Expression of miR-196b is not exclusively *MLL*-driven but is especially linked to activation of *HOXA* genes in pediatric acute lymphoblastic leukemia

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Deregulation of microRNA may contribute to hematopoietic malignancies. MicroRNA-196b (miR-196b) is highly expressed in *MLL*-rearranged leukemia and has been shown to be activated by *MLL* and *MLL*-fusion genes.

Design and Methods

In order to determine whether high expression of miR-196b is restricted to *MLL*-rearranged leukemia, we used quantitative stem-loop reverse transcriptase polymerase chain reaction to measure the expression of this microRNA in 72 selected cases of pediatric acute lymphoblastic leukemia i.e. *MLL*-rearranged and non-*MLL*-rearranged precursor B-cell and T-cell acute lymphoblastic leukemias. We also determined the expression of *HOXA*-genes flanking *miR-196* by microarray and real-time quantitative polymerase chain reaction. Furthermore, we used CpG island-arrays to explore the DNA methylation status of *miR-196b* and *HOXA*.

Results

We demonstrated that high expression of miR-196b is not unique to *MLL*-rearranged acute lymphoblastic leukemia but also occurs in patients with T-cell acute lymphoblastic leukemia patients carrying *CALM-AF10*, *SET-NUP214* and inversion of chromosome 7. Like *MLL*-rearrangements, these abnormalities have been functionally linked with up-regulation of *HOXA*. In correspondence, miR-196b expression in these patients correlated strongly with the levels of *HOXA* family genes (Spearman's correlation coefficient ≥ 0.7; $P \leq 0.005$). Since *miR-196b* is encoded on the *HOXA* cluster, these data suggest co-activation of *miR-196b* and *HOXA* genes in acute lymphoblastic leukemia. Up-regulation of miR-196b coincides with reduced DNA methylation at CpG islands in the promoter regions of *miR-196b* and the entire *HOXA* cluster in *MLL*-rearranged cases compared to in cases of non-*MLL* precursor B-cell acute lymphoblastic leukemia and normal bone marrow ($P<0.05$), suggesting an epigenetic origin for miR-196b over-expression. Although patients with *MLL*-rearranged acute lymphoblastic leukemia are highly resistant to prednisolone and L-asparaginase, this resistance was not attributed to miR-196b expression.

Conclusions

High expression of miR-196b is not exclusively *MLL*-driven but can also be found in other types of leukemia with aberrant activation of *HOXA* genes. Since miR-196b has been shown by others to exert oncogenic activity in bone marrow progenitor cells, the findings of the present study imply a potential role for miR-196b in the underlying biology of all *HOXA*-activated leukemias.

Key words: miR-196b, *HOXA*, acute lymphoblastic leukemia.

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Introduction

MicroRNA (miRNA) were discovered to be small non-protein coding RNA molecules that post-transcriptionally regulate the expression of many protein-coding genes by complementary binding to their targeted mRNA.[1] Subsequently, the bound mRNA is cleaved or, in the majority of cases, its translation into protein is repressed.[2] Despite their name, miRNA play major roles in biological processes such as cell proliferation, differentiation and apoptosis and aberrant activities of miRNA have been found in a variety of malignancies. For example, let-7 has been identified as a tumor suppressor since let-7 down-regulates the expression of the oncoprotein Ras. Consequently, reduced expression of let-7 miRNA was associated with an increased expression of Ras in patients with lung cancer, which may explain their unfavorable prognosis.[3,4]

Recent studies also showed that aberrantly expressed miRNA contribute to hematopoietic malignancies. For example, over-expression of the oncogenic miR-17-92 polycistron accelerated the formation of lymphomas in an *Eμ-Myc* transgenic mice model.[5] This miR-17-92 cluster is often amplified in human B-cell lymphomas, suggesting a role in lymphomagenesis.[6] Enforced expression of miR-155 in *Eμ-miR-155* transgenic mice resulted in preleukemic pre-B-cell proliferation followed by mature B-cell leukemia, implying a leukemogenic contribution of miR-155.[7] Epigenetic silencing of miR-124a caused up-regulation of its target CDK6 in acute lymphoblastic leukemia (ALL), which may drive proliferation of leukemia cells, and is associated with higher relapse and mortality rates among patients with ALL.[8]

