MiR-15a and miR-16-1 cluster functions in human leukemia

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Contributed by Carlo M. Croce, January 16, 2008 (sent for review December 6, 2007)

MicroRNAs (miRNAs) are short noncoding RNAs regulating gene expression that play roles in human diseases, including cancer. Each miRNA is predicted to regulate hundreds of transcripts, but only few have experimental validation. In chronic lymphocytic leukemia (CLL), the most common adult human leukemia, *miR-15a* and *miR-16-1* are lost or down-regulated in the majority of cases. After our previous work indicating a tumor suppressor function of *miR-15a/16-1* by targeting the *BCL2* oncogene, here, we produced a high-throughput profiling of genes modulated by *miR-15a/16-1* in a leukemic cell line model (MEG-01) and in primary CLL samples. By combining experimental and bioinformatics data, we identified a *miR-15a/16-1*-gene signature in leukemic cells. Among the components of the *miR-15a/16-1* signature, we observed a statistically significant enrichment in AU-rich elements (AREs). By examining the Gene Ontology (GO) database, a significant enrichment in cancer genes (such as *MCL1*, *BCL2*, *ETS1*, or *JUN*) that directly or indirectly affect apoptosis and cell cycle was found.

cancer | microRNA | targets

MicroRNAs (miRNAs) are short noncoding RNAs of ≈19–24 nt, that regulate gene expression by imperfect base-pairing with complementary sequences located mainly, but not exclusively, in the 3′ UTRs of target mRNAs. MiRNAs represent one of the major regulatory family of genes in eukaryotic cells by inducing translational repression and transcript degradation (1–4). Different algorithms such as TargetScan (5), PicTar (6), and Diana microT (7) have been developed to identify miRNA targets, but only few of these predictions have been experimentally validated, supporting the rationale for a combination of bioinformatics and biological strategies to this aim. Two independent studies predicted that 20–30% of human genes could be controlled by miRNAs (8, 9). Deviations from normal miRNA expression patterns play roles in human diseases, including cancer (for reviews see refs. 10–15).

The *miR-15a/16-1* cluster resides at chromosome 13q14.3, a genomic region frequently deleted in B cell chronic lymphocytic leukemias (CLLs), and the two members of the cluster are cotranscribed and down-regulated in the majority of CLL patients (16). CLL is a disease with a frequent association in families (10–20% of patients have at least one first-degree relative with CLL) (17). Previously, we identified germ-line or somatic mutations in several miRNAs (including *miR-16-1*) in ≈15% of CLL patients, with the majority of the patients having a known personal or family history of CLL or other hematopoietic and solid tumors (18). These findings, together with the identification of an abnormal *miR-15a/16-1* locus in the NZB strain of mice that naturally develop CLL (19), suggest that this cluster might play also a role in familial CLL.

Among the targets of *miR-15a* and *miR-16*, we identified the antiapoptotic protein Bcl2, which is overexpressed in the malignant, mostly nondividing B cells of CLL (20), and in many solid and hematologic malignancies (21). Restoration of miR-15-a/16-1 induces apoptosis in MEG-01, a cell line derived from acute megakaryocytic leukemia (22). These data support a role for *miR-15a* and *miR-16-1* as tumor-suppressor genes (TSGs) in CLLs and perhaps in other malignancies in which these genes are lost or down-regulated.

Here, to investigate the mechanism of action of *miR-15a* and *miR-16-1* as tumor suppressors in leukemias, we analyzed the effects of *miR-15a* and *miR-16-1* on transcriptome and proteome in MEG-01 leukemic cells. This approach allowed us to validate a number of target genes, whose expression was also investigated in cases of CLL.

Results

***In Vivo* Effects of *miR-15a/miR-16-1* Transfection into MEG-01 Leukemic Cells.** We reported that *miR-15a/16-1* cluster induces apoptosis of MEG-01 cells by activating the intrinsic apoptosis pathway as identified by activation of the APAF-1–caspase9–PARP pathway (22). To further investigate the effect of these miRNAs, we tested their tumor-suppression function *in vivo*. Ten million viable MEG-01 cells, transfected *in vitro* with pRS15/16, pRS-E, or mock transfected, were inoculated s.c. in the flanks of immunocompromised "nude" mice (5 per group). As shown in Fig. 1*A*, the *miR-15a/16-1* cluster inhibits the growth of MEG-01 tumor engraftments. After 28 days, tumor growth was completely suppressed in three of five (60%) mice inoculated with *miR-15a/16-1*-transfected MEG-01 (Fig. 1*B*). At day 28, the average tumor weights for the untreated and empty vector-treated mice were 0.95 ± 0.5 g and 0.58 ± 0.39 g, respectively; in mice inoculated with *miR-15a/16-1*-treated cells, the average was 0.020 ± 0.01 g ($P < 0.003$) (Fig. 1*C*). Thus, the results of these experiments demonstrate the tumor-suppressor function of *miR-15a/16-1* cluster in MEG-01 leukemia cells.

