

Involvement of SRSF1 in Alternative Splicing of FPGS and Methotrexate Resistance in Children with Acute Lymphoblastic Leukemia

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ABSTRACT

Methotrexate (MTX) is a key component in treatment of childhood ALL. Impaired polyglutamation is a known mechanism of MTX-resistance. To date, a spectrum of splicing alterations was identified for folypoly- γ -glutamate synthetase (FPGS), the enzyme which catalyzes polyglutamation. The serine/arginine-rich splicing factor 1 (SRSF1) is involved in both constitutive and alternative splicing. We found an association between the expression of SRSF1 isoforms, ASF1 and ASF3, and alternative splice variants of FPGS. Moreover, in a subgroup of patients with deficient polyglutamation, the ratio of ASF3 to ASF1 was associated with survival. Therefore splice regulators are potential prognostic markers for both patient stratification and personalized medicine in childhood ALL.

Keywords

Acute Lymphoblastic Leukemia, Alternative splicing, Splicing factor, SRSF1, FPGS, Drug resistance

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of blood cancer in children. The 5-year overall survival rates of childhood ALL currently reach as high as 90%.¹ However, still in a large proportion of these children, treatment failure results in relapse, which is largely caused by drug resistance. Since methotrexate (MTX) is a key component used during treatment of pediatric ALL, it is important to unravel the mechanisms behind MTX resistance and treatment failure in children with ALL.²

Polyglutamation is an important metabolic conversion, which influences the mode of action of MTX. Folypoly- γ -glutamate synthetase (FPGS) catalyzes polyglutamylolation of folates and antifolates, such as MTX. Interpersonal differences and sometimes loss of FPGS activity has been reported in patient samples of children with ALL.³ Because of the frequent lack of any substantial decrease in FPGS mRNA levels in combination with decreased FPGS activity, the contribution of posttranscriptional mechanisms to decreased FPGS activity is of interest. To date, a spectrum of FPGS splicing products was characterized in both ALL patient samples of children and cell lines.⁴ Moreover, high levels of partial intron 8 retention (8 PR), one of the FPGS splicing alterations

identified in ALL, is significantly associated with overall survival and event free survival in patients with impaired accumulation of long chain MTX polyglutamates.⁵ Therefore it is essential to characterize the mechanisms behind this phenomenon.

The process of alternative splicing is tightly regulated by a range of factors, including splicing factors. Pilot analysis points to differences in expression of serine/arginine-rich splicing factor 1 (SRSF1) isoforms between MTX resistant and sensitive cell lines. In general, SRSF1 is a well-documented splicing factor essential for both constitutive and alternative splicing. SRSF1 promotes spliceosome assembly and facilitates splice site selection.⁶ ASF1, ASF2 and ASF3 (alternative splice factor 1,2 and 3) are isoforms of SRSF1 which themselves are generated by alternative splicing. ASF2 and ASF3 are predicted to lack the serine/arginine rich (RS) domain and 12 residues immediately upstream of it, due to an early stop codon inserted by intron retention, in comparison to ASF1. The RS domain is thought to be required for protein-protein interaction of SR proteins with each other and with other components of the splicing machinery.^{7,8}

In the present study we investigate the role of SRSF1 in regulation of FPGS splicing and MTX resistance. ASF2 isoform is rather uncommon, most likely degraded by the nonsense-mediated decay pathway (NMD), and therefore unlikely to influence the splicing of FPGS. Therefore, this study primarily focuses on the differences in expression between isoforms ASF3 and ASF1 in relation to alternatively spliced fragments of FPGS and MTX sensitivity.

MATERIAL AND METHODS

Antibodies and Reagents

Mouse monoclonal SF2/ASF antibody was obtained from Santa Cruz (Dallas, TX, USA). Infrared secondary antibodies (anti-mouse and anti-rabbit) were obtained from Li-Cor (Lincoln, NE, USA).

