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Journal of Hematology & Transfusion

Research Article

Upregulation of Mir-29 in Normal Karyotype Aml Showing Dnmt3a Mutation

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Submitted: 15 July 2016

Accepted: 04 August 2016

Published: 10 August 2016

ISSN: 2333-6684

Keywords

- Acute myeloid leukemia; DNMT3A; miRNA; Gene expression

Abstract

Background: Acute myeloid leukemia (AML) represents a heterogeneous disorder with recurrent chromosomal alterations and molecular abnormalities. A number of molecular abnormalities have been described involving several genes such as *FLT3, NPM1, CEBPA, IDH1, IDH2* and *DNMT3A* among AML with normal kariotype (NK-AML). *DNMT3A*, a member of DNA methyltransferases, is mutated in approximately 22% of de novo NK-AML; the mutation at codon R882 (R882-DNMT3A mutations) confers unfavorable outcome in this setting. Our previous data demonstrated distinctive miRNA expression patterns in some genetic groups, the aim of the study is to investigate about the miRNA profile and its role in the pathogenesis of DNMT3A mutated AML.

Methods and Results: To indagate about miRNA signature in NK-AML R882-DNMT3A mutated we performed quantitative real-time PCR (TaqMan Human MicroRNA Array, A); we studied the expression of 365 known human miRNA in 9 selected de-novo AML cases showing DNMT3A mutations. We compare miRNA expression data with our previous results obtained in 31 AML DNMT3A unmutated and we focused on an up-regulation of mir-29 family: miR-29a, miR-29b and miR-29c. So we decide to investigate expression levels of these miRNAs in additional 50 new DNMT3A mutated AML patients and we confirm the up-regulation of 2 of them: miR-29a and miR-29c. The most interesting issue is that miR-29s have been demonstrated to directly target 3'-UTR of DNMT3A and DNMT3B resulting in a global hypomethylation but also miR-29s is able to directly suppress the major DNA demethylases, TET1 and TDG. In order to better understand the pathogenesis of the subgroup of AML DNMT3A mutated and the existing correlation between miR-29s and its targets, we evaluated the expression levels of miR-29s targets DNMT3A, DNMT3B, TET1, TET2 and TDG in 50 AML DNMT3A mutated patients and in 50 patients AML DNMT3A wild type (control group) using qRT-PCR with specific TaqMan assay (Applied Biosystems). Results obtained revealed a no significant differences in expression of DNMT3A, DNMT3B and TDG; however we found significant down-regulation of the demethylases TET1 and TET2.

Conclusion: These data suggest that miR-29s act as crucial regulators of DNA methylation and probably in presence of DNMT3A mutations and TET1 down-regulation may cause a perturbation of methylation pattern. This issue may have important implications for treatment and response to hypomethylating drugs in patients affected by alterations in *DNMT3A*.

ABBREVIATIONS

AML: Acute Myeloid Leukemia; NK-AML: Normal Karyotype

INTRODUCTION

Acute myeloid leukaemia is a cytogenetically heterogeneous disorder with acquired recurrent chromosomal alterations detected in about 55% of adult patients. In the remaining cases showing normal karyotype (NK-AML) a number of molecular abnormalities with a prognostic value have been described such as mutations in genes *FLT3*, *MLL*, *CEBPA*, *NPM1, WT1, IDH1, IDH2* and *DNMT3A* [1,2]. *DNMT3A*, a member of DNA methyltransferases, is mutated in approximately 22% of de novo AML patients with normal karyotype leading to adverse overall survival (OS), independently from age and presence of *FLT3* mutations. There are two major types of *DNMT3A* mutations: the first type is the highly recurrent set of mutations at codon R882 (R882-DNMT3A mutations); the second type is represented by all the other mutations in this gene (no-R882-DNMT3A mutations) [3,4]. The heterogeneity of AML can also be resolved according to their microRNA signatures [5-10]. MicroRNA (miRNA) are 19–24 nucleotides noncoding RNA which regulate the expression of target mRNAs at transcriptional and translational level; miRNA deregulation has been found in different human diseases, including cancer, leukaemia, diabetes, immuno- or neurodegenerative disorders [11-13]. Their role in normal hematopoiesis elucidated by recent studies, reveal specific variations of the miRNome during the commitment and development of the hematological stem cells in the different lineage [14-16]. Our previous data demonstrated distinctive miRNA expression patterns in some genetic groups [10]. The aim of this study is to investigate about the miRNA profile and its role in the pathogenesis of DNMT3A mutated AML.

MATERIALS AND METHODS

Patients and specimens

100 cases of AML, genetically characterized at "Cervello

Hospital" of Palermo, were enrolled. Diagnosis of AML was established according to WHO classification criteria by standard morphological, cytogenetic, immunophenotypic and molecular studies [17]. AML patients were selected to obtain comparable genetic groups: 50 DNMT3 mutated AML and 50 AML with other genetic alterations; patient's characteristics are shown in (Table 1). Genes and *miR-29s* expression levels were evaluated in samples from bone marrow obtained at diagnosis or from samples of peripheral blood showing more than 70% leukemic cells. All AML samples were taken for diagnostic purposes from patients registered in the GIMEMA protocols AML12 (ISRCTN number 17833622) and AML 1310 (Eudra CT number 2010-023809-36) who gave informed written consent for usage of biological material not necessary only for diagnostic purposes but also for scientific purposes. GIMEMA protocols were approved by Medical Ethics Committee of AOOR Villa Sofia Cervello "Comitato Etico Palermo 2".

Cytogenetic and molecular analysis

Cytogenetic studies from bone marrow specimens were performed according standard procedures. For each culture 15-20 metaphases were karyotyped. Chromosomal abnormalities were classified according to the International System for Cytogenetic Nomenclature (ISCN) [18]. Molecular studies of leukemic cells were performed to detect gene fusion-transcripts associated with AML as described by EAC protocols [19]. *FLT3* and *NPM1* gene mutations analysis were performed according to Meshinchi S, et al. (2001) [20], and to Falini B, et al. (2005) [21], respectively. DNMT3A mutation were studied by standard sequencing of exon 23 and DNMT3A-R882-mutated samples were examined using Sequencing Analysis and SeqScape software analysis on a AB3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

miRNA profile

Total RNA was extracted from blast cells from bone marrow or peripheral blood at diagnosis by the QIAsymphony System (QIAGEN). To study miRNA expression levels, we used a low-density array system, the TaqMan Custom Array-TLDA human miRNA panel (4342265, Applied Biosystems). This technology is a method for quantitative RT-PCR (Q-RT-PCR) that can simultaneously assay the expression levels of up to 365 different miRNA genes on a single card (about 50% of the miRNA currently known, the composition of the array is viable at www.appliedbiosystems.com). The low-density arrays were performed in accordance to manufacturer's protocols as previously described [10]. We utilized our previous data for the control categories (CD34 healthy donors and AML DNMT3A Wt) and we performed 3 new arrays with a pool of three cases each for a total of 9 new diagnosed DNMT3A mutated AML. Data were quantified using the SDS 2.3 software and normalized using the RNU48 as endogenous control. The cycle threshold (Ct) value, which was calculated relatively to the endogenous control, was used for our analysis (ΔCt). The 2-ΔΔCT (delta-delta-Ct algorithm) method was used to calculate the relative changes in gene expression among different groups. Heat map was constructed by RStudio software using ΔCt values; in particular, to increase the stability of results we applied filtering criteria that included miRNA which were reliably quantifiable in at least 70% of single categories.

miRNA expression

Total RNA was extracted from blast cells derived from bone marrow at diagnosis by the QIA symphony System (QIAGEN). To study *miR*-29s expression levels, we used TaqMan assay for *miR-29a*, *miR-29b* and *miR-29c* (002447, 000413, 000587 Applied Biosystems). Briefly, reverse transcription reactions were performed for each sample using the High Capacity cDNA Archive Kit (Applied Biosystems) with the specific primer 5X for single *miR-29s*. One μL of cDNA from each sample was added to a 6.5 μL of TaqMan® Universal PCR Master Mix and 0.65 μL of 20X *miR-29s* probe in a final volume of 13 μL. Q-PCR amplifications were performed on an ABI 7900HT Q-RT-PCR; data were quantified using the SDS 2.3 software and normalized using the RNU48 as endogenous control. The cycle threshold (Ct) values, which were calculated relatively to the endogenous control, were used for our analysis (ΔCt). The 2-ΔΔCT method was used to calculate relative changes in gene expression among different samples.

miR-29 targets prediction analysis

To predict potential target genes of miR-29s we utilized miRBase (http:// http://www.mirbase.org/). miRBase is a searchable database of published miRNA sequences and annotations. This resources is linked to TargetScanHuman application, allowing us to identify target gene of 29 miRNAs family simultaneously, applying a "consensus" aggregate P_{CT} score> 0.8. This score reflected the Bayesian estimate of the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance or any other reason not pertinent to miRNA targeting.

miR-29s targets gene expression

To determinate mRNA expression levels of miR-29s targets we used Q-RT-PCR with the 7900 TAQMAN system and pre-designed available assay (Assay on Demand, Applied Biosystems). Q-RT-PCR data were quantified using the SDS 2.3 software and normalized using the *ABL* as endogenous control. To calculate relative changes in gene expression among different samples we used the 2-ΔΔCT method.

Statistical analysis

Results from each TaqMan run were quantified separately. To increase the accuracy of the results, we applied filtering criteria that included gene control expression which were reliably quantifiable (cutoff < 35 Ct). Undetermined values of Ct were estimated at 50 Ct (the last cycle of the reaction). Resulting ΔCts were used for our analysis. Differential expressions of miRNA and mRNA targets were defined using the Relative Expression Software Tool (REST) proposed by Pfaffl (2002) [22]. Fold change was estimated by REST. Namely, a gene was defined differentially expressed when estimated p-value was < 0.05.

To asses correlation between targets and miR-29s we utilized Spearman's rank correlation test also.

RESULTS AND DISCUSSION

Significantly differentially expressed miRNAs in DNMT3A-mutated AML

To identify the most significantly differentially expressed

miRNA between purified CD34+ hematopoietic progenitor cells from healthy donors and AML DNMT3A mutated samples, we performed a supervised analysis by REST. We identified a set of 38 differential expressed miRNA, 2 of them being decremented and 36 upregulated in DNMT3A mutate samples (Table 2). Another step was finalized to study the differentially expressed miRNA in AML DNMT3A mutated compared to different genetic categories of AML, for this reason, CD34 samples were excluded from further analysis. Unsupervised hierarchical cluster analysis, generated on all 365 miRNAs, failed to clearly group the DNMT3A mutated in the same cluster (data not show). To find miRNAs with statistically significant differences in expression level among the major AML genetic groups and DNMT3A mutated group, we performed a REST analysis and we observed 83 differentially expressed miRNAs. Unsupervised hierarchical cluster analysis, generated on the differentially expressed miRNAs, clearly grouped the DNMT3A mutated in the same cluster, in particular those samples DNMT3A+ve/NPM1neg are grouped in the same subcluster (Figure 1). Furthermore we observed 20 miRNAs up-regulated with a stronger significant p-value (Table 3). Due to the relevant role on epigenetic regulation of miR29s, that we found over expressed in DNMT3A compared to both control groups (CD34 and AML DNMT3a Wt), we decide to investigate expression levels of these miR29s in additional 50 newly diagnosed DNMT3A mutated AML patients.

Up-regulation of miR-29s in DNMT3A-R882-mutated AML

The analysis was finalized to study the expression level of *miR-29s* in a cohort of 100 AML including 50 DNMT3A mutated and 50 DNMT3A Wt samples; patient's characteristics are shown in (Table 1). Statistical analysis performed by t-test, showed up-regulation of *miR-29s* ($p<0.02$) in DNMT3A mutated AMLs compared with AML without DNMT3A mutations. We evaluated the expression of single family member's *miR-29a*, *miR-29b* and *miR-29c* in our cohort. ΔCt values of single samples (*miR-29s* Ct - RNU48 Ct) of both groups are reported respectively in (figure 2A, 3A and 4A). ΔCt values are plotted in ascending order that inversely correlate to expression levels. (Figure 2A and 4A) clearly showed a lower ΔCt in DNMT3A mutated group. Statistical analysis performed by REST showed a 2,4 fold up-regulation of *miR-29a* ($p<0.01$) in DNMT3A mutated AMLs compared with AML without DNMT3A mutations (Figure 2B), no difference in expression of *miR-29b* (Figure 3B) and a 7,5 fold up-regulation of *miR-29c* ($p<0.001$) in DNMT3A mutated AMLs compared with AML without DNMT3A mutations (Figure 3B).

miR-29 targets prediction analysis

We used TargetScanHuman to predict target gene of miR-29 family and we found 679 miRNAs with a $P_{CT}> 0.8$ score. Next in this selection we looked for targets with a central role in the

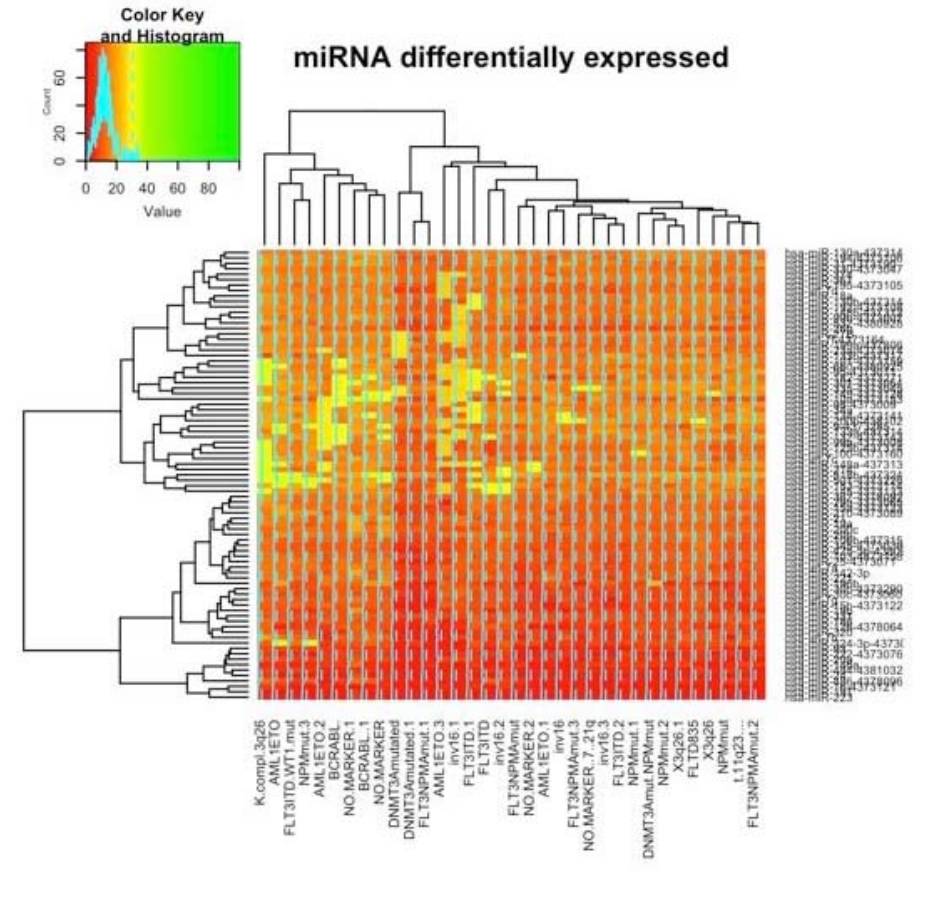


Figure 1 Heatmap of differentially expressed miRNAs in 31 DNMT3A Wild type (Wt) and 9 DNMT3A mutated patients.

Table 1: Patient's characteristics.	
Patients DNMT3A mutated	**Total 50**
Mean Age	66 (range 43-89)
Male/Female	23/27
DNMT3A mutated	16
DNMT3A /NPM1 mutated	23
DNMT3A /NPM1/FLT3 mutated	1
DNMT3A /IDHs mutated	8
DNMT3A /NPM1/IDHs mutated	2
DNMT3A / CEBPA mutated	0
Patients DNMT3A unmutated	**Total 50**
Mean age	50 (range 11-88)
Male/Female	25/25
NK/No marker	16
NPM1 mutated	5
NPM1/FLT3 mutated	2
FLT3 mutated	7
MLL rearrangement	2
CBF leukemia	3
IDH	2
CEBPA	2
Monosomy 7	1
Trisomy 8	3
Complex Karyotype	5
5q aberrations	2
Abbreviations: NK, normal karyotype; No marker = negative for molecular screening of fusion-transcripts associated to AML.	

determination of methylation cell status and we identified the demethylases: TET2 (P_{CT}> 0.99), TET1 (P_{CT}> 0.99) and TDG (P_{CT}: 0.82) and the methylases DNMT3B (P_{CT}: 0.94) andDNMT3A (P_{CT}: 0.80).

Expression levels of miR-29s targets in AML blast cells

We tested the expression of predicted and known to be *mirR-29s* targets genes: the methylases DNMT3A and DNMT3B, the demethylases *TET1,* TET2 and TDG in our cohort of 100 AML patients. mRNA levels of target genes were obtained by Q-RT-PCR, ΔCt values of single samples (target gene Ct – ABL Ct) of both groups are reported respectively in (Figure 5A,C and 6A,C,E). ΔCt values are plotted in ascending order that inversely correlate to expression levels. TET1 and TET2 expression are reported in (Figure 6A & 6C) that clearly showed a higher ΔCt in DNMT3A mutated group. We found that *DNMT3A and DNMT3B* genes do not exhibit a significant inverse correlation with *miR-29s* up-regulation (Figure 5 B,D), on the contrary demethylases *TET1* and *TET2,* but not *TGD,* exhibit an inverse trend compared with *miR-29s* up-regulation, being down-regulated in DNMT3A-mutated AML versus others, with a fold of 0.6 fold ($p<0.009$) and of 0.36 ($p<0.001$) respectively (Figure 6 B,D,F). Moreover we found an inverse correlation between *TET2* and *miR-29a* with a Spearman's rank correlation coefficient of -0,39. We also found low negative value (<-0,3) of Spearman's rank correlation coefficient among TET1 vs *miR-29a* and TET1 *and* TET2 vs. *miR29c.*

DISCUSSION

In AML, the association of microRNA expression profile and AML genetic categories has been investigated in recent studies [5-11], indeed AML genetic heterogeneity can also be resolved according to their variable miRNA expression signatures. We used quantitative real-time RT-PCR to study the expression of 365 known human miRNAs in a cohort of 9 primary selected AML DNMT3A mutated cases and we compare their miRNA profile to those obtained, in our previous data [10], from AMLs characterized by common cytogenetic and molecular alterations and from three controls of purified CD34+ hematopoietic progenitor cells from healthy donors.

Not surprisingly, AML DNMT3A mutated cases could be distinguished, based on profile of expressed miRNA, from the other genetic groups by an unsupervised clustering method. The miRNA profile in DNMT3A mutated AML was investigated by Marcucci et al. [4], by microarray experiments but the authors did not found any signature associated with DNMT3A mutations and they suggest that these features could be confuse due to the positive association of DNMT3A mutations with NPM1 mutations and the negative association with CEBPA mutations. For the first time, we describe miRNA expression profile derived from a quantitative technology associated with high-risk AML carrying DNMT3A mutations and we found a group of 83 deregulated miRNA and among them two members of the miR-29 family, miR-29a and miR-29c.

The human miR-29 family consists of 3 members: miR-29a, miR-29b and miR-29c; despite the identical seed sequence, recent studies suggest a context-dependent pattern for miR-29s expression and regulation. Consistently, a variety of physiological and disease conditions have been correlated to a differential expression and subcellular organization of miR-29 family members [23].

In this report, to clarify the role of *miR-29*s deregulation in DNMT3A mutated AML, we consolidate the data that *miR-29a* and *miR-29c,* but not *miR-29b,* are up-regulated in DNMT3A mutated AML. We used quantitative real-time RT-PCR to study *miR-29s* expression in a large cohort of 100 primary AML characterized by common cytogenetic and molecular alterations, including 50 DNMT3A-mutated AML. According to miRNA profile data, we found *miR-29a and miR-29c* to be specifically up-regulated in this category. The way leading to aberrant expression of miRNAs is not yet completely understood. The mechanisms through which DNMT3A mutation associates with increased *miR-29s* expression are almost undefined, although exists indirect evidence for epigenetic regulation of miRNAs from DNA methyltransferase (DNMTs) [24,25]. Moreover miR29a/29b-1 cluster is a candidate for DNA methylation dependent regulation and is epigenetically activated in CLL [26].

Growing evidence supports a role for microRNAs (miRNAs) as both targets and effectors in aberrant mechanisms of DNA methylation [27]. Studies in AMLs have provided a wide scenario supporting the role of miR-29s as epi-miRNAs. Disturbances in expression levels of these epi-miRNAs have been linked to

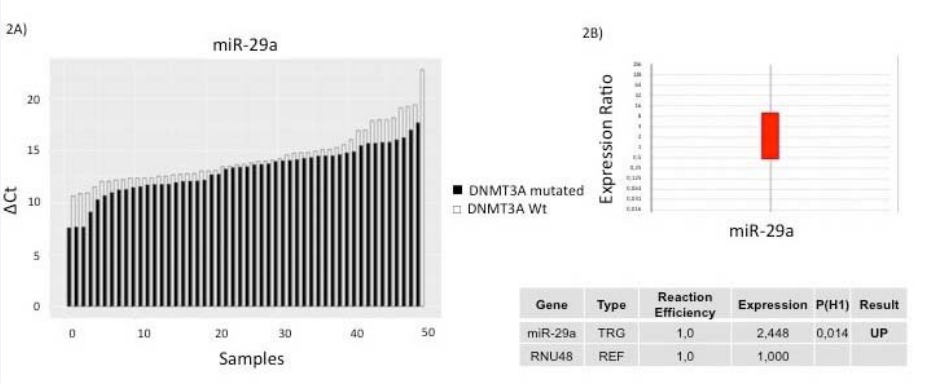


Figure 2 A) Histogram of relative expression levels of miR-29a in 50 DNMT3A Wt (white bars) and 50 DNMT3A mutated patients (black bars). The Y axes indicate ΔCt values (target miRNA Ct - RNU48 Ct) in ascending order that inversely correlated to level of expression. B) Graphic representation of miR-29a expression data obtained by REST analysis showing a miR-29a up-regulation (2,448-fold, p-value = 0.014) in DNMT3A mutated AML cases compared with the cohort of patients without DNMT3A mutation. Data are showed as expression ratio mean value between the two groups. The figure shows box plots of gene expression where the top and bottom of each box indicate the 75th and 25th percentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.

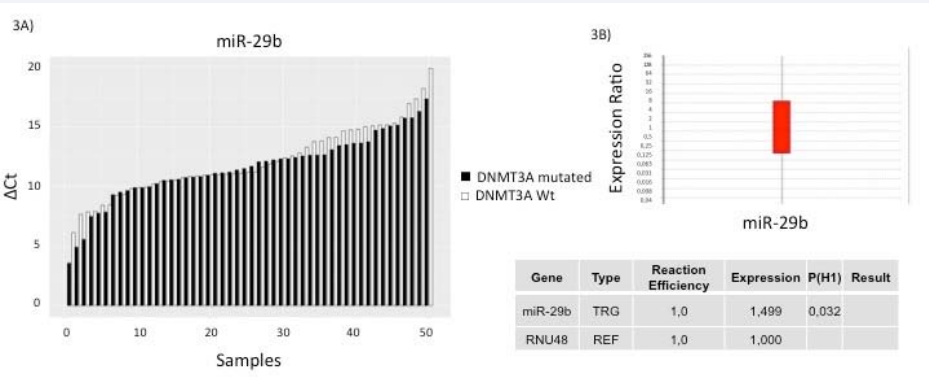
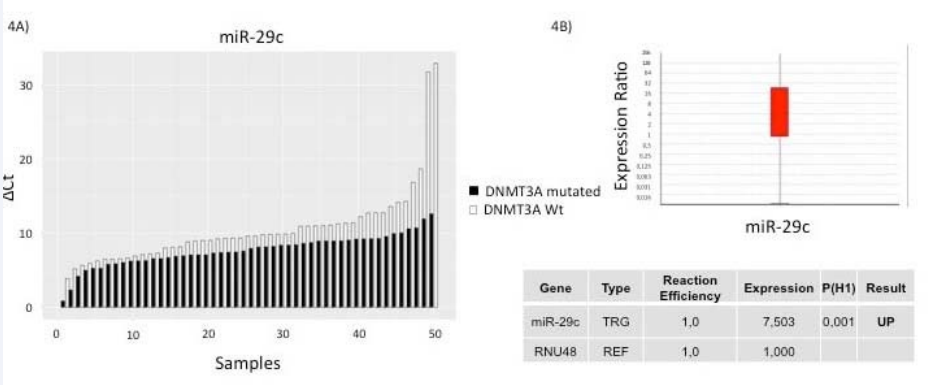


Figure 3 A) Histogram of relative expression levels of miR-29b in 50 DNMT3A Wt (white bars) and 50 DNMT3A mutated patients (black bars). The Y axes indicate ΔCt values (target miRNA Ct - RNU48 Ct) in ascending order that inversely correlated to level of expression. B) Graphic representation of miR-29b expression data obtained by REST analysis showing the levels of miR-29b in DNMT3A mutated AML cases compared with the cohort of patients without DNMT3A mutation. Data are showed as expression ratio mean value between the two groups. The figure shows box plots of gene expression where the top and bottom of each box indicate the 75th and 25th percentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.

Table 2: miRNA differentially expressed between AML DNMT3A mutated and CD34 healthy donors.

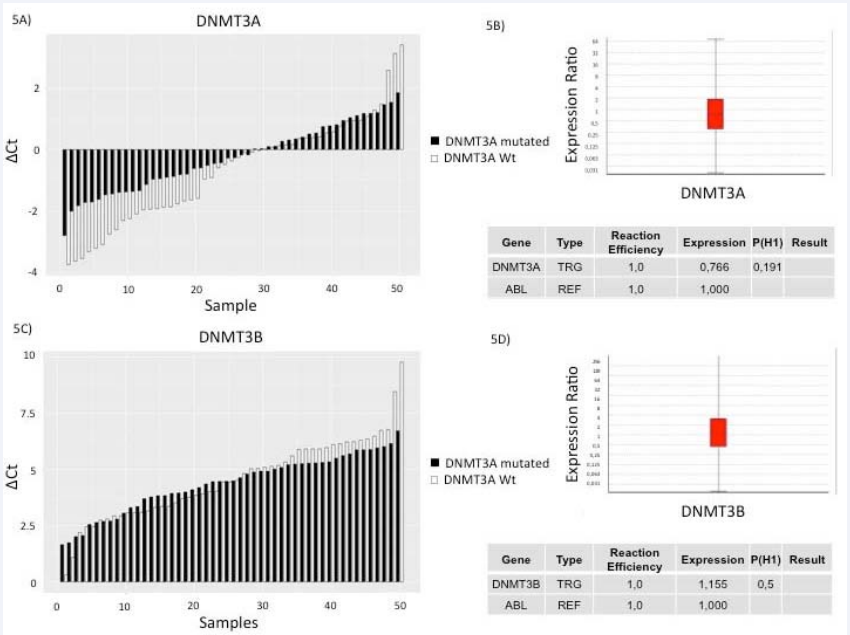
miRNA	p value REST	Fold expr. REST	miRNA	p value REST	Fold expr. REST
hsa-miR-210	p<0.001	10205	**hsa-miR-642**	p<0.001	114,8
hsa-miR-106b	p<0.001	9992	**hsa-miR-618**	p<0.001	113
hsa-miR-133a	p<0.001	4982,2	**hsa-miR-200b**	p<0.001	112,4
hsa-miR-29a	p<0.001	3594,6	**hsa-miR-29c**	p<0.001	109,4
hsa-miR-99a	p<0.001	3563,3	**hsa-miR-152**	p<0.001	78,7
hsa-miR-194	p<0.001	3507,5	**hsa-miR-518b**	p<0.001	27,7
hsa-miR-660	p<0.001	2893,2	**hsa-miR-16**	p<0.001	17,2
hsa-miR-501	p<0.001	2530,7	**hsa-let-7e**	p <0.031	45038,5
hsa-miR-362	p<0.001	2488,8	**hsa-miR-34a**	p <0.031	3.885
hsa-miR-132	p<0.001	2448,3	**hsa-miR-320**	p <0.031	103
hsa-miR-195	p<0.001	1754,8	**hsa-let-7b**	p< 0.031	5,3

hsa-miR-183	p<0.001	1274,7	**hsa-miR-218**	p< 0.032	179,4
hsa-miR-130b	p<0.001	1234,1	**hsa-miR-9**	p< 0.033	348,8
hsa-miR-101	p<0.001	889,8	**hsa-miR-19b**	p< 0.033	5,3
hsa-miR-224	p<0.001	877,2	**hsa-miR-301**	p< 0.035	495,7
hsa-miR-296	p<0.001	630,3	**hsa-miR-191**	p< 0.035	55
hsa-miR-27a	p<0.001	320,6	**hsa-miR-21**	p< 0.036	7947
hsa-miR-130a	p<0.001	304,5	**hsa-miR-345**	p< 0.039	32034,2



Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
miR-29c	TRG	1,0	7,503	0,001	UP
RNU48	REF	1,0	1,000		

Figure 4 A) Histogram of relative expression levels of miR-29c in 50 DNMT3A Wt (white bars) and 50 DNMT3A mutated patients (black bars). The Y axes indicate ΔCt values (target miRNA Ct - RNU48 Ct) in ascending order that inversely correlated to level of expression. B) Graphic representation of miR-29c expression data obtained by REST analysis showing a miR-29c up-regulation (7,503-fold, p-value = 0.001) in DNMT3A mutated AML cases compared with the cohort of patients without DNMT3A mutation. Data are showed as expression ratio mean value between the two groups. The figure shows box plots of gene expression where the top and bottom of each box indicate the 75th and 25th percentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.



Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
DNMT3A	TRG	1,0	0,766	0,191	
ABL	REF	1,0	1,000		

Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
DNMT3B	TRG	1,0	1,155	0,5	
ABL	REF	1,0	1,000		

Figure 5 A) and C) Histogram of relative expression levels of methylases DNMT3A and DNMT3B in 50 DNMT3A Wt (white bars) and 50 DNMT3A mutated patients (black bars). The Y axes indicate ΔCt values (target gene Ct - ABL Ct) values in ascending order that inversely correlated to level of expression. B) and D) Graphic representation of DNMT3A and DNMT3B expression data obtained by REST analysis showing the levels of DNMT3A and DNMT3B genes in DNMT3A mutated AML cases compared with the cohort of patients without DNMT3A mutation. Data are showed as expression ratio mean value between the two groups. The figure shows box plots of gene expression where the top and bottom of each box indicate the 75th and 25th percentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.

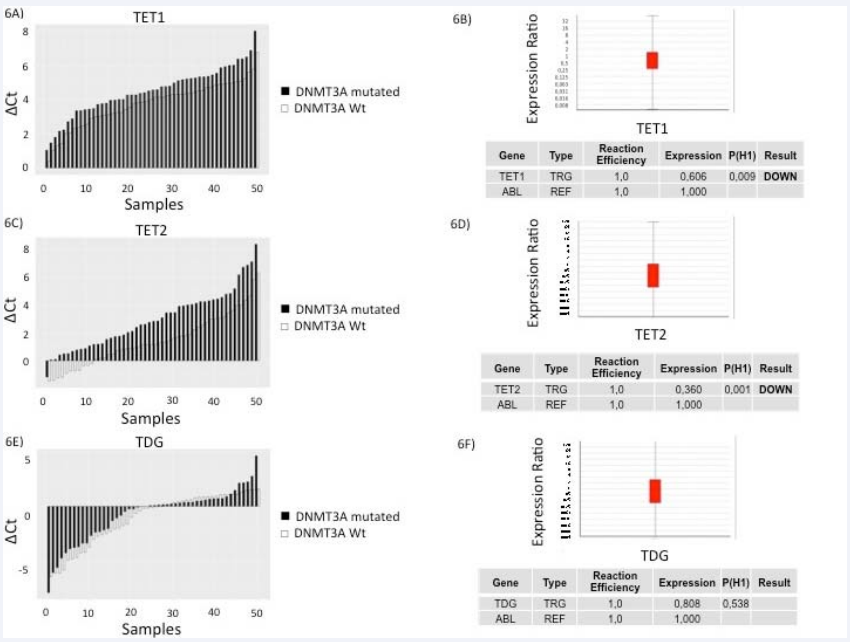
tumorigenesis and tumor aggressiveness. Members of the miR-29 family directly target the *de novo* DNA methyltransferases, DNMT3A and DNMT3B and then miR-29s can repress DNA methylation [28] but they are also able to suppress tumorigenesis by preserving the DNA methylation status. Indeed it was demonstrated that miR-29s repress two important components of the DNA demethylation pathway, namely TET1 and TDG [29]. Therefore, miR-29s repress the activities of both DNA methyltransferases and DNA demethylases, which have opposing functions in the control of DNA methylation.

We, further, corroborated *miR-29s* findings in our cohort of 100 AML samples demonstrating an inverse behavior between their up-regulation and the down-regulation of the mRNA level of their targets TET1 and TET2.

These data suggest that miR29s act as a crucial regulator of DNA methylation and probably in presence of DNMT3A mutations and TET1 down regulation may cause a perturbation of methylation pattern.

Increasing knowledge in cancer epigenetics, as well as the availability of experimental tools to study epigenetic modifications, suggests that epigenetic alterations are also involved in the development of human cancer [30]. In general, human tumors are globally hypomethylated and this contributes to genomic instability, transposon activation and accumulation of mutations, however, locus-specific hypermethylation occurring at CpG islands located in the promoter region of TF genes, have been detected in many hematologic neoplasias like AML [31]. DNMT3A constitutes one of the enzymes mainly responsible for *de novo* methylation. DNMT3A is essential for normal hematopoietic cell differentiation and defects in DNMT3A and DNMT3B lead to impaired self-renewal capacity in mouse hematopoietic stem cell (HSC) [32]. *DNMT3A* mutations are among the most common mutations in AML, most of the DNMT3A mutations are in heterozigous and located in the catalytic domain at the R882 aminoacidic residue. R882H mutation has recently been shown to act as a dominant negative that inhibits wild-type DNMT3A [33] and its presence have been linked to decrease methylation levels of some genes such as two homeobox genes HOXA11 and HOXB2 [34,35] and the HOX cofactor MEIS1 [36]. However, no clear epigenetic signature has so far emerged that could explain its role in the pathogenesis of AML DNMT3A mutated. We hypotize that the ipomethylation associated to DNMT3A R882H mutation may cause the epigenetic reactivation of *miR-29s*; overexpression of miR-29s perturbs the global methylation pattern downregulating their target TET1 and TET2.

