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Kais Hussein, Wiebke Dralle, Katharina Theophile, Hans Kreipe, Oliver Bock. Megakaryocytic expression of miRNA 10a, 17-5p, 20a and 126 in Philadelphia chromosome-negative myeloproliferative neoplasm. *Annals of Hematology*, 2008, 88 (4), pp.325-332. 10.1007/s00277-008-0602-9 . hal-00477980

HAL Id: hal-00477980

<https://hal.science/hal-00477980>

Submitted on 30 Apr 2010

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Megakaryocytic expression of miRNA 10a, 17-5p, 20a and 126 in Philadelphia chromosome-negative myeloproliferative neoplasm

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Received: 2 July 2008 / Accepted: 25 August 2008 / Published online: 5 September 2008
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Abstract Micro RNA (miRNA) are small non-coding RNA molecules which have a post-transcriptional inhibitory regulation function, e.g. in megakaryopoiesis. A characteristic of Philadelphia chromosome-negative myeloproliferative neoplasm (Ph⁻ MPN) is the abundance of morphologically aberrant megakaryocytes. Based on previously published in vitro megakaryocytic differentiation assay data, we selected miRNA 10a, 17-5p, 20a and 126 and potential target proteins (HOXA1, RUNX1) for analysis of laser-microdissected bone marrow megakaryocytes from Ph⁻ MPN and controls ($n=66$). Furthermore, we tested a potential influence of cyto-reductive treatment on miRNA expression in bone marrow cells during the course of Ph⁻ MPN ($n=18$). In summary, miRNA 17-5p, 20a and 126 are constitutively expressed in Ph⁻ MPN megakaryopoiesis while low or absent miRNA 10a appeared to correlate with strong megakaryocytic HOXA1 protein expression. No association to thrombocytosis, JAK2^{V617F} mutations or cyto-reductive treatment (bone marrow cells) were observed.

Keywords Micro RNA · miRNA · Philadelphia-chromosome-negative chronic myeloproliferative neoplasm · Megakaryocytes · Laser microdissection

Introduction

Epigenetic regulation of gene expression at different levels, e.g. gene promoter methylation, represents a fundamental mechanism of physiological cell homeostasis. A few years ago, the epigenetic micro RNA (miRNA) system was identified as a post-transcriptional inhibitory regulator of mRNA translation [1–3]. These small non-amino acid-coding RNA molecules originate from miRNA-gene-derived hairpin primary miRNA transcripts of ~80 nucleotides which are reduced in length during several intermediate stages and finally result in ~20 nucleotide-long single-strand and now biologically active mature miRNA. Mature miRNA are capable of semi-complementary binding to the 3'-untranslated region of target mRNA which results in suppression of mRNA translation/protein generation. This imperfect miRNA/mRNA base-pairing explains why one miRNA may have several potential mRNA targets and vice versa one mRNA may be targeted by more than one miRNA [1–3].

Only a few approaches have been performed to gain a deeper insight into aberrant miRNA regulation in leukaemia of myeloid origin [4–7], e.g. Philadelphia chromosome-negative chronic myeloproliferative neoplasm (Ph⁻ MPN), including polycythaemia vera, PV, primary myelofibrosis, PMF, and essential thrombocythaemia, ET) [4–6]. Characteristically 50–95% of Ph⁻ MPN cases are positive for the

Electronic supplementary material The online version of this article (doi:10.1007/s00277-008-0602-9) contains supplementary material, which is available to authorized users.

Research grant: Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung 10-2191 (O.B., H.K.); Deutsche Forschungsgemeinschaft-DFG BO 1954/1-1 (O.B., H.K.); Hochschul-interne Leistungsförderung-HiLF 11/07, Medizinische; Hochschule Hannover (K.H.)

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Janus kinase 2 G1849T/V617F point mutation (JAK2^{V617F}) [8] and ~5% of PMF and ET cases show a G1544A/T/W515K/L point mutation of the thrombopoietin receptor/myeloproliferative leukaemia virus oncogene (MPL^{W515K/L}) [9] and one of the most prominent morphological features is an aberrant megakaryopoiesis [10]. Megakaryocytes show abnormal lobulated nuclei and cellular clustering, typically in PMF, and enlarged cytoplasm in ET or pleomorphism in PV.

