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

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Background/aim: The canonical Wntless-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 methylations 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*.

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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 İstanbul and Cerrahpaşa Medical Faculties of İstanbul 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 $33 \times 10^9/L$ (min. 1, max. 600). Twelve of the patients were translocations carriers; 5 of them 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 İstanbul Medical Faculty of İstanbul 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 RNeasy Protect Kit. RNA samples were treated using DNase (1 U/ μ g) 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 (χ^2) 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. $P \leq 0.05$ (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.

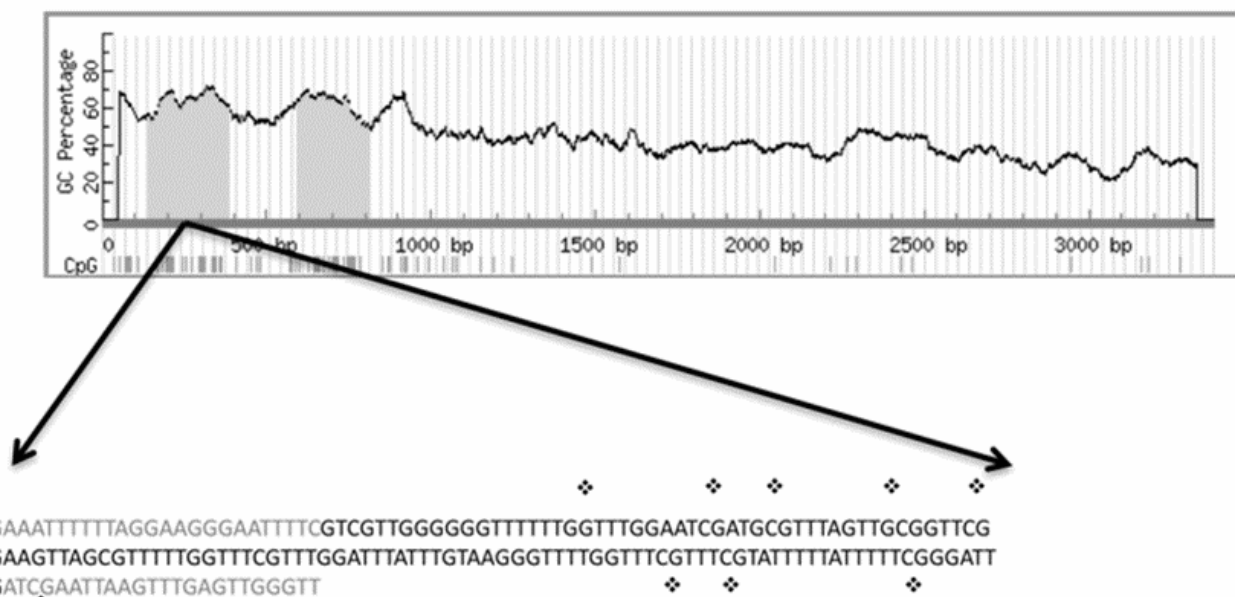


Figure 1. CpG island range of *DKK1* promoter determined by MethPrimer program. Lighter letters represent primer sequences, ♠ represents CpG islands.

