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MYELOID NEOPLASIA

Acquired copy number alterations of miRNA genes in acute myeloid leukemia are uncommon

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Key Points

- Somatic copy number alterations of miRNA genes are uncommon in de novo and secondary AML.
- *MIR223* silencing in AML occurs through both genetic and epigenetic mechanisms.

Altered microRNA (miRNA) expression is frequently observed in acute myelogenous leukemia (AML) and has been implicated in leukemic transformation. Whether somatic copy number alterations (CNAs) are a frequent cause of altered miRNA gene expression is largely unknown. Herein, we used comparative genomic hybridization with a custom high-resolution miRNA-centric array and/or whole-genome sequence data to identify somatic CNAs involving miRNA genes in 113 cases of AML, including 50 cases of de novo AML, 18 cases of relapsed AML, 15 cases of secondary AML following myelodysplastic syndrome, and 30 cases of therapy-related AML. We identified a total of 48 somatic miRNA gene-containing CNAs that were not identified by routine cytogenetics in 20 patients (18%). All these CNAs also included one or more protein coding genes. We identified a single case with a hemizygous deletion of *MIR223*, resulting in the complete loss of miR-223 expression. Three other cases of AML were identified with very low to absent miR-223 expression, an miRNA gene known to play a key role in myelopoiesis. However, in these cases, no somatic genetic alteration of *MIR223* was identified, suggesting epigenetic silencing. These data show that somatic CNAs specifically targeting miRNA genes are uncommon in AML. (*Blood*. 2013;122(15): e44-e51)

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression posttranscriptionally by binding to target messenger RNAs (mRNAs).[1] Although miRNAs are frequently dysregulated in acute myelogenous leukemia (AML),[2-9] the mechanism of dysregulation remains poorly understood. It is known that the majority of human miRNA genes are present in fragile sites and genomic regions frequently altered in cancer.[10] Point mutations of miRNA genes appear to be rare in human cancers. While single nucleotide polymorphisms (SNPs) in miRNAs that affect expression have been reported,[11,12] there are only rare examples of recurring somatic point mutations in miRNA genes in human cancer.[13,14] Conversely, somatic copy number alterations (CNAs) that include miRNA genes have been reported in several human cancers.[15-18] However, whether miRNA genes are frequently and specifically targeted in AML by deletion or amplification is largely unknown. To address this issue, we performed a comprehensive analysis of somatic CNAs involving miRNA genes in 113 cases of AML (50 cases of de novo AML, 18 cases of relapsed AML, 15 cases of secondary AML following myelodysplastic syndrome, and 30 cases of therapy-related AML [t-AML]) by using custom miRNA-specific, high-resolution array-based comparative genomic hybridization (aCGH) and whole-genome sequence data.

Methods

Human subjects

All AML samples were obtained from a study at Washington University to identify genetic factors contributing to AML initiation and progression. Approval for these studies was obtained from the Washington University institutional review board. After obtaining written informed consent for the patients in accordance with the Declaration of Helsinki, a bone marrow sample and a 6-mm punch biopsy of skin (for analysis of matched normal cells) were obtained.

aCGH

A custom high-resolution aCGH platform (3×720K array; NimbleGen, Madison, WI) was generated to interrogate CNAs of all known miRNA genes at the time these studies were performed (835 miRNAs [miRBase, version 14.0] for the 30 t-AML samples and 1027 miRNAs [miRBase, version 15.0] for the 18 relapsed AML samples) and 44 miRNA processing genes (Table 1). Each gene and 40 kb of its flanking genome were interrogated with densely

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Table 1. miRNA processing genes

Gene	Chromosome	Start	Stop
ADAR	1	152 811 158	152 857 306
DDX20	1	112 089 713	112 121 721
EIF2C1	1	36 053 645	36 167 440
ILF2	1	151 891 138	151 920 103
LIN28	1	26 599 856	26 638 806
PAPD3	1	52 651 535	52 801 331
NOP58	2	202 828 760	202 886 629
PACT	2	178 994 395	179 034 110
TERC	3	170 955 092	170 975 542
GAR1	4	110 946 115	110 975 342
NPH2	5	177 499 072	177 523 567
PAPD4	5	78 933 999	79 028 227
RNASEN	5	31 426 359	31 578 039
TERT	5	1 296 287	1 358 162
XPO5	6	43 588 047	43 661 790
EIF2C2	8	141 600 446	141 724 828
PIWIL2	8	22 178 755	22 279 529
TRIM32	9	118 479 402	118 513 400
ADARB2	10	1 208 073	1 779 718
PAPD1	10	30 628 736	30 688 273
PIWI4	11	93 930 122	94 004 234
HNRNPA1	12	52 950 755	52 975 297
IPO8	12	30 663 189	30 750 018
PIWI1	12	129 378 567	129 432 826
RAN	12	129 912 736	129 937 316
TARBP2	12	52 170 972	52 196 482
DICER1	14	94 612 318	94 687 808
NOP10	15	32 411 209	32 432 654
TNRC6A	16	24 638 550	24 755 048
DDX5	17	59 914 836	59 942 946
GEMIN4	17	584 411	612 251
FBL	19	7 502 445	7 541 588
HNRNPL	19	45 006 934	45 038 894
ILF3	19	44 008 868	44 044 819
KHSRP	19	10 615 937	10 674 093
PTBP1	19	6 354 119	6 385 822
UPF1	19	738 392	773 327
NOP56	20	18 793 744	18 850 039
DDX17	22	2 571 254	2 597 039
DGCR8	22	37 199 389	37 242 291
NHP2L1	22	18 437 834	18 489 400
PIWI3	22	40 389 883	40 424 859
DKC1	X	23 435 001	23 510 683
FMR1	X	153 634 225	153 669 157

Coordinates are based on NCCI36/HG18 assembly.

tiled probes at either 30 to 40 bp (miRNA genes) or 80 bp (miRNA processing genes). This array also contained dense tiling of probes designed to interrogate 170 DNA repair genes. In addition, probes uniformly spaced throughout the genome at approximately 8600-bp intervals were included. Two micrograms of genomic DNA from unfractionated bone marrow (tumor) and paired normal tissue (skin) was fragmented, labeled, and hybridized to the array as previously described.[19] Log2 ratios of fluorescent intensity for tumor/skin were generated for each probe. Abnormal segments (ie, putative regions of CNAs) were identified by using segmentation algorithms from NimbleGen (segments) and Partek (segmentation). Segments generated by segmentation algorithms were prioritized on the basis of the number of probes and the log2 ratio of each segment (score = log10 [number of probes per segment] × log2 ratio) and manually reviewed, as previously described.[19] To identify CNAs within miRNA genes and miRNA processing gene loci, plots of log2 values for each probe spanning the locus with 0.5 to 5 Mb flanking DNA were manually reviewed by 4 independent reviewers. Next, we collapsed contiguous segments generated by segmentation algorithms and identified boundaries by using segment boundaries and manual review. For 18 of the 30 t-AML patients, an independent iScan platform was available, and it confirmed 100% of the aCGH calls.

Analysis of whole-genome sequencing data

We recently reported the sequence of 50 de novo AML genomes[20] and 15 genomes of patients with secondary AML following myelodysplastic syndrome.[21,22] The sequence data were analyzed to identify potential somatic CNAs as previously described.[20] However, there is a high false-positive rate with CNAs identified in this fashion.[21] Thus, we also performed aCGH by using the Affymetrix 6.0 SNP array to independently call somatic CNAs in all cases. We included for further analysis only those CNAs that were identified by both platforms.

Real time RT-PCR

Total RNA was reverse transcribed by using the TaqMan microRNA Reverse Transcription Kit per manufacturer's instructions (Applied Biosystems). Real time reverse transcription-polymerase chain reaction (RT-PCR) for the indicated miRNA and RNU48 (as a control) were performed by using the relevant TaqMan MicroRNA assay.

Quantitative genomic PCR

Quantitative PCR was performed by using SYBR Green Master Mix (Applied Biosystems) and 50 ng of genomic DNA. PCR primers were designed to amplify *MIR223* and *MIR181b*. *MIR181b* was included as a diploid gene copy number control, since no somatic CNAs of this gene were identified in any of the samples. *MIR223* primers were 5′-CTTTACCTGCTTATCTTCAGGATC TCT-3′ and 5′-CGTACGCGCCCCCATCAGCACTCT-3′. *MIR181b* primers were 5′-GTCTCCCATCCCCTTCAGAT-3′ and 5′-TTTGCCTTTTCTAAA ACATGCTC-3′. Technical triplicates were performed for each sample.

Results and discussion

A total of 113 patients with AML were studied, including 50 cases of de novo AML (Table 2), 18 cases of relapsed de novo AML (Table 2), 15 cases of secondary AML following myelodysplastic syndrome (Table 3), and 30 cases of t-AML (Table 4). The median age of the de novo AML patients was 54.5 years (range, 21 to 82 years), and the median blast percentage was 75% (range, 35% to 100%). A normal karyotype was identified in 37 (74%) of 50 patients. The median age of the relapsed AML patients was 57.5 years (range, 24 to 77 years). The median blast percentage was 59% (range, 12% to 95%). A normal karyotype was identified in 6 (40%) of 15 patients with relapsed AML. The patients with secondary AML were older, with a median age of 66 years (range, 26 to 77 years). The median time to progression from myelodysplastic syndrome to AML was 400 days (range, 28 to 1751 days), and the median blast percentage in the bone marrow was 43% (range, 21% to 89%). A normal karyotype was identified in 43% of cases, and abnormalities involving chromosome 5 or 7 were observed in 43%. The median age of patients with t-AML was 59 years (range, 26 to 80 years). Twelve of the t-AML patients (40%) were treated for breast cancer, 6 (20%) for non-Hodgkin lymphoma, 2 (6.7%) for Hodgkin lymphoma, 2 (6.7%) for multiple myeloma, and 8 (20%) for other diseases. Most of the t-AML patients (76.7%) were treated for their primary cancer with a combination of chemotherapy that included topoisomerase inhibitors and/or alkylating agents. The median blast percentage in the bone marrow was 76% (range, 31% to 95%). Cytogenetic analysis revealed −5/−5q and/or −7 in seven patients (23%), translocations involving chromosome 11q23 (*MLL* gene rearrangement) in 6 patients (20.0%), and a normal karyotype in 6 patients (20%).

