PhD Thesis

Somatic *TP53* mutations characterize preleukemic stem cells in acute myeloid leukemia

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee" at Medical University of Graz, Austria.

Ridhima Lal

13/03/2017

I dedicate this thesis to my grandparents, Mrs Mohini saxena & late Dr. Bhasker raj Saxena who have always loved, inspired and motivated me.

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Abstract

Acute myeloid leukemogenesis is a multistep process involving mutagenesis, epigenetic dysregulation and copy number alterations (CNAs). In this work we hypothesized that somatic *TP53* mutations are early events of leukemogenesis. Employing a NSGS mouse model, we show that transplanted human *TP53* mutated acute myeloid leukemia (AML) cells led to high engraftment rates with a predominant blast phenotype. However, differentiation into granulocytes and B-lymphocytes was also observed in the majority of mice analyzed. Importantly, the specific *TP53* mutation was shown in up to 100% of engrafted cells. Investigating samples from patients with AML and somatic *TP53* mutations, highly purified T-lymphocytes revealed the specific aberration in 75% of specimens. Furthermore, investigating early phases of specimens from patients with secondary or therapy-related AML showed the leukemia-specific *TP53* mutation detectable in 80% of antecedent hematological disorders. In CFU-GM assays derived from sorted Lin-/CD34+/CD38-/CD99- single cells, *TP53* mutations were frequently observed with a paucity of cooperating mutations (median, 1; range, 0-3). Apart from one concomitant *DNMT3A* mutation, all mutations developed sequentially in the *TP53* mutated clone. In addition, a multitude of CNAs with a median of 37 losses and 34 gains per *TP53* mutated AML sample were detected. Loss of heterozygosity at the *TP53* locus in bulk leukemia but not colony cells suggested that CNAs are late leukemogenic events. Somatic *TP53* mutations were associated with significantly reduced overall and relapse-free survival rates of intensively treated AML patients. Analysis of sequential AML specimens revealed the *TP53* mutational load of comparable levels at diagnosis and relapsed/refractory stages. Our results indicate that in AML, somatic *TP53* mutations characterize preleukemic stem cells, are initiating leukemogenic events and act as mediators of resistant disease. They extend previous data and confirm recent claims of *TP53* mutated AML as a distinct disease entity.

Zusammenfassung

Akute myeloische Leukämie (AML) ist eine heterogene, aggressive Neoplasie, die auf dem Boden von genetischen Mutationen, epigenetischer Dysregulation und Aberrationen der chromosomalen Kopienzahl (CNA) entsteht. In dieser Arbeit prüften wir die Hypothese, dass somatische *TP53* Mutationen bei der AML frühe, leukämogene Ereignisse darstellen. Mittels eines NSGS Xenograftmodells konnten wir zeigen, dass transplantierte, humane AML Zellen zu einem Engraftment in der Mehrzahl der Mäuse führten, wobei die Zellen einen vorwiegend blastären Phänotyp aufwiesen. Nichtsdestoweniger zeigte sich auch eine Differenzierung in reife, humane Granulozyten und B-Lymphozyten. Wesentlich war, dass die patientenspezifische *TP53* Mutation in bis zu 100% der angewachsenen, humanen Zellen nachgewiesen werden konnte. Wir haben sodann T-Lymphozyten aus dem peripheren Blut von AML PatientInnen bei Diagnosestellung untersucht und fanden die jeweilige *TP53* Mutation in 75% der analysierten Fälle. Überdies haben wir Proben von frühen Phasen von PatientInnen mit sekundärer oder Therapie-assoziierter Leukämie untersucht, die ebenfalls die spezifische, somatische *TP53* Mutation in 75% aufwiesen. In CFU-GM Kulturen, die aus Einzelzellen gewonnen wurden, die dem Lin-/CD34+/CD38-/CD99- Phänotyp entsprachen, wurde die *TP53* Mutation häufig detektiert. Interessanterweise fanden sich nur wenige kooperierende Mutationen (median, 1; Spanne 0-3), die sich allesamt in den *TP53* mutierten Klonen entwickelten. AMLs mit somatischen *TP53* Mutationen zeigten jedoch eine große Zahl von CNAs - sowohl was Verluste (median, 37; Spanne, 27-80) als auch Zugewinne (median, 34; Spanne, 21-113) betraf. Nachgewiesene Verluste der Heterozygotie am *TP53* Lokus in AML Zellen, jedoch nicht in Kolonien, deuteten darauf hin, dass CNAs nachgeschaltete Ereignisse darstellen. Somatische *TP53* Mutation waren mit signifikant schlechterem Gesamtüberleben und relapsfreiem Überleben assoziiert, was eine Kohorte von intensiv therapierten AML PatientInnen betraf. Eine Analyse von sequentiellen AML Proben zum Zeitpunkt der Diagnose und einer refraktären bzw. relapsierten Erkrankung erbrachte, dass die spezifische *TP53* Mutationslast zu beiden Zeitpunkten vergleichbar war. Diese Daten zeigten, dass somatische *TP53* Mutationen bei AML präleukämische Stammzellen charakterisieren, dass sie initiale Ereignisse darstellen und in starkem Masse zu refraktärer Erkrankung beitragen. Sie erweitern unser Wissen um diese Form der AML und bestärken Forderungen, *TP53* mutierte AML als eine spezifische Entität zu klassifizieren.

Peer reviewed publications derived from the doctoral work

Lal R, Lind K, Heitzer E, Ulz P, Aubell K, Kashofer K, Middeke JM, Thiede C, Schulz E, Rosenberger A, Hofer S, Feilhauer B, Rinner B, Svendova V, Schimek MG, Rücker FG, Hoefler G, Döhner K, Zebisch A, Wölfler A, Sill H. Somatic *TP53* mutations characterize preleukemic stem cells in acute myeloid leukemia. **Blood** 2017 Mar 3. pii: blood-2016-11-751008. doi: 10.1182/blood-2016-11-751008

Schulz E, Lind K, Renner W, Petersen BS, Quehenberger F, Dills C, Hofer S, **Lal R**, Höfler G, Schlenke P, Ehninger G, Schetelig J, Middeke JM, Stölzel F, Sill H. The *TP53* Pro72Arg SNP in de novo acute myeloid leukemia – results of two cohort studies involving 215 patients and 3759 controls. **Br J Haematol** 2017 Jan 20. doi: 10.1111/bjh.14527

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 Nov 3;128(18):2270-2272

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1 Introduction

1.1.1 Clinical features and classification of acute myeloid leukemia

Acute myeloid leukemia (AML) is the most prevalent type of acute leukemia in adults accounting for 25% of all leukemias. It is an aggressive clonal disorder of hematopoietic stem and precursor cells characterized by incomplete maturation of blood cells ultimately leading to hematopoietic failure. AML is predominantly a disease of the elderly with greater incidence rates in patients above 65 years. Mortality rates in AML are high with an age adjusted mortality of 2.7/100,000 people (Deschler, Lubbert 2006). Patients in good general condition are administered intensive therapies including allogeneic stem cell transplantation whereas older or unfit patients are only given non-intensive regimens or best supportive care (Dohner, Weisdorf et al. 2015) .

AML development could originate in a former healthy individual and is designated as *de novo* AML. Genetic instability caused by a preceding clonal disorder like myelodysplastic /myeloproliferative neoplasms (MDS/MPN) could also lead to transformation into AML and is referred to as secondary AML (sAML) (Szotkowski, Rohon et al. 2010). AML could also emerge following a primary malignancy as a consequence of cytotoxic drug therapy referred to as therapy-related AML (tAML) (Sill, Olipitz et al. 2011). Even poorer outcomes are observed in patients with tAML which can be attributed to many factors. Firstly, due to treatment for preceding disorders by which the hematopoietic reservoir would be damaged. Secondly, patients are also prone to the relapse of the prior malignancy. Lastly, molecular mutations such as *TP53* mutations are found at a higher frequency in tAML patients which is known to contribute to drug resistance (Granfeldt Ostgard, Medeiros et al. 2015).

Clinical symptoms in AML patients are usually non-specific and include fatigue and weakness caused by anemia, bleeding due to thrombocytopenia and infection as well as impaired wound healing attributed to neutropenia. About 10% of AML patients exhibit considerable upsurge in the white blood count levels (>100,000/μL) being at risk for leukostasis and even poorer prognosis. However, in older patients, pancytopenia is more common due to bone marrow dysfunction (Armitage J 2004).

AML is diagnosed by the existence of 20% or more blasts in the peripheral blood or in the bone marrow (Dohner, Estey et al. 2017). About 50 years earlier it was considered an absolutely fatal disease, however, with the advancement of novel therapies and better supportive care it is presently curable in up to 35-40% of cases below the age of 60 years and between 5-15% in older patients (>60 years).Clonal heterogeneity in AML was discovered over 30 years ago though only in the recent 15 years it has been thoroughly elucidated (Dohner, Weisdorf et al. 2015).

The first attempt to classify AML was carried out by the French-American-British system (FAB), which was ingrained in 1976 (Bennett, Catovsky et al. 1976). The FAB system classified AML into eight categories namely from M0-M7 based on the morphology and maturation stage of myeloid cells (Table 1). However, the main drawback of this classification is attributed to lack of prognostic importance (Bennett, Catovsky et al. 1976).

Table 1: French–American-British (FAB) classification (adapted from Tenen 2003)

FAB classification with description	Comments
MO: Undifferenciated	Myeloperoxidase negative;myeloid markers positive
M1: Myeloblastic without maturation	Some evidence of granulocytic differenciation
M2: Myeloblastic without maturation	Maturation at or beyond the promyelocytic stage of differenciation; can bedivided into those with t(8;21) AML1-ETO fusion and those without
M3: Promyelocytic	Acute promyelocytic leukaemia; most cases have t(15;17)Promyelocytic leukaemia-retinoic acid receptor α or another translocation involving retinoic acid receptor α
M4: Myelomonocytic	
$M4_{EO}$: Myelomonocytic with bonemarrow eosinophillia	Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5: Monocytic	
M6: Erythroleukemia	
M7: Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

The second classification system was introduced by World Health Organization (WHO) in 2001 and this was further revised in 2008. The WHO revised version (Table 2) of classification published in 2016 incorporated recurrent cytogenetic aberrations and morphology, molecular abnormalities, immunophenotype and clinical manifestations for AML (De Kouchkovsky, Abdul-Hay 2016). The WHO classification reduced the blast

percentage from 30% to 20% in peripheral blood or in bone marrow necessary for the diagnosis of AML. However, auxiliary patients harboring clonal recurring cytogenetic abnormalities such as t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22), and t(15;17)(q22;q12) would be diagnosed as AML even if they have lower blast percentages (Arber, Orazi et al. 2016, Vardiman, Harris et al. 2002).

Table 2: The revised World Health Organization classification 2016 (adapted from Dohner, Estey et al. 2017)

WHO classification	Criteria
Myeloid neoplasms with germline predispostion	
Acute myeloid leukemia and related neoplasms	
Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* AML with inv(16)(p13.1q22) or t(16:16)(p13.1;q22);*CBFB-MYH11* Acute promyelocytic leukemia with *PML-RARA* AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A* AML with t(6;9)(p23;q34.1);*DEK-NUP214* AML with inv(3)(q21.3q26.2)ort(3;3)(q21.3;q26.2); *GATA2*, *MECOM(EVI1*) AML(megakaryoblastic)with t(1;22)(p13.3;q13.3);*RBM15-MKL1* Provisional entity: AML with *BCR-ABL1* AML with mutated *NPM1* AML with biallelic mutations in *CEBPA* AML with mutated *NPM1* Provisional entity: AML with mutated *RUNX1*
Acute myeloid leukemia with myelodysplasia related changes	
Therapy related myeloid neoplasms	
Acute myeloid leukemia,not otherwise specified(NOS)	AML with minimal differenciation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferationsrelated to Down's syndrome	Transient abnormal myelopoiesis Myeloid leukemia associated with Down's syndrome
Blastic plasmacytoid dendritic cell neoplasm	
Acute leukemias of ambiguous lineage	Acute undifferenciated leukemia Mixed phenotype acute leukemia with t(9,22)(q34.1;q11.2);*BCR-ABL1* Mixed phenotype acute leukemia with t(v;11q23.3);KMT2A rearranged Mixed phenotype acute leukemia ,B/myeloid,NOS Mixed phenotype acute leukemia ,T/myeloid,NOS Provisional entity: Natural killer cell (NK) lymphoblastic leukemia/lymphoma

The latest update to the preexisting WHO classifications includes a new interim entity "AML with *BCR-ABL1*" in the category AML with recurrent genetic abnormalities. This inclusion was made to distinguish patients harboring the aberration to be administered with tyrosine kinase inhibitors. "AML with mutated *NPM1*" and "AML with biallelic mutations of *CEBPA*" have been incorporated as distinct disease entities. Another new interim entity "AML with mutated *RUNX1*" was made owing to diverse clinic-pathological characteristics endowed with poor outcomes (Dohner, Estey et al. 2016).

Further changes were made to category "AML not otherwise specified (NOS)" by excluding the "acute erythroid leukemia" subclass, and introducing "pure erythroid leukemia" now obliging to ≥30% proerythroblasts with >80% immature erythroid progenitors. Additionally, a new category emerged owing to identification of myeloid neoplasms arising due to hereditary predispositions (Dohner, Estey et al. 2016).

1.1.2 Risk stratification

Assessment of a patient´s prognosis is extremely valuable for treatment decisions in AML. Apart from leukemia-specific factors, risk stratification for treatment options depends also on the age of the patient and the presence of comorbidities.

Age and comorbidities

Age plays an important role in determining the outcome in AML. The median age at AML diagnosis is 65 years, and the disease is infrequent in individuals below 40 years. An increased occurrence of MDS with age could be one reason for higher incidence rates of AML in the elderly since a substantial number of MDS cases transforms into AML. AML occurs at an incidence rate of 1.8/100,000 persons in individuals below 65 years. However, the incidence rate dramatically increases to 17/100,000 persons in patients above 65 years (Deschler, Lubbert 2006).Older patients exhibit inferior survival rates as compared to the younger ones, however, scoring of co-morbidities involved play an extremely important role in designing treatment options in both age categories (Dohner, Estey et al. 2010, Deschler, Lubbert 2006).

Karyotyping

A vast heterogeneity of molecular aberrations has been observed in AML which is contributed by chromosomal aberrations, mutations in genes, differential gene expressions and miRNAs. Karyotyping is considered the most significant factor for prognosis in AML; about 749 different chromosomal aberrations have been described in AML (Mitelman F, Johansson B, Mertens F, 2017, Kumar 2011).

In over 55% of adult AML patients, chromosomal aberrations have been observed, making karyotyping of AML of utmost importance for the prediction of the disease outcome and overall survival (Orozco, Appelbaum 2012). According to the novel European Leukemia Net (ELN) classification, AML risk groups are classified as favorable, intermediate and adverse risks (Table 3).(Dohner, Estey et al. 2017) The favorable risk group consists of patients with t (8; 21), t (15; 17), or inv (16), normal karyotype AML with *NPM1* mutations without *FLT3-ITD* or low ratios as well as biallelic *CEPBA* mutations. Patients belonging to these risk groups exhibit high complete remission (CR) and good overall survival (OS) rates.

The intermediate risk group according to the latest classification has been reduced to one group rather than the prior classification of intermediate risk group I and II. The reason for the generalization was owed to the extensive studies proving no noteworthy prognostic difference in elderly patients who predominantly compose the two groups. AML patients with *NPM1* mutations and no or very low *FLT3-ITD* mutational frequency belong as a subcategory. AML patients without *NPM1* mutations with low *FLT3-ITD* mutational load are another subcategory belonging to the intermediate risk group. Patients with t (9; 11) (p21.3; q23.3); *MLLT3-KMT2A* and cases where cytogenetic changes are not categorized under favorable or adverse risks belong also to the intermediate risk category.

The adverse risk group consists of AMLs with complex or monosomal karyotypes conferring a very poor outcome and low overall survival. Patients harboring three or more chromosomal abnormalities with inexistence of t (8; 21), t (15;17), or inv(16) are classified as complex karyotype. Aberrations in chromosomes 5 and 7 also play a role in the disease outcomes since patients with aberrations in these chromosomes had a much lower OS than the patients without those. Chromosomal losses at 17p with/without mutations of *TP53* gene are observed in about two-thirds of AML with complex karyotypes. Monosomal karyotypes are defined as aberrations harboring either two monosomies (excluding X or Y

chromosomes) or one monosomy with another structural aberration. These patients exhibit the worst prognosis and extremely poor overall survival rates (Orozco, Appelbaum 2012).

Table 3: Risk stratification by karyotyping in AML (adapted from Dohner, Estey et al. 2017)

Risk category	Genetic abnormalities
Favorable	t(8;21)(q22;q22.1);*RUNX1-RUNX1T1*
	inv(16)(p13.1q22)or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3-ITD* or with *FLT3-ITD*^{low}
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3-ITD high*
	Wild-type *NPM1* without *FLT3-ITD* or with *FLT3-ITD*^{low}
	(without adverse – risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, *MECOM(EVI1)*
	-5 or del(5q); -7;-17/abn(17p)
	Complex karyotype,monosomal karyotype
	Wild-type*NPM1* and *FLT3-ITD* ^{high}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

Gene mutations

In extension to the cytogenetic profiling and mutations described above, it is also important to include further gene mutations for assessment of prognosis since they may influence outcomes (Dohner, Estey et al. 2010, Papaemmanuil, Gerstung et al. 2016)

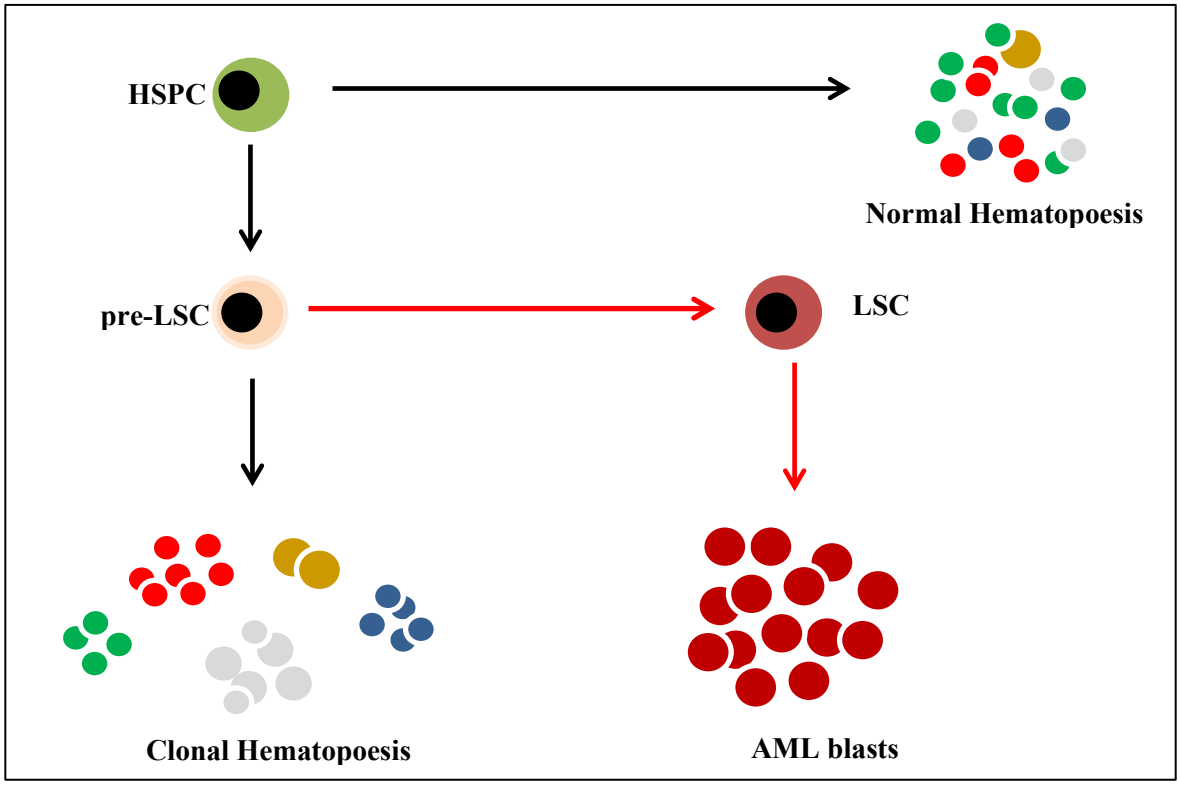
In patients belonging to the favorable risk group with t (8; 21), a gene mutation in *c-KIT* exponentially reduces overall survival and increases the chance of a relapse (Paschka, Marcucci et al. 2006). The *TP53* gene mutations confer the worst overall survival. The extended study conducted by the Cancer genome Atlas which employed 200 AML samples research reported that an AML genome on an average harbors 13 mutations with 5 recurrently mutated genes (Cancer Genome Atlas Research Network 2013).

