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# 소아 급성림프모구백혈병에서 *CDH1*, *p16*, *DAPK* 유전자의 DNA 메틸화

한승민 · 권승연 · 김효선 · 한정우 · 유철주

연세대학교 의과대학 소아과학교실, 연세암연구소

# Aberrant DNA Methylation of *CDH1*, *p16* and *DAPK* in Childhood Acute Lymphoblastic Leukemia

**Seung Min Hahn, M.D., Seung Yeon Kwon, M.D., Hyo Sun Kim, M.D., Jung Woo Han, M.D. and Chuhl Joo Lyu, M.D., Ph.D.**

*Department of Pediatrics, Yonsei Cancer Research Center, Yonsei University College of Medicine, Seoul, Korea*

**Background:** Hypermethylation of tumor suppressor gene has been reported in various types of leukemia with potential involvement in the inactivation of regulatory cell cycle and apoptosis genes.

**Methods:** To evaluate the methylation status at initial diagnosis and morphologic complete remission (CR) period in childhood acute lymphoblastic leukemia (ALL), we analyzed the methylation status of three key genes (*CDH1*, *p16* and *DAPK*) in 43 childhood ALL patients and 7 healthy bone marrow (BM) donors.

**Results:** *CDH1* was methylated in 26 (60.4%) patients, *p16* in two (4.6%) patients and *DAPK* in six (13.9%) patients at the time of diagnosis. Twenty nine (67.4%) patients had methylation of at least one gene. None of the healthy BM donors showed methylation of the above genes. Age was the only factor which showed significant association with the presence of DNA methylation ($P$=0.03). None of the other clinicopathological factors showed association with initial methylation status. At the time of morphologic CR, all patients who had aberrant DNA methylation at the time of diagnosis had no detectable residual methylation.

**Conclusion:** Since hypermethylation was found in around two thirds of pretreatment ALL patients and none in healthy BM donor, we suggest hypermethylation of some important genes is a biologic marker of childhood ALL. We recommend that further studies with a large number of patients should be conducted.

**Key Words:** DNA methylation, Pediatric acute lymphoblastic leukemia

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**Corresponding Author:** Chuhl Joo Lyu
Department of Pediatrics, Yonsei University College of Medicine, Yonsei-ro 50, Seodaemun-gu, Seoul 120-752, Korea
Tel: +82-2-2228-2060
Fax: +82-2-393-9118
E-mail: cj@yuhs.ac

## Introduction

Hypermethylation of DNA, which involves the addition of a methyl group to the carbon 5 position of the cytosine ring in a cytosine-guanine (CpG) pair, is known to be the most common and well-described epigenetic mechanism. CpG pairs are usually underrepresented in most of the mammalian genome. However, short regions rich in CpG content known as CpG islands are found in the proximal promoter regions of almost half of the genes in the human genome, and are generally unmethylated in normal cells

[1-3]. Methylation of these promoter-associated areas has been associated with gene silencing which serves as an alternative mechanism by inactivating functionally relevant genes related to diverse human pathologies including cancer and aging [4,5]. In cancer, the aberrant hypermethylation of CpG islands surrounding gene promoter regions is the most well-investigated epigenetic change to occur in tumors. CpG island hypermethylation has been described in various types of human malignancies, and is associated with inappropriate transcriptional silencing of functionally important cancer related genes, including tumor-suppressor genes [6,7].

Hypermethylation of promoter-associated CpG islands also has been found in acute lymphoblastic leukemia (ALL). Initial studies in ALL were targeted on the analysis of single genes such as *calcitonin* [8-10], *p73* [11,12], *p15* [13-15], *E-cadherin (CDH1)* [16], and so on. Further studies focusing upon hypermethylation of multiple genes have proved that concurrent methylation of multiple genes is a common feature in both adult and childhood ALL [17,18]. The studies in Philadelphia chromosome (Ph)-negative adult ALL and acute myeloid leukemia (AML) have shown that residual methylation at remission could predict poor prognosis, suggesting that DNA methylation could be a potential biomarker in ALL [19,20]. Furthermore, many studies implicated that methylation pattern in childhood ALL patients at diagnosis were different from that of adults [21-24]. To study these issues, we examined methylation status of three key genes in leukemia, *CDH1*, *p16* and *DAPK*, at the time of initial diagnosis and at the time of morphologic complete remission (CR). *CDH1* is a member of membrane glycoprotein, its function as a tumor suppressor and its loss due to methylation enables disaggregation of cancer cells from one another and increases their metastatic potential. *p16* is a important member of cyclin dependent kinase inhibitor, regulated by a feedback loop with Rb. *DAPK* is a $Ca^{+2}$/Calmodulin regulated serine/threonine kinase, associated with the cytoskeleton, and participates in a wide range of apoptotic pathways.

We also aimed to investigate the correlation of methylation status with clinicopathological features.

## Materials and Methods

### 1) Patients and samples

We studied 43 patients (24 males, 19 females) who were diagnosed as de novo childhood ALL between January 2006 and February 2010. Diagnosis was established according to the standard morphologic, cytochemical, and immunophenotypic criteria. All patients were risk stratified and treated with protocols based on recognized prognostic factors, including cytogenetic analysis. Criteria for CR were defined as restoration of normal hematopoiesis with a blast cell fraction of less than 5% by light microscopic examination of the bone marrow (BM) [25] after 4 weeks of induction chemotherapy. The median age at the time of diagnosis was 4.8 years (range, 0.9-17 years). Patients were grouped according to the National Cancer Institute (NCI) risk classification criteria [26] for statistical analyses. Other clinical characteristics of the patients are described in Table 1. The median follow up period of the patients was 5.4 years (range, 4.1-7.8 years). Seven healthy individuals were included in this study for comparison. All of them were BM donors for their siblings. The median age of the donors was 8.2 years (range, 3.5-22 years).

Study samples were obtained from BM aspirates from the above patients at the time of diagnosis (prior to chemotherapy) and at the time of morphologic CR (after 4 weeks of induction chemotherapy). Patients with sample from one of the time points were not included in this study. All 43 patients included in this study had achieved CR after induction chemotherapy. BM aspiration samples were also obtained from 7 healthy individuals who were BM donors for their siblings. Consent for sample collection was obtained from all patients and healthy individuals following the institutional guidelines. This study was reviewed and approved by the Institutional Review Board.

### 2) Methylation specific PCR (MSP)

To study methylation patterns at the time of initial diagnosis and CR in childhood ALL, we analyzed the methylation status of three key genes related to its pathogenesis, *CDH1*, *p16* and DAPK, in 43 patients. Gene selection was

based on the previous methylation studies in ALL [17,21,27, 28]; we selected the three genes that are known to be frequently methylated in childhood ALL [18,29].

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation, and genomic DNA was extracted with QIAmp DNA blood mini kit (QIAGEN, CA, USA) following procedures according to the manufacturer's instructions. Genomic DNA was modified using the sodium bisulfate as previously described by Herman et al. [30]. One microgram of genomic DNA was treated with sodium bisulfate to convert all unmethylated cytosine residues to uracil using EZ DNA Methylation kit (Zymo Research, CA, USA) following the manufacturer's instructions. Modified DNA was stored at −70°C until used. PCR was performed using specific primers for *CDH1*, *p16* and *DAPK* genes in 43 pairs of samples from the patients and 7 samples from healthy donor. Two hundred nanograms of sodium bisulfate modified DNA was used as a template in PCR reaction containing 25 pmoles of each primer, 200 μM each dNTP, 1 U AmpliTaq Gold DNA polymerase with 10X PCR buffer and 25 mM $MgCl_2$ solution (Applied Biosystems, CA, USA) in a final volume of 25 μL. PCR conditions and primers specific for methylated and unmethylated genes were the same as those used in the literature by Gutierrez et al. [18]. After amplification, 10 μL of the PCR product was separated on a 2% agarose gel containing ethidium bromide. For each MSP reaction, a CpGenone universal methylated DNA (Millipore, MA, USA) and commercial normal human genomic DNA [Human Genomic DNA: Female (Promega, MI, USA)] was used as controls for methylation and unmethylation. Distilled water was used as a negative control for PCR in each set of reaction. Specificity of each reaction was demonstrated when methylated gene consistently yielded a band from universally methylated DNA.

### 3) Statistical analysis

For statistical purposes, patients were classified into two different groups: unmethylated (no methylated genes) and methylated (at least one methylated gene). Association of methylation status with patient characteristics were analyzed using $\chi^2$ test or Fisher exact test. Overall survival (OS) was measured from the day of diagnosis until death from any cause and was censored only for patients known to be alive at last contact. Disease-free survival (DFS) was measured from the day that CR was established until either relapse or death without relapse, and it was censored only for patients who were alive without evidence of relapse at the last follow-up. Comparisons among the groups were performed by analysis of variance with SPSS ver. 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Estimated 5-year disease free survival and overall survival were based on the Kaplan-Meier method, and differences were tested using the log-rank test. An effect was considered statistically significant if the *P*-value was 0.05 or less.

## Results

### 1) Methylation pattern at initial diagnosis and CR

Among 43 patients, methylation frequency of each gene at the time of initial presentation was as follows: 60.4% (n=26) for *CDH1*, 4.6% (n=2) for *p16*, and 13.9% (n=6) for *DAPK*. 67.4% (n=29) of all patients had methylation of at least one gene, whereas 32.6% (n=14) of the patients had no methylation at all. Five (11.6%) patients had methylation of two genes. Methylation of *CDH1* showed considerably high frequency of methylation as previously reported [18]. However, *p16* and *DAPK* were rarely methylated in this study.

To evaluate the dynamics of DNA methylation changes as a result of therapy, we also analyzed the methylation status of the above three genes at the time of morphologic CR with the paired samples. All patients who showed methylation at the time of initial presentation (n=29) revealed clear absence of methylation of all three genes at the time of CR. One patient who did not have methylation at initial presentation revealed methylation of *CDH1* gene at the time of CR. The patient was an eight year-old girl who experienced bone marrow and CNS relapse after six months, and finally died of disease progression. Except this one patient, neither residual methylation nor new methylation was detected at the time of CR in childhood ALL patients.

### 2) Clinical characteristics and methylation profile

We studied the association between DNA methylation

**Table 1.** Clinical characteristics and methylation profile of the patients

| Features | | All patients | | *CDH1* | | *p16* | | *DAPK* | | Methylation | | Nonmethylation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | N | % | N | % | N | % |
| Gender | Male | 24 | 55.8 | 15 | 62.5 | 2 | 8.3 | 3 | 12.5 | 17 | 70.8 | 7 | 29.2 |
| | Female | 19 | 44.2 | 11 | 57.9 | 0 | 0 | 3 | 15.8 | 12 | 63.2 | 7 | 36.8 |
| Age[a] | <2 | 4 | 9.3 | 1 | 25.0 | 0 | 0 | 1 | 25.0 | 1 | 25.0 | 3 | 75.0 |
| | 2-10 | 31 | 72.1 | 18 | 58.0 | 2 | 6.5 | 3 | 9.7 | 21 | 67.7 | 10 | 32.3 |
| | ≥10 | 8 | 18.6 | 7 | 87.5 | 0 | 0 | 2 | 25.0 | 7 | 87.5 | 1 | 12.5 |
| WBC count | <50 | 32 | 74.4 | 20 | 62.5 | 2 | 6.3 | 5 | 15.6 | 23 | 71.9 | 10 | 31.3 |
| ($\times 10^9$/L) | ≥50 | 11 | 25.6 | 6 | 54.5 | 0 | 0 | 1 | 9.1 | 6 | 54.5 | 4 | 36.4 |
| NCI risk group | SR | 22 | 51.2 | 12 | 54.5 | 2 | 9.1 | 1 | 4.5 | 14 | 63.6 | 8 | 36.4 |
| | HR | 21 | 48.8 | 14 | 66.7 | 0 | 0 | 5 | 23.8 | 15 | 71.4 | 6 | 28.6 |
| Cytogenetic/molecular | *TEL-AML*1, t(12;21) | 11 | 25.6 | 8 | 72.7 | 1 | 7 | 0 | 0 | 8 | 72.7 | 3 | 27.3 |
| abnormalities | *E2A-PBX*1, t(1;19) | 2 | 4.7 | 2 | 100 | 0 | 0 | 2 | 100 | 2 | 100 | 0 | 0 |
| | *mBCR-ABL*, t(9;22) | 4 | 9.3 | 2 | 50.0 | 0 | 0 | 0 | 0 | 2 | 50.0 | 2 | 50.0 |
| | Hyperdiploidy | 9 | 20.9 | 7 | 77.8 | 0 | 0 | 0 | 0 | 6 | 66.7 | 3 | 33.3 |
| State of Disease | | | | | | | | | | | | | |
| Complete remission | | 31 | 72.0 | 17 | 54.8 | 2 | 6.5 | 3 | 9.7 | 22 | 71.0 | 10 | 32.3 |
| Relapse | | 12 | 27.9 | 6 | 50.0 | 0 | 0 | 1 | 8.3 | 3 | 25.0 | 1 | 8.3 |
| Death | | 15 | 34.9 | 7 | 46.7 | 0 | 0 | 2 | 13.3 | 4 | 26.7 | 3 | 20.0 |
| Total patients | | 43 | 100 | 26 | 60.4 | 2 | 4.6 | 6 | 13.9 | 29 | 67.4 | 14 | 32.6 |