In general, the 5-year disease-free survival is 85% for children with ALL on contemporary treatment protocols. However, patients with some subtypes such as those with a rearrangement of the *Mixed Lineage Leukemia* (*MLL*) gene and *BCR-ABL1*-positive ALL have a much worse 5-year disease-free survival rate of approximately 50%.[9-11] We previously observed aberrant miRNA expression patterns in different genetic subtypes of pediatric ALL.[12] One of the miRNA that was most aberrantly expressed in *MLL*-rearranged cases was miR-196b. The expression level of miR-196b was up-regulated by 500- to 800-fold in the majority of cases of *MLL*-rearranged precursor B-ALL and in about one-third of selected T-ALL cases compared to precursor B-ALL patients without *MLL* translocations. In addition, the expression of miR-196b in these cases of leukemia was also higher than in normal bone marrow cells.[12] Interestingly, in a recent report it was postulated that miR-196b may be involved in leukemogenesis and that its expression is induced by normal MLL and MLL fusion products such as MLL-AF4.[13] The MLL-AF4 fusion is most frequently found in infants with *MLL*-rearranged leukemia,[14] who were included in our previous study.[12] MLL normally regulates the expression of the homeobox domain (*HOX*) gene family which plays an important role in regulating normal hematopoiesis.[15] The *HOXA* cluster genes, and especially *HOXA4*, *HOXA5*, *HOXA9* and *HOXA10*, are over-expressed in *MLL*-rearranged ALL.[16-18] However, aberrant expression of *HOXA* genes is not restricted to *MLL*-rearranged precursor B-ALL cases and has also been reported to occur in T-ALL patients carrying *MLL*- or *HOXA*-rearrangements e.g. inversion of chromosome 7, or fusion products including CALM-AF10 and SET-NUP214.[19,20] As *miR-196b* is mapped between the *HOXA9* and *HOXA10* genes on chromosome 7p15.2, the level of expression of miR-196b may be linked to *HOXA* gene transcription, irrespective of the *HOXA* activating mechanism (MLL fusions or other factors). To test this hypothesis, we measured the expression levels of miR-196b and *HOX* gene-family members in cases of *MLL*-rearranged and non-*MLL*-rearranged precursor B-ALL and T-ALL carrying different genetic abnormalities leading to *HOXA* gene activation. In addition, since the level of expression of various miRNA may be regulated by gene methylation,[21] we investigated the methylation status upstream of the *miR-196b* locus in *MLL*-rearranged cases. Since *MLL*-rearranged ALL cases are often highly resistant to prednisolone and L-asparaginase,[22] two drugs that form major components of current ALL treatment, we also investigated whether miR-196b expression levels were linked to responsiveness to these two drugs.

Design and Methods

Patients' samples

Leukemic cell samples from children with newly diagnosed ALL were obtained after informed consent from the children's parents or guardians and approval by the institutional review board. The immunophenotype of the samples was determined by flow cytometry (T-ALL or precursor B-ALL), and the genetic subtype by fluorescence *in situ* hybridization (FISH) and/or reverse transcriptase polymerase chain reaction (PCR).[11,20] In total 12 *MLL*-rearranged precursor B-ALL i.e. five patients carrying t(4;11), six patients carrying t(11;19) and one positive for t(9;11), 38 non-*MLL* precursor B-ALL and 22 T-ALL cases were included. The *HOXA*-linked T-ALL subgroup consisted of selected cases characterized by the fusion genes *MLL-AF6* (n=2), *CALM-AF10* (n=5) and *SET-NUP214* (n=3) as well as one case with an inversion of chromosome 7 [inv(7)(p15q35)]. The *HOXA*-negative T-ALL group consisted of *TAL/LMO*-rearranged (n=4), *TLX3*-rearranged (n=2) and T-ALL cases negative for the above-mentioned abnormalities (n=5). Mononuclear cells were isolated from bone marrow or peripheral blood samples using sucrose density centrifugation.[23,24] The percentage of leukemic cells was determined on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. If the percentage was below 90%, samples were enriched by eliminating non-malignant cells with immunomagnetic beads.[23,24]

Quantitative stem-loop real-time polymerase chain reaction analysis of miRNA and HOXA expression levels

Total RNA was extracted with TRIzol reagent (Invitrogen, Leek, the Netherlands) according to the manufacturer's guidelines with minor modifications as described before.[25] The 2100 bioanalyzer (Agilent, Amstelveen, the Netherlands) was used to determine the quality of total RNA. All RNA samples had an RNA integrity number of 7.5 or greater. MiR-196b expression was measured by real-time quantitative PCR (RT-qPCR) using a specific stem-loop primer and probe combination designed by Applied Biosystems, USA.[26] Endogenous small nucleolar RNA 1 (RNU24) was used as the reference for small RNA-input. The levels of expression of *HOXA3*, *HOXA9* and *HOXA10* transcripts were quantified relative to the level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using cDNA synthesized from total RNA, as described previously.[20] Primer and probe sequences are presented in *Online Supplementary Table S1*. All RT-qPCR were performed on an Applied Biosystems 7900HT system. Details on sample prepara-

tion, primers, probes and the real-time procedure are given in the *Online Supplementary Design and Methods*.

Gene expression microarray analysis

Affymetrix U133A and U133 plus 2.0 GeneChips (Santa Clara, CA, USA) were used to determine the expression of all *HOXA*, *HOXB* and *HOXC*-family genes in pediatric ALL cases, according to the manufacturer's guidelines. Data extraction and normalization procedures of the 22,283 probe sets that both arrays have in common have been extensively described elsewhere.[23] The data collected are part of a larger data set that has been deposited in NCBI's Gene Expression Omnibus (GEO)[27] and is accessible via GEO numbers GSE13351 and GSE13425.[23]

Assessment of methylation status

The methylation status of *miR-196b* and the *HOXA* cluster was assessed by the differential methylation hybridization (DMH) procedure using 244K CpG island microarrays (Agilent Technologies, Santa Clara, USA). The microarray labeling and hybridization procedures were performed according to Yan *et al.*[28] as described elsewhere.[29] The high-resolution microarrays contain 243,497 60-mer oligonucleotide probes, including numerous CpG island probes related to miRNA. For the present study, the probes containing multiple CpG islands located at chromosome 7p15 in the 5' promoter region of the *miR-196b* and *HOXA* cluster genes were used. A pool of genomic DNA derived from ten healthy individual (five males and five females, Promega Benelux BV, Leiden, the Netherlands) constituted a common reference. Data were extracted using Agilent Feature Extraction 9.5.3 software. Subsequently, the data were normalized and differential methylation assessed in the R and Bioconductor Statistical environment, as described elsewhere.[29] Methylation data are presented as ratios of the patient's signal divided by the common reference signal. For nine *MLL*-rearranged cases, both miRNA methylation and matching miRNA expression levels were measured. Unprocessed genome-wide DNA methylation data were uploaded in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE18400 as part of a previous study.[29]

Drug resistance assay

Responsiveness to prednisolone or L-asparaginase was determined by a 4-day *in vitro* methyl thiazolyl tetrazolium (MTT) drug resistance assay as described elsewhere.[30,31] The concentration ranges tested were 0.008-250 µg/mL for prednisolone and 0.003-10 IU/mL for L-asparaginase. The concentration of prednisolone or L-aspariginase that was lethal to 50% of the ALL cells (LC_{50}) was taken as a measure of the cellular drug resistance. LC_{50} values are known to be predictive of clinical outcome[30] and are used to adapt treatment regimens.[32,33]

Statistics

Differences in the distribution of variables between groups of patients were analyzed by the Mann-Whitney U test. Correlations between miRNA and mRNA levels were determined using the Spearman's correlation coefficient (Rs). *P* values were two-tailed and considered statistically significant when less than 0.05.

Results

The expression of miR-196b was measured in 72 pediatric ALL cases at diagnosis. Figure 1 shows that miR-196b was highly expressed in nine out of 12 *MLL*-rearranged ALL cases and in 14 out of 22 cases of T-ALL. In particular, all *CALM-AF10* (n=5), *MLL-AF6* (n=2), *SET-NUP214* (n=3) and inv(7) (n=1) positive T-ALL cases showed high levels of expression of miR-196b comparable to the levels found in *MLL*-rearranged cases (Figure 1). Since these specific chromosomal abnormalities are linked to the activation of *HOXA* genes[19,20] and since *miR-196b* is mapped between *HOXA9* and *HOXA10*, we quantified the expression of the *HOXA9* and *HOXA10* transcripts by RT-qPCR. A strong correlation between the level of expression of miR-196b and *HOXA9* and *HOXA10* expression ($Rs \geq 0.7$; $P \leq 0.005$) was found in *MLL*-rearranged precursor B-ALL as well as in cases of T-ALL (Figure 2). Figure 2 and *Online Supplementary Figure S1* illustrate that patients with low levels of expression of miR-196b (such as non-*MLL* precursor B-ALL and the majority of T-ALL cases) also had low expression of *HOXA* genes. In those cases in which the levels of miR-196b were higher (such as the majority of *MLL*-rearranged precursor B-ALL and one third of T-ALL cases), *HOXA* levels were also elevated. The levels of *HOXA9* and *HOXA10* were also significantly correlated with each other, as shown in Figure 2C and 2F ($Rs \geq 0.90$; $P<0.0001$). These data suggest co-expression of miR-196b with *HOXA* cluster genes. To determine whether this was only restricted to the two adjacent *HOXA* genes, we also investigated the expression pattern of other *HOX*-family genes using a second technique. i.e. the Affymetrix human genome microarray platform. The results confirmed the strong correlation between the level of expression of miR-196b and that of *HOXA9* ($Rs = 0.8$; $P \leq 0.0001$) and *HOXA10* ($0.8 \leq Rs \leq 0.9$; $P \leq 0.0001$) and also revealed that miR-196b levels correlated with the expression levels of nearly all other *HOXA* genes represented on the array platform ($0.5<Rs<0.8$, $P<0.05$, Figure 3A for all cases and *Online Supplementary Figures S2 and S3* for *MLL*-rearranged ALL and T-ALL cases, separately).

