HDAC2-dependent miRNA signature in acute myeloid leukemia

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Acute myeloid leukemia (AML) is a multifactorial and highly heterogeneous malignancy, whose incidence rises with age [1]. The evolution of the disease is characterized by uncontrolled progenitor cell proliferation and block of differentiation. To date, although many genetic mutations in AML have been identified, prognosis has markedly improved in recent years but still remains poor [2]. It is well known that epigenetic mechanisms regulate gene expression and consequently define pathways involved in the physiopathogenesis of AML. Among the many epigenetic regulators, histone deacetylases (HDACs) are tightly involved in AML etiology [3]. Notably, HDAC2 is highly overexpressed in solid and hematological cancers, including AML [4–9]. HDAC2 silencing by enzymatic inhibition has a substantial impact on leukemia cell proliferation and immune regulation, as described in our previous work [10], where we established an HDAC2-knockdown AML clone to better understand the role of cancer cell proliferation dynamics with and without treatment with the well-studied HDAC inhibitors (HDACi) suberanilohydroxamic acid (SAHA) and entinostat (also known as MS-275). Several epigenetic drugs, including HDACi, are in fact actively undergoing clinical investigation as single agents or mainly in combination with consolidated chemotherapeutics [11]. Similarly, miRNAs are now recognized as epigenetic regulators of transcripts in nearly all physiological processes and human cancers,

Acute myeloid leukemia (AML) arises from a complex sequence of biological and finely orchestrated events that are still poorly understood. Increasingly, epigenetic studies are providing exciting findings that may be exploited in promising and personalized cutting-edge therapies. A more appropriate and broader screening of possible players in cancer could identify a master molecular mechanism in AML. Here, we build on our previously published study by evaluating a histone deacetylase (HDAC)2-mediated miRNA regulatory network in U937 leukemic cells. Following a comparative miRNA profiling analysis in genetically and enzymatically HDAC2-downregulated AML cells, we identified miR-96-5p and miR-92a-3p as potential regulators in AML etiopathology by targeting defined genes. Our findings support the potentially beneficial role of alternative physiopathological interventions.

Keywords: epigenetics; HDAC2; immunoregulation; leukemia; miRNA; SAHA

Abbreviations
AML, acute myeloid leukemia; FC, fold change; FDR, false discovery rate; HDACi, histone deacetylase inhibitor(s); HDACs, histone deacetylases; MHC, major histocompatibility complex; SAHA, suberanilohydroxamic acid.
including AML [12]. The key involvement of miRNAs in crucial biological pathways hints at their functional role in complex molecular gene networks in cancer. Recently, the potential use of cellular and circulating miRNAs as biomarkers for AML diagnosis/prognosis, and as therapeutic targets has been widely explored, and many miRNAs were found to be associated with HDAC2 dysfunction in leukemia [13].

Here, we identified a cluster of common up- and downregulated miRNAs in both SAHA-treated and HDAC2-downregulated cells. By miRNA target network computational analysis, we defined an HDAC2-mediated miRNA signatures in AML by genetic and enzymatic HDAC2 deficiency in a U937 leukemic cell line. We propose a crucial role of miR-96-5p and miR-92a-3p and related target genes and their relationship with HDAC2 in AML. Here, we corroborated our previous findings and strongly suggested an HDAC2-mediated regulation of the immune system in AML, involving major histocompatibility complex (MHC) class II genes and specific miRNAs, via finely tuned molecular mechanisms.

Materials and methods

Cell culture and treatment

U937 leukemic cells were kept in RPMI-1640 medium (EuroClone, Pero, Milan, Italy) supplemented with 10% heat-inactivated FBS (Gibco, Monza and Brianza, Italy), 100 units·mL⁻¹ penicillin G (EuroClone), 100 μg·mL⁻¹ streptomycin (EuroClone), 2 mM l-glutamine (EuroClone), 250 mg·mL⁻¹ amphotericin B (EuroClone) and 50 mg·mL⁻¹ G-418 sulfate (Sigma-Aldrich, Milan, Italy). The cells were incubated at 37 °C at a fixed concentration of CO₂ (5%). The HDACi SAHA (Merck, Rome, Italy) was dissolved in dimethyl sulfoxide (Sigma-Aldrich) and used at a final concentration of 5 μM for 6 h of treatment.

Stable transfection of sh2 vector

Silencing of HDAC2 in U937 cells was performed as previously described [10].

RNA isolation and miRNA expression analysis

Total miRNA-enriched RNA was isolated and miRNA expression levels were analyzed by real-time PCR as previously reported [14].