Table 2. Clinical characteristics of the patients with de novo or relapsed AML

UPN	AML diagnosis	FAB subtype	Sex	Age, y*	% BM blast	Cytogenetics
933124	De novo	M1	F	57	100	46,XX[20]
807970	De novo	M1	M	38	86	46,XY[20]
123172	De novo	M1	M	56	90	46, XY[20]
831711	De novo	M1	F	57	64	46, XX[19]
849660	De novo	M1	M	22	71	46,XY[30]
808642	De novo	M1	M	61	49	46,XY[20]
509754	De novo	M1	F	21	91	46, XX[20]
327733	De novo	M1	F	32	94	46, XX[20]
709968	De novo	M3	M	25	91	46,XY,t(15;17)(q22;q21)[20]
863018	De novo	M3	M	62	82	46,XY,t(15;17)(q22:q21)[11]/46,XY[9]
478908	De novo	M3	M	50	74	46,XY,t(15;17)(q22;q21)[20]
344551	De novo	M3	M	48	65	46,XY,t(15;17)(q22:q21)[11]/46,XY[8]
673778	De novo	M3	M	53	42	46,XY,t(15;17)(q22;q21)[19]/46,XY[1]
321258	De novo	M3	F	31	40	46,XX,t(15;17)(q22;q21)[11]/46,XX[9]
758168	De novo	M3	F	25	93	46,XX,t(15;17)(q22;q21)[20]
455499	De novo	M3	F	29	85	46,XX,t(15;17)(q22;q21)[12]/46,XX[8]
103342	De novo	M2	F	61	43	46, XX[20]
113971	De novo	M2	F	57	43	46, XX[15]
142074	De novo	M4	M	60	89	46, XY[15]
179223	De novo	M2	F	82	53	46, XX[20]
224143	De novo	M1	F	67	76	46, XX[20]
225373	De novo	M2	F	71	70	46, XX[14]
246634	De novo	M4	M	79	58	46,XY[20]
254137	De novo	M2	F	31	63	46, XX[20]
273919	De novo	M2	M	25	56	46, XY[20]
335640	De novo	M5	F	67	85	46, XX[20]
400220	De novo	M4	F	34	71	46, XX[20]
426980	De novo	M2	M	68	64	46, XY[20]
440422	De novo	M0	M	69	82	46, XY[20]
445045	De novo	M2	M	75	63	46, XY[20]
452198	De novo	M5	M	55	97	46, XY[15]
456892	De novo	M4	M	58	58	46, XY[18]
545259	De novo	M1	F	30	86	46, XX[20]
548327	De novo	M1	M	51	85	46, XY[20]
573988	De novo	M4	F	67	75	46, XX[16]
700717	De novo	M0	M	45	75	46,XY[20]
702808	De novo	M5	F	75	41	46,XX[18]
753374	De novo	M2	M	29	45	46,XY,15pstk+[20]
775109	De novo	M5	M	45	81	46,XY[20]
804168	De novo	M1	M	53	86	46,XY[20]
816067	De novo	M5	F	35	87	46, XX[20]
817156	De novo	M2	M	54	67	46,XY[19]
869586	De novo	M4	M	23	51	46,XY[20]
906708	De novo	M4	F	76	91	46,XX[20]
907786	De novo	M5	F	81	53	46,XX[20]
991612	De novo	M2	M	63	35	46,XY[20]
202127	De novo	M3	F	68	85	46,XX,t(15;17)(q22;q21)[20]
529205	De novo	M3	M	59	79	46,XY,t(15;17)(q22;q21)[20]
501944	De novo	M3	F	40	90	46,XX,t(15;17)(q22;q21.1)[19]/47,idem,+8 [1]
943309	De novo	M3	M	35	90	47,XY,del(7)(q22),+8,t(15;17)(q22;q21)[18]/46, XY,del(7)(q22),t(15;17)(q22;q21)[2]
142074	Relapsed	M4	M	61	65	46, XY[15]
255108	Relapsed	M0	M	62	80	47,XY,+8 [19]
375182	Relapsed	M5	M	57	79	Not available
387919	Relapsed	M1	F	58	20	46, XY, +3 [3], 46,XY [17]
400220	Relapsed	M4	F	35	60	46, XX[20]
426980	Relapsed	M2	M	71	12	46, XY[20]
452198	Relapsed	M5	M	57	20	46, XY[15]
573988	Relapsed	M4	F	68	54	Failed
593890	Relapsed	M2	M	36	95	47,XY,+21 [6]/46,XY[13]
708512	Relapsed	M4	F	65	38	50 XX, +4,+6,+8, +19 [4]/ 47 XX, + i4(q10)[12].
758168	Relapsed	M3	F	27	92	46,XX,t(15;17)(q22;q21)[20]

BM, bone marrow; F, female; FAB, French-American-British; M, male; UPN, unique patient number.

*Age at presentation of initial diagnosis of AML.

