bodies showed a clear band for PKM1/2, but not PKM2, in normal lymphocytes (Figure 2E). In samples from ALL patients, however, equivalent expression levels of PKM1/2 and PKM2 were present (Figure 2F). Analogous to the Q-PCR results, no significant difference in protein levels were observed between prednisolone-resistant and prednisolone–sensitive cases, suggesting that pyruvate kinase isoform M2 is not responsible for glucocorticoid resistance in childhood leukemia. Whether the difference in expression between different isoforms of pyruvate kinase that was detected between normal bone marrow and leukemic cells reflects a difference in glycolytic rate is not known, since patient cells do not grow *in vitro* and we can not detect glucose consumption. Thus, although pyruvate kinase might play a role in the regulation of glycolysis in childhood ALL, glucocorticoid resistance is unlikely to be caused by selective expression of PKM2.

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References

1. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med 2006;354:166-78.
2. Pieters R, Klumper E, Kaspers GJ, Veerman AJ. Everything you always wanted to know about cellular drug resistance in childhood acute lymphoblastic leukemia. Crit Rev Oncol Hematol 1997;25:11-26.
3. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood 1998;92:259-66.
4. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol 2003;21:3262-8.
5. Hulleman E, Kazemier KM, Holleman A, Vanderweele DJ, Rudin CM, Broekhuis MJ, et al. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood 2009;113:2014-21.
6. Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 2006;66:8927-30.
7. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 2008;452:230-3.
8. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990; 76:2327-36.
9. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin Cancer Biol 2005;15:300-8.
10. Dombrauckas JD, Santarsiero BD, Mesecar AD. Structural basis for tumor pyruvate kinase M2 allosteric regulation and catalysis. Biochemistry 2005;44:9417-29.
11. Koss K, Harrison RF, Gregory J, Darnton SJ, Anderson MR, Jankwski JA. The metabolic marker tumour pyruvate kinase type M2 (tumour M2-PK) shows increased expression along the metaplasia-dysplasia-adenocarcinoma sequence in Barrett's oesophagus. J Clin Pathol 2004; 57:1156-9.

Detection of twelve nucleotides insertion in the *BCR-ABL* kinase domain in an imatinib-resistant but dasatinib-sensitive patient with bi-phenotypic acute leukemia

Although targeted inhibition of BCR-ABL by imatinib (IM) is an effective therapy for patients with Philadelphia chromosome-positive leukemias, a minority of patients, most of them in advanced phase, acquire mutations in the BCR-ABL kinase domain (KD) leading to relapse.[1-5] These mutations consist, almost exclusively, in single nucleotide (nt) substitutions. Rare cases of splicing events inducing deletion or insertion of multiple nucleotides into ABL KD have been described. A deletion of 27 amino acids (aa) induced by the L248V mutation activating a cryptic splice site has been identified[6] as well as cases resulting from the insertion of 35 nt from intron 8 leading to a frameshift.[7,8] In the latter, this insertion could account for up to 62% of IM-resistant CML patients in chronic phase.[8] Here, we describe a novel mutation acquired at the moment of the IM resistance, consisting in an insertion of 12nt, and leading to the conservation of the open reading frame (ORF).