Quantitative real-time PCR

Real-time PCR was performed using the VILO cDNA Synthesis Kit (Invitrogen, Monza and Brianza, Italy) to convert RNA into cDNA. 1X SYBR Green PCR Master Mix (BioRad, Segrate, Milan, Italy) was used according to the manufacturer's instructions, using 50 ng of cDNA. Primers for qRT-PCR were: TRIβ3 Fw: 5'-CCACGC TCCTCTAGCGCTTTTT-3' and TRIβ3 Rev: 5'-CGACACACG CTGTCGATCCG-3'; SLC37A3 Fw: 5'-TGTCAGAAG TCGGATGAA-3' and SLC37A3 Rev: 5'-GATTCATG TTTCTTCGCAT-3'; TBC1D8 Fw: 5'-TACTCTCACTGCT CGTGGAAA-3' and TBC1D8 Rev: 5'-GCTCTTCTCTCT TGCGTTGAGT-3'; FAM49A Fw: 5'-GATGGCCCAAATCG AA TGTCCC-3' and FAM49A Rev: 5'-GCCATCCCACTTC CATGCAGA-3'. Data were normalized with GAPDH Fw: 5'-GGAGTCAACCGATTTGTCGT-3' and GAPDH Rev: 5'-GCTTCCCGTCTCAGCCCTGA-3'.

miRNA microarray profiling and data analysis

miRNAomes of U937 scramble vector (scr) and HDAC2 knockdown (shHDAC2) cells were analyzed. Each sample was prepared according to Agilent’s miRNA Microarray System protocol. Total RNA (100 ng) was dephosphorylated with calf intestine alkaline phosphatase (GE Healthcare Europe, Rome, Italy), denatured with DMSO (Sigma-Aldrich), and labeled with Cyanin 3-pCp by T4 RNA ligase (GE Healthcare Europe). The labeled RNA was purified and then hybridized to Human miRNA Microarray (v1) 8x15K (G4470B; Agilent, Cernusco sul Naviglio, Milan, Italy) for 20 h at 55 °C with rotation. After hybridization and washing, the arrays were acquired with an Agilent Scanner and data extracted using agilent feature extraction software (Cernusco sul Naviglio, Milan, Italy), as specified by the manufacturer. Microarray quality control reports generated by the agilent feature extraction software. Using R/BioConductor [15] and limma package, probe level raw intensity was processed. The ‘normexp’ limma method was used for background correction and data normalization was carried out. Differential expression was performed using Student’s t-test. The selected miRNA list was obtained by applying a false discovery rate (FDR) < 0.05; each value was converted to log2.

Microarray data are available in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE129154.

Computational prediction of miRNA target genes

Target gene prediction of differentially expressed miRNAs was performed using the mirNet database. All miRNA entries are annotated according to the latest miRBase (release 22) (http://mirbase.org/) [16]. Target genes were then selected. miRNA target interaction data were downloaded from 11 well-annotated databases, miRTarBase, TarBase, miRecords, SM2miR, Pharmaco-miR, miR2Disease, Phe nomiR, StarBase, EpimiR, miRDB, and miRanda, selecting the Homo sapiens species.

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microRNA signatures mediated by HDAC2 in AML
Gene set enrichment and functional annotation analysis

The relative abundance of ‘Biological Process’ (BP), Pathways (by KEGG), oncogenic and immunologic signatures Gene Ontology terms in each of the selected lists was analyzed using the Molecular Signatures Database v6.2 (MSigDB) in Gene Set Enrichment Analysis (GSEA) software (http://software.broadinstitute.org/gsea/msigdb) for Annotation, Visualization and Integrated Discovery.

Gene expression microarray profiling and data analysis using the Agilent platform

Gene expression profiles of U937 scramble vector (scr) and HDAC2 knockdown (shHDAC2) cells were analyzed by Whole Human Genome Two-Color Microarray (G4112F; Agilent), following the manufacturer’s protocol. Microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE37529 [10]. Probe-level raw intensity was processed using R/BioConductor and limma package. Background correction was performed using ‘normexp’ limma method and data normalization was carried out in two steps: LOWESS normalization within array to correct systematic dye bias and quantile normalization between arrays to detect systematic nonbiological bias. Ratios representing the relative target mRNA intensities compared to control RNA probe signals were derived from normalized data. For each P-value, the Benjamini–Hochberg procedure was used to calculate the FDR in order to avoid the problem of multiple testing.

Results

Differentially expressed miRNA profiling in HDAC2-defective AML

We built on our previous epigenetic study [10] by evaluating the impact of HDAC2 deficiency on miRNA expression in AML. miRNome analysis was performed in HDAC2-silenced (shHDAC2) and relative scramble control (scr) U937 stable clones, previously obtained and retested for mRNA and HDAC2 protein expression levels (Fig. 1A,B). In addition, we treated scr cells with the well-known HDACi SAHA for 6 h (scrSAHA6h) to enzymatically mimic HDAC2 silencing. Volcano plots display differentially expressed miRNAs in shHDAC2 and scrSAHA6h compared to scr cells (Fig. 1C,D). Student’s t-test analysis revealed the presence of 29 and 14 differentially expressed miRNAs in shHDAC2/scr and scrSAHA6h/scr cells, respectively, by applying an FDR significance threshold < 0.05 (Tables 1 and 2). Comparative analysis showed that 11 miRNAs are commonly regulated both when HDAC2 is genetically silenced and enzymatically inhibited (Fig. 2A). Among these, miR-801 and miR-923 were
As shown in Tables 3 and 4, many genes involved in immunoregulatory mechanisms are perturbed, further confirming our previous finding that the immune system is affected in an HDAC2-defective AML clone (in both genetic and enzymatic conditions).

**Identification of HDAC2-dependent miRNA targets**
To validate target prediction analysis, we performed a very stringent intersection analysis between the commonly altered genes in shHDAC2/scr and scrSAHA6h/scr cells (GSE37529) [10] (Table 5) and the predicted miRNA hits. Among the targets of the six upregulated miRNAs, we identified five predicted gene targets (TRIB3, SLC37A3, EMP1, SCD, IL1B) also regulated in gene expression profiles of shHDAC2/scr and scrSAHA6h/scr cells (Fig. 4A). Only one downregulated hit corresponding to TRIB3 gene displayed an according trend compared to the related regulating miRNA, mir-96-5p. Figure 4B shows TRIB3 microarray expression fold change (FC) in log2 in shHDAC2 and scrSAHA6h compared to scr cells. In contrast,
distinguishing between the targets of the three downregulated miRNAs, we identified four predicted gene targets (SLC37A3, TBC1D8, SCD, FAM49A) regulated in gene expression profiles of shHDAC2/scr and scrSAHA6h/scr cells (Fig. 5A). SLC37A3, TBC1D8, and FAM49A are upregulated hits targeted
by miR-92a-3p. Figure 5B shows the microarray expression FC in log2 of three upregulated target genes in both shHDAC2 and scrSAHA6h compared to scr cells. SCD was excluded as it showed a different trend in the two conditions (FC = 1.03 in scrSAHA6h/scr; FC = 1.46 in shHDAC2/scr). These data are in line with trends in mRNA regulation, target prediction, and miRNA expression levels.

Validation of miRNAs and target genes in HDAC2-defective U937 cells

Following miRNA microarray profiling and computational prediction of miRNA target genes, we investigated the expression levels of miRNAs and their corresponding target genes. We analyzed miR-92a-3p and miR-96-5p expression levels by real-time PCR (Fig. 6A). Gene expression levels of TRIB3, a target of miR-96-5p, were analyzed in scr and shHDAC2 cells as well as in scr cells untreated or treated with SAHA for 6 h. TRIB3 relative expression was downregulated in both HDAC2-deficient and scr U937 cells treated with SAHA, suggesting a correlated response due to HDAC2 enzymatic inhibition and genetic silencing (Fig. 6B). The expression levels of SLC37A3, FAM49A, and TBC1D8, target genes of hsa-miR-92a-3p, were also analyzed (Fig. 6C). According to miRNA expression, all target genes were upregulated in shHDAC2 as well as in scrSAHA treated cells, related to scr. Notably, miRNA-mRNA regulation in HDAC2-downregulated cells was comparable after both enzymatic and pharmacological inhibition by SAHA, supporting the hypothesis that upregulated expression levels of HDAC2 indicate dysfunction of these regulators in AML.

Table 3. Immunologic signatures of predicted target genes of three downregulated miRNAs identified by applying an FDR q-value significance threshold ≤ 0.001.

| Gene set name                                                                 | No. of genes in gene set (K) | No. of genes in overlap (k) | k/K   | P-value | FDR q-value |
|--------------------------------------------------------------------------------|-------------------------------|-----------------------------|-------|---------|-------------|
| GSE2405_0H_VS_9H_A_PHAGOCYTYPHILUM_STIM_NEUTROPHIL_DN                          | 200                           | 59                          | 0.295 | 1.42E-37 | 6.93E-34    |
| GSE9006_HEALTHY_VS_TYPE_1_DIABETES_PBMCI_1MONTH_POST_DX_UP                     | 200                           | 56                          | 0.28  | 2.18E-34 | 5.31E-31    |
| GSE2405_0H_VS_24H_A_PHAGOCYTYPHILUM_NEUTROPHIL_STIM                            | 200                           | 51                          | 0.255 | 2.68E-29 | 4.35E-26    |
| GSE38920_TGFBEATA1_VS_TGFBEATA3_IN_IL6_TREATED_CD4_TCELL_UP                   | 200                           | 48                          | 0.24  | 2.21E-26 | 2.15E-23    |
| GSE9006_HEALTHY_VS_TYPE_1_DIABETES_PBMCI_4MONTH_POST_DX_UP                     | 200                           | 48                          | 0.24  | 2.21E-26 | 2.15E-23    |
| GSE27241_VIT_VS_RORST_KO_TH17_POLARIZED_CD4_TCELL_TREATED_WITH_DIGOXIN_UP     | 170                           | 44                          | 0.2588| 9.27E-26 | 7.53E-23    |

Table 4. Immunologic signatures of predicted target genes of six upregulated miRNAs in AML cells identified by applying an FDR q-value significance threshold ≤ 0.001.

| Gene set name                                                                 | No. of genes in gene set (K) | No. of genes in overlap (k) | k/K   | P-value | FDR q-value |
|--------------------------------------------------------------------------------|-------------------------------|-----------------------------|-------|---------|-------------|
| GSE42021_TREG_PLN_VS_CD24INT_TREG_THYMUS_UP                                  | 200                           | 50                          | 0.25  | 5.54E-32 | 2.70E-28    |
| GSE27434_VIT_VS_DNMT1_KO_TREG_DN                                             | 200                           | 47                          | 0.235 | 7.55E-29 | 1.84E-25    |
| GSE229220_PROGESTERONE_VS_TGFBI1_AND_PROGESTERONE_TREATED_CD4_TCELL_UP       | 199                           | 43                          | 0.2161| 6.19E-25 | 1.01E-21    |
| GSE14769_UNSTIM_VS_60MIN_LPS_BMDM_DN                                         | 200                           | 42                          | 0.21  | 7.15E-24 | 5.81E-21    |
| GSE20500_RETINOIC_ACID_VS_RARA_ANTAGONIST_TREATED_CD4_TCELL_DN               | 200                           | 42                          | 0.21  | 7.15E-24 | 5.81E-21    |
| GSE39020_TGFBEATA3_IL6_VS_TGFBEATA3_IL6_IL23A_TREATED_CD4_TCELL_UP           | 200                           | 42                          | 0.21  | 7.15E-24 | 5.81E-21    |
| GSE3920_IFNA_VS_IFNG_TREATED_ENDOTHELIAL_CELL_UP                               | 166                           | 38                          | 0.2289| 3.76E-23 | 2.61E-20    |
| GSE13411_SWITCHED_MEMORY_BCELL_VS_PLASMA_CELL_UP                               | 200                           | 41                          | 0.205 | 6.47E-23 | 3.50E-20    |
| GSE4748_CTRL_VS_LPS_STIM_DC_3H_UP                                            | 200                           | 41                          | 0.205 | 6.47E-23 | 3.50E-20    |
| GSE12003_MIR223_KO_VS_WT_BM_PROGENITOR_4D_CULTURE_UP                           | 200                           | 40                          | 0.2   | 5.68E-22 | 1.97E-19    |
miRNAs are known to play a critical and functional role in a broad range of key molecular processes via sophisticated regulation of distinct targets, orchestrating a molecular intracellular balance of gene expression. miRNA activity and expression are affected in cancer. Altered miRNAs in AML are involved in a variety of biological pathways [18], and a better understanding of their signatures might help unravel the complexity associated with the emergence of this disease. Since HDAC2 deregulation affects cell proliferation, apoptosis, and immune system in AML, focusing on specific miRNAs altered by this epigenetic regulator may identify potential markers for determining the best strategies in AML treatment. To date, many miRNAs were found directly targeted by HDAC2 in several cancers such as colorectal cancer [19], hepatocellular carcinoma [20], breast cancer [21], and AML [22]. In AML, differentially expressed miRNAs have a prognostic and functional role associated with cytogenetics, molecular features, molecular markers, morphology, and clinical outcome [23]. In a previous study, we found that HDAC2 gene is considerably upregulated in AML ex vivo patient samples and cell lines. Following HDAC2 silencing and enzymatic inhibition using the epigenetic-based drug SAHA, we observed a pivotal HDAC2-dependent modulation of chromatin architecture leading to transcriptional changes promoting mainly activation of an immune response. Specifically, HDAC2 acts directly at epigenetic level by regulating the promoter regions of specific allelic forms of MHC class II genes (HLA-DRA and HLA-DPA1). Here, based also on our previous findings, we elucidated miRNA-HDAC2 crosstalk and its involvement in AML state. Interestingly, a stringent computational analysis between the transcriptome and miRNome profiles in shHDAC2/scr and scrSAHA6h/scr cells identified a crucial role for miR-96-5p and miR-92a-3p, and defined their target gene regulation enclosing convergent pathways already identified in our previous work. We investigated upregulated miR-96-5p, which has an oncogenic role in several cancer types [24–26]. Low expression levels of miR-96-5p were found in a specific cohort of AML patients, suggesting that this epigenetic marker could be considered a prognostic factor for AML at diagnosis [27]. miR-96-5p upregulation acts as an antiapoptotic factor in bladder cells, as it negatively regulates specific targets such as CDKN1A, which is involved in cell cycle regulation and DNA damage pathway in bladder cancer [28]. Our data show that TRIB3 is targeted by miR-96-5p. The protein encoded by TRIB3 gene is a potential kinase that negatively regulates NF-kB and Akt1 pathways, affecting cell proliferation and apoptosis, and promoting ubiquitination-dependent degradation of several key proteins. TRIB3 is strongly expressed in AML with t(8;21) and t(15;17) translocations, as well as in M2/M3 AML subtypes [29], although its specific role in leukemogenesis is still elusive. However, evidence suggests that TRIB3 contributes to acute promyelocytic leukemia progression by PML-RARα stabilization via specific binding to SUMOylation motifs, thereby acting on PML-RARα degradation and differentiation [30]. In addition, we found and subsequently investigated the downregulation of miR-92a-3p targeting SLC37A3, TBC1D8, and FAM49A genes. High expression levels of miR-92a-3p are associated with acute megakaryoblastic leukemia and affect genes controlling apoptosis and cell proliferation [31]. High expression levels of this miRNA were also found in both AML and acute lymphoblastic leukemia cells compared to normal blasts [32]. We identified SLC37A3 as one of the targets of miR-92a-3p. This transmembrane protein is localized in the endoplasmatic reticulum and is involved in sugar transport. The SLC37A3 gene is one of the four sugar-phosphate

| Gene symbol | ANXA1 | ARHGEF3 | BMF | CX3CR1 | EMP1 | EPAS1 | FAM117A | FAM49A | FPR1 | GRB10 | H1F0 | HLA-DMB | IL1B | IL4i1 | LOH11CR2A | LPAAT-THETA | MAFB | MMP1 | RNF149 | SCD | SLC37A3 | SYTL3 | TBC1D8 | TMEM118 | TRIB3 |
|-------------|-------|---------|-----|--------|------|-------|---------|---------|-------|-------|------|---------|------|------|-------------|-----------------|------|-------|-------|-----|--------|------|--------|--------|------|

Table 5. Genes commonly altered in shHDAC2/scr and scrSAHA6h/scr AML cells identified by applying an FDR significance threshold < 0.05.

Discussion

miRNAs are known to play a critical and functional role in a broad range of key molecular processes via sophisticated regulation of distinct targets, orchestrating a molecular intracellular balance of gene expression. miRNA activity and expression are affected in cancer. Altered miRNAs in AML are involved in a variety of biological pathways [18], and a better understanding of their signatures might help unravel the complexity associated with the emergence of this disease. Since HDAC2 deregulation affects cell proliferation, apoptosis, and immune system in AML, focusing on specific miRNAs altered by this epigenetic regulator may identify potential markers for determining the best strategies in AML treatment. To date, many miRNAs were found directly targeted by HDAC2 in several cancers such as colorectal cancer [19], hepatocellular carcinoma [20], breast cancer [21], and AML [22]. In AML, differentially expressed miRNAs have a prognostic and functional role associated with cytogenetics, molecular features, molecular markers, morphology, and clinical outcome [23]. In a previous study, we found that HDAC2 gene is considerably upregulated in AML ex vivo patient samples and cell lines. Following HDAC2 silencing and enzymatic inhibition using the epigenetic-based drug SAHA, we observed a pivotal HDAC2-dependent modulation of chromatin architecture leading to transcriptional changes promoting mainly activation of an immune response. Specifically, HDAC2 acts directly at epigenetic level by regulating the promoter regions of specific allelic forms of MHC class II genes (HLA-DRA and HLA-DPA1). Here, based also on our previous findings, we elucidated miRNA-HDAC2 crosstalk and its involvement in AML state. Interestingly, a stringent computational analysis between the transcriptome and miRNome profiles in shHDAC2/scr and scrSAHA6h/scr cells identified a crucial role for miR-96-5p and miR-92a-3p, and defined their target gene regulation enclosing convergent pathways already identified in our previous work. We investigated upregulated miR-96-5p, which has an oncogenic role in several cancer types [24–26]. Low expression levels of miR-96-5p were found in a specific cohort of AML patients, suggesting that this epigenetic marker could be considered a prognostic factor for AML at diagnosis [27]. miR-96-5p upregulation acts as an antiapoptotic factor in bladder cells, as it negatively regulates specific targets such as CDKN1A, which is involved in cell cycle regulation and DNA damage pathway in bladder cancer [28]. Our data show that TRIB3 is targeted by miR-96-5p. The protein encoded by TRIB3 gene is a potential kinase that negatively regulates NF-kB and Akt1 pathways, affecting cell proliferation and apoptosis, and promoting ubiquitination-dependent degradation of several key proteins. TRIB3 is strongly expressed in AML with t(8;21) and t(15;17) translocations, as well as in M2/M3 AML subtypes [29], although its specific role in leukemogenesis is still elusive. However, evidence suggests that TRIB3 contributes to acute promyelocytic leukemia progression by PML-RARα stabilization via specific binding to SUMOylation motifs, thereby acting on PML-RARα degradation and differentiation [30]. In addition, we found and subsequently investigated the downregulation of miR-92a-3p targeting SLC37A3, TBC1D8, and FAM49A genes. High expression levels of miR-92a-3p are associated with acute megakaryoblastic leukemia and affect genes controlling apoptosis and cell proliferation [31]. High expression levels of this miRNA were also found in both AML and acute lymphoblastic leukemia cells compared to normal blasts [32]. We identified SLC37A3 as one of the targets of miR-92a-3p. This transmembrane protein is localized in the endoplasmatic reticulum and is involved in sugar transport. The SLC37A3 gene is one of the four sugar-phosphate
Fig. 4. Intersection analysis between commonly altered genes in shHDAC2/scr and scrSAHA6h/scr cells and predicted targets of upregulated miRNAs. (A) Venn diagram showing the five common altered target genes of six upregulated miRNAs in shHDAC2/scr and scrSAHA6h/scr cells, also predicted by computational analysis. (B) Microarray expression FC in log2 in shHDAC2 and scrSAHA6h cells compared to scr cells for TRIB3 downregulated hit targeted by miR-96-5p. The FDR are reported on histogram bars.

Fig. 5. Intersection analysis between commonly altered target genes in shHDAC2/scr and scrSAHA6h/scr cells associated with downregulated miRNAs. (A) Venn diagram showing the four common altered target genes of three downregulated miRNAs in shHDAC2/scr and scrSAHA6h/scr cells, also predicted by computational analysis. (B) Microarray expression FC in log2 in shHDAC2 and scrSAHA6h cells compared to scr cells for SLC37A3, TBC1D8, and FAM49A upregulated hits targeted by miR-92a-3p. The FDR are reported on histogram bars.
exchanger family members, but its functional activity is not yet clear. Evidence suggests that SLC37A3 might be involved in physiopathological regulation in pancreatic but also in immune system [33]. The methylation levels of this gene affect glucose blood degree, suggesting its potential role in epigenetic modifications via a mechanism that still requires further investigation, and its involvement in obesity-related metabolism. We also identified FAM49A as a miR-92a-3p-target by computational analysis. This target gene was detected as a downregulated protein in bladder cancer cells [34]. FAM49A is a consensus PU.1-activated target gene. PU.1 is an E26 transformation-specific family transcription factor widely involved in hematopoiesis. FAM49A is a direct functional regulator of myeloid, dendritic cell, B cell and a differentiation factor of earliest stages of T-cell and terminal erythroid cell [35]. The third hsa-miR-92a-3p target which we investigated is TBC1D8. This gene is a member of the Tre2/Bub2/Cdc16 (TBC) domain protein family, characterized by highly conserved TBC domains [36]. TBC1D8 was found among differentially expressed genes in pre-B acute lymphocytic leukemia samples with ALL1/AF4, E2A/PBX1, and BCR/ABL molecular rearrangements, and positively controls cell proliferation [37]. Other studies identified TBC1D8 as a target of IL4 in chronic lymphocytic leukemia and normal B cells [38]. In this work, we propose the existence of a mechanistic cross-talk between miRNAs and HDAC2 in an epigenetic superstructure regulating pathogenesis and progression of AML. All our findings converge in identifying HDAC2 and miRNA interplay in specific biological processes (Fig. 6), which potentially affects regulation of gene expression, cell cycle, apoptosis, response to stress and response to organic substance. These mechanisms are robustly altered in leukemogenesis, further confirming our previous findings. We mostly speculate on the immunologic signature of predicted target genes of both downregulated and upregulated miRNAs (Tables 4 and 5) identifies immune cells such as CD4 and CD8T in a specific gene set. It is not surprising that some hits were also associated with type I diabetes, as MHC class II genes are related to this disease [39]. Since MHC class II genes regulate initiation of immune response, our data characterize a specific signature also involving endothelial, thymic, epithelial, and B cells in gene sets. The epigenetic drug SAHA was used as a therapeutic agent in this and in our previous study. This drug plays a critical role as an immunomodulating agent by enhancing cancer cell
immunogenicity. We and other authors reported that HDAC1 make cancer cells more responsive to immunotherapy by increasing the expression levels of tumor antigens, and drive gene expression toward a proapoptotic mechanism in cancer [40,41]. Finally, taken together, our findings identified miR-96-5p and miR-92a-3p as prospective epi-regulators in AML. This HDAC2-dependent miRNA signature in AML highlights the potentially beneficial effects of treatment with epigenetic drugs alone or in combination with other therapies (including immunotherapy) acting via a targeted mechanism involving the perturbation of genes affecting cell cycle, proliferation, apoptosis, and immune system. To date, achieving greater insights into leukemogenesis has allowed us to make progress toward the prevention and treatment of this devastating disease. Given that many disease agents including those that are not strictly biological (such as smoking, obesity, exposure to certain types of radiation or other substances) are not always controllable and vary continuously throughout a person’s lifetime, the need for multifaceted therapeutic approaches is imperative.

