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Discordant Disease Course in a Monozygotic Twin Pair with Juvenile Myelomonocytic Leukemia

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Abstract

Juvenile myelomonocytic leukemia is a rare neoplastic disorder occurring in early childhood often showing an aggressive progression. We report a case of a twin pair with concordant JMML but an extremely different disease course. Both twins presented with somatic aberrations of chromosome 7 and mutations in *PTPN11*. Analysis of sorted BM and PB cell populations revealed the clonal nature of the disease and indicated that genomic aberrations arise from common hematopoietic precursor cells. *PTPN11* mutations in oral swab specimens of both patients during the active phase of the disease were attributed to monocytes infiltrating the oral mucosa and not to the presence of mosaic tissue mutations. This study provides evidence that the discordant clinical disease course in the twins is associated with a distinct gene expression profile.

Keywords

Juvenile myelomonocytic leukemia, Monozygotic twins, Discordant disease

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myelodysplastic/myeloproliferative disorder, characterized by the malignant transformation of myeloid progenitors in the stem cell compartment [1]. Clinical features like hepatosplenomegaly, lymphadenopathy, pallor, fever and skin rash, peripheral blood monocyte counts >1x10^9/L, bone marrow blast counts <20% and the absence of *BCR-ABL1* rearrangements are usually indicative of JMML [2], yet diagnosis can be established with certainty only after laboratory workup has confirmed the presence of clonal abnormalities, including monosomy 7 and/or point mutations resulting in the activation of the RAS/MAPK cascade [3].

Leukemia is not generally considered to be inherited but its incidence in twins is high and this has been related to a common clonal origin of the disease. Generally, accepted basis of concordance of leukemia is that following the genomic alterations in one twin fetus, these aberrations spread to the co-twin via a common monochorionic placenta [4]. Seventyfive percent of monozygotic twins are monochorionic hence presenting a high probability of blood cell exchange [5,6].

Clinicians and parents facing a severely diseased child of a twin pair are frequently concerned about concordance of the disorder even in the absence in the co-twin of specific clinical features or any clear evidence as to the constitutional genetic background of the disease.

The clonal origin of JMML has been demonstrated in patients with *RAS* mutations [7] and *NF1* deletions [8]. However, cell differentiation stages where aberrations occur are not univocal. As to JMML patients with *RAS* mutations, one patient presented aberrations in both the myeloid lineage and the B-lineage, whereas the mutations were restricted only to the myeloid lineage in two other patients [7]. One case of JMML with *NF1* harbored the mutation in the

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myeloid population and in the derived T-cell lymphoma, suggesting the pluripotent stem cell as the cell of origin [8].

In a murine model of myeloproliferative disease a conditional knock-in mutation of *Ptpn11E76K* in pan hematopoietic cells resulted in the activation of hematopoietic stem cells (HSCs) and myeloid progenitors [9].

With respect to the timing of appearance of the mutation during hematopoietic stem cell differentiation, no data are currently available for JMML patients with the most common *PTPN11* mutation (i.e. 35% of patients).

This report presents the results of a two-year study on a monozygotic twin pair suffering from concordant juvenile myelomonocytic leukemia (JMML), carrying the same *PTPN11* mutation and a rare 46, XY, -7 +mar karyotype but presenting a very different disease course. A clinical case report described the outcome of allogeneic hematopoietic stem cell transplant in this twin pair [10].

Methods

Materials and methods are described in full detail in the Supplemental Appendix.

In summary, we used the following approaches to analyze the specimens of a twin pair with JMML: microarray gene expression profiling and classification of the diagnostic samples, using the diagnostic classifier model [11]; cytogenetics, including karyotyping, FISH and aCGH to define the specific chromosome 7 aberration at relapse of Twin_01; flow cytometric sorting of whole bone marrow and peripheral blood in subpopulations, according to cell lineage origin and differentiation state; Sanger sequencing and Amplicon Ultra Deep 454 sequencing of *PTPN11* mutations to determine mutant allele frequencies; human leukocyte antigen (HLA) and short tandem repeats (STR) analyses of monozygosity; specific STR analysis of chromosome 7 to determine origin and extent of chromosome 7 aberrations.

Results and Discussion

A twin pair with concordant JMML

This study refers to a pair of monozygotic, monochorionic twins born at 36 weeks of gestation. At first presentation (age 19 month) Twin_01 was suffering from fever, low platelet count, anemia and hepatosplenomegaly. Peripheral blood (PB) smears showed monocytosis and low blast count (3% of blasts). A few months later, the bone marrow specimen showed dysplasia in myeloid cell lineages. The co-twin (Twin_02) presented no clinical symptoms of JMML at the same age but laboratory analysis revealed blood morphology and blood count data compatible with the diagnosis of JMML. Symptoms appeared 4 months later.

Analysis of the HLA system and STR analysis confirmed the monozygotic origin of the twins (Supplemental Figure 1). According to differential diagnostic criteria, each twin received a diagnosis of JMML with a common *PTPN11* mutation and abnormal chromosome 7 [10]. Disease timeline and progression is shown in Figure 1, whereas treatment details were reported earlier [10].

