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MicroRNA as a prognostic biomarker for survival in childhood acute lymphoblastic leukemia: a systematic review

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Abstract

Recent studies suggest abnormal microRNA (miRNA) expression may have potential prognostic value in childhood acute lymphoblastic leukemia (ALL). In this systematic review, we searched different databases (PubMed, ASH, ASCO, and SIOP) for studies published from 2008 to 2018 that evaluated the prognostic impact of miRNAs in childhood ALL. We also used DIANA-miRPath v3.0 to further characterize the functional role of the significant prognostic miRNAs identified in our systematic review. Here we evaluate 15 studies with a total of 38 different miRNAs and 1545 children with B-cell ALL (B-ALL) or T-cell ALL (T-ALL) recruited over approximately 3 decades (1984–2016) with different treatment protocols and ethnicities. Out of the 15 studies examined, 14 reported 32 dysregulated miRNAs with significant prognostic impact in pediatric ALL patients. Only one Brazilian study reported no significant prognostic effect of 7 miRNAs, while the seventh miRNA (miR-100) showed prognostic significance in a Chinese study. Using DIANA-TarBase v7.0 of DIANA-miRPath v3.0, pathway enrichment analysis revealed 25 miRNAs modulated 24 molecular pathways involved in cancer development. To remove the effect of salvage therapy, 9 studies carried out multivariate cox regression analysis for both relapse-free survival and disease-free survival to develop a panel of 23 miRNAs acting as independent prognostic biomarkers. To enhance the clinical application, utility, and validity of the miRNAs discussed here, their potential prognostic value should be confirmed in larger cohort studies within different ethnicities and different ALL protocols adjusted for other contemporary validated prognostic factors in childhood ALL.

Keywords Acute lymphoblastic leukemia · MicroRNA · Prognostic biomarker

1 Introduction

Childhood acute lymphoblastic leukemia (ALL) is the most common childhood cancer with high overall survival at 80–90% [1]. This high survival is primarily attributable to decades of outstanding advancement in the knowledge of leukemic cell biology, which has shed light on the remarkable progress in antileukemic therapy development and risk-adapted treatment [2]. This is along with collaborative multicenter clinical trials and progress in supportive care. However, the relapse rate in pediatric ALL patients remains significant at 10–20%, with disappointing survival outcome using contemporary salvage therapy [3]. Identification of prognostic biomarkers will aid in both increasing the accuracy of patients' risk stratification as well as reducing the chances of relapse by optimizing therapy at the early stages of treatment.

With great advances in technologies used for gene expression analysis, molecular profiling of novel microRNAs (miRNAs) in childhood ALL has been accessible. MiRNAs are 19–22 ribonucleotide long noncoding RNA. They mediate posttranscriptional regulation of many human genes and consequently have preclinical and clinical research applications across many diseases [4–6]. Recently, miRNAs have been found to have both diagnostic and therapeutic clinical utility [7]. Many studies have been published on individual miRNAs

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or a panel of miRNAs as reliable prognostic biomarkers in childhood ALL. Therefore, evaluating miRNA databases for candidates with clinical application in childhood ALL bridges basic research and clinical application. This systematic review aims to identify all studied miRNAs with prognostic significance in childhood ALL. This panel can be validated in future large cohort studies.

2 Methods

2.1 Systematic search

This systematic review was registered in International prospective register of systematic reviews (PROSPERO) with identification number CRD42019136496. We searched the PubMed database in addition to SIOP, ASH, and ASCO abstract meetings from 2008 to 2018. Citation databases (Web of Science and Scopus) were used to identify publications that have cited previous publications. The following combinations were used in PubMed (MEDLINE), (((microRNA) OR miRNA) OR miR) and childhood acute lymphoblastic leukemia.

The research was limited to full-text English literature only. The eligibility criteria for included studies was as follows: (i) time since publication ≤ 18 years and (ii) measurement of at least one survival curve for overall survival (OS), leukemia-free survival (LFS), disease-free survival (DFS), and relapse-free survival (RFS) with or without hazard ratio (HRs) and 95% confidence intervals (CIs). The main exclusion criteria were (i) the data in cell lines or any nonhuman experimental approach (ii) reviews or letters without primary data and retracted or duplicated papers (iii) frequency of research evaluating prognostic value of miRNAs in tissue of four or less (iv) articles published in language other than English.

Two investigators (WMR and MMH) independently conducted a standardized research protocol and data extraction. The following information was extracted: the surname of the first author/year of publication, sample size/country, age, protocol used/recruitment period, follow-up duration, blast cells cutoff, RNA extraction method/source of sample, platform (miRNA assay method), miRNA studied, endpoint, and dysregulated miRNA-associated poor survival. Any conflict regarding inclusion of certain studies were settled by discussion. Studies were divided according to the ethnicity of the country/population studied (Asian, European, North American, African, and Mixed).

