Cryptic ins(2;11) with clonal evolution showing amplification of 11q23–q25 either on hsr(11) or on dmin, in a patient with AML-M2

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TO THE EDITOR

Genomic amplification is a frequent aberration in malignant proliferation that usually leads to an inappropriate expression of one or more oncogenes located within the amplicon. Cytogenetically, it appears like homogeneously staining regions (hsr) or double minute chromosomes (dmin). In contrast to solid tumors, genomic amplification is rarely detected in hematological malignancies. The estimated incidence of cytogenetically detectable gene amplification in acute myeloid leukemia (AML) is approximately 1%. Recently, 11q23 over-representation has been described as a new cytogenetic entity in myeloid malignancies, and in these cases, the *MLL* gene (11q23) was consistently amplified. The oncogenic role of amplification in myeloid malignancies has not been demonstrated so far, although duplication or amplification of the *MLL* gene in t-AML has been significantly associated with complex karyotypes, deletion 5q, and prior therapy with alkylating agents.[1] Van Limbergen *et al*[2] found *MLL* amplification together with 5q- only in AML patients, and that was significantly associated with an extremely short survival. In most cases, the 11q23 amplified region includes the *MLL* gene (11q23.3), although is not restricted to this locus. Several studies using FISH or other molecular techniques in patients with myeloid neoplasias (10 cases with AML, and seven cases with MDS), have allowed the identification of the genes included in the amplified region as dmin and/or hsr such *FGF3* (11q13.1), *THRSP* (11q13.5), *DDX6* (11q23.3), *ETS1* (11q24.3) and *FLI1* (11q24.3).[1,3–5] Only three cases (two AML and one MDS) have been reported to have amplification of the 11q23–24 region not including the *MLL* gene.[6,7] Moreover, Zatkova *et al*[5] found that deletions within the amplicon 11q13–24 were a quite common event (four out of 13 patients analyzed), suggesting that duplications and inversions affecting the 11q arm might play a role in the highly complex variability nature of the 11q amplicons, and that other genes with possible oncogenic potential role might be implicated in the amplification of this region. Here, we report the clinical, cytogenetic and molecular data of a patient with AML-M2 which presented a cryptic ins(2;11)(q3?6;q23.3) with a clonal evolution to 11q23–q25 amplification, either on hsr(11) or on dmin, in two divergent clones. The patient had the insertion in all clones, even in the one with no 11q amplification, suggesting that this rearrangement might play a role in the leukemogenic progression, and in the amplification process.

A 81-year-old white man was admitted with a history of syncope, weight loss, faintness and weakness, anorexia, and diffuse muscular pain. Physical examination revealed asthenia, cold sweat, dizziness, paleness, and pain in the left hypochondrium. There was no lymphadenopathy, splenomegaly, or petechiae. His medical history included type II diabetes, moderate kidney failure, hypertension, and the diagnosis of a prostate adenocarcinoma 6 years before. The patient had antidiabetic treatment and hormone therapy. A complete blood count showed hemoglobin, 88 g/l; white blood cells, 1.7×10^9; and 94×10^9/l platelets. Biochemical results were within normal ranges, although creatinine was lightly elevated. Bone marrow aspirate revealed a hypocellular marrow with 32% blasts. Blasts were large, with irregular nucleus in a rather coarse granular chromatin and evident nucleoli; cytoplasm was moderately abundant and basophilic with many azurophilic, peroxidase-positive granules; there was prominent nuclear and cytoplasmic vacuolation, and a single Auer rod. The blast cells were positive for markers cMPO, CD34, CD33, HLA-DR, CD11b and CD45. He was diagnosed as having AML-M2. In accordance with the family, the patient received palliative treatment, and he died 3 weeks after admission.