Whereas miR-196b is encoded within the *HOXA* cluster, another family member, miR-196a, which differs by only one nucleotide from miR-196b, is encoded by *miR-196a-1* located in the *HOXB* cluster (17q21.32) and *miR-196a-2* located in the *HOXC* cluster (12q13.13). The high homology between miR-196a and miR-196b may hamper the discriminative power of the stem-loop RT-qPCR procedure (and any other quantifying method) for determining solely miR-196b expression levels. However, no significant correlations were found between expression levels of miR-196b and those of members of the *HOXB* and *HOXC* cluster, except for *HOXB5*, *HOXB7*, *HOXB9*, and *HOXB13* (Figure 3B and 3C). Only one out of two and one out of three probe sets designed for *HOXB5* and *HOXB7*, respectively, showed a significant correlation with miR-196b ($0.4 \leq Rs \leq 0.6$; $P<0.05$, Figure 3B). These correlations were less significant and less strong than the association observed between expression levels of miR-196b and family members of the *HOXA*-cluster. This suggests that miR-196b and the *HOXA* cluster are more likely to be co-transcribed than miR-196b and *HOXB* or *HOXC* family genes.

Since the expression of miRNA genes and protein-coding genes can be affected by DNA methylation of promoter regions, we analyzed the methylation state of the 5' region upstream of *miR-196b* and the promoter region of all *HOXA* cluster genes. The methylation of the 5'region of *miR-196b* (Figure 4A) and of the entire *HOXA* cluster (Figure 4B) was reduced in *MLL*-rearranged cases compared to in cases of B-ALL without a *MLL*-translocation as well as in normal bone marrow ($P \leq 0.01$ and $P<0.05$, respectively), which may explain the increased expression

levels of both miR-196b and the *HOXA* cluster in these cases.

We also investigated whether the level of expression of miR-196b was linked to the sensitivity of the *MLL*-rearranged ALL and T-ALL patients to prednisolone and L-asparaginase, since resistance to these drugs is indicative of an unfavorable outcome.[30,31] Figure 5A and 5C show that the *in vitro* cytotoxicity (LC_{50} values) for both drugs did not differ between patients with high and low expression levels of miR-196b. In correspondence, patients who were sensitive, intermediately sensitive or resistant to prednisolone or L-asparaginase did not have significantly different levels of miR-196b (Figure 5B and 5D, $P>0.05$).

Discussion

In this study we demonstrated a strong association between the expression levels of miR-196b and genes belonging to the *HOXA* cluster in pediatric ALL. This co-transcription was not restricted to *MLL*-rearranged cases, but was also found for T-ALL cases characterized by activation of *HOXA* genes due to non-*MLL* mechanisms. Hypomethylation of CpG islands in the 5' upstream/promoter regions of miR-196b and *HOXA* cluster genes, as demonstrated in *MLL*-rearranged cases, may explain the high expression levels of this cluster and embedded miR-196b. *In vitro* resistance to prednisolone and L-asparaginase could not be explained by differential levels of miR-196b expression.

Popovic *et al.* reported that the expression of miR-196b is regulated by MLL and MLL fusion products.[13] In correspondence, we observed high-level expression in *MLL*-

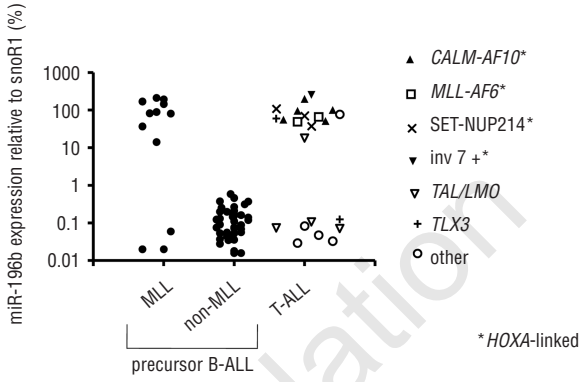

Figure 1. Expression of miR-196b in pediatric ALL. The miR-196b level was measured in leukemic cells of 12 cases of *MLL*-rearranged precursor B-ALL, 38 of non-*MLL* precursor B-ALL and 22 T-ALL. * Refers to T-ALL cases that have genetic aberrations that are associated with activation of *HOXA* cluster genes. Dots represent the individual miR-196b levels as a percentage of the expression level of the endogenous reference, snoRNA-1. *MLL*-rearranged *versus* non-*MLL* precursor B-ALL $P=0.003$; T-ALL *versus* non-*MLL* precursor B-ALL $P=0.001$.