2.2 Pathway enrichment analysis:

We used DIANA-TarBase v7.0 of DIANA-miRPath v3.0 [8] for better understanding of the functional role of the significant prognostic miRNAs identified in our systematic review. We entered all significant prognostic miRNAs in the search bar of DIANA-miRPath v3.0 to identify both significant genes and molecular function in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) categories, respectively. To identify the significant pathways and molecular function modulated by the same miRNAs, we created HeatMap (as an exploratory tool) of dysregulated miRNAs vs pathways or categories.

3 Results

3.1 Baseline characteristics of studies

We have retrieved 206 records from PubMed using specified research terms. Our search in SIOP, ASH, and ASCO abstract meetings from 2008 to 2018 resulted in one study with eligible characteristics. Figure 1 summarizes the search strategy used. Out of the fifteen eligible studies, only four studies used microarray platform in the test cohort (discovery) followed by qRT-PCR in the validation cohort [9–12]. Six studies used TRIzol method for RNA extraction [9, 12–16]. The source of specimens in all studies except 3 [10, 13, 17] was bone marrow with different cutoff values (table 1). Out of the 14 studies, there were two studies that tested miRNAs in precursor B-ALL patients only [11, 17]. The total number of pediatric ALL patients across the 15 studies was 1545. Studies that included the same cohort in another study or subsequent analysis were not counted twice [9, 11, 18] (Table 1). Patients of European ethnicity represent the largest cohort (36.15%) in 6 studies [10, 13, 18–21] followed by Asian ethnicity (31.16%) in 5 studies (Fig. 2). Studies in this systematic review included ALL patients recruited over approximately 3 decades (1984–2016). During these 3 decades, different treatment protocols were used (Table 1).

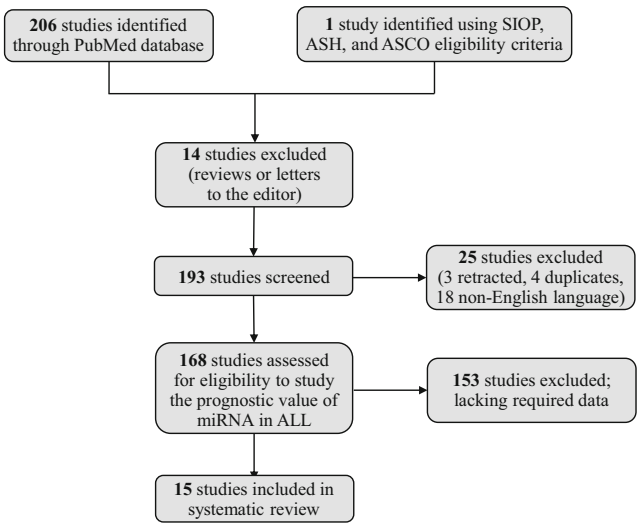

Fig. 1 Flow chart for the systematic search

Table 1 Characteristics of ALL studies included in the systematic review

No.	Study	Sample size [B-lineage, T-lineage], country	Median age	Protocol, recruitment period	Median follow-up duration	% Blast cells cutoff	RNA extraction method, source	Platform	miRNA studied	Endpoint	miRNA dysregulation
1	de Oliveira *et al.* 2012[#]	**128** [108, 20] Brazil	5.2 years (13 months–17 years)	**GBTLI-ALL99** Jan. 2002–May 2005	56 months	≥ **70**	Trizol reagent BM	RT-qPCR	miR-92a, miR-100, miR-125a-5p, miR-128a, miR-181b, miR-196b, let-7e	EFS	NS
2	Mei *et al.* 2014	Test cohort: 38 [34, 4] China Validation cohort: 76 [66, 10] China	n/a n/a	CCLG ALL 2008 Apr. 2008–Oct. 2009	61 months (1–68 months) 48 months (159 months)	≥ 70	mirVana miRNA Isolation Kit BM mirVana miRNA Isolation Kit BM	RT-qPCR	miR-210	LFS EFS OS	Downregulation
3	Mei *et al.* 2017	91 [81, 10] China	5 years (1–14 years)	CCLG ALL 2008 Mar. 2008–July 2010	37.2 months (1–50 months)	≥ 70	mirVana miRNA Isolation Kit BM	RT-qPCR	miR-210	LFS EFS OS	Downregulated
4	Piatopoulou *et al.* 2017	125 [110, 15 Greece	5 years	BFM May 2006 Oct. 2016	79 months	≥ 85 (sub--analysis for B-lineage)	TrI-Reagent BD, BM	RT-qPCR	miR-125b-5p	DFS OS	Downregulated
5	Piatopoulou *et al.* 2018[#]	125 [110, 15] Greece	5 years	ALL-BFM 95 and ALL IC-BFM 2009 guidelines. May 2006–Oct. 2016	86.0 months	n/a	TrI-Reagent BD, BM	RT-qPCR	miR-143-3p, miR-182-5p	DFS OS	Upregulated
6	Schotte *et al.* 2011[$]	81 [70, 11] Netherlands	n/a	Multiple protocols used recruitment period n/a.	5 years	> 90	TRIzol reagent BM, PB	Stem-loop RT-qPCR microRNA array	miR-33, miR-215, miR-369-5p, miR-496, miR-518d, miR-599 miR-10a, miR-134, miR-214, miR-484, miR-572, miR-580, miR-624 miR-627	DFS	Upregulated Downregulated
7	Yan *et al.* 2013[@]	Test cohort: 63 [56, 7] Malaysia, Singapore Validation cohort: 73 [69, 4] Malaysia, Singapore	n/a	Ma-Spore ALL 2003 July 2002–Mar. 2011	EFS = 4.74 years OS = 5.35 years	≥ 90	Test: TRIzol reagent BM Validation: miRNeasy Mini Kit BM	Test: Agilent human miRNA Microarray V2 Validation: RT-qPCR	miR-335	OS	Downregulated
8	Han *et al.* 2011	Test cohort: 18 [11, 6, 1 unk] China Validation cohort 104 [81, 14, 9 unk] China	6.0 years (0.25–14.5 years)	ALLIC BFM 2002 (*modified*) recruitment period n/a.	36 months	n/a	TRIzol method BM	Test: Affymetrix miRNA microarray platform Validation: RT-qPCR	miR-708, miR-27a, miR-223	RFS	Downregulated

9	Organista-Nava *et al.* 2015	111 [93, 11, 7 unk] Mexico	7.73 years	96091, 96092 or CIE-10:C9.1.0 *(Cancer Institute from Guerrero State)* Sep. 2005–Jul. 2013	n/a	NA	TRIzol method/ BM	RT-qPCR	miR-24	OS	Upregulated
10	Mosakhani *et al.* 2012†	79 [68, 11] Finland	n/a	NOPHO-ALL-2000 (age > 1 year) Interfant (< 1 year) /2000-2006	n/a	> 80 (quantified from core biopsies)	MiRNeasy FFPE Mini Kit/core biopsy	Test: Agilent miRNA microarray system Validation: RT-qPCR	miR-423-5p	EFS OS	Downregulated
11	Labib *et al.* 2017	75 Egypt	n/a	NS Feb. 2011–May 2016	5 years	> 50	Qiagen miRNeasy Serum/Plasma Kit/Serum	RT-qPCR	miR-21	RFS OS	Upregulated
12	Kaddar *et al.* 2009	93 [62, 31] France	6.5 years (0.2–1-7.7 years)	EORTC 58831, 58832, 58881 1994–2002	n/a	> 80	RNA Plus/BM	Northen blot / RT-qPCR	miR-16	DFS	Upregulated
13	Avigad *et al.* 2016	Test cohort: 48 [35, 13] Israel and Czech Republic	0.3–19 years	ALL BFM-based protocols [INS-84, INS-89, INS-98, ALL-IC-BFM 2003] 1984–2005	92 months (8–361 months)	≥ 80	miRNeasy Mini Kit/BM	Test: miRXplore™ microarray	miR-151-5p	RFS	Downregulated
		Validation cohort 1: 138 [138, 0] Israel and Czech Republic						Validation(1&2) RT-qPCR	miR-451		Downregulated
		Validation cohort 2: 33 [33,0] Netherlands	5.7 years (1.2–1-5.6 years)	DCOG ALL-9 1997–2004					miR-1290		Upregulated
14	Li *et al.* 2013	111 [93, 18] China	6.0 years (1–14.5 years)	n/a n/a	5 years	n/a	TRIzol method/BM	RT-qPCR	miR-100	OS LFS	Downregulated
									miR-99a		Downregulated
15	Agirre *et al.* 2009	179 Spain	5 years (0.3–14 years)	ALL protocols of the PETHEMA [ALL-89, ALL-93, ALL-96] Jan. 1989–Dec. 2006	14 years	> 70	Ultraspec/BM	RT-qPCR	miR-124-3p (*previously miR-124-a*)	OS DFS	Downregulated

#Overexpression of miR-143-3p/miR182-5p at the end of induction (EOI) day 33 of BFM protocol showed significant association with DFS and OS. Out of 125 ALL patients, patients' group with available matched BM specimen at the EoI (n = 83) was followed up for a similar period (median 91.0 months)

$Survival correlation with the selected miRNAs expression was calculated for 78 patients out of the total cohort (81 patients)

@38 patient samples were used for both test and validation

†The test and validated cohort of ALL patients are the same

Abbreviations: *BFM* Berlin–Frankfurt–Munster protocol, *BM* bone marrow, *CCLG* The Chinese Children's Leukemia Group, *DCOG* The Dutch Childhood Oncology Group, *DFS* disease-free survival, *GBTLI* Brazilian Childhood Leukemia Treatment Group, *INS* Israel National study, *LFS* leukemia-free survival *n/a* not available, *unk* lineage unknown, *NOPHO* The Nordic Society of Pediatric Hematology and Oncology, *NS* not significant, *PETHEMA* Programa Espanol de Tratamiento en Hematologia, *RFS* relapse-free survival, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *OS* overall survival