Karyotyping in a sample of the patient was done on unstimulated short-term bone marrow (BM) cultures. Fluorescence *in situ* hybridization (FISH) was performed on BM metaphases using six BACs located from 11q13.2 to 11q25. The order of the probes according to the current mapping data is: centromere –RP11-699M19 (that covers *CCND1*), RP11-241D13 (*ATM*), RP11-635F12 (*ZNF145*), RP11-640N11 (*GRIK4*), RP11-1007G5 (*ETS1*), RP11-654C20 (*OPCML*)- telomere (Figure 1a). Six BACs located on 2q36 and 2q37 were designed to analyze the 2q region. The probes, from centromere to telomere are: RP11-395N3 (that covers *IRS1*), RP11-395A23 (*SP100*), RP11-174L18 and RP11-400N9 (*SHIP*), and RP11-275G7 and RP11-110K9 (*HDAC4*). The clones were obtained from libraries from BACPAC Resources (Children's Hospital Oakland, Oakland, CA, USA). The probes were labeled with Spectrum Green® and Spectrum Orange®, (Vysis, Downers Grove, IL, USA) by nick translation and used pairwise. A commercial probe for *MLL*, LSI *MLL* Dual Color, was used (Vysis, Downers Grove, IL, USA). Primers were designed for specific assays of gene expression for *GRIK4* (GRIK4-F (exon 17): 5′-AGCGCAAATGGTGGGAAGGA-3′, and GRIK4-R (exon 19): 5′-TGCGCAGCTCGGTCACCATC-3′; AT: 65°C) and for the *MLL* gene (MLL-F (exon 3): 5′-CTGCCTTTCCACTCCTTCATCT-3′, and MLL-R (exon 5): 5′-TCCTGGGGTGCCTTGTTTCT-3′; AT: 60°C). All reactions were carried out on BM of the patient and on BM and PB from a healthy donor; the *GRIK4* gene expression was carried out as well on the brain cell line SK-N-MC (American Type Culture Collection No. HTB-10), as a positive control. Expression levels of *MLL* and *BCR* on BM cells from the patient and from a healthy donor were compared using the *BCR* gene as an internal control (BCR-F: 5′-GAGAA-GAGGGCGAACAAG-3′, and BCR-R: 5′-CTCTGCTTAATTC-CAGTGGC-3′). Serial dilutions of the sample from the healthy donor were analyzed to control that all reactions were kept in the linear phase of amplification.

G-banding on BM cells of the patient at diagnosis showed three different clones: clone 1: 46,XY,del(5)(q31q35), +8,−20[15], clone 2: 46,idem,10–17dmin[3] and clone 3: 45,XY,del(5)(q31q35),hsr(11)(q23),−20[12]. This karyotype suggests that all clones could have come from a previous one with 5q- and monosomy 20. From this one the other clones would have progressed: clone 1 (5q−, +8, −20), from which would derive clone 2 (5q-, +8, −20, dmin); and clone 3, that is hypodiploid (5q-, hsr(11q), −20) (Figure 1b). Therefore, the 11q amplification

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REGION	GENE	PROBE	FISH results CLONE 1	CLONE 2	CLONE 3
11q13.2	*CCND1*	RP11-699M19	chr 11(x1), chr 11(x1)	chr 11(x1), chr 11(x1)	chr 11(x1), hsr(11)(x1)
11q22.3	*ATM*	RP11-241D13	chr 11(x1), chr 11(x1)	chr 11(x1), chr 11(x1)	chr 11(x1), hsr(11)(x1)
11q23.3	*ZNF145*	RP11-635F12	chr 11(x1), chr 11(x1)	chr 11(x1), chr 11(x1)	chr 11(x1), hsr(11)(x1)
11q23.3	*MLL*	LSI MLL DUAL COLOR	chr 11(x1), chr 11(x1)	chr 11(x1), 4dmin	chr 11(x1), hsr(11)(amplified)
11q23.3	*GRIK4*	RP11-640N11	der(2)(x1), chr 11(x1), chr 11(x1)	der(2)(x1), chr 11(x1), 4-6dmin	der(2)(x1), chr 11(x1), hsr(11)(x1)
11q24.3	*ETS1*	RP11-1007G5	chr 11(x1), chr 11(x1)	chr 11(x1), 3-8dmin	chr 11(x1), hsr(11)(amplified)
11q25	*OPCML*	RP11-654C20	chr 11(x1), chr 11(x1)	chr 11(x1), 16-20dmin	chr 11(x1), hsr(11)(amplified)

