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Molecular and pathologic characterization of AML with double Inv(3)(q21q26.2)

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Abstract

The inv(3)(q21q26.2) altering a single chromosome 3 homolog is an established myeloid malignancy-associated entity. Comparatively, double inv(3) cases involving both homologs are exceedingly rare with 13 reports across AML, CML and MDS. This scarcity was confirmed by finding only 2 new cases out of 34,898 bone marrows collected during a 55 year period at a large medical center (0.0005%). The double inv(3) was detected by karyotype and confirmed by FISH on both homologs in a 41 year old female and a 72 year old male with AML. In the latter case, a 2.26-fold increase in MECOM RNA level was found using an NGS myeloid gene panel. Chromosomal microarray analysis identified segmental copy-neutral loss-of-heterozygosity (CN-LOH) at 3q21 extending to near the q-arm terminus. This is the third report of distal 3q CN-LOH, substantiating that the double inv(3) arises through somatic repair of acquired segmental LOH. Long term clinical and genetic evaluation revealed no discernible morphologic difference between single and double inv(3) cases, conventional chemotherapy resistance and rapid dominance of the double inv(3) clone. The two new cases are consistent with relatively longer survival of double inv(3) patients in the absence of concurrent chromosome 7 loss compared to those with both abnormalities. Importantly, the first known outcome data of bone marrow transplantation in double inv(3) AML is also presented.

Keywords Inversion 3, Double inversion 3, Chromosomal microarray, Copy-neutral loss-of-heterozygosity, Acute myeloid leukemia, 3q, MECOM expression.

Introduction

Inversion or translocation of chromosome 3, specifically inv(3)(q21q26.2/t(3;3)(q21;q26.2), is a recurrent finding in myeloid malignancies. These rearrangements usually result in elevated expression of the proto-oncogene *MECOM* (*EVI1)* at 3q26.2, through juxtaposition with a distal *GATA2* enhancer [1,2]. Acute myeloid leukemia (AML) with inv(3)/t(3;3) is classified by the WHO as a distinct entity which can present *de novo* or arise from prior myelodysplastic syndrome (MDS) and is associated with aggressive disease, minimal or no response to chemotherapy, and short survival [3,4]. Such cases represent approximately 1-2% of AML and are frequently characterized by anemia, normal to elevated platelet counts, and bone marrow hyperplasia with multilineage dysplasia of non-blast cells, particularly dysmegakaryopoeisis [4]. The frequent presence of multilineage dysplasia suggests multipotent stem cell involvement. Additional genetic aberrations most frequently include monosomy 7/deletion 7q and occasionally deletion 5q, which can be part of a complex karyotype [2,4,5]. Inv(3)/t(3;3) is also well established in blast phase chronic myeloid leukemia (CML) [6].

Hematological malignancies with inversion of both chromosome 3 homologs are exceedingly rare and have only been described in 13 cases; 11 of AML [2,3,5–9], one of MDS

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[10] and one of CML [11]. Many of these reports have a paucity of available pathologic and clinical information, and SNP array data with a reportable finding is only available for two previous cases. The current study presents two new double inv(3) AML cases with long term pathologic and clinical follow up, genetic evolution evaluation, and chromosomal microarray analysis on one of the cases informing the genetic mechanism.

Methods and materials

Patients

In accordance with the Institutional Review Board, two cases involving karyotypes with double inv(3) were identified for inclusion in this study out of 34,898 bone marrow samples analyzed at a cytogenetics laboratory in a tertiary medical center from 1961–2016. Additional results for Patient 2 were obtained from a second tertiary medical center.

Chromosome and fluorescence *in situ* hybridization (FISH) analysis

Conventional chromosome analysis of 20 metaphases on 24 h unstimulated and 48 h GMCSF-stimulated bone marrow cultures was performed employing standard methods. FISH was applied using standard protocols and the following commercially available probes (Abbott Molecular): *BCR*/*ABL1*(dual-color dual-fusion), *RUNX1T1*/*RUNX1* (dual-color dual-fusion), EGR1 (5q31, SpectrumOrange), D7S486 (7q31, SpectrumOrange), and D20S108 (20q12, SpectrumOrange). The double inv(3) was detected using either manually mixed *EVI1* break-apart (3q26.2, SpectrumOrange) and *RPN1* break-apart (3q21, SpectrumGreen) or pre-mixed *RPN1*/*EVI1* (dual-color dual-fusion).

