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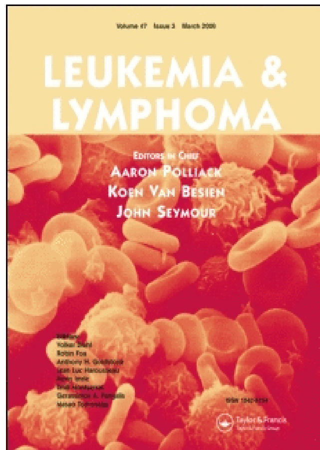

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LETTER TO THE EDITOR

A new allelic discrimination assay using locked nucleic acid-modified nucleotides (LNA) probes for detection of *JAK2* V617F mutation

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Discovery of an acquired mutation in the Janus kinase 2 (*JAK2* V617F) in patients with Philadelphia chromosome negative (Ph-) myeloproliferative diseases (MPD) was reported by multiple groups of authors in 2005 [1]. The presence of the mutation confirms clonality in MPD, and according to some authors, it may be relevant even prognostically [2]. The reported proportions of patients carrying this mutation are highly variable, depending on the respective MPD entity and on the sensitivity of the method used for its detection [3].

The newly developed allelic discrimination assay that we wish to report on is based on real-time RT-PCR [4,5]. It is very simple, high throughput and enables detection of 2% of the mutated allele. It uses two dual labelled TaqMan probes with locked nucleic acid (LNA)-modified nucleotides. LNA is a nucleic acid analog that contains a 2′-O, 4′-C methylene bridge. This bicyclic structure locks the ribose group into a C3′-endo conformation. Introduction of LNA monomers into oligonucleotides increases thermal stability of heteroduplexes within +3/+8°C per modification. This translates in increased specificity of the allelic discrimination [6].

The probes differ at the polymorphic site; one of them is complementary to the wild-type *JAK2* allele and the other to the mutated one. Each probe is labelled with a different reporter dye on its 5′-end (6-carboxyfluorescein (FAM) for the probe complementary to the wild type allele, 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) for the mutant one in our hands) and a black hole quencher dye (BHQ1) is attached to the 3′-end of both the probes. During PCR cycling, the probes specifically hybridize to the targeted sequences (mutated and wild type, respectively). Then the Taq polymerase cleaves the probe with its 5′-3′ nuclease activity, so that the reporter dye and quencher dye become separated [Figure 1(A)]. This leads to an increase of fluorescence intensity. Patients homozygous for the *JAK2* mutation show only JOE signal, whereas increase of only the FAM signal indicates wild type homozygous genotype. Heterozygous patients show various ratios of both signals. To verify the results obtained by our new method, all samples were tested by the common allele-specific (AS)-PCR developed by Baxter et al. [7].

Peripheral blood samples from 261 patients with already diagnosed or suspected Ph-MPD were obtained. Patients with WHO-defined polycythaemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) were included in the study, as well as patients with undifferentiated MPD (MPD-U), secondary polyglobulia (SP) and secondary thrombocytosis (ST). Mononuclear cells from peripheral blood were removed using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO) density gradient, and erythrocytes were lysed using red cell lysis solution (155 mmol NH_4Cl, 10 mmol NH_4HCO_3, 0.1 mmol EDTA) in order to obtain relatively pure granulocytes. The contamination by other cells was estimated in smears of the separated cells and routinely did not exceed 10%. This granulocyte-rich cell fraction was washed twice with phosphate-buffered saline (137 mmol NaCl, 2.7 mmol KCl, 4.3 mmol $Na_2HPO_4 \cdot 7H_2O$, 1.4 mmol KH_2PO_4), and aliquots of 5×10^6 cells

