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WT1 gene is overexpressed in myeloproliferative neoplasms, especially in myelofibrosis

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ABSTRACT

Classical Philadelphia-negative myeloproliferative neoplasms include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). They are characterized by the presence of driver mutations of *JAK2*, *CALR* or *MPL* genes. Overexpression of *WT1* is used as a marker of minimal residual disease in acute myeloid leukemia, especially after allogeneic stem cell transplantation (SCT). We investigated *WT1* expression at diagnosis in 152 MPN patients and showed that the *WT1* transcript was overexpressed in PMFs and PVs compared to controls. In particular, *WT1* transcript levels were higher in PMF than in ET and PV. *WT1* transcript levels were significantly increased during myelofibrotic transformation of ET or PV. Using multivariate linear regression, high *WT1* transcript levels in PMF were associated with age over 65, splenomegaly and thrombocytopenia. The ROC curve analysis showed that a level of *WT1* transcript > 10 *WT1* copies/10^4*ABL1* enabled the diagnosis of PMF with a specificity of 95.8% (PMF vs ET; ROC AUC = 0.91). In myelofibrosis, studying follow-ups of *WT1* transcript showed that this marker is of interest after allogeneic SCT. These results demonstrate that *WT1* overexpression is a simple marker of myelofibrosis in MPN and could be used during patient follow-up.

1. Introduction

Philadelphia-negative classical myeloproliferative neoplasms (MPN) are chronic hematopoietic disorders that include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). PMF is defined by medullar fibrosis observed with bone marrow biopsy which is graded from MF0 to MF3 according to the WHO classification [1]. The overall survival is better in PV and ET than in PMF, with a median survival over 15 and 20 years respectively versus 6 years for PMF [2]. For PV and ET, the short-term course is marked by the risk of thrombosis whereas the long-term outcome is marked by the risk of progression to post-PV or post-ET myelofibrosis (MF) or acute myeloid leukemia (AML). PMF patients have a higher risk of leukemic transformation [1]. The prognosis of PMF is accessed with scores such as the International Prognostic Scoring System (IPSS) based on age, constitutional symptoms, hemoglobin level, leukocytes count and circulating blasts [3]. Philadelphia-negative MPNs are mostly caused by driver mutations in Janus kinase 2 (*JAK2*), calreticulin (*CALR*) or thrombopoietin receptor (*MPL*) genes [4–6]. *JAK2* mutations are present in > 95% of patients with PV and around 55% of those with ET and PMF. *CALR* and *MPL* mutations are specific to ET and PMF and are found in 25% and 5% of patients with ET, and in 30% and 8% of

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patients with PMF, respectively [5,7]. Patients with none of these driver mutations are called triple negative. Progressively, correlations between molecular and clinical characteristics have been identified in MPNs [8–10].

First noted for its role in a renal neoplasm, Wilms' tumor 1 gene (*WT1*) is now known for its function in organ development and is involved in many diseases. *WT1* is a transcription factor that directs the development of several organs and tissues through the Wnt4 pathway [11,12]. It can act as either a tumor suppressor or an oncogene and its involvement in normal and malignant hematopoiesis is unclear [13]. Among the multiple existing *WT1* splicing variants, some may be of interest in blood disorders [14]. In addition, an epigenetic role of WT1 has recently been demonstrated as the cofactor of TET2 in DNA methylation control [15,16].

WT1 is expressed in a small percentage of bone marrow CD34+ cells and has been shown to be highly overexpressed in most de novo acute myeloid leukemias (AML) and myelodysplastic syndromes [17,18]. *WT1* is consequently often used as a molecular marker for minimal residual disease in AML especially after allogeneic stem cell transplantation [19–21].

WT1 expression in MPNs has been sparsely studied. In 2007, Guglielmelli et al. showed by a transcriptomic micro array approach that the *WT1* level was higher in PMF-derived CD34+ cells than in PV and ET ones [22]. More recently, Gallo et al. reported a positive correlation between *WT1* transcript levels and the IPSS score at diagnosis in PMF patients [23].

The aim of this work was to determine whether *WT1* expression can be a useful marker for prognosis and monitoring disease progression in MPN patients.