Methylation, patients with DNA methylation of at least one gene; nonmethylation, patients with no methylation at all; WBC, white blood cell; NCI, National Cancer Institute; SR, standard risk; HR, high risk.
[a]$P=0.03$ (differences were tested using the log-rank test).

status and clinicopathological characteristics, including age, gender, initial WBC count, pretreatment cytogenetic analysis and NCI risk group. Methylation profiles of the patients at initial presentation according to various clinicopathological characteristics are summarized in Table 1.

Methylation of *CDH1* was observed in 62.5% (15/24) of the male patients and 57.9% (11/19) of the female patients. By age, methylation of *CDH1* was shown in 25.0% (1/4) of patients younger than two years of age, 58.0% (18/31) of patients between two and 10 years, and 87.5% (7/8) of patients older than 10 years.

Methylation of *CDH1* gene was also found in 62.5% (20/32) of patients whose initial WBC counts were less than 50,000/ $\mu$L and in 54.5% (6/11) of those with more than 50,000/ $\mu$L of WBCs. The same gene was methylated in 54.5% (12/22) of patients with standard risk and 66.7% (14/21) of patients with high risk. Frequencies of *CDH1* methylation in patients who had cytogenetic abnormalities were as follows: 72.71% (8/11) for TEL-AML1 positive patients, 50.0% (2/4) for minor BCR-ABL positive patients, and 100% (2/2) for E2A-PBX1 positive patients.

Methylation of *p16* was rarely found; only two out of 43 patients showed methylation. Both two patients were male, aged between 2-10 years, and had standard risk. Methylation of *DAPK* was found in 12.5% (3/24) of males and 15.8% (3/19) of females. Methylation was observed in 25.0% (1/4) of patients under age of two, 9.7% (3/31) of patients between two and ten years old, and 25.0% (2/8) of patients older than 10 years Five out of six patients who had methylation of *DAPK* showed initial WBC count lower than 50,000/ $\mu$L. A majority of patients (5/6) with *DAPK* methylation were high risk group patients.

Only age factor was significantly associated with the presence of methylation ($P$=0.03). Methylation was observed in 25.0% (1/4) of patients under age of two, whereas 12.5% (1/8) of patients older than 10 years showed nonmethylation. Since methylation of *CDH1* accounted for a large majority of aberrant methylation of the studied genes, while only few or several cases had methylation of *p16* and *DAPK*, *CDH1* methylation predominantly contributed to the above results. Factors such as gender, initial WBC count, cytogenetic abnormalities, and NCI risk group did not show any

significant association with methylation status.

Since all patients who had methylation at the time of initial diagnosis showed clear absence of methylation at the time of CR, methylation profile at the time of CR was not available.

Estimated 5-year OS of all patients was 80.4% for standard risk patients and 65.6% for those with high risk. Estimated 5-year OS for methylated patients was 77.3% and 83.2% for nonmethylated ones. Five year DFS for methylated patients was 75.5% and 81.8% for the nonmethylated group. There was no statistical significance for OS and DFS between methylated and nonmethylated patients.

