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**PU.1 expression is restored upon treatment of chronic myeloid leukemia patients**

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**Abstract**

The PU.1 transcription factor is a crucial regulator of hematopoiesis which expression is altered in various leukemic processes. Our previous work in chronic myeloid leukemia (CML) cells demonstrated that interferon-α up-regulated PU.1 expression. Here we show that expression of PU.1 is severely impaired in patients with CML at diagnosis. However, the PU.1 suppression is abrogated in patients in remission, after interferon-α or imatinib treatment. These effects are not found in patients with other myeloproliferative diseases such as polycythemia vera or essential thrombocythemia. PU.1 could, therefore, be used as an additional marker of the response to the treatment of the CML.

*Keywords:* PU.1, CML patients, interferon-α, imatinib

## 1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder. The disease evolves from an initial chronic phase with accumulation of myeloid progenitors and mature cells in the blood, to a fatal blast crisis with a block in the differentiation of the myeloid or lymphoid lineages (reviewed in [1-3]). CML is characterized by the expression of the BCR-ABL oncoprotein, a fusion protein with constitutive tyrosine kinase activity. BCR-ABL is expressed through the disease, but the progression to blast crisis requires additional molecular alterations [3,4]. Before the introduction of the BCR-ABL inhibitor imatinib, therapeutic options of CML included drugs such as busulfan, hydroxyurea and interferon-α (IFN-α) and allogenic bone marrow transplantation. Treatment with IFN-α delays the progression to blast crisis and prolongs patient survival [5]. Nowadays, imatinib is the first-line therapy for CML, producing an elevated rate of molecular response and event-free survival [5-7]. Possible additional benefits of the combination of imatinib with IFN-α have been described [8].

PU.1 is a transcription factor of the Ets family which is essential for myeloid and lymphoid cell development (for recent reviews see [9-12]). PU.1 controls the expression of genes important for the determination of both myeloid and lymphoid lineages ([9] and references therein). Moreover, PU.1 is involved in various hematopoietic malignancies. The overexpression of PU.1 in erythroid precursors induces erythroleukemia in transgenic mice [13,14], whereas a reduction in the PU.1 expression level is associated to acute myeloid leukemias (AML) [11,12], indicating that the PU.1 role in leukemic processes depends on its expression levels. However, to our knowledge there are no reports on PU.1 expression in myeloproliferative diseases, including CML.

Our previous work demonstrated that interferon upregulated PU.1 mRNA and protein expression in CML cells [15]. In this report we analyzed PU.1 expression in samples of patients with three different myeloproliferative disorders (CML, polycythemia vera and essential thrombocythemia) and we found that PU.1 is repressed in CML patients at diagnosis but PU.1 expression increased upon treatment with interferon-α or imatinib that restore normal hematopoiesis.

## 2. Materials and Methods

*2.1. Cell lines, cultures and transfections*

K562 cells (ATCC), originally derived from a patient with CML in blastic crisis, were grown in RPMI 1640 medium supplemented with 8% fetal calf serum (Biochrome). Exponentially growing K562 cells were treated with 2000 U/ml of human interferon-α2a (Roche), or with 1 μM Imatinib mesylate (provided by Novartis, Basel, Switzerland). For stable transfections, 32D murine myeloid cells (provided by A. Bigas, IDIBELL, Barcelona) were electroporated with the following expression vectors: pSRα-BCR-ABL, pSRα-vector (provided by T. Skorski, Temple University, Philadelphia) and pM5Neo-ts-bcr-abl (provided by J.H. Kabarowski, Institute of Cancer Research, London). 32D cells ($5x10^6$) were electroporated in 0.8 ml of RPMI containing 30 μg of the different plasmids at 260 V and 1 mFa with a Bio-Rad electroporator. 48 hours after electroporation, G418 (500 μg/ml) was added and selected cells were expanded and analyzed. For siRNA transfection, K562 cells were electroporated at the above conditions using different amounts (40 to 320 pmol) of siRNA per $2x10^6$cells. To specifically inhibit BCR-ABL expression a 21-base siRNA directed against the fusion sequence was used [16].To exclude secondary effects in the transfected cells, an scrambled siRNA at the same concentrations, and mock transfected cells were used as controls. The sequences were: b3a2-1 siRNA, sense 5'-GCA GAG UUC AAA AGC CCU UdTdT-3' and antisense 5'-AAG GGC UUU UGA ACU CUG CdTdT-3'; scrambled-siRNA, sense 5'-UUG UAC GGC AUC AGC GUU AdTdT-3' and antisense 5'-UUA CGC UGA UGC CGU ACA AdTdT-3' (all from Dharmacon). Cells were harvested 48h after transfection and the reduction in BCR-ABL mRNA was analyzed by qRT-PCR.

