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14q32/miRNA clusters loss of heterozygosity in acute lymphoblastic leukemia is associated with up-regulation of BCL11a

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For Peer Review

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5 **14q32/miRNA clusters loss of heterozygosity in acute lymphoblastic leukemia is associated**
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7 **with up-regulation of BCL11a**
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36 **Short title:**

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38 **14Q32/MICRORNA LOH IN ALL**
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42 **ABSTRACT**

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44 The present study evaluated the loss and expression level of miRNAs 14q32 clusters in ALL
45 patients with cryptic deletions at 14q32 chromosomal band to investigate their involvement in this
46 disease. We demonstrate that a subset of ALL cases bearing 14q32 LOH showed a down-
47 regulation of miRNA 14q32 clusters, which is directly linked to the submicroscopic chromosomal
48 deletion. As a consequence of miRNAs deregulation we reported an inverse correlation with the
49 expression of their target *BCL11a*, a transcription factor involved in lymphoid differentiation. These
50 results suggest that 14q32/miRNA clusters LOH may be another mechanism involved in lymphoid
51 B cell transformation and differentiation and therefore, could be used as a diagnostic marker and
52 therapeutic target in subsets of ALL.
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INTRODUCTION

Genome-wide analyses of DNA copy number abnormalities and loss of heterozygosity (LOH) have provided important insights into the pathogenesis of newly diagnosed acute lymphoblastic leukemia (ALL) (1). If one assume that LOH may impact on the mechanisms of leukemogenesis, definition of its real role is to be considered of interest: one intriguing model may be that cryptic deletions may influence gene expression pattern by loss of microRNA or as a consequence of gene dosage (2).

MicroRNAs (miRNAs) are 19–24 nucleotide non-coding RNA which regulate the expression of target mRNAs both at transcriptional and translational level through perfect or partial base-pairing, mainly at the 3'-untranslated region (3'-UTR) of the target messenger. The physiologic roles for miRNAs have recently started being elucidated by showing that their functions span from development, differentiation, cell cycle regulation to aging and metabolism.

The first report linking miRNA and cancer involved the deletion of a cluster of two miRNAs, miR-15a and miR-16-1, that was found to be located within the commonly deleted region at 13q14 in chronic lymphocytic leukaemia (CLL) (3). Interestingly, miR-15a and miR-16-1 are down-regulated in the majority (70%) of CLL samples and the expression level was inversely correlated with that of *BCL2* (4). Recently, involvement of miRNA in ALL has been documented (5-9).

MicroRNAs are frequently located in clusters (10), moreover, plus than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs may play an important role in the pathogenesis of cancer.² Two clusters including more than 40 miRNAs are located at 14q32 chromosomal region, this chromosomal region is involved in physiological rearrangement of immunoglobulin heavy chain (IGH) locus in precursor B lineage cells, albeit chromosomal translocation involving IGH locus are rare event in non mature B lineage ALL this region may be prone to error of the rearrangement machinery leading to loss of genetic material.

The present study evaluated the loss and expression level of miRNAs 14q32 clusters in ALL patients with cryptic deletions at 14q32 chromosomal band to investigate their involvement in this disease.

METHODS

Sample selection

A total of 50 consecutive patients diagnosed with B lineage ALL at our institution between 2001 and 2009 were eligible for this study: these were 27 males and 23 females with a median age of 36.7 years (range 12-72). The diagnosis of ALL was established according to standard morphological criteria and the immunologic definition was based on the criteria defined by the European Group for the Immunological Characterization of Leukemia (EGIL) (11). Cytogenetic studies from bone marrow specimens were performed according standard procedures. Chromosomal abnormalities were classified according to the the International System for Cytogenetic Nomenclature (ISCN) (12). Karyotype was available in 35 of the 50 cases (70%). Molecular studies from leukemic cells were performed to detect gene fusion-transcripts associated with ALL as described by EACP protocols (13). Informed consent was obtained from all patients enrolled and the study was approved by Ethics Committee.

