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Morgana acts as an oncosuppressor in chronic myeloid leukemia

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-morgana haploinsufficiency causes in mice a lethal and transplantable CML like myeloid neoplasm

-morgana is underexpressed in aCML and in a subgroup of CMLs where predicts a worse response to Imatinib but sensitivity to ROCK inhibitors

Abstract

We recently described *morgana* as an essential protein able to regulate centrosome duplication and genomic stability, by inhibiting ROCK. Here we show that *morgana* +/- mice spontaneously develop a lethal myeloproliferative disease resembling human atypical chronic myeloid leukemia (aCML), preceded by ROCK hyperactivation, centrosome amplification and cytogenetic abnormalities in the bone marrow (BM). Moreover, we found that *Morgana* is underexpressed in the BM of patients affected by atypical CML, a disorder of poorly understood molecular basis, characterized by non-recurrent cytogenetic abnormalities. *Morgana* is also underexpressed in the BM of a portion of patients affected by Philadelphia positive CML (Ph+ CML) caused by the BCR-ABL oncogene and in this condition *Morgana* underexpression predicts a worse response to Imatinib, the Ph+ CML standard treatment. Thus, *Morgana* acts as an oncosuppressor with different modalities: on one hand, *Morgana* underexpression induces centrosome amplification and cytogenetic abnormalities, on the other, in Ph+ CML, it synergizes with BCR-ABL signaling, reducing the efficacy of Imatinib treatment. Importantly, ROCK inhibition in the BM of patients underexpressing *Morgana* restored the efficacy of Imatinib to induce apoptosis, suggesting that ROCK inhibitors, in combination with Imatinib treatment, can overcome suboptimal responses in patients in which *Morgana* is underexpressed.

Introduction

CML is a neoplastic disorder of the hematopoietic stem cells that accounts for 15–20% of newly diagnosed cases of adult leukemia. The causative molecular event in CML is the genomic reciprocal translocation t(9;22)(q34;q11), known as Philadelphia (Ph) chromosome. The consequence of this genomic rearrangement is the formation of the *BCR-ABL* fusion gene, resulting in the production of a chimeric protein with aberrant tyrosine kinase activity. Inhibition of BCR-ABL kinase activity through Tyrosine Kinase Inhibitors (TKI) treatment is successfully used in Ph+ CML to induce remission from the pathology. However, treatment with TKI does not completely eradicate CML,

due to the insensitiveness of CML stem cells to these drugs.¹⁻³ Therefore the identification of pathways that can cooperate with BCR-ABL in the development or in the maintenance of CML is mandatory in order to identify additional targets to achieve synthetic lethality in the aim of curing CML. Furthermore, five percent of CML patients lacks BCR-ABL and is affected by a so called atypical CML, a disease with a poorly understood etiology. Recurrent mutations of SETBP1 are described in about 24% of aCML.⁴ Due to the absence of BCR-ABL, aCML patients are not eligible to Imatinib treatment and therefore the identification of specific oncogenic mechanisms is essential to develop a molecular therapy. Typically, aCML is characterized by a high incidence of non-recurrent cytogenetic abnormalities, often due to aneuploid karyotypes,⁵⁻⁷ suggesting that genes involved in maintenance of genomic stability could be involved in this pathogenesis.

We recently characterized *morgana/chp-1* as an essential protein in mouse embryonic development, involved in the regulation of centrosome duplication and genomic stability.⁸ In fact, *morgana +/-* mouse embryonic fibroblasts (MEFs), expressing 50% of the normal *morgana* level, show a higher frequency of supernumerary centrosomes, multipolar spindles, aneuploid and polyploid karyotypes.⁸ At a molecular level, *morgana* forms a complex with ROCKI and ROCKII⁸ and, by inhibiting ROCK II kinase activity, suppresses centrosome overduplication.^{8,9,10} Supernumerary centrosomes represent an important characteristic in cancer onset and progression¹¹ leading to multipolar mitosis, genomic instability¹² and chromothripsis.¹³ Accordingly, *morgana +/-* MEFs acquire transformed features over time *in vitro*.⁸ In addition, *morgana +/-* mice show increased susceptibility to tumor development in response to chemical mutagens.⁸ Here we show that *morgana +/-* mice develop with age a fatal and transplantable myeloproliferative disease similar to human aCML, presenting centrosome amplification as well as cytogenetic abnormalities in the BM. Notably, low *morgana* expression levels were found in the BM of aCML affected patients and in a portion of Ph+ CML. In this latter condition low *morgana* levels cooperate with BCR-ABL oncogenic signal in promoting ROCK activity, reducing sensitivity to Imatinib treatment.

Methods

Mice

The *morgana* gene (*chordc1*) was inactivated by homologous recombination in mouse ES cells as described.⁸ The genetic background of the *morgana* +/- and the wild type mice analyzed was C57BL/6×129SV. The use of animals was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and was approved by the Animal Care and Use Committee of the University of Torino.