Transcriptional Effects of Exogenous Expression of *miR-15a* and *mirR-16-1*. To characterize the molecular basis of *miR-15a/16-1* tumor suppression in leukemias, we first investigated the effect of miRNAs

Author contributions: G.A.C., A.C., and M. Fabbri contributed equally to this work; G.A.C. and C.M.C. designed research; G.A.C., A.C., M. Fabbri, M. Ferracin, S.E.W., M.S., C.T., N.Z., R.G., R.I.A., H.A., X.L., C.-g.L., T.J.K., and M.N. performed research; L.R. contributed new reagents/analytic tools; G.A.C., A.C., M. Fabbri, M. Ferracin, C.T., S.V., T.J.K., M.N., and C.M.C. analyzed data; and G.A.C., A.C., M. Fabbri, and C.M.C. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The microarray data were submitted to the Minimum Information About a Microarray Experiment (MIAME) Database (accession no. E-MEXP-1482).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0800121105/DC1.

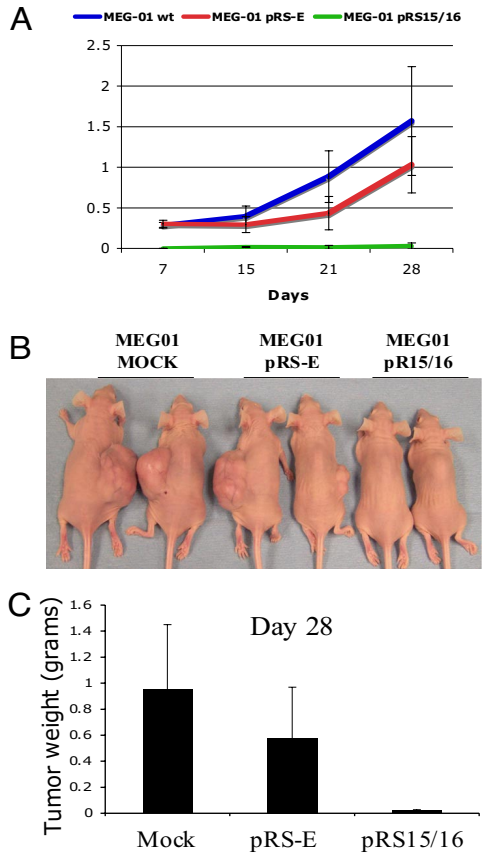

Fig. 1. *MiR15a/16-1* cluster inhibits the growth of MEG-01 tumor engraftments in nude mice. (*A*) Growth curve of engrafted tumors in nude mice injected with MEG-01 cells pretransfected with pRS-E or pRS15/16 or mock transfected. (*B*) Comparison of tumor engraftment sizes of mock-, pRS-E-, and pRS15/16-transfected MEG-01 cells 28 days after injection in nude mice. (*C*) Tumor weights ± SD in nude mice.

on genome-wide transcription of protein-coding genes. We transiently transfected the pRS15/16 vector into MEG-01 cells. This vector contains a genomic region encoding for both miRNAs as described (22). Transfection with the empty vector (pRS-E) was used as control. The success of transfection was assessed by measuring the expression levels of *miR-15a*, and *miR-16-1* by quantitative (q)RT-PCR as described in ref. 18 (data not shown). Genome-wide transcriptome was investigated by using Affymetrix microarray. The microarray analysis clearly shows a different pattern of gene expression among pRS15/16- and pRS-E-transfected cells [supporting information (SI) Fig. 3]. After transfection with *miR-15a/16-1* cluster, 355 probes (265 genes) were significantly up-regulated and 5,304 probes (3,307 genes) down-regulated (SI Table 5). The cluster analysis, performed with the differentially expressed genes, shows a clearly distinct gene expression profile between pRS15/16- and pRS-E-transfected cells (SI Fig. 3). Among the down-regulated probes, 140 (85 genes) are predicted as targets of miR-15/16 by three of the most used software algorithms (TargetScan, PicTar, and MiRanda), that are built on different prediction criteria and, therefore, used in combination, give the highest probability of target identification. If we consider only one prediction program, we found that 370, 332, and 312 transcripts, respectively, are predicted to be direct targets of these miRNAs (SI Fig. 3, SI Table 6). Among the up-regulated genes, there are no commonly predicted targets. Therefore, the *miR-15a/16-1* cluster seems to regulate, directly or indirectly, ≈14% (265 genes up- and 3,307 down-regulated) of the 25,000 total predicted genes in the human genome (23) (SI Fig. 4).

Table 1. Cluster distribution of ARE-mRNAs deregulated in MEG-01 cells after *miR-15a/16-1* cluster transfection

ARE cluster	Up-regulated genes, *n* (%)	Down-regulated genes, *n* (%)
I	1 (2.8)	4 (0.6)
II	0 (0)	8 (1.2)
III	5 (13.9)	76 (11.4)
IV	4 (11.1)	84 (12.6)
V	26 (72.2)	494 (74.2)

AU-Rich Elements (AREs) Are More Frequently Found Among *miR-15a/miR-16-1* Down-Regulated Genes, in MEG-01. Because for *miR-16-1* both a direct interaction in the "seed" region of the target mRNAs (22) and an ARE-mediated mRNA instability (24) have been reported, we investigated the frequency of ARE-containing mRNAs among the *miR-15a/16-1*-deregulated transcripts. As shown in SI Table 7, the number of genes containing AREs in their 3′ UTR was 36 of 265 (13.6%) up-regulated genes, and 666 of 3,307 (20.1%) among the down-regulated genes. This difference was statistically significant, with a χ^2 value of 6.674 ($P = 0.0098$). Among the 85 genes that are predicted targets of *miR-15a/16-1*, 28 (32.9%) contain AREs, whereas among the remaining 3,222 down-regulated genes that are not commonly predicted targets, 638 (19.8%) mRNAs contain AREs (χ^2 value = 8.89, $P = 0.003$). According to the number of motifs in the ARE stretch, the ARE-mRNAs can be clustered into five groups, containing five (cluster I), four (cluster II), three (cluster III), and two (cluster IV) pentameric repeats, whereas cluster V contains only one pentamer within the 13-bp ARE pattern as described (25). The ARE-cluster distribution of the *miR-15a/16-1* deregulated genes is shown in Table 1. These results indicate that AREs are more frequently found among down-regulated targets of *miR-15a/16-1*, especially the commonly predicted targets, further confirming the influence of AREs in miR-16 targeting.

Gene Ontology (GO) of Genes Deregulated by *miR-15a/16-1* Cluster. Genes found to be differentially expressed in MEG-01 cells after transfection with pRS15/16 versus pRS-E were analyzed with the GeneSpring Gene Ontology browser tool to identify the Gene Ontology categories most represented in down-regulated genes (Table 2 and SI Table 8). These results show that the *miR-15a/16-1* cluster directly or indirectly affects the expression of many cell cycle-related genes. In particular, many genes involved in the different transition checkpoints of the cell cycle are targeted by the

Table 2. Most significant GO categories after *miR-15a/16-1* cluster transfection in MEG-01 cells.

GO ID	GO description	*P**
GO:7049	Cell cycle	2.7E-13
GO:278	Mitotic cell cycle	1.7E-12
GO:87	M phase of mitotic cell cycle	1.5E-11
GO:7067	Mitosis	1.6E-10
GO:51301	Cell division	2.7E-10
GO:75	Cell cycle checkpoint	1.8E-05
GO:82	G_1/S transition of mitotic cell cycle	3.2E-04
GO:7095	Mitotic G_2 checkpoint	2.2E-03
GO:6916	Antiapoptosis	4.4E-03
GO:31575	G_1/S transition checkpoint	4.8E-03
GO:31572	G_2/M transition DNA damage checkpoint	8.6E-03
GO:31576	G_2/M transition checkpoint	8.6E-03
GO:43069	Negative regulation of programmed cell death	1.3E-02
GO:43066	Negative regulation of apoptosis	1.7E-02

**P* values establish whether there is a significant enrichment of down-regulated genes belonging to that GO category when compared with all genes present on the arrays.

Table 3. Examples of proteins down-regulated by the *miR-15a/16-1* cluster identified by proteomics in MEG-01 cells

Group	Protein	Gene description	*Z*-Score	Comments
Cell growth & cell cycle	Ruvbl1	RuvB-like 1; TATA binding protein interacting protein 49 KDa	2.01	—
	Sugt1	Suppressor of G2 allele of SKP1	2.43	—
	Cdc2	Cell division cycle 2, G_1 to S and G_2 to M	2.43	—
	Psf1	GINS complex subunit 1 (Psf1 homolog)	2.43	—
Antiapoptotic	Grp78	Heat shock 70-kDa protein 5 (glucose-related protein, 78 kDa)	2.43	—
	Bcl2	B-cell CLL/lymphoma 2	2.43	Predicted and validated target of miR-15a/16 (22)
	Pdia2	Protein disulfide isomerase family A, member 2	2.43	—
Oncogenesis	Wt1	Wilms tumor 1	2.43	Predicted target of miR-15a/16; Validated qRT-PCR in MEG-01
	MageB3	Melanoma antigen family B, 3	2.43	—
	Rab9B	RAB9B member RAS oncogene family	2.16	Predicted target of miR-15a/16
Others	Cdh26	Cadherin-like 26	2.43	—
	Crhbp	Corticotropin releasing hormone-binding protein	2.43	Predicted target of miR-16
	Actr1A	ARP1 actin-related protein 1 homolog A, centractin alpha	2.43	Predicted target of miR-15a/16
	Cshl1	Chorionic somatomammotropin hormone-like 1 precursor	2.43	Predicted target of miR-16
	Hla-B	Major histocompatibility complex, class I, B	2.43	—
	Tpi1	Triosephosphate isomerase 1	2.43	Predicted target of miR-15a/16
	Hsp90AB1	Heat shock protein 90-kD protein 1, β	2.43	—
	Cfl2	Cofilin 2	1.72	Predicted target of miR-16
	AldoA	Aldolase A, fructose-bisphosphate	2.43	—

For complete version, see SI Table 9. *Z*-score, probability of identification of the protein (2.43 = 99%, 1.28 = 90%).

miRNAs. Consistent with our previous finding that BCL2 is a target of *miR-15a/16-1*, in this GO ontology analysis, the category "antiapoptosis" (GO:6916) is significantly represented among the down-regulated transcripts.

Effect of *miR-15a* and *miR-16-1* on MEG-01 Proteome. Because both transcriptional and translational levels of miRNA-dependent gene regulation have been described (26), to investigate the effects of *miR-15a/16-1* on MEG-01 cells at the protein level, we analyzed the proteins differentially expressed between MEG-01 cells transfected with pSR15/16 or pRS-E vector 48 h after transfection. By proteomics analysis, we identified proteins whose intensity was reduced 4-fold or more in the pRS15/16 group with respect to the pRS-E group. We isolated 27 different proteins (Table 3 and SI Table 9). Interestingly, *BCL2*, which we had already shown as a target of *miR-15a/16-1* (22), and *WT1*, another predicted target of these miRNAs, were identified. The targeted proteins have a variety of biological functions and can be grouped into four groups. The first group includes proteins that play a role in regulation of cell growth and cell cycle (Ruvbl1, Anxa2, Rcn1, Cct7, Sugt1, Cdc2, Psf1), another category is formed by antiapoptotic proteins (Grp78, Bcl2, Pdia2), and proteins involved in human tumorigenesis, either as oncogenes, or as tumor-suppressor genes (Wt1, MageB3, Rab9B). The remaining 14 proteins have different biological functions, and we identified them as "others." Among the 27 experimentally identified down-regulated proteins, 8 (29.6%) are predicted targets of *miR-15a*/16 by at least one of the prediction algorithms. Finally, among this group of eight proteins, two (Bcl2, and Cfl2) were present also in the group of down-regulated mRNAs.

Validation of the Results in the MEG-01 Cell Line. To validate the results obtained by transcriptomic or proteomic analyses, we assayed the expression of nine genes (four identified by the EST microarray, two by proteomics, and three identified by neither of the techniques and therefore considered as negative controls), by qRT-PCR in MEG-01 cells transfected with pRS15/16 or pRS-E (control). As shown in Fig. 2*A*, the transfection with *miR-15a/16-1* reduces the expression of both microarray identified mRNAs (*PDCD4*, *RAB21*, *IGSF4*, *SCAP2*) and proteomics identified proteins (Bcl2, Wt1). *MiR-15a/16-1* transfection does not affect the expression of any of the control genes (*IGF1*, *ACE*, and *ERBB2*).

We also performed the luciferase assay on one of the validated genes (*IGSF4*) and demonstrated that the *miR-15a/16-1* cluster directly targets *IGSF4* (Fig. 2*B*). The direct interactions with *BCL2* and DMTF1 were proved by us and others (7, 22). Therefore, we were able to consistently confirm the MEG-01 profile of down-regulated genes and identified another direct target of *miR-15a/16-1* in this leukemic model.

Variation of Expression of *miR-15a/mir 16-1* Targets in Primary CLLs. Because MEG-01 is a leukemia cell model with abnormal 13q14 and loss of the miR15a/16-1 cluster (similar to CLL) but is a megakaryocytic established leukemic cell line, we decided to investigate the effects of the different expression of *miR-15a/16-1* cluster also in primary CLLs. Therefore, to verify whether some of the targets of *miR-15a/16-1* identified in MEG-01 cells were inversely correlated to the expression of these two miRNAs in CLL patients, we selected a group of 16 CLL samples in whom the expression of *miR-15a/16-1* had already been determined by miRNA microarray analysis in our previous studies (18, 27). We have shown that a signature of 13 miRNAs distinguished between indolent and aggressive CLL and that loss of the *miR-15a/16-1* cluster is a characteristic of indolent CLLs (18). First, we validated the expression of *miR-15a/16-1* by qRT-PCR and confirmed the microarray data by qRT-PCR (data not shown). Among the considered 16 patients, 8 have higher expression of *miR-15a/16-1*, with respect to the other 8 patients ($P = 7.7 \times 10^{-6}$ at microarray analysis, $P = 0.019$ at qRT-PCR analysis). The comparison between eight CLLs with high and low *miR-15a/16-1* expression by EST oligonucleotide microarray analysis showed 678 Affymetrix probes (539 genes) significantly differentially expressed among the two groups (SI Table 10). Overall, 82 of 539 genes (15.2%) are ARE mRNAs, and 4 are predicted as targets by all three bioinformatics algorithms.

