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Research Paper

STUDY OF MicroRNA EXPRESSION AS A MOLECULAR BIOMARKER IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Medical Science

KEYWORDS : miRNAs; Paediatric acute lymphoblastic leukemia; miRNA-196b;miRNA-100.

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of leukemias that results from the proliferation of lymphoblasts that originates from the lymphocyte progenitor cell of the bone marrow or the thymus. It includes many subtypes that show different clinical behaviors and need different therapy schemes. Many studies in the last few years have shown that differential expression of candidate miRNAs in normal compared to transformed cell provides important insights into the pathogenesis of cancer including leukemia. We aimed to evaluate the expression profile of the microRNAs associated with childhood ALL: miR-100& miR-196b and their association with biological/prognostic features in 40 consecutive samples of newly diagnosed childhood ALL by quantitative real-time PCR. FISH was done for only selected ALL cases for t(12;21) and MLL rearrangement, when required.Both miRNAs analyzed (miRNAs 100 and 196b) were significantly underexpressed in ALL patients relative to control. There was an increased incidence of T-ALL in cases showing miR196b expression. Cases showing increased miR-196b expression were significantly overexpressed in CNS affected patients.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is defined as the proliferation of lymphoblasts that originates from the lymphocyte progenitor cell of the bone marrow or the thymus (Kinney*et al*, 1999). It is a hematological malignant disorder that originates in a single B- or T-lymphocyte progenitor. It is considered the most common type of childhood cancer. The majority of the cases are B-lineage ALL, which represents 85% of childhood ALL and 75% of adult ALL (Copelan*et al*, 1995 and Baak *etal*, 2008) . The current World Health Organization Classification of hematopoietic neoplasms designates these disorders as B- or T-lymphoblastic leukemia/ lymphoma (Ambros, 2004) .

Understanding the dynamics of gene expression is vital in elucidating cell biological and pathological processes. In cancer, alterations in gene expression pattern are more common than mutation (Sager, 1997). The measurements of certain RNA-based markers and/or their expression profiles have been very useful in providing better understanding of molecular mechanisms, better refinement of disease classifications and improvement in diagnosis and prognosis of some cancers.

MicroRNAs (miRNAs) has been discovered as an abundant class of small non-protein coding RNAs, which can have important roles in development, cell proliferation, differentiation, and apoptosis (Bartel, 2004 and He L, 2004).

Evidence is emerging that miRNAs can function as oncogenes (OGs) and tumor suppressors (TSGs) so having a role in the pathogenesis of the oncogenic process (He L 2005. Johnson 2005, Esquela-Kerscher 2006, Calin 2006 and Wu W 2007).

The differential expression of candidate miRNAs in normal versus transformed cell provides important insights into the pathogenesis of cancer. Various studies showed that miRNAs are consistently associated with different cancers, either acting as OGs or TSGs, and are now collectively called as oncomiRs (Schickel, 2008 and Gartel, 2008).

MiRNAs has proven to be associated with specific cytogenetic changes and different clinical outcomes of different subtypes of leukemia, demonstrating that miRNAs have the potential to be used for clinical diagnosis, prognosis and cancer therapy (Zhang H*et al* 2009, Labbaye 2012, Calin *etal* 2005,Garzon R *etal* 2008, Gimenes-Teixeira HL *etal* 2013, Jongen-Lavrencic M *etal* 2008 and Fernando TR *etal* 2012).Besides the described pattern of expression in normal progenitor cells, these miRNAs have also been found to be differentially expressed in different subtypes of ALL.(Schotte D *etal* ,2009).

Although microRNA-profiling appears to differentiate among distinct cytogenetic groups of the differrent subtypes of leukemia, the specific signatures differ among studies, which may be due to the lack of standardization of the different analytical methods (Guido Marcucci *etal* ,2011).

For MicroRNA profiling, three major approaches are currently well-established: quantitative reverse transcription PCR (qRT-PCR), hybridization based methods (for example, DNA microarrays) and high-throughput sequencing (that is, RNA-seq).