1.1.3 Clonal heterogeneity in AML

Hematopoietic stem and precursor cells (HSPCs) are self-renewing cells with multilineage differentiation potential giving rise to functional mature blood cells. Long term HSPCs are capable of limitless self-renewal while in the short term HSPCs it is restricted to a limited period of roughly 8 weeks (Passegue, Jamieson et al, Lapidot, Sirard et al. 1994). Leukemia stem cells (LSCs) are capable of initiating and sustaining the process of leukemogenesis. LSC develop due to the mutations in normal HSPCs. These transformed cells retain the ability of self-renewal but proliferate aberrantly resulting in leukemia (Schepers, Campbell et al. 2015, Valent, Bonnet et al. 2012).

LSCs were first identified in AML by employing in vivo xenograft mice models (Lapidot, Sirard et al. 1994, Bonnet, Dick 1997). LSCs are dormant at the G0 phase of the cell cycle thereby escaping their elimination by apoptosis following chemotherapy. Since they differ from AML blast cells, they survive the conventional therapies resulting in relapsed disease which is the major concern in AML treatment. Initial phenotyping analysis revealed the existence of LSCs in the $Lin^{-}CD34^{+}CD38^{-}$ compartment (Pollyea, Gutman et al. 2014). However, subsequent work was able to identify LSCs in the CD34- compartment, too (Figure 1) (Bonnet, Dick 1997).

Figure 1: Normal hematopoiesis and AML leukemogenesis



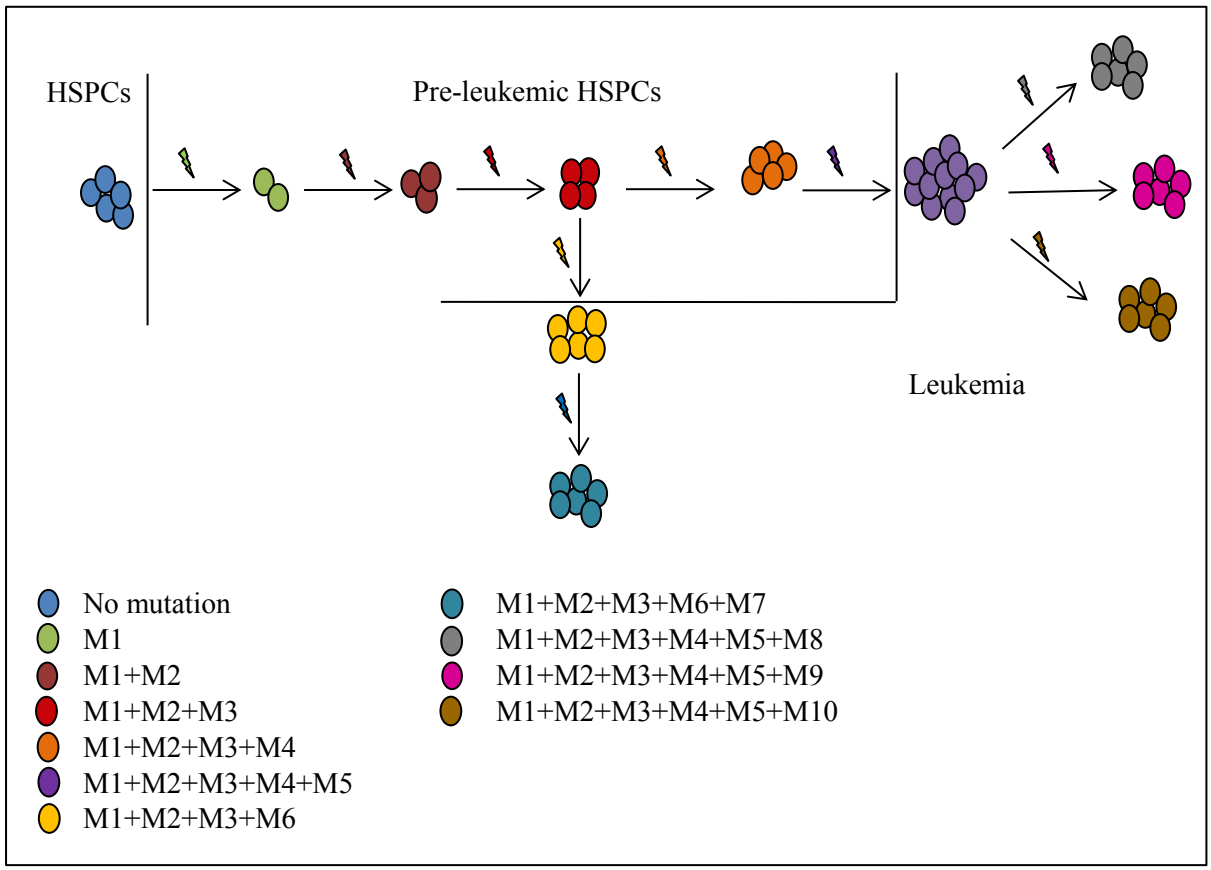
The investigation conducted by The Cancer Genome Atlas (TCGA) which employed coding genomes from over 200 AML patients revealed that approximately 30 gene mutations were found in over 2% of the patients. The authors reported an average of 13 gene mutations in an AML genome and detected 23 genes that were significantly mutated with a proven involvement in AML pathogenesis. These genes included FLT3, *NPM1*, *DNMT3A*, *IDH2*, *IDH1*, and *CEBPA* with newly identified genes such as *U2AF1* and *EZH2* (Cancer Genome Atlas Research Network 2013). Consequently, eight functional subgroups have been defined that comprise AMLs with mutations in DNA methylator, chromatin modifier, spliceosome and cohesion complex genes, myeloid transcription factors, tumor suppressors as well as *FLT3* and *NPM1* (Papaemmanuil, Gerstung et al. 2016).

Mutations in the genes responsible for epigenetic modifications such as *DNMT3A, IDH2, TET2, and ASXL1* occur early in the course of leukemic transformation. Alterations in these genes transpired in the founding clone however solitarily these mutations were not sufficient for leukemogenesis (Abdel-Wahab, Levine 2013).

A clonal progression model by Jan and Majeti (Jan, Snyder et al. 2012) suggests that multiple mutations are needed for the transformation of HSPCs into frank leukemia. This model suggests that the HSPC compartment consists of normal cells and transformed cells harboring some mutations which on accumulating further genetic aberrations over time turn into a leukemic clone (Figure 2). Transformed HSPCs harboring few but not all the mutations are described as "preleukemic stem cells" (Corces-Zimmerman, Majeti 2014). It is therefore important to detect the initiating oncogenic event termed as "driver mutations" which confers selective growth dominance and powers the leukemogenic process. The driver mutation is retained in the blast cells signifying as this being the primary step of leukemia development (Shlush, Zandi et al. 2014). Passenger mutations do exist in a leukemic cell but they - unlike driver mutations - do not establish the malignant clone but are associated with leukemia progression (Bozic, Antal et al. 2010).

DNMT3A, *TET2*, *NPM1* are some of the reported driver mutations in AML while *FLT3* mutations are documented to be occurring later during disease progression. Therefore, there is an immediate need to understand the genomic landscape to develop targeted therapies in AML (Corces-Zimmerman, Majeti 2014, Chan, Majeti 2013).

Figure 2: Clonal heterogeneity model (adapted from Jan, Majeti 2013)



1.1.4 Epigenetics of AML

Alterations in gene expression without a change of the genetic code are referred to as epigenetic modification which plays an important role in the pathogenesis of many neoplastic disorders including AML. Frequent recurrent alterations in gene expression can, nevertheless, be traced to somatic mutations in particular genes like *DNMT3A*, *TET2*, *IDH1* and *ASXL1*, respectively.

DNMT3A (DNA - cytosine - methyltransferase 3A) belongs to the family of DNA methyltransferases which consists of genes responsible for the methylation of CpG dinucleotides. *DNMT3A* is located on chromosome 2p32 and consists of 23 exons and a protein size of 130kD. Aberrations of *DNMT3A* leads to enhanced self-renewal, impaired differentiation and therefore flawed hematopoietic function. *DNMT3A* mutations are heterozygous in AML, and the dominant negative function of the R882 mutation has been demonstrated previously. Mutation R882 is a hotspot mutation which occurs in the RD domain with the mutated protein hindering the DNA methylation process. Studies have also found a correlation between *DNMT3A* mutations and *NPM1* mutations, where they co-exist in about 60% of the AML patients (Eriksson, Lennartsson et al. 2015). It is one of the most frequently mutated genes in AML, without current evidence of a prognostic impact (Ley, Ding et al. 2010).

The *TET2 (*Ten eleven translocation 2*)* gene is a member of the *TET* family of dioxygenase enzymes and is responsible for catalysis of 5-methylcytosine conversion to 5-hyderxymethylcytosine (5HMC). Mutations in the *TET2* gene diminish the catalytic activity of DNA demethylation causing reduction in 5HMC levels thereby contributing to leukemogenesis (Eriksson, Lennartsson et al. 2015). *TET2* mutations show higher incidence rates in intermediate or cytogenetically normal risk group (Wouters, Delwel 2016, Figueroa, Abdel-Wahab et al. 2010).

Mutations in the isocitrate dehydrogenase 1 and 2 genes (*IDH1/2*) are observed in about 10% of AMLs, and commonly occur in patients with intermediate risk including the ones with normal cytogenetics. *IDH1* & *IDH2* genes bring about the conversion of isocitrate to alpha-ketoglutarate and if there are mutations in these genes it leads to the production of 2-hydroxyglutarate. 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate on which the *TET2* genes are dependent (Wouters, Delwel 2016).

Mutations in *ASXL1* (additional sex comb like genes) have been identified in AML patients and more importantly as early events in leukemogenesis. *ASXL1* regulates the activity of the polycomb and trithorax genes responsible for histone methylation. Occurrence of *ASXL1* mutations in AML varies between 3% and 25%, it is also found to co-occur with *RUNX1* mutations (A Eriksson et al, 2015).

1.1.5 Treatment options and disease outcomes in AML

Cytogenetics, molecular genetics and age as well as co-morbidities play a very important role in accessing the clinical outcome and in the decision process regarding administration of effective treatments. AML treatment is a two-step process; first a complete remission (CR) is aimed for by induction treatment followed by post-induction treatment to maintain a CR which is defined as less than 5% blasts in the bone marrow with no indication of extramedullary AML. In younger patients (<60 years of age) about 70-80% achieve CR (Burnett, Wetzler et al. 2011, Dohner, Estey et al. 2016). Induction therapy mainly involves a seven day treatment with cytarabine followed by three day course of an anthracycline (daunorubicin, idarubicin). Consolidation therapy is offered to patients in order to prevent a relapse of the disease. Chemotherapy and allogenic hematopoietic stem cell transplantation are usually provided as a part of consolidation therapy. Chemotherapy regimen includes two to four cycles of intermediate dose of cytarabine; allogenic hematopoietic stem cell transplantation (HSCT) is limited to availability of appropriate donors (De Kouchkovsky, Abdul-Hay 2016). Thereby, elimination of residual LSCs by HSCT is not only due to the conditioning regimen, but also an immunological mechanism ("Graft-versus-leukemia effect"). However, in up to 30%, this is associated with graft-versus-host reactions which may become life-threatening (Showel, Levis 2014).

Older patients incapable of tolerating intensive treatments are provided with non-intensive therapies including hypomethylating agents (azacitidine, decitabine) or best supportive care (Deschler, Lubbert 2006). Treatment with hypomethylating agents is associated with improved survival and quality-of-life (Dombret, Seymour et al. 2015). In particularly healthy older patients, intensive treatment may be administered where daunorubicin at 45-50mg/m^2 and cytarabine 100-200 mg/m^2 is applied. About 45-60% of older patients treated with intensive therapy attain CR (Burnett, Wetzler et al. 2011). The survival rates in

elderly AML patients are meager with less than 20% of patients survive 5 years (Ferrara, Schiffer 2013).

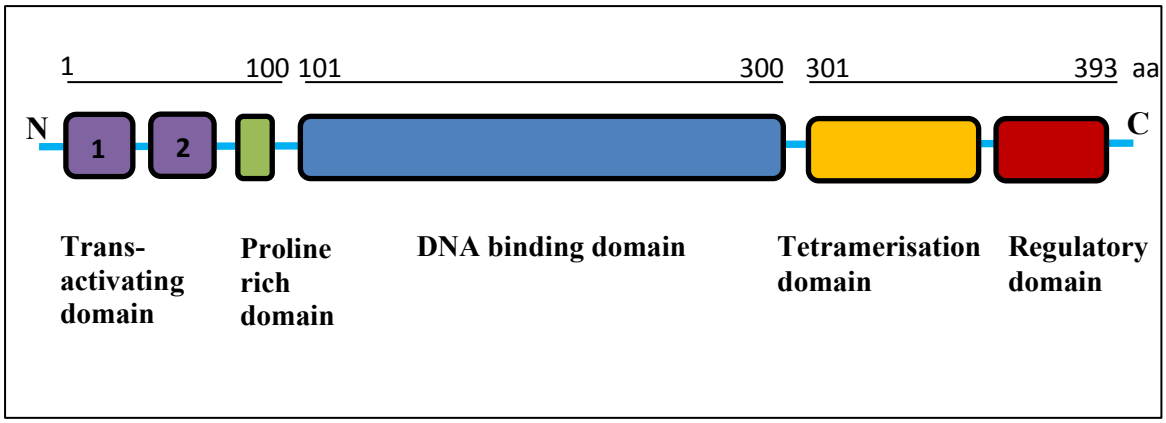
A multitude of new targeted treatments have emerged to improve the prognosis of AML patients. Among those, sorafenib and midostaurin are multi-kinase inhibitors which were investigated in patients with *FLT3* mutated AMLs. Monoclonal antibodies are being designed to target receptors such as CD33 and CD123 (Dohner, Weisdorf et al. 2015, Dohner, Estey et al. 2017). Novel hypomethylating agents include guadecitabine; immunological approaches focus on strategies engaging chimeric antigen receptor T-cells (Stein, Tallman 2016, Dohner, Estey et al. 2017).

1.1.6 The *TP53* gene

The *TP53* gene is one of the utmost comprehensively studied genes in biology and cancer. The p53 protein was first discovered in 1979 by David lane and Lionel Crawford as a partner of SV40, a simian retrovirus, and therefore considered as an oncogene (Lane, Crawford 1979). However, the tumor suppressive properties of *TP53* were demonstrated years later. The *TP53* family consists of three genes, namely p53, p63 and p73, respectively. Multiple splicing variants generated by alternate promoters give rise to several *TP53* isoforms which express different protein domains of the gene (Murray-Zmijewski, Lane et al. 2006, Dotsch, Bernassola et al. 2010, Joerger, Rajagopalan et al. 2009).

p53 consists of 393 amino acids with a protein mass of 53 kDa (43,7kDa on SDS gel) spanning across 20kb comprising of 11 exons (Levine, Oren 2009). The *TP53* gene exhibits a rather conservative structural design consisting of a N-terminal domain, DNA binding domain and a C-terminus domain. The N-terminus entails a transactivation domain (TAD) and a proline rich domain, the latter being essential for *MDM2* mediated *TP53* stability. Subsequent 102-192 amino acid assembly originates in the DNA binding domain, and is vital for the sequence–specific interaction of the DNA. The majority of the *TP53* mutations are clustered in this region. The C-terminus region includes amino acids 301-393, responsible for oligomerization, regulation, nuclear signal localization and nuclear export (Figure 3) (Teufel, Freund et al. 2007).

Figure 3: *TP53* structural organization (adapted from Teufel, Freund et al. 2007)



1.1.7 The *TP53* gene as the guardian of the genome

TP53 is a tumor suppressor gene regarded as the "guardian of the genome" for its fundamental role in cycle arrest and apoptosis through transcriptional regulation (Freed-Pastor, Prives 2012). Activation of *TP53* can be triggered by a series of stress signals such as DNA damage, ribosome dysfunction, hypoxia and activation of an oncogene (Brady, Attardi 2010). *TP53* activation involves three stages namely stabilization, sequence specific DNA binding and activation of target genes. *TP53* stabilization is accomplished by revoked binding with MDM2, which is attained by phosphorylation of the amino terminal or by p14ARF .Following a successful stabilization, *TP53* as a tetramer binds with its response elements, facilitated by the tetramerization domain located in the C terminus subsequently activating its target genes (Zilfou, Lowe 2009).

