Dysregulation of the DKK1 gene in pediatric B-cell acute lymphoblastic leukemia

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Received: 14.07.2015 ● Accepted/Published Online: 16.03.2016 ● Final Version: 27.02.2017

Background/aim: The canonical Wingless-type (WNT) pathway is involved in normal hematopoietic cell development and deregulated WNT signaling is implicated in the development of hematological malignancies. Dickkopf 1 (DKK1) acts as a modulator of the β-catenin regulated canonical pathway. Activation of DKK1 leads to apoptosis and growth suppression, whereas silencing by promoter hypermethylation results in abnormal WNT activation. The secreted inhibitor Dickkopf can antagonize WNT signaling by competitively binding to low density lipoprotein receptors (LRPs) 5 and 6.

Materials and methods: We studied DKK1 gene promoter methylation and investigated DKK1, β-catenin, LRP5, and LRP6 mRNA levels in B-cell acute lymphoblastic leukemia (B-ALL) patients (n = 90). Methylation-specific PCR and bisulfite sequencing were used for methylation profiling and quantitative real-time PCR was used for mRNA detection.

Results: The DKK1 gene was examined for its promoter methylation and only 33% of patients were found methylated. On the other hand, B-ALL cases showed high expression of DKK1 (P = 0.01), LRP5 (P = 0.04), and LRP6 (P = 0.02) compared to normal bone marrow cells.

Conclusion: DKK1 methylation exists in some of cases but is not sufficient for WNT pathway activation alone in pediatric B-ALL.

Key words: B-cell acute lymphoblastic leukemia, methylation, β-catenin, DKK1, WNT pathway

1. Introduction
WNT signals play a role in the self-renewal of hematopoietic stem cells and T- and B-lymphocyte development. WNT antagonists are active components in tuning WNT signals (1). The DKK family encodes secreted proteins that antagonize β-catenin regulated signaling by inhibiting the WNT coreceptors Lrp5 and Lrp6. The Dkk1 protein binds to Lrps and leads to the disassociation of Lrp-5/6 from frizzled receptors (Fz), which are the specific receptors of WNT ligands that prevent the formation of a functional WNT receptor complex. DKK1 is the most extensively studied Dickkopf family member. Different levels of expression in tumor cell lines or tissues were shown. Elevated levels of DKK1 are found to influence several important steps in the mobilization, engraftment, and proliferation of hematopoietic stem cells (2). Tian et al. showed that short-term exposure to low levels of Dkk1 induces moderate proliferation of mesenchymal stem cells, whereas long-term exposure to high levels of Dkk1 causes a loss of cell viability (2). Additionally, DKK1 has also been identified as a potential target gene of p53. Evidence of a p53 binding site in the promoter region of DKK1 and its induction may mediate p53 tumor suppression by antagonizing the WNT signaling pathway (3).

DKK1 has been reported as a tumor suppressor gene and promoter methylation were found in several human cancers such as gastrointestinal cancer (4), cervical cancer (5), and breast cancer (6), as well as leukemias. DKK3 gene hypermethylation is reported in acute lymphoblastic leukemia (ALL) and a correlation was found with good prognosis in T-cell ALL patients (7). Suzuki et al. revealed that DKK1 methylation is involved in leukemogenesis and can be used as a prognostic marker in acute myeloid leukemia (AML) (8). Impairment of the DKK1 gene by genetic and epigenetic mechanisms may also accompany blastic transformation in ALL. This idea generated our study to investigate the DKK1 expression and promoter methylation status in pediatric ALL patients and its relationship with the WNT cascade partners LRP5 and LRP6.
2. Materials and methods

2.1. Patients and controls
A total of 90 pediatric B-cell acute lymphoblastic leukemia (B-ALL) patients who diagnosed at the Istanbul and Cerrahpaşa Medical Faculties of Istanbul University were included in this study. Bone marrow samples were obtained at the time of diagnosis. Patients were diagnosed according to the criteria of the French-American-British (FAB) group. The median age was 10.0 years (min. 1 month, max. 16 years) and the median WBC count was 33 × 10⁹/L (min. 1, max. 600). Twelve of the patients were t(12;21)-positive, 4 of them were t(4;11)-positive, and 3 of them were t(9;22)-positive. Additional clinical features such as tumor lysis (n = 6), lymphadenopathy (n = 22), splenomegaly (n = 47), and hepatomegaly (n = 43) were detected among the patients. In addition to B-ALL patients, a group of T-ALL (n = 15) patient samples, a B-ALL cell line (FLEB 14-4), and three T-ALL (MOLT4, JURKAT, and TALL-1) cell lines were also examined.

Sixteen healthy peripheral blood samples, normal bone marrow (n = 6), total thymus tissue, and CD19-positive cells were used as control groups. To obtain CD19-positive cells, mononuclear cells were isolated from 5 healthy individuals and sorted by CD19-specific magnetic beads (MACS, Miltenyi Biotech, Germany) as described by the manufacturer. The purity was checked by flow cytometry. The ethical committee of the Istanbul Medical Faculty of Istanbul University approved this study and informed consent was obtained from all patients and healthy controls.

2.2. RNA isolation and cDNA synthesis
Bone marrow and/or peripheral blood samples were homogenized in RTL buffer (QIAGEN GmbH, Germany). Total RNA was isolated by the QIAGEN RNasy Protect Kit. RNA samples were treated using DNAsse (1 U/μg) to avoid possible DNA contamination resulting from isolation. RNA quality and quantity were checked by 1% ethidium bromide-stained agarose gel. cDNA synthesis was performed by using random hexamers (Roche Diagnostics, Mannheim, Germany) and MMLV reverse transcriptase (MBI Fermentas, Lithuania) according to the manufacturer's protocol.

2.3. Analysis of gene expression by real time quantitative RT-PCR
The expression levels of DKK1, β-catenin, LRP5, and LRP6 were detected by quantitative real-time PCR (QRT-PCR) carried out on a LightCycler Instrument 480 (Roche Diagnostics), with the LightCycler 480 Fast Start SYBR Green I Master Kit (Roche Diagnostics). Primers (5 pmol) and 200 ng of cDNA were used in each run and each sample was studied in duplicate. The specificity of product amplification was confirmed by melting curve analyses and agarose gel electrophoresis. The PCR program was as follows: initial denaturation at 95 °C for 7 min; amplification for 5 s at 95 °C, 10 s at 56–60 °C, and 10 s at 72 °C for 45 cycles; and melting curve for 15 s at 60 °C for one cycle. The 3 reference genes (β-actin, Cyclophilin, and ABL) were used for normalization as described by Vandesompele et al. (9).

2.4. Bisulfite treatment and methylation specific polymerase chain reaction (MS-PCR)
Following the DNA isolation, sodium bisulfite (NaBis) treatment was performed as described by Frommer et al. (10). NaBis-treated DNA was purified by the Gene Clean III Kit (Qbiogene, USA) according to the manufacturer's instructions. After the bisulfite treatment, MS-PCR was used to amplify the promoter region of the DKK1 gene in all samples. MethPrimer (http://www.urogene.org/methprimer/index1.html) was used to determine the CpG island range of the DKK1 gene and 130–300 bp and 600–800 bp of DKK1 was found in the CpG-rich region of this gene. Previously described primer sequences (11) and the CpG island range of the DKK1 gene are given in Figure 1.

PCR conditions were as follows: 95 °C for 10 min for initial denaturation; 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min for 35 cycles; and a final extension at 72 °C for 5 min. PCR yields were evaluated based on methylation status using a 3% agarose gel. One DNA sample was treated with SssI methylase enzyme (New England Biolabs, USA) to obtain a fully methylated positive control in each run.

