Genome wide molecular analysis of minimally differentiated acute myeloid leukemia

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ABSTRACT

Background

Minimally differentiated acute myeloid leukemia is heterogeneous in karyotype and is defined by immature morphological and molecular characteristics. This originally French-American-British classification is still used in the new World Health Organization classification when other criteria are not met. Apart from *RUNX1* mutation, no characteristic molecular aberrations are recognized.

Design and Methods

We performed whole genome single nucleotide polymorphism analysis and extensive molecular analysis in a cohort of 52 patients with minimally differentiated acute myeloid leukemia.

Results

Many recurring and potentially relevant regions of loss of heterozygosity were revealed. These point towards a variety of candidate genes that could contribute to the pathogenesis of minimally differentiated acute myeloid leukemia, including the tumor suppressor genes *TP53* and *NF1*, and reinforced the importance of *RUNX1* in this leukemia. Furthermore, for the first time in this minimally differentiated form of leukemia we detected mutations in the transactivation domain of *RUNX1*. Mutations in other acute myeloid leukemia associated transcriptions factors were infrequent. In contrast, *FLT3, RAS, PTPN11* and *JAK2* were often mutated. Irrespective of the *RUNX1* mutation status, our results show that RAS signaling is the most important pathway for proliferation in minimally differentiated acute myeloid leukemia. Importantly, we found that high terminal deoxynucleotidyl transferase expression is closely associated with *RUNX1* mutation, which could allow an easier diagnosis of *RUNX1* mutation in this hematologic malignancy.

Conclusions

Our results suggest that in patients without *RUNX1* mutation, several other molecular aberrations, separately or in combination, contribute to a common minimally differentiated phenotype.

Key words: AML-M0, loss of heterozygosity (LOH), *RUNX1*, acute myeloid leukemia.

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Introduction

Minimally differentiated acute myeloid leukemia (AML-M0) is a distinct entity but heterogeneous in manifestation.[1] It accounts for less than 5% of all cases of AML, is mostly seen in elderly patients and has a remarkably poor prognosis.[1-3] AML-M0 blasts show low expression of myeloperoxidase, express at least one myeloid surface antigen (CD13, CD33, CD15) and have immunophenotypic characteristics of early progenitor cells such as expression of CD34, terminal deoxynucleotidyltransferase (TdT), HLA-DR and CD117.[1-3] Co-expression of lymphoid-associated antigens is sometimes seen but does not preclude diagnosis.[4]

The incidence of abnormal karyotypes is more frequent in AML-M0 (71 to 81%) than in other subtypes.[2,3,5] Complex aberrant karyotypes are detected in approximately 20% of AML-M0 cases, and unbalanced chromosomal changes involving -5/del(5q),-7/del(7q), +8, +11 and +13 are the most frequent.[2,3,5] Nevertheless, unlike for other AML subtypes, no characteristic translocation has been described for AML-M0.

The most frequently recurring molecular alterations observed in AML-M0 are mutations in *RUNX1* (alias *AML1*).[6] These are mainly biallelic or dominant-negative point mutations, other than translocations, and are detected in 15 to 35% of cases.[6] *RUNX1* is a transcription factor essential for hematopoiesis that binds DNA through its Runt domain.[7] *RUNX1* is described as both a tumor suppressor gene (TSG), as in AML-M0,[8] and as an oncogene, as it is frequently involved in chromosomal translocations in various hematologic malignancies.[9] Other genes found to be mutated in AML-M0 include *FLT3*, *RAS* and *PTPN11*.[10-14] However, these mutations are considered to be, rather than surrogates, collaborating abnormalities with mutations in transcription factors such as *RUNX1*.[15]

In this study we aimed to identify new molecular alterations that could explain the etiology of AML-M0. Together with cytogenetic and immunophenotypic analyses, we analyzed a unique cohort of 52 AML-M0 samples by whole genome single nucleotide polymorphism (SNP) screening to reveal regions of loss or gain that could contain putative TSG or oncogenes. We also performed extensive mutation analysis in genes known to be mutated frequently in AML.

Design and methods

Patients' material

This study was performed with archived material from diagnostic cryo-preserved bone marrow aspirates from 52 patients (numbered from 1 to 9 and 18 to 60), classified morphologically and immunophenotypically as having AML-M0 (*presented in Table 1 and in an Online Supplementary file*). Material was collected from the medical centers at the Robert-Rössle-Clinic, Charité Berlin, Germany; University of Leiden, the Netherlands; University of Groningen, the Netherlands; Erasmus University, Rotterdam, the Netherlands; and University of Vienna, Austria. All samples were handled in a coded fashion and research was conducted following the medical ethical guidelines of the national organization of scientific societies (FEDERA; *Code for Proper Secondary Use of Human Tissue in The Netherlands*). Pure tumor cell populations were sorted by flow cytometry from mononuclear cells isolated from bone marrow or peripheral blood at the time of diagnosis.[8] T cells from each sample (with the exception of those from patients 38 and 49) were expanded using previously described conditions as a source for control material.[8,16] DNA was isolated from the sorted tumor cells and T cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from the mononuclear cell fraction using the QIAamp RNA Blood Mini Kit (Qiagen). cDNA was prepared using SuperScript® First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR) (Invitrogen, Breda, the Netherlands).

