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Contribution: G.M.-B. and C.B.B. designed the study, collected and analyzed data, and wrote the manuscript; S.A.W. performed histopathological analysis and helped analyze data; F.R., T.K., J.C., N.D., K.T., C.D., E.J., G.B., M.K., S.K., and H.K. included patients and wrote the manuscript; S.P. collected and analyzed the data; K.H.Y. and C.B.-R. performed histopathological analysis; K.P. performed all the sequencing studies and participated in writing the manuscript; and G.G.-M. and M.A. designed the study, analyzed data, and wrote the manuscript.

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To the editor:

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

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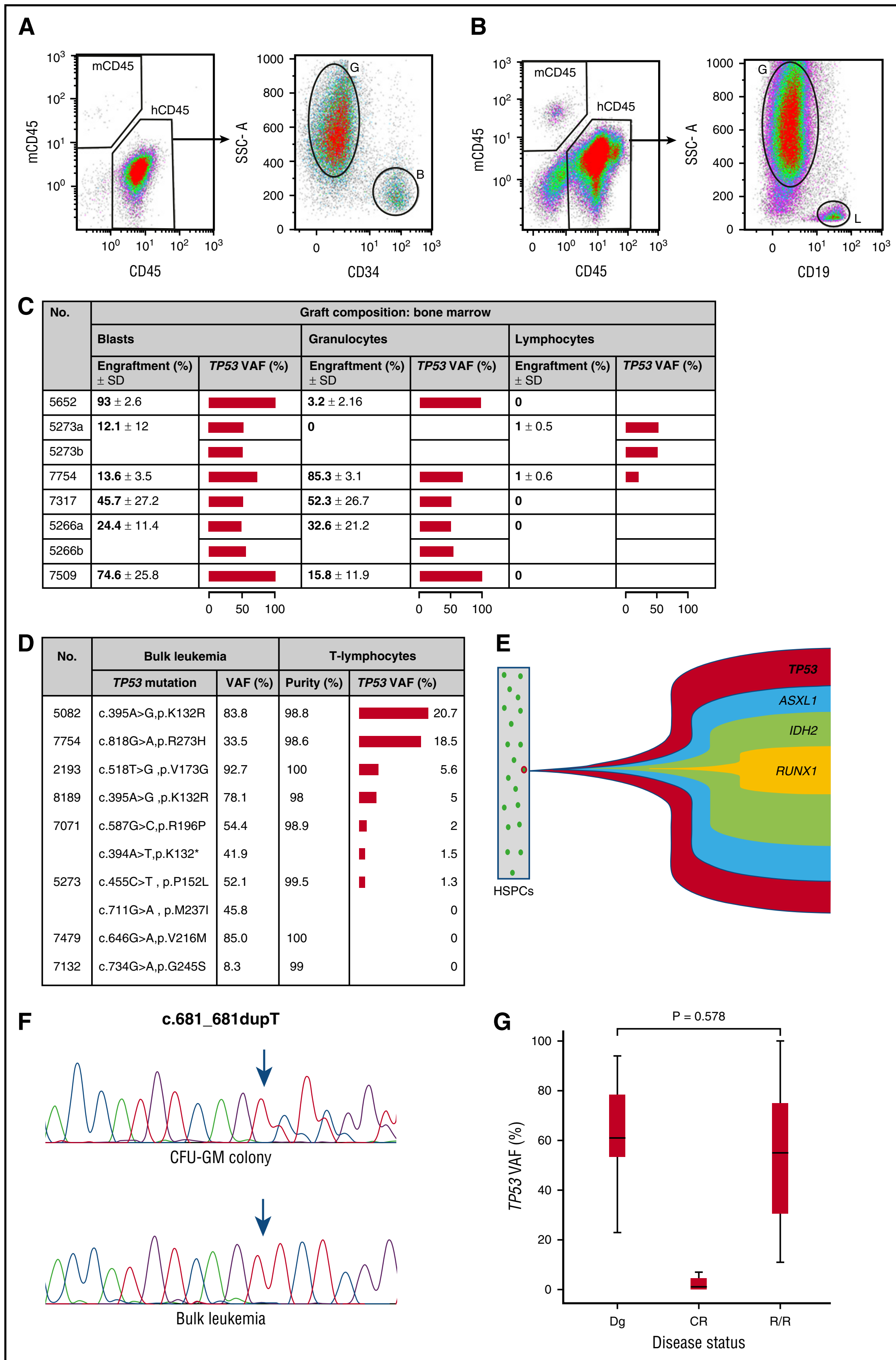
Acute myeloid leukemia (AML) is an aggressive malignancy with a variety of genetic and epigenetic aberrations pinpointing a multistep process of leukemogenesis.[1] It is hierarchically organized, with bulk leukemic cells derived from leukemia-initiating cells that possess self-renewal capacity and are capable of establishing leukemia in vivo.[2] Recently, preleukemic stem cells (preLSCs) have been described in



No.	Graft composition: bone marrow					
	Blasts		Granulocytes		Lymphocytes	
	Engraftment (%) ± SD	*TP53* VAF (%)	Engraftment (%) ± SD	*TP53* VAF (%)	Engraftment (%) ± SD	*TP53* 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**	

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

Figure 1. In AML, somatically acquired *TP53* mutations characterize preleukemic stem cells, are initiating genetic events, and mediate resistant disease. (A) FACS analysis of bone marrow of an NSGS mouse engrafted with 1×10^6 unpurified, human *TP53*-mutated AML cells showing both blast cells as well as maturation into granulocytes. (B) Engrafted human cells with a blast-cell and B-lymphocyte phenotype. (C) Graft composition of mouse bone marrow. Human blasts were characterized by a sideward scatter (SSC) low/CD34$^+$/CD45^{dim}/CD19$^-$ phenotype, granulocytes by a SSChigh/CD33$^+$/CD34$^-$/CD19$^-$, and B lymphocytes by an

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AML. These clonal cells, derived from hematopoietic stem and progenitor cells (HSPCs), initiate the leukemogenic process but retain their ability to differentiate into mature blood cells. Importantly, preLSCs can survive chemotherapy, suggesting that they constitute a reservoir for leukemia recurrence.[3-5]

TP53 is an essential tumor suppressor gene located on chromosome 17p13.1. Germ line *TP53* mutations characterize the Li-Fraumeni syndrome and occur at low frequencies in patients with AML.[6,7] Somatic *TP53* mutations have been detected in up to 20% of AMLs, often associated with a complex karyotype. Most importantly, patients with AML with *TP53* mutations show resistance to intensive treatments, with inferior survival rates.[8-10] On the basis of these facts, "AML with *TP53* mutations, chromosomal aneuploidy, or both" has been proposed as a distinct AML subtype, and testing for *TP53* mutations has been incorporated into the 2017 European LeukemiaNet recommendations.[1,11]

We hypothesized that somatic *TP53* mutations are early leukemogenic events. By transforming HSPCs into preLSCs, *TP53* mutations contribute substantially to the development of AML and its therapeutic resistance.

The study was approved by the ethical committee of the Medical University of Graz (Graz, Austria). Initially, we analyzed 150 diagnostic AML specimens for *TP53* mutations (methods described in the supplemental Data, available on the *Blood* Web site).[6,7] Thirty-nine somatic *TP53* mutations were detected in 32 specimens (missense, 29 [74%]; nonsense, 8 [21%]; and splice site, 2 [5%]), and loss of the wild-type allele was observed in 5 (16%) of 32 (supplemental Table 1; supplemental Figure 1).

