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RESEARCH

Expression of Certain Leukemia/Lymphoma Related microRNAs and its Correlation with Prognosis in Childhood Acute Lymphoblastic Leukemia

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Abstract In spite of the improved efficacy of therapy, it still fails in 15–20 % of childhood acute lymphoblastic leukemia (ALL) patients. Recently, altered expression of certain miRNAs (miRs) have been described in ALL with potential effect on prognosis. Presence of certain miRs (miRNA-16, −21, −24, −29b, −128b, −142-3p, −155, −223) was characterized in human lymphoma and leukemia cells by real-time PCR. Expression of miRs in pediatric ALL patients (n=24) was measured before chemotherapy, at conventional response checkpoints and at relapse. Correlation between altered miR expression and response to prednisolone at day 8 of therapy and long term prognosis was statistically analysed. Overexpression of "oncomiR/inflammamiR"-21 – which is characteristic in different tumors—was missing in human ALL cells. However, higher expression of miR-128b and lower expression of miR-223 is generally characteristic for human ALL cell lines and ALL cells isolated from pediatric patients. Correlation was shown between miR-128b expression and prognosis, prednisolone response and survival data in childhood ALL. Expression of miR-128b and miR-223—both are leukemia specific—changed in parallel with percentage of bone marrow blasts in remission and during relapse. Therefore, we suggest that overexpression of miR-128b and downregulation of miR-223 shows a significant correlation with treatment response and prognosis in childhood ALL.

Keywords miRNA-128b miRNA-223 · Childhood ALL · Prognosis · Relapse

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignant disorder in pediatrics, which takes about 30 % of newly diagnosed childhood malignancies. Annual incidence of childhood acute lymphoblastic leukemia (ALL) is about 4–5 cases per 100,000 children [1]. Intensive combination therapy remarkably improved the prognosis [2]. However, success of the treatment still fails in 15–20 % of patients. New predictive factors and targets are needed especially for high risk patients with a higher potential for failure of treatment. Recently, miRNAs has gained remarkable attention in different cancer types [3–5], including haematological malignancies [6, 7].

MiRNAs (miR) are a class of small (16–29 nucleotides) noncoding RNAs. Many human miRNAs have been identified, which target dozens of genes. The importance of miRNAs in cancer is highlighted by the observation that many miRNAs are located in cancer-associated genomic regions. Recent studies have shown the important role of certain miRs in human carciogenesis [8]. MiRNAs could be divided into different groups: oncogenic, tumor suppressor-like and other subtypes. Data on miRNA expression provide information about lineage and differentiation status of cancer cells. Altered expression levels of different miRs have been described in connection with several solid tumors [3–5] and hematologic

malignancies [7], including ALL [9–11]. In previous studies about miRNA expression profile in ALL higher expression of miR-128a, miR-128b and lower expression of a miR-223, let-7b was shown [12, 13]. A possible correlation was mentioned between different expression of certain miRNAs and prognosis of ALL patients [14].

For our study different miRNAs were selected (miRNA-16, −21, −24, −29b, −128b, −142-3p, −155, −223) with potential role in human solid and hematologic malignancies. Altered expression of certain miRNAs has already been described in lymphoma or acute leukemia.

High expression of miR-21 has been found in a wide variety of human cancers [15] and haematological malignancies [7]. Its potential targets are PTEN [16], PDCD4 and STAT3 [17]. Increased expression of miR-155 has also been described in different tumor types [7, 18, 19]. It is involved in activation, proliferation and survival of B-cells and has also powerful effect on T-cell activation. Based on sequence homology potential targets of miR-155 are c-MYC, PIK3R1 (negative regulator of PI3K) and SHIP1 [19–21].

Reduced expression of tumorsupressor miR-16 had been confirmed in various malignancies (e.g. CLL, ALL, anaplastic large cell lymphoma, myeloma multiplex) [7]. It regulates negatively the cell cycle progression presumably by inhibiting BCL2 and TIAM1 [22]. MiR-24 is an important cell cycle regulator; its overexpression inhibits cell proliferation by G2/M arrest [48] and negatively regulates differentiation of CD34+ hematopoietic cells [7], inhibits B-cell development and promotes myeloid development of hematopoietic progenitors, directly downregulates the expression of MAPK phosphatase-7 and enhances phosphorylation of both JNK and p38 kinases [23].

The members of the miR-29 family serve in several cases as tumor suppressors. Decreased expression of miR-29b has been confirmed in AML, osteosarcoma and small cell lung cancer. Potential target of miR-29b is MCL-1, ABL-1 (BCR/ABL) and some anti-apoptotic BCL-2 family members. Overexpression of miR-29b induces apoptosis of multiple myeloma cells and of CML cells. Its downregulation showed correlation with more aggressive forms of cancer or with later relapse. [24–26].

MiR-142-3p is involved in the development and differentiation of hematopoietic stem cells through interferon regulatory factor 7 (irf7)-mediated signaling. It has a crucial role in T-lymphocyte development and potentially in T-cell leukemogenesis regulating cAMP, PKA, GRα [27]. Its overexpression had been detected in ALL and AML patients [28]. MiR-142-3p decreases GRα protein expression leading to glucocorticoid resistance. Its decreased expression had been shown in MLL-AF4 ALL [29].