Table 2. (continued)

UPN	AML diagnosis	FAB subtype	Sex	Age, y*	% BM blast	Cytogenetics
804168	Relapsed	M1	M	54	81	46,Y,t(X;6)(q22;q23)?t(1;12;7;3)(p36.1;q13;p11.2;p21)[17],46,XY[3],ish,der3,t(3,;17)(p53+),de(12)t(1;12)(1pter+)
817156	Relapsed	M2	M	55	58	46,XY[19]
869586	Relapsed	M4	M	24	54	Failed
869922	Relapsed	M2	F	56	50	46,XX[20]
923966	Relapsed	M5	M	61	79	47,XY,t(9;11)(p22;q23),+8[7]/45,XY,t(9;11)(p22;q23),-8[7]/46,XY[4]
962561	Relapsed	M4	F	77	32	46,XX,+13,-21[3],46,XX[17]
972783	Relapsed	M0	M	72	66	46,XY,der(15)t(15;17)(p11.2q11.2),der(17)t(15;17)del(17)p(1.3)[3]/47,idem,+mar[1]

BM, bone marrow; F, female; FAB, French-American-British; M, male; UPN, unique patient number.
*Age at presentation of initial diagnosis of AML.

We interrogated paired tumor/normal samples for somatic CNAs by using aCGH or whole-genome sequencing data. The t-AML and relapsed AML cases were analyzed by using a custom CGH array that contained densely spaced oligomers (every 30 to 40 bp spacing) for all miRNA genes that were identified in miRBase at the time this study was performed (835 miRNAs in miRBase, version 14.0, were included in the arrays for the 30 t-AML samples and 1027 miRNAs in miRBase, version 15.0, for the 18 relapsed de novo AML samples). A total of 40 kb of genomic DNA flanking the miRNA precursor gene was targeted. We also included probes for 44 genes involved in miRNA processing (Table 1). In each case, genomic DNA isolated from a skin biopsy was used to distinguish inherited CNAs from somatic CNAs. To call a somatic CNA, we required that a minimum of 25 contiguous probes show differential hybridization. Thus, for miRNA genes, we theoretically should be able to identify somatic CNAs of approximately 1 kb. A total of 64 CNAs that were not apparent by routine cytogenetics were identified in 14 patients (all with t-AML). CNAs were judged to be cytogenetically apparent if any part of the contiguous segment was contained within a chromosomal loss, gain, or interstitial chromosomal deletion identified by routine metaphase cytogenetics. For interstitial deletions, coordinates of the cytogenetic banding were estimated by using National Center for Biotechnology Information (NCBI) Map Viewer, Build 36. Twenty-six of these somatic CNAs, identified in 11 of the 48 patients, contained one or more miRNA genes (Table 5). No cytogenetically unapparent somatic CNAs involving miRNA processing genes were identified in any case.

To expand our analysis, we next analyzed whole-genome sequencing and aCGH data for 50 cases of de novo AML and 15 cases of secondary AML to identify somatic CNAs. For these samples, the Affymetrix 6.0 SNP array was used. We required that the CNAs be identified by both whole-genome sequencing and by aCGH. Given the lower probe density of the Affymetrix 6.0 SNP array, we estimated that the lower size limit of somatic CNA detection for this approach was approximately 18 kb. Four somatic CNAs involving miRNA genes were identified in 4 de novo AML patients, all with a normal karyotype (Table 5). In the secondary AML cases, we identified 18 somatic CNAs in 5 patients, only one of which had

Table 3. Clinical characteristics of patients with secondary AML

UPN	Sex	Age, y	MDS FAB	Time to AML, days	% BM blast	Cytogenetics
461282	M	70	RAEB	1751	69	45,XY,del(5)(q22q33),-17, del(20)(q11.2)[14]/46,XY[4]
667720	F	67	RAEB	644	Not done	46,XX[19]/45,XX,-7[1]
859640	F	64	RA	252	25	47,XX,+13[3]/46,XX[17]
610184	F	46	RA	314	38	41-44,XX,add(1)(p36.3),del(5)(q13q33),-7,-13,dic(16;21)(p13.3;p11.2),add(17)(p13), −18, −22,+mar[cp17]/84,idemx2[cp2]/44,XX,-17, −22[1]
182896	M	77	RA	1047	51	47,XY,add(4)(p16),del(5)(q15q33), −7,+8,del(9)(q22),+22,+2mar[1]/54,XY,+3,+8,+8,+9,-12,+15,+19,+20,-21,+22,+2-3mar[cp11]/46,XY[8]
266395	M	64	RAEB	75	66	46,XY[17]
288033	F	30	RAEB	28	43	46,XX[20]
298273	M	26	RAEB-T	131	35	46,XY[20]
689147	F	69	RAEB	421	Not done	48,XX,+1,del(5)(q15;q33),+11,i(22)(q10)[20]
891669	M	66	RA	323	75	46,XY,inv(3)(q21q26.2)[20]
169510	M	58	RAEB	796	28	46,XY[20]
989382	M	69	RA	1332	89	Unknown
178647	M	61	RA	368	23	46,XY[20]
759134	M	67	RA	400	21	46,XY[20]
838538	M	67	RAEB	437	51	40~46,XY,add(X)(p22.1),-2,del(5)(q22q35),del(7)(q22),+8,-12,-16,+mar[19]/46,XY[1]

FAB, French-American-British; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation.

