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Description of a novel JAK3-P132A mutation in Acute Megakaryoblastic Leukemia and demonstration of previously reported JAK3 mutations in normal subjects

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ABSTRACT

Gain-of-function (GOF) mutations of Janus kinase 2 (JAK2) are frequently seen in myeloproliferative disorders (MPD), meanwhile JAK3 activating substitutions were found in few megakaryocytic cell lines and in primary myeloid leukemia (AMKL). Here, we sought to discover novel leukemogenetic mutations in *de novo* acute myeloid leukemia of Non-Down Syndrome (N-DS) patients by DNA sequencing. A total of 191 normal Caucasian individuals were studied to define single nucleotide polymorphisms (SNP) within the JH2 and JH6 domains. Although known activating substitutions were observed in rare AML [V722I (2/134) or P132T (1/119)], all samples were wild-type (WT) for the oncogenic A572V (119/119). Interestingly, a novel homozygous mutation (P132A) was discovered in a AMKL patient and *in vivo* studies demonstrated that its ectopic expression was oncogenic in a mouse xenotransplantation model. This study defines a novel JAK3 mutation among N-DS AML patients and demonstrates that normal individual can also display germline JAK3 substitutions, previously proven to have oncogenic properties, *in vitro* and *in vivo*. The discovery of these substitutions in normal donors encourages future studies to define new risk factors among MPD patients.

INTRODUCTION

Acute myeloid leukemia (AML) is relatively rare heterogeneous group of processes characterized by uncontrolled proliferation of clonal neoplastic hematopoietic myeloid elements, which accumulate in the bone marrow, peripheral blood, and often in other lymphoid and non-lymphoid organs, impairing the production of normal blood cells. Among AML, several groups can be distinguished according to cytological, cytochemical and immunophenotyping/differentiation stages [1], or by virtue of unique chromosomal aberrations [2]. Nowadays, it is well established that the molecular stratification of AML patients provides critical information on the clinical progression and evolution, allowing a more objective/quantitative molecular monitoring of the tumor burden, with relevant prediction of clinical relapse rates and/or the delivery of targeted therapies [3].

Like other neoplastic processes, the development of AML is associated with the overtime accumulation of acquired genetic alterations and epigenetic changes. Numerous recurrent structural and numeric cytogenetic aberrations have been identified in unique subset of AML patients [4,5].

The pathogenetic signaling pathways involved in the maintenance of the neoplastic phenotype of AML have in part been elucidated. Critical player appears to be the signal transducer and activator of transcription 5 (STAT5), that is constitutively phosphorylated in at least 70% of AML patients [6], [7], suggesting the presence of deregulated tyrosine kinase (TK) signaling and/or mutated tyrosine kinases. Activating TK mutations have indeed been discovered in a subset of AML samples, as point mutations within the coding region of the c-KIT (5%) gene, mutations or internal tandem duplications of FLT3 (30%), and rare somatic mutations of JAK2, JAK3 and PDGFR. Nevertheless, in many AML it is unclear which molecular mechanisms are responsible for STAT5 phosphorylation, suggesting that other defects may exist.

Here, we have searched for the presence of novel putative JAK3 mutations in a well-characterized panel of AML from adult Caucasian individuals. A group of normal control individuals was investigated to underline the presence of JAK3 SNP within this ethnic population. This approach has identified a novel homozygous JAK3 point mutation in position 132 in a single case of AMKL,

which can sustain the growth of BaF3 cells in absence of exogenous IL3, *in vivo*. These findings further confirm that JAK3 mutations may have a tumorigenic role in a small subset of AML patients. Moreover, we have discovered that many of the oncogenic JAK3 mutations seen in Down Syndrome (DS) AMKL can be detected in N-DS AML and in normal individuals as well. These later finding suggests that these substitutions may represent SNP, which may be linked to a higher risk of myeloid disorders. The molecular characterization of JAK3 further stratifies AML patients and might allow the design of novel tailored therapies..

MATERIAL AND METHODS

Clinical Samples

A panel of 134 well-characterized cases of untreated Acute Myeloid Leukemia (AML 1 to 5), 28 AMKL and 6 AML-M6 were selected among the Hematopathological files of the Pathology Laboratories of the University of Turin, Brescia and the San Raffaele Scientific Institute. Age of AML patients ranged from 23 to 94 years, with a mean of 60 years. Diagnoses were assigned according to the WHO classification and/or the FAB classification. After clinical diagnosis, unutilized fresh peripheral blood and/or bone marrow aspirate samples were used for research. Archived paraffin-embedded materials were utilized for DNA extraction in selected cases (28 AMKL samples). A total of 191 peripheral blood mononuclear cell collections from normal blood donors were included as controls (Blood Bank Collection, Turin).

A general informed consent was obtained according to the local ethical committees guidelines of each participating Institution. Samples were numerically identified maintaining patients' anonymity.

DNA And RNA Extraction

Formalin-fixed, paraffin-embedded tissue blocks were also used for the study. Formalin-fixed, paraffin-embedded samples were de-paraffinized with a xylene-ethanol protocol [8]. Briefly, tissue specimens were incubated overnight at 55°C in lysis buffer with Proteinase K (20 mg/mL), and

DNA was obtained by phenolisopropanol extraction. Total RNA was extracted from cells or tissues and used as template for the cDNA transcription accordingly to the BIOMED guidelines [8,9].

