



## Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome

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### Summary

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**Background** Children with Down's syndrome have a greatly increased risk of acute megakaryoblastic and acute lymphoblastic leukaemias. Acute megakaryoblastic leukaemia in Down's syndrome is characterised by a somatic mutation in *GATA1*. Constitutive activation of the JAK/STAT (Janus kinase and signal transducer and activator of transcription) pathway occurs in several haematopoietic malignant diseases. We tested the hypothesis that mutations in *JAK2* might be a common molecular event in acute lymphoblastic leukaemia associated with Down's syndrome.

**Methods** *JAK2* DNA mutational analysis was done on diagnostic bone marrow samples obtained from 88 patients with Down's syndrome-associated acute lymphoblastic leukaemia; and 216 patients with sporadic acute lymphoblastic leukaemia, Down's syndrome-associated acute megakaryoblastic leukaemia, and essential thrombocythaemia. Functional consequences of identified mutations were studied in mouse haematopoietic progenitor cells.

**Findings** Somatically acquired *JAK2* mutations were identified in 16 (18%) patients with Down's syndrome-associated acute lymphoblastic leukaemia. The only patient with non-Down's syndrome-associated leukaemia but with a *JAK2* mutation had an isochromosome 21q. Children with a *JAK2* mutation were younger (mean [SE] age 4·5 years [0·86] vs 8·6 years [0·59],  $p<0\cdot0001$ ) at diagnosis. Five mutant alleles were identified, each affecting a highly conserved arginine residue (R683). These mutations immortalised primary mouse haematopoietic progenitor cells in vitro, and caused constitutive Jak/Stat activation and cytokine-independent growth of BaF3 cells, which was sensitive to pharmacological inhibition with JAK inhibitor I. In modelling studies of the *JAK2* pseudokinase domain, R683 was situated in an exposed conserved region separated from the one implicated in myeloproliferative disorders.

**Interpretation** A specific genotype-phenotype association exists between the type of somatic mutation within the *JAK2* pseudokinase domain and the development of B-lymphoid or myeloid neoplasms. Somatically acquired R683 *JAK2* mutations define a distinct acute lymphoblastic leukaemia subgroup that is uniquely associated with trisomy 21. JAK2 inhibitors could be useful for treatment of this leukaemia.

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### Introduction

The oncogenic role of aberrations in chromosome number, the most common abnormalities in cancer, is poorly understood. Children with constitutional trisomy 21 (Down's syndrome) have 20-fold increased risk of developing acute leukaemia, including acute lymphoblastic leukaemia and acute megakaryoblastic leukaemia,<sup>1</sup> suggesting that this trisomy is leukaemogenic.

Acute megakaryoblastic leukaemia in Down's syndrome is characterised by somatic mutations in the X-linked gene *GATA1*, suggesting cooperation with trisomy 21.<sup>2-5</sup> By contrast, the pathogenesis of acute lymphoblastic leukaemia, the most common type in children with Down's syndrome, remains elusive. Overall, about 2% of children diagnosed with acute lymphoblastic leukaemia have Down's syndrome. Acute lymphoblastic leukaemia in Down's syndrome is of exclusively B-cell origin, and, trisomy 21 or tetrasomy 21 is the most common

chromosomal abnormality in the sporadic B-cell precursor childhood form of this leukaemia.<sup>6</sup> Because cytogenetic abnormalities that are commonly detected in the sporadic childhood acute lymphoblastic leukaemia are less frequent in the Down's syndrome-associated form of the leukaemia,<sup>7,8</sup> the existence of somatic genetic events that work together, similar to the *GATA1* mutation in acute megakaryoblastic leukaemia associated with Down's syndrome has been postulated, but not yet proven.

Mutations of the Janus kinase (*JAK2*) gene on chromosome 9p24 that activate the encoded tyrosine kinase have been reported in most patients with myeloproliferative disorders.<sup>9-13</sup> The valine 617 is mutated in most cases (V617F) but mutations in other *JAK2* residues are implicated in some patients. Mutations that activate *JAK2* or *JAK3* rarely arise in acute myeloid leukaemia and Down's syndrome-associated acute megakaryoblastic leukaemia.<sup>14,15</sup> In addition to the

myeloid lineages, the JAK/signal transducer and activator of transcription (STAT) signalling pathway is important in early B-cell development.<sup>16</sup> The TEL-JAK2 translocation rarely arises in acute lymphoblastic leukaemia.<sup>17</sup> A case report of a somatic *JAK2* mutation in a patient with Down's syndrome and this leukaemia has been reported.<sup>18</sup> Together, these findings suggest that *JAK2* might be implicated in lymphoid neoplasms.

To test the hypothesis that mutations in the *JAK2* gene cooperate with trisomy 21, we screened diagnostic DNA samples from patients with acute lymphoblastic leukaemia associated with Down's syndrome.

## Methods

### Patient samples

We extracted DNA from diagnostic bone marrow samples obtained from 88 patients with acute lymphoblastic leukaemia associated with Down's syndrome that had been stored in European laboratories of childhood leukaemia study groups (webtable 1 shows clinical data). These patients represent about half of those diagnosed with acute lymphoblastic leukaemia in Down's syndrome during that period (overall period was from 1989–2007; the specific range differed among the centres; webtable 2). To identify mutations associated with other haematological malignant diseases, we screened genomic DNA extracted from diagnostic bone marrow samples obtained from 109 patients with sporadic B-cell precursor childhood acute lymphoblastic leukaemia, 11 with Down's syndrome-associated acute megakaryoblastic leukaemia, 96 with essential thrombocythaemia negative for *JAK2* V617F mutation, and 23 leukaemia cell lines (cell lines used are listed in webtable 3).

We obtained the samples after informed parental consent for patients participating in the Berlin-Frankfurt-Munster (BFM) leukaemia treatment protocols. The study was approved by the Sheba Medical Centre and the ethical committee of the Israeli Health Ministry.

### *JAK2* mutation analysis

We used intronic primers of human *JAK2* sequence (accession number NM\_004972) to amplify exons 10–25 of the gene with PCR, and analysed the fragments by denaturing high-performance liquid chromatography (WAVE, Transgenomic, Omaha, NE, USA; for detailed methods see webappendix 1; webfigure 1).<sup>3,19</sup> We sequenced the fragments with abnormal chromatography patterns. For expression analyses (RT-PCR), we extracted RNA with Trizol (Invitrogen, Carlsbad, CA, USA), and generated full-length cDNA with the Reverse-iT 1st Strand Synthesis Kit (ABgene, Epsom, UK; webtable 4 shows the primers sequences used).

### Site-directed mutagenesis, BaF3 proliferation assays, and western blotting

The mouse *Jak2* cloned into the MSCViresGFP retrovirus (encoding the green fluorescent protein [GFP]) was used

as a template for the generation of mutations—ie, V617F, R683G, R683S, R683K, and I682-D686del—by site-directed mutagenesis (QuikChangeTM II XL, Stratagene, Amsterdam, Netherlands). The plasmid sequences were verified by sequencing. The positive controls were the myeloproliferative disorder-associated V617F *Jak2* and the I682-D686del *Jak2* variants reported previously.<sup>15</sup> We transduced parental BaF3 cells (a mouse pro-B cytokine-dependent cell line) and those expressing erythropoietin or thrombopoietin receptors (BaF3/EpoR or BaF3/TpoR, respectively)<sup>20</sup> with the retroviruses containing wild-type *Jak2*, mutant *Jak2*, or *BCR-ABL* cDNAs. We purified the GFP-positive cell population by flow-cytometric sorting 2–4 days later and then cultured the cells for less than a week.

To investigate the functional consequences of the *JAK2* mutations, we expressed the wild-type or mutant mouse *Jak2* in parental BaF3 cells or those transfected with either erythropoietin or thrombopoietin receptors (BaF3/EpoR or BaF3/TpoR, respectively). We cultured BaF3/EpoR or BaF3/TpoR cells in RPMI-1640 containing 10% fetal calf serum and either erythropoietin (1 U/mL) or interleukin 3 (1 ng/mL), respectively. To assay for factor-independence, we washed the cells three times, cultured them (100 million per L) in the absence of exogenous cytokine for 5 days, and counted the cells that were viable every day with Trypan Blue exclusion or Casy counter (Scharfe, Reutlingen, Germany). For western blot analyses, we harvested BaF3/EpoR cells after 15 h of erythropoietin deprivation and used anti-*JAK2* (C-20, Santa Cruz, CA, USA), anti-STAT5, anti-phospho *JAK2* Tyr 1007-1008 (Cell Signaling, Beverly, MA, USA), anti-phospho STAT5 Tyr 694 (Epitomics, Burlingame, CA, USA), and anti- $\alpha$ -tubulin (Sigma, Saint Louis, MO, USA).

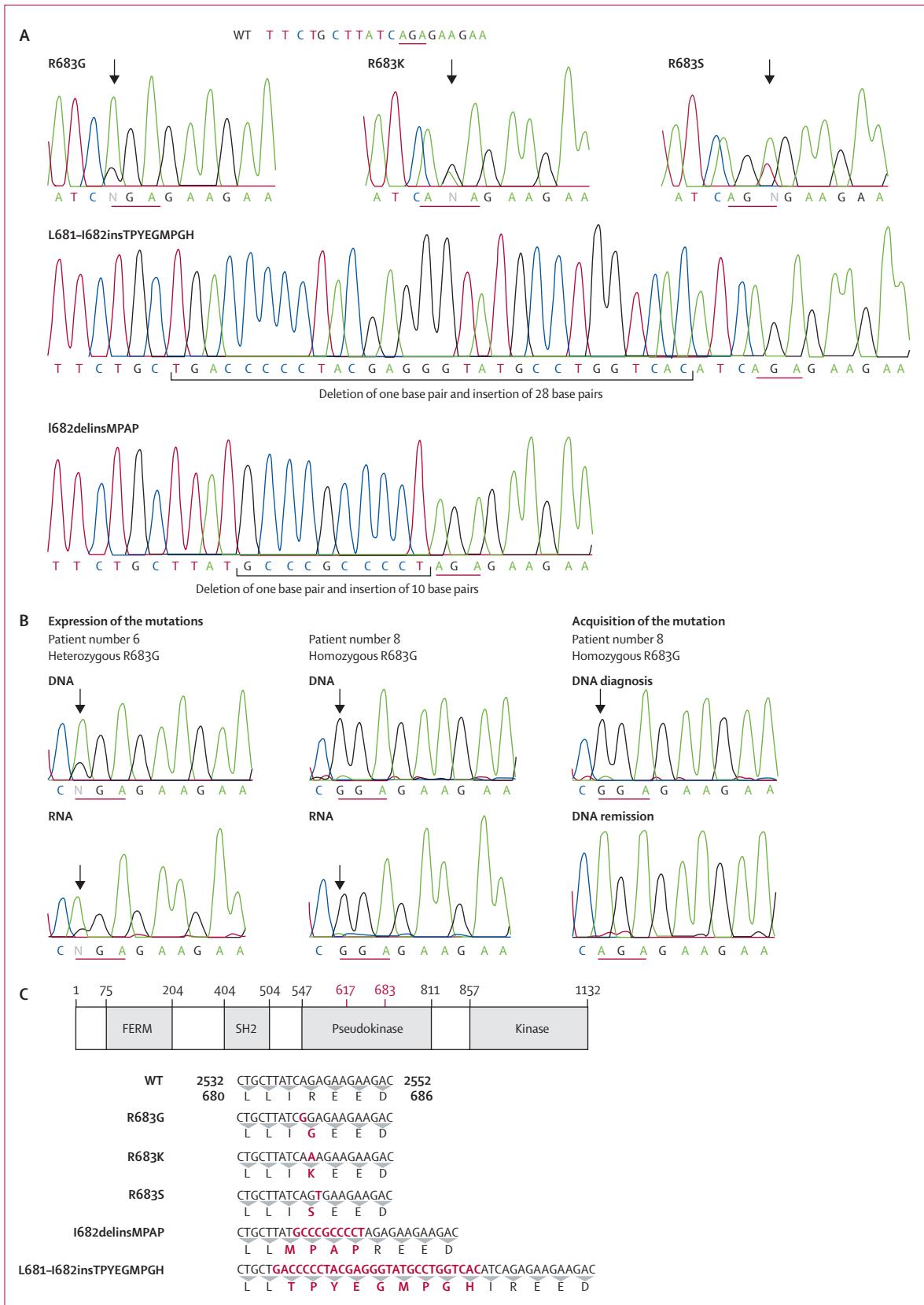
### Methylcellulose cultures of primary mouse haemopoietic progenitor cells

We extracted and purified haematopoietic progenitor cells (cKit+Lin-) from bone marrow of 6-week-old C57BL/6 mice with the lineage-cell depletion kit (Miltenyi Biotec, Auburn, CA, USA) and magnetic activated cell sorter (Miltenyi biotec). We transfected 293T cells with MSCViresGFP retrovirus expressing wild type and mutated *Jak2* (R683S or V617F); harvested the retrovirus-containing supernatants after 48 h and concentrated them by centrifugation for 1 h at 16 000 g. We transduced purified haemopoietic progenitor cells with the retroviruses and cultured the cells in duplicate in 1·1 mL of methylcellulose at 37°C in 35 mm plates (10<sup>4</sup> cells per plate) 48 h after infection. For B-cell assays, we plated cells in Methocult M3231 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with interleukin 7 (20 ng/mL), Flt-3 ligand (10 ng/mL), and stem-cell factor (100 ng/mL). For myeloid assays, we plated cells in Methocult M3534 (Stem Cell Technologies) containing interleukin 3 (10 ng/mL), interleukin 6

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See Online for webtables 1–4, webappendix 1, and webfigure 1



**Figure 1: Mutations of JAK2 exon 16 in patients with Down's syndrome-associated acute lymphoblastic leukaemia**

(A) Examples of sequences with heterozygous mutations. R683 is coded by the AGA codon (underscored red). The inserted sequences are within parentheses (both del-ins mutations were heterozygous; however, only the mutated allele is shown after allele separation). WT=wild-type JAK2 sequence. N=sites with both wild-type and mutant nucleotides at the same position. del=deletion. ins=insertion. (B) Examples of two mutated JAK2 sequences with homozygous and heterozygous 2541A→G transition that caused a R683G substitution. The same alleles occurring in the genomic DNA are expressed in the RNA from diagnosis but not in remission samples, showing that this mutation is a somatic mutation expressed only in the leukaemic clone. (C) Alignment of wild-type and mutated exon 16 JAK2 domains. Numbers show the positions of nucleotides and corresponding aminoacids; the R683 and V617 mutations and the aminoacid substitutions are shown in red.

(10 ng/mL), stem-cell factor (50 ng/mL), and granulocyte-macrophage colony-stimulating factor (10 ng/mL). After 6–10 days, we scored, pooled, and counted cells in colonies containing 50 cells or more. We did secondary assays and subsequent rounds by replating  $1 \times 10^4$  to  $3 \times 10^4$  cells per mL under identical culture conditions.

### Pharmacological inhibition of JAK2

To examine if cells expressing mutated R683 JAK2 are sensitive to pharmacological inhibition of the JAK/STAT pathway, we incubated BaF3/EpoR or BaF3/TpoR cells (100 million per L) expressing R683G, R683S, I682-D686del, and V617F *Jak2* mutants cultured without cytokines with DMSO in the absence or presence of the JAK inhibitor I (Calbiochem, La Jolla, CA, USA), a commercially available inhibitor of the pathway. Controls were BaF3/EpoR cells expressing BCR-ABL and the BCR-ABL-positive cell line K562. We counted the viable cells after 48 h (for BaF3/EpoR) or 72 h (for BaF3/TpoR). Data from three independent experiments were combined for analysis. We calculated the normalised viability by dividing the cell number at each inhibitor concentration by the cell number with vehicle alone. For the western blot analysis, the cells were grown for 5 h in the absence or presence of the JAK inhibitor I.

### Structural modelling of JAK2 pseudokinase domain

To suggest a molecular interpretation of altered aminoacids in the protein, we modelled the three-dimensional structure of the JAK2 pseudokinase (JH2) domain. We used ConSurf<sup>21</sup> and a multiple-sequence alignment of homologous proteins to calculate the evolutionary conservation of each residue and project the conservation grades on the model structure (see webappendix 2 for detailed methods).

### Statistical analysis

We used Fisher's exact test to compare categorical variables, and Mann-Whitney *U* test to compare continuous variables. We plotted survival curves with the Kaplan-Meier procedure, and compared the numbers of viable cells present in BaF3-cell proliferation assays with an unpaired Student's *t* test.

### Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Results

*JAK2* mutations in diagnostic bone marrow specimens from patients with Down's syndrome-associated acute lymphoblastic leukaemia showed clustering around a

Patient number	JAK2 mutation	Sex	Age (years)*	WBC (per L)*	Current status	Time to first event (months)‡	Time of total follow-up (months)
1	R683S	M	3.21	$209 \times 10^8$	1st CCR	..	72.03
2	R683S	F	1.99	$1240 \times 10^8$	1st CCR	..	46.50
3	R683S	F	4.11	$692 \times 10^8$	1st CCR	..	57.70
4	R683S	M	4.85	$98 \times 10^8$	Dead‡	9.83	9.83
5	R683S	M	3.54	$118 \times 10^8$	1st CCR	..	8.53
6	R683G	F	2.99	$24 \times 10^8$	2nd CCR	27.90	62.87
7	R683G	M	4.99	$305 \times 10^8$	1st CCR	..	127.23
8	R683G	F	4.02	$112 \times 10^8$	1st CCR	..	82.23
9	R683G	M	19.87	$860 \times 10^8$	Dead‡	23.13	24.07
10	R683G	M	4.14	$1940 \times 10^8$	Dead‡	0.47	0.47
11	R683G	F	3.43	$234 \times 10^8$	1st CCR	..	33.27
12	R683G	M	2.87	$273 \times 10^8$	1st CCR	..	9.30
13	R683G	M	2.85	$1390 \times 10^8$	1st CCR	..	6.97
14	R683K	F	4.63	$331 \times 10^8$	1st CCR	..	39.37
15	I681-I682insTPYEGMPGH	F	2.05	$1490 \times 10^8$	1st CCR	..	101.10
16	I682delinsMPAP	F	2.78	$77 \times 10^8$	1st CCR	..	61.13

WBC=white blood cells. M=male. F=female. R683G=2541A→G. R683S=2543A→T. R683K=2542G→A.

L681-I682insTPYEGMPGH=2537del-T-ins-GACCCCTACGAGGGTATGCTGGTCAC.

I682delinsMPAP=2540del-C-ins-GCCCCCCC. 1st CCR=continuous complete remission. 2nd CCR=continuous complete remission after relapse. \*At diagnosis. ‡First relapse or death. #Death of patient from disease or during induction.

Table 1: clinical characteristics of patients with Down's syndrome-associated acute lymphoblastic leukaemia and mutated JAK2

	Wild type (N=72)	R683 mutation (N=16)	p value
Sex			1.000
Male	35 (49%)	8 (50%)	
Female	37 (51%)	8 (50%)	
Age (years)*			<0.0001
Mean (SE)	8.6 (0.59)	4.5 (1.05)	
Median (range)	7.68 (1.74–20.22)	3.49 (1.99–19.87)	
White blood cell count (per L)*			0.066
Mean (SE)	$340 \times 10^8$ ( $58 \times 10^8$ )†	$587 \times 10^8$ ( $152 \times 10^8$ )	
Median (range)	$148 \times 10^8$ (14–2590×10 <sup>8</sup> )†	$289 \times 10^8$ (24–1940×10 <sup>8</sup> )	
Genetic subgroups of ALL‡			0.197
Any	10 (14%)	0	
None	62 (86%)	16 (100%)	

Data are number (%), unless otherwise indicated. ALL=acute lymphoblastic leukaemia. \*At diagnosis. †Percentages are for 69 patients with available data. ‡TEL-AML1, BCR-ABL, MLL-4, or high hyperdiploid.

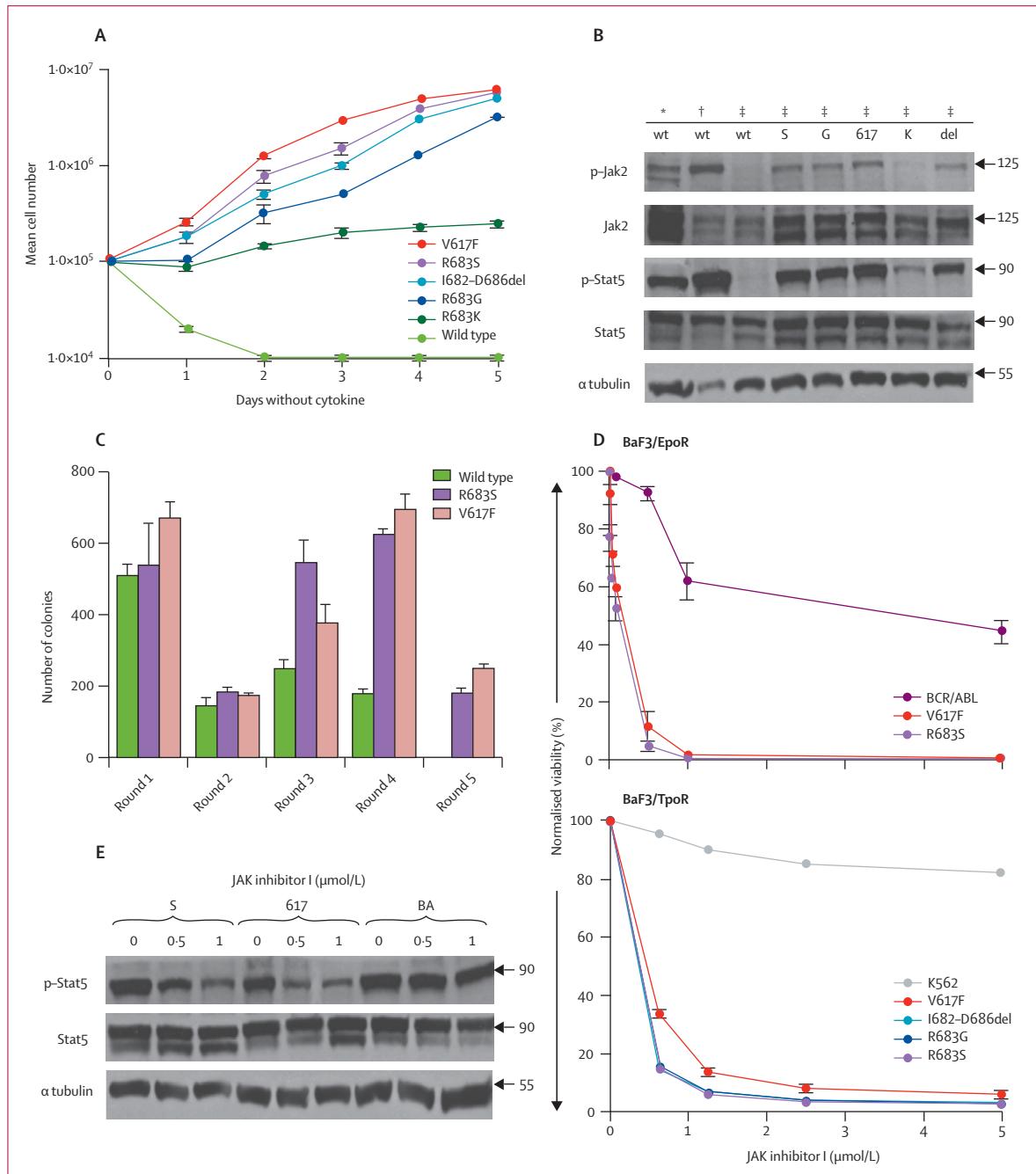
Table 2: Clinical and diagnostic characteristics of patients with Down's syndrome-associated acute lymphoblastic leukaemia with and without JAK2 mutations

conserved arginine at position 683 (R683) in 16 (18%) of 88 patients (table 1; figure 1). We confirmed all the mutations by subcloning and sequencing them. We did not find any additional mutations in *JAK2* exon 16 in all 72 samples without the R683 mutations. The most frequent of the five types of missense mutations was a 2541A→G transition (ie, AGA to GGA) that caused

See Online for webappendix 2

substitution of R683 with glycine (R683G) in eight patients. A 2543A→T transversion (ie, AGA to AGT), causing a serine substitution (R683S), occurred in

five patients; a 2542G→A transition (ie, AGA to AAA) in one case resulted in a lysine substitution (R683K). Complex inframe deletion and insertion events occurred



**Figure 2: Expression of Jak2 R683 and V617F mutants in mouse hematopoietic progenitor cells**

(A) Cytokine withdrawal assay of BaF3 cells expressing erythropoietin receptor (BaF3/EpoR) and wild type or mutant (V617F, R683S, R683G, R683K, or I682-D686del) mouse Jak2. Error bars represent SE. (B) Constitutive activation of the Jak/Stat pathway in BaF3/EpoR cells expressing Jak2 mutants. The arrows indicate the proper sizes and the molecular weight of the proteins in kDa. wt=wild type Jak2. S=R683S. G=R683G. 617=V617F. K=R683K. del=I682-D686del. \*Cells harvested in steady state (1 U/ml erythropoietin). †Cells harvested after 15 h of erythropoietin deprivation followed by 15 min of erythropoietin stimulation (10 U/ml). ‡Cells harvested after 15 h of erythropoietin deprivation. (C) Colony-forming and replating assays of primary mouse bone marrow lineage-depleted hematopoietic progenitor cells transduced with wild type or mutant (V617F, R683S) mouse Jak2. (D) Effect of JAK inhibitor I on BaF3/EpoR or BaF3/TpoR cells expressing Jak2 mutants (R683G, R683S, I682-D686del, and V617F). (E) Effect of JAK inhibitor I on Stat5 phosphorylation in BaF3/EpoR cells expressing R683 Jak2 mutants when incubated for 5 h. The arrows indicate the proper sizes and the molecular weight of the proteins in kDa. S=R683S. 617=V617F. BA=BCR-ABL1.

immediately upstream of R683 in patients 15 and 16 (figure 1; table 1). Although these DNA rearrangements did not directly affect R683, they caused a large insertion immediately before this residue, and we have thus included them among the R683 mutations (figure 1; table 1). We did not detect the previously reported I682-D686del mutation.<sup>18</sup>

With the exception of one patient (number 8; table 1), the mutations were heterozygous. We believe that these *JAK2* mutations were somatically acquired because they were absent in remission samples (patients 1–8, 11–14, and 16; table 1). In patient number 6, from whom we had obtained bone marrow samples during diagnosis, remission, and subsequent relapse, the same R683G mutation occurred only in the diagnosis and relapse samples. RT-PCR analysis of three patients (numbers 6, 8, and 15; table 1) for whom we had RNA confirmed that the mutant *JAK2* allele was expressed (figure 1, webfigure 2).

In our series of samples from patients with sporadic B-cell precursor acute lymphoblastic leukaemia, only one individual, a 20-month-old child, had the R683G mutation. Cytogenetic analysis at diagnosis showed the presence of isochromosome 21q as the only abnormality.

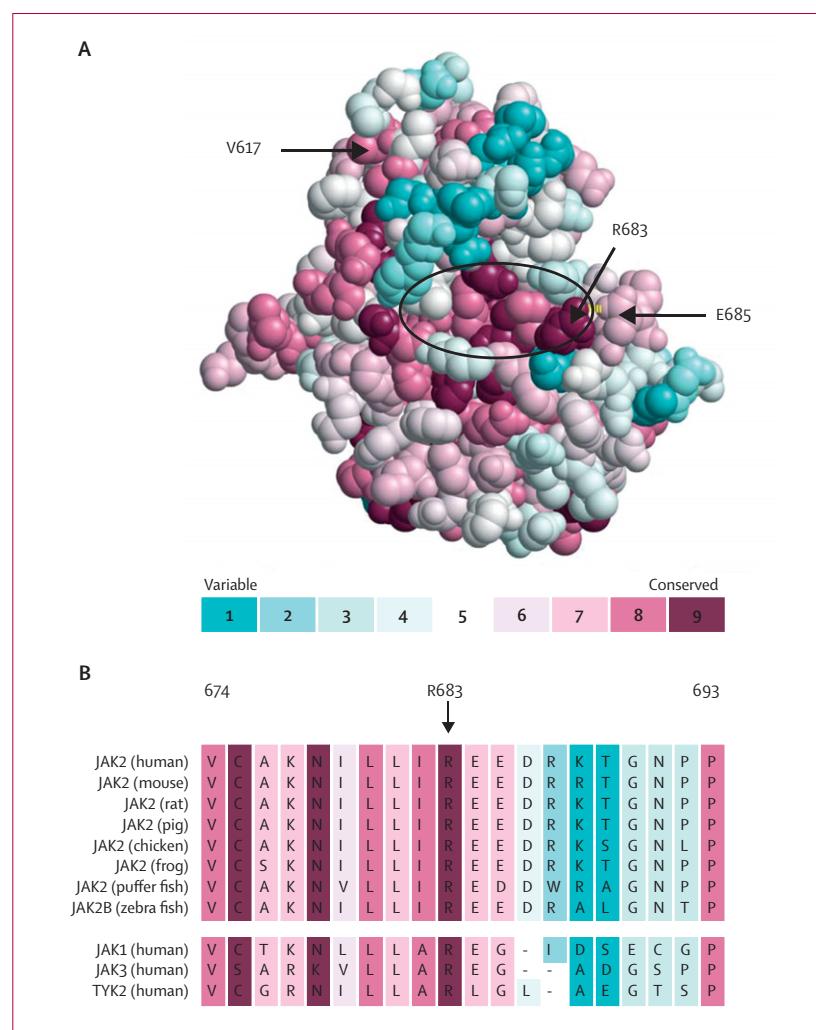
All patients with Down's syndrome-associated acute lymphoblastic leukaemia had B-cell precursor leukaemias. Patients with a mutant *JAK2* allele were diagnosed at a younger age than those with the non-mutated gene (table 2, webfigure 3). With the exception of one patient (diagnosed at 19 years), all the others with the *JAK2* mutation were younger than 5 years at diagnosis. By contrast, 27 (38%) of 72 patients with non-mutated *JAK2* were older than 10 years. The numbers of peripheral blood blasts at presentation were generally higher in patients with mutated *JAK2* (table 2). The frequency of the typical cytogenetic aberrations of childhood acute lymphoblastic leukaemia was low in the whole group (10 [11%] of 88). These aberrations—ie, *TEL-AML1*, *MLL-AF4*, *BCR-ABL*, and high hyperdiploidy—were identified in the non-*JAK2* mutated leukaemias (webtable 1), suggesting they might be mutually exclusive with the *JAK2* mutations. The presence of the *JAK2* mutation had no effect on event-free survival of this heterogeneously treated group of patients (webfigure 4).

Expression of the *Jak2* mutants, but not the wild-type gene, conferred cytokine-independent proliferation of BaF3/EpoR and BaF3/TpoR cells and constitutive tyrosine phosphorylation of Jak2 and Stat5 (figure 2; webfigure 5). The conservative R683K mutation was the least potent mutation in the cell proliferation assay. Proliferation of the cells in the absence of exogenous cytokines required the expression of erythropoietin or thrombopoietin receptors, and it was not seen in the parental cells (webfigure 5). These findings suggest that these *JAK2* mutations are all gain-of-function, activating biochemical pathways associated with JAK/STAT signalling.

Lineage-cell depleted haematopoietic progenitor cells (cKit+Lin-) transduced with wild-type or mutated V617F or R683S *Jak2* did not form colonies when plated on methylcellulose with cytokines promoting B-cell growth from the second replating onwards (data not shown). By contrast, both V617F and R683S *Jak2* mutations provided growth advantage in replating experiments when grown in myeloid conditions (figure 2).

Interleukin-3-dependent BaF3/TpoR or erythropoietin-dependent BaF3/EpoR cells expressing the R683 *Jak2* mutants were highly sensitive to JAK inhibitor I (IC<sub>50</sub> <0·5 µM) compared with BaF3 or K562 cells expressing

See Online for webfigures 2–5



**Figure 3:** ConSurf analysis of evolutionary conservation profile of the structural model of pseudokinase domain of *JAK2*

(A) The evolutionary conservation grades of the amino acids are presented with the colour bar at the bottom. V617 and R683 (shown by arrows) were assigned high conservation grades of 8 and 9, respectively. Of note, R683 is situated on the rim of a conserved patch of exposed amino acids in an apparent binding-pocket (encircled by an ellipse) distinct from V617. Furthermore, R683 seems to be salt-bridged to the conserved E685 residue (shown by an arrow and dashed yellow lines). Thus, a mutation of R683 might hinder the ability of the proteins to bind the substrate or a hypothetical protein domain that binds through this interface. (B) A segment (residues 674–93) of the multiple-sequence alignment of *JAK2* human homologues. The alignment is coloured according to the ConSurf conservation colouring scheme (colour bar). An arrow indicates position 683 of human *JAK2*; the R683 is completely conserved.

BCR/ABL, which were at least 20 times less sensitive ( $>5\text{ }\mu\text{M}$ , not reached; figure 2). Growth of cells expressing the R683 *Jak2* mutants was dose-dependently inhibited by the JAK inhibitor I.

Homology-based structural modelling suggests that R683 is one of the most highly conserved aminoacids of the pseudokinase domain, and is located within a deep and narrow putative binding pocket that includes other conserved aminoacids (figure 3). In the multiple-sequence alignment analysis, this position accommodated arginine exclusively throughout the homologues. Furthermore, R683 seems to be salt-bridged with E685, which is also conserved. Thus, any mutation of R683, even to the electrostatically similar lysine (R683K), is likely to interfere with that salt-bridge, change the stereochemistry and physicochemical properties of the binding pocket, and alter the protein's capacity to bind substrates or one of the other domains of JAK2. Our model is consistent with our findings because all the R683 JAK2 mutants caused constitutive activation of JAK/STAT pathway. The wild-type Jak2 is probably less stable than the mutant Jak2 protein (figure 2).

R683, which is specifically mutated in acute lymphoblastic leukaemia in Down's syndrome, and V617, commonly mutated in myeloproliferative disorders, are located on different conserved surfaces of the JAK2 pseudokinase domain (figure 3). Thus, these residues might mediate different interactions that could explain the association of these mutations with distinctly different leukaemia phenotypes.

## Discussion

Somatically acquired mutations centred around the R683 residue in the pseudokinase domain of JAK2 affect about a fifth of patients with Down's syndrome-associated acute lymphoblastic leukaemia. The mutations resulted in the activation of the JAK2 protein, as shown by their proproliferative effect on transduced haematopoietic progenitor cells and constitutive phosphorylation of Jak2 and its target Stat5 in mouse pro-B cells.

Our data suggest that the R683 mutations are specific to this B-cell precursor leukaemia in constitutional trisomy 21. The only patient without Down's syndrome but with the R683G mutation in the leukaemic cells had an isochromosome 21q, a rare abnormality in the sporadic form of this disease. Thus, the possibility exists that this patient was a Down's syndrome mosaic with three copies of the whole long arm of chromosome 21 as previously reported.<sup>22</sup> The young age of this patient is in agreement with the ages of patients with Down's syndrome-associated acute lymphoblastic leukaemia who have the JAK2 R683 mutations.

The R683 JAK2 mutations are gain-of-function driver mutations because they reduce the cytokine-dependency of mouse BaF3 cells. Similar to previous findings,<sup>9,23</sup> coexpression of a homodimeric type 1 cytokine receptor—eg, thrombopoietin or erythropoietin receptor—with the

*Jak2* mutants was needed for proliferation of BaF3 cells. These receptors are thought to provide a structural scaffold for the mutant JAK2 protein. However, neither receptor is expressed in B-lymphoid progenitors or their mature progeny, suggesting that the lymphoid-associated R683 mutants use the intracellular domains of other receptors to exert their effects. Transduction of primary haematopoietic progenitor cells with the R683S-mutated *Jak2* provided a survival advantage when cultured in pro-myeloid but not pro-B lymphoid growth conditions. These results might be caused by technical limitations of B-cell precursor in-vitro proliferation assays; however, Maligne and colleagues<sup>18</sup> reported that transplantation of bone marrow cells transduced with JAK2 containing the I682-D686del mutation resulted in a myeloproliferative disorder that was indistinguishable from that caused by the V617F mutations. Together, these results suggest that trisomy 21 might have a specific role in conferring a growth advantage on B-cell precursor cells with acquired JAK2 R683 mutations. For example, induction of the expression of a specific type 1 cytokine receptor in B-cell progenitors might be involved.

The specific association between constitutional trisomy 21 and R683 JAK2 mutations is similar to that of *GATA1* mutations in the acute megakaryoblastic leukaemia associated with Down's syndrome. The elucidation of the genes on chromosome 21 that bring about these specific oncogenic collaborations has been a challenge because of the ubiquitous overexpression of several genes from the trisomic chromosomes.<sup>24</sup> Research suggests that acute megakaryoblastic leukaemia in Down's syndrome is caused by the overexpression of several chromosome 21 genes that enhance fetal megakaryopoiesis and hence cooperate with mutated *GATA1*, which regulates the same developmental process.<sup>4,25</sup> A similar multigene mechanism might exist for acute lymphoblastic leukaemia in Down's syndrome. The nature of this mechanism (eg, lymphoid-specific R683 interacting proteins or an effect on B-cell differentiation) is presently unknown, and its elucidation will need future development of experimental models of the disorder.

Perhaps the most surprising observation of our study is the strict genotype-phenotype specificity of the mutations. Tissue specificity of inherited mutations is known;<sup>26</sup> however, lineage specificity of different somatic mutations of the same oncogene is very rare, and is a good example of how mutations in different portions of an isolated domain of a signalling molecule lead to different phenotