(qChIP) assay was performed to directly measure the DNA-binding function.[15] This assay confirmed an absence of DNA-binding affinity to the DNA sequences derived from human PC-HC in the mutated IKAROS protein (Supplementary Figure S3b). On the basis of previous analyses on specific single amino acid substitutions in IKAROS, we are aware that only six amino acid regions (154, 159, 162, 180, 184 and 188) within the second or third N-terminal zinc finger are critical sites for both high-affinity DNA binding and PC-HC localization with regard to IKAROS function.[3] A previous structural study also indicated the importance of amino acid regions and mentioned that the N159 region involves residues for pericentromeric targeting and protein–DNA interactions according to homology models. Consistent with these findings, the IKAROS mutation in our patient was highly damaging to the protein function.

The IKAROS mutated sites and accompanying somatic mutations in leukemic cells may contribute to the differences in clinical manifestations, as well as leukemic phenotypes, as was observed in our patient and previously reported patients. In summary, the present case provides the first definitive evidence on the ability of an IKAROS heterozygous mutation to cause both immunodeficiency and *NOTCH1*-driven T-ALL in humans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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OPEN

JAK2-V617F activates β1-integrin-mediated adhesion of granulocytes to vascular cell adhesion molecule 1

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Chronic myeloproliferative neoplasia (CMN) represents a group of clonal disorders characterized by excessive proliferation of one or more of the myeloid, erythroid or megakaryocytic cell lineages. CMN includes several subentities such as polycythemia vera (PV), essential thrombocytosis (ET), primary myelofibrosis (PMF) and others. An activating somatic point mutation of the *JAK2* gene (JAK2-V617F) was found in the majority (95%) of PV patients and

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in 50% of ET and PMF patients, respectively.[1–3] The clinical course of CMN patients is characterized by increased risk of thrombosis, splenomegaly and an inflammatory response syndrome.[4,5] Clinical studies using JAK kinase inhibitors have shown considerable improvement of splenomegaly, constitutive symptoms and overall survival.[4–6] JAK2-V617F activates proliferative and anti-apoptotic signaling pathways, thereby driving clonal proliferation of myeloid cells. In addition, JAK2-V617F was reported to activate Lu/BCAM-mediated erythrocyte adhesion through Rap1/Akt signaling in PV, a mechanism that may explain the increased risk of thrombosis in PV patients.[7] However, in platelets of ET patients, impairment of the PI3 kinase/Rap1/integrin $\alpha_{IIb}\beta_3$ pathway was demonstrated and was unrelated to the mutation status of JAK2.[8] In leukocytes of CMN patients, the effect of JAK2-V671F on integrin function and on adhesion is unknown.

Therefore, the aim of this study was to explore whether JAK2-V617F activates β1 integrin-mediated adhesion of granulocytes in CMN. Besides β2 and β3, the β1 integrin chain is expressed on human granulocytes, forming the major heterodimer VLA-4 (very late antigen-4) in combination with α4 integrin.[9,10] Integrins play essential roles in leukocyte activation by mediating rolling and firm adhesion to the endothelium, transmigration and trafficking into tissues.[9,10] In non-stimulated leukocytes, VLA-4 is expressed in a closed, inactive conformation. Upon external stimuli (for example, chemokines as SDF1α) VLA-4 rapidly undergoes a conformational change, thereby enhancing both the affinity and the avidity for its natural ligand, the VCAM1 molecule.

We first tested adhesion of granulocytes isolated from peripheral blood of JAK2-V617F-positive CMN patients on VCAM1-coated surface. Despite similar levels of β1 integrin expression as compared with healthy controls (Supplementary Figure 1a), primary JAK2-V617F-positive granulocytes showed an overall increase in adhesion to immobilized VCAM1 (Figure 1a). To test for an involvement of JAK2-V617F in activation of integrins, we assessed binding of soluble recombinant VCAM1/Fc (sVCAM1) to the granulocytes. Here the granulocytes of CMN patients demonstrated a significant increase in sVCAM1 binding as compared with healthy donors (Figure 1b, right). As several JAK2-mutated CMN samples showed values comparable to healthy donor controls, we assessed for a potential influence of mutational burden on the phenotype. Here sVCAM1 binding closely correlated with the JAK2-V617F allelic ratio, which is highly variable depending on stage and clinical CMN subtype (Figure 1b, left).[11] To further study JAK2-V617F-mediated β1 integrin activation in more detail, 32D myeloid progenitor cells ectopically expressing Epo-R plus either JAK2-WT or JAK2-V617F were generated.[1–3,12] As for the patient granulocytes, 32D JAK2-V617F cells displayed a strong increase in static adhesion to immobilized VCAM1 (Figure 1c). The enhanced adhesion was reversed by inhibition of JAK2 kinase activity (Figure 1c) and not due to altered expression levels of the β1 integrin (CD29) (Supplementary Figure 1b). The adhesion assay employed here was performed using (human-)Fc-tagged and Fc-free VCAM1 in parallel. No differences could be observed, indicating that the human-Fc-tag does not result in unspecific binding on murine cells (data not shown). In the sVCAM1 binding assay, JAK2-V617F led to a sixfold increase in soluble ligand binding as compared with JAK2-WT cells (Supplementary Figure 1c). Pharmacological inhibition of JAK2-V617F downregulated sVCAM1 binding in a time-dependent fashion (Supplementary Figure 1d, and data not shown), without affecting β1 integrin surface expression and cell viability (data not shown).