Analysis of several peripheral blood-cell fractions in Ph⁻ MPN revealed a myeloid lineage-specific altered expression of microRNA, e.g. down-regulation of miRNA 339 [5] and up-regulation of miRNA 26b [6] in platelets, but no Ph⁻ MPN-derived bone marrow cells, particularly megakaryocytes in their natural intertrabecular environment, have been analysed [4–6].

During differentiation of non-leukaemic CD34⁺ cells towards a megakaryocytic phenotype several miRNA, including miRNA 126 (localised on chromosome region 9q34), members of the miRNA 17-5p-92 cluster (miRNA 17-5p and 20a, both 13q31) and particularly miRNA 10a (17q21), were down-regulated [11]. Investigation of potential miRNA targets, such as homeobox gene (HOX) transcription factors, which are involved in embryogenesis, haematopoiesis and myeloid leukaemogenesis [12], revealed an up-regulation of HOXA1 mRNA and protein during in vitro megakaryopoiesis, which was reciprocal to miRNA 10a down-regulation and vice versa miRNA 10a over-expression in MEG01 and K562 cell lines reduced HOXA1 expression [11]. A potential target of the miRNA 17-5p-92 cluster and miRNA 126 is the Runt-related transcription factor 1 (RUNX1, also designated acute myeloid leukemia 1, AML1) [11] which is one of the central transcription factors in normal and leukaemic megakaryopoiesis [13, 14]. In monocytic differentiation, it has been shown that RUNX1 is inhibited by the miRNA 17-5p-92 cluster and in a negative feed-back regulation mechanism RUNX1 protein binds to the promoter of miRNA 17-5p-92 cluster genes [15].

Based on these in vitro-derived results on non-neoplastic megakaryocytic differentiation [11], we performed an in situ analysis of laser-microdissected bone marrow megakaryocytes from Ph⁻ MPN patients in order to clarify whether the aberrant morphological differentiation is associated to aberrant expression of miRNA 10a, 126, 17-5p and 20a and their potential targets HOXA1 and RUNX1.

Materials and methods

Bone marrow study group and ethical background

The study protocol of this retrospective analysis was approved by the local Ethics Committee, Hannover Medical School. All formalin-fixed and paraffin-embedded (FFPE)

bone marrow trephines from a total of 96 cases were retrieved from the archival files of the Institute of Pathology, Hannover Medical School.

Megakaryocytic miRNA expression was analysed in 66 cases: 41 randomly selected Ph⁻ MPN prior to any treatment at first presentation and <5% myeloblasts (11 PMF in cellular/prefibrotic phase, 11 PMF in fibrotic phase, nine PV, ten ET), seven follow-up biopsies from Ph⁻ MPN with elevated myeloblasts (~5% myeloblasts in one PV and two PMF, 10–20% in three PMF and ~30% in one PMF) and 18 non-malignant control cases. Cases were diagnosed from 2001–2006 according to histomorphology and clinical data. Myelofibrosis was classified according to presence and extent of fibre deposition, osteosclerosis, and intra-sinusoidal haematopoiesis, as described [16]. In all control cases under investigation, biopsies were taken for exclusion of neoplastic BM infiltration and showed normal or mild hyperplastic non-leukaemic haematopoiesis. A summary of clinical data is provided in Table 1.

For evaluation of a potential effect of cytoreductive treatment on miRNA expression in bone marrow cells, a second group of 30 cases was analysed retrospectively (diagnosed from 1999–2006; follow-up biopsies of seven hydroxyurea-treated and 11 untreated Ph⁻ MPN and 12 control cases other than for the megakaryocytic analysis).

Laser microdissection for in situ cell sorting of megakaryocytes

FFPE bone marrow sections (~5 µm) were deparaffinised, rehydrated and stained with methylene blue. Subsequently, laser microdissection (PALM Laser-MicroBeam System, P.A.L.M., Wolfraatshausen, Germany) of ~200 megakaryocytes per case was performed for further RNA extraction [17, 18]. Due to the relatively small number of megakaryocytes, biopsies of normal haematopoiesis and limited sample volumes, a subfraction of controls were pooled (three individuals per pool) for analysis of miRNA 17-5p and 20a.