2.5. Statistical analysis
Relative expressions were calculated according to the mathematic model based on the crossing points (12). Differences between the relative expression levels of cases and controls were tested by Mann–Whitney U test. Proportional differences between groups were analyzed by chi-square (χ²) or Fisher exact tests. The correlation between methylation status and clinical parameters (sex, age, WBC count at diagnosis, etc.) was examined by the use of regression analysis. The Kaplan–Meier method was used to estimate survival rates. P ≤ 0.05 (two-sided) was considered statistically significant. The log rank test was used for overall survival analyses. All statistical analyses were done with SPSS 10.0.

3. Results

3.1. DKK1 promoter methylation in cell lines and ALL patients
To check the epigenetic changes in DKK1, the promoter methylation status of the DKK1 promoter was analyzed in patients, as shown in Figure 2A. B-ALL (FLEB 14-4) and T-ALL (MOLT4, JURKAT, and TALL-1) cell lines were found methylated. Methylation was confirmed by methylation-specific sequencing as shown in Figure 2B.
Thirty-three percent of the B-ALL patients (30/90) were found methylated for the DKK1 gene and no methylation was found in control samples (n = 15). The methylation rate of T-ALL cases was much lower (4/14, 20%).

3.2. DKK1, LRP5, and LRP6 genes are highly expressed in B-ALL patients
To evaluate the potential effects of hypermethylation of the DKK1 gene, the relative mRNA levels of DKK1 and its directly interacted genes (β-catenin, LRP5, and LRP6) were determined in T-cell and B-cell ALL patients. Seventy-one percent of B-ALL patients showed prominent DKK1 mRNA levels. Briefly, B-ALL patients showed significantly higher DKK1 expression (P = 0.01) compared to CD19-positive cells and expression levels of LRP5 (P = 0.04) and LRP6 (P = 0.02) were found increased (Figure 3A). There was no difference in DKK1 (P = 0.58), LRP5 (P = 0.11), or LRP6 (P = 0.07) gene expressions in T-ALL patients (Figure 3B). The expression levels did not differ between B-cell and T-cell ALL patients for DKK1 (P = 0.38), LRP5 (P = 0.18), and LRP6 (P = 0.26).

3.3. Clinical correlation
Clinical and laboratory findings including sex (P = 0.97), phenotype (P = 0.28), age (P = 1.00), WBC count at diagnosis (P = 0.86), material (P = 0.90), translocation (P = 0.16), and outcome (P = 0.40) of the patients did not show differences between methylated and unmethylated groups (Table). The median follow-up was 6 years (min. 1 month, max. 12 years). Kaplan–Meier estimate of probability of survival according to DKK1 gene methylation existence showed no significant difference (P = 0.60; 95% CI, 4.8–5.9). There was also no statistical difference between methylated and unmethylated patients in 150-month overall survival analyses (P = 0.94; Figure 4).

4. Discussion
DKK1 acts as a tumor suppressor and differential expressions and methylations have been reported in different cancer types (13). Methylation of DKK1 helps to inactivate tumor suppressive apoptotic or growth-arresting events and may have prognostic impacts on B-cell and T-cell ALL (14). Our previous findings also showed that WNT5A is highly methylated in T-ALL but not B-ALL samples (15).

In this study, we examined DKK1 promoter methylation status in representative cell lines and pediatric ALL cases and showed that 33% of B-ALL patients had promoter methylation of the DKK1 gene. The mechanisms responsible for activation and regulation of DKK1 expression in leukemia are not known. However, there are some studies that propose various mechanisms of DKK1 activation. Tumor tissues, which have high levels of methylation, are expected to be silenced and this presumably results in a growth advantage and clonal expansion to the affected cells. Epigenetic changes in the DKK1 gene could generate unexpected activating or inactivating events.