DNA isolation and chromosomal microarray analysis

DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) bone marrow clot sections from Patient 1 using the QIAamp DNA FFPE Tissue kit (Qiagen,Valencia CA) according to the manufacturer's protocol. Tumor content was confirmed through corresponding H&E review by a pathologist. CMA was performed using the OncoScan FFPE Express 2.0 assay (Affymetrix, Santa Clara, CA) following the manufacturer's protocol. Application of the OncoScan Nexus Express Software (Biodiscovery, El Segundo, CA) to the resulting data included analysis of both copy number and loss of heterozygosity events on chromosome 3 with data visualization of the LogR ratio and B allele frequency (BAF) plots.

Next generation sequencing analysis

MECOM expression level was assessed in patient 2 using the Oncomine Myeloid Research Assay gene targeted NGS panel (Ion Torrent, USA). Standard procedures were employed with *MECOM* expression level normalized to the average expression of the control genes *EI2B1, FBXW2, PSMB2, PUM1, and TRIM27.*

Results

Patient 1

A 41-year old woman with a history of stroke, hypothyroidism, headache, and pineal gland cyst with one month of night sweats, anemia, and palpable splenomegaly presented to a tertiary clinical center with leukopenia, macrocytic anemia and thrombocytosis. FISH for *BCR-ABL1* fusion was negative.

A diagnostic biopsy was performed five months later, delayed due to ischemic stroke. At that time there was moderate macrocytic anemia, and absolute neutropenia with 9% circulating blasts (Table 1). Additional detailed pathology and clinical information is presented in Supplemental Table 1. The bone marrow was hypocellular (30%) with panhypoplasia. Blasts were increased, 17% by aspirate differential count and 20% by CD34 immunohistochemistry on the core biopsy, which was diagnostic of acute myeloid leukemia. The blasts were of intermediate size and without morphologically distinct features. By flow cytometry, they expressed CD13, CD34, CD45, CD117, and HLA-DR, and lacked CD33 and monocytic and lymphoid markers. Megakaryocytes were dysplastic, some small with monolobate nuclei and other with multiple separated nuclei. There was also subtle non-diagnostic dyspoiesis in the erythroid and granulocytic lineages and treated with induction chemotherapy.

A Day 14 marrow biopsy showed morphologically persistent AML with less than 5% cellularity. Re-induction with an anthracycline and cytarabine-based regimen occurred on Day 16. The patient's course was complicated by pancytopenia and bleeding. A Day 47 bone marrow biopsy showed 5% total cellularity with 2% blasts. Despite initiation of G-CSF therapy, the patient remained pancytopenic and transfusion dependent.

Three months following the initial induction (day 111), a repeat bone marrow biopsy showed 5% total cellularity. The karyotype showed an abnormal clone (10 of 20 metaphases) containing a double inversion of chromosome 3 and including two subclones, one each containing t(X;15) and t(3;10). (46,XX,inv(3)(q21q26.2) × 2[4] /46,idem,t(X;15)(p11.4;q22)[3] /46,idem,t(3;10)(q12;q24)[3]/ 46,XX[10]). Fluorescence *in situ* hybridization (FISH) analysis using manually mixed break-apart probes sets for *EVI1* and *RPN1* demonstrated rearrangement of both chromosome 3 homologs in 44% of cells and no single fusion was observed. Representative images of chromosome 3 abnormalities by karyotype and FISH are presented (Fig. 1). Chromosomal microarray analysis on DNA isolated from bone marrow clot sections identified mosaic CN-LOH of 3q, specifically arr[hg19] 3q21.3q29(128,236,447–198,022,430) × 2 hmz (Fig. 2). Based on these results, FISH for the *MECOM (EVI1)* region was retrospectively performed on the two early karyotypically normal bone marrow samples, which confirmed the presence of a double inv(3) clone in both initial samples at a frequency of 28% and 20% (Table 2). All genetic testing results are summarized in Supplemental Table 2. Within a month of the last testing, the patient received a haplo-matched stem cell transplant from her sister complicated by slow engraftment, pancytopenia and mucosal bleeding. She had no evidence of recurrent disease in a bone marrow biopsy performed one year after diagnosis.

Table 1 Summary of clinical and pathologic characteristics at diagnosis of current patients and cases with double inv(3)(q21q26.2) reported in the literature.

Case	Patient 1 (current)	Patient 2 (current)	Gu et al., 2015 (Patient 1)	Gu et al., 2015 (Patient 2)	Gu et al., 2015 (Patient 3)	De Braekeleer et al., 2013 (Patient 1)	De Braekeleer et al., 2013 (Patient 2)	Toydemir et al., 2010	Lugthart et al., 2010 (31_HO04)	Lahortiga et al., 2004 (Case 1)	Lee, 1999	Secker-Walker et al., 1995 (Case 8)	Secker–Walker et al., 1995 (Case 10)	Levy et al., 1994 (Patient 3)	Walter et al., 1990 (Case 5)
Age (years)/ Gender	41 (Female)	72 (Male)	72 (Male)	64 (Female)	56 (Female)	62 (Male)	67 (Female)	36 (Male)	NA (Female)	65 (Male)	83 (Female)	80 (Male)	39 (Male)	NA (Female)	55 (Female)
Referring diagnosis	Leukopenia, macrocytic anemia, and thrombocyto-sis	Severe anemia and pancytopenia	MDS (RAEB-2 with 15% blasts)	Monocytosis and CMML Type 2	Pancytopenia/ thrombocy-topenia	AML (M1)	AML with multilineage dysplasia	CML with resistence to imatinib	AML	AML (M4)	AML	MDS (RAEB)	AML (M4)	CML	MDS (RA)
Final diagnosis	AML	AML (M0)	AML(M6A)	AMML	AML	AML (M1)	AML with multilineage dysplasia	CML with resistence to imatinib	AML	AML (M4)	AML	Hypoplastic AML (M1)	AML (M4)	AML (M4)	MDS (RA)
Survival post-diagnosis (months)	17+	16	23	12	5	9	4	NA	NA	24	NA	63.8 (13.3 months following 3q finding)	3 (3 months following 3q finding)	NA	~60 (32 months following 3q finding)
Follow up post-diagnosis (months)	17	16	4	3	3.5	9	4	NA	NA	NA	NA	63.8	3	NA	~60
Treatment	SWOG S1203, anthracycline and cytarabine-base regimen, G-CSF	Standard 3+7 chemotherapy, 5+2 re-induction, azactidine, Xel-janz/tofactinib, decitabine	cytarabine, imatinib, vorinostat	decitabine	Idarubicin, cytarabine	Cytarabine and idarubicin	Failed induction therapy	Imatinib mesylate	NA	NA	NA	NA	NA	NA	Regular transfu-sions
Stem cell transplant	yes	yes	no	no	no	NA	no	NA	NA	NA	NA	NA	NA	NA	no
Complete remission	yes	no	no	no	morphological	yes	no	NA	NA	NA	NA	NA	NA	NA	no

Abbreviations: NA, not available, RAEB-2, refractory anemia with excess blasts 2, CMML, chronic myelomonocytic leukemia, CML, chronic myelogenous leukemia, RA, refractory anemia, AML, acute myelogenous leukemia.

Table 2 Summary of genetic testing results of current patients and cases with double inv(3)(q21q26.2) reported in the literature.

Case	Karyotype	FISH	SNP array	Other molecular
Patient 1 (current)	Initial karyotype and repeat one week later were 46,XX[20] (retrospectively FISH confirmed 28% and 20% cells with RPN1/EVI1 rearrangement on both homologues, respectively)	*RPN1*/EVI1 fusion on both homologues. Normal 5q, 7q, 20q and BCR/ABL1.	Mosaic CN-LOH of 3q, specifically arr[hg19] 3q21.3q29(128,236,447–198,022,430) $\times$ 2 hmz	NA
Patient 2 (current)	Initial karyotype 46,XY[20] and three weeks later 46,XY,inv(3)(q21q26.2) $\times$ 2 [16]/46,XY[4]	*RPN1*/*EVI1*fusion on both homologues. Normal MDS panel and *RUNX1T1/RUNX1*.	NA	*JAK3* p.Val722Ile(+), *DNMT3A*(+), *FLT3*(−), *NPM1*(−)
Gu et al., 2015 (Patient 1)	46,XY,inv(3)(q21q26.2) [13]/46,idem,del(7) (q22)[1]/ 46,XY[7]	NA	arr[hg19] 3q13.21q29 (10,344,387–197,802,470) $\times$ 2 hmz	*FLT3*(−), *KRAS*(−), *NRAS*(−)
Gu et al., 2015 (Patient 2)	Outside hospital reported normal chromosomes. Current study 46,XX,inv(3)(q21q26.2) $\times$ 2[18]/ 46,XX[2]	Outside hospital reported normal FISH for 5/5q, 7/7q, 8 and 20q	NA	*FLT3*(−), *KRAS*(−), *NRAS*(−)
Gu et al., 2015 (Patient 3)	46,XX,inv(3)(q21q26.2) $\times$ 2 [3]/45,idem,−7 [14]/46,XX[3]	*EVI1* rearrangement of both homologues	Monosomy 7 (<10%)	*FLT3*(−), KRAS(−), NRAS(−), *PTPN11*(missense at <10%), *CEBPA*(germline variant).
De Braekeleer et al., 2013 (Patient 1)	45,XY,inv(3)(q21q26) $\times$ 2,−7 [20]/46,XY[2]	*EVI1* rearrangement of both homologues (breakpoints 3q21 and 3q26)	NA	NA
De Braekeleer et al., 2013 (Patient 2)	46,XX,inv(3)(q21q26), add(5)(q1?3)[3]/ 45,idem,−7[7]/45, idem,inv(3)(q21q26),−7[12]	Two clones with *EVI1* rearrangement, either single homologue or double homologue (breakpoints at 3q21 and 3q26)	NA	NA

(*continued on next page*)

Table 2 (*continued*)

Case	Karyotype	FISH	SNP array	Other molecular
Toydemir et al., 2010	46,XY,inv(3)(q21q26.2) × 2,del(7)(q22q34)[20]	*EVI1* inversion of both homologues; complex BCR/ABL1 rearrangement	arr [hg18]3q13.2q29(112,422,681–198,963,099) × 2 hmz,7q21.2q32.3 (92,452,780–131,082,318) × 1, 9q34.12q34.13 (132,630,823–134,459,997) × 3, 14q32.33q32.33 (105,522,074–105,854,077) × 2 hmz, 22q11.2q11.2(23,994,249–24,252,536) × 2 hmz	*BCR*/*ABL1* fusion transcript (subclone with missense c.944C–>*T* or *p*.T315I)
Lugthart et al., 2010 (31_HO04)	47,XX,inv(3)(q21q26), + inv(3)(q21q26)[a]	NA	NA	NA
Lahortiga et al., 2004 (Case 1)	46,XY,inv(3)(q21q26) × 2[30]	Complex rearrangement[b]	NA	NA
Lee, 1999	45,XX,inv(3)(q21q26) × 2,−7 (80% abnormal, 20% normal cells)[a]	NA	NA	NA
Secker–Walker et al., 1995 (Case 8)	46,XX,inv(3)(q21q26), inv(3)(q21q26)[a]	NA	NA	NA
Secker–Walker et al., 1995 (Case 10)	45,XY,inv(3)(q21q26),inv(3)(q21q26),−7[a]	NA	NA	NA
Levy et al., 1994 (Patient 3)	46,XX,inv(3)(q21q26) × 2,*t*(9;17;22)[a]	Rearrangement 3′ of *EVI1*in both homologues	NA	NA
Walter et al., 1990 (Case 5)	46,XX,inv(3)(q21q26),inv(3)(q21q26)[a]	NA	NA	NA

Abbreviations: NA = not available, CN-LOH = Acquired copy-neutral loss of heterozygosity.

[a] Total number of abnormal cells studied not provided.

[b] Each inv(3) had insertion of a fragment from 3q21 into 3q26 and a second insertion of a fragment from 3q26 into 3q21.

Note: Nomenclature for abnormalities presented as described in publications.

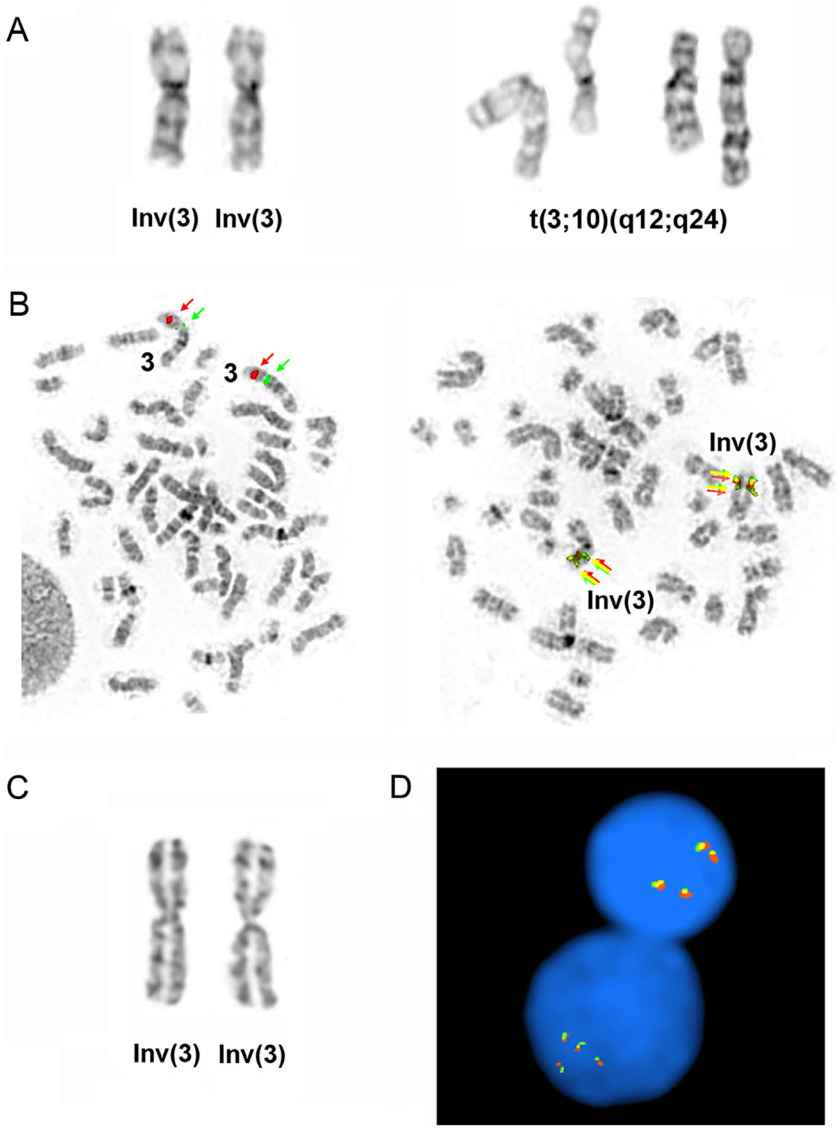

Fig. 1 Representative images of chromosome 3 abnormalities. (A) Karyotype analysis on Case 1 showed a double inv(3) stemline and a subclone that included a t(3;10) involving one of the inverted chromosome 3 homologs. (B) FISH on Case 1 using a probe mixture of *EVI1* break-apart (at 3q26.2; Orange) and *RPN1* break-apart (at 3q21; Green) identified some normal cells (left), which confirmed accurate probe placement, and cells with *RPN1*/*EVI1* fusions on both homologs that represent the stemline (right). (C) Karyotype analysis on Case 2 demonstrated the stemline to have only a double inv(3), which was retained during the clonal evolution that occurred throughout the disease course. (D) FISH on Case 2 using a dual-color, dual-fusion probe set confirmed the presence of *RPN1*/*EVI1* fusions on both chromosome 3 homologs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Patient 2

A 72 year old male presented to the emergency room for an apparently unrelated issue, was found to be pancytopenic with severe anemia, and was transfused 4 units or pRBCs and *u* unit of platelets. His history included basal cell carcinoma at age 69, long dormant ulcerative colitis, non-insulin dependent diabetes, multiple abdominal and chest lipomas, and tobacco use/smoking over 25 years ago. Family history was notable for CML in his mother. The diagnostic bone marrow was less than 10% cellular with 44% blasts and dysplastic hypolobated megakaryocytes. Although lineage assignment was not possible based on the absence of myeloperoxidase, CD3 and CD19 or other B-cell markers, a diagnosis of acute myeloid leukemia with minimal differentiation (corresponding to FAB-M0) was favored at the time of diagnosis (Table 1), which was based on expression of some myeloid markers (CD13 (subset, dim) and CD117). A concomitant complete blood count showed pancytopenia without circulating blasts. Chromosome analysis demonstrated 46,XY[20] and FISH for MDS was also normal while mutation analysis identified *JAK3 p.*Val722Ile and a *DNMT3A* alteration (Table 2). Additional detailed pathology clinical information is presented in Supplemental Table 1. Mutation analysis on peripheral blood near this time was also negative for *FLT3* ITD and *NPM1* mutations. All genetic testing results are summarized in Supplemental Table 2.

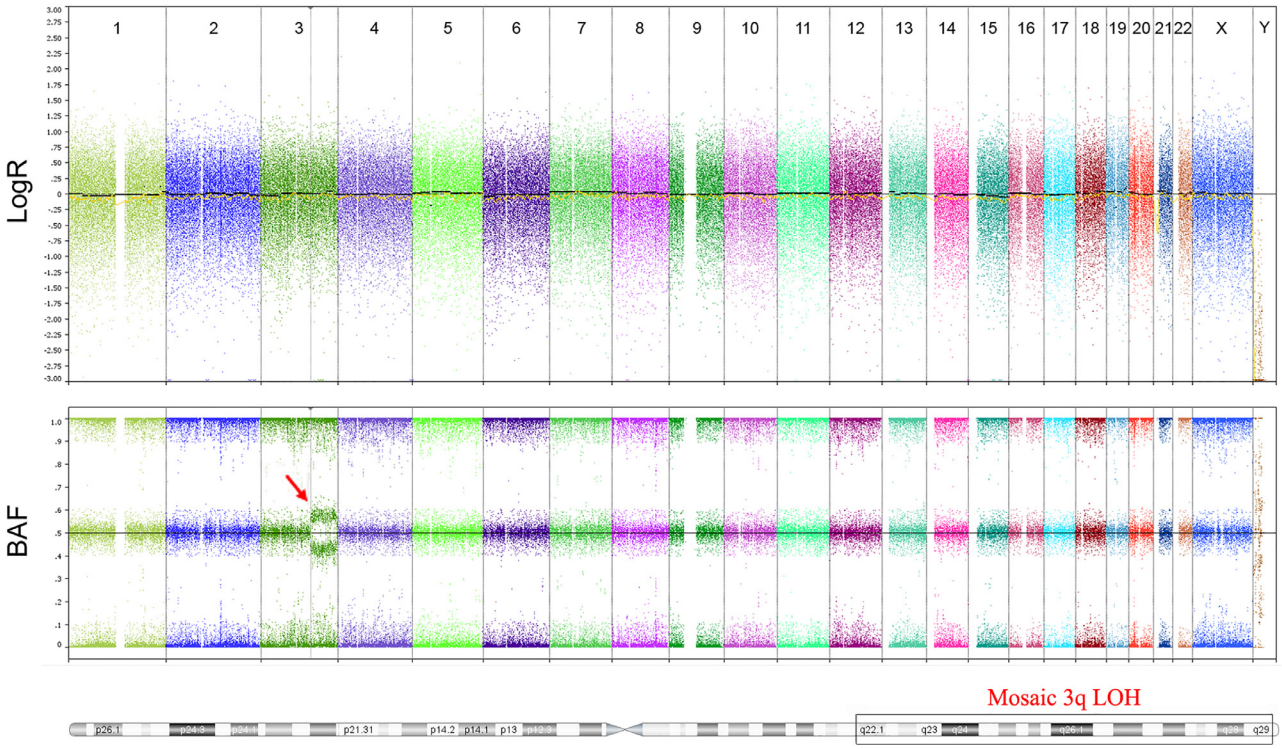

Fig. 2 CN-LOH of 3q detected in Case 1 using chromosomal microarray. Whole genome view showing log ratio (LogR) and B-allele frequency (BAF) plots with 3q CN-LOH denoted by a red arrow. The CN-LOH region is approximately 69.7 Mb at 3q21.3-q29 (chr3:128,236,447-198,022,430; hg19). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The patient initially received standard 7+3 induction chemotherapy. A post-induction marrow biopsy was 10% cellular with persistent disease (28% blasts), minimal maturing hematopoiesis and no evident dysplasia or megakaryocytes. The associated karyotype was 46,XY,inv(3)(q21q26.2) × 2[16]/46,XY[4] (Fig. 1). Persistent disease remained after 5 + 2 re-induction therapy as well, with pancytopenia, marked marrow hypocellularity (<5%), and increased blasts. However, circulating blasts were now present (6%), and some dysplastic small megakaryocytes were identified along with normal appearing megakaryocytes using CD61 on the bone marrow biopsy.

Based on the presence of persistent disease after re-induction, the patient underwent combination therapy with azacitidine and the Jak1/3 inhibitor Xeljanz/tofacitinib. Disease persisted after one cycle and three cycles of this therapy, with marrow biopsies at these times showing hypocellular marrow, 10-11% blasts, and low level persistent abnormal karyotype (one abnormal metaphase at each time). Due to a perceived lack of efficacy, therapy was changed to decitabine. A marrow biopsy approximately one month into decitabine therapy showed persistent disease with a hypocellular marrow and at least 20% blasts. The concurrent karyotype was 46,XY,inv(3)(q21q26.2) × 2[4]/46,idem,t(2;21)(q21;q11.2)[15]/46,XY[4], consistent with increasing tumor burden and possible clonal evolution. The patient was pancytopenic and RBC transfusion dependent, receiving treatment about once every 10 days.

At evaluation prior to bone marrow transplant, the patient remained pancytopenic with a hypocellular (5-10%) marrow with greater than 50% blasts (confirmed by CD34 on the biopsy).