RNA extraction and miRNA-expression assay

Deparaffinised laser-microdissected megakaryocytes and five to ten 10-µm sections from FFPE biopsies were digested overnight at 55°C in 1:1 lysis solution and proteinase K (100 µL volume for megakaryocytes and 1,000 µL volume for bone marrow cells) [18]. After organic extraction by phenol/chloroform, total RNA was precipitated from the aqueous phase with isopropanol and glycogen for at least 24 h at -20°C [18]. Megakaryocytic or bone marrow cell-derived total RNA (10 ng each), pretreated with RNase-free DNase (1 U/µg RNA; RQ1, Promega, Madison, WI, USA), were used for real-time reverse transcriptase-PCR (RT-PCR)-based expression analysis of human miRNA hsp-miR-10a

Table 1 Clinical parameters and JAK2^{V617F}/MPL^{W515L} status of Ph⁻ MPN cases and non-malignant control cases

Diagnosis (n, gender)	Age (years)	n JAK2 ^{V617F} /MPL ^{W515L} (allele burden in mutated cases)	Haemoglobin (g/dl)	Leukocytes (x10 ⁹ /l)	Thrombocytes (x10 ⁹ /l)
Pre-fibrotic phase PMF(11, 7♀/4♂)	75(47–84)	5/11 JAK2 ^{V617F} (40%, 29–63%) 1/11 MPL ^{W515L} (55%)	12.3(9.8–14.5)	11.3(3.2–24)	1134*** (530–1641)
Fibrotic phase PMF(11, 6♀/5♂)	64(56–76)	5/11 JAK2 ^{V617F} (48%, 34–74%)	11.3(6.5–12.5)	10.3(4.43–31.9)	393(191–478)
PMF, follow-up, ≥5% myeloblasts (6, 3♀/3♂)	69(57–78)	4/6 JAK2 ^{V617F} (52%, 38–85%)	10(8.37–10.2)	46.6(12.4–70.08)	95(29–1276)
PV(9, 4♀/5♂)	65(50–74)	8/9 JAK2 ^{V617F} (46%, 22–80%)	16.9(14.2–20.0)	10.1(5–15.4)	424.5** (234–962)
PV, follow-up, ~5% myeloblasts(1♀)	60	JAK2 ^{V617F} (81%)	9.2	15.2	208
ET(10, 4♀/6♂)	54(28–72)	6/10 JAK2 ^{V617F} (26.5%, 14–39%)	14.0(10.0–14.8)	9.3(6.5–17.1)	676*(320–968)
Control(18, 7♀/11♂)	55.5(28–75)	18/18 JAK2 ^{wild-type} /MPL ^{wild-type}	14.8(8.7–17.9)	5.8(2.5–20.6)	211.5(84–369)

At disease presentation in non-fibrotic Ph⁻ MPN, the number of platelets were significantly higher than in control haematopoiesis (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Median disease duration in PMF follow-up cases was 1.5 years (range 1–7 years) and 14 years in the PV follow-up case. Median values are followed by range
♀ Female, ♂ male

(MIMAT0000782), hsp-miR-17-5p (miRBase Accession number MIMAT0001114), hsp-miR-20a (MIMAT0001786) and hsp-miR-126 (MIMAT0000832), as recommended by the manufacture's instructions (TaqMan MicroRNA Expression Assays, Applied Biosystems, Foster City, CA, USA). The provided miRNA-specific primer design allowed the detection of the biologically active, mature miRNA fragments. RT-PCR was performed with an ABI PRISM 7500 Sequence Detector (Applied Biosystems Foster City, CA, USA). Target miRNA-expression levels were normalised to the expression of small nuclear RNA, C/D box 49A (RNU 49; GenBank Accession number NR002744) as an endogenous control. Negative control was performed using water instead of reverse transcriptase. Linearity of amplification for target and control genes could be demonstrated over a broad concentration range enabling relative quantification using the $\Delta\Delta\text{CT}$ -method as described [19]. Expression levels of miRNA in a given sample of Ph⁻ MPN represent the average of two measurements and are illustrated relative to the mean of expression in the control group which was arbitrarily set to 1.