Polymerase Chain Reaction Amplification And DNA Sequencing Analysis

The entire JAK3 JH2 domain (including A572V and V722I) and the exon 3 of the JH6 domain, spanning the amino acid site P132T, were sequenced. Amplification of JAK3 exons was performed by polymerase chain reaction (PCR) with specific oligonucleatide primers (Table 1 of supplementary material). DNA sequencing was executed using the cDNA [AML (100) and control sample (191)] or the genomic DNA from fixed tissue bone marrow biopsies [AML-7 (28) or AML-6 (6)]. Sequencing reactions were carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and an ABI 310 automated capillary system (Applied Biosystems) (see supplementary methods).

cDNA Constructs And Mutagenesis

The JAK3 cDNA (Clone: HsCD00021445, Harvard Medical School) was subcloned into the pcDNA3 (Invitrogen) expression vector at *EcoR*V site. $JAK3^{A572V}$, $JAK3^{V722I}$ and $JAK3^{P132A}$ mutated (mut) cDNA were generated by PCR-based mutagenesis (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. $JAK3^{WT}$ and mut-JAK3 cDNA were then subcloned (*Hind*III/*Xho*I) into a EGFP retroviral vector (Pallino) [10]. The oligonucleotide primers list used for the JAK3 mutagenesis is available in the Table II of supplementary methods.

Cell Culture And DNA Transfection

Human embryonic kidney (HEK) 293T, NIH3T3 and murine IL-3-dependent BaF3 cells were cultured, respectively, in Dulbecco modified Eagle medium (DMEM) or in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin,. WEHI-conditioned medium (10%) was used as a source of IL-3. Cells were transfected using the Effectene kit, following the manufacture's instructions (Qiagen, Valencia, CA).

For the focus assay, NIH3T3 cells ($2.5x10^5$/100 mm plate) were first transfected (2 µg of total DNA), then selected with G418 (400 µg/ml, pcDNA3 vectors) or puromycin (2 µg/ml, retroviral vectors). Three weeks post transfection, NIH3T3 cells were fixed and stained with crystal violet.

Murine IL-3-dependent BaF3 cells were electroporated using Cell Line Nucleofector Kit V (Amaxa Biosystems, USA) and selected with puromycin (2 µg/mL, 5 days).

For cytokine independent growth assays, JAK3 BaF3 cells were electroporated, then extensively washed with PBS, and subsequently resuspended in complete media in absence of IL-3 (seeded at 10^6/ml in duplicate in 12-well plates). Viable cells were determined by trypan blue or by TMRM staining exclusion [11].

Western Immunoblotting Analysis

BaF3 and electroporated JAK3 BaF3 cells were cultured in RPMI 1640 medium with or without IL-3 (12h). NIH3T3 cells were transiently transfected with the different JAK3 constructs and harvested 48h after transfection. Cells were lysed (4°C for 30 min.) using a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitors (Roche, Mannheim, Germany). Cell lysates were collected by centrifugation at 15,000 x g, and supernatants were analyzed for protein concentration with a Bio-Rad D_c protein assay kit (Bio-Rad Laboratories) and stored at -80° C. Forty micrograms of proteins were run on SDS-PAGE under reducing conditions.

For immunoblotting, proteins were separated by SDS/PAGE, transferred to nitrocellulose and incubated with the specific antibody. Immune complexes were detected with secondary antimouse or antirabbit peroxidase-conjugated antibodies (Amersham) and visualized by enhanced chemiluminescence reagent (Amersham) according to the manufacturer's.

The following antibodies were used: anti-ALK (1:4000; Zymed, Seattle, WA), monoclonal anti-phospho-Tyr (PY20; 1:1000; Transduction Laboratories, Lexington, KY, USA), anti-phospho-Stat3 (Y705) (1:1000; Cell Signaling Technology), anti-Stat3 (1:1000; Cell Signaling Technology), anti-phospho-Erk1/2 (1:500; Cell Signaling Technology), anti-Erk1/2 (1:1000; Santa Cruz

Biotechnology), anti-phospho-AKT (1:1000, Cell Signaling Technology), anti-AKT (1:1000; Cell Signaling Technology), anti-phospho-Shc (Y317) (1:1000; Cell Signaling Technology), anti-Shc (1:1000; Santa Cruz Biotechnology), anti-Stat5 (1:1000 Cell Signaling Technology), anti-phospho-Stat5 (1:1000 Cell Signaling Technology), anti-phospho-JAK3 (1:1000, Santa Cruz Biotechnology), anti-JAK3 (1:1000, Santa Cruz Biotechnology), anti-actin (1:1000; Chemicon).

***In Vivo* Tumor Growth Analysis**

Immune-compromised mice (FoxChase, C.B-17) were injected s.c. with neomycin or puromycin selected (400 µg/ml or 2 µg/mL, for 7 days, respectively) JAK3 transfected NIH3T3 cells (1 x 10^6 cells/0.2ml PBS). Puromycin selected BaF3 cells ($4x10^6$/0.2 ml PBS) were inoculated s.c. in both flanks of NOD/Scid mice. Animals were followed overtime and sacrificed at earliest sign of distress. Tumor growth was scored weekly over a period of 9 weeks for FoxChase, C.B-17 and 26 weeks for NOD/Scid mice.