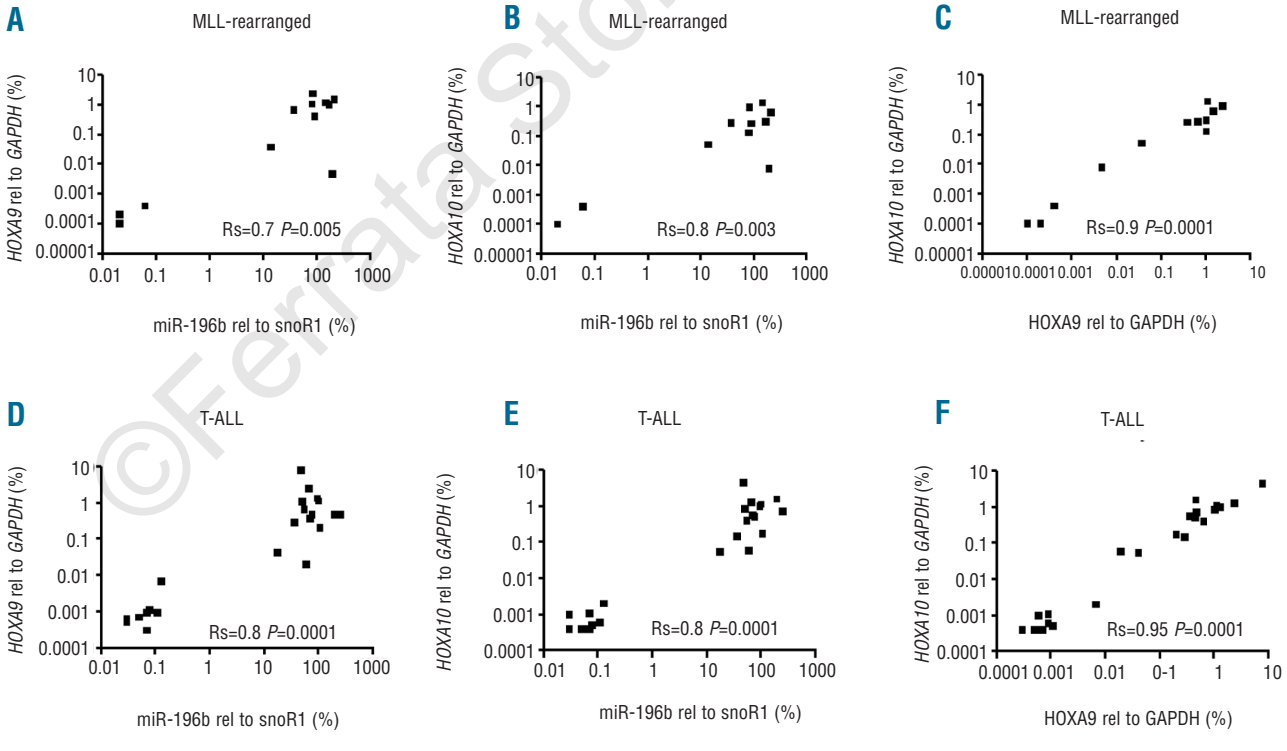

Figure 2. Correlation between expression levels of *miR-196b*, *HOXA9* and *HOXA10* in ALL patients. The expression levels are compared between miR-196b and *HOXA9* (A, D), between *miR-196b* and *HOXA10* (B, E), as well as between *HOXA9* and *HOXA10* (C, F) in 12 *MLL*-rearranged precursor patients (upper panel, A-C) and 22 T-ALL patients (lower panel D-F). The expression level of miR-196b is normalized for the expression level of snoRNA-1 as measured by quantitative stem-loop RT-qPCR whereas the expression of *HOXA9* and *HOXA10* transcripts is normalized for *GAPDH* mRNA expression levels as measured by quantitative RT-qPCR. Rs and *P* values are indicated in each panel.