2. Patients and methods

2.1. Patients

We studied a total of 152 patients affected by myeloproliferative neoplasm according to the 2008 World Health Organization (WHO) criteria: 39 PV, 71 ET and 42 PMF. A bone marrow biopsy was available for all triple negative patients. Fifteen patients affected by acute myeloid leukemia were included as a comparison group. All MPN and AML patients were analyzed at diagnosis before any treatment. As control group, 30 non-MPN patients were analyzed. The latter group included patients for whom a diagnosis of MPN was suspected (polycythemia, thrombocytosis or leukocytosis) and was finally rejected based on clinical-biological parameters, including search for MPN driver mutation. Biological and clinical data at diagnosis were collected retrospectively. All patients provided written informed consent for the use of remnant DNA or RNA for investigational purposes in accordance with the Declaration of Helsinki.

2.2. Molecular testing, CD34+ quantification and LDH measurement

Diagnosis samples from all patients were analyzed for driver mutations of *JAK2*, *CALR* and *MPL* genes, using respectively the MutaQuant kit (Qiagen), fragment analysis and MutaScreen kit (Qiagen). For *WT1* quantification, total RNA was extracted from blood leukocytes using NucleoSpin® RNA (Macherey-Nagel) and then reverse transcribed to cDNA. *WT1* expression was assessed by Real-time Quantitative Polymerase Chain Reaction (RQ-PCR) as previously described [21]. RQ-PCR was performed in an ABI Prism 7500 system (Applied Biosystems). Calibration curves with plasmids containing *ABL1* and *WT1* target sequences were used (Ipsogen WT1 ProfileQuant Kit, Qiagen). All PCR analyses were performed in duplicate. The *WT1* transcript values obtained by RQ-PCR were normalized with respect to the number of *ABL1* transcript and expressed per 10^4 *ABL1* copies. The *WT1* expression was transformed into a logarithm to ensure that values follow a normal distribution for statistic analysis.

The determination of circulating CD34+ cells was obtained through flow cytometry with a standardized simple platform technique (BD™ Stem Cell Enumeration Kit, BD Bioscience; Stem-Kit™ CD34 HPC Enumeration kit, Beckman Coulter).

For MPN patients, serum LDH levels were obtained with the kit ADVIA® Chemistry XPT.

2.3. Statistical analysis

Patients' characteristics were summarized as numbers (percentage) for qualitative variables, and with mean ± standard deviation, or median – [Inter-Quartile Range (IQR)], as appropriate, for continuous variables.

Patients' characteristics were compared using the Fisher exact test for categorical variables and the Mann-Whitney or Kruskal-Wallis tests for continuous variables (followed by Dunn's tests for multiples comparisons). For multiple comparisons, *P*-values were adjusted using the Hochberg procedure, which allows a control of the Family-wise Error Rate at 5% [24,25]. *WT1* expression for diagnosis of PMF was studied using Receiver Operating Curves (ROC). We then performed a linear regression to model the *WT1* expression by thoroughly checking the assumptions on residuals. Survival estimates were obtained with the Kaplan-Meier method and statistical tests were performed using a log-rank test, with a check of hazard proportionality assumptions. All tests were two-sided, with a Type-I error rate at 5%.

Statistical analysis were performed using the R software version 3.4.1, and the multitest package for multiple comparisons procedures [26,27].

3. Results

3.1. WT1 expression in MPN at diagnosis

Patients' clinical and biologic characteristics at diagnosis are summarized in Table 1. Compared to control patients (0.58 *WT1* copies/10^4*ABL1*), PV and PMF patients had an increase in *WT1* transcript expression with the following median values (*WT1* copies/10^4*ABL1*): PV: 3.23 ($P < 0.001$) and PMF: 38.02 ($P < 0.001$) (Fig. 1). No difference was shown between ET and controls (median values in ET group: 1.2 *WT1* copies/10^4*ABL1* ($P = 0.069$)). This last result might be due to a heterogeneous expression of *WT1* in ET. Indeed, *JAK2* and *CALR* mutated ET had higher levels of *WT1* compared to both triple negative ET and controls (respectively $P = 0.0016$ and $P < 0.001$, Supplemental Fig. 1). On the opposite, in PMF, *WT1* was overexpressed equally among the different driver mutation groups (Supplemental Fig. 2).

WT1 levels in PMF were statistically higher than in ET and PV ($P < 0.001$ and $P < 0.001$ respectively) but lower than in AML (1900 *WT1* copies/10^4*ABL1*, $P < 0.001$). We were unable to highlight a difference of *WT1* expression between the different degrees of bone marrow fibrosis in both ET (MF0 vs focal MF1, $P = 0.47$) and PMF patients (MF1 vs MF2–3, $P = 0.18$). PV patients had a significantly higher level of *WT1* transcript than ET patients ($P < 0.001$).