Clonal origin of JMML in twins with *PTPN11* mutation and aberrant chromosome 7

Historical records on monozygotic twins with concordant leukemia revealed a high probability of a common origin for the disease, leukemia stem cells in one twin fetus being transmitted to the co-twin via a common placenta [4]. Table 1 shows 6 cases of concordant

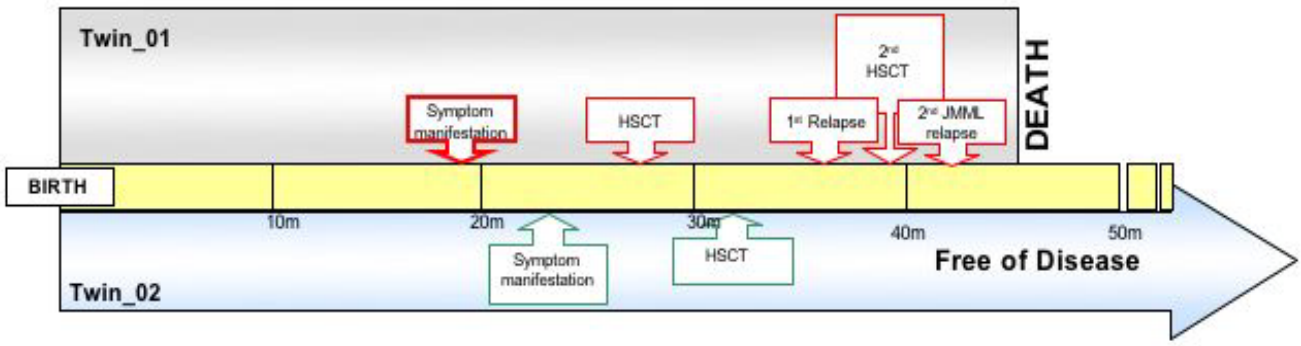

Figure 1: Timeline of the disease course in both twins. Twin_01 timeline from birth to death at 45 months. Twin_02 timeline from birth to follow-up at 48 months, in complete remission.

Table 1: Historic record of JMML (previously named JCML or jCMML) twin cases.

JMML/JCML/(j)CMML MZ type	Karyotype Twin_01	Karyotype Twin_02	Reference	Comments
(j)MML⁺ monochorionic	46,XY	46,XY; at final stage of the disease Ph+	Arch. of disease in childhood, 1977, 52: 72-74	Both twins deceased after a similar course of the disease
(j)MML	No Karyotypic marker	No Karyotypic marker	Cancer, 1984, 54: 675-686	Twin sister disease after a different course of disease
JMML No data monozygotic type	46,XX	46,XX	Cancer Genet Cytogenet 1989, 38: 205-213	Karyotypes of both twins evolved with hyperdiploid clones; Both twins deceased after a similar course of the disease
JCML Monozygosity 98%	46,XY, -7, +mar	46,XY, -7, +mar	Leukemia 1997, 11: 306-312	Both twins responded well to therapy
CMML ND monozygotic type	46,XY, t(7;16)(p10;q10)*	46,XY, t(7;16)(p10;q10)	BLOOD, 1995, 85: 1742-1750	Twin_01 deceased at 5 month after diagnosis Twin_02 is alive at 11 month after diagnosis**
JCML ND on twin type	ND	ND	BJH, 1995.90: 353-357	In one twin B-lineage clonality was demonstrated
JMML monochorionic- biamniotic	46,XX with *NRAS* mutation	Free of disease	Pediatric Blood Cancer 2008, 50: 665-667	Discordant JMML in two twins

⁺This twin pair was included here since the Ph+ karyotype was found only in the terminal phase of the disease and because it is the 'oldest' case reporting a twin pair with a myelomonocytic leukemia. *constitutional translocation also present in the father without hematologic abnormalities; ** alive at time of publication (1995).

JMML in monozygotic twins, suggesting a common clonal origin of the disease, even though, in most cases, no information concerning chorionic/amniotic type and genomic aberrations is provided [12-17]. In addition, absence of disease concordance has also been noted in at least one case of JMML (Table 1) [18].

We present here the case of a twin pair with confirmed monozygosity and monochorionic placentation, a $PTPN11_{E76K}$ mutation and a karyotype with -7 mar+. Array CGH had delineated diploid chromosome 7, p12.1 harboring sequences of 3 genes and known noncoding RNAs in each of the twins [10]. The presence of the same rare aberration of der 7 of maternal origin along with the same mutations of *PTPN11* strongly corroborates the hypothesis that both events occurred *in utero* in one twin fetus first, the aberrations later being transmitted to the co-twin via the common placenta. Previous detection of *KRAS* and *PTPN11* mutations in Guthrie cards of JMML patients is in line with their prenatal occurrence [19]. The possibility that the above aberrations in the twins could have occurred *in utero* as unrelated events is refuted.

Non-lineage restriction of genetic JMML markers

The clonal origin of JMML has long been recognized [7,8], but the involvement of all three major lineages (i.e. myeloid, B and T lymphoid) is a new finding.

The loss of both arms of chromosome 7 and the *PTPN11* mutation in all subpopulations of the myeloid lineage as well as in the B and T-lineage show that both JMML inciting events occurred in the common hematopoietic precursors (Supplemental Figure 2, Supplemental Table 1). We speculate that these common hematopoietic stem cells resided in the hypoxic BM niche of Twin_01 and that slightly differentiated progenitors left the niche and reached Twin_02 through common placental circulation.