Table 4. Clinical characteristics of patients with t-AML

UPN	Sex	Age, y	Prior disease or cancer	Alk	XRT	Topo	Other chemo	Latency (years)*	% BM blast	Cytogenetics
180365	F	54	AML	Y	Y	Y	Y	~7.8	83	47,XX,+8[18]/46,XX[2]
180866	M	66	Multiple myeloma	Y	Y	Y	Y	3.2	Not done	47,XY,+i(8)(q10)[3] / 47,XY,+8[17]
189941	F	42	Ovarian/breast	Y	Y	Y	Y	5.6	76	45, XX,add(3)(q27),del(3)(q12),-4,del(5)(q12q33),-7,+add(18)(p11.1),+mar,+mar1[cp19]/46,XX[1]
205133	F	59	Breast	Y	Y	N	Y	7.0	36	46,XX[30]
266608	M	80	Renal cell carcinoma	N	N	N	Y	6.4	33	46,XY[20]
317821	F	42	Non-Hodgkin lymphoma	Y	Y	Y	Y	1.1	80	36-46,XX,+der(1;7)(p10;q10),add(1)(q42),del(7)(q11.2),der(?)t(?;7)(?::7q11.2->7qter)[cp10]
377512	F	74	Non-Hodgkin lymphoma	N	N	Y	Y	2.4	31	38-51,XX,add(1)(p13),del(1)(p36.1),del(1)(q12),der(2)t(2;15)(q37;q11.2), add(3)(q29)-5,del(7)(q31),add(8)(p23),r(8)(?p22q24),add(13)(p11.2), iso(13)(q10),add(19)(q13.4),add(20)(p?13),iso(21)(q10)[cp20]
458613	M	28	Hodgkin lymphoma	N	Y	N	N	1.5	90	46,XY,inv(16)(p13.1q22)[18] / 46,XY[2]
476081	F	68	Breast	Y	Y	Y	Y	1.6	66	46,XX[20]
476204	F	51	Breast	Y	Y	Y	Y	6.8	87	46,XX[20]
482711	F	57	Breast	Y	Y	Y	Y	11.8	44	44-45,XX,der(4)t(4;?)(q22;?)[3],-5[10],add(6)(q13)[6, del(7)(q22)[10], del(12)(p11.2)[4],-17[6],+mar[4],+2mar[3],add(19)(q13)[4] / 46,XX[10]
501254	F	67	Breast	Y	Y	Y	Y	1.4	95	46,XX,t(11;19)(q23;p13)[16] / 47,idem,+8[4]
514901	F	63	Breast	N	Y	N	Y	1.15	95	46,XX,t(6;11)(q27;q23)[20]/46,XX[7]
530447	M	43	Hodgkin lymphoma	Y	Y	Y	Y	12.8	40	44,XY,-3,-5,-7,add(9)(p21),add(17)(q25),+mar1[8] / 45,sl,+mar2[11]/48,sdl1,+21,+22,+r[1]
548417	F	77	Breast	Y	N	Y	Y	7.3	95	46,XX[20]
557772	M	60	Multiple myeloma	Y	N	Y	Y	3.3	47	49,XY,-5,+8,+11,-17,-17,+21,+22,+2mar[19] / 50,idem,+mar[1]
572162	F	59	Breast	Y	Y	Y	Y	4.75	79	46,XX,t(3;12)(p13;p13)[5]/46,XX[15]
644242	F	56	Breast	Y	Y	Y	Y	4.0	62	46,XX,t(8;21)(q22;q22)[19]
658208	M	50	Multiple sclerosis	N	N	Y	N	2.8	94	45,X,-Y,t(8;21)(q22;q22)[19]
706395	F	45	Lung	Y	Y	Y	Y	1.9	90	46,XX,t(9;11)(p21;q23)[16] / 46,idem,der(1)(t(1;?)(p13;?)[2] / 46,XX[2]
751407	M	74	Rheumatoid arthritis	N	N	N	Y	1.5	61	85,XXY,-Y,-2,-5,-7,-16,-17,-18 [10]/46,XY[10]
779828	M	79	Prostate	N	Y	N	N	2.1	76	46,XY[20]
811184	M	26	Non-Hodgkin lymphoma	Y	N	Y	N	NK	56	42~46, XY, der(11)t(11;15)(p11.2;q11.1), t(11;19)(q23;p13), del(13)(q22), −15, add(22)(q11.1)[cp7]/46,XY[6]
856024	M	26	Non-Hodgkin lymphoma	Y	Y	Y	Y	1.1	90	46,XY,der(12)t(1;12)(q25;p13),add(12)(q24.2)[18] / 46,XY[2]
860923	F	71	Non-Hodgkin lymphoma	Y	Y	N	Y	6.9	71	92,XXXX[6] / 46,XX[14]
864484	M	39	Testicular	N	Y	N	Y	1.1	92	42-48,XY,-2,inv(7)(p15q11.2),-11,-13,del(13)(q12q21),-17,der(19)t(?;19)(?;p13.1),+mar1,+mar2,+mar3,+mar4,+mar5[cp20]
925964	F	58	Uterine	N	N	Y	Y	2.2	38	46,XX,inv(11)(p15q22~23)[19] / 46,XX[1]
942008	M	69	Non-Hodgkin lymphoma	Y	Y	Y	Y	13.3	67	45,der(X)t(X;16)(p22.1;p13.2),add(X)(q26),Y,t(3;9)(p13;q34),del(5)(q13q31),inv(6)(p21.1q25),der(7)(7pter->q21~q22;?),der(16)t(X;16)(p22.1;p13.2)[2] / 92,der(X)t(X;16)(p22.1;p13.2),add(X)(q26)x2,Y,t(3;9)(p13;q34)x2,del(5)(q13q31)x2,inv(6)(p21.1q25)x2,der(7)(7pter->q21~q22;?)x2,der(16)t(X;16)(p22.1;p13.2)x2[1] / 46,XY[27]
982895	F	47	Breast	N	Y	N	Y	~3	93	46,XX,t(9;11)(p22;q23)[7]/47, idem, +8[13]
983545	F	61	Breast	Y	Y	N	Y	1.4	41	49,XX,ins(6)(?q13?),+8,+8,t(9;11)(p22;q23),+der(9)t(9;11)(p22;q23),del(13)(q12q14)[7] / 49,XX,idem,+del(13)(q12q14)[7] / 46,XX[5]