The last bone marrow evaluation before transplant also showed persistent disease (hypocellular at 10% and elevated blasts at 56%) and a complex abnormal karyotype: 45-46,XY,der(2)t(2;21)(q21;q11.2)[9],inv(3)(q21q26.2) × 2[9], + 15[4],add(15)(q22)[9], −21[9], + mar1[3][cp9]; FISH was consistent with the karyotype, scoring negative for *t*(8;21) and positive for double inv(3), specifically 69% of cells had two copies of the *RPN1*/*EVI1* fusion using a dual-fusion probe set (Fig. 1).

The RNA expression level of MECOM was also assessed using a NGS myeloid gene panel and found to be abnormally elevated in this patient compared to the average expression of 5 control genes (35,258 versus 15,584; 2.26-fold increase). In addition, evaluation of the diagnostic bone marrow of 15 patients with RPN1/MECOM fusion-negative AML, as confirmed by chromosomes and FISH, did not show significant overexpression (data not shown); a single outlier had a 1-fold increase in expression compared to the controls, which was considerably lower than the level identified in patient 2 and suggestive of a different genetic mechanism.

The patient subsequently underwent allogeneic bone marrow transplant, and within one month chimerism studies showed 100% donor, followed by 60% donor three weeks later and 70% donor a week subsequently. Bone marrow analysis approximately two months after transplant showed 30% cellularity, 50% blasts, and marked granulocytic and megakaryocytic hypoplasia. Chromosome analysis results were 46, XY,der(2)t(2;21)(q21;q11.2),inv(3)(q21q26.2), + 15, −21[3]/46,

idem,inv(3)(q21q26.2)[3]/46,XY[14] and FISH confirmed double inv(3) resulting in two *RPN1*/*EVI1* fusions in 76% of cells. The patient died 3 months after this analysis and 16 months after initial diagnosis at the age of 73 years old.

Discussion

While heterozygous inv(3)(q21q26.2) is a well-established entity in myeloid malignancies, double inv(3) has only been reported in 13 cases, the majority being AML [2,3,5–11]. We present two new AML cases with clinical, pathologic and genetic results in comparison to previously published reports, most of which have a relative paucity of reported data. The frequency of such cases was proven to be exceedingly low, present in 2/34,898 consecutive bone marrows (0.0005%). No diagnostic morphologic differences were apparent between double and single inv(3) AML cases, indicating genetic analysis is necessary to distinguish these cases. Of note, both cases were hypocellular at diagnosis. Although marrow cellularity is often not specified in previous reported cases, those described were all either hypercellular or normocellular for age [5,7,8,10]. The significance of this finding is therefore unclear. Also described is only the third known double inv(3) case to have a reportable finding by SNP array, which further informs the formative mechanism of the abnormality.

The three potential mechanisms for the development of the double inv(3) are independent inversion of each homolog, loss of a normal chromosome 3 homolog and subsequent duplication of the inv(3) homolog, or segmental deletion followed by somatic repair using the other homolog as a template. Application of chromosomal microarray testing to Case 1 showed a SNP pattern consistent with mosaic segmental CN-LOH beginning at the 3q21 breakpoint and extending towards the telomere of the long arm with normal heterodisomy on the short arm. This result does not support either of the first two potential mechanisms because there is not whole chromosome heterozygosity or homozygosity, respectively. The segmental CN-LOH finding is similar to the results of two previous double inv(3) cases analyzed by microarray [7,11], strongly suggesting a recurrent somatic repair mechanism. Since the breakpoints appear to have some variability and the involved DNA region is large (~69.7 Mb in Case 1), the segmental CN-LOH is likely repaired through nonhomologous end-joining (NHEJ) rather than another somatic double-stranded DNA repair mechanism (i.e., gene conversion or non-allelic homologs recombination). However, one additional published double inv(3) case analyzed by microarray was normal, likely due to insufficient tumor or clonal fraction, although the occurrence of independent inversions of each chromosome 3 cannot be ruled out [7].

The double inv(3) has been suggested to arise during clonal evolution, representing a subclone of a single inv(3) stemline based on the presence of both cell lines in patient 2 of De Braekeleer et al. and patient 1 of Gu et al. [5,7]. The mechanism would involve an inverted deletion of a single 3q that is repaired using the normal homolog as a template, creating the first cell line. The second cell line would evolve when the normal homolog has an inverted deletion that is repaired using the first inverted chromosome as a template. The complexity of this mechanism is consistent with the rarity of double inv(3) AML. Although all such cases likely arise this way, the majority, including the newly described Cases 1 and 2, demonstrate only the double inv(3). It is particularly illuminating that the double inv(3) but not single inv(3) was found by FISH in Case 1 three months before karyotype analysis could detect any abnormal clone. This finding supports very early clonal evolution and/or rapid dominance of the double inv(3) clone compared to the single inv(3) abnormality.