Karyotyping

Cytogenetic analysis was performed on GTG-banded chromosomes and the karyotype was assigned according to the criteria of the International System for Human Cytogenetic Nomenclature (ISCN).[17] A complex aberrant karyotype is defined in this study by at least five abnormalities.

Single nucleotide polymorphism analyses

SNP analysis was performed using the GeneChip Mapping 10k 2.0 array (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Briefly, 250 ng of total genomic DNA was digested with XbaI restriction enzyme and ligated to a universal adapter. The ligated fragments were then PCR-amplified using primers complementary to the universal adapters. PCR products were purified, fragmented by DNase I, labeled with biotinylated dATP and hybridized to the array. The arrays were scanned using a GeneChip Scanner (Affymetrix). Affymetrix GeneChip 5.0 genotyping software was used to examine the SNP hybridization patterns and to make SNP calls. The resulting data were analyzed with the dChip software package.[18]

Mutation screening

RUNX1, *FLT3* internal tandem duplications (ITD) and *FLT3* D835 mutation screening was performed as previously described.[8,19] *KIT* D816V mutations (exon 17) were screened using the HinfI restriction assay.[20] All patients were screened for these mutations.

Melting curve analysis was performed for all patients to detect mutations in *NRAS* (codons 12/13 and 61), *KRAS* (codon 61), *JAK2* (codon 617), *PTPN11* (exons 3 and 13), *PTPN6* (exons 3 and 13) and *SPI1* (exons 1 to 4). The above mentioned regions were amplified in reactions containing LCGreen PLUS (Idaho Technology, Salt Lake City, UT, USA) using the primers and conditions described in the *Online Supplementary Appendix*. Subsequently, melting curves of the PCR products were generated in a LightScanner HR 96 (Idaho Technology). Aberrant melting curves were subjected to DNA sequencing to confirm mutations, as previously

described.[8] Standard PCR techniques were used to amplify *RUNX1* (exons 6 to 8), *NRAS* (codon 61), *CEBPA, CEBPD, SPI1* (exon 5), *TET2, PURA, PILRA, PILRB* from genomic DNA and *RUNX1, CSF2RA* and *IL3RA* from cDNA (*Online Supplementary Appendix*). Subsequent DNA sequencing was performed as previously described.[8] *CEBPA* and *CEBPD* sequencing reactions included, in addition to standard reagents, 1.6 M of betaine (Sigma-Aldrich, Saint-Louis, MO, USA).

NPM1 exon 12 and *CEBPB* mutations were studied by fragment analysis. After amplification (the conditions and primers are presented in the *Online Supplementary Appendix*), the PCR products were analyzed on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with GeneScan 500 ROX (Applied Biosystems) as a size standard. Detected mutations were confirmed by sequencing.[8]

The tumor specificity of mutations detected in genomic DNA of AML tumor cells was confirmed by sequencing the respective control DNA (expanded T cells) for the absence of mutations.

Results

Minimally differentiated acute myeloid leukemia shows karyotype heterogeneity

The cohort of 52 patients was not selected for any cell-biological feature other than the diagnosis of AML-M0. The cohort had a median age of 61 years and consisted of 47 *de novo* cases, 1 therapy-related case and 4 cases of secondary leukemia (Table 1).

Karyotypes were available for 49 cases (Table 1). Six (12%) cases had complex aberrant karyotypes, 14 (29 %) cases were normal and 17 (35%) cases were associated with a single abnormality. The remaining 12 cases carried two or three chromosomal abnormalities. The predominant single abnormalities were trisomy 13, trisomy 8 and monosomy 7. Overall the most frequent abnormalities detected were del(5q), trisomies 8 and 13 and monosomy 7/ del(7q) (Table 3).

We also detected several chromosomal abnormalities that might involve known transcription factors, including the del(16)(q22) (patient 42), frequently associated with *CBFB*, the inv(3)(q21q26) and t(2;3)(p23;q27) (patients 51 and 4), associated with *EVI1*, and the previously reported translocations involving *ETV6* in cases 2, 9 and 43 (Table 1).[21]

Single nucleotide polymorphism array analysis revealed several potential new regions involved in minimally differentiated acute myeloid leukemia

We compared DNA isolated from flow-sorted leukemic cells to control DNA of the same patients using the Affymetrix GeneChip 10K mapping array. Loss of genetic information, detected as loss of heterozygosity (LOH) and/or copy number changes, was found in several chromosomes (Tables 1 and 2). Twenty-three regions were found to be affected in 37 AML-M0 patients. Most patients had losses or gains additional to those found by karyotype analysis. Interestingly, of the 14 patients with a normal diploid karyotype, nine displayed LOH on at least one chromosome (Table 1).