Quantitative reverse transcription PCR-based methods: An appealing aspect of this approach is that it is an established method, sensitive and specific. It can be also used for absolute quantification (Ach, R. A ,2008).Microarrays were among the first methods to be used for parallel analysis of large numbers of miRNAs, and several variations of the approach have been developed, including different approaches for fluorescent labelling of the miRNA in a biological sample for subsequent hybridization to DNA based probes on the array(Ach, R. A , 2008).

In the light of these facts known about miRNAs, the present study was conducted to review the role of miRNAs in leukemia, mainly focusing on studying the differential expression of both

miRNA-100 and miRNA- 196b as biomarkers for diagnosis and prognosis in ALL using quantitative reverse transcription PCR (qRT-PCR) .

In our study , we have tried to investigate the molecular link if any between these differentially expressed miRNAs (miRNA-100 & miRNA-196b) and the important genes involved in the molecular circuitry of ALL including mainly MLL rearrangement and TEL/AML translocation(using FISH analysis).

SUBJECTS AND METHODS

The present study was conducted in the Department of Clinical Pathology Faculty of Medicine, Alexandria University, Egypt. Bone marrow (BM) samples and Peripheral blood (PB) samples were obtained at time of diagnosis. Informed consent was obtained from patients and controls according to the Ethical Committee for Human Research in the Alexandria University Hospitals. Samples were collected from forty patients with newly diagnosed ALL from the Department of Pediatrics, Shatby Hospital, Faculty of Medicine, Alexandria University. They were collected between August 2013 And June 2014, their age ranged from 12 months to 11 years. The diagnosis was made by standard morphological analysis and flow cytometry in BM/PB samples at diagnosis, with all patients presenting more than 50% of blasts cells. Control PB samples were obtained from ten healthy subjects with matched age and sex and with no known malignancy. Patient group received only one cycle of induction chemotherapy in the form of intravenous vincristine 1.5mg/m2 on days 0, 7, 14&21, intramuscular L-Asparaginase 6000 IU/m2/thrice/week and daily steroids then assesssement of the response was performed by bone marrow aspirate on day 14 and day 28, counting from the start of induction therapy. Minimal residual disease (MRD) was detected by immunophenotyping on days 14 and 28.

Measurement of the ratio of miRNA-196b and miRNA-100 in PB/BM samples from the ALL patients comparing it with that from the control samples was carried out using quantitative RT-PCR (Livak, K.J. and Schmittgen, T.D, 2001)

Extraction of total RNA

Total RNA isolation and small RNA enrichement using miRNeasy Mini Kit (QIAGEN, Maryland, USA, Cat No.217004) was carried out according to the manufacturer's instructions. The concentration and purity of RNA were measured at 260 & 280 & 230 nm using Nanodrop 2000/2000c Spectrophotometer (Thermo Scientific, USA). Ratio of A_{260}/A_{280} = 1.8-2.1 and A_{260}/A_{230} =1.8-2.1 indicates highly pure RNA.

Quantitative Reverse Transcription PCR (QRT-PCR)

Extracted RNA was reverse transcribed into complementary DNA (cDNA) using microRNA-specific primers (miRNA 100 &miRNA 196b) using miScript II RT Kit (QIAGEN) according to the manufacturer's protocol. Reverse transcription was carried out in a 20µl reaction contained 10ng of RNA samples. The reactions were incubated in Biometra thermocycler for 60mins at 37°C then for 5mins at 95°C and then held at 4°C.

Real time PCR was performed using Rotor-Gene Q 3000 Real Time Cycler. Real time was performed in 25µl reaction mixture included 2µl RT product, 2.5µl Universal primer,12.5µl of Syber green Master Mix and 2.5µl of specific primer and 5.5µl nuclease free water . Reactions were incubated at 95°C for 5mins followed by 40 cycles of 94°C for 15s ,55°C for 30s and 70°C for 30s. SNORD 68 was used as an endogenous reference for normalizing the expression levels of mi-RNA 100 and mi-RNA 196b.

