Diplomarbeit

Untersuchung von AML-spezifischen *TP53* Mutationen in mesenchymalen Stromazellen des Knochenmarks in hypoxischer *ex vivo* Zellkultur

eingereicht von

Dipl. Biochem. Marian Müller

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unter der Anleitung von

Univ.-Prof. Dr. Heinz Sill
Assoz.-Prof. Dr. Andelko Hrzenjak

Graz, 04.12.2019

Eidesstattliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig und unabhängig sowie ohne fremde Hilfe verfasst habe. Ich habe keine anderen als die angegebenen Quellen verwendet und ich habe die verwendeten Quellen entweder wörtlich oder inhaltlich als solche kenntlich gemacht. In dieser gesamten Arbeit und in allen zugehörigen Publikationen habe ich die Richtlinien von „Good Scientific Practice" beachtet.

Graz, 04.12.2019 *Marian Müller eh*

Publikationen, die aus der Tätigkeit an der Klin. Abteilung für Hämatologie/ Univ.-Klinik für Innere Medizin resultierten:

Zebisch A, Lal R, **Müller M**, Lind K, Kashofer K, Girschikofsky M, Fuchs D, Wölfler A, Geigl JB, Sill H. Acute myeloid leukemia with *TP53* germ line mutations. **Blood** 2016; 128(18):2270-2272.

Müller M, Graf R, Kashofer K, Macher S, Wölfler A, Zebisch A, Hrzenjak A, Heitzer E, Sill H. detection of AML-specific *TP53* mutations in bone marrow-derived mesenchymal stromal cells cultured under hypoxic conditions. **Ann Hematol** 2019;98(8):2019-20. doi: 10.1007/s00277-019-03680-4.

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Abstract

Acute myeloid leukaemia (AML) is a heterogeneous, aggressive malignancy affecting mostly elderly patients. The disease is characterised by malignant transformation of hematopoietic stem and progenitor cells (HSPCs) leading to a block in their differentiation capacity. Malignant transformation of single HSPCs is caused by a multitude of genetic as well as epigenetic alterations forming the basis of AML subentities like acute promyelocytic leukemia, AML with *NPM1* or *TP53* mutations. Over the last years, the role of the bone marrow microenvironment in AML has increasingly been elucidated with respect to both, pathogenesis and therapeutic resistance. In the following thesis, we focused on genetic analysis of mesenchymal stromal cells (MSCs), an essential part of the bone marrow microenvironment, in patients with *TP53* mutated AML - a subentity showing an exceedingly poor outcome with 3-year survival rates of <10%.

Diagnostic bone marrow specimens from a total of 14 patients with *TP53* mutated AML were analyzed – 13 with somatic mutations and 1 with a germline mutation serving as control specimen. These specimens were characterized cytogenetically and by targeted deep sequencing of a panel of 39 myeloid-associated genes. *Ex vivo* cultures of mononuclear bone marrow cells were performed under low oxygen conditions with the addition of human platelet lysates. Adherent cells representing MSCs were further subjected to FACS sorting to obtain pure cell populations. Using error-corrected, high-resolution next-generation sequencing, patient-specific *TP53* and cooperating mutations were analyzed.

The bone marrow specimens showed a complex karyotype in 12/14 (86%) cases, whereas cooperating mutations were rare (median, 1; range, 0-3). MSCs were cultivated up to 4 passages and their adipogenic, chondrogenic, and osteogenic differentiation capacity was demonstrated. In purified MSCs, no cooperating gene mutations were detectable. However, the leukemia-specific *TP53* mutation was detected in 2/13 specimens at low variant allele frequencies (VAFs) (0.25 and 0.1%, respectively) and confirmed using biological replication. As expected, MSCs with the germ-line *TP53* mutation showed a VAF of 47.1%.

The data presented here further confirm that *TP53* mutations are early events in acute myeloid leukemogenesis, possibly having their origin in a common mesodermal ancestral cell. Clinically, they may also have consequences as MSCs generated from AML bone marrows are increasingly used as a source of constitutional material.

Zusammenfassung

Akute myeloische Leukämie

Die akute myeloische Leukämie (AML) ist eine heterogene neoplastische Erkrankung mit altersabhängiger Inzidenz, die durch Transformation von hämatopoetischen Stamm- und Vorläuferzellen (HSPC) gekennzeichnet ist. Die ungehemmte Proliferation dieser transformierten Leukämiezellen führt zu Suppression der normalen Hämatopoese mit entsprechendem numerischem Defizit an Leukozyten, Erythrozyten und Thrombozyten, das sich im peripheren Blut als Panzytopenie unterschiedlichen Ausmaßes widerspiegelt.