The outcome of cell fate is decided on the type of the cell, environmental conditions and type of stress. In case of low stress or repairable damage, p53 initiates a transitory cell cycle arrest followed by prompting a DNA repair mechanism thereby preventing propagation of mutations (Levine 1997). p53 arrests cell division by restraining G1 phase of cell cycle by transactivating p21, a gene responsible for cell cycle arrest. p53 can also disrupt cell cycle progression by perturbing the cyclinB1/cdc2 complex responsible to stimulate G2 to mitosis stage. Furthermore p53 is also known to transactivate 14-3-3 σ and inhibits localization of cyclinB1/cdc2 complex thereby arresting the cell cycle (Zilfou, Lowe 2009). In case of acute stress, p53 launches irretrievable apoptosis or cell senescence mechanisms (Yee, Vousden 2005). Apoptosis by p53 is prompted either nuclear or cytoplasmically (Galluzzi, Morselli et al. 2008). p53 transactivates apoptotic genes such as

PUMA, PIG3, CD95. p53 directly can also initiate apoptosis cytoplasmically through MOMP (Mitochondrial Outer Membrane Permeablization) by modulating *BCL-2* family genes consisting of proapoptotic factors such as *BAX, BAK, BH3* (Brady, Attardi 2010, Wolff, Erster et al. 2008).

p53 curbs the accumulation of reactive oxygen species by increasing the expression of antioxidant genes. p53 stimulates oxidative phosphorylation and impedes glycolysis thereby shielding the cells from tumor transformation, a phenomenon termed Warburg effect (Brady, Attardi 2010). Autophagy can be directly induced by *TP53* activating AMPK which inhibits mTOR thereby stimulating autophagy. Other downstream target genes in addition to *AMPK* exist which include *DRAM, BAX, PUMA* and have recently been shown to play a role in p53 mediated autophagy. These functions attributed to *TP53* christened it as the "guardian of the human genome" (Bieging, Mello et al. 2014).

1.1.8 *TP53* germline mutations

Li-Fraumeni syndrome (LFS) and Li-Fraumeni like syndrome (LFL) are cancer predisposition syndromes as a consequence of inheritance of *TP53* mutation in one of the two alleles exposing the individual to a more than 80% lifelong risk of neoplasms with an early commencement of several cancers. In patients with Li-Fraumeni syndrome, cancers such as breast cancer, sarcomas, adrenocortical carcinoma, brain tumors, leukemia, ovarian cancer, colorectal cancer are common (Olivier, Hollstein et al. 2010, Li, Fraumeni et al. 1988, Bougeard, Sesboue et al. 2008, Tinat, Bougeard et al. 2009).

The "classic" definition of LFS includes probands with sarcoma below the age of 45 years having an cancer affected first degree family member and a first degree or second degree relative below 45 years affected by any cancer (Varley, Evans et al. 1997). Families who exhibit some but not all the features of a "classical LFS" are termed as Li-Fraumeni like syndrome (Birch, Hartley et al. 1994)

The cancer risk in patients belonging to the "classical LFS" is significantly higher where in 50% of the cases cancer development is seen by 40 years of age. Interestingly, females harboring *TP53* germline mutations have a higher chance of developing cancer and at a much younger age as compared to the males (Hwang, Lozano et al. 2003). In about 80% of LFS families and 20% of LFL families germline *TP53* mutations have been reported.

(Kamihara, Rana et al. 2014) .Over 200 germline mutations are documented in the COSMIC (http://cancer.sanger.ac.uk/cosmic), IARC (www.p53.iacr.fr) and *TP53* website databases (www.p53.free.fr) (Kato, Han et al. 2003).

Inclusion of the category "Myeloid neoplasms with germ line predisposition" as a separate entity in the 2016 WHO classification demonstrated also the importance of germline mutations in myeloid malignancies including those in *TP53*. A study published recently by our group demonstrated the occurrence germline mutations in *TP53* gene preferably in tAML patients that often belonged to Li-Fraumeni families. The study consisted of 186 AML patients with *de novo* AML, sAML and tAML cases. In 1.1% of the patients a *TP53* germline mutation was detected. Focusing on an extended cohort of tAML patients, we could show that *TP53* germline events occurred in 5.6% of patients and are significantly associated with previous radiotherapy. (Zebisch, Lal et al. 2016).These clinical data are in line with experimental ones of radiation hypersensitivity of *TP53* mutant mice (Kemp, Wheldon et al. 1994).

In humans, about 80 *TP53* polymorphisms have been reported, amongst which proline (P) to arginine (R) located at codon 72 the most is extensively studied. This polymorphism exists in the in the proline rich region of the gene and might have an effect on the SH3 region (Olivier, Hollstein et al. 2010). Numerous differences between the two polymorphisms exist. R72 has more proficiency in apoptosis while P72 has a proven ability in DNA repair and cell cycle arrest. Likewise individuals with R72 have an improved outcome in comparison to the P72 following administration of chemotherapy (Pietsch, Humbey et al. 2006) . Reports also suggest a higher *TP53* mutation incidence in those individuals with proline-arginine heterozygosity than in arginine homozygous (LOH) individuals (Rivlin, Brosh et al. 2011).However a recent cohort study conducted by our group which involved 215 *de novo* AML patients and 3759 control samples reported no significant difference in the overall survival or increased risk towards the development of *de novo* AML in patients with a *TP53* codon 72 variation (Schulz, Lind et al. 2017).

1.1.9 Somatically acquired *TP53* mutations

In addition to inherited mutations, *TP53* mutations can occur spontaneously through lifetime of a person leading to development of different cancers. The most prevalent

somatic mutations are the ones occurring in *TP53* gene in about 50% of human cancers (Olivier, Hollstein et al. 2010).Over 17,000 somatic mutations are described in the IARC and *TP53* mutation databases. The majority of them (97%) are bundled up in the DNA binding domain thereby affecting the DNA binding ability of the gene. (Kato, Han et al. 2003).

The majority of the *TP53* mutations described are missense mutations caused by single nucleotide substitutions typically clustered in the highly conserved DNA binding region of the gene. Mutations causing disruption of the DNA binding region of the protein are termed as contact mutations and mutations causing a conformational change bringing about instability of p53 are termed structural mutations. Hotspot mutations occur within the following codons of the DNA binding region of *TP53* gene: R175H, Y220C, R237H, G245S, R248W, R249S, and R282W (Rivlin, Brosh et al. 2011). In solid tumors, the incidence of *TP53* mutations in the DNA binding region has previously been associated with lower treatment response rate and reduced survival rates (Hou, Chou et al. 2015).

The role of the *TP53* gene in HSPCs homeostasis is extensively reported where it guards the cell from DNA damage by lowering the level of reactive oxygen species (Sablina, Budanov et al. 2005, Asai, Liu et al. 2011). Experiments with mice carrying a *TP53* mutation indicate faster development of hematopoietic malignancies (Pant, Quintas-Cardama et al. 2012).

1.1.11 Role of *TP53* in AML

The role of p53 in HSPCs homeostasis is extensively reported where it guards the cells from DNA damage. Role of *TP53* gene in hematopoiesis of mice devoid of *TP53* show an increased HSPC pool with a propensity for accumulation of other mutations. On the other hand, enhanced p53 function is also reported to cause radiation hypersensitivity in super *P53* mice by damaging the stemness of HSPCs ultimately resulting in hematopoietic failure (Pant, Quintas-Cardama et al. 2012).

Human hematological malignancies exhibit *TP53* associated genetic alterations at a relatively low frequency of 10-20% in comparison to solid tumors. Nevertheless, *TP53* is one of the recurrently mutated genes in the AML genome (Cancer Genome Atlas Research Network 2013, Rucker, Schlenk et al. 2012, Haferlach, Dicker et al. 2008, Bowen, Groves

et al. 2009). In AML patients harboring complex karyotypes, *TP53* mutations are the most frequent alterations indicating a significant role in leukemogenesis. Therefore, "AML with *TP53* mutations, chromosomal aneuploidies, or both" has been proposed as a distinct AML sub entity (Dohner, Estey et al. 2017). *TP53* mutations in AML also occur predominantly as missense mutations. Whereas in *de novo* AML, *TP53* mutations are being observed at a frequency of 10%, in tAML they occur up to 30% (Renneville, Roumier et al. 2008).

AML patients with *TP53* mutations show significantly reduced CR rates and according to a recent study, an inferior overall survival with no patients surviving beyond 3 years (Haferlach, Dicker et al. 2008). Thereby, the main reason of treatment failure is refractory or relapsed AML. This is corroborated by further reports showing similar poor survival of this cohort of patients even when allogeneic HSCT was performed (Haferlach, Dicker et al. 2008, Dohner, Gaidzik 2011, Middeke, Herold et al. 2016). Studies have also implicated an association between mutations of the *TP53* gene with aberrations of chromosome 17p. Loss of heterozygosity (LOH) at 17p13, the *TP53* locus, is observed at a frequency of up to 45%, preferably in complex karyotype AMLs indicating loss of the normal p53 functions with GOF properties of the remaining, mutant allele (Jasek, Gondek et al. 2010).

2. Hypothesis & aims of the study

Based on clinical observations as well as the fact that "AML with *TP53* mutations, aneuploidies or both" has recently been proposed as a distinct AML subtype, we hypothesized that *TP53* mutations are "driver events" of acute myeloid leukemogenesis. They are early events which together with further cooperating mutations gives rise to a dominant AML clone that contributes significantly to relapsed disease.

2.1 Specific aim I: Screening for somatic *TP53* mutations in primary specimens. We examined diagnostic samples form AML patients for somatically acquired *TP53* mutations. We then probed for their early occurrence in antecedent hematological disorders in respective samples of sAML and tAML patients.

2.2 Specific aim II: Assessment at what stage somatic *TP53* mutations are acquired.

a) *In vivo* xenograft models. NSGS mice models were employed to investigate the engraftment and differentiation potential of human AML specimens with somatic *TP53* mutations.

b) T-lymphocytes from AML patients with somatic *TP53* mutations. As these cells are long lived, they were used to detect somatic mutations affecting preleukemic stem cells.

c) *In vitro* clonogenic assays. The Lin-/CD34+/CD38-/CD99- HSPC population from AML samples harboring somatic *TP53* mutations were assayed to examine the existence of *TP53* mutations in these cells. We have also investigated cooperating mutations occurring in these AML specimens.

2.3 Specific aim III: Assessment of the prognostic impact of somatic *TP53* mutations. We assessed event free survival and overall survival of intensively treated AML patients with and without *TP53* mutations. We also analyzed sequential samples obtained at diagnosis, in remission and at refractory/relapsed stages with respect to the *TP53* mutational load.

3. Materials and methods

3.1 Patient cohort and sample preparation

3.1.1 Inclusion criteria for the study. 150 specimens were selected and analysed in this study. This cohort comprises patients with *de novo* AML exhibiting high risk karyotypes (n=39), secondary AML (n=51) and therapy-related AML (n=60). Matched non-tumoral control specimens were also obtained from the respective patients. Written informed consent for bio banking of the specimens was procured from each patient and the study was approved by the ethics committee of the Medical University of Graz (MUG), Austria. For the assessment of *TP53* mutations in the antecedent haematological disorders, sAML and tAML patient samples from MUG Graz and the German-Austrian AML study group, Ulm, Germany, were used. Serial sample analysis for the assessment of *TP53* mutational frequency at diagnosis, complete remission and relapse was performed in samples obtained from biobanks of MUG and the Study Alliance Leukemia Group, Dresden, Germany.

3.1.2 Preparation of bone marrow and peripheral blood samples. Mononuclear cells from peripheral blood were isolated using Lymphoprep™ (Stem Cell Technologies, 07801). 35ml of the blood was layered carefully on 15ml sterile lymphoprep™ in a 50ml tube and centrifuged at 2500rpm for 25 minutes. Following, the upper layer was aspirated out leaving behind the undisturbed mononuclear interphase layer. This layer consists of blast cells, lymphocytes and monocytes. The cells were then resuspended in PBS counted and frozen down in sterile DMSO for later use. Cells from the bone marrow were extracted using lysing buffer by filling it up to 50ml in a falcon. Following this, the falcon was left undisturbed for about 5 minutes for the lysis. The falcon was then centrifuged at 1200 RPM at room temperature and the procedure repeated twice for complete lysis. The supernatant was then discarded and the cells were resuspended gently and washed twice with PBS. An aliquot was used for cell counting followed by freezing down in sterile DMSO.

3.1.3 Primary culture and cryopreservation of fibroblasts. Primary fibroblasts were used to unambiguously assess the somatic status of *TP53* mutations as described previously by our group (Schulz, Valentin et al. 2012). The skin biopsy sample was first

washed in sterile fresh complete DMEM medium containing 1% penicillin-streptomycin for 1 minute and finally washed again in sterile fresh media. The biopsy sample was then cut further into smaller sections and one section each transferred into a well of a 6 well culture plate. We added 1ml fresh sterile FCS into each well and waited till the sections stick to the plate, then removed the FCS carefully by slanting the plate and air dried it in the hood. It is very important to have the pieces stuck firmly to the plate. Fresh complete DMEM medium was added to the wells and incubated for over a week. The fibroblasts were cultured till the desired numbers of cells obtained following which the DNA is extracted using DNeasy® Blood & Tissue Kit (Qiagen, 51304).

3.1.4 FACS sorting of CD3+ T cells. Frozen leukemia samples were thawed, washed and re-suspended in PBS. C-Chip disposable hemocytometer (DigitalBio, DHC-N01) was used to enumerate the number of viable cells. The cells were incubated for 7 minutes in 1% sheep serum (Sigma-Aldrich, S2263) at room temperature to inhibit nonspecific binding. The leukemia cells were then stained with specific human antibodies namely CD45 (Beckman Coulter), CD33 (Beckman Coulter), CD34 (BD Pharmingen), and CD3 (BD Pharmingen) for 15 minutes at room temperature. The stained cells were washed to eliminate unbound antibodies followed by re-suspension with 200µl of PBS. T-cells were sorted using FACSAria (BD Biosciences) with a CD45+CD3+ phenotype to a purity of 99% which was assessed by Flow cytometry (FACSAria, BD Biosciences). DNA was extracted from these cells using QIAamp DNA Micro Kit (Qiagen).Targeted deep sequencing as described below was performed in duplicates.

Table 4: Antibody panel for isolation of T cells

Antibody	Dye	1 million cells
CD45	Crome O	7.5
CD33	FITC	5.0
CD34	PE	5.0
CD3	PerCP	7.5

3.1.5 *In vitro* clonogenic and genotyping assays: Selected leukemia samples were thawed, washed and re-suspended in PBS. The viable cell count was determined by trypan blue and cells at a density of 5x10^6 (in 250 µl) were stained with the antibodies. A multicolor gating strategy was used to discriminate preleukemic stem cells from blast cells using the antibody panel listed in Table 5. Nonspecific antibody binding was avoided by incubating the cells in 1% sheep serum (Sigma-Aldrich) for 7 minutes followed by staining with specific antibodies for 15 minutes. The cells were then washed with PBS to remove unbound antibodies and followed by resuspension in PBS. CD99 (BD Pharmingen) and or TIM3 (R&D systems) were used to exclude positively stained leukemia cells during the sorting. The lineage markers included CD3 (BD Pharmingen), CD19 (Beckman coulter) and CD20 (BD Pharmingen). CD34 (Beckman Coulter) and CD38 (Beckman Coulter) was used to identify ancestral cells.

The preleukemic stem cell population which resides in the Lin-CD34+/CD38-/CD99- compartment (Martelli, Pettirossi et al. 2010, Jan, Snyder et al. 2012) was sorted into a 96 well plate (Thermo Scientific) containing 100ul of cytokine supplemented MethoCult™ H4034 media (Stem Cell Technologies) containing 1% penicillin and streptomycin (Sigma Aldrich). The 96 well culture plates were maintained in a humidified incubator at 37°C for 14 days after which the CFU-GM colonies are enumerated by microscopy. Genomic DNA from the colonies was extracted using QIAamp DNA Micro Kit (Qiagen, 56304) and was PCR amplified using specific primers. Sanger sequencing was performed for each colony DNA for specific *TP53* as well as cooperating mutations obtained from eight samples.

Table 5: Antibody panel for isolation of preleukemic stem cell populations

Antibody	Company	Dye	Per 50µl/1x10^6 cells
CD90	Coulter, IM1839U	FITC	1
CD99	BD Pharmingen, 555689	PE	7,5
TIM3	R&D Systems, FAB2365P	PE	2,5
CD3	BD, 345766	PerCp	7,5
CD19	Coulter, A07771	PC5	2
CD20	BD, 345794	PerCp	5
CD38	Coulter, A54189	PC7	5
CD34	Coulter, A86354	A-AF700	5
control	BD, 345815	FITC	2,5
control	BD, 345816	PE	7,5
control	BD, 345817	PerCP	2,5
control	Coulter, IM2663	PC7	2
control	Coulter, A71118	A-AF700	5

3.1.6 May-Grünwald-Giemsa staining. It is a common staining used for blood and bone marrow smears. We employed this technique to visualize the type of blood cells obtained from *in vitro* clonogenic assays after 14 days of culture. 100*ul* of the cells from the 96 well was fixed on a slide by employing cytospins. The slides are air dried for 1 day and following which the staining is performed. The slides are first immersed in May-Grünwald Eosin-Methylenblau (Merck Millipore) for 4 minutes in a slide holder following submergion in distilled water for 1 minute after which the holder is dipped in diluted Giemsa Azur Eosin-Methylenblau stain (1.8ml May-Grünwald Eosin-Methylenblau with 50ml of Giemsa-Buffer) for 20 minutes. The tray is then washed with water till the excess stain comes out and the water is no more colored. The slides are then mounted using Eukitt Mounting Medium (Sigma-Aldrich, 25608-33-7) and microscopy is performed.

3.2 Mutational screening and analysis

3.2.1 DNA/RNA isolation and purification. DNA purification from the frozen leukemias specimens was performed using the DNeasy® Blood & Tissue Kit (Qiagen, 51304). The DNA from the respective nontumor controls was isolated from paraffin slides using the Qiagen FFPE DNA extraction kit (Promega, A2351) and from swabs as well as fibroblasts using the QIAamp DNA Micro Kit (Qiagen, 56304). RNA from the sample was isolated using the RNA mini prep Kit (Qiagen, 74106).

3.2.2 Quantification of the nucleic acids. The DNA/RNA obtained were spectrophotometrically quantified based on their optical densities. BioPhotometer plus (Eppendorf) was used to quantify the nucleic acids. A 260/280nm ratio was used as a parameter to evaluate the purity with a ratio of 1.80 regarded highly pure.

3.2.3 Polymerase Chain Reaction (PCR) for mutational analysis. It is an *in vitro* technique of DNA amplification using a thermostable DNA polymerase of a specific DNA molecule occurring in a backdrop of 10^5 cells. HotStarTaq Master Mix was used for the *TP53* PCR (Qiagen, 203445) which is a mixture of HotStarTaq DNA Polymerase, QIAGEN PCR Buffer, and dNTPs. *HotStarTaq* DNA polymerase is a modified DNA polymerase with high specificity since it inhibits nonspecific annealing and primer dimer formations which usually occur at low temperatures. A typical PCR reaction consisted of 1.25 U/reaction of HotStarTaq Master Mix, 25ng of genomic DNA, primers at a concentration of 0.2 μM of each primer (Applied Biosystems, Warrington, UK) and PCR water in a volume of 25 μL. Q buffer (Qiagen, 1005485) was supplemented for primers of exons 2, 3 and 7. A separate PCR reaction devoid of the genomic DNA was used as a negative control. The synthesised primers include a M13 forward (TGTAAAACGACGGCCAG) and reverse tag (CAGGAAACAGCTATGAC) respectively. This a common tag used which is known to simplify Sanger sequencing reaction set up (Daigle, Simen et al. 2011).