Cell Culture

The human T-cell acute lymphocytic leukemia cell lines CCFR-CEM (ATCC, Manassas, VA, USA) and its two MTX-resistant sublines, CCRF CEM/R30dm and CCRF-CEM/RFC-, were maintained in RPMI-1640 medium containing 10% FCS and 1% penicillin/streptomycin. The MTX-resistant cell lines used in this study include: CEM-CCRF/R30dm which shows diminished FPGS activity (only 1-3 percent) while mRNA levels are not affected, was kindly provided by John McGuire. CCRF-CEM/RFC-displays decreased RFC activity caused by 2 inactivating mutations and thereby affects transport of MTX into the cell.

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Leukemic Patient Specimens

Bone marrow and/or peripheral blood samples were collected from 71 children with ALL at diagnosis after obtaining a written informed consent. Mononuclear cells were purified using Ficoll density separation. Cryopreserved cells were thawed and subsequently used for RNA isolation as described below.

Drug exposure

One million exponentially growing cells were exposed to a dose range (0nM, 100nM, 250nM, 500nM and 1000nM) of MTX for 24 hours. After exposure, cells were harvested by centrifugation and used for RNA extraction. For western blot analysis, twenty million cells from the logarithmic phase of growth were exposed to 1µM of MTX for 24 hours. Next to exposed cells, untreated control cells were harvested by centrifugation and washed two times with PBS. Nuclear and cytoplasmic proteins were extracted as described below.

RNA isolation, PCR and qRT-PCR assay

Total RNA was isolated using the RNeasy kit according to the instructions of the manufacturer (Qiagen, Venlo, The Netherlands). One µg of the obtained RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV; Invitrogen) in a reaction buffer containing random hexamer primers (Roche, Basel, Switzerland), dNTPs (Roche), and a ribonuclease inhibitor Rnasin (Promega, Madison, WI, USA). A polymerase chain reaction (PCR) was performed, using 1µl of the forward and reverse primers (table 1) and Reddy Mastermix (Thermo Scientific, Waltham, MA, USA).

Table 1. Primers and Sequences used for PCR

SF2-ex3	5'-ATGGCACTGGTGTCTGGAG-3'
SF2-ex3/4	5'-TCCTTGGGGAGTAAGTGCAC-3'

Quantitative real-time was performed using the LightCycler 480 SYBR green 1 master Real-Time PCR system (Roche). Primers specifically amplifying ASF1 and ASF3 isoforms of SRSF1 were used (table 2). Relative gene expression was calculated for each sample, as the ratio of ASF1 or ASF3 expression to GUS. CCRF-CEM wild type cells were used to normalize the expression of our target gene in patient samples.

Table 2. Primers and Sequences used for Real-Time PCR

ASF1_fw	5' ATCTCATGAGGGAGAACTGCC 3'
ASF1_rev	5' GTAAGTGGCACTCCTGCTGT 3'
ASF3_fw	5' ACACGTATTCTTTTCTTTGACCAGA 3'
ASF3_rev	5' TCTGGCAATTTACTTGGACAACC 3'

Protein Isolation and Western Blot analysis

Nuclear and cytoplasmic proteins were isolated directly after cells were harvested. Pellets were resuspended in 400 µl of Buffer A (10mM Hepes, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT and protease inhibitor) and incubated on ice for 15 minutes. Subsequently, 25µl of Nonidet P40 (10%) was added to lyse cell membranes. After 30 seconds of centrifugation, the supernatant, containing the cytosolic fraction was collected. Next, 100 µl of Buffer B (20mM Hepe, pH7.9, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and protease inhibitor)

was added to the remaining pellet. Tubes were incubated in the cold room, while shaking, for 30 minutes. Finally, after 2 minutes of centrifugation at 4°C, the supernatant was collected which corresponds to nuclear proteins. Protein concentrations of the obtained extracts were assessed using Biorad DC protein assay (Hercules, CA, USA). Western blot was performed as described previously by Franke et al.⁹

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics 21 software. Associations between SRSF1 expression and FPGS splicing alterations as well as variables related to drug resistance were analyzed using Spearman's Rho test. Median values were used as cut-off values to dichotomize variables, while cut-off values for clinical factors were selected based on historical values. Kaplan-Meier analysis was used in the univariate analysis of event free survival and overall survival in relation to the ratio of ASF3 to ASF1. Events included in event free survival were relapse and death. All tests were considered significant when p-value <0.05.