Using ROC curve analysis, we defined the cut-off of 10 *WT1* copies/10^4*ABL1* to distinguish PMF from PV or ET, showing good sensitivity and specificity of 73.8% and 89.1% respectively (ROC AUC = 0.88). The specificity increased to 95.8% when we focused on PMF versus ET (ROC AUC = 0.91). When we combined *WT1* overexpression with CD34-positive circulating cells (data available in 63/71 ET and 37/42 PMF; CD34 cut-off previously described of 10 cells/μL), the sensitivity increased to 89.5% with a specificity of 95.2% [28].

3.2. Clinical-biological features associated with WT1 expression in myelofibrosis

We then focused on the 42 patients with PMF. *WT1* levels were higher in patients with intermediate-1 IPSS ($P = 0.0067$) or

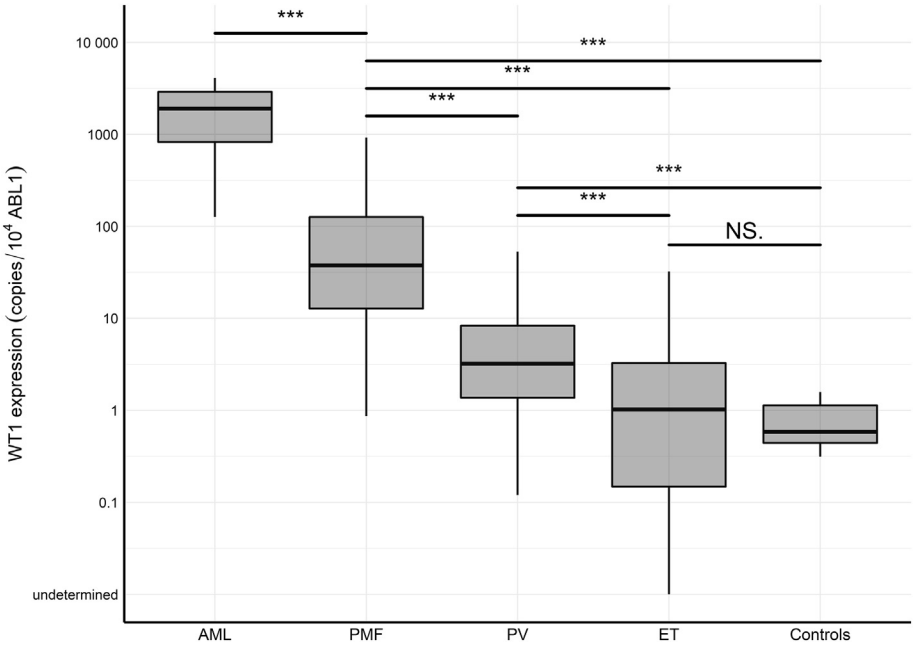

Fig. 1. *WT1* expression levels in MPN at diagnosis. *WT1* levels were expressed as a ratio to *ABL1* quantification. AML: acute myeloid leukemia, PMF: primary myelofibrosis, PV: polycythemia vera, ET: essential thrombocythemia.
*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: non significative result. *P*-values were corrected with Hochberg method.

Table 1
Clinical and biologic characteristics of MPN patients.