Table 6: PCR programs employed for the amplification of *TP53* and cooperating mutations

Program A	Time/cycles	Temperature
Initial denaturation	15min	95°C
Denaturation	30sec/50	94°C
Annealing	45sec	61°C
Extension	45sec	72°C
Final extension	10min	72°C

Program B	Time/cycles	Temperature
Initial denaturation	15min	95°C
Denaturation	30sec/50	94°C
Annealing	45 sec/20	63°C
	-0.5°C/every 3 cycles	60°C
	45sec/30	
Extension	1min	72°C
Final extension	10min	72°C

Program C	Time/cycles	Temperature
Initial denaturation	15min	95°C
Denaturation	30sec/50	94°C
Annealing	30sec	60°C
Extension	30sec	72°C
Final extension	10min	72°C

Program D	Time/cycles	Temperature
Initial denaturation	15min	95°C
Denaturation	30sec/50	94°C
Annealing	45sec/20	58.5°C
	-0.5°C/every 3 cycles	55°C
	45sec/30	
Extension	1min	72°C
Final extension	10min	72°C

Table 7: *TP53* primer sequences used in this study

Gene	Primer name	Primers	Program used	Amplicon size (bp)
TP53	Exon 2&3_Fw	tctcatgctggatccccact	A	344
	Exon2&3_Rev	agtcagaggaccaggtcctc		
TP53	Exon 4_Fw	tgaggacctggtcctctgac	B	413
	Exon 4_Rev	agaggaatcccaaagttcca		
TP53	Exon 5&6_Fw	tgttcacttgtgccctgact	B	467
	Exon 5&6_Fw	ttaacccctcctcccagaga		
TP53	Exon 7_Fw	cttgccacaggtctccccaa	C	237
	Exon 7_Rev	aggggtcagaggcaagcaga		
TP53	Exon 8&9_Fw	ttgggagtagatggagcct	B	445
	Exon8&9_Rev	agtgttagactggaaacttt		
TP53	Exon 10_Fw	caattgtaacttgaaccatc	D	260
	Exon 10_Rev	ggatgagaatggaatcctat		
TP53	Exon 11_Fw	agaccctctcactcatgtga	B	245
	Exon 11_Rev	tgacgcacacctattgcaag		

Table 8: Primer sequences used for analysis of cooperating mutations

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)
BCL11A	CCCAACACGGAGAACGTGTA	CCGGACCACTAATATGGGGC	238 + tag
ATP1A1	ATAGAGCTGTGCTGTGGTTCC	CCGTGTACCCATCGATCTTCA	114 + tag
DNMT3A	TGTGTGGTTAGACGGCTTCC	CCCCCATGTCCCTTACACAC	229 + tag
FLT3	CCCACGGGAAAGTGGTGAAG	CACCACAGTGAGTGCAGTTGT	168 + tag
PTPN11	GCCTCCCTTTCCAATGGACT	TGGACCAACTCAGCCAAAGT	126 + tag
SRSF2	TCCGCGGACCTTTGTGAGG	CGACGCTATGGATGCCATGGA	234+ tag
NF1_m1	CGTACTCCTGGAGCCTCTCT	TTCAGTGTCAGGGTTCCACA	150+ tag
NF1_m2	GGGACTTCAAATACGGACCA	GATGTTCAGAGCTGCCTTCA	221+ tag
ASXL1	GGACCCTCGCAGACATTAAA	TACACTTTCCAGGGGTGCTC	228+ tag
IDH2	CAATGGTGATGGGCTTGGTC	GTTGAAAGATGGCGGCTGCA	188 + tag
RUNX1	GGATGCACTTACTTCGAGGTTCT	CATTCTGTTTTGTTCTCTATCGTGTCC	150+ tag

Reverse transcriptase-PCR. This technique was used to assess whether novel mutations found during this project are being expressed. The isolated RNA was transcribed to cDNA using the GeneAmp RNA PCR Kit (Applied Biosystems). Specific primers were designed employing Primer3plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and the PCR was carried out using program C.

3.2.5 Screening of *TP53* mutations by Sanger sequencing. SigmaSpin™ Post-Reaction clean-up kit (Sigma-Aldrich, S5059) was used to clean up the PCR reactions. The sequencing reaction was set up using BigDye® Terminator v1.1 Cycle sequencing kit (Applied Biosystems, 4336774) .A typical sequencing reaction consisted of 1µL of cleaned Product, 4 µL of 2.5X ready reaction mix, 4µL of 5x BigDye Sequencing Buffer, 0.5 µM of forward and reverse primer respectively in different tubes. The sequencing reactions were then run on ABI PRISM 310 Genetic Analyzer (POP6) or PRISM 3700 DNA Analyzer (POP7) at the central research facility (ZMF, Medical University Graz). Alternatively, the sequencing was outsourced to Microsynth, Switzerland, by providing PCR products; the purification was also performed at Microsynth. The raw sequences were

downloaded from the server following which they were analysed. The sequences were base called and analyzed using sequence analysis software SeqScape V 2.7 which is designed to detect mutations when aligned with reference sequences of the particular gene.

The mutations nomenclature was reconfirmed by the Mutalyzer (https://www.mutalyzer.nl/) online tool. The pathogenicity of the detected mutations was determined by the IARC *TP53* (www.p53.iacr.fr), database, COSMIC database (http://cancer.sanger.ac.uk/cosmic) and the *TP53* website (www.p53.free.fr).

3.2.6 Targeted deep sequencing. Targeted deep sequencing was performed as described previously to determine the *TP53* mutant allele frequency of diagnostic AML samples and of peripheral blood T-lymphocytes of AML patients. It was further used to detect and quantify cooperating mutations in *TP53* AML specimens (Geiger, Hatzl et al. 2015). Briefly, using the Ion AmpliSeq™ *TP53* and AML Research Panels, respectively (both Thermo Fisher Scientific), samples were sequenced to a depth of >2000x. Mapping to the human reference genome (hg 19) and variant calling were performed using the Ion Torrent Suite Software (Thermo Fisher Scientific, open source, GPL,https://github.com/iontorrent/. All called variants were annotated using ANNOVAR and custom Perl scripts (Wang, Li et al. 2010). Sequence information was obtained from purified T-lymphocytes in duplicates and from control samples exhibiting a *TP53* wild-type status. Cooperating mutations identified in *TP53* mutated AML samples were confirmed by Sanger sequencing.

3.2.7 Whole exome sequencing. DNA was extracted from bulk leukaemia cells of AML patients used for colony analysis. This DNA was further tagged using Nextera Rapid Capture Exomes Preparation Kit (Illumina) resulting in adapter-tagged libraries. Rapid capture hybridization was employed to obtain single stranded DNA and biotin labelled probes for the specific DNA region. Streptavidin beads were used to magnetically pull down the biotin labelled enriched DNA and eluted. Burrows-wheeler aligner was used for the alignment of pair end reads with the human reference genome (h19). Genome Analysis Tool Kit (GATK, version 3.3.0) was employed for the realignment of PCR duplicates (marked by Picard) for identification of earlier documented indels. Picard and GATK were used in setting of mate information. VarScan and MuTect programs were used for the identification of somatic single nucleotide polymorphisms (SNPs). Pile up file was created for VarScan using “SAMtools mpileup” command followed by “somatic” command with a threshold of 10%.The resultant SNPs were annotated by ANNOVAR and the variants with low reads were labelled as artifacts. High confidence SNPs were also annotated using

ANNOVAR and the mutations in cancer gene censes were prioritized. SNPs identified by whole exome sequencing were further confirmed by Sanger sequencing. Copy number variation analysis: Enriched exome sequencing was performed followed by analysis employing CNVkit (version 0.7.10) (Talevich, Shain et al. 2016).Comparison was drawn between non-tumor samples and tumor samples and the data sets were referenced with a batch command. VarScan copy number and copy Caller function were used for the identification of the CNAs. Circos package was used to visualize the data (Krzywinski, Schein et al. 2009). To unambiguously detect CNVs segments containing fewer than 100 probes was cast-off and the adjacent sections were fused provided the log 2 ratios were between -0.2 which represents the chromosomal loss and 0.2 which embodies chromosomal gains.

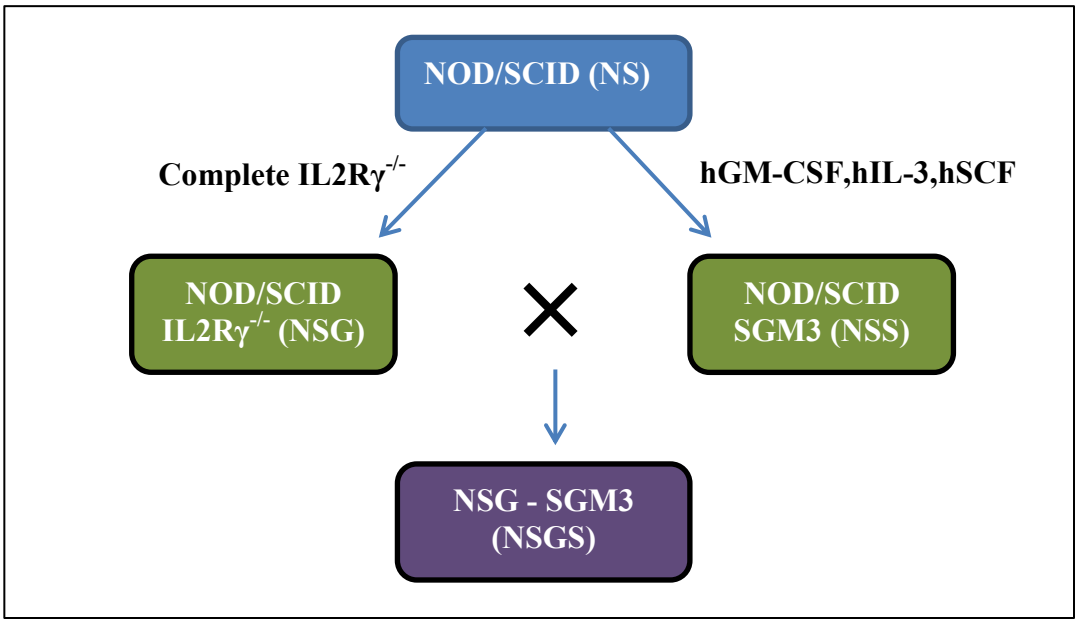
3.2.8 Ultra-deep sequencing. The *TP53* mutational load in the engrafted human cells isolated from NSGS mice was accessed using the Safe-Sequencing system. Amplification of the gDNA was carried out by Phusion polymerase (Thermo Fisher) via amplicon specific 12 base unique identifier (UID). Following PCR cycles the amplicons were purified with Ampure XB beads (Beckman Coulter) with elution in nuclease free water. Illumina specific adapters and indices were incorporated in a second PCR comprising of 35 cycles. The products obtained were purified, checked for quality control and quantified using an Agilent Bioanalyser DNA 7500 chip (Agilent Technologies). Samples were then equimolarly pooled and sequenced on Illumina MiSeq in 2 x 150bp pair-ends run. UID was then used to group reads into read family. FastQ-file was used and aligned to human reference genome (hg19) by Burrows-Wheeler Aligner and SAMtools. Integrated Genome Viewer (IGV) was then used to identify the mutations.

3.3 Xenotransplantation studies

3.3.1 Characterization of the NSGS mice model. Animals experiments have been approved by the Austrian ministry for science and economy (GZ: BMWFW-66.010/0162-WF/V/3b/2015).To minimalize animal suffering all the mice handling and experiments was followed stringently according to FELASA recommendations. ARRIVA guidelines were used for the reporting of animal study (Kilkenny, Browne et al. 2010). NOD-scid IL2rγ^{null} mice (NSGS mice) were purchased from Charles river-Jackson Laboratory were employed for this study since they do not necessitate irradiation and are devoid of

interleukin 2. NSGS mice encompass three transgenes, human interleukin 3, human granulocyte/monocyte stimulation factor and human stem cell factor which support better engraftment of hematopoietic cells (Figure 4) (Wunderlich, Chou et al. 2010).

Figure 4: NSGS mice model (adapted from Wunderlich, Chou et al. 2010)

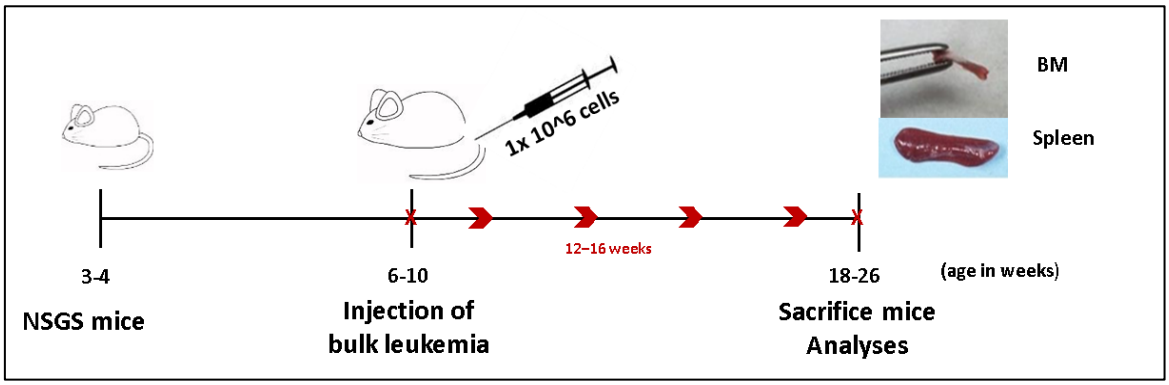


3.3.2 Tail vein injection of bulk leukemic sample. The mice were housed under quarantine condition and were acclimatized before the transplant. NSGS mice at the age of 6-10 weeks were used for the experiments. The cryopreserved bulk leukemia cells from the patient harboring somatic *TP53* mutation were thawed, resuspended with PBS and 2% FBS. Any clumps of cells were filtered out using a 70 µM cell strainer and care was also taken to use samples only above 90% viability before the injection. CD3+ cells were depleted out using anti-human CD3 magnetic particles (BD Biosciences, 552593) for the prevention of graft versus host disease. 25µl of anti-human CD3 magnetic particles for 5x10^6 cells was used, mixed and incubated at room temperature for 30 minutes.1X BD IMag Buffer (BD Biosciences , 552362) was used to obtain a cell suspension of 3ml and this was placed on the magnetic separator for 10 minutes for the separation of the negative cell fraction. $1x10^6$ bulk leukemia cells were transplanted via lateral tail vein injection into unconditioned NSGS (Wunderlich, Chou et al. 2010) as depicted in Figure 5.

3.3.3 Necropsy and tissue harvesting. Mice were sacrificed by performing cervical dislocation at 18 to 26 weeks after xenotransplantation. The scarified mice were fixed to a surgical base, disinfected and the femora, sternum and spleen were recovered.

The tail was shock frozen and stored at -20°C if required. The recovered tissues were stored at -70°C.

Figure 5: Experimental design of xenotransplantation studies



3.3.4 Preparation of spleen. The spleen was first cut into smaller sections followed by performing tissue digestion. The tissue digestion is performed using 3ml total mixture consisting of RPMI (Sigma-Aldrich, R8758), 1mg/ml collagenase and 1µg/ml DNase in a centrifuge tube. This is kept in a 37°C water bath for 15 minutes following which the tissue is mashed and passed through a 70 µM cell strainer. The falcon is then filled up with a mixture of HBSS (containing 10% FCS+ 1%Penstrep). The tube is then centrifuged at 4°C, 1500rpm for 5 minutes. The cell pellet is then resuspended with 2ml pharmalyse and incubated at room temperature for 5 minutes. 18ml of HBSS (containing 10% FCS+ 1% penstrep) is then added to the tube and centrifuged at 4°C, 1500rpm for 5 minutes. The cells are then resuspended in 1 ml HBSS (containing 10% FCS+ 1% penstrep) and counted.

3.3.5 Preparation of bone marrow. The bones and sternum were thoroughly cleaned with sterile tissues and collected in a mortar consisting of HBSS (containing 10% FCS+ 1% penstrep) solution. The tissues are then mashed thoroughly and filtered through a 70 µM cell strainer. The tube is centrifuged at 4°C, 1500rpm for 5 minutes. The supernatant is discarded and cell pellet is resuspended in 2ml 1X pharmlyse and incubated at room temperature, following which the cells are centrifuged at 4°C, 1500rpm for 5 minutes and the cell pellet is resuspended in 1ml buffer and counted.

Table 9: Antibody panel for human engraftment analysis

Antibody	Company	Dye	Per 50µl/1x10^{6}cells
CD45	Coulter B36294	Crome O	2.5
CD33	Coulter IM1135U	FITC	5.0
CD 19	Coulter IM3628	PC7	2.5
CD34	Coulter IM2709U	ECD	5.0
mCD45	Pharmingen 553081	PE	1.0
CD3	BD 34566	PerCP	7.5

3.3.6 Human graft analysis. The mouse CD45 cells were depleted with IMAG depletion system using biotinylated antibody. 50 µl per 1x10^7 cells of biotinylated CD45 was used (BD Biosciences, 553078). IMAG buffer (BD Biosciences, 552362, 100 ml) was added 10 fold to the volume of cell pellet. Centrifugation was carried out at 4°C, 1200rpm for 5 minutes. Concentration of 50 µl per 1x10^7 cells of streptavidin particles (BD Biosciences, 557812, 5 ml) was added to the cells and incubated at 4°C for 30 minutes. The cells were further resuspended with 2ml of IMAG buffer and placed on the magnetic strip for 7 minutes for effective separation. The magnetic separation was repeated twice to obtain pure population.

The human engraftment was elucidated by >1% human CD45 positivity of analyzed cells and blast engraftment by SSC low/CD34+/CD33-/CD19-orSSClow/CD34+/CD33+/CD19-The mature granulocytic engraftment was interpreted by SSC high/CD33+/CD34-/CD19-cells and the mature B-cell by SSC low/CD19+/CD34-/CD33-compartment.The DNA was then extracted using Qiagen micro kit for *TP53* mutation analysis.

3.4 Assessment of follow-up

3.4.1 Estimation of survival parameters. For those AML patients of the MUG cohort that had received "7+3 induction" and postremission therapy +/- allogeneic HSCT, response assessment was performed. Distribution of *TP53* wild-type and *TP53* mutated cases in the different AML and cytogenetic subgroups were tested using Pearson´s χ^2 test. Fisher´s exact test was used to test for an association of *TP53* mutations with sex. Correlation of

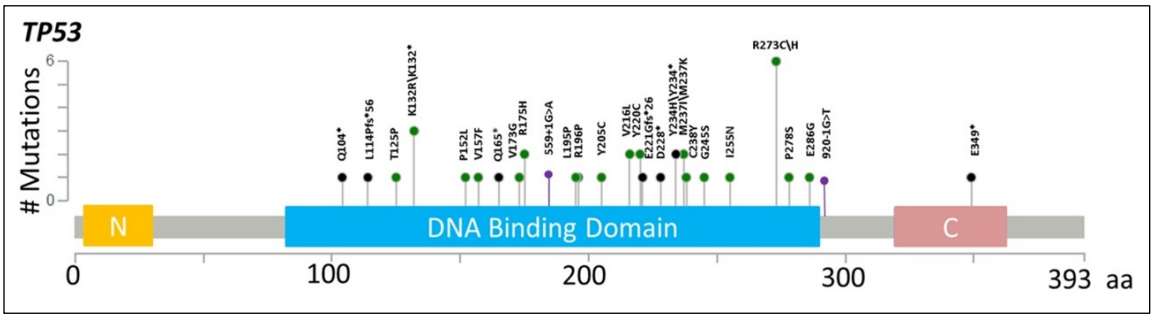
TP53 mutations with all continuous variables was tested employing Mann-Whitney-Wilcoxon test. IBM-SPSS 22.0 was sued for analysis. The clinical endpoints investigated were complete remission (CR) after induction therapy, overall survival (OS) and event-free survival (EFS) as defined previously (Dohner, Estey et al. 2010). Kaplan-Meier curves and the log-rank test were used to compare patients with *TP53* mutations versus those with a *TP53* wild-type status. Univariate and multivariate Cox regression was performed to identify significant prognostic variables assessing age, white blood cell count and the cytogenetic risk group as explanatory variables in addition to the *TP53* status. Statistical analysis was done in the R package survival (https://www.r-project.org/) and a *P* value <0.05 considered statistically significant.