Immunohistochemistry

Immunohistochemical stainings were performed on formalin-fixed, paraffin-embedded tissues. Sections from neutral buffered formalin fixed tissues were stained with hematoxylin and eosin and immunohistochemistry was performed on a semi-automated immunostainer with a primary antibody specific for phospho-JAK3 (1:1000, Santa Cruz Biotechnology) [11].

RESULTS

JAK3 Somatic Mutations In Untreated Adult Primary AML Patients

Previous studies have shown that activating mutations in JAK3 can contribute to the pathogenesis of some AMKL [12]. To discover novel JAK3 mutations and to further study the pathogenetic role of JAK3 in AML, we selected a panel of *de novo* processes comprehensive of all subsets in Non Down Syndrome (N-DS) Caucasian adult patients. Normal DNA samples (191) were used as control.

Since the majority of known JAK3 mutations were described within the JH2 domain and the third exon of the JH6 domain, we first focused on these regions. No mutations in position 572 were seen either in normal as well as in primary AML samples [12]. An oncogenic JAK3^{A572V} substitution was first discovered in a CMK cell line, and two additional activating (gain-of-function GOF) mutations, in position 132aa and 722aa, were detected in primary DS and N-DS AMKL patients. Amino acid 132 and 722 are located within the JAK3 JH6 and in the JH2 pseudokinase domains, respectively (Figure 1A). Here, we have expanded these findings identifying similar substitutions in 3 AML samples [V722I (2/134) or P132T (1/119)] (Figure 1B and 1C). The pathogenetic role of these substitutions, however, remains unclear, and no data are available regarding their possible presence in normal control individuals. Interestingly, the JAK3 sequencing of normal DNA from PBMC has unexpectedly demonstrated the presence of the same substitutions, with a frequency similar to that observed in AML patients, suggesting that they may represent Single Nucleotide Polymorphisms (V722I 5% and P132T 1%) (Table I). Among our AMKL cases, we have however discovered a single case (1 of 28) carrying a novel homozygous mutation at 132aa position, corresponding to a transversion of C to G leading to an amino acid substitution (proline at 132 to alanine, P132A) (Figure 1D). This substitution was not observed within our control group (191), excluding a novel putative SNP.

Transforming Properties Of JAK3 Mutations

To investigate the role of the JAK3/STAT pathway, we examined the transforming properties of different JAK3 substitutions, after transfection into NIH3T3 cells (see "Material and Methods"). Transfected cells were first selected (G418 or puromycin) and then cultured in low fetal calf serum media. Western blotting analysis confirmed the ectopic expression of each mutants and their constitutive phosphorylated status (Figure 2A). Nevertheless, two weeks after selection, a similar number of foci were scored for each JAK3 constructs (Figure 2B) and no synergist effect was observed when individual JAK3 plasmids were co-transfected with oncogenic c-myc or K-Ras12mut constructs (data not shown). Overall these findings suggest that JAK3 mutations in this

context do not display a definitive transforming property. This hypothesis is supported by the lack of any detectable activation of STAT1, STAT5 proteins, known downstream molecules of JAK3 [13], contrary to TEL-JAK fusion [14]. Similar findings were seen when the phosphorylation status of phosphoinositol-3-kinase-Akt, Shc and MAP kinase (p42/44) were studied (Figure 2C).

Since the cellular context may be essential to dissect the oncogenic property of any given oncogene, these original findings do not exclude the oncogenic role of mut-JAK3 proteins. To overcome this putative limitation and to confirm the known transforming property of the $JAK3^{A572V}$, we enforced the mut-JAK3 expression in BaF3 cells, after electroporation. Puromycin selected cells were followed analyzing the EGFP expression of the target cells in the presence or in the absence of IL-3. As expected [12], the ectopic expression of $JAK3A^{572V}$ led to a IL-3-independent growth, whereas $JAK3^{WT}$ positive cells remained dependent on IL-3 for a sustained growth and cell survival (Figure 3A). These findings confirm that the cellular context/model plays a critical role.

The mutation in position 572aa lays within the JH2 domain of JAK3, a region known to negatively regulate the kinase activity of the enzyme [15]. When we analyzed JAK3 expression and its phosphorylation status in $JAK3^{A572V}$ cells, we confirmed that its forced expression was associated with the presence of constitutively phosphorylated JAK3, irrespective of the IL-3 stimulation (Figure 3B). In agreement, the phosphorylation status of STAT5, MAP kinase (p42/44) and in minor extent of the phosphoinositol-3-kinase-Akt signaling pathway were constitutively activated in $JAK3^{A572V}$ cells but not in $JAK3^{WT}$, $JAK3^{AV722I}$ or $JAK3^{P132A}$ BaF3 cells (Figure 3B). As expected, transfected $JAK3^{A572V}$ BaF3 cells could survive and proliferate in the absence of IL3, contrary to $JAK3^{V722I}$ or $JAK3^{P132A}$ cells, which failed to grow and underwent cell apoptosis (Supplementary Figure 1A and data not shown).