The 57-year old female patient was diagnosed in July 2006 with bi-phenotypic acute leukemia (hyperleukocytosis at 48 G/L with 47% of blasts exhibiting myeloid and lymphoid features: $CD13^+$, $CD33^+$, $CD19^+$, $CD10^+$ and $CD22^+$). The procedures followed were in accordance with the Helsinki Declaration as revised in 2008. A karyotypic analysis demonstrated a t(9;22)(q34;q11) as sole anomaly and molecular analysis detected *M*-BCR-ABL transcript. Hyper C-VAD and IM at 800 mg/day were used as induction regimen leading to complete remission. Consolidation therapy with alternating high-dose methotrexate plus cytarabine and Hyper C-VAD plus IM were given. She achieved a complete hematologic remission, a complete cytogenetic response and a major molecular response (*BCR-ABL/ABL* IS 0.06%). In April 2007, as she didn't have any HLA-matched donor she underwent high-dose therapy with cyclophosphamide plus total body irradiation (12Gy) conditioning regimen followed by the autologous transplantation of G-CSF collected PBSC. As the *BCR-ABL/ABL IS* rose to 0.09% IM was reintroduced at 600 mg/day leading to a sustained drop of the transcript level to 0.035% six months later (Figure 1). Then the transcript ratio rose rapidly within three months (0.7%; 2.4%; 8.1%). A mutation screen performed in April 2008 revealed an insertion of 12nt in 100% of the BCR-ABL transcripts and no other mutation. This mutation induced the insertion of 4 aa (A, F, G and S) between I293 and K294 (Figure 2). Retrospective analyses revealed that the mutation could be detected by a sensitive RQ-PCR on the cDNA five months before relapse, even while the patient experienced a molecular response (*BCR-ABL/ABL IS* at 0.035%). The proportion of the mutated clone in the setting of minimal residual disease assessed by nested PCR-RFLP analysis was 100% at this time (Figure 1). Comparison of this 12nt with

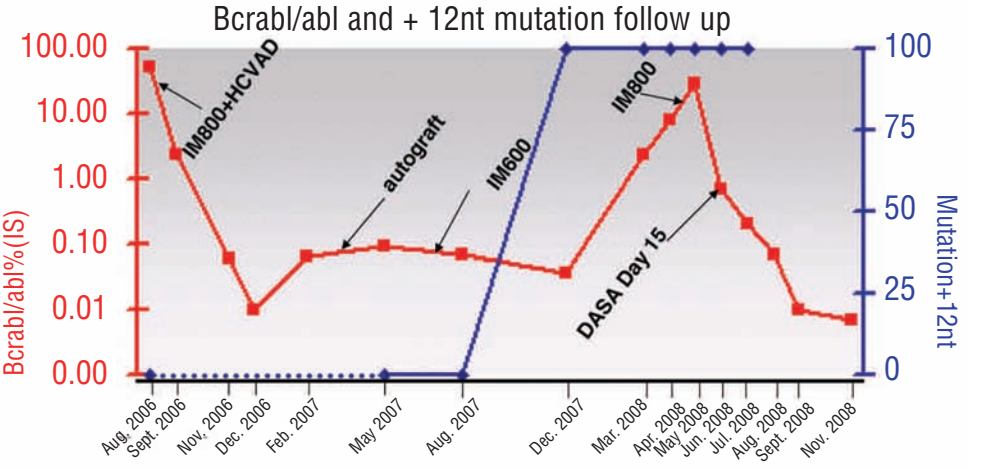

Figure 1. Quantification of *BCR-ABL* transcripts and mutated transcripts follow-up with time. Reverse transcription (RT) and quantitative real time-PCR (RQ-PCR) to quantify *BCR-ABL* fusion transcript and *ABL* control gene were performed according to the ELN recommendations.[9,10] The mutation was detected by direct sequencing (on both strands) from the relapsed patient's sample. It was quantified by RQ-PCR from available cDNA samples and from genomic DNA extracted from the patient's epithelial mouth cells or from the patient's leukemic cells at the time of relapse with a fluorescent probe (UPL N#10, Roche diagnostics) and a reverse allele specific oligonucleotide: TTTGGACC-CAAAAGCAATCT. The forward primers used were: CCGTGAAGACCTTGAAGGAG and GCACATGCAAGCCAGCTTTG for cDNA and gDNA, respectively. A serial 10-fold dilution series of mutated cDNA from the relapse sample (ranging from 10^6 to 10^1 copies) was amplified and the assay was found to be linear over at least five orders of magnitude (slope -3.41, intercept 36.61). Assessment of the proportion of the mutated transcripts was performed by quantification of the specific bands from each NlaIV digested fragments by restriction fragment length polymorphism (RFLP) analysis as previously described.[11,12] Red lines with squares represent the levels of total *BCR-ABL* transcripts expressed as *BCR-ABL* ratios in % according to the international scale (log red scale, left hand side). Blue lines show the *BCR-ABL* mutated transcripts expressed in % of total *BCR-ABL* transcripts (linear blue scale, right hand side).[11]

