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Brief communication

Complexity of miR-223 regulation by CEBPA in human AML

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ABSTRACT

microRNA-223 (miR-223) can trigger normal granulopoiesis. miR-223 expression is regulated by two distinct CEBPA (CCAAT/enhancer binding protein-alpha) sites. Here, we report that miR-223 is largely suppressed in cells from acute myeloid leukemia (AML) patients. By sequencing, we found that miR-223 suppression in AML is not caused by DNA sequence alterations, nor is it mediated by promoter hypermethylation. The analysis of the individual contribution of both CEBPA sites to miR-223 regulation identified the site upstream of the miR-223 primary transcript as the predominant regulatory element. Our results suggest that miR-223 suppression in AML is caused by impaired miR-223 upstream factors.

1. Introduction

microRNAs (miRNAs) are non-coding RNAs comprising 20–22 nucleotides. By silencing target mRNAs, they exert key roles in transcriptional regulation [1]. In particular, microRNA-223 (miR-223) is regulating normal granulopoiesis: miR-223 is preferentially expressed in myeloid cells [2–4], it can trigger granulocytic differentiation [5,6], and it plays a crucial role for maturation and for maintaining granulocytic function [3]. In addition, miR-223 blocks the differentiation towards other blood cells such as erythrocytes [7].

The suppression of miR-223 has been associated to various types of myeloid leukemias [8,9]. However, only for AML patients carrying the t(8;21) chromosomal translocation, a model for miR-223 suppression has been proposed so far involving epigenetic silencing of miR-223 through the t(8;21) fusion protein AML1-ETO [6].

Two groups have investigated the regulation of miR-223. Both groups identified a transcriptional activation by CEBP (CCAAT/enhancer binding protein) family members, although the proposed mechanisms are different. Fazi et al. [5] introduced a minicircuit involving CEBPA (CEBPalpha) and NFIA (nuclear factor I-A) competing for a regulatory binding site 700 bp upstream of the pre-miR-223 sequence. Fukao et al. [10] demonstrated miR-223 regulation through a CEBP/PU.1 site located 3420 bp upstream of the pre-miR-223 locus. Based on comparative genomic studies, the authors concluded that the CEBP/PU.1 site is located in a highly conserved promoter region directly upstream of the pri-miR-223 transcription start site, whereas the regulatory CEBPA/NFIA site is located in a non-conserved intronic region of the pri-miR-223 transcript. However, the relative impact of both regulatory sites on miR-223 expression remained to be clarified.

Here, we investigated both miR-223 regulatory regions for alterations in their genomic DNA sequences. These studies intended to further elucidate the mechanisms leading to miR-223 suppression in AML patients. Moreover, we aimed to investigate the individual contribution of both regulatory sites to miR-223 expression.

2. Patients, material and methods

2.1. Patients, controls and cell lines

Peripheral blood or bone marrow samples of 119 AML patients at diagnosis including all FAB-subtypes and peripheral blood donations from 121 healthy volunteers were used. Mononuclear cells were harvested using Ficoll gradient (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). DNA and (mi)RNA were extracted using extraction kits from QIAGEN (Hombrechtikon, Switzerland). Mature monocytes and granulocytes from 6 healthy volunteers were isolated from peripheral blood using EasySep selection kits #18088-CD14 and #18682-CD66b, respectively (RoboSep,

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StemCell Technologies, Vancouver, Canada). Informed consent of patients and volunteers was obtained according to the declaration of Helsinki, and characteristics are summarized in Supplemental Table 1.

Leukemic Kasumi-1 cells stably transfected with an inducible estrogen receptor CEBPA fusion protein (CEBPA-ER) [11] were cultured in phenol red-free RPMI-1640 medium supplemented with 10% foetal calf serum, and selected with 1 μg/ml Puromycin. The CEBPA-ER fusion protein was induced using 1 μM β-estradiol for up to 5 days. H1299 lung cancer and U937 leukemic cell lines were from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 medium with 10% foetal calf serum. Reagents were from Sigma–Aldrich (Buchs, Switzerland).

2.2. Nucleotide sequencing

Both regulatory sequences and the entire pre-miR-223 sequence including the mature miR-223 were amplified by PCR and sequenced using ABIprism technology (ABI 3730xl, BigDye Terminator v1.1 reagents; Applied Biosystems, Rotkreuz, Switzerland). All primer sequences are listed in Supplemental Table 2.

2.3. Promoter luciferase assays

870 bp upstream of the pre-miR-223 start site including the polymorphic CEBPA site were cloned into the PXP2 luciferase vector, co-transfected with expression plasmids of CEBPA or NFIA using Lipofectamine 2000 (Invitrogen, Basel, Switzerland), and detected with the Dual Luciferase Reporter Assay (Promega, Dübendorf, Switzerland).

2.4. Real-time PCR

Expression of miR-223 – and of miR-93/-191 for normalization [12] – was assessed using miScript SYBR green PCR Kit and primer assays for mature miRNAs: hs-miR-223 #MS_3871, hs-miR-93 #MS_3346, hs-miR-191 #MS_3682 (Qiagen, Hombrechtikon, Switzerland), and 7900HT Fast Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). Primer sequences for pri- or pre-miR-223 detection are listed in Supplemental Table 2. miR-223 expression in patient and control samples was normalized according to the geometric mean value of miR-93 and miR-191 expression [12]. miR-93 was used for normalization of the experiments with Kasumi-CEBPA-ER cells.

2.5. EMSA (electrophoretic mobility shift assay)

Antibodies detecting CEBPA and NFIA were sc-61X and sc-5567X (Santa Cruz, Heidelberg, Germany). Sequences of the oligonucleotides are listed in Supplemental Table 2.

2.6. Western blot analysis

Detection of CEBPA and CEBPE were performed using antibodies #39306 (Active Motif, Rixensart, Belgium) and sc-158 (Santa Cruz, Heidelberg, Germany). For loading control, the β-actin antibody MAB1501 (Chemicon/Milipore, Zug, Switzerland) was used.

2.7. URLs (uniform resource locators) and statistical analysis

The following URLs were used: http://genome.ucsc.edu/ for conservation analysis of the miR-223 locus (Assembly March 2006), and http://www.urogene.org/methprimer/index1.html for prediction of CpG islands in both miR-223 regulatory sites. Frequencies of the sequence variations were evaluated by Chi-square test, and differences in promoter activities and miR-223 levels were analyzed by *t*-test.

3. Results and discussion

miR-223 is crucial for triggering myeloid differentiation of progenitor cells [5] and for maintaining granulocyte function [3]. In addition, suppression of miR-223 has been reported in various types of leukemias [8,9]. To investigate the mechanism of miR-223 suppression in AML, we assessed miR-223 expression in leukemic cells of 54 AML patients at diagnosis (Fig. 1). In comparison to mature granulocytes, miR-223 expression was largely suppressed in all AML FAB subtypes, thereby confirming previous reports [13]. Remarkably, even AML subtypes with partial differentiation along the granulocyte lineage (FAB M1/2) expressed miR-223 at similar low levels as observed in the subtypes along the monocytic lineage (FAB M4/5) or as in mature monocytes. A possible mechanism for miR-223 suppression has been reported so far only for AML patients with the t(8;21) translocation [6]. In these patients, miR-223 transcription is epigenetically suppressed by the t(8;21) fusion protein AML1-ETO.

The regulation of miR-223 expression has been previously investigated by two groups proposing two different mechanisms: miR-223 regulation may be mediated through a conserved CEBP/PU.1 site upstream of the pri-miR-223 transcription start [10] as well as through a non-conserved CEBPA/NFIA responsive element 700 bp upstream of the pre-miR-223 in an intronic sequence of the pri-miR-223 transcript [5]. To elucidate the individual contribution of these regulatory sites and their role in miR-223 suppression in AML patients, we first analyzed both elements for DNA sequence alterations by PCR screening (Fig. 2A).

Remarkably, no mutations were detected in the mature miR-223 and in the pre-miR-223 sequence in 119 AML patients and in 121 healthy volunteers. Also, no alterations were detectable in the conserved CEBP/PU.1 site [10]. Interestingly, we found a single nucleotide A to G transition within the CEBPA/NFIA site in the non-conserved region, affecting one of the two CEBPA binding sites previously shown to regulate miR-223 transcription (Fig. 2B) [5]. Importantly, the NFIA binding site was not affected by this sequence alteration. The alteration was detectable in 15.1% of the AML samples (18/119) and in 16.5% of the healthy volunteers (20/121), with

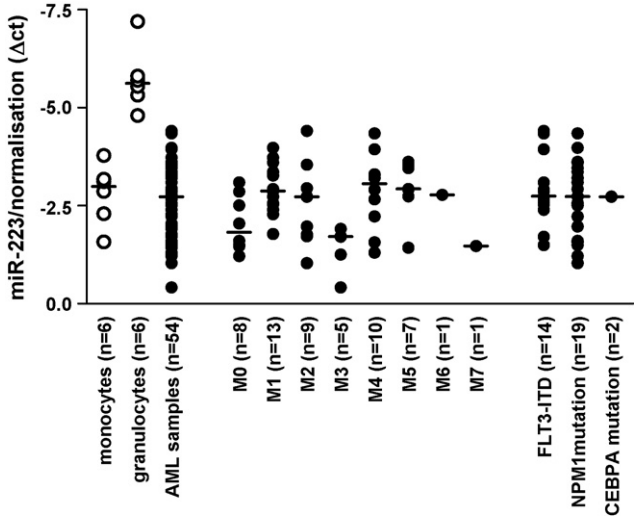

Fig. 1. miR-223 expression in AML, in mature granulocytes and monocytes. Mature miR-223 expression from 54 AML patients samples is presented, according to the FAB subtypes (M0–M7) or stratified according to molecular abnormalities (FLT3-ITD, NPM1, and CEBPA mutations). Expression levels are given as Δct-values (ct(miR-223) – ct(normalization)).