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miR-21 is overexpressed in NPM1-mutant acute myeloid leukemias

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ABSTRACT

MicroRNAs (miRs) play a key role in the pathogenesis of human malignancies and particularly in acute myeloid leukemias (AMLs) and are increasingly recognized as potential biomarkers and therapeutic targets. miR-21 is dysregulated in several types of cancers, including some hematologic malignancies, and plays a key role in carcinogenesis, disease recurrence and metastasis. However, no studies have specifically investigated the role of miR-21 in AMLs.

In this study we analyzed the expression of miR-21 and of its target PDCD4 (Programmed Cell Death 4) during normal hematopoietic differentiation and in AMLs. Our results showed that: (i) miR-21 expression is strongly up-modulated during normal granulo/monocytic differentiation, while PDCD4 protein level is concomitantly downmodulated; (ii) miR-21 is frequently overexpressed in AML blasts, in association with a marked PDCD4 protein downmodulation; (iii) miR-21 expression level is particularly elevated in NPM1mutant AMLs.

Together, these findings suggest that deregulated miR-21 expression may contribute to disease pathogenesis in NPM1-mutated AMLs.

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1. Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous disease, and, despite recent advances in the field, remains a frequently incurable tumor. Several mechanisms underlying the molecular pathogenesis of AML have been investigated. It is now known that AML results from mutations in classes of genes that affect both proliferation/survival and differentiation/apoptosis. Changes in transcriptional control of hematopoietic cells and mutations involving different proto-oncogenes or tumor suppressors are responsible for the deregulation of normal myelopoiesis (Cancer Genome Atlas Research Network, 2013). Such changes ultimately result in the activation of cellular pathways that mediate signals, which promote cell growth and/or maintain survival, leading to leukemogenesis [1–3].

Programmed cell death, also called apoptosis, plays a key role in many biological processes, including embryonic development, cell differentiation, tissue turnover, tumor development [4]. The evasion from the physiologic mechanisms of cell death is one of the hallmarks of the cancer cell [5].

Programmed Cell Death 4 (PDCD4) is a gene initially identified as being up-regulated after initiation of programmed cell death [6]. PDCD4 protein protects from apoptosis by lowering and/or suppressing the translation of procaspase-3 mRNA [7]. In addition to its role in apoptosis, PDCD4 exerts many other biological functions and growing evidence indicates that it acts as a tumor suppressor gene [6].

The decrease of PDCD4 observed in many tumors has been ascribed to the expression of miR-21, a microRNA that directly downregulates PDCD4 expression [8,9]. A large number of studies have shown that miR-21 is overexpressed in glioblastoma, carcinomas of the lung, prostate, breast, pancreas, colon, head and neck, stomach, esophagus and liver, compared to normal adjacent non-neoplastic tissues (reviewed in [10]); miR-21 is also up-regulated in blood cancers such as chronic lymphocytic leukemia [11], lymphoma and multiple myeloma. These observations emphasize the concept that miR-21 is the only miRNA that is up-regulated in all types of tumors, supporting its role as an ubiquitous oncogene [12]. Importantly, a meta-analysis of all published studies clearly showed that the elevated miR-21 expression is significantly

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associated with poor survival in patients with various types of carcinoma [10]. It is worth noting that the pro-oncogenic role mediated by miR-21 overexpression is not only related to suppression of PDCD4, but also to the functional targeting of other genes (such as SPRY1 and PTEN) involved directly or indirectly in the control of apoptosis [13].

Surprisingly, few studies concerning the expression of miR-21 and its possible prognostic significance and association with specific molecular abnormalities have been reported in acute myeloid leukemia (AML) patients: thus, Garzon and coworkers reported that miR-21 was one of the microRNAs most up-regulated in NPM1-mutated AMLs [14], while Jongen-Lavrencic and coworkers reported that miR-21 was generically overexpressed in AML samples, compared to the normal hematopoietic counterpart, and it is particularly deregulated in AMLs with C/EBPA and inv(16) mutations [15].

In general, the clonal chromosomal abnormalities and genetic alterations observed in the leukemic cells from AML patients can be used to categorize cases into discrete groups with distinct prognostic outcome [16,17]. However, the prognosis of most AML patients is dismal, and a high incidence of adverse cytogenetic features (particularly in elderly patients) contributes to confer chemoresistance to leukemic cells to chemotherapeutic drugs and accounts for such unfavorable outcomes [18,19]. Furthermore, a number of cytogenetically undetectable genetic mutations have significant impact on AML prognosis, refining the disease risk stratification, especially in patients with a normal karyotype. Among these, mutations in the nucleophosmin (NPM1), CCAAT/enhancer binding protein alpha (CEBPA), and c-kit genes, as well as the internal tandem duplications of Fms-like tyrosine kinase 3 gene (FLT3-ITD) are listed as molecular predictors of AML prognostic outcome in both the European Leukemia Net (ELN) classification and the National Comprehensive Cancer Network (NCCN) guidelines.

NPM1 is a nucleus-cytoplasm shuttling protein already known to be involved in chromosome rearrangements in acute leukemia and lymphoma [20,21]. The newly reported subtle mutations, which are mostly detectable in AML with normal cytogenetics (AML-NC), consist of insertions/deletions in *NPM* exon 12 that encodes the nucleic acid-binding domain (NABD) at the C terminal of the nucleophosmin (NPM) protein. The abnormal mutated NPM1 protein shows an aberrant cytoplasmic localization, whereas the wild-type protein is mainly located in the nucleolus and the nuclear membrane.

Mutations in the nucleophosmin (*NPM1*) gene represent the most common genetic alteration in AML with normal karyotype (AML-NK) and, in the absence of a concomitant *FLT3*-ITD mutation, are considered to confer good prognosis [22].

The enormous progress achieved in the understanding of the molecular basis of AMLs is essential not only for a better understanding of the pathogenesis of this heterogeneous group of leukemias, but also for their classification and, importantly, for the identification of new targets for development of specific therapies. Several studies indicated that miRNAs may be used to develop a panel of diagnostic and prognostic biomarkers and that deregulation of miRNA expression characterizes different subtypes of AMLs [23,24].

