Oncogenic Potential of the miR-106-363 Cluster and Its Implication in Human T-Cell Leukemia

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Abstract

We previously reported the identification of the Kis2 common retrovirus integration site, located on mouse chromosome X, in radiation leukemia virus–induced T-cell leukemias. Tumors with a provirus at the Kis2 locus overexpressed a novel non-coding RNA (ncRNA) with a complex splicing pattern and no polyA tail. Database upgrade revealed the presence of a microRNA (miRNA) cluster, miR-106-363, just downstream of the Kis2 ncRNAs. We found that Kis2 ncRNAs are the primary miRNA of miR-106-363, and we present evidence that Kis2 ncRNA overexpression in mouse tumors results in miR-106a, miR-19b-2, miR-92-2, and miR-20b accumulation. We show the oncogenic potential of those miRNAs in anchorage independence assay and confirm primary miR-106-363 overexpression in 46% of human T-cell leukemias tested. This overexpression contributes in rising miR-92 and miR-19 levels, as this is the case for miR-17-92 cluster overexpression. Furthermore, we identified myosin regulatory light chain–interacting protein, retinoblastoma-binding protein 1-like, and possibly homeodomain-interacting protein kinase 3 as target genes of this miRNA cluster, which establishes a link between these genes and T-cell leukemia for the first time. [Cancer Res 2007;67(12):5699–707]

Introduction

In the last few years, the RNA world has been enlightened by the emergence of microRNAs (miRNAs). Those tiny RNAs of ~21 nucleotides are involved in the attenuation of translation by base pairing to the 3′-untranslated region (UTR) of their target mRNAs (see ref. 1 for review). These miRNAs are part of a long transcript, termed pri-miRNA, which are transcribed by the RNA polymerase II, capped, and polyadenylated (2). Nearly half of the pri-miRNAs are noncoding RNAs (ncRNAs). The miRNAs are occasionally part of an intron of the ncRNA. Most of the time, ncRNAs are spliced. Once the pri-miRNA is transcribed, it is processed in the nucleus by the RNase III Drosha into smaller RNA fragments called pre-miRNA (3). These are exported in the cytoplasm where they are processed by the RNase III enzyme Dicer to produce the miRNAs (4).

In humans, it is estimated that 20% to 30% of all the genes are targeted by miRNAs, with an average of 200 genes for each miRNA (5, 6). Because a single miRNA can target several genes, and because a gene could be targeted by different miRNAs, gene expression regulation by those tiny RNA seems to be a huge complex network.

Interestingly, miRNAs seem to be critical for cellular proliferation, differentiation, and apoptosis. A growing number of studies report the involvement of miRNAs in cancer and these are referred to as “oncomirs.” The first miRNAs implicated in cancer were miR-15a and miR-16-1, which are often deleted or down-regulated in B-cell chronic lymphocytic leukemia (7). Other examples came from miR-143 and miR-145, which are down-regulated in colorectal cancer (8). Another well-known miRNA implicated in cancer as a tumor suppressor is let-7, being down-regulated in human lung cancer (9). Indeed, Johnson et al. (10) showed that let-7 targets the key oncogene Ras both in Caenorhabditis elegans and in human cell lines. Furthermore, the human miRNA cluster miR-17-92 is amplified in large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and primary cutaneous B-cell lymphoma and acts as an oncogene collaborating with myc (11). Interestingly, inhibition of miR-17-92 in a cervical cancer cell line caused the up-regulation of the E2F1 oncogene (12). Thus, it seems that this miRNA cluster can also act as a tumor suppressor. Recently, miR-17-5p has been involved in breast cancer through targeting of the AIB1 oncogene (13).

In our laboratory, we use murine retroviruses as molecular bait to find oncogenes. One of our retrovirus tools is radiation leukemia virus (RadLV), which specifically induces T-leukemia. A few years ago, we found a common integration site of RadLV on mouse chromosome X called Kis2 and we reported the overexpression of an unknown gene located nearby in the rearranged tumors (14). This gene, called Kis2, contains three different transcription start sites (TSS) and produces at least five major spliced RNA species, all of which are over-expressed in the analyzed tumors. All the RNA species seemed to lack an open reading frame and were consequently classified as ncRNAs.

Recently, owing to the database update, we discovered that a cluster of six miRNAs (miR-106a-363) lies directly 3′-end to the Kis2 ncRNAs. Interestingly, this cluster is closely related to the miR-17-92 cluster located on mouse chromosome 14, which has been implicated in B-cell leukemia. Recently, this locus was shown to be targeted by the SL3-3 retrovirus, which induces mostly T-cell leukemia in mice (15). However, despite its high homology with the miR-17-92 cluster, the oncogenic potential of miR-106-363 has never been studied, and it has never been implicated in leukemia.

In this article, we report that Kis2 ncRNAs are the pri-miRNAs for the miR-106-363 cluster in mouse. We further provide strong evidence of their oncogenic potential and their implication in human leukemias. We finally found that myosin regulatory light chain–interacting protein (Myilp), retinoblastoma-binding protein 1-like (Rbp1-like), and possibly homeodomain-interacting protein kinase 3 (Hipk3) could be considered as target genes and consequently relate them for the first time to T-cell leukemia.

Materials and Methods

Cell culture. NIH/3T3 cells were maintained in DMEM supplemented with 10% calf serum (Invitrogen). Medium was changed every 3 days, avoiding cell confluence.
Human leukemias and normal individuals. Human leukemia cells from blood or bone marrow were obtained from the Banque de Cellules Leucémiques du Québec. Peripheral blood mononuclear cells (PBMC) used as control were obtained from four different adult blood samples. Adult thymus RNA was purchased from Sigma. All RNAs were extracted from cell samples with Trizol (Invitrogen) and submitted to a DNase treatment before reverse transcription-PCR (RT-PCR) analysis (Ambion).

**Anchor independence assay.** Anchor independence assay in soft agar was done as follows: NIH/3T3 cells were transfected with 10 nmol/L of either mmu-miR-106a, mmu-miR-19b, mmu-miR-92, mmu-miR-20b (Dharmacon), or a mix of the four miRNAs (mix assays at two different concentrations: 2.5 or 10 nmol/L of each miRNAs) using LipofectAMINE 2000 reagent (Invitrogen). Equal amount of an unrelated miRNA (Dharmacon) was used as a negative control. Bottom layer (0.6% agarose in DMEM) was poured into six-well plates, and 48 h after transfection, 10^3 cells were mixed with a top layer (0.5% agarose in DMEM) and poured over the bottom layer. The top layer was covered with 1.5 mL DMEM and changed twice weekly. After 3 weeks, colonies were counted. The experiment was done twice in triplicates (n = 6).

**Detection of miRNAs by Northern blot.** Total RNA was extracted from frozen normal and leukemic tissues with the Trizol reagent. For Northern analysis, 15 μg of total RNA were separated on a 12.5% polyacrylamide/8 mol/L urea gel and transferred to a nylon membrane (Osmonics) using a Transblot semidry apparatus (Bio-Rad). Hybridization was done as described (14). Probes included oligonucleotides complementary to mouse miR-106a, miR-20b, miR-19b, and miR-92, which were 32P labeled using the T4 polynucleotide kinase (New England Biolabs). After washing, membranes were revealed with a phosphorimager (Bio-Rad).

**Detection of miRNAs by padlock rolling circle amplification.** Padlock rolling circle amplification was done as described by Jonstrup et al. (16), with 0.5 μg of total RNA mixed with 2.5 fmol of P-pus padlock specific to either hsa-miR-106a, hsa-miR-19b, or hsa-miR-20b. U6 was done using FirstChoice RLM-RACE kit (Ambion). To isolate the 5’ ends of mouse pri-miR-106-363 (Kis2), reverse transcriptase was primed with the mir-18beta primer (5’-GCTCTAGAAGAACGCTGGCAGCAGCTTC-3’) and the nested PCR was done with the oligo B primer (5’-GACATGCACAAGGACACAGGAGCAGTTCC-3’). PCR program was 94°C for 2 min and 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 2 min followed by 10 min at 72°C. The primer used for 5’-RACE was Kis (5’-GAGTCTCTAAGGCAGCTGAATTCCTTAC-3’). Amplified products were cloned in pDrive vector for sequencing.

**Luciferase assay.** 3’-UTRs were amplified from mouse genomic DNA using High-Fidelity Taq Polymerase (New England Biolabs) and cloned downstream of the luciferase coding sequence (XbaI site) in the pGL3 control vector (Promega). Primers used to amplify 3’-UTR were the following: Dock4, 5’-GTCATCTTCTTATGCTGGCAT-3’ (forward) and 5’-CAATTCAGCAACAACTAGT-3’ (reverse); Rbp1-L, 5’-CACCCAGACTTTTGGCTAAAGC-3’ (forward) and 5’-CAGAGCCCTTTTGTTGTACCTTAC-3’ (reverse); Myl5, 5’-ATGCTGCCTGCATGCTGAGC-3’ (forward) and 5’- CCTTCAGCCTTATGGAAGC-3’ (reverse) and 5’-CATGCTCTGCTTGTTGTTGA-3’ (reverse); methyl CpG-binding protein 2 (Mecp2), 5’-GGTATTGTGTGTGCTTGCTGGAAGG-3’ (forward) and 5’-CAT- GTTCCTTCTTCTGCTTCTGTTGA-3’ (reverse) and Kruppel-like factor 12 (Klf12), 5’-GAAGGTTGTTGGTGTTGTTTGGTGTTGTT-3’ (forward) and 5’-GTTCATGGTGTGGTGTTTGGTGTTGTT-3’ (reverse).

**Detection of miRNAs by Northern blot.** Total RNA was extracted from frozen normal and leukemic tissues with the Trizol reagent. For Northern analysis, 15 μg of total RNA were separated on a 12.5% polyacrylamide/8 mol/L urea gel and transferred to a nylon membrane (Osmonics) using a Transblot semidry apparatus (Bio-Rad). Hybridization was done as described (14). Probes included oligonucleotides complementary to mouse miR-106a, miR-20b, miR-19b, and miR-92, which were 32P labeled using the T4 polynucleotide kinase (New England Biolabs). After washing, membranes were revealed with a phosphorimager (Bio-Rad).

**Results**

**Kis2 ncRNAs are the pri-miRNA for the miR-106-363 cluster.** Previously, we showed that the RadLVL/VL3 integration at the Kis2 locus (mouse chromosome X, 48,989,600–489,987,200 bp) gives rise to overexpression of several Kis2 ncRNAs (14). Although we were investigating the potential role of those ncRNAs in tumor development, a database upgrade allowed us to discover that six miRNAs lied directly downstream of the Kis2 ncRNAs: miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92-2, and miR-363 (Fig. 1A). Together, they constitute the miRNA cluster, called miR-106-363, which is highly homologous to the miR-17-92 cluster located on mouse chromosome 14 (Fig. 18). In particular, both miR-92-2 and miR-19b-2 from the miR-106a-363 cluster are identical to miR-92-1 and miR19b-1 from the miR-17-92 cluster. Although miR-106a, miR-20b, and miR-18b share high homology with miR-17-5p, miR-20a, and miR-18a from the miR-17-92 cluster, respectively, they are not identical and are thus specific to the miR-106-363 cluster.