MiR-128 has an important role in brain development. Its aberrant expression could be detected in certain leukemias and some solid tumors (e.g. NSCLC). Overexpression of miR-128b was described in ALL, however, it is downregulated in AML [12, 13], as well as in MLL-AF4 ALL. Moreover, miR-128 can play an important role in the regulation of PI3K-AKT-mTOR signaling pathway through downregulation of PTEN [30].

MiR-223 is a hematopoietic tissue-specific miRNA, with lower expression in lymphoid cells and with highly increased expression in the myeloid lineage [12, 13]. It can alter cancer cell phenotypes not only in hematological malignancies but also in solid tumors. Its potential targets in lymphoid cells could be important in the regulation of cell cycle or different signaling mechanism (e.g. E2F1 [7], CEBPα [31], E2A [32] etc.).

The aim of our study was to determine the expression profile of presented miRNAs in ALL cell lines and childhood ALL cells. Moreover, correlation of certain miRNA expressions with the clinical and prognostic data of patients were analyzed. Changes in miRNA expression were followed during monitoring the efficacy of the treatment and potential role of miRNA's as a prognostic factor was determined.

Materials and Methods

Patients

Peripheral blood and bone marrow samples were collected from children with ALL (n=51). Diagnosis was made upon the treatment protocol (clinical features, cytomorphologic, flow cytometric, cytogenetic and molecular genetic analyses of blast cells). Some samples were not evaluable for further analysis because of the low number of isolated lymphoblasts. Samples from all phases of treatment (collected at diagnosis, and at conventional response checkpoints [on days 15, 33), and before beginning of protocol M) were available from 24 patients (female 6; male 18).

Peripheral blood and bone marrow samples from 15 children with pre-B ALL (BCP-ALL) from 9 children with T-ALL, and from non-leukemic children as controls were used for further studies. Data were analysed statistically. Clinical data were collected prospectively from the time of diagnosis. Average age of patients at diagnosis was 5.9 years (range 2–16.4 years). All ALL patients were treated according to the ALL IC-BFM 2002 protocol. Risk group stratification was based on criteria listed in the protocol. 6 (25 %) children were stratified to standard (SR), 12 (50 %) to intermediate (IR) and 6 (25 %) to high risk arm (HR).

Prognosis evaluation was based on initial WBC count, age at diagnosis, prednisolone response on day 8, percentage of bone marrow blasts at day 15 and 33, immunophenotype, cyto- and molecular genetics. Long time prognosis depended also on relapse during follow-up. Most patients with initially good prognosis stayed in remission during the observation

period, however, one of them relapsed. The mean follow-up time from diagnosis was 28.1 months (range: 6–44 months). All relevant clinical and laboratory data as well as results of cytogenetic and molecular genetic analysis of the 24 patients are summarized in Table 1.

Samples were obtained with informed consent and all protocols were approved by the Institutional Ethical Review Board *(TUKEB – Scientific and Research Ethics Council, Hungary – no. 53/2011).* Written informed consent was obtained from the parents on behalf of the children participants involved in this study.

Primary ALL Cells, Cell Lines and Culture Conditions

Samples from peripheral blood and bone marrow were used. Normal B-cells were isolated by MACS CD19 micromagnetic beads (Miltenyi Biotec, Auburn, CA), and T-cells by nylon wool fiber (Wako Chemicals) according to Wohler [66]. The isolated lymphoblasts/mononuclear cells (by ficoll gradient centrifugation - Histopaque 1077, Sigma Aldrich, St. Louis, MO) and normal T- and B-cells were stored at −80 °C.

Human leukemia cell lines (Nalm6 – B-cell precursor leukemia, clone ACC 128, German Tissue Collection of Microorganisms and Cell Culture [DSMZ]; Mn60 – B-cell leukemia established from acute B-ALL, clone ACC 138, DSMZ; Jurkat – acute T-cell leukemia, clone E6-1, American Type Culture Collection [ATCC]; CCRF-CEM – acute T lymphoblastic leukemia, clone #82112105, European Collection of Cell Culture [ECACC]; HL60 – promyelocytic leukemia cell line, CCL 240 clone, ATCC) and lymphoma cell line (KMH2 – Hodgkin lymphoma cell line, clone ACC 8, DSMZ) were used in miRNA expression studies as well.

RPMI 1640 Medium (Sigma) supplemented with 10–20 % fetal calf serum (Sigma, Gibco), 0.03 % L-glutamine and 100 μg/ml streptomycin (Sigma) were used for cell culturing at 37 °C in a 5 % CO_2 humidified atmosphere.

Isolation of miRNA, cDNA Transcription, Quantitative Real-Time PCR and Data Analysis

MicroRNAs (miRs) were isolated using mirVana ™ miRNA Isolation Kit (Ambion) and the isolated miRNA samples were stored at −70 °C until use. TaqMan MicroRNA Reverse Transcription Kit and Taqman MicroRNA Assays stem-loop RT primers [(hsa-miR-16 (Assay ID: 000391), hsa-miR-21 (Assay ID: 000397), hsa-miR-24 (Assay ID: 000402), hsa-miR-29b (Assay ID: 000413), hsa-miR-128b (Assay ID: 000589), hsa-miR-142-3p (Assay ID: 000464), hsa-miR-155 (Assay ID: 000479), hsa-miR-223 (Assay ID: 000526), RNU6B (Assay ID: 001093), Applied Biosystems, Foster City, CA, US] were used in Mastercycler personal (Eppendorf) according to the protocol.