Alk, alkylator chemotherapy; chemo, chemotherapy; N, no; Topo, topoisomerase chemotherapy; XRT, radiation therapy; Y, yes.
*Latency, time (years) from treatment of original cancer.

a normal karyotype. In total, we identified cytogenetically unapparent somatic CNAs involving miRNA genes in 18% of patients with AML. In AML with a normal karyotype, somatic CNAs involving miRNA genes were identified in only 5 (9.1%) of 55 cases. The most common recurring somatic CNA (present in 3 cases of AML) is an approximately 1.3-Mb deletion at 17q11.2, which includes *MIR4733*, *MIR4724*, *MIR193a*, *MIR4725*, and *MIR365b* (Table 5). However, as is the case for all of the somatic CNAs identified in this study, the 17q11.2 CNA includes several protein coding genes.

Table 5. CNAs containing miRNA genes not identified by routine cytogenetics

UPN	AML Diagnosis	Chr	Breakpoint start	Breakpoint end	Call	CNA (bp)	miRNA genes in the CNA
327733	De novo	16	30 514 514	31 420 587	d	906 073	4519, 762
113971	De novo	2	24 395 064	25 807 518	d	1 412 454	1301
869586	De novo	17	26 063 968	27 437 770	d	1 373 802	4733, 4724, 193a, 4725, 365b
906708	De novo	9	81 151 141	87 703 853	d	6 552 712	7-1
169510	Secondary	6	118 096	26 790 111	d	26 672 015	6720, 4645, 3691, 5683, 5689, 4639, 548a-1
169510	Secondary	6	26 790 111	48 691 459	a	21 901 348	3143, 877, 4640, 4646, 1236, 6721, 3135b, 219-1, 5004, 3934, 1275, 5690, 3925, 4462, 4641, 4647, 4642, 586
182896	Secondary	12	2 128 232	78 142 425	a	76 014 193	31 miRNAs
182896	Secondary	12	79 457 892	87 807 120	a	8 349 228	617, 618, 4699
182896	Secondary	12	95 700 444	121 346 369	a	25 645 925	1251, 135a-2, 4495, 4303, 1827, 3652, 3922, 4496, 619, 4497, 3657, 1302-1,620, 4472-2, 1178, 4498, 4700
182896	Secondary	12	121 996 058	123 901 827	d	1 905 769	4304, 3908
182896	Secondary	17	25 505 826	27 326 775	d	1 820 949	4733, 4724, 193a, 4725, 365b
182896	Secondary	21	13 395 102	33 441 194	a	20 046 092	3156-3, 3118-5, 99a, 7c, 125b-2, 548x, 6130, 155, 4759, 4327
182896	Secondary	21	36 524 064	46 921 386	a	10 397 322	6508, 4760, 3197, 5692b, 6070
182896	Secondary	Y	0	57 427 648	a	57 427 648	3690-2, 6089-2
610184	Secondary	2	2 784	13 404 817	d	13 402 033	4261, 4429, 548s, 4262, 3681, 3125
610184	Secondary	2	27 745 709	30 891 590	d	3 145 881	4263
610184	Secondary	7	1 273 675	2 400 101	a	1 126 426	4655
610184	Secondary	17	526	5 781 507	d	5 780 981	3183, 22, 132, 212, 1253
838538	Secondary	1	61 736	225 115 792	a	225 054 056	121 miRNAs
838538	Secondary	17	527	51 162 464	d	51 161 937	53 miRNAs
838538	Secondary	17	51 162 465	78 643 088	a	27 480 623	33 miRNAs
891669	Secondary	17	26 117 586	27 302 527	d	1 184 941	4733, 4724, 193a, 4725, 365b
180365	Therapy-related	2	123 372 048	132 969 208	d	9 597 160	663b, 4783, 4784
180365	Therapy-related	5	121 883 092	138 624 717	d	16 741 625	4633, 4460, 3936, 1289-2, 3661, 4461, 5692c-1, 874
189941	Therapy-related	3	169 461 120	170 271 699	d	1 513 091	551b
189941	Therapy-related	3	171 702 102	173 816 191	d	2 114 089	569
189941	Therapy-related	12	11 708 326	22 796 431	d	11 088 105	1244-2, 613, 614, 3974
317821	Therapy-related	1	120 308 171	220 764 934	a	100 456 763	3118-1, 3118-2, 3118-3, 6077-1, 5087, 6077-2, 4257, 554, 5698, 190b, 4258, 92b, 555, 9-1, 9-5b, 765, 4259, 5187, 4654, 556, 3658, 921, 1255b-2, 557, 3119-1, 3119-2, 1295, 214, 3120, 199a-2, 488, 4424, 3121, 4426, 1278, 4735, 181b-1, 181a-1, 5191, 1231, 135b, 29c, 29b-2, 205, 4260, 3122, 215, 194-1, 664
317821	Therapy-related	3	144 186 839	199 381 715	a	55 194 876	5186, 3919, 15b, 16-2, 1263, 551b, 569, 4789, 4448, 1224, 5588, 548aq, 1248, 28, 944, 3137, 570, 4797, 922
377512	Therapy-related	2	236 856 627	241 034 230	d	4 177 603	4440, 4441, 4269, 2467, 4786
377512	Therapy-related	15	18 422 770	22 846 333	d	4 423 563	3118-4, 5701-1, 3118-6, 5701-2, 1268a, 4509-1, 4508
482711	Therapy-related	6	73 561 217	77 720 182	a	4 158 965	4282, 4463
482711	Therapy-related	19	7 917 000	8 565 000	a	648 000	4999
482711	Therapy-related	19	9 458 030	12 415 444	a	2 957 414	5589, 4322, 1181, 1238, 638, 4748, 199a-1
482711	Therapy-related	19	13 331 909	19 078 761	a	5 746 852	24-2, 27a, 23a, 181c, 181d, 639, 1470, 3188, 3189
530447	Therapy-related	9	28 278 165	29 708 951	d	1 430 786	876, 873
557772	Therapy-related	21	9 892 286	46 915 712	a	37 023 426	3156-3, 3118-5, 99a, let-7c, 125b-2, 548x, 6130, 155, 4759, 4327, 6501, 802, 6508, 4760, 3197, 5692b, 6070
706395	Therapy-related	10	42 100 384	57 162 870	d	15 062 486	5100, 3156-1, 4294, 605, 548f-1
811184	Therapy-related	1	188 612 922	247 171 197	a	58 558 275	4426, 1278, 4735, 181b-1, 181a-1, 5191, 1231, 135b, 29c, 29b-2, 205, 4260, 3122, 215, 194-1, 664, 320b, 4742, 5008, 3620, 4666a, 1182, 4427, 4671, 4753, 1537, 4428, 3123, 4677, 3916, 3124
811184	Therapy-related	12	33 393	16 168 160	d	16 134 767	3649, 200c, 141, 1244-3, 613, 614
811184	Therapy-related	13	40 292 732	71 225 257	d	30 932 525	3168, 5006, 3613, 16-1, 15a, 5693, 4703, 759, 1297, 5007, 3169, 548x, 4704