Data Analysis

The threshold cycle data (Ct) and baselines were determined using autosettings. The relative quantification of miRNA-100 and of miRNA-196b was calculated using the comparative $C_T$method ($2^{-\Delta\Delta Ct}$) where ΔΔCt is the difference of ΔCt value between the leukemia and the control (ΔΔCt=ΔCt leukemia miRNA - ΔCt control miRNA), and ΔCt is the difference of CT value between the target (miRNA-100 or miRNA-196b) and endogenous reference SNORD68. (ΔCt= CT miRNA-100or miRNA-196b- SNORD68).

FISH Analysis

The FISH Assay was performed according to the manufacturer's instructions. Slides for FISH analysis of MLL rearrangement and t(12;21) were prepared from air-dried unstained peripheral blood or bone marrow aspirate smears on superfrost charged slides. The slides were fixed in freshly prepared methanol: glacial acetic acid at a ratio of 3:1, for 20 minutes. The slides were left at room temperature for at least 1 hour (up to 24 hours). The slides were then stored at -20°C until used. Slides were pretreated before applying the probe by immersion in a coplin jar of 2xSSC, pH=7.0 at 37°C for 30 minutes. Then the slides were dehydrated in a series of ethanol solutions of increasing concentration (70%, 90%, and 100%) 5 minutes each then were left to air-dry for probe application. Ten µL of the ready to use probe were applied in the dark to the selected area of the slide. A glass coverslip (22mm x 22mm) was then applied and was sealed with fixogum. The slides were then placed in the VysisHYBrite™ (Abbott Molecular/Vysis, Des Plaines,IL).The slides were co-denatured (the samples and probe DNA) at a melting temperature of 75 °C for 5 minute and then hybridized at 37 °C overnight (16-18 hours).When the hybridization time is completed, the slides were removed from the HYBrite, the rubber cement was removed, and the coverslips were slid off the slides carefully. This was followed by wash in 1 x wash buffer I (Pre-warmed in a glass coplin jar to 72C in water bath) for 2 min at 72°C (±1°C) without agitation. Then the slides were washed in 1 x wash buffer II for 1 min at room temperature followed by dehydration in 70%, 90% and 100% ethanol for 1 min each and left to air-dry.15 µl DAPI counterstain were applied to the target area and the coverslip (24mm x 40mm) was applied, and the slides were left for 15 minutes before analysis. Finally, the slides were examined under the fluorescent microscope using BX-51/61 Olympus epifluorescent microscope equipped with appropriate filter sets for visualizing spectrum green or red and DAPI fluorescence signals. Image capture was done using a color digital JAI progressive scan CCD camera (Olympus, Japan), and the software CytoVision (Applied Imaging, UK). .

Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Kirkpatrick LA,2013) Qualitative data were described using number and percent. Quantitative data were described using mean and standard deviation, median, minimum and maximum. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparisons between two independent populations were done using independent t-test. For abnormally distributed data ,comparisons between different categories were done using Mann Whitney or Kruskal Wallis test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

The study was carried out on forty subjects with newly diag-

nosed ALL as the patient group and ten healthy subjects as a control group.

Haematological profile

Hemoglobin ranged from 4.0 to 12.5 g/dL in the ALL patients and from 10.0 to 14.7 g/dL in the controls with a mean of 7.5 ± 2.40 and 11.8 ± 1.3g/dL in ALL patients and controls respectively. Hemoglobin was significantly lower in the patients' group compared to the control group (p<0.001). White blood cell count (WBC) ranged from 1.8 $x10^9$/L to 134 $x10^9$/L in ALL patients and from 4.0$x10^9$/L to 17.3 $x10^9$/L in the control group with a median of 18$x10^9$/L and 11.5 $x10^9$/L in patients and controls respectively. WBC count was significantly higher in ALL patients compared to controls (p<0.001). Platelet count ranged from 4.0 $x10^9$/L to 547.0 $x10^9$/L in patient group and from 120.0$x10^9$/L to 66. **(Table 1)**

Table 1: Comparison between the two studied groups as regards CBC parameters.

