Aberrant *EPHB4* gene methylation and childhood acute lymphoblastic leukemia

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Abstract. The present study aimed to investigate the association between aberrant DNA methylation of the promoter region of the ephrin type-B receptor 4 (*EPHB4*) gene and the development of childhood acute lymphoblastic leukemia (ALL). Bisulfite sequencing polymerase chain reaction (BSP) was performed to determine the methylation density of cytosine-guanine pair islands in the promoter region of *EPHB4*, in bone marrow samples from 40 children with ALL. The mRNA and protein expression levels of *EPHB4* were detected using reverse transcription-quantitative polymerase chain reaction and western blot analysis. A total of 10 children with idiopathic thrombocytopenic purpura (ITP) were recruited as controls. The results revealed that the average methylation density of the bone marrow samples from the patients with ALL was significantly higher, compared with the patients with ITP (P=0.046). The relative mRNA expression levels of *EPHB4* in the patients with ITP (25.08±4.03) and the patients with ALL without methylation (12.33±2.16) were significantly higher, compared with that observed in the patients with ALL with methylation (6.48±2.73; P<0.01). Pearson analysis revealed a significant negative linear correlation between *EPHB4* gene methylation and its expression levels (r=-0.957; P<0.01). Western blot analysis indicated that *EPHB4* protein expression levels were low in the methylated ALL samples. An evaluation of the two-year disease-free survival (DFS) of the patients with ALL was performed, which revealed that the patients with unmethylated ALL exhibited a significantly higher two-year DFS rate, as compared with patients with methylated ALL (P=0.036). These results suggest that the methylation of the *EPHB4* gene is prevalent in childhood ALL and may result in expressional inactivation, which consequently promotes ALL pathogenesis and is associated with an unfavorable prognosis. Therefore, the *EPHB4* gene may function as a potential tumor suppressor in childhood ALL.

Introduction

Leukemia is the most prevalent malignant childhood tumors, accounting for 35% of all malignant tumors in patients <15 years old with an incidence rate of 4 in 100,000. There are ~15,000 new cases of leukemia in China every year. Acute lymphoblastic leukemia (ALL), as the most prominent leukemia subtype, accounts for 75% of pediatric leukemia cases (1). ALL is characterized by the accumulation of immature lymphoblastic cells due to genomic abnormalities, blocking the differentiation of early lymphoid progenitors (2). The genetic changes that underlie the pathogenesis of ALL have previously been examined in order to elucidate their relative contributions (3). However, it has been established that aberrant epigenetic alterations, in particular the DNA methylation of promoter-associated cytosine-guanine pair (CpG) islands (CGIs), are frequent events in the progression of ALL and are associated with an unfavorable prognosis (4-7). A number of previous studies have indicated that the epigenetic silencing of tumor suppressor genes due to the DNA methylation of CGIs is an important mechanism underlying leukemogenesis in ALL (8-10).

The erythroprotein-producing hepatoma amplified sequence (Eph) receptors and their ephrin ligands comprise the largest subfamily of receptor tyrosine kinases and are involved in various cellular developmental processes, including embryonic, hematopoietic and vascular development, as well as tumorigenesis (11,12) Previous studies have suggested that Eph receptors exhibit a tumor suppressor function in certain types of cancer, including colorectal and breast cancer (13,14). Aberrant DNA methylation has been reported to be an important contributor to the inactivation of Eph receptors and ephrin genes, a process which subsequently promotes tumor progression (6,15,16). Kuang *et al* (17) performed a comprehensive analysis of Eph/ephrin methylation using bisulfite pyrosequencing, revealing that 15 of the Eph/ephrin family genes are frequently hypermethylated and are associated with gene

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inactivation in ALL bone marrow tissues and leukemia cell lines. These genes include ephrin type-B receptor 4 (*EPHB4*), which is hypermethylated in ALL, resulting in transcriptional silencing (16). In addition, apoptosis induction and the growth suppression of tumor cells are observed following the restoration of *EPHB4* gene expression levels using lentiviral transduction (16).