We were first interested to determine if Kis2 ncRNAs could represent the pri-miRNA of this miRNA cluster. By doing 5’-RACE and 3’-RACE analysis with oligonucleotides used in the miRNA region, we indeed found the Kis2 TSS2 and TSS3 as 5’-end (Fig. 1C). We also found a new potential 5’-end, termed TSS4, although we did not observe the corresponding transcript by

### References


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The 3'-RACE analysis revealed that expressed sequence tag (EST) AI464896 was part of the 3'-end of the gene (Fig. 1A and C) and it contained the polyadenylation signal AATAAA. However, complete miR-106-363 pri-miRNAs were never observed in Northern blots, suggesting that they are rapidly and efficiently processed by Drosha. Therefore, the Kis2 RNA species observed on Northern blots are likely the result from Drosha processing. Thus far, we identified eight Kis2 ncRNA species, with...
five being overexpressed in analyzed tumors (Kis2 t1, Kis2 t2, Kis2 t3, Kis2 t4, and Kis2 t5; Fig. 1C) and easily detectable in Northern blot (14). The three other Kis2 RNA species (t3s, t6, and t7) could be detected by RT-PCR only.

**Overexpression of the miRNAs from the miR-106-363 cluster in Kis2-rearranged tumors.** We next verified the expression profile of the miR-106-363 cluster in RadLV-induced tumors and found some disparities in miR-106a, miR-20b, miR-19b-2, and miR-92-2 expression. The results showed that expression levels are not equivalent for each miRNA from the cluster. For example, miR-106a is clearly overexpressed in three tumors (139.1, 255.3, and 255.6) with a retroviral integration at the Kis2 locus (termed rearranged tumors), although slightly overexpressed in the two other rearranged tumors (k31.5 and 132.9; Fig. 2A and B). In contrast, the signal for miR-20b is very weak compared with the other miRNAs and seems only slightly overexpressed when compared with nonrearranged tumors, miR-92 and miR-19 accumulation due to Kis2 ncRNA overexpression is more difficult to appreciate because these miRNAs are also products of miR-17-92. Nevertheless, miR-92 seems overexpressed in three of five rearranged tumors (255.3, 255.6, and 139.1). For miR-19, the highest expression level is seen with tumors 255.6 and 139.1 when compared with nonrearranged tumors 159.2, k31.1, k20.2, and k24.2. The nonrearranged tumors 255.7 and 116.1 also express large levels of miR-19 but do not overexpress Kis2 ncRNAs (data not shown). This suggests that, in this case, high levels of miR-19 could be attributable to miR-17-92.

Moreover, no signals were observed for miR-18b and miR-363 (results not shown).

We conclude that the overexpression of Kis2 ncRNAs in mice following retroviral integration could result in high accumulation of miRNAs from the miR-106-363 cluster and, particularly, miR-106a. However, overexpression of both Kis2 ncRNAs and miRNAs is not always correlated.

The **miR-106-363 cluster induces anchorage independence.** Despite its close homology with the miR-17-92 cluster, the oncogenic potential of the miR-106-363 cluster has never been shown. To investigate the oncogenic potential of miR-106-363 cluster, we tested its ability to induce anchorage independence in NIH/3T3 cells. Cells transfected with either miR-106a, miR-20b, miR-19b-2, or miR-92-2 were submitted to a soft agar assay. After 3 weeks, colonies could be seen with each of the tested miRNA, whereas no colonies were observed in the negative control (Fig. 2C and D). Interestingly, the four tested miRNAs were not equivalent in inducing anchorage independence. We observed that miR-106a and miR-20b induced twice as much colonies than miR-92-2 or miR-19b-2 (Fig. 2C and D). It should be pointed out that, when all four miRNAs were transfected together, no increase in colony number was observed compared with each miRNAs transfected alone.

![Figure 2](cancerres.aacrjournals.org)
Oncogenic Potential of the miR-106-363 Cluster

We conclude that, in addition to miR-106a, miR-20b, miR-19b-2, and miR-92-2 overexpression in tumors, their capacity to induce anchorage independence growth strongly supports an oncogenic potential for those miRNAs.

