Aven overexpression: Association with poor prognosis in childhood acute lymphoblastic leukemia

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Abstract

Aven expression has recently been identified as an anti-apoptotic protein. In this study, Aven expression in 91 children with acute lymphoblastic leukemia (ALL) was investigated for possible correlation with clinical features at diagnosis and treatment outcome. Aven expression was found to be higher in patients ≥10 years old or <1 year (P=0.003), and in patients with unfavorable cytogenetic abnormalities (P<0.001). Aven expression was also significantly higher in relapsed patients in the standard-risk group. Aven overexpression was an independent poor prognostic factor. These findings demonstrate that Aven expression can predict prognosis in childhood ALL.

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1. Introduction

Apoptosis is an active biological mechanism leading to programmed cell death. During the last decade, a complex network of pro- and anti-apoptotic proteins, that strictly govern the regulation of apoptosis pathways have been identified [1–4]. Regulation of a strict balance between life and death at the cellular level is the core function of proteins involved in apoptosis. For example, a loss of apoptosis might result in a wide variety of diseases including cancer. Cellular defects that interfere with the normal apoptotic process are frequently involved in cancer development and progression [5]. Furthermore, many studies have demonstrated the role of apoptosis regulators in rendering tumor cells resistant to apoptosis in vitro and in vivo. In addition, up-regulation of anti-apoptotic proteins favors tumor survival [6–8]. It is well known that a variety of anti-apoptotic proteins, such as inhibitors of apoptosis proteins and Bcl-2 family proteins are expressed in different tumor cells; expression of these proteins may be related to unfavorable clinical features at diagnosis and a poor response to treatment [8–12].

Recently, Chau et al. identified a novel apoptosis inhibitor, named Aven [13]. Aven binds to both Bcl-xL, an anti-apoptotic Bcl-2 family member, and to the caspase regulator, Apaf-1. The Aven protein appears to act synergistically and thus enhances the anti-apoptotic function of Bcl-xL; in addition, it was also shown to interfere with the ability of Apaf-1 to self-associate, suggesting that Aven independently impairs Apaf-1-mediated caspase activation. Although it is known that Aven acts as anti-apoptotic regulator, little is known about its clinical relevance in patients with different tumors. In the only clinical study of Aven expression, Paydas et al. [14] showed that Aven expression was higher in adult acute leukemia patients with relapse than in those without relapse. This is the only report on the relationship between the expression level of Aven and clinical parameters in malignancy.
ALL is the most common malignancy in children. It accounts for one-fourth of all childhood cancers and approximately 75% of all cases of childhood leukemia. The parameters of initial leukocyte count and age at diagnosis have traditionally provided the most reliable basis for patient stratification, because they are readily available and are relatively independent predictors of prognosis [15]. Immunophenotypic and cytogenetic abnormalities of leukemic cells are also important factors to be considered for prognosis, and are used in the design and analysis of modern therapeutic trials for childhood ALL [16, 17]. Recently, as a result of the accumulation of knowledge concerning the molecular biology of malignancy, new diagnostic modalities are beginning to be incorporated into diagnostic and therapeutic strategies [18–20]. One of these modalities, the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) allows messenger RNA expression levels to be determined. This technique allows researchers to examine the expression patterns of genes at the RNA level. If specific patterns of gene expression can be correlated with clinical features in childhood ALL, refinements of current prognosis-based stratification systems would be possible.

In this study, we evaluated the expression of Aven in childhood ALL, in an attempt to determine possible relationship between Aven expression and clinical features at diagnosis and treatment outcome. Quantitative RT-PCR was used to study Aven expression. We found that Aven overexpression is correlated with the presence of unfavorable prognostic factors at diagnosis and a poorer treatment outcome. This is the first study to conclude that Aven expression is associated with prognosis in childhood ALL.

2. Materials and methods

2.1. Patients and treatment

Ninety-one children younger than 15 years of age who were newly diagnosed with ALL from May 2000 to May 2005 at the Samsung Medical Center were included in the study. A diagnosis of ALL was made based on morphologic assessment of Wright-Giemsa stained smears of bone marrow aspirate. Complete immunophenotyping and cytogenetic analysis including fluorescent in situ hybridization (FISH) for (t(9; 22), 11q23 rearrangement and t(12; 21) were a part of the routine evaluation. Ploidy was determined directly by the classic method of counting the modal number of chromosomes in a metaphase karyotype preparation.

