Monitoring of the JAK2-V617F mutation by highly sensitive quantitative real-time PCR after allogeneic stem cell transplantation in patients with myelofibrosis

Nicolaus Kröger,1 Anita Badbaran,1 Ernst Holler,2 Joachim Hahn,2 Guido Kobbe,3 Martin Bornhäuser,4 Andreas Reiter,5 Tatjana Zabelina,1 Axel R. Zander,1 and Boris Fehse1

1Bone Marrow Transplantation, University Medical Center Hamburg-Eppendorf, Germany; 2Department of Hematology/Oncology, University Hospital Regensburg, Germany; 3Department of Hematology/Oncology, University Hospital Düsseldorf, Germany; 4Department of Hematology/Oncology, University Hospital Dresden, Germany; 5Department of Hematology/Oncology, University Hospital Mannheim, Germany

The JAK2-V617F mutation occurs in about 50% of patients with myelofibrosis and might be a reliable marker to monitor residual disease after allogeneic stem cell transplantation. We describe a new, highly sensitive (≤ 0.01%) real-time polymerase chain reaction (PCR) to monitor and quantify V617F-JAK2-positive cells after dose-reduced allogeneic stem cell transplantation. After 22 allogeneic stem cell transplantation procedures in 21 JAK2-positive patients with myelofibrosis, 78% became PCR negative. In 15 of 17 patients (88%), JAK2 remained negative after a median follow-up of 20 months. JAK2 negativity was achieved after a median of 89 days after allograft (range, 19-750 days). A significant inverse correlation was seen for JAK2 positivity and donor-cell chimerism (r = -0.91, P < .001). Four of 5 patients who never achieved JAK2 negativity fulfilled during the entire follow-up all criteria for complete remission recently proposed by the International Working Group, suggesting a major role for JAK2 measurement to determine depths of remission. In one case, residual JAK2-positive cells were successfully eliminated by donor lymphocyte infusion. In conclusion, allogeneic stem cell transplantation after dose-reduced conditioning induces high rates of molecular remission in JAK2-positive patients with myelofibrosis, and quantification of V617F-JAK2 mutation by real-time PCR allows the detection of minimal residual disease to guide adoptive immunotherapy. (Blood. 2007;109:1316-1321)

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CML or the heavy chain rearrangement in patients with myeloma, in which sensitivity exceeds $10^{-4}$ to $10^{-6,12,14}$, we herein describe a highly sensitive real-time PCR method to detect the JAK2-V617F mutation in patients with myelofibrosis. Using this novel technique, we studied MRD kinetics in patients who underwent allogeneic stem cell transplantation after a dose-reduced conditioning regimen containing busulfan (10 mg/kg), fludarabine (180 mg/m2), and anti-thymocyte-globulin (60 mg/kg body weight [BW]) to determine the incidence and clinical impact of molecular remissions.

### Patients, materials, and methods

#### DNA samples

DNA was isolated from 200 μL whole blood using QIAamp columns (Qiagen, Hilden, Germany). This procedure regularly results in 3 μg to 8 μg DNA in a final volume of 200 μL. DNA (10 μL; 150-400 ng) was used in each PCR reaction. Given a mean number of 5000 nucleated cells per microliter, each 10-μL DNA sample contained about 50,000 cells. Based on our experience, blood samples with normal cell contents result in $c_{t}$ values of 19 to 22 for the hematopoietic cell kinase (HCK) control PCR. Higher $c_{t}$ values thus indicate lower DNA contents (or impaired DNA quality) and, consequently, decreased sensitivity of the assay. Although all patient samples were accepted, those samples with $c_{t}$ values higher than 26 were marked “low DNA content or bad DNA quality.”

JAK2-V617F mutation analysis was performed before stem cell transplantation and in most cases every 2 to 3 months after transplantation. We carried out our V617F mutation genotyping assay on DNA samples extracted from peripheral blood. We used peripheral blood because it is easier to access than bone marrow. Furthermore, due to bone marrow fibrosis aspiration is possibly impossible.

Four centers participated in this study. After isolation from blood samples DNA was stored at $-80^\circ\text{C}$ until analysis.

#### Real-time TaqMan PCR

Real-time quantitative PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) or an MJ research PTC-200 (Biorad, Munich, Germany). After initial denaturation (10 minutes or 3 minutes at 95°C in the ABI Prism 7700 and PTC-200, respectively), PCR was carried out for 45 cycles at standard conditions (94°C for 15 seconds; 60°C for 60 seconds).21 Ready-to-use (i.e., Taq Supermixes with Rox (Biorad) were used. Primers and TaqMan probes were selected using PrimerExpress software (Applied Biosystems). Primers and probes were purchased from MWG Biotec (Ebersberg, Germany) and Applied Biosystems.

Duplex PCRs were carried out to facilitate reliable quantification of minimal residual disease based on the Δ$C_{t}$ method.22 Briefly, $c_{t}$ values as obtained with the HCK gene-specific PCR (ie, DNA content control) were subtracted from the $c_{t}$ values for the JAK2-V617F–specific DNA. This difference was plotted against the known concentration of the standard dilutions (see “Results”) to generate a standard curve. Unknown concentrations were determined plotting the respective Δ$C_{t}$ values against the standard curve.

Standard curves for quantification were generated by mixing the JAK2-V617F–positive UKE1 cell line22 at defined percentages (10%, 3%, 1%, 0.3%, 0.1%, and 0.01%) into buffy coat–derived mononuclear cells (MNCs) from a voluntary healthy donor. MRD quantification was carried out using Excel software (Microsoft, Seattle, WA).

The UKE1 cell line was first described by Fiedler et al22 and derived from a patient with transformed myelodysplastic syndrome (MDS). Using the JAK2-V617F–specific as well as an analogous wild-type–specific PCR we determined the UKE1 cell line to be heterozygous for the JAK2-V617F mutation (not shown).

The following primer/probe combinations were eventually used (see “Results”) for MRD quantification by real-time PCR (the deliberately mutated nucleotide is underlined, final concentrations are indicated in brackets): JAK2 (V617F): forward (150 nM) 5′ TTA TGG ACA ACA GTCT ACA AAA CAA TTC 3′; reverse (150 nM) 5′ CTT ACT CTC GCC ACA AAA 3′; FAM probe (200 nM): 5′ TGG TAC TT TTT TTT TCC TTA GTT TCT TTT TGA AGC AGC A 3′; HCK: forward (25 nM) 5′ TAT TAG CAC CAT CCA TAG GAG GCT T3; reverse (15 nM) 5′ GTT AGG GAA AGT GGA GCG GAA G 3′; VIC-probe (200 nM): 5′ TAA CGC GTC CAC GAA GGA TGC GAA 3′.

### Patients’ characteristics

Patients up to 70 years of age with histologically proven primary or secondary osteomyelofibrosis according to the Italian Criteria for Diagnosis of Myelofibrosis24 were allowed to participate in the treatment protocol, which was approved by the ethics committee of the Ärztekammer Hamburg, Germany; all patients gave written informed consent, in accordance with the Declaration of Helsinki. JAK2-positive patients’ characteristics are summarized in Table 1. Twenty-one of 41 (51%) patients with myelofibrosis had a point mutation of JAK2 tyrosine kinase (V617F) and received a total of 22 allogeneic stem cell transplantations. JAK2-positive patients did not differ significantly regarding characteristics or overall survival from JAK2-negative patients (data not shown). Preliminary results of the treatment protocol were reported recently.10 Fifteen male and 6 female patients with a median age of 55 years (range, 32-63 years) were enrolled. Fifteen patients were diagnosed with primary myelofibrosis whereas 6 patients developed myelofibrosis after polycythemia vera (n=5) or thrombocythemia (n=1). Two patients with primary myelofibrosis had already developed secondary acute leukemia (sAML) either in remission (n=1) or refractory (n=1) at time of transplantation. According to the Lille score4 (excluding the patients with sAML), 4 patients had low risk with constitutional symptoms; 13 patients had an intermediate risk, and 2 patients had a high risk. According to the Cervantes score3 (excluding the 2 patients with AML), 5 patients were classified as low risk with constitutional symptoms, whereas 14 patients were scored as high risk. Five donors were HLA-identical siblings, whereas 16 patients received grafts from matched unrelated donors. All patients received peripheral-blood stem cells, and the median number of transplanted CD34-positive cells was $8 \times 10^{6}$ per kg BW (range, 0.9 $\times 10^{6}$–15.6 $\times 10^{6}$ per kg BW). No manipulation of the graft was performed. Conditioning consisted of busulfan (10 mg/kg BW administered orally, given as 4 mg/kg BW, or 8 mg/kg BW administered

<table>
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<th>Value</th>
<th>Number of patients</th>
<th>Sex</th>
<th>Myelofibrosis</th>
<th>Secondary AML from primary myelofibrosis</th>
<th>Lille score, excluding sAML</th>
<th>Cervantes score, excluding sAML</th>
<th>Acute GVHD, %</th>
<th>Chronic GVHD, %</th>
<th>Median age of the patients (range, 32-63 years)</th>
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<td>2</td>
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</table>
intravenously, given as 3.2 mg/kg BW, divided in 4 doses daily on days −5 and −4, and in 2 doses on day −3; fludarabine (30 mg/m², given daily intravenously from day −9 to day −4); ATG (rabbit; Fresenius, Bad Homburg, Germany) was given at a dose of 10 mg/kg BW for related and 20 mg/kg BW for unrelated donors over 12 hours on days −3, −2, and −1, followed by allogeneic peripheral-blood stem cell transplantation on day 0. Granulocyte colony-stimulating factor (Lenograstim, Fa. Chugai, Germany) was given intravenously at a dose of 5 μg/kg BW after allogeneic transplantation from day +5 and continued until sustained neutrophil engraftment. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine A (3 mg/kg, given from day −100 to day +100 after transplantation). The dose of cyclosporine A was adjusted to serum levels (200 ng/mL-300 ng/mL). Cyclosporine A was tapered between day −80 and discontinued until day +100 if no signs of GVHD were observed. In patients who received stem cells from unrelated donors, cyclosporine A was tapered between day +140 and day +180. Methotrexate (10 mg/m²) was given intravenously on days +1, +3, and +6 after transplantation. Because of elevated liver enzymes, 2 patients received mycophenolate mofetil (2 × 1 g from day +1 until day +28). Donor-cell chimerism analysis was performed by real-time PCR based on sequence polymorphism as described. 

Acute GVHD was treated with high-dose steroids, and extensive chronic GVHD was treated with cyclosporine A and steroids. All patients were nursed in single rooms equipped with HEPA-filters. Antibiotic prophylaxis consisted of ciprofloxacin; antifungal prophylaxis of fluconazole and, in case of prior mycotic infections, ofitraconazole or amphotericin B. Acyclovir was given as herpes prophylaxis from day +1 until day +180. Cytopermanentivirus (CMV)–seropositive patients with unrelated donors received CMV prophylaxis with ganciclovir after stable engraftment until day +100. Pneumocystis carinii prophylaxis consisted of either trimethoprim and sulfamethoxazole on 3 days weekly or of monthly pentamidine inhalation.

Statistical methods

Survival curves for JAK2-negative survival and for overall survival were estimated by the Kaplan-Meier method. The log rank test was performed for statistical analysis for time-dependant analyses of survival. Correlation was performed by Spearman test. A P value of less than .05 was considered significant. Overall survival was calculated from transplantation until death from any cause. JAK2-negative survival was calculated from transplantation until JAK2 positivity or death from any cause.