Lab investigations Patients		Group		Z	P value
		Controls			
Hb	Minimum	4.0	10.0	4.1	0.000*
	Maximum	12.5	14.7		
	Mean	7.5	11.8		
	SD	2.4	1.3		
	Median	7.0	11.5		
WBCS	Minimum	1.8	4.0	2.5	0.014*
	Maximum	134.0	17.3		
	Mean	25.1	11.1		
	SD	28.4	4.1		
	Median	18.0	11.5		
Plt	Minimum	4.0	120.0	4.1	0.000*
	Maximum	547.0	667.0		
	Mean	79.4	320.1		
	SD	99.5	199.4		
	Median	33.0	235.0		

Z: Mann-Whitney test * P < 0.05 (significant)

Immunophenotyping

According to the Immunophenotyping done to ALL patients, 82.5% (33 cases)of the cases were B-ALL and 17.5% (7 cases) of the cases were T-ALL. According to the results of MRD done by immunophenotyping 85% of the ALL cases were negative for MRD on Day14 and 95 % of ALL cases were negative for minimal residual disease (MRD) on day 28. While only 15% of the cases were positive for MRD on day 14 and 5% of the cases were positive for MRD on day 28.**(Table 2)**

Table 2: Distribution of ALL patients according to MRD

MRD	Phase				P value
	Day 14		Day 28		
	No	%	No	%	
Negative	34	85.0	38	95.0	0.219
Positive	6	15.0	2	5.0	

MiRNAs expression and its correlation to studied parameters

Regarding the relative quantitation of the studied microRNAs,MiRNAs 100 and 196b deregulation were significantly underexpressed in ALL patients relative to control group. **(Figure 1&2)(Table 3).**

Table 3: Comparison between miRNA 100 & 196b expression between both patients and control

miRNA	Group		Z	P value
	Patients	Controls		
miR196			2.0	0.045*
Minimum	0.01	0.35		
Maximum	98.40	71.50		
Mean	8.38	7.71		
SD	20.08	22.41		
Median	0.21	0.59		
miR100			2.535*	0.011*
Minimum	0.02	0.24		
Maximum	27.30	1.89		
Mean	1.97	1.31		
SD	4.96	0.48		
Median	0.59	1.21		

Z: Mann-Whitney test * P < 0.05 (significant)

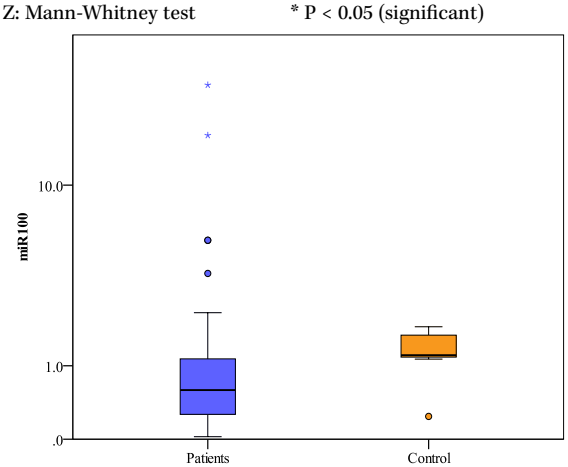

Figure 1.Comparison of the ratio of miRNA-100 signal intensity to SNORD68signal intensity by qRT-PCR among patients and controls.

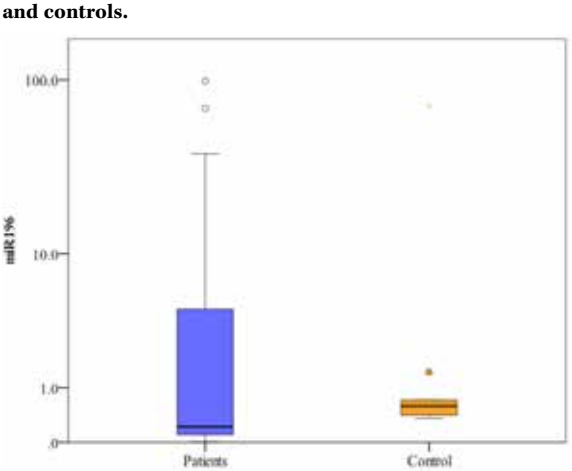

Figure 2.Comparison of the ratio of miRNA-196b signal in-

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tensity to SNORD68 signal intensity by qRT-PCR among patients and controls.