*2.2. Patient samples*

Fresh leukemic mononuclear cells from CML patients were isolated from bone marrow by Ficoll-Hypaque density centrifugation. The diagnosis of CML patients (65% male/ 35% female, median age 50 years, range 23-75 years) was confirmed by the presence of Philadelphia chromosome and the BCR-ABL mRNA by RT-PCR. CML sampling was as follows: 43 samples at diagnosis (all in chronic phase), 32 samples after treatment with IFN-α

(3-5x$10^6$ units/$m^2$/day) and 31 samples after treatment with imatinib mesylate (400 mg daily). Half of the imatinib treated patients had been previously treated with IFN-α. For CML patients, serial samples at diagnosis and post-treatment at different times were collected. Patients were evaluated for hematological, cytogenetic and molecular responses for a minimum of 12 months. Samples from patients with myeloproliferative diseases at the time of diagnosis were also taken. The 33 patients in the study group were divided into patients with essential thrombocythemia (n=18, male/female=10/8, median age 71.4 years, range 52-93 years, 43% positive for the JAK2 V617F mutation) and patients with polycythemia vera (n=15, male/female=10/5, median age 73.1 years, range 60-87; 80% positive for the JAK2 V617F mutation). JAK2 V617F mutation was analyze as described [17]. Bone marrow samples from 16 hematologically normal individuals were collected as control samples. Samples were from Hospital Marqués de Valdecilla (Santander, Spain), Hospital Dr. Negrín and Hospital Insular de Gran Canaria (Las Palmas, Spain). This study was approved by the ethics committees and informed consent was obtained from all patients according to procedures approved by the respective institutions.

*2.3. RNA extraction and expression analysis*

Total RNA was extracted with the RNeasy kit (Qiagen). For RT-PCR analysis, 2 μg of total RNA were reverse transcribed with the iScript™ cDNA synthesis kit (BioRad). cDNA was amplified by semiquantitative PCR (Sigma REDTaq™ PCR reaction mix) or by real-time-PCR (Bio-Rad iQ™ SYBR green supermix). The expression levels of PU.1 were normalized by the internal control ribosomal protein RPS14. The following primers were used: PU.1 (human cDNA sequence [18]) forward 5'-GCCATAGCGACCATTACTGG-3', reverse 5'-TCTTCTTGCCTGTCTCC-3' RPS14: forward 5'-GGCAGACCGAGATGAATCCTC-3', reverse 5'-CAGGTCCAGGGGTCTTGGTCC-3'. For BCR-ABL determination a standardized protocol for TaqMan-based qRT-PCR was used [19]. Northern analysis with probes for BCR-ABL and PU.1 were performed as described in a previous report [20].

*2.4. Statistical analysis*

Comparison tests were performed using the Mann-Whitney U-test. A level of $p<0.05$ was accepted as statistically significant. The data were analyzed using SPSS-14 software.

**3. Results**

*3.1.PU.1 expression in CML cells upon treatment with interferon-*α *or interferon-*α *plus imatinib*

We previously reported that IFN-α upregulated PU.1 expression in CML-derived cells [15]. As most of the patients studied in the present work have been sequentially treated with IFN-α and imatinib, we asked for the effect of imatinib on the PU.1 expression in a CML derived cell line (K562) previously exposed to IFN-α. As shown in Fig. 1, PU.1 mRNA expression showed a marked increase in K562 cells upon IFN-α treatment (8-fold by real-time RT-PCR) and imatinib treatment did not impair PU.1 upregulation by IFN-α in cells treated consecutively with both drugs. A modest reduction in BCR-ABL levels was observed when cells were treated with both drugs as compared to treatment with one drug (see quantification of Fig. 1).

