



## MicroRNA microarrays on archive bone marrow core biopsies of leukemias—Method validation

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### ARTICLE INFO

#### Article history:

Received 15 April 2010

Received in revised form 12 August 2010

Accepted 12 August 2010

Available online 9 September 2010

#### Keywords:

Bone marrow core biopsy

Bone marrow aspirate

miRNA profiling

ALL

CML

### ABSTRACT

Due to availability of bone marrow core biopsies (CB) in many pathology laboratories, we evaluated the quality and the biological information of the miRNA profiling using 9 acute lymphoblastic leukemia (ALL) and 9 chronic myeloid leukemia (CML) matched CB and bone marrow aspirates (BA). Technical replicates showed reproducible results across platforms and clustered together in hierarchical clustering analysis; and matched samples showed similar biological content having common differentially expressed miRNAs against the same control samples. We showed, that CBs, which have undergone decalcification in addition to formalin-fixation, are suitable for miRNA profiling.

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## 1. Introduction

MicroRNAs (miRNAs), small molecules around 22 nucleotides in size, are important in posttranscriptional gene regulation [1]. They are involved in crucial biological processes both in normal tissue development and growth and in diseases, such as malignancies, including leukemias [2,3]. One miRNA may regulate numerous target genes and one target gene may, respectively, be regulated by several miRNAs [4]. They have functions resembling oncogene and/or tumor suppressors depending on their target genes [5–7] and about one half of them are located in oncogenomic chromosomal areas [4,8,9]. Importantly, miRNAs may open a new avenue for targeted treatments of malignancies [4,10]. Conventionally, miRNAs are studied by polymerase chain

reaction methods, but the array technology have made it possible to conduct global analyses of all miRNA simultaneously and to profile and to cluster them according to various parameters [11].

As the miRNAs are small, they have been reported to be intact in formalin-fixed paraffin-embedded (FFPE) tumor tissues [12,13]. These have been shown to be reliable source of miRNAs [14–18] and have enabled utilization of vast archival FFPE tissue material. Similarly, in most hematological units bone marrow core biopsies are taken from posterior iliac crest and embedded in paraffin, and stored in normal archives. Core biopsies (CB) and marrow aspirates (BA) are obtained in the same procedure through one skin incision, but slightly altering the angle of the needle in between. As compared to marrow aspirate, the core biopsy contains trabecular bone and a higher ratio of stromal elements, mainly adipose and connective tissue, but less contaminating peripheral blood cells.

The aim of our study was to determine whether the formalin-fixed and decalcified material is feasible for miRNA array analyses. Therefore, we selected leukemia patients from whom fresh-frozen BAs and CBs were available. Besides the description of

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the methodology to be used for miRNA profiling, we present miRNA profiling from Philadelphia-positive (as a sole aberration) chronic myeloid leukemia (CML) and acute lymphoblastic leukemias (ALL).

## 2. Patients and methods

### 2.1. Samples

A total of 18 matched archival CBs and BAs samples from which 9 CML (median 54 years) and 9 ALL (median 6 years) at diagnosis were used for the current study. As controls we used 8 bone marrow samples from healthy donors, obtained from the archived May–Grünwald–Giemsa-stained bone marrow smear. The patients were treated at the Department of Hematology, Helsinki University Central Hospital, and at the Hospital for Children and Adolescents, University of Helsinki according to the contemporary protocols. Karyotype analyses were conducted for all patients, while array comparative genomic hybridization (aCGH) by Agilent 44K or 244K platform system (Agilent Technologies, CA, USA), as described elsewhere [19], was performed only for ALL cases (Supplementary Table).

### 2.2. Core biopsy preparations

Bone marrow core biopsies were fixed in 10% neutral phosphate buffered formalin (Oy FF-Chemicals Ab, Finland) for at least 24 h prior to decalcification. The decalcification was performed using neutral decalcification solution (0.27 M EDTA, 0.3 M sodium hydroxide, 10% formalin purchased from HUSLAB Media Production Unit, Finland) using 3 h or 17 h overnight microwave treatment. The treatment (either 3 h or 17 h) was repeated until tissue was softened properly, however at most 5 days. The biopsies were processed overnight and the embedding into paraffin was performed using the standard laboratory protocols. As an exception, rapidly processed samples were fixed with microwave for 20 min using the Milestone's Rapid Histoprocessing-1 (RHS-1) equipment (Milestone, Italy) (samples 2,4,9,10,12 and 14).

### 2.3. Sections for miRNA extraction

CB samples were cut using Leica RM2155 microtome (Leica Microsystems GmbH, Germany). For each block a new sterile blade was used to avoid contamination and to cut five sections of 20- $\mu$ m thick.

### 2.4. RNA purification

From the CBs total RNA was extracted using the miRNeasy FFPE Kit (Qiagen, CA, USA), according to the manufacturer's protocol. Briefly, five 20- $\mu$ m sections per sample were deparaffined with xylenes (Merck, Germany), followed by lyses/digestion of the sample with proteinase K and heat-treatment. After removal of genomic DNA the sample was transferred into the RNase MiniElute column keeping the small RNA fractions and washed with absolute ethanol. The total RNA was eluted in 14  $\mu$ l RNase-free water.

As for the BAs, after the frozen whole bone marrows had melted at room temperature, the miRNeasy Mini Kit (Qiagen) was used, according to the manufacturer's protocol, to extract total RNA. Briefly, QIAzol Lysis reagent was added into 2 ml melted sample in order to lyse and homogenize the cells. After the dissociation of nucleoprotein complexes, chloroform (99.0–99.8% from Merck) was added and mixed well (important step for phase separation). The upper, aqueous phase was mixed with absolute ethanol. After washing, the RNA was eluted into 30  $\mu$ l RNase-free water.

To detach the cover slides bone marrow smear slides were kept in xylene for a few days. After washing the slides with decreasing alcohol series and buffered saline the cells were scratched with a scalpel from the slides, and transferred immediately into 1.5 microcentrifuge tube. The total RNA was extracted using the same protocol as for BAs (miRNeasy Mini Kit, Qiagen).

All total RNA samples were kept at -70 °C from 2 days to 2 months until further processing. The RNAs were quantified with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE, USA). The miRNA integrity and quantity was assessed with the Agilent 2100 Bioanalyzer by performing RNA Nano 6000 and the Small RNA Chips for all the samples, according to the manufacturer's protocol (Agilent Technologies).

### 2.5. miRNA microarray

The samples were processed using Agilent miRNA Complete Labeling and Hybridization Kit (Protocol Version 2.1, Agilent Technologies). Briefly, 100 ng of starting material was dephosphorylated and then labeled with Cyanine 3-pCp. Complete drying of the sample with a vacuum concentrator at the medium-high heat setting followed this. Next, the samples were resuspended in nuclease-free water and hybridized on the Agilent Human miRNA Microarray V2 (contains probes for 723 human and 76 human viral miRNAs from the Sanger database v.10.1). Duplicates were performed for 3 BAs and one CB sample. The slides

were scanned with the high-resolution Microarray Scanner (G2539A), and images were processed with the Feature Extraction Software v.9.5 (Agilent Technologies). Further, data was analyzed using the Chipster Software Version 1.4.2 (<http://chipster.csc.fi/>). The data was normalized according to the default settings in Chipster for Agilent miRNA platform. In the preprocessing of the data the coefficient of variation was used to measure miRNA expression level variation across the samples. To focus on the miRNAs that showed remarkable variation among the samples and to reduce multiple testing, we selected the miRNAs with 10% largest variation for a closer analysis. Two-group tests were then performed on the preprocessed data to obtain most differentially expressed miRNAs with  $p < 0.05$  (corrected for multiple testing using default Chipster settings, Benjamini–Hochberg test).

### 2.6. Statistical analysis

Statistical analysis was performed with the Chipster (<http://chipster.csc.fi/>) and SPSS (v.16.0 for Windows, SPSS Inc.) software packages. To assess variations due to differences between sample types, bivariate correlations, Pearson's coefficient, were calculated between technical replicates (mean here a duplicate of the same patient sample). The calculations were performed for both sample types (BAs and CBs) to determine and to compare technical reproducibility of the method. Normalized (using Chipster's inbuilt parameters for Agilent's miRNA microarrays) miRNA expression values of matched BA and CB samples were used for visual comparison in scatter plots.

## 3. Results

### 3.1. Quality and quantity of the total RNA

The RNA yield (CBs 50–600 ng/ $\mu$ l, BAs 100–1000 ng/ $\mu$ l) for all samples was enough to proceed with labeling and hybridizations. The RNA purity ratio (mean optical density) 260/280 was 2.0 for BAs and 1.8 for CBs, these ratios may be explained by use of the xylene in the RNA extraction, as previously reported [16–18,20–21]. Sample analysis with the 2100 Bioanalyzer using Small RNA Chips (Agilent Technologies) showed good recovery of the miRNA fraction (Fig. 1).

### 3.2. Technical replicates

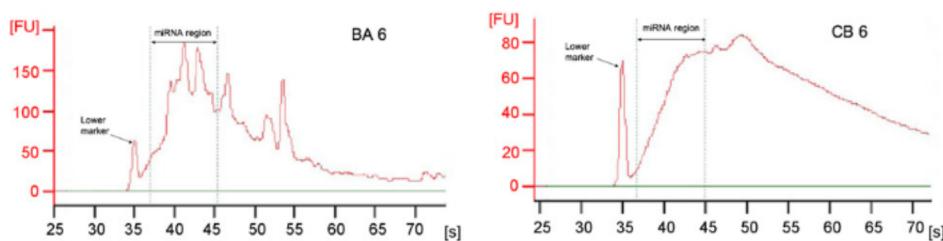
For technical reproducibility, miRNA expression profiles were performed in duplicate for 3 matched samples (CB/BA 2, CB/BA 4 and CB/BA 10). The source material was all from the same patients, 2 ALL (2, 4) and 1 CML (10). As we expected the miRNA quality predicted the Pearson's correlation values.

Scatter plot analysis was used to determine the distributional similarity of the technical replicates (Fig. 2). The miRNA expression profiles between technical replicates correlated highly, with Pearson's coefficient above 0.94 in all cases. The correlation coefficient of unrelated samples was dependent on the sample type (CB or BA) and the leukemia subtype (ALL or CML). The samples derived from the same source material (CB or BA) and representing the same leukemia subtype generally had higher correlation (on average coefficient of 0.97) compared with the samples representing the same leukemia subtype but originating from different source materials (with average coefficient of 0.75). Unsupervised hierarchical clustering was used to visualize the similarity of the technical replicates that, generally, clustered together (Fig. 3).

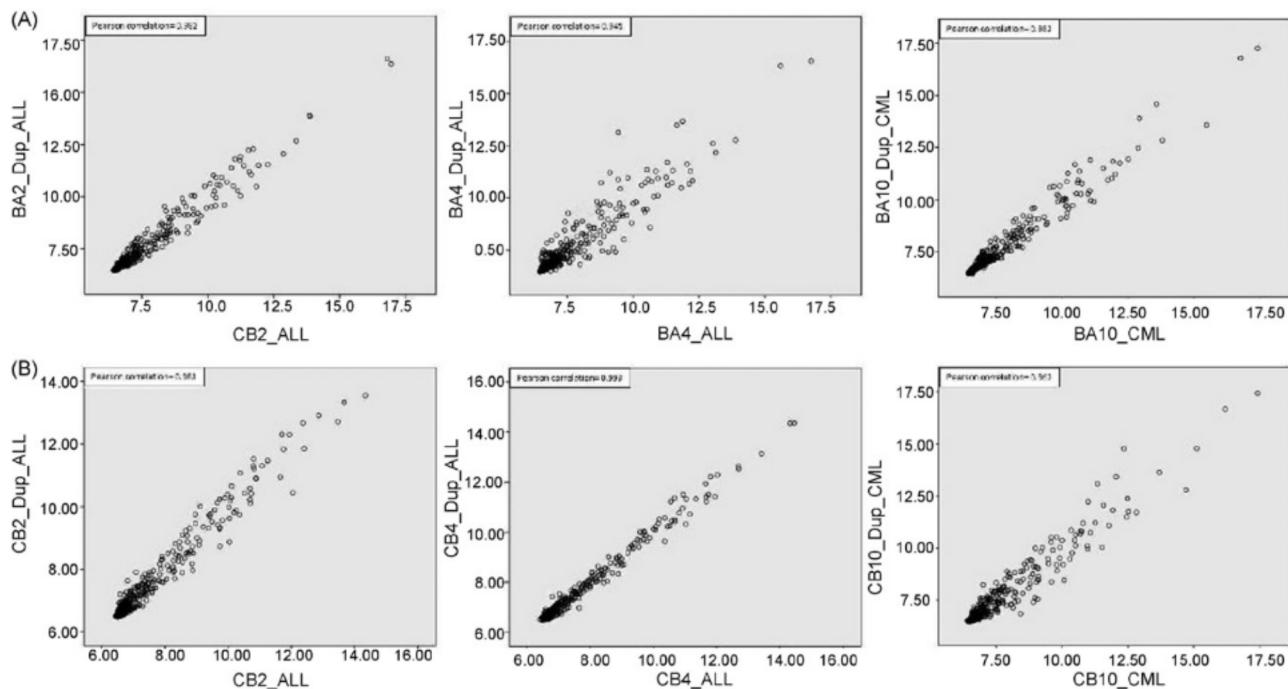
The technical reproducibility of the data was also evaluated in terms of the expressed miRNAs. The number of miRNAs expressed (marked as present by feature extraction algorithms) was determined for all samples. Generally, more miRNAs were expressed in CB samples. The average difference in the number of miRNAs, expressed by the technical replicates, ranged from 49 (CB samples) to 62 (BA samples).

### 3.3. miRNA profiles

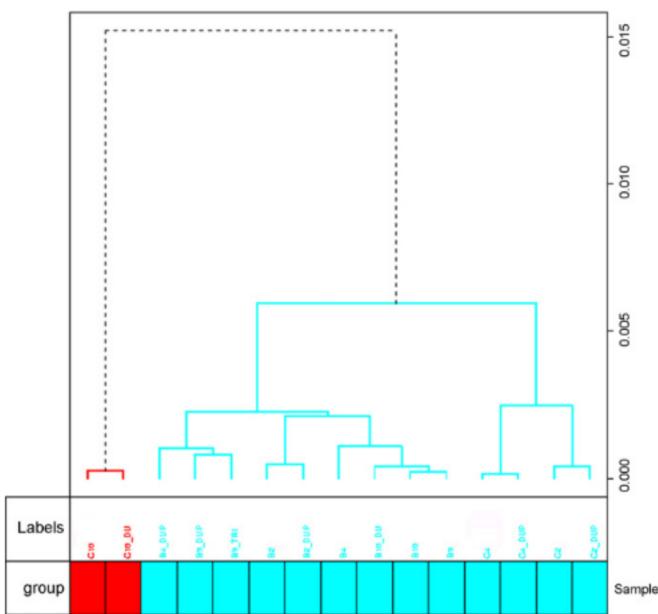
Between matched CBs and BAs correlations were found with mean Pearson's coefficient of 0.84 for ALL samples and 0.77 for



**Fig. 1.** The electropherogram images of the BA 6 and in the matched CB 6 sample performed with the Small RNA assay on the Agilent 2100 Bioanalyzer, which illustrate fluorescence (X-axis) over time (Y-axis). The first peak is the lower marker used as an internal reference. The vertical lines delimit the miRNA detection region, and the Agilent 2100 expert software draws them automatically based on its default settings.