	PV	ET	PMF
	(n = 39)	(n = 71)	(n = 42)
Age (median [range]) (years)	69.4 [60.4; 79.2]	67.2 [55.1; 76.4]	68.4 [57.5; 77.0]
Sex (M/F)	20/19	31/40	26/16
WBC (x 10^9/L)	11.53 [8.89; 14.88]	8.15 [6.71; 9.98]	9.43 [6.70; 18.43]
Hemoglobin level (g/dL)	18.0 [16.8; 19.2]	13.9 [13.2; 15.2]	12.8 [9.9; 14.2]
Platelets count (x 10^9/L)	419 [338; 719]	735 [631; 864]	464 [222; 722
JAK2 V617F positive (n (%))	39 (100%)	44 (62%)	23 (55%)
CALR positive (n (%))	0 (0%)	13 (18%)	13 (31%)
MPL positive (n (%))	0 (0%)	2 (3%)	3 (7%)
Triple negative (n (%))	0 (0%)	12 (17%)	3 (7%)
Bone marrow biopsy			
Grade 0 (n (%))	11 (28%)	45 (63%)	0
Grade 1 (n (%))	7 (18%)	10 (14%)	10 (24%)
Grade 2 (n (%))	0	0	24 (57%)
Grade 3 (n (%))	0	0	6 (14%)
No bone marrow biopsy available (n (%))	23 (54%)	16 (23%)	2 (5%)
IPSS			
Low (n (%))	–	–	9 (21%)
Intermediate-1 (n (%))	–	–	15 (36%)
Intermediate-2 (n (%))	–	–	16 (38%)
High (n (%))	–	–	2 (5%)
Median follow-up (years [range])	4,0 [1,6; 6,2]	3,0 [1,2; 6.1]	4.5 [1,5; 6.4]
Leukemic transformation (n)	1	0	1
Progression in post-PV MF and post-ET MF (n)	3	6	–
Death (n)	5	4	11

intermediate-2/high IPSS ($P < 0.001$) than in those with a low IPSS. In order to investigate the clinical-biological parameters associated with *WT1* overexpression in PMF, we performed a linear regression with the following parameters: age > 65 years, gender, anemia (< 100 g/L), leukocytosis (> 25 G/L), thrombocytopenia (< 150 G/L), circulating blasts (> 1%) and splenomegaly. The cut-offs we used were those defined in the IPSS prognostic classification [3]. Results are summarized in Table 2. Older age, circulating blasts, splenomegaly and thrombocytopenia were associated with higher *WT1* expression levels. The most significant association was thrombocytopenia which resulted in an increase of 104 *WT1* copies/10^4*ABL1* the value of *WT1* expression (with other clinical-biological parameters constant).

Table 2
Parameters associated with *WT1* overexpression in PMF.
Results of the linear regression model (n = 41 because of one patient with *WT1* expression undetectable), significant associations of clinical-biological parameters are in bold. **: P < 0.01, ***: P < 0.001.

Clinical-biological parameters	β	CI-95%	*P*-value
Intercept	−1.62	[−2.21; −1.04]	< 0.001***
Age (> 65y)	**0.60**	**[0.17; 1.03]**	**0.008****
Sex (Male)	0.22	[−0.16; 0.62]	0.251
Circulating blasts (> 1%)	0.38	[−0.12; 0.89]	0.130
Anemia (hemoglobin < 10 g/dL)	0.39	[−0.06; 0.85]	0.090
Splenomegaly	**0.67**	**[0.24; 1.02]**	**0.003****
Thrombocytopenia (platelets < 150 G/L)	**1.02**	**[0.40; 1.63]**	**0.002****

In order to study the impact of *WT1* levels at diagnosis on the prognosis of PMF, we defined the threshold of 40 *WT1* copies/10^4*ABL1* (median of the *WT1* expression in PMF patients) as high expression for *WT1*. With a median follow-up of 54 months, we did not demonstrate that *WT1* overexpression had an impact on survival ($P = 0.19$).

3.3. *WT1* expression during follow-up of MPN

During the follow-up (median follow-up: 3.7 years), 10 patients developed hematological transformation (6 post-PV MF, 3 post-ET MF and 1 post-PV AML) with a median time of transformation of 8.3 years [3–13 years]. It is worth mentioning that three of them already had a quantification of *WT1* above 10 copies/10^4*ABL1* at the time of diagnosis. These 3 patients were 2 PV with no fibrosis at diagnosis and one patient for whom bone marrow biopsy was not performed at diagnosis but who had CD34 circulating cells at 4.26/μL (a thresholds of < 10 or 15 cells/μL exclude PMF [28,29]). We performed *WT1* quantification for these 9 patients at the time of transformation that showed a significant increase in all patients ($P = 0.004$; Fig. 2A).