LOH was most frequently observed in chromosomes 21, 17, 7 and 5. As regards chromosome 21, copy neutral LOH (also known as uniparental disomy [UPD] or [partial] isodisomy) was found in 14 cases and a hemizygous deletion in 3 cases (Figure 1A). Homozygous deletions of the region on chromosome 21 harboring the tumor suppressor gene *RUNX1* were detected in three of the 17 cases with LOH (Table 2, Figure 1A). LOH in chromosomes 5 (9 patients) and 7 (9 patients), in contrast to that in chromosome 21, was mainly due to deletions. In chromosome 17 the minimal regions of overlap in LOH between the patients comprised two separate regions, one including *TP53* and the other *NF1* (Table 2, Figure 1B). LOH was also detected in chromosomes 3 (5 patients), chromosome 4 (4 patients) and chromosome 12 (4 patients). LOH in chromosome 12 resulted from a hemizygous deletion that included the *ETV6* locus, as previously reported.[21] Microdeletions (smaller than 2 Mb) were present in three patients, affecting chromosomes 3, 4, 8 and 11 (Table 2). The remaining examples of LOH were restricted to a limited number of patients (Table 2). In addition, two cases showed gain of genomic regions in chromosomes 3 and X/Y (Table 2).

Mutation analysis of candidate genes did not reveal new targets

Following the results of the SNP analysis we screened several candidate genes, located within the minimal common regions of LOH or gain, for mutations. Genes were selected based on their potential relevance for leukemia, and included *PURA, PILRA, PILRB, TET2* and *CEBPD* for regions of LOH, and *IL3RA* and *CSF2RA* for a region of amplification near the pseudo-autosomal region of chromosomes X and Y (Table 2). In most cases, mutation screening was restricted to the samples showing LOH or copy number gain (*Online Supplementary Table S2*). No mutations were detected in any of these candidate genes. However, *CSF2RA* and *IL3RA,* which are involved in hematopoietic development,[22] were shown to be highly up-regulated by gene expression microarray analysis (*data not shown*).

RUNX1 mutations in minimally differentiated acute myeloid leukemia are not restricted to the RUNX1 runt domain

Mutations in *RUNX1* were detected in 18 patients (35%) (Table 1 and *Online Supplementary Table S1*). In concordance with the SNP analysis, 13 of these mutations were homozygous/biallelic (Table 1 and Figure 1A). Patient 38 had a homozygous mutation but without detectable LOH (Table 1). The most common *RUNX1* mutations found in exons 3, 4 and 5 were base substitutions in or near to the DNA binding runt domain (Table 1). We also detected four insertions that resulted in truncated proteins as well as one insertion/duplication and one deletion. In addition, exons 6, 7 and 8 of *RUNX1* were sequenced in samples for which a mutation was expected based on the LOH results and other analyses. In two samples (from patients 26 and 41) we found a frame shift mutation. In only one patient (patient 52) with UPD (Figure 1A), a *RUNX1* mutation remained

Table 1. Molecular, clinical, immunological and cytogenetic features of the patients.