Die maligne Transformation einzelner HPSCs wird dabei durch genetische Veränderungen verursacht. Ein Kennzeichen von AML ist, dass sie sowohl ohne assoziierte Vorerkrankungen als *de novo* AML, als sekundäre AML (s-AML), d.h. durch Fortschreiten einer myeloproliferativen oder myelodysplastischen Erkrankung oder als Therapie-assoziierte AML (t-AML) nach Chemo- und / oder Radiotherapie früherer Malignome auftreten kann. Gemäß aktuellen Richtlinien der Weltgesundheitsorganisation ist die Diagnose der AML durch ≥20% Blasten im Knochenmark oder peripheren Blut definiert (1, 2).

Ca. 85% aller akuten Leukämien bei Erwachsenen und 17,5% aller akuten Leukämien bei Kindern sind AML. Die Inzidenz der AML beträgt 3-4 neue Fälle/ 100.000 Einwohner/ Jahr, das mediane Erkrankungsalter liegt zwischen 63 und 67 Jahren (3). Zwar ist die genaue Ätiologie der AML unbekannt, jedoch werden verschiedene Ursachen wie exogene Noxen und erbliche Faktoren mit der AML in Verbindung gebracht (3).

Der Karyotyp der leukämischen Blasten stellt den wichtigsten Prognoseparameter auf das Therapieansprechen und Überleben dar. 45% der AML-Patienten besitzen einen normalen Karyotyp, während 55% unterschiedliche chromosomale Aberrationen aufweisen. Als weitere Prognosefaktoren sind somatische Mutationen in spezifischen Genen relevant. So finden sich in bis zu 20% klonale *TP53* Mutationen, welche mit einem komplexen Karyotyp assoziiert sind. Diese Patienten und Patientinnen weisen trotz allogener Stammzelltransplantation eine schlechte Prognose mit 3-Jahres-Gesamtüberleben von <10% auf (4, 5).

Das Tumorsuppressor-Gen *TP53* bei AML

Im menschlichen Genom befindet sich das *TP53* Gen auf dem kurzen Arm des Chromosoms 17 (17p13.1). Das Gen umfasst 20 kb und 11 Exons mit einem nichtkodierenden Exon 1 und einem sehr langen ersten Intron von 10 kb. Die kodierende Sequenz enthält fünf funktionelle Regionen, vorwiegend in den Exons 2, 5, 6, 7 und 8, die eine hohe Konservierung zwischen verschiedenen Spezies von Vertebraten zeigen. Das Tumorsupressor-Gen *TP53* spielt eine Schlüsselrolle bei der Reaktion der Zelle auf erlittene DNA-Schäden durch Einleitung von Zellzyklusarretierung und DNA-Reparaturprozessen bzw. den Übertritt der Zelle in die Seneszenz und Apoptose bei nicht reparablen Alterationen.

Während Keimbahnmutationen im *TP53* Gen ein essentielles Charakteristikum für das autosomal-dominant vererbte Li-Fraumeni-Syndrom (LF) und Li-Fraumeni-like (LFL) Syndrom darstellen, konnten bisher in mehr als der Hälfte von humanen, malignen Erkrankungen somatisch erworbene *TP53* Mutationen nachgewiesen werden. In den vergangenen Jahren wurde das Modell der klonalen Expansion von präleukämischen Stammzellen (preLSC) postuliert, deren Transformation von normalen HSPC über preLSC hin zu Leukämiezellen über sogenannte "Treibermutationen" gesteuert wird. Wie vorhergehende Arbeiten an der Klinischen Abteilung für Hämatologie Graz aufzeigen konnten, stellen somatische *TP53*-Mutationen initiale leukämogene Ereignisse dar (6).

Hämatopoetische Stammzellnische des Knochenmarks

Im Jahr 1978 definierte Schofield die hämatopoetische Stammzellnische als Region im Knochenmark (KM), welche eine wichtige spezialisierte Mikroumgebung für die Hämatopoese darstellt (7). Diese Knochenmarknische besteht aus zwei verschiedenen Stammzellpopulationen – den hämatopoetischen Stammzellen und den nicht-hämatopoetischen Stammzellen, zu denen auch die mesenchymalen Stamm- / Stromazellen gerechnet werden (MSC) (8, 9). Das Konzept der KM-Nische und ihre Rolle für die Hämatopoese wurde über die Jahre weiterverfolgt und es konnte gezeigt werden, dass die von MSC beeinflusste lokale Gewebeumgebung die wesentlichen Faktoren für die HSPC-Repopulation und Differenzierung liefert (10, 11).

Mesenchymale Stamm-/Stroma Zellen (MSC)

MSC stellen eine der zellulären Hauptkomponenten der Mikroumgebung des Knochenmarks dar. In jüngsten Studien konnte von mehreren Forschungsgruppen gezeigt werden, dass MSC über die Bildung von Zytokinen sowie direkten Zell-Zell-Kontakt die hämatopoetische Knochenmarknische modulieren und hierüber einen Einfluss auf die Hämatopoese sowie Proliferation und Therapieresistenz leukämischer Zellen ausüben (11, 12). Der therapeutische Wert von humanen MSC des Knochenmarks ist jedoch durch deren geringe Anzahl von 10 bis 100 „Kolonie-formierenden Einheiten – Fibroblasten“ (CFU-F) pro 10 Millionen mononukleärer Zellen sowie einer niedrigen Proliferationsrate und einer eingeschränkten Lebensdauer während der *ex-vivo* Expansionskultur eingeschränkt (12, 13). Als mögliche Ursache für die beobachteten Effekte der *ex-vivo* Zellkultur von MSC wird angeführt, dass diese häufig bei Luftsauerstoffgehalten von 21% durchgeführt wird und nicht den physiologischen Bedingungen der Knochenmarknische mit einem Sauerstoffgradienten von pO_2 1%-7% entspricht (8, 12). Verschiedene Gruppen haben in den letzten zehn Jahren die Wirkung eines niedrigen Sauerstoffgehalts von 2.5% bis 5% pO_2 auf MSC aus Nabelschnurblut und Knochenmark in der Zellkultur untersucht. Für MSC von Patienten und Patientinnen, welche keine hämatopoetischen Erkrankungen aufweisen, wird ein positiver Einfluss eines reduzierten Sauerstoffpartialdrucks auf *ex-vivo* Zellkulturen von MSC diskutiert (14–18).

Hypothese und Methodik

Wie beschrieben, weisen AML-Patienten und Patientinnen mit klonalen *TP53* Mutationen eine sehr schlechte Prognose mit einem 3-Jahres-Gesamtüberleben von <10% auf. In vorangehenden Arbeiten konnte gezeigt werden, dass *TP53* Mutationen initiale, leukämogene Ereignisse in der Pathogenese der AML mit *TP53* Mutationen darstellen, welche preLSC charakterisieren (6).

Überdies ist bekannt, dass zwischen KM-Stammzellen und Stroma enge Interaktionen bestehen, die sowohl für die normale wie auch maligne Hämatopoese von essenzieller Bedeutung sind. In der Literatur wird auch beschrieben, dass MSCs von Patienten und Patientinnen mit myeloproliferativen Erkrankungen unter normoxischen Kulturbedingungen reduzierte *in vitro* Proliferations- und Differenzierungsfähigkeiten zeigen (14). Offene Frage bestehen jedoch dahingehend, inwieweit MSC AML-assoziierte somatische Mutationen aufweisen und inwieweit sich MSC und hämatopoetische Zellen gemeinsame somatische AML-spezifische Mutationen teilen und ihren Ursprung in einer für hämatopoetische und mesenchymale Zellen gemeinsamen Vorläuferzelle haben, wie es bereits für somatische *KIT* D816V Mutationen bei indolenter systemischer Mastozytose gezeigt wurde (19).

Das Kernziel dieser Studie war daher die Untersuchung von MSC des Knochenmarks von AML-Patienten und Patientinnen auf das Vorhandensein von zuvor in hämatopoetischen Zellen nachgewiesenen somatischen *TP53*-Mutationen. Hierdurch sollte unsere Ausgangshypothese bestätigt werden, dass HSPC bzw. preLSC und MSC des Knochenmarks einer gemeinsamen Ursprungszelle entstammen und mögliche initiale AML-assoziierte somatische *TP53*-Mutationen in einem frühen Stadium der Embryogenese auftreten. Zusätzlich zur Analyse der Existenz somatischer *TP53* Mutationen in MSC des Knochenmarks wurden die MSC Proben der AML Patientinnen und Patienten auf die Existenz von weiteren spezifischen kooperierenden Mutationen hin analysiert.

Es wurden pseudonymisierte Patientenproben beiderlei Geschlechts verwendet, welche aus Knochenmarkspunktionen gewonnen wurden. Die Zusammenfassung der klinischen Daten, die *ex-vivo* Kultur der AML Proben zur Generierung von MSC, deren Differenzierung sowie die DNA Extraktion zur genetischen Analyse wurde meinerseits eigenständig unter Supervision durchgeführt. Die humangenetische Analyse von 14 AML Proben mittels des „High-resolution Safe-Sequencing System“ erfolgte in Kooperation mit

dem Humangenetischen Institut der Medizinischen Universität Graz. Eine AML-Patientin mit Li-Fraumeni-Syndrom und *TP53* Keimbahnmutation diente uns als Positivkontrolle.

Ergebnisse und Diskussion

Patientenspezifische *TP53* Mutationen sowie kooperierende Mutationen wurden sowohl in hämatopoetischen Zellen als auch in aufgereinigten MSC mittels des "High-resolution Safe-Sequencing System" in Kooperation untersucht. Dabei wurden in den AML-Proben somatische *TP53* und kooperierende Mutationen bei varianten Allelfrequenzen (VAF) zwischen 1,5% und 91,2% gefunden. Demgegenüber gelang uns der Nachweis von Leukämie-spezifischen *TP53* Mutation in aufgereinigten MSC des Knochenmarks bei zwei von 13 Patienten und Patientinnen (14%) mit einer VAF von 0,25% und 0.1%. Unter Verwendung von biologischen Replikaten wurden diese Ergebnisse bestätigt. In keiner der von uns analysierten Proben wurden Leukämie-spezifische kooperierende Mutationen in MSC nachgewiesen.

Der Nachweis von somatischen Leukämie-spezifischen *TP53* Mutationen in MSC von AML-Patienten und Patientinnen deutet darauf hin, dass diese Leukämie-spezifischen Mutationen in gemeinsamen mesodermalen Vorläuferzellen von HSPC und MSC auftreten, was unsere Eingangshypothese unterstützt, dass *TP53* Mutationen ein Frühereignis in der Entstehung dieser AML darstellen.

Diese Beobachtung ist auch von klinischer Bedeutung. MSC werden zunehmend als Quelle für konstitutionelle DNA verwendet. Der Nachweis von Leukämie-spezifischen Mutationen in diesen Zellen könnte somit zu falsch-positiven Resultaten führen. Somit sollte das Augenmerk in dieser Hinsicht wiederum auf kultivierte Hautfibroblasten gerichtet werden.

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LETTER TO THE EDITOR

Detection of AML-specific *TP53* mutations in bone marrow–derived mesenchymal stromal cells cultured under hypoxia conditions

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Dear Editor,

TP53 mutations are early events in the pathogenesis of acute myeloid leukemia (AML) and *TP53*-mutated AML has recently been classified as a distinct subentity [1–3]. An increasing number of reports postulate that the bone marrow (BM) microenvironment of patients with myeloid malignancies contributes to both leukemogenesis and therapeutic resistance [4]. As disease-specific, somatic aberrations have been reported in cells of the BM microenvironment in these disorders [5, 6], we hypothesized that BM-derived mesenchymal stromal cells (BM-MSCs) are also affected by leukemia-specific mutations in patients with *TP53*-mutated AML.

The study was approved by the ethics committee of the Medical University of Graz, Austria, and written informed consent was obtained from all patients. Diagnostic, vitally frozen BM specimens from 13 AML patients with somatic *TP53* mutations were used for BM-MSC culture (Supplementary Table 1) [7]. One specimen from a patient with Li-Fraumeni-syndrome suffering from therapy-related AML served as a positive control. In accordance with previous reports, these leukemia specimens revealed a complex karyotype (12/14; 86%) and a paucity of cooperating gene mutations (median, 1; range, 0–3) [3]. As outlined in detail in the "Supplementary Methods," ex vivo culture of mononuclear BM cells was performed under low oxygen conditions (3% pO_2 and 5% CO_2 at 37 °C) with the addition of human platelet lysate. Adherent cells representing BM-MSCs were cultivated up to a maximum of 4 passages. To obtain pure cell populations, they were further subjected to cell sorting by FACS (FACSAria, BD) using the human monoclonal antibodies CD 73, CD105 (Bioscience), CD90 (Biolegend), and CD34 (Biolegend), CD45, CD14, and HLA-DR (all Beckman Coulter), respectively. In addition, their adipogenic, chondrogenic, and osteogenic differentiation capacity as a characteristic feature of BM-MSCs was demonstrated (Supplementary Fig. 1) [8]. Patient-specific *TP53* and cooperating mutations were analyzed in both AML and purified BM-MCS specimens, using the error corrected, high-resolution "Safe-Sequencing System" method as described previously [1, 3]. In AML specimens, somatic *TP53* and cooperating mutations were found at variant allele frequencies (VAFs) between 1.5 and 91.2%. In purified BM-MSCs, the leukemia-specific *TP53* mutation was detected in 2/13 patients (15%) at VAFs of 0.2% each and confirmed using biological replicates (0.2% and 0.1%, respectively) (Fig. 1). However, apart from one single nucleotide polymorphism in *TET2* (c.100C > T, p.L34F [rs111948941], sample #7479), no leukemia-specific, cooperating mutation was detected in BM-MSCs in any of the specimens analyzed (Supplementary Table 2).

The detection of somatic, leukemia–specific *TP53* mutations in BM-MSCs of AML patients may indicate that these mutations have arisen in common mesodermal ancestors of hematopoietic stem and progenitor cells and BM-MSCs [9]. It further supports

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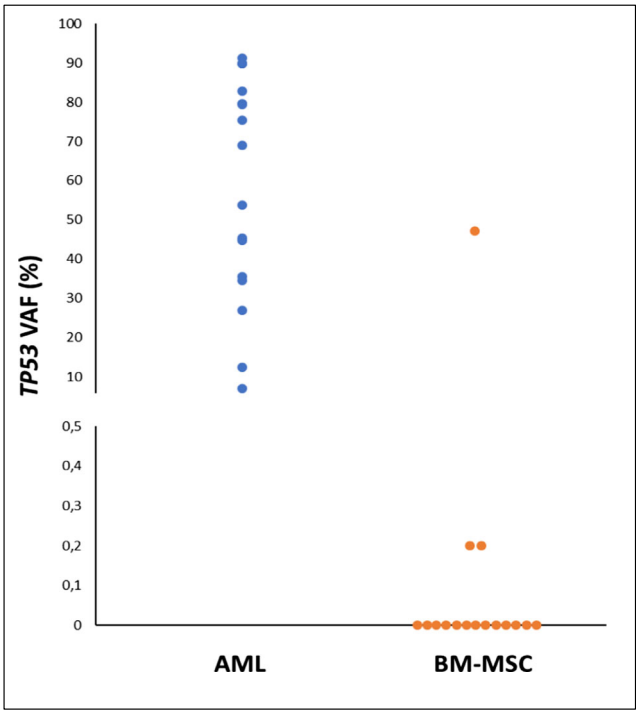

Fig. 1 Variant allele frequencies (VAFs) from primary leukemia specimens and purified bone marrow–derived mesenchymal stromal cells (BM-MSCs) from patients with *TP53*-mutated acute myeloid leukemia (AML). The BM-MSC specimen with a VAF of 47.1% was derived from a patient with Li-Fraumeni syndrome suffering from therapy-related AML serving as a positive control

the concept of *TP53* mutations being early events of acute myeloid leukemogenesis. The demonstration of BM-MSCs affected by leukemia-specific mutations—albeit at low VAFs—might also have practical implications as these cell types are increasingly used as a source of germline, control DNA [10]. Future work will focus on the functional role of the bone marrow microenvironment in this distinct AML subentity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary Data

Detection of AML-specific *TP53* mutations in bone marrow-derived mesenchymal stromal cells cultured under hypoxia conditions

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Patients and Methods

Primary leukemia specimens

At the Division of Hematology, Medical University of Graz, Graz, Austria (MUG), diagnostic bone marrow (BM) specimens from patients with acute myeloid leukemia (AML) are processed by Ficoll-Hypaque density gradient centrifugation and vitally frozen in liquid nitrogen. They are molecularly characterized by targeted deep sequencing assessing the coding regions of the *CEBPA*, *BCOR*, *DDX41*, *DNMT3A*, *ETV6*, *GATA2*, *NF1*, *PHF6*, *SF3B2*, *SFRP1*, *SRP72*, *STAG2*, *TP53* and *ZRSR2* genes, respectively, as well as mutational hot-spots of *NPM1, ASXL1, BRAF, CALR, CBL, CSF3R, ETNK1, EZH2, FLT3, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAT3, TET2, U2AF1* and *WT1* using the Ion Torrent Ampliseq Panel for "Myeloid Neoplasms".

Isolation of bone marrow-derived mesenchymal stromal cells (BM-MSC)

A total of 14 BM specimens from patients with newly diagnosed *TP53* mutated AML were used for this study (Supplementary Table 1). Between $1.0x10^6$ and $9.5x10^6$ cryopreserved, mononuclear cells (MNCs) were thawed per specimen, washed with calcium-free phosphate buffered saline (PBS) and re-suspended in αMEM (Sigma) supplemented with 10% human platelet lysate (provided by Department for Blood Group Serology and Transfusion Medicine, MUG), 1% L-Glutamine solution (Sigma), 1% Penicillin-Streptomycin (Sigma) and 2 U/ml Heparin (Biochrom) in accordance with previously published protocols.[1]

For *ex vivo* expansion, BM-MNCs were seeded in tissue flasks at a density of $0.8 x10^5$ to $1.6x10^5$ cells/cm^2 and cultured under low oxygen conditions (3% pO_2 and 5% CO_2 at 37°C). After 72 hours, cell culture medium and non-adherent cells were removed. Adherent cells were washed once with calcium-free PBS. Fresh culture medium was added to adherent cells which were cultivated for further 3 to 5 days (1^{st} passage). Fifty percent of culture medium was exchanged every other day. When cells reached 70%-90% confluence, they were detached using TrypLE Express (Gibco) and transferred to a new flask. Adherent cells were cultivated up to maximum of 4 passages.

Cell sorting

Cultured BM-MSCs were further sorted by FACS (FACSAria, BD) in cooperation with the core facility "Imaging/Flow Cytometry" at the Center for Medical Research, MUG. Re-suspended cells were labelled by using a broad spectrum of monoclonal antibodies (MoAbs) as previously described:[2] CD 73, CD105 (Bioscience), and CD90 (Biolegend) were used as positive markers and CD34 (Biolegend), CD45, CD14 and HLA-DR (all Beckman Coulter) as negative markers, respectively. Detached cells were blocked with blocking buffer (10% FBS in calcium-free PBS) for 10 minutes on ice, subsequently re-suspended in staining buffer (3% FBS in calcium-free PBS) with a maximum cell concentration of $1.0x10^7$ cells/ml and incubated with MoAbs for 25 min at 4°C in the dark.

Adipogenic, chondrogenic and osteogenic differentiation

The adipogenic, chondrogenic and osteogenic differentiation capacity of BM-MSCs were tested using commercially available assays (MesenCult™, STEMCELL Technologies) (Supplementary Figure 1). For all three assays, *in-vitro* cultures were performed at 20% pO_2.

For adipogenic differentiation, BM-MSCs were plated at a density of $6.0x10^3$ cells/cm^2 and cultured in the MSC medium described above until they reached confluence of 90%-100%. Thereafter, the medium was replaced by "MesenCult™ Adipogenic Differentiation Medium" (#05412 Human) and cells were cultured for additional 20 days with a medium change every 3 days. During that time, lipid vacuoles were formed. Adipogenic differentiation was assessed by Oil Red O staining.

For chondrogenic differentiation, $2.0x10^6$ BM-MSCs were re-suspended in 2 ml of "MesenCult™-ASF Chondrogenic Differentiation Medium" (#05455). Cell suspensions of 0.5 ml were transferred into Falcon tubes and centrifuged for 5-10 min at 300x g and BM-MSC incubated at 5% CO_2 and 37°C for 3 days. Incubation was carried out for 6 to 21 days with the medium changed every 3 days. When cells have reached chondrogenic differentiation, pellets were fixed in 10% formalin for 30 minutes at room temperature, followed by paraffin embedding. Sections of 6 µm were stained with Alcian Blue and Nuclear Fast Red.

For osteogenic differentiation, BM-MSCs were plated in triplicates in six-well plates at a cell density of $6.0x10^3$ cells/cm^2. When reaching confluence of 70%-80%, the culture medium was replaced by "MesenCult™ Osteogenic Stimulatory Medium" (#05465 without β-glycerophosphates). During the subsequent cultivation period of 1 week, BM-MSCs formed a multilayer and β-glycerophosphates were added. The cells were then cultivated for further 4 weeks with a medium change every 3 days. During that time, calcium deposition could be observed. Osteogenic differentiation was finally assessed by Alizarin Red S staining.

Error-corrected, next generation sequencing

Patient-specific *TP53* and cooperating mutations were analyzed in purified BM-MSCs and re-analyzed in leukemia specimens, respectively, using the high-resolution Safe-Sequencing System (Safe-SeqS) method as previously described.[3-5] Primers spanning the respective mutations were designed using primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). 10-20 ng of DNA were amplified using 1U Phusion DNA Polymerase (Thermo Fisher), 0.25 mM dNTPs and 02.µM amplicon-specific primers in 10 cycles of amplicon-specific PCR. To remove first-round primers, the PCR products were purified using Ampure XP beads (Beckman Coulter) and eluted in 15 µl nuclease-free H_2O. In a second round of PCR, Illumina specific adapters and indices were attached to the 5' ends for 35 cycles. After the second round of amplification, PCR fragments were again purified using Ampure XP beads (Beckman Coulter) and eluted in 12 µl of nuclease-free H_2O. For quality control and quantification, samples were run on an Agilent Bioanalyzer DNA 7500 chip (Agilent Technologies). All samples from one patient were pooled equimolarly and sequenced on an Illumina MiSeq in a 2x150 bp paired-end run.

Generated reads were grouped to read families according to the unique identifier (UID) added to the target-specific primer. Reads containing an "N" in the UID were discarded. After grouping, a consensus sequence of each read family was generated by picking a base that occurs in at least 80% of all the reads assigned to a family at that position. If no consensus was found, "N" was used as the consensus output base at that position. Based on the grouping output, a new fastq-file was generated form the consensus sequences of each read family comprising at least 5 reads and containing a maximum of 2 N positions at the forward and reverse consensus sequence, respectively. If after read collapsing, the sequencing depth was less than 1000x and no mutation was detected, we additionally

included read families with 1-4 reads for assessing the presence of a respective mutation. Forward and reverse sequences were merged using FLASH and the resulting FastQ file was aligned to the human reference genome (hg19) using BWA and samtools. Alignments were visualized in IGV to detect variations.

To test the limit of detection of Safe-SeqS, we used a serial dilution of a cell line harboring the *TP53* mutation c.743G>A, p.R248Q. This *TP53* mutation could be detected at a variant allele frequency as low as 0.2%. Moreover, sequencing of genomic DNA of 10 *TP53* wild-type specimens revealed no false-positive reads when considering only read families comprising of at least 5 reads and an error rate of 0.88% when all read counts were taken into account, respectively.

In cases of detection of leukemia-specific mutations in BM-MSCs, biological replicates were analyzed using DNA extracted from a different passage of BM-MSCs.

References

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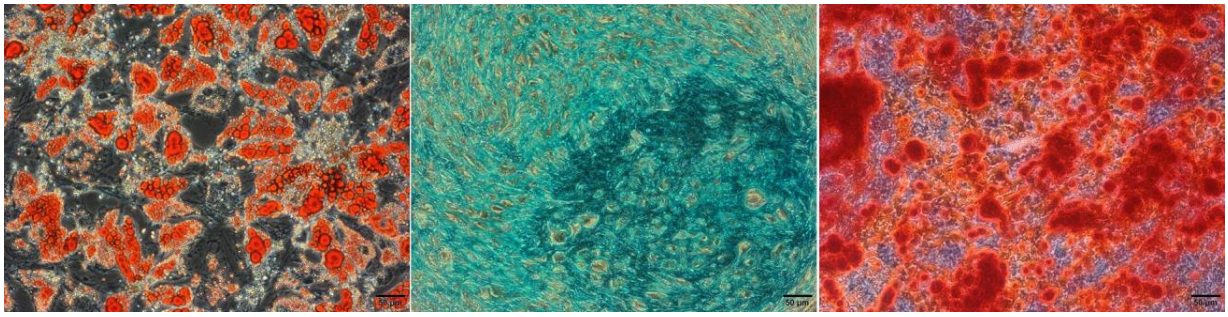
Results

Sample #	Sex	Age	Type of leukemia	WBC [G/l]	Cytogenetics
7351	F	73	tAML	2.18	44~46,XX,-7,-11,-17,+2mar[12]/46,XX[7]
7479	M	73	sAML	1.32	86~91,XXY,-Y,-2,-3,-3,-4,-5,-6,-7,-8,-10,-10,-11,-12,-14,-16,-16,-17,-17,-18,-21,-22,+11~19mar[cp11]/46,XY[3]
7484	F	78	sAML	17.45	50~51,XX,+1,del(5)(q15q33),+6,+8,+21[cp20]
7680	M	72	sAML	4.14	43~46,XX,+1,del(3)(p14),-7,-13,add(16)(q22),17 [cp15]
7754	F	74	*de novo*	11.50	43~45,X,-X,del(5)(q12q33),-12,-16,-17,+1~4mar [cp15]
8074	M	77	sAML	4.27	45~46,XY,der(5)t(5;12)(5pter->5q14::12q13->12qter)[7],inv(5)(p15q13)[6],del(12)(q13)[7][cp7].ish der(5)t(5;12)(EGR1-,wcp12+)/46,XY[1]
8189	M	68	tAML	7.33	34~45,X,-Y,-5,-7,-9,-12,-13,-14,-15,-16,-17,-18,-20,-22,+3~9mar[cp7]/46,XY[8]
8281	M	75	*de novo*	4.11	44~47,XY,-2,del(3)(q21),del(5)(q12),-7,+?8,-10,-11,-13,-13,-15,-18,+3~6mar[cp13]/46,XY[2]
8286	F	75	sAML	2.56	45,XX,-7[3]/46,XX[17]
8353	F	59	AML	3.24	43~44,XX,del(5)(q31q35),-7,-18[cp19]
8717	M	72	*de novo*	1.02	44~49,X,-Y,-4,-6,-7,-11,-17,-17,-18,+8~11mar[cp12]/46,XY[5]
8851	F	78	sAML	2.60	45,XX,?del(5)(q13q32),del(6)(q25),-18,-20,-20,+2mar[cp6]/46,XX[3]
8931	M	85	tAML	4.07	42~44,XY,t(1;2)(p34;p32)[4],der(5)t(5;13)(q11.2;q22)[16],dic(12;16)(p11.2;p11.2)[16],-13[16],-16[16]/46,XY[2]
8239	F	46	tAML	16.15	41~44,XX,-7,-11,-12,-14,-16,-17,-21,-22,+5~6mar[cp20]/46,XX[1]

Supplementary Table 1: Clinical and cytogenetic data of 14 AML patients with somatic *TP53* mutations and one patient with Li-Fraumeni syndrome suffering from tAML (#8239). Abbreviations: #, number; WBC, white blood cell count; F, female; M, male; *de novo*, *de novo* AML; tAML, therapy-related AML; sAML, secondary AML.



Supplementary Figure 1. *In-vitro* differentiation of bone marrow-derived mesenchymal stromal cells of patients with *TP53* mutated AML. Left, adipogenic differentiation (Oil Red O staining); middle, chondrogenic differentiation (Alcian Blue and Nuclear Fast Red staining); right, osteogenic differentiation (Alizarin Red S staining).

Sample #	Mutations	Cell type	VAF (%)	# mutated read groups	# wild-type read groups
7351	*TP53*: c.614A>G, p.Y205C NM_000546.3	AML	27.0	11.527	31.180
		BM-MSC	0.0	0	14.428
7479	*TP53*: c.646G>A, p.V216M	AML	79.6	12.005	3.071
		BM-MSC	0.0	0	888
	TET2: c.100C>T, p.L34F NM_001127208	AML	50.1	1.064	1.061
		BM-MSC	49.1	25.219	26.106
7484	*TP53*: c.469G>T, p.V157F	**AML**	**82.8**	**57.398**	**11.916**
		BM-MSC	**0.2** **0.2**	**39** **6**	**18.077** **3.075**
	DNMT3A: c.2657A>G, p.Q886R NM_022552	AML	38.2	2.861	4.634
		BM-MSC	0.0	3	7.772
7680	*TP53*: c.710T>A, p.M237K	AML	89.8	15.725	1.778
		BM-MSC	0.0	0	18.088
	DNMT3A: c.1979A>G, p.Y660C	AML	47.0	390	439
		BM-MSC	0.0	0	27974
	KRAS: c.173C>T, p.T58I NM_033360	AML	42.3	112.294	153.261
		BM-MSC	0.0	0	2.148
	CEBPA: c.588_589GG>TT NM_001285829	AML	46.0	28.486	33.399
		BM-MSC	0.0	0	13.607
7754	*TP53*: c.818G>A, p.R273H	AML	75.3	952	313
		BM-MSC	0.0	0	50.790
	TP53: c.749C>T, p.P250L	AML	7.1	262	3.444
		BM-MSC	0.0	1	6.589
	NRAS: c.35G>C, p.G12A NM_002524.5	AML	1.8	6.353	346.729
		BM-MSC	0.0	16	418.482
	NRAS: c.38G>A, p.G13D	AML	1.5	5.256	346.140
		BM-MSC	0.0	0	1.945
8074	*TP53*: c.743G>A, p.R248Q	AML	34.6	948	1.794
		BM-MSC	0.0	3	18.070

8189	*TP53*: c.395A>G , p.K132R	AML	79.5	3.642	939
		BM-MSC	0.0	0	2.343
	NRAS: c.183A>T, p.Q61H	AML	24.5	407	1.255
		BM-MSC	0.0	2	39.880
	TET2: p.S1848*	AML	4.7	13.272	271.507
		BM-MSC	0.0	1	20.812
8281	*TP53*: c.824G>A, p.C275Y	AML	35.6	96	174
		BM-MSC	0.0	0	5.177
8286	*TP53*: c.434T>C, p.L145P	AML	12.5	202	1.413
		BM-MSC	0.0	0	11.321
8353	*TP53*: c.97-1G>A, p.S33fs	AML	53.7	1.716	1.477
		BM-MSC	0.0	0	3.186
	RUNX1: c.292delC, p.L98fs NM_001754.4	AML	31.4	100	218
		BM-MSC	0.0	0	16.155
8717	*TP53*: c.838A>G, p.R280G	AML	91.2	234.781	22.560
		BM-MSC	0.0	0	55.922
8851	*TP53*: c.746G>C, p.R249T	**AML**	**45.3**	**16.935**	**20.409**
		BM-MSC	**0.2** **0.1**	**453** **28**	**241.361** **28.400**
	TP53: c.393_395del, p.N131K	AML	44.7	9.595	11.857
		BM-MSC	0.0	0	2.407
8931	*TP53*: c.841G>T, p.D281Y	AML	68.9	1.949	880
		BM-MSC	0.0	2	80.637
	TET2: c.1924C>T, p.Q642*	AML	36.4	63	110
		BM-MSC	0.0	0	868
8239	*TP53*: c.467G>C, p.R156P	**AML**	**89.8**	**90.165**	**10.213**
		BM-MSC	**47.1**	**91.058**	**102.077**

Supplementary Table 2. Analysis of *TP53* and cooperating mutations in AML specimens and cultured bone marrow-derived mesenchymal stromal cells (BM-MSCs). The *TET2* single nucleotide polymorphism c.100C>T, p.L34F (rs111948941), found in specimen #7479, was also detected in purified MSCs. Specimen #8239 served as a positive control and was derived from a patient with Li-Fraumeni syndrome suffering from therapy-related AML. Abbreviations: #, number; VAF, variant allele frequency.