Next, we investigated a potential change to the open, high-affinity conformation of β1 integrin chains by using the high-affinity conformation-specific antibody 9EG7. Supplementary Figure 2a shows a significant increase in binding of 9EG7 in 32D JAK2-V617F cells, indicating a change from the bent to the open conformation of the β1 integrin chain.

Considering the potential of JAK2-V617F to induce production of chemokines/cytokines, which in turn may cause increased ligand binding of integrins, we co-cultured 32D JAK2-WT and JAK2-V617F cells. The presence of the mutant had no apparent effect on sVCAM1 binding in JAK2-WT cells, indicating a cell intrinsic effect of JAK2-V617F on integrin activation (Supplementary Figure 2b).

As the small GTPase Rap1 has been reported to play a role in β1 integrin-mediated adhesion,[13] we employed pull-down experiments of activated Rap1. In 32D JAK2-V617F cells, a strong increase in Rap1 activation was observed (Figure 2a, left), which was suppressed following pharmacological inhibition of JAK2

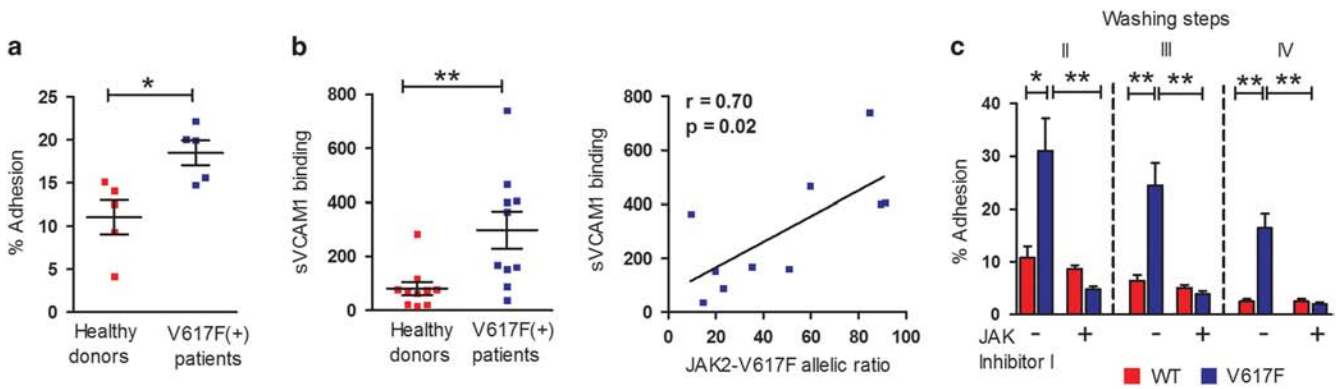

Figure 1. Peripheral blood was obtained from healthy volunteers and JAK2-V617F-positive patients (PV, ET and PMF) who were untreated with JAK inhibitors after informed consent and upon approval by the local ethics committee (protocol no MD115108). Mononuclear cells were removed by Ficoll-Paque density gradient centrifugation, followed by lysis of erythrocytes with BD FACS Lysing solution (BD Biosciences, Franklin Lake, NJ, USA). (**a**) Static adhesion assay of primary granulocytes from healthy donors ($n = 5$) and JAK2-V617F-positive patients ($n = 5$) on immobilized VCAM1 (R&D Systems, McKinley, MN, USA, ADP5-050) was performed as described before for ICAM1.[15] (**b**) sVCAM1 binding assay using soluble VCAM1/Fc (R&D Systems, 862-VC) in primary granulocytes from healthy donors ($n = 10$) and JAK2-V617F-positive patients ($n = 10$) as described previously for ICAM1[15] (right). Correlation of sVCAM1 binding of granulocytes isolated from JAK2-V617F-positive patients with JAK2-V617F allelic burden of peripheral blood cells (left). (**c**) Static adhesion of 32D JAK2-WT (WT) and JAK2-V617F (V617F) cells on immobilized VCAM1 (R&D Systems, 862-VC) in the absence and presence of JAK inhibitor I treatment (shown are results obtained upon subsequent washing steps II, III and IV) as described before for ICAM1.[15] Cells were treated either with DMSO (−) or with JAK inhibitor I (200 nM) (+) for 16 h. Three independent experiments were performed. $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$ (unpaired, two-tailed Student's *t*-test).