Expression analysis of miR-106-363 cluster in human T-leukemias. We next wondered if the implication of the probe (miR-92-2, miR-20b, and miR-19b) was evaluated using the padlock approach and rolling circle amplification approach described by Jonstrup et al. (16). This technique has the advantage to discriminate between highly similar miRNAs, and it could be used quantitatively. Our results show that miR-106-363 overexpression was amplified compared with controls in 6 of 13 tumors. When we look at the expression of individual miRNAs, we found that miR-92-2 and miR-19b-2 were detected in all the tested tumors. These results are consistent with the fact that miR-92 and miR-19b are also products of the miR-17-92 cluster. When looking at the miR-17-92 expression, it seems more regularly expressed among both tumors and controls than the miR-106-363 cluster. Thus, miR-17-92 should be responsible for the miR-92 and miR-19b signals in all tumors where miR-106-363 is not detected. However, the signals of both miR-92-2 and miR-19b-2 seem stronger in those tumors that show miR-106-363 overexpression (e.g., LALT, LALT3b, LALT3j, or LAPTj1). This suggests that miR-106-363 overexpression contributes to the increase of miR-92 and miR-19b as well. No signal was detected for miR-20b.

Surprisingly, miR-106a was detected in only two of six tumors overexpressing miR-106-363. We expected an increased expression of miR-106a in those six tumors, as it was the strongest signal seen by Northern blot in mouse tumors (Fig. 2A). This result highlights the existence of a regulation for mature miRNA production as already mentioned by others (see Discussion). We conclude that overexpression of miR-106-363 very likely participates in the oncogenic process leading to human leukemias (as previously shown for miR-17-92). However, overexpression of the pri-miRNA of a miRNA cluster is not synonymous with overexpression of all the miRNAs produced from the cluster and careful examination of each tumor is required.

Identification of potential target for the miR-106-363 cluster. To find targets related to the miR-106-363 cluster in a tumoral context, we hypothesize that those miRNAs should exert their oncogenic potential by targeting tumor suppressor miRNAs. Because four miRNAs from the miR-106-363 cluster are expressed at the same time in our tumors, we focussed on targets common to miR-106a, miR-19b-2, miR-20b, and miR-92-2. We thought that each of them could target the same genes to cooperate for an efficient inhibition. We first adopted an in silico approach and worked with the prediction Web sites from the computational biology center of the Memorial Sloan-Kettering Cancer Center1 and TargetScan.2 Target predictions are based on the miRNA “seed,” which corresponds to nucleotide 2 to 8 starting from the miRNA 5′-end. Indeed, high-quality pairing of the miRNA seed seems to be important for stability of the miRISC complex (19, 20) as well as for biological function (21, 22). Based on this property, one should consider that any target prediction related to miR-106a should be envisaged for miR-20b as well, as they

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1 http://www.microrna.org
2 http://www.targetscan.org
both possess the same seed (Figs. 1 and 4). Among the common target predictions (146 genes predicted), we selected genes based on their known tumor suppressor activity or genes whose down-regulation could lead to tumor development (Table 1). Furthermore, we limited our choice in this study to the highest prediction scores and target sites perfectly conserved between human, mouse, and rat. We next tested the ability of all four miRNAs to target the 3′-UTR of the selected gene in a luciferase assay. We found that three of six tested genes showed significantly reduced luciferase activity: Mylip, Hipk3, and Rbp1-like (Fig. 5A). The latter was selected because of a high prediction score, although this transcript was not targeted in its 3′-UTR by miR-92-2. The three other genes (Mecp2, Klf12, and Dock4) did not seem to be targeted by miR-106a, miR-20b, miR-19b-2, or miR-92-2 in our assay (results not shown). In contrast to our predictions, not all four tested miRNAs could modulate gene expression (Fig. 4). In the case of Rbp1-like, only miR-106a/miR-20b exerted a significant inhibitory effect. This could be correlated with the presence of two target sites for miR-106a/miR-20b in its 3′-UTR (Fig. 4). Surprisingly, miR-19b-2 seemed to have no effect on Rbp1-like 3′-UTR despite the prediction of a target site (Fig. 4). As expected, no targeting by miR-92-2 was observed. All four miRNAs displayed an inhibitory effect on Mylip 3′-UTR (Fig. 5A). Similarly, miR-92-2 efficiently targeted Hipk3 3′-UTR, whereas miR-106a/miR-20b and miR-19b-2 seemed to have a lower but significant effect.