Mutation load of *PTPN11* in whole BM and PB samples diverged from the maximum of 50% for heterozygous mutations revealing a considerable presence of cells without *PTPN11* mutations (Supplemental Appendix); similarly, the FISH analysis had shown the presence of nuclei with a normal karyotype. Comparable results were found in all cell subpopulations, which may suggest that also normal cells are present in the hematopoietic niche of the BM. The high percentage of non-mutated cells observed in PB samples may indicate that mutated hematopoietic cells are extremely prone to infiltrate different tissues.

Distinct gene expression signatures in Twin_01 and Twin_02

The twin pair showed two distinct gene expression signatures: the diagnostic classifier revealed an AML-like signature in Twin_01 and a non AML-like signature in Twin_02 (Supplemental Table 2, Supplemental Appendix). Remarkably, Twin_01 relapsed twice following HSCT and deceased, whereas Twin_02 remained in remission after HSCT.

The highly discordant clinical disease course in the twins (Figure 1) is in close concordance with our results on 44 cases with JMML. This study showing that patients with an AML-like signature have a poor prognosis, whereas patients with an non-AML-like signature have a favorable prognosis following HSCT [11]. The co-occurrence of monosomy 7 and *PTPN11* mutation in the twins with an AML-like and a non-AML-like signature was also previously found to be present in patients with distinct GEP-based classification [11].

As described at diagnosis, the BM of Twin_01 presented with the same $PTPN11_{E67K}$ mutation, as well as the loss of the long and short arms of chromosome 7, der7, p12.1 and an AML-like GEP signature, also at relapse (Supplemental Figure 3).

Mosaic tissue mutations vs. disease related infiltration with mutated leukocytes

Constitutional mutation syndromes have been related to JMML along with recurrent mutations including *NF1 (Neurofibromatosis type 1)*; *PTPN11, KRAS, NRAS (Noonan syndrome) and CBL* [20-22]. Also mosaicism in non-hematopoietic tissues for *KRAS* and *NRAS* has been recently reported in JMML patients [23]. Even though Noonan syndrome phenotypical features were absent in the twins, we analyzed other tissues looking for *PTPN11* mutations in order to rule out the hypothesis that the genomic aberrations in the common hematopoietic progenitor cells may have occurred also in common mesentoderm progenitor cells.

We analyzed fibroblasts, hair follicles and oral swabs to fully exclude the presence of mutations beyond the hematopoietic compartment. No mutation of *PTPN11* was detected in either fibroblasts or hair bulbs. We did find *PTPN11* mutations in oral swab during the active phase of the disease (Supplemental Figure 4) but not following HSCT, when the twins were in remission. We assume that the *PTPN11* mutations in both patients during the active phase of the disease with very similar mutant allele frequency can be attributed to monocytes infiltrating the oral mucosa and not to the presence of mosaic tissue mutations [23] (Supplemental Figure 5).

With respect to the absence of *PTPN11* mutations, also two copies of chromosome 7 were found in fibroblasts, hair bulbs and during remission in oral swabs. Aberrations of chromosome 7 were detected only in the active phase of the disease.

Implications for clinicians and translational research

Our study of a twin pair with JMML and identical genetic and karyotypic features and historical reports of JMML in twins, leads us to infer that the disease triggering mutations were already present at birth in each monozygotic twin. To the best of our knowledge, only one monochorionic-diamniotic twin pair has been described so far and only one of the twins suffered from JMML [18]. This condition may be due to the absence of transfer of the *NRAS* mutated clone from one twin to the other during prenatal life.

In the case reported here, the seeding of cells with both inciting genetic aberrations in the twin pair occurred long before the onset of the disease at the age of two, as inferred from the common prenatal origin. Also the presence of the mutations was demonstrated in Twin_02 before the disease became clinically manifest. For this reason, along with historical data (Table 1) pointing to extremely high concordance of JMML in twins, our data indicate that an immediate diagnostic workup for differential diagnosis of JMML in twins is mandatory, in case one of them presents with clinical features suggestive of JMML.

Mutant allele frequency levels in total BM and PB specimens and in the sorted cell populations (Supplemental Appendix, Supplemental Table 1) also showed that nonmutated cells can be found at all levels of differentiation. This suggests that there is still room for investigation into the therapeutic strategies aiming to spare healthy cells and eliminate mutated cells.

Monocytic leukemias are highly invasive and spread to other tissues, especially to the spleen, skin and mucosa. As a consequence, oral swabs taken in the active phase of the disease as well as other tissues that are subject to monocyte infiltration are not the most appropriate specimens, if we aim to look for any constitutional or mosaic tissue mutations, since the mutations may be attributed to infiltrating mutated leucocytes. In case of positive oral swab specimens it is advisable to repeat the mutation analysis on different tissues.

Finally, non-AML-like signatures in one of the twins with JMML warrants success of current therapies, whereas AML-like signatures along with a high incidence of relapses highlight the need to eliminate the mutated common hematopoietic cells in the bone marrow niche.