**Fig. 2.** Scatter plots of technical replicates. X- and Y-axes are signal intensities. (A) Comparison of BA replicates with respective Pearson's coefficient. (B) Comparison of CB sample replicates with respective Pearson's coefficient.

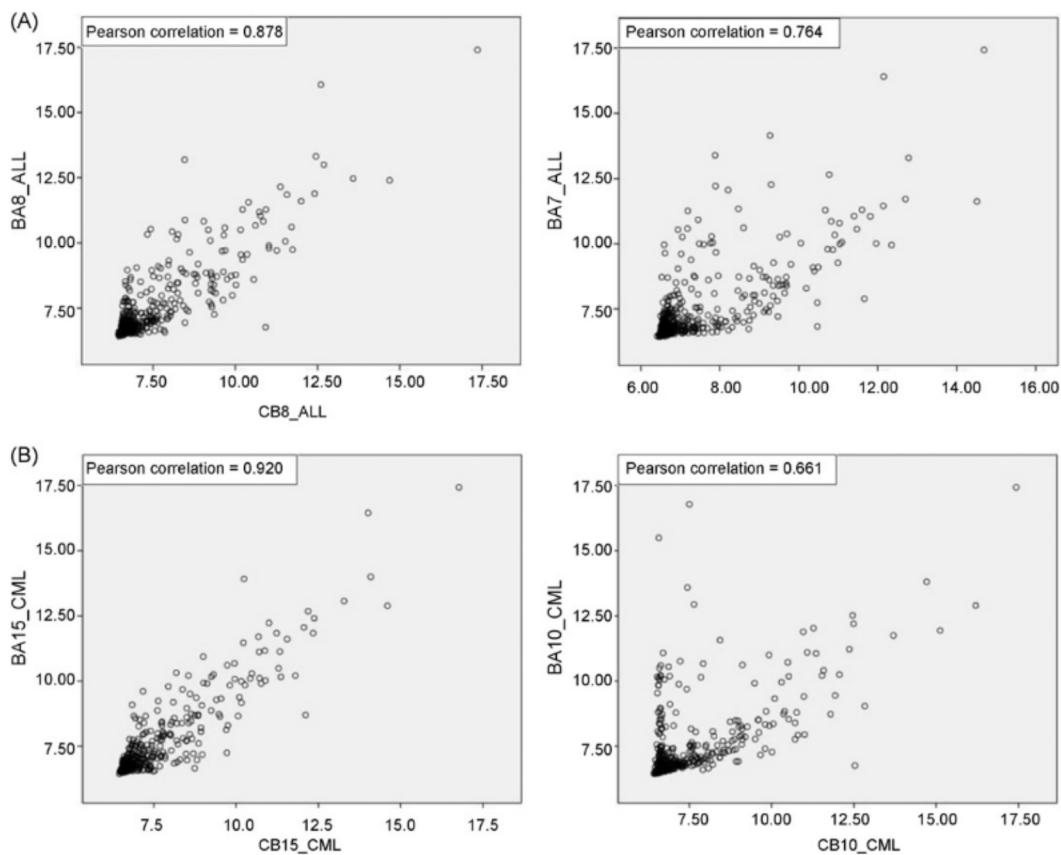


**Fig. 3.** Hierarchical clustering indicating that the technical replicates generally clustered together.

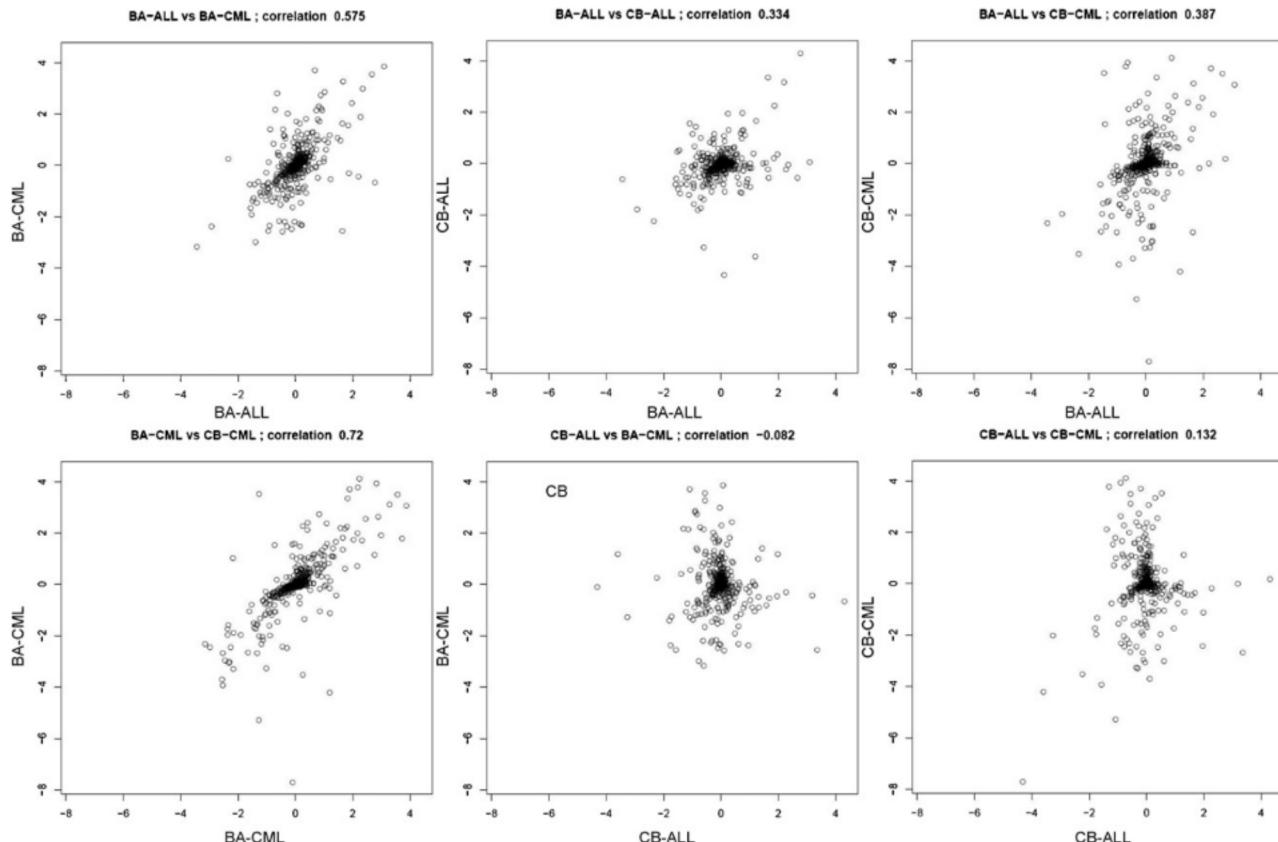
CML samples, respectively. Scatter plots for matched sample pairs with the best correlations from ALL and CML samples are shown in Fig. 4, while Table 1 summarizes the Pearson's correlation values for each matched sample. Correlation is used to measure global similarity between the absolute expression profiles over all miRNAs, including those that did not show statistically significant differential expression between the two conditions. Note that individual miRNAs can have systematic differences in their average expression