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MTSS1 is a critical epigenetically regulated tumor suppressor in CML

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Abstract

Chronic myeloid leukemia (CML) is driven by malignant stem cells that can persist despite therapy. We have identified Metastasis suppressor 1 (Mtss1/MIM) to be downregulated in hematopoietic stem and progenitor cells from leukemic transgenic SCLtTA/Bcr-Abl mice and in patients with CML at diagnosis, and Mtss1 was restored when patients achieved complete remission. Forced expression of Mtss1 decreased clonogenic capacity and motility of murine myeloid progenitor cells and reduced tumor growth. Viral transduction of Mtss1 into lineage depleted SCLtTA/Bcr-Abl bone marrow cells decreased leukemic cell burden in recipients, and leukemogenesis was reduced upon injection of Mtss1 overexpressing murine myeloid 32D cells. Tyrosine kinase inhibitor (TKI) therapy and reversion of Bcr-Abl expression increased Mtss1 expression but failed to restore it to control levels. CML patient samples revealed higher DNA methylation of specific Mtss1 promoter CpG sites that contain binding sites for Kaiso and Rest transcription factors. In summary, we identified a novel tumor suppressor in CML stem cells that is downregulated by both Bcr-Abl kinase-dependent and -independent mechanisms. Restored Mtss1 expression markedly inhibits primitive leukemic cell biology in vivo, providing a therapeutic rationale for the Bcr-Abl-Mtss1 axis to target TKI resistant CML stem cells in patients.
Introduction

Chronic myeloid leukemia (CML) is a model disease reflecting the biology of a stem cell driven neoplasm. The reciprocal translocation t(9;22) gives rise to the cytoplasmic Bcr-Abl protein that activates various signaling pathways resulting in increased proliferation and differentiation, protection from apoptosis and altered adhesion properties of CML cells\textsuperscript{1, 2}. Moreover, these cells evolve mechanisms that facilitate disease progression and support their survival under stressful conditions\textsuperscript{3, 4}. The detection of Bcr-Abl and subsequent evidence that it is sufficient to induce CML in vivo\textsuperscript{5} paved the way for the development of targeted therapy. Today, the implementation of tyrosine kinase inhibitors (TKI) that bind to and inhibit the Abl kinase has resulted in high molecular response rates\textsuperscript{6}. However, while the majority of newly diagnosed patients in chronic phase (CP) respond well to TKI treatment, about one third of the patients develops primary or secondary resistance due to Bcr-Abl mutations or intolerance to TKIs. Moreover, even in patients without mutations, mechanisms such as low persisting Bcr-Abl levels in patients on TKI and/or a lack of oncogene addiction have been described to induce CML stem cell resistance in CP\textsuperscript{7-9}. Genetically, disease progression is associated with the acquisition of further chromosomal aberrations or mutations\textsuperscript{10}. At the epigenetic level, DNA promoter methylation of specific genes, often tumor suppressors, has been associated with disease progression\textsuperscript{11-13}.

The metastasis suppressor 1 (Mtss1) protein has recently been described to exert tumor suppressive function and its downregulation is associated with poor prognosis in various cancers\textsuperscript{14-19}. The structure of the multidomain protein suggests a scaffolding function\textsuperscript{20, 21}, and Mtss1 was found to interact with multiple partners to regulate actin dynamics and suppress cell migration and motility in various tumors\textsuperscript{22-24}. The mechanisms that induce these effects remain poorly understood.

Here, we identified Mtss1 to be suppressed in murine leukemic LSK (lin\textsuperscript{−};c-kit\textsuperscript{+};Sca-1\textsuperscript{+}) cells as well as primary cells from CML patients. Mtss1 downregulation was mediated via Bcr-Abl kinase dependent and independent mechanisms. Forced expression of the tumor suppressor
in CML cells reduced leukemic cell growth and motility in vitro and affected malignant cell propagation and leukemogenesis in vivo.
Materials and Methods

DNA constructs

Murine Mtss1 cDNA was amplified using cDNA library no. 968, clone IRAVp968D0272D6 (German Resource Center for Genome Research) as a template and the following primer pair: Mtss1DoEcoRV-AAGATATCCTAAGAGAAGCGCGGTGAGC and Mtss1UpFlagBamHI-AAAGGATCCATGGACTACAAGGACGACGACGATAAGGAGGCTGTGATCGAGAAGGGATGCAGCGCGCTTGGAGG. PCR product and pENTRY1A were digested using BamHI and EcoRV, and positive clones were fully sequenced and shuttled into the pMY destination vector using LR Clonase (Life Technologies).

Cell culture

32Dcl3 (hereafter named 32D) and K562 (ACC-411, ACC-10, DSMZ) cells were cultured as described previously25. Mononuclear (MNC) cells were cultured in serum-free BIT medium (Stem Cell Technologies) in the presence of growth factors (5GF) as described previously 26. All applied cell lines were routinely tested for mycoplasma contamination. The application of primary human samples was approved by the local ethics board of the medical faculty RWTH Aachen (EK127/12, EK206/09) and by the West of Scotland Research Ethics Service (REC reference: 15/WS/0077, expiry 05 JUNE 2020). Informed consent was obtained from all subjects.

Retroviral transduction

Retroviral transductions were performed as described previously25. Briefly, Plat-E-packaging cells were transfected with pMY-IRES-GFP (green fluorescent protein) vectors containing the gene of interest and supernatants were collected after 24h. Virus was centrifuged onto retronectin-coated 6-well plates, and cells were subsequently added and cultured overnight.
Transduction was repeated 2 to 3 times on 2 to 3 consecutive days using serum-free BIT9500 cell culture media (Stem Cell Technologies) that was supplemented with IL3, IL6 and mSCF (10ng/ml, 5ng/ml and 50ng/ml).

**Real-time quantitative RT-PCR (qRT-PCR)**

The process of RNA isolation, cDNA synthesis and qRT-PCR have been previously described\(^2\). Commercially available TaqMan assays (Applied Biosystems) were used for human (HS00207341_m1) or murine Mtss1 (Mm00460614_m1) and murine DNTM3B (Mm01240113_m1) expression analysis. For human DNMT3B, we used FAM(6-Carboxyfluorescein)-labeled probe #21 from a universal probe library (Roche Diagnostics) combined with Primer DNMT3BUp-AGTCTGCTAAGCTACACACAGGAC and DNMT3BDo-AAGAGCTTTGGCATGACTG.

**Migration Assay**

Co-culture of 32D and M2-10B4 cells was performed for 16h and time-resolved video microscopy was subsequently performed for 120min, using a ZEISS microscope Axiovert 40C, linked to a CCD video camera (Hamamatsu). Image acquisition (60s interval for 120min) was controlled by HiPic or WASABI software. Cell contours were segmented with Amira software and these data served as the basis for the quantification of the migratory behavior. Migration was quantified as the movement of the cell center with time. We calculated the cell speed (in \(\mu m/min\)) as a three point difference quotient and the translocation (in \(\mu m\)) as the net distance covered within the experiment as described previously\(^2\). The directionality was derived from the quotient of translocation and total path length.
Mice and genotyping

C3H/HeJ mice were purchased from the Jackson Laboratory. Genotyping of SCLtTA/Bcr-Abl double transgenic (dtg) mice was described previously\textsuperscript{27}. FVB/N CD45.2 recipients were bred in-house. Animal experiments were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, LANUV Az. 84-02.04.2013.A072).

Bone marrow transplantation and cell injection

BM cells were harvested from SCLtTA/BCR-ABL and control mice (SCLtTA stg or wt). Cells were lineage-depleted using magnetic activated cell sorting (MACS, Miltenyi Biotec) and infected as described. For transplantation, these cells were injected along with $1 \times 10^5$ FVB/N CD45.2 spleen cells into irradiated FVB/N CD45.2 mice. Mice were treated with cotrimoxazole (Ratiopharm) until 2 weeks after transplant. For analysis of tumor formation or CML development, 32D Bcr-Abl:ev or 32D Bcr-Abl: Mtss1 cells were injected subcutaneously or intravenously into 3Gy-irradiated or non-irradiated C3H/HeJ mice. Peripheral blood was collected from the retro-orbital plexus.

Flow cytometry analysis

PB, BM, and spleen cells were isolated and analyzed as described previously\textsuperscript{27}. In brief, cells were incubated with CD45.1, CD45.2, and Gr-1 antibodies (BD Biosciences). For LSK cells we used tricolor labeled CD3, CD4, CD8a, B220, Gr-1 (Caltag/Invitrogen), CD11-b, Ter119 (eBioscience) combined with CD117-APC, Sca-1-biotin and anti-streptavidin-PE-Cy7, (BD Biosciences) antibodies. FACS was performed using a FACS Navios (Beckman Coulter).

Methylation analyses

Cells were washed daily and supplemented with 5-aza-2’-deoxycytidine (5-Aza-CdR; Sigma-Aldrich) to a final concentration of 0.2µM for 7 days. For bisulfite sequencing, gDNA was
isolated using QIAamp DNA Blood Mini Kit (Qiagen) and processed using EZ DNA Methylation Kit (Zymo Research Corporation) according to the manufacturers’ protocols. The Mtss1 promoter region was amplified as described, and processed using Blunting Enzyme Mix (NEB Inc.), ligated into EcoRV digested pBluescript-KS(+) and sequenced. For each patient, 9 to 12 single PCR products were analyzed.

Preparation of cell lysates, SDS-PAGE and immunoblotting

Western blotting was performed as previously described and proteins were detected via chemoluminescence (Fusion SL, PeqLab).

Colony formation assay

32D cells were mixed with methylcellulose (MethoCult M3231 or M3434 Stem Cell Technologies) at a density of 200 cells/ml. Colonies were counted 7 days after plating using a light microscope.

Motif Matching

We obtained position frequency matrices from Jaspar. The motif matching procedure was performed using MOODS. The threshold corresponded to a p-value of 0.0005. We post-processed all putative binding sites by removing the matches with bit-score log-odds ratio less than 4, with regard to the corresponding bit-score threshold.

Chromatin immunoprecipitation (ChiP)

ChiP experiments using KCL-22 cells were performed and analyzed as described previously. KAISO (6F8) or REST (H-290) antibody from Santa Cruz Biotechnology and
following Primers were used: MTSS1-for: ACTACAAGCGGGCTCTGG and MTSS1-rev: CCCACCCCTACAATGGCTG.

Statistical analysis
Statistical analyses were performed using Student two-sided t test (normal distribution) or Mann-Whitney U test (when normal distribution was not given). P < 0.05 was considered statistically significant. The exact sample size (n) represents the number of biological triplicates and is given in the respective figure legend or in the Results section of the manuscript. Error bars are given as SEM. Groups with similar variation have been statistically compared.