As already observed with the anchorage independence growth assay, no synergistic effect from miRNA targeting was observed on Mylip and Hipk3 3′-UTR when all four miRNAs were present.

Similar results were obtained with a more concentrated miRNA mixture in NIH/3T3 cells. To confirm our results from luciferase assays, we verified if endogenous Mylip, Rbp1-like, and Hipk3 protein levels could be affected in NIH/3T3 by the miR-106-363 cluster. Transfections of miR-106a and miR-20b clearly down-regulated levels of Mylip and Rbp1-like proteins (Fig. 5B). However, miR-92-2 and miR-19b-2 seemed to be less efficient in inhibiting endogenous Mylip production, although they seemed to have no effect on Rbp1-like production. This lack of effect of miR-92-2 and miR-19b-2 on Rbp1-like expression correlates with the results observed in our luciferase assay. Unfortunately, the Hipk3 protein could not be detected with commercial antibodies, and thus, miRNA inhibition could not be confirmed.

### Table 1. **miR-106-363** target genes predicted and tested in our luciferase assay

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dock4*</td>
<td>GTPase activator</td>
</tr>
<tr>
<td>Mylip</td>
<td>Identified as a neuron outgrowth inhibitor</td>
</tr>
<tr>
<td>Rbp1-like*</td>
<td>Inhibits cell proliferation</td>
</tr>
<tr>
<td>Hipk3</td>
<td>Fas/FADD-interacting serine/threonine kinase</td>
</tr>
<tr>
<td>Klf12</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>Mecp2</td>
<td>Methylation-mediated silencing</td>
</tr>
</tbody>
</table>

*Gene known as tumor suppressor.
confirmed at the protein level. However, the correlation between the luciferase results and Mylip and Rbp1-like protein levels strongly suggests that Hipk3 is likely to be a real target of miR-106-363.

According to our results, we conclude that Mylip and Rbp1-like are true targets of the miR-106-363 cluster. More specifically, Rbp1-like seemed only targeted by miR-106a/miR-20b, whereas Mylip could be targeted by the four miRNAs with miR-106a/miR-20b being more efficient.

**Mylip and Rbp1-like are down-regulated in RadLV tumors overexpressing miR-106-363 cluster.** We next decided to verify the level of Mylip and Rbp1-like proteins in our RadLV-induced tumors that overexpressed the miR-106-363 cluster. Western blot analysis for Mylip and Rbp1-like showed lower protein levels in those tumors that overexpressed the four miRNAs compared with negative tumors (Fig. 5C). As miR-106a is strongly overexpressed in the tumors, these results are consistent with our previous results that show a more efficient inhibition of Mylip and Rbp1-like with miR-106a/miR-20b (Fig. 5B). Interestingly, tumor 139.1, which showed the highest miRNA levels, also exhibited the lowest protein level for both Mylip and Rbp1-like (Fig. 24).

Targeting of Rb by miR-106a in human has already been reported (23). Because the miR-106a target site in Rb 3'-UTR is perfectly conserved between human and mouse, we also looked at the expression of Rb. We observed varying levels of protein among the tumors analyzed (Fig. 5C). Low levels of Rb were detected not only in those tumors overexpressing miR-106a but also in tumors that did not. Thus, if the Rb protein can be targeted by miR-106a as shown by others, our results show that miR106a overexpression is not always correlated with Rb reduction. As Rb is often down-regulated in tumors, miRNAs are probably not the only mechanism explaining a reduction in Rb protein level.