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

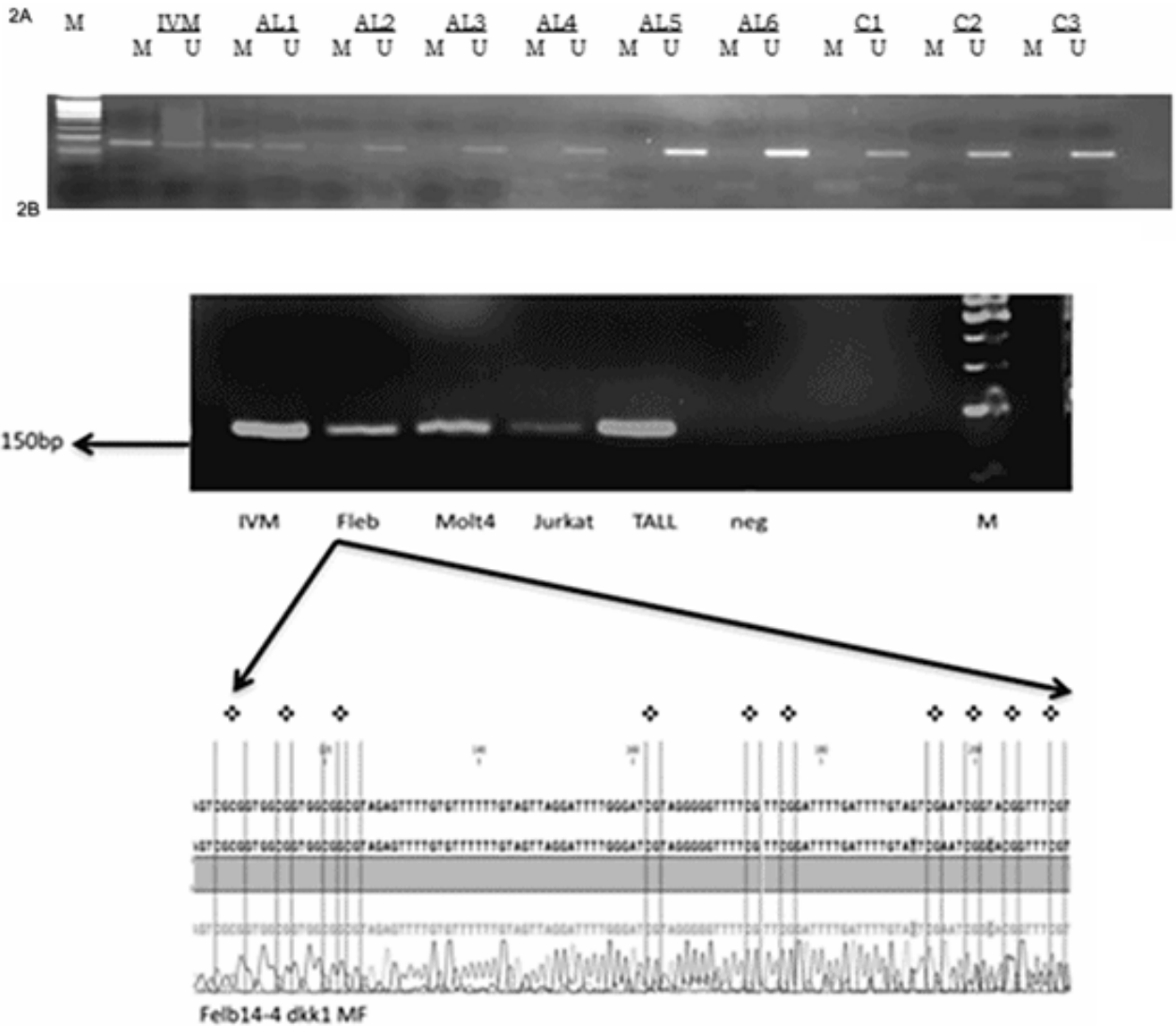


Figure 2. A) *DKK1* methylation status in acute leukemia patients and controls. Methylation-specific PCR (MS-PCR) results on agarose gel electrophoresis; M: DNA ladder, IVM: in vitro methylated sample, AL1-AL6: patient samples, C1-C3: control samples. B) *DKK1* gene methylation-specific PCR results in B- and T-ALL cell lines and confirmation of *DKK1* methylation of FLEB 14-4 cell line by methylation-specific sequencing.

