the telomerase-positive signals were quantified using the Gel Doc 200 gel analytical system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Negative and heat-inactivation controls were also amplified in each experiment.

Assessment and quantification of telomerase activity. A TRAPeze® XL telomerase detection kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to quantify the telomerase activity according to the method of Kim *et al* (25). Specifically, a standard curve was constructed from the measurements of the reactions using the kit's TSR8 quantification control template to calculate the amount of telomerase primers with telomeric repeats extended by telomerase in each cell extract. The standard curve constructed using the TSR8 template was used to validate quantitative results of testing samples. A total of 5,000 bone marrow mononuclear cells from each patient were subjected to the telomeric repeat amplification protocol (TRAP) assay, and the reaction products (50 μl of each) from the TRAP assay were mixed with 150 μl buffer 1 (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 2 mM $MgCl_2$) and transferred to a 96-well plate. The optical density fluorescence value (535 nm) was then measured using a fluorescence microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Each unit of total product generated (TPG) corresponded to the number of telomerase primers.

Statistical analysis. All experiments were repeated 3 times. SPSS software (version 19.0; SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses and the results are presented as the mean ± standard deviation. A Kruskal-Wallis one-way analysis of variance was performed to analyze nonparametric variance to compare the ALL risk groups, and the Mann-Whitney U test was used to analyze nonparametric differences between the ALL group and the ITP group, and the Mann-Whitney U test was also used to analyze nonparametric differences the patients with ALL that exhibited *CDKN2B* methylation and those that did not. Association of *CDKN2B* methylation status with telomerase activity was assessed using the χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

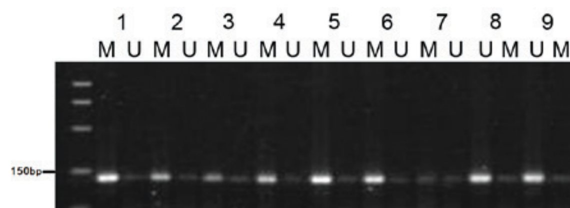

Figure 1. *CDKN2B* methylation status in childhood ALL and ITP. Genomic DNA from bone marrow mononuclear cells was subjected to MSP analysis of *CDKN2B* methylation. Lanes 1-7, MSP results of *CDKN2B* methylation in seven patients with ALL; lanes 8 and 9, MSP results of *CDKN2B* methylation from two ITP controls. All samples were amplified using MSP with methylation-specific primers and unmethylation-specific primers. *CDKN2B*, cyclin-dependent kinase inhibitor 2B; ALL, acute lymphoblastic leukemia; ITP, immune thrombocytopenia; MSP, methylation-specific polymerase chain reaction; M, methylated *CDKN2B*; U, unmethylated *CDKN2B*.

Results

Methylation of p15 CpG islands and telomerase activity in childhood ALL. Of the 72 children with ALL examined, there were 31 cases that exhibited *CDKN2B* methylation and 41 that did not, which resulted in a *CDKN2B* methylation frequency of 43.10%. However, no *CDKN2B* methylation was detected in the ITP controls (Fig. 1). Furthermore, levels of telomerase activity were between 1 and 140 TPG units in the children with ALL (39.52±39.33 TPG units), whereas telomerase activity levels of the 12 ITP controls were between 0 and 7.5 TPG units (2.49±2.27 TPG units), indicating that telomerase activity was significantly upregulated in ALL compared with controls (P=0.002; Table II). Fig. 2 presents an image of the gel from the telomerase activity assay).

There were 31 cases that exhibited *CDKN2B* methylation and 41 that did not in 72 children with ALL examined. Telomerase activity levels were 49.09±44.43 and 29.99±32.43 in patients with ALL that exhibited *CDKN2B* methylation and those that did not, respectively, revealing a significant association between increased telomerase activity and *CDKN2B* methylation in patients with ALL (P=0.041). The maximum level of telomerase activity in the ITP controls was 7.5 TPG units, therefore twice this amount was used as the threshold value, i.e., 15 TPG units. Takaishi *et al* (26) demonstrated the predictive worth of this threshold value in the evaluation of telomerase activity in precancerous hepatic nodules. In the present study, telomerase hyperactivity was considered to be indicated by an activity level of >15 TPG units. A significant increase in telomerase hyperactivity was identified in patients with ALL that exhibited *CDKN2B* methylation (22/31; 71.0%) compared with patients with ALL that did not (17/41; 41.5%) (P=0.013; Table III).

Discussion

Cell cycle progression is highly regulated and there are key checkpoints to control cell growth, proliferation and apoptosis.

Table II. Association of telomerase activity with various ALL risk groups and controls.

	n	Mean ± standard deviation	Kruskal-Wallis one-way ANOVA	Mann-Whitney U-test
Control	12	2.49±2.27		P=0.002
ALL cases group	72	39.52±39.33		
SR	27	24.99±28.90	P=0.023	
IR	39	43.60±40.38		
HR	6	78.33±37.16		

The Kruskal-Wallis one-way ANOVA P-value was calculated among the different risk groups. The Mann-Whitney U-test P-value was calculated between the ALL cases and the controls. ALL, acute lymphoblastic leukemia; SR, standard-risk group; IR, intermediate-risk group; HR, high-risk group.

Table III. Association of telomerase activity with *CDKN2B* methylation.

Telomerase activity	Methylated *CDKN2B*	Unmethylated *CDKN2B*	Total	χ^2	P-value
Decreased	9	24	33		
Increased	22	17	39	6.19	0.013

CDKN2B, cyclin-dependent kinase inhibitor 2B.