levels. Therefore, comparison of the absolute expression values is expected to yield positive correlations even between unrelated conditions. Importantly, the correlations between repli-

**Table 1**  
Pearson's coefficient values of matched BA and CB samples.

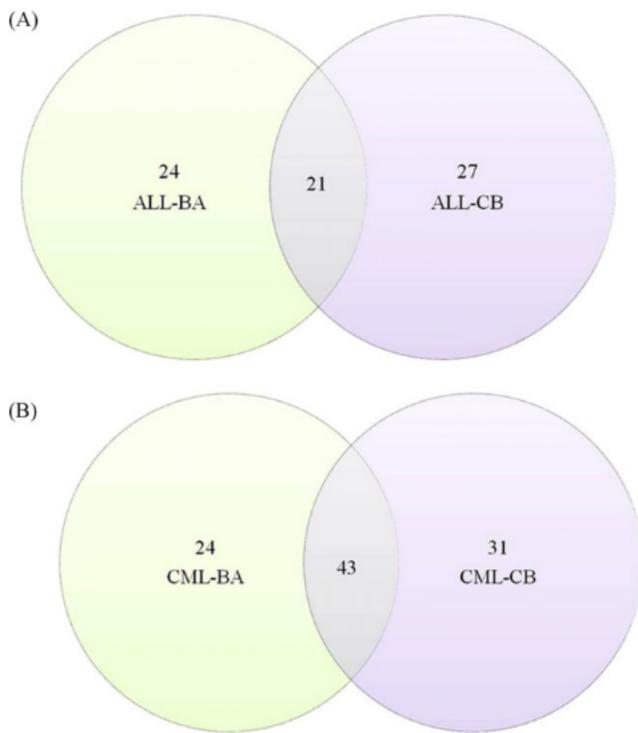
ALL samples	Pearson's coefficient	CML samples	Pearson's coefficient
CB1 vs. BA1	0.89	CB10 vs. BA10	0.66
CB2 vs. BA2	0.81	CB11 vs. BA11	0.80
CB3 vs. BA3	0.83	CB12 vs. BA12	0.74
CB4 vs. BA4	0.82	CB13 vs. BA13	0.72
CB5 vs. BA5	0.86	CB14 vs. BA14	0.74
CB6 vs. BA6	0.84	CB15 vs. BA15	0.92
CB7 vs. BA7	0.76	CB16 vs. BA16	0.80
CB8 vs. BA8	0.89	CB17 vs. BA17	0.79
CB9 vs. BA9	0.84	CB18 vs. BA18	0.77



**Fig. 4.** Scatter plots of matched BA and CB samples. X-axes represent the BA sample signal intensity and Y-axes the CB sample signal intensity. (A) Scatter plots of the best and worst ALL correlation sample pairs. (B) Scatter plots of the best and worst CML correlation sample pairs.