TETs, methylcytosine dioxygenase protein, are enzymes that convert 5methylCytosina (5mC) to 5-hydroxymethylcytosine (5hmC). 5hmC is not mantained by DNMT1 causing passive demethylation during cell division [37-39]. In myeloid blast cells carrying DNMT3A mutation may be present, despite the ipomethylated genome status, some specific hypermethylation locus located in the promoter region of transcription factor (TF) genes involved in the myeloid differentiation that physiologically will be reactivated during development and cell division, the deregulation of the TETs activity, in this setting, may impair this mechanism. Because DNMT3A mutations seems to cause a reduction of DNA methyltransferase activity, the results of



Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
TET1	TRG	1,0	0,606	0,009	DOWN
ABL	REF	1,0	1,000		

Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
TET2	TRG	1,0	0,360	0,001	DOWN
ABL	REF	1,0	1,000		

Gene	Type	Reaction Efficiency	Expression	P(H1)	Result
TDG	TRG	1,0	0,808	0,538	
ABL	REF	1,0	1,000		

Figure 6 A), C) and E) Histogram of relative expression levels of demethylases TET1, TET2 and TDG in 50 DNMT3A Wt (white bars) and 50 DNMT3A mutated patients (black bars). The Y axes indicate ΔCt values (target gene Ct - ABL Ct) in ascending order inversely that correlated to level of expression. B), D) and F) Graphic representation of TET1, TET2 and TDG genes expression data obtained by REST analysis showing a TET1 and TET2 up-regulation (0,606 fold; p-value=0.009 and 0,36 fold; p value 0,001 respectively) in DNMT3A mutated AML cases compared with the cohort of patients without DNMT3A mutation. Data are showed as expression ratio mean value between the two groups. The figure shows box plots of gene expression where the top and bottom of each box indicate the75th and 25thpercentiles, respectively, whereas the dotted-line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles.

Table 3: The 20 differentially expressed miRNA between AML DNMT3A mutated and DNMT3A unmutated showing the lowest p value.

miRNA	p value REST	Fold expr. REST
miR-210	p< 0.001	270,78
let-7b	p< 0.001	245,32
miR-145	p< 0.001	194,13
miR-133a	p< 0.001	193,03
miR-34	p< 0.001	147,15
miR-532	p< 0.001	52,39
miR-126	p< 0.001	52,03
miR-320	p< 0.001	48,18
let-7d	p< 0.001	40,60
miR-25	p< 0.001	31,63
miR-425-5p	p< 0.001	26,61
miR-194	p< 0.001	26,03
miR-15b	p< 0.001	20,58
let-7g	p< 0.001	20,25
let-7a	p< 0.001	13,63
miR-21	p< 0.001	12,79
miR-20b	p< 0.001	12,76
miR-29a	p< 0.001	11,15
miR-191	p< 0.001	10,84
miR-345	p< 0.001	10,62

therapeutic benefit provided by demethylating agents [40] is puzzling and may be explain by the putative reactivation of TF that play a key role on myeloid differentiation.

The performed study suggests that the DNMT3A R882H loss of function mutation may contribute to the significant upregulation of miR29s and consequent downregulation of demethylase TETs. This complex deregulation would contribute to epigenetic perturbations of the genome in patients with DNMT3A mutation. This issue may have important implications for treatment and response to hypomethylating drugs in patients affected by alterations in DNMT3A. In this scenario, epi-miRNAs are emerging as excellent candidate epi-therapeutics for their prominent role in the regulation of the epigenetic machinery.

CONCLUSION

In conclusion our study provides data on *miR-29* family members overexpression associated with DNMT3A mutated AML and describes an opposite trend between these miRNAs and their targets TET1 and TET2 and in particular show an inverse correlation between *miR-29a* and TET2. These mechanisms may be involved in the molecular pathogenesis of DNMT3A mutated AML and suggest a molecular pathway that, starting from DNMT3A mutation, through *miR-29* family members, alter methylation pattern in this disease, providing a rationale for treatment with hypomethylating drugs.

ACKNOWLEDGEMENTS

We thank AML patients for their valuable involvement with this study. This work was supported by a grant of Associazione Italiana contro le leucemie, linfomi e mielomi - Palermo (AIL).

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Cite this article

Randazzo V, Salemi D, Agueli CCannella S, Marfia A, et al. (2016) Upregulation of Mir-29 in Normal Karyotype Aml Showing Dnmt3a Mutation. J Hematol Transfus 4(2): 1048.