Patients were assigned to the standard-risk group if the leukocyte number was <50,000/µL and the age was 1–9 years at diagnosis. Otherwise, patients were assigned to a high-risk group. In the standard-risk patients, the treatment protocol was modified from the Children’s Cancer Group (CCG)-1891 [21] and CCG-1952 protocols [22]. The regimen used was switched to one modified from the CCG-1882 protocol [23] from consolidation chemotherapy if percentage of bone marrow leukemic blasts on day 7 was >25% during induction chemotherapy. If the percentage of leukemic blasts on day 14 was still >25%, the protocol used for the high-risk patients was restarted. In high-risk patients, the treatment protocol was modified from the CCG-106B [24] and CCG-1901 protocols [25]. If a patient, regardless of risk group, had one or more of the following: a leukocyte number of >100,000/µL, an age of <1 year, presence of t(9; 22), or the 11q23 rearrangement, an additional two courses of consolidation chemotherapy (ifosfamide + etoposide/high-dose methotrexate + high-dose cytosine arabinoside) was given after the first consolidation chemotherapy. Patients with an appropriate stem cell donor proceeded to hematopoietic stem cell transplantation and patients without a stem cell donor proceeded to maintenance chemotherapy. The protocols used were approved by the institutional review board and informed consent was obtained from parents or guardians.

2.2. RNA isolation and real-time quantitative RT-PCR

Mononuclear cells (MNCs) were isolated from 2 ml of bone marrow aspirate at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using a QiAamp RNA Blood kit (Qiagen, USA) to remove chromosomal DNA, complementary DNA (cDNA) was synthesized using oligo (dT)15 mer primer using SuperScript III Reverse Transcriptase (Invitrogen, USA) and stored at −20 °C until use.

The mRNA expression levels of Aven and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by quantitative RT-PCR using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA). Quantitative RT-PCR amplification was performed using pre-developed assays-on-demand gene expression sets for Aven gene (Hs00220565_m1, GeneBank accession number NM_020371, Applied Biosystems), and TaqMan® GAPDH Control Reagents (Applied Biosystems) for the GAPDH gene in combination with the TaqMan® Universal PCR Master Mix.

All reactions were performed in triplicate using 20 µl samples containing 50 ng of cDNA. The reaction protocol used involved heating for 2 min at 50 °C and 10 min at 95 °C, followed by 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C). Analysis was performed using ABI PRISM 7000 Sequence Detection software. Aven expression levels in unknown samples were calculated as ratios of Aven versus GAPDH. Quantitations of Aven and GAPDH mRNA were performed using standard curves made from known serial dilutions of standard RNA obtained from A549 cells. Standard curves were generated by assuming a linear relationship between the first cycle number at which the fluorescence signal significantly increased (Ct value) and the logarithm of starting quantity. A negative control without a template was included in each experiment.
2.3. Statistical analysis

Differences in Aven expression with respect to common prognostic factors (i.e. gender, age, leukocyte count, immunophenotype, and numerical or structural cytogenetic abnormalities) and treatment outcome (occurrence of relapse) were analyzed using the Mann–Whitney U-test. Expression levels are presented as median values. Patients were categorized into two groups according to Aven expression levels (≥median or <median). The relationship between the relapse free survival rate (RFS) and Aven expression levels in each risk group was estimated using the Kaplan–Meier method. Univariate and multivariate analyses comprising prognostic factors with relapse free survival was estimated using the Cox regression test. Statistical significance was accepted for P-values of <0.05.

3. Results

3.1. Patients characteristics

Ninety-one children were included in this study. Their clinical characteristics and Aven expression levels are presented in Table 1. The median age of the 53 boys and 38 girls was 59 months (range 3–174), and their median leukocyte count was 8,890/\(\mu l\) (range 310–452,500). The number of standard-risk and high-risk patients were 55 and 36, respectively, and 75 (82.4%) had the precursor B cell immunophenotype. The common translocations identified by conventional chromosomal analysis and/or FISH examination were: \((9; 22)\) in five patients, \((8; 14)\) in one, \((12; 21)\) in five, \((9; 19)\) in five and \((11q23)\) in 10. The number of patients with hypodiploidy (≤45), near tetraploidy and hyperdiploidy (≥47) were 6, 1 and 19, respectively. Fifteen patients received allogeneic hematopoietic stem cell transplantation at the first complete remission.