Patient status	Age (years)	AML-M0	Karyotype[j]	Other chromosomes[j]	Mutations in transcription factors[a]	Other mutations[a]	Inferred genes	Immunophenotype (%) of cells CD34	CD33	MPO	TdT
1[b,d]	65		46,XX,i(17)(q10), del(20)(?)	12		JAK2 V617F; PTPN11 E76K	*ETV6*[e]; *TP53*[e]		71	71	0,5
2[b,d]	67	Secondary (PV)	47,XX,t(4;12)(q12;p13),-21, +2r(21)	4, 22	ETV6 t(4;12)[d]	NRAS Q61H; PTPN11 D61H	*RUNX1*[e]	95	5	0,5	0,4
3[b]	43		46,XX					86	50		79
4[b]	68		46,XY,t(2;3)(p23;q27)	3, 4			*EVI1/MDS* t(2;3)[g] + hemizygous dele	50	44		0
5[b]	61		Complex	Complex		PTPN11 D61Y	*TP53*[e]	58	67		0
6[b,d]	37		46,XY	17	ETV6 [S107fs]+[V345_Y346insR][d]		*NF1*[e]	56	70	10	3
7[b,c]	68		47,XY, +13, i(17)(q10)	1, 21	**RUNX1 W79C**		*FLT3*[f]; *TP53*[e]	74	3		83
8[b]	68		46,XX	5, 17			*NF1*[e]	0	84		12
9[b,d]	47	Secondary	Complex	Complex		KRAS G12D	*ETV6*[e]	77	74	1	1
18[b]	83		45,XY,-7, del20(q1?2)	21	**RUNX1 del**			91	35		87
19[b]	68		46,XY	4, 21	**RUNX1 del**	FLT3 ITD		75	10		81
20	73		47,XY,+8	3				97	86	6	10
21[d]	59		46,XY	17	ETV6 R360Xd			95	5	1	2
22	77		47,XY,+9					93	42	2	95
23	62	Secondary (PV)	46,XY	9		**JAK2 V617F**		60	77	2	6
24	5		45,XY,-7	21	**RUNX1 R139Q**	NRAS G13D		89	70	74	58
25	59		Complex	Complex			*RUNX1*[e]; *TP53*[e]; *NF1*[e]	91	69	1	7
26	65		47,XY,+8	7, 21	**RUNX1 A297fs**			76	39	5	58
27[c]	58		n.d.	21	**RUNX1 R80H**		*FLT3*[f]	86	18	3	76
28	69	Secondary (MDS)	47,XY,+8		RUNX1 D48Y	JAK2 V167F		88	35	1	13
29	45		46,XX,del(7)(q22)	3		FLT3 ITD		76	72	12	22
30[c]	55		47,XY,+13	21	**RUNX1 W79C**	PTPN11 G60V	*FLT3*[f]	89	21	4	27
31[c]	68		96,XXYY,+13,+13,der(17) t(16;17)(p11;p11)x2,+19,+19		RUNX1 A115fs		*FLT3*[f]; *TP53*[e]	92	56	4	45
32[c]	64		47,XY,+13	21	**RUNX1 D171V**		*FLT3*[f]	91	5	3	68
33	31		Complex	Complex			*RUNX1*[e]; *TP53*[e]	71	71	8	1
34	22		46,XY,t(11;19)(q13;p13)			FLT3 ITD		96	98	6	0
35	35		46,XY	8	CEBPA [D69fs (+) K313dup]	NRAS G13D	*CEBPD*[e]	94	95	96	16
36[c]	65		47,XY,+13		RUNX1 R142fs		*FLT3*[f]	94	11	0	83
37	74	Therapy related	Complex	Complex	**RUNX1 M106fs**			82	33	12	77
38	64		n.d.		**RUNX1 [L55fs (+)** V128_A129insEY_E111_V128dup]	FLT3 ITD		45	68	8	0
39	74		47,XX,+14	5, 7, 21	**RUNX1 del**			89	54	4	41
40	49		46,XY					56	8	3	0
41	51		46,XY		RUNX1 R319fs			78	67		44
42	57		47,XX,del(16)(q22), +21			FLT3 D835V	*CBFB* del(16)(q22)[g]	70	18	3	1
43[d]	64		46,XY, t(4;12)(q12;p13)		ETV6 t(4;12)[d]			71	51	8	81
44	60		45,XY,-7	5		PTPN11 E76V		60	20	0	
45[d]	29		n.d.	Complex			*ETV6*[e], *NF1*[e]	90	55		
46[c]	81		47,XX,+13,16qh+c				*FLT3*[f]	90	0	2	
47	75		46,XX	21	**RUNX1 K83N**			85	0	0	
48	71		46,XY					95	2	6	
49	78		47,XX,+8			FLT3 ITD		95	0	1	
50	86		46,XX,t(9;11)(q34;p11.2)	4, 11, 19				75	15	2	
51	33		46,XY,inv(3)(q21q26)			FLT3 ITD	*EVI1* inv(3)(q21q26)[g]	95	60	1	
52	24		46,XX,hexaploid	21		FLT3 ITD	*RUNX1*[h]	95	1	0	
53[c]	86		46,XY,der(13)t(13;21)(q32~34;q22),+der(13),-21	21	**RUNX1 del**		*FLT3*[f]	70	32	8	
54	47		Complex	complex			*NF1*[e]	97	84	0	
55	62		46,XY	7, 21	**RUNX1 V105fs**			88	46	0	49
56	49		49,X,idic(Y)(q12)x3	X/Y		NRAS Q61R		93	94	1	0,1
57	57		50,X,idic(Y)(q12)x4					2	80		0,1
58[d]	50		47,XY,idic(21)(q10), +add(21)(p1?1)	19	ETV6 F103fsd	FLT3 ITD		91	83	1	
59	40		46,XY			NPM1 W288fs; KRAS G12D		1	87	2	1
60	59		46,XX	5		FLT3 ITD; PTPN11 E76Q		80	94	4	1

[a]mutations in bold are homozygous; [b]published in Silva et al. (2003)[8]; [c]published in Silva et al.,(2007)[19]; [d]published in Silva et al. (2008)[21]; [e]hemizygous locus loss detected by SNP analysis; [f]:FLT3 over expression associated with trisomy 13 (Silva et al., 2007)[19]; [g]inferred from cytogenetic data; [h]inferred from UPD; [i]in case of multiple karyotypes the one confirmed by copy number analysis is shown; [j]chromosomes with LOH or copy number changes detected by SNP analysis only, see table 2 for complete information; n.d. : not done; PV : polycythemia vera; MDS: myelodysplastic syndrome.