For the amplification TaqMan Gene Expression Master Mix and TaqMan MicroRNA Assay TM primers (miR-16, −21, −24, −29b, −128b, −142-3p, −155, −223, Applied Biosystems) were used. The quantitative real-time PCR assay was performed on a 7300 Real Time PCR System (Applied Biosystems). MiR's expression values were calculated as the mean of the three parallels, and normalized to the level of RNU6B endogene control, which is frequently used as a reference in miRNA experiments of lymphoid malignancies [68, 69]. Results were obtained as threshold cycle (Ct) values. The data were evaluated using 7500 software v.1.3.0-, and DataAssist software v.2.0 (Applied Biosystems).

Statistical Analysis

Data of 16 patients with good and 8 patients with poor prognosis were statistically analysed. Mean arithmetic values (x) and standard deviation (SD) were calculated and compared with Student's *t*-test (normal distribution; Statistica 9.0) or Mann–Whitney *U* test (asymmetrical distribution; Statistica 9.0). The cutoff value for relative expression of miR-128b—which segregates patients into good and poor prognostic groups with appropriate specificity and sensitivity—was calculated by ROC curve using covariance analysis (SPSS). Chi^2 test and Fisher's exact probability test were used according to sample size for categorical data comparison. Statistical analysis was performed with Stat Soft STATISTICA 9.0 (Statsoft, Tulsa, OK) and SPSS 15.1 software (SPSS Inc.), $p<0.05$ was considered statistically significant.

Results

MiRNAs Relative Expression in Human Lymphoma and Leukemia Cellsrom

Expression levels of miRs (miR-16,-21, −24, −29b, −128b, −142-3p, −155, −223) were determined by real-time PCR. Measurements were carried out on PMNC and/or on T-cells from healthy individuals, on B-cells derived from tonsilla palatina and on MNC isolated from peripheral blood and bone marrow from non-leukemic and ALL patients. MiRNA expression profile was not significantly different in peripheral blood and bone marrow samples derived from the same patient. MiRNA profile of PMNC samples or isolated normal lymphoid cells from different non-leukemic donors showed no significant difference. Based on these, current miR expression of human PMNC was considered 100 % (or 1) and the miRNA expression in leukemic cells of patients was given as relative expression of normal PMNC.

Leukemia cells derived at diagnosis from pediatric B- or T-ALL patients ($n=24$) and different human ALL cell lines

Table 1 Data of patients at diagnosis and during follow-up period

Patient no.	Age[a], sex[b] (F/M)	Lineage	Blast In BM	WBC 10^9/L	Immunophenotype	Karyotyping[c]	Prognosis	Blasts in BM on day 15	Blasts in BM on day 33	Protocol	Current status[d]
1	14.6/M	T	80 %	20.56	pre-T, latecorticalis	Normal	Good	0 %	<1 %	IR	Remission
2	16.4/M	T	98 %	218.00	pre-T	ampl. ABL	Poor	78 %	6.30 %	HR	Death (early remission, disease progression)
3	4.5/M	T	90 %	500.16	pre-T, with proT-cell phenotype	Normal	Poor	80 %	10 %	HR	Remission (after transplatation)
4	4.3/M	T	97 %	19.35	pre-T, late corticalis CD1a neg.	Normal	Good	3 %	0 %	IR	Remission
5	2.6/M	T	98 %	164.12	pre-T	Normal	Poor	2.0 %	0 %	HR	Remission
6	11.2/M	T	90 %	144.00	pre-T	Normal	Good	0 %	0 %	IR	Remission
7	5.5/F	T	95 %	271.54	pre-T, corticalis	Normal	Good	<1 %	0 %	IR	Remission
8	6/F	T	70 %	40.3	pre-T, late corticalis	Normal	Good	<0.3 %	<0.3 %	IR	Remission
9	3.5/M	T	95 %	121.48	pre-T	ETV6/RUNX1 fusion	Good	3.70 %	1.20 %	IR	Remission
10	2/F	BCP	75 %	11.4	pre-B	Normal	Good	0 %	0 %	SR	Remission
11	3/M	BCP	90 %	53.86	pre-B	BCR/ABL fusion	Poor[e]	<1 %	0 %	HR	Death (TX, early relapsion, disease progression)
12	4.6/M	BCP	90 %	18.18	pre-B, common	Hyperdiploid	Good	0 %	0.4 %	SR	Remission
13	2.8/M	BCP	90 %	6.20	pre-B	Hyperdiploid	Good	1.4 %	0 %	SR	Remission
14	3.3/M	BCP	90 %	94.90	pre-B	Hyperdiploid	Good	0 %	0 %	SR	Remission
15	4.2/F	BCP	85 %	6.50	pre-B	Hyperdiploid	Good	0 %	0 %	SR	Remission
16	2/M	BCP	85 %	89.30	pre-B	Hyperdiploid	Poor	5.8 %	0 %	HR	Remission
17	10.3/F	BCP	90 %	44.83	pre-B	ETV6/RUNX1 fusion, del. ETV6	Good	0 %	0.10 %	IR	Remission
18	9.3/M	BCP	100 %	13.81	pre-B	BCR/ABL fusion	Poor	5.20 %	0 %	HR	Death (transplantation related mortality)
19	4.2/F	BCP	95 %	216.3	pre-B	del. p16[f]	Poor	10 %	0 %	IR	Death (disease progression)
20	2.5/M	BCP	100 %	119.8	pre-B	Hyperdiploid[f]	Good	0 %	0 %	IR	Remission
21	8/M	BCP	91 %	6.22	pre-B common	Hyperdiploid	Good	<1 %	0 %	SR	Remission
22	11.6/M	BCP	92 %	66.76	pre-B common	Normal	Poor	6 %	0 %	IR	Late relapse, remission
23	4/M	BCP	67 %	19.59	pre-B common	Normal	Good	2.90 %	0 %	IR	Remission
24	2.2/M	BCP	100 %	3.01	pre-B	ETV6/RUNX1 fusion	Good	12.00 %	0 %	IR	Remission