A higher miRNA 196b expression among cases showing splenomegaly. A higher MiR-196b expression was also found in T-ALL but with no statistical significance. Positive correlations were found between WBC count and miR-100 expressions levels in ALL patients. The comparison between ALL patients according to WBC count revealed that miR-100 were more overexpressed in cases with WBC count $<50x10^9$/L compared to cases with WBC count $>50x10^9$/L but not statistically significant while cases showing mi-R196b were more overexpressed in cases with WBC count > $50x10^9$/L compared to cases with $<50x10^9$/L.

The comparison between ALL patients according to CNS involvement revealed that miR-196b were significantly overexpressed in patients showing CNS involvement compared to patients showing no CNS involvement. Cases with miRNA196b expression showed significant increased incidence of MRD in day 28 compared to cases that showed miRNA100 expression. **(Table 4)**

Table 4: Relation between miRNA expression and prognostic factors

prognostic markers	miR196b			miR100		
	Minimum	Maximum	Median	Minimum	Maximum	Median
CNS						
No	0.006	98.4	0.203	0.02	27.30	0.59
Yes	11.6	38	16.3	0.18	5.54	0.72
Z (P)	2.3 (0.022)*			0.488 (0.626)		
WBCs						
<50,000	0.006	98.4	0.355	0.02	27.30	0.59
>50,000	0.036	30	14.545	0.12	0.67	0.34
Z (P)	0.05 (0.964)			1.027 (0.304)		
MRD14						
Negative	0.006	69.5	0.21	0.02	27.30	0.59
Positive	0.203	98.4	49.3015	0.23	3.78	2.01
Z (P)	1.1 (0.264)			0.310 (0.756)		
MRD28						
Negative	0.006	69.5	0.1865	0.02	27.30	0.59
Positive	0.07	98.4	13.95	0.18	3.78	0.55
Z (P)	2.1 (0.037)*			0.0 (1.000)		

Z: Mann-Whitney test * P < 0.05(significant)

Interphase FISH analysis
In total, 17 cases out of 40 ALL cases were selected for Interphase-FISH analysis of t(12;21)(p13;q22) and MLL rearrangement done on peripheral blood or bone marrow aspirate samples.

I-FISH analysis of t(12;21)(p13;q22):
I-FISH analysis of t (12; 21)(p13;q22)using Poseidon™ TEL/AML t(12;21) Dual Color probe only was done for 8 ALL cases. Cases showing higher miRNA 100 expressions were subjected to FISH analysis.

Only two cases out of the 8 selected cases showed amplification of the green signal (AML 21q22).One of the cases showed 60% of the cells with amplification of (AML 21q22).The other case showed 30% of the cells with amplification of (AML 21q22). None of the selected cells showed fusion of the red and green signals.

I-FISH analysis for MLL rearrangement:

FISH analysis for MLL rearrangement using Kreatech MLL/AFF1 t(4;11) Fusion Dual color Breakapart probe(Cat# KBI-10404)was only done for 9 ALL cases according to miRNA 196b expression.

Out of the 9 cases that were selected according the miRNA196b expression, 2 cases show positivity for MLL rearrangement, while the remaining 7 cases were negative.