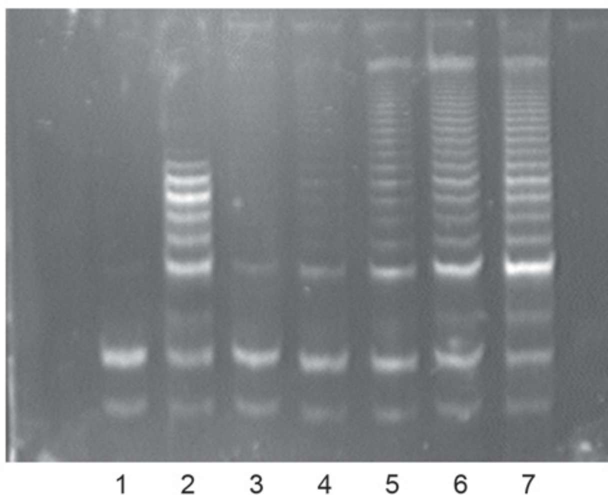

Figure 2. Telomerase activity of childhood ALL and ITP, detected by the Ag-staining method, Telomerase activity was considered positive when a ladder of products was observed with 6-bp increments. Telomerase activity was not present in the heat-inactivation control and the ITP control (lane 1 and lane 3), Telomerase activity was detected three of the four ALL samples (lanes 5-7). ALL, acute lymphoblastic leukemia; ITP, immune thrombocytopenia.

The G_1/S checkpoint is critical for cells to enter the G_2 phase and proliferate, and the activity of CDKs facilitates the entry of cells into S-phase (27). The protein encoded by the *CDKN2B* gene is able to inhibit kinase activity and thereby suppress cell proliferation (28). Previous studies have demonstrated that *CDKN2B* is a tumor-suppressor gene (29,30), although the underlying molecular mechanisms by which *CDKN2B* is modulated and transcribed remain to be characterized in ALL. In the present study, aberrant hypermethylation of the *CDKN2B* gene promoter CpG islands was assessed in ALL samples and it was identified that *CDKN2B* methylation occurred in 41.26% of children with ALL, whereas there was no *CDKN2B* methylation in the bone marrow of control samples. These results indicated that *CDKN2B* methylation is a common event and associated with the development of childhood ALL. The methylation of gene promoters contributes to silencing of gene expression, thus aberrant *CDKN2B* hypermethylation may inhibit *CDKN2B* transcription and expression, and lead to loss of cell cycle control and ALL cell proliferation. Furthermore, telomerase levels and activity were evaluated in ALL samples, and it was demonstrated that telomerase activity was significantly upregulated in ALL compared with controls. In wild-type cells, telomeres, the ends of linear chromosomes, shorten with each round of DNA replication (each cell cycle), and loss of telomeric DNA may lead to cellular senescence, a state in which cells are unable to divide (31). To prevent this phenomenon, cancer and stem cells are able to maintain their telomeres; for example, by expressing telomerase, an enzyme that is able to extend telomeres (32). Activity of telomerase is primarily associated with velocity of cell division, cell cycle progression and cell differentiation. Upregulation of telomerase in cells is considered to be responsible for immortalization and carcinogenesis, and it may be a molecular marker of vigorous cell proliferation (33). Therefore, the results of the present study further confirmed the importance of telomerase in human malignancy, by demonstrating up-regulated telomerase activity in ALL, and are consistent with a previous study (34).

Inactivation of tumor suppressor genes or activation of telomerase frequently occurs in human cancer (35,36). The cell cycle regulator *CDKN2B* has been demonstrated to be an important tumor suppressor gene with a direct effect on inhibition of cell cycle progression (37,38). In the present study, *CDKN2B* suppression was demonstrated to be associated with increased telomerase levels and activity. Previous studies have demonstrated that overexpression of genes coding for tumor protein 53, transcription factor E2F, CDKN2A, CDKN1A or CDKN2B individually may induce cell premature growth arrest, which was accompanied by inhibition of telomerase activity in head and neck squamous cell carcinoma and human glioma cell lines (39-41). This previous evidence led to the investigation of the potential association of *CDKN2B*

hypermethylation with upregulated telomerase expression in childhood ALL. The results of the present study demonstrated that patients with ALL that exhibited methylated *CDKN2B* were associated with telomerase hyperactivity compared with patients with ALL that did not. Furthermore, the mean telomerase activity in patients with ALL that exhibited methylated *CDKN2B* was significantly increased compared with patients with ALL that did not, suggesting that inactivation of *CDKN2B*-dependent pathways, potentially in conjunction with telomerase activation, may be a critical step in the promotion of ALL progression and ALL cell immortalization.

The results of the present study suggested that hypermethylation of *CDKN2B* promoter CpG islands and hyperactivity of telomerase are common events in childhood ALL, and that hypermethylation of *CDKN2B* promoter CpG islands is associated with upregulation of telomerase level and activity. Furthermore, an association between the telomerase activity and ALL risk categories was identified. However, the present study represents a proof-of-principle, and further in-depth work is required to reveal the underlying molecular mechanisms of the association between telomerase and CDKN2B, and how they coordinate to promote ALL development and progression. The results of the present study have identified the association between *CDKN2B* CpG island methylation and upregulated telomerase activity in children with ALL. Further studies are required to investigate whether telomerase and CDKN2B molecules may be used as biomarkers for predicting the progression or treatment success of ALL, and whether targeting these two molecules is useful as a novel strategy in the clinical management of ALL.

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