*3.2. PU.1 expression is impaired in CML patients at diagnosis and is recovered after treatment with IFN-α or imatinib*

The above results prompted us to analyze the PU.1 expression in samples from CML patients at diagnosis and CML patients treated either with IFN-α or with imatinib or both. PU.1 mRNA expression was analyzed by real-time RT-PCR. Relative PU.1 mRNA expression was found to be significantly lower in CML samples at diagnosis than in samples from healthy donors used as reference values ($p<0.05$) (Fig. 2). Interestingly, PU.1 expression recovered in patients upon treatment with IFN-α or with imatinib, reaching levels clearly higher than in samples of patients at diagnosis ($p<0.001$ for both treatments) (Fig. 2).

*3.3. PU.1 expression in serial samples from CML patients at diagnosis and post-treatment*

Whereas the former results show a difference in PU.1 expression between untreated and treated groups of patients, they do not formally demonstrate that in a particular patient the levels of PU.1 increase in response to treatment. Thus we extended our study to serial samples from CML patients at diagnosis and at different times after treatment with IFN-α (16 samples) and upon imatinib treatment (18 samples). All IFN-α treated patients were at least in

hematological remission at the time of sampling although, as expected, the expression of BCR-ABL was detectable by nested RT-PCR in most of the cases. The PU.1 mRNA expression, analyzed by semiquantitative RT-PCR, on samples from the CML patients treated with IFN-α is shown in Fig. 3a. In most of the cases (13/16) PU.1 expression was very low or undetectable at diagnosis (Fig. 3a) and increased upon treatment to similar or higher levels than healthy donors used as reference values (Fig. 3b). These findings were further confirmed and validated by real-time RT-PCR (Fig. 3c).

As imatinib is today the first-line drug in CML treatment, we also analyzed PU.1 expression in serial samples from 18 CML patients at diagnosis and after imatinib treatment. The results showed that, in the same patient, PU.1 expression was low in samples at diagnosis and clearly increased following imatinib treatment (Fig. 3d). BCR-ABL mRNA quantification and cytogenetic analysis at the time of sampling indicated that, upon imatinib treatment, 67% of the patients had reached at least major molecular response (12/18), 22% complete cytogenetic response (4/18) and 11% obtained hematological response (2/18). Although no correlation could be found between PU.1 up-regulation and the remission status, PU.1 levels were consistently lower at diagnosis than upon treatment.

*3.4. Reduced PU.1 expression is not found in other myeloproliferative diseases*

In order to ascertain whether the low PU.1 expression was specific to CML patients or could also be found in other myeloproliferative syndromes, we analyzed PU.1 expression levels in patients with polycythemia vera (PV) and essential thrombocytemia (ET) by semiquantitative RT-PCR (Fig. 4a). The results showed similar PU.1 expression levels in samples from PV, ET and comparable to those found in healthy donors. No correlation between the status of JAK2 V617F mutation and PU.1 expression in PV or TE samples was found. The quantification of the relative PU.1 expression showed significant differences ($p<0.05$) only in CML at diagnosis, where PU.1 levels were dramatically reduced in comparison with the other myeloproliferative syndromes and healthy donors (Fig. 4b).

*3.5. PU.1 expression upon ectopic expression or inhibition of BCR-ABL*

The former results could be explained if PU.1 is repressed by active BCR-ABL, which is only present in untreated CML cells. To test this possibility we generated BCR-ABL transfectants in murine myeloid 32D cells (which do not express endogenous BCR-ABL). However, no changes in PU.1 expression were observed in two 32D sublines with enforced expression of BCR-ABL, achieved by two different BCR-ABL expression vectors (Fig. 5a), as compared to the empty vector-transfected subline. This is in agreement with a previous report showing that up-regulation of PU.1 by G-CSF in 32D cells was not affected by BCR-ABL expression [21].

In a complementary approach to explore whether BCR-ABL directly represses PU.1 expression, we assayed PU.1 expression after silencing the BCR-ABL gene in K562 cells. K562 cells were transfected with different amounts of b3a2-1 siRNAs directed against the fusion site b3a2 of BCR-ABL present in K562 cells. siRNA reduced BCR-ABL mRNA levels in a dose-dependent manner up to 70-80% as compared to cells transfected with a control scrambled siRNA (Fig. 5b-top panel), which is in agreement with previous reports [16]. The b3a2-1 siRNA, but not the scrambled siRNA, inhibited the proliferation of K562 cells (not shown). The results showed that PU.1 expression levels did not increase upon down-regulation of BCR-ABL in K562 (Fig. 5b-bottom panel). On the contrary, we detected a reduction of PU.1 of around 50%, although the extent of PU.1 repression did not paralleled that of BCR-ABL, which experimented a higher repression with the specific siRNAs.