Animal studies
Statistical analyses of data including animal studies were performed as described above. Blinding or randomization were not applied. Single animals were not excluded from analyses.
Results

Mtss1 is downregulated in murine and human CML stem cells

We previously developed and applied an inducible double transgenic SCLtTA/Bcr-Abl mouse model that closely reflects human CML. Expression profiling using the stem cell enriched LSK population (GSE18446) revealed a significant 2.35-fold downregulation of the potential tumor suppressor gene Mtss1. In order to confirm Mtss1 protein downregulation, we performed Western blot analysis using lineage-depleted BM cells from SCLtTA/Bcr-Abl mice that had been induced to express Bcr-Abl for four weeks (Figure 1a). We previously confirmed Bcr-Abl expression in these cells. Mtss1 protein was completely absent upon Bcr-Abl induction in leukemic lineage-depleted progenitor cells, while 3 of 4 control mice expressed high levels of Mtss1. Moreover, we confirmed significant 5.8-fold Mtss1 RNA downregulation in LSK cells using qRT-PCR in a cohort of mice that had expressed Bcr-Abl for 3 weeks (Figure 1b). Next, we assessed the effect of Bcr-Abl expression on Mtss1 levels in the murine myeloid progenitor cell line 32D. Again, oncogenic expression dramatically reduced Mtss1 expression at RNA (Supplementary Figure S1, left) and protein level (Supplementary Figure S1, right). To study if our data could be translated to human CML, we performed microarray analyses from publicly available datasets. These datasets comprised four Mtss1 probe sets detecting distinct predicted transcripts. First, we checked for tissue specific expression patterns in the Gene Expression Profiler database and out of the four probe sets, Mtss1_203037_s_at showed highest expression in hematopoietic cells, including the CD34+CD38- stem cell compartment. Interestingly, this probe set revealed a significant Mtss1 downregulation in blast cells from newly diagnosed CML patients compared to healthy donor (HD) CD34+ control samples (Figure 1c, Supplementary Figure S2a). Next, we performed Western blot analysis using primary MNCs from CML patients. At the time of diagnosis, Mtss1 protein was absent in all patients (Figure 1d). We then looked at Mtss1 levels in remission samples from two patients: after 9 months of TKI therapy in one patient (CML01) and 12 months of therapy in another patient (CML02). In CML01 and CML02 Bcr-
Abl levels declined from 47% and 32% to undetectable levels by nested PCR and 0.023%, respectively. In both patients, Mtss1 was markedly increased during remission. The same was evident in a third dataset comparing Mtss1 expression at diagnosis (Bcr-Abl expression 77%) and in remission (MR<sup>4</sup> and Bcr-Abl not detectable by nested PCR) from two different CML patients, CML03/04. In line with these data, Mtss1 RNA was also downregulated in CML patients at diagnosis when compared to healthy control samples (Supplementary Figure S2b). In another CML patient (CML05) who did not respond to TKI therapy and developed a T315I mutation, Mtss1 expression was repeatedly absent (Bcr-Abl at diagnosis: 99.61%, +9months: 25.13%). This patient progressed to CML-BC. Absence of Mtss1 protein was also confirmed in a second CML-BC patient (CML06). This patient relapsed after allogeneic transplantation and did not respond to TKI therapy. Subsequently a complex aberrant karyotype was verified in this patient. For samples for which residual RNA was available, we performed qRT-PCR, including normal controls, which confirmed Mtss1 downregulation in these patients (Supplementary Figure S2c).

Mtss1 overexpression affects leukemic cell motility, tumor growth and CML development

To study the potential function of Mtss1 in Bcr-Abl positive hematopoietic cells, we retrovirally infected 32DBcr-Abl cells using FLAG tagged Mtss1 or empty vector (ev) control retrovirus. 32DBcr-Abl:Mtss1 and 32DBcr-Abl:ev control cells showed no growth differences in suspension cell culture (data not shown), but CFU (colony forming unit) capacity was markedly decreased upon Mtss1 overexpression (Figure 2a), in the presence or absence of added cytokines. This decrease in CFU capacity in 32DBcr-Abl:Mtss1 cells was not due to Mtss1 induced differentiation. Even in the presence of 40ng/ml G-CSF for 10 days, 32DBcr-Abl:ev or Mtss1 cells did not exceed 2% or 0.5% Gr-1 positivity respectively (Supplementary Figure S3). As Mtss1 is involved in cell motility, we next analyzed the capacity of these cells to migrate upon co-culture using the BM stromal derived cell line M2-10B4. These stromal
cells had been engineered to overexpress hG-CSF and hIL3. 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 were seeded on a confluent monolayer, and migration was monitored for up to 120 min by means of time-lapse video microscopy. Mtss1 overexpression had a profound impact on cell motility (Figure 2b). 32DBcr-Abl:ev cells moved with high speed (8.9 ± 2.0 µm/min) and directionality (0.58 ± 0.05) across the stromal cell layer. In contrast, Mtss1-overexpressing cells had a dramatically reduced speed and directionality (1.8 ± 0.2 µm/min and 0.12 ± 0.02), respectively. To analyze the effect of Mtss1 expression in vivo, we injected 3x10^5 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells subcutaneously into syngeneic C3H/HeJ mice after total body irradiation using 3 Gy. Mice were sacrificed for analysis 25 days after injection and tumors that resulted from Mtss1 overexpressing cells were 2.2-fold smaller compared to ev controls (Supplementary Figure S4a upper panel). We confirmed persisting Mtss1 overexpression in these tumors via detection of FLAG-tagged Mtss1 protein (Supplementary Figure S4a lower panel). QRT-PCR capable of detecting transduced and endogenous Mtss1 confirmed that expression was high in tumors that resulted from 32DBcr-Abl:Mtss1 injection but low in tumors that developed from 32DBcr-Abl:ev cells (Supplementary Figure S4b). The spleen size of 32DBcr-Abl:Mtss1 injected mice was 1.6-fold decreased compared to controls (Supplementary Figure S4c), but this did not reach statistical significance. In order to study if Mtss1 overexpression can impair leukemic development, we next transplanted freshly transduced 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells that express GFP to allow for leukemic cell tracking. 5x10^5 cells were injected into non-irradiated C3H/HeJ recipients. The disease in this model develops rapidly, and mice were therefore analyzed 11 days after transplantation. Mtss1 overexpression significantly reduced the numbers of leukemic cells in PB by 2.8-fold (Figure 2c). Analysis of BM and spleen confirmed a significant 1.8- and 2.4-fold decrease of leukemic cells in the respective organ, upon Mtss1 overexpression (Figure 2c). Moreover, splenomegaly was reduced in mice that had received Mtss1 positive cells (Figure 2d, left). Using an in vivo imaging system (IVIS), we were able to detect GFP positive leukemic cells in the spleen. These malignant cells were not evenly spread but clustered in focal areas. Interestingly, overexpression of Mtss1
decreased leukemic spleen cell burden (Figure 2d, right). We detected reduced Bcr-Abl expression in BM and spleen of Mtss1 overexpressing cell recipients (Supplementary Figure S5a). Again, this reflected a decrease in leukemic cell burden, as both cell lines had similar Bcr-Abl expression levels upon injection (Supplementary Figure S5b). Histology of the spleen confirmed malignant cell infiltration and this was diminished upon transplantation of Mtss1 overexpressing cells (Supplementary Figure S5c). The decrease in leukemia development was not due to alterations in the capacity of these cells to home to the BM. However, there was a 2-fold decrease of cell homing to the spleen, which was not significant suggesting that Mtss1 overexpression could compromise homing to the vascular niche (Supplementary Figure S6).