Acknowledgments

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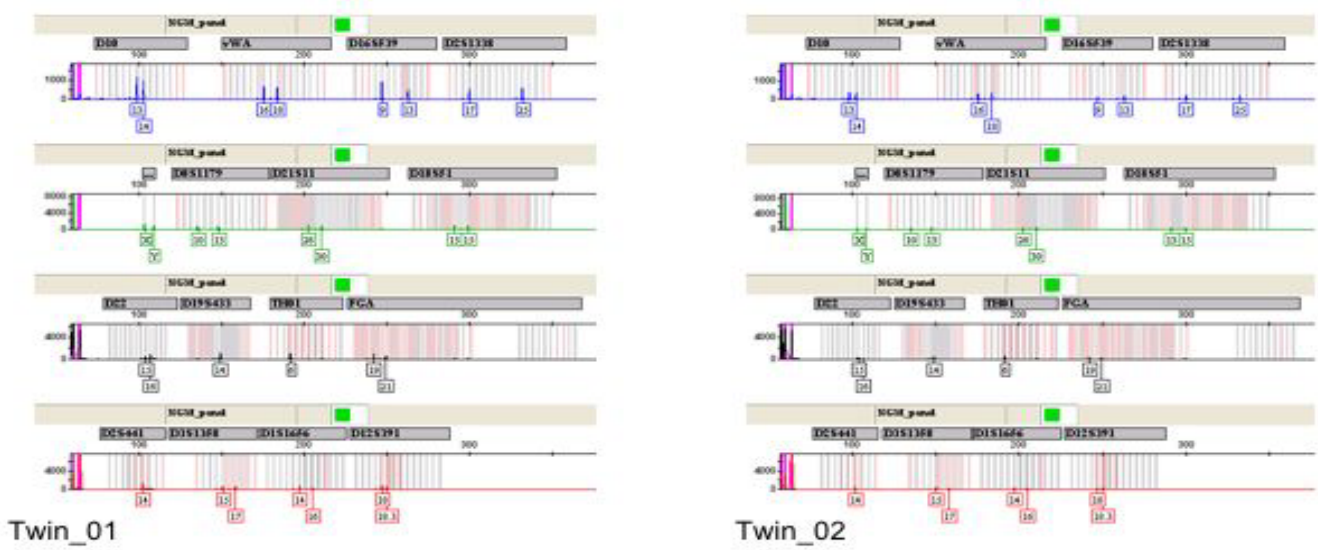
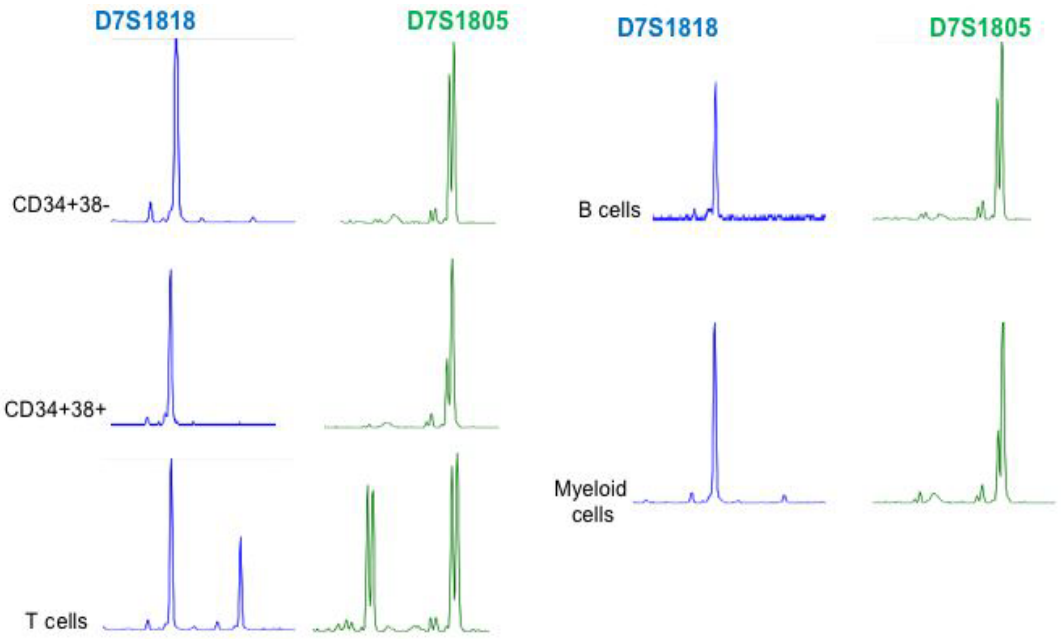


