Overexpression of Apollon, an Antiapoptotic Protein, Is Associated with Poor Prognosis in Childhood *De novo* Acute Myeloid Leukemia

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Abstract

Purpose: The genes that encode inhibitor of apoptosis proteins are frequently overexpressed in human cancers and can be associated with resistance to therapy. The overexpression of Apollon, a member of inhibitor of apoptosis proteins, is intuitively expected to be associated with unfavorable clinical features in malignant diseases; however, there have been no clinical studies reporting the prognostic relevance of Apollon expression in human malignancies. This study was done to investigate the clinical relevance of the expression of Apollon in childhood *de novo* acute myeloid leukemia.

Experimental Design: In 55 pediatric patients with *de novo* acute myeloid leukemia, the level of Apollon expression was determined by using quantitative reverse transcriptase – PCR and was analyzed with respect to the patients' clinical features and treatment outcomes.

Results: Apollon expression was found to be higher in patients with a leukocyte number of $\geq$10,000/μL, patients with extramedullary disease, and patients with the French-American-British classification subtype M7. In addition, Apollon overexpression ($\geq$median expression) was associated with an unfavorable day 7 response to induction chemotherapy and also associated with a poorer 3-year relapse-free survival rate (48.3 $\pm$ 11.2% versus 78.7 $\pm$ 8.5%, $P = 0.040$).

Conclusion: This is the first study demonstrating the prognostic implication of the Apollon expression in human cancers, indicating that Apollon overexpression may be used as a poor prognostic marker in childhood acute myeloid leukemia through validation by further studies.

Acute myeloid leukemia (AML) comprises a heterogeneous group of hematologic malignancies that arise within the bone marrow precursors of the myeloid, monocyte, erythroid, and megakaryocytic cell lineages. Although AML makes up only 15% to 20% of childhood leukemia, it still accounts for >30% of the deaths from leukemia. The predictors of favorable prognosis in AML include favorable cytogenetics [e.g., $t(15;17)$, inv(16), and $t(8;21)$ (1–3)] and a good initial treatment response (4–7). Recently, the accumulation of knowledge on the molecular biology of malignancies has led to new diagnostic modalities to be incorporated into various diagnostic and therapeutic strategies in AML. One of these modalities is the quantitative reverse transcriptase–PCR, which can be used to determine the mRNA expression levels and allows researchers to examine the expression patterns of a large number of genes at the RNA level. It will be possible to refine current prognosis-based stratification systems if specific patterns of gene expression can be correlated with the clinical features in childhood AML.

Apoptosis is an active biological mechanism that leads to programmed cell death. The failure of apoptosis might result in the development of a wide variety of diseases, including cancer. Moreover, the up-regulation of antiapoptotic proteins would certainly be advantageous for tumor survival (8–11). Over the last decade, a complex network of proapoptotic and antiapoptotic proteins, which strictly regulate the apoptosis pathways, has been revealed. In particular, a group of proteins known as the inhibitor of apoptosis proteins (IAP) were identified (12–15). The IAPs are a family of proteins that has one to three baculovirus IAP repeat (BIR) domains and inhibits apoptosis by direct binding and inhibiting caspase.

Apollon (also known as BRUCE or BIRC6, BIR-containing protein 6) is a large IAP that contains BIR and ubiquitin-conjugating enzyme domains at the aminoterminals and carboxyterminals, respectively. The gene encoding Apollon, BIRC6, is located on the chromosome band 2p22. In 1999, Chen et al. isolated a complementary DNA, encoding a novel IAP by using PCR with degenerated primers from BIR domains, which they termed *Apollon* (16). They found that Apollon was up-regulated in some brain tumor cell lines that are resistant to

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certain DNA-damaging agents, and antisense oligonucleotides against *Apollon* enhanced the extent of apoptosis induced by these agents (16). Apollon not only inhibits Smac-induced apoptosis but also binds to pro–caspase-9 and inhibits its cleavage (17–19), which initiates the caspase cascade (20). From this context, Apollon overexpression is considered to be associated with poor prognosis in malignancies; however, there have been no reports of its prognostic relevance based on clinical data.