Microsatellites analysis

Diagnostic samples from bone marrow and/or peripheral blood containing a high proportion of leukemic cells (>80%) were used for molecular studies. We used epithelial cells from buccal mucosa at diagnosis, or peripheral blood at clinical remission as a source of constitutional DNA for each patient. LOH was assessed by using oligonucleotide primers for 4 highly polymorphic microsatellite repeat markers (STR) mapped ,according to NCBI Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview>), at 14q32 nearby the mirNA-clusters (spanning from 101.3 to 101.5 kb) : D14S65 (at 97.6 kb), D14S1426 (at 100,6 kb), D14S985 (at 101,2 kb) and D14S292 (at 104.5 kb) (see Supplementasry on line material figure 1s). Commercial primers with fluorescent dye phosphoramidites FAM, TET or NED were used (Applied Biosystem, CA, USA). PCR-amplification of microsatellite sequences was performed using forward primers labelled with fluorescent dye phosphoramidites (FAM or NED) and analyzed by capillary electrophoresis as

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3 previously described (14). Only heterozygous loci were considered useful. LOH was scored as
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5 positive when the degree of reduction in allelic signal intensity was greater than 70% in one of the
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7 alleles of the blast population compared with control DNA. All samples showing LOH were
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9 subjected to repeat amplification and analysis for data confirmation. The presence of, at least, a
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11 double intensity signal of one of the alleles in leukemic cells was defined as genomic amplification
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13 (see Supplementasry on line material figure 2s).
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20 **miRNA expression.** We analyzed the miRNA level expression, by the MicroRNA TAQman assay
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22 (Applied Biosystems), of 18 miRNA included in the microRNA-clusters mapped at 14q32
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24 (<http://www.mirbase.org>) : *miR-376b*, *miR-369-3p*, *miR-329*, *miR2-99-3p*, *miR-368*, *miR-493*, *miR-*
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26 *453*, *miR-656*, *miR-433*, *miR-127*, *miR-432*, *miR-379*, *miR-494*, *miR-410*, *miR-376a*, *miR-412*, *miR-*
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28 *382*, *miR-544*. This technology is a method for quantitative RT-PCR (Q-RT-PCR) that assay the
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30 RNA expression levels of mature miRNA genes. The assay were performed in accordance to
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32 manufacturer's protocols. Briefly, 250-500 ng of total RNA was reverse transcribed and the
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34 reactions were performed for each sample using the Multiplex RT Human Pool for TaqMan miRNA
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36 (pools 3,5,6 and 7) and the High Capacity cDNA Archive Kit (Applied Biosystems). One μL of
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38 cDNA from each sample were added to a 6.5 μL of TaqMan® Universal PCR Master Mix , 0.65 μL
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40 of primer/probe MicroRNA TAQman assay mix (20X) in a final volume of 13 μL . The RT-PCR
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42 amplification were performed on an ABI 7900HT . Reverse-transcriptase PCR data were quantified
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44 using the SDS 2.3 software and normalized using the RNU48 as endogenous control. The cycle
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46 threshold (Ct) value, which was calculated relatively to the endogenous control were used for our
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48 analysis (ΔCt). The $2^{-\Delta\Delta\text{CT}}$ method was used to calculate relative changes in gene expression among
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50 different patient groups.
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***BCL11a* gene expression**

From many predicted targets of miRNA clusters at 14q32 we selected BCL11a, because this gene is predicted to be a targets of several microRNAs of 14q32 clusters as predicted by the currently available major prediction programs, such as: miR-376a (<http://www.microrna.org>), miR-544 (<http://www.microrna.org>), miR-656 (<http://www.microrna.org>; <http://diana.cslab.ece.ntua.gr/microT/>), miR-432 (<http://www.microrna.org>), miR-494 (<http://www.microrna.org>) and miR-410 (<http://www.targetscan.org>; <http://diana.cslab.ece.ntua.gr/microT/>). We used Q-RT-PCR to determine RNA level through the 7900 TAQMAN systems and pre-designed available assay (Assay on Demand, Applied Biosystems). Q-RT-PCR data were quantified using the SDS 2.3 software and normalized using the *ABL* gene as endogenous control. In order to calculate relative changes in gene expression among different sample we used the $2^{-\Delta\Delta CT}$ method.