Taken altogether, these results indicate that the activity of the BCR-ABL oncoprotein is not directly responsible for the reduced level of PU.1 mRNA seen in CML patients.

## 4. Discussion

Here we describe three novel findings: i) the expression of PU.1 is severely impaired in patients with CML, ii) PU.1 suppression is abrogated following IFN-α or imatinib therapy in CML patients, iii) these effects are not found in patients with other myeloproliferative diseases.

The PU.1 transcription factor is a crucial regulator of hematopoiesis and loss of PU.1 function has been found related to human and experimental AML (reviewed in [10-12]).

Inactivating mutations in the PU.1 gene have been found in radiation-induced murine myeloid leukemias [22]. Furthermore, the graded reduction of PU.1 can induce AML in mice [23] and PU.1-deficient adult mice developed myeloid leukemia [24]. Several leukemogenic proteins such as AML1-ETO [25], the mutant FLT3-ITD receptor [26] and PML-RARα [27] have been described as interfering with the PU.1 function. Taken together these data suggest that PU.1 has tumor suppressor activity in myeloid cells [12].

Our results in CML patients show that PU.1 expression is low in samples corresponding to leukemic cells at diagnosis while it increases up to normal levels after treatment with drugs that led to the re-establishment of normal hematopoiesis, independently of their mechanism of action. The clinical responses of CML patients towards IFN-α or imatinib are different. Imatinib induces a rapid response, as a consequence of its activity against differentiated CML progenitors, while IFN-α effects are slow but durable, since its activity seems to be directed at the CML stem cells [28]. In contrast to IFN-α, we found that imatinib did not upregulate PU.1 in K562 cells. However, K562 is a clonal cell line derived from blast crisis CML and thus it cannot be compared to the heterogeneous cell population from CML patients in chronic phase.

We found no differences in PU.1 expression in patients treated only with IFN-α, only with imatinib or with both IFN-α and imatinib. In spite that PU.1 levels were consistently lower at diagnosis than upon treatment, no correlation could be found between PU.1 up-regulation and the levels of BCR-ABL. This suggests that PU.1 up-regulation observed in treated CML is not a direct consequence of the inhibition of BCR-ABL activity, but a result of the suppression of leukemic cells and the concomitant recovery of normal hematopoiesis. This hypothesis is further supported because: i) imatinib did not upregulate PU.1 in K562 cells, ii) silencing of BCR-ABL by siRNA in K562 cells did not induce PU.1, and iii) enforced expression of BCR-ABL did not repress PU.1 in murine myeloid cells. Our results are consistent with the findings recently reported in promyelocytic leukemia, where PU.1 is upregulated after treatment of promyelocytic leukemia with ATRA [27].

PU.1 gene expression and protein function are tightly controlled by a complex network of transcription factors (reviewed in [9]). Although the precise mechanisms remain unknown, there are several potential mechanisms that could explain the suppression of PU.1 in CML

patients: i) transcriptional control of PU.1 expression is mainly mediated by the upstream regulatory element (URE). For example, AML1 (Runx1) regulates PU.1 through the URE [29] and the leukemogenic oncoprotein AML1-ETO inhibits PU.1 [25]. Thus, a similar scenery could be operative in CML, ii) PU.1 protein function is regulated by its interaction with transcription factors such as ICSBP (Interferon Consensus Sequence Binding Protein). Interestingly, downregulation of ICSBP was also found in patients with CML, and this reduction could be reversed by treatment with IFN-α (reviewed in [3]), iii) JunB is a critical downstream effector of PU.1 [30]. Downregulation of JunB has been reported in CML patients and inactivation of JunB seems important for CML development [3], and iv) PU.1 gene might be silenced epigenetically in CML, eg, by methylation of regulatory elements as it has been reported in human myeloma cells [31].

Notably, the reduced PU.1 expression in patients of CML in chronic phase at diagnosis was not found in patients with PV or ET, suggesting that the inhibited PU.1 expression is not an effect common to all myeloproliferative disorders –on the contrary it appears to be specific to CML. Although the analysis of BCR-ABL by quantitative PCR represents nowadays an excellent maker for minimal-residual-disease studies, we propose that PU.1 could be used as an additional and complementary marker of the response to the treatment of the CML.