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Author contributions

LA supervised the study; MC and CD designed, performed experiments, and wrote the manuscript; GS performed experiments; AC analyzed GE and miRNA data.

References

1 De Kouchkovsky I and Abdul-Hay M (2016) Acute myeloid leukemia: a comprehensive review and 2016 update. Blood Cancer J 6, e441.
2 Saultz JN and Garzon R (2016) Acute myeloid leukemia: a concise review. J Clin Med 5, 33.
3 Ahmadzadeh A, Khodadi E, Shahjahani M, Bertacchini J, Vosoughi T and Saki N (2015) The role of HDACs as leukemia therapy targets using HDI. Int J Hematol Oncol Stem Cell Res 9, 203–214.
4 Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP and Gottlicher M (2004) Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 5, 455–463.
5 Schuler S, Fritsche P, Diersch S, Arlt A, Schmid RM, Saur D and Schneider G (2010) HDAC2 attenuates TRAIL-induced apoptosis of pancreatic cancer cells. Mol Cancer 9, 80.
6 Song J, Noh JH, Lee JH, Eun JW, Ahn YM, Kim SY, Lee SH, Park WS, Yoo NJ, Lee JY et al. (2005) Increased expression of histone deacetylase 2 is found in human gastric cancer. APNIS 113, 264–268.
7 Shan W, Jiang Y, Yu H, Huang Q, Liu L, Guo X, Li L, Mi Q, Zhang K and Yang Z (2017) HDAC2 overexpression correlates with aggressive clinicopathological features and DNA-damage response pathway of breast cancer. Am J Cancer Res 7, 1213–1226.
8 Zhao J, Xie C, Edwards H, Wang G, Taub JW and Ge Y (2017) Histone deacetylases 1 and 2 cooperate in regulating BRCA1, CHK1, and RAD51 expression in acute myeloid leukemia cells. Oncotarget 8, 6319–6329.
9 Yang H, Maddipoti S, Quesada A, Bohannan Z, Cabrero CM, Colla S, Wei Y, Estecio M, Wierda W, Bueso-Ramos C et al. (2015) Analysis of class I and II histone deacetylase gene expression in human leukemia. Leuk Lymphoma 56, 3426–3433.
10 Conte M, Dell’Aversana C, Benedetti R, Petraglia F, Carissimo A, Petrizzi VB, D’Arco AM, Abbondanza C, Nebbioso A and Altucci L (2015) HDAC2 deregulation in tumorigenesis is causally connected to repression of immunogen. Blood C 6, 886–901.
11 Saygin C and Carraway HE (2017) Emerging therapies for acute myeloid leukemia. J Hematol Oncol 10, 93.
12 Wallace JA and O’Connell RM (2017) MicroRNAs and acute myeloid leukemia: therapeutic implications and emerging concepts. Blood 130, 1290–1301.
13 Trino S, Lamorte D, Caivano A, Laurenzana I, Tagliaferri D, Falco G, Del VL, Musto P and De Luca L (2018) MicroRNAs as new biomarkers for diagnosis and prognosis, and as potential therapeutic targets in acute myeloid leukemia. Int J Mol Sci 19, E460.
14 Dell’Aversana C, Giorgio C, D’Amato L, Lania G, Matarese F, Saeed S, Di Costanzo A, Belsito Petrizzi V, Ingenito C, Martens JHA et al. (2017) miR-194-5p/ BCLAF1 deregulation in AML tumorigenesis. Leukemia 31, 2315–2325.
15 Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5, R80.
16 Kozomara A, Birgaoanu M and Griffiths-Jones S (2019) miRBase: from microRNA sequences to function. Nucleic Acids Res 47, D155–D162.
17 Fan Y, Siklenka K, Arora SK, Ribeiro P, Kimmims S and Xia J (2016) miRNet – dissecting miRNA-target interactions and functional associations through network-based visual analysis. Nucleic Acids Res 44, W135–W141.
18 Gabra MM and Salmena L (2017) microRNAs and acute myeloid leukemia chemoresistance: a mechanistic overview. Front Oncol 7, 255.
19 Mao QD, Zhang W, Zhao K, Cao B, Yuan H, Wei LZ, Song MQ and Liu XS (2017) MicroRNA-455 suppresses the oncogenic function of HDAC2 in human colorectal cancer. Braz J Med Biol Res 50, e6103.
20 Kim HS, Shen Q and Nam SW (2015) Histone deacetylases and their regulatory microRNAs in hepatocarcinogenesis. J Korean Med Sci 30, 1375–1380.
21 Xia X, Wang X, Zhang S, Zheng Y, Wang L, Xu Y, Hang B, Sun Y, Lei L, Bai Y et al. (2019) miR-31 shuttled by halofuginone-induced exosomes suppresses MFC-7 cell proliferation by modulating the HDAC2/cell cycle signaling axis. J Cell Physiol 234, 18970–18989.