Statistical analysis. Results from each sample were quantified separately. In order to increase the stability of the results, we applied a filtering criteria that included 10 miRNA which were reliably quantifiable (cutoff < 35 Ct) in at least 70% of single categories. Undetermined values of Ct were estimated as 50 Ct (the last cycle of the reactions), in order to remove differences due to sampling, the mean of RNU48 was used as internal normalization. Statistical analysis of the relative expression results was performed by the Relative Expression Software Tool (REST) proposed by Pfaffl (2002) (15). The mathematical model used in this software is based on the PCR efficiencies (E) and the difference (Δ) obtained by subtracting the threshold cycle (Ct) value of the target gene from the Ct value of the endogenous control (ΔCt housekeeping-sample). Fold change and p-value were estimated by REST. Namely, a miR was defined differentially expressed when estimated p-value was <0.05.

RESULTS

Identification of LOH at 14q32

DNA from leukemic blasts and normal cells from 50 ALL patients were analyzed for LOH by using 4 highly polymorphic microsatellites repeat markers mapping at band 14q32 : D14S65, D14S1426, D14S985 and D14S292. Informative allelotypes were found in 172 of 200 examined STR patterns (86%). Balanced 14q32 allelotypes were found in 39 cases, 14q32 imbalance was detected in 11 case. No difference in age, WBC and genetic characteristics was observed between balanced and unbalanced cases ((see Supplementasry on line material table 1). Patients showing imbalance at 14q32 included 3 cases with hyperdiploidy karyotype and microsatellite amplification and 8 cases showing LOH at one or more STR analyzed who did not exhibited at the conventional cytogenetic analysis aberration at 14q32 (tab.2). Six cases showed more than one LOH event or LOH at STR mapped nearest to the miRNA clusters, the remaining 2 cases showed a single LOH at D14S65 (case 3) and D14S292 (case 1), this STR mapped respectively upstream and downstream the miRNA 14q32 clusters (see Supplementasry on line material figure 1s, table 2).

To ensure that allelic loss did not depend on potential technical pitfalls , LOH was confirmed in a second STR amplification.

Expression of miRNA 14q32 clusters in ALL patients. We performed a miRNA expression analysis of 18 mammalian miRNAs included in miRNA 14q32 clusters on 40 ALL samples (due to the unavailability of RNA from leukemic blasts at diagnosis we were unable to perform gene expression analysis on 10 ALL samples). Samples analyzed included 31 ALL specimens showed balanced 14q32 STR pattern (LOH-ve), 6 14q32 STR unbalanced samples showing LOH (LOH+ve case 1,3,4,6,7 and 8 tab.2) and 3 cases showing amplification at 14q32. We selected between the LOH+ve

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3 group 4 samples (case 4,6,7 and 8 tab.2) showing extended LOH region (more than one LOH event)
4 mapped nearby the miRNA 14q32 clusters confirming the deletion of the chromosomal region, to
5 perform comparative miRNAs expression analysis, and we excluded the remaining cases 1 and 3
6 which showed LOH only at D1465 or D14S292 that mapped upstream or downstream to the
7 miRNA clusters. As documented by duplication of experiments, expression data were highly
8 reproducible (data not show), standardization was carried out using RNU48 expression. According
9 pre-filtering criteria (see methods) we were able to analyzed the level expression of 10 miRNAs:
10 *miR-433*, *miR-127*, *miR-432*, *miR-379*, *miR-494*, *miR-410*, *miR-376a*, *miR-412*, *miR-382* and *miR-*
11 *544*. We performed a comparative analysis of the two population LOH-ve and LOH+ve and we
12 observed that all miRNA evaluated, except *miR-410*, showed a lower level of expression in the
13 LOH+ve group (fold change ranging from 0.001 to 0.24). Moreover 4 miRNAs, *miR-382* (decreased
14 fold 0.001 p-value <0.01) , *miR-412* (fold 0.03 p-value = 0.04), *miR-433* (fold 0.012 p-value <0.01)
15 and *miR-127* (fold 0.03 p-value = 0.01) resulted down-regulated at statistical REST analysis (Figure
16 1).

