High *IGSF4* expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23)

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Abstract

Pediatric MLL-rearranged acute monoblastic leukemia with t(9;11)(p22;q23) has favorable outcome compared to other MLL-rearranged AML. The biological background for this difference remains unknown. Therefore, we compared gene expression profiles (GEP, Affymetrix HGU133+2.0) of 26 t(9;11)(p22;q23) patients with 42 other MLL-rearranged AML patients to identify differentially expressed genes. IGSF4, a cell-cell adhesion molecule, was found to be highly expressed in t(9;11)(p22;q23) patients, which was confirmed by RT-qPCR and Western Blot. IGSF4 expression within t(9;11)(p22;q23) patients was 4.9 times higher in FAB-M5 versus other FAB-types (p=0.001). Methylation status investigation showed that high IGSF4 expressing t(9;11)(p22;q23) patients with FAB-M5 have no promoter hypermethylation, whereas all other cases do. Cell line incubation with demethylating agent decitabine resulted in promoter demethylation and increased expression of IGSF4. Downregulation of IGSF4 by siRNA did not affect proliferation nor drug sensitivity. In a cohort of 79 MLL-rearranged AML cases we show significant better overall survival (OS) for cases with high IGSF4 expression (5-yr OS 0.70 vs 0.37, p=0.03) In conclusion, we identified IGSF4 overexpression to be discriminative for t(9;11)(p22;q23) patients with FAB-M5, regulated partially by promoter methylation and resulting in survival benefit.
**Introduction**

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease. Currently, apart from response to treatment, the most important prognostic factor is cytogenetic aberrations. Well known cytogenetic abnormalities that predict differences in survival are t(15;17)(q22;q21) (*PML-RARA*), t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16)(p13q22) (*CBF-MYH11*) and Mixed Lineage Leukemia (MLL)-rearranged AML.\(^1\)\(^2\) Intensive chemotherapy has improved survival rate over the past decades (5 year event free survival (EFS) 60%). Future therapeutic strategies should be directed towards outcome as well as towards limitation of short and long term toxicity.\(^3\) It is anticipated that such strategies can be based on molecular targeting of abnormally expressed genes in specific genetic types of pediatric AML.\(^5\)

In recent years, more than 60 different translocation partners of the *MLL*-gene have been described.\(^6\) In pediatric *MLL*-rearranged AML the most common translocations are t(9;11)(p21;q23) (*MLL-AF9*) (approximately 50% of patients), t(10;11)(p12;q23) (*MLL-AF10*), t(6;11)(q27;q23) (*MLL-AF6*) and t(11;19)(q23;p13.3) (*MLL-ENL*).\(^1\)\(^2\) Of interest is that t(9;11) has been linked with favorable outcome.\(^7\)\(^9\) Recently, we identified that superior prognosis in the t(9;11) cases was restricted to those with French-American British morphology classification (FAB) M5 phenotype.\(^10\)

So far the underlying biological factors that determine the differences in clinical outcome of *MLL*-rearranged AML cases based on translocation partner are not known as scarce information is available on the molecular aberrations. Therefore, the aim of this study was to investigate the biological background of t(9;11)(p22;q23) AML with and without FAB M5 compared to AML with other *MLL*-translocation partners.
Materials and methods

Patients
Viably frozen diagnostic bone marrow or peripheral blood samples from 269 de novo and 8 secondary pediatric AML cases were provided by the Dutch Childhood Oncology Group (DCOG), the AML ‘Berlin-Frankfurt-Münster’ Study Group (AML-BFM-SG), the Czech Pediatric Hematology Group (CPH), and the St. Louis Hospital in Paris, France. Samples were chosen to represent all common cytogenetic groups and were selected based on availability of high-quality RNA. Each study group performed central morphological reviews according to the FAB classification. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, after Erasmus MC Institutional Review Board approval according to national law and regulations.

The samples included 33 pediatric MLL-rearranged cases with t(9;11)(p22;q23) and 52 with other MLL-rearrangements, the other 192 samples represented all other common AML cytogenetic groups (Table 1). Among the 8 secondary AML cases, 3 harbored a t(9;11)(p22;q23). These 3 cases were all classified as FAB-M5. The 5 other secondary AML cases did not harbor an MLL-rearrangement.