a, amplification; Chr, chromosome; d, deletion.

Table 5. (continued)

UPN	AML Diagnosis	Chr	Breakpoint start	Breakpoint end	Call	CNA (bp)	miRNA genes in the CNA
811184	Therapy-related	17	42 399 786	78 637 123	a	36 237 337	5089, 152, 1203, 10a, 196a-1, 3185, 6129, 6165, 3614, 142, 4736, 454, 301a, 4729, 21, 4737, 633, 3064, 5047, 6080, 4315-2, 634, 548d-2, 635, 4524a, 3615, 3678, 4738, 636, 4316, 4739, 1268b, 4730, 657, 3065, 338, 1250, 4740, 3186, 4525
856024	Therapy-related	1	120 321 638	247 171 198	a	126 849 560	3118-1, 3118-2, 3118-3, 6077-1, 5087, 6077-2, 4257, 554, 5698, 190b, 4258, 92b, 555, 9-1, 9-5b, 765, 4259, 5187, 4654, 556, 3658, 921, 1255b-2, 557, 3119-1, 3119-2, 1295, 214, 3120, 199a-2, 488, 4424, 3121, 4426, 1278, 4735, 181b-1, 181a-1, 5191, 1231, 135b, 29c, 29b-2, 205, 4260, 3122, 215, 194-1, 664, 320b, 4742, 5008, 3620, 4666a, 1182, 4427, 4671, 4753, 1537, 4428, 3123, 4677, 3916, 3124
856024	Therapy-related	12	33 393	17 253 192	d	17 219 799	200c, 141, 1244-3, 613, 614
856024	Therapy-related	12	120 756 138	132 283 286	d	11 527 148	4304, 3908, 5188, 4419b, 3612
856024	Therapy-related	17	44 017 170	78 637 124	a	34 619 954	196a-1, 3185, 6129, 6165, 3614, 142, 4736, 454, 301a, 4729, 21, 4737, 633, 3064, 5047, 6080, 4315-2, 634, 548d-2, 635, 4524a, 3615, 3678, 4738, 636, 4316, 4739, 1268b, 4730, 657, 3065, 338, 1250, 4740, 3186, 4525
864484	Therapy-related	14	53 281 577	57 637 143	d	4 355 566	5580, 4308
864484	Therapy-related	X	64 736 865	65 165 635	d	428 770	223

a, amplification; Chr, chromosome; d, deletion.