The *EPHB4* gene was originally identified to be abundantly expressed in human bone marrow CD34+ cells, and is highly expressed by primary T cells (18). Previous studies have reported that the interaction between *EPHB4* and ephrin-B2 is involved in the inhibition of T-cell proliferation by activating the Src, phosphoinositide 3-kinase (PI3K), Abelson murine leukemia (Abl) and N-terminal kinase signaling pathways (19). It has also been suggested that T cells are important in mediating antitumor immune responses; therefore, insufficient priming of CD4+ T cells may result in impaired specific anti-leukemia immunity and subsequently enhance the invasive ability of leukemic cells (20,21). The role of *EPHB4* in the modulation of T-cell physiology implies that *EPHB4* gene methylation may be associated with ALL pathogenesis (22). A previous study demonstrated that the *EPHB4* gene was highly methylated and transcriptionally silenced in CEM cell lines, whereas the suppression of cell growth and promotion of apoptosis was observed upon addition of 5-aza-2'-deoxycytidine, a demethylating agent (23). Therefore, the *EPHB4* gene is a promising candidate tumor suppressor in ALL. In the present study, the DNA methylation status of promoter-associated CGIs in the *EPHB4* gene, and the mRNA and protein expression levels of *EPHB4*, were analyzed in newly diagnosed cases of childhood ALL. Furthermore, the two-year disease-free survival (DFS) of the patients was examined in order to reveal the clinical relevance of aberrant *EPHB4* methylation and its role in the pathogenesis and prognosis of childhood ALL.

Materials and methods

Clinical samples. The present study included 40 newly diagnosed patients with ALL who were treated at the Division of Hematology and Oncology at Shenzhen Children's Hospital, Department of Pediatrics (Shenzhen, China), between 1st October 2010 and 30th September 2012. Bone marrow biopsy samples were obtained from the patients with ALL at the time of initial diagnosis, prior to chemotherapy administration. All patients participated in the ongoing Multicenter Trial of GD-2008 ALL protocol for childhood ALL (trial no. NCT00846703). All the patients were diagnosed and classified according to the ALL International Berlin-Frankfurt-Münster 2002 protocol (24) and the duration of follow-up was 2 years (patient characteristics are summarized in Table I). Treatments administered to the patients with ALL were carried out according to the GD-2008 ALL protocol (25) which is based on a modification of the ALL IC-BFM 2002 protocol. For all patients with ALL the duration of therapy was 104 weeks. During the first stage of remission induction, all patients were treated with vincristine intravenously (1.5 mg/m²/day; Shanghai Hualian Pharmacy Co., Ltd., Shanghai, China), dexamethasone orally or intravenously (6 mg/m²/day; SPH Sine Pharmaceutical Co., Ltd., Shanghai,

Table I. Patient characteristics.

Characteristic	ALL	ITP
Mean age, years (range)	4.2 (0.5-12.6)	5.5 (2.0-10.5)
Sex		
Male	18 (40)	4 (10)
Female	22 (40)	6 (10)
Median WBC count, 10^9/l	15.8	9.3
Immunophenotype		
T-ALL	8 (40)	
B-ALL	32 (40)	
Complete remission	38 (40)	
Relapse	3 (40)	

ALL, acute lymphoblastic leukemia; ITP, idiopathic thrombocytopenic purpura; T, T cell; B, B cell; WBC, white blood cell; PCR, polymerase chain reaction.

China), daunorubicin intravenously guttae (30 mg/m²/day; Zhejiang Hisun Pharmaceutical Co., Ltd., Zhejiang, China) and L-asparaginase intravenously guttae (5,000 IU/m²/day; GuangZhou BaiYunShan Pharmaceutical Holdings Co., Ltd, Guangzhou, China), following prednisone prophase. In the second stage of remission induction, patients were treated with cyclophosphamide intravenously guttae (1,000 mg/m²/day; Shanghai Hualian Pharmacy Co., Ltd.), Ara-c intravenously or intramuscularly (75 mg/m²/day; Pfizer, Inc., New York, NY, USA.), 6-mercaptopurine orally (6-MP; 60 mg/m²/day; Zhejiang Zhebei Pharmaceutical Co., Ltd., Zhejiang, China) and methotrexate intrathecally (age-adapted dose; SPH Sine Pharmaceutical Co., Ltd.). Oral 6-MP (25 mg/m²/day) and intravenous methotrexate (5,000 mg/m²) were used in consolidation therapy following induction therapy. The subsequent reintroduction and maintenance therapy was based on the same scheme as the introduction and consolidation stages. Control bone marrow samples were obtained from 10 patients with idiopathic thrombocytopenic purpura (ITP) of a similar age range to the patients with ALL, who did not exhibit hematological malignancy or any other tumorous disease. The Ethics Committee of Shenzhen Children's Hospital approved the study. Written informed consent for recruitment to the study was provided by patients and/or their parents, following the Shenzhen Children's Hospital Ethical Guidelines in accordance with the Declaration of Helsinki. The bone marrow samples were aspirated and stored in heparinized tubes; mononuclear cells were separated using Ficoll-Paque density centrifugation at 300-540 x g for 15 min at 4°C (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) and stored at -80°C.

DNA extraction and bisulfite sequencing polymerase chain reaction (BSP). DNA was extracted from the mononuclear cells using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). The genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen, Inc., Valencia, CA, USA), according to a previous protocol (26). Bisulfite treatment induces deamination of

Table II. Primer sequences.

A, Primers used for bisulfite pyrosequenscing

Primer	Sequence	Amplification product size	Amplified region	Covered CpG sites
EPHB4 forward (5'-3')	GTTTGTTTTGGGGGTTTTTGGGTTTTAG	329bp	(-305, +24)	31
EPHB4 reverse (5'-3')	AACAAAAACTAAACTACACTAAAACTAAACC			

B, Primers for quantitative polymerase chain reaction

Primer	Sequence
EPHB4 forward (5'-3')	GTCCTGGTGGTCATTGTGGT
EPHB4 reverse (5'-3')	TGTCCGTGTTTGTCCGAATA
GADPH forward (5'-3')	AGAAGGCTGGGGGCTCATTTG
GADPH reverse (5'-3')	AGGGGCCATCCACAGTCTTC

CpG, cytosine-guanine pair; bp, base pair; *EPHB4*, ephrin type-B receptor 4.

unmethylated cytosines, converting unmethylated CpG sites to uracil-guanine pairs (UpG) with no effect on the methylated sites (27). Previously described primer sequences for *EPHB4* (23) were used for bisulfite pyrosequencing [reaction mixes (final volume, 150 μl) included: 30 μl RNA-free water, 35 μl DNA Protect buffer (Qiagen, Inc.), DNA (between 1 ng and 2 μg), 85 μl bisulfite mix] and primer sequences are provided in Table II. The thermocycling conditions were as follows: 95℃ for 10 min; 40 cycles of 95℃ for 30 sec, 60℃ (~5℃ below the melting temperature of the primers) for 15 sec and 72℃ for 1 min. To evaluate the pyrosequencing results of the mononuclear cell samples, BSP was performed using the same forward and reverse primers as applied for pyrosequencing. The PCR products were cloned into a pMD19-T vector (Takara Biotechnology Co., Ltd., Dalian, China), and individual clones were sequenced at BGI Genomic Organization (Shenzhen, China). Five clones were sequenced in each ALL and ITP sample. The methylation density was calculated as the mean value of the five clones.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA (1 μg) was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse transcription was performed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Inc.) and random hexamer primers (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), according to the manufacturer's protocols. SYBR®-Green Real-Time PCR Master mix (Toyobo Co., Ltd., Osaka, Japan) and the aforementioned *EPHB4* primers in Table II were used for RT-qPCR analysis of mRNA expression levels, according to the manufacturer's protocol (Toyobo, Co., Ltd.). Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (28) and normalized to the reference gene GAPDH with primers as described in Table II. The thermocycling conditions were as follows: 95℃ for 10 min; 40 cycles of 95℃ for 30 sec, 60℃ (5℃ below the melting temperature of the primers) for 15 sec and 72℃ for 32 sec.

Western blot analysis. The protein samples were analyzed by western blotting using a previously described method (29). Briefly, the mononuclear cell samples were washed with PBS and lysed using a radioimmunoprecipitation assay buffer containing a protease inhibitor (1:100; Thermo Fisher Scientific, Inc.), 50 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 1% Na deoxycholate, 0.5 M EDTA and 0.1% NP-40. The protein lysates were rotated using a rotator at 50 rmp at 4℃ for 1 h and the insoluble proteins were subsequently removed by centrifugation at 13,400 x g for 10 min at 4℃. A bicinchoninic acid protein assay kit was used to determine the soluble protein concentration. Protein products (8-10 μg/μl; 50-60 μg total) were separated using 10% SDS-PAGE on a 10% gel and subsequently transferred overnight onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) using SDS-transfer buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked for 1 h using a western-blocking reagent (Bio-Rad Laboratories, Inc.) at room temperature, prior to protein detection using a specific monoclonal *EPHB4* antibody (1:1,000; cat. no. sc-130081; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4℃, followed by incubation with an horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1,000; cat. no. 6120-05; SouthernBiotech, Birmingham, AL, USA) overnight at 4 ℃. The Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Shanghai, China) was used to visualize the blots, according to the manufacturer's protocol, with a 5 min exposure to SuperRX X-ray film (Fujifilm Investment Co., Ltd., Shanghai, China). Protein band density was quantified used ImageJ (version 2.1.4.7, National Institutes of Health, Bethesda, MD, USA). Anti-GAPDH (1:1,000; cat. no. ab9485; Abcam, Cambridge, UK) antibody, incubated at 4℃ for 1 h, was used as a loading control.

Statistical analysis. Statistical analysis was performed using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA). Results

were presented as the mean ± standard deviation of values from each group. The χ^2 test and Fisher's exact test were used to compare gene methylation frequencies between the ALL group and the ITP control group. One-way analysis of variance with the Bonferroni correction was performed to determine the variation in relative mRNA expression levels between methylated ALL, unmethylated ALL and ITP groups. Pearson correlation analysis was applied to determine the correlation between DNA methylation and *EPHB4* mRNA expression levels. Kaplan-Meier analysis was performed to analyze the association between the gene methylation status and DFS in patients with ALL, and the log-rank test was used to examine the variation between the methylated and unmethylated ALL groups. P<0.05 was considered to indicate a statistically significant difference.

Results

EPHB4 *methylation status in patients with ALL, compared with the control ITP group.* BSP was used to detect the methylation profile of *EPHB4* in patients with ALL and ITP. The methylation density (proportion of methylated CpG sites within a specific promoter region) of the *EPHB4* gene in the ALL bone marrow samples ranged from 7.94 to 36.50%, and the average methylation density was 15.05%. A 15% methylation density was used as the cut-off value to classify a bone marrow sample as methylated (16). The number of methylated cases in the ALL group was 12 (30%), whereas the *EPHB4* gene was determine to be unmethylated in all the ITP bone marrow samples, in which the methylation density ranged from 0-7.14% and the average methylation density was 3.47%. The frequency of *EPHB4* gene methylation was significantly higher in the ALL samples compared with the ITP samples (P=0.046). The status of methylation density and the bisulfite sequencing results for *EPHB4* are presented in Figs. 1 and 2. The sequence data for the *EPHB4* gene have been submitted to the GenBank® database under the accession number NM_004444.

The expression levels of the EPHB4 *gene in the patients with ALL and the control ITP group.* In order to investigate the association between *EPHB4* DNA methylation and gene expression, mRNA levels were detected using RT-qPCR in the ALL and the control ITP bone marrow samples. The relative *EPHB4* mRNA expression levels in the ITP patients and the patients with unmethylated ALL (25.08±4.03 and 12.33±2.16, respectively) were significantly higher (P<0.01), compared with those observed in the patients with methylated ALL (6.48±2.73). The mRNA expression levels in the ALL samples were markedly lower (P<0.01), as compared with the ITP samples (Fig. 3). Pearson correlation analysis revealed a negative linear correlation between *EPHB4* gene methylation and its expression (r=-0.957; P<0.001; Fig. 4). Additionally, western blot analysis was performed to examine the protein expression levels of the *EPHB4* gene, demonstrating that its protein production was negatively associated with the methylation level of the gene (Fig. 5). Collectively, these results suggested that the DNA methylation of the *EPHB4* gene is associated with the suppression of its expression.
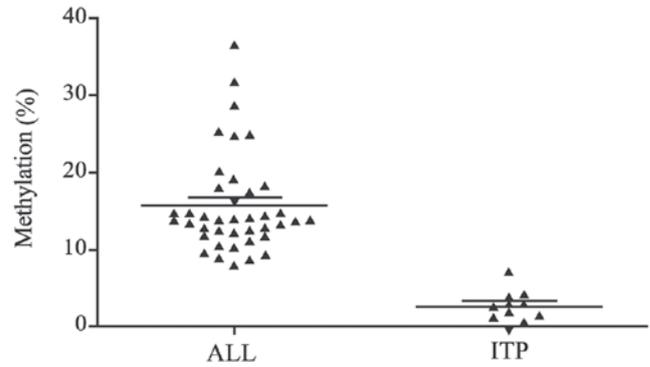

Figure 1. Methylation density of the ephrin type-B receptor 4 (*EPHB4*) gene in ALL and ITP groups. The average methylation density was 15.05% and 3.47% in the ALL and the ITP groups, respectively. The frequency of *EPHB4* gene methylation was significantly increased in the ALL samples compared with the ITP samples (P=0.046) A 15% methylation density was used as the threshold value of methylation. ALL, acute lymphoblastic leukemia; ITP, idiopathic thrombocytepenic purpura.
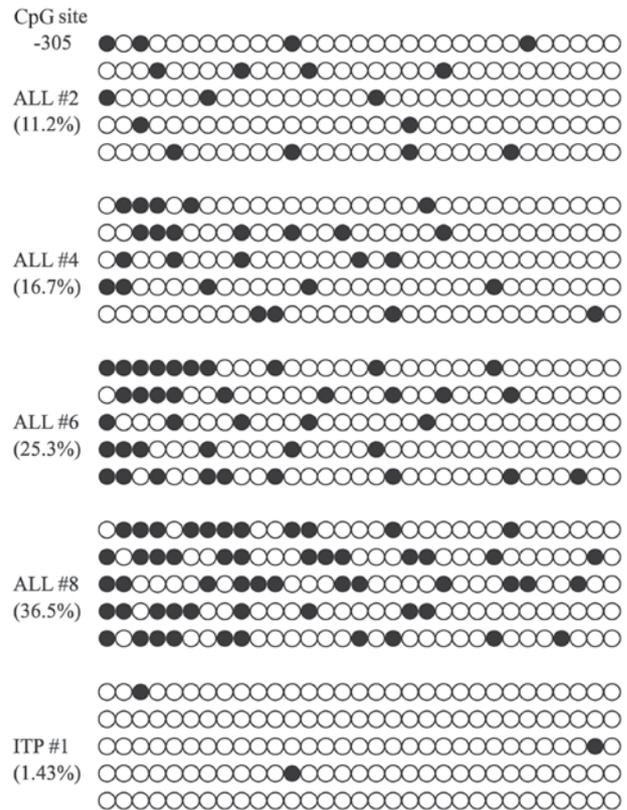

Figure 2. Methylation status of the promoter region of the ephrin type-B receptor 4 (*EPHB4*) gene as analyzed by bisulfite sequencing polymerase chain reaction. Each row of circles indicates the sequence of an individual clone; ○ represents an unmethylated CpG site and ● represents a methylated CpG site. EPHB4 was more frequently methylated in ALL samples compared with ITP samples. CpG, cytosine-guanine pair; ALL, acute lymphoblastic leukemia; ITP, idiopathic thrombocytopenic purpura.

The impact of EPHB4 gene methylation on the prognosis of patients with ALL. To evaluate the clinical impact of *EPHB4* methylation on patients with ALL, the present study analyzed the association between EphB4 methylation and the following variables: Age, sex, white blood cell count,
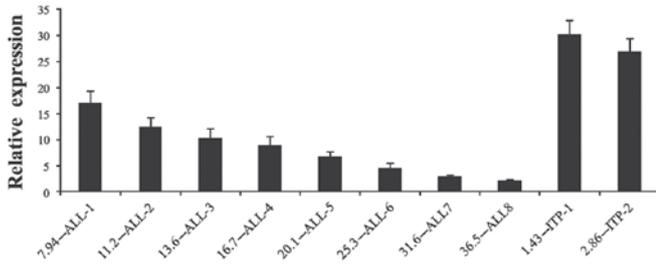
Figure 3. Relative EPHB4 mRNA expression levels, as determined by reverse transcription-quantitative polymerase chain reaction, in bone marrow samples representing various methylation levels in ALL and ITP. mRNA expression is presented as the fold change relative to GAPDH expression. Methylation densities (%) of representative samples are provided on the x-axis. The relative EPHB4 mRNA expression level was significantly increased in ITP samples (P<0.01, means of all ITP samples vs. means of all ALL samples). ALL, acute lymphoblastic leukemia; ITP, idiopathic thrombocytopenic purpura; EPHB4, ephrin type-B receptor 4.
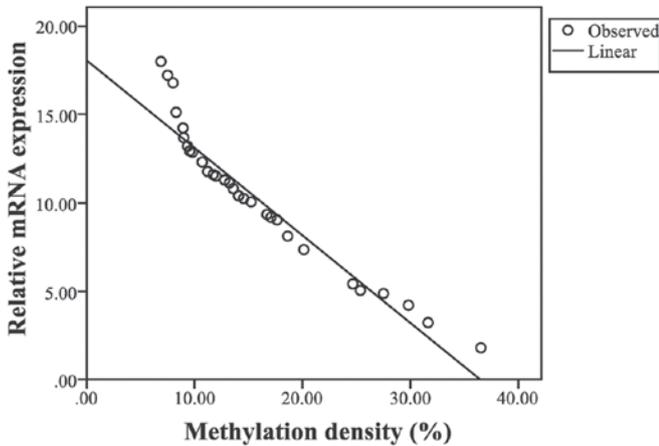

Figure 4. Pearson correlation analysis of EPHB4 gene methylation and expression levels, revealing a negative linear correlation between EPHB4 gene methylation and its expression (r=-0.957; P<0.001). EPHB4, ephrin type-B receptor 4.
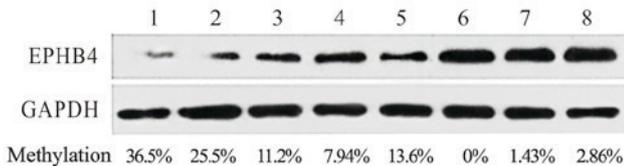

Figure 5. Western blot analysis of EPHB4 protein expression levels in the ALL and ITP bone marrow samples (ALL samples: 1-5; ITP samples: 6-8). The methylation density of each sample is presented below its corresponding lane. The protein production of EPHB4 gene was negatively associated with gene methylation level. ALL, acute lymphoblastic leukemia; ITP, idiopathic thrombocytopenic purpura; EPHB4, ephrin type-B receptor 4.

French-American-British classification, immunophenotype and Berlin-Frankfurt-Münster risk classification. No significant correlation was observed between EPHB4 gene methylation and any of these variables (Table III). Kaplan-Meier analysis was performed to investigate the impact of gene methylation status on the overall survival of patients with ALL. These patients were divided into methylated and unmethylated groups by using a methylation density of 15% as the cut-off value.

The two-year DFS rate of the patients with unmethylated ALL (92.3±7.4%) was significantly higher, compared with that of the methylated patients with ALL (68.4±1.58%; P=0.036; Fig. 6). These results suggest a trend toward a poorer prognosis in patients with EPHB4 gene methylation. However, an increased observation time and a larger sample size are required in order to evaluate the predictive role of EPHB4 methylation in ALL prognosis.

Discussion

DNA hypermethylation in promoter CGIs has been revealed as a predominant mechanism, by which tumor suppressors are inactivated in various types of cancer (8). The potential mechanisms underlying the loss of EPHB4 expression include promoter hypermethylation, chromosomal abnormalities and transcriptional repression (30). Gene inactivation caused by promoter hypermethylation in the Eph family has been implicated in the pathogenesis and progression of various tumors (30). In the present study, the methylation status of the EPHB4 gene was detected in bone marrow samples from 40 newly diagnosed patients with ALL, and 10 patients with ITP. Using BSP, it was demonstrated that the EPHB4 gene was methylated significantly more frequently in the ALL samples, compared with the ITP controls (P=0.046). The methylation density in the ALL bone marrow samples ranged from 7.94 to 36.50%, the average methylation density was 15.05% and methylation was present in 30% of the samples. By contrast, the EPHB4 gene was classified as unmethylated in all the ITP controls, with the methylation density ranging from 0-7.14%. These data suggest that EPHB4 gene methylation is prevalent in patients with ALL, concordant with a previous study (17). The present study provided additional evidence that EPHB4 gene methylation may be important in the development of childhood ALL; however, the methylation level in the present study was slightly lower compared with that detected by Kuang et al (17). This may be due to variation in patient ethnic background or in the CpG sites examined. A previous study reported that the expression levels of EPHB4 varied significantly between Caucasian and African-American patients (31). Therefore, a larger scale study, including additional geographic regions and performed under homogenous conditions, is required to demonstrate that the hypermethylation of EPHB4 gene is a frequent event during the pathogenesis of childhood ALL.