$JAK3^{A572V}$ And $JAK3^{P132A}$ Induce Tumor Formation *In Vivo*

Since the oncogenic properties of any given gene may be dissected only in unique/specific models [16-19] and because it is known that the gene dosage plays a critical role in tumorigenesis, not only for canonical tumor suppressor genes [20] but also for classical oncogenes [16,17], we decided to

apply an *in vivo* mouse xenograft model to further characterize the transforming potential of mut-JAK3. Immunocompromised mice models are less stringent than *in vitro* approaches and provide favorable conditions for the growth of cell with weak transformed phenotype [18,19]. Thus, neomycin selected $JAK3^{WT}$, $JAK3^{P132A}$, $JAK3^{A572V}$ and $JAK3^{V722I}$ NIH3T3 cells were first injected s.c. into immune-compromised mice (FoxChase (C.B-17) SCID mice; $1x10^6$ cells/injection). As negative controls, we used non-transfected cells or pcDNA3 neomycin selected cells. NPM-ALK or kinase dead mutant NPM-ALK^{K210R} NIH3T3 cells were selected as additional controls. Tumor growth was rapidly observed in mice injected with $JAK3^{A572V}$ NIH3T3 cells. On the contrary, animals carrying $JAK3^{P132A}$ cells developed s.c. tumor masses only after several weeks after the challenge (>33). Moreover, the tumor frequency and size of $JAK3^{A572V}$ and $JAK3^{P132A}$ tumors were noticeably different as compared to positive control mice (Figure 3C). Similar results were obtained using puromycin selected JAK3 NIH3T3 cells (data not shown). These findings were further confirmed using puromycin selected JAK3 BaF3 cells. As shown in Figure 3D, both $JAK3^{A572V}$ or $JAK3^{P132A}$ positive cells could generate tumor masses overtime, and as for the NIH3T3 cells, $JAK3^{P132A}$ BaF3 cells required a very long period of time in establishing detectable tumorgrafts. The analysis of $JAK3^{A572V}$ and $JAK3^{P132A}$ positive tumours confirmed the expression of phosphorylated JAK3 species in the neoplastic cells whereas surrounding reactive lymphocytes were negative (Supplementary Figure 2). Notably, no tumorgrafts were established after the injection of $JAK3^{V722I}$ positive cells.

DISCUSSION

The tumorigenic role of constitutively active JAKs has been previously documented in some clonal myeloid proliferative disorders and in AML [21]. In this study, we have confirmed the oncogenic properties of $JAK3^{A572V}$ and discovered a novel oncogenic JAK3 mutation. No $JAK3^{A572V}$ substitutions [22] were however found in our AML panel, confirming previous data and suggesting that this mutation may have emerged during the *in vitro* cell line establishment. Our screening

however has detected a new JAK3^{P132A} substitution, as a homozygous alteration. Interestingly, when the JAK3^{P132A} construct was ectopically expressed in BaF3 cells could not lead to detectable/constitutive phosphorylation changes and sustain growth of BaF3 cells *in vitro* in absence of IL3. However, the JAK3^{P132A} could promote tumor growth *in vivo*. We have also observed that previously known JAK3 substitutions could be found in DNA samples from normal blood donors, at a frequency similar to that observed in AML patients. These data suggest that these substitutions may represent single nucleotide polymorphism. These data indicate that the oncogenic potential of JAK3 changes in myeloid elements should be carefully dissected and additional studies are necessary to fully underscore the relevance of JAK3 substitutions in these disorders. The presence of JAK3 changes in healthy individual may identify a novel risk group.

The constitutive activation of JAK/STAT pathways has been frequently documented in chronic myeloid proliferative disorders as result of the expression of novel chimeric proteins [23,24], which contribute to the cellular transformation [25] and to the maintenance of the neoplastic phenotype [26,27]. In this context, the oncogenic role of JAK3 was suggested by its frequent expression and/or activation in several cancers even in the absence of known mutations [28] and by its co-association with activated STATs. Originally, JAK3 mutations were described in patients with severe combined immunodeficiency, resulting in proteins with a loss-of-function (LOF), demonstrating the essential role of this kinase in T-cell development [29-38]. More recently, aminoacid substitutions of JAK3 have been documented in a subgroup of AML in Down Syndrome (DS) and non-DS adult individuals often carrying AMKL [22,39-41], in some AMKL cell lines and in a single high-risk childhood acute lymphoblastic leukemia patient [42].

It is well established that JAK3 deletion and/or substitution within the JH1 lead to LOF [40], while changes/deletions within the JH2, JH6 and JH7 regions can display LOF or gain-off-function (GOF) properties [15]. Walters *et al.* have proposed that mutations A572V, A573V and M576L may abolish the auto-inhibitory properties of the JH2 domain [12,15].