To gain insight into the properties of ancestral cells affected by somatic *TP53* mutations, a NOD-*scid* $IL2r\gamma^{null}$ mouse model engineered to express human stem-cell factor, granulocyte-macrophage colony-stimulating factor and interleukin-3 (NSGS mice) was employed (data supplement).[12] Unpurified bulk leukemia cells of 6 *TP53*-mutant AMLs were injected into 5 to 6 mice each, which were analyzed 12 to 16 weeks thereafter. The mean human $CD45^+$ engraftment rate per AML specimen was between 31% and 63% in bone marrow and 4% and 52% in spleen, respectively (supplemental Figure 2A-B). A majority of cells engrafted revealed a blast phenotype. Nevertheless, differentiation into granulocytes was observed in 83% of specimens in the bone marrow and 67% in the spleen. Differentiation into B lymphocytes occurred in 33% of specimens in the bone marrow and 17% in the spleen (Figure 1A-B). Importantly, in all human cell types engrafted, the patient-specific somatic *TP53* mutation was detected in up to 100% as assessed by ultradeep sequencing (Figure 1C; supplemental Figure 2C; supplemental Table 2). To corroborate these findings, highly purified T lymphocytes obtained from patients at AML diagnosis were analyzed (data supplement). As shown in Figure 1D, a median purity of 99.2% (range, 98% to 100%) of $CD45^+/CD3^+$ cells was obtained. Using targeted deep sequencing, the leukemia-specific *TP53* mutation was detected in 75% of specimens at a median variant allele frequency of 5% (range, 1.3% to 20.7%). These data indicate that somatic *TP53* mutations in AML affect preLSCs that retain their ability to differentiate into mature blood cells in both experimental animals and patients with AML. They support a role for *TP53* mutations as early events of acute myeloid leukemogenesis. A number of mutated genes in preLSCs of patients with AML have been described to date, including *DNMT3A*, *TET2*, and *IDH1/2*.[4,5] Interestingly, these genes act as epigenetic modifiers, whereas one of the fundamental roles of p53 is related to cell-cycle control, DNA repair, and apoptosis. Dysregulation of these pivotal functions might be an alternative mechanism in establishing a proleukemogenic state in HSPCs.[13]

Next, we determined cooperating genetic aberrations of *TP53*-mutated AMLs. Bulk leukemia cells were analyzed by whole-exome sequencing and targeted deep sequencing, respectively. Cooperating mutations identified were then assessed in CFU-GM colonies derived from sorted $Lin\text{-}CD34^+/CD38^-/CD99^-$ single cells (data supplement; supplemental Figure 3A). The patient-specific *TP53* mutation was present in the vast majority of colonies (median, 97%; range, 45% to 100%); however, only a paucity of cooperating mutations in cancer gene census genes was detected (median, 1; range, 0-3). Some of them (*DNMT3A*, *IDH2*, *RUNX1*) have been described as early events in AML before. Notably, in all specimens analyzed, cooperating mutations developed sequentially or concomitantly in the *TP53*-mutated clone (Table 1; supplemental Figure 3C). Whole-exome sequencing revealed an abundance of copy-number alterations, with a median of 37 chromosomal losses (range, 27-80) and 34 chromosomal gains (range, 21-113) per sample (Table 1; supplemental Figure 3B). The fact that loss of heterozygosity at the *TP53* locus was shown in bulk leukemia cells but not in CFU-GM colonies (Figure 1F) supports previous concepts of copy-number alterations as secondary events after the onset of *TP53* mutations.[14,15] From 20 patients with secondary or therapy-related AML exhibiting somatic *TP53* mutations, material from antecedent hematological disorders was available. Using Sanger and targeted deep sequencing, respectively, the particular *TP53* variant could be shown in 18 of them (90%; supplemental Table 3). These data showing *TP53* mutations as initiating events are in line with reports on therapy-related AML indicating 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.[16,17] Using a mouse model with bone marrow chimeric wild-type and $TP53^{+/-}$ HSPCs, Wong et al[17] further demonstrated that haploinsufficient p53 cells preferentially expanded after cytotoxic exposure. Recently, clinical studies showed that patients with a primary malignancy and clonal hematopoiesis of undeterminate potential (CHIP) at the time of initial antineoplastic treatment and autologous stem cell transplantation, respectively, are at increased risk of therapy-related myeloid neoplasms as compared with those without CHIP. *TP53* mutations were frequently detected clonal aberrations in these patients, with the mutant clone expanding substantially over time.[18-20] Furthermore, *TP53* mutations are among those found in healthy individuals with CHIP who also exhibited an increased risk of developing various blood cancers.[21,22]

Finally, we assessed the role of somatic *TP53* mutations with respect to resistant disease (data supplement). When analyzing

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Figure 1 (continued) $SSClow/CD34^-/CD33^-/CD19^+$ phenotype. The horizontal bar depicts mean *TP53* variant allele frequencies (VAFs). (D) Highly purified peripheral blood $CD45^+/CD3^+$ cells were obtained at AML diagnosis, and the *TP53* VAFs were assessed using ultradeep sequencing. Samples 7071 and 5273, respectively, exhibited 2 different somatic *TP53* mutations. Note that in each case analyzed and scored positive, the *TP53* VAF exceeded the minute impure fraction of sorted T lymphocytes, thereby excluding results biased because of contamination of AML cells. (E) Data obtained from colony-forming unit–granulocyte, monocyte (CFU-GM) colonies derived from specimen 5652 revealed the *TP53* mutation as the initiating event (positive in 38 of 38 colonies), followed by an *ASXL1* mutation (37 of 38), *IDH2* mutation (20 of 38), and *RUNX1* mutation (19 of 38). All cooperating mutations developed sequentially in the *TP53*-mutated clone. The exact mutation type is shown in Table 1. (F) Loss of heterozygosity at the *TP53* locus of samples from UPN 7317. In CFU-GM colonies, a heterozygous *TP53* c.681_681dupT mutation is shown, whereas in bulk leukemia cells, the wild-type allele was lost, resulting in a hemizygous state. (G) Quantitative assessment of the *TP53* mutational load by the ultradeep sequencing, indicating comparable levels between diagnostic specimens and those obtained at relapsed or refractory (R/R) phase (P = .578 by the exact permutation test for related samples). B, blast cells; CR, complete remission; Dg, diagnosis; G, granulocytes; h, human; L, lymphocytes; m, mouse; SD, standard deviation.

Table 1. Analysis of concomitant genetic aberrations in AML with somatic *TP53* mutations

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

Concomitant mutations were initially determined by whole-exome sequencing and targeted deep sequencing (samples 5652 and 7139), respectively, of bulk leukemia cells and then assessed in CFU-GM colonies derived from sorted Lin-$CD34^+$/$CD38^-$/$CD99^-$ single cells. A total of 423 colonies were assessed, with a median of 58.5 colonies per samples (range, 11-112). Copy-number alterations were assessed by whole-exome sequencing of bulk leukemia cells. The length of gains and losses refers to the total number of alterations observed per sample.

NA, not available; VAF, variant allele frequency of *TP53* as determined by targeted deep sequencing of diagnostic specimens.

59 patients who received intensive treatments for their AML (supplemental Table 4), the estimated 5-year overall survival rates for *TP53* wild-type and *TP53*-mutated cases were 18% and 0% ($P = .008$), and the 5-year event-free survival rates were 16% and 0% ($P = .033$), respectively (supplemental Figure 4A-B), confirming previous reports.[8-10] Ultradeep sequencing of sequential bone marrow specimens from patients with *TP53*-mutated AML at the time of diagnosis, complete remission, and relapsed or refractory disease revealed a decrease of the median *TP53* variant allele frequency from 67.5% at diagnosis to 1% at complete remission. However, at relapsed or refractory stages, it rose again to 45.5%, comparable to diagnostic levels ($P = .578$; Figure 1G; supplemental Table 5).

In summary, we show that somatic *TP53* mutations characterize preLSCs in AML, using both a xenograft mouse model and primary AML specimens. *TP53* mutations represent initiating mutations in this type of leukemia and are mediators of resistant disease in AML. These data add further evidence to recent claims of *TP53*-mutated AML as a distinct disease entity and have implications for the development of targeted treatment approaches.

The online version of the article contains a data supplement.

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Contribution: H.S. designed and supervised the study; R.L., K.L., E.H., P.U., K.A., K.K., E.S., A.R., B.F., S.H., B.R., V.S., M.G.S., A.Z., A.W., and H.S. acquired data; J.M.M., C.T., F.G.R., and K.D. provided patient samples and clinical data; R.L., K.L., E.H., P.U., K.K., E.S., V.S., M.G.S., G.H., K.D., A.Z., A.W., and H.S. analyzed and interpreted data; R.L. and H.S. wrote the manuscript; and all authors reviewed and approved the manuscript.

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