The effect on transcription of double inv(3) was explored using a targeted NGS panel in patient 2. MECOM RNA was overexpressed by 2.26-fold compared to expression of control genes. In a cohort of 15 cases with AML at diagnosis and no evidence of chromosome 3 inversion, 14 did not show MECOM overexpression. However, one of these cases demonstrated a 1-fold increase, which is consistent with reports of *MECOM* (*EVI1*) transcriptional activation in approximately 8–15% of AML patients without chromosomal defects affecting the *MECOM* locus [12, 13]. The NGS panel did not have coverage to detect single base pair mutations in the *MECOM* gene.

Whatever the mechanism of double inv(3) formation and concurrent increased MECOM expression, the resulting clinical manifestations have not been well explored to date due to a paucity of cases and limited descriptive information available for most of those that have been reported. Review of all known patients does suggest the double inv(3) contributes to general aggressiveness and high rates of conventional chemotherapy resistance. This is illustrated in Case 1 where the double inv(3) clone percentage in the bone marrow increased as treatment intensity and diversity progressed, even when overall bone marrow cellularity significantly decreased. A poor outcome was clearly demonstrated in Case 2 where clonal persistence without remission leading to death within 16 months occurred despite applying standard 3 + 7 chemotherapy induction, reinduction with 5 + 2, azacitidine, Xeljanz/tofactinib, decitabine and bone marrow transplant.

The two new double inv(3) cases are the only reported examples of bone marrow transplant outcome data in this subset of AML. While Case 2 had a poor response to transplant, dying within 3 months, Case 1 was disease-free 12 months after transplant. However, extrapolation from *de novo* AML cases with single inv(3)/*t*(3;3) would suggest that transplant is not curative. One study indicated early relapse (<1 month, 2 months and 10 months) and death without having achieved remission in three single inv(3)/*t*(3;3) cases [14], while another study of 11 similar patients showed that supplementation of chemotherapy with allogeneic stem cell transplantation resulted ultimately in death but there was significantly prolonged survival compared to chemotherapy alone (13.8 versus 8.0 months) [15]. These results overall suggest bone marrow transplant could be considered in treatment of double inv(3) AML patients as a method to potentially extend survival time with more data needed to fully understand the short and long term impact.

In the reported cases of non-transplanted AML, overall survival (OS) analysis did not show an adverse effect of having a doubled inv(3) relative to a single inv(3) clone (median OS of 12 months and 8.9 months, respectively). However, within the double inv(3) patient subset, the four cases with concurrent monosomy 7 were associated with significantly worse outcome when compared to the five lacking this abnormality (median OS of 4.5 months and 23 months, respectively) [7]. Multivariate analysis demonstrated similar findings in single

inv(3)/t(3;3) AML, where decreased relapse-free and overall survival were exacerbated in the 66% of cases with concurrent monosomy 7 [2]. Contrarily, no additional cytogenetic features were shown to independently correlate with the poor prognosis in another study of single inv(3)/*t*(3;3) AML [15]. The two new double inv(3) cases in the current study, which both lacked chromosome 7 anomalies and had an overall survival of 16 and $>$ 17 months, are consistent with the previously reported timeframe for non-deletion 7 cases; this finding supports the hypothesis that chromosome 7 deletion may adversely impact AML patients in the setting of a double inv(3) clone.

In summary, we describe pathologic, clinical and genetic results on two new AML patients with double inv(3), significantly expanding the known AML cases up to 13 total. Chromosomal microarray analysis identified segmental CN-LOH on 3q in one of the new cases, making it the third such finding and strongly informing the genetic mechanism of double inv(3) formation. The comparatively longer survival and lack of concurrent chromosome 7 anomalies in the two new cases are further suggestive of a worse prognostic impact of the combined anomalies. When this is considered alongside the lack of discernible difference in morphologic criteria between single and double inv(3) cases, it re-emphasizes the importance of incorporating genetic analysis into the work-up of acute myeloid leukemia for accurate diagnosis and risk-stratification. Finally, we present the first two cases with outcome data following bone marrow transplantation.

Conflict of interest

The authors have no conflicts of interest to declare.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2018.08.007.

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