Materials
Leukemic cells were isolated and enriched as previously described. All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. A minimum of 5x10⁶ leukemic cells was lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at -80°C. Isolation of genomic DNA and total cellular RNA was performed as described before.
Leukemic samples were routinely investigated for MLL-rearrangements by standard chromosome-banding analysis and/or FISH. If translocation with one of the common partners (MLL-AF9, MLL-AF10, MLL-AF6, MLL-ENL and MLL-ELL) was suspected, Reverse Transcriptase PCR (RT-PCR) was performed (Primers are described in Table S1). Of the 85 MLL-rearranged cases, 33 harbored a t(9;11)(p22;q23), 19 a t(10;11)(p12;q23) and 15 a t(6;11)(q27;q23). The remaining 18 cases were confirmed with Long Distance Inverse PCR (LDI-PCR) as MLL-other.

**Gene expression profiling**

Gene expression profiling (GEP) was performed on the RNA of a cohort of 237 de novo and 8 secondary pediatric AML samples. We included 14 additional cases of MLL-rearranged AML (5 of which carried a t(9;11)) for GEP to increase group size. Integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). cDNA and biotinylated cRNA was synthesized, hybridized and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s guidelines. Data-acquisition was performed using Expresso (Bioconductor package Affy) and probe-set intensities were normalized using the variance stabilization normalization (Bioconductor package VSN) in the statistical data analysis environment R, version 2.7.0.14-15 Expression levels were log-transformed during this normalization. An empirical Bayes linear regression model was used to compare the signatures for the t(9;11) cases to all other MLL-rearranged AML cases.16 Moderated T-statistics p-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg.17 IGSF4 was identified from a top-50 differentially expressed gene list. For the expression analysis of IGSF4 probe set 209031_at was used.

**Quantitative real-time PCR**

Quantitative real-time PCR (RT-qPCR) was performed on cDNA of 95 pediatric AML patient
samples, selected on availability of remaining cDNA, produced as previously described. Within this group, 57 were classified as MLL-rearranged leukemia of which 24 harbored a t(9;11) (Table 1). An ABI PRISM 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) was used to validate the GEP results. Primers used for IGSF4 are described in Table S1. For expression analysis of IGSF4 SYBRgreen was used. The expression of the genes was compared to GAPDH, with primers and probe as previously described (sequences are shown in Table S1). The average cycle threshold (Ct) value was used to calculate mRNA expression levels of IGSF4 relative to the expression level of the reference gene (GAPDH) using the comparative Cycle time (ΔCt) method.  

**Western Blot**

For Western Blot 10 leukemia samples were selected based on availability of material, of which 3 harbored a t(9;11), 3 harbored another MLL-translocation and 4 had a karyotype other than MLL (AML-other, containing a case with t(8;21), one with inv(16), one with t(15;17) and one with a normal karyotype). Cell pellets stored at –80°C were quickly thawed and resuspended in 100 µL lysis buffer composed of 25 mM Tris (tris(hydroxymethyl)aminomethane) buffer, 150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin (Sigma-Aldrich, St Louis, MO, USA), 10 mM sodium fluoride, and 20 µL freshly prepared sodium pervanadate. Subsequently, cell lysis was allowed for 30 minutes on ice. Cell lysates were cleared by centrifugation for 15 minutes at 10 000 g (13 000 rpm) and 4°C. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA) with different concentrations of bovine serum albumin (BSA) as standards. Cell lysates containing 20ng of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western Blots were probed
with goat polyclonal IgG anti-TSLC1 (synonym of IGSF4, sc-25077, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-beta-actin (ab6276, Abcam, Cambridge, MA, USA) antisera. Subsequently, the blots were labeled with peroxidase-conjugated anti-goat antibody (sc-2020, Santa Cruz Biotechnology) or anti-mouse antibody (DAKO, Glostrup, Denmark). Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology) was used to detect luminescence using the Syngene chemigenius (Syngene, Cambridge, United Kingdom).

**Methylation Specific PCR**

To investigate the methylation status of *IGSF4* Methylation Specific PCR (MS-PCR) was used. Fourteen leukemia samples were selected, 5 samples with a t(9;11) based on their high IGSF4 expression with GEP and RT-qPCR. These samples with t(9;11) were compared to 5 MLL-rearranged samples with other translocation partners and 4 other AML samples. The primers as described by Overmeer et al. were used, using three different areas of the promoter (designated 1 M/U, 5 M/U and 9 M/U) (sequences are shown in Table S1).20 Unmodified genomic DNA was used to test the specificity of the primers for bisulfate converted DNA. One DNA sample was first treated with DNA methylase SSS1 and methyl donor SAM (M0226S New England Biolabs, Ipswich, MA, USA) and then bisulfate converted, creating a hypermethylated sample (M) as a control for the methylation specific primers. As a control for the unmethylated specific primers bisulfate converted DNA of healthy, adult male donors was used (U).

The specificity of the methylation (M) and unmethylation (U) specific primers was tested on a dilution range with a mix of M and U DNA (Figure S1). The dilution ranges indicated that the combination of 9M and 9U is the most specific.
Demethylating agents

Cell lines ML-2 (MLL t(6;11)), HL-60 (AML other) and MONO-MAC-1 (MLL t(9;11)) (DSMZ GmbH, Braunschweig, Germany) were cultured with and without demethylating agent decitabine. ML-2 and HL-60 were selected for their low expression of IGSF4 on RT-qPCR, MONO-MAC-1 was used as a control, since it shows high IGSF4 expression. Decitabine concentration was chosen after an in vitro drug assay with decitabine concentrations ranging from 0.125- 4.0 μM and was determined for each cell line to be the approximate 50% lethal concentration (LC50). ML-2 was cultured with a concentration of decitabine of 2 μM, HL-60 and MONO-MAC-1 with a concentration of 4 μM. Decitabine and culturing medium (RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen, Breda, The Netherlands), 10% Fetal Calf Serum (FCS) (Integro, Zaandam, The Netherlands), penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 0.125 μg/ml (PSF) (Invitrogen)) were refreshed daily. The experimental condition started with 100x10⁶ cells. Cell counts were determined on a daily basis and cells were maintained in culture at a concentration of 0.5x10⁶ cells/ml. Cell samples of both test and control conditions were taken from the medium every other day for the first 6 days and daily thereafter. They were washed with Phosphate Buffered Saline (PBS) and samples for protein studies were frozen at -80°C as dry cell pellets, for DNA and RNA extraction cells were lysed in Trizol reagent and stored at -80°C. The experiment ended as soon as all remaining experimental cells were apoptotic.

siRNA transfection

The MONO-MAC-1 (t(9;11)) and NOMO-1 (t(9;11)) cell line (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium supplemented with 10% FCS and PSF, and grown as suspension cultures at 37°C in humidified air containing 5% CO2. Cells from both cell lines (10x10⁶) were transfected by electroporation in 400 μl RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) containing 250nM of either a mix of equal amounts of IGSF4
siRNAs (Thermo Fisher Scientific Dharmacon ON-TARGETplus LQ-016565, Lafayette, CO, USA) or Non-targeting siRNA (Thermo Fisher Scientific Dharmacon ON-TARGETplus D-001810-01-05, Lafayette, CO, USA), in 4 mm electroporation cuvettes (BioRad, Hercules, CA, USA) (sequences are described in Table S1). Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a rectangle pulse of 300 V for 10 ms. To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of siRNA. After incubating for 15 min at room temperature, the cells were diluted in 10 ml RPMI 1640 supplemented with 10% FCS and PSF and incubated at 37°C and 5% CO2. They were maintained in culture for 72 hours. Cell counts were determined daily (t= 6 hours, t= 24 hours, t= 48 hours, t= 72 hours). Cell samples of both test and control conditions were taken from the medium at every time point. They were washed with Phosphate Buffered Saline (PBS) and lysed in Trizol reagent and stored at -80°C. DNA content and cell cycle phase were assessed using PI staining and measured by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