Protocol: ALL-IC-BFM 2002

SR Standard risk group, *IR* Intermediate risk group, *HR* High risk group

[a] Age at diagnosis in years

[b] Gender, F/M, female/male

[c] Karyotyping includes G-banding and FISH analysis (ETV6/RUNX1, BCR/ABL, MLL, CEP9/p16 probes)

[d] Current status (at the end of study)

[e] Patient 11 had initially good prognosis (according to treatment response criteria, see methods), but after the retrospective analysis (occurrence of relapses during follow-up time) was modified the prognosis of this patient to poor prognosis

[f] 21 trisomie

(CEM, Jurkat, Nalm6 and Mn60) showed generally similar alterations in miRNA expression. In our study we could not find significant overexpression in the expression of well known "oncomiRs" (miR-21 and −155). None of the studied leukemia cells and cell lines showed significant alteration in miR-21 expression. Higher expression level of miR-155 could only be detected in one cell line (CEM, T-ALL). Slightly increased expression of miR-155 could only be shown in B-ALL patients [5.5x (0.54–11.7)]. Samples isolated from pediatric T- and B-ALL patients - results comparable to those in cell lines - showed in both types low miR-29b [0.19x (0.001–1.05)], miR-21 [0.15x (0.003–0.73)] and miR-223 [0.31x (0.003–1.56)] expression.

Lower expression of miR-24 [0.05x (0.01–0.13)] was significant in isolated leukemia cells from T-ALL patients and all cell lines (except Jurkat) showed similar low expression. Overexpression of miR-16 was characteristic only in T-ALL cell lines. No significant alteration in miR-142-3p expression could be detected in any of the studied leukemia cell lines, however, decreased expression of miR-142-3p could be shown in leukemia cells from T-ALL patients. The most characteristic change in miRNA expression was the highly significant overexpression of miR-128b, which could be observed in human ALL cell lines (>500x in the first three cell lines) and all samples derived from leukemia patients [409x (17.3-1048)] (Fig. 1).

Correlation Between miR-128b Expression and Prognostic Factors, as well as Clinical Data of Childhood ALL Patients

The difference in expression profile of miR-128b on day 0 (before treatment) was determined in PMNC and/or BMNC derived from ALL patients (n=24) in order to find potential correlation with good or poor prognosis. The miR-128b expression was significantly higher in the initial samples of patients with good prognosis (both in B- and T-ALL) compared to children with poor prognosis (Fig. 2a). Upon the prognosis of patients and the expression of miR-128b, a relative expression value (80x higher expression compared to normal PBMNC) was determined by ROC analysis (sensitivity: 75 %, specificity: 87.5 %) and our ALL patients were divided into two groups: patients with higher (80x<, n=16) and lower (80x>, n=8) miR-128b expression (Fig. 2b). Significant correlation was shown between the lower (80x>) miR-128b expression and the poor prognosis (p<002—Fisher exact probability test), as well as the poor prednisolone reponse on day 8 (p<001—chi^2 test). A longer disease free time period of patients showed a significant correlation with higher expression levels of miR-128b (p<0.011—chi^2 test). We could not find any correlation between miR-128b and age, gender, immunophenotype, initial WBC count and karyotype (Fisher exact probability and chi^2 test were carried out case number dependently).

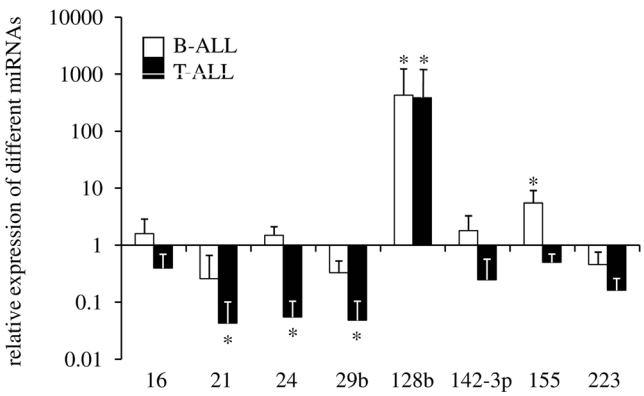

Fig. 1 miRNA expression of ALL cells derived from pediatric patients. The mean of relative expression of miR-16, −21, −24, −29b, −128b, −142-3p, −155, −223 in leukemia cells separated from bone marrow samples (n=15 B-ALL, n=9 T-ALL patients) on day 0, compared to the miR expression of normal PBMNC (actual miRNA expression of normal PMNC was considered to be 100 % - 1 in comparison; *p<0.05)

MiRNS Expression Alteration During Follow-Up of ALL Patients

The expression levels of the studied miRNAs changed in parallel with the decreasing blast (bone marrow) count in all pediatric ALL patients (n=24) during treatment. All lower expressed miRNAs—at diagnosis—showed increasing expression and all overexpressed miRNAs decreased during therapy. The most characteristic difference was the alteration of miR-128b (decrease) and miR-223 (increase) expression. It was also observed, that bone marrow samples of relapsed patients have a significantly higher level of miR-128b compared to values measured at initial diagnosis (Fig. 3.)

Discussion

Data about the studied miRNAs in connection with childhood ALL are missing or they are controversial [7, 8, 14, 33, 34].

According to previous data, miR-21 and miR-155 could play an important role in T- and especially B-cell differentiation and function (isotype switch and generation of memory cells) [6]. In our study no significant increase in miR-21 and miR-155 expression was observed in ALL. However, overexpression of these onco- and/or inflamma-miR is described in most of the lymphoid malignancies (DLBCL, HL, Burkitt lymphoma etc.). Therefore our results confirm that the data of those studies in which the change in the expression of these two miRs is not characteristic in ALLs. It is possible, that the slight increase of miR-155 expression in the cells of B-ALL patients could corroborate the previously described regulatory role of this miR in B-lymphoid cells [35].

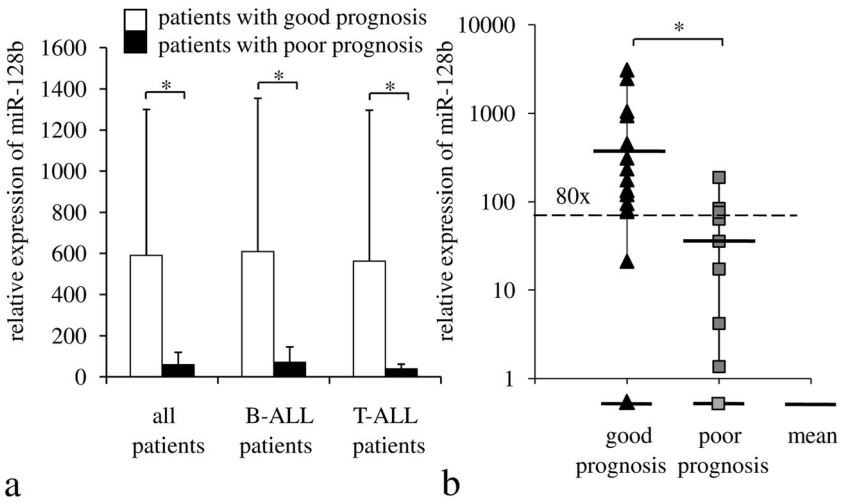

Fig. 2 miR-128b expression in ALL patients with different prognosis. **a** The mean of relative expression of miR-128b in ALL patients with poor and good prognosis. **b** Based on miR-128b expression and prognostic data of patients a cut off value (80x) was calculated by ROC analysis (sensitivity 75 %, specificity: 87.5 %) between higher and lower relative expression of miR-128b in the studied ALL cases

MiR-24 could have an important role in the negative regulation of tumor proliferation, its overexpression can cause a block in the G2/M phase of cell cycle [36, 42]. We showed decreased miR-24 expression in human leukemia cell lines and specially in samples derived from children with T-ALL. These results suggest its possible role in the regulation of T-cell proliferation through loss of function.

We also found decreased expression of miR-29b in ALL cells. MiR-29b has important role in B-cell function. It could help the survival of the leukemic clone (as previously were described in several leukemia subtypes) through loss of negative feedback of the BCR/ABL fusion protein or through overexpression of antiapoptotic proteins [24–26].

Decreased expression of miR-16 is already known in certain hematologic malignancies [7, 8]. Previously, correlation was found between ovexpression of miR-16 and the poorer prognosis of patients [37]. However, our results could not show any significant change in the expression of miR-16. All these three miRs (miR-29b, −16, −24) were previously described with importance in leukemogenesis through the known targets and/or regulatory proteins [22, 36, 38–41].

Changes in miRNA expression in relapsed ALL are in the focus of interest in several groups [42, 43]. These suggest that the low miR-223 expression could be a possible predictor of ALL relapse. We confirmed this, since miR-223 expression was almost undetectable in ALL cells isolated at diagnosis. The expression level recovers to normal during treatment as patients achieve remission and decreases again during relapse.

Our most interesting result is the extreme overexpression of miR-128b in childhood ALL samples, which shows a significant decrease of expression during treatment until the expression level of normal mononuclear cells is reached in remission. Opposite direction was observed in the case of miR-223. Based on others' results, it could be assumed that the increased miR-128b expression by PTEN inhibition while the decreased miR-223 expression through the regulation of IGFR signal activity could contribute to cell proliferation and survival [44, 45]. These observations correlate to the described and published high mTOR activity in ALL cells [48].