We also performed a *WT1* quantification during follow-up in 11 patients in chronic phase (2 ET, 1 PV and 8 PMF with a median follow-

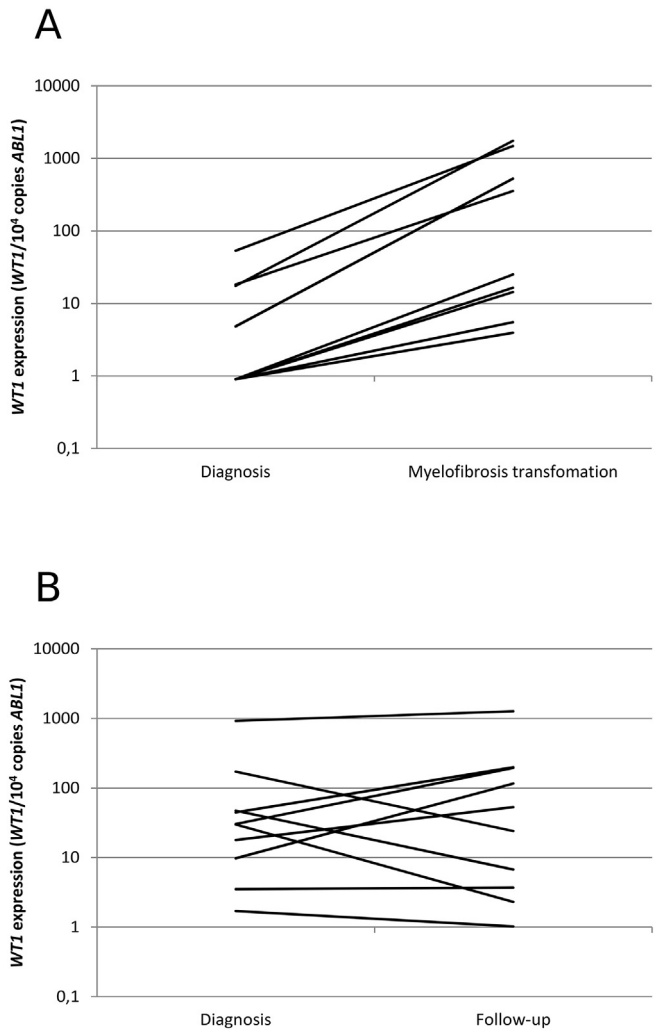

Fig. 2. *WT1* follow-up in patients that evolved to post-PV MF and post-ET MF (panel A) and in patients at chronic phase without hematological progression (panel B).

up of 6 years) and found stable *WT1* expression ($P = 0.21$; Fig. 2B). Six of these 11 patients were treated with pegylated-interferon from whom we observed an increased expression level in 3 patients and a decreased expression in the 3 others. The evolution of *WT1* quantification was compared to *JAK2*V617F allele burden for the four *JAK2*-mutated patients treated with pegylated-interferon (Supplemental Fig. 3). For three of them, the evolution of markers was similar. For the last, evolution was marked by a decrease in *WT1* expression while *JAK2*V617F expression increased (Supplemental Fig. 3).

Finally, we studied the follow-up of *WT1* expression and *JAK2*V617F allele burden in a patient treated by allogenic stem cell transplantation and later by donor lymphocyte infusions. Both markers decreased drastically after allograft. During follow-up, *WT1* rate increased at the same time as *JAK2* mutation, motivating donor lymphocyte infusion which resulted in molecular remission for both markers (Fig. 3).

4. Discussion

In this work, we found that the *WT1* transcript was overexpressed in all MPNs compared to controls. Furthermore, we showed that the expression level was higher in patients with PMF, especially in those with poor prognostic features.

Guglielmelli et al. have previously described a higher *WT1* expression level in PMF patients compared to PV, ET and healthy controls [22], but they did not find any difference between PV, ET and controls for *WT1* expression using the comparative cycle threshold (CT) method. In our study, we found that PV and PMF had an overexpression of *WT1* compared to controls and we found that PV had a slightly higher *WT1* expression than ET. These results could be explained because we quantified *WT1* expression using a range of commercial plasmids (Ipsogen WT1 kit, Qiagen). The use of a range of plasmids eliminates variations related to differences in efficacy between two series of PCR. Gene quantification using a plasmid range allows a higher sensitivity and precision in low values than the ΔΔCt method. Our choice of controls seems relevant to us because they were patients with abnormal blood counts close to MPN but for whom this diagnosis was then excluded.