**In vitro drug resistance**

After transfection in vitro drug resistance for daunorubicin (DNR; Cerubidine, Sanofi Aventis, Gouda, The Netherlands), cytosine arabinoside (ARA-C; Cytosar, Pharmacia, Woerden, The Netherlands), Cladribine (2-CDA; Leustatin, Janssen-Cilag, Tilburg, The Netherlands), and Etoposide (VP16; Toposin, Pharmachemie, Haarlem, The Netherlands) and as a control also Vincristine (VCR; Pharmachemie), L-Asparaginase (ASP; Paronal, Nycomed Christiaens, Breda, The Netherlands), Prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands) and Dexamethason (DXM; Erasmus MC, Rotterdam, The Netherlands) was determined using the 2-, 3- and 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.21 Six concentrations of each drug were tested in
duplicate. The ranges of the final concentrations of these drugs were as follows: DNR: 0.002 - 2.0 µg/ml; ARA-C: 0.01 – 10 µg/ml; 2-CDA: 0.0004 - 4 µg/ml; VP16: 0.05 – 50 µg/ml; VCR: 0.05 - 50 µg/ml; ASP: 0.003 - 10 IU/ml; PRED: 0.008 - 250 µg/ml and DXM: 0.0002 – 6 µg/ml.

**Statistical Analyses**

Statistical workup of GEP data is described under ‘gene expression profiling’. For comparison of the gene expression in different groups the Mann-Whitney test was used. For assessment of correlation of the results from gene expression profiling and RT-qPCR, Spearman’s Correlation coefficient was used. All MLL-rearranged de novo AML cases with available follow-up data were included for survival analysis. Probabilities of overall survival, event-free survival (events: non remitter, relapse, secondary malignancy, death from any cause) and cumulative incidence of relapse (events: non remitter, relapse) were estimated by the method of Kaplan and Meier and compared by the Log-rank Test. Median *IGSF4* expression in the t(9;11) group was used to split all MLL cases in high and low *IGSF4* expression. The Cox Proportional hazards model analysis was applied to determine the association of *IGSF4* overexpression with overall and event free survival adjusted for prognostic factors as described for pediatric AML (white blood cell count (WBC), age and karyotype). All analyses were performed with SPSS Statistics version 16.0 (SPSS Inc. Chicago, IL, USA). All used tests were two-tailed and a p-value of less than 0.05 was considered significant.
Results

Gene expression profiling

In a comparison of t(9;11)(MLL-AF9) AML with other MLL-rearranged AML cases, IGSF4 was among the highly differentially expressed genes. Recent literature described IGSF4 as a tumor suppressor gene in solid tumors, but so far no data are available about the function of IGSF4 in leukemia.22-24 As this gene was never described to be expressed in pediatric AML, we decided to further study IGSF4 specifically.

We chose probe set 209031_at, which revealed the most significant differences, to compare differential expression in AML subgroups. Patients with t(9;11) (n=26) had a 4.1 fold higher median IGSF4 mRNA expression as compared to patients with other MLL-rearrangements (n=42) (8.8 Arbitrary Units (AU) vs 7.4 AU, p<0.001) (Figure 1a). IGSF4 expression was also significantly higher in t(9;11) when compared to non MLL-rearranged AML cases with other karyotypes (n=192) (fold change 7.0, median expression of 8.8 vs 6.8 AU, p<0.001) (Figure 1a).

Within the t(9;11) group, expression of IGSF4 was 4.9 fold higher in FAB-M5 (n=21) versus other FAB-types (n=4) (median: 8.9 AU vs 7.3 AU, p=0.001) (Figure 1b). This difference, associated with FAB-classification, was also present in MLL-rearranged AML patients with other translocation partners (n=23 vs n=19) (median 7.8 vs 7.0, fold change 2.4, p=0.009), but not in the AML-other group (n=16 vs n=166) (median 6.8 vs 6.8, p=0.20) (Figure 1b). All cases with unknown FAB-type were excluded from these analyses (t(9;11) n=1, AML-other n=10).