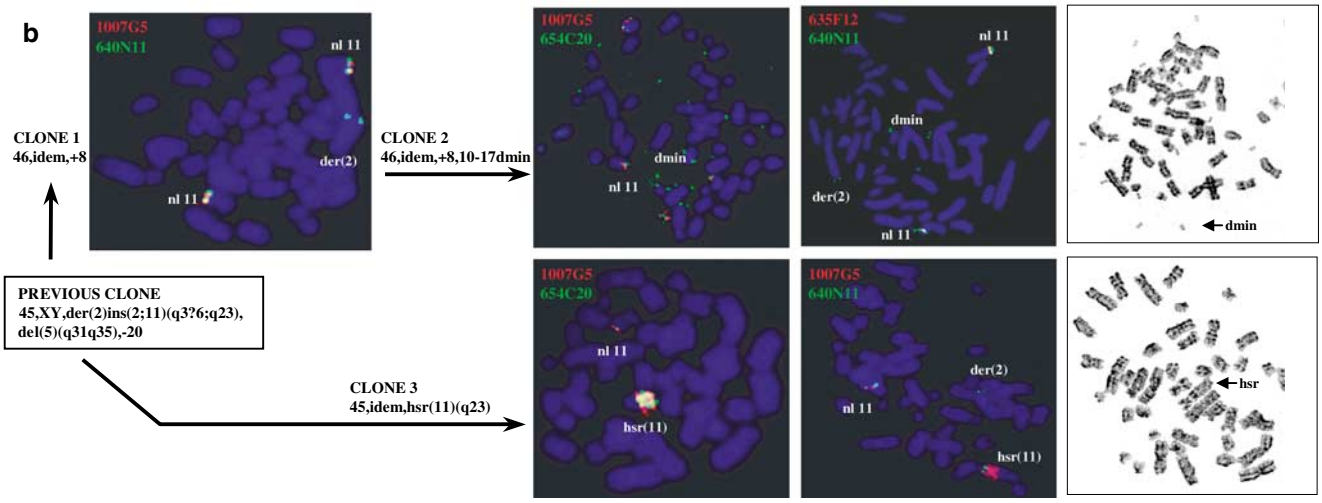

Figure 1 FISH analysis of a patient with AML-M2. (a) A map of the region from 11q13.2 to 11q25 showing the relative position of the clones used in this study, and the genes involved. In FISH results, the chromosome where each probe hybridizes in the three different clones is indicated. (b) Images of FISH and G-banding metaphases of the three clones, showing the hypothetical clone progression. The probes and color used for their detection are indicated.

in dmin does not derive from the clone with hsr, indicating that the mechanism of amplification could be independent. Cytogenetically divergent clones could be of interest in the discussion of the clonal evolution, and for an evaluation of the role of different chromosome aberrations in malignant transformation and tumor progression. To our knowledge, this is the third time that a patient showing two divergent clones containing either hsr or dmin with *MLL* amplification is reported.[3,4]

FISH experiments using the *MLL* probe showed amplification of this gene in clones 2 and 3, the clones with dmin (3–17) and hsr. To delimit the amplified region, a panel of six clones from 11q13.2 to 11q25 was chosen. The amplification spanned from *MLL*, on 11q23.3 (117.8–117.9 Mb), to *OPCML*, on 11q25 (Figure 1a). The probes that cover *MLL*, *ETS1*, and *OPCML* were amplified in both hsr and dmin, whereas there were only two copies of the probes centromeric to *MLL*, including RP11-635F12 (113.45–113.65 Mb), also located on 11q23.3. However, BAC RP11-640N11, that covers the 3′ region of the *GRIK4* gene, showed a different signal pattern: we detected amplification on the dmin but not on hsr, showing the high variability in the amplification in this region. Furthermore, with this probe an extra signal in the long arm of chromosome 2 was found in all three clones, even in clone 1 that had neither dmin nor hsr (Figure 1b). To characterize the cryptic rearrangement between chromosomes 2 and 11, we performed FISH analysis with six probes located on 2q36–q37. The probes were found neither deleted nor split, and no material from chromosome 2 was found on 11q, suggesting a cryptic der(2)ins(2;11)(q3?6;q23.3orq24?) in all malignant cells that could play a role in the mechanism of amplification.

The precise mechanism by which gene amplification occurs in human cancers is unknown. Experimentally, it appears that several types of genetic abnormalities are required to allow gene amplification to take place: loss of cell-cycle control by inactivation of p53, DNA damage or instability, and some stimulus to progress as a result of oncogene activation or other mechanisms. Unfortunately, it is difficult to draw firm conclusions about early events in the amplification process (reviewed in Hogarty and Brodeur[8]). Some information can be obtained by structural analysis of the amplified unit, as well as by analysis of the genomic configuration of the locus that was amplified. In this sense, it is useful to delimit the size of the amplicon, and the genes involved. In our patient, the amplification was from 11q23.3 (less than 5 Mb upstream *MLL*) to 11q25. Interestingly, hsr and dmin were found in two different clones, indicating there is an independent mechanism involved, or that both events could have derived from similar mechanisms with different behavior during the cell cycle. As expected, in the hsr(11q) the amplicon was arranged in tandem. The extrachromosomal dmins are structures that lack centromeres or