The smallest somatic CNA identified in this study is a 429-kb deletion on chromosome X that includes *MIR223* and two other genes, *MSN* and *VSIG4* (Figure 1A). It occurred in a male patient with t-AML with complex cytogenetics (Table 4, unique patient number [UPN] 864484). Quantitative PCR of genomic DNA isolated from the bone marrow of this patient confirmed a hemizygous deletion of *MIR223* (Figure 1B). As expected, the hemizygous deletion of *MIR223* in this patient resulted in the complete loss of miR-223 expression (Figure 1C). miR-223 is one of the most highly expressed miRNAs in human CD34$^+$ cells,[23] and its expression increases with myeloid differentiation.[24] Accordingly, miR-223 has been implicated in granulocytic differentiation. Fazi et al[24] showed that enforced expression of miR-223 in acute promyelocytic leukemic cells induces granulocytic differentiation. Conversely, loss of *Mir223* is associated with a myeloproliferative-like phenotype in mice.[25]

To determine whether loss of miR-223 expression was a common occurrence in AML, we performed real-time RT-PCR on bone marrow RNA from an additional 28 cases of AML and from CD34$^+$ cells isolated from 5 healthy donors (Figure 1C). We identified three cases in which miR-223 expression was below the 90% confidence interval based on normal CD34$^+$ cells. Two of these samples (UPN 2_37 and 731274) were from male patients. Quantitative PCR performed on genomic DNA isolated from their leukemic bone marrow showed no deletion of *MIR223* (Figure 1B). The third sample with very low miR-223 expression (UPN 189941) was from a female patient. The sequence of her leukemic genome was recently reported and revealed no point mutation or CNA of *MIR223*.[26] Thus, in all of these cases, an epigenetic mechanism is the likely cause of miR-223 silencing. Indeed, UPN 2_37 (a 46-year-old male with M1 AML) had a t(8;21) translocation producing the *AML-ETO* fusion oncogene,

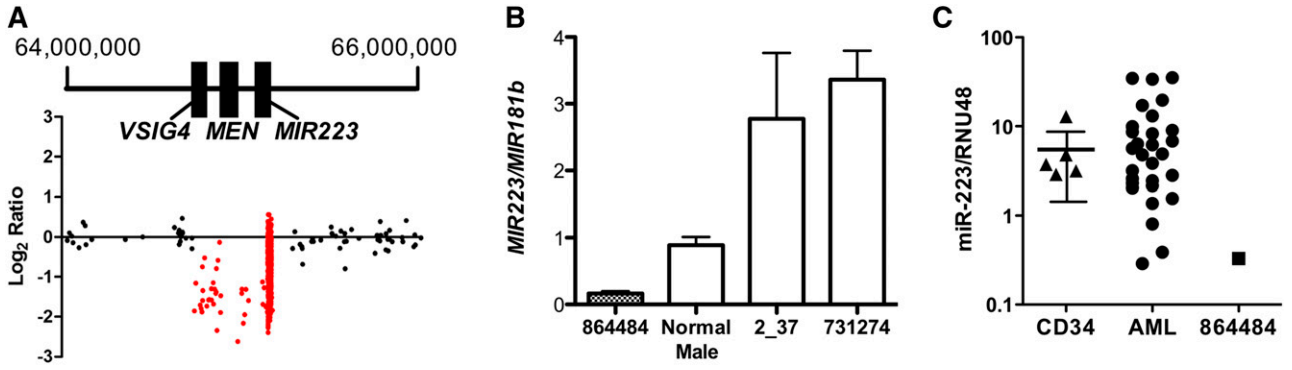

Figure 1. Hemizygous loss of *MIR-223* in a patient with AML. (A) Log2 ratio dot plots of paired tumor and normal DNA from patient UPN 864484 analyzed by using the custom CGH array. A discrete deletion of approximately 429 kb on chromosome X is depicted. Genomic coordinates are based on NCIBI36/HG18 assembly. (B) Quantitative PCR for *MIR223* and *MIR181b* (control gene) was performed by using genomic DNA from the indicated source. Shown is the ratio of *MIR223* to *MIR181b* signal. Data represent the mean ± standard error of the mean of triplicate measurements. (C) miR-223 expression relative to RNU48 is shown for CD34$^+$ cells isolated from healthy donors (CD34) and leukemic bone marrow from patient UPN 864484 or 28 other patients with AML. The 90% confidence interval is shown for CD34$^+$ cells.

which has been shown to epigenetically silence *MIR223*.[14,27] Our data suggest that the deletion of *MIR223* represents another, albeit uncommon, mechanism to decrease miR-223 expression in AML.

Although miRNAs are frequently dysregulated in AML, it appears that genetic alterations in miRNA are relatively rare. Results from whole genome sequencing of 24 cases of de novo AML identified recurring point mutations in a single miRNA gene.[21] Specifically, point mutations in *MIR142* were identified in 2% of cases of de novo AML. Our study suggests that small somatic CNAs involving miRNA genes that are not apparent by standard cytogenetics are uncommon. Thus, it appears that epigenetic, rather than genetic, mechanisms are responsible for most cases of miRNA dysregulation.

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Authorship

Contribution: G.R., M.A.J., M.J.W., and D.C.L. designed the custom comparative genomic hybridization array; G.R., M.A.J., J.S., R.E.D.J.P., D.S., M.T., A.H.G., and M.J.W. contributed to data analysis; T.J.L. provided crucial reagents (acute myelogenous leukemia samples); and D.C.L. was responsible for the overall design and analysis of all studies and edited the final manuscript.

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