3.2. Aven overexpression was correlated with unfavorable prognostic factors

The presence of Aven overexpression was found to be correlated with the presence of unfavorable prognostic factors at diagnosis (Table 1 and Fig. 1). Aven expression was significantly higher in patients ≥10 years old or <1 year (\(P=0.003\), Fig. 1a). When an unfavorable cytogenic feature was defined as the presence of one or more of the following: hypodiploidy (≤45), near tetraploidy, \((9; 22)\), or the \(11q23\) rearrangement, Aven expression was found to be significantly higher in patients with unfavorable cytogenetic features (\(P=0.001\), Fig. 1b). In addition, Aven expression was significantly higher in patients with an unfavorable structural cytogenetic abnormality defined as the presence of \((9; 22)\) or an \(11q23\) rearrangement (\(P=0.006\)). Of the frequent translocations, i.e. \((9; 22)\), \(11q23\) rearrangement and \((12; 21)\), Aven expression was highest in the presence of the \(11q23\) rearrangement and lowest in \((12; 21)\) (Fig. 1c). Aven expression was also significantly higher in patients with an unfavorable numerical cytogenetic abnormality, defined as the presence of hypodiploidy (≤45) or near tetraploidy. In addition, Aven expression was higher in patients with hypodiploidy (≤45) than in those with diploidy or hyperdiploidy (≥47), and this was particularly true for those patients with a chromosome number ≥51 (Fig. 1d). Aven expression was also elevated in patients with a non-precursor B cell immunophenotype or leukocyte count of ≥50,000/\(\mu l\); there were no significant associations between Aven expression and these findings.

### Table 1

<table>
<thead>
<tr>
<th>Differential expression of Aven according to common prognostic factors at diagnosis and treatment outcome</th>
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<tr>
<td>Risk factors and outcome</td>
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<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>≤10 or &lt;1</td>
</tr>
<tr>
<td>Leukocyte ((\mu l))</td>
</tr>
<tr>
<td>&gt;50000</td>
</tr>
<tr>
<td>Risk group</td>
</tr>
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<tr>
<td>Immunophenotype</td>
</tr>
<tr>
<td>Others 16</td>
</tr>
<tr>
<td>Cytogenetic abnormality</td>
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<tr>
<td>Favorable or normal 74</td>
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<td>Treatment outcome</td>
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<td>Relapse 8</td>
</tr>
</tbody>
</table>

Expression levels are presented as median values. Differences in Aven expression were analyzed using the Mann–Whitney U-test. Statistical significance was accepted for P-values of <0.05.

- A favorable structural cytogenetic abnormality was defined as the presence of hypodiploidy (≤45) or near tetraploidy.
- A favorable numerical cytogenetic abnormality was defined as the presence of hypodiploidy (≤45). Near tetraploidy, \((9; 22)\), or the \(11q23\) rearrangement.
- An unfavorable combined cytogenetic abnormality was defined as the presence of one or more of the following: hypodiploidy (≤45), near tetraploidy, \((9; 22)\), or the \(11q23\) rearrangement.
Fig. 1. Aven expression as associated with age and the presence of chromosomal abnormalities. Aven expression was significantly higher in patients ≥10 years old or ≤1 year (a). When an unfavorable cytogenetic feature was defined as the presence of one or more of the following: hypodiploidy (≤45), near tetraploidy, t(9;22), or the 11q23 rearrangement, Aven expression was significantly higher in patients with an unfavorable cytogenetic feature than in those with favorable or normal cytogenetic features (b). Of the frequent translocations, Aven expression was highest in the presence of the 11q23 rearrangement and lowest in t(12;21) (c). Aven expression was higher in patients with hypodiploidy (≤45) than in those with hyperdiploidy, and this was particularly true for those with chromosome number ≥51 (d).

3.3. Aven overexpression was correlated with a poorer treatment outcome

Higher expression levels of Aven were also found to be correlated with a poorer treatment outcome. Aven overexpression was related to the occurrence of relapse in the standard-risk patients ($P=0.049$), but not in the high-risk patients (Table 1 and Fig. 2a). Similarly, RFS was lower in the standard-risk patients who exhibited overexpression of Aven ($\geq$median) ($P=0.090$, Fig. 2b); however, this was not found in the high-risk patients. Univariate and multivariate analyses comprising common prognostic factors with relapse-free survival demonstrated that Aven overexpression is an independent poor prognostic factor in childhood ALL (Table 2).

<table>
<thead>
<tr>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Male</td>
<td>2.470</td>
</tr>
<tr>
<td>Age ≥10 or &lt;1</td>
<td>2.971</td>
</tr>
<tr>
<td>WBC &gt;50000/μl</td>
<td>2.012</td>
</tr>
<tr>
<td>Non-precursor B cell</td>
<td>0.634</td>
</tr>
<tr>
<td>t(9;22), 11q23 rearrangement</td>
<td>1.587</td>
</tr>
<tr>
<td>Hypodiploidy (≤45)</td>
<td>3.011</td>
</tr>
<tr>
<td>Aven expression level</td>
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</table>

Prognostic impact of each variable was estimated using Cox regression analysis. Statistical significance was accepted for $P$-values of <0.05. HR, hazard ratio; CI, confidence interval.
Fig. 2. Aven expression and treatment outcome. Aven overexpression was significantly related with the occurrence of relapse in the standard-risk patients (a). Similarly, RFS was lower in patients with overexpression of Aven (≥ median expression) in the standard-risk patients (b).