Results

Sensitivity and specificity of JAK2-V617F mutation polymerase chain reaction

The mutated differs from the wild-type JAK2 allele by just one nucleotide exchange (G>T) leading to the valine to phenylalanine (V>F) transition. Using PrimerExpress we designed a TaqMan PCR where the reverse primer terminates at the 3’ nucleotide corresponding to this point mutation. Thus, this reverse primer should bind with higher affinity to the mutated than to the wild-type allele. In fact, the primer/probe combination initially suggested by the software for our standard duplex-PCR conditions (see “Patients and methods”) allowed detection of the mutated allele with much higher sensitivity than detection of the wild-type allele (data not shown). However, those primers would not be useful for MRD detection because of the limited specificity, resulting in relatively high background signals of the wild-type allele.

To increase the specificity while conserving optimal sensitivity of the MRD-specific PCR we generated a set of primers shortened each time by one nucleotide at their 5’ end. In parallel, all those shortened primers were designed to contain an additional mutation at the third-to-last 3’ position. We then systematically tested all primer variants using dilutions of the JAK2-V617F–positive UKE1 cell line into healthy donor–derived MNCs. This allowed us to identify the reverse primer, combining high specificity with a sensitivity that has so far not been reported. Indeed, to test specificity, 60 DNA samples from healthy donors were investigated in 3 independent PCR reactions. On all 3 plates, in addition to the usual no-template controls, both a 10% and a 0.01% positive control were included. Of the 60 DNA samples, 56 (93.3%) tested negative in all test reactions. Three (5%) samples tested positive in 1 of 3 reactions; the respective ct values were 43.44, 41.61, and 44.72. One sample (1.7%) became positive in 2 of 3 wells (ct values: 42.34 and 40.36). According to our definition, none of the 60 healthy donor DNA would be regarded positive for the JAK2 mutation. At the same time, the mutant JAK2-V617F allele was regularly detected at dilutions of at least 1 in 10 000 (ie, 0.01%) (Figure 1).

To document reproducibility of results in the very low concentration range, 5 DNA probes from different patients with low amounts of JAK2 mutation (0.01% and < 0.01%) were tested in 4 independent polymerase chain reactions (each time in triplicate). For each single probe, excellent reproducibility was found with a maximal Δct variation of 1.24 (data not shown). A further increase of sensitivity might be possible if only granulocytes were analyzed. However, since the JAK2 mutation could also be found in other hematopoietic cells, the specificity will be lowered.

JAK2 monitoring after allogeneic stem cell transplantation

After 21 patients underwent 22 allogeneic stem cell transplantations, 256 PCR measurements of the JAK2-V617F tyrosine kinase mutation were performed. After 17 of 22 (78%) allogeneic stem cell transplantations, the JAK2-V617F mutation status became negative. In 15 of 17 patients, JAK2-V617F mutation status remained negative after a median follow-up of 20 months (range, 0.01% positive control were included. Of the 60 DNA samples, 56 (93.3%) tested negative in all test reactions. Three (5%) samples tested positive in 1 of 3 reactions; the respective ct values were 43.44, 41.61, and 44.72. One sample (1.7%) became positive in 2 of 3 wells (ct values: 42.34 and 40.36). According to our definition, none of the 60 healthy donor DNA would be regarded positive for the JAK2 mutation. At the same time, the mutant JAK2-V617F allele was regularly detected at dilutions of at least 1 in 10 000 (ie, 0.01%) (Figure 1).

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3-51 months). One of the 2 patients who became positive underwent transplantation for refractory AML after myelofibrosis. His JAK2-V617F negativity lasted only 6 weeks, and one month after becoming PCR positive this patient experienced clinical relapse. The second patient remained JAK2 positive for more than 12 months after allografting (patient no. 20), but became negative after DLI (1 × 10^6 CD3^+ cells/kg). PCR negativity lasted more than 7 months before the JAK2 mutation became detectable again (Figure 2). This patient also experienced mild skin GVHD after DLI.

In 15 patients, a close monitoring for the JAK2 mutation was performed after allografting. JAK2 negativity was achieved after a median of 89 days after allografting (range, 19-750 days). The one patient who became JAK2-V617F negative after 750 days (patient no. 20), was treated by DLI (Figure 2). Excluding this patient and focusing on the impact of initial engraftment, JAK2 negativity was always achieved before day 180 after allografting (range, 19-176 days; Figure 3).

Five patients (22%) never became JAK2-V617F negative after allografting. One of those patients received a second allograft from another unrelated donor after conditioning with treosulfan and fludarabine, resulting in JAK2-V617F negativity (patient no. 19) (Table 2). A second patient died of treatment-related liver toxicity on day 133 after unrelated stem cell transplantation (patient no. 10). A third patient showed only a transient decrease in JAK2-V617F-positive cells and is going to be prepared for a second allograft. The fourth patient (patient no. 2) remained PCR positive on a low molecular level (0.004%-0.018%) after allogeneic stem cell transplantation for more than 36 months. Now, 3 years after allogeneic transplantation, an increase of JAK2 positivity (2%) has been noted. So far, no increase of fibrosis in bone marrow histology, constitutional symptoms, splenomegaly, or abnormalities in peripheral-blood count have been observed. The fifth patient with secondary myelofibrosis after polycythemia vera did not respond to DLI and is going to be prepared for a second allograft (patient no. 14).

A highly significant inverse correlation was seen for JAK2 positivity and donor-cell chimerism: r = −0.91, P < .001.

After a median follow-up of 19 months (range, 3-51 months), the estimated 4-year JAK2-mutation–negative and overall survivals are 70% (95% CI: 50%-90%) and 88% (95% CI: 74%-100%), respectively (Figure 4).

Discussion

Monitoring residual disease by molecular markers after allogeneic stem cell transplantation has become an important tool to determine the depths of remission as well as to guide immunologic treatment strategies such as DLI. Here we have introduced a novel, highly sensitive, real-time PCR assay to quantify minimal residual disease in patients with myelofibrosis and other diseases characterized by the V617F mutation of the JAK2 gene.

The point mutation on exon 14 (V617F) of the Janus kinase 2 (JAK2) gene has been found in 35% to 50% of patients with myelofibrosis. Therefore, for about half of the patients with myelofibrosis who underwent allogeneic stem cell transplantation, monitoring the quantity of the JAK2-V617F mutation in blood granulocytes by real-time PCR would be a suitable method to determine response and residual disease. However, the currently available methods have a sensitivity of only 1% to 5%.

We have here developed a novel real-time TaqMan PCR with a high sensitivity of at least 10^−4 (0.01%). To do so, we made use of the amplification refractory mutation system (ARMS) initially proposed by Newton et al. This system is based on the deliberate introduction of an additional mutation in the 3′ region of the mutation-specific PCR primer. The additional mutation should increase the specificity of the mutation-specific primer by decreasing the probability of binding the unmutated allele. Indeed, for the optimal primer set design based on ARMS we found very low nonspecific amplification of the wild-type JAK2 allele. At the same time, amplification of the V617F mutated allele was efficient enough to allow the detection of at least 1 mutated cell in 10,000 normal cells.

Based on this highly sensitive real-time PCR technique we quantitatively evaluated the presence of the mutated JAK2-V617F allele in 21 patients after allogeneic stem cell transplantation. JAK2 negativity was achieved by 78% of the patients after reduced conditioning allografting. In 88% of these patients, JAK2 remained undetectable after a median of 20 months. The data correlated very well with the clinical course in those patients.