**Mtss1 impairs cell growth of leukemic progenitor cells in vivo**

Next, we studied the effect of Mtss1 overexpression using our transgenic SCLtTA/Bcr-Abl mice that lose Mtss1 protein upon Bcr-Abl expression (Figure 1a). Transduction of SCLtTA/Bcr-Abl BM allows for differentiation during culture and thus long-term engraftment of transduced cells is difficult to achieve. Therefore, we used a number of approaches varying radiation dose, cell numbers and transduction methodology. We repetitively retrovirally infected lineage negative (lin-) cells from non-induced SCLtTA/Bcr-Abl and control mice using Mtss1 or ev retrovirus. In vitro CFU assays that were performed in the presence of cytokines revealed a non-significant 20% decrease in clonogenic potential upon Mtss1 overexpression in these cells (data not shown). In a first in vivo experiment, we transplanted 5x10⁴ FACS-sorted Bcr-Abl negative cells that were infected with Mtss1 or ev (control:Mtss1 and control:ev) as well as 8x10⁴ FACS-sorted Bcr-Abl positive cells, infected with Mtss1 or ev (Bcr-Abl:Mtss1 and Bcr-Abl:ev) in 6Gy irradiated recipients that did not receive tetracycline to allow for Bcr-Abl expression in the leukemic cells. PB was drawn 13 days after transplantation and revealed a significant 1.8-fold decrease of GFP positive cells in SCLtTA/Bcr-Abl:Mtss1 transplanted recipients compared to SCLtTA/Bcr-Abl:ev controls.
This decrease was accompanied by a reduction of Bcr-Abl positive Gr1+/GFP+ cells showing that restored Mtss1 expression markedly decreases leukemic cell propagation in vivo and does not enhance differentiation of myeloid cells. Over time we lost all GFP positive cells and to increase the number of infected transplanted transgenic cells, we next avoided sorting and separated donor from wt cells by using CD45.1 (donor) CD45.2 (recipients) strain specific isoform expression (Supplementary Figure S8). Combined with the retroviral vector encoded GFP expression, this allowed for separation of infected transgenic cells (CD45.1+/GFP+) from non-infected transgenic cells (CD45.1+/GFP−) as well as from wt recipient cells (CD45.2+). Transduction efficiency of lin− SCLtTA/Bcr-Abl BM cells using ev was 70.5% and therefore 1.3-fold higher than Mtss1-transduced BM (55.3% transduced cells). We injected 3x10^5 GFP+ cells per recipient that had been irradiated using 8Gy. Again, the total number of GFP+ cells declined in both groups. However, Mtss1 infected Bcr-Abl cells decreased significantly more rapidly than ev transduced Bcr-Abl cells. The difference between ev and Mtss1 was 1.3-fold by the time of transduction and increased to 2.1-fold at day 26 in the PB. Mtss1 overexpression continued to exert a negative effect on leukemic cell growth as Mtss1 overexpressing cells further declined and were 3.6-fold decreased in PB on day 78 (Figure 3a). 82 days after transplantation, we sacrificed the recipients for analysis. There was a significant 3- and 17-fold decrease of Mtss1 overexpressing BM and spleen cells, respectively, as compared to controls (Figure 3b).

Finally, we again infected lin− SCLtTA/Bcr-Abl BM cells using Mtss1 or ev retrovirus and FACS-sorted GFP+ cells. Western blot analysis confirmed Mtss1 overexpression in transduced progenitor cells (Figure 3c). For short-term in vivo analysis, we injected 1.3x10^5 sorted cells into 9Gy-irradiated mice and analyzed these recipients 13 days after transplantation. Although expression of Bcr-Abl did not differ between the groups (Supplementary Figure S9), the percentage of Mtss1 overexpressing cells was significantly diminished in BM, showing a 2.8-fold reduction (Figure 3d), again confirming the adverse effects of restored Mtss1 expression on leukemic progenitor cell propagation. In vitro
analysis showed that apoptosis was not increased in total SCLtTA/Bcr-Abl BM cells upon Mtss1 overexpression (Supplementary Figure S10).