A Signature of *miR-15a/16-1* Down-Regulated Transcripts. We selected genes that were low in miR-15/16 high-expressor CLLs and high in miR-15/16 low-expressor CLLs, which were intersected with

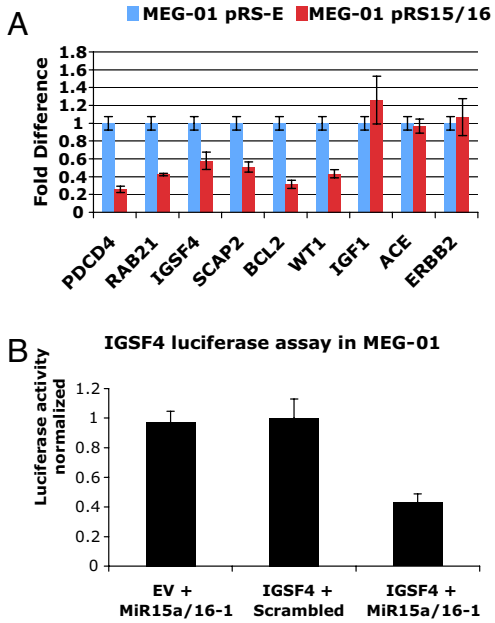

Fig. 2. Validation of some of the targets of *miR-15a/16-1* identified by microarray or proteomics in MEG-01. (*A*) qRT-PCR validation of *PDCD4*, *RAB21*, *IGSF4*, *SCAP2* (down-regulated in the microarray), *BCL2*, and *WT1* (down-regulated in proteomics). *IFG1*, *ACE*, and *ERBB2* are negative controls. The results were normalized to pRS-E-transfected cells. Samples were normalized with β-tubulin. (*B*) Luciferase assay of *IGSF4* in MEG-01 cells, showing that the *miR-15a/16-1* cluster directly targets this gene.

genes down-regulated in MEG-01 cells after transfection with pRS15/16. A signature of 60 genes (70 probes) emerged (Table 4 and SI Table 11). Thirteen of these genes (21.7%) are ARE-mRNAs, distributed in cluster III (7.8%), IV (7.8%), and V (84.6%). No statistically significant enrichment in ARE-mRNAs was observed in this signature with respect to both the total of down-regulated mRNAs in MEG-01 ($P = 0.76$) and the total of repressed transcripts in patients with high expression of *miR-15a/16-1* ($P = 0.14$). We performed the GO analysis of these 70 transcripts and found, among the significantly represented categories, some of those previously identified in transfected MEG-01 and involved in regulation of cell cycle and apoptosis, such as "antiapoptosis" (GO:6916), "negative regulation of apoptosis" (GO:43066), and "negative regulation of programmed cell death" (GO:43069) (SI Table 12). The consistency of the results in MEG-01 and in CLL patients confirms the validity of our *in vitro* model and identifies GO categories and a panel of protein coding genes, whose expression is consistently controlled by the cluster.

Discussion

In this study, we show that *miR-15a/16-1* exert a tumor suppressor function *in vivo* by inhibiting the growth of tumor engraftments of leukemic cells in nude mice. To investigate the molecular bases of *miR-15a/16-1* tumor-suppressor function, we performed an extensive microarray analysis of the deregulated genes after transfection of MEG-01 cells with pRS15/16, a vector expressing *miR-15a/16-1*, and using the same empty vector (pRS-E) as a control. Interestingly, we confirmed some of the targets observed by other groups in different models, such as *CDK6*, *CDC27*, and *RAB11FIP2* (28) in solid tumor cell lines and *ACVR2A* in *Xenopus laevis* (29). We matched our experimentally identified down-regulated genes with the targets of *miR-15a/16-1* commonly predicted by three of the most widely used algorithms for the identification of miRNA-targets (PicTar, TargetScan, MiRanda), and found 85 genes (2.6%) in common. Interestingly, by matching our results with a computational method that identifies miRNA targets by predicting miRNA regulatory modules (MRMs) or groups of miRNAs and target genes that are believed to participate cooperatively in posttranscriptional gene regulation (30), we found 5 of 13 (38.5%) miR-15/16 MRM predicted genes (*ATP2B1*, *FBXW7*, *PPM1D*, *SON*, and *WT1*) among our differentially expressed genes. This percentage represents the highest among all of the considered prediction algorithms. As expected, among the 265 experimentally up-regulated mRNAs, none is predicted as target of *miR-15a*/16. This finding could be explained by indirect effects, for example by the regulation of transcription factor(s) targeted by these two miRNAs. The effects of the exogenous expression of *miR-15a/16-1* in MEG-01 cells was also investigated by proteomics 48 h after the transfection. We also studied different time-from-transfection intervals to analyze the effects of *miR-15a/16-1* at a transcriptional (24 h) or translational (48 h) level, because after 24 h, mRNA silencing is maximal, but secondary transcriptional effects due to protein depletion are minimal (31). Our proteomic approach was able to detect 27 targets of *miR-15a/16-1*, approximately one-third of which are also predicted targets. Interestingly, 25% (two of eight) of the predicted targets were down-regulated both in the transcriptome and in the proteome. Among the *miR-15a/16-1* down-regulated genes, we demonstrated that *IGSF4* is a direct target of the cluster. *IGSF4* was originally identified as a tumor-suppressor gene in lung cancer and is involved in cell adhesion (32, 33). Sasaki *et al.* (34) have demonstrated that *TSLC1/IGSF4* acts as an oncoprotein involved in the development and progression of adult T cell leukemia (ATL). It can be hypothesized that by directly silencing *IGSF4*, *miR-15a/16-1* could exert a more general antileukemic effect.