In this study, the authors analyzed the expression of Apollon in 55 children with *de novo* AML using quantitative reverse transcriptase–PCR to determine the relationship between the Apollon expression and the clinical features at diagnosis and treatment outcomes. The results revealed a strong association between Apollon overexpression and the unfavorable clinical features in childhood AML. This is the first clinical study demonstrating the prognostic implications of Apollon expression in malignancies.

Materials and Methods

Patients and treatment. Children younger than 15 years of age who were newly diagnosed with *de novo* AML from July 2000 to April 2006 at the Samsung Medical Center were enrolled in this study. The diagnosis of AML was made based on a morphologic assessment of the Wright-Giemsa stained smears of the bone marrow aspirates along with special stains and immunophenotyping by flow cytometry. Laboratory investigation included conventional and molecular cytogenetic analyses.

All patients with AML other than acute promyelocytic leukemia were treated according to the modified KBRMS protocol (Table 1). The patients first received 10 days of induction chemotherapy, in which the dose of behenoyl 1-β-D-arabinofuranosylcytosine for the last 3 days was modified according to the bone marrow response on day 7. Discontinuation of the chemotherapy was allowed in patients who experienced sepsis with unstable vital signs before the completion of the induction regimen if at least 7 days of induction chemotherapy had been provided. If complete remission (CR) was not achieved after the primary induction chemotherapy regimen, an additional course of induction chemotherapy using high-dose 1-β-D-arabinofuranosylcytosine was given. Once CR had been achieved, patients with an appropriate stem-cell donor received consolidation chemotherapy until the hematopoietic stem-cell transplantation. An entire course of consolidation chemotherapy was given in patients without an appropriate stem-cell donor.

For patients with acute promyelocytic leukemia, the induction regimen was composed of all *trans*-retinoic acid (45 mg/m^2/day from day 0 until CR was achieved) and idarubicin (12 mg/m^2/day on days 1, 3, 5, and 7). Once CR had been achieved, the patients received three courses of consolidation chemotherapy (first, second, and fourth consolidation chemotherapy according to the modified KBRMS protocol) along with daily all *trans*-retinoic acid (45 mg/m^2/day). The patients then received the maintenance chemotherapy (all *trans*-retinoic acid, 45 mg/m^2/day for 15 days every 12 weeks; 6-mercaptopurine, 50 mg/m^2/day daily; and methotrexate, 10 mg/m^2/day weekly) over a 2-year period.

The Institutional Review Board of Samsung Medical Center approved this study, and informed consent was obtained from parents or guardians for both the laboratory studies and treatment.

RNA isolation and real-time quantitative reverse transcriptase–PCR. Mononuclear cells were isolated from 2 mL of the bone marrow aspirate at diagnosis by Ficoll density gradient centrifugation. The total RNA was extracted from the mononuclear cells using a QIAamp RNA blood kit (Qiagen) according to the manufacturer's protocol. After treatment with DNA-free (Ambion) to remove the chromosomal DNA, the complementary DNA was synthesized using oligo (dT) 15-mer primers by SuperScript III reverse transcriptase (Invitrogen) and stored at -20°C until use. The mRNA expression levels of Apollon and

Table 1. The modified KBRMS protocol

Regimen	Drug	Dose (mg/m^2/d)	Schedule
Primary induction	BH-AC	300	i.v., days 0-6
		300	i.v., days 7-9 (if <5% BM blasts on day 7)
		400	i.v., days 7-9 (if 6-25% BM blasts on day 7)
		500	i.v., days 7-9 (if >25% BM blasts on day 7)
	Idarubicin	12	i.v., days 0-2
	6-Thioguanine	100	p.o., days 0-6
	Cytarabine	20	i.t., day 0 (if age is <1 y)
		30	i.t., day 0 (if age is between 1 and 2 y)
		50	i.t., day 0 (if age is between 2 and 3 y)
		70	i.t., day 0 (if age is >3 y)
Secondary induction	Cytarabine	6,000	i.v., days 0-3
	Cytarabine	20-70	i.t., day 0 (different dose according to age)
1st consolidation	BH-AC	200	i.v., days 0-4
	Idarubicin	12	i.v., days 0 and 1
	Cytarabine	20-70	i.t., day 0 (different dose according to age)
2nd consolidation	BH-AC	200	i.v., days 0-4
	Mitoxantrone	12	i.v., days 0 and 1
	Cytarabine	20-70	i.t., day 0 (different dose according to age)
3rd consolidation	Etoposide	100	i.v., days 0-4
	Amsacrine	100	i.v., days 0 and 1
	Cytarabine	20-70	i.t., day 0 (different dose according to age)
4th consolidation	Etoposide	100	i.v., days 0-4
	Mitoxantrone	12	i.v., days 0 and 1
	Cytarabine	20-70	i.t., day 0 (different dose according to age)