**Mtss1 suppression involves Bcr-Abl kinase activity**

As our data demonstrate a tumor suppressing function for Mtss1, we next analyzed if Bcr-Abl inhibition could completely restore Mtss1 expression levels in vitro and in vivo. Firstly, we treated 32DBcr-Abl cells as well as 32D control cells with imatinib for 18h and this significantly increased Mtss1 levels in Bcr-Abl positive, but not negative cells (Figure 4a). However, treatment could not restore Mtss1 expression to control levels of Bcr-Abl negative 32D cells and this was true despite complete Bcr-Abl kinase inhibition (Supplementary Figure S11). We then tested Bcr-Abl mediated Mtss1 regulation in human CML and applied imatinib treatment to K562 cells and this markedly increased Mtss1 expression (Supplementary Figure S12). Next, we tested Mtss1 expression in primary human MNCs as well as purified CD34 positive cells. For latter one we previously showed complete Bcr-Abl inhibition upon 16h of imatinib treatment in 5GF. Upon imatinib and dasatinib treatment both cell types showed Mtss1 upregulation in response to Bcr-Abl kinase inhibition in 5GF (Supplementary Figure S13, Figure 4b). To analyze the effect of short-term TKI therapy in vivo, we assessed Mtss1 levels in BM cells of CML mice that were treated with imatinib for four weeks (Figure 4c). Transplantation of SCLtTA/Bcr-Abl BM cells in comparison to Bcr-Abl negative control cells reduced Mtss1 levels in BM by 13.9-fold in vehicle-treated animals. Imatinib treatment significantly increased Mtss1 levels in Bcr-Abl positive mice. However, this increase was mild showing a 3.8-fold rise of transcript level and these mice still had a 3.2-fold Mtss1 reduction compared to Bcr-Abl negative control animals. We then analyzed Mtss1 expression in SCLtTA/Bcr-Abl mice that had been reverted for Bcr-Abl expression (Figure 4d) and this confirmed a trend of persisting Mtss1 downregulation upon inhibition of Bcr-Abl kinase activity for 48 days. These data suggest that Mtss1 suppression might not depend
exclusively on Bcr-Abl kinase activity and is not mediated via Bcr-Abl kinase independent mechanisms of the oncogenic protein.

Mtss1 downregulation is mediated by epigenetic mechanisms

A mechanism that has been described to induce Mtss1 downregulation in cancer is DNA promoter methylation\textsuperscript{29, 38}. Moreover, the de novo methyltransferase DNMT3B has recently been shown to be involved in Mtss1 suppression\textsuperscript{14}. We therefore analyzed the potential role of Mtss1 promoter methylation in CML. Treatment with the demethylating agent 5-Aza-CdR increased Mtss1 expression by 3.1-fold in 32D-Bcr-Abl, but only 1.5-fold in parental 32D cells, and 12.1-fold in K562 cells (Figure 5a). Interestingly, TKI treatment decreased DNMT3B RNA expression in 32D-Bcr-Abl and K562 cells and simultaneously increased Mtss1 levels, as detected by qRT-PCR (Supplementary Figure S14, Figure 5b). Bisulfite sequencing of a previously defined Mtss1 promoter region\textsuperscript{29} confirmed excessive promoter methylation in K562 cells showing 100% methylation in 24 out of 29 CpG sites and this was reduced to 8 CpG sites in imatinib and 14 CpG sites in 5-Aza-CdR treated cells (Supplementary Figure S15). Therefore, we next analyzed Mtss1 promoter methylation using primary CML cells (n=6) versus normal (n=7). In line with a previous report showing excessive hypermethylation in cancer cell lines as compared to the primary human malignancy\textsuperscript{39}, Mtss1 promoter methylation was increased in the CML cell line. Primary CML samples demonstrated a significant increase in Mtss1 promoter methylation at specific CpG sites compared to healthy controls (Figure 5c). Sequence based motif analysis indicated two motifs, REST (Re1-silencing transcription factor) and ZBTB33 (zinc finger and BTB domain containing 33, also termed KAISO) that match to the region with the highest difference between the methylation levels of CML and control and both are known to act as transcriptional repressors. Interestingly, we could confirm binding of both transcription factors to this Mtss1 promoter region, using ChiP experiments and the human CML cell line KCL-22 (Figure 5d).
Discussion

Reactivation of tumor suppressors in CML has previously been shown to strongly affect malignant cell biology and disease progression\(^\text{11, 40-42}\). Here, we identified Mtss1 to be downregulated in murine and human leukemic progenitor and stem cells and forced expression markedly decreases CML cell motility and disease development in vivo. Mtss1 suppression involves DNA promoter methylation in primary CML-CP samples and TKI therapy in SCLTA/Bcr-Abl mice only partially restores Mtss1 levels.

Mtss1 has a multidomain protein structure that suggests a cytoskeletal associated function\(^\text{20, 21}\), and both overexpression and knock down of Mtss1 globally impairs cellular architecture, resulting in altered cell motility, adhesion, and/or proliferation in normal and malignant cells\(^\text{22-24}\). Mtss1 function appears to depend on the cell type or context as it acts as a tumor suppressor\(^\text{14, 43, 44}\) but also exerts oncogenic effects\(^\text{45}\).

Our data reveal that, while Mtss1 expression did not affect proliferation in pure suspension cell cultures, overexpression markedly decreased the clonogenic potential and cell motility of CML cells. Moreover, Mtss1 overexpression reduced leukemic progenitor cell growth and CML development in vivo. Decreased cell motility and reduced in vivo proliferation upon Mtss1 overexpression have also been described in a breast cancer mouse model\(^\text{46}\). In hepatocellular carcinoma (HCC), Mtss1 overexpression decreased the proliferation and clonogenic potential, while Mtss1 knockdown increased tumor formation in vivo\(^\text{14}\). The tumor suppressive function of Mtss1 involves an altered bidirectional communication between the cell and its tumor niche. Using a mouse model of targeted Mtss1 disruption, Yu et al. demonstrated a predisposition for lymphoma development in aging mice\(^\text{43}\). These mice showed an altered B-cell differentiation potential and a pathologic communication between B-cells and their microenvironment, with Mtss1 knockout cells failing to internalize the chemokine receptor CXCR5 upon exposure to its ligand, CXCL13, a chemokine released from splenic stromal cells\(^\text{43}\). CML cells are characterized by a premature release into the blood and this can be mediated via altered chemotaxis, e.g. via CXCR4 expression, or altered cell interaction with extracellular matrix proteins involving integrins, selectins or...
cadherins. In CML, adhesion mediated suppression of proliferation is impaired and taken together it is conceivable if not likely that Mtss1 overexpression inhibits colony growth, cellular migration and subcutaneous tumor growth in response to cell-cell or cell-matrix interaction. However, the mechanism of how Mtss1 expression increases adhesion and reduces growth of malignant cells remains to be clarified.