**Fig. 5.** Correlations between log-transformed fold-change values from BA and CB of both leukemia subtypes.



**Fig. 6.** (A) Venn diagram showing the miRNAs detected in ALL samples: 24 were detected only in BA ALL sample, 27 of them were also detected only in CB ALL samples; and 21 miRNAs were common to both ALL sample types. (B) Venn diagram showing the miRNAs detected in CML samples: 24 were detected only in BA CML sample, 31 of them were exclusively detected only in CB CML samples; and 43 miRNAs were common to both CML sample types.

cate measurements are higher than between unrelated conditions. This confirms that the two sample types yield reproducible miRNA expression measurements. In addition, the overlap between the most significant findings (miRNAs with differential expression between case and control samples) confirms that the findings are largely reproducible between BA and CB. The differential gene expression changes correlated positively between the two groups, although some miRNAs have statistically significant differential expression against the control samples in only one of the groups. Fig. 5 shows the correlations between log-transformed fold-change values from BA and CB.

#### 3.4. miRNA expression profiles in CML and ALL

We used standard statistical methodology (*t*-test) to determine differentially expressed miRNAs in patient samples against control samples. The tests were conducted separately for BA and CB samples. The differentially expressed miRNAs were then compared to find similarly expressed miRNAs in the two types of samples.

Compared to control samples, CBs from ALL patients showed 48 differentially expressed miRNAs, of which 27 were differentially expressed only in these samples, while in BAs altogether 45 differentially expressed miRNAs were observed of which 24 only in these samples. As for the miRNAs common in both types of samples (Fig. 6A), 10 showed upregulated and 11 were downregulated (Table 2).

CB samples of CML class showed 74 differentially expressed miRNAs, whereas in BA samples 67 differentially expressed miRNAs were found compared to control samples. Similarly, in CML samples, we found 43 common miRNAs (Fig. 6B), with 16 upregulated ones and 27 downregulated ones (Table 3), while 24 miRNAs were seen only in BA samples and 31 miRNAs in CBs.

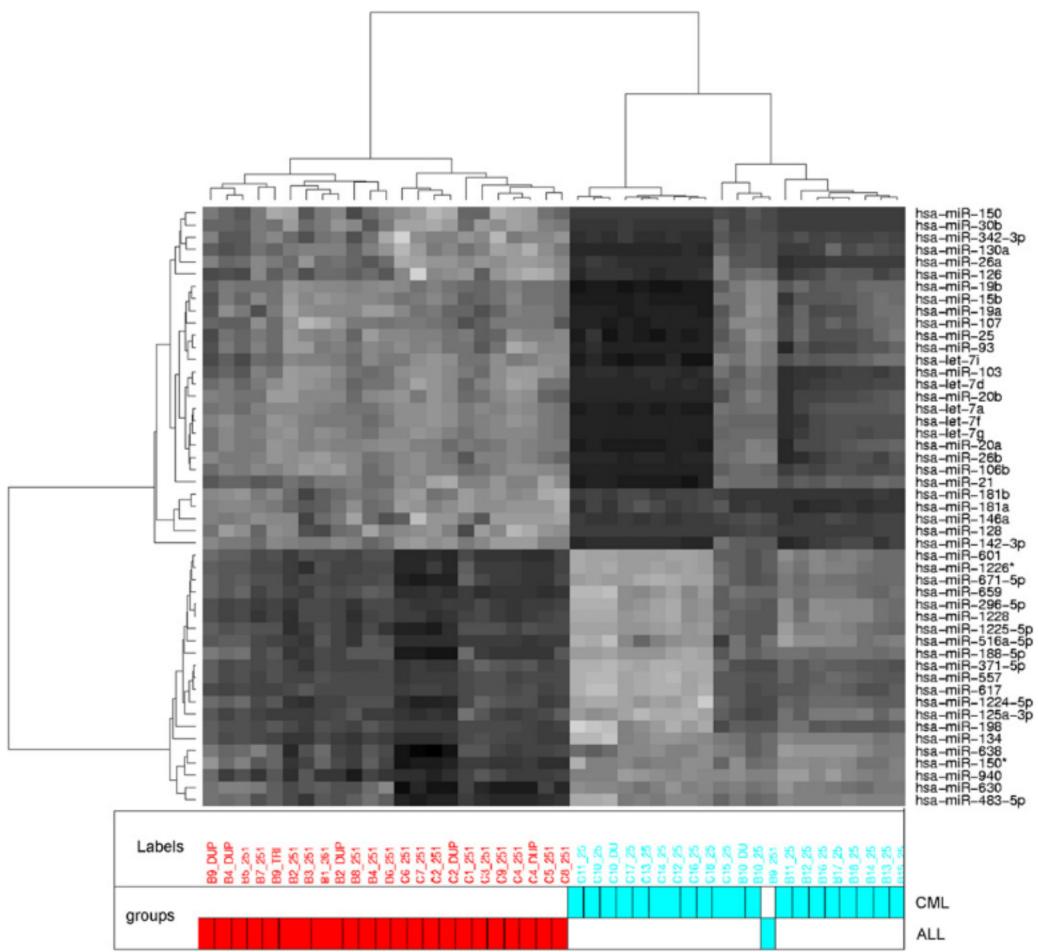
**Table 2**  
Shared differentially expressed miRNAs in both the CB and the BA ALL sample types.

