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A novel mutation in the miR-128b gene reduces miRNA processing and leads to glucocorticoid resistance of MLL-AF4 acute lymphocytic leukemia cells

Ai Kotani1, Daon Ha2, Diana Schotte3, Monique L. den Boer3, Scott A. Armstrong4, and Harvey F. Lodish2,5,*

1 Division of Molecular Therapy Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan 2 Department of Biology Massachusetts Institute of Technology, Cambridge, MA USA 3 Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children’s Hospital, Rotterdam, GJ Netherlands 4 Division of Hematology/Oncology, Children’s Hospital and Harvard Medical School, Boston, MA USA 5 Whitehead Institute for Biomedical Research, Cambridge, MA USA

Abstract
MLL-AF4 Acute Lymphocytic Leukemia has a poor prognosis, and the mechanisms by which these leukemias develop are not understood despite intensive research based on well-known concepts and methods. MicroRNAs (miRNAs) are a new class of small noncoding RNAs that post-transcriptionally regulate expression of target mRNA transcripts. We recently reported that ectopic expression of miR-128b together with miR-221, two of the miRNAs downregulated in MLL-AF4 ALL, restores glucocorticoid resistance through downregulation of the MLL-AF4 chimeric fusion proteins MLL-AF4 and AF4-MLL that are generated by chromosomal translocation t(4;11). Here we report the identification of new mutations in miR-128b in RS4;11 cells, derived from MLL-AF4 ALL patient. One novel mutation significantly reduces the processing of miR-128b. Finally, this base change occurs in a primary MLL-AF4 ALL sample as an acquired mutation. These results demonstrate that the novel mutation in miR-128b in MLL-AF4 ALL alters the processing of miR-128b and that the resultant downregulation of mature miR-128b contributes to glucocorticoid resistance through the failure to downregulate the fusion oncogenes.

Keywords
miRNA; processing; mutation; steroid resistance; miR-128

Introduction
microRNAs (miRNAs) are a recently discovered class of small noncoding RNAs that are 18–24 nucleotides (nt) long that downregulate target genes at a post-transcriptional level. The majority of miRNA genes are transcribed by RNA polymerase II into long primary (pri) miRNA transcripts, processed by the nuclear nuclease Drosha into ~60 bp hairpins termed precursor (pre) miRNAs, and further cleaved in the cytosol by the Dicer nuclease into mature miRNAs (Fig. 1). Mature miR-NA is then incorporated into the multiprotein RNA-induced silencing complex (RISC), exerting post-transcriptional repression of target miRNAs, either by inducing mRNA degradation or blocking mRNA translation or both.2,3

*Correspondence to: Harvey F. Lodish; lodish@wi.mit.edu.
Each miRNA is thought to have several target mRNAs, and computational predictions suggest that more than a third of all human genes are targets of miRNAs.\textsuperscript{4} In animals, miRNAs control many developmental and physiological processes. For example, abnormal expression of certain miRNAs leads to developmental arrest in \textit{C. elegans}.\textsuperscript{5} In the hematopoietic system, miR-181a downregulates several phosphatases that regulate the sensitivity of T cells to antigens, and overexpression of miR-181 in hematopoietic stem/progenitor cells significantly increases B cell production. Overexpression of miR-150 leads to a block in B cell formation at the proB to preB cell transition by downregulating c-myb among other targets.\textsuperscript{6–9}

Many studies show specific alterations in miRNA expression profiles that correlate with particular human tumor phenotypes.\textsuperscript{10,11} Downregulation of specific miRNAs in certain cancers implies that some miRNAs act as tumor suppressors. For example, reduced expression of let-7 family members, which directly downregulate the expression of Ras and other proto-oncogenes, occurs in lung cancer. miR-15 and -16, negative regulators of bcl-2, exhibit reduced expression in B cell chronic lymphocytic leukemia.\textsuperscript{12,13} On the other hand, increased expression of miR-17-92 and miR-155 often occurs in B cell lymphomas,\textsuperscript{14,15} implying that these miRNAs are oncogenes.\textsuperscript{15,16}