The expression of miR-21 and PDCD4 during normal hematopoietic differentiation has not been investigated, while this information is fundamental to better evaluate possible expression abnormalities of this miR occurring in AMLs. In this context, the only available observation refers to the regulatory effect of the zinc finger growth factor independent-1 (GFI-1) on myelopoiesis, which seems to be related to the effects exerted by this transcription factor on miR-21 expression [25]. In this study it was reported an increase of miR-21 expression in the transition between CMP and GMP in Lin^- cells purified from bone marrow, and that its forced expression alters myeloid colony formation [25].

Here, we evaluated both miR-21 and PDCD4 expression in normal and malignant granulo/monocytic cells. Our results provided clear evidence that: (i) miR-21 expression is strongly up-modulated during normal granulo/monocytic differentiation, while PDCD4 protein level is concomitantly downmodulated; (ii) miR-21 is frequently overexpressed in AML blasts, in association with a marked PDCD4 protein downmodulation; (iii) miR-21 level of expression in NPM1mut/AML allowed the identification of other subgroups of AMLs with defined immunophenotype.

2. Materials and methods

2.1. Leukemic cells

AML fresh leukemic blasts were isolated from diagnostic bone marrows obtained from 91 unselected AML patients, using Ficoll–Hypaque density-gradient centrifugation and immediately processed. Leukemias were classified by morphological and cytochemical criteria according to FAB classification (i.e. *1 M0*, *15 M1*, *22 M2*, *18 M3*, *22 M4*, *12 M5*, *1 M6*). Analysis was performed after informed patient consent, obtained in accordance with the Declaration of Helsinki with ethical approval of the IRB from the Policlinico Tor Vergata of Rome (Italy).

2.2. HPCs purification, granulocytic and monocytic culture

Cord blood (CB) was obtained after informed consent from healthy full-term placentas according to institutional guidelines. Human $CD34^+$ cells were purified from CB by positive selection using the midi-MACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladabach, Germany) according to the manufacturer's instructions. The purity of $CD34^+$ cells was assessed by flow cytometry using a monoclonal phycoerythrin (PE)-conjugated anti-CD34 antibody and was routinely over 95% (range, 92–98%).

$CD34^+$ progenitor cells were cultured in serum-free medium in the presence of 1 U/ml IL-3, 0.1 ng/ml GM-CSF combined with saturating level of G-CSF (500 U/ml) (PeproTech, Rocky Hill, NJ, USA) to induce granulocytic differentiation and in the presence of M-CSF (10 ng/ml), IL-6 (10 ng/ml) and FLT3 ligand (50 ng/ml) to induce monocytic differentiation.

2.3. Analysis of FLT3 and NPM1 mutational status

Total RNA was extracted from Ficoll–Hypaque isolated mononuclear cells using the method of Chomczynsky and Sacchi [26]. RNA was reverse-transcribed using random examers primers. To determine the mutational status of Flt3 and NPM1 genes, a multiplex RT-PCR assay, followed by capillary electrophoresis, was used (NFmPCR assay [27]). This assay was previously reported and validated in a large number of AML samples [27].

2.4. RNA extraction and RT-PCR

Total RNAs were extracted using Trizol reagent and reverse transcribed by Moloney murine Leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo (dT).

PDCD4 mRNAs were detected by SYBR Green system and normalized with GAPDH using the following primers:

PDCD4: for 5′-TATGATGTGGAGGAGGTGGATGTGA-3′, rev 5′-CCTTTCATCCAAAGGCAAAACTACAG-3′, GAPDH: for 5′-ACCTGACCTGCCGTCTAGAAAA-3′, rev 5′-CCTGCTTCACCACCTTCTTGA-3′.

Real-time PCR for miR-21 was performed using TaqMan® MiRNA Assays protocol (assay ID 000397). Briefly, reverse transcriptase reaction was performed using 10 ng of total RNA and 50 nM miRNA specific stemloop RT primers. Real-time PCR was performed using standard protocol. Normalization was performed by using RNU6B primer kit (ID 001093, Applied Biosystems).

All qRT-PCR reactions were run in duplicate and the analysis was performed using an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA).

2.5. Proteic extracts and Western blotting assays

Whole cell extracts were obtained as previously described [28]. Protein were loaded onto SDS-PAGE, transferred onto Hybond-C paper (Amersham Biosciences, Little Chalfont, UK), incubated with PDCD4 monoclonal rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) and normalized with anti-actin antibody (Oncogene Research, San Diego, CA, USA). Detection was performed using ECL detection kit (Pierce, Rockford, IL, USA). The expression levels were analyzed by Scion Image Software (Scion, Frederich, MD, USA).

2.6. Immunophenotypic analysis of leukemic cells

Analysis of cell surface antigens was performed by flow cytometry using a FACScan Flow Cytometer (Becton Dickinson, Bedford, MA, USA). The following antibodies directed to membrane antigens were used for standard immunophenotypic analysis of AML blasts: anti-CD3, -CD7, -CD11a, -CD11b, -CD11c, -CD13, -CD14, -CD15, -CD18, -CD19, -CD33, -CD34, -CD36, -CD38, -CD41, -CD45, -CD61, -CD64, -CD71, -CD90, -CD116, -CD117, -CD235 and -HLA-DR (all from Pharmingen/Becton Dickinson, San Jose, CA, USA). In addition, in this study we used the following monoclonal antibodies (mAbs) to characterize AML blasts: anti-CDw123 (Pharmingen/Becton Dickinson) -CD116, -CDw131 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), -CD133 (Miltenyi Biotec), anti-FLT3 mAb purchased from Serotec (Oxford, UK). Cells were labeled and analyzed as previously reported [29].

3. Results

3.1. Expression of miR-21 and PDCD4 during normal granulocytic and monocytic differentiation

To evaluate the expression of PDCD4 and miR-21 during normal granulocytic (G) or monocytic (Mo) differentiation, we purified $CD34^+$ hematopoietic progenitor cells (HPCs) from human cord blood and induced them, under appropriate growth factor combinations, to differentiation and maturation along the G and Mo unilineage cultures. In these serum-free unilineage cultures, PDCD4 is highly expressed in quiescent $CD34^+$ cells at both mRNA and protein levels, as evaluated by real time qRT-PCR and Western blot, respectively (Fig. 1).

The data obtained by qRT-PCR showed that the elevated expression of PDCD4 mRNA observed in $CD34^+$ cells is followed by a marked downmodulation in the initial steps of differentiation (days 0–5), remaining expressed at low levels thereafter in the G differentiation. In the Mo cultures the PDCD4 mRNA levels showed a similar kinetic of modulation (Fig. 1).

The analysis of total proteins extracted from each lineage at different days of culture, corresponding to discrete sequential stages of differentiation/maturation process, showed that PDCD4 protein is rapidly downmodulated during G differentiation, being virtually undetectable from day 7 onward; a similar kinetics of PDCD4 expression was observed during Mo differentiation, even though slightly delayed (Fig. 1).

Conversely, quantitative analysis of miR-21 expression by qRT-PCR showed relatively low levels in quiescent $CD34^+$ cells that are markedly up-modulated along both the G and Mo differentiation (Fig. 1).

3.2. PDCD4 and miR-21 expression in AMLs

The mRNA expression of PDCD4 was evaluated by real-time RT-PCR in leukemic blasts derived from 91 AML patients and in CD34 positive cells purified from cord blood of normal donors (Fig. 2A). The analysis of PDCD4 mRNA values, normalized with respect to GAPDH, and plotted on a logarithmic scale as $2^{-\Delta CT}$, displayed a very high variability of expression in these patients, compared to its more homogeneous expression in $CD34^+$ cells. In particular, about 10% of AML patients displayed PDCD4 mRNA values markedly lower than the rest of AML patients.

PDCD4 was described as a target of miR-21 in many tumors [14,15]. We then explored miR-21 expression in a subset of 26 cases of AMLs, whose main biological properties are reported in Table 1, and found that expression of miR-21 ($2^{-\Delta CT}$) was higher in the majority of AMLs than in normal $CD34^+$ cells (Fig. 2B), suggesting a deregulation of the mechanisms regulating miR-21 expression in leukemic cells with respect to normal $CD34^+$ cells. Based on these findings we carried out Western blot assays to analyze PDCD4 protein expression on cellular extracts derived from leukemic blasts of 11 AML cases, with high and low expression of miR-21, respectively (Fig. 2C). Protein levels were quantified by densitometric analysis of Western blot assays (Fig. 2C). Comparing densitometry values of PDCD4 protein with the percentage of miR-21 expression for the same leukemic samples, we found a strong inverse correlation (Fig. 2D) between these two parameters, thus suggesting the existence of a direct regulatory network involving miR-21 and its target PDCD4.

The distribution of the various AMLs in the linear regression analysis of miR-21 and PDCD4 levels showed the existence of two AML subpopulations, one characterized by high miR-21 and low PDCD4 levels and the other one by low miR-21 and high PDCD4 values (Fig. 2D). According to this distribution, it was possible to identify a miR-21 cutoff level (about 275) suitable to distinguish AMLs into a high miR-21 expression group and a low miR-21 expression group.

3.3. miR-21 levels are particularly elevated in nucleophosmin-mutated (NPM1mut) AMLs

Various recurrent genetic abnormalities have been observed in AMLs. Among them, particularly frequent are the mutations of *NPM1* and *FLT3* (*FLT3*-ITD). Thus, we have analyzed *NPM1* and *FLT3*-ITD mutational status in the subgroups of AML patients characterized for miR-21 levels, showing that: (a) 38% of AML patients in the high miR-21, but only 12% in the low miR-21 expression group have NPM1 mutations; (b) 31% of AML patients in the high miR-21 and 50% in the low miR-21 expression group displayed FLT3-ITD mutations; (c) miR-21 levels are significantly higher in *NPM1*-mut AMLs (856 ± 321) compared to *NPM1*-wt AMLs (342 ± 71, $p = 0.03$) (Fig. 3a); (d) miR-21 levels were lower in *FLT3*-ITD AMLs (314 ± 87) than in *FLT3*-wt (634 ± 178, $p = 0.1$) AMLs (Fig. 3a). The inspection of individual miR-21 levels in *NPM1*-wt and *NPM1*-mut AMLs allowed the identification of three groups of AMLs: a first one, displaying *NPM1*-mut and high miR-21 levels, a second one with *NPM1-wt* and low/normal miR-21 levels and a third one with *NPM1-wt* and high miR-21 levels. We have then explored the occurrence of some differences at the level of the immunophenotype among these three different groups of AMLs: *NPM1*-mut AMLs displayed significantly higher percentages of CD11b, CD14 and CD116-positive cells than the other two AML subgroups (Fig. 3); (b) the three AML subgroups displayed similar percentages of $CD34^+$ cells, although it can be noted that the percentage of these cells was slightly lower in NPM1-mutated AMLs than in the other two AML subgroups (Fig. 4); (c) the *NPM1*-wt group with low/normal miR-21 levels displayed a higher percentage of CD117-positive cells than the other two AML subgroups ($p = 0.08$; Fig. 4); (d) CD133 exhibited a trend toward a lower expression in NPM1-mutated AMLs than in the other two AML subgroups (Fig. 4). We have also analyzed the distribution of other parameters, such as WBC counts at diagnosis, in the three AML subgroups and we observed a slightly lower level of WBCs among AML patients NPM1-WT with miR-21 high than in the other two AML subgroups (Fig. 4).

Given these findings, we attempted to verify the possible occurrence in the whole AML population of a correlation between miR-21 levels and the percentage of CD11b and CD14 positive cells. This analysis showed that there is a significant correlation between the level of miR-21 and CD11b expression ($p = 0.002$) and an almost significant correlation between the level of miR-21 and CD14 ($p = 0.06$). It is important to note that the correlation between miR-21 levels and the percentage of CD11b positive cells is significant also in the group of AML patients with NPM1-wt ($p = 0.05$, data not shown). According to these observations we have concluded that in AMLs the association between miR-21 levels and the expression of myeloid maturation markers CD11b and CD14 had two components, one related to the presence of NPM1 mutations and the other independent on NPM1 mutations and correlated with miR-21 levels.

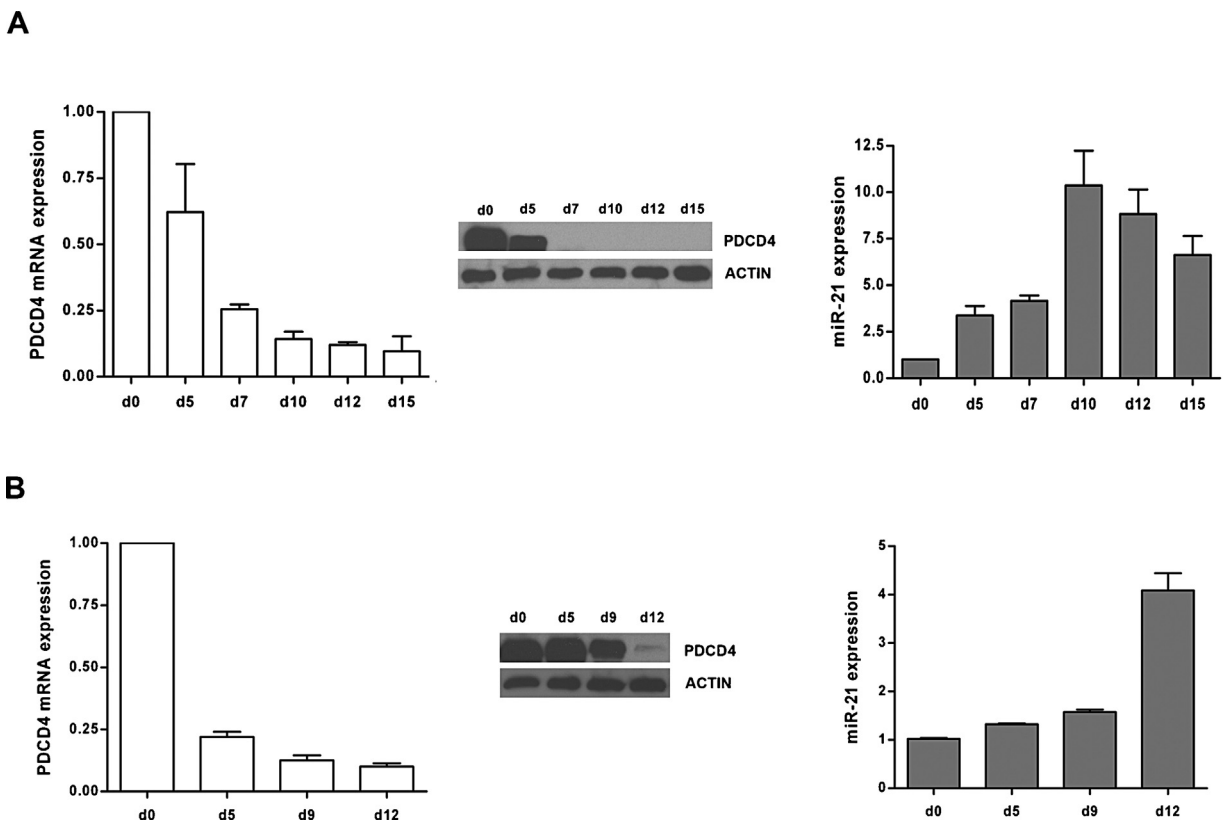

Fig. 1. PDCD4 and miR-21 expression in purified cord blood (CB) CD34⁺ HPCs induced to (A) G and (B) Mo differentiation. *Left panels*: qRT-PCR analysis of PDCD4 mRNA normalized for GAPDH at the indicated days in HPCs grown in liquid phase unilineage G or Mo cultures. *Middle panel*: western blot assays of PDCD4 protein expression using anti-PDCD4 antibody. Actin was used as control. *Right panel*: qRT-PCR analysis for miR-21, normalized for RNU6B expression, performed in the same samples at sequential days of G and Mo cultures. Data are the mean of three independent experiments.

In order to confirm our results on a larger set of data, we have analyzed the data on miR-21 contained in the TCGA data set. These data offer the opportunity to perform different types of analyses, but we mainly limited the analysis to miR-21 levels among AML patients with NPM1 mutations. Interestingly, this analysis showed that miR-21 levels were significantly higher among NMP1-mutated AMLs (42 patients), compared to NPM1-wt AMLs (157 patients) ($P \leq 0.01$). However, it must be pointed out that in AML patients with NPM1 mutations miR-21 levels are heterogeneous, suggesting the existence of two AML populations, low and high (data shown

Table 1
Main characteristics of 26 AML patients, including miR-21 levels.

Patient	FAB	WBC ($\times 10^9$/L)	% Blasts	Molecular abnormalities	miR-21 (ΔCT)
1	M5	40	65	FLT3-ITD AML1/ETO	290
2	M2	1.2	60	–	133
3	M1	36	88	FLT3-ITD NMP1-mut	45
4	M4	102	90	–	455
5	M4	78	65	–	79
6	M1	61	94	FLT3-ITD	46
7	M4	15	48	–	255
8	M1	264	100	FLT3-ITD	75
9	M2	175	83	FLT3-ITD NPM1-mut	393
10	M4	40	70	–	333
11	M1	60	96	–	526
12	M4	184	62	FLT3-ITD	455
13	M5	49	57	FLT3-ITD NMP1-mut	484
14	M1	2.3	72	–	428
15	M2	1.1	63	FLT3-ITD	729
16	M2	48	82	NPM1-mut	1669
17	M4	59	90	–	1164
18	M1	2.6	92	–	473
19	M4	125	46	–	54
20	M4	42	66	NPM1-mut	2418
21	M4	24	58	–	270
22	M4	81	64	NPM1-mut	327
23	M2	9	81	NPM1-mut	295
24	M5	10	72	–	411
25	M3	15	84	–	1003
26	M3	29	78	–	107

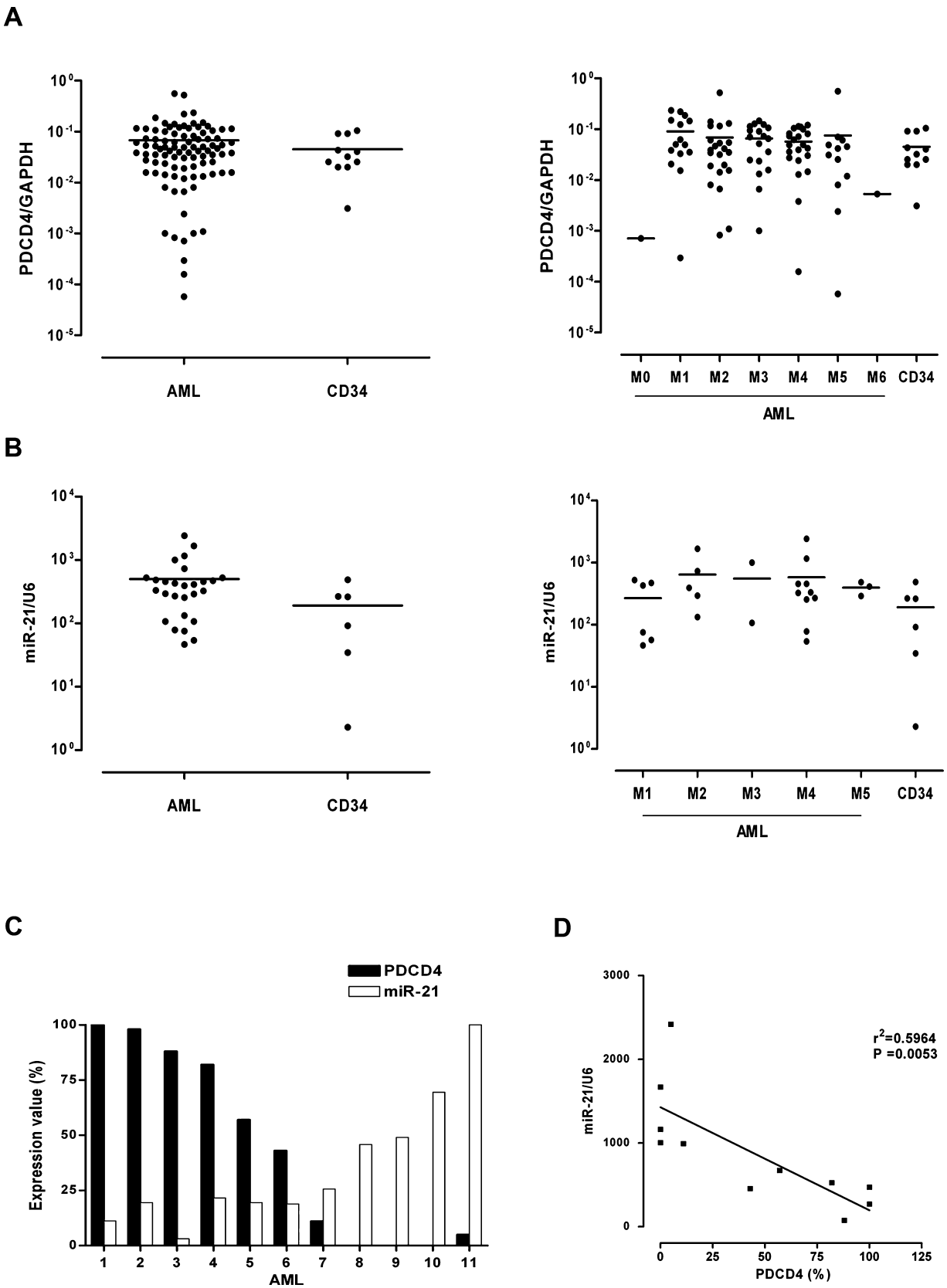

Fig. 2. PDCD4 and miR-21 expression in acute myeloid leukemias (AMLs). (A, left) qRT-PCR analysis of PDCD4 mRNA expression in 91 AML blasts, and in CD34⁺ cells purified from 11 normal donors. Samples were normalized with GAPDH expression. (A, right) qRT-PCR analysis of PDCD4 mRNA expression in 91 AML blasts, subdivided according to FAB classification into M0, M1, M2, M3, M4, M5 and M6, and in CD34⁺ cells purified from 11 normal donors. Samples were normalized with GAPDH expression. (B, left) qRT-PCR of miR-21 expression in a sub-group of 26 AML blasts, subdivided according to FAB classification into M0, M1, M2, M3, M4, M5 and M6 and in 6 samples of normal CD34⁺ cells. (B, right) qRT-PCR of miR-21 expression in a sub-group of 26 AML blasts, and in 6 samples of normal CD34⁺ cells. (C, left) Co-expression of PDCD4 protein and miR-21 in 11 AML patients. PDCD4 levels, detected by western blot assays and normalized with actin expression, were quantified by densitometric analysis and expressed as percentage relative to the highest value. (D) miR-21 was negatively correlated with PDCD4 protein in 11 AML samples.

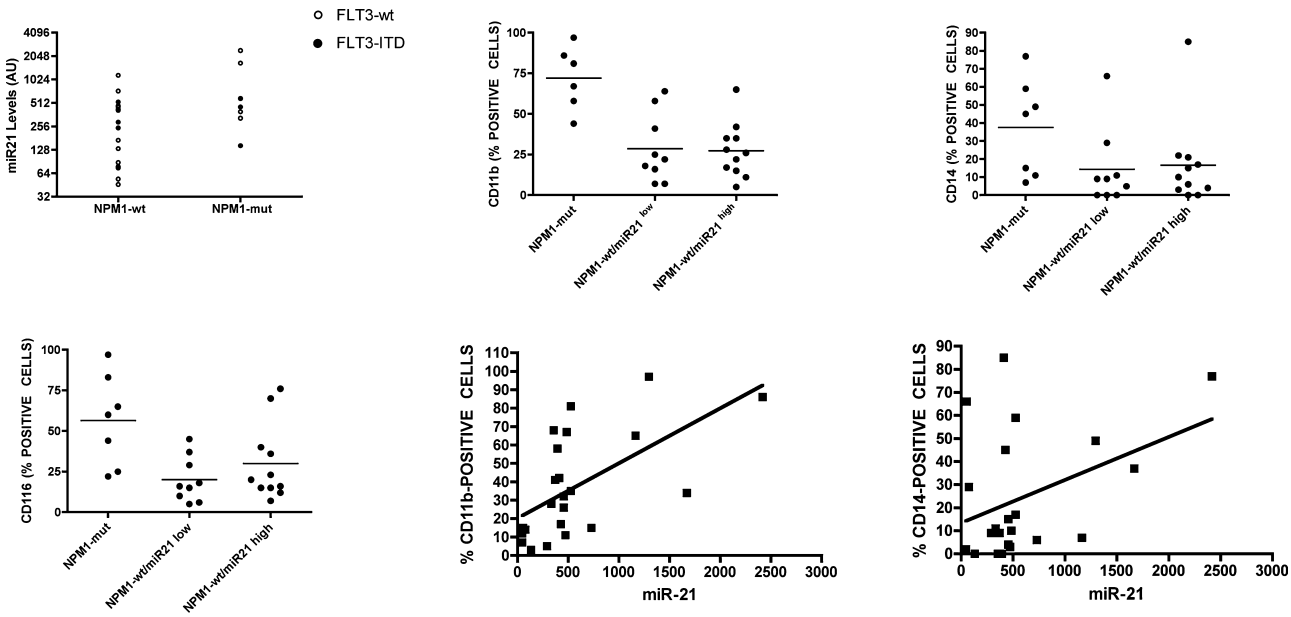

Fig. 3. miR-21 levels are particularly elevated in NPM1-mutated AMLs.
Top panel, left: miR-21 levels in NPM1-WT and NPM1-mutated AMLs. In each of these two AML groups the FLT3-ITD AMLs are indicated. *Top panel, middle and right and bottom panel left:* Percent of CD11b, CD14 and CD116-positive cells in three subgroups of AMLs: NPM1-mutant, NPM1-wt/miR-21 low and NPM1-wt/miR-21 high. *Bottom panel, middle and right:* Correlation between miR-21 levels and CD-11b or CD14-positive cells.

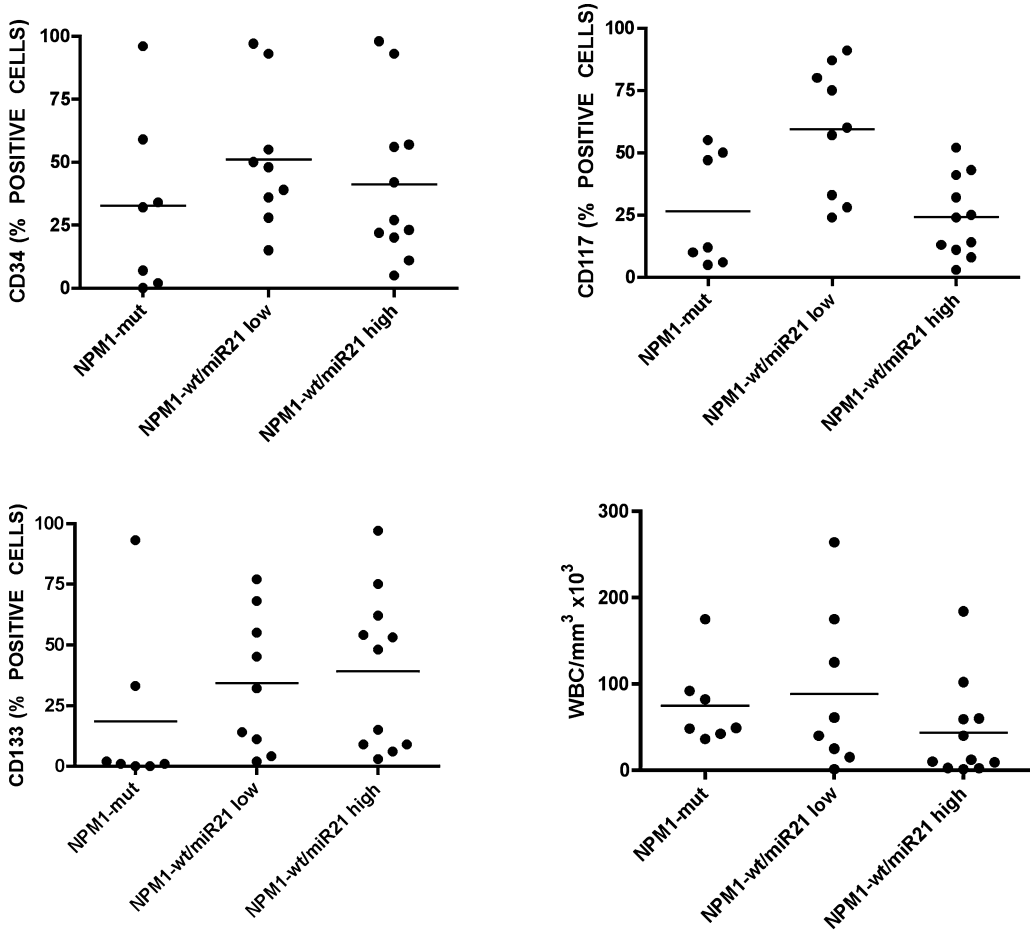

Fig. 4. Expression of membrane markers of stem/progenitor cells and number of WBCs at diagnosis in AML patients subdivided according to miR-21 levels and to the presence of NPM1 mutations. *Top panel and bottom panel left:* Percent of CD34, CD117 and CD133-positive cells in three subgroups of AMLs: NPM1-mutant, NPM1-wt/miR-21 low and NPM1-wt/miR-21 high. *Bottom panel right:* Number of WBCs at diagnosis in the three subgroups of AMLs.

in the supplementary Fig. S1). Since in the analysis of our patients we observed that the AML patients displaying particularly high miR-21 levels pertain to the group of patients with NPM1-mut and FLT3-wt (see Table 1), we decided to compare also miR-21 levels among NPM-mut/FLT3-wt (22 patients), NPM1-mut/FLT3-mut (20 patients) and NPM1-WT/FLT3-mut (30 patients) and we observed that the first ones exhibited miR-21 levels higher than the second and the third ones (Fig. S1; $p < 0.05$). miR-21 levels were similar in the NPM1-mut/FLT-mut and NPM1-wt/FLT3-mut AML groups (Fig. S1).