Abbreviations: BH-AC, behenoyl 1-β-D-arabinofuranosylcytosine; BM, bone marrow; p.o., oral.

Table 2. The expression levels of Apollon and the treatment outcomes with respect to the clinical characteristics at diagnosis

Clinical characteristics	*n*	3-y RFS ± SE (%)	Median expression	*P*
Sex				
Female	21	72.3 ± 10.6	3.796	
Male	34	58.1 ± 10.0	4.842	0.478
Age (y)				
<5	20	50.3 ± 12.8	4.818	
≥5	35	71.4 ± 8.7	3.796	0.146
Leukocyte (/μL)				
<10,000	22	64.9 ± 10.9	1.840	
≥10,000	33	62.7 ± 10.2	5.401	0.004
Extramedullary disease				
Absent	43	71.6 ± 14.0	3.730	
Present	12	62.6 ± 8.4	17.102	<0.001
FAB classification				
M1-M6	49	66.7 ± 7.7	3.796	
M7	6	40.0 ± 21.9	7.062	0.049
Cytogenetics				
Favorable	18	90.9 ± 8.7	3.321	
Others	37	51.9 ± 9.0	4.720	0.190
FLT3-ITD/TKD mutations				
Absent	43	65.9 ± 7.8	4.223	
Present	9	47.6 ± 22.5	6.124	0.352
Not examined	3	100		
Day 7 response to treatment				
Favorable	31	75.7 ± 8.9	2.072	
Unfavorable	12	35.0 ± 15.4	9.317	0.035
Undetermined*	12	62.5 ± 17.1	4.842	
Induction of remission				
Yes	43	68.2 ± 7.7	3.796	
No	8	45.0 ± 18.8	11.099	0.312
Not evaluable †	4		5.416	

NOTE: The expression levels are presented as the median values. Differences in the level of Apollon expression were analyzed using the Mann-Whitney *U* test. A *P* value of <0.05 was considered significant.
Abbreviations: ITD, internal tandem duplication; TKD, tyrosine kinase domain.
* Day 7 bone marrow examination was not done in 12 patients (five with acute promyelocytic leukemia, three with severe infection, and four who experienced early death).
† Four patients died from toxicity during induction chemotherapy.

glyceraldehyde-3-phosphate dehydrogenase were determined by quantitative reverse transcriptase–PCR using the ABI PRISM 7000 sequence detector system (Applied Biosystems). Quantitative reverse transcriptase–PCR amplification was done using predeveloped assay-on-demand gene expression sets for the *Apollon* gene (Hs00212288_m1, GeneBank accession number NM_016252.2, Applied Biosystems) and TaqMan glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems) for the glyceraldehyde-3-phosphate dehydrogenase gene in combination with the TaqMan Universal PCR Master Mix (Applied Biosystems). All the reactions were done in triplicate using 20-μL samples containing 50 ng of complementary DNA. The reaction protocol involved heating for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). The data were analyzed using the ABI PRISM 7000 Sequence Detection software. The levels of Apollon expression in unknown samples were calculated as a ratio of Apollon to glyceraldehyde-3-phosphate dehydrogenase. The levels of Apollon and glyceraldehyde-3-phosphate dehydrogenase mRNA expression were quantified using the standard curves generated from known serial dilutions of the standard RNA obtained from A549 cells by assuming a linear relationship between the first cycle number, at which the fluorescence signal significantly increased (Ct value) and the logarithm of the starting quantity. A negative control without a template was included in each experiment.