Figure 5. **A,** analysis of different 3'-UTRs in a luciferase assay. Targeting of the 3'-UTR of Mylip, Rbp1-like, and Hipk3 by miR-106a, miR-20b, miR-19b-2, and miR-92-2 was tested in a luciferase assay in NIH/3T3 cells. As negative control, the experiment was done with an unrelated miRNA. Each miRNA has been assessed individually or together (2.5 nmol/L mix). Experiment was done twice in triplicate. **B,** Western blot of the Rbp1-like and Mylip protein levels in NIH/3T3 cells transfected with either miR-106a, miR-19, miR-92, miR-20b, or a mix of the four miRNAs each at 2.5 nmol/L (mix) or with an unrelated miRNA (neg ctrl). Experiment was done in triplicate. **C,** Western blot of Mylip, Rbp1-like, and Rb protein levels in tumors overexpressing (*) or not the miR-106-363 cluster. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a loading control.
In summary, our results strongly suggest that MylIp and Rbp1-like RNAs are targeted by the miR-106-363 cluster in mouse tumors associated with its overexpression.

Discussion

In this article, we report that the overexpressed Kis2 ncRNAs resulting from the retroviral integration within the locus are the pri-miRNAs for the miR-106-363 cluster in mouse, thus revealing this cluster for the first time as an oncogene in T-leukemia. Indeed, we showed its ability to induce anchorage independence in NIH/3T3 cells and its overexpression in 46% of human T-cell leukemias analyzed.

Our results are also supported by reports indicating that miRNAs from the miR-106-363 cluster are overexpressed in some solid tumors (e.g., miR-106a and miR-92-2 in colon and prostate; ref. 23). Furthermore, miR-106a, miR-92, and miR-19 have recently been reported to be overexpressed in K562 and U937 leukemia cell lines (24). All together, these results reveal the oncogenic potential of the miR-106-363 cluster both in leukemias but also in other cancer types.

One of the most striking clues about the oncogenic abilities of the miR-106-363 cluster is its close homology with the miR-17-92 cluster and, particularly, miR-19 and miR-92, which are 100% identical in both clusters. Our results show that, whereas miR-17-92 seems to be constitutively expressed in normal cell, this is not the case for miR-106-363, which could be strikingly overexpressed in tumors, thus contributing in rising up miR-19 and miR-92 levels. As a consequence, when miR-92 or miR-19 is detected, one should consider not only the miR-17-92 implication but also the miR-106-363 implication. This contribution of two distinctively regulated loci in producing identical miRNAs has also been described for the miR-15/miR-16 cistron (25). Similar to our observations with miR-17-92 and miR-106-363, one locus expresses constitutively miR-15-16, whereas the other is induced during development only.

When miRNAs are not identical, they could share high homology (e.g., miR-17-5p and miR-106a or miR-106a and miR-20b). This redundancy between several miRNAs is not yet understood and has consequences on target predictions. To date, most predictions are based on 5' miRNA seed, which tends to be a common signature between several miRNAs (e.g., miR-17-5p, miR-106a, and miR-20b). Consequently, target predictions are nearly identical for those three miRNAs, and one could speculate that overexpression of the miR-106-363 cluster would have nearly the same effects than miR-17-92 overexpression. However, if highly similar miRNAs can share similar targets, it is likely that their small nucleotidic differences could confer some specificity to each miRNA in terms of targeting. Some studies showed clearly the importance of miRNAs 5'-end but also highlight modulatory effects from the 3'-end (21, 22).