In a breast cancer mouse model, Mtss1 overexpression impaired cell migration and reduced metastasis via inactivation of the small GTPase RhoA. Interestingly, RhoA is activated in Bcr-Abl positive cells and mediates transformation in vivo. GTPases have been shown to regulate homing and mobility of normal and malignant HSC. In an in vitro model of breast cancer, Mtss1 loss enhanced cell invasion, and this involved upregulation of the tyrosine phosphatase PTP' and subsequent Src activation by dephosphorylating an inhibitory Src-kinase phosphotyrosine residue. Interestingly, phosphorylation of Src-kinase Lyn at the activating phosphotyrosine residue facilitates the imatinib-induced migration of CML cells to the BM.

As forced Mtss1 expression reduced the leukemic potential of primitive CML cells, as demonstrated in this study, it would be extremely interesting to investigate the therapeutic potential of preventing or reversing Mtss1 suppression in combination with TKI therapy in the future. Mtss1 has previously been described to be activated by Sonic Hedgehog (Shh) signalling and can subsequently associate with Gli transcription factors to potentiate Shh signalling. Our own confocal microscopy data revealed that Mtss1 localization was restricted to the cytoplasm (Supplementary Figure S16) suggesting that Mtss1 does not associate with Gli in our model. Bcr-Abl inhibition did not increase expression of the Shh target gene Gli1 in K562 cells (data not shown) suggesting that TKI induced upregulation of Mtss1 did not enhance Shh signalling. However, conversely we did observe that IM-induced upregulation of Mtss1 expression was impaired by simultaneous Shh inhibition (Supplementary Figure 17) which implies that Shh signalling is involved in Mtss1 gene regulation. The mechanisms that induce inactivation of tumor suppressors in CML vary and include binding of inhibitory proteins and/or enhanced protein degradation as well as
downregulation by genetic or epigenetic mechanisms\textsuperscript{41}. DNA promoter methylation of tumor suppressor genes has been described to occur upon CML progression\textsuperscript{11-13}, but it also already exists in CML-CP. Interestingly, the adhesion promoting molecule Cadherin 13 has been shown to be downregulated via DNA promoter methylation in CML-CP and this correlated with poor response to treatment\textsuperscript{66}. Our data reveal DNA methylation of the Mtss1 promoter in CML-CP patients at the time of diagnosis. It has been described that DNMT3B is responsible for Mtss1 suppression in HCC\textsuperscript{14}, and here we show that DNMT3B expression was inversely correlated with Mtss1 expression in treatment-naïve cells as well as after TKI treatment, suggesting that DNMT3B is involved in Mtss1 downregulation in CML cells. Likewise, suppression of Mtss1 by DNA methylation has also been described for bladder and gastric cancer\textsuperscript{29, 38}. Along the same line, we previously demonstrated Mtss1 suppression in FLT3-ITD positive AML and low Mtss1 expression correlated with high DNMT3B levels in primary AML samples, suggesting that Mtss1 suppression could be a common mechanism in oncogenic tyrosine kinase induced leukemia\textsuperscript{19}.

In a phase II clinical trial, 63% of CML-CP patients had objective responses to 5-Aza-CdR (decitabine) demethylating therapy, as well as 55% in CML-AP and 28% in CML-BC\textsuperscript{57}. The potency of demethylating agents varies and in line with this clinical trial, we used 5-Aza-CdR for our studies that has previously been shown to be more potent than 5-Azacytidine\textsuperscript{58}. Clinical trials elucidating the role of TKI therapy combined with decitabine have been performed for imatinib-refractory CML-AP and CML-BC but did not show improved responses as compared to decitabine alone\textsuperscript{59}. Our data demonstrate methylation of the Mtss1 promoter already in CML-CP patients providing a rationale to further explore the therapeutic benefit of demethylation agents combined with TKI therapy in early phase CML.

In addition to decitabine, exploration of more specific DNMT3B inhibitors, such as nanaomycin A, is in progress\textsuperscript{60}. Nanaomycin A is an antibiotic belonging to the antracycline group and similar compounds such as doxorubicin or daunomycin have been shown to exert powerful antitumor activity.
The exact mechanism on how Mtss1 promoter methylation results in decreased expression needs to be clarified. However, we identified increased methylation in specific CpG sites in CML, and these CpG sites harbor transcription factor binding sites for KAISO and REST, two transcriptional repressors. Like BCL-6 or PLZF, KAISO is a member of POZ-ZF transcription factors that are involved in the process of cancer development and CML stem cell survival. Interestingly, KAISO preferentially binds to methylated DNA to enable recruitment of the histone deacetylase-containing nuclear corepressor complex (NCoR) that enhances chromatin condensation and therefore gene silencing. In CML, KAISO expression has solely been described in the context of a CML-BC cell line model, and here, KAISO inactivation reduced granulocytic differentiation by suppression of CEBPalpha. Despite the described biochemical characteristics of the Mtss1 protein, its role in different tissues is only poorly understood. Here, we demonstrate that Mtss1 functions as a tumor suppressor in CML, providing a rationale for enhancing Mtss1 expression in CML in order to target the TKI resistant stem cell population.
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Authorship contributions

MS, AS, IGC, CMT, SL, TLH, THB and SK designed research. MS, OH, MMR, NC, NK, CS, SH, EGG, SKi, TB, AH, VH and MC performed research. MS, OH, IGC, AS and SK analyzed data. MS, TLH, THB and SK wrote the manuscript.