Determination of JAK2^{V617F} and MPL^{W515K/L} mutations by Pyrosequencing®

DNA was extracted from FFPE bone marrow cells with the DNeasy kit (Qiagen, Hilden, Germany). Subsequently, 25 ng of DNA were used for PCR amplification of JAK2 and MPL fragments (GenBank AL161450; U68161) and pyrosequencing® in a PSQ 96MA instrument (Biotage, Uppsala, Sweden) was performed as described [17, 20]. JAK2^{V617F} cell line HEL, MPL^{W515L} PMF patient sample and JAK2^{wild-type}/MPL^{wild-type} cell line HL-60 were used as controls.

Immunohistochemical detection of HOXA1 and RUNX1 proteins in bone marrow cells

FFPE bone marrow sections (~1 μm) were deparaffinised, rehydrated and pretreated in an autoclave prior to overnight incubation with antisera (1:200 anti-HOXA1 polyclonal rabbit antibody, Lifespan Biosciences, Seattle, WA, USA; 1:150 anti-RUNX1 monoclonal mouse antibody, Abnova, Taipei, Taiwan). Immunostaining was performed with a ZytoChem-Plus HRP Polymer Kit (Zytomed Systems, Berlin, Germany), visualised by DAB-brown (Dako, Glostrup, Denmark) and counterstained with haematoxylin. CRK, a potential target of all four miRNA under investigation, was also analysed, but despite variable pretreatment protocols, no immunostaining was detectable (1:50 anti-CRK monoclonal mouse antibody, BD biosciences, Franklin Lakes, NJ, USA).

The percentage and staining intensity of immunolabelled megakaryocytes was estimated and compared to the corresponding megakaryocytic miRNA-expression levels.

The staining intensity was divided into four categories: intense brown/black staining (+++), weak or very weak brown staining (+), brown staining between +++ and + (++) and absent staining (-).

Statistical analyses

To analyse differences of miRNA expression, mutant allele burden and correlation with clinical parameters in the study group, the Kruskal–Wallis test was performed followed by Dunn's post-test for pair-wise group differences. *P* values ≤ 0.05 were considered as statistically significant.

Results

Megakaryocytes in Ph⁻ MPN and non-malignant haematopoiesis constitutively express miRNA 17-5p, 20a and 126 but low miRNA 10a

Megakaryocytic expression levels of miRNA 10a, 17-5p, 20a and 126 were not significantly different among Ph⁻ MPN entities or Ph⁻ MPN and control cases (Fig. 1). Compared to miRNA 10a miRNA 17-5p, 20a and 126 had higher constitutive expression levels. We observed a tendency of lower or undetectable expression levels of miRNA 10a in all Ph⁻ MPN entities (highest median relative expression level was 0.29 in ET) and controls (median 0.0), although at least one individual fibrotic stage PMF case (<5% myeloblasts but prominent promegakaryocytes and megakaryoblasts, JAK2^{wild-type}/MPL^{wild-type}) revealed elevated miRNA 10a expression (22.5; case #5, supplementary Table 1). No follow-up samples were available, but 1 month later, a secondary acute myeloid leukaemia (AML) was diagnosed and the 66-year-old female patient died 9 months after Ph⁻ MPN diagnosis.

To test a potential association to beginning leukaemic transformation, we analysed megakaryocytes in follow-up samples from Ph⁻ MPN with $\geq 5\%$ myeloblasts but no significant change could be observed. In the 14-year follow-up, trephine of a 60-year-old female patient which suffered from fibrotic stage JAK2^{V617F} PV (~5% myeloblasts, 14% peripheral blast cells) marked up-regulation was observed for megakaryocytic miRNA 10a (4,469,452.45 times) but not for the miRNA 17-5p, 20a and 126 (#22, supplementary Table 1).