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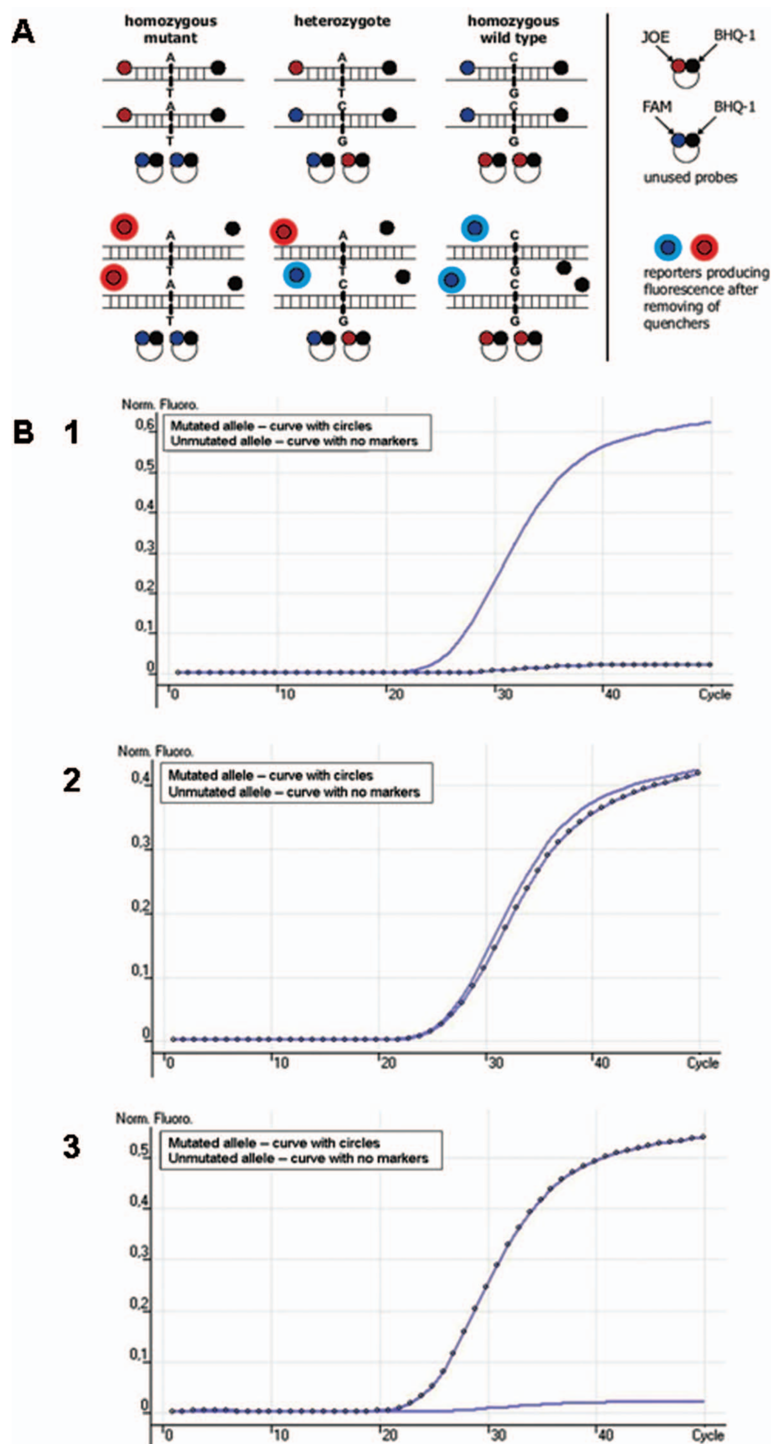

Figure 1. (A) Principle of genotyping using Taqman probes. (B) Allelic discrimination curves produced by the RotorGene 3000A analysis software. The *y*-axis represents the intensity of fluorescence, and the *x*-axis represents the number of amplification cycles. Example of amplification curves of (1) sample carrying only wt allele of *JAK2*, (2) heterozygous mutant patient, and (3) homozygous mutant patient.

were prepared. After lysis with the TRIzol Reagent (Invitrogen, Carlsbad, CA), RNA, DNA and proteins were isolated according to manufacturer's instructions. For the detection of *JAK2* mutation, cDNA was used, as it was previously shown that this approach was more sensitive than the use of genomic DNA [8]. One microgram of RNA was reverse transcribed into cDNA as described previously [9].

The primers and probes for the newly developed allelic discrimination real-time RT-PCR assay were

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designed according to generally accepted guidelines [10]. We had tested three pairs of probes (IBA, Göttingen, Germany) with different LNA modified nucleotides and finally chose the pair showing the best allelic discrimination and also the highest intensity of fluorescence. The sequences of probes and primers are as follows (LNA nucleotides are capitalized):

JAK2 wt 5′-FAM-cacaGaCAcAtActc-BHQ1-3′
JAK2 mut 5′-JOE-ctccACagAAaCatac-BHQ1-3′
JAK2 forward 5′-agcagcaagtatgatgagc-3′
JAK2 reverse 5′-gatgctctgagaaaggcat-3′

Real-time RT-PCR was performed in the total volume of 20 μl using the RotorGene 3000A cycler (Corbett Research, Sydney, NSW, Australia). The reaction mixture consisted of 1 μl of sample cDNA, 500 nmol of both forward and reverse primers (Invitrogen), 300 nmol of both dual labelled probes, 200 μmol dNTPs, 1× PCR reaction buffer, 4 mmol $MgCl_2$, 1 U Platinum Taq DNA Polymerase (Invitrogen) and sterile water up to the final volume of 20 μl. A wide range of different $MgCl_2$ concentrations was tested; the best results were obtained with 4 mmol. The PCR cycle parameters were as follows: an initial denaturing step of 95°C for 5 min and 50 cycles consisting of 95°C for 10 s, 60°C for 30 s, followed by measuring of fluorescence acquisition (FAM and JOE) and 72°C for 25 s. All samples including negative (1 μl of sterile water was added instead of sample cDNA) and positive controls were run in duplicates, in order to check reproducibility. The allelic discrimination curves were evaluated and printed as a result report by RotorGene 3000A Allelic Discrimination software [Figure 1(B)].