Here, we have identified a new mutation within the JH6 domain at codon 132 with a proline to

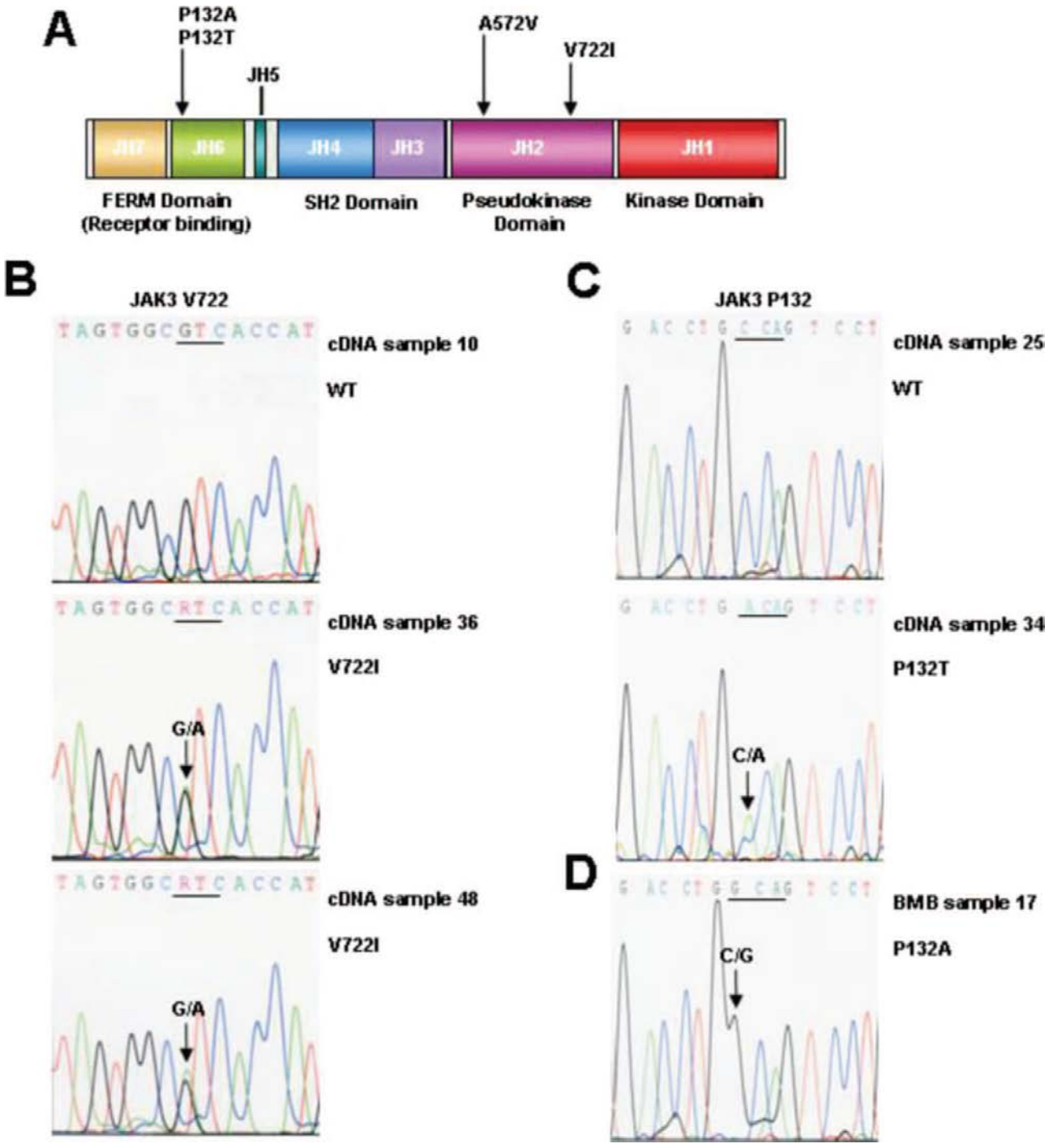
alanine change, originally described by Walters *et al* as a proline to threonine substitution.. Our AMKL P132A patient displayed this substitution on both alleles. More importantly, contrary to the P132T, the new P132A and A572V were not detected among control samples. P132A presence in homozygosis tends to exclude a novel SNP and its low oncogenic penetrance justifies the requirement of a two allelic mutation to induce transformation

To identify the putative pathogenetic role of JAK3 mutations investigators have taken advantage of *in vitro* assays, often using BaF3 cells as a model. Our data in NIH3T3 indicate that different models may provide unique information and that within different experimental contexts each models have significant limitations. Nevertheless, it is temping to speculate that the mut-JAK3 oncogenic properties may be subtle and require additional and/or specific alterations (trisomy 21, GATA mutations etc.) to lead to a definitive transformed phenotype (i.e. in NIH3T3 cells). This hypothesis is supported by the fact that JAK3 mutations can be frequently seen in DS patients and the discovery that the new P132A was found in homozygosis. Nevertheless, since some of these activating mutations are seen in normal individuals, additional studies are needed to identify if any relationship between these markers and a propensity to myeloid transformation may exist.

It is plausible that the abrogation of JAK-STAT signaling may represent a novel therapeutic strategy in AML disorders. Toward this end, JAK3 specific inhibitors are available, although they have been used mainly in controlling tissue rejection or in the treatment of autoimmune disorders. Their potential application in oncology remains, however, highly problematic and possibly unfeasible.