Overexpression of miR-128b in leukemia has already been described [46], but none of these studies reported its expression levels in relapsed ALL and correlation with the

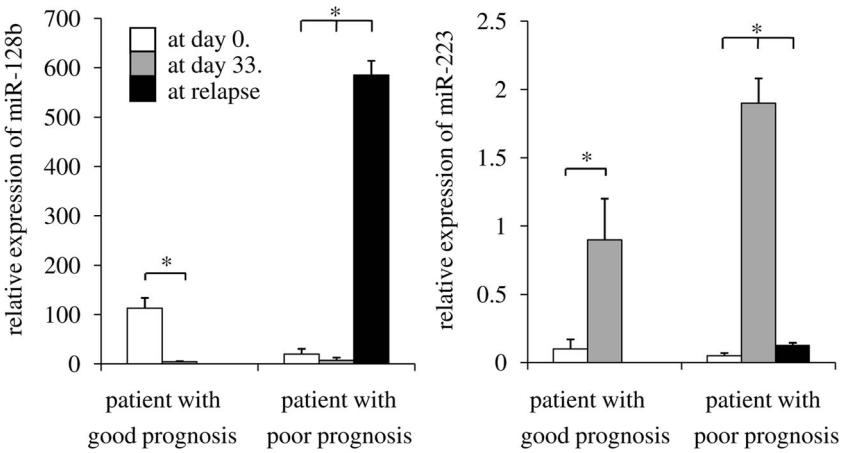

Fig. 3 Relative expression of miR-128b and miR-223 in follow-up samples of childhood ALL patients. Representative results show restored expression of miR-128b and miR-223 in bone marrow cells during treatment at protocol check points in a patient with good prognosis. However, this restitution disappeared in the samples of patient with poor prognosis at relapse

prognosis. We found correlation between the good prognosis and the expression of miR-128b. Higher expression at diagnosis predicted a better prognosis. It is particularly interesting that enhancing miR-128b expression in MLL-AF4 translocation possessed ALL cell lines, and the glucocorticoid sensitivity of these cells could be increased [47].

The data (higher miR-128b expression correlates with good prednisolon response and good prognosis) suggest a correlation between miR-128b expression changes and steroid sensitivity of ALL cells. MLL-AF4 is typically present in infant ALL, however, none of our patients was under 1 year-old. Moreover, our currently presented miRNA results on identical samples used in one of our previously published study [48] related to mTOR activity (p-4EBP1, p-S6 expression) help to confirm the above described possibility. Using Spearman's rank correlation significant inverse correlation was observed both with p-4EBP1 OD values indicating higher mTOR activity (1.1<) and lower miRNA-128b expression (80x>) in ALL samples (data are not shown). Besides these results, it should be considered that both mTOR activity and miR-128b expression were higher in ALL than in normal lymphocytes, however, the highest mTOR activity and the lowest miR-128b correlated to the worse prognosis. Further studies are needed to verify previous and recent results.

We found altered expression of miRNAs which could have relevant correlation with prognosis and prednisolon response in children with ALL. In certain cases this could help to predict early relapse of ALL.

To conclude our real-time PCR results, it was demonstrated that increased expression of "oncomiR"-21 and −155 is not characteristic for human ALL cells, while the overexpression of miR-128b and downregulation of miR-223 are typically characteristic of human ALL cell lines and childhood ALL cells. It has been confirmed that the expression of miR-128b in ALL cells correlates with the prognosis, the prednisolone response on day 8 and the survival. Changes in expression of miR-128b and miR-223, due to their leukemia cell specificity, effectively follow the proportion of leukemia cells in bone marrow samples.

Taken together, our results suggest that determination of miR-128b expression could help for predicting prognosis and prednisolon response, in order to select patients with poor prognosis before treatment, and to follow response to chemotherapy during treatment. This monitorization of the level of miR-128b expression from peripheral blood samples may also help to detect early relapse.

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Conflict of interest We declare that all authors have no financial or other conflict of interest that might bias their work.