Table 2. Summary of minimal common regions of chromosomal losses, gains and uniparental disomy detected by LOH and copy number analyses.

Chrom. region	Aberration[d]	Patient(s)[e]	Proximal SNP	Distal SNP	Size (Mb)	Candidate genes
1 p32.2-pter (36.33)	UPD	7	rs1926910	telomere	~56.041	
3q25.33-q26.2	UPD (2), Loss (2)	5, **20, 29,** 45	rs958985	rs721128	10.088	*IL12A*, *KPNA4*, *NMD3*, *PDCD10*
3q26.2	Loss	4	rs1488106	rs1920116	0.721	*EVI1*, *MDS*
3q26.2-qter (29)	Gain	29	rs974944	telomere	~29.135	
4q24	UPD (2), Loss (1)	2, 4, **19**	rs1528382	rs1374530	1.314a	*TET2*[b]
4q31.22-qter (35.2)	UPD	**4, 19, 50**	rs720485	telomere	~45.271	
5q31.2-q32	UPD (1), Loss (8)	5, 8, 9, 25, 33, 37, **39,** 44, 60	rs2351463	rs724603	7.435	*PURA*[b]
7q32.1-qter (36.3)	UPD (2), Loss (7)	5, 18, 24, **26,** 29, 39, 44, 45, **55**	rs721691	telomere	~29.980	*CDC26*, *FAM40B*
8qcen(11.1)-q11.21	Loss	35	centromere	rs1384217	~2a	*CEBPD*[b], *MCM4*
9p21.2-pter(24.3)	UPD	**23**	rs721672	telomere	~25.733	*JAK2*[b]
11q12.2-q13.2	UPD, Loss	**25,** 50	rs1593480	rs1938684	9.168	
11q14.1-q14.2	UPD, Loss	25, 50	rs62388	rs1378879	1.533	
12p13.31-p13.2	Loss	1, 9, 33, **45**	rs747726	rs252028	3.257	*ETV6*[b,f]
16q21-q23.1	Loss	42	rs588037	rs725710	11.489	*CBFB*
17p13.1-p13.2	Loss	1, 5, 7, 25, 31, 33	rs1379867	rs724809	5	*TP53*
17q11.2	Loss	6, 8, 25, 45, 54	rs719601	rs1394385	2.958a	*NF1*
17q21.31-qter(q25.3)	UPD	**21, 45, 54**	rs1981998	telomere	~35	
19q12-qter(13.43)	UPD	**50**	rs9304866	telomere	~30	
19p13.2pter(23.3)	UPD	**58**	rs2009518	telomere	~9.661	
20q11.23-q13.2	Loss	1, 18, 33	rs910760	rs2208006	14.207	
21q22.12	UPD (13), Loss (4)	2, **7, 18, 19, 24,** 25, **26, 27, 30, 32,** 33, **37, 39, 47, 52,** 53, **55**	rs2409561	rs1573304	1.042c	*RUNX1*[b,c]
22q11.21-qter(q13.33)	UPD	**2, 9, 25**	rs878825	telomere	~28.609	
X/Yp22.33	Gain	56	rs9334	telomere	2.832	*CSF2RA*[b], *IL3RA*[b]

Chromosomal regions presented, proximal SNP, distal SNP and size refer to the minimally common regions between patients. Trisomies or monosomies were not considered for this table. Patients showing complex aberrant karyotypes were not used to define minimally common regions with exception of 12p13 and 17p13. [c]confirmed by MLPA analysis; [b]gene screened for mutations; [c]defined by homozygous deletions; [d]UPD equals copy-neutral LOH, Loss equals hemizygous deletion; [e]patients with UPD in bold; [f]published in Silva et al., (2008)[21].

undetected. In addition to patients 18, 39 and 53 (Figure 1A, Table 2), *RUNX1* appeared to be homozygously deleted in patient 19.[8]

Mutations in other acute myeloid leukemia- associated transcription factors are infrequent

We screened all patients for mutations in *CEBPA*, *CEBPB* and *SPI1*. We found biallelic *CEBPA* mutations in patient 35 (Table 1 and *Online Supplementary Table S1*). No mutations were detected in *CEBPB* or *SPI1*. All patients were also screened for exon 12 insertions in *NPM1*. Patient 59 showed a four nucleotide insertion (Table 1 and *Online Supplementary Table S1*). This patient did not express CD34 and had a normal karyotype, as reported in other cases with *NPM1* mutation.[23]

RAS-related genes are frequently mutated in minimally differentiated acute myeloid leukemia

We detected 25 mutations in 22 patients (42%) in genes related to the RAS and JAK signaling pathways (Tables 1 and 3; *Online Supplementary Table S1*). *FLT3* mutations were the most frequent mutations, i.e., nine *FLT3* ITD and one *FLT3* D835. Activating mutations in *RAS* genes were present in six patients (11.5%), two in *KRAS* and four in *NRAS*. *PTPN11* mutations were observed in 6 patients (11.5 %). Activating mutations in codon 617 of *JAK2* were found in three patients, two of whom had a previous history of hematopoietic disorders. In patient 23, the *JAK2* mutation was homozygous, in line with UPD detected in chromosome 9 (Table 1). *PTPN11* mutations coexisted with *RAS, FLT3* and *JAK2* mutations in one case each (Table 1).