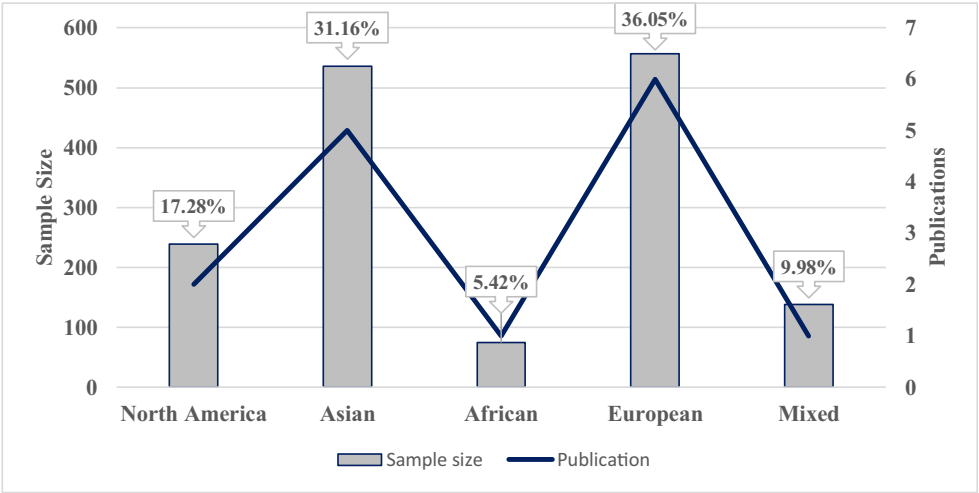

Fig. 2 The number of publications and sample size according to ethnicity of 15 eligible studies

3.2 Dysregulated miRNAs

We identified 38 miRNAs across the 15 studies examined in this systematic review. Only one Brazilian study reported no significant prognostic effect of 7 miRNAs (miR-92a,, miR-125a-5p, miR-128a, miR-181b, miR-196b, let-7e, miR-100) [14]. Yet, the seventh miRNAs (miRNA-100) in the same study showed a significant prognostic effect in the Chinese study [16]. Thirty-two dysregulated miRNAs with significant prognostic impact in pediatric ALL patients were identified in 14 studies. The total number of up- and downregulated miRNAs was 12 and 20, respectively (Table 1). All studies showed a significant association between tested miRNAs with survival at ALL diagnosis, excluding one study [18]. This study showed significant association between overexpression of miR-143-3p/miR182-5p at the end of induction (EOI) day 33 of Berlin-Frankfurt-Münster (BFM) protocol and both DFS and OS. Only 1 study reported the epigenetic alteration of miR-124-3p (previously known as miR-124a) in ALL. Epigenetic downregulation of miR-124-3p induces upregulation of its target cyclin-dependent kinase 6 (CDK6) in the cell cycle machinery. This is associated with phosphorylation of retinoblastoma gene. Consequently, it results in cell proliferation.

Out of the 14 studies, only 10 carried out multivariate cox regression analysis and reported 25 significant dysregulated miRNAs as an independent prognostic biomarker in childhood ALL (Table 2). They reported 13 down- and 9 upregulated miRNAs in addition to 1 panel of miRNA (2 up- and 1 downregulated miRNA).

3.3 Pathway enrichment analysis and target genes:

All genes and the associated KEGG pathways modulated by the 25 miRNAs were identified by DIANA-TarBase v7.0 of DIANA-miRPath V3.0 (Table S1). Furthermore, 25 miRNAs modulate 24 molecular pathways involved in cancer development as identified in the reference database (Table 3). Pathways in cancer (hsa05200) was the molecular pathway with the highest number of both modulated genes (294 genes) and miRNAs (24 miRNAs). Overall, this is an evidence that these miRNAs are involved in cancer development including ALL.

HeatMap in both Figs. 3 and 4 represents the dysregulated miRNAs with the significant KEGG pathways and GO molecular function, respectively. Using experimentally validated miRNA interactions derived from DIANA-TarBase v7.0, the results showed 7 miRNAs, out of the 32 miRNAs, with no targeted genes in the reference database. The seven miRNAs are composed of 2 upregulated (miR-496 and miR-215) and five downregulated (miR-210, miR-134, miR-580, miR-599, and miR-627)

4 Discussion

Out of 207 records retrieved via PubMed, SIOP, ASH, and ASCO search, only 15 studies were eligible to be included in this systematic review. The total number of patients in this systematic review was 1545 ALL pediatric patients. A total 38 miRNAs in 15 studies were identified. Only one Brazilian study reported no significant prognostic effect of 7 miRNAs. Thus, 32 dysregulated miRNAs with significant prognostic impact in pediatric ALL patients were reported from 14 studies. Out of the 14 studies, only 10 studies carried out multivariate cox regression analysis and reported 25 significant miRNAs as an independent prognostic biomarker in childhood ALL. Pathway enrichment analysis using DIANA-miRPath V3.0 database has revealed the functional role of the dysregulated miRNAs to modulate different KEGG pathways and GO categories involved in cancer. Overall, the thirty-two dysregulated miRNAs with significant prognostic effect in