3.4.2 Assessment of serial specimens from *TP53* mutated AMLs. Serial assessment of the *TP53* mutational load was performed by ultradeep sequencing using bone marrow specimens either obtained at diagnosis, CR and relapse or at diagnosis and refractory disease following induction treatment. These samples were derived from the leukemia biobanks of MUG and the Study Alliance Leukemia Group, Dresden, Germany. Two-sample inference for related samples was performed by exact non-parametric permutation tests which were calculated in the StatXact package (Cytel Software).

4. Results

4.1 Screening of diagnostic AML specimens for somatic *TP53* mutations. The *TP53* gene was analysed for mutations in a selected AML cohort employing Sanger sequencing of the coding region of *TP53* from exon 2 to exon 11. Two samples carrying the *TP53* mutation in matched nontumor material as well were excluded from the study as they represented a *TP53* germline event. The frequency of somatic *TP53* mutations was found to be 21% with 32 AML patients harbouring 39 mutations among the 150 patient samples analysed. In 7 patients, 2 *TP53* mutations were identified. The frequency of mutations in *de novo* AML patients was 18% (7/39), in sAML patients 20% (10/51) and in tAML patients 25% (15/60).According to previous studies, missense mutations are most prevalent in human cancers which predominantly occur in the DNA binding domain of the gene (Petitjean, Achatz et al. 2007). Our results are in accordance with former literatures since the majority of the *TP53* mutations identified in the cohort are missense mutations with a frequency of 95% (29/39). We also identified 2 nonsense mutations (8/39) and 2 splice site mutations (2/39), respectively (Figure 6). C-bio portal (http://www.cbioportal.org/) was used to visualise the data obtained by using the MutationMapper programme. Wild type allelic loss was observed in 16% (5/32) patients with *TP53* mutations with hemizygous *TP53* mutational state in 4/5 cases whereas only 1/5 patients exhibited a homozygous state. Of the 118 patients with WT *TP53* status, none exhibited allelic loss at the 17p13 locus.

Figure 6: Type and distribution of *TP53* mutations in the AML cohort studied
(Figure originally published in Lal et al. Blood 2017)

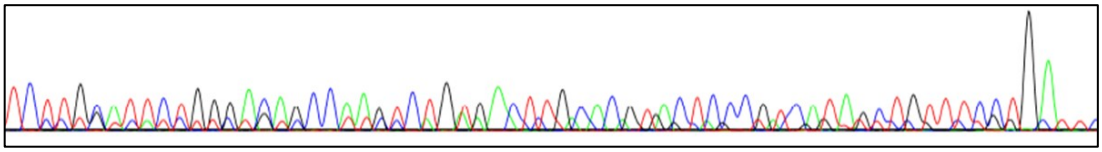


Mutations marked with green are missense mutations; red dots represent nonsense mutations; black dots represent splice-site mutations; purple dots represent two diverse types of mutations at the same location. The height of the bars represents the number of times the specific mutation occurs in different samples. Abbreviation: N, N-terminus domain of *TP53* gene; C, C terminus domain of *TP53* gene; aa, amino acid.

We also identified one novel mutation – *TP53* c.276_339dup, pL114Pfs*56. The mutation is consequence of 63 base pairs duplication causing a frameshift which is transcribed into mutated mRNA (Figure 7a). Its significance was assessed using three mutation prediction tools (Figure 7b).

Figure 7: cDNA analysis of the novel mutation, patient 5799

A



B

No	Mutation	MutPred	SIFT	Polyphen-2
5799	c.276_339dup, p.L114Pfs*56	0.650	0.05 Damaging	0.101 Benign

(A) Mutated *TP53* sequence from AML blasts. Duplication of 63 base pairs was observed in this patient. (B) Prediction algorithms for the pathogenicity of the novel mutation. MutPred, SIFT, Polyphen -2 analyses. Scores with g >0.75 and p <0.05 are referred to as confident hypotheses. SIFT scores, the amino acid substitution is predicted damaging if the score is <=0.05, and tolerated if the score is >0.05. Polyphen-2 numerical score range from 0.0 (benign) to 1.0 (damaging).

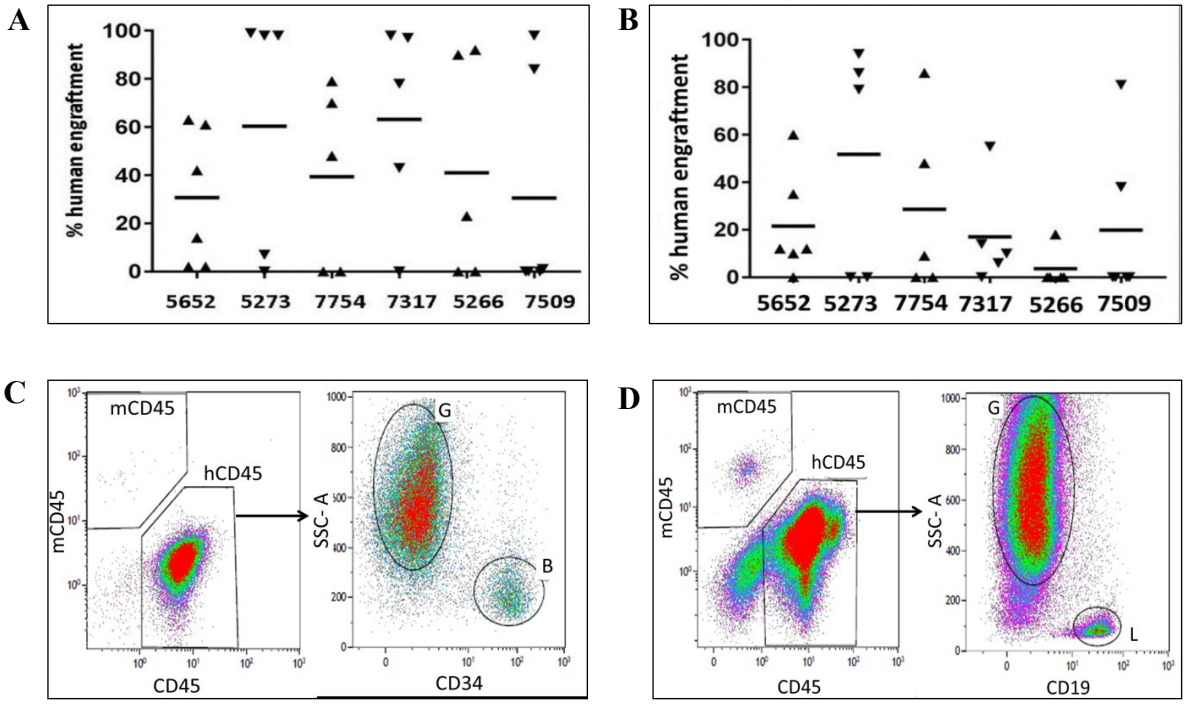
4.2 Xenotransplantation assays to study the engraftment potential of *TP53* mutated AML specimens

A proof of the occurrence of *TP53* mutation in functional preleukemic stem cells as an early event is their detection in hematopoietic cells engrafted in immunocompromised mice. For that purpose, immunodeficient non-obese diabetic/ severe combined immunodeficient/ interleukin-2 receptor-γ null (NSGS) mice was used. These mice do not require any irradiation and have been earlier demonstrated to have improved human engraftment potential (Jan, Snyder et al. 2012, Shlush, Zandi et al. 2014, Buss, Ho 2011). The bulk leukemia cells were transplanted into the tail vein of NSGS mice and analysis performed following 12 weeks. Engraftment was defined by >1% human CD45 cells in both, spleen and bone marrow (Wunderlich, Chou et al. 2010).

Six AML samples with somatic *TP53* mutations were injected into 5-6 mice each. The mean human CD45+ engraftment rate per AML specimen was between 31% and 63% in

bone marrow and 4% and 52% in spleen, respectively (Figure 8A, B). The majority of cells engrafted revealed a blast phenotype. Nevertheless, differentiation into granulocytes was observed in 5/6 (83%) specimens in the bone marrow and 4/6 (67%) in the spleen, respectively. Differentiation into B-lymphocytes occurred in 2/6 (33%) specimens in the bone marrow and 1/6 (17%) in the spleen, respectively (Figure 8 C, D).

Figure 8: Xenograft studies of *TP53* mutated AML specimens
(Figure originally published in Lal et al. Blood 2017)



(A) Engraftment rates in mouse BM. (B) Engraftment rates in the spleen. (C) Leukemic engraftment seen in the hCD45+/hCD34+ population and mature myeloid cells in the hCD45+/hCD33+ compartment. (D)Leukemic engraftment seen in mature myeloid cells in the hCD45+/hCD33+ compartment and mature lymphoid cells in the hCD45+/hCD19+ compartment. Abbreviation: h, human; m, mouse; G, granulocytes; B, blast cells; SSC-A, side scatter A.

In all human cell types engrafted, the patient-specific, somatic *TP53* mutation was detected in up to 100% as assessed by ultra-deep sequencing. With sample no 5652 (c.524G>A, p.R175H) 6 mice were injected, from which engraftment was seen both in the bone marrow and spleen in 4/6 mice. The leukemia specific *TP53* mutational allele frequency observed in the engrafted blast population was 100%. Interestingly, *TP53* mutations were also found in mature granulocytic cells in up to 98%. In sample 5273, 4/5

mice presented a human engraftment in the bone marrow and in 3/5 mice, it was also observed in the spleen. The *TP53* mutant allele frequency found in blast cells was at 52% for the mutation 1 (c.455C>T, p.P152L) and 50.5% for the 2nd mutation (c.711G>A, p.M237I). *TP53* mutations were also found in mature B-lymphocytes at 53% and 51%, respectively. In sample 7754 (c.818G>A, p.R273H), engraftment was detected in 3/5 mice with a *TP53* mutational frequency of 80.3% in blast cells. Here, *TP53* mutations were found in both, mature granulocytes at 73.5% and in B-lymphoid cells at 9%. In sample 7317 (c.681_681dupT, p.D228*), 4/5 mice showed an engraftment in both bone marrow (Figure 9) and spleen (Figure 10). The analysis of the immature blast cells reveals the incidence of the particular *TP53* mutation at a frequency of 51% and in mature granulocytes of 50%. For sample 5266, 3/5 mice presented with a human engraftment in the bone marrow and 1/5 in the spleen. Leukemia specific *TP53* mutations were seen at a frequency of 49.5% for mutation 1 (c.493C>T, p.Q165*) and 55.6% for mutation 2 (c.581T>C, p.L195P), in immature blasts and 51.5% in mature granulocytes. Finally, with sample 7509 (c.469G>T, p.V157F), six mice were injected with unpurified leukemia cells; engraftment was detected in the bone barrow of 3 mice and in the spleen of 2 mice. The *TP53* mutational frequency was observed at 100% in the blast population and in mature granulocytic cells.

These data indicate that somatic *TP53* mutations in AML affect pre LSCs that are capable of engrafting NSGS mice and retain their ability to differentiate into mature blood cells.

Figure 9: *TP53* mutant allelic frequencies in blast, granulocytic and B cell populations from NSGS mice
(Figure originally published in Lal et al. Blood 2017)

No.	Graft composition: bone marrow					
	Blasts		Granulocytes		Lymphocytes	
	Engraftment (%) ± SD	*TP53*^{mut} VAF (%)	Engraftment (%) ± SD	*TP53*^{mut} VAF (%)	Engraftment (%) ± SD	*TP53*^{mut} VAF (%)
5652	93 ± 2.6		3.2 ± 2.16		0	
5273a	12.1 ± 12		0		1 ± 0.5	
5273b						
7754	13.6 ± 3.5		85.3 ± 3.1		1 ± 0.6	
7317	45.7 ± 27.2		52.3 ± 26.7		0	
5266a	24.4 ± 11.4		32.6 ± 21.2		0	
5266b						
7509	74.6 ± 25.8		15.8 ± 11.9		0	
		0 50 100		0 50 100		0 50 100

Horizontal red bars indicate the variant allele frequencies. Abbreviation: VAF, variant allele frequency; SD, standard deviation; No., Sample description.

Figure 10: *TP53* mutant allelic frequencies in blast, granulocytic and B cell populations from NSGS mice in spleen
(Figure originally published in Lal et al. Blood 2017)

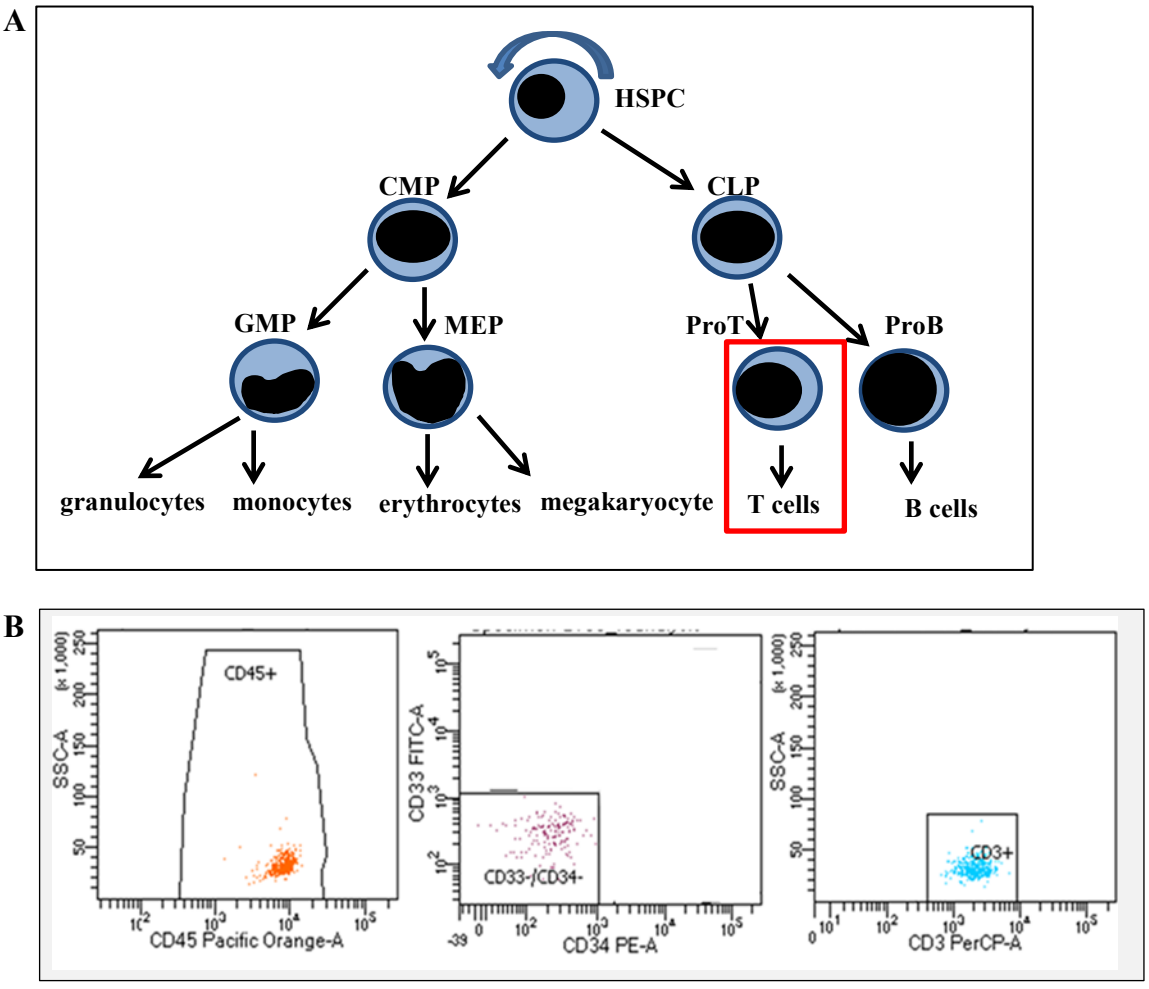
No.	Graft composition: spleen					
	Blasts		Granulocytes		Lymphocytes	
	Engraftment (%) ± SD	*TP53*^{mut} AF (%)	Engraftment (%) ± SD	*TP53*^{mut} AF (%)	Engraftment (%) ± SD	*TP53*^{mut} AF (%)
5652	**32.3** ± 15.8		**0**		**0**	
5273a	**10.9** ± 3.3		**0**		**3.6** ± 3.5	
5273b						
7754	**0.1**		**88.5**		**0**	
7317	**27.9** ± 27.9		**63.2** ± 26.2		**0**	
5266a	**18.6**		**10.3**		**0**	
5266b						
7509	**74.3** ± 12.8		**20** ± 8.3		**0**	
		0 50 100		0 50 100		0 50 100

Horizontal red bars indicate the variant allele frequencies. Abbreviation: VAF, variant allele frequency; SD, standard deviation; No., Sample description.

4.3 *TP53* mutations are present in T lymphocytes from patients with AML

T-cells were used for the approach since these cells originate from HSPCs. If the *TP53* mutations are detected in the T-cells, this would indicate a very early manifestation of these mutations (Figure 11A). T-cells are long lived and are present in an appropriate number in the peripheral blood even of leukemia patients and, therefore, constitute a perfect population for this investigation. They exhibit a $CD3^+$ phenotype and have been previously used in the analysis of preleukemic stem cells as a non tumoral control (Corces-Zimmerman, Majeti 2014).

Figure 11: T cell analysis for the presence of leukemia specific *TP53* mutations



(A) Representative figure of T cell development (adapted from Daser, Rabbitts 2004). (B) FACS sorting of highly pure (CD45+CD3+) T cells for the analysis of *TP53* mutations.

Eight AML patients with confirmed somatic *TP53* mutations were used for sorting of CD45+CD3+ positive T-cells (Figure 11B). Stained cells were sorted twice to achieve a high purity and the post sorting median purity obtained was 99.2% (range 98%-100%). Deep targeted sequencing was performed to identify leukemia specific *TP53* mutations in the samples. Interestingly, in 75% (6 out of 8) of the patients analyzed the identical *TP53* mutation found in the leukemia specimen was detected in the respective T-cells fractions. The frequency of *TP53* mutations in T cells detected was at a 5% median allelic frequency (ranging between 1.3% to 20.7%) (Figure 12). The identification of *TP53* mutations in the non-leukemic T-cell fraction from AML patients further indicates their early occurrence in AML leukemogenesis.

