Asparagine synthetase expression is linked with L-Asparaginase resistance in TEL-AML1 negative, but not in TEL-AML1 positive pediatric acute lymphoblastic leukemia

Wendy A.G. Stams¹, Monique L. den Boer¹, Amy Holleman¹, Inge M. Appel¹, H. Berna Beverloo², Elisabeth R. van Wering³, Gritta E. Janka-Schaub⁴, William E. Evans⁵ and Rob Pieters¹,³

¹Erasmus MC / University Medical Center Rotterdam / Sophia Children’s Hospital, Division of Pediatric Oncology/Hematology, Rotterdam The Netherlands.
²Erasmus MC / University Medical Center Rotterdam, Department of Clinical Genetics, Rotterdam, The Netherlands.
³Dutch Childhood Oncology Group, The Hague, The Netherlands.
⁴COALL study group, Hamburg, Germany.
⁵St. Jude Children’s Research Hospital, Department of Pharmaceutical Sciences, Memphis, Tennessee.

Corresponding author:

Monique L. den Boer
Erasmus MC - Sophia Children’s Hospital
Division of Pediatric Oncology / Hematology
Dr. Molewaterplein 60
3015 GJ Rotterdam, The Netherlands
Tel.: 0031 10 4089379
Fax.: 0031 10 4089433

Email: m.l.denboer@erasusmc.nl
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ABSTRACT

Resistance to L-Asparaginase in leukemic cells may be caused by an elevated cellular expression of asparagine synthetase (AS). Previously, we reported that high AS expression did not correlate to L-Asparaginase resistance in TEL-AML1 positive B-lineage ALL. In the present study, we confirmed this finding in TEL-AML1 positive patients (n=28) using microarrays. In contrast, 35 L-Asparaginase resistant TEL-AML1 negative B-lineage ALL patients had a significant 3.5-fold higher AS expression than 43 sensitive patients (p=0.0004). Using RTQ-PCR, this finding was confirmed in an independent group of 39 TEL-AML1 negative B-lineage ALL patients (p=0.03). High expression of AS was associated with poor prognosis (4- yrs pDFS 58% ± 11%) compared to low expression (4- yrs pDFS 83% ± 7%; p=0.009). We conclude that resistance to L-Asparaginase and relapse-risk are associated with high expression of AS in TEL-AML1 negative but not in TEL-AML1 positive B-lineage ALL.
INTRODUCTION

L-Asparaginase (L-Asp) is an enzyme widely used in chemotherapeutic protocols for treatment of children with acute lymphoblastic leukemia (ALL). *In vitro* and *in vivo* resistance to L-Asp correlates with a relative poor prognosis in ALL\(^1,2\). L-Asp depletes asparagine and glutamine in blood cells\(^3\). Impaired capacity to synthesize sufficient amounts of asparagine due to reduced asparagine synthetase (AS) levels is thought to explain the L-Asp-sensitivity of ALL cells to L-Asp treatment\(^4,5\). L-Asp-resistance is suggested to be caused by an elevated cellular level of AS\(^6\).

TEL-AML1 positive ALL patients are more sensitive to L-Asp compared to TEL-AML1 negative patients\(^7,8\). However, we found a 5-fold higher expression of AS in the TEL-AML1 positive ALL patients compared to TEL-AML1 negative ALL patients\(^7\), recently confirmed by Krejci et al.\(^9\). Therefore, within TEL-AML1 positive ALL resistance to L-Asp is not associated with AS expression levels. Due to limited sample size in our previous study, it was unclear if this conclusion could be generalized for all other B-lineage ALL patients. Therefore, we investigated the relationship between AS expression and L-Asp-resistance in two independent and larger groups of TEL-AML1 negative ALL patients.
MATERIALS AND METHODS

Patient samples
Bone marrow and/or peripheral blood samples from 117 untreated children with TEL-AML1 negative and 28 untreated children with TEL-AML1 positive B-lineage ALL at initial diagnosis were collected at the Erasmus MC - Sophia Children’s hospital, the Dutch Childhood Oncology Group and the German COALL study group. Leukemic cell samples of patients were used after proper informed consent was given. In addition, samples from 17 TEL-AML1 negative ALL patients used in our previous study were included7. Within 24 hours after sampling, mononuclear cells were isolated and total cellular RNA was extracted from a minimum of 5 x 10^6 (≥ 90% leukemic cells) using Trizol reagent, as described before7.