The EPHB4 expression profile was subsequently evaluated using RT-qPCR. The relative mRNA expression levels of EPHB4 in patients with ITP and ALL without methylation were significantly higher, compared with those detected in the patients with methylated ALL (P<0.01). Pearson analysis revealed a significant negative linear correlation between EPHB4 gene methylation and its expression levels (r=-0.957; P<0.01; Fig. 3). As presented in Fig. 4, there were decreased EPHB4 protein expression levels, as examined by western blot analysis, in the methylated ALL samples, with higher levels of expression in the ITP samples. A previous study demonstrated that the EPHB4 gene in the CEM T-cell line was able to be restored by the methyltransferase inhibitor 5-aza-2'-deoxycytidine, resulting in increased mRNA expression levels and protein production (23). Furthermore, decreased cell proliferation and increased cell

Table III. Correlations between *EPHB4* methylation status and clinical variables.

Clinical variables	*EPHB4* methylation status			
	+	-	χ^2	P-value
Age, years				0.833
<6	8	16	0.045	
>6	4	12		
Sex, n				0.267
Male	7	11	1.231	
Female	5	17		
WBC count				0.499
<50x10^9/l	9	25	0.458	
≥50x10^9/l	3	3		
FAB classification, n				0.348
L1	7	18	2.111	
L2	3	9		
L3	2	1		
Immunophenotype, n				0.204
T-ALL	4	3	1.616	
B-ALL	8	25		
BFM risk, n				0.956
SR	4	8	0.091	
IR	6	15		
HR	2	5		

EPHB4, ephrin type-B receptor 4; WBC, white blood cell; FAB, French-American-British; BFM, Berlin-Frankfurt-Münster; SR, standard risk; IR, intermediate risk; HR, high risk; ALL, acute lymphoblastic leukemia; T, T cell; B, B cell.

apoptosis were observed upon the reintroduction of *EPHB4* gene expression (21). Taken together, these results support the hypothesis that promoter hypermethylation and epigenetic gene silencing may be an important molecular mechanism in the progression of ALL, and that the *EPHB4* gene may be considered as a candidate tumor suppressor. Regarding the overall mechanisms underlying Eph family gene inactivation, the role of transcriptional regulation appears to be more complex, and the variation in the *EPHB4* gene caused by chromosomal abnormalities has yet to be elucidated (30). Previous studies have reported that *EPHB2* is suppressed by *REL* but induced by Wnt, β-catenin and T-cell factor family transcription factors in colorectal cancer (32,33). However, the effects of β-catenin on *EPHB2* and *EPHB4* gene expression vary according to the stage of colorectal cancer progression (32,33). In the present study, only the mechanism of methylation-associated gene silencing was investigated. However, other mechanisms, including mutations and chromosomal changes in mRNA stability, also regulate Eph and ephrin expression in cancer, which may affect the process of *EPHB4* gene inactivation, leading to diverse gene methylation levels between individuals (30).
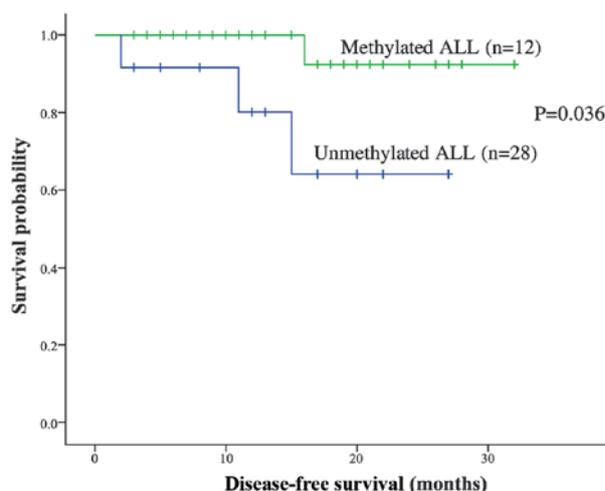