We did not find any mutations in exons 3 or 13 of *PTPN6*, a *PTPN11*-related gene, or in *KIT*. A summary of the results is presented in Table 3 and detailed information on the detected mutations is given in *Online Supplementary Table S1*.

RUNX1 mutations are associated with terminal deoxynucleotidyl transferase expression in minimally differentiated acute myeloid leukemia

Data on protein expression of TdT were available for 40 cases (Table 1). Twenty-two (55%) cases demonstrated high TdT expression (>10% of cells). Notably, we found a strong association between high protein expres-

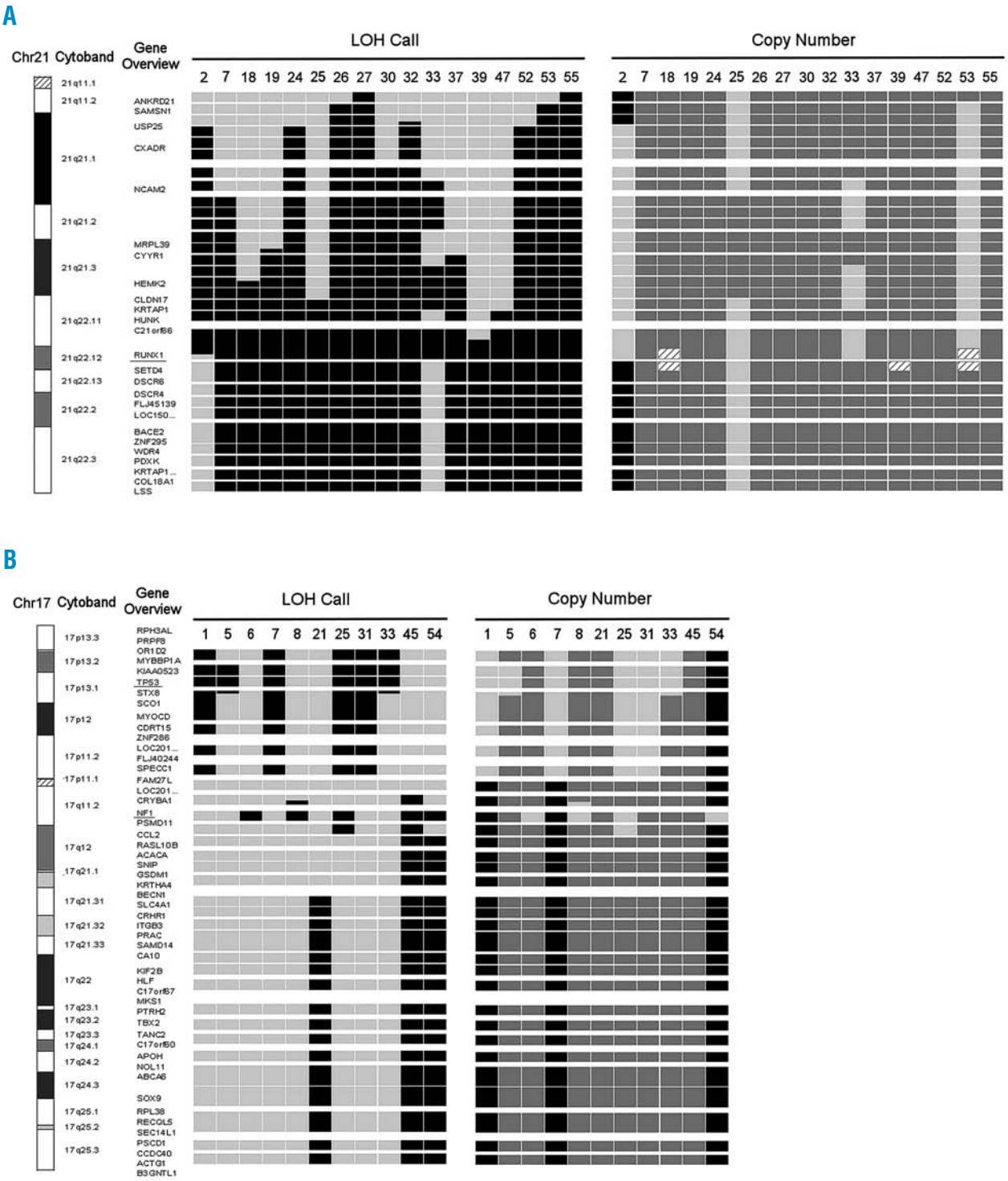

Figure 1. Single nucleotide polymorphism analysis of patients showing chromosome 17 or 21 abnormalities. (A) LOH in chromosome 21 was detected in 17 patients. LOH was related to a hemizygous deletion in four patients (2, 25, 33, 53) and UPD (copy neutral) in 13, as can be seen by the copy number call. Homozygous deletions were detected in three patients (18, 39, 53) and affected the *RUNX1* locus. Chromosome representation, cytoband and gene distribution (overview) are at the left side of the panel. Candidate genes are underlined. The left heat map shows inferred LOH calls based on a hidden Markov model considering haplotype (using the paired normal). Each column represents one patient's result. Each box represents the combined call for one or more SNP between tumor sample and respective control (T cells). The right heat map represents chromosome copy number inferred using the paired normal as reference and a median smoothing. Dark gray boxes represent two copies, light gray boxes represent one copy (deletion) and black boxes represent three copies for each chromosome locus. Homozygous deletions are represented by striped boxes (see copy number heat map). (B) LOH at chromosome 17 was detected in 11 patients. Two main regions of LOH were detected: one including the *TP53* locus, shared by patients 1, 5, 7, 25, 31, and 33, the other including the *NF1* locus, in patients 6, 8, 25, 45 and 54. In addition three patients showed extensive LOH of the q arm of chromosome 17. Legend as in panel (a).

sion of TdT and presence of a *RUNX1* mutation (one-sided Fisher's exact test; using a 10 % expression cut-off p=0.00002 or a 20 % cut-off p=0.00001). The only exceptions to this association were patients 28 and 38 who, although carrying *RUNX1* mutations, showed no or low expression of TdT, and patients 3, 22 and 43 who showed high TdT expression without having *RUNX1* mutation (Table 1).

Discussion

In the last years, the approach to the classification of AML has shifted from a morphological to a molecular basis. Although the cohort of patients studied here was selected based on morphological criteria, recent results using gene expression profiling have shown that AML-M0 patients cluster together as a separate group of AML-M0.