Immunohistochemistry

Immunohistochemistry experiments were performed on formalin-fixed, paraffin-embedded tissues using the following antibodies: anti-myeloperoxidase (ab45977, Abcam, 1µg/ml), anti-P-MLC2 (LS-C16676, LifeSpan Biosciences) and anti-*morgana* P1/PP0 antibody (10µg/ml).^{8,14} *Morgana* expression level was defined low when staining intensity was lower compared to normal BM specifically in the myeloid progenitor cells. Slides were scored independently by two pathologists. Furthermore, IHC scoring was also performed using IHC Profiler software.¹⁵

Flow cytometry

Hematopoietic cells were obtained from mouse BM, peripheral blood and spleen. After lysis of red blood cells, cells were stained with the indicated antibodies (see supplemental Methods). For each analysis, a total of at least 10,000 cells were analyzed. For intracellular *morgana* staining Lin⁺ and Lin⁻ BM cells were incubated with antibodies and treated with FIX & PERM (Caltag). *Morgana* was stained using FITC-coniugated P1/PP0 (5µg/ml). Flow cytometric analyses were carried out on a FACSCalibur using CellQuest Software (Becton Dickinson).

Bone marrow transplantation

Sub-lethal total body irradiations were obtained in nude mice with a dose of 5 Gy. Lin⁻ BM cells were purified using Lineage cell depletion kit mouse (Miltenyi Biotec) from BM cells obtained from femurs and tibias of *morgana* +/- and wild type littermates. Next, 1×10⁶ Lin⁻ cells were injected into the tail vein of irradiated nude mice.

BM transduction

BM cells from 5-FU-treated (150 mg/kg) *morgana* +/+ and +/- donor mice were transduced with a double spinoculation protocol in presence of interleukin-3, interleukin-6 and stem cell factor, as described.¹⁶ p210-BCR-ABL vector was kindly provided by Prof. K. Ito.¹⁷

Colony assay

2×10⁴ mouse BM cells were plated on M3434 semisolid methylcellulose medium (StemCell Technologies) and scored 12-14 days later. For serial replating assays, primary colonies were recovered from the methylcellulose medium. Cells were replated in M3434 media followed by serial replating every 8-10 days.

Immunofluorescence

BM cells were fixed with PBS 4% PFA after cytopspin and stained with anti- γ -tubulin (T5192 Sigma), anti- α -tubulin (ab15246, Abcam) or anti-*morgana* (P1/PP0; 10 μ g/ml). Immunocomplexes were detected with anti-rabbit IgG Alexa Fluor 488 (A11008, Invitrogen), anti-mouse IgG Alexa Fluor 568 and 488 (A11004, Invitrogen). Cells were visualized with apotome software (Zeiss).

Western Blot Analysis

BM cells were washed with cold PBS and lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β -glycerolphosphate, and 1 mM

orthovanadate and Protease Inhibitor Cocktail (Sigma). Western Blot was performed as described¹⁴ with the indicated antibodies (see supplemental Methods).

Cell lines and Morgana silencing

K562 cell line was purchased from American Type Culture Collection (ATCC). Morgana knockdown was performed by infecting K562 cells with pGIPZ lentiviral particles expressing turboGFP and two different shRNAs targeting morgana (Open Biosystems).¹⁴

Patient bone marrow samples

Primary leukemia cells were obtained from the BM of patients with myeloproliferative disorders (chronic phase aCML and Ph⁺ CML) after appropriate informed consent and Institutional Review Board approval (Institutional Ethics Committee Approval # 81/2011). All samples were collected at diagnosis, prior to initiation of treatment.

K562 and BM cell treatment

K562 and patient BM cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 ug/mL streptomycin at 37°C in 5% CO₂. BM cells were treated for 96h with Imatinib (10μM) or Fasudil (10μM) or a combination of the two drugs. K562 were cultured for 48h with Imatinib (1μM), Fasudil (10μM), Y27632 (20μM) or a combination of them. Effective concentration (IC₅₀) values were calculated by regression analysis, using the dose-response curves generated from the experimental data, using GraphPad PRISM 5 Software. Mouse BM cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 ug/mL streptomycin at 37°C in 5% CO₂ in presence of interleukin-3 (6 ng/ml; 213-13, Peprotech), interleukin-6 (2.5 ng/ml; 216-16, Peprotech) and stem cell factor (50 ng/ml; 250-03, Peprotech) for 48h in presence of Fasudil (10μM), LY294002 (Calbiochem) and BEZ235 (Chemdea).

Statistics

Two-sided Student's *t* test and one-way ANOVA was calculated using GraphPad Prism software. *P* values < 0.05 were considered statistically significant. Data presented with column graphs and error bars represent the average \pm SE.

Results

Morgana haploinsufficiency causes a myeloproliferative neoplasm in mice

We assessed the susceptibility of *morgana* +/- mice to spontaneous tumor formation by monitoring them for 20 months. From 12 months of age onwards, 60% of *morgana* +/- mice showed clear pathological signs, such as rapid weight loss, hunched posture and severe hypokinesia and died as a result (Figure 1A). Necroscopic examination did not reveal the presence of solid tumors, but histopathological analysis disclosed the presence of myeloid infiltrates in different organs. *morgana* +/- and wild type littermates were therefore subjected every 2 months to peripheral blood analysis and where overt pathological signs were evident, they were considered moribund, euthanized and subjected to blood and hematopoietic organ examinations. *morgana* +/- moribund mice showed the presence of immature myeloid cells in peripheral blood (Figure 1B) and clear signs of leukocytosis, anemia, anisocytosis, and circulating nucleated red blood cells, while no abnormalities were detected in healthy *morgana* +/- or wild type mice (Figure 1C-D and supplemental Figure 1A). Notably, expansion of monocytes and eosinophils was not observed (supplemental Figure 1B), while, as reported in other MPD murine models¹⁸, an increase of reticulocytes was present (supplemental Figure 1C-E). *morgana* +/- diseased mice also showed hepatomegaly (Figure 1E and supplemental Figure 1F) and in 50% of cases, the presence of myeloid infiltrations in the liver, as demonstrated by positive myeloperoxidase staining (Figure 1F). Moreover, they invariably showed splenomegaly (Figure 1G and supplemental Figure 1G) and a significant increase of Mac-1⁺ Gr-1⁺ and Mac-1⁺ c-Kit⁻ cells, paralleled by a reduction in CD3⁺

cells in the spleen, compared with control mice (Figure 1H). BM of *morgana* +/- diseased mice showed a strong expansion of Mac-1⁺ Gr-1⁺ and Mac-1⁺ c-Kit⁻ cells and a reduction of B220⁺ cells when compared with wild type mice, demonstrating the expansion of the myeloid population to the detriment of the lymphoid compartment (Figure 1I). Interestingly, in two *morgana* +/- diseased mice, we detected the presence of a pool of Mac-1⁻ c-Kit⁺ cells in peripheral blood, suggesting that the myeloproliferative disease was evolving into blast crisis (supplemental Figure 1H).

Finally, to distinguish leukemia from less aggressive disturbances of hematopoiesis, we performed BM transplantation experiments.¹⁹ Lineage negative BM cells were purified from BM of *morgana* +/- diseased mice and wild type littermates and injected into the tail vein of sub-lethally irradiated nude mice. After 3-6 months *morgana* +/- recipient mice showed clear pathological signs, demonstrating that the hematologic neoplasm of *morgana* +/- mice is transplantable (Figure 1L, supplemental Figure 1I-L and supplemental Table 1). Notably, in a secondary recipient mouse the pathology progressed toward a more aggressive phenotype as demonstrated by the presence of a pool of c-Kit⁺ cells in peripheral blood (Figure 1L), suggesting the clonal evolution of the disorder. Overall these data indicate that with age, *morgana* +/- mice develop a fatal and transplantable myeloproliferative disease closely resembling human CML.

To characterize the origin of the disease, we analyzed possible pathological signs in *morgana* +/- mice at 8 months. No differences were found in peripheral blood counts (supplemental Figure 2A), in BM subcellular populations (supplemental Figure 2B), in the presence of dysplastic megakaryocytes in the BM (supplemental Figure 2C). Moreover, *morgana* +/- and wild type mice did not differ for hematopoietic stem cell number, basal apoptotic level, proliferation and P-MLC2 expression levels (supplemental Figure 2D-G). BM cells from healthy *morgana* +/- mice and wild type littermates were subjected to a colony assay to assess their clonogenic potential. The frequency of erythroid, granulocytic and monocytic colonies in *morgana* +/- mice was comparable to that of wild type mice (supplemental Figure 2H); however a slight, but significant, increase in *morgana* +/- cell replating potential was noticed (supplemental Figure 2I-L), indicating that *morgana* +/- cells

can acquire hyperproliferative capacity. These observations correlate with the increased proliferation rate of *morgana* +/- MEFs,⁸ suggesting that low levels of *morgana* favor cellular proliferation.

We previously demonstrated that *morgana* acts as a ROCK inhibitor in primary fibroblasts and that *morgana* underexpression causes centrosome amplification and genomic instability.⁸ Analysis of the phosphorylation status of the ROCK substrates MLC-2 and MYPT1 in the BM of *morgana* +/- mice, prior to disease onset, demonstrated that a low *morgana* expression level enhances ROCK signaling in BM cells (Figure 2A). In addition, AKT phosphorylation is significantly lower in *morgana* +/- mice (Figure 2A), implying a role for *morgana* in regulating AKT activation also in BM other than in breast cancer cells.¹⁴ However, *morgana* +/- BM cells did not show a higher sensitivity to PI3K inhibitors (supplemental Figure 2M). Moreover, *morgana* +/- mice have a higher number of BM cells with greater than two centrosomes when compared with wild type mice both before (Figure 2B) and after (Figure 2C) disease onset. Notably, in *morgana* +/- diseased mice, the number of aberrant metaphases drastically increased (Figure 2D).

Morgana is underexpressed in human aCML and in a subgroup of Ph+ CMLs

To analyze the relevance of *Morgana* in human pathology, we first evaluated its expression in normal human BM by immunohistochemistry (IHC). This analysis indicated that *Morgana* is expressed at different levels according to the BM cell type. In particular, *Morgana* is highly expressed in the myeloid progenitor compartment with a subsequent reduction during differentiation into mature neutrophils. *Morgana* is also highly expressed in megakaryocytes, while mature erythroid colonies and red blood cells show undetectable expression (Figure 3A). Similar results were obtained in mouse BM, where Western blot and FACS analysis confirmed high levels of *morgana* expression in progenitor and staminal BM cells (Figure 3B-C).

The phenotype observed in *morgana* +/- mice, resembling human chronic myeloid leukemia, prompted us to investigate *Morgana* levels in human CML and aCML. Both diseases are

myeloproliferative disorders with similar clinical manifestations. While the hallmark of CML is the Philadelphia chromosome, coding for the chimeric protein BCR-ABL, aCML lacks the expression of this fusion product. Given that *BCR-ABL* translocation does not occur in mice²⁰ and that the myeloproliferative disease affecting *morgana* +/- mice strongly resembles CML, we decided to analyze Morgana expression levels by IHC in five BM from aCML patients and one case of the closely related myelodysplastic/myeloproliferative neoplasm (MDS/MPN). Strikingly, all five aCML samples tested expressed a very low/undetectable Morgana level (Morgana^{low}) in the myeloid compartment, while the MDS/MPN sample showed normal Morgana expression (Figure 3D). Notably, P-MLC2 detection by IHC was inversely correlated with Morgana expression levels in BM from aCML patients compared with the MDS/MPN (case 6) (Figure 3E). These observations together with *morgana* +/- mouse phenotype, strongly suggests that Morgana plays a role in human aCML pathogenesis. Interestingly, CSF3R mutations were absent in all samples tested, while SETPB1 was found mutated in one aCML patient. To assess if Morgana gene (*CHORDC1*) mutations can be responsible for low morgana expression levels in aCML patients, we screened exome sequencing data obtained from 16 aCML patients.⁴ However, no mutations were detectable, suggesting that more complex regulatory mechanisms are involved in Morgana underexpression in this pathology.

To evaluate the possibility that *morgana*^{low} leukemia cells were addicted to ROCK signaling we treated BM from *morgana* +/+ and *morgana* +/- diseased mice with Fasudil (10µM) for 48h and we analyzed cell viability. ROCK inhibition promoted apoptosis induction only in *morgana* +/- BM (Figure 3F), suggesting that ROCK inhibitors may have a therapeutic value in aCML.

Given that oncosuppressor downregulation often cooperates with activated oncogenes in tumor growth and progression, we expanded our analysis to Philadelphia positive CML. IHC analysis on 19 BM patients revealed that Morgana was clearly underexpressed in 16% of patients (Figure 4A and B and supplemental Figure 3A and 3B). These data were also confirmed by immunofluorescence on CD34⁺ purified cells (supplemental Figure 3C).

Given that *morgana* +/- diseased mice showed centrosome amplification in BM cells, we assessed whether *Morgana*^{low} Ph+ CML patients showed similar features. It is known that BCR-ABL expression alone induces centrosome amplification (see also Figure 4C).^{21,22,23} However, immunofluorescence analysis of CD34⁺ purified cells from the BM of Ph+ CML patients highlighted the presence of a significantly higher number of cells with supernumerary centrosomes in *Morgana*^{low} patients (Figure 4C).

Given that centrosome amplification causes aneuploidy, we analyzed metaphases from Ph+ CML and aCML collected at the moment of diagnosis and we observed a correlation between *Morgana* underexpression and the percentage of aneuploid cells. In particular, BM of *Morgana*^{low} patients showed the highest levels of aneuploidy (Figure 4D).

Low *morgana* in Ph+ CML predicts a suboptimal response to Imatinib that can be rescued by inhibiting ROCK

Imatinib blocks the activity of BCR-ABL and of its downstream effector ROCK, leading to CML cell apoptosis.^{24,25,26,27} Given that low *Morgana* levels lead to ROCK activation also in Ph+ CML BM cells (Figure 5A and supplemental Figure 3D), we reasoned that *Morgana* underexpression, by sustaining ROCK activity, may cause a suboptimal response to Imatinib treatment. Indeed, as shown in Figure 5B, our follow-up analysis in a cohort of 12 Ph+ CML patients without BCR-ABL mutations affecting Imatinib resistance highlighted a significant correlation between *Morgana* underexpression and a suboptimal response to Imatinib (detected by patients' molecular response). Strikingly, a *Morgana*^{low} CML patient progressed into blast phase in four months after the diagnosis. Furthermore, another *Morgana*^{low} patient displayed Ph chromosome in 13% of metaphases after 18 months of treatment. Real time analysis on 102 Ph+ CML patients indicated that 30% of them expressed *Morgana* less than half of the mean of *Morgana* mRNA levels in normal BM (supplemental Figure 4A and B). The follow up analysis on 22 patients confirmed that *Morgana*^{low} patients displayed a suboptimal response to Imatinib (supplemental Figure 4C).

These data suggest that low Morgana levels can predict response to Imatinib treatment. These observations led us to investigate therapeutic implications in Morgana^{low} CML.

Mali et al. demonstrated recently that BCR-ABL causes a constitutive activation of ROCK that sustains cell transformation and survival.²⁴ We then tested if low Morgana level impacts on the response to Imatinib by sustaining ROCK activity. First, we interfered morgana in K562 cells and we tested their response to Imatinib treatment by determining their dose response curves (Figure 5C). IC₅₀ value for K562 empty was 1,0μM, while for K562 silenced for morgana was 3,65μM (shmorgana1) and 3,63μM (shmorgana2). Moreover, cells downregulated for Morgana showed a reduced apoptotic response to Imatinib (Figure 5D). However, when cells were treated with a combination of Imatinib and the ROCK inhibitors Fasudil or Y27632, the apoptotic response was restored to normal levels (Figure 5D). Signaling analysis demonstrated that morgana downregulation promotes ROCK activation also in K562 CML cell line, as demonstrated by increased MLC2 and MYPT1 phosphorylation (Figure 5E). It has been reported that ROCKI can phosphorylate PTEN, increasing its stability and activity.^{28,29} Moreover, we recently demonstrated that morgana overexpression, by inhibiting ROCK kinase activity, results in reduced PTEN levels.¹⁴ Accordingly, in morgana^{low} K562, PTEN was expressed at higher levels. However, AKT phosphorylation was not significantly lower, likely due to BCR-ABL dependent PI3K hyperactivation (Figure 5E). STAT5 has been involved in Imatinib sensitivity.³⁰ However, STAT5 phosphorylation, albeit showing a slightly upregulation in K562 interfered for morgana, was not significantly different (Figure 5E).

Moreover, combined treatment with Imatinib and the ROCK inhibitor Fasudil, significantly increased apoptosis in Morgana^{low} Ph+ CML BM cells (Figure 5F). To eliminate the variability due to the heterogeneity of response to Imatinib in cells of different patients, we calculated the apoptosis fold induction of cells treated with Imatinib and Fasudil versus Imatinib alone for each individual patient and we observed a significant increase in the percentage of apoptosis in Morgana^{low} compared to BM cells expressing a normal level of Morgana (Figure 5G).

However, as for BCR-ABL addiction, *morgana* haploinsufficiency could not be exploited to eradicate leukemia stem cells. In fact, the apoptotic response to Fasudil was not significantly different in BCR-ABL KSL derived from *morgana* +/- versus wild type mice (supplemental Figure 3E-G).

Overall, these data indicate that Morgana underexpression, through ROCK hyperactivation, causes a suboptimal response to TKI in Ph+ CML cells that can be rescued by ROCK inhibitors, pointing to a potential efficacy of ROCK inhibitors in the chronic treatment of Ph+ CML.

Discussion

Here we show that *morgana*, a chaperone protein³¹⁻³⁵ regulating ROCK activity^{8,10,14} is a tumor suppressor gene involved in myeloid leukemogenesis. In particular, *morgana* +/- mice spontaneously develop a fatal Ph negative CML-like myeloproliferative disease. This disease is indeed characterized by dramatic expansion of myeloid compartment, less than 2% of basophils, less than 10% of monocytes, fewer than 20% of blasts, anemia, splenomegaly and absence of fibrosis in the BM. This disease was transplantable into nude mice and eventually able to evolve into acute leukemia. Murine CML-like MPD is associated with centrosome amplification and cytogenetic abnormalities in the BM. Morgana haploinsufficiency in BM cells leads to ROCK hyperactivation and centrosome amplification. Supernumerary centrosomes cause aneuploidy and chromotripsis, which ultimately lead to tumor onset and progression.^{13,36,37} Accordingly, BM cells from diseased *morgana* +/- mice showed a high level of aneuploidy. The phenotype of *morgana* +/- mice prompted us to investigate whether *morgana* is involved in human Ph-positive CML and atypical CML. aCML has been proposed to represent a clinically distinct entity from the unclassifiable MDS/MPN, for which the identification of specific markers are needed to better classify, stratify and treat patients with myeloid disorders.³⁸ aCML shares with Philadelphia positive CML a similar clinical presentation but lacks BCR-ABL and therefore cannot benefit from TKI specific targeted therapy. The only therapeutic option for these patients is the treatment with

conventional cytoreductive drugs. aCML is characterized by somatic mutations at low recurrence of several different genes (TET2, CBL, EZH2, SETBP1, CSF3R, ETNK1 and others)^{4,39,40,41,42,43,44} and a high incidence of non-recurrent cytogenetic abnormalities, often due to aneuploid karyotypes,^{5,6,7} suggesting that genes that regulate genomic stability maintenance could be involved in the pathogenesis of aCML. Here we show that Morgana is underexpressed in the BM of all aCML patients tested, suggesting that Morgana underexpression could represent a common feature in aCML. Given that Morgana underexpression correlates with centrosome amplification and aneuploidy, it is tempting to speculate that Morgana plays a role in the onset of this disease in patients. Given that ROCK is hyperactivated in Morgana^{low} aCML BM, ROCK inhibitors, already in clinical use for other applications, could represent a new potential therapeutic approach in aCML. Accordingly, Fasudil treatment of BM cells induces an apoptotic response only in *morgana* +/- diseased mice, suggesting the dependence of these cells to ROCK signaling.

Further, we show that Morgana underexpression in Ph+ CMLs synergizes with BCR-ABL signaling in inducing centrosome amplification and genomic instability. It is known that BCR-ABL fusion protein activates different signaling molecules,^{45,46} including ROCK²⁴ and that BCR-ABL expressing cells show addiction to BCR-ABL²⁷ and ROCK signaling.^{24,25,26} Indeed, inhibition of BCR-ABL kinase activity through Imatinib treatment is successfully used in Ph+ CML to induce apoptosis in BCR-ABL expressing cells. However, while the majority of Ph+ CML patients show a good response to Imatinib, about 25-30% show insufficient therapeutic effect.^{47,48} Our follow-up analysis demonstrated that Morgana^{low} patients show a worse response during Imatinib treatment. Notably, suboptimal response is highly predictive of worse overall survival.⁴⁹ Thus, the identification of Morgana expression level at the onset of the disease may represent an important prognostic tool to predict a suboptimal response to Imatinib. Given that Morgana underexpression confers Imatinib resistance via ROCK hyperactivation, the combined treatment with Imatinib and a ROCK inhibitor restored a normal apoptotic response in Morgana^{low} Ph+ CML cells, suggesting a new potential therapeutic treatment.

In conclusion, our data show that Morgana behaves as a tumor suppressor in the pathogenesis of both aCML and Ph⁺ CML, via its ability to regulate ROCK activity. Morgana underexpression, promoting ROCK hyperactivation, can have a dual action: it favors genomic instability and, at the same time, it sustains cell survival and proliferation,²⁴ further resulting in myeloproliferation.

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Authorship Contributions

AD, SR, FF, RF CP, SC,GC, AC, IF performed experiments, UF and MP performed and supervised the histopathological analysis and immunohistochemistry, EG performed cytogenetic analysis on human BM samples, BM performed mouse blood examinations, JCC and AM performed mice histopathological examination, RP and CG analyzed exome sequencing data, ET, LS, EH provided discussions and advice, PPP and GT contribute to experimental design and critical analysis of the results, GS and GR provided patient samples and critical analysis of data, AM and MB designed the research, coordinated and directed the study, wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

1. Perl A, Carroll M. BCR-ABL kinase is dead; long live the CML stem cell. *J Clin Invest.* 2011;121:22-25.
2. Redner RL. Why doesn't imatinib cure chronic myeloid leukemia? *Oncologist.* 2010;15:182-186.
3. Morotti A, Panuzzo C, Fava C, Saglio G. Kinase-inhibitor-insensitive cancer stem cells in chronic myeloid leukemia. *Expert Opin Biol Ther.* 2014;14:287-299.
4. Piazza R, Valletta S, Winkelmann N, et al. Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. *Nat Genet.* 2013;45:18-24.
5. Guo-yu H, Chao-hui Y, Kui T, Zhen-zhen C. Atypical chronic myeloid leukaemia with trisomy 13: a case report. *Chin Med Sci J.* 2011;26:254-256.
6. Hernandez JM, del Canizo MC, Cuneo A, et al. Clinical, hematological and cytogenetic characteristics of atypical chronic myeloid leukemia. *Ann Oncol.* 2000;11:441-444.
7. Burgstaller S, Reiter A, Cross NC. BCR-ABL-negative chronic myeloid leukemia. *Curr Hematol Malig Rep.* 2007;2:75-82.
8. Ferretti R, Palumbo V, Di Savino A, et al. Morgana/chp-1, a ROCK inhibitor involved in centrosome duplication and tumorigenesis. *Dev Cell.* 2010;18:486-495.
9. Hanashiro K, Brancaccio M, Fukasawa K. Activated ROCK II by-passes the requirement of the CDK2 activity for centrosome duplication and amplification. *Oncogene.* 2011;30:2188-2197.
10. Ferretti R, Sbroglio M, Di Savino A, et al. Morgana and melusin: two fairies chaperoning signal transduction. *Cell Cycle.* 2011;10:3678-3683.
11. Fukasawa K. Oncogenes and tumour suppressors take on centrosomes. *Nat Rev Cancer.* 2007;7:911-924.
12. Ganem NJ, Godinho SA, Pellman D. A mechanism linking extra centrosomes to chromosomal instability. *Nature.* 2009;460:278-282.

13. Crasta K, Ganem NJ, Dagher R, et al. DNA breaks and chromosome pulverization from errors in mitosis. *Nature*. 2012;482:53-58.
14. Fusella F, Ferretti R, Recupero D, et al. Morgana acts as a proto-oncogene through inhibition of a ROCK-PTEN pathway. *J Pathol*. 2014.
15. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One*. 2014;9:e96801.
16. Gavrilescu LC, Van Etten RA. Murine retroviral bone marrow transplantation models for the study of human myeloproliferative disorders. *Curr Protoc Pharmacol*. 2008;Chapter 14:Unit14 10.
17. Ito K, Bernardi R, Morotti A, et al. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature*. 2008;453:1072-1078.
18. Kaufmann KB, Grunder A, Hadlich T, et al. A novel murine model of myeloproliferative disorders generated by overexpression of the transcription factor NF-E2. *J Exp Med*;209:35-50.
19. Kogan SC, Ward JM, Anver MR, et al. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*. 2002;100:238-245.
20. Saglio G, Storlazzi CT, Giugliano E, et al. A 76-kb duplison maps close to the BCR gene on chromosome 22 and the ABL gene on chromosome 9: possible involvement in the genesis of the Philadelphia chromosome translocation. *Proc Natl Acad Sci U S A*. 2002;99:9882-9887.
21. Patel H, Gordon MY. Abnormal centrosome-centriole cycle in chronic myeloid leukaemia? *Br J Haematol*. 2009;146:408-417.
22. Kramer A. Centrosome aberrations--hen or egg in cancer initiation and progression? *Leukemia*. 2005;19:1142-1144.
23. Giehl M, Fabarius A, Frank O, et al. Centrosome aberrations in chronic myeloid leukemia correlate with stage of disease and chromosomal instability. *Leukemia*. 2005;19:1192-1197.

24. Mali RS, Ramdas B, Ma P, et al. Rho kinase regulates the survival and transformation of cells bearing oncogenic forms of KIT, FLT3, and BCR-ABL. *Cancer Cell*. 2011;20:357-369.
25. Kuzelova K, Hrkal Z. Rho-signaling pathways in chronic myelogenous leukemia. *Cardiovasc Hematol Disord Drug Targets*. 2008;8:261-267.
26. Burthem J, Rees-Unwin K, Mottram R, et al. The rho-kinase inhibitors Y-27632 and fasudil act synergistically with imatinib to inhibit the expansion of ex vivo CD34(+) CML progenitor cells. *Leukemia*. 2007;21:1708-1714.
27. Sharma SV, Gajowniczek P, Way IP, et al. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell*. 2006;10:425-435.
28. Li Z, Dong X, Wang Z, et al. Regulation of PTEN by Rho small GTPases. *Nat Cell Biol*. 2005;7:399-404.
29. Vemula S, Shi J, Hanneman P, Wei L, Kapur R. ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability. *Blood*. 2010;115:1785-1796.
30. Warsch W, Kollmann K, Eckelhart E, et al. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood*. 2011;117:3409-3420.
31. Shirasu K, Lahaye T, Tan MW, Zhou F, Azevedo C, Schulze-Lefert P. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. *Cell*. 1999;99:355-366.
32. Brancaccio M, Menini N, Bongioanni D, et al. Chp-1 and melusin, two CHORD containing proteins in vertebrates. *FEBS Lett*. 2003;551:47-52.
33. Hahn JS. Regulation of Nod1 by Hsp90 chaperone complex. *FEBS Lett*. 2005;579:4513-4519.
34. Wu J, Luo S, Jiang H, Li H. Mammalian CHORD-containing protein 1 is a novel heat shock protein 90-interacting protein. *FEBS Lett*. 2005;579:421-426.

35. Michowski W, Ferretti R, Wisniewska MB, et al. Morgana/CHP-1 is a novel chaperone able to protect cells from stress. *Biochim Biophys Acta*. 2010;1803:1043-1049.
36. Holland AJ, Cleveland DW. Chromoanagenesis and cancer: mechanisms and consequences of localized, complex chromosomal rearrangements. *Nat Med*. 2012;18:1630-1638.
37. Gordon DJ, Resio B, Pellman D. Causes and consequences of aneuploidy in cancer. *Nat Rev Genet*. 2012;13:189-203.
38. Wang SA, Hasserjian RP, Fox PS, et al. Atypical chronic myeloid leukemia is clinically distinct from unclassifiable myelodysplastic/myeloproliferative neoplasms. *Blood*. 2014;123:2645-2651.
39. Muramatsu H, Makishima H, Maciejewski JP. Chronic myelomonocytic leukemia and atypical chronic myeloid leukemia: novel pathogenetic lesions. *Semin Oncol*. 2012;39:67-73.
40. Cazzola M, Malcovati L, Invernizzi R. Myelodysplastic/myeloproliferative neoplasms. *Hematology Am Soc Hematol Educ Program*. 2011;2011:264-272.
41. Kosmider O, Gelsi-Boyer V, Ciudad M, et al. TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. *Haematologica*. 2009;94:1676-1681.
42. Grand FH, Hidalgo-Curtis CE, Ernst T, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood*. 2009;113:6182-6192.
43. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. *N Engl J Med*. 2013;368:1781-1790.
44. Gambacorti-Passerini CB, Donadoni C, Parmiani A, et al. Recurrent ETNK1 mutations in atypical chronic myeloid leukemia. *Blood*. 2014
45. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat Rev Cancer*. 2005;5:172-183.
46. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat Rev Cancer*. 2007;7:441-453.

47. Rea D, Etienne G, Corm S, et al. Imatinib dose escalation for chronic phase-chronic myelogenous leukaemia patients in primary suboptimal response to imatinib 400 mg daily standard therapy. *Leukemia*. 2009;23:1193-1196.
48. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol*. 2008;26:3358-3363.
49. Marin D, Ibrahim AR, Lucas C, et al. Assessment of BCR-ABL1 transcript levels at 3 months is the only requirement for predicting outcome for patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors. *J Clin Oncol*. 2012;30:232-238.

Figure Legends

Figure 1. Evaluation of the spontaneous onset of hematopoietic neoplasm in *morgana* +/- mice.

(A) Survival curves of *morgana* +/+ and *morgana* +/- mice. Kaplan-Meier analysis demonstrates a significant difference in survival (*morgana* +/+: n= 21 and *morgana* +/-: n= 25). (B) Representative picture of peripheral blood smears of diseased *morgana* +/- mice. (Scale bar, 10 μ m) (C-D) White Blood Cells (WBC) count and Hemoglobin (HGB) values in the peripheral blood of diseased *morgana* +/- mice compared with healthy *morgana* +/- and wild type littermates. (E) Liver/body weight ratios (liver index) of *morgana* +/- diseased and wild type mice. (F) Myeloid infiltration in the liver of *morgana* +/- diseased mice as confirmed by myeloperoxidase staining (Scale bar, 100 μ m). (G) Spleen/body weight ratios (spleen index) of *morgana* +/- diseased and wild type mice. (H-I) Flow cytometric analysis of (H) spleen and (I) BM single cell-suspension from diseased *morgana* +/- and wild type littermates and relative quantifications (n= 6 mice/group). (L) Flow cytometric analysis on the peripheral blood of representative *morgana* +/- and +/+ donor, primary and secondary recipient mice are shown. (*P<0.05; **P<0.01; ***P<0.001).

Figure 2. Molecular pathogenesis of *morgana* +/- myeloproliferative disease. (A) Western blot analysis of BM protein extracts from *morgana* +/+ and *morgana* +/- mice, prior to disease onset, stained with antibodies against *morgana*, ROCK I and II, phosphorylated MLC2 (P-MLC2), total MLC2, P-MYPT1, total MYPT1, P-AKT, total AKT, P-STAT5, total STAT5 and actin, used as a loading control. The graphs show the densitometric quantification of P-MLC2, P-MYPT1 and P-AKT bands normalized to total MLC2, MYPT1 and AKT (n=3 mice/group). Note that ROCK I, ROCK II and STAT5 phosphorylation were similar in *morgana* +/+ and +/- BM. (B) Percentages of BM cells with more than two centrosomes in 8-month-old *morgana* +/+ (n=6) and *morgana* +/- (n=5) mice. (C) Percentages of BM cells with more than two centrosomes in *morgana* +/- diseased mice and *morgana* +/+ littermates (n=4 mice/group). (D) Percentages of aneuploid metaphase spreads obtained from BM of *morgana* +/+ and *morgana* +/- diseased mice (n=4 mice/group). (*P<0.05; **P<0.01).

Figure 3. Morgana is underexpressed in aCML patients' BM. (A) Morgana IHC staining on normal human BM. (B) Western blot analysis of *morgana* in different BM populations and in total mouse BM. (C) Morgana fluorescence intensity in different mouse BM populations as assessed by flow cytometry. (D) Morgana IHC staining on BM biopsies of four aCML patients. (Scale bar, 100 μ m). (E) P-MLC2 IHC staining on a BM biopsy from aCML patient expressing low Morgana level (case 1) and a case of MDS/MPD expressing normal Morgana level (case 6) (Scale bar, 100 μ m). The graph shows the IHC quantification of P-MLC2. (F) Percentages of apoptosis in BM cells derived from *morgana* +/+ and *morgana* +/- diseased mice after 48h treatment with Fasudil (10 μ M). (*P<0.05).

Figure 4. Morgana is underexpressed in a subgroup of CML Ph+ patients. (A) Morgana IHC staining on BM biopsies of representative Morgana normal (case 7 and 8) and Morgana low (case 9 and 10) Ph+ CML patients. (Scale bar, 100 μ m). (B) The graph indicates the percentage of Ph+

CML patients expressing Morgana at normal (84%) and low (16%) levels as assessed by IHC analysis on BM sections (n=19). (C) Percentages of CD34⁺ BM cells with more than two centrosomes in CML patients at diagnosis, expressing normal or low Morgana levels (n=4 patients/group) and healthy donors (n=3). Black bar indicates low Morgana expression levels. (D) Percentage of aneuploid metaphases in Morgana normal and Morgana low Ph⁺ CML and Morgana low aCML BM cells compared to normal BM cells. Black bars indicate low Morgana expression levels. (*P<0.05).

Figure 5. Morgana underexpression synergizes with BCR-ABL and negatively impacts on CML treatment efficacy. (A) Western blot analysis of BM protein extracts from two representative Morgana normal and Morgana low Ph⁺ CML patients stained with antibodies against morgana, phosphorylated MLC2 (P-MLC2), total MLC2 and tubulin. The graphs show the densitometric quantification of morgana bands normalized to tubulin and P-MLC2 bands normalized to total MLC2 (n=4 patients/group). (B) Correlation between normal and low Morgana expression levels and patients' molecular response during TKI treatment in CML patients stratified for the levels of expression of Morgana by IHC (Morgana normal: n= 9 Morgana^{low}: n=3). (C) Dose-response curves of K562 infected with an empty vector (empty) or with vectors carrying two different shRNAs against morgana (shmorgana1 and 2) treated for 48h with different Imatinib concentration (0,005-0,1-0,5-1-5-10-20-50μM) analyzed by MTT assay (mean r²=0,98). Viability was expressed as a percentage of untreated cells. (D) Percentages of apoptotic K562 empty or shmorgana1 in response to 48h treatment with Imatinib (1μM), Imatinib (1μM) + Fasudil (10μM) and Imatinib (1μM) + Y27632 (20μM). (E) Western blot analysis of K562 empty and shmorgana stained with antibodies against morgana, ROCKI, ROCKII, P-MYPT1, MYPT1, P-MLC2, MLC2, PTEN, P-AKT, AKT, P-STAT5, STAT5 and tubulin. (F) Apoptosis quantification of BM cells from Ph⁺ patients cultured in the presence of Imatinib (10μM), the ROCK inhibitor Fasudil (10μM) or a combination of the two drugs (Morgana normal: 6 patients, Morgana low: 9 patients). Bars

represent the percentages of Annexin V-positive cells for each treatment. (G) Mean of apoptosis fold-induction calculated on BM cells treated with Imatinib and Fasudil versus Imatinib alone for each individual patient. (*P<0.05, **P<0.01, ***P<0.001).