Supplementary information is available at Leukemia´s website


Figure Legends

Figure 1: Mtss1 is suppressed in CML stem cells. a) Lineage negative BM cells from control mice and from SCLtTA/Bcr-Abl that had been induced to express Bcr-Abl for four weeks were isolated using magnetic bead cell sorting. Protein lysates from these cells were analyzed for Mtss1 by Western blot (n= 4/4). b) LSK cells were isolated via FACS from induced SCLtTA/Bcr-Abl and control mice. Expression of Mtss1 was assessed by qRT-PCR and is shown as percent of GAP-DH (n=3/3). c) RNA expression of Mtss1 in normal healthy donor (HD) CD34 positive cells (n=74) and CML blasts in chronic phase (n=76; GSE#4170). d) Mtss1 levels determined by Western blot in CML-CP patients’ blood mononuclear cells at diagnosis and in complete hematologic remission (upper panel) versus CML-BC (lower panel).

Figure 2: Mtss1 overexpression affects leukemic cell motility, solid tumor growth and CML development. a) 200 cells were seeded in methylcellulose containing cytokines (IL3, IL6 and mSCF) or no cytokines. Numbers of CFU were counted after 7 days of culture (n=3/3). b) Migration of individual cells was tracked for up to 120min (upper panel) and shown are the mean values of speed (lower left) and directionality (lower right) from individual cells each in three independent experiments. Cell motility was analyzed using Amira® software, n 32DBcr-Abl:ev=27, n 32DBcr-Abl:Mtss1=31. c) C3H/HeJ mice were transplanted using 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells (n=5/5). PB, BM and spleen cells were subjected to flow cytometry analysis 11 days after transplantation. d) Spleen size of 32DBcr-Abl:Mtss1 and 32DBcr-Abl:ev transplanted recipients (left) and leukemic cell burden analysis in spleen by IVIS imagine systems detecting fluorescing (GFP positive) leukemic cells (right). *p<0.05, ***p<0.001

Figure 3: Mtss1 impairs leukemic progenitor cell growth in vivo. a) PB was analyzed by flow cytometry for GFP positive cells 26 and 78 days after transplantation of 3x10^5 GFP/CD45.1^+ cells that had been transduced to express Mtss1 or ev control (n=8/8). b) BM and spleen cells were analyzed at autopsy 82 days after transplantation. c) Expression of
Mtss1 was confirmed by Western blot using protein lysates from infected, sorted lineage negative BM cells. d) Percent of GFP positive cells was analyzed in BM and spleen 13 days after transplantation, in recipients of 1.3x10^5 FACS-sorted SCLtTA/Bcr-Abl:ev or SCLtTA/Bcr-Abl:Mtss1 cells (n=3/4). *p<0.05, **p<0.01

**Figure 4: Suppression of Mtss1 is mediated via Bcr-Abl activity.** a) 32D and 32DBcr-Abl cells were subjected to imatinib (IM 5µM) treatment for 18h, and Mtss1 expression was assessed by qRT-PCR (n=3/3). b) CD34 positive cells from CML patients at diagnosis (n=3) were treated with imatinib (5µM, n=3) or dasatinib (Da 150nM, n=3) for 24h. Mtss1 levels were assessed by qRT-PCR. c) Recipients of SCLtTA/Bcr-Abl or control BM cells were treated with imatinib or vehicle control for four weeks and Mtss1 expression was assessed in isolated BM cells (n=3/3). d) Expression of Mtss1 in SCLtTA/Bcr-Abl and control mice upon 25 days of induction (n=3/3) and after reversion (n=4/4) of Bcr-Abl expression for 48 days. *p<0.05; **p<0.01

**Figure 5: Mtss1 is downregulated via DNA promoter methylation.** a) 32D, 32DBcr-Abl (B/A) and K562 cells were subjected to 0.2µM 5-aza-2'-deoxycytidine (5-Aza-CdR) treatment and Mtss1 expression was determined at the indicated time points (n=3/3). b) Mtss1 and DNMT3B expression were analyzed in imatinib treated K562 cells (18h, 5µM) by qRT-PCR and are shown as % of GAP-DH (n=3/3). c) Genomic DNA isolated from PB-derived cells of six CML patients at diagnosis and seven healthy controls was isolated and analysed for Mtss1 promoter methylation using bisulfite sequencing (n=6/7). d) ChIP for KAISO and Rest binding to the Mtss1 promoter region was performed using KCL-22 cell line (n=3/3). *p<0.05; **p<0.01; ***p<0.001
Expression of Mtss1 in Bcr-Abl positive versus negative 32D cells. 32D empty vector (ev) and 32DBcr-Abl cells were analyzed for Mtss1 expression using qRT-PCR (left, n=3/3) and Western blot (right).
Expression of Mtss1 in primary patient material. a) Microarray analyses for Mtss1 expression in CD34+ healthy donor (HD) versus CML blasts at diagnosis. Mtss1 downregulation was evident using probe set 203036_s_at, reflecting a transcript that is predicted to be expressed in a subset of B-cells. Transcripts 210359_s_at and 210360_s_at are predicted to be absent or expressed at low levels in CD34+CD38- cells. b) Expression of Mtss1 was analyzed in MNCs of healthy donor (HD) vs CML at diagnosis by qRT-PCR. c) Analysis was performed by qRT-PCR from red cell lysed PB and Mtss1 expression is shown as percent of GAP-DH. ***p<0.001
32DBcrAbl:ev and 32DBcr-Abl:Mtas1 cells were cultured at a density of 2x10^5 to 4x10^5 cells/ml in BIT9500 (Stem Cell Technologies) medium in the presence (+) or absence (-) of 40ng/ml G-CSF (Immunotools). Analysis was performed by FACS for Gr1-expression (Ly-6G/Ly-6C, PE-Cy5, Biolegend) at the indicated time points. (n=3/3), *p<0.05
32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells were injected subcutaneously into C3H/HeJ mice (n=4/4) and induced tumor formation at the injection site. a) Tumor weight was measured upon autopsy 25 days after cell injection (upper panel) and protein lysates were isolated from tumors, to determine expression of Bcr-Abl, Mtss1, β-Actin and GFP by immunoblotting (lower panel) b) Expression analysis of Mtss1 in tumors was performed by qRT-PCR and Mtss1 expression is shown as percent of GAP-DH. c) Spleen weight of C3H/HeJ recipients transplanted with 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells. * p<0.05, **p<0.001
C3H/HeJ mice were transplanted using 32DBcr-Abl:ev or 32DBcr-Abl:Mtss1 cells. a) Expression of Bcr-Abl was analyzed in BM and spleen by qRT-PCR and is shown as percent of GAP-DH. b) Expression of Bcr-Abl and Mtss1 in transplanted cell lines before injection was performed by qRT-PCR and is presented as % of GAP-DH. c) Histology of spleens was performed using hematoxylin and eosin (HE) staining *p<0.05, **p<0.001; ns=not significant
Homing assay was performed by intravenous injection of 7.5×10^5 32DBcr-Abl^ev and 32DBcr-Abl:Mtss1 cells into syngeneic C3H/HeJ mice that had been irradiated using 8 Gy, 2 days prior to cell injection. Percent of leukemic cells in BM and spleen were analyzed by FACS for GFP^+ cells 20h after injection and are shown as percent of total living. (n=6/6)
Mtss1 overexpression decreases leukemic cells in peripheral blood (PB). Lineage depleted single transgenic Bcr-Abl negative control BM cells and SCLtTA/Bcr-Abl cells were infected using empty vector (ctrl:ev and Bcr-Abl:ev) or Mtss1 retrovirus (ctrl:Mtss1 and Bcr-Abl:Mtss1) and transplanted into wt recipients. PB was drawn 13 days after transplantation and analyzed by FACS for GFP+ and Gr1/GFP+ cells after AKC lysis. ***p<0.001; *p<0.05
Experimental design: SCLtTA/Bcr-Abl BM cells expressing CD45.1 isoform were lineage depleted and infected using Mtss1 (Bcr-Abl/Mtss1) or ev (Bcr-Abl/ev) retrovirus. Retroviral vector encoded GFP expression allowed for distinction of infected from non-infected cells. FVB/N mice expressing the CD45.2 isoform were used as recipients. Unsorted cells were transplanted by tail vein injection.
Expression of Bcr-Abl in recipients of 1.3x10^5 SCLtTA/Bcr-Abl;ev or SCLtTA/Bcr-Abl;Mtss1 cells. RNA was isolated upon autopsy and subsequently analyzed by qRT-PCR.
SCLiT/A Bcr-Abl BM cells were infected using Mtss1 or empty vector (ev) retrovirus and transduced cells were isolated via FACS-sorting. These cells were then cultured using serum-free BIT9500 (IL-3 10ng/ml, IL-6 5ng/ml and mSCF 50ng/ml) for 96 h and subsequently analysed for apoptotic and necrotic cells by Annexin-APC, 7AAD FACS staining (n=4 each group).
32D, 32DBcrAbl and K562 cells were cultured for 16h using RPMI + 10% FCS in the presence of indicated imatinib (IM) concentrations. Western blot was performed using antibodies that allow for detection of p-cAbl (Y412, 247C7, Cell Signaling), pSTAT5 (Y694, 9351S, Cell Signaling), STAT5 (C-17, Santa Cruz) and β-Aktin (R-22, Santa Cruz).
K562 cells were subjected to imatinib (IM 5μM) treatment for 18h, and Mtss1 expression was assessed by qRT-PCR, n=3/3, *p<0.05
MNCs cells from CML patients at diagnosis (n=3) were treated with imatinib (5μM) or dasatinib (Da 150nM) for 24h. Mtss1 levels were assessed by qRT-PCR. *p<0.05
Mtss1 and DNMT3B expression were analyzed in imatinib treated 32D and 32DBcr-Abl cells (18h, 5μM) by qRT-PCR and are shown as % of GAP-DH. **p<0.01
Bisulfite sequencing of the Mtss1 promoter in K562 cells that had been treated with 5-Aza-CdR (0.2μM, 7 days) or imatinib (IM, 5μM, 18h). PCR was performed using bisulfite treated gDNA. PCR product was ligated into EcoRV digested pBluescript-KS(+) after blunting. Positive clones were subsequently sequenced. Each square represents a single Mtss1 promoter CpG site in untreated controls (n=4), imatinib (n=5) or 5-Aza-CdR (n=6) treated samples.
The subcellular localization of MTSS1 was analysed in HEK T-Rex BCR-ABL cells stably expressing MTSS1-flag. These cells were grown on polylysine-coated glass cover slips and BCR-ABL expression was induced by doxycycline (10 ng/ml) 10 h before fixation with 3.5 % PFA for 20 min. Fixation of cells and immunostaining has been described previously (Vogt M et al. J Cell Sci 2011). Primary antibodies were diluted 1:100 (c-Abl, sc-131; flag, Sigma-Aldrich), secondary Alexa Fluor-405- and Alexa Fluor-555- (Life Technologies, Paisley, UK) conjugated antibodies 1:1000 and applied for 45 min. DRAQ5 (Biostatus, Leicestershire, UK) was added to the secondary antibody. The cover slips were mounted with ImmuMount (Thermo Scientific, Pittsburgh, PA, USA). Images were generated with a Zeiss LSM 710 confocal microscope (Zeiss, Jena, Germany). The cells were examined with a Zeiss LD C-apolochromat 40 x /1.1 water objective. Confocal images represent confocal slices of ~1 μm and were analysed with the ZEN 2012 software (Zeiss, Jena, Germany). DIC, differential interference contrast. Scale bars, 10 μm.
K562 cells were treated with IM or the Smoothened antagonist LDE225 for 16h. Expression of Mtss1 was determined by qRT-PCR and is shown as percent of GAP-DH. (n=3/3), *p<0.05 ***p<0.001