Statistical analysis. The differences in Apollon expression with respect to the clinical factors at diagnosis [i.e., gender, age, leukocyte count, the presence or absence of extramedullary disease, French-American-British (FAB) classification (21), and structural cytogenetic abnormalities] and the treatment outcome (day 7 response to induction chemotherapy and induction of remission with the primary induction chemotherapy regimen) were analyzed using a Mann-Whitney *U* test. The expression levels are presented as median values. The patients were categorized into two groups according to the level of Apollon expression (≥median versus <median). The proportion of relapsed patients in the two groups of patients was compared using a Pearson χ^2 test. The event-free survival and relapse-free survival (RFS) rates along with SE were estimated using the Kaplan-Meier method. An event was defined as a disease relapse or treatment-related death. The differences in the survival rates according to the Apollon expression levels (≥median versus <median) were compared using a log-rank test. *P* values of <0.05 were considered significant.

Results

Patient characteristics. Fifty-five children were enrolled in this study. Table 2 shows the patients' clinical characteristics and Apollon expression levels. The median age of the 34 boys

and 21 girls was 82 months (range, 2-179 months), and their median leukocyte count was 20,460/μL (range, 440-345,950). When the disease was classified according to the FAB classification, 1 patient had AML M0, 30 had M1/M2, 5 had M3, 12 had M4/M5, and 6 had M7. Three patients had Down syndrome, and one of them had AML M7. The common structural abnormalities identified by conventional chromosomal analysis and/or fluorescence *in situ* hybridization studies were *t*(8;21) in 11 patients, t(15;17) in 5, 11q23 rearrangement in 11, and inv(16) in 2. Among 12 patients with extramedullary disease, 11 patients had chloroma (scalp in 5, orbit in 3, and skin in 3) and one had a central nervous system involvement of