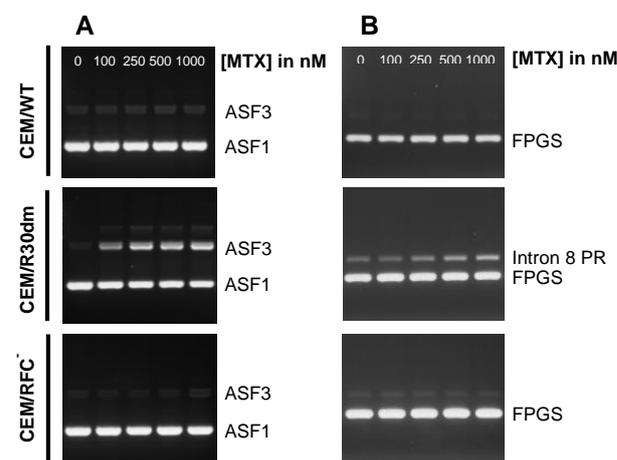


Figure 1; Shifts in splicing of SRSF1 and FPGS genes in ALL cell lines upon MTX exposure. A. SRSF1 is expressed in several isoforms with the main two being ASF1 and ASF3. The lower band represents ASF1 while the upper band corresponds to ASF3. Upon exposure to MTX, ASF3 appears in CCRF-CEM/R30dm. B. A FPGS splicing analysis by standard PCR spanning exon 5-exon 9 detected a gradual increase of intron 8 partial retention in CCRF-CEM/R30dm after exposure to a range of MTX.

RESULTS

Expression of SRSF1 isoforms is associated with FPGS splicing alterations in cell lines

Expression of FPGS splicing alterations and SRSF1 at the mRNA and protein level was studied in CCRF-CEM and its MTX-resistant subline CCRF-CEM/R30dm, a cell line displaying aberrant FPGS splicing and diminished FPGS activity. Since elevated levels of intron 8 PR have been reported for CCRF-CEM/R30dm, we first investigated SRSF1 expression in both these cell lines. CCRF-CEM/RFC-, a MTX resistant subline displaying decreased RFC activity, was included in this study to confirm that differences in SRSF1 expression are specific for CCRF-CEM/R30dm, and not related to general MTX resistance.

A

		8 PR	I5	I6	I5I6	E5	E6
Ratio	p	.000	.001	.001	.001	.043	.009
ASF3/ASF1	r	.407	.390	.393	.393	.240	.307
Frequency		71	71	71	71	71	71

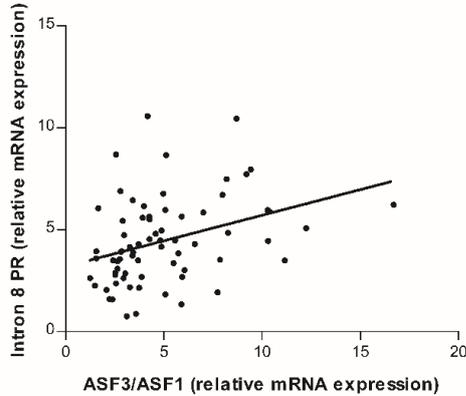
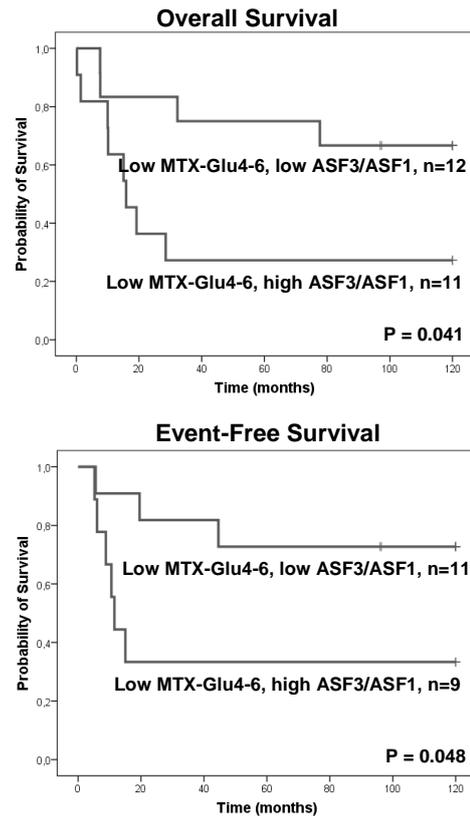
B