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**Figure Legends**

Fig. 1. PU.1 mRNA expression in K562 cells upon treatment with IFN-α or Imatinib. Northern hybridization analysis of K562 cells, treated with 2000 U/ml human interferon-α2a (IFN), 1 μM Imatinib mesylate, or first with IFN-α and then with 1 μM Imatinib mesylate (IFN+Imatinib) for the indicated days. The same membrane was consecutively hybridized with the PU.1 and BCR-ABL probes. The lower panel shows the 28S rRNA stained with ethidium bromide to assess RNA loading and integrity. A representative experiment out of three with essentially the same result is shown. The ratios of the intensity of the PU.1 and BCR-ABL mRNA bands over the intensity of the corresponding 28S rRNA bands were determined and expressed as -fold change relative to the untreated cells (designated as 1).

Fig. 2. PU.1 mRNA expression in samples from CML patients. Analysis by real-time RT-PCR of PU.1 mRNA expression in samples from normal healthy donors and CML patients at diagnosis and following IFN-α or Imatinib treatments. Data refer to PU.1 expression values normalized to the levels of RPS14 in each case. Mean value of healthy donors was set at 1. Bars represent mean ± SEM of the indicated number of samples.

Fig. 3. PU.1 mRNA expression in serial samples from CML patients at diagnosis and after treatment with IFN-α or Imatinib. (a) Analysis by semiquantitative RT-PCR of PU.1 mRNA expression in serial samples from patients at diagnosis and after treatment with IFN-α. For each patient (1 to 16) the sample number 1 corresponds to diagnosis, numbers 2 to early (4 to 12 months) IFN-α treatment and numbers 3 or 4 to late (13 to 24 months) IFN-α treatment. The expression of PU.1 was compared to the reference (RPS14 gene). PCR protocols were standardized to ensure that PCR-amplifications were in the exponential phase (26 cycles for PU.1 and 22 cycles for RPS14). The experiments were repeated at least three times with similar results. (b) Semiquantitative RT-PCR of PU.1 mRNA expression in samples from 11 healthy donors. The expression of PU.1 was compared to the reference (RPS14 gene). (c) Analysis by real-time RT-PCR of PU.1 mRNA expression in serial samples from 13 CML

patients at diagnosis and after early (4 to 12 months) and late (13 to 24 months) IFN-α treatment. Data refer to PU.1 expression values normalized to the levels of RPS14. Value at diagnosis was set at 1. Different symbols indicate different patients. (d) Analysis by real-time RT-PCR of PU.1 mRNA expression in serial samples from 18 CML patients at diagnosis and after imatinib treatment (6 to 12 months). Data were analyzed as described in (c).

Fig. 4. PU.1 mRNA expression in samples from patients with polycythemia vera and essential thrombocythemia. (a) Analysis by semiquantitative RT-PCR of PU.1 mRNA expression in samples from 15 patients with polycythemia vera and 18 patients with essential thrombocythemia. W, water (instead of RNA), HD, healthy donor. The expression of PU.1 was compared to the expression of RPS14 gene. (b) Quantification of the PU.1 mRNA expression results on samples from healthy donors (HD), patients with polycythemia vera (PV), essential thrombocythemia (ET) and CML at diagnosis (CML). The relative expression of PU.1 refers to the ratio of the intensity of the PU.1 bands over the intensity of the corresponding RPS14 bands after densitometric analysis. Mean value of healthy donors was set at 1. Bars represent mean ± SEM of the indicated number of samples.

Fig. 5. PU.1 mRNA expression upon ectopic expression or down-regulation of BCR-ABL. (a) Northern hybridization analysis of murine myeloid 32D cells stably transfected with the pSRα-bcr-abl, pSRα (vector) and pM5Neo-ts-bcr-abl constructs. Cells expressing ts-bcr-abl were incubated at 32ºC (permissive temperature) for BCR-ABL kinase activation. The same membrane was consecutively hybridized with the PU.1 and BCR-ABL probes. The lower panel shows the 28S rRNA stained with ethidium bromide to assess RNA loading and integrity. (b) Analysis by real-time RT-PCR of BCR-ABL (top panel) and PU.1 (bottom panel) mRNA levels in K562 cells transfected with the indicated amounts of b3a2-1 BCR-ABL siRNA or scrambled siRNA. Data refer to BCR-ABL or PU.1 expression values normalized to the levels of RPS14 or GUS and expressed as percentage of the values obtained with the scrambled siRNA.