Table 2 List of significant dysregulated miRNAs as independent prognostic marker in the multivariate cox regression analysis of all studies in the systematic review

No.	1st Author/ year	miRNA expression	Multivariate analysis, HR (95%CI)	End point	Other prognostic factors (covariates)
(I) Downregulated miRNAs (n = 13)					
1	Mei *et al.* 2017	miR-210	0.029 (0.002–0.461)	RFS	*CAS8AP2,* MRD day 33, prednisone response, CNS involvement, BCR-ABL, TEL-AML1, MLL rearrangement, E2A-PBX1
2	Piatopoulou *et al.* 2017	MiR-125b	3.891(1.195–12.67) 2.963 (1.079–8.135)	DFS OS	Prednisone response (BFM day 8), poor vs good, bone marrow response (BFM day 15); M2–M3 vs M1, immunophenotype, T-ALL vs B-ALL WBC count, _50 000 cells/μl vs < 50 000 cells/μl; Age, < 1 or _10 years vs 1–9 years; Gender, male vs female; BFM risk group, high risk vs low/intermediate risk
3	Yan *et al.* 2013	miR-335	Test cohort: 7.55 (2.37–24.01) Validation cohort: 2.84 (1.07–7.52)	OS	Age, WBC, BCR-ABL1, NCI criteria, MRD risk group at day 33
4	Han *et al.* 2011	miR-223	24.22 (3.73–157.28)	RFS	Age (10-year increase), immunophenotyping (T-ALL), 33-day remission status (≥ 50%), MLL
5	Schotte *et al.* 2011	miR-10a, miR-134, miR-214 miR-484, miR-572, miR-580, miR-624, miR-627	0.82 (0.69–0.97) 0.73 (0.56–0.96) 0.73 (0.59–0.90) 0.81 (0.69–0.94) 0.59 (0.41–0.85) 0.81 (0.65–0.99) 0.79 (0.67–0.93) 0.68 (0.49–0.93)	DFS	ALL subtypes: *MLL, TEL-AML1, BCR-ABL, E2A-PBX1*, hyperdiploid, T-ALL
6	Agirre *et al.* 2009	miR-124-3p	NA	DFS	NCI poor risk, T-cell phenotype, WBC > 50 × 10^9/L, BCR/ABL positivity
(II) Upregulated miRNAs (n = 9)					
7	Piatopoulou *et al.* 2018	miR-143, -182 overexpression at day 33	6.907 (1.208–39.47) 2.939 (0.537–16.09)	DFS OS	Prednisone response (BFM day 8), bone marrow response (BFM day 15), M2–M3 vs M1; immunophenotype, T-ALL vs B-ALL; WBC count < vs > 50 × 10^3/L; Age, < 1 or ≥ 10 years vs 1–9 years; gender, male vs female, BFM risk group, high risk vs low risk vs intermediate risk
8	Schotte *et al.* 2011	miR-33, miR-215, miR-369-5p, miR-496, miR-518d, miR-599	1.32 (1.02–1.69) 1.30 (1.01–1.67) 1.30 (1.01–167) 1.52 (1.15–2.00) 1.43 (1.01–1.04) 1.39 (1.01–1.89)	DFS	ALL subtypes: *MLL, TEL-AML1, BCR-ABL, E2A-PBX1*, hyperdiploid, T-ALL.
9	Labib *et al.* 2017	miR-21	4.01 (1.74–9.34) 4.63 (1.96–10.98)	DFS OS	Age, CNS infiltration, Platelet (× 10^9/l)
(III) Panel of miRNAs (up- and down-regulated) (n = 1)					
10	Avigad *et al.* 2016	miR-151-5p, miR-451, miR-1290	8.34 (2.97–23.3)	RFS	Age, WBCs, prednisone response

Abbreviations: BFM Berlin-Frankfurt-Münster protocol, *DFS* Disease- free survival, *HR* Hazard Ratio, *MLL* mixed lineage leukemia gene, *RFS* Relapse-free survival, *OS* overall survival

pediatric ALL patients should be investigated as a prognostic panel in larger cohort study along with other prognostic factors of childhood ALL.

Patients from European descent represented the highest sample size (36.15%) in a total 6 studies followed by Asian descent (31.16%) in a total 5 studies. This coincides with a recent report by Sirugo *et al* regarding the predominant representation of European population and subsequent lack of ethnic diversity in genetic association studies [22]. The underrepresentation of ethnically various populations leads to misunderstanding of the genetic architecture of human disease, along with a delay in clinical translation of basic research. Consequently, treatment protocols and diagnostic/prognostic biomarkers validated in predominantly European cohorts may be blindly applied to other populations in clinical practice. Therefore, genetic research studies including different population especially African population should be encouraged through funding agencies to avoid this racial bias. Moreover, international collaboration amongst research groups could

Table 3 Summary of the significant molecular KEGG pathways involved in cancer development modulated by the selected miRNAs