No correlation of megakaryocytic miRNA expression to thrombocytosis or Jak2/MPL mutation status

As expected [10], cellular phase PMF, PV and ET showed significantly elevated platelet counts when compared to non-malignant controls ($p < 0.001$, $p < 0.01$, $p < 0.05$, respectively,

Table 1) but thrombocytosis (or any other clinical parameter under investigation, including JAK2^{V617F} status/allele burden) did not show any correlation to megakaryocytic expression levels of miRNA 10a, 17-5p, 20a and 126. Furthermore, there was no hint of an outstanding difference in the MPL^{W515L} PMF case regarding expression levels of miRNA 10a (0.31), miRNA 17-5p (0.35), miRNA 20a (3.64) or miRNA 126 (4.0), respectively.

Megakaryocytic miRNA 10a levels reciprocally correlates with HOXA1 protein expression in megakaryocytes

An inhibitory effect of the biologically active miRNA under investigation on their potential target genes was predicted by immunohistochemical evaluation of the corresponding post-translation protein expression (additional mRNA expression analysis was not performed due to limited sample volumes). The subfraction of 34/66 cases was selected according to the median and range of expression levels of miRNA 10a (supplementary Table 1).

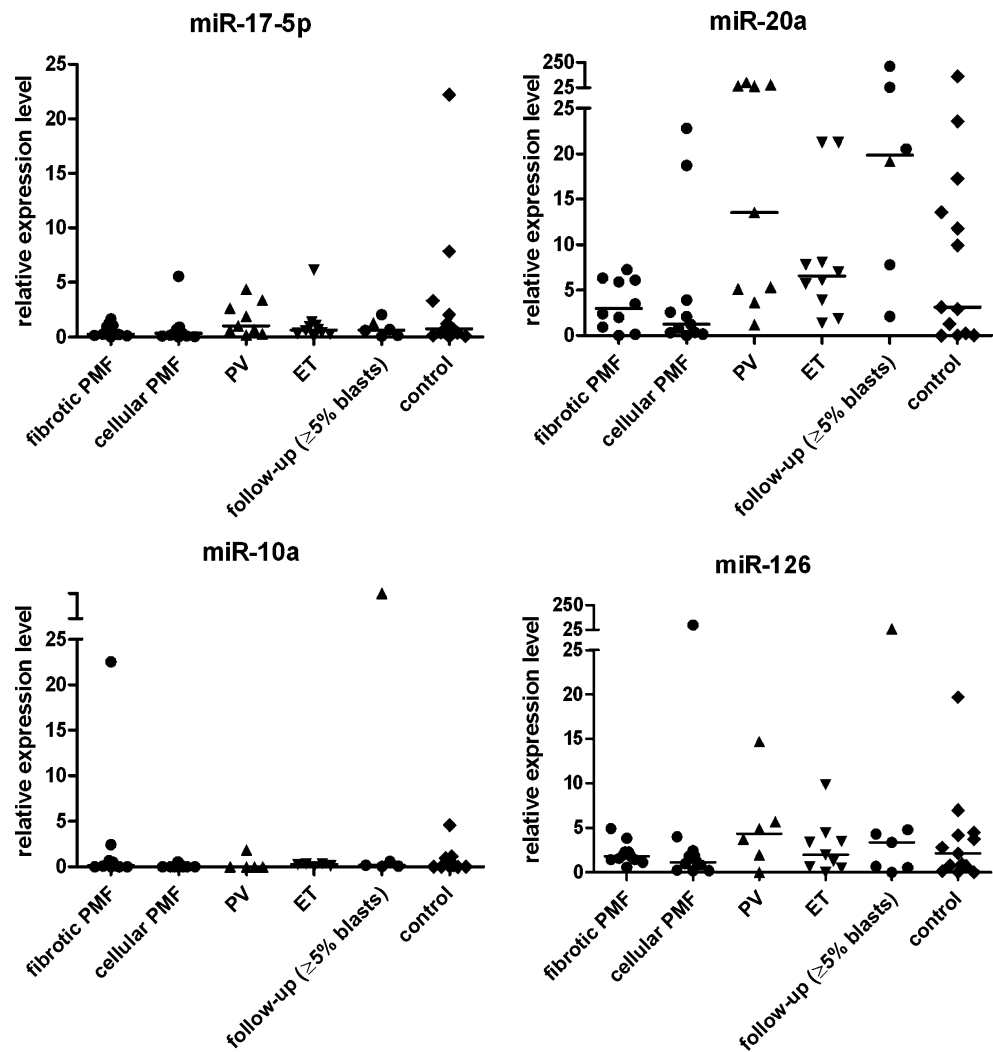
Ph⁻ MPN and control cases with absent or low miRNA 10a expression levels showed marked cytoplasmatic HOXA1 protein expression in 80–95% of all megakaryocytes (Fig. 2 a and b). Vessel walls and, occasionally, a few progenitor cells were also stained HOXA1-positive but no other haematopoietic or stromal cells. In contrast, in the PMF with high miRNA 10a levels and the follow-up PV about a quarter of megakaryocytic cells did not express HOXA1 protein (Fig. 2 c and d; #5 and #22 in supplementary Table 1). In three out of six patients, follow-up PMF with elevated myeloblasts (#23, #27, #28, supplementary Table 1), megakaryocytic HOXA1 staining was reduced, too, but the corresponding megakaryocytic miRNA 10a was expressed at low levels. False-negative staining can be excluded because strongly and weakly stained cells showed an intermingled pattern (which might reflect a cell cycle-dependent expression) and vessel wall served as an internal control.