DISCUSSION
MicroRNAs have a role in regulation of various biologic functions and pathways such as cell proliferation, differentiation, apoptosis and metabolism. Previous studies suggested a direct link between microRNAs and certain diseases including cancer (Yu DC LQ ,2011). MicroRNAs can act as OGs or TSGs, depending on the target genes that they regulate and control (Iorio MV FM *etal* 2005,Wu WK LC *etal* 2010, Garzon R HC *etal* 2009, Iorio MV CP *etal* 2009, Visone R PF 2008, Wu W SM *etal* 2007 and Zhang HH WX *etal* 2007).Existing data from previous studies show the potential clinical utility of miRNAs as diagnostic, prognostic and predictive markers for aggressive and metastatic cancers (White NM FE *etal* 2011). However, few studies have been conducted until now concerning the role of miRNA in childhood ALL (Schotte D 2009, Tassano E *etal* 2010, Gefen N *etal* 2010 and Kaddar T *etal* 2009). These studies have shown that differential miRNA expression analysis may help to understand the development of different phenotypes and the biological functions of these miRNAs in childhood ALL.

The primary aim of the present study was to assess the expression pattern of miR-100 and miR196b for childhood ALL. In addition, we tried to evaluate the potential role of miRNAs in ALL pathogenesis, identify the relations between miRNAs and other clinicopathological features of ALL patients and determine miRNAs relation with other prognostic markers as CNS involvement, WBC count, MRD and certain chromosomal translocations to explore the usefulness of miRNAs expression in predicting prognosis in ALL patients.

Peripheral blood samples/bone marrow aspirate samples were obtained from newly diagnosed cases of childhood ALL. Expression of the selected miRNAs panel was analyzed by RQ-PCR using the comparative C_t method after normalization for the expression of SNORD68 as endogenous controls. Flow cytometry and morphological studies was used for diagnosis of ALL cases. Patients were followed up for 6 months from the time of diagnosis. Expression levels of miRNAs were then compared with different clinicopathological and laboratory parameters.

In the present study, ALL cases showed significantly lower miRNA 100 & 196b expression levels as compared to control cases. Despite the differences in the study design, de Oliveira et al,(de Oliveira J.C CSSb ,2012) also found that miRNA100 &miRNA 196b expression was significantly lower in ALL cases compared to controls using qRT-PCR (de Oliveira J.C CSSb, 2012).

A meta-analysis was done to provide a comprehensive evaluation of the role of miRNA-100 expression on the overall survival rate among different types of carcinomas. The results indicated that lower expression of miR-100 in cancerous tissue could significantly predict poorer survival in various carcinomas(Chen J1 ZB etal, 2014).In conclusion, the findings from this study suggest that miR-100 expression is related with overall survival in cancer patients and could be a useful clinical prognostic factor for those patients. (Chen J1 ZB *etal* , 2014).

MiR-100 expression was elevated in bone marrow from pediatric patients with newly diagnosed AML, compared with normal controls; increased expression of miR-100 was significantly associated with advanced clinical features of pediatric AML patients (Jin Bai1 AG *etal* 2012). Our results are also supported by Li XJ,

et al (Li XJ LX *etal*, 2013) who showed that miR-100 and miR-99a were down-regulated in 111 ALL patients, especially in high-risk groups; their expression levels were correlated with the patient's 5-year survival. However, in our study we only followed up the patients for 6 months.

Recently, miRNA profiling studies have indicated that miRNA-196 (miR-196) is overexpressed in different types of cancer. In addition, increasing numbers of studies showed that miR-196b plays important roles in development and immunity through targeting of specific genes. Further investigations and researches of these newly discovered functional roles of miR-196b may lead to potential clinical applications of miR-196b in the management of several human diseases (Changyi Chen YZ *etal*, 2011).

In this study, microRNA 196b expression was shown to be significantly lower among ALL cases compared to the control group however there is an increased incidence of microRNA196b among T-ALL cases compared to B-ALL cases but not statistically significant. Bhatia et al.,supported our study that indeed B-cell ALL patients had conspicuously significant lower levels of miR-196b expression as compared to that found in control subjects (Bhatia S *etal*, 2010).

In agreement to our results, Bhatia et al proved a significant down-regulation in the expression of miR-196b in EB-3 cell line and B-cell leukemia patients as compared to the normal B-cell counterparts (Bhatia S *etal*, 2010).On the contrary, a recent study showed that miR-196b expression is significantly more up-regulated in AML than ALL and that, again, miR-196b up-regulation is negatively associated with overall survival of AML patients. (Wang Y etal, 2010).