[24] In the present study we aimed to identify TSG and oncogenes that might contribute to the AML-M0 phenotype. Whole genome SNP analysis revealed various new regions of LOH containing known and candidate TSG. Conversely, chromosomal gains were rare. Many of the LOH regions described here could not have been found by standard cytogenetic techniques as they were UPD (Table 1 and 2). Two LOH regions containing known TSG were on chromosome 17. LOH at 17p13.1 (*TP53*) was clearly independent from that at 17q11.2 (*NF1*). This result confirms similar findings in AML.[25,26] However, our results suggest that both regions have equal importance in AML-M0, since the number of occurrences for each region is comparable. *TP53* has an important role in the maintenance of chromosomal stability and its deletion has been linked to AML with complex karyotype.[26] In concordance, three AML-M0 patients with *TP53* loss also had complex karyotypes. *NF1* is involved in negative regulation of the RAS pathway (see below) and is found to be mutated in patients with hematopoietic disorders.[27] Interestingly, a third region of LOH was detected in chromosome 17 implying that another TSG is present at 17q (Table 2).

In a number of minimal LOH regions without known TSG, we screened several candidate genes or transcripts for mutations, including *CEBPD* at chromosome 8, *TET2* at chromosome 4, and others at chromosomes 3, 5 and 7 (Table 2). Candidate genes were selected based on the likelihood of them having a role in AML-M0 or, in the case of the region containing *TET2*, because it was described as a microdeletion in four patients.[28] No mutations were found in any of the candidate genes. However, for some of the LOH regions detected, hemizygous deletions may already be sufficient for the neoplastic process, without requiring a mutation in the other allele.[29] In fact, several reports suggest that haploinsufficiency of one or more genes, especially in chromosome 5 and 7, contributes to AML.[26,30,31] Of note, some of the areas of LOH detected by us, in particular the ones with UPD, were too large to be screened efficiently for TSG. Importantly, genome wide SNP analysis also showed that the genomic region on chromosome 21 containing the TSG *RUNX1* is the most frequently affected region in AML-M0 (Figure 1A). The complete loss of *RUNX1* or biallelic *RUNX1* mutations observed in the majority of patients with UPD in chromosome 21 was in line with mitotic recombination being the mechanism of homozygosity.[8] On the other hand, deletion of one *RUNX1* allele appears to co-exist with complex patterns of LOH (Table 1 and 2). In all, we detected 18 cases (35 %) with *RUNX1* mutation of which 15 were biallelic. This number is higher than in previous studies.[6] Most of the mutations affected the runt domain and are considered to result in loss of DNA binding ability.[32,33] Interestingly, two of the *RUNX1* mutations were found in the transactivation domain. To our knowledge, C-terminal *RUNX1* mutations have not been reported in AML-M0, although a few cases in other AML subtypes have been reported and they are frequent in myelodysplastic syndrome.[6,34,35]

Table 3. Summary of molecular findings and main cytogenetic abnormalities.