Figure 12: *TP53* mutational frequency detected in highly purified T lymphocytes
(Figure originally published in Lal et al. Blood 2017)

No.	Bulk leukemia		T-lymphocytes	
	TP53 mutation	VAF (%)	Purity(%)	*TP53* VAF (%)
5082	c.395A>G,p.K132R	83.8	98.8	20.7
7754	c.818G>A,p.R273H	33.5	98.6	18.5
2193	c.518T>G ,p.V173G	92.7	100	5.6
8189	c.395A>G ,p.K132R	78.1	98	5
7071	c.587G>C,p.R196P	54.4	98.9	2
	c.394A>T,p.K132*	41.9		1.5
5273	c.455C>T , p.P152L	52.1	99.5	1.3
	c.711G>A , p.M237I	45.8		0
7479	c.646G>A,p.V216M	85.0	100	0
7132	c.734G>A,p.G245S	8.3	99	0

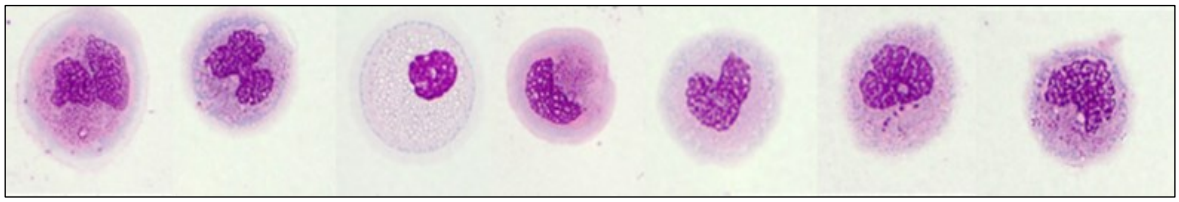
The horizontal red bars indicate the frequency of leukemia specific *TP53* mutations found in T cells. Two samples, namely 7071 and 5273, harbored two *TP53* mutations. Abbreviation: VAF, variant allele frequency; No., Sample description

4.4 Somatic *TP53* mutations are early events of acute myeloid leukemogenesis

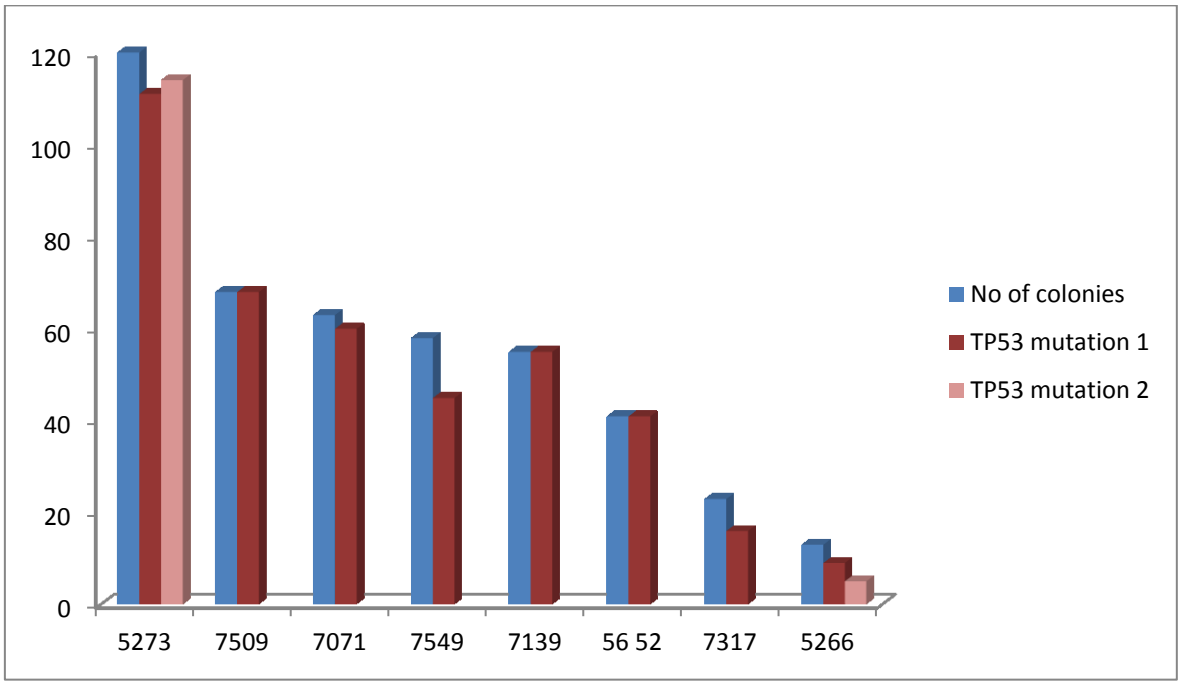
To determine cooperating genetic aberrations of *TP53* mutated AMLs, bulk leukemia cells were analyzed by whole exome sequencing (WES) (n=6) and targeted deep sequencing (n=2), respectively, the latter approach focusing on mutations in the *CEBPA*, *DNMT3A*, *GATA2*, *TET2*, *ASXL1*, *BRAF*, *CBL*, *FLT3*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1* and *WT1* genes. *TP53* and cooperating mutations identified were then assessed in each CFU-GM colony derived from sorted, Lin-CD34^{+}/CD38^{-}/CD99^{-} single cells. The *in vitro* clonogenic assays were performed for three *de novo* AML, three tAML and two sAML samples. A total of 423 CFU-GM colonies were obtained with a median of 58.5 colonies per AML samples (range 11-112). May-Grünwald-Giemsa staining revealed the differentiated status of the cells (Figure 13A). Notably, the *TP53* mutation was observed in the majority of the colonies at a median *TP53* allelic frequency of 97 % (ranging from 45%-100%) (Table 10, Figure 13B).

Figure 13: *In vitro* clonogenic assays

A



B



(A) May-Grünwald-Giemsa staining of one of the CFU-GM colony displaying mature monocytes. (B) Eight samples, 3 denovo AML; 3 t-AML and 2 sAML samples were analyzed. Samples 5266, 5273 & 7071 harbor 2 mutations while the other samples harbor 1 mutation each. The blue bars indicate the total number of colonies obtained; red and pink bars indicate the first mutation and the brick red bars indicate the second *TP53* mutation.

4.4.1 Identification of co-operating mutations in *TP53* mutated AMLs

Interestingly, very few concomitant mutations are found to transpire in patients harboring somatic *TP53* mutations with a median of 1 cooperating mutation per AML sample (range 0-3). The single cells obtained from the colonies were then genotyped by Sanger sequencing (Table 10 and Figure 14).

In sample number 5266, an *ATP1A1* mutation in addition to the *TP53* mutation was identified in the bulk leukemia sample. Single cell genotyping from this sample showed the existence of *ATP1A1* mutation in 6 /11 colonies. *ATP1A1* belongs to the ATPase family and is responsible for ion transport (Lingrel, Orlowski et al. 1990) Mutations in the *ATP1A1* gene with a high frequency have been identified in the CD34+ cells of CML patients nonresponding to imatinib mesylate (Jiang et al., 2010, blood). The analysis discloses that the *TP53* mutation 1 occurs first followed by the *ATP1A1* mutation followed by the second *TP53* mutation.

Sample 7549 harbored 2 mutations in addition to *TP53*, namely *BCL11A* and *SRSF2,* in bulk leukemia cells. Genotyping of single colonies from this sample revealed the *SRSF2* mutation in 38/60 colonies and the *BCL11A* one in 26/60 colonies. The results indicate the first mutation event in *TP53* followed by *SRSF2* and then *BCL11A*.

Sample 5652, was subjected to deep targeted sequencing for the identification of somatic mutations harbored in the bulk leukemia. Three additional mutations were identified, namely *ASXL1*, *RUNX1 and IDH2*. Genotyping of the colonies resulted in the detection of the *ASXL1* mutation in 37/38 colonies, the *IDH2* mutation in 20/38 colonies and *RUNX1* mutation in 19/38. The mutational pattern in this sample signals the presence of *TP53* as the first event followed by *ASXL1*, *IDH2* and lastly *RUNX1* mutations.

In sample 7071, two concomitant mutations in addition to *TP53* were found, namely *FLT3* and *PTPN11*. The colony genotyping led to the detection of *FLT3* in 57/62 colonies and

PTPN11 in 2/62 colonies. In this case, both the *TP53* mutations occur initially followed by *FLT3* and finally *PTPN11*.

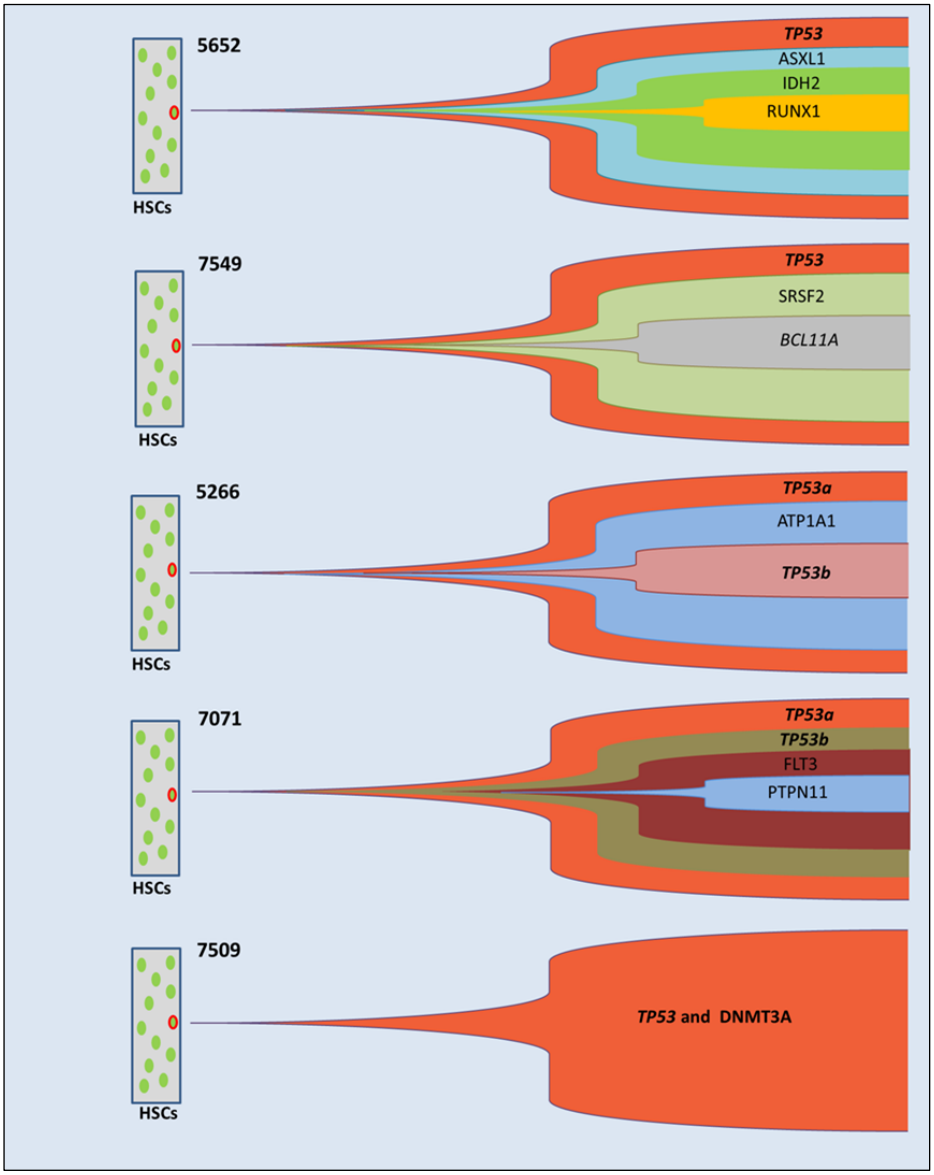
Interestingly in sample 7509, the *DNMT3A* mutation occurred in all the 67 colonies together with the *TP53* mutation. It is well documented that *DNMT3A* is an early lesion and has been proved to be harbored in preleukemic stem cells which is coherent with our finding (Corces-Zimmerman, Majeti 2014). Sample 5273, 7317 and sample 7319 did not show the presence of concomitant mutations.

Table 10: Analysis of co-operating mutations in acute myeloid leukemia with somatic *TP53* mutations

(Table originally published in Lal et al. Blood 2017)

No.	*TP53* mutations		Concomitant mutations	
	Type	Mutated colonies	Type	Mutated colonies
7549	p.E221Gfs*26	44/60 (73%)	***SRSF2*** p.P95L ***BCL11A*** p.P702L	38/60 (63%) 26/60 (43%)
5266	p.L195P p.Q165*	10/11 (91%) 5/11 (45%)	***ATP1A1*** p.N483S	6/11 (55%)
5273	p.M237I p.P152L	107/112 (96%) 106/112 (95%)	None	
7317	p.D228*	18/18 (100%)	***NF1*** p.T676fs	18/18 (100%)
7509	p.V157F	67/67 (100%)	***DNMT3A*** p.Q886R	67/67 (100%)
7071	p.R196P p.K132*	62/62 (100%) 60/62 (97%)	***FLT3*** p.D835H ***PTPN11*** p.D61Y	57/62 (92%) 2/62 (3%)
5652	p.R175H	38/38 (100%)	***ASXL1*** p.G658* ***IDH2*** p.R140Q ***RUNX1*** p.T178T	37/38 (97%) 20/38 (53%) 19/38 (50%)
7139	p.I255N	55/55 (100%)	None	

Figure 14: Genotyping of CFU-GM colonies
(Figure originally published in Lal et al. Blood 2017)



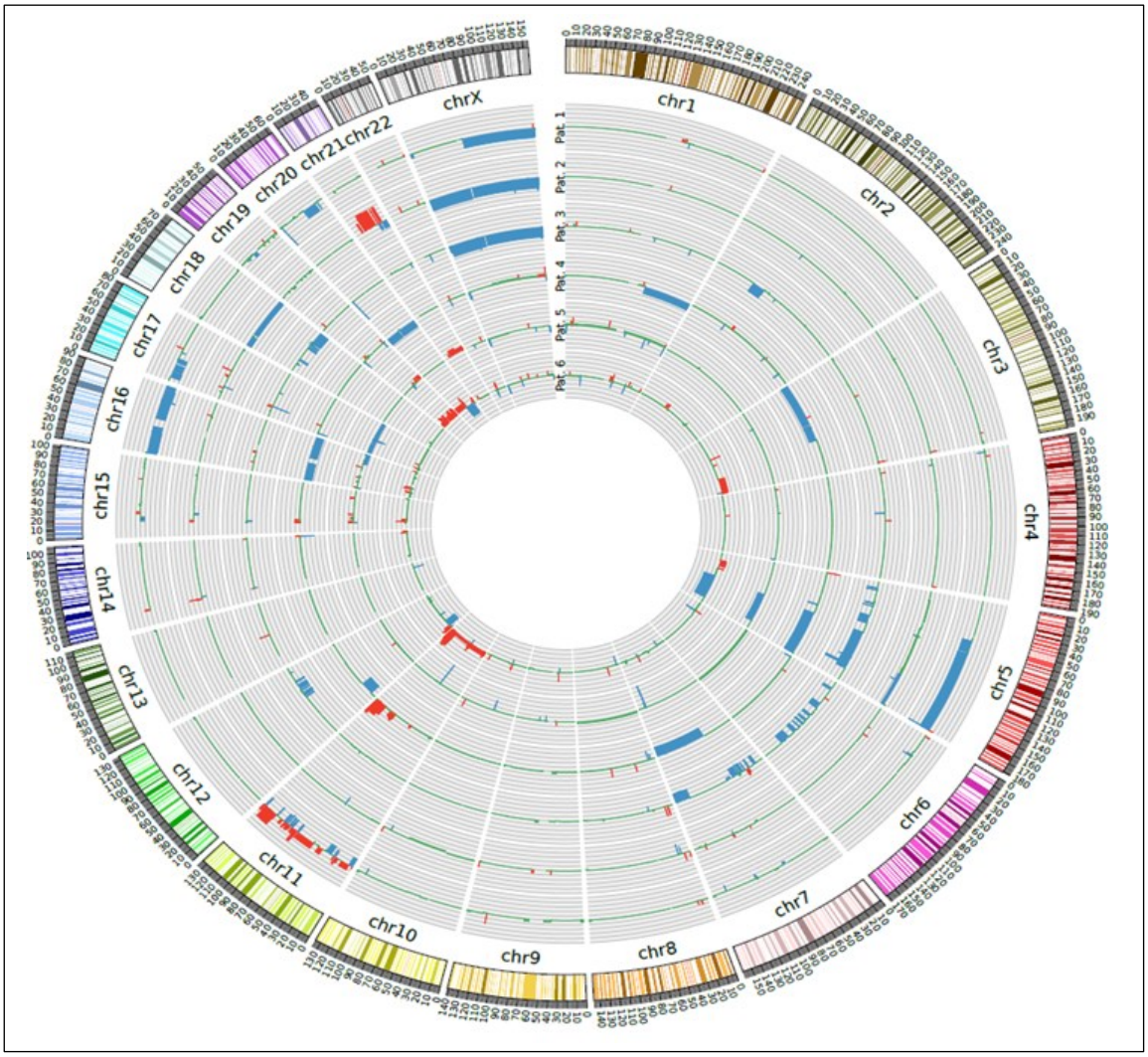
The majority of the samples assessed revealed an occurrence of *TP53* mutation as a founding event during AML leukemogenesis in patients harboring somatic *TP53* mutations.

4.4.2 Identification of leukemia-specific copy number aberrations in *TP53* mutated AMLs.

Many copy number variations were observed in the diagnostic AML samples with losses occurring at a median of 37 (ranging 27-80) and gains occurring at a median of 34 (ranging 21-113) per AML specimen (Figure 15 & Table 11). Losses frequently occurred at

chromosome 5q and 21q while gains were typically observed on chromosome 11q. Copy number alterations are known to occur as a secondary event post *TP53* mutations (Rausch, Jones et al. 2012).