The \textit{MLL} gene is located at 11q23, a site frequently involved in chromosomal translocations in aggressive human lymphoid and myeloid leukemias. As a result of these chromosomal translocations a portion of \textit{MLL} becomes fused with one of more than forty different partner proteins, yielding a diverse collection of chimeric fusion proteins. MLL rearrangements are a common genetic alteration found in human leukemia. Of these MLL associated leukemias, MLL-AF4 acute lymphocytic leukemia (ALL), resulting from a balanced translocation between MLL and AF4, occurs in approximately 50% of ALL cases in infants, 2% in children and 5–6% in adults.\textsuperscript{17} MLL-AF4 ALL is associated with steroid resistance and has a poor prognosis.\textsuperscript{18} Overexpression of MLL-AF4 in lymphoid cells reduces cell growth and induces resistance to etoposide-mediated cytotoxicity.\textsuperscript{19,20} The reciprocal fusion protein, AF4-MLL, may also have oncogenic activity and may be an important hit for leukemogenesis.\textsuperscript{21,22} The detailed roles of these fusion proteins in leukemogenesis are not well understood.

We focused on miR-128b, which together with miR-221 are two miRNAs commonly downregulated in MLL rearranged ALL compared to other types of ALL. We hypothesized that these miRNAs normally downregulate several proteins that contribute to leukemia development. Recently, we show that re-expression of miR-128b sensitizes two MLL-AF4 ALL cell lines to killing by both low and high concentrations of glucocorticoids, and cooperates with miR-221 in this process\textsuperscript{23} (Fig. 2). Furthermore miR-128b downregulates MLL, AF4, as well as both MLL-AF4 and AF4-MLL fusion genes, and miR-221 downregulates CDKN1B, a downstream transcriptional target of MLL and MLL-AF4,\textsuperscript{18,23} thus explaining the oncogenic effects of downregulating miR-128b and miR-221.

However, we do not understand the mechanism(s) by which expression of these miRNAs is downregulated in MLL-AF4 cells. Methylation of the miRNA promoters and aberrant processing of miRNAs have been are suggested to cause such downregulation. In this extraveiw, we report the identification of an A13G point mutation in the miR-128b gene in RS4;11, an MLL-AF4 ALL cell line. This mutation causes a significant block in the processing of miR-128b and a reduction in the level of the mature miR-128b. The downregulation of miR-128b caused by the mutation, and the restoration of glucocorticoid sensitivity by the overexpression of miR-128b, showed that this acquired mutation might play a role in the resistance of MLL-AF4 ALL cells to steroid treatment.
Results

A point mutation in the pri-miR-128b segment of the miR-128b gene in RS4;11 MLL-AF4 cells

In trying to understand why the level of miR-128b and miR-221 might be reduced in MLL-AF4 ALL patients, we cloned and sequenced the miR-128b and the miR-221 genes from RS4;11, a cell line derived from an MLL-AF4 ALL patient. Figure 3A shows that the cells have a single nucleotide change, A13G, located 13 bp from the 3′ end of mature miR-128b. This base substitution has been reported neither as a polymorphism nor as a mutation (International HapMap Project, Entrez SNP, 25).

The A13G mutation reduces the processing efficiency of pri-miR128b

To identify the molecular effects of the A13G mutation, expression vectors were constructed encoding a 316 bp segment of either the wild type (miR-128b-Wt) or the mutated allele (miR-128b-A13G) of a pri-miR-128b gene. The vectors were transfected into 293T cells, which express a low level of endogenous miR-128b (data not shown). The A13G mutation greatly reduced the extent of processing of the miR-128b precursor (Fig. 3B). In cells expressing miR-128b-A13G, the relative densities of the pri-, pre- and mature miR-128b “bands” were 1.3, 2.7 and 1.0, respectively, while in miR-128b-Wt-expressing cells, these values were 0, 0.9 and 1.0, respectively. This result suggests that the processing of the miR-128b precursor is impeded by the A13G mutation. To confirm and extend this conclusion, we appended a 240bp pri-miR-221 coding sequence to the 5′ side of miR-128b-Wt or -A13G; thus mature miR-221 serves as an internal control to assess the efficiencies of processing of the cotranscribed miR-128b-WT and miR-128b-A13G precursors (Fig. 3C top right). While the expression levels of pre-miR-128b showed no noticeable difference between cells expressing the wild-type and A13G miR-128b genes, a significantly lower level of mature miR-128b was detected in cells transfected with the mutant miRNA gene compared to the wild-type (Fig. 3C left). Normalized to the expression levels of pre- and mature miR-221, there was a 2-fold reduction in processing of the A13G mutant pri miRNA to the mature miRNA (when compared to wild type; Fig. 3C bar graph).

To study the effects of the A13G mutation in an MLL-AF4 ALL cell, miR-128-b-Wt and -A13G genes were transduced into RS4;11 cells by retroviral infection. As shown by northern blot analysis, cells expressing exogenous miR-128b-Wt exhibited an increased level of mature miR-128b compared to that of control cells, while cells expressing exogenous miR-128b-A13G expressed almost the same level of mature miR-128b as control cells (Fig. 3D). No bands corresponding to unprocessed pri-miR-128b and only faint bands of pre-miR-128b could be detected in the northern blot analysis (data not shown). These results were consistent with the results of quantitative PCR measurements of mature miR-128b: cells transduced by miR-128b-Wt expressed mature miR-128b at a level five-times that of control cells, whilst cells transduced by miR-128-b-A13G expressed mature miR-128b at a level only twice that of the endogenous miR-128b (Fig. 3E upper). In contrast, quantitative PCR measurements of pri-miR-128b, showed a 17-fold increase in the level of pri-miR-128b in cells expressing miR-128b-A13G compared to the control, but a 5-fold increased level in cells transduced with miR-128b-Wt (Fig. 3E middle). The PCR products of both pri-miR-128bs were sequenced to verify the correct sequence (data not shown). The processing efficiency was calculated by dividing the expression level of mature miR-128b by that of the pri-miR-128b. The processing efficiency in cells expressing exogenous miR-128b-A13G was only one-tenth that of cells expressing miR-128b-Wt (Fig. 3E lower).
We previously reported that mature miR-128b increases DEX induced apoptosis. In Figure 3F, miR-128b-A13G over-expressing RS4;11 cells, which express mature miR-128b at a level only twice that of control cells, showed an increase in DEX-induced apoptosis over control cells, but at all DEX concentrations the extent of DEX-induced apoptosis was significantly lower than in cells expressing miR-128-b-Wt. Taken together, the A13G mutation reduces the processing of the miR-128b primary transcript into mature miR-128b RNA, and thus reduces the effectiveness of miR-128b in restoring glucocorticoid sensitivity to MLL-AF4 cells.

The A13G mutation is found in some primary human MLL-rearranged ALL tumor cells

In work done using samples collected at the Erasmus MC/Sophia Children’s Hospital, Rotterdam, 18 human primary MLL-rearranged ALL tumor cell samples were screened for the presence of the A13G mutation in the miR-128b gene. These samples were collected at the time of diagnosis before chemotherapy, but direct sequence analysis of the PCR product of the miR-128b gene showed that none contained mutations. In preliminary experiments, we amplified by PCR the genomic DNA segment of patient No.1 collected at the Dana Farber Cancer Institute and sequenced individual clones. One of 77 clones harbored the A13G mutation, suggesting that a small population of the leukemic cells bear the mutation.

To increase the sensitivity of detection of the mutant allele we developed a nested PCR strategy (Fig. 4A) in which the primary PCR product was digested with the Tsp45I digestion (Fig. 4B lower left). Restriction enzyme Tsp45I, which is predicted to cleave the normal but not the A13G mutant allele. Among the six MLL-rearranged ALL primary patient samples from the Dana Farber Cancer Institute analyzed, together with three normal samples, patients 1 and 5 yielded a significant amount of undigested DNA after the 2nd Tsp45I digestion (Fig. 4B upper left). Subsequent DNA sequence analysis revealed that patient 5 indeed contained the A13G mutant allele while patient 1 did not (Fig. 4B right). Other patient samples yielded no notable amount of undigested DNA after the 2nd Tsp45I digestion (Fig. 4B lower left).

That no mutant clones were detected in patient 1 by this analysis contradicted our preliminary sequence analysis that indicated that patient 1 indeed contained the A13G mutation. To resolve this issue, an examination of the sequence analysis of the 1st PCR product from patient 1 revealed that the mutant clone had other mutations in the miR-128b gene that generated a new Tsp45I restriction site. The new restriction site was located inside the 2nd nested PCR product, indicating that the mutant clone in patient 1 could not be amplified by the 2nd nested PCR (data not shown). Thus we conclude that the novel A13G mutation in pri-miR-128b was found in not only RS4;11, but also in two primary samples of the six MLL-rearranged ALL tumor DNAs tested. Clearly the occurrence of the mutant allele in a total leukemic population is rare at the time of presentation of the disease (Fig. 4A right middle). Thus the A13G mutation likely occurred after the initial chromosomal translocation.

Discussion

Genetic alteration in miRNAs

Recently several studies illustrating mutations and polymorphisms in primary miRNAs have been published. Calin reported that in 92 chronic lymphocytic leukemia (CLL) patient sample, 5 mutations were identified; one of them was homozygous, and displayed complete abolishment of processing of both miR-15 and miR-16. Other groups identified as many as 24 mutations in members of the let-7 family, miR-15, -16, -124 genes in genomes of various cancer cell lines, but concluded that none of them affected the processing of micro
RNAs. However, they did not analyze miRNA processing quantitatively as we did in this study. Similarly, Iwai etc., reported many polymorphisms in primary miRNAs from the 96 Japanese healthy individuals, but these have not been assessed yet for functional alterations. Our observations suggest that some of these polymorphisms might alter the processing of miRNAs and lead to disease.\(^{25-27}\)

We identified the A13G point mutation in the miR-128b gene in RS4;11, a cell line derived from an MLL-AF4 ALL patient. This novel mutation significantly reduced the processing of miR-128b both in transiently transfected 293T cells and stably transduced derived from relapsed or refractory MLL-AF4 ALL RS4;11 cells. The A13G point mutation in the miR-128b gene is present both in the RS4;11 cell line and in two out of six primary MLL-rearranged ALL patient samples. Clearly, because the mutation is present in only a small population of leukemic cells derived from MLL-rearranged leukemia, the mutation cannot be responsible for the initiation of the disease. miR-128b is significantly downregulated in primary MLL rearranged tumors, before chemotherapy.\(^{10}\) Since this would lead to upregulation of expression of both MLL-AF4 and AF4-MLL fusion proteins, selection for cells with downregulated miR-128b, whether caused by the A13G mutation or otherwise, could occur early in disease progression. It is likely that there is also selection for this mutation, or other genetic or epigenetic changes that cause downregulation of miR-128b, during later stages of leukemogenesis, especially during steroid treatment, since down-regulation of mature miR-128b causes the tumor to be refractory to dexamethasone chemotherapy and relapse. Further studies are needed, using cells tumors after chemotherapy, to better understand the significance of and selection for the A13G mutation and other mechanisms that cause miR-128b downregulation.

**Materials and Methods**

Methods for culturing and studying RS4;11 cells were detailed in our previous paper.\(^{23}\)

**Determination of A13G mutation of pri-miR-128b in primary samples of tumor DNA**

In order to reduce the ratio of normal alleles to the mutant alleles, a genomic 394-bp pri-miR-128b PCR product, generated using Pfx polymerase (Forward primer, 5′-TGT TCT TAA GGC TAG GGA ACC A-3′; Reverse primer, 5′-CGG GAA TTC CGG CCG TCA GGC AGT CTT CAG C-3′), was digested with 5 units of Tsp45I overnight at 65°C and visualized on an agarose gel. The PCR product of normal sequence is digested by TspI45, which recognizes the sequence, GTSAC, and two small DNA fragments of 246 and 148 bp were generated (Fig. 4). If the PCR product contains the A13G mutation, however, the Tsp45I failed to digest it because the recognition site was disturbed by the mutation (GTSGC), leaving an undigested 394-bp fragment. The 394-bp undigested product was purified and placed into the 2nd-round semi-nested PCR reaction (Forward primer, 5′-GGT TGT TTC AAT ATC GTG CTA AAA-3′; Reverse primer, 5′-CCG GAA TTC CGG CCG TCA GGC AGT CTT CAG C-3′). The PCR conditions were the same as that described for pri-miR-128b. After checking by agarose gel electrophoresis that the expected 367-bp fragment was of acceptable quality, the product was digested again with 5 units of Tsp45I for 3 hrs at 65°C. After purifying the undigested 367-bp DNA, we sequenced the fragment, which was expected to be enriched for the A13G mutant allele.

**Use of human samples**

ALL samples were obtained from patients enrolled on Dana Farber Cancer Institute IRB approved childhood ALL trials between 1995 and 2005 and Erasmus MC-Sophia Children’s Hospital IRB approved Interfant-study trials between 1999 and 2007. In all cases the studies were preapproved by the appropriate Institutional Regulatory Board (IRB). Samples were...
taken from bone marrow or peripheral blood and had >80% blasts. Mononuclear cells were obtained by density centrifugation, and the DNA was obtained by standard methods.