Real-time quantitative PCR

Gene expression results were confirmed with RT-qPCR in 78 cases. An additional 17 cases of
which no GEP-data were available were used to expand the number of cases. The median relative expression of $IGSF4$ in patients with t(9;11) was 7.4 fold higher compared to $MLL$-rearranged patients with another translocation (6.4% vs 0.9%, $p=0.008$) (Figure 2). Relative mRNA expression of $IGSF4$ in other AML patients was 396 fold lower than in t(9;11) patients (0.02% vs 6.4%, $p<0.001$).

A correlation coefficient was calculated comparing the expression of $IGSF4$ by GEP and RT-qPCR. Because of the use of SYBRgreen in the RT-qPCR reaction Ct-values $>32$ can be considered noise. The remaining 56 pairs resulted in a highly correlated Spearman $R=0.839$ ($p=0.01$) (Figure S2).

**Western Blot**

The IGSF4 antibody specifically identified two different isoforms that have previously been described.$^{25}$ We did not find a difference in isoform 1 expression, but the expression of isoform 2 was higher in t(9;11) positive patients than in the other patients (Figure 3).

**Methylation Specific PCR**

In the selected $MLL$-rearranged AML cases with t(9;11), the $IGSF4$ promoter was unmethylated. In contrast, in other $MLL$-rearranged cases and cases without an $MLL$-rearrangement the $IGSF4$ promoter was methylated (Figure 4). This difference between cytogenetic groups was also found in the cell lines (Figure 4).

**Treatment with demethylating agent**

To study the role of promoter methylation in the regulation of $IGSF4$ expression, AML cell lines were cultured with and without decitabine. RT-qPCR showed an increase of $IGSF4$ RNA
expression, up to at least 1000-fold on day 8-9 for the treated hypermethylated cell lines ML-2 (t(6;11)(q27;q23)) and HL-60 (AML-other) when compared to their non treated counterparts (Figure 5a). Bisulfate treated DNA tested on MS-PCR showed methylation status changes, most significantly with selected primers 9M and 9U (Figure 5c). The control cell line MONO-MAC-1 (t(9;11)) that has a high IGSF4 mRNA expression and moderate methylation, showed a 10-fold increase of expression during treatment with decitabine (Figure 5b). In this cell line MS-PCR showed moderate methylation at the start of treatment which was lost during treatment (Figure 5c).

**Transfection**

Transfection of siRNAs targeting IGSF4 by electroporation in the cell line MONO-MAC-1 resulted in 50-70% silencing of IGSF4 mRNA in repeated experiments (Figure 6). NOMO-1 was more difficult to transfect than MONO-MAC-1, and therefore was not used in further experiments. No significant differences were found between transfected and control conditions in MONO-MAC-1 cells, neither in apoptosis or cell cycle arrest (Figure S3) nor in cell proliferation (data not shown).

**In vitro drug resistance**

No significant differences in drug toxicity were found after 2, 3 or 4 days of consecutive culturing of transfected MONO-MAC-1 cells with the most commonly used cytostatic drugs in AML and ALL (Figures S4 and S5).

**Outcome**

Five year overall survival of MLL-rearranged patients with high IGSF4 expression is 70%, which is significantly better than an overall survival of 37% in MLL-rearranged patients with low IGSF4 expression (n= 79, p= 0.03) (Figure 7a-c). This group included 28 patients with t(9;11). When
analyzed separately this group proved to be too small to show significant survival differences (data not shown). Using the Cox proportional hazards model, no correlation with outcome could be shown after adjustment for known prognostic factors (WBC, age) (data not shown).
Discussion

In pediatric MLL-rearranged AML, t(9;11) is the most common genetic aberration. Recently we showed that prognosis of this patient group largely depends on the morphologic FAB classification, i.e. patients with t(9;11) with FAB M5 had a significantly better prognosis than patients with other FAB types. However, so far the biological background for this survival difference is largely unknown. In order to study differentially expressed genes in this specific group, we performed gene expression profiling and identified IGSF4 as a discriminative gene.