## Discussion

There has been much evidence that DNA methylation plays a key role in cancer development [6,31]. An explosive amount of data has indicated the importance of epigenetic processes, especially those resulting in the silencing of key regulatory genes in all stages of cancer development [32]. In ALL, study of epigenetic alterations was a relatively obscure field of research before; however, it has shown a considerable progress in the last decade. Studies on methylation of multiple genes in both adults and child patients have shown that multiple promoter CpG islands are common features in both groups [17,18,29,33]. However, difference in the patterns of methylation between pediatric and adult ALL has been suggested in several studies. While the report of Garcia-Manero et al. [33] indicated that there were no obvious differences in terms of the frequency of methylation observed in childhood and adult ALL, another study showed that epigenetic inactivation of *p73/p15/p57* which was observed in close to 25% of adult patients was extremely rare in the younger patients [21]. Nakamura et al. [22] reported that methylation of *p16*, a rare event in primary ALL [27,34], has been shown to be present in pediatric cases with *MLL* alterations. Methylation of *FHIT* gene showed a similar pattern [23,24]. Even though the above results need to be confirmed in other larger group of patients, they suggest that prognostic differences between the age groups could be related in part to different methylation patterns [35].

Several studies have focused on whether DNA methylation could be used as a marker of residual disease. One study of methylation profiles of five genes (*ER, MDR1, p73, p15* and *p16*) in a group of patients before therapy and at the time of morphologic relapse showed that a gain of methylation may have a role in relapse/resistance mechanisms in adult ALL, which can suggest a potential role of DNA methylation profile in implicating relapse and resistance [4]. Another study conducted in adult Ph chromosome-negative ALL patients showed that 28% of patients had detectable residual methylation at remission, and the presence of residual *p73* methylation was associated with a significant shorter duration of first CR and OS [19]. As many authors suggested the different characteristics of methylation between adult and pediatric patients, methylation pattern at remission status in childhood ALL can differ from those of adults.

In this study, we have analyzed the methylation patterns of *CDH1*, *p16* and *DAPK* genes at the time of initial diagnosis and at the time of CR in 43 childhood ALL. Aberrant DNA methylation was most frequently observed in *CDH1* gene (60.4%), while it was rare in *p16* and DPAK gene had several such cases. As Gutierrez et al. [18] reported high frequency (72%) of *CDH1* methylation in childhood ALL, this data supports this result. Age was the only factor which showed statistically significant association with the presence of DNA methylation of the studied genes ($P$=0.03). Even though patients under age of two years are known to have poor prognosis, a majority of patients did not show any methylation. Most patients over 10 years had methylation. This result can be supported by the concept that methylation increases with aging [36], and may suggest a role of more significant mechanism in leukemogenesis other than epigenetic mechanism in those young children under age of two. However, since this study included only a very small number of genes among many different cancer-related genes in limited number of patient, further survey is definitely required.

Most significant result of this study is that all patients who had DNA methylation at initial presentation showed clear absence of methylation at CR after induction chemotherapy. Unlike the results of the analysis of residual meth-

ylation in Ph-negative adult patients, none of the childhood ALL patients at clinical remission showed residual or newly developed methylation of the studied genes. Since there was no patient with residual methylation at clinical remission, we were unable to evaluate the correlation of clinicopathological factors with methylation status at CR in this study. It also was difficult to identify if residual methylation status could predict relapse or poor prognosis. Several limitations are included in this study. First, the selection of genes for this study might not represent methylation profiles in childhood ALL. Since there are no representative genes known to carry the most relevant epigenetic role in childhood ALL, we selected the three genes based on prior results from other studies as we mentioned before. By including more genes which have a potential epigenetic role in childhood ALL, we might have more obvious results of methylation status at CR. The second problem is the limitation in detecting methylation. This study was conducted only with MSP which is limited to qualitative analysis. Since we did not undergo quantitative studies, the analysis according to the level of methylation was unavailable. We believe that further qualitative analysis is certainly needed. Next, the follow up period was relatively short to evaluate the significance of methylation on survival outcome. Further studies of a larger patient number with the more selected genes and a long term follow up will give us better understanding of the driving mechanisms and also therapeutic interventions for childhood ALL at diagnosis, treatment and relapse.

In conclusion, we have analyzed methylation status of *CDH1*, *p16* and *DAPK* genes in childhood ALL at the time of initial diagnosis and at CR using MSP method. Methylation of *CDH1* was frequently detected, showing significant association with age. Since all of the DNA methylation presented at the time of diagnosis revealed total unmethylation at CR and all healthy individuals showed complete unmethylation. Further studies with larger populations and more relevant genes should be conducted in methods that include quantitative ones.

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