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References


**Figure 1.**
Processing machinery of miRNA. miRNA genes are transcribed by RNA polymerase II or III into long primary (pri) miRNA transcripts, processed by the nuclear nuclease Drosha into ~60 bp hairpins termed precursor (pre) miRNAs, and further cleaved in the cytosol by the Dicer nuclease into mature miRNAs. Mature miRNAs are then incorporated into the multiprotein RNA-induced silencing complex (RISC), exerting post-transcriptional repression of target mRNAs, either by inducing mRNA cleavage, mRNA degradation or blocking mRNA translation or both.
Ectopic expression of miR-128b and miR-221 restores glucocorticoid sensitivity to RS4;11 cells. RS4;11 cells were infected with bicistronic retroviruses encoding GFP as well as a specific miRNA. Sorted GFP-positive control- (termed MDH), let7b-, miR-221- or miR-128b-transduced cells are treated (DEX⁺) or not (DEX⁻) with 10 μM dexamethasone for 40 hours, followed by FACS analysis. The percentage of viable cells is determined by dividing the number of Annexin V(−) GFP (+) cells by the number of total cells analyzed. Taken from ref. 23.
Figure 3.
A point mutation in pri-miR-128b in RS4;11 cells blocks the processing of pri-miR-128b to mature miR-128b. (A) DNA chromatograms illustrating the genomic sequence of miR-128b DNA from RS 4;11 cells. Nucleotides are indicated by capital letters. +13 indicates the A13G base substitution. Note that the wt and mutant sequence are both present. The base substitution is located 13 bp from the 3’ end of mature miR-128b miRNA. (B) Northern blot analysis of primary, precursor and mature miR-128b expression in 293T cells transiently transfected by miR-128b-Wt and -A13G. Arrows indicate (top to bottom) primary (pri), precursor (pre), and mature miR-128b respectively. The relative intensities of each band were normalized to the mature miR-128b by using Multigage software and are indicated above each band. (C) Northern blot analysis of precursor and mature miR-221 (left) and miR-128b (middle) in 293T cells transiently transfected by a vector encoding both miR-221 and miR-128b-Wt or miR-128b A13G. The graph shows the relative intensities of precursor and mature miR-128b normalized to those of miR-221 using Multigage software (right). (D) Northern blot analysis of mature miR-128b expression in RS4;11 cells transduced by miR-128b-Wt, -A13G, and control (C) vector. Expression of 5S RNA is used as the loading control. The graph shows that the relative intensities of each band normalized to the 5S loading control using Multigage software. (E) Quantitative PCR for mature miR-128b in RS4;11 cells transduced by miR-128b-Wt, -A13G and control (upper). The position of primers that detect both unprocessed and processed pri-miR-128b (middle upper). The arrows with solid lines and dotted lines indicate the position of the primers for PCR and RT, respectively. Quantitative PCR for the unprocessed and processed pri-miR-128b in RS4;11 cells transduced by miR-128b-Wt, -A13G, and control vector (middle lower). The processing efficiency is calculated as the ratio of expression levels of mature and primary miR-128b (lower). (F) Sorted GFP (+) miR-128b-Wt-, -A13G- or control-transduced cells.
are treated with an increasing concentration of dexamethasone for 40 hrs, followed by FACS analysis. GFP (+) cells are assessed for viability by Annexin V staining as the same way as in Figure 2.
Figure 4.
Detection of the A13G mutant miR-128b allele in a primary MLL-AF4 ALL tumor. (A) Schematic of the procedure used to enrich the mutant alleles is shown along with the gel images that are analyzed at each step. After the 1st genomic PCR, the target bands are seen in all samples. The arrow indicates the expected size of the PCR product. After digestion of the 1st PCR products by Tsp45I, which cleaves the wild-type but not the A13G mutant DNA, no bands corresponding to the undigested PCR product, which corresponds to the mutant allele, are visible in any of these samples. The arrows (top to bottom) indicate the predicted sizes of DNAs corresponding to the undigested and the two digested products. After the 2nd semi-nested PCR, all samples show the expected amplified species; the arrow indicates the expected size of the PCR product. 1–9 indicated patients 1 to 9; a–f, normal control a, b, c; P, positive control that contains 10% of mutant and 90% wild type DNA. (B) After the digestion of the 2nd PCR products by Tsp45I, patients 1 and 5 show an undigested DNA, which corresponds to the mutant allele, as indicated by the upper arrows (upper left). Other patient samples show no undigested DNA (i.e., wild-type sequence; lower left). The DNA sequence analysis demonstrated that the PCR product from patient 5, as well as the positive control (P) show a G base at position 13, that is, the A13G mutation, while patient 1 showed only an A base at this position (right). The arrow indicates the position of A13G mutation.