Furthermore, some interesting questions arise from miRNA clusterization. Do those miRNAs collaborate in regulating the same gene? Alternatively, do they regulate different genes in identical or distinct pathways? Because cancer is a multistep process involving collaboration of several genes and pathways, miRNA clusters seem to be ideal oncogenic candidates. To date, most of the miRNAs implicated in cancer are indeed part of miRNA cluster [e.g., miR-15/miR-16 (7), miR-372/miR-373 (26), or miR-143/miR-145 (8)]. We show in our study that miR106a/miR-20b, miR-92, and miR-19 could commonly target distinct sites of the MylIp gene. However, in contrast to let-7 targeting of Ras, where Ras inhibition is proportional to the number of let-7 target sites (10), this "common targeting" does not seem to occur synergistically in our hands when the four miRNAs are present in the cell. Common targeting could operate at other levels, and each miRNA quantity could also be important.

In light of this, microarray expression analysis of miRNAs often revealed differential expression among miRNAs from clusters. Our results also revealed quantity differences between miRNAs produced from the miR-106-363 cluster, with miR-106a being not detected in a subset of human leukemias despite the presence of the pri-miRNA. In the same way, miR-363 and miR-18 are not detected in mice analyzed tumors, and miRNAs from the miR-106-363 cluster are not overexpressed at the same level. This highlights the emergent regulation hypothesis of miRNA cluster processing. This is shown in the recent study by Thomson et al. (25) who show that the processing of pri-miRNAs by Drosha is a key regulation step in miRNA production. The block could occur not only at the Drosha step, but each miRNA from a same cluster could be differentially processed.

As a miRNA could target a lot of genes at the same time, and as the overexpression of a miRNA cluster gives rise to accumulation of several miRNAs, it is difficult to identify important targets in tumorigenesis. A combination of targeting events by miRNA cluster is probably the more realistic view, and protein array technology is the better way to handle it. Nevertheless, we attempted to identify miR-106-363 targets among existing predictions that could be related to tumor development, and we found that MylIp, Rbp1-like, and most likely Hipk3 are target genes of this cluster.

Mylip is an ezrin-radixin-moesin (ERM)-like protein that interacts with myosin regulatory light chain and its only known function is to inhibit neurite outgrowth (27). However, ESTs of this gene are found in a broad variety of tissues, including thymus and lymph nodes. Its ERM domain retained our attention because of its close homology with the ERM domain of the tumor suppressor NF2/Merlin. ERM proteins, such as Merlin, link the actin cytoskeleton to membrane-bound proteins and are involved in regulation of cell motility, cell shape, contact inhibition, and cell attachment (28). We show here for the first time that Mylip represents a novel interesting candidate gene whose function as a tumor suppressor in human leukemias deserves to be investigated.

The Rbp1-like gene is already linked to cancer, although this information is still controversial. First, Rbp1-like is recognized by IgG antibodies isolated from a breast cancer patient and used as a marker (29). Second, it shares high homology with retinoblastoma-associated protein 1 (Rbp1), and both proteins are part of the mSin3A corepressor complex involved in histone deacetylation (30). Moreover, overexpression of either Rbp1 or Rbp1-like caused a profound inhibition of cell proliferation and induced expression of a senescence marker (31), thus conferring tumor suppressor activities to Rbp1-like. Our results about Rbp1-like targeting by miR-106a/miR-20b in tumors provide an additional clue to its potential tumor suppressor role. Furthermore, Rbp1-like targeting by miR-17-5p/miR-20a should be considered in tumors over-expressing this miRNA.

Finally, Hipk3 has been identified as a Fas-interacting kinase (32). Overexpressing Hipk3 in mammalian cells impaired Fas ligand–induced activation of Jun NH2-terminal kinase without affecting cell death. However, Möller et al. (33) reported that Hipk3 associates in the nucleus with its highly homologous tumor suppressor Hipk2 to
form the Hipk domains. We believe that it would be interesting to investigate the tumoral suppressor aspect of Hipk3 and further confirm its targeting by the miR-106-363 cluster.

In conclusion, our work highlights the importance of miR-106-363 cluster in leukemia and raises important questions about its close relationship to miR-17-92 cluster. In addition, it provides three new candidate genes with tumor suppressor potential, which should be considered as targets of the miR-17-92 cluster as well.

References

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