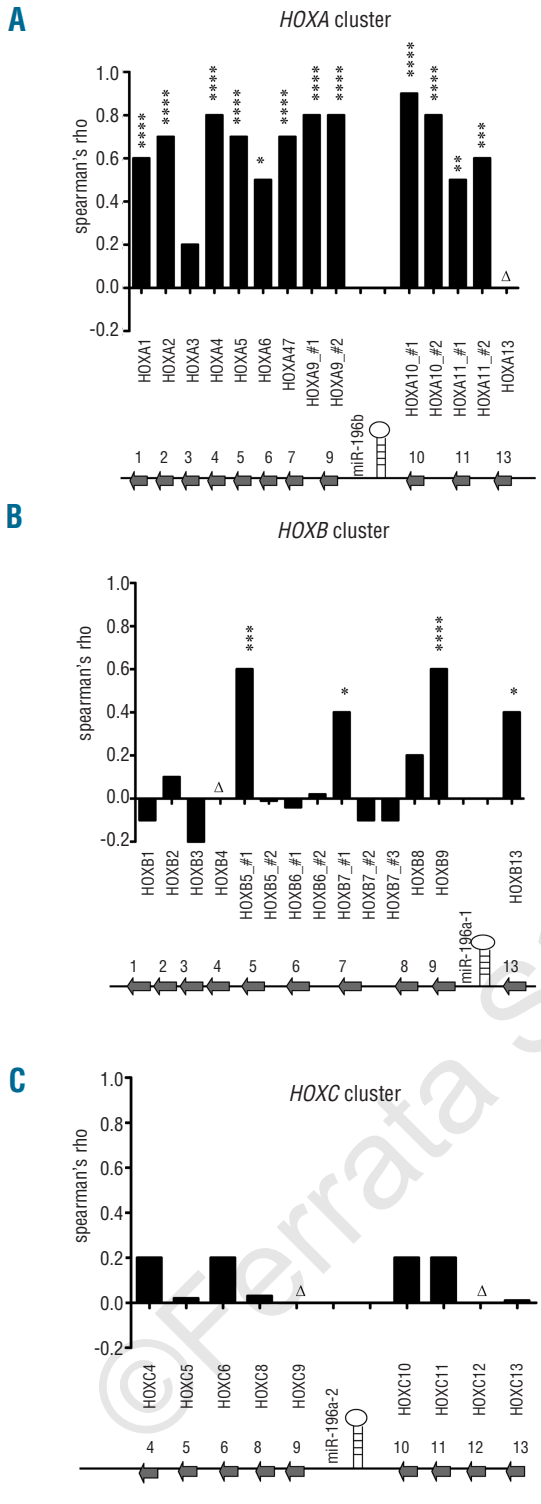

Figure 3. MiR-196b and *HOXA* cluster genes are co-transcribed in pediatric ALL. The expression levels of miR-196b were compared to the expression of different members of the *HOXA* (A), *HOXB* (B) and *HOXC* (C) cluster in 12 *MLL*-rearranged B-ALL patients and 18 T-ALL patients. Spearman's correlation coefficient was calculated and plotted as bars. # 1, 2 and 3 refer to the different probe sets for the specified genes on the Affymetrix U133A platform (*Online Supplementary Table S2*). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001. Genomic location of miR-196b on 7p15.2 within the *HOXA* cluster (A), miR-196a-1 on 17q21.32 within the *HOXB* cluster (B) and miR-196a-2 on 12q13.13 within the *HOXC* cluster (C) is indicated at the bottom of each graph. Δ indicates genes for which no probe sets were available on the U133A microarray.

rearranged cases. However, we demonstrated that high expression of miR-196b is not restricted to *MLL*-rearranged cases but can also be found in patients with other cytogenetic abnormalities that are known to activate *HOXA* cluster genes, i.e. *CALM-AF10*, *SET-NUP214* and inv(7)(p15q35). The mechanism by which the *HOXA* cluster is transcriptionally activated may differ between these patients. It has been demonstrated that CALM-AF10, SET-NUP214 and MLL fusions recruit the DOT1L histone methyltransferase that facilitates gene transcription of the *HOXA* cluster by dimethylation of histone H3 lysine 79 residues (H3K79). The H3K79 dimethylation possibly allows further epigenetic modification that opens up the entire *HOXA* locus.[20,34-36] We here demonstrate CpG island hypomethylation of the *HOXA* cluster in *MLL*-rearranged patients suggesting the existence of additional mechanisms driving *HOXA* expression. Moreover, inv(7) cases have elevated *HOXA10* and *HOXA11* expression due to the rearrangement of the T-cell receptor beta locus into this region of the *HOXA* cluster.[36] Taken together these findings indicate that high levels of expression of miR-196b and *HOXA* cluster genes are not exclusively *MLL*-driven but can also be due to other routes of *HOXA* locus activation. It should also be noted that not all *MLL*-rearranged cases have high expression levels of miR-196b

