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Genetic analysis of chronic myeloid leukemia treated with dasatinib and rapidly developing AML with monosomy 7 in Philadelphia-negative cells

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Abstract

Despite the recent success of tyrosine kinase inhibitors (TKI) in the treatment of chronic myeloid leukemia (CML), some patients (2-17%) develop clonal cytogenetic changes in the Philadelphia (Ph-) negative cell population. A fraction of these patients, in particular those displaying trisomy 8 or monosomy 7, are at risk of developing a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Consequently, there is a need to characterize the clinical features of such cases and to increase our understanding of the pathogenetic mechanisms underlying the emergence of clonal cytogenetic changes in Ph-negative cells. So far, most cases reported have received treatment with imatinib. Herein, we describe a patient with CML who developed monosomy 7 in Ph-negative cells during dasatinib therapy. Twenty months after dasatinib initiation, the patient developed MDS that rapidly progressed into AML. Genome-wide 500K SNP array of the monosomy 7 clone, revealed no acquired submicroscopic copy number changes. Given the strong association between monosomy 7 and mutations of genes involved in the RAS pathway in juvenile myelomonocytic leukemia, we also screened for pathogenetic variants in *KRAS*, *NRAS*, and *PTPN11*, but did not detect any changes.

Key words: SNP array, monosomy 7, CML, RAS-mutations, dasatinib

1. Introduction

The leukemogenic event in chronic myeloid leukemia (CML) occurs at the level of the hematopoietic stem cell through a reciprocal chromosomal translocation, t(9;22)(q34;q11). The derivative chromosome 22, referred to as the Philadelphia chromosome (Ph), leads to the formation of a *BCR-ABL1* fusion protein that encompasses a constitutive tyrosine kinase activity crucial for the transforming capacity and the development of CML (1, 2).

Growth arrest and apoptosis in Ph-positive cells can be achieved by specifically blocking the tyrosine kinase activity of the BCR-ABL1 fusion protein (3). Through this action, imatinib mesylate (Glivec® or Gleevec®), a specific tyrosine kinase inhibitor (TKI), inhibits disease progression to accelerated phase (AP) or blast crisis (BC) in over 90% of the CML patients (4). However, tyrosine kinase inhibition fails to eradicate the quiescent Ph-positive hematopoietic stem cells and clinical relapse is a common consequence of ceased therapy (2).

Noteworthy, a subset of patients (2-17%) treated with imatinib show clonal cytogenetic changes in the Ph-negative cell population (5-21). These cytogenetic changes include trisomy 8 and monosomy 7 that are frequently observed in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), which has raised concerns about their clinical implications. In addition, limited information is available as to whether treatment with more potent, second generation TKIs, may result in similar clonal cytogenetic aberrations in Ph-negative cells and if the clinical implications of such findings may differ depending on the type of TKI treatment administered.

Herein, we describe a CML patient who initially was treated with imatinib, but failed to obtain an adequate cytogenetic response. Upon dasatinib treatment, the patient developed monosomy 7 as the sole cytogenetic change in Ph-negative cells and rapidly progressed first into MDS and then to AML with unusual bone marrow morphology. To identify possible submicroscopic changes in the Ph-negative cells displaying monosomy 7, we performed 500K

single nucleotide polymorphism (SNP,) array analysis at three different time points during the disease course. In addition, mutational analysis of genes involved in RAS signaling (*KRAS2*, *NRAS* and *PNPT11)* was performed.

2. Materials and Methods

2.1 Chromosome, Fluorescent In Situ Hybridization, and Real-time PCR Analyses

Chromosome analysis was performed using standard procedures and karyotypes were described according to ISCN (2009). Complete cytogenetic response (CCR) was defined as 0% Ph positive metaphases in the bone marrow cells, major cytogenetic response (MCR) as 1-35%, minor cytogenetic response as 36-95%, and no response as 96-100%. Fluorescent in situ hybridization (FISH) analysis was performed as previously described (22) using the probes LSI BCR/ABL1 ES, for the detection of t(9;22)(q34;q11), and LSI D7S486 and CEP 7 probes for chromosome 7, all from Vysis (Downers Grove, IL). Hybridization was performed according to the manufacturer's instructions. Slides were mounted, counter stained, and analyzed using a fluorescent microscope (Cytovision, Applied Imaging Corporation, Newcastle, UK). A total of 250 interphase nuclei were scored for fluorescent signals. In addition, metaphase-FISH was performed using the probes LSI CBFB Dual Color Break Apart (Vysis), for the detection of inv(16)(16q22), and Poseidon Repeat Free PDGFRB Break Probe (Kreatech, Amsterdam, The Netherlands), for the detection of 5q33/*PDGFRB*-rearrangement. Real-time PCR analysis of the *BCR/ABL1* fusion transcript, using *ABL1* as a reference gene, was performed according to protocols outlined by the Europe Against Cancer (EAC) program (23).

2.2 Case History

The patient, a 55-year-old female, was diagnosed with CML at the end of June 2004. Clinical and genetic features are briefly summarized in Table I. At the time of diagnosis, all 25 metaphases in the bone marrow displayed a t(9;22)(q34;q11) and according to the Sokal prognostic scoring system, the patient was classified into the high risk group. After initial treatment with hydroxyurea (HU) for two months, imatinib was started at an initial daily dose of 400 mg in September 2004. No cytogenetic response was obtained despite elevated doses of imatinib (up to 800 mg) as 22 of 23 examined bone marrow cells were still Ph-positive after 14 months of treatment (Table 1). Mutation analysis of the kinase domain of BCR/ABL1 was negative. Because of the poor response to imatinib and a persistent thrombocytopenia, the patient was included in a cohort in which CML patients in accelerated phase obtain dasatinib therapy. Treatment with dasatinib was started in February at daily dose of 100-140 mg. Real-time PCR analysis, five months after initiation of dasatinib therapy, showed a *BCR/ABL1* to *ABL1* ratio of 8% (Table 1). A major cytogenetic response (MCR) was observed 13 months after the start of dasatinib, however, concomitantly, a de novo Ph-negative clone with monosomy 7 was present in Ph-negative cells. The karyotype at this occasion was: 45,XX,-7[17]/46,XX,t(9;22)[6]/46,XX[2] (Table 1). Retrospective FISH-analysis showed that the monosomy 7 clone was present in 11% of the investigated nuclei already three months after the initiation of dasatinib, but was not detectable at the last follow-up during treatment with imatinib. May-Grunwald-Giemsa (MGG)-staining of a bone marrow aspirate, taken 12 months after the start of treatment with dasatinib, revealed reduced cellularity with very few but normal-appearing megakaryocytes and normoblastic erythropoiesis. Myelopoietic cells displayed slight cytoplasmic hypogranulation and in addition, a few neutrophils showed pelgeroid nuclei. However, no other signs of dysplasia were evident and therefore, a formal

MDS diagnosis could not be established. Real time PCR analysis in July 2007, 17 months after start of dasatinib treatment, showed a *BCR/ABL1* to *ABL1* ratio of 1% (Table 1). Cytogenetic analysis of a bone marrow aspirate displayed a clone with monosomy 7 as the sole change, detected in 23/25 metaphases without Ph-positive cells being present. One month later, in August 2007, the disease progressed with severe anemia, thrombocytopenia and neutropenia. Peripheral blood smears exhibited basophilia and eosinophilia with occasional blasts together with atypical monocytes and promonocytes. Bone marrow examination showed normal cellularity with dysplastic megakaryocytes, but pronounced basophilia and eosinophilia (Fig. 1). Pelgeroid nuclei and cytoplasmic hypogranulation was evident in the myeloid series with the absence of ringed sideroblasts. The blood and bone marrow picture at this time was consistent with MDS or an unclassifiable myelodysplastic-myeloproliferative disorder.

In October 2007, 20 months after the initiation of dasatinib, the disease deteriorated further and progressed to AML. Peripheral blood smears revealed more than 30% blasts. Bone marrow examination showed hypercellularity with a blast count of roughly 20%, with a minor fraction having features of megakaryoblasts (Fig. 1). No Auer rods were present. The maturing myeloid cells had dysplastic features and pronounced basophilia and eosinophilia could be observed. Cytogenetic examination revealed the karyotype: 45,XX,-7[24]/46,XX[1]. FISH analysis showed 90% cells with monosomy 7, but no presence of Ph-positive cells. The patient entered an allogeneic stem cell transplantation program, but succumbed shortly after induction therapy in a fungal sepsis.

2.3 500 K SNP-Array and Mutational Analysis

DNA was extracted from bone marrow aspirates at the time of initial diagnosis, three and 13 months following the initiation of dasatinib therapy (Table 1). SNP array analysis was

performed using the Affymetrix mapping 500K array set (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After hybridization, the raw image files were imported into the Affymetrix Genotyping Console where SNP-genotypes were calculated using dynamic modeling. The CEL-files containing raw signal values and the files containing genotype calls were imported into dChip (http://www.dchip.org). The raw signal values were normalized using an invariant set of probes, and the "expression value" of each SNP was calculated using model based expression. Copy numbers were calculated using median smoothing with an 11-SNP window and compared to a reference copy number, calculated from all samples by trimming the 25 extreme values in both ends (Fig. 1). In addition, manual inspection was performed for 36 SNPs located on 4q12 region to investigate if a cryptic deletion was present, indicative of a PDGFRA/FIP1L1-rearrangement. DNA obtained from CD34-positive cells from two CML cases in chronic phase was used as reference samples.

To identify possible mutations in *KRAS*, *NRAS* and *PTPN11*, primers for amplifying *KRAS* codons 12, 13 and 61, *NRAS* codons 12, 13 and 61, and *PTPN11* exons 3 and 13 were designed using the OLIGO 7.0 software (Molecular Biology Insights, Cascade, CO; primer sequences available upon request). Genomic DNA was subjected to 35 cycles of polymerase chain reaction (PCR) of 30 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C. PCR products were purified by standard methods and sequenced bidirectionally using a ABI 3130 (Applied Biosystems, Foster city, CA). The sequence data was analyzed using SeqScape (Applied Biosystems, Foster city, CA) and the BLAST program (www.ncbi.nlm.nih.gov).

3. Results and Discussion

The introduction of tyrosine kinase inhibitors has revolutionized the treatment of chronic phase CML and according to the latest update of the International Randomized Study of Interferon vs STI571 (IRIS), the estimated event free survival at six years for patients receiving imatinib was 83% and the overall survival close to 90% (4). Despite this remarkable success, some aspects of treatment with TKIs still remain challenging. A substantial number of patients receiving imatinib display problems associated with intolerance or resistance. These obstacles can partly be circumvented by second generation TKIs such as dasatinib or nilotinib (24). Another intricate feature is the emergence of clonal abnormalities in Ph-negative cells observed in a subset (2-17%) of CML patients receiving imatinib. The vast majority of these chromosomal aberrations resemble those seen in MDS and AML such as trisomy 8, monosomy 7, 20q- and –Y (1, 13). In the more recent studies, including larger number of patients, the frequency of clonal cytogenetic changes in Ph-negative cells has been reported to be in the order of 5-10% (6, 10, 12).

To date, approximately 40 patients with monosomy 7 in Ph-negative cell population as the sole abnormality or in more complex karyotypes, have been reported in CML patients receiving imatinib treatment (5, 6, 8-21). Of these 40 patients, 22 displayed monosomy 7 as the sole abnormality in the absence of other clonal changes. Notably, almost half of these 22 cases experienced a disease progression towards MDS or AML. Furthermore, the appearance of a monosomy 7 clone was followed by rapid disease deterioration (3-6 months) in the vast majority of published cases. Although a selection bias, in favor of reporting cases with monosomy 7 and disease progression into MDS/AML is likely to be present, available data

thus suggest that monosomy 7 is an ominous sign when seen in Ph-negative cells under imatinib treatment.

Currently, limited information is available as to whether newer, more potent, TKIs may be associated with an increased frequency of clonal evolution in Ph-negative cells, and, if present, at what frequencies such patients may develop MDS/AML. Fabarius *et al* (2007) found that five out of 71 patients (7%) treated with dasatinib showed clonal cytogenetic changes in Ph-negative cells. None of these displayed morphological signs of MDS/AML, but all showed trisomy 8 as the cytogenetic change in the Ph-negative cells. Recently, however, Athanasiadou *et al* (2008) reported a case of CML receiving treatment with dasatinib and displaying monosomy 7 in Ph-negative cells. With a follow-up time of six months after the emergence of monosomy 7, and on continued treatment with dasatinib, no signs of MDS/AML were present upon bone marrow examination (7).

The patient described herein received the second generation TKI dasatinib due to poor response to imatinib. Clinically, she obtained a cytogenetic and hematologic response three months following the initiation of dasatinib. A major cytogenetic response was observed 13 months after the start of dasatinib, but at the same time monosomy 7 was detected in Ph-negative cells in 17/25 investigated cells (Fig. 2). At 17 months, the monosomy 7 clone was detected in 23/25 metaphases, with morphological bone marrow investigation revealing features consistent with MDS. Three months later the disease progressed into AML. The morphological features of the AML were remarkable with pronounced eosinophilia and basophilia exceeding 30% of the total cell count. This picture is rarely seen in context with AML, but rather in patients with CML blast crises (BC) or in AML displaying a t(6;9)(p22;q34) with a *DEC-NUP214 (DEC-CAN)* fusion gene (25). However, no signs of chromosomal rearrangements involving these chromosomal bands were present. Nor could the presence of pronounced eosinophilia be explained by rearrangements of *MYH11/CBFB*,

PDGFRB or *PDGFRA/FIP1L1* as methaphase-FISH and SNP-array analysis, respectively, failed to detect such changes. Future studies of similar cases, including detailed morphological bone marrow analysis, will hopefully reveal if the unusual morphology seen in the present case is present also in other CML patients displaying monosomy 7 in Ph-negative cells.

The pathogenetic mechanisms underlying the occurrence of clonal Ph-negative abnormalities in CML patients are unknown. One likely mechanism would be that imatinib by itself induces or favors the acquisition and selection of clonal cytogenetic changes, e.g., monosomy 7 in normal hematopoietic progenitor cells (9). This could be explained by the (side) effect of imatinib on the normal ABL1 protein, which is involved in DNA damage and repair control (26). In this context, a recent report by Pitini *et al* (2009) lends support that the latter may be the case. Thus, it was reported that a patient diagnosed with a gastrointestinal stromal tumor (also referred to as GIST) after 10 months of treatment with imatinib, developed an MDS (RAEB-1) with monosomy 7 that two months later transformed into AML. This suggests that imatinib directly may induce aneuploidy in normal hematopoietic progenitors, which could be more pronounced in CML when hematopoiesis is being restored from a limited pool of Ph-negative stem cells.

To date, no study has specifically addressed if genome-wide submicroscopic copy number changes are present in the bone marrow cells of CML patients developing monosomy 7. Therefore, high resolution SNP array analysis was performed on samples taken at the time of diagnosis and three and 13 months following dasatinib therapy to identify possible genomic changes either located within the remaining chromosome 7 or at other loci. However, no copy number changes apart from the monosomy 7 were detectable (Fig. 2).

Another hematologic disorder displaying monosomy 7 is juvenile monomyelocytic leukemia (JMML.) In JMML, recent studies have shown that monosomy 7 cooperates with

deregulated signaling via the RAS pathway and that 80% of the reported cases display activating mutations in the genes encoding *NRAS* and *KRAS*, malfunctional neurofibromin (NF1), or mutations in the *PTPN11* gene (27). We therefore sequenced codons 12, 13 and 61 of *NRAS* and *KRAS*, as well as exons 3 and 13 of *PTPN11* gene, genomic regions reported to display changes in JMML, but no alterations were detected. Furthermore, we searched for signs of uniparental disomy at the *NF1* locus in 17q, since this is one of the mechanisms by which the RAS pathway becomes activated in NF1 patients developing JMML (28). Twelve SNPs covering the *NF1* gene were investigated, but no signs of uniparental disomy could be detected. Thus, monosomy 7 in this patient with CML does not seem to be associated with the same spectrum of molecular abnormalities as seen in JMML.

To our knowledge, this is the first case of CML that upon dasatinib treatment displayed monosomy 7 and rapidly developed severe pancytopenia followed by AML. Apart from the monosomy 7, no genomic copy number changes were detectable using high resolution SNP arrays, nor were any mutations in the RAS pathway identified. Our case, together with available literature data, suggest that until otherwise proven, patients displaying monosomy 7 under treatment with TKIs should be closely monitored cytogenetically and clinically for signs of MDS and AML. Although the clinical experience of the occurrence of monosomy 7 in TKI-treated CML is still limited, the course in our case indicates that at least when accompanied by dysplastic morphological changes, this should be regarded as a poor prognostic sign. In our opinion, such patients should early be considered for allogeneic stem cell transplantation, or possibly for treatment with decitabine (29) or azacitidine (30).

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Figure legends

Figure 1 Bone marrow morphology at the time of MDS and AML diagnosis. **A** May-Grunwald-Giemsa (MGG)-staining of bone marrow smears from August 2007, showing prominent basophilia and eosinophilia. The morphological examination at this time was consistent with MDS or an unclassifiable myelodysplastic-myeloproliferative disorder. Original magnification 400x. **B** MGG-staining of bone marrow cells at the time of AML diagnosis in October 2007 with a myeloblast, megakaryoblast, an eosinophilic myelocyte, and normal-appearing basophilic granulocytes at the top left and right, respectively. Original magnification 1000x.

Figure 2 Genetic analyses of the presently described CML case. **A** Chromosome analysis displaying monosomy 7 at 33 months following the diagnosis of CML. **B** Interphase FISH at the same time point using the probes LSI *D7S486* and CEP 7. The interphase cells on the right and left show only one red and green signal, consistent with the presence of only one copy of chromosome 7. The interphase cell seen in the middle displays two red and two green signals indicating two copies of chromosome 7. **C** SNP array copy number data (blue denotes loss

and red gain of genetic material) of chromosome 7. 1, sample taken at the time of diagnosis; 2, sample taken three months after initiation of dasatinib; 3, sample taken 13 months after the start of dasatinib treatment. C1 and C2 represent reference samples.

Table 1 Summary of clinical data, cytogenetics, interphase FISH and quantitative RT-PCR analysis

Clinical Status of the disease	**Time from the diagnosis** (months)	**Treatment**	**Hb**[b] (g/dL)	**WBC** ($x10^9$/L)	**Thrombocytes** ($x10^9$/L)	**Karyotype** **G-band**	**Ph-positive cells** (FISH)	**Monosomy 7 positive cells** (FISH)	**Quantative RT- PCR** **([BCR/ABL]/[ABL1]%)** [c]
[a]**CML**	**0**	No	7.5	311	270	46,XX, t(9;22)(q34;q11)[20]/ 90-100idemx2[cp5]	93%	0%	nd
CML	**8**	Imatinib	12.8	3.1	202	46,XX, t(9;22)(q34;q11)[15]	nd	nd	nd
CML	**15**	Imatinib	10.9	2.9	124	46,XX, t(9;22)(q34;q11)[22]/46,XX[1]	96%	0%	nd
[a]**CML**	**23**	Dasatinib	10.0	5.3	33	nd	5%	11%	nd
CML	**25**	Dasatinib	11.7	4.8	40	nd	nd	nd	8%
CML	**29**	Dasatinib	10.4	2.6	71	46,XX,t(9;22)(q34;q11)[10]/46,XX[15]	nd	nd	7%
[a]**CML**	**33**	Dasatinib	10.2	5.5	36	45,XX,-7[17]/46,XX,t(9;22)(q34;q11)[6]/ 46,XX[2]	14%	30%	3%
CML/MDS	**37**	Dasatinib	9.9	5.6	35	45,XX,-7[23]/46,XX[2]	0%	nd	1%
CML/AML	**40**	No	7.5	2.3	<10	45,XX,-7[24]/46,XX[1]	0%	90%	nd

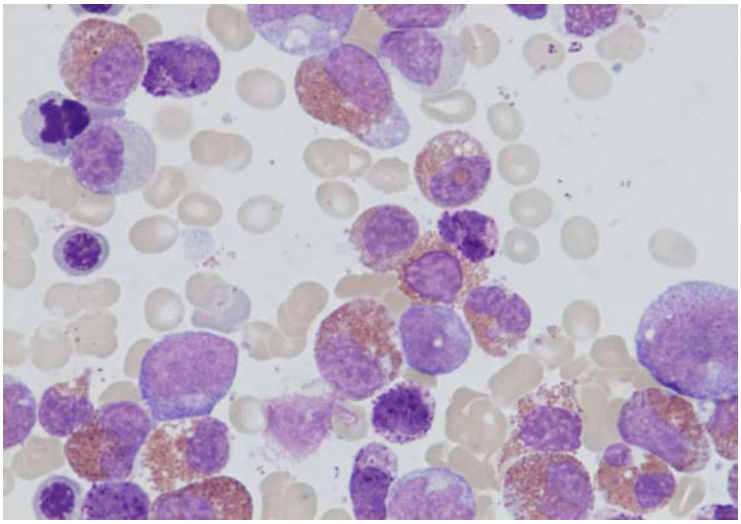
Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; ND, not determined

[a]SNP array and mutational analyses performed.

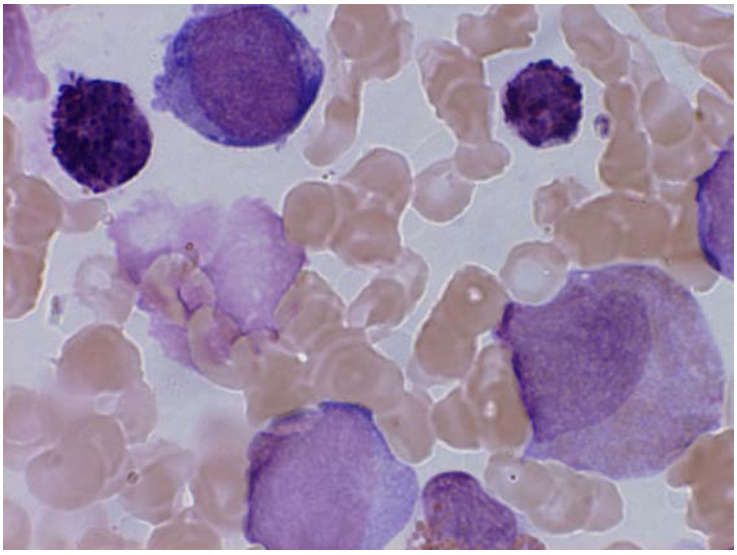
[b]Hb value transfusion dependent.

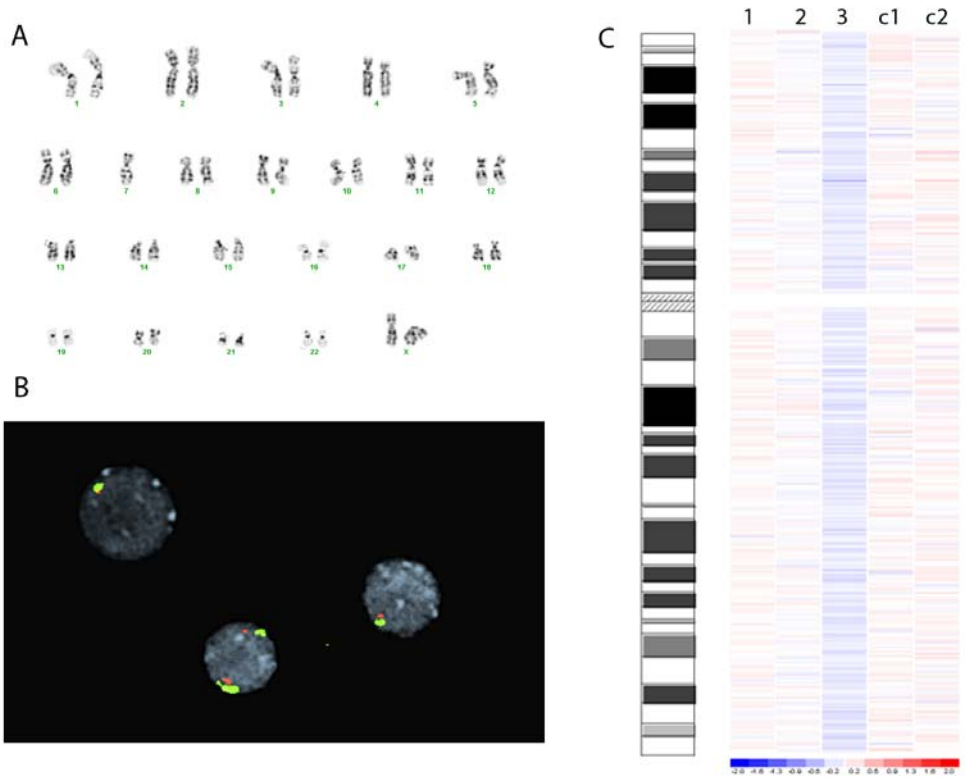
[c]Analyses performed on peripheral blood.

A



B




A
B
C
1
2
3
c1
c2