The present study next focused on the potential relation between miRNA100 & 196b expression levels and various clinicopathological charactaristics to evaluate the prognostic potential of miRNA 100 & 196b. There is a significantly higher miRNA 196b expression levels in cases showing splenomegaly than those without splenomegaly. Presence of organomegaly indicates bad prognosis which might indicate that miRNA 100 & 196b expression could be used as a prognostic marker.

We demonstrated the comparison between ALL patients according to CNS involvement revealed that miR-196b were significantly overexpressed in cases showing CNS involvement compared to ALL patients with no CNS involvement.

J.Bai et al performed large retrospective studies in order to show the association of miR-100 expression with clinicopathologic features and clinical outcome of pediatric AML patients. Overexpression of miR-100 was significantly associated with the presence of extramedullary disease, FAB classification subtype M7, and unfavorable day 7 response to induction chemotherapy(. Chen J1 ZB etal ,2014).

Micro RNA 196b was proved to be a potential poor prognostic factor in other types of cancer. C.Zhang et al., found that *miR-196a* and *miR-196b* expression were both upregulated in osteosarcoma tissues compared to the corresponding noncancerous bone tissues on contrary to our results in acute lymphoblastic leukemia (Chun Zhang CY etal ,2014)

In our study, FISH analysis for of t(12;21)(p13;q22) was done for 8 out of 40 ALL cases.. Cases showing high miRNA 100 expressions were subjected to FISH analysis. Only two cases out of the 8 selected cases showed amplification of the green signal (AML 21q22). One of the cases showed 60% of the cells with amplification of (AML 21q22).The other case showed 30% of the cells with amplification of (AML 21q22) .None of the selected cells showed fusion of the red and green signals.

FISH analysis for MLL rearrangement was also done for 9 out of 40 ALL cases based on the miRNA-196b expression. Out of the 9 cases, 2 cases show positivity for t(4;11)(q21;q23**)**, while the remaining 7 cases were negative.

Popovic*et al.* demonstrated overexpression of miR-196b was found specifically in patients with MLL-associated leukaemia based on analysis of 55 primary leukaemia samples; these demonstrated increased proliferative capacity and survival, as well as a partial block in differentiation in bone marrow progenitor cells (Popovic R *etal* ,2009).

D Schotte et al showed that TEL-AML1–positive ALL patients were distinguished from those with other (non-*TEL-AML1*) genetic subtypes by approximately 5- to 1700- fold up-regulation of various miRNA including miR-99a, miR-100, miR-125b, miR-383 and let-7c (Schotte D DMR etal,2011).

In addition, D Schotte et al ,demonstrated that different genetic subtypes of ALL have unique miRNAs expression profiles which point to several miRNAs with potential oncogenic and tumor suppressive activity in ALL (Schotte D DMR etal,2011).

Due to the small number of cases carrying MLL rearrangements and TEL/AML translocation, additional studies on a higher number of cases are necessary to corroborate these data.

To conclude, our study showed that miR-196b expression was found to be higher in T-ALL and miR-100 expression was higher in patients with a WBCcount $<50x10^9/L$ at diagnosis. Also miRNA-100 expression showed significant higher expression among cases showing hepatomegaly while cases showing miRNA-196b showed higher expression among cases of splenomagaly.MiRNA-196b expression showed significant overexpression among cases showing CNS involvement and cases showing positive MRD on day 28.These findings suggest a potential role of these microRNAs in specific subtypes of pediatric ALL and in the development of different phenotypes. Further studies are needed to corroborate and extend our results.

In general, the study provides information on miRNAs emphasis on the importance of considering more than one miRNA to assess the tumor burden, predict the clinical outcome & prognosis in ALL patients. Therefore, it could help in better tumor staging, risk stratification and tailored clinical management enhancing therapeutic successes, and increasing the life expectancy of ALL patients.

CONFLICT OF INTERESTS

The authors hereby declare that they have no conflicts of interest. This research was completely funded by the authors. All the authors of this paper have no financial relationship with the organization where the research was carried out.

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