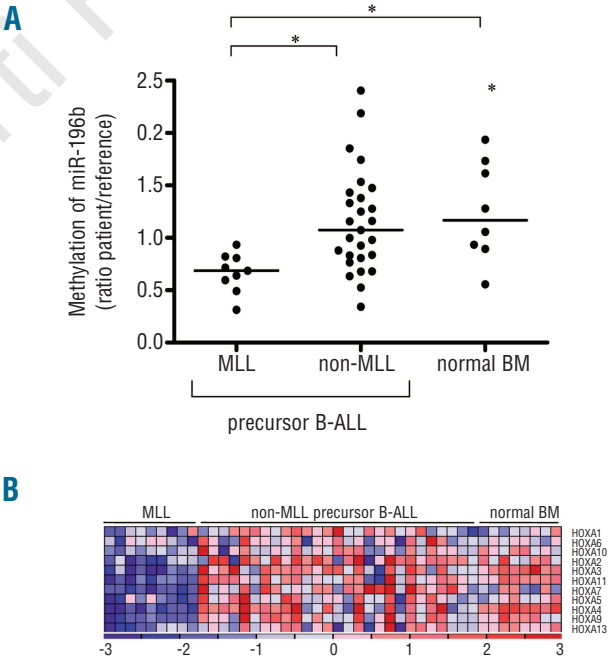

Figure 4. The promoters of *miR-196b* and *HOXA* genes have a lower level of methylation in *MLL*-rearranged precursor B-ALL patients than in non-*MLL* precursor B-ALL patients and normal bone marrow. (A) Methylation status of the probe covering multiple CpG islands (y-axis) within the 5' region of miR-196b (27,175,990-27,176,034) was analyzed in nine *MLL*-rearranged precursor B-ALL patients, 27 non-*MLL* precursor B-ALL and eight normal bone marrow (BM) samples. Dots represent individual patients **P*≤0.01. (B) Heat map displaying methylation status of the *HOXA* cluster in the same patients as for Figure 4A. Columns represent patients' samples and rows represent the different *HOXA* cluster genes. Relative DNA methylation levels are shown in red (high) and blue (low). Gene names are listed at the right. The heat map was generated in GenePattern version 3.1.2.[43] *MLL*-rearranged *versus* non-*MLL* precursor B-ALL, *P*<0.0001; *MLL*-rearranged precursor B-ALL *versus* normal BM, *P*<0.0002.

and *HOXA* cluster genes (Figures 1 and 2). This corresponds with the fact that two distinct subgroups, which are separated based on the expression signature of *HOXA* cluster genes, have been found in *MLL*-rearranged ALL.[17,37]

We demonstrated that the expression of miR-196b was strongly correlated with that of most members of the *HOXA* cluster (Figure 3) whereas there was a less pronounced correlation with the *HOXB* and *HOXC* cluster genes in pediatric ALL cells. *HOXA3* microarray-based expression levels did not correlate with miR-196b expression levels. This lack of correlation was due to a less optimal array-probe design for probe set 208604_s_at, exemplified by the fact that microarray and quantitative Taqman-based RT-qPCR data did not correlate for *HOXA3* whereas these data were highly correlated for other *HOXA* cluster genes such as *HOXA9* and *HOXA10* (*Online Supplementary Figure S4*). Since we cannot rule out the possibility of non-optimal design for other array-probes, correlations between miR-196b and additional *HOX* genes may have been missed. However, since the *miR-196b* gene is positioned between *HOXA9* and *HOXA10* and is transcribed from the same DNA strand as the *HOXA* cluster, the high co-expression between the *miRNA* and *HOXA* genes suggests co-transcriptional activation. Correspondingly, the expression levels of both miR-196b and *HOXA9* are restored upon re-expression of *Mll* in *Mll*-deficient mouse embryonic fibroblasts.[13] A similar co-activation may explain the strong association for miR-10a (positioned between *HOXB4* and *HOXB5*) as well as miR-196a (encoded between *HOXB9* and *HOXB13*) and the *HOXB* cluster as observed in acute myeloid leukemia.[38,39] Recent studies suggest that miRNA, in general, are often expressed at lower levels in cancer cells than in their normal counterparts.[40] In the case of ALL, this phenomenon may be caused by a high frequency of CpG island hypermethylation.[21] However, the fact that we observed that miR-196b and *HOXA*-genes are highly co-transcribed may indicate that this region has reduced DNA methylation. We demonstrated that the level of methylation of the CpG islands in the 5' region of miR-196b and in the promoter region of the entire *HOXA* cluster is lower in *MLL*-rearranged cases than in precursor B-ALL patients without *MLL*-rearrangements and in healthy individuals. Since *MLL*-rearranged ALL is characterized by hypermethylation of CpG islands across the genome,[29] the hypomethylation of the *miR-196b/HOXA* region is remarkable. Whether this locus displays a similar methylation status in *HOXA*-linked T-ALL cases needs to be explored.