As reported [15], RUNX1 protein was localised in the nucleus, predominantly in megakaryocytes but also in erythropoiesis (Fig. 2 e and f). No inverse correlation to megakaryocytic expression levels of miRNA 17-5p, 20a or 126, potential regulators of RUNX1, could be observed (supplementary Table 1).

Cytoreductive therapy has no major effect on miRNA 10a, 17-5p, 20a and 126 expressions in Ph⁻ MPN bone marrow cells during the course of disease

Paralleling the results on megakaryocytic miRNA, prior to treatment in unsorted bone marrow cells of Ph⁻ MPN and controls, the expression levels of all four miRNA revealed

Fig. 1 Point plot illustration of megakaryocytic expression of miRNA 17-5p, 20a, 126 and 10a in Ph⁻ MPN. Black bars indicate median values. Note that follow-up samples comprised of one PV (filled upright triangle) and six PMF (filled circle) with 5–30% myeloblasts. Ph⁻ MPN cases were not divided according to the individual JAK2^{V617F} and MPL^{W515L} mutation status, because statistical analysis revealed no significant correlation



no significant difference, neither between Ph⁻ MPN entities nor when compared to control cases. During the course of disease, no significant alterations of miRNA expression could be linked to treatment with hydroxyurea, myelofibrotic progress or haematological parameters (data not shown).

Discussion

Little is known about miRNA regulatory function in the pathogenesis of myeloid leukaemias. So far, all our knowledge on miRNA expression in Ph⁻ MPN is based on analyses of in vitro-differentiated cells and peripheral blood cells [4–6]. In this approach, we have analysed for the first time, a subset of miRNA from bone marrow cells and megakaryocytes in normal haematopoiesis and in Ph⁻ MPN entities.

Paralleling previous findings on CD34⁺ megakaryocytic differentiation assay [11], in our collective of non-leukaemic

and Ph⁻ MPN megakaryopoiesis low or not detectable miRNA 10a expression levels were frequently evident and appeared to correlate to strong HOXA1 protein expression in the majority of megakaryocytes. In non-leukaemic CD34⁺ bone marrow cells, before the in vitro differentiation process had been initiated towards megakaryocytic cells, high miRNA 10a/low HOXA1 in immature cells [11] might reflect a more progenitor-like molecular characteristic which is lost during megakaryocytic differentiation. At least in some individual Ph⁻ MPN patients, elevated miRNA 10a expression and corresponding low HOXA1 protein might recapitulate megakaryopoiesis towards a more primitive phenotype during the AML transformation process. Regarding this, in contrast to previous in vitro experiments [11] these patient sample-derived results do not prove a causative relationship. It has to be taken into account that in a subfraction of follow-up cases with elevated blasts counts reduced HOXA1 expression was not paralleled by increased miRNA 10a expression in megakaryocytes. Vice versa, increased megakaryocytic miRNA 10a expression in one