No.	KEGG pathway	*p* value	No. of genes	No. of miRNAs
1	Proteoglycans in cancer (hsa05205)	1.03E-12	164	23
2	Cell cycle (hsa04110)	4.08E-09	102	23
3	Pathways in cancer (hsa05200)	2.34E-06	294	24
4	Hippo signaling pathway (hsa04390)	4.32E-06	112	22
5	Chronic myeloid leukemia (hsa05220)	1.06E-05	62	24
6	TGF-beta signaling pathway (hsa04350)	1.12E-05	64	22
7	Regulatory pathways of stem cell pluripotency (hsa04550)	0.0004	105	23
8	Adherens junction (hsa04520)	0.000597	57	22
9	HIF-1 signaling pathway (hsa04066)	0.001583	82	23
10	ErbB signaling pathway (hsa04012)	0.001583	67	24
11	Acute myeloid leukemia (hsa05221)	0.001649	47	21
12	FoxO signaling pathway (hsa04068)	0.002306	100	23
13	RNA transport (hsa03013)	0.003077	124	23
14	Insulin signaling pathway (hsa04910)	0.003077	105	24
15	mTOR signaling pathway (hsa04150)	0.0043	49	21
16	Viral carcinogenesis (hsa05203)	0.004519	141	24
17	MAPK signaling pathway (hsa04010)	0.004611	178	23
18	Central carbon metabolism in cancer (hsa05230)	0.005454	51	21
19	TNF signaling pathway (hsa04668)	0.005505	82	23
20	AMPK signaling pathway (hsa04152)	0.006182	92	23
21	Steroid biosynthesis (hsa00100)	0.007855	16	10
22	p53 signaling pathway (hsa04115)	0.01019	54	21
23	Wnt signaling pathway (hsa04310)	0.01019	97	22
24	Transcriptional misregulation in cancer (hsa05202)	0.0148	125	24

also increase representation of other ethnically various populations.

In this systematic review, 3 studies did not associate the prognostic value of tested miRNAs to certain protocols [13, 16, 17], while 2 studies associated the results of the prognostic value of certain miRNAs to ALL BFM-based protocols [11, 18]. In general, using miRNA as prognostic biomarker requires clinical validation in addition to clinical utility. Thus, studies investigating the prognostic effect of miRNA should be validated using multiple different protocols.

Only 10 studies carried out multivariate cox regression analysis to prove the independency of tested miRNAs as prognostic biomarkers with contemporary validated prognostic factors in childhood ALL (Table 2). We have to clarify that both RFS and DFS are the same as defined by NCI Dictionary of Cancer Terms-National Cancer Institute at National Institutes of Health. So, to remove the effect of the salvage therapy, we can enlist the significant dysregulated miRNAs as an independent prognostic biomarker in the multivariate cox regression analysis for DFS/RFS only. The result is a panel of 23 independent prognostic miRNAs. This panel reported by 9 studies and composed of 13 downregulated miRNAs (miR-210, miR-125b, miR-223, miR-10a, miR-134, miR-214, miR-484, miR-572, miR-580, miR-624, miR-627, miR-151-5p, miR-451) and 10 upregulated miRNAs (miR-143, miR-182, miR-21, miR-33, miR-215, miR-369-5p, miR-496, miR-518d, miR-599, miR-1290). We failed to run meta-analysis for these 9 studies due to two reasons. First, we didn't find clinically important confounders in the multivariate cox regression analysis in 2 studies [13, 17]. Second, two studies reported significant hazard ratios only for combined rather than single miRNAs [11, 18]. In general, the clinical utility and the clinical validity of this panel as independent prognostic biomarker should be confirmed in further large study.

The 14 studies included in this systematic review reported miRNAs as a reliable prognostic biomarker. Two studies [9, 15] in the systematic review used OS as an end point in testing prognostic value of tested miRNAs (miR24 and miR335). Using OS as an end point will reflect the effect of the overall treatment including both the treatment protocol and the salvage therapy used. Most contemporary childhood ALL treatment protocols are risk-adapted stratification. Current prognostic factors/