The expression of *WT1* was an easy-to-practice marker for PMF at the time of diagnosis showed good performance with good specificity. Moreover, when combined with the measurement of circulating CD34+ cells, sensitivity increased to 89.5%. This biomarker could therefore be helpful for diagnosis in some difficult cases or when a bone marrow biopsy is non-contributory or is impracticable. Furthermore, the *WT1* expression increased in all 9 patients who progressed to post-PV MF and post-ET MF. Other blood test as serum lactate dehydrogenase (LDH) levels were previously described as specific of bone marrow fibrosis including in prefibrotic stage [30] but the test's sensibility is still debated [31]. In our study, 7 of the 9 patients had increased LDH levels at the time of myelofibrotic progression. Therefore, *WT1* quantification could be useful in case transformation to post-PV MF and post-ET MF is suspected. Nevertheless, a histological confirmation remains necessary.

In our retrospective study, the diagnosis of MPN was made according to the WHO 2008 classification. We were therefore unable to study the expression of *WT1* in prefibrotic PMF that was introduced in the WHO 2016 classification. It would be of interest to investigate differences in *WT1* levels between ET, prefibrotic PMF and PMF whose differential diagnosis can be difficult [32,33].

As described by Gallo et al., we found that hyperexpression of the *WT1* gene in PMF is associated with the IPSS prognostic score [23]. In addition, we showed herein that this overexpression was associated with age but also with the presence of splenomegaly and thrombocytopenia that are not part of the IPSS score.

Gallo et al. also showed that *WT1* expression increased during leukemic transformation of MPN [23]. We showed here that transformation into post-PV MF and post-ET MF is also associated with an increase in *WT1* expression. Conversely, in patients without hematologic progression, *WT1* levels were stable. Specifically, the follow-up of 4 patients under interferon therapy showed that *WT1* expression mostly follows the evolution of *JAK2* allele burden.

In conclusion, we showed that *WT1* is overexpressed in MPN, in particular in myelofibrosis where its expression was associated with poor prognostic clinical-biological factors. *WT1* expression can be a helpful additional diagnostic marker and follow-up marker if progression to post-PV MF and post-ET MF is suspected. We quantified *WT1* expression from total blood leukocytes that are easily available in routine lab practice. However, studying the expression of *WT1* in clonal granulocytes or sorted CD34+ cells would also be interesting. Finally, it can be a marker of minimal residual disease in patients who underwent allogeneic stem cell transplantation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2018.12.004.

Authors contribution

DLP, OB and VU designed the study. LC, RJC and ABE performed the experiments. LC, JR, ABO, BR and DLP analyzed the data. FB, AZ, ABL, MT, CO and MHB followed the patients and brought clinical data. LC, OB, VU and DLP wrote the paper.

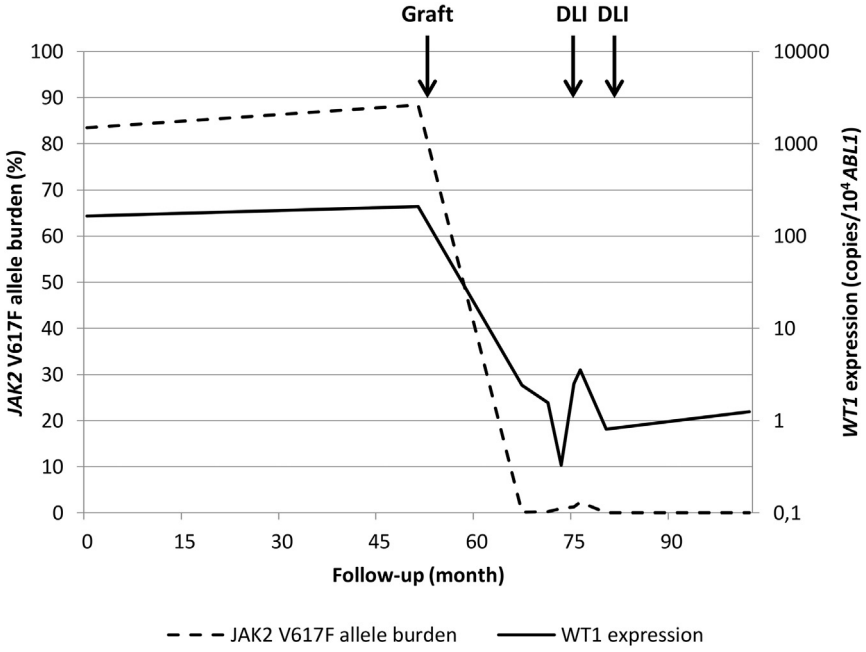

Fig. 3. Evolution of *WT1* transcript quantification and *JAK2*V617F allele burden in a patient after allogenic graft and donor lymphocytes injection (DLI).

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