Upregulated miRNAs	p-Value	Downregulated miRNAs	p-Value
hsa-miR-142-3p	0.001	hsa-miR-768-5p.v11.0	<0.001
hsa-miR-146a	0.004	hsa-miR-125b	0.006
hsa-miR-222	0.012	hsa-miR-223	0.007
hsa-miR-142-5p	0.022	hsa-miR-22	0.012
hsa-miR-150*	0.032	hsa-miR-27a	0.027
hsa-miR-144	0.041	hsa-miR-15b	0.032
hsa-miR-155	0.043	hsa-miR-574-5p	2.800
hsa-miR-181b	1.480	hsa-miR-595	3.110
hsa-miR-181a	3.800	hsa-miR-371-5p	3.800
hsa-miR-128	4.890	hsa-miR-32*	7.240
		hsa-miR-148a	7.400

The miRNA expression difference between ALL and CML was determined, similarly, using a two-group test. Firstly, CB and BA samples were separately analyzed. CBs of ALL and CML showed 60 differentially expressed miRNAs ( $p < 0.05$ ) while 33 miRNAs were differentially expressed ( $p < 0.05$ ) in BA samples. The miRNA expression profiles of the two sample sets shared 23 miRNAs that classified the samples into two groups according to leukemia subtypes. Of 23 miRNAs, 16 were upregulated in ALL and downregulated in CML while the other 7 showed reverse expression. Secondly, the CB and BA samples were combined for each leukemia subtype and the differentially expressed miRNAs were determined resulting in 65 miRNAs ( $p < 0.05$ ), of which 49 miRNAs clearly classified the samples into the two leukemia subtypes (Fig. 7). Interestingly, 2 miRNAs (miR-142-3p and miR-15b) were present also in the differentially expressed miRNAs shared by both sample types in both leukemia subtypes. Both of these miRNAs were downregulated in CML samples compared to ALL and control ones. Similarly, the miR-142-3p was found to be upregulated in ALL samples compared to CML and control samples, while the miR-15b was upregulated against CML but it showed slight under-expression compared with control samples.

**Table 3**  
Shared differentially expressed miRNAs in both the CB and the BA CML sample types.

Upregulated miRNAs	p-Value	Downregulated miRNAs	p-Value
hsa-miR-188-5p	<0.001	hsa-miR-103	<0.001
hsa-miR-296-5p	<0.001	hsa-miR-144	0.006
hsa-miR-575	<0.001	hsa-miR-126	<0.001
hsa-miR-483-5p	<0.001	hsa-miR-768-5p.v11.0	<0.001
hsa-miR-601	<0.001	hsa-miR-185	3.530
hsa-miR-370	<0.001	hsa-miR-20a	7.005
hsa-miR-1225-5p	3.890	hsa-miR-29a	1.006
hsa-miR-150*	<0.001	hsa-miR-107	7.870
hsa-miR-135a*	<0.001	hsa-miR-20b	4.080
hsa-miR-125a-3p	1.700	hsa-miR-22	2.910
hsa-miR-1226*	4.200	hsa-miR-363	0.024
hsa-miR-134	<0.001	hsa-miR-19a	0.004
hsa-miR-1228	1.006	hsa-miR-15b	1.100
hsa-miR-630	<0.001	hsa-miR-150	<0.001
hsa-miR-659	<0.001	hsa-miR-26a	3.005
hsa-miR-516a-5p	1.0E-6	hsa-miR-144*	6.600
		hsa-miR-140-3p	6.200
		hsa-miR-106b	<0.001
		hsa-miR-93	1.510
		hsa-miR-768-3p.v11.0	<0.001
		hsa-miR-17	<0.001
		hsa-let-7d	<0.001
		hsa-let-7a	1.006
		hsa-miR-26b	<0.001
		hsa-let-7f	1.006
		hsa-let-7g	<0.001
		hsa-miR-142-3p	1.200



**Fig. 7.** Heatmap showing that 49 miRNAs with  $p < 0.05$  classified leukemia samples into 2 subtypes (here ALL and CML).

#### **4. Discussion**

Our results indicated that small miRNAs remain intact after paraffin-fixation and decalcification procedure, and the expression profiling results are not significantly different from BA specimens, even though mRNA was badly degraded. It is well known that formalin causes cross-linkage between proteins and nucleic acids altering DNA and RNA quantity and quality [21,22] and, therefore, making it very difficult to conduct reliable gene expression profiling.

Earlier studies from FFPE material have also shown that miRNAs remain intact in formalin-fixed samples [12,13]. Li et al. [23] hypothesized that the cross-links between RNA and protein inhibit the yield and integrity of the RNA, and that the RISC (RNA induced silencing complex) protects the miRNAs and they are not, thereby, so sensitive to degradation. To our knowledge, no previous reports exist revealing the suitability of the decalcified material for miRNA profiling.