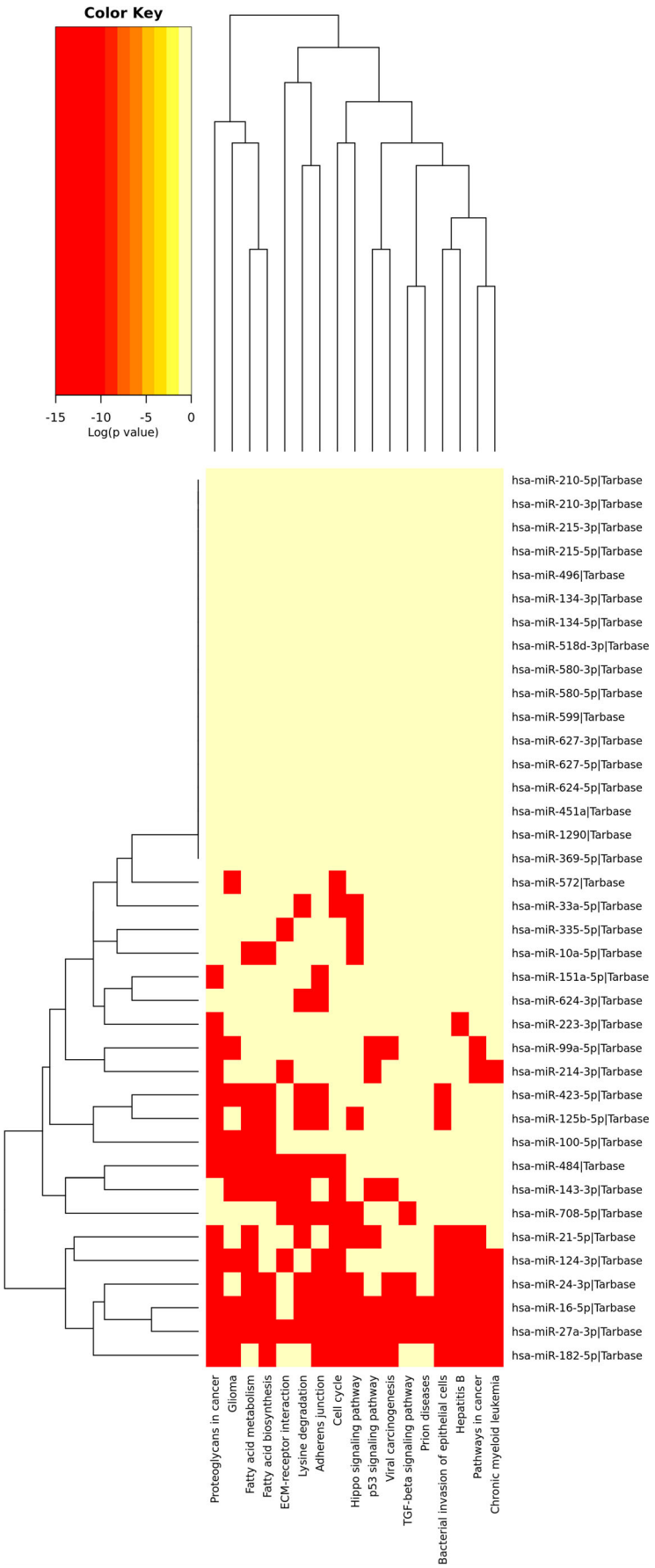

Fig. 3 HeatMap of KEGG pathway vs significant dysregulated miRNAs

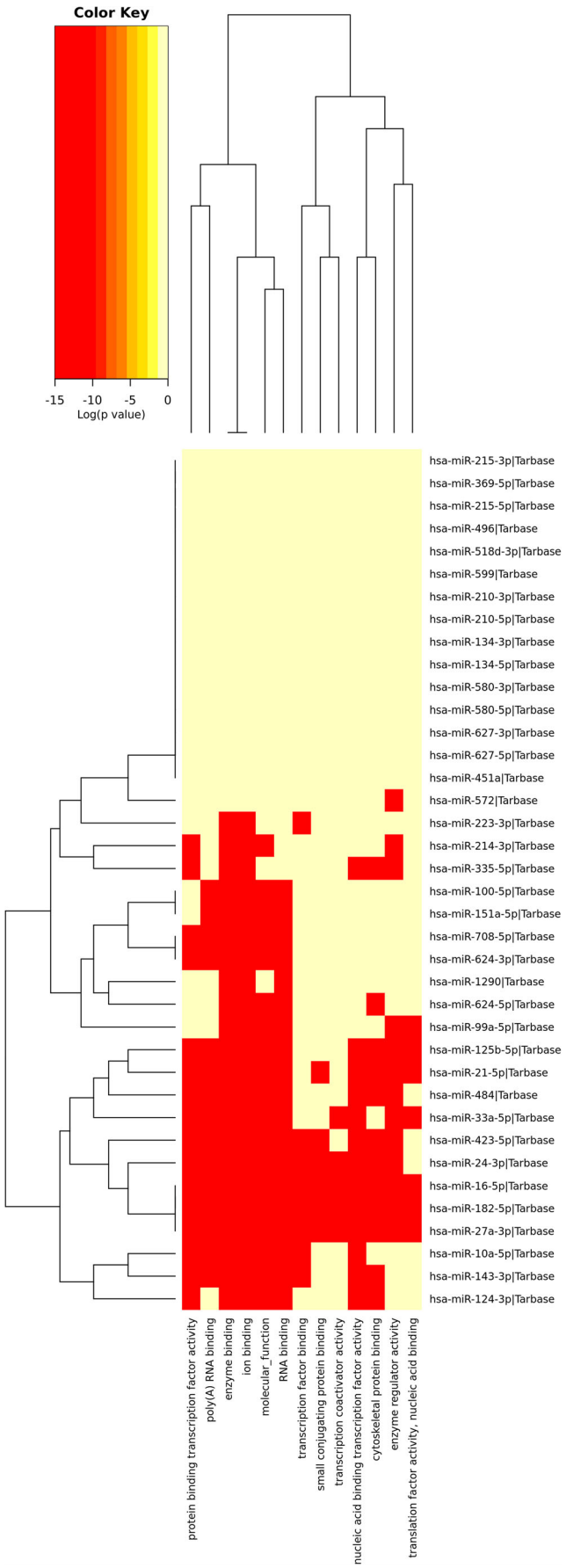

Fig. 4 HeatMap of GO molecular function vs significant dysregulated miRNAs

features [23] enable clinicians to stratify ALL patients at diagnosis into their corresponding group. Yet, there is no standard salvage therapy for ALL patients. Therefore, the prognostic impact of both miR-24 and miR-335 should be evaluated in further study using relapse-free survival (RFS) and disease-free survival (DFS) as an end point.

Induction of apoptosis in ALL protocols is enhanced either directly by glucocorticoid (GC) [24] or indirectly via other antileukemic therapies [25, 26]. Many miRNAs included in this systematic review are associated with GC response. MiR-143, miR-182, and miR-16 target B-cell lymphoma 2 (BCL2) expression as the apoptotic cell machinery [18, 27]. BCL2 family proteins are known to play an important role in GC-induced apoptosis of malignant lymphocytes [28]. Also, miR-335 regulates MEK/ERK pathway in GC-induced apoptosis [9]. In addition, miR-708 was reported to be associated with the response to GC treatment and with ALL risk stratification [12]. Furthermore, a significant correlation between low miR-151-5p levels and poor prednisone response and BFM high-risk classification was identified [11]. Both miR-100 and miR-99a regulate glucocorticoid receptor (GR) signaling pathway through their target called FK506-binding protein 51 (FKBP51). So, developing therapeutic methods to reintroduce synthetic miRNAs to ALL patients or/and modifying the underlying signaling pathway that is associated with GC-induced apoptosis may be potential therapeutic strategies to overcome GC resistance and improve the treatment outcome for pediatric ALL patients.

Although miRNA-100 showed no prognostic impact in childhood ALL in one study [14], other study showed its prognostic impact [16]. Also, Zhang *et al* demonstrated the increased miR-210 expression in high-risk group is associated with a poorer outcome in pediatric ALL, which is in contrast with the findings of Mei *et al.* study [29–31]. These discrepancies in findings can be attributed to the difference in the methods of extraction and detection of miRNAs, in addition to the risk classification groups in treatment protocols.

5 Recommendations

Recently, microRNA research has become a hot topic due to growing evidence of miRNA dysregulation in many cancer types. However, using miRNAs as a prognostic biomarker remains a challenge. The presence of certain consensus methodology in setting up miRNA research studies enables researchers to perform systematic reviews and/or meta-analyses. Moreover, it will enhance the clinical translation and application of the findings. In addition to previously published recommendations [29] regarding both sample source and