We also studied by microarray the down-regulated mRNAs in eight CLL patients with high levels of *miR-15a/16-1* with respect to eight CLL patients with low levels of these two miRNAs and identified a signature of 60 genes in common between CLLs and MEG-01 transfected with *miR-15a/16-1*. This signature (which includes ≈2% of the down-regulated genes in MEG-01 and ≈11% of those repressed in patients) contains oncogenes such as *MCL1*, *JUN*, *SCAP2*, *TRA1*, *PDCD6IP*, *RAD51C*, and *HSPA1A*/1B, which could explain the oncosuppressor effect of *miR-15a/16-1* observed in MEG-01 both *in vitro* (22),and *in vivo* (present work). *MCL1* is an antiapoptotic *BCL-2* family member that contributes to B cell survival in CLL and has been associated with resistance to chemotherapy (35, 36). Despite the fact that *MCL-1* expression is not different in ZAP 70-positive (aggressive) vs. ZAP 70-negative (indolent) B-CLL cells (37), it represents a relevant therapeutic target in both acute and chronic lymphoid malignancies, because its silencing is sufficient to promote apoptosis in ALL and CLL cells and increase sensitivity to rituximab-mediated apoptosis (38). Interestingly, *miR-29b* has also been identified to target Mcl1 in a cholangiocarcinoma model (39), and many pieces of evidence converge in defining a role of the *miR-29* family as TSGs in both solid (40) and hematologic malignancies (41). Our findings give a rationale to an association of *miR-15a/16-1* and *miR-29s* in the treatment of CLL. Moreover, a sustained signaling through the B cell receptor promotes survival of B-CLL cells both by induction of *MCL1* and, to a less extent, by activation of *c-JUN* NH_2-terminal kinase (*JNK*) (42). Therefore, by targeting both *MCL1*, and *c-JUN* transcripts, the impact of the *miR-15a/16-1* cluster on the survival of B-CLL cells could be even more robust. The presence of BCL2 in the proteomics list confirms our previous statement of a post-transcriptional regulation of this target (22). Moreover the repression of *LARS* (leucyl-tRNA synthetase), involved in the same pathway of *RARS* (arginyl-tRNA synthetase), and the presence of *RARS* among the down-regulated genes in MEG-01 confirms our previous hypothesis that this pathway could be targeted by *miR-15a/16-1* (16). Interestingly, the signature includes also many important tumor-suppressor genes (*RNASEL*, *HACE1*, *CEP63*, *CREBL2*, *MSH2*, *TIA1*, and *PMS1*) and reveals an intriguingly

Table 4. Examples of the CLL signature of *miR-15a/16-1* down-regulated genes by microarray

Gene symbol	Map	Gene name	*P*	
			CLL	MEG-01
HSDL2	9q32	Hydroxysteroid dehydrogenase like 2	0.00178	0.00254
SLC35A1	6q15	Solute carrier family 35 (CMP-sialic acid transporter), member A1	0.00178	0.00341
ECHDC1	6q22.33	Enoyl coenzyme A hydratase domain containing 1	0.00323	0.00582
CARD8	19q13.32	Caspase recruitment domain family, member 8	0.00446	0.00078
OMA1	1p32.2-p32.1	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)	0.00446	0.0158
UGP2	2p14-p13	UDP-glucose pyrophosphorylase 2	0.00446	0.000629
CREBL2	12p13	cAMP responsive element binding protein-like 2	0.00457	0.0199
Cep63	3q22.1	Centrosome protein Cep63	0.0049	0.0137
PNN	14q21.1	Pinin, desmosome-associated protein	0.0049	0.00359
TRA1	12q24.2-q24.3	Tumor rejection antigen (gp96) 1	0.00496	0.0211
SLC35B3	6p24.3	Solute carrier family 35, member B3	0.00601	0.0208
RHOT1	17q11.2	Ras homolog gene family, member T1	0.00695	0.0197
LARS	5q32	Leucyl-tRNA synthetase	0.00696	0.00239
RAD51C	17q22-q23	RAD51 homolog C (*S. cerevisiae*)	0.0075	0.00334
WASPIP	2q31.1	Wiskott–Aldrich syndrome protein interacting protein	0.00783	0.0108
MCL1	1q21	Myeloid cell leukemia sequence 1 (BCL2-related)	0.00863	0.011
ASXL2	2p24.1	Additional sex combs like 2 (Drosophila)	0.00875	0.000503
ARFIP1	4q31.3	ADP-ribosylation factor interacting protein 1 (arfaptin 1)	0.0114	0.0108
HERC6	4q22.1	Hect domain and RLD 6	0.0116	0.00107
TIA1	2p13	TIA1 cytotoxic granule-associated RNA-binding protein	0.0116	0.0116
VPS45A	1q21-q22	Vacuolar protein sorting 45a (yeast)	0.0117	0.000788
HLC-8	17q25.1	Lung cancer-related protein 8	0.0124	0.0164
HACE1	6q21	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1	0.0125	0.0115
ARV1	1q42.2	ARV1 homolog (yeast)	0.0156	0.000825
NT5C2L1	6q22.1	5′ nucleotidase, cytosolic II-like 1	0.0172	0.0139
PDCD6IP	3p23	Programmed cell death 6 interacting protein	0.0214	0.00276
GTF2H1	11p15.1-p14	General transcription factor IIH, polypeptide 1, 62 kda	0.0217	0.00125
MSH2	2p22-p21	mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)	0.0242	0.00192
JUN	1p32-p31	v-jun sarcoma virus 17 oncogene homolog (avian)	0.0281	0.00059
ALDH6A1	14q24.3	Aldehyde dehydrogenase 6 family, member A1	0.0297	0.00798
SCAP2	7p21-p15	Src family-associated phosphoprotein 2	0.0298	0.0108

For complete version, see SI Table 12. In bold: ARE-mRNAs; *P*, difference between groups with high and low *miR-15a/16-1* expression.

possible explanation for the link between *miR-15a/16-1* expression and CLL prognosis. We described that in CLL patients with unmutated IgV_H, and high expression of *ZAP-70* (poor prognosis), the levels of *miR-15a/16-1* are higher than in CLL patients with a better prognosis (18). The observed coexistence of oncogenes and TSGs in *miR-15a/16-1* CLL signature could give a molecular explanation as to why high levels of these two miRNAs are associated with CLLs with a worse prognosis (18). High *miR-15a/16-1* levels could down-regulate many TSGs and consequently negatively affect many oncosuppressor pathways, therefore leading to a more oncogenic phenotype.

Recently, it has been demonstrated that *miR-16* is critically involved in ARE-mediated mRNA instability (24). In MEG-01 cells, we found that ARE-mRNAs are significantly more represented among the down-regulated genes (20.1%) than among the up-regulated (13.6%, $P = 0.0098$). Although the identified signature is not enriched with ARE-mRNAs, it shows a predominance (84.6%) of cluster V ARE-mRNAs (which reflects the higher number of members of this cluster in both MEG-01 and patients), indicating that a higher number of pentameric AU-repeat does not correspond to a higher silencing effect by *miR-15a/16-1*. Finally the GO analysis of the deregulated genes indicates that *miR-15a/16-1* impacts strongly on metabolic pathways, on nucleic acid-binding pathways, and the activities of translation factors. It has been shown that in solid tumor cell lines *miR-16*-down-regulated transcripts are enriched with genes whose silencing causes an accumulation of cells in G_0/G_1 and that this function does not depend on AU-rich elements (28). Accordingly, we found that some of the described *miR-16* targets whose disruption triggered G_0/G_1-cell accumulation were down-regulated also in our cell model (*CDK6*, *CDC27*, *RAB11FIP2*) and that some of the previously described GO categories [namely "mitotic cell cycle" (GO:278), and "cell cycle" (GO:7049)] are represented also in our data. In contrast with the previous report, we found a statistically significantly higher number of ARE-mRNAs among the down-regulated targets with respect to the up-regulated. These differences may reflect cell-specific functions of *miR-15a/16-1*, whereas the common finding that *miR-15a/16-1* targets "cell cycle"-involved genes, both in solid and in hematologic tumor models, suggests a more general and robust effect of the cluster on this group of genes. In conclusion, our work describes *miR-15a/16-1* deregulated genes in both a leukemic cell model and in primary CLLs, and identifies a signature of common genes whose silencing characterizes the *miR-15a/16-1*-induced phenotype in CLL. These findings could have important significance for the development of therapeutic approaches for CLLs.