Microarray analysis
RNA processing and hybridization to the U133A GeneChip® oligonucleotide microarray (Affymetrix) was performed according to manufacturer’s protocol. Data analysis was performed as described before10. For this study, we limited the analysis to AS data in \textit{in vitro} L-Asp-sensitive and resistant TEL-AML1 positive (23 and 5 patients respectively) and TEL-AML1 negative patients (43 and 35 patients respectively).

Real-time quantitative PCR (RTQ-PCR)
The mRNA expression level of AS and an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference, were quantified using real-time PCR analysis (Taqman chemistry) with efficiencies ≥ 95, as described before7. The relative expression level of AS to GAPDH is calculated using the comparative cycle time method7.

In vitro L-Asp cytotoxicity assay
In \textit{vitro} L-Asp (Paronal, Christiaens B.V., Breda, The Netherlands) cytotoxicity was determined using the MTT assay as described previously7. Cut-off criteria for sensitive (LC_{50}<0.033 IU/ml), intermediate sensitive and resistant (LC_{50}>0.912 IU/ml) towards L-
Asp were as described previously\textsuperscript{11}. These 3 groups were associated with prognosis of ALL patients\textsuperscript{11}.

\textit{Statistics}

Differences in mRNA expression between groups of patients were analyzed using the Mann-Whitney U test. The correlation between AS expression and L-Asp-resistance was calculated using the Spearman’s rank correlation test. Disease-free survival (pDFS) was calculated from the date of diagnosis to the date of non-response, relapse or last contact using the Kaplan-Meier method and compared by log-rank test. Multivariate analysis was performed with the Cox proportional-hazard regression model.
RESULTS AND DISCUSSION

AS expression was analyzed with micro-array technology in 78 TEL-AML1 negative and 28 TEL-AML1 positive ALL patients. No difference in AS expression between L-Asp-resistant (n=5, median=828 AU, P25-P75: 164-1871) and L-Asp-sensitive (n=23, median=516 AU, P25-P75: 141-689; p=0.3) patients was found within TEL-AML1 positive ALL patients, which is in concordance with earlier findings from our lab and others\(^7,9\). However, in TEL-AML1 negative ALL patients a significant 3.5 fold difference in AS expression was shown between L-Asp-resistant (n=35; median=946 AU, P25-P75: 293–1617) and sensitive (n=43; median=244 AU, P25-P75: 92–787) patients (p=0.0004) (Figure 1).

The original TEL-AML1 negative ALL patient group (n=17) from our previous study\(^7\) was enlarged to a total of 39 patients (different patients than used for micro-array analysis) and AS expression was analyzed by RTQ-PCR. A significant positive correlation was found between AS expression and L-Asp-resistance (R\(_s=0.39; p=0.017\)). Patients were divided in 3 subgroups based on \textit{in vitro} cellular cytotoxicity to L-Asp, i.e.: 10 sensitive, 15 intermediate sensitive and 14 resistant patients to L-Asp. A significant 3.5-fold higher AS expression was shown in L-Asp-resistant compared to L-Asp-sensitive TEL-AML1 negative ALL patients (p=0.03; Figure 2), which is in concordance with our micro-array data (Figure 1).

The mRNA expression level of AS does not necessarily correlate with protein level and enzyme activity of AS. However, Hutson et al.\(^{12}\) showed in human leukemia cell lines that complete amino acid deprivation resulted in a simultaneous increase in AS mRNA, protein, and enzymatic activity, suggesting that mRNA corresponds to protein and activity levels.