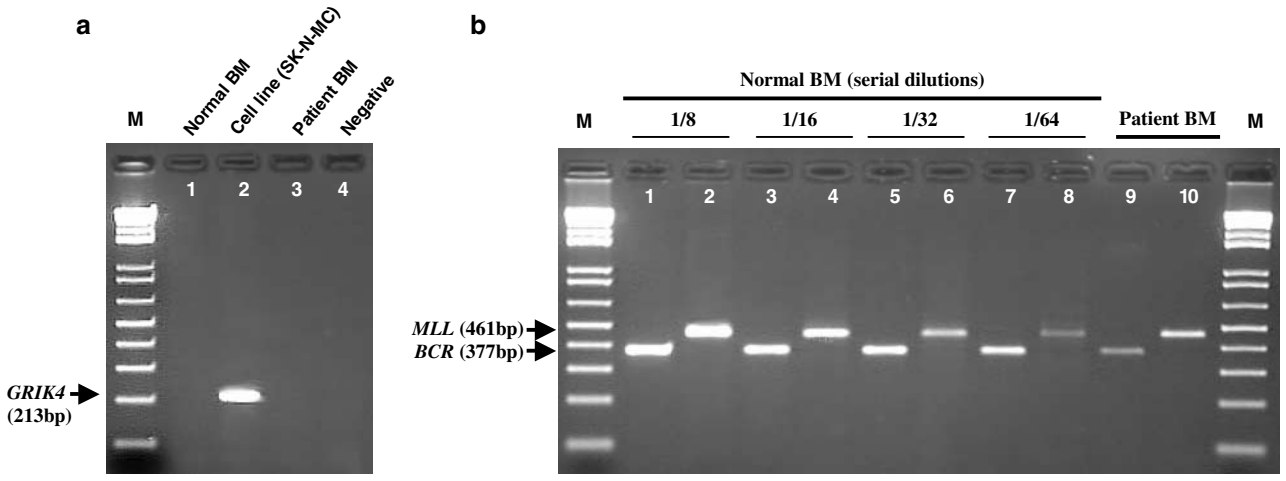

Figure 2 Analysis of the expression of *GRIK4* and *MLL* in the patient. (a) Analysis of the expression of *GRIK4* (213 bp): Line 1: Normal BM; Line 2: SK-N-MC brain cell line; Line 3: BM of the patient; Line 4: negative control; M: 1 kb plus molecular weight marker. (b) Expression pattern of *BCR* (377 pb) and *MLL* (461 pb) in normal BM, and in BM from the patient. *BCR* amplification was used as an internal control. Lines 1, 3, 5 and 7: *BCR* amplification in a 1/8, 1/16, 1/32 y 1/64 dilution in normal BM; lines 2, 4, 6, and 8: *MLL* amplification on a 1/8, 1/16, 1/32 and 1/64 dilution in normal BM; line 9: *BCR* amplification in the patient BM; line 10: *MLL* amplification in the patient. M: 1 kb plus molecular weight marker.

kinetochores, and it is likely that to remain stable they are closed circular molecules.[8] They apparently segregate randomly in the two daughter cells after cell division. The mechanism of dmin formation probably involves the duplication of a large chromosomal region, followed by deletion and circularization to form dmin. The number of FISH signals we found in the dmins (clone 2) were different depending on the probes used, there were more signals in the more telomeric regions (Figure 1). This suggests that the mechanism of amplification as dmin in our patient could be the duplication and deletion of different overlapping regions, always including the more telomeric region. The dmin may contain amplicon of different size which suggests additional rearrangement at the time of dmin replication, during cell cycle, with loss of sequences. That could explain we detected more copies of the telomeric probes in the dmin.

The only gene contained in the chromosome 11 probe that goes to the der(2) is *GRIK4* (glutamate receptor, ionotropic, kainate 4). This gene encodes a protein that belongs to the glutamate-gated ionic channel family, and is a major excitatory neurotransmitter in the mammalian central nervous system, through activation of ligand-gated ion channels and G protein-coupled membrane receptors. This gene is highly expressed in brain but neither in BM nor PB. To check if *GRIK4* was ectopically expressed in BM of our patient, we performed a RT-PCR. As expected, we found a strong expression of the *GRIK4* gene in the SK-N-MC brain cell line, but no expression was detected either in normal BM, normal PB, or in the patient sample (Figure 2a). We also performed RT-PCR on RNA from BM cells using specific primers for *MLL*. This analysis showed that the *MLL* gene was overexpressed in the patient as a consequence of the amplification. Although a quantitative analysis was not possible because there was no material left, comparison in normal BM and in the leukemic cells, using the *BCR* gene expression as a control, suggests that *MLL* is overexpressed in our patient (Figure 2b). Therefore, overexpression of *MLL*, but not the ectopic expression of *GRIK4*, could have contributed to the leukemic transformation in this patient, and not all genes contained in the amplicon were overexpressed.