Materials and Methods

Cell Culture and Patient Samples. The human megakaryocytic MEG-01 cell line was purchased from the American Type Culture Collection and grown in 10% FBS RPMI medium 1640, supplemented with 1× nonessential amino acids and 1 mmol of sodium pyruvate at 37°C and 5% CO_2. For the patient study, we used 16 CLL samples obtained after informed consent from patients diagnosed with CLL at the CLL Research Consortium institutions. Briefly, blood was obtained from CLL patients and mononuclear cells were isolated through Ficoll/Hypaque gradient centrifugation (Amersham Pharmacia Biotech) and processed for RNA extraction according to the described protocols (18). For all of the samples, the microarray expression data were known as reported in ref. 18, and we further performed confirmation with qRT-PCR.

***In Vivo* Studies.** Animal studies were performed according to institutional guidelines. MEG-01 cell lines were transfected *in vitro* with *p*-Retrosuper vector (43) expressing *miR-15a/miR-16-1* (pRS15/16). Untransfected (mock) or cells transfected with the same empty plasmid (pRS-E) served as tumorigenic controls. At 24 h after the transfection, 10^7 viable cells were injected s.c. into the left flanks of 5-week-old female nude mice (Charles River Breeding Laboratories), five mice per transfected or control cell line. Tumor diameters were measured on days 7, 15, 21,

and 28. After 28 days, the mice were killed, necropsies were performed, and tumors were weighted. Tumor volumes were calculated by using the equation V (in mm^3) = $A \times B^2/2$, where A is the largest diameter, and B is the perpendicular diameter.

***In Vitro* Transfection.** MEG-01 cells were transiently transfected with 1 μg/ml (final concentration) pRS-15/16 or pRS-E vector by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

Microarray Hybridization and Data Analysis. Two samples obtained from the MEG01 cell line transfected with pRS-15/16 and pRS-E vector, each one in triplicate, and 16 CLL samples were analyzed by microarray using Human Genome U133A Plus 2.0 GeneChip arrays (Affymetrix). The .CEL files generated by the GeneChip scanner were imported in GeneSpring GX 7.3 software (Agilent Technologies) and further processed. Details about the microarray experiment are described in *SI Text*.

MiRNA Target Prediction. The analysis of miRNA predicted targets was determined by using the algorithms TargetScan (http://genes.mit.edu/targetscan/), PicTar (http://pictar.bio.nyu.edu/), and miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl).

Adenylate Uridylate-Rich Elements (ARE)-Containing Genes Identification. The ARE-mRNA database version 3.0 (ARED; http://rc.kfshrc.edu.sa/ared/), as described (44), was used (for details see *SI Text*).

Two-Dimensional PAGE and Protein Identification by Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) and Mass Spectrometry (MS). MEG-01 cells were transiently transfected for 48 hr with 1 μg/ml (final concentration) pRS15/16 or pRS-E vector by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions and the details of the two-dimensional PAGE, and protein identification by MALDI-TOF and MS are described in *SI Text*.

qRT-PCR. qRT-PCR analysis for miRNAs was performed in triplicate with the TaqMan MicroRNA assays kit (Applied Biosystems) according to the manufacturer's instructions and as described (45). For normalization, 18S RNA was used; qRT-PCR analyses for other genes of interest were performed by reverse transcription of RNA to cDNA with gene-specific primers and IQ SYBR green Supermix (Bio-Rad) according to the manufacturer's instructions. β-Tubulin was used for normalization.

Luciferase Reporter Assay. For luciferase reporter experiments, a IGSF4 3′ UTR segment of 237 bp was amplified by PCR from human cDNA and inserted into the pGL3-control vector with SV40 promoter (Promega) by using the XbaI site immediately downstream from the stop codon of luciferase. Details about the microarray experiment are described in *SI Text*. The experiments were performed in triplicate.

ACKNOWLEDGMENTS. We thank Dr. D. Vandre for performing the proteomics analysis and Dr. S. Yoon for the prediction data. This work was supported by National Cancer Institute grants (to C.M.C., T.J.K., and T.D.S.), by a CLL Global Research Foundation grant, by funding from the University of Texas System Regents Research Scholar Award and the University of Texas M. D. Anderson Research Trust (G.A.C.), by grants from the Italian Ministry of Health (to C.M.C.), and by the Italian Ministry of University Research and the Italian Association for Cancer Research (to M.N.). A.C. was supported by an American–Italian Cancer Foundation Fellowship. M. Ferracin was a recipient of a fellowship from the Fondazione Italiana per la Ricerca sul Cancro.

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