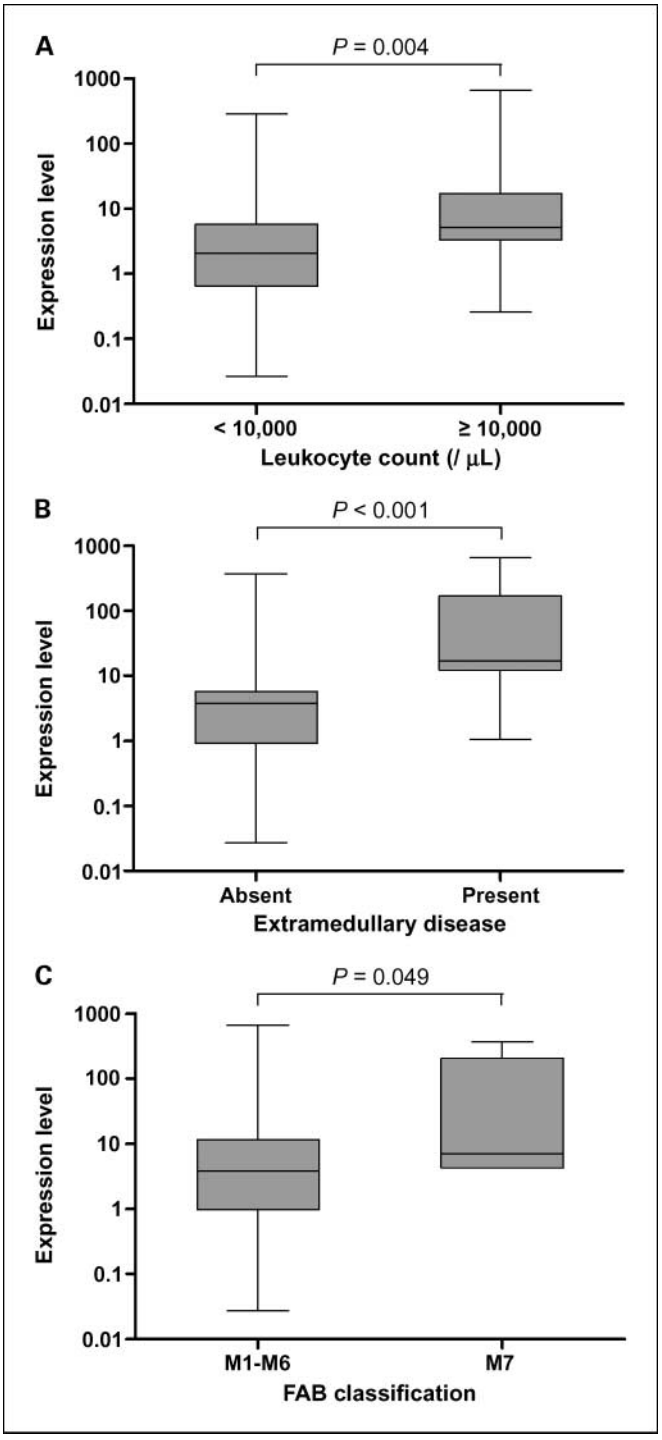

Fig. 1. The level of Apollon expression according to the characteristics at diagnosis. The level of Apollon expression was higher in patients with a leukocyte number of ≥10,000/μL (*A*), patients with extramedullary disease (*B*), and patients with FAB M7 (*C*) than in patients with a leukocyte number of <10,000/μL, patients without extramedullary disease, and patients with the FAB subtype other than M7.