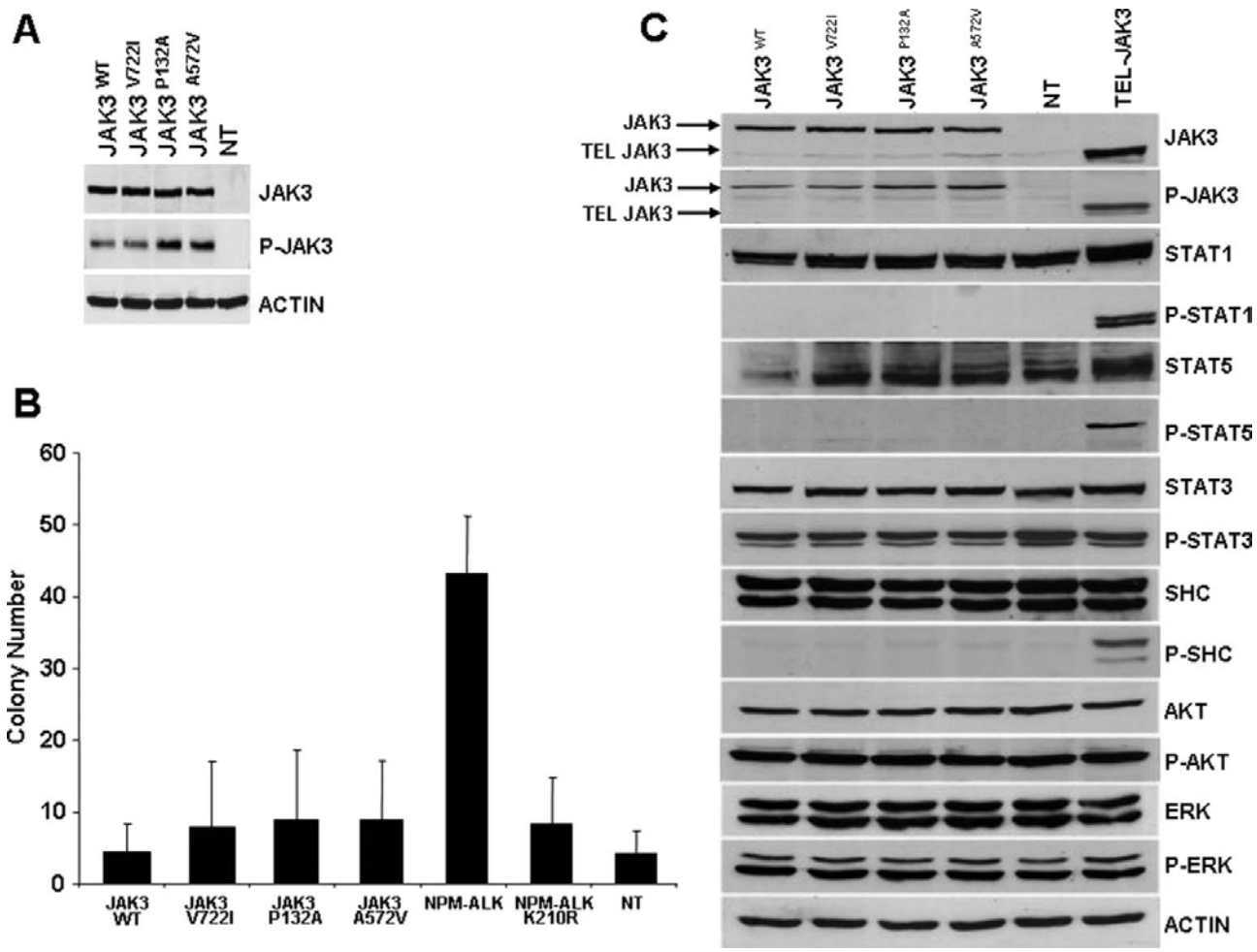
FIGURE LEGENDS

Figure 1. DNA Sequencing Of JAK3 In AML Patients



A) JAK3 mutations in myeloproliferative disorders. Schematic structure of human JAK3 showing the JH domains and the positions of aminoacid substitutions studied. The corresponding nucleotide changes in the JAK3 cDNA sequence are shown. DNA sequence analysis shows JAK3 somatic mutations in AML cells corresponding to the indicated aminoacid: $JAK3^{V722I}$ (B) and $JAK3^{P132T}$ (C) residues. The novel $JAK3^{P132A}$ mutation was found in a single M7 patient (D).

Figure 2. Transforming Activity Of Transfected NIH3T3 Cells With Different JAK3 Constructs.