References

1. Kaatsch P (2010) Epidemiology of childhood cancer. Cancer Treat Rev 36:277–285
2. Pui CH, Evans WE (2006) Treatment of acute lymphoblastic leukemia. N Engl J Med 354:166–178
3. Baranwal S, Alahari SK (2010) miRNA control of tumor cell invasion and metastasis. Int J Cancer 126:1283–1290
4. Shin VY, Chu KM (2014) MiRNA as potential biomarkers and therapeutic targets for gastric cancer. World J Gastroenterol 20: 10432–10439
5. Zhu J, Zheng Z, Wang J, Sun J, Wang P, Cheng X, Fu L, Zhang L, Wang Z, Li Z (2014) Different miRNA expression profiles between human breast cancer tumors and serum. Front Genet 5:149
6. Vasilatou D, Papageorgiou S, Pappa V, Papageorgiou E, Dervenoulas J (2010) The role of microRNAs in normal and malignant hematopoiesis. Eur J Haematol 84:1–16
7. Lawrie CH (2013) MicroRNAs in hematological malignancies. Blood Rev 27:143–154
8. Wang Y, Lee CG (2009) MicroRNA and cancer-focus on apoptosis. J Cell Mol Med 13:12–23
9. Dong C, Ji M, Ji C (2009) MicroRNAs and their potential target genes in leukemia pathogenesis. Cancer Biol Ther 8:200–205
10. So AY, Zhao JL, Baltimore D (2013) The Yin and Yang of microRNAs: leukemia and immunity. Immunol Rev 253:129–145
11. Schotte D, Akbari Moqadam F, Lange-Turenhout EA, Chen C, van Ijcken WF, Pieters R, den Boer ML (2011) Discovery of new microRNAs by small RNAome deep sequencing in childhood acute lymphoblastic leukemia. Leukemia 25:1389–1399
12. Rainer J, Ploner C, Jesacher S, Ploner A, Eduardoff M, Mansha M, Wasim M, Panzer-Grümayer R, Trajanoski Z, Niederegger H, Kofler R (2009) Glucocorticoid-regulated microRNAs and mirtrons in acute lymphoblastic leukemia. Leukemia 23:746–752
13. Mi S, Lu J, Sun M, Li Z, Zhang H, Neilly MB, Wang Y, Qian Z, Jin J, Zhang Y, Bohlander SK, Le Beau MM, Larson RA, Golub TR, Rowley JD, Chen J (2007) MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. Proc Natl Acad Sci U S A 104:19971–19976
14. de Oliveira JC, Scrideli CA, Brassesco MS, Morales AG, Pezuk JA, QueirozRdeP YJA, Brandalise SR, Tone LG (2012) Differential miRNA expression in childhood acute lymphoblastic leukemia and association with clinical and biological features. Leuk Res 36:293–298
15. Wang Y, Gao X, Wei F, Zhang X, Yu J, Zhao H, Sun Q, Yan F, Yan C, Li H, Ren X (2014) Diagnostic and prognostic value of circulating miR-21 for cancer: a systematic review and meta-analysis. Gene 533: 389–397
16. Mirnezami AH, Pickard K, Zhamg L, Primrose JN, Packham G (2009) MicroRNAs: key players in carcinogenesis and novel therapeutic targets. Eur J Surg Oncol 35:339–347
17. Löffler D, Brocke-Heinrich K, Pfeifer G, Stocsits C, Hackemüller J, Kretzschmar AK, Burger R, Gramatzki M, Blumert C, Bauer K, Cvijic H, Ullmann AK, Stadler PF, Horn F (2007) Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood 110:1330–1333
18. Jurkovicova D, Magyerkova M, Kulcsar L, Krivjanska M, Krivjansky V, Gibadulinova A, Oveckova I, Chovanec M (2014) miR-155 as a diagnostic and prognostic marker in hematological and solid malignancies. Neoplasma 61:241–251