In non-malignant cells, IGSF4 is known to play a role in cell-cell adhesion, cell polarity and as a signaling molecule for NK- and T-cell cytotoxicity. Recently, Kawano et al. showed that IGSF4 participates in the ErbB2/ErbB3 pathway as a competitive antagonist of ErbB2 in complex formation with ErbB3. Loss of IGSF4 expression resulted in AKT pathway stimulation which resulted in improved cell movement and survival. We could not confirm similar pathway activation in our pediatric AML dataset using microarray analysis (data not shown). In non small cell lung carcinoma (NSCLC), IGSF4 was found to be located in an area with a common loss of heterozygosity. Transferring this gene in A549 cells (NSCLC cell line) inhibited tumor formation in nude mice. In neuroblastoma and cervical carcinoma, aberrant promoter methylation of IGSF4 influenced tumor growth.

We found a high IGSF4 mRNA expression in MLL-rearranged pediatric patients with t(9;11) which was associated with increased protein expression, in combination with a hypomethylated promoter region of IGSF4. This indicates that indeed epigenetic regulation plays a major role in the expression of IGSF4 in pediatric AML as was further illustrated by cell line studies with demethylating agents. The expected effect of IGSF4 on proliferation could not be shown in the
siRNA experiments in a t(9;11) cell line. Our in vitro studies however do not represent the normal cell environment. Future studies using a design including environmental factors (like homing assays) are more potent to show proliferative advantage caused by differential expression of this cell surface protein. We are not the first group to report gene silencing by methylation in pediatric AML. CCAAT/enhancer binding protein (CEBPA) is a well known gene that is linked to mutations as well as methylation differences and whose expression predicts survival in AML.28-29 In this context, we might consider designing clinical studies to assess whether the outcome of patients with epigenetic silencing can be improved by adding demethylating agents.

So far only one study in AML cell lines reported on IGSF4, showing hypermethylation of its promoter region in MLL-rearranged AML cell lines versus cell lines without an MLL-rearrangement.30 In adult T-cell leukemia IGSF4 overexpression resulted in a proliferation advantage.31 However, the precise role of IGSF4 in hematopoiesis and leukemogenesis is currently unknown. It remains to be determined whether cell-cell adhesion plays a role in IGSF4+ leukemia like in solid tumors. The finding by Boles et al. that expression of IGSF4 protein on the cell surface is a trigger for NK- and CD8+ T-cell mediated cytotoxicity, might support our finding of overexpression of IGSF4 in a group with a more favorable outcome.26 Normally, circulating leukocytes do not express high levels of IGSF4. If Boles’ hypothesis proves to be true in pediatric AML, we would expect blasts with high IGSF4 expression to be more easily recognized by the immune system. Blasts with low IGSF4 expression are able to evade this mechanism. As low expression is often derived from promoter hypermethylation, these patients might benefit from demethylating agents. In conclusion, we hypothesize that silencing of IGSF4 could be considered as a secondary event, causing the leukemic blasts to be immunologically silent and thereby allowing longer survival.
The ErbB-RAC-AKT pathway, influenced by IGSF4 interaction with ErbB3, could also be of interest for leukemias, since this pathway is linked to proliferation and apoptosis.\textsuperscript{27}

We found that \textit{IGSF4} was mainly and most apparently expressed in monoblastic t(9;11) rearranged patients. As this subgroup of \textit{MLL}-rearranged pediatric AML has recently been identified as important prognostic group\textsuperscript{10}, the role of \textit{IGSF4} deserves further attention. Interestingly, the \textit{IGSF4} expression also seems to be determined by the cell type (M5) in which the maturation arrest occurs. This reflects a unique novel collaboration of a specific (epi-)genetic aberration and type II mutations (i.e. MLL) together with maturation status in pediatric AML.

In this retrospective study, which included AML samples from differently treated pediatric patients, we show there was no significant difference for EFS between cases with high and low IGSF4 expression. However, there was a significant difference in overall survival favoring patients with high IGSF4 expression, due to a better salvage rate following relapse in these patients. Further studies in larger prospective cohorts will be necessary to determine the full role of \textit{IGSF4} in pediatric AML.