Both *MLL*-rearranged and presumably *HOXA*-linked *CALM-AF10*-positive T-ALL patients have a poor clinical outcome.[11,41] It has previously been shown that both *MLL*-rearranged and T-ALL pediatric ALL cases are more resistant to prednisolone and L-asparaginase, as determined by *in vitro* drug cytotoxicity assays.[42] These two drugs are extensively used in the treatment of pediatric ALL and resistance to them is indicative of an unfavorable prognosis.[30] However, we did not find evidence that miR-196b contributes to resistance to these drugs since patients with high miR-196b expression were not more resistant to both drugs than patients with low miR-196b expression levels. In contrast to a role in drug responsiveness, miR-196b may have leukemogenic potential since ectopic expression of miR-196b resulted in increased proliferation and reduced differentiation of c-Kit$^+$ bone marrow cells of mice.[13]

In conclusion, we found that aberrant over-expression of miR-196b is not restricted to *MLL*-rearranged ALL cases (T-ALL or precursor B-ALL) but also occurs in T-ALL patients with other genetic abnormalities that activate the *HOXA* gene cluster. This observation is of great importance since miR-196b is known to have oncogenic activity[13] and may, therefore, play a role in the biology underly-

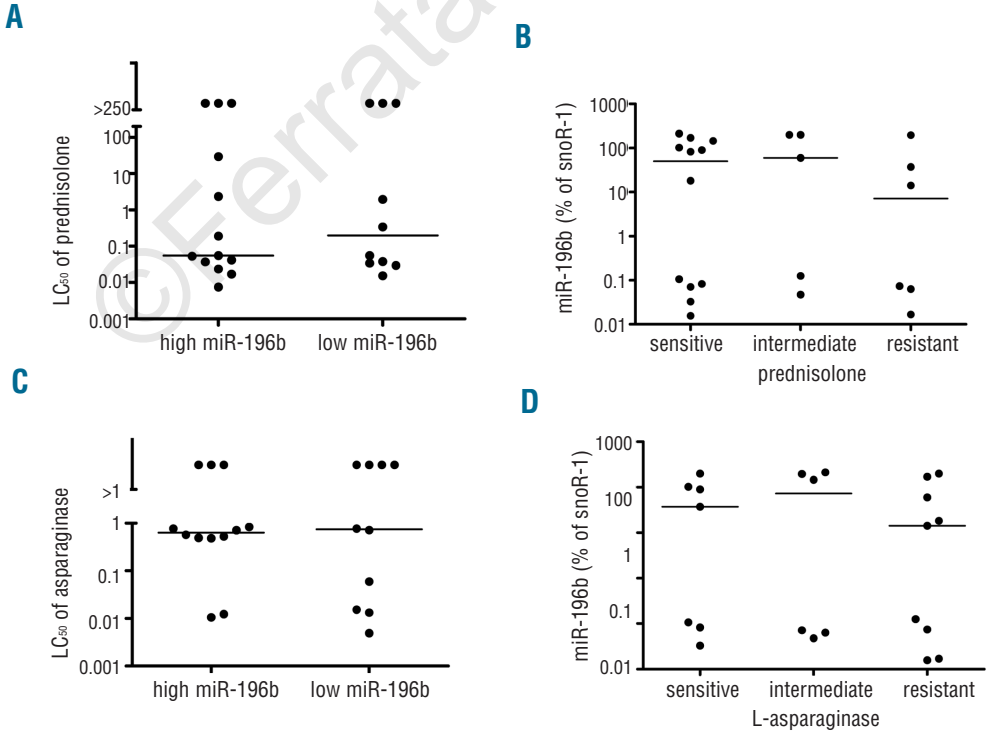

Figure 5. Expression levels of miR-196b are not associated with resistance to prednisolone and L-asparaginase in pediatric ALL cells. ***In vitro*** **cytotoxicity (represented by LC_{50} value) of prednisolone (A) and L-asparaginase (C) was measured in 11** ***MLL*****-rearranged precursor B-ALL and 11 T-ALL patients. Patients were separated according to their miR-196b expression level into two groups, i.e. low miR-196b (<1% of snoR-1) and high miR-196b (>1% of snoR-1). In B and D miR-196b expression is plotted against degree of resistance towards prednisolone (B) and L-asparaginase (D), i.e. sensitive, intermediately sensitive or resistant samples based upon previously established cut-off values.[30,44]** ***P*****>0.05 for all comparisons.**

ing *HOXA*-activated precursor B-ALL and T-ALL. The high expression of miR-196b has no effect on the level of cellular responsiveness to prednisolone and L-asparaginase in pediatric ALL. The role of miR-196b in leukemogenesis and survival of *HOXA*-expressing ALL deserves further studies since targeting miR-196b by 'antagomirs' reduced the proliferation capacity of *MLL*-rearranged bone marrow cells of mice.[13]

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