Figure 2; Involvement of SRSF1 in both FPGS splicing and MTX resistance in ALL patient samples. A. Non-parametric correlations and level of significance derived with a Spearman Rho, describe the relation of FPGS splicing alterations. R - correlation coefficient, P - p-value (Spearman's Rho test), N - the number of patients evaluated per particular test. B. An association between the ratio ASF3 to ASF1 and intron 8 PR has been found. C. Associations between the ratio ASF3 to ASF1 and MTX resistance in ALL patients displaying low levels of long-chain MTX polyglutamates.

Exposure to MTX induced a clear difference in expression of isoform ASF3, in CCRF-CEM/R30dm cells in parallel to the gradual increase of FPGS intron 8 PR. Both control cell lines, CCRF-CEM and CCRF-CEM/RFC-, did not show any differences in ASF3 or FPGS intron 8 PR expression after exposure to MTX (Figure 1). To confirm SRSF1 expression on protein level, proteins isolated from the cytoplasmic and nuclear fractions were analyzed. While ASF1 expression was clearly present in the nuclei of these cell lines, the detection of ASF3 isoform appears to be unstable and could not always be observed. Therefore the results are inconclusive and need further validation (data not shown).

Expression of SRSF1 isoforms and FPGS intron 8 PR are correlated in childhood ALL patients

To explore clinical relevance of our current findings 71 childhood ALL patient samples were screened for SRSF1 isoform expression by Real-Time PCR. Subsequently, SRSF1 isoforms expression profiles were compared with the FPGS splicing profile and clinical data of ALL patients. Expression of ASF1, ASF3 and the ratio of ASF3 to ASF1 were correlated to FPGS splicing profiles using a Spearman Rho test. The ratio of ASF3 to ASF1 was found to be positively correlated to several splicing alterations (Figure 2a). Interestingly, intron 8 PR showed the strongest association with the ratio of ASF3 to ASF1 (Figure 2a, 2b), followed by retention of intron 5,6 (I5, I6), a simultaneous retention of both these introns (I5I6) as well as exon 6, and exon 5 skipping (E6, E5). Thereby, 6 out of 11 identified alterations seemed to be linked with the relative expression of SRSF1 isoforms. Increased levels of ASF3 relative to

C

ASF1 correlated to a higher ratio of FPGS splicing alterations over the wildtype FPGS. Moreover, FPGS splicing alterations showed stronger associations with ASF3 alone then with ASF1.

The relation of SRSF1 expression with the clinical outcome of childhood ALL

To determine the relation of the ratio ASF3 to ASF1 with treatment outcome, a Kaplan-Meier analysis of overall survival as well as the event free survival was performed. No significant results were found in the complete patient cohort for either the total SRSF1 expression, as well as for ASF1 and ASF3 variants alone. Previously, in a subgroup of patients with low levels of long chain MTX polyglutamation, an association between intron 8 PR and survival was found. Therefore we studied if SRSF1 isoform expression is also related to survival in this group of patients. Just as for intron 8 PR, in patients displaying lower levels of long-chain MTX polyglutamates, increased ratio ASF3 to ASF1 were indicative of a worse prognosis for event free survival as well as overall survival (Figure 2c).

DISCUSSION

In the present study we showed that the expression of SRSF1 isoforms is associated with alternative FPGS splicing in childhood ALL. The levels of ASF3 mRNA increased together with FPGS 8 PR after exposure to MTX in MTX-resistant, FPGS-deficient cell line. This observation was further supported by an association of SRSF1 variant expression to FPGS splicing alteration in childhood ALL patient samples on mRNA level.

Moreover, the ratio of ASF3 to ASF1 is related to both overall and event-free survival in ALL patients with low level of MTX polyglutamates. Interestingly, intron 8 PR of FPGS showed exactly the same association.

To date, it is unclear if the relation between SRSF1 and FPGS splicing is causative or if it is merely an association due to regulation by a common factor. However, our observations point to a relation of SRSF1 expression to both alternative splicing of FPGS and MTX resistance and therefore suggest differences in regulation by ASF1 or ASF3. Because the RS domain is thought to be important for protein-protein interactions, ASF3 could attract other factors in the process of spliceosome assembly and thereby direct the splicing machinery to an alternative splice site.⁹ For instance, SRSF1 is predicted by the Human Splicing Finder (HSF)¹⁰ tool to bind to a spectrum of enhancer motifs in intron 8 of FPGS. Both ASF1 and ASF3 contain an RNA recognition motif (RRM),^{8,9} therefore both isoforms could select different alternative enhancer motifs and thereby affect alternative splicing.

Since we observed differences in expression of SRSF1 on mRNA level between CCRF-CEM and its MTX resistant subline, CEM/R30dm, one would expect differences in expression of ASF1 and ASF3 on protein level as well. This needs to be further evaluated to confirm that ASF3 has potential to influence the regulation of FPGS splicing. Moreover, differences in expression of both isoforms in the nuclear fraction should be investigated. Since the RS domain is responsible for shuttling between the nucleus and the cytoplasm,^{7,8} the lack of it in ASF3 isoform could have significant impact on its subcellular localization and consequently function.

Although ASF3 appears in *in vitro* studies, expression of this isoform alone does not seem to be significantly associated to FPGS splice variants in patient samples on mRNA level. Overall, the ratio of ASF3 to ASF1 is related to FPGS splicing aberrations, and therefore both isoforms seem to be important in this phenomenon. A role of both isoforms in aberrant FPGS splicing is supported by the suggestion of dimerization of SRSF1 by Zuo et al.⁸ The function of SRSF1 could be affected by the quantity of ASF3/ASF1 heterodimers in comparison to ASF1 homodimers in MTX sensitive cell lines. Though the main functions of SRSF1 involve regulation of splicing, the interaction of SRSF1 with a variety of proteins presumably enables it to regulate several cellular functions, e.g. translation and processing of small non coding RNA's (miRNA's).⁶ Therefore, SRSF1 could even affect the translation of FPGS protein or influence its expression by miRNA's without affecting mRNA levels. For all functions, the difference between both isoforms ASF1 and ASF3, e.g. the difference between RS domain-dependent and RS domain-independent splicing, should be elucidated. Differences in function can be studied by functional experiments, for instance a model overexpressing ASF1 or ASF3 isoforms or siRNA mediated knockdown. In the present study CCRF-CEM were transduced with lentiviral constructs expressing either ASF1 or ASF3 isoform. In this pilot experiment a

three-fold increase in ASF3 mRNA expression resulted in a slight upregulation of intron 8 PR (data not shown).

Taken altogether, our findings suggest that expression of ASF1 and ASF3 isoforms is related to FPGS splice alterations and MTX resistance. The emerging role of alternative splicing and splicing regulators in cancer and chemotherapy resistance could generate new therapeutic avenues or improvements to the current treatment of childhood ALL. Therefore, both FPGS splice variants and/or splicing regulators are interesting potential prognostic markers for future personalized treatment and patient stratification.

ROLE OF THE STUDENT

From February till June 2014 I worked as intern at the department of Pediatric Oncology/Hematology in the Cancer Center Amsterdam. After this period I was hired as junior researcher to finalize my dataset. I carried out all experiments and analyzed the data provided in this paper. I also actively participated in the design of particular experiments. Weekly meetings with lively discussions directed the project towards the success described in this paper.

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