From CB no more than one half of the paraffin block was used for miRNA extraction, and the rest of the valuable clinical material was saved, importantly, for other diagnostic purposes.

The technical replicates, in the current study using decalcified CB samples consistently showed a good correlation. The Pearson's correlation coefficient between the replicates was  $>0.94$ . In addition, technical replicates clustered together in the unsupervised clustering analysis, confirming reproducibility of the method (Fig. 2).

Similarly, consistently higher Pearson's coefficient correlation, 0.98 for ALL and 0.97 for CML, were observed in systematic comparison of matched BAs and CBs compared to non-matched ones

suggesting that miRNA content is well retained in the decalcified CB samples. Previously it has been questioned whether FFPE samples reliably retain miRNA content seen in FF samples [24,25]. The similarity of the matched BAs and CBs and the fact that the global profiling of each sample type for both leukemia subtypes against control samples produced common differentially expressed miRNAs (**Tables 2 and 3**) confirm, according to our study, that the samples share similar biological ground.

We analyzed the miRNA expression profiles of CML and ALL for both sample types (BA and CB) compared to control miRNA extracted from bone marrow smears of healthy persons. Selection of the reference or control is problematic, as it is well known that the distribution of different hematopoietic cells is very different in normal bone marrow than in leukemic one. As for our cases, they represented morphologically and karyotypically (**Supplementary Table**) a heterogeneous group of childhood ALL. This heterogeneity/homogeneity is to be taken into consideration when interpreting the results. Thus, the heterogeneity may, for example, explain the fact that CML samples, in our study, showed more differentially expressed miRNA than ALL samples compared to control ones. Moreover, the comparison of our CML and ALL miRNA profiles with those of others' is difficult, as previous studies have mainly been conducted on cell lines or stem cells [26–33].

The numbers of differentially expressed miRNAs in CB and BA samples were similar within the leukemia subtype. The fact that the profiles in both sample types were not identical was very much expected as cellular components in the samples were to some extent different: compared to marrow aspirate, the core biopsy contained trabecular bone and a higher ratio of stromal

elements, mainly adipose and connective tissue, but less contaminating peripheral blood cells. Variation in cellular components between different specimens was assumingly smaller in CB than that in BA, as the amount of contaminating blood cells varied between different aspirates.

CML samples shared 43 miRNAs, of which 16 were upregulated and 27 downregulated. When exploring BCR-ABL-dependent miRNAs, Venturini et al. [26] carried out one of the few miRNA expression profilings on CML and found miR-17-92 polycistron to be downregulated. Interestingly, three members of this cluster (miR-17, miR-19a and miR-20a) were, in our study, among the downregulated miRNAs.

Abnormal expression of miR-15a, miR-16, miR-142, miR-155, miR-181, miR-221, and let-7a has been reported in the K562 cells line [27], but, in our analysis, only miR-142 and let-7a had abnormal expression. The function and the targets regulated by single miRNAs, especially in encoded polycistronic transcripts, are mostly unknown. Bueno et al. [28] reported decreased expression of miR-203 (situated at chromosome 14 in humans), due to loss of one of the alleles and hypermethylation promoter CpG, for the remaining allele in ALL and CML cases carrying the BCR-ABL1 fusion gene. Aguirre et al. [29] tested 157 miRNAs and, according to their study, hsa-miR-10a, hsa-miR-150, and hsa-miR-151 were downregulated in the mononuclear and in the CD34+ cells of CML patients, whereas hsa-miR-96 was upregulated in CML cells, compared to health controls. In our study we could not see downregulation of these miRNAs, which can be explained by the use of whole bone marrow instead of stem cells.

In our study, BA and CB ALL samples shared 21 miRNAs, among these miR-222 and miR-142-3p that were shown to be upregulated in pre-B-ALL patients. These miRNAs have been reported to target ALL associated genes such as *KIT* by miR-222 and *BCLAF1* by miR-142-3p [30]. Also upregulation of miR-181b was shown in ALL miRNA profiling study by Zanette et al. [31]. Our results are in consistent with previous studies determining the miRNA signature of ALL [30,32,33].

We showed, for the first time, that core biopsy samples, which have undergone decalcification process in addition to formalin-fixation, are technically and biologically reliable in miRNA profiling. Our results contribute, moreover, to the increasing evidence of the feasibility and utility of CB samples in miRNA profiling. The miRNA expression profiles of the two leukemia types, ALL and CML, presented in this paper include miRNAs (miR-17-92, miR-155, miR-223, miR-181a, miR-142) with important roles in normal hematopoietic differentiation and leukemogenesis.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

None.

**Contributions.** IB and MG have equally contributed to this study. SK, as a senior researcher, designed the study and participated in writing the manuscript. IB and SM performed the laboratory work, IB wrote the manuscript. MG designed and performed the data analysis and also participated in writing the manuscript; LL participated in designing the statistical analysis and preparing the manuscript. EE, US-P, M-LK-J and AR participated in designing the study and provided clinical data.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2010.08.005.

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