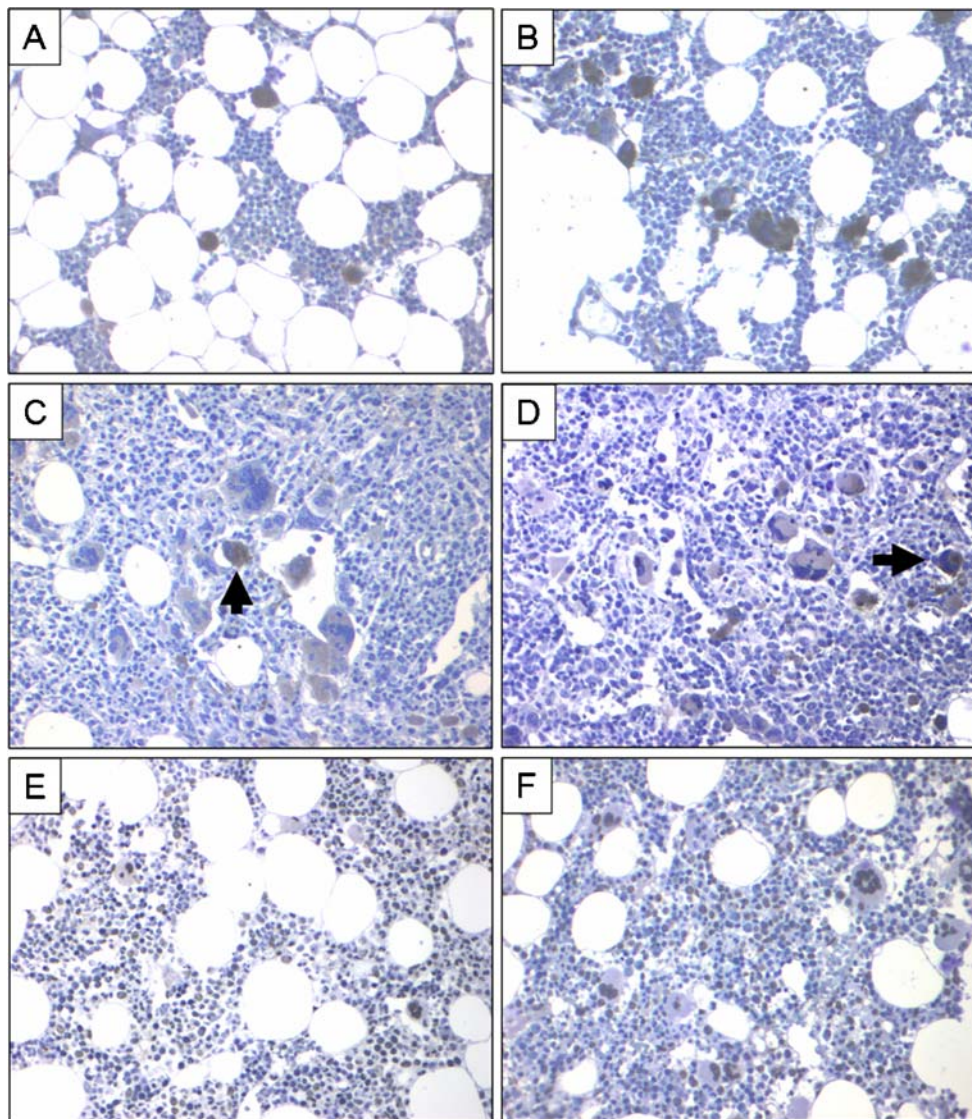


Fig. 2 Protein expression of miRNA 10a target gene HOXA1 and miRNA 17-5p/20a target gene RUNX1 in bone marrow cells. Low or absent megakaryocytic miRNA 10a expression appeared to correlate with strong HOXA1 immunostaining in almost all control cases (**a**) and Ph⁻ MPN (**b**, a representative cellular PMF at presentation is depicted). In one fibrotic stage PMF case (**c**) which transformed shortly afterwards into AML and in one PV in acceleration stage (**d**, 14-year follow-up biopsy), elevated megakaryocytic miRNA 10a expression levels seemed to correlate with lower detection rate and lower immunostaining intensity of HOXA1 protein. As an internal control, some megakar-