	Cases	Percentage of total
Confirmed mutations		
RUNX1	20	39
ETV6[a]	5	10
CEBPA	1	2
FLT3	10	19
RAS	6	12
PTPN11	6	12
JAK2	3	6
NPM1	1	2
Main cytogenetic abnormalities		
Del(5q)	8	15
-7/del(7q)	7	14
+8	5	10
+13	8	15

a :published in Silva et al., (2008)[21].

As over 60% of the AML-M0 cases retained normal *RUNX1*, we screened the cohort for mutations in other hematopoietic transcription factors frequently implicated in AML. Mutations in *CEBPA*, *CEBPB* and *NPM1* were rare, showing that AML-M0 is largely unrelated to these provisional World Health Organization (WHO) subgroups.[36] In fact, only a few patients (patients 35, 51 and 59) classified in this study as having FAB AML-M0 would not be considered to be part of the WHO subgroup with minimally differentiated leukemia. Contrary to a study showing a high (23%) frequency of mutation in AML-M0,[37] we and others found no mutations in *SPI1*.[13,38] It seems that mutations of these transcription factors are not an alternative to *RUNX1* mutation in AML-M0. However, as we previously reported, *ETV6* mutations are infrequent alternatives to *RUNX1* mutation in this cohort.[21]

FLT3, RAS and *PTPN11* are genes of the *RAS* pathway and mutations in these genes heve been reported to collaborate with *RUNX1* mutations in the pathogenesis of AML by providing a proliferative advantage to the cells.[10-14] We detected a higher frequency of *RAS* (12%) and *PTPN11* (12%) mutations in AML-M0 than in previ-

ous studies (Table 3).[11-13] Mutation frequencies for *FLT3* (19%) were in accordance with other published data for AML and AML-M0.[10,39-41] It is possible that the number of cases involving these genes is even higher, since we sequenced only mutational hotspots. Interestingly, mutations in *FLT3*, *RAS* and *PTPN11* were absent in patients showing a deletion of the *NF1* region. This result is in line with the view that deletion of *NF1* might be an alternative to activation of the RAS signaling pathway in AML-M0. Finally, we detected three cases with mutation in *JAK2*, another gene involved in cell proliferation (Table 3). *JAK2* mutations occur frequently in myeloproliferative disorders and less commonly in myelodysplastic syndrome and *de novo* AML.[42,43] Though two of our cases had a previous history of hematopoietic disorders, this result could indicate some relation between AML-M0 and *JAK2* mutation (Table 1).

Contrary to previous findings, mutation of RAS pathway-related genes did not correlate with *RUNX1* mutation.[14,44] We also did not find a positive association between -7/del(7q) and *RUNX1* mutation or a negative one between del(5q) and *RUNX1* mutation as previously reported in myelodysplastic syndrome,[44] although a similar trend was noticeable. In fact, the only mutation associated with mutations in *RUNX1* was trisomy 13, as observed by us and others.[19,34] Trisomy 13 is also correlated with higher *FLT3* expression and is probably another factor contributing to proliferative advantage in AML-M0.

TdT expression is a common characteristic of CD34$^+$ immature AML and is associated with a poor prognosis.[45] Remarkably, *RUNX1* mutation and TdT expression were strongly correlated. TDT encodes a DNA polymerase normally expressed in pre-B and pre-T lymphocytes during early differentiation.[46] Recently, we found, by gene expression profiling, that AML-M0 *RUNX1* mutants showed a characteristic B-lymphocyte signature, which could explain this correlation.[24] TdT expression in leukemia is frequently assessed by immunophenotyping. Thus screening for *RUNX1* mutations in AML-M0 at diagnosis might become possible by measuring TdT.

In conclusion, whole genome SNP analysis confirmed our previous findings that events leading to partial UPD are a major cause of mutation homozygosity in AML-M0.[8] In fact, recent reports show that UPD is common in AML, myelodysplastic syndrome and myeloproliferative disease,[47-50] suggesting that this is a general mechanism leading to loss of TSG activity in hematologic disorders. The observed heterogeneity in chromosomal losses in AML-M0 without *RUNX1* mutation suggests that not one but several genes may be alternatives to *RUNX1* mutation. Conversely, mutations related to cell proliferation, though genetically diverse, affect mainly the RAS pathway. Combining trisomy 13 and hemizygous loss of *NF1* with the mutations found in *FLT3, NRAS, JAK2* and *PTPN11*, we detected a strikingly high frequency (63%) of cell proliferation-related mutations. Importantly, we showed a strong association between TdT expression and *RUNX1* mutation suggesting that TdT expression may serve as a surrogate marker for *RUNX1* mutation in AML-M0.

Authorship and Disclosures

FPGS contributed to the conception and design of the study; acquisition, analysis and interpretation of data, and drafted the article; IA, BM, GB-M, HW, RV contributed to the acquisition and analysis of data; HV, EWAM, PJMV, HCK-N, WRS, W-DL and MG-G contributed to interpretation of data, critical manuscript revision, conception and design of the study. All authors revised the article critically for important intellectual content approved the final version to be published.

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