Figure 15: Circos blot of CNVs in selected AML samples
(Figure originally published in Lal et al. Blood 2017)



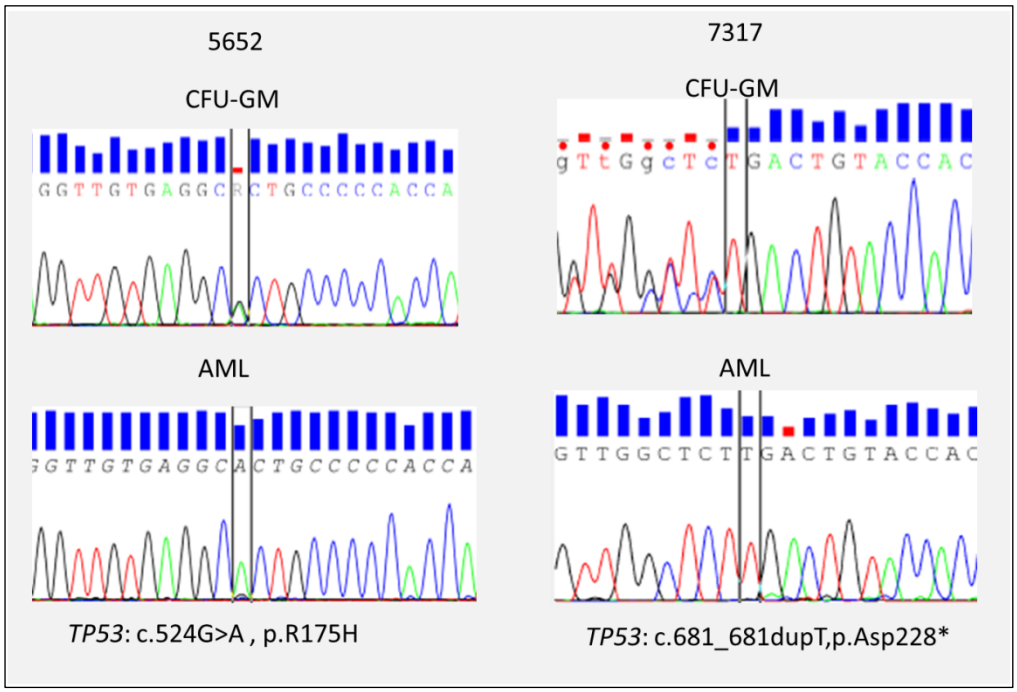
The outermost circle represents different chromosomes. Every circle depicts one AML patient with different losses and gains in different chromosomes. The copy number losses are depicted in blue while the copy number gains in red; the height of the plot depicts the copy number ratio. Abbreviation: Chr, chromosome.

Table 11: Analysis of co-operating mutations and copynumber alterations in acute myeloid leukemia with somatic *TP53* mutations
(Table originally published in Lal et al. Blood 2017)

No.	***TP53* VAF (%)**	***TP53* and concomitant mutations: number of mutated clones**	**Copy number alterations**			
			losses		**gains**	
			n	**length** (bp)	**n**	**length** (bp)
7549	65.9	*TP53*: p.E221Gfs*26 44/60 (73%) ***SRSF2***: p.P95L 38/60 (63%) ***BCL11A***: p.P702L 26/60 (43%)	28	285.789.803	34	32.999.479
5266	44.0	*TP53*: p.L195P 10/11 (91%) ***ATP1A1***: p.N483S 6/11 (55%) *TP53*: p.Q165* 5/11 (45%)	80	402.787.349	21	9.619.914
5273	52.0	*TP53*: p.M237I 107/112 (96%) *TP53*: p.P152L 106/112 (95%)	36	572.663.152	34	58.851.203
7317	88.1	*TP53*: p.Asp228* 18/18 (100%)	52	367.103.483	45	87.828.354
7509	64.0	*TP53*: p.V157F 67/67 (100%) ***DNMT3A***: p.Q886R 67/67 (100%)	38	182.926.187	30	55.245.921
7071	54.4	*TP53*: p.R196P 62/62 (100%) *TP53*: p.K132* 60/62 (97%) ***FLT3***: p.D835H 57/62 (92%) ***PTPN11***: p.D61Y 2/62 (3%)	27	131.583.940	113	316.102.979
5652	89.9	*TP53*: p.R175H 38/38 (100%) ***ASXL1***: p.G658* 37/38 (97%) ***IDH2***: p.R140Q 20/38 (53%) ***RUNX1***: p.V179C fs* 34 19/38 (50%)	na	na	na	na
7139	51.9	*TP53*: p.I255N 55/55 (100%)	na	na	na	na

In sample 7317, a heterozygous *TP53* mutation c.681_681dupT was detected in the CFU-GM colonies obtained from sorted single preleukemic stem cells. Interestingly, loss of heterozygosity was observed at this locus in bulk leukemia cells. Similarly, in sample 5652, *TP53* mutation c.524G>A occurs in a heterozygous state in CFU-GM colonies and in leukemia it is present as a homozygous mutation (Figure 16). These results further strengthen the hypothesis of early occurrence of *TP53* somatic mutations in AML which may induce gross chromosomal aberrations.

Figure 16: Loss of heterozygosity in *TP53* gene
(Part of the figure originally published in Lal et al. Blood 2017)



AML Samples 5652 and 7317 exhibit a heterozygous mutation in GM-CFU, while in the diagnostic AML specimen a homozygous condition is observed. Abbreviation: CFU-GM, colony forming unit granulocyte, monocyte

4.5 *TP53* mutations are early events in secondary AML and therapy-related AML.

Myelodysplastic syndromes are clonal hematopoietic disorder characterised by high risk of transformation into AML. (Jadersten, Saft et al. 2011). *TP53* mutations are frequently found in MDS and MPN patients harbouring a chromosomal 5q deletion and contribute enormously to disease progression and poor prognosis. *TP53* mutations also exhibit a resistance towards lenalidomide treatment (Cazzola, Della Porta et al. 2013, Jadersten, Saft et al. 2011).

The early occurrence of *TP53* mutations in the sAML (14 patients) and tAML (6 patients) cohort was assessed by analysing initial diagnostic material available from MDS/MPN phases. The median transformation time from diagnosis was 218 days (ranging 21-2094). Sanger sequencing or deep targeted sequencing was employed to analyse the presence of mutations in the diagnostic material obtained. Leukaemia specific somatic *TP53* mutations were found to be harboured in 18/20 (90%) patients from whom the early phase samples were available (Table 12). These findings again indicate the early manifestation of *TP53* mutations of sAML or t-AML patients.

Table 12: Analysis of early phases in sAML/tAML patients with *TP53* mutations
(Table originally published in Lal et al. Blood 2017)

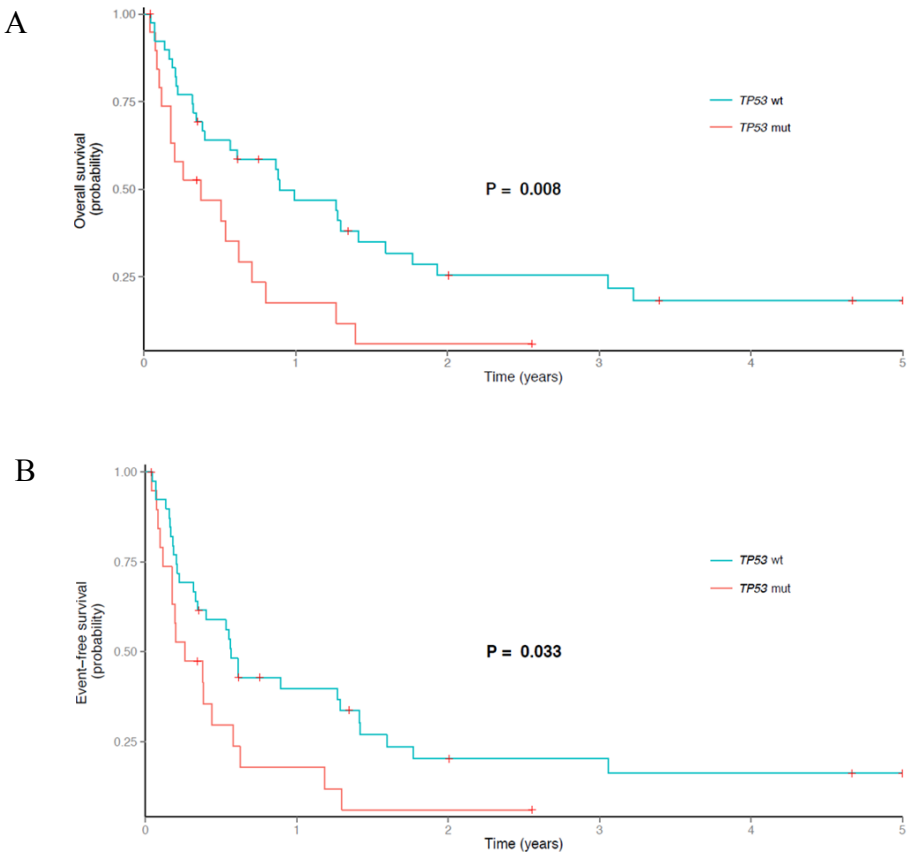
No.	Diagnosis	*TP53* Mutation	AHD	Mutation detectable	Tool	*TP53* VAF	Latency period (days)
7324	sAML	c.559+1G>A, p.?	MDS	Yes	Targeted deep sequencing	44	248
		p.Y234*		No	Targeted deep sequencing	n.a.	
7509	sAML	p.V157F	MDS	Yes	Sanger sequencing	n.a.	21
7071	sAML	p.K132*	MDS	Yes	Sanger sequencing	n.a.	221
		p.R196P		Yes	Sanger sequencing	n.a.	
6512	sAML	p.T125P	MDS	Yes	Sanger sequencing	n.a.	133
7317	tAML	p.D228*	tMDS	Yes	Sanger sequencing	n.a.	320
6216	tAML	p.R273H	tMDS	Yes	Targeted deep sequencing	69.8	78
		p.E286G		Yes	Targeted deep sequencing	7.3	
5970	tAML	p.Y220C	tMDS	Yes	Targeted deep sequencing	32	47
5273	tAML	p.P152L	tMDS	Yes	Sanger sequencing	n.a.	92
		p.M237I	tMDS	Yes	Sanger sequencing	n.a.	
7132	tAML	p.G245S	tMDS	Low detection levels	Targeted deep sequencing	n.a.	575
8196	tAML	p.Q104X	tMDS	No	Targeted deep sequencing	n.a.	38
U02	sAML	p.C176G	MDS	Yes	Sanger sequencing	n.a.	2094
U05	sAML	p.I195N	MDS	Yes	Sanger sequencing	n.a.	269
U09	sAML	p.H179R	MDS	Yes	Sanger sequencing	n.a.	158
U10	sAML	p.R248W	MDS	Yes	Sanger sequencing	n.a.	105
U14	sAML	p.M160I	MDS	Yes	Sanger sequencing	n.a.	224
		p.A161S	MDS	Yes	Sanger sequencing	n.a.	
U15	sAML	p.V173M	MDS	No	Sanger sequencing	n.a.	1644
U25	sAML	c.622_665+20dup,	MDS	Yes	Sanger sequencing	n.a.	215
U39	sAML	p.Q136E	MDS	Yes	Sanger sequencing	n.a.	126
U41	sAML	p.R248W	MDS	Yes	Sanger sequencing	n.a.	391
U43	sAML	p.R248Q	MDS	Yes	Sanger sequencing	n.a.	162

The latency period is calculated between diagnosis of the early phase (MDS) and diagnosis of sAML and tAML, respectively. Abbreviations: VAF, variant allele frequency; MDS, myelodysplastic syndromes; tMDS, therapy-related MDS.

4.6 Prognostic value of somatic *TP53* mutations in AML

CR rates, overall survival (OS) and event-free survival (EFS) were estimated by analyzing 59 AML patients who have been treated with high-dose chemotherapy +/- allogeneic HSCT. The median follow up time for the intensively treated AML patients was 207 days. The CR rate was 72.5% for *TP53* wild-type and 52.6% for *TP53* mutated cases (*P*=0.656), the estimated 5-year OS rate 18% and 0% (*P*=0.008) and the 5-year EFS rate 16% and 0% (*P*=0.033), respectively (Figure 17A, B). In uni- and multivariate analyses, incorporating the *TP53* status, age, white blood cell count and cytogenetic risk group, *TP53* mutations remained the most significant prognostic parameter for both, OS (multivariate, hazard ratio [HR] 2.89 [CI, 1.50-5.58]) and EFS (multivariate, HR 2.32 [CI, 1.23-4.40]).

Figure 17: Survival analysis in AML patients treated with induction therapy
(Figure originally published in Lal et al. Blood 2017)



(A) The figure is depictive of the overall survival analysis in comparison between *TP53* mutated and wild type patients. (B) The figure is depictive of the event free survival analysis in comparison between *TP53* mutated and wild type patients.

To further assess the contribution of *TP53* mutations to relapsed disease, we measured the mutational load in serial samples from the respective AML patients. The median blast count at diagnosis was 42% and 31.5% at relapse/refractory (R/R) phases of AML (Table 13).

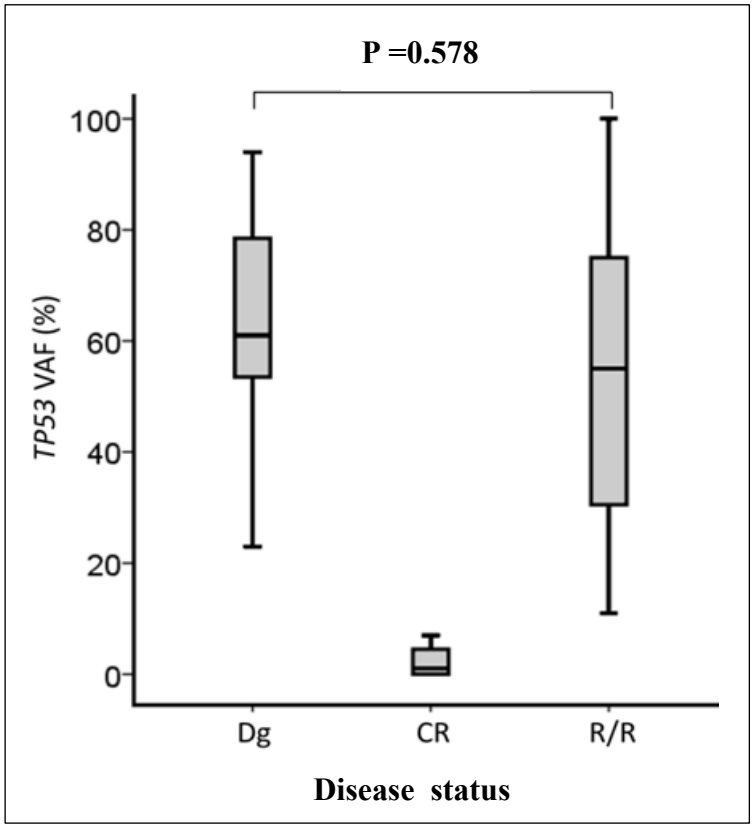
Table 13: Sequential analysis of *TP53* mutated AML samples acquired from different phases during the course of the disease
(Table originally published in Lal et al. Blood 2017)

Pat. No.	No.	*TP53* mutation	AML phase	Blast count (%)	*TP53* VAF (%)
1	7324	c.559+1G>A	Diagnosis	36	49
	7345		Remission	3	2
	7388		Relapse	35	36
2	7549	p.E221Gfs*26	Diagnosis	22	74
	7577		Refractory	28	82
3	8392	c.920-1G>T	Diagnosis	48	94
	8434		Refractory	12	25
4	D3669/09	p.R282_R283insQ	Diagnosis	90	23
	D6320/09		Relapse	53	55
5	D7404/05	p.V274L	Diagnosis	66	61
	D2419/06		Remission	n.a.	0
	D1096/08		Relapse	12	11
6	D7864/07	p.R175H	Diagnosis	77	83
	D1596/08		Remission	n.a.	0
	D3723/08		Relapse	77	68
7	D8658	p.I255T	Diagnosis	30	58
	D8427		Remission	0	7
	D8520		Relapse	98	100

Following induction treatment, the median *TP53* mutant allele frequency decreased from 67.5% at diagnosis to 1% at CR. However, at R/R stages, it rose again to 45.5% comparable to diagnostic levels ($P = 0.578$) (Figure 18). These data confirm and extend previous notions that *TP53* mutations act as mediators of resistant disease in AML.

Figure 18: Sequential sample analysis in AML patients

(Figure originally published in Lal et al. Blood 2017)



Assessment of *TP53* variant allele frequencies from diagnosis, remission and relapse/refractory stages. Abbreviations: VAF, variant allele frequency; Dg, diagnosis; CR, complete remission; R/R relapse/refractory stage.

5. Discussion

The current thesis investigated in its central part the characterization of preleukemic stem cells in AMLs with somatically acquired *TP53* mutations. Preleukemic stem cells have been reported recently as a distinct type of ancestral cells (Jan, Snyder et al. 2012, Jan, Majeti 2013, Kreso, Dick 2014, Corces-Zimmerman, Majeti 2014, Pollyea, Gutman et al. 2014) .The hypothesis was based on clinical observations that *TP53* mutations are driver events in AML leukemogenesis that contribute significantly to relapsed diseases. The results obtained will also play a role in designing therapies targeting mutant p53 or aberrant signaling pathways for attaining improved survival of such patients.

The initial analysis for obtaining the mutational status of the *TP53* gene in 150 selected AML patients revealed a somatic mutation frequency of 21%. Arguably, the *TP53* mutation frequency found here is higher than previously reported in the study conducted by the "The Cancer Genome Atlas" which included 200 samples from patients with *de novo* AML. (Cancer Genome Atlas Research Network 2013). However, AML investigative studies involving tAML and complex karyotype cases revealed a higher occurrence of *TP53* mutations of up to 21% in these subgroups (Schulz, Valentin et al. 2012, Shih, Chung et al. 2013,(Haferlach, Dicker et al. 2008) and the *TP53* mutation frequency found in our cohort can be attributed to the presence of these particular specimens.

The majority of the *TP53* mutations found in our cohort were missense mutations (29/39, 74%) arising predominantly in the DNA binding domain of the gene. Of the 39 mutations identified in our cohort 37 (95%) were found to be clustered in this region of *TP53* which is in accordance with previous literatures (Olivier, Hollstein et al. 2010). The DNA binding domain is a sequence specific region responsible for binding to other genes mediating essential p53 functions. Mutations in this region obliterate its DNA binding capacity thereby contributing to oncogenesis (Gohler, Jager et al. 2005). Six hotspot mutations are located in the DNA binding domain of the gene which are described in many human malignancies including AML (Rucker, Schlenk et al. 2012, Grossmann, Schnittger et al. 2012, Rivlin, Brosh et al. 2011). Mutations arising at the hotspot positions are classified into contact mutations or structural mutants. Contact mutants (R248, R273) alter the structure of the DNA binding domain thereby disrupting direct DNA binding. Structural mutants (R282, R249, G245, R175) bring about contortions generating dents

which interferes in protein conformation affecting its folding ability and, thereby, abrogating the DNA binding ability (Pierre Hainaut, 2010,(Mello, Attardi 2013). Identification of hot spot mutations in 8 of our 150 samples (5 contact mutants and 3 structural mutants) indicates the presence of frequently occurring mutations affecting essential functions of p53.