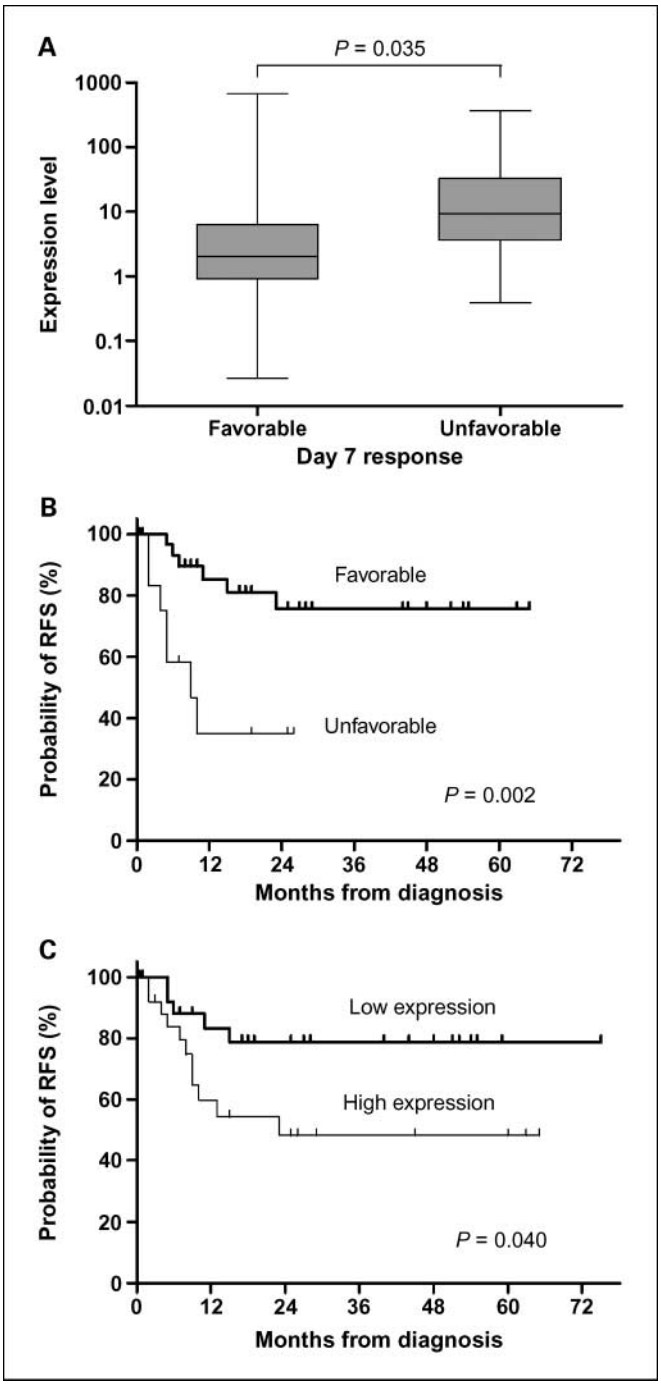

Fig. 2. *A,* the level of Apollon expression was higher in patients with an unfavorable day 7 response than in those with a favorable day 7 response. *B,* the RFS rate was higher in patients with a favorable day 7 response than in those with an unfavorable response. *C,* the RFS rate was lower in patients with Apollon overexpression than in those without Apollon overexpression.

leukemic cells. The FLT3 gene mutation was found in 9 among 52 patients, which revealed five patients had internal tandem duplications and four had point mutations in the tyrosine kinase domain. The median proportion of bone marrow leukemic blasts at diagnosis was 73.0% (range, 25-98%). The proportion of patients with >50% bone marrow leukemic blasts was 74.5%. There was no difference in the proportion of bone marrow leukemic blasts between the patients with Apollon overexpression (≥median) and those without (P = 0.873). Forty-three (78.2%) patients achieved CR after primary induction chemotherapy. Thirty patients received an allogeneic stem cell transplant (6 related and 24 unrelated; 4 peripheral blood, 14 bone marrow, and 12 cord blood stem cells) at the first CR.

Apollon overexpression: association with the unfavorable clinical features at diagnosis. Apollon overexpression was found to be associated with the unfavorable clinical features at diagnosis (Table 2; Fig. 1). The median level of Apollon expression in 55 patients was 4.255 (range, 0.027-671.675). The level of Apollon expression was higher in patients with a leukocyte number of ≥10,000/μL (P = 0.004), patients with extramedullary disease (P < 0.001), and patients with a FAB M7 (P = 0.049) than in those with a leukocyte number of <10,000/μL, those without extramedullary disease, and those with a FAB subtype other than M7. The level of Apollon expression in three patients with Down syndrome was not significantly different from that in the remaining patients. Whereas four of six patients with the FAB M7 subtype died from relapse (3) or treatment-related toxicities (1), the only patient with Down syndrome is still alive. There was no difference in the level of Apollon expression between FAB M3 and the other subtypes. The level of Apollon expression was higher in patients with unfavorable cytogenetic abnormalities defined by the absence of t(8;21), t(15;17), and inv(16), albeit without a statistical significance. The level of Apollon expression was higher in patients with 11q23 abnormalities or *FLT3* gene mutations than in patients lacking those genetic aberrations (median 5.401 versus 4.239 and 6.124 versus 4.223, respectively), which was not statistically significant (Table 2).

Apollon overexpression: association with a poorer treatment outcome. The median follow-up duration in 33 live patients was 30 months (range, 8-76 months). The leukemia relapsed in 16 patients, and treatment-related mortality was observed in six patients. The 3-year RFS and event-free survival rates (±SE) in all 55 patients were 64.1 ± 7.4% and 56.3 ± 7.1%, respectively. Apollon overexpression was associated with an unfavorable early response to induction chemotherapy. The level of Apollon expression was higher in patients with an unfavorable day 7 response (defined as >5% leukemic blasts on the bone marrow aspirate on day 7, the presence of a leukemic cell cluster on the bone marrow tissue section on day 7, or a persistence of circulating leukemic blasts in the peripheral blood on day 7) than in those with a favorable day 7 response (absence of an unfavorable response; P = 0.035; Table 2 and Fig. 2A). The RFS rate was higher in patients with a favorable day 7 response than in those with an unfavorable response (75.7 ± 8.9% versus 35.0 ± 15.4%, P = 0.002; Fig. 2B). The level of Apollon expression was higher in patients who did not achieve CR after chemotherapy with the primary induction regimen than those who did; however, the difference was not significant. The relapse of disease was more frequent in patients with Apollon overexpression (11 of 28) than those without (5 of 27; P = 0.090). Similarly, the 3-year RFS rate was lower in patients with Apollon overexpression than in those without (48.3 ± 11.2% versus 78.7 ± 8.5%, P = 0.040; Fig. 2C).