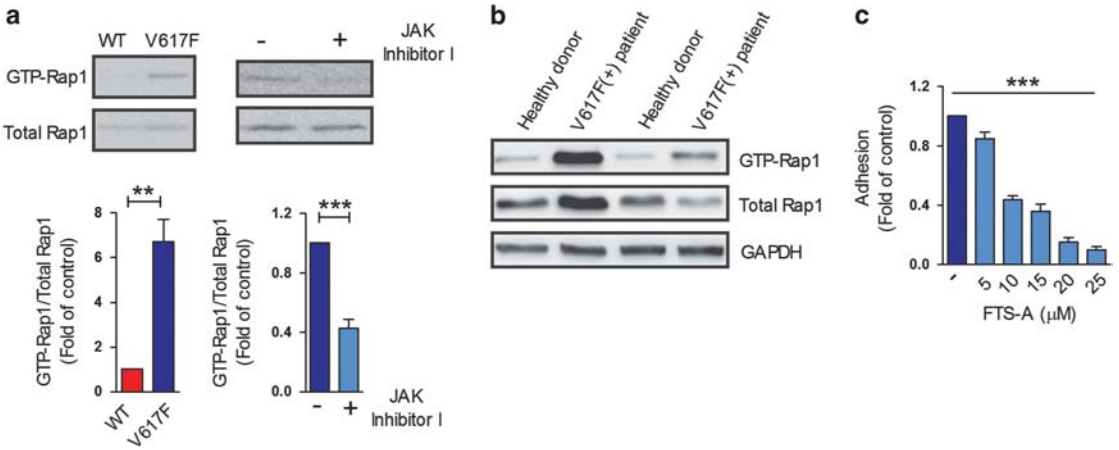

Figure 2. (**a**) Rap1 activation in 32D cells expressing either JAK2-WT or JAK2-V617F detected by pull-down of GTP-bound Rap1 as indicated by the manufacturer (Thermo Scientific, Waltham, MA, USA) (right). Inhibition of Rap1 activation by JAK inhibitor I treatment (0.5 μM; 4 h) in 32D JAK2-V617F cells; (−) indicates DMSO control (left). Lower panels show quantitative analysis of Rap1 activation. (**b**) Rap1 activation in primary human granulocytes isolated from JAK2-V617F-positive patients. Immunoblots show two independent cases of increased Rap1 activation in primary granulocytes isolated from healthy donors and JAK2-V617F-positive patients, respectively. (**c**) Effect of Rap1 inhibitor FTS-A treatment (16 h) on static adhesion of 32D JAK2-V617F cells on immobilized VCAM1; (−) indicates DMSO control. Three independent experiments were performed. $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$ (unpaired, two-tailed Student's *t*-test).

kinase (Figure 2a, right). Prominent Rap1 activation was also observed in primary JAK2-V617F-positive granulocytes (Figure 2b). Employing the Rap1 inhibitor FTS-A,[14] we observed dose-dependent reduction in adhesion without apparent influence on cell death (Figure 2c, and data not shown). Although shRNA-targeting of Rap1 was only moderately effective in our experiments, the reduction in adhesive capacity correlated with the efficacy of RNAi-mediated inactivation of Rap1 (Supplementary Figures 2c and d). Thus, in granulocytes and 32D myeloid progenitors, Rap1 is activated by JAK2-V617F as described for erythrocytes of PV patients[7] and may play an important role in JAK2-V617F-activated β1 integrin adhesion.

Together, our findings indicate a novel role for JAK2-V617F in activation of β1 integrins and enhanced adhesion of granulocytes and 32D myeloid progenitors to VCAM1-coated surfaces. As VCAM1 is abundantly expressed on endothelial cells, this newly identified characteristic may play a critical role in abnormal interaction of granulocytes with the endothelium in JAK2-V617F-positive CMN.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

NG, BE, TMS and FS performed the experiments, analyzed the data and contributed to writing of the manuscript. DW provided essential materials. NG, BE, SK, BS, FHH and TF designed the research, analyzed the data and wrote the manuscript.