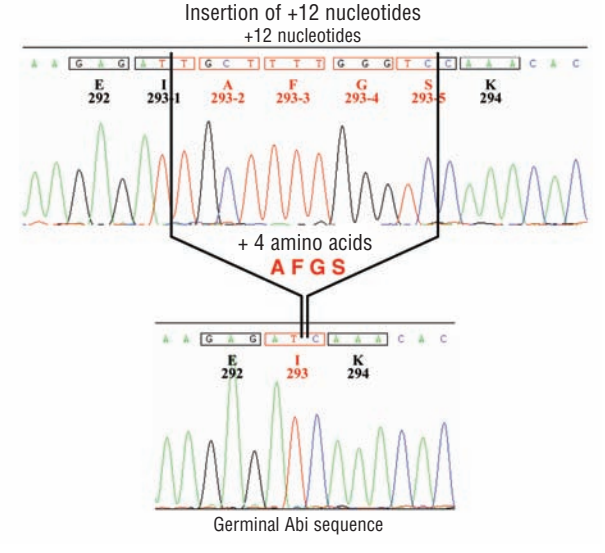

Figure 2. Sequence of the mutated transcripts. Upper reading frame: primary sequence data of the 12 nucleotide insertion mutation with the corresponding red sequences in nucleotides and amino acids. Amino acid numbers from ABL Ia isoform (*Genbank accession n. NM_005157*). Lower reading frame: wild type sequence. Black indicates guanine (G); blue, cytosine (C); red, thymidine (T); and green, adenosine (A).

those in the human data bank revealed its presence in many genomic regions but none into ABL (9q34) or BCR genes. Unfortunately, the small length of this sequence does not make it possible to deduce its genomic origin. Despite this, this insertion does not seem to be a constitutional polymorphism because it was present at the genomic level in DNA extracted from leukemic cells but was missing in genomic DNA extracted from the genotypically distinct patient's epithelial mouth cells. Moreover, retrospective analysis by sensitive specific RQ-PCR on the cDNA from the patient did not detect any mutated clone either in previous samples from December 2007 nor at the time of diagnosis which definitively excludes constitutional polymorphism and suggests that the mutational mechanism was induced or selected by IM therapy. Usually, alternative splicing events give rise to mixed spliceoforms at mRNA levels and were often due to punctual mutations inducing cryptic splice sites[6] but in this case all *BCR-ABL* transcripts carried only the insertion as sole anomaly and the screening for other genomic mutations performed by sequencing amplified PCR fragments 307nt upstream and 225nt downstream of the insertion did not show any other mutation. The increase of IM up to 800mg/day during six weeks led to the loss of hematologic response whereas molecular monitoring performed monthly after dasatinib introduction (70 mg twice a day one month later) showed a strong reduction of the *BCR-ABL/ABL* ratio to 0.7, 0.2, 0.07, 0.01 and 0.007%, respectively.

In view of these results, this insertion which correlated with IM resistance was not a polymorphism and must be induced by an unknown recombination mechanism rather than alternative splicing events. We would like to underline that the mutation is highly sensitive to dasatinib and we strongly suggest that it requires the introduction of second generation TKIs.