Besides DKK1 methylation, expression levels of DKK1 were also examined in pediatric ALL. DKK1 overexpression is shown to be a frequent finding in breast cancer (16), mesothelioma (17), hepatoblastoma, and Wilms tumor.
Figure 2. A) DKK1 methylation status in acute leukemia patients and controls. Methylation-specific PCR (MS-PCR) results on agarose gel electrophoresis; M: DNA ladder, IVM: in vitro methylated sample, AL1–AL6: patient samples, C1–C3: control samples. B) DKK1 gene methylation-specific PCR results in B- and T-ALL cell lines and confirmation of DKK1 methylation of FLEB 14-4 cell line by methylation-specific sequencing.

Figure 3. Relative DKK1, β-catenin, LRP5, and LRP6 expression levels in B-ALL and T-ALL patients and the controls (CD19-positive cells for normal B-cell population and total thymus for normal T-cell population) by QRT-PCR.
Table. Clinical characteristics and the methylation status of acute lymphoblastic leukemia patients.

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<tr>
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<th>Acute lymphoblastic leukemia patients (n = 104)</th>
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<tbody>
<tr>
<td></td>
<td>Methylated</td>
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<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>33.30</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>33.70</td>
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<tr>
<td>T-cell</td>
<td>20.00</td>
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<tr>
<td>Age</td>
<td></td>
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<tr>
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<td>31.50</td>
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<tr>
<td>WBC count</td>
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<tr>
<td>&lt;10 × 10⁹/L</td>
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<tr>
<td>10–50 × 10⁹/L</td>
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<tr>
<td>&gt;50 × 10⁹/L</td>
<td>55.30</td>
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<tr>
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<td>30.60</td>
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<tr>
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<tr>
<td>Outcome</td>
<td></td>
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<tr>
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<td>30.20</td>
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<td>Ex</td>
<td>50.00</td>
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WBC, White blood cell count at diagnosis; CR, complete remission; Ex, exitus. P < 0.05 represents a statistically significant value.

![Figure 4](image-url)  
**Figure 4.** Overall survival analyses in ALL patients.
patients (18). This study showed that 71% of B-ALL cases had upregulated DKK1 gene expression. DKK1 expression was reported to induce the proliferation of human adult bone marrow stem cells. In adult human mesenchymal stem cells from bone marrow stoma, high levels of DKK1 allow the cells to reenter the cell cycle by inhibiting the canonical WNT pathway (19). In our previous study, deregulated WNT/β-catenin signaling was shown as a novel oncogenic event in childhood T-ALL (20), whereas this is not the case for B-ALL patients (unpublished data). Here, DKK1 expression was not significant in T-ALL, supporting our previous findings due to the canonical WNT inhibitor role of DKK1.

LRP5 and -6 proteins are essential coreceptors for the Dickkopf antagonist function in WNT signaling. B-ALL samples, which did not express β-catenin, have high DKK1, LRP5, and LRP6 mRNA expressions. On the other hand, it has been suggested that Dkk proteins might have WNT-independent functions, such as Dkk1 overexpression inducing growth suppression (13).

Clinical features did show any association between the expression and methylation patterns among the pediatric acute leukemia patients. Survival analyses also did not show any difference.

Although the mechanism(s) by which DKK1 contributes to acute leukemia is not clear, we can conclude that Wnt involvement in T-cell and B-cell leukemia is different. These results imply a link between genetic and epigenetic changes during the blastic transformation in leukemia. We may propose that DKK1 has dual functions; expression of this gene promotes β-catenin-mediated transcription activation and hypermethylation of DKK1 in bone marrow leukemic blasts may affect leukemia pathogenesis by a different mechanism.

Acknowledgments

The authors are grateful to Dr Tiraje Celkan, Dr Zeynep Karakaş, and Dr Gönül Aydoğan for the patient material. This work was funded by the Research Fund of İstanbul University (Project no: 355 / 03062005), the Turkish Society of Hematology, the Scientific and Technological Research Council of Turkey (Project no: 106S112), and the T.R. Prime Ministry State Planning Organization (Project no: 2005K120430).

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