19. Seddiki N, Brezar V, Ruffin N, Lévy Y, Swaminathan S (2014) Role of miR-155 in the regulation of lymphocyte immune function and disease. Immunology 142:32–38
20. Huang X, Shen Y, Liu M, Bi C, Jiang C, Igbal J, McKeithan TW, Chan WC, Ding SJ, Fu K (2012) Quantitative proteomics reveals that miR-155 regulates the PI3K-AKT pathway in diffuse large B-cell lymphoma. Am J Pathol 181:26–33
21. Pedersen IM, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, Rickert RC, Gronbaek K, David M (2009) Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. EMBO Mol Med 1:288–295
22. Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, Alder RI, Alder H, Volinia S, Rassenti L, Liu X, Liu CG, Kipps TJ, Negrini M, Croce CM (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci U S A 105:5166–5171
23. Zaidi SK, Dowdy CR, van Wijnen AJ, Lian JB, Raza A, Stein JL, Croce CM, Stein GS (2009) Altered Runx1 subnuclear targeting enhances myeloid cell proliferation and blocks differentiation by activating a miR-24/MKP-7/MAPK network. Cancer Res 69:8249–8255
24. Garzon R, Heaphy CE, Havelange V, Fabbri M, Volinia S, Tsao T, Zanesi N, Kornblau SM, Marcucci G, Calin GA, Andreeff M, Croce CM (2009) MicroRNA 29b functions in acute myeloid leukemia. Blood 114:5331–5341
25. Zhang YK, Wang H, Leng Y, Li ZL, Yang YF, Xiao FJ, Li QF, Chen XQ, Wang LS (2011) Overexpression of microRNA-29b induces apoptosis of multiple myeloma cells through down regulating Mcl-1. Biochem Biophys Res Commun 414:233–239
26. Li Y, Wang H, Tao K, Xiao Q, Huang Z, Zhong L, Cao W, Wen J, Fen W (2013) miR-29b suppresses CML cell proliferation and induces apoptosis via regulation of BCR/ABL1 protein. Exp Cell Res 319:1094–1101
27. Lv M, Zhang X, Jia H, Li D, Zhang B, Zhang H, Hong M, Jiang T, Jiang Q, Lu J, Huang X, Huang B (2012) An oncogenic role of miR-142-3p in human T-cell acute lymphoblastic leukemia (T-ALL) by targeting glucocorticoid receptor-alpha and cAMP/PKA pathways. Leukemia 26:769–777
28. Wang XS, Gong JN, Yu J, Wang F, Zhang XH, Yin XL, Tan ZQ, Luo ZM, Yang GH, Shen C, Zhang JW (2012) MicroRNA-29a and microRNA-142-3p are regulators of myeloid differentiation and acute myeloid leukemia. Blood 119:4992–5004
29. Dou L, Li J, Zheng D, Li Y, Gao X, Xu C, Gao L, Wang L, Yu L (2013) MicroRNA-142-3p inhibits cell proliferation in human acute lymphoblastic leukemia by targeting the MLL-AF4 oncogene. Mol Biol Rep 40:6811–6819
30. Palumbo T, Faucz FR, Azevedo M, Xekouki P, Iliopoulos D, Stratakis CA (2013) Functional screen analysis reveals miR-26b and miR-128 as central regulators of pituitary somatomammotrophic tumor growth through activation of the PTEN-AKT pathway. Oncogene 32:1651–1659
31. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, Bozzoni I (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. Cell 123:819–831
32. Liu TY, Chen SU, Kuo SH, Cheng AL, Lin CW (2010) E2A-positive gastric MALT lymphoma has weaker plasmacytoid infiltrates and stronger expression of the memory B-cell-associated miR-223: possible correlation with stage and treatment response. Mod Pathol 23:1507–1517
33. Mosakhani N, Sarhadi VK, Usvasalo A, Karjalainen-Lindsberg ML, Lahti L, Tuononen K, Saarinen-Pihkala UM, Knuutila S (2012) MicroRNA profiling in pediatric acute lymphoblastic leukemia: novel prognostic tools. Leuk Lymphoma 53:2517–2520
34. de Oliveira JC, Brassesco MS, Scrideli CA, Tone LG, Narendran A (2012) MicroRNA expression and activity in pediatric acute lymphoblastic leukemia (ALL). Pediatr Blood Cancer 59:599–604
35. Vigorito E, Kohlhaas S, Lu D, Leyland R (2013) miR-155: an ancient regulator of the immune system. Immunol Rev 253:146–157
36. Akbari Moqadam F, Boer JM, Lange-Turenhout EA, Pieters R, den Boer ML (2014) Altered expression of miR-24, miR-126 and miR-365 does not affect viability of childhood TCF3-rearranged leukemia cells. Leukemia 28:1008–1014
37. Kaddar T, Chien WW, Bertrand Y, Pages MP, Rouault JP, Salles G, Ffrench M, Magaud JP (2009) Prognostic value of miR-16 expression in childhood acute lymphoblastic leukemia relationships to normal and malignant lymphocyte proliferation. Leuk Res 33:1217–1223
38. Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW, Fernandez-Zapico ME (2010) Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. J Cell Biochem 110:1155–1164
39. Mraz M, Kipps TJ (2013) MicroRNAs and B cell receptor signaling in chronic lymphocytic leukemia. Leuk Lymphoma 54:1836–1839
40. Xi Y, Li J, Zan L, Wang J, Wang G, Ning Y (2013) Micro-RNA-16 expression in paraffin-embedded specimen correlates with overall survival of T-lymphoblastic lymphoma/leukemia. Hum Pathol 44:1011–1016
41. Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GS, Banerjee D, Bertino JR (2007) A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. Proc Natl Acad Sci U S A 104:13513–13518
42. Han BW, Feng DD, Li ZG, Luo XQ, Zhang H, Li XJ, Zhang XJ, Zheng LL, Zheng CW, Lin KY, Zhang P, Xu L, Chen YQ (2011) A set of miRNAs that involve in the pathways of drug resistance and leukemic stem-cell differentiation is associated with the risk of relapse and glucocorticoid response in childhood ALL. Hum Mol Genet 20:4903–4915
43. Zhang H, Luo XQ, Zhang P, Huang LB, Zheng YS, Wu J, Zhou H, Qu LH, Xu L, Chen YQ (2009) MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. PLoS One 4:e7826
44. Alqurashi N, Hashimi SM, Wei MQ (2013) Chemical Inhibitors and microRNAs (miRNA) Targeting the Mammalian Target of Rapamycin (mTOR) Pathway: Potential for Novel Anticancer Therapeutics. Int J Mol Sci 14:3874–3900
45. Jia CY, Li HH, Zhu XC, Dong YW, Fu D, Zhao QL, Wu W, Wu XZ (2011) MiR-223 suppresses cell proliferation by targeting IGF-1R. PLoS One 6:e27008
46. Wang Y, Li Z, He C, Dongmei W, Xianggui Y, Jianjun C, Jie J (2010) MicroRNAs expression signatures are associated with lineage and survival in acute leukemias. Blood Cells Mol Dis 44:191–197
47. Kotani A, Ha D, Hsieh J, Rao PK, Schotte D, den Boer ML, Armstrong SA, Lodish HF (2009) miR-128b is a potent glucocorticoid sensitizer in MLL-AF4 acute lymphocytic leukemia cells and exerts cooperative effects with miR-221. Blood 114:4169–4178
48. Nemes K, Sebestyén A, Márk A, Hajdu M, Kenessey I, Sticz T, Nagy E, Barna G, Váradi Z, Kovács G, Kopper L, Csóka M (2013) Mammalian target of rapamycin (mTOR) activity dependent phospho-protein expression in childhood acute lymphoblastic leukemia (ALL). PLoS One 8:e59335