Based largely on \textit{in vitro} observations in (non-) human leukemia cell lines, it has been hypothesized that elevated AS activity is a cause of L-Asp-resistance in human leukemia cells lines\(^6,12-17\). Only a few studies have reported this relation between AS expression and L-Asp-sensitivity in patient materials\(^{18,19}\). We and others showed previously that AS expression is not related to L-Asp-sensitivity in TEL-AML1 positive ALL\(^7,9\). We now confirmed this with a second method (i.e. micro-array analysis). In

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contrast, we show in two independent groups of patients studied by two different techniques (micro-array and RTQ-PCR) that elevated AS expression is related to L-Asp-resistance in TEL-AML1 negative ALL patients. This is in line with the *in vitro* observations in (non-) human leukemia cell lines\textsuperscript{6,12-17}.

The 78 TEL-AML1 negative patients analyzed by micro-array had a median follow-up of patients at risk of 4.4 (range: 0.6-9.1) years. The clinical characteristics of L-Asp-resistant and sensitive patients were respectively: WBC of median 25.6 $\times 10^9$/L (P25-P75: 7.7-52.0) and 26.8$\times 10^9$/L (P25-P75: 8.8-73.2) $p=0.8$, age of median 8.0 (P25-P75: 4.5-11.5) and 4.0 years (P25-P75: 2.0-7.3) $p<0.0001$. High AS expression (median value) was associated with a poor prognosis (4-yrs pDFS 58%±11%) compared to low AS expression (4-yrs pDFS 83%±7%; $p=0.009$). Furthermore, the AS expression in patients who relapsed (946 AU, P25-P75: 305-2153) was 2.5 fold higher compared to patients who did not relapse so far (379 AU, P25-P75: 101-932; $p=0.01$). Multivariate analysis including WBC and age revealed that high AS expression is independently related to a poor prognosis (Hazard ratio: 3.0, 95% CI: 1.1-7.9, $p=0.03$). In contrast, we recently showed that the expression of AS in TEL-AML1 positive ALL is not related to outcome\textsuperscript{20}. Krejci et al.\textsuperscript{9} even found the opposite: a relation between high AS expression and a good prognosis in TEL-AML1 positive ALL patients. These data suggest that the role of AS for L-Asp-resistance and therapy is different between both genetic subtypes. Since the TEL-AML1 genotype is associated with an increased L-Asp-sensitivity\textsuperscript{7,8}, the mechanism of action (and hence cause of resistance) may be different. A possibility might be the higher expression levels of the pro-apoptotic protein CD95 in TEL-AML1 positive ALL cells compared to TEL-AML1 negative ALL cells\textsuperscript{21}, since the CD95-mediated pathway is activated by cell shrinkage which can be mediated by L-Asp\textsuperscript{22}. An alternative explanation might be that TEL-AML1 positive ALL cells are not able to provide sufficient amounts of the AS substrates, aspartate and glutamine, due to different amino acid metabolism and/or transmembrane amino acid transporters that contribute to the function of AS\textsuperscript{23}. Further detailed studies are required to understand the observed difference between TEL-AML1 negative and positive subtypes.
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REFERENCES:

LEGENDS

Figure 1.
**AS expression in TEL-AML1 negative ALL determined by micro-array**
AS expression in TEL-AML1 negative ALL patients sensitive (n=43) and resistant to L-Asp (n=35; p=0.0004) as determined by micro-array analysis. Circles represent individual patients; bars represent the median values. AU=Arbitrary units defined as scaled fluorescence measured on micro-array.

Figure 2.
**AS expression in TEL-AML1 negative ALL determined by RTQ-PCR**
AS expression in TEL-AML1 negative ALL patients sensitive (n=10) and resistant to L-Asp (n=14; p=0.03) as determined by RTQ-PCR. AS mRNA expression is indicated in arbitrary units (AU), and defined as the mRNA expression of AS relative to GAPDH *100. Circles represent individual patients; bars represent the median values.
Figure 1.

Asparagine synthetase (micro-array) versus L-Asparaginase cytotoxicity in TEL-AML1 negative B-lineage ALL
Figure 2.

Asparagine synthetase (RTQ-PCR) versus L-Asparaginase cytotoxicity in TEL-AML1 negative B-lineage ALL

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