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Figure(s)

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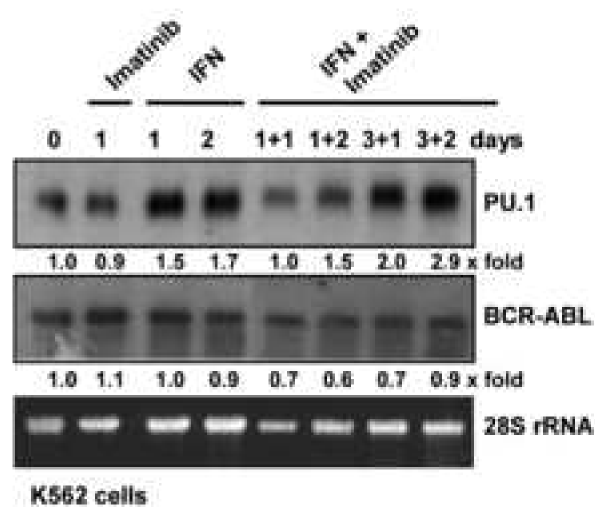


Figure 1

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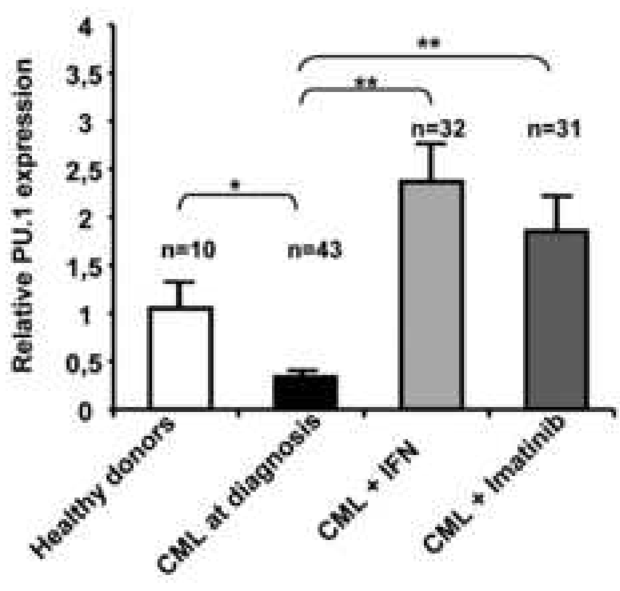


Figure 2

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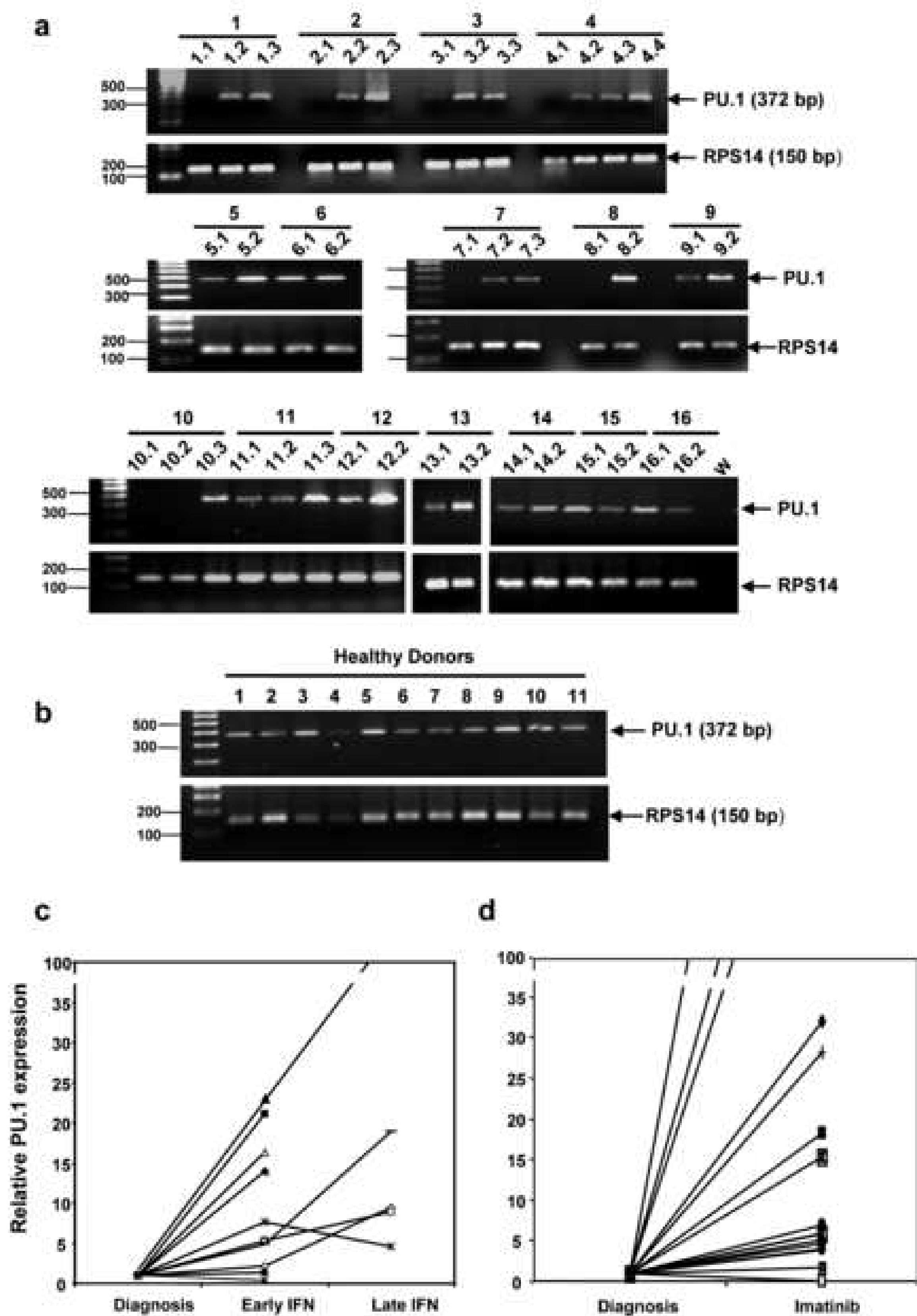


Figure 3

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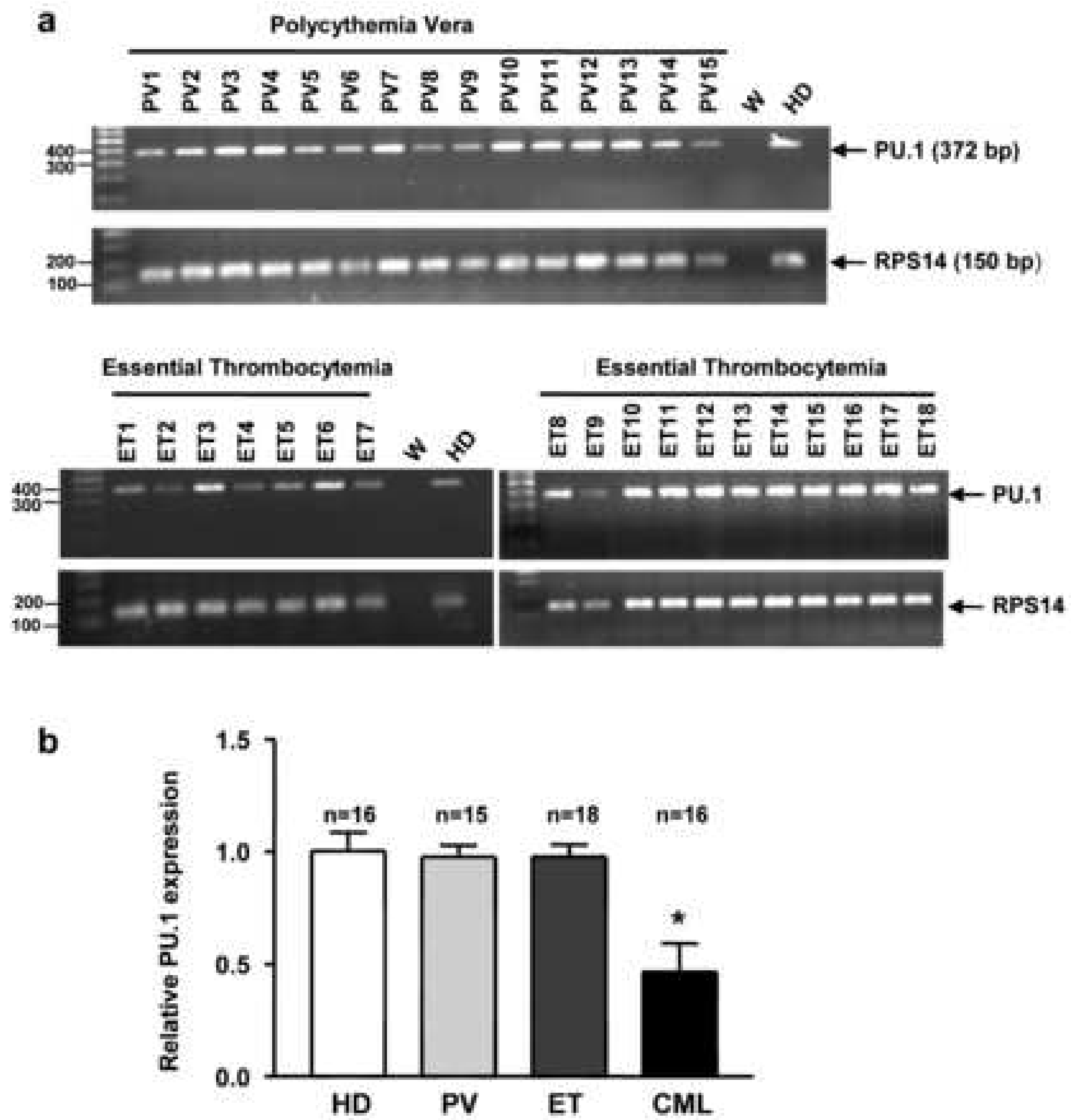


Figure 4

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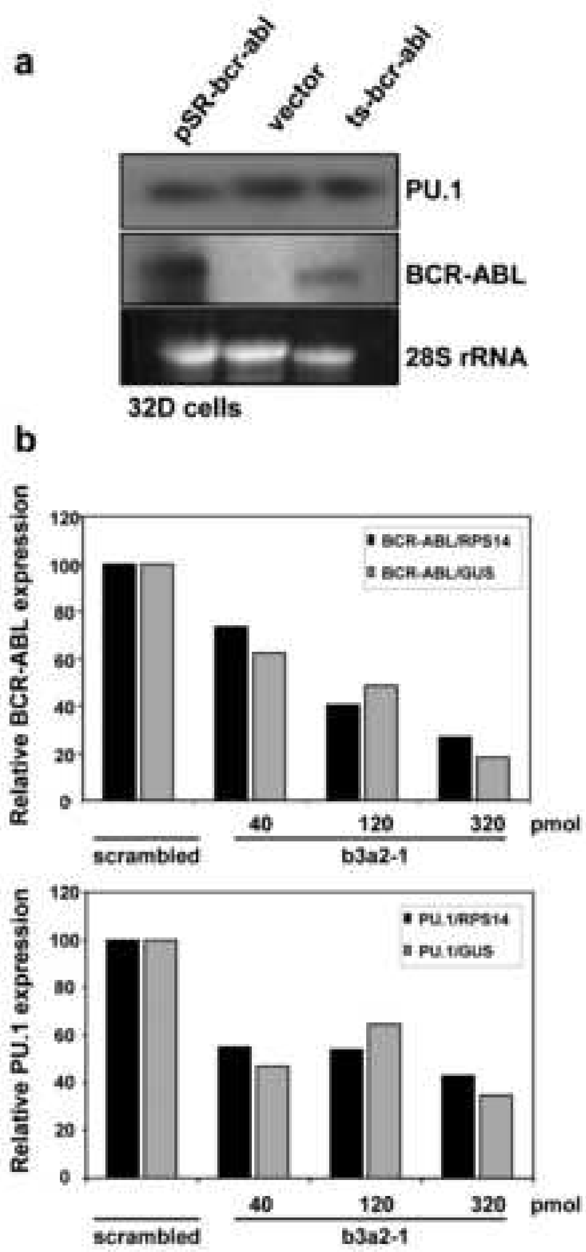


Figure 5

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