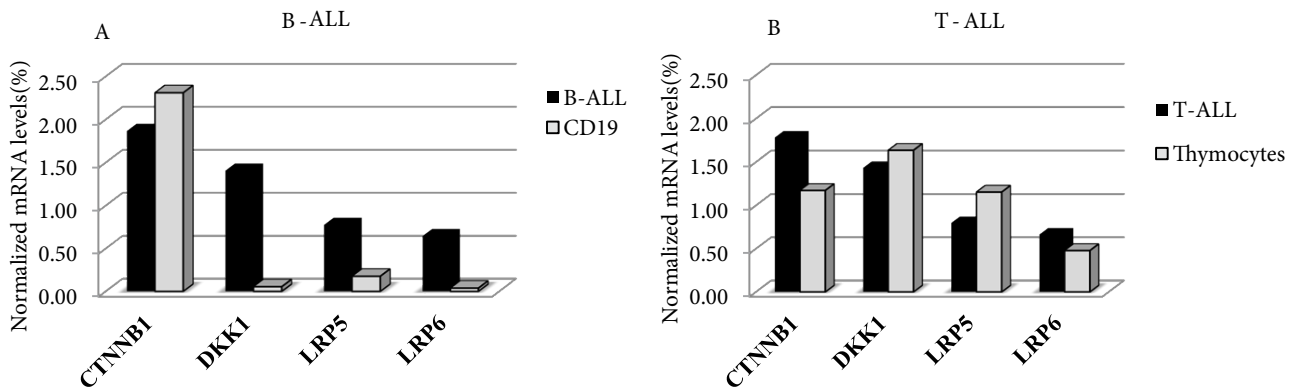


Figure 3. Relative *DKK1*, β -catenin, *LRP5*, and *LRP6* expression levels in B-ALL and T-ALL patients and the controls (CD19-positive cells for normal B-cell population and total thymus for normal T-cell population) by QRT-PCR.

Table. Clinical characteristics and the methylation status of acute lymphoblastic leukemia patients.

Acute lymphoblastic leukemia patients (n = 104)				
		Methylated	Unmethylated	
		n (%)	n (%)	P-value
Sex				
	Male	33.30	66.60	0.97
	Female	27.00	73.00	
Phenotype				
	B-cell	33.70	66.30	0.28
	T-cell	20.00	80.00	
Age				
	Children	31.50	68.50	1.00
WBC count				
	<10 × 10 ⁹ /L	12.80	24.50	0.86
	10–50 × 10 ⁹ /L	31.90	56.00	
	>50 × 10 ⁹ /L	55.30	18.90	
Material				
	Bone marrow	30.60	69.30	0.90
	Blood	32.00	68.00	
Outcome				
	CR	30.20	69.79	0.40
	Ex	50.00	50.00	

WBC, White blood cell count at diagnosis; CR, complete remission; Ex, exitus.
 P < 0.05 represents a statistically significant value.

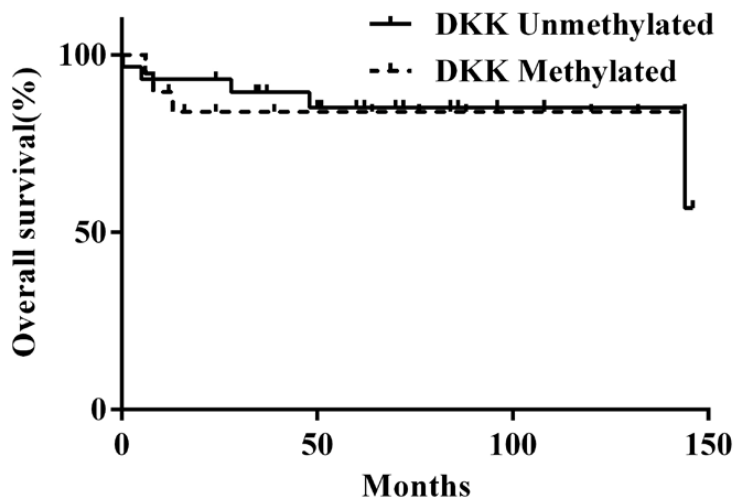


Figure 4. Overall survival analyses in ALL patients.

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 stroma, 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.

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