4. Discussion

Over the past decade, a complex network of regulators that strictly govern the regulation of apoptotic pathways has been identified. New apoptosis regulators are continuously being identified. Resistance of tumor cells to apoptosis may pose serious clinical problems and be associated with high-risk features at diagnosis as well as poor response to treatments such as chemotherapy and radiotherapy. It is well known that a variety of anti-apoptotic proteins are expressed in different tumors and that their expressions may be related to unfavorable features at diagnosis and/or a poor response to treatment. However, little is known about Aven in this respect, since Chau et al. reported it as an anti-apoptotic regulator. In the only available report by Paydas et al., Aven expression was observed to be higher in patients with adult acute leukemia (33 acute myeloid leukemias and 26 ALLs) when compared to normal controls. Furthermore, Aven expression was noted to be higher in patients who had disease relapse. One limitation of this report was the fact that both acute myeloid leukemia and ALL patients were studied as one group; although these disorders are biologically different. Therefore, they could not show significant correlations between Aven expression and clinical features at diagnosis or treatment outcome in either disease. No other study has reported on the clinical association of Aven with clinical characteristics and patient response to treatment.

In this study, in an attempt to investigate a possible correlation between Aven expression and clinical features in childhood ALL, we adopted a quantitative RT-PCR approach. Aven expression was found to be significantly higher in patients ≥10 years old or <1 year and in patients with unfavorable cytogenetic abnormalities. Both unfavorable structural and numerical abnormalities were significantly correlated with Aven overexpression. In addition, we also found that Aven expression appears to be correlated with treatment outcome. Aven overexpression was significantly correlated with the occurrence of relapse in the standard-risk patients. Similarly, RFS was lower in the standard-risk patients overexpressing Aven. In addition, univariate and multivariate analyses of common prognostic factors, with relapse free survival, demonstrated that Aven overexpression is an independent poor prognostic factor in childhood ALL. It is interesting that the occurrence of relapse was associated with Aven overexpression only in the standard-risk patients. However, unfavorable prognostic factors at diagnosis were consistently related with Aven overexpression in all patients both in the standard and high-risk groups. These findings suggest that the intense treatment protocol applied to high-risk patients may override the risk associated with Aven overexpression. Therefore, these findings suggest that Aven overexpression is associated with both the presence of unfavorable clinical features at diagnosis and a poorer treatment outcome.

Translocation is the most common structural cytogenetic abnormality in childhood ALL. A variety of chromosomal translocations have been linked to rearrangements and an altered regulation of cellular oncogenes. These translocations are thought to play a pivotal role in the leukemogenic process. Some translocations, via altered gene expression, confer a growth advantage on cells of a particular phenotype. It is interesting that Aven overexpression was found to be strongly correlated with unfavorable structural cytogenetic abnormalities including (9; 22) and the 11q23 rearrangement. These are clinically important structural cytogenetic abnormalities in childhood ALL and are associated with the poorest prognosis [26–29]. Function of fusion proteins, i.e. products of these translocations, may be related to Aven overexpression. However, in contrast to Aven overexpression in the presence of these unfavorable translocations, Aven expression was significantly lower in the presence of (12; 21), the most common translocation with the best prognosis [30,31].

Aven expression in this study was not only significantly higher in patients with hypodiploidy (≤45) or near tetraploidy but also lower in patients with hyperdiploidy (≥47), especially ≥51. The ploidy of the childhood ALL karyotype has long been known to be a prognostic determinant [32]. Independent of other prognostic factors, hypodiploidy appears to have important prognostic implications that have not changed with current therapy [33,34]. Although the absolute number of chromosomes chosen as the “cut-point” for analy-
sis may vary slightly between studies, children with higher ploidy (>51), except near tetraploidy, have the best prognosis [35–37]. It is interesting to note that Aven overexpression in this study was consistently correlated with unfavorable numerical cytogenetic abnormalities in childhood ALL.

This is the first report to show that Aven over expression is related to unfavorable prognostic factors at diagnosis and a poorer treatment outcome in childhood ALL. Our findings demonstrate that Aven expression can predict prognosis in childhood ALL. Thus, further studies on a larger group of patients with different malignancies are now needed for confirmation and further understanding.

Acknowledgement

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References