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39 ***miRNA target genes expression.*** Recent findings indicate that animal miRNAs may not only repress
40 protein synthesis but also induce mRNA degradation of a large portion of targets. We selected the
41 *BCL11a* gene that is predicted to be a targets of several microRNAs of 14q32 clusters down-
42 regulated in our LOH+ve cases such as *miR-376a*, *miR-432*, *miR-494*, *miR-656* and *miR-544*. In
43 order to validate the findings of miRNA expression associated with the presence of LOH, we tested
44 the mRNA levels of selected target gene *BCL11a* in the same ALL patients grouped by genetic
45 characteristics (LOH-ve vs LOH+ve). We found that *BCL11* showed a significant inverse
46 correlation with miRNA expression resulting up-regulated in patients LOH+ve (9.7 fold, p-
47 value=0.013 at REST analysis) (Figure 2).
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DISCUSSION

In haematological malignancies a link between miRNA and cancer was initially described in CLL by Calin et al (2002) (3). A cluster of two miRNAs, miR-15a and miR-16, was found to be located within the deleted region at 13q14, and down-regulated in the majority (70%) of CLL samples. Notably, *miR-15a* and *miR-16-1* down-regulation contribute to malignant transformation by up-regulating BCL2 (3,4). Recently, involvement of miRNA in ALL has been documented identifying that miRNA expression profiles are ALL subtype-specific (6-8) and suggesting a pathogenetic role of miRNA in ALL (5,9).

The present study evaluated the loss and expression level of miRNAs in ALL patients with cryptic deletions at 14q32 chromosomal band to investigate their involvement in this disease. Among the candidate microRNAs located at fragile sites, miRNA clusters at 14q32 have been already reported, but their direct implication still remained hypothetical. The present study evaluated the loss and expression level of miRNAs 14q32 clusters in ALL patients with cryptic deletions at 14q32 chromosomal band to investigate their involvement in this disease. Our data demonstrate that in a subset of ALL cases bearing 14q32 LOH, there is an down-regulation of miRNA 14q32 clusters, which is directly linked to the submicroscopic chromosomal deletion. This conclusion is reinforced by the very low level of expression (from 0.24 to 0.001 fold) of nine miRNAs, included in 14q32 clusters, in leukemic cells carrying the deletion as compared with the pool of patients without deletions. Our data suggest a crucial role for miRNA clusters at 14q32, which were deleted in a subset of ALL cases bearing 14q32 deletions.

The down-regulation of the miRNA clusters could influence the expression level of different target genes modifying crucial cellular pathways. From many predicted targets of miRNA clusters at 14q32 individuated by the currently available major prediction programs, we selected *BCL11a*, this gene is predicted to be a targets of several miRNA of clusters down-regulated in our LOH+ve cases such as *miR-376a*, *miR-432*, *miR-656*, *miR-410*, *miR-494* and *miR-544*.

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3 In order to validate the findings of miRNAs expression associated with the presence of LOH, we
4 tested the mRNA levels of selected target gene *BCL11a* in the same ALL patients grouped by
5 genetic characteristics (LOH-ve vs LOH+ve). We found that *BCL11a* showed a significant inverse
6 correlation with miRNA expression resulting up-regulated in patients LOH+ve (9.7 fold, p 0.013 at
7 REST analysis). *BCL11a* codifies for a transcription factor involved in lymphoid differentiation, this
8 gene functions upstream of the transcription factors EBF1 and PAX5 in the B cell pathway (16) and
9 it is known its involvement in lymphoid malignancies through either chromosomal translocation
10 leading to over-expression or amplification (17).
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24 We report herein the first cryptic deletion down-regulating a microRNA clusters in ALL. As a
25 consequence of miRNAs deregulation we reported the inverse correlation with the expression of
26 their target *BCL11a*. These results suggest that 14q32/miRNA clusters LOH may be another
27 mechanism involved in lymphoid B cell transformation and differentiation and therefore, could be
28 used as a diagnostic marker and therapeutic target in subsets of ALL.
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38 **Conflict of interest statement**