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.11.001.

4. Discussion

MicroRNAs are small non-coding RNA molecule that regulates gene expression *via* post-transcriptional mechanisms. A leukemogenetic role for miRNAs in AMLs was established through numerous profiling and functional studies allowing a diagnostic use of miRNAs to discriminate AMLs from ALLs, to identify AML subgroups, and to discover some miRNAs with a clear prognostic value [23].

Expression profiling of primary AML specimens to characterize global miRNA expression has shown that miR-21 is generally up-regulated in AMLs compared with normal CD34$^+$ cells [15]. Garzon and coworkers have compared the miRNA signature of *NPM1*-mut and *NPM*-wt AMLs and have identified a miRNA signature specific for *NPM1*-mut AMLs; interestingly, miR-21 was among the miRNAs most up-regulated in the *NPM1*-mut AMLs [14].

In the present study we have provided additional evidence showing a frequent enhanced expression of miR-21 in AMLs compared to normal CD34$^+$ cells, supporting a preferential miR-21 overexpression at the level of NPM1-mut AMLs. In particular, we observed that according to miR-21 levels and to the *NPM1* mutational status, three AML subgroups can be identified: (a) a *NPM1*-mut AML group showing elevated miR-21 levels; (b) a *NPM1*-wt group showing low miR-21 levels; (c) a *NPM1*-wt AML group showing high miR-21 levels. Compared to AML groups with *NPM1*-wt, the *NPM1*-mut AMLs were characterized by some peculiar immunophenotypical features, consisting of high CD11b, CD14 and CD116 expression. Our observations are in line with recent studies showing high expression of mature-myeloid cells antigens in *NPM1*-mut AMLs [30,31].

Importantly, since our results on miR-21 expression were based on the analysis of only 26 AML patients, we have explored the TCGA data set, where data on miR expression of 199 AML patients are available. This analysis confirmed that miR-21 levels were significantly higher in NPM1-mut AMLs than in NPM1-wt AMLs. Interestingly, this large data set of AMLs allowed also to perform an additional analysis taking into account both NPM1 and FLT3 mutations: according to this analysis, NPM1-mut/FLT3-wt AMLs displayed significantly higher miR-21 levels than NPM1-mut/FLT3-mut or NPM1-WT/FLT3-mut AMLs. In line with these findings, we also observed that the highest levels of miR-21 expression were observed among NPM1-mut/FLT3-wt AML patients. These observations are in line with many recent studies indicating different biological properties and clinical outcome of NPM1-mut/FLT3-wt with respect to NPM1-mut/FLT3-ITD AMLs [32].

Since the data observed in NPM1-mut AMLs suggested a preferential expression of miR-21 in AMLs displaying a more mature granulo-monocytic phenotype, we attempted to evaluate the occurrence of a possible relationship between miR-21 and CD11b expression and we observed a significant, direct correlation between these two parameters. In line with this observation, we observed that miR-21 expression is strongly up-modulated during normal granulopoiesis and monocytopoiesis. In line with our findings, previous studies carried out in leukemia cell line HL60 have shown that ATRA-induced granulocytic differentiation of these cells is accompanied by a marked miR-21 up-modulation [33]. The low expression of miR-21 in CD34$^+$ progenitor cells and its up-modulation during granulo-monocytopoiesis are controlled by the transcription factor GFI1 and are required for the normal process of granulo-monocytic differentiation.

As mentioned in Section 1 miR-21 was found to be overexpressed in a variety of malignancies, where its level is of prognostic value. The mechanism through which miR-21 overexpression favors tumor development and progression is largely unknown, but certainly a key role is played by the targeting of some tumor suppressor genes, such as PTEN, tissue inhibitor of metalloproteinase-3 and PDCD4. In fact, several literature data suggest that the reduced apoptotic activity, induced by miR-21 overexpression through targeting of proteins such as PDCD4 and PTEN (increasing AKT activity) could play a relevant role in the potential oncomiR activity of miR-21 [34]. In line with this hypothesis, it was shown that increased miR-21 contributes to NK-cell leukemogenesis through a reduction of apoptotic activity mediated by targeting of PDCD4 and PTEN [34]. Furthermore, increased miR-21 expression is involved in the molecular mechanisms determining daunorubicin resistance in K562 cells [35]. This hypothesis is also supported by the observation that in NPM1-mutated AMLs PTEN is a functionally important target of nucleophosmin [36].

In primary AML samples we observed a highly significant inverse correlation between miR-21 expression and PDCD4 protein levels, thus indicating that AMLs expressing high miR-21 levels display very low/absent PDCD4 levels. Interestingly, this inverse relationship between miR-21 and PDCD4 levels was observed also during the normal granulo-monocytic differentiation.

A recent study provided evidence that AML subtypes characterized by activation of homeobox (HOX) transcription factors exhibit increased miR-21 and miR-196a expression; importantly, inhibition of these two microRNAs by specific antagomirs inhibited *in vitro* leukemic colony forming activity and depleted *in vivo* leukemia-initiating activity of HOX-based leukemias in murine AML models [33].

Conflict of interest statement

All authors have no conflicts of interest.

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Author's contributions: RR and VL performed the research and analyzed the data; GC, RT and EP performed all the cord blood CD34$^+$ purification and culture experiments; FLC analyzed all the data on leukemias and contributed to write the paper; UT designed the research study, analyzed the data and wrote the paper.

References

[1] Shih AH, Abdeh-Wahab O, Patel JP, Levine PL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer 2012;12:599–612.
[2] White BS, Di Persio JF. Genomic tools in acute myeloid leukemia: from the bench to the bedside. Cancer 2014;120:1134–44.
[3] Shivarov V, Bullinger L. Expression profiling of leukemic patients: key lessons and future directions. Exp Hematol 2014;42:651–60.
[4] Hotchiss RS, Strasser A, McDunn J, Swanson PE. Cell death. N Engl J Med 2009;361:1570–83.
[5] Kelly G, Strasser A. The essential role of evasion from cell death in cancer. Adv Cancer Res 2011;111:39–96.
[6] Laukat-Buttgerert B, Goke R. The tumor suppressor PDCD4: recent advances in the elucidation of function and regulation. Biol Cell 2009;101:309–17.

[7] Eto K, Goto S, Nakashima W, Uza Y, Abe SI. Loss of programmed cell death 4 induces apoptosis by promoting the translation of procaspase-3 mRNA. Cell Death Differ 2012;19:573–81.
[8] Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, et al. MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene 2008;27:4373–9.
[9] Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasion and metastasis in colorectal cancer. Oncogene 2008;27:2128–36.
[10] Fu X, Han Y, Wu Y, Zhu X, Lu X, Mao F, et al. Prognostic role of microRNA-21 in various carcinomas: a systematic review and meta-analysis. Eur J Clin Invest 2011;41:1245–53.
[11] Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. MicroRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood 2010;116:945–52.
[12] Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, et al. Reprogramming of miRNA networks in cancer and leukemia. Genome Res 2010;20:589–99.
[13] Becker Buscaglia LE, Li Y. Apoptosis and the target genes of microRNA-21. Chin J Cancer 2011;30:371–80.
[14] Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. Proc Natl Acad Sci USA 2008;105:3945–50.
[15] Jongen-Lavrencic M, Sun SM, Dikstra MK, Valkj PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. Blood 2008;111:5078–85.
[16] Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. Cancer 2009;115:3842–7.
[17] Dohner H, Estey E, Amadori S, Appelbaum F, Buchner T, Burnett A, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel on behalf of the European Leukemia Net. Blood 2010;115:453–74.
[18] Mrozek K, Marcucci G, Nicolet D, Maharry KS, Becker H, Whitman SP, et al. Prognostic significance of the European Net standardization system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. J Clin Oncol 2012;30:4515–23.
[19] Grossmann V, Schnittger S, Kohlmann A, Eder C, Roller A, Dicker F, et al. A novel hierarchical prognostic model of AML solely based on molecular mutations. Blood 2012;120:2963–72.
[20] Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc$^+$ AML): biologic and clinical features. Blood 2007;109:874–85.
[21] Falini B, Martelli MP, Bolli N, Sportoletti P, Liso A, Tiacci E, et al. Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity? Blood 2011;117:1109–20.
[22] Schneider F, Hoster E, Unterhalt M, Schneider S, Dufour A, Benthaus T, et al. The FLT3-ITD mRNA level has a prognostic normal karyotype. Blood 2012;119:4383–6.
[23] Gordon J, Wang J, Rosko J. MicroRNA in myeloid malignancies. Br J Haematol 2013;162:162–76.
[24] Schotte D, Pieters R, Den Boer ML. MicroRNAs in acute leukemia: from biological players to clinical contributors. Leukemia 2012;26:1–12.
[25] Velu CS, Baktula AM, Grimes HL. Gfi 1 regulates miR-21 and miR-196b to control myelopoiesis. Blood 2009;113:4720–8.
[26] Chomczynsky P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol chloroform extraction. Anal Biochem 1987;162:156–9.
[27] Noguera NI, Ammatuna E, Zangrilli D, Lavorgna S, Divona M, Buccisano F, et al. Simultaneous detection of NPM1 and FLT3-ITD mutations by capillary electrophoresis in acute myeloid leukemia. Leukemia 2005;19:1479–82.
[28] Lulli V, Romania P, Morsilli O, Cianciulli P, Gabbianelli M, Testa U, et al. MicroRNA-486-3p regulates gamma-globin expression in human erythroid cells by directly modulating BCL11A. PLOS ONE 2013;8:pe60436.
[29] Testa U, Riccioni R, Militi S, Coccia E, Stellacci E, Samoggia P, et al. Elevated expression of IL-3Ralpha in acute myelogenous leukemia is associated with enhanced blast proliferation, increased cellularity, and poor prognosis. Blood 2002;100:2980–8.
[30] Nomedadea J, Bussaglia E, Villemor N, Martinez C, Esteve J, Tormo M, et al. Immunophenotype of acute myeloid leukemia with NPM mutations: prognostic impact of the leukemic compartment size. Leuk Res 2011;35:163–8.
[31] Liu YR, Zhu HH, Ruan GR, Qin YZ, Shi HX, Lai YY, et al. NPM1-mutated acute myeloid leukemia of monocytic or myeloid origin exhibit distinct immunophenotypes. Leuk Res 2013;37:737–41.
[32] Port M, Bottcher M, Thol F, Ganser A, Schlenk R, Wsem J, et al. Prognostic significance of FLT3 internal tandem duplication, nucleophosmin 1, and CEBPA gene mutations for acute myeloid leukemia patients with normal karyotype and younger than 60 years: a systematic review and meta-analysis. Ann Hematol 2014;93:1279–86.
[33] Velu C, Chaubey A, Phelan J, Horman S, Wunderlich M, Guzman M, et al. Therapeutic antagonists of microRNAs deplete leukemia-initiating cell activity. J Clin Invest 2014;124:222–36.
[34] Yamaka Y, Tagawa H, Takahashi N, Watanabe A, Guo YM, Iwamoto K, et al. aberrant overexpression of microRNAs activate AKT signaling via down-regulation of tumor suppressors in natural killer-cell lymphoma/leukemia. Blood 2009;114:3265–75.
[35] Bai H, Xu R, Cao Z, Wei D, Wang C. Involvement of miR-21 in resistance to daunorubicin by regulating PTEN expression in the leukemic K562 cell line. FEBS Lett 2011;585:402–8.
[36] Noguera NI, Song MS, Divona M, Catalano G, Calvo KL, Garcia F, et al. Nucleophosmin/B26 regulates PTEN through interaction with HAUSP in acute myeloid leukemia. Leukemia 2013;27:1037–43.