22 Lai TH, Ewald B, Zecovic A, Liu C, Sulda M, Papaioannou D, Garzon R, Blachly JS, Plunkett S and Sampath D (2016) HDAC inhibition induces microRNA-182, which targets RAD51 and impairs HR repair to sensitize cells to sapacitabine in acute myelogenous leukemia. Clin Cancer Res 22, 3537–3549.
23 Marcucci G, Mrozek K, Radmacher MD, Garzon R and Bloomfield CD (2011) The prognostic and functional role of microRNAs in acute myeloid leukemia. Blood 117, 1121–1129.
24 Ress AL, Stiegelbauer V, Winter E, Schwarzenbacher D, Kiesslich T, Lux S, Jahn S, Deutsch A, Bauernhofer T, Ling H et al. (2015) MiR-96-5p influences cellular growth and is associated with poor survival in colorectal cancer patients. Mol Carcinog 54, 1442–1450.
25 Shi Y, Zhao Y, Shao N, Ye R, Lin Y, Zhang N, Li W, Zhang Y and Wang S (2017) Overexpression of microRNA-96-5p inhibits autophagy and apoptosis and enhances the proliferation, migration and invasiveness of human breast cancer cells. Oncol Lett 13, 4402–4412.
26 Bao YH, Wang Y, Liu Y, Wang S and Wu B (2017) MiR-96 expression in prostate cancer and its effect on the target gene regulation. Eur Rev Med Pharmacol Sci 21, 4548–4556.
27 Zhao J, Lu Q, Zhu J, Fu J and Chen YX (2014) Prognostic value of miR-96 in patients with acute myeloid leukemia. Diagn Pathol 9, 76.
28 Wu Z, Liu K, Wang Y, Xu Z, Meng J and Gu S (2015) Upregulation of microRNA-96 and its oncogenic functions by targeting CDKN1A in bladder cancer. Cancer Cell Int 15, 107.
29 Li K, Wang F, Cao WB, Lv XX, Hua F, Cui B, Yu JJ, Zhang XW, Shang S, Liu SS et al. (2017) TRIB3 promotes APL progression through stabilization of the oncoprotein PML-RAaRalpha and inhibition of p53-mediated senescence. Cancer Cell 31, 697–710.e7.
30 Sharifi M and Salehi R (2016) Blockage of miR-92a-3p with locked nucleic acid induces apoptosis and prevents cell proliferation in human acute megakaryoblastic leukemia. Cancer Gene Ther 23, 29–35.
31 Tanaka M, Oikawa K, Takanashi M, Kudo M, Ohyashiki K, Ohyashiki K and Kuroda M (2009) Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PLoS One 4, e5532.
32 Cappello AR, Curcio R, Lappano R, Maggiolini M and Dolce V (2018) The physiopathological role of the exchangers belonging to the SLC37 family. Front Chem 6, 122.
33 Yang G, Xu Z, Lu W, Li X, Sun C, Guo J, Xue P and Guan F (2015) Quantitative analysis of differential proteome expression in bladder cancer vs. normal bladder cells using SILAC method. PLoS One 10, e0134727.
34 Rothenberg EV, Hosokawa H and Ungerback J (2019) Mechanisms of action of hematopoietic transcription factor PU.1 in initiation of T-cell development. Front Immunol 10, 228.
35 Frasa MA, Koessmeier KT, Ahmadian MR and Braga VM (2012) Illuminating the functional and structural repertoire of human TBC/RABGAPs. Nat Rev Mol Cell Biol 13, 67–73.
36 Chiaretti S, Li X, Gentleman R, Vitale A, Wang KS, Mandelli F, Foà R and Ritz J (2005) Gene expression profiles of B-lineage adult acute lymphocytic leukemia reveal genetic patterns that identify lineage derivation and distinct mechanisms of transformation. Clin Cancer Res 11, 7209–7219.
37 Ruiz-Lafuente N, Alcaraz-Garcia MJ, Sebastian-Ruiz S, Gomez-Espuch J, Funes C, Moraleda JM, Garcia-Garay MC, Montes-Barqueros N, Minguela A, Alvarez-Lopez MR et al. (2014) The gene expression response of chronic lymphocytic leukemia cells to IL-4 is specific, depends on ZAP-70 status and is differentially affected by an NFkappaB inhibitor. PLoS One 9, e109533.
38 Wong FS and Wen L (2003) The study of HLA class II and autoimmune diabetes. Curr Mol Med 3, 1–15.
39 Vanneman M and Dranoff G (2012) Combining immunotherapy and targeted therapies in cancer treatment. Nat Rev Cancer 12, 237–251.
40 Conte M, De Palma R and Altucci L (2018) HDAC inhibitors as epigenetic regulators for cancer immunotherapy. Int J Biochem Cell Biol 98, 65–74.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Predicted targets of three downregulated miRNAs obtained from miRNet database (n = 1711).
Table S2. Predicted targets of six upregulated miRNAs obtained from miRNet database (n = 1418).