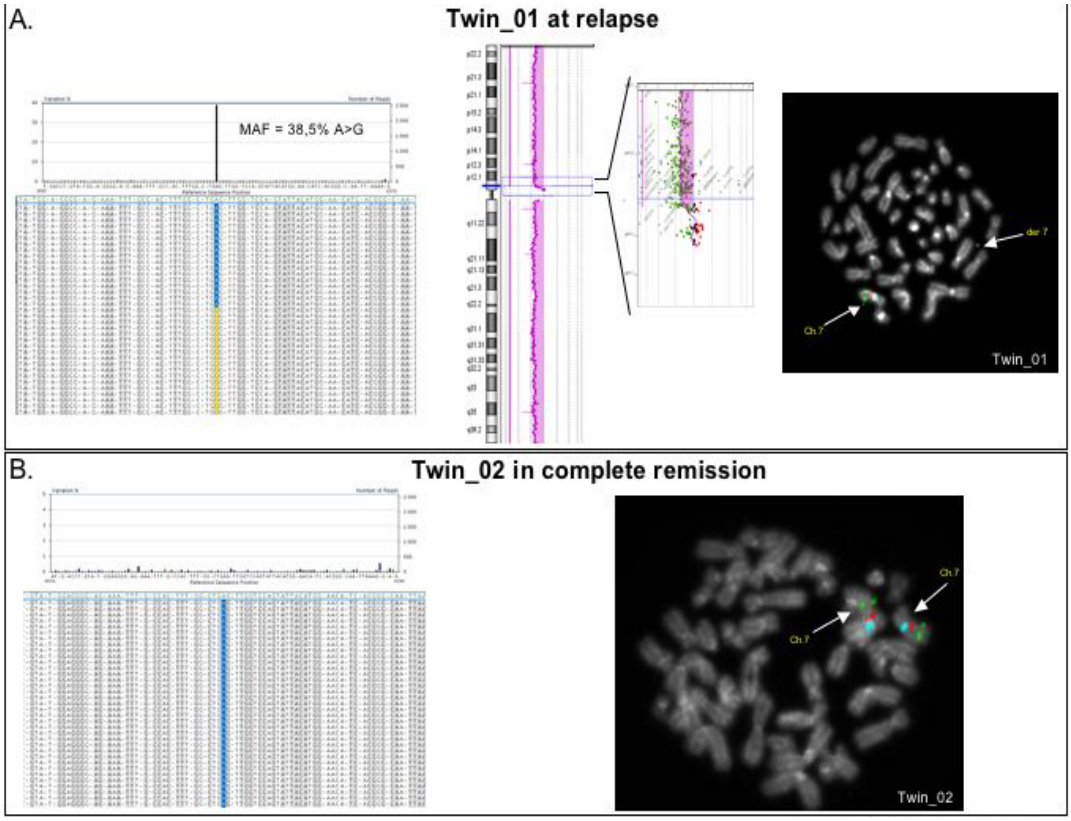
Supplemental Figure 1: Short tandem repeats (STR) profiles of both twins in fibroblast cells.

STR approach for forensic human identification was used to identify with high accuracy the monozygosity of the twins. Identical profiles were obtained from both twins.



Supplemental Figure 2: Short Tandem Repeat analysis to detect monosomy 7 in subpopulations of total bone marrow in Twin_02

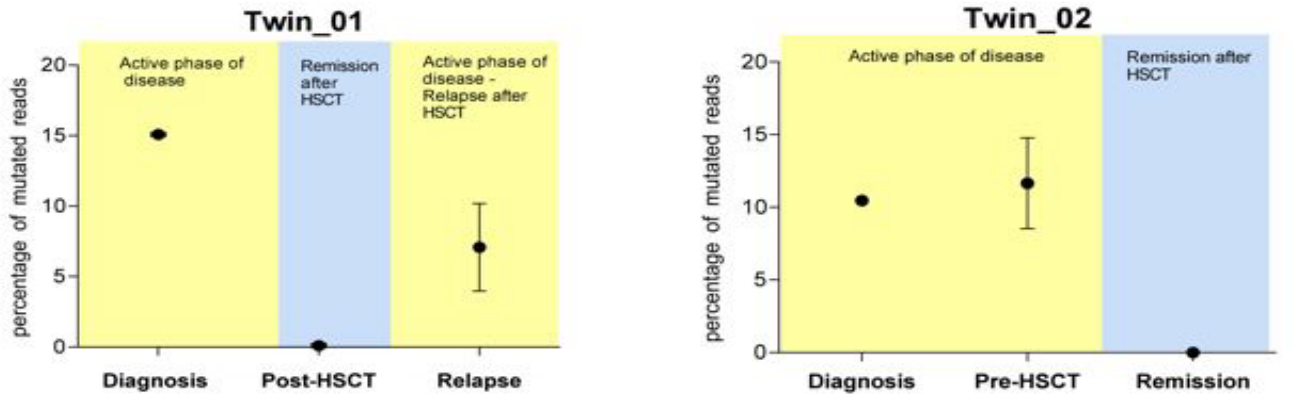
Detection of monosomy 7 was performed using two markers of chromosome 7, i.e. D7S1818 and D7S1805. In distinct subpopulations, loss to various extent of material of chromosome 7 was revealed. Twin_01 showed similar losses of chromosome 7 in subpopulations at diagnosis and the same losses were revealed at relapse.



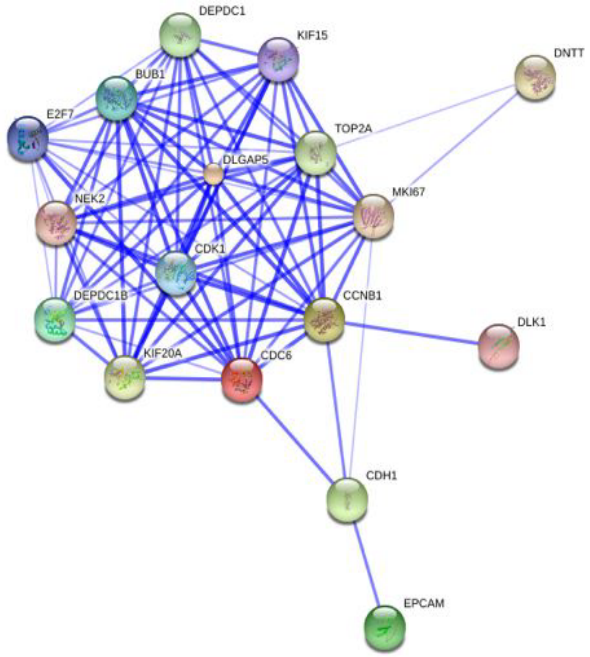
Supplemental Figure 3: Molecular features of the twins after HSCT.

A. Twin_01 at relapse. The molecular features of Twin_01 found at diagnosis remained unchanged at relapse. Ultra deep sequencing confirmed the same PTPN11 mutation at (E76K) and FISH analyses revealed the presence of der 7 with the conserved p12.1 region, including 41 transcripts (array CGH profile, in the middle). On the top, left-hand side of the figure, we show reads obtained with 454 for BM of Twin_01 at relapse. Substitution of A in G was identified with a MAF of 38,5%. B. Twin_02 in complete remission. Twin_02 ultra deep sequencing of PTPN11 mutation in exon 3 and FISH analysis showed normal features of the hematopoietic cells. On the bottom, left-hand side of the figure, we show reads obtained with 454 for BM of Twin_02 in complete remission after HSCT; no mutated reads were obtained compared to reference sequence of exon 3.

FISH analysis in both twins (right-hand side of the figure) was performed using probe CEP 7 Spectrum Aqua (Blue: B); Williams Region Probe FISH Probe Kit: ELN (7q11.23), Spectrum Orange (Red: R) / D7S486, D7S522 (7q31) Spectrum Green (Green: G). In the metaphase, chromosome 7 (signals B,R,G), and “der 7” (smaller signal) were identified, as indicated by the arrows.



Supplemental Figure 4: *PTPN11* mutations in oral swabs in the twins. Using 454 technologies, the presence of *PTPN11* mutations in the oral swabs was detected only in the active phase of the disease (for Twin_01 at diagnosis and relapse and at diagnosis only in Twin_02). No *PTPN11* mutations were identified after HSCT or during the remission phase. On the x-axis, oral swabs at different time points are indicated, i.e. at diagnosis, pre-HSCT, post-HSCT and at relapse after HSCT. On the y-axis, the percentage of mutated reads identified using 454 technology is indicated. Each point gives the mean values of three independent oral swabs.



Supplemental Figure 5: Network diagram of protein and their interactions. Representative protein-protein interaction network of cell cycle enriched genes differentially expressed in Twin_01 and Twin_02 generated from STRING database. The connecting lines indicate functional relationships and direct protein-protein interactions.

Supplemental Table 1: *PTPN11* mutation in sorted and unsorted cells derived from BM and PB in both twins.

	Twin_02 at diagnosis					
	BM			**PB**		
	Percentage of total population	Percentage of mutated reads	Percentage of mutated cells	Percentage of total population	Percentage of mutated reads	Percentage of mutated cells
Total unsorted cells	-	41,62%	84%	-	22,71%	46%
Myeloid cells	61,6 %	45,34%	90%	56%	25,43%	52%
Stem cells	0,8%	46,1%	92%	0,2%	nd	Nd
Progenitor cells	3,9%	46,7%	94%	0,6%	nd	nd
B-cells	0,1%	47,37%	95%	0,4%	nd	nd
T-cells	6,1%	20,28%	40%	31%	27,21%	55%

	Twin_01 at relapse					
	BM			**PB**		
	Percentage of total population	Percentage of mutated reads	Percentage of mutated cells	Percentage of total population	Percentage of mutated reads	Percentage of mutated cells
Total unsorted cells	-	40,24%	81%	-	34,26%	69%
Myeloid cells	67,7%	50%	100%	71,9%	48%	96%
Stem cells	1,6%	47,87%	96%	1,1%	49,51%	98%
Progenitor cells	5,1%	47,89%	96%	2,2%	49,3%	98%
B-cells	8,8%	41,94%	84%	0,6%	41,94%	84%
T-cells	6,4%	3,6%	7,2%	4,4%	0,7%	1,4%

Supplemental Table 2: Genes differently expressed between Twin_01 vs Twin_02 at diagnosis selected with a Fold Change >1,5.

Genes down-regulated in AML-like signature versus Non AML-like signature	
Probe set	**Gene Symbol**
1552713_a_at	*SLC4A1*
1556037_s_at	*HHIP*
1556598_at	*ARPP21*
1556599_s_at	*ARPP21*
1558322_a_at	*PAQR9*
1559172_at	*NA*
1559394_a_at	*NA*
1559520_at	*GYPA*
1561137_s_at	*GYPE*
1563849_at	*SH2D4B*
1569191_at	*ZNF826P*
204114_at	*NID2*
204681_s_at	*RAPGEF5*
204865_at	*CA3*
205267_at	*POU2AF1*
205268_s_at	*ADD2*
205278_at	*GAD1*
205413_at	*MPPED2*
205837_s_at	*GYPA*
205838_at	*GYPA*
205856_at	*SLC14A1*
206465_at	*ACSBG1*
206591_at	*RAG1*
207220_at	*ART4*
207459_x_at	*GYPB*
207854_at	*GYPE*
208383_s_at	*PCK1*
209172_s_at	*CENPF*
210429_at	*RHD*
210487_at	*DNTT*
211207_s_at	*ACSL6*
211820_x_at	*GYPA*
211821_x_at	*GYPA*
213484_at	*NA*
213683_at	*ACSL6*
214295_at	*KIAA0485*
214407_x_at	*GYPB*
214761_at	*ZNF423*
214769_at	*CLCN4*
215117_at	*RAG2*
215819_s_at	*NA*
216317_x_at	*RHCE*
216833_x_at	*GYPB*
219148_at	*PBK*
220068_at	*VPREB3*
220474_at	*SLC25A21*
221349_at	*VPREB1*
221627_at	*TRIM10*
223463_at	*RAB23*
224370_s_at	*CAPS2*
224520_s_at	*BEST3*
225060_at	*LRP11*
225275_at	*EDIL3*
226806_s_at	*NFIA*
227497_at	*SOX6*
227498_at	*SOX6*
227529_s_at	*AKAP12*
227846_at	*GPR176*
228377_at	*KLHL14*
229151_at	*SLC14A1*
229725_at	*ACSL6*
230493_at	*SHISA2*
230597_at	*SLC7A3*
230720_at	*RNF182*
232204_at	*EBF1*
232932_at	*NA*
234059_at	*NA*
234980_at	*TMEM56*
236081_at	*SNCA*
236305_at	*RFESD*
236759_at	*NA*
237515_at	*TMEM56*
239142_at	*RFESD*
239205_s_at	*NA*
239206_at	*CR1L*
239680_at	*WDR76*
239913_at	*SLC10A4*
240136_at	*NA*
241542_at	*NA*
241929_at	*NA*
242197_x_at	*CD36*
242496_at	*NA*
Genes up-regulated in AML-like signature versus Non AML-like signature	
1552908_at	*C1orf150*
202311_s_at	*COL1A1*
205990_s_at	*WNT5A*
206385_s_at	*ANK3*
210889_s_at	*FCGR2B*
232737_s_at	*ENPP3*
236028_at	*IBSP*

Supplemental Appendix

Materials and Methods

Written informed consent in accordance with the Declaration of Helsinki and the Local Ethical Committee was obtained prior to receiving Bone marrow (BM) as well as peripheral blood (PB), oral swab, fibroblast and hair bulb samples from a monozygotic twin pair with JMML. PB specimens of the patients' parents were also included.

Microarray experiments

Total RNA was extracted from total BM using Trizol (Invitrogen, Karlsruhe, Germany). RNA quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE).

For microarray experiments, *in vitro* transcription, hybridization and biotin labeling were performed according to GeneChip 3'IVT Express kit protocol (Affymetrix, Santa Clara, CA). Human Genome U133 Plus 2.0 arrays were used (Affymetrix, Santa Clara, CA). Microarray data (.CEL files) were generated using default Affymetrix microarray analysis parameters of GeneChip Command Console Software (AGCC). Gene expression-based classification was obtained using Diagnostic Classifier (DC) model [1-3]. CEL files can be found at GEO repository (GEO, http://www.ncbi.nlm.nih.gov/geo/; Series Accession Number GSE45736). Data were normalized using RMA performed in R (http://www.r-project.org). To identify differently expressed probe sets the fold change (FC) was calculated as the ratio between the values of Twin_01 and Twin_02 for all probe sets of the array. A FC>1.2 was chosen as indicative of genes with distinct gene expression levels in the twins (at diagnosis and at relapse). In a separate analysis, Gene Set Enrichment Analysis was performed using GSEA v2.0 with probes ranked by signal-to-noise ratio and statistical significance determined by 1000 gene set permutations. Gene set permutation was used to enable direct comparisons between selected common genes and FC>1.2 in the twin pair (Twin_01 at diagnosis vs. Twin_02 at diagnosis and Twin_01 at diagnosis vs. Twin_01 at relapse) [4]. Network diagrams of gene interactions were performed using STRING database[5].

Cytogenetics, karyotyping, FISH and aCGH

FISH analyses were performed according to the protocol

recommended by the manufacturer, using CEP 7 probe and Williams Region Probe ELN / D7S486, D7S522 FISH Probe Kit (Abbott Molecular/Vysis, Des Plains, IL).

Molecular karyotyping was performed using Agilent Human Genome Microarray Kit 244A (Agilent Technologies, Santa Clara, CA, USA) according to the Agilent protocol. Anomalies present in approximately 30% of the cells were the detection limit. Gains or losses ≤ 20Kb were not considered, due to technical resolution limits.

Sorting lineage cells

Bone marrow and peripheral blood cells were incubated with different combinations of anti-human antibodies and analyzed on a MoFlo XDP (Beckman Coulter, Milano, Italy). Relative percentages of different subpopulations, based on live gated cells (as measured by the physical parameters of side scatter and forward scatter), were calculated. We have sorted different BM and PB subpopulations, based on CD34 (clone 8G12, PE conjugated, Becton Dickinson, San Jose, CA) vs. CD38 (clone HIT2, PE-Cy5 conjugated, Becton Dickinson, San Jose, CA) for stem-like and progenitors cells, CD3 (clone SK7, APC conjugated, Becton Dickinson, San Jose, CA) for T-lymphocyte cells, CD19 (clone J4.119, PE-Cy7 conjugated, Beckman Coulter, Milano, Italy) for B-lymphocyte cells and CD16 (clone 3G8, FITC conjugated, Beckman Coulter, Milano, Italy), CD11b (clone Bear1, PE conjugated, Beckman Coulter, Milano, Italy), CD14 (clone RM052, PE conjugated, Beckman Coulter, Milano, Italy), CD15 (clone 80H5, FITC conjugated, Beckman Coulter, Milano, Italy) and CD33 (clone D3HL60, Cy5-conjugated, Beckman Coulter, Milano, Italy) lineage) for myeloid lineage. Cells to be analyzed and sorted were re-suspended in an adequate volume of Running Buffer (PBS 1x, BSA 0.5% and EDTA 5mM). Sorted cells were collected in a tube containing growth medium. After sorting, an aliquot of the sorted cells was run on the sorter to check the purity of the populations.

Sequencing and Amplicon Deep sequencing of *PTPN11* mutations

DNA was extracted using of the Puregene DNA isolation kit (QIAGEN).

From 5 to 20ng/ul of genomic DNA extracted from different tissues was processed for the generation of PCR amplicons suitable for deep sequencing, according to the manufacturer's protocol (Roche, Applied Science). Fusion primers with different MID were designed to amplify exon 3 of *PTPN11* gene (Forward_*PTPN11*_ex3_AAAATCCGACGTGGAAGATG; reverse_*PTPN11*_ex3_TCTGACACTCAGGGCACAAG).

PCR product was purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany), quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) and equimolar pooled together for the emPCR (library at 1 x 10^7molecules/ul). All data were generated using GS Junior Sequencer Instrument software version 2.3 (Roche, Applied Science). Image processing and amplicon pipeline analysis were performed using default settings of the GS RunBrowser software version 2.3 (Roche Applied Science). Sequence alignment and variant detection were performed using the GS Amplicon Variant Analyzer software version 2.3 (Roche Applied Science). Sanger sequencing was performed using BigDye chemistry (Applied Biosystems, Weiterstadt, Germany) [5].

Human leucocyte antigen analysis

Donor-host bone marrow cell chimerism was determined by serology for HLA-A and B antigens and by high-resolution DNA typing for DRB1 antigens after HSCT from allogenic donors [6].

STR analysis of monozygosity and monosomy 7

Analysis of human identification was performed using Short Tandem Repeat (STR) system used in forensic genetics [7]. Autosomal STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA, D10S1248, D221045, D2S441, D1S1656, D12S391) and Amelogenin were amplified using the AmpFI STR NGM® (Applied Biosystem, Warrington, UK). AmpFI STR NGM® profiles were obtained from DNA: the 29-cycle amplification was made according to the manufacturer's protocol in a 25ul of final reaction volume, consisting of 10ul SGM plus reaction mix, 5ul NGM primer set and 10ul of gDNA at 0.1ng/ul. Amplified fragments were analyzed on ABI Prism 310 Genetic Analyzer (Applied Biosystem) and genotyping was carried out using GeneMapperID v3.2 Software. The identity of each allele was determined by comparison to an allelic ladder.

To detect the origin of the abnormal chromosome 7 (-7, mar+) and the extent of cells with two normal chromosomes 7 we used several markers (D7S2202, D7S3048, D7S1820, D7S796, D7S1839, D7S1818 and D7S1805) on chromosome 7 using capillary electrophoresis on PB and BM samples of the patients and on PB samples of the parents. We then used markers D7S1818 and D7S1805 on sorted cells from PB and BM. Peak analysis was made using Peak Scanner™ Software v1.0 (Applied Biosystem).

Mutant allele frequency detection

We used ultra deep 454 sequencing technology (Roche) to detect the variation frequencies of *PTPN11* mutation. Considering *PTPN11* a heterozygous mutation in these JMML patients, we calculated the percentages of mutated cells as twice the percentage of variation frequency. Percentages of mutated cells of total bone marrow, peripheral blood and each specific sub-populations were obtained after sorting.

Supplemental Results

Mutant allele frequency of *PTPN11* in hematopoietic lineages

PTPN11 mutation was screened in all hematopoietic subpopulations after sorting of BM and PB cells on specific antibodies to isolate stem and progenitor cells, myeloid lineage, T- and B-cells.

Ultra deep mutation detection rate allows obtaining a quantitative resolution of variant allele frequencies and a number of mutated cells in a bulk of cells in the total bone marrow or peripheral blood. At least 2000 reads per amplicon of all specimens were achieved, thus giving significant power to the mutation allele frequency detection. Mutant allele frequency (MAF) in total BM was within a range of 41,62% - 38,79% in the BM. Moreover, lower MAFs were observed in the total peripheral blood (range 34,26% - 22,71%) (Supplemental Table 1). A mutant allele frequency of 50% is expected if all cells harbour a heterozygous mutation.

Analysis of sorted cells of the BM revealed high mutated allele frequencies for all maturation stages of the myeloid lineage, stem and progenitor cells and B- cells with more than 90% heterozygous mutated cells. T-lineage cells showed lower MAFs with less than 50% of mutated cells. The same scenario was observed in the peripheral blood where all subpopulations showed a MAF ranging between 22,71%-27,21% pointing at 45%-54% of mutated cells in the sub-populations.

Gene expression profiling

Gene expression-based classification using DC model classifier identified different signatures in the twins. Variance analysis of expression values of probe sets selected 1132 probe sets with a FC >1.2 (log2) between Twin_01 and Twin_02. Twin_01 showed a positive enrichment of genes related to molecular adhesion; otherwise genes more down expressed in Twin_01 than in Twin_02 represent genes related to cell cycle (mitotic phase), cell proliferation, cytoskeleton organization and B cell development as previously reported for JMML patients with an AML-like signature [2].

On the basis of these results, we speculate that Twin_01 showed a mitotic arrest of cells and a block of proliferation that could reflect, at least in part, the high risk of relapse after HSCT due to an incomplete ablation of hematopoietic cells in the BM niche before HSCT. In the context of these results, analysis of the relapse specimen of Twin_01

identified a set of 17 genes with an even more pronounced down-regulation compared to diagnosis. Using protein interaction database, a densely interconnected network among the set of genes with known roles in mitotic cell cycle regulation was discovered (Supplemental Figure 5) [5].

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