Due to the low number of patients, a detailed subanalysis is not possible, but it is of interest that from 6 patients with secondary myelofibrosis (after polycythemia vera [PT] or essential thrombocythemia [ET]) only 2 achieved JAK2-V617F negativity. A third patient with myelofibrosis after polycythemia vera achieved JAK2 negativity after GVHD due to a second allograft from an alternative donor. The decrease of JAK2 mutation after GVHD is a further hint for the graft-versus-myelofibrosis effect of allogeneic stem cell transplantation. More evidence for the graft-versus-myelofibrosis effect comes from another patient (Figure 2) who became, at least for 7 months, JAK2-V617F negative after DLI.
We further observed a highly significant inverse correlation between JAK2 positivity and donor-cell chimerism ($r = 0.91$), which suggests that changes in donor-cell chimerism might also detect incomplete remission or early relapse.

It is of note that 4 of 5 patients who never achieved V617F-JAK2 negativity fulfilled the overall criteria for complete response according to the European Myelofibrosis Network, or the criteria for complete remission recently proposed by the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT). This suggests that quantification of JAK2-V617F–positive cells after allogeneic stem cell transplantation may be a much better tool to define complete remission. However, longer follow-up is necessary to determine the impact of residual molecular disease since one patient (no. 2) remained JAK2 positive on a low level for more than 3 years after allograft without bone marrow fibrosis or clinical signs of progression. In contrast, those 2 patients who achieved JAK2 negativity but became positive again, also showed clinical progress.

In conclusion, measurement of JAK2 mutation by using high sensitivity, real-time PCR in JAK2-positive patients with myelofibrosis allows monitoring of residual disease after allogeneic stem cell transplantation and might help to guide adoptive immunotherapy strategies such as donor lymphocyte infusion. Because it is possible that JAK2 mutation is a secondary event, the true reliability of V617F as a marker of minimal residual disease needs to be evaluated with a longer follow-up.

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**Acknowledgments**

We thank the staff and the technicians of the BMT units for providing excellent care of our patients.

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**Table 2. Patients' results**

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<th>Patient no.</th>
<th>Sex</th>
<th>Age, y</th>
<th>Donor</th>
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<th>Myelofibrosis</th>
<th>Acute GVHD, grade</th>
<th>Chronic GVHD</th>
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<td>M</td>
<td>61</td>
<td>MUD</td>
<td>Intermediate</td>
<td>Secondary (ET)</td>
<td>3</td>
<td>Lim</td>
<td>118</td>
<td>5 + alive, JAK2 neg</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>53</td>
<td>Rel</td>
<td>Intermediate</td>
<td>Secondary (PV)</td>
<td>2</td>
<td>Lim</td>
<td>750 (after DLI)</td>
<td>40 + alive, JAK2 pos</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>46</td>
<td>MUD</td>
<td>Intermediate</td>
<td>Primary</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>3 + alive, JAK2 neg</td>
</tr>
</tbody>
</table>

Unknown indicates that no sample immediately after allo-SCT was available.

*The patient died.

GVHD indicates graft-versus-host disease; Rel, HLA-identical sibling; Lim, limited; MUD, matched unrelated donor; PV, polycythemia vera; sAML, secondary acute myeloid leukemia; TRM, transplant-related mortality; ET, essential thrombocythemia; SCT, stem cell transplantation; DLI, donor lymphocyte infusion.

†Same patient; two different SCTs.

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**Figure 4. JAK2-V617F–negative and overall survival after allogeneic stem cell transplantation in JAK2-V617F–positive patients with myelofibrosis.**

(A) JAK2-V617F–negative survival; (B) overall survival; n = 21.
References


Monitoring of the JAK2-V617F mutation by highly sensitive quantitative real-time PCR after allogeneic stem cell transplantation in patients with myelofibrosis

Nicolaus Kröger, Anita Badbaran, Ernst Holler, Joachim Hahn, Guido Kobbe, Martin Bornhäuser, Andreas Reiter, Tatjana Zabelina, Axel R. Zander and Boris Fehse