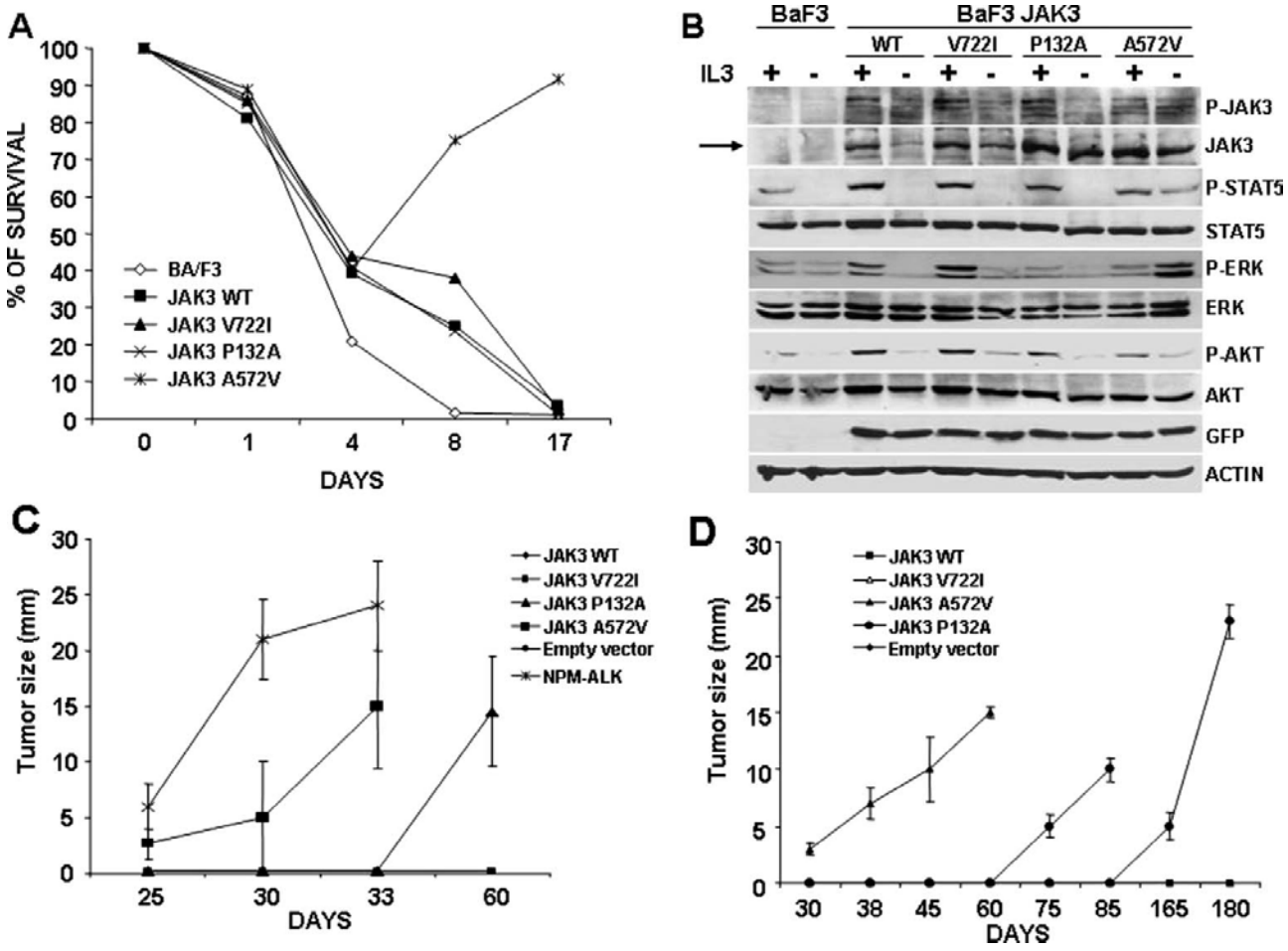
A) Expression of JAK3 constructs. NIH3T3 cells were transiently transfected using the Effectene method with the indicated JAK3 constructs. After transfection, cells were harvested and P-JAK3 and JAK3 expression were determined by Western blotting.

B) NIH3T3 cells were first transfected, then cells were grown in the G418 (data not shown) or puromycin media. Three weeks post transfection cells were fixed and stained with crystal violet. Data indicate the means and corresponding S.D. of multiple samples. Similar results were obtained in three independent experiments.

C) Activation of signal transduction pathways by JAK3 mutants. Western blot analysis of JAK3 proteins and their downstream effectors in wild-type or mutated JAK3-expressing NIH3T3 cells

48h after transfection. Cell lysates were subjected to immunoblot analysis with the indicated antibodies.

Figure 3. Oncogenic Properties Of Mut JAK3.



A) JAK3^{V722I} and JAK3^{P132A} do not sustain the BaF3 cell growth in the absence of IL-3. Three independent experiments were executed. A representative experiment is shown.

B) Activation of signal transduction pathways by JAK3 mutants. BaF3 cells were electroporated with JAK3 constructs and then puromycin selected (for 5 days). Western blot displays JAK3 proteins and their downstream effectors in wild-type or mutated JAK3-expressing BaF3 cells after IL-3 depletion (for 12 h). Cell lysates were subjected to immunoblot analysis with the indicated antibodies.

C) JAK3^{A572V} or JAK3^{P132A} transfected NIH3T3 cells lead to tumor formation *in vivo*. Neomycin selected NIH3T3 cells expressing JAK3^{WT}, JAK3^{V722I} JAK3^{P132A} or JAK3^{A572V} were injected s.c. into FoxChase (C.B-17) SCID mice (1 x 10^6 cells/mouse). Tumor formation and survival were monitored over time.

D) *In vivo* expression of JAK3^{A572V} or JAK3^{P132A} causes a lethal malignancy. Puromycin selected BaF3 cells expressing JAK3^{WT}-GFP, JAK3^{V722I}-GFP JAK3^{P132A}-GFP or JAK3^{A572V}-GFP were injected s.c. into SCID/Beige mice (4 x 10^6 cells/mouse). Tumor formation and survival were monitored over time. The two different lanes of JAK3^{P132A} represent two sets of mice which developed tumor masses at different time points.

TABLE I. **Patient Details And Results**. The percentages of JAK3 mutation detected in this study are indicated.

Table I. JAK3 mutations within AML and normal control

	A572V (JH2)	V722I (JH2)	P132T (JH6)	P132A (JH6)
BOM M7 (DNA)(28)	0/19	0/28	0/28	1/28
BOM M6 (DNA) (6)	-	0/6	-	-
AML BM (cDNA) (100)	0/100 (0%)	2/100 (2,0%)	1/91 (1,1%)	0/91 (0%)
CONTROLS(DNA) (191)	0/183 (0%)	10/191 (5,2%)	2/190 (1,0%)	0/190 (0%)

SUPPLEMENTARY FIGURES

Figure 1. JAK3^{A572V} Cells Do Not Required Exogenous IL-3.

The growth of BaF3 cells in the presence (+) or the absence of cytokines (-) was determined over time. Only JAK3^{A572V} transfected cells are able to generate detectable colonies in the IL-3 deficient media.

A) Percentage of viable wt BaF3 (upper panel) and JAK3^{A572V} BaF3 cells after IL3 depletion (lower panel). These analyses are representative of two independent experiments (duplicate).

B) Activation of signal transduction pathways in JAK3^{A572V} mutant. JAK3^{A572V} stable cells (> 15 days in absence of IL3) were analyzed by Western blot for JAK3 proteins and their downstream effectors performed with the indicated antibodies. Control cells have been included for comparison (BaF3 cells blots as in Figure 3B)

Figure 2. Phospho-JAK3 Is Detectable In JAK3^{A572V} And JAK3^{P132A} Transfected Tumor Cells

Formalin-fixed, paraffin-embedded sections from JAK3^{A572V} and JAK3^{P132A} positive tumours (top left and right, respectively) were stained with H&E and immuno-stained with a specific antibody against phospho-JAK3 by immunohistochemistry. Images were obtained after magnification (400).

SUPPLEMETARY MATERIAL AND METHODS

PCR Amplification And Analysis.

PCR conditions were as follow: 5 min at 95 °C; 10 cycles of 30 sec at 95 °C, 30 sec at 53 °C, 1 min at 72 °C; 20 cycles of 30 sec at 95 °C, 30 sec at 55 °C, 1 min at 72 °C, followed by 5 min at 72 °C for cDNA amplification or 3 min at 92 °C; 3 cycles of 15 sec at 92 °C, 30 sec at 64 °C, 30 sec at 72 °C; 3 cycles of 15 sec at 92 °C, 30 sec at 61 °C, 30 sec. at 72 °C; 3 cycles of 15 sec at 92 °C, 30 sec at 58 °C, 30 sec at 72 °C; 26 cycles of 15 sec at 92 °C, 30 sec at 57 °C, 30 sec at 72 °C for DNA amplification.

Amplified products were first analyzed by electrophoresis in a 2% agarose gel with ethidium bromide and visualized by UV light and then purified using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Sequencing reaction was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and the analysis was performed on an ABI 310 automated capillary system, following the manufacturer's instructions (Applied Biosystems). All samples were sequenced in both directions and the DNA sequences were compared to published germ-line data bases using a Basic Local Alignment Search Tool (BLAST).

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The authors declare no conflict of interest.

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Table I. JAK3 mutations within AML and normal control individuals

	A572V (JH2)	V722I (JH2)	P132T (JH6)	P132A (JH6)
BOM M7 (DNA)(28)	0/19	0/28	0/28	1/28
BOM M6 (DNA) (6)	-	0/6	-	-
AML BM (cDNA) (100)	0/100 (0%)	2/100 (2,0%)	1/91 (1,1%)	0/91 (0%)
CONTROLS(DNA) (191)	0/183 (0%)	10/191 (5,2%)	2/190 (1,0%)	0/190 (0%)

Supplementary Table I. Primers used for JAK3 amplification

JH2-for	TCAGCCCCAATCCCAATAC
JH2-internal-rev	CCAGGGTCACTCAGCTTGAT
JH2-internal-for	GCCCTCAACTATCTGGAGGA
JH2-rev	AGGTGTGGGGTCTGAGAGG
Exon15-for	CTCACCGACAGGATCCCC
Exon15-rev	GATCCAGGGCACTGATGG
Exon3-for	TACTTCCCCAATTGGTTTGG
Exon3-rev	CTGGGCAAAGAGGTGCTC
A572V-for	CCTAAGGCAGGTCTGTGAGC
A572V-rev	GAGATGCCGGTACGACACTT

Supplementary Table II. Oligonucleotide primers for the JAK3 mutagenesis

V722I-for	5’-GTGTTTAGTGGCATCACCATGCCCATC-3’
V722I-rev	5’-GATGGGCATGGTGATGCCACTAAACAC-3’
P132A-for	5’-TCCTTGACCTGGCAGTCCTGGAGCAC-3’
P132A-rev	5’-GTGCTCCAGGACTGCCAGGTCAAGGA-3’
A572V-for	5’-TCATTCCTGGAAGTAGCGAGCTTGATG-3’
A572V-rev	5’-CATCAAGCTCGCTACTTCCAGGAATGA-3’