yocytes showed strong HOXA1 staining (arrows in **c** and **d**). RUNX1 protein expression in normal haematopoiesis (**e**) and Ph⁻ MPN (**f**, a representative PV at presentation is depicted) showed no inverse correlation to their potential regulator miRNA 17-5p, 20a and 126. Original magnification in all micrographs: $\times 200$. Images were produced with a DP71 Camera (Olympus, Germany) on an Axiophot microscope with a Plan-Neofluar 20 \times /0.50 objective (both Zeiss, Germany) and were processed with Soft Imaging System software (Olympus, Germany)

control case was not paralleled by decreased HOXA1 expression or sign of any leukaemic transformation. Furthermore, in de novo AML (compared to healthy controls) [21] or megakaryoblastic cell lines (compared to non-neoplastic CD34⁺-derived megakaryocytic cells) [11], miRNA 10a is not differentially expressed, indicating that deregulation of miRNA 10a/HOXA1 homeostasis might be an event which could be restricted to the beginning phase of transformation and might require additional genetic or epigenetic deregulat-

ing events on the background of a leukaemic clone. For instance, at least in the promyelocytic leukaemia cell line NB4, the HOXA1 gene is epigenetically regulated by CpG methylation and histone acetylation [22], indicating that regulation by miRNA 10a might be only one of several mechanisms.

In a patient suffering from Ph⁺ chronic myeloid leukaemia (CML), miRNA 17-5p-92 cluster genes were up-regulated in the chronic phase of the disease but decreased during the

AML phase [7], but no comparable effect was observed in megakaryocytes from Ph⁻ MPN follow-up cases with increased blast counts or secondary AML. Most recently, Shen et al. demonstrated a negative-regulatory function of miRNA 126 on HOXA9 expression in vitro and in vivo [23]. HOXA9 is a known regulator of haematopoiesis, and in peripheral blood cells from CML patients its expression is elevated in the acceleration stage [24].

In this approach, we investigated the RUNX1 protein expression but, in contrast to miRNA 10a/HOXA1, no correlation to potential regulator miRNA 17-5p, 20a or 126 expression levels could be detected.

In the cohort under investigation, thrombocytosis in Ph⁻ MPN was not associated to miRNA 10a, 17-5p, 20a and 126 expression levels. While miRNA 339 had been reported to be differentially expressed in Ph⁻ MPN-derived platelets [5] in a preliminary study by our group, this miRNA species did not seem to be aberrantly expressed in Ph⁻ MPN megakaryocytes (unpublished data). Furthermore, there are no data on clinical correlation which link aberrant miRNA 339 expression to thrombocytosis and further analysis of peripheral blood platelets and corresponding megakaryocytes might clarify whether miRNA expression and function are preserved in bone marrow and peripheral blood.

Similar to previous studies [4–6], in this cohort, no significant link between miRNA expression and JAK2^{V617F} mutated and unmutated PV, PMF and ET could be detected [4, 6]. Only by rearrangement of the cohort according to the allelic burden, including unmutated controls, was a significant JAK2^{V617F} correlation to miRNA 182 and 183 achieved [4].

As yet, no investigations on potential therapy-related effects on miRNA expression in Ph⁻ MPN have been published. In this first retrospective approach, we chose unsorted bone marrow cells because hydroxyurea is a non-megakaryocytic specific myelosuppressive agent, but for miRNA 10a, 17-5p, 20a and 126, no significant changes were observed.

In conclusion, similar to results on non-neoplastic megakaryopoiesis, miRNA 17-5p, 20a and 126 are constitutively expressed in Ph⁻ MPN megakaryocytes while miRNA 10a is low or absent and appeared to correlate to HOXA1 expression.

Acknowledgements The authors wish to thank Ms. Sabine Schröter and Ms. Anna-Lena Becker for their skilful work in the laboratory.

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