In conclusion, we report the analysis of a patient with AML-M2 and amplification of the 11q23–q25 region on dmin and hsr in two divergent clones. Our analysis revealed that the amplified region was not restricted to the *MLL* gene, but spanned from 11q23.3 to 11q25. The probe RP11-640N11, that covers the *GRIK4* gene, was amplified in the dmin, but not in the clone with hsr, and was inserted on 2q36 in all clones analyzed. Molecular analysis showed that *MLL*, but not *GRIK4* was overexpressed in the patient. These results suggest that the process of gene amplification might be based on initial structural aberrations affecting the 11q arm that could have a role in the progression. The biological significance of the two mechanisms of amplification in the disease process remains to be elucidated.

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Prevention of venous thromboembolism with low molecular-weight heparin in patients with multiple myeloma treated with thalidomide and chemotherapy

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TO THE EDITOR

In multiple myeloma (MM) patients, treatment with thalidomide has proven its efficacy as monotherapy, but also combined with dexamethasone and chemotherapy. However, in combination with these drugs thalidomide may increase the incidence of deep venous thrombosis and pulmonary embolism up to 30%.[1]

The pathogenesis of these thrombotic events is poorly understood and it is also not known how to prevent thalidomide-associated venous thromboembolism (VTE).[2]

In patients who were treated in the prospective, multicenter phase III HOVON-50/GMMH-HD3 study, we evaluated the incidence of VTE in newly diagnosed MM patients during induction therapy with thalidomide, doxorubicin and dexamethasone when using the low molecular-weight heparin (LMWH) nadroparine in prophylaxis dosage.

Patients, age 18–65 years, with newly diagnosed MM, Salmon & Durie stage II or III, were eligible for inclusion. Informed consent was obtained from all patients. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board of each participating hospital. Patients were randomly assigned to induction chemotherapy consisting of three cycles of vincristine (0.4 mg, i.v. on days 1–4), doxorubicin (9 mg/m^2, i.v. on days 1–4) and dexamethasone 40 mg orally (days 1–4, 9–12, 17–20), (VAD) arm A. Patients assigned to arm B received thalidomide instead of vincristine (TAD). Cycle 2 starts at day 29, cycle 3 at day 57. Thalidomide was given as 200 mg orally, starting at day 1 of the first TAD cycle and was stopped 2 weeks before chemotherapy for stem cell mobilization was started. The thalidomide dose could be escalated to maximally 400 mg in case of good tolerability. Patients in arm B started with standard dosage thrombosis prophylaxis consisting of subcutaneously LMWH nadroparine 2850 IE anti-Xa or 5700 anti-Xa in case of weight above 90 kg. Prophylaxis was started at day 1 of the first TAD cycle until 1 week before start of chemotherapy for stem cell mobilization. Stem cells were mobilized after cyclophosphamide 1000 mg/m^2 i.v. day 1, doxorubicin 15 mg/m^2, i.v. on days 1–4, dexamethasone 40 mg orally on days 1–4 (CAD) and G-CSF 5 mg/kg twice daily until collection. After induction therapy all patients received one or two courses of high-dose melphalan (HDM) 200 mg/m^2 with autologous stem cell rescue. Patients randomized to arm A received maintenance therapy with α-interferon (3×10^6 IU, thrice weekly) and patients randomized to arm B received thalidomide 50 mg/day without VTE prophylaxis.

The incidence of VTE was a secondary end point of the study and had to be reported directly by fax to the datacenters as a serious adverse event. The reporting hospitals were then contacted for further details. As control a separate questionnaire was sent to all participating hospitals.

All types of venous thrombosis and pulmonary embolism were included and diagnosis was made by Doppler ultrasonography or spiral pulmonary computer tomography.

The time to occurrence of the first VTE, T_{VTE}, was calculated from the date of randomization. Patients who died within 6 months without VTE were censored at the date of death. T_{VTE} was estimated with the actuarial method of Kaplan and Meier and 95% confidence intervals were calculated, Kaplan–Meier curves of T_{VTE} were generated to illustrate differences between the two treatment arms, and the log-rank test was used to compare the two curves. The reported *P*-values are two-sided, and a significance level of $\alpha = 0.05$ was used.

Inclusion started in November 2001 and as of May 1, 2003, 412 patients were included, 201 patients in Arm A and 211 patients in Arm B. The data were analyzed as of January 16, 2004.

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