Figure 6. Kaplan-Meier survival analysis of patients with ALL based on the methylation status of the promoter region of ephrin type-B receptor 4. The two-year disease-free survival of the patients with unmethylated ALL (92.3±7.4%; n=12) was significantly higher, compared with the patients with methylated ALL (68.4±1.58%; n=28; P=0.036). ALL, acute lymphoblastic leukemia.

A previous study, based on >9 years of follow-up data, revealed that *EPHB4* is a valuable prognostic marker in colorectal cancer as low *EPHB4* expression levels are significantly associated with a shorter survival time (34). Kuang *et al* (17) investigated the association between the methylation of Eph receptors and overall survival in patients with ALL, observing that patients with methylation of numerous Eph genes exhibited a shorter median survival time. However, to the best of our knowledge, the impact of *EPHB4* gene expression on the prognosis of childhood ALL has yet to be investigated. The present study revealed, using Kaplan-Meier survival analysis, that patients with a methylated *EPHB4* gene exhibited a shorter disease-free survival time, as compared with patients with an unmethylated *EPHB4* gene (P=0.036), establishing a correlation between the methylation status of the *EPHB4* gene and an unfavorable prognosis. This suggests that *EPHB4* methylation is a potential prognostic factor for childhood ALL. However, longer-term observation is required for a thorough elucidation of the role of *EPHB4* in the prognosis of childhood ALL.

The current understanding of the mechanisms underlying the tumor suppressor effect of the *EPHB4* receptor is primarily based on its forward signaling pathway, which is involved in tumor pathogenesis and progression (22,35). Previous studies have indicated that the *EPHB4* gene exhibits a bidirectional role in the regulation of tumorigenesis in epithelial cancer, depending on the presence or absence of the ephrin-B2 ligand (29,30). The binding of ephrin-B2 to the *EPHB4* receptor leads to the modulation of certain signaling pathways underlying tumor suppression, including the Harvey rat sarcoma-extracellular-signal related kinase, phosphoinositide 3-kinase-protein kinase B (Akt) and Abl-Crk signaling pathways, inhibiting tumor cell proliferation, migration and invasion (30,36,37). Previous studies have indicated that this bidirectional signaling mechanism may serve a particular role in inducing tumor dormancy, which consequently reduces tumor progression (30,38,39). According to research by

Kuang *et al* (17), the modulation of the Akt signaling pathway is attributed to *EPHB4*-mediated cell growth inhibition in ALL cell lines following stimulation by ephrin-B2. However, this result does not incorporate data from bone marrow samples from patients with ALL. The function of the downstream signaling pathway of *EPHB4* during leukemogenesis, and the interactions between *EPHB4* and its ligand, have not yet been fully elucidated. Further clinical studies in addition to long-term survival observations, are required in order to provide a clear profile of the involvement of the *EPHB4* gene in ALL pathogenesis. The established knowledge of the suppressive effect of the *EPHB4* gene on oncogenesis, and the potential positive correlation between *EPHB4* gene expression levels and a favorable ALL prognosis (19,29,40), indicates that the *EPHB4* gene may be a candidate tumor suppressor and prognostic biomarker in childhood ALL. Due to the reversible status of gene methylation, the present study has revealed the promising value of *EPHB4* as a novel target for demethylation treatment.

In conclusion, the present study demonstrated that *EPHB4* gene methylation is a frequent event in childhood ALL. The methylation-associated inactivation of the *EPHB4* gene leads to transcriptional silencing, resulting in reduced or absent gene expression levels. Further survival analysis performed in patients with ALL revealed a trend of positive association between *EPHB4* gene inactivation and poor prognosis. Therefore, it is hypothesized that the *EPHB4* gene may function as a tumor suppressor in ALL pathogenesis, and may be utilized as a novel prognostic biomarker during ALL therapy. However, larger scale investigations are required in order to clarify the function of Eph/ephrins in the development of ALL and hematological malignancies.

Acknowledgements

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