The plasmids containing mutated and wild type PCR products of *JAK2*, respectively, were constructed in order to check the sensitivity of the new assay. 364-bp long PCR products were prepared using forward and reverse primers according to Baxter et al. [7]. These PCR products were purified using phenol/chloroform method and than T/A ligated into pGEM-T Easy vector (Promega, Madison, WI) and transformed into the XL1-Blue competent cells (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Plasmids were isolated using Concert Rapid Plasmid Purification Midiprep System (Gibco BRL); their concentration was measured by Beckman DU-600 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) and sequences were verified by control sequence analysis. Plasmids containing *JAK2* mutated and wild type alleles in the starting concentration of 10 pg/μl were admixed in different ratios (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, and 1% of mutated allele) to compare the sensitivities of both the above mentioned assays. An analogical experiment was performed with mixtures of RNA isolated from granulocytes of a homozygous mutant patient and RNA of a patient without mutation. Using our real-time PCR method, we were able to positively detect 2% of mutated allele in both the diluted plasmid and patients' RNAs. The same sensitivity of detection (2% of mutated allele in either plasmid or RNA) was reached when using the method developed by Baxter et al. [7]. However, when using the latter method, we were not able to confirm the heterozygous status of the mutation by sequencing in samples with the proportion of the mutant allele $<15-20\%$.

The results in the 261 patient samples tested were virtually equal when tested by both methods (i.e., the original method of Baxter et al. [7] and our novel real-time RT-PCR). In both of the assays, *JAK2* mutation was found in the same 127 out of 261 patients (48.7%). Thirteen of the 127 *JAK2* mutations (10.2%) were homozygous, and nine of them were found in PV patients.

In ET, *JAK2* mutations were demonstrated in 20/52 (38.5%) patients, and none of them was homozygous. This corresponds with previous findings that in ET, homozygosity of the *JAK2* mutant allele is rare in spite of the relatively frequent occurrence of the heterozygous state [11,12].

Out of 65 patients suffering from PV, 64 had mutations (98.5%); in nine of them (14.1%), the mutation was homozygous. Seventeen of 37 (45.9%) individuals with IMF had *JAK2* mutations (three were homozygous, including one patient who had carried heterozygous mutation in 2002 and developed a homozygous mutant state within three years). Of 35 MPD-U patients (i.e., patients with a known diagnosis of MPD, but not precisely distinguished by WHO criteria), 22 had *JAK2* mutations (62.9%). Within patients with thrombocythemia of unknown reason (i.e., patients that might have MPD with thrombocythemia or ST), 3/18 had mutated *JAK2* genes, whereas none of the five patients with well-defined ST had their *JAK2* genes mutated. Within patients with polyglobulia of unknown reason (i.e., patients that might have PV, SP, or idiopathic erythrocytosis), 0/22 had mutated *JAK2* genes, and also none of the seven patients with well-defined SP had their *JAK2* genes mutated. Of four patients with an overlap of MPD and myelodysplastic syndrome, one had mutated *JAK2* genes. Among the remaining 16 patients with miscellaneous diagnoses, no case with *JAK2* mutation was found.

In conclusion, the TaqMan allelic discrimination assay yields the same results as the AS-PCR and is therefore also suitable for routine clinical laboratory testing. In contrast to the latter, it is very simple, less

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laborious and time-consuming because it does not require sequencing or *Bsa*X I restriction analysis to distinguish between homozygotes and heterozygotes. We admit, however, that it is not possible to differentiate patients carrying heterozygous cells only from those having a mixture of wild type and homozygous mutant cells by other method than single cell cloning analysis. Theoretically, this may be a problem particularly in patients with PV, in whom both of these possibilities might occur [13]. Moreover, quite frequently these patients have all three types of cells (V617F homozygous, heterozygous, and homozygous wild type). In contrast, heterozygous patients with ET hardly ever harbour homozygously mutated cells. These patients always have heterozygous cells admixed in different ratios with wild type cells [13].

Furthermore, using the TaqMan allelic discrimination assay, the manipulation with post-PCR samples is not necessary, which strongly decreases the risk of cross contamination. The same pairs of probes and primers may be used for amplification on genomic DNA as well. This could, on one hand, save the time required for sample preparation, but may reduce the sensitivity of the assay on the other [8]. Last but not least, according to our local conditions, the detection of *JAK2* mutation by TaqMan allelic discrimination assay is three times cheaper than direct sequencing. We recommend our method to laboratories examining relatively high numbers of samples.

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