In conclusion, we found \textit{IGSF4} mRNA and protein to be differentially expressed in \textit{MLL} rearranged pediatric monoblastic AML patients with the highest expression in t(9;11) M5 AML. This expression seems to be largely regulated by promoter hypermethylation. Further studies are needed to be able to determine the biological role and prognostic relevance of \textit{IGSF4} expression in pediatric AML.
Authorship Contributions: EAC, JEK, AAD-vO and BVB designed the study and performed the research. JS, AB, VdH, ESJMdB, DR, GJLK and JC contributed materials and clinical data. EAC, JEK, BVB, MLdB, RP, CMZ and MMvdH-E analyzed data and wrote the paper. CMZ, MMvdH-E, and RP supervised the study.

Disclosure of Conflicts of Interest: nothing to disclose.

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References


Tables

Table 1: Clinical characteristics of GEP cohort of initial pediatric AML samples

<table>
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<tr>
<th></th>
<th>Original GEP cohort (n=245)</th>
<th>Additional cases (n=32)</th>
<th>Total (n=277)</th>
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<td><strong>Sex</strong></td>
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<tr>
<td>male</td>
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<td>female</td>
<td>108 (44)</td>
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<td><strong>Age (median, range, years)</strong></td>
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<td>3.0 (0.4-17.3)</td>
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<td><strong>WBC x 10⁹/l (median, range)</strong></td>
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<td>M0</td>
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GEP: gene expression profiling array, WBC: white blood cell count, FAB: French American British morphology classification, RT-qPCR: Quantitative real-time PCR. Numbers reflect number of cases (%) unless otherwise specified.
Figure legends

Figure 1: *IGSF4* gene expression in pediatric AML as determined by gene expression arrays.

Graphs showing the expression of probe set 209031_at, representing the *IGSF4* gene, after log transformation. Bars represent the median expression in each group.

**a**: Significant differences are shown between patients with a t(9;11) (n= 26) and patients with another *MLL*-rearrangement (MLL other, n=42) (8.8 vs 7.4, p<0.001) or AML patients without an *MLL*-rearrangement (AML other, n=192) (8.8 vs 6.8, p<0.001).

**b**: Expression of probe set 209031_at with all groups divided based on morphology, i.e. FAB M5 vs other FAB-types (non FAB M5). All cases with unknown FAB-type were excluded from this analysis (t(9;11) n=1, AML-other n=10). We detected a significant difference for median expression within the patients with a t(9;11) (n= 21 vs n=4) (8.9 vs 7.3, p=0.001) and the patients with other *MLL*-rearrangements (n= 23 vs n=19) (7.8 vs 7.0 p=0.009). This difference was not detected in the remaining patients without an *MLL*-rearrangement (AML other, n=16 vs n=166) (6.8 vs 6.8, p=0.20).

Figure 2: *IGSF4* gene expression in pediatric AML as determined by RT-qPCR.

Graph showing the expression of *IGSF4* on mRNA level measured with RT-qPCR. Bars represent the median expression in each group. Significant differences are observed between patients with a t(9;11) (n=24) and patients with another *MLL*-rearrangement (MLL other) (n=33) (6.4 vs 0.9, p=0.008) or AML patients without a *MLL*-rearrangement (AML other) (n=38) (6.4 vs 0.02, p<0.001).
Figure 3: Protein expression analysis of IGSF4 with Western Blot

Sections of Western Blot showing data from 3 cell lines and 10 patient samples. Two described isoforms (iso 1 and iso 2) are shown at 48 kDa and 45 kDa respectively. The lower panel shows loading control with beta-actin. The protein expression of IGSF4 isoform 2 in patients with a MLL t(9;11) is higher than protein expression of IGSF4 isoform 2 in the other groups. The first lane shows the ladder, the other lanes contain cell lysates from cell lines and patients (separated by the thin line). At the thick line one lane was spliced out.