Discussion

The resistance of tumor cells to apoptosis may pose serious clinical problems and be associated with high-risk features at diagnosis as well as a poor response to various treatments, such as chemotherapy and radiotherapy. A variety of antiapoptotic proteins are expressed in different tumors, and their expression levels may be related to the unfavorable features at diagnosis and/or a poor response to treatment. However, the clinical relevance of these biological regulators remains largely elusive, and particularly, little is known about Apollon in this respect since Chen et al. first described the protein as an antiapoptotic regulator (16). From this context, this study was done to investigate the possible relationship between Apollon expression and the clinical features in childhood *de novo* AML for the first time in human cancers.

In this study, Apollon overexpression was found to be associated with initial clinical characteristics at diagnosis. For example, the level of Apollon expression was higher in patients with a leukocyte count of ≥10,000/μL and patients with extramedullary disease, although these patients did not have a poorer RFS. This suggests that Apollon overexpression is associated with the high-proliferative nature of leukemic blasts. Considering that the survival in patients with the FAB M7 subtype is very poor (22), it is notable that the level of Apollon expression was also higher in these patients than in patients with the other FAB subtypes. In addition, the level of Apollon expression in patients with favorable cytogenetics [t(8;21), t(15;17), and inv(16)] was lower, albeit without a statistical significance. Lastly, Apollon overexpression was consistently associated with lower 3-year RFS rates (Table 2). Overall, Apollon overexpression is associated with the unfavorable clinical features at diagnosis in childhood AML.

The early response to induction chemotherapy has proved to play an important role in children with acute lymphoblastic leukemia (23–25). In childhood acute lymphoblastic leukemia, the failure of achieving blast clearance from the bone marrow aspirates after 1 or 2 weeks of remission induction chemotherapy (23, 24) or the persistence of circulating blasts after 1 week of multiagent chemotherapy (25) indicates a poor prognosis. Although it is likely that the initial response to induction chemotherapy may also be predictive of the outcome in childhood AML, there are currently limited data available to draw any conclusion. Some studies have evaluated the response to induction chemotherapy by assessing the degree of residual leukemic infiltration in the bone marrow after 6 or 14 days of chemotherapy (4–7). In this study, the early response to induction chemotherapy was evaluated on day 7, and the unfavorable day 7 response was strongly associated with a poorer RFS. Here again, it is of note that Apollon overexpression was associated with an unfavorable day 7 response. In addition, the level of Apollon expression was higher in patients who did not achieve CR after primary induction chemotherapy than in those who did, although the difference was not statistically significant. Collectively, it was suggested that Apollon overexpression is associated with delayed blast

clearance from the bone marrow or peripheral blood and the resistance of blasts to apoptotic stimuli provided by the chemotherapeutic agents, eventually leading to a poorer RFS.

The IAP family proteins inhibit the apoptosis induced by a variety of stimuli, and therefore, their overexpression is expected to be associated with the unfavorable clinical features in a variety of malignancies, including AML. However, the clinical significance of IAP overexpression in acute leukemia is not completely consistent with what was expected from previous *in vitro* studies. For example, IAP overexpression was not always associated with the unfavorable clinical features in acute leukemia (26). Furthermore, it was recently reported that the high expression of Livin, also a member of IAP family proteins, is an independent favorable prognostic factor in childhood acute lymphoblastic leukemia (27). This suggests that the role of IAP in leukemogenesis or in the maintenance of leukemic cells might be different from that which has been previously recognized. However, the findings from this study showed that the clinical significance of Apollon overexpression is consistent with what has been previously recognized from *in vitro* studies.

This is the first report demonstrating that Apollon overexpression is associated with unfavorable clinical features at diagnosis and a poorer treatment outcome in childhood AML. Through validation by further studies, Apollon expression may be used as a prognostic marker in childhood AML. Studies involving a larger group of patients with different malignancies, particularly with respect to other apoptosis-related molecules, will be needed to reveal the pathophysiologic and clinical relevance of Apollon expression in human cancers.

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