leukemic blast, we propose some statistical analysis recommendations that should be taken in consideration to optimize published data on miRNAs in cancer (see below). Overall, to enhance the clinical application, utility, and validity of the 32 miRNAs identified here as prognostic biomarkers, these should be validated in larger cohort studies with ethnically diverse cohorts and different ALL protocols along with contemporary validated prognostic biomarkers in childhood ALL.

1. **Standardization of the cutoff determining method:** Several methods were applied to detect the optimal cutoff point for miRNA expression in this systematic review. These ranged from using the median to using ROC and X-tile algorithms. Mosakhani *et al* used K-means and Euclidean distance to divide groups based on unsupervised clustering technique [10], while Han *et al* applied hierarchical clustering on the data and the separation coincided precisely with disease at diagnosis, at relapse, and in CR, using median as the cutoff [12]. All but 2 studies identified high and low expression groups. Standardization of the cutoff determining method is highly recommended to facilitate the critical comparison of results across different studies. Using the ROC curve method is highly recommended, as it will yield more accurate results than simply using median or quartile values.
2. **Multivariate regression analysis:** The essence of multivariate regression is to adjust for potential confounders so as to ensure that the risk factor in question (miRNA expression) is an independent prognostic factor. When we omit well-known risk factors from the multivariate analysis, we just can't accurately assess risk factor independence. Moreover, several risk factors added to cox regression models were not "Day 0" risk factors, as for example, MRD level, steroid response. These factors vividly violate the proportional hazard assumption of the cox regression model and consequently threaten the validity of the cox regression results. Time-dependent cox regression is highly recommended to be used instead.
3. **Sample size:** Sufficient sample size is needed for miRNA studies to ensure accuracy of results. This is especially important in exploratory studies, (e.g., microRNA profiling) where reliable miRNAs are identified for the first time within the study. Using small sample size and upon application of subgroup analysis, the problem is aggravated, and survival estimates are less reliable. This can be evident by a wide confidence interval reported.
4. **Correction of multiple testing:** Several articles had multiple testing of different miRNAs. This increases the likelihood of false positive results. Correction of multiple testing is highly recommended and has been adopted in one study[13].

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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