39 All authors disclose any financial and personal relationships with other people or organizations that
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44

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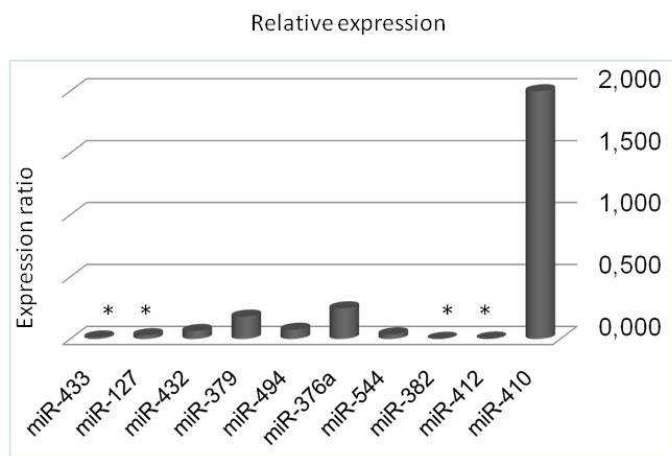
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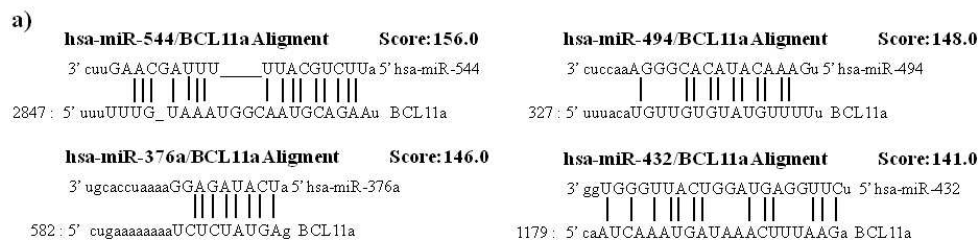
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Figure 1 Graphic representation of miR-433, miR-127, miR-432, miR-379, miR-494, miR-376a, miR-544, miR-382, miR-412 and miR-410 relative expression data obtained by REST analysis: Data are showed as expression ratio mean value between the LOH+ve group (case 4,6,7 and 8 tab.2) and the control group (LOH-ve) and showed a miRNA down-regulation in ALL cases with 14q32 LOH as compared with the cohort of patients without deletion. * p-value < 0.05
254x190mm (96 x 96 DPI)



b)

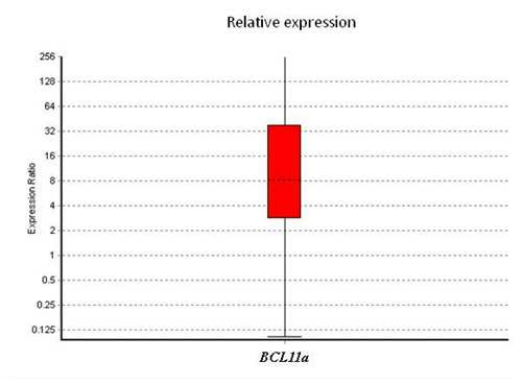


Figure 2 a) mir544/BCL11a alignment as reported by <http://www.microrna.org> ; b) Graphic representation of BCL11a expression data obtained by REST analysis showing a BCL11a up-regulation (p-value= 0.013) in ALL cases with 14q32 LOH as compared with the cohort of patients without deletion. Data are showed as expression ratio mean value between the LOH+ve group and the control group (LOH-ve) . The figure shows box plots of gene expression where the top and bottom of each box indicate the 75th and 25th percentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.