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References

1. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 2003;102:276-83.
2. Ernst T, Erben P, Muller MC, Paschka P, Schenk T, Hoffmann J, et al. Dynamics of BCR-ABL mutated clones prior to hematologic or cytogenetic resistance to imatinib. Haematologica 2008;93:186-92.
3. Jabbour E, Kantarjian H, Jones D, Talpaz M, Bekele N, O'Brien S, et al. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. Leukemia 2006;20:1767-73.
4. Soverini S, Martinelli G, Rosti G, Bassi S, Amabile M, Poerio A, et al. ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia. J Clin Oncol 2005;23:4100-9.
5. Nicolini FE, Corm S, Le QH, Sorel N, Hayette S, Bories D, et al. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). Leukemia 2006; 20:1061-6.
6. Gruber FX, Hjorth-Hansen H, Mikkola I, Stenke L, Johansen T. A novel Bcr-Abl splice isoform is associated with the L248V mutation in CML patients with acquired resistance to imatinib. Leukemia 2006;20:2057-60.
7. Laudadio J, Deininger MW, Mauro MJ, Druker BJ, Press RD. An intron-derived insertion/truncation mutation in the BCR-ABL kinase domain in chronic myeloid leukemia patients undergoing kinase inhibitor therapy. J Mol Diagn 2008;10:177-80.
8. Lee TS, Ma W, Zhang X, Giles F, Cortes J, Kantarjian H, et al. BCR-ABL alternative splicing as a common mechanism for imatinib resistance: evidence from molecular dynamics simulations. Mol Cancer Ther 2008;7:3834-41.
9. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003;17:2318-57.
10. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 2006;108:28-37.
11. Hayette S, Michallet M, Baille ML, Magaud JP, Nicolini FE. Assessment and follow-up of the proportion of T315I mutant BCR-ABL transcripts can guide appropriate therapeutic decision making in CML patients. Leuk Res 2005; 29:1073-7.
12. Nicolini FE, Hayette S, Corm S, Bachy E, Bories D, Tulliez M, et al. Clinical outcome of 27 imatinib mesylate-resistant chronic myelogenous leukemia patients harboring a T315I BCR-ABL mutation. Haematologica 2007;92:1238-41.

Elevated profile of Th17, Th1 and Tc1 cells in patients with immune thrombocytopenic purpura

T-lymphocyte abnormalities are considered important in the pathogenesis of chronic immune thrombocytopenic purpura (ITP). Both $CD4^+$ (Th) and $CD8^+$ (Tc) T lymphocytes can be functionally divided into type 1 (T1) and type 2 (T2) subsets based on the secretion of cytokines. Since Semple[1] discovered an early Th0 and Thl cell activation in children with chronic ITP, it has become evident a higher Th1 response was closely related to the etiology and status of chronic ITP.[2] Until now, there have been few studies on the Tc cell profile in ITP, and we only find one report which suggests that Tc1 cell response was predominant in active ITP patients.[3] Th17 cells characterized by the production of IL-17 have recently been identified as a unique subset of Th cells.[4] Considerable evidence suggests Th17 cells have been linked to the development of autoimmune diseases,[5,6] so we presume that Th17 cells may be of importance in ITP. To further investigate the role of Th17, Th1 and Tc1 cells in the pathogenesis of ITP, we examined the levels and correlation of Th17, Th1 and Tc1 cells in ITP patients by intracellular cytokine analysis.

Thirty adult chronic ITP patients (16 women and 14 men; mean age 36, range 17-80 years) were enrolled by diagnostic criteria for ITP,[7] and the platelet count ranged between 1 and 30×10^9/L, with a median count of 11×10^9/L. Patients with complications, i.e. viral hepatitis, diabetes, hypertension, cardiovascular diseases, pregnancy, active infection, or connective tissue diseases, were excluded. The control group consisted of 30 adult healthy volunteers matched for sex and age with the study population and platelet counts ranged from 136 to 298×10^9/L, with the median count of 225×10^9/L. Informed consent was obtained from each patient and the study was approved by the Medical Ethical Committee of Qilu Hospital of Shandong University.

Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. Briefly, heparinized peripheral blood (400 μL) with an equal volume of RPMI 1640 medium was incubated for 4 h at 37°C, 5% CO_2 in the presence of 25 ng/mL phorbol myristate acetate (PMA), 1 μg/mL ionomycin, and 1.7 μg/mL Monensin (Alexis Biochemicals, San Diego, CA). After incubation, the cells were stained with PE-Cy5-conjugated anti-CD3 and FITC-conjugated anti-CD8 to delimitate $CD4^+$ T cells because CD4 was down-modulated when cells were activated by PMA.[8] After the sur-