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OPEN

Clinical utility of gene panel-based testing for hereditary myelodysplastic syndrome/acute leukemia predisposition syndromes

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Myelodysplastic syndrome (MDS) and acute leukemia (AL) are clinically diverse and genetically heterogeneous groups of hematological malignancies. Hereditary forms of MDS/AL were considered rare, but have been increasingly recognized in recent years.[1–3] Pathogenic variants in a single gene can predispose carriers to an increased lifetime risk of primary MDS and/or AL. Hereditary MDS/AL can occur in the context of familial MDS/AL that have MDS/AL as the principal clinical feature, or arise from inherited bone marrow failure syndromes (IBMFS), such as Fanconi anemia (FA), dyskeratosis congenita/telomerase biology disorders (TBD), Diamond–Blackfan anemia and severe congenital neutropenia.[1,4] Within the past decade, nearly a dozen adult-onset familial MDS/AL syndromes have been defined. These include thrombocytopenia with associated myeloid malignancies caused by germ line mutations in *RUNX1*, *ANKRD26* and *ETV6*; *GATA2*-associated syndromes (Emberger syndrome; MonoMAC syndrome; immunodeficiency); familial MDS and acute myeloid leukemia caused by mutations in *CEBPA*, *DDX41* and *SRP72*; and TBD due to mutations in *TERT* or *TERC*.[2] Although the majority of patients with classic IBMFS are diagnosed in childhood, some patients have no or only subtle extra hematopoietic manifestations and may present in adulthood with MDS or AL.[2,5]

A few studies have shown that genetic abnormalities exist in 11–37% of families with hereditary MDS/AL.[6–10] The recognition of patients with a hereditary predisposition to MDS/AL is particularly important for hematopoietic stem cell transplantation donor selection, pre-transplant planning and post-transplant care.[11] The correct clinical diagnosis is also important to avoid the risk of life-threatening toxicities with inappropriate therapy, for long-term cancer surveillance and prognosis, and for identification of at-risk or affected family members.[5] Clinical guidelines for the care of MDS/AL predispositions are now emerging.[1–3] To reflect the increasing recognition and clinical awareness of hereditary hematological malignancies, the World Health Organization (WHO) has included germ line predisposition to myeloid malignancies in the forthcoming WHO classification guidelines.[12] However, the application of genetic testing on hereditary MDS/AL in clinical practice has never been systematically reported.

Given the phenotypic overlap of the known hereditary MDS/AL predisposition syndromes, a gene panel-based approach to genetic testing is preferred, as it offers the ability to analyze multiple genes simultaneously and cost-effectively. Our College of American Pathologists certified and Clinical Laboratory Improvement Amendments-licensed laboratory is the first to provide comprehensive clinical testing via a combination of multiple next-generation sequencing and array comparative genomic hybridization-based panel tests to evaluate genetic predisposition to MDS/AL. Multiple gene panels are available, including a familial MDS/AL panel, IBMFS panel, and panels for FA, dyskeratosis congenita/TBD, Diamond–Blackfan anemia and severe congenital neutropenia (Table 1 and Supplementary Table 1). Cultured skin fibroblasts are the preferred tissue for germ line mutation testing in patients with hematological malignancy as they provide higher quality and quantity of DNA compared to hair roots and nail clippings. The targeted next-generation sequencing was performed using Illumina technology (San Diego, CA, USA). The high-density exon-targeted array comparative genomic hybridization is custom designed using Agilent Technology (Santa Clara, CA, USA). The variant interpretation follows the standards and guidelines for the interpretation of sequence variants from the American College of Medical Genetics and Genomics.[13]

A total of 197 patients (110 females and 87 males) were referred to our laboratory for MDS/AL predisposition gene panel testing from October 2014 to June 2016. The patient age at the time of testing ranged from 1 to 84 years in 65 children and 132 adults. Seventy-eight patients were referred for testing for the familial MDS/AL panel, 86 for the IBMFS panel, 15 for the dyskeratosis congenita/TBD panel and 12 for multiple panel testing. In addition, a total of six patients were referred for specific testing of FA, Diamond–Blackfan anemia and severe congenital neutropenia (Table 1).

The overall molecular diagnostic rate was 19% (37 of 197) with 15% in children and 21% in adults (Table 1). Pathogenic/likely pathogenic variants were identified in 14 (18%) patients tested on the familial MDS/AL panel, 13 (16%) patients tested on the IBMFS panel, 5 (33%) patients tested on the dyskeratosis

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