Figure 4: Methylation status of IGSF4 tested by MS-PCR

Figure showing results of methylation specific PCR in AML patients and cell lines. Three separate regions of the promoter were investigated; region 1, 5 and 9. Each column shows results for a specific primer pair (M: methylated, U: unmethylated). The upper part of the figure shows the IGSF4 methylation status of several patients (indicated by number), the lower part shows methylation status of cell lines and methylated and unmethylated control DNA. On the left the identity of each sample is indicated, on the right the cytogenetic group each patient or cell line belongs to is shown. In patients with t(9;11) (n=5) no bands are seen with the methylated specific primers and heavy bands are seen with the unmethylated specific primers. In contrast, the other MLL patients (n=5) and other AML patients (n=5) do show a band with the methylated primer. This difference is also seen in the cell lines.

Figure 5: relative expression and promoter methylation of IGSF4 in cell lines after culture with decitabine

Graph showing IGSF4 mRNA expression in cell lines ML-2, HL-60 and MONO-MAC-1 at
different time points during culture with demethylating agent decitabine (DAC).

**a:** Solid lines correspond to untreated conditions of cell lines ML-2 (t(6;11)) and HL-60 (AML-other), dotted lines reflect values from treated conditions of the same cell lines. Shown is >1000-fold upregulation of \textit{IGSF4} expression during culture with decitabine, whereas in control conditions expression remained stable.

**b:** Treatment of cell line MONO-MAC-1 with decitabine. The solid line reflects values from untreated condition, the dotted line from corresponding treated samples. A 10-fold upregulation was found over a treatment period of 8 days.

**c:** Figure showing results for methylation specific PCR in cell lines cultured with demethylating agent decitabine for 2-9 days. Left panel provides results for 9M primers, the right panel for 9U primers. Under control conditions ML-2 and HL-60 mainly show a methylated promoter region. Shortly after start of treatment unmethylated bands are visible and methylated bands decrease in intensity. MONO-MAC-1 shows both bands at the start of the experiment, and the methylated band clearly weakens during treatment.

**Figure 6: IGSF4 knock-down in MONO-MAC-1 after transfection**

The figure shows \textit{IGSF4} expression levels after transfection with \textit{IGSF4} siRNAs measured by RT-qPCR relative to levels measured in corresponding samples transfected with non-targeting siRNA. Time points are given in hours after transfection. Shown are the means of 4 experiments. Error bars represent standard error of the mean.

**Figure 7: Survival plots for patients with high and low IGSF4 expression**

Plots showing overall survival (pOS) (panel a), event free survival (pEFS) (panel b) and cumulative incidence of relapse (CIR) (panel c) for all \textit{MLL}-rearranged patients (n=26) \textit{IGSF4}
high) vs n=53 (IGSF4 low)). Median expression in the t(9;11) group was chosen as a cut-off for the division in high and low IGSF4 expression. The solid line represents patients with high IGSF4 expression, the dotted line represents patients with low IGSF4 expression. NR: non remitter.
Figure 2

IGSF4 mRNA expression

Expression relative to GAPDH (%)

- t(9;11)
- MLL other
- AML other

p<0.001

p=0.008
Figure 3

Iso 1 and iso 2

B-actin

Cell lines: t(9;11), MLL-other, AML-other

Patients: t(9;11), MLL-other, AML-other
Figure 4

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- t(9;11)
- MLL-other
- AML-other
- t(9;11)
- MLL-other
- AML-other
Figure 5

(a) IGSF4 mRNA expression relative to GAPDH for ML2, ML2 Co, HL60 DAC, and HL60 Co over 0 to 8 days.

(b) IGSF4 mRNA expression relative to GAPDH for Monomac DAC and Monomac Co over 0 to 8 days.

(c) Gel images showing expression levels at time points t=2, t=4, t=6, t=7, and t=8 for ML2 and HL60, and t=2, t=4, t=6, t=7, and t=8 for Monomac.
Figure 6

IGSF4 knock-down after siRNA transfection
High /IGSF4 expression in pediatric M5 acute myeloid leukemia with t(9;11)(p22;q23)