In AML, *TP53* mutations are often allied with loss of heterozygosity leading to a homo- or hemizygous mutational state (Rucker, Schlenk et al. 2012, Jasek, Gondek et al. 2010) .As reported by Rucker et al in their study involving 234 AML patients exhibiting a complex karyotype, 168 patients harbored *TP53* mutations with 79 (47%) patients harboring homozygous *TP53* mutations and 43 (26%) patients harboring heterozygous mutations in the gene (Rucker, Schlenk et al. 2012). A similar study was conducted by Jasek, Gondek et al demonstrating a *TP53* LOH frequency of 45% in patients with complex karyotypes with 69% of them harboring a *TP53* mutation as compared to patients with a normal karyotype where the *TP53* mutational frequency was as low as 3.1% (Jasek, Gondek et al. 2010). *TP53* mutations showing loss of the remaining wild-type allele render the mutant p53 protein gain-of-function properties (Oren, Rotter 2010). In our study, wild type allelic loss was observed in 5/32 (16%) of our AML specimens only, with hemizygous mutations in 4 samples and a homozygous mutational state in 1 sample, respectively. Interestingly, the remaining 118 wildtype samples did not show any allelic loss at 17p13 at all. Most likely, our analysis was conducted employing conventional karyotyping and FISH analysis thereby owing lower sensitivities of these methods as compared to high-resolution array techniques.

We also identified two germline *TP53* mutations in our cohort of 150 AML samples (1.3%). Germline mutations in the *TP53* gene gives rise to Li-Fraumeni or Li-Fraumeni-like syndrome predisposing an individual to various cancers including hematological malignancies (Varley, Evans et al. 1997). In accordance with a previous report of our group, germline *TP53* mutations in AML show a higher occurrence in patients with tAML following ionizing radiation for a primary malignancy (Zebisch, Lal et al. 2016, Schulz, Valentin et al. 2012). The study involved a cohort of 186 AML patients which included *de novo* AML, sAML and tAML cases. Two of the 186 (1.1%) patients harbored a germline mutation in the *TP53* gene. The analysis was furthermore extended to a tAML specific cohort involving 107 patients with 6 (5.6%) patients identified to be harboring a germline *TP53* mutation. Investigation in mice harboring mutations at the phosphorylation site of

Trp53 demonstrates reduced levels of adult stem cells thereby hampering regeneration (Liu, Ou et al. 2010). Additionally, higher mortality and predisposition towards various tumors were observed in mice harboring *Trp53* germline mutations with an increase in murine bone marrow cells observed. The increase in mouse bone marrow cells was attributed to reduced apoptosis and, obviously, these cells are prone to acquire further mutations (TeKippe, Harrison et al. 2003).

Xenotransplantation studies were employed to investigate whether somatic *TP53* mutations affect preleukemic stem cells in AML. This method has been widely used to investigate the ability of leukemia initiating cells (LICs) towards generation of human AMLs in immunodeficiency mice. The first evidence in this regard was shown using a "Severe Combined Immuno-Deficiency" mouse model (SCID) where LICs on transplantation exhibited homing into the bone marrow and initiated leukemogenesis (Lapidot, Sirard et al. 1994). These mice lacked T-cells and B-cells, however, NK cells were present at a high frequency therefore affecting the engraftment efficiency (Greiner, Hesselton et al. 1998). NOD/SCID IL2RG (NSG) mice were then generated with an interleukin 2 knockout leading to a paucity of NK cells in addition to T- and B-lymphocytes. They are considered as a gold standard in xenotransplantation studies owing to their great engraftment potentials due to diminished natural killer cell activity (Shultz, Ishikawa et al. 2007). Nevertheless, a further improved mouse model (NSGS) was then developed that exhibited greater human myeloid engraftment efficiencies. NSGS mice, in addition, exhibit transgenic expression of human cytokines namely stem cell factor, and GM-CSF, IL-3 (Wunderlich, Chou et al. 2010). Importantly, there is no need for total body irradiation which potentially harms the bone marrow microenvironment, prior to transplantation. Investigation for the engraftment rates between NS, NSG and NSGS mice were performed by injecting 1 million leukemic cells harboring a MLL G12D mutation via tail vein of non-irradiated mice. NSGS mice exhibited significantly higher engraftment rates as compared to the NS and NSG mice, respectively (Wunderlich, Chou et al. 2010, Klco, Spencer et al. 2014). In addition, serial transplantations performed to test the "fitness" of LICs, also revealed similar observations (Wunderlich, Chou et al. 2010). Therefore, NSGS mice prove to be an optimal model to study human leukemia stem cell properties.

The *in vivo* xenograft analysis to study the engraftment potential of the preleukemic stem cells potentially affected by somatic *TP53* mutations was performed in 6 AML samples. Of the samples used, 5 AML revealed a complex karyotype, and for each sample 5-6 mice

were injected with the bulk leukemia cells. The mean human engraftment in mice was assessed by >1% hCD45+ cells (Klco, Spencer et al. 2014). The assessment for the presence of granulocytes was carried out by the occurrence of CD45+CD33+ cells and for B-cells by the presence of CD45+CD19+ phenotype (Jan, Snyder et al. 2012, Wunderlich, Chou et al. 2010) The mean human engraftment obtained in our mice cohort ranged between 31% to 63% in the bone marrow and between 4% to 52% in the spleen with a predominant immature blast population (CD45+CD34). Interestingly, mature granulocytes (CD33+) were detected in the bone marrow of 5 engrafted mice and in the spleen of 4 engrafted mice. A mature B-lymphocytes (CD19+) population was detected in the bone marrow of 2 engrafted mice and in the spleen of 1 engrafted mouse. Importantly, the patient specific *TP53* mutation was detected to as high as 100% frequency in the blast cells using the highly sensitive ultra-deep sequencing technology. Surprisingly, *TP53* mutations were also detected in mature human granulocytes and in B-lymphocytes. The fact of a human engraftment showing patient-specific *TP53* mutations and the differentiation properties of these cells unambiguously demonstrate that the particular mutations affect, indeed, preleukemic stem cells in AML.

To further confirm our results we aimed at assessing T-cells from patients with AML for the presence of specific somatic *TP53* mutations. Recently, the presence of *DNMT3A* mutations in preleukemic stem cells which propagates into T-cells as well as into frank AML was demonstrated by Slush et al. According to their study, T-cells were used as a non-leukemic control but interestingly occurrence of *DNMT3a* mutations was also detected there. In total, 12 AML samples were analyzed in that study revealing a *DNMT3a* mutation in 4 of them. Other mutations such as *FLT3-ITD* were found in the diagnostic AML bulk samples but were absent from T-cell samples implying subsequent mutational events (Shlush, Zandi et al. 2014). In our study, T-cells were isolated from 8 diagnostic AML samples using FACS sorting. The stained cells expressing a CD45+CD3+ phenotype were analyzed for the presence of patient specific somatic *TP53* mutations. The median purity of T-cells obtained was 99%. Targeted deep sequencing revealed an incidence of the patient specific somatic *TP53* mutations of 75% (6/8 of the samples examined). The median somatic *TP53* mutant allele frequency (MAF) was 5% and ranged between 1.3% to as high as 20.7% In all the samples analyzed, the MAF exceeded the impure cell fraction thereby eliminating a bias due to contaminating blast cells. Consequently, identification of leukemia specific *TP53* mutations in the T-cells isolated from AML samples support our

hypothesis that *TP53* mutations are early events in AML leukemogenesis occuring in preleukemic stem cells.

The clonal architecture of the primary AML specimens harboring somatic *TP53* mutations was studied by performing *in vitro* clonogenic assays to understand the progression of HSCs into preleukemic stem cells and further bulk leukemia cells. In a recent study conducted by the Majeti group, they assessed the mutational acquisition pattern in preleukemic stem cells. They proposed a model where serial mutations accumulate in the hematopoietic stem cells leading to leukemogenesis. Six de novo AML samples were employed and preleukemic stem cells carrying a Lin-CD34+CD38-CD99- or Lin-CD34+CD38-TIM3- phenotype was separated and analyzed for presence of mutations. Identification of somatic mutations in the HSPCs which gave rise to AML was demonstrated (Jan, Snyder et al. 2012).We analyzed 8 AML samples with 3 *de novo* AMLs, 3 tAMLs and 2 sAMLs by separating preleukemic stem cells carrying a Lin-CD34+/CD38-/CD90+/CD99- phenotype, culturing and sequencing them. We obtained over 428 colonies from the samples analyzed and detected *TP53* mutations in 401/428 (94%). We then wanted to examine the occurrence of concomitant mutations which in synchrony with *TP53* mutations contribute in the formation of frank leukemia. We identified only a small number of concomitant mutations with an average of 1.0 per AML sample (range, 0-3). Interestingly, in 3 samples no co-operating mutation was identified. This is in line with a study involving 235 AML patients with 33 patients harbouring a *TP53* mutation demonstrating that the incidence of concomitant mutations was also very low with just 1 patient harboring *MLL/PTD* mutation and 1 patient exhibiting a *FLT3* mutation. (Hou, Chou et al. 2015).

Importantly, our analysis unambiguously determined that the majority of leukemia specific *TP53* mutations were initial events followed by the other cooperating mutations. All the cooperating mutations that were identified exist only in the *TP53* mutated colonies. However, *TP53* mutations and *DNMT3a* mutations were found at the same frequencies in CFU-GM colonies obtained. *DNMT3a* is a methyl transferase which modulates the methylation of CpG islands. *DNMT3a* mutations have been previously identified to occur in the preleukemic stem cell populations (Chan, Majeti 2013).

Other mutations identified in our colony assays are *ASXL1, RUNX1, IDH2, SRSF2, BCL11a, FLT3, PTPN11 and NF1.* The *ASXL1* gene belongs to a family of polycomb proteins which play an important role in epigenetic regulation by chromatin remodeling.

They act as transcription repressors thereby playing a vital role in stem cell renewal and mutation in this gene has been demonstrated to confer poor prognosis and are often found to be mutated in myeloid malignancies (Sauvageau, Sauvageau 2010). Mutations in the exon 12 of *ASXL1* have been reported to co-occur with other gene mutations such as *TET2, IDH1, IDH2 and RUNX1* (Gelsi-Boyer, Brecqueville et al. 2012). In sample 5652, *ASXL1* was identified to co-occur with *IDH2* and *RUNX1* mutations.

IDH2 belongs to a family of enzymes which catalyze the conversion of isocitrate to alpha-ketoglutarate. Mutations in the gene lead to the formation of 2–hydroxyglutarate which aids in tumor progression (Larsson, Cote et al. 2013). *RUNX1* belongs to Runt related family and plays a significant role in hematopoiesis, it is also described that *RUNX1* plays a major role in differentiation of myeloid progenitors into granulocytes. Mutations in the gene have been implicated to impede the differentiation of granulocytes causing an upsurge in cell proliferation (Ito 2004). *SRSF2* belongs to family of spliceosome genes responsible in RNA processing by splicing non-coding introns from the coding exons from pre-mRNA therefore mutations in this gene hamper RNA processing consequently giving rise to aberrant mature mRNA (Wu, Kuo et al. 2012, Zhang, Lieu et al. 2015). The *BCL11A* gene is recognized as a crucial controller of hematopoiesis and mutations in the gene leads to improper lymphoid maturation and escalation of proliferation in the cells with *BCL11a* deletions or mutations (Powers, Satija 2015). *FLT3* belongs to the family of tyrosine kinases associated with stem cell proliferation and is found mutated at a high incidence rate in AML (Smith, Levis et al. 2004). However, these mutations occur later in leukemogenesis and can be lost during relapse (Corces-Zimmerman, Majeti 2014). *PTPN11* is a type of tyrosine phosphatase involved in intracellular signaling pathways and hematopoiesis .Mutations in *PTPN11* are found occasionally in AML, however, the majority of mutations in this gene were found in juvenile myelomonocytic leukemias. Mutations in *PTPN11* result in gain of function thereby causing an abnormal stimulation of signaling pathways ultimately leading to increased proliferation and imparting survival advantage in affected cells (Tartaglia, Martinelli et al. 2005). *NF1* is known as neurofibromatosis type 1 gene and encrypts neurofibromin .It is a tumor suppressor gene which is a negative regulator of *RAS* in AML. Therefore, mutations in *NF1* cause overactive *RAS* signaling leading to enhanced cell proliferation ultimately contributing to leukemogenesis (Cutts, Sjogren et al. 2009).

In addition to the gene mutations identified in our AML samples analyzed, a large number of copy number alterations with chromosomal losses and gains were detected including the ones at the *TP53* locus. Whole exome sequencing of bulk leukemia samples revealed alterations with chromosomal losses occurring at a median of 37 (27-80) and gains at 34 (21-113) indicating augmented chromosomal variations. The majority of chromosomal losses were observed on chromosome 5q and 21q and gains were observed on chromosome 11a. This is in line with a previous report describing *TP53* mutations to initiate chromotrypsis, a phenomenon describing catastrophic chromosomal rearrangements (Rausch, Jones et al. 2012). In samples 7317 and 5652 sequencing of CFU-GM colonies and leukemia samples displayed a loss of heterozygosity in the leukemia sample but not in colony DNA indicating that *TP53* mutations are, indeed, initial events followed by allelic losses.

We also aimed at analyzing the *TP53* mutational status in samples obtained from an early phase of secondary and therapy-related AMLs. As endorsed in a study related to MDS patients, *TP53* is implicated in disease progression and transformation into AML. Sub clonal *TP53* mutations with a median clone size of 11% were observed in 18% of the patients analyzed. Interestingly, *TP53* mutations were associated with progression into AML, with 9/10 AML patients exhibiting <2% BM progenitor cells harboring *TP53* mutations. (Jadersten, Saft et al. 2011). A recent study also reported the relationship between *TP53* mutations occurring in AML contributing to disease progression into AML. 85 MDS patients were analyzed for the study and 30 of them exhibited ≥1% of *TP53* mutated BM progenitor cells allied with reduced overall survival and rapid disease progression. An increase in *TP53* levels was observed in 8/21 patients in just 3 months with a reduced overall survival rates (Saft, Karimi et al. 2014). We assessed 20 MDS phase samples from sAML/tAML patients with patient specific AML *TP53* mutations occurring in 18/20 (90%) preceding phases. The median time of transformation into AML in the cohort of *de novo* MDS patients was 218 days. The results from our analysis, therefore, confirm the presence of *TP53* mutations as an early event in AML leukemogenesis thereby further validating the possibility of its role as a driver event.

These data of *TP53* mutations as initiating events are in line with two reports on therapy-related AML showing that the leukemia-specific *TP53* aberration was already present at low levels in normal bone marrow before commencement of cytotoxic treatments for the primary malignancy (Schulz, Kashofer et al. 2015, Wong, Ramsingh et al. 2015). Using a

mouse model with bone marrow chimeric wild-type and *TP53*$^{+/-}$ HSPCs, Wong et al. corroborated their clinical observation by demonstrating that haploinsufficient p53 cells preferentially expanded following exposure to the DNA alkylator N-ethyl-N-nitrosourea. Recently, clinical studies using a case-control design demonstrated that patients with a primary malignancy and clonal hematopoiesis of undeterminate potential (CHIP) at the time of initial cytotoxic treatment and autologous stem cell transplantation, respectively, are at increased risk of therapy-related myeloid neoplasms as compared to those without CHIP. *TP53* mutations were frequently detected clonal aberrations in these patients with the mutant clone expanding substantially over time (Montalban-Bravo, Takahashi et al. 2017, Gillis, Ball et al. 2017, Gibson, Lindsley et al. 2017). Furthermore, *TP53* mutations are among those found in healthy individuals with CHIP who also exhibited an increased risk of developing various blood cancers. (Genovese, Kahler et al. 2014, Jaiswal, Fontanillas et al. 2014).

Serial sample analysis was performed to understand the persistence of somatic *TP53* mutations at relapse. The bone marrow samples from diagnosis, remission and relapse/refractory phases were available from 7 patients. A decrease in the *TP53* mutational load was observed following induction therapy. The median mutant allele frequency at diagnosis was observed to be 67.5% and as low was 1% during remission. Nonetheless, the mutational load increased to 45.5% at relapse/refractory phase of the disease. Our analysis determines that the leukemia specific *TP53* mutations found at the diagnosis are retained in obviously dormant preleukemic stem cells and pinpoint towards the important role of this cell compartment with respect to resistant disease.

We also performed univariate and multivariate analysis to confirm the effect *TP53* mutations has on overall survival and event free survival in AML patients. *TP53* mutations are associated with reduced overall survival. In a study involving 80 AML patients, a reduced overall survival with a median of 4.6 months was observed in patients with *TP53* mutations whereas the median overall survival in *TP53* wild type AML patients was 35.6 months. (Grossmann, Schnittger et al. 2012). Another independent study demonstrated the relationship between *TP53* mutations and poor prognosis. The authors evidenced the occurrence of *TP53* mutations in complex karyotype AML patients with lower event free survival, inferior remission rates and reduced overall survival with no patient surviving beyond 48 months (Rucker, Schlenk et al. 2012).We assessed 59 patients treated with intensive therapy from our cohort of 150 AML patients. Median follow up for the patients

was 207 days. Complete remission was observed in 72.5% of the *TP53* WT cases whereas in *TP53* mutated cases it was 52.6%. The estimated 5 year overall survival and event free survival was 0% in *TP53* mutated patients whereas in WT *TP53* patients it was 19% & 16% respectively. On multivariate analysis, age, WBC count, cytogenetic risk group, *TP53* mutational status were the most important prognostic parameters.

In summary, we have shown that somatic *TP53* mutations in AML characterize preleukemic stem cells employing an *in vivo* mouse model and analyzing primary AML specimens. *TP53* mutations occur early during leukemogenesis with limited cooperating mutations but an abundance of copy number alterations. We also evidenced that *TP53* mutation mediates resistance thereby contribute substantially to relapsed disease in AML. Our results additionally confirm the recent claims that AML with *TP53* mutations should be recognized as a separate disease entity.

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7. Appendix

7.1 List of tables

7.2 List of figures

7.3 Abbreviations

AML	Acute myeloid leukemia
ASXL1	Additional sex comb like genes
ATP1A1	ATPase Na+/K+ Transporting Subunit Alpha 1
CFU-GM	Colony forming unit granulocyte, monocyte.
CR	Complete remission
DNMT3A	DNA (cytosine) methyltransferase 3A
EFS	Event-free survival
ELN	European Leukemia Net
FAB	French-American-British system
GOF	Gain of function
HR	Hazard ratio
HSPCs	Hematopoietic stem and precursor cells
IARC	The International Agency for Research on Cancer
IDH1/2	Isocitrate dehydrogenase 1 and 2
LFL	Li-Fraumeni like syndrome
LFS	Li-Fraumeni syndrome
LOH	Loss of heterozygozity
LSCs	Leukemia stem cells
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasms
NSGS	NOD-scid IL2rγnull mice
OS	Overall survival
POP6	ABI PRISM 310 Genetic Analyzer
POP7	PRISM 3700 DNA Analyzer
R/R	Relapse/refractory
sAML	Secondary AML
SD	Standard deviation
SNP	Single nucleotide polymorphism
SRSF2	Serine And Arginine Rich Splicing Factor 2
TAD	Transactivation domain
tAML	Therapy related AML
TCGA	The Cancer Genome Atlas
TET2	Ten eleven translocation 2
VAF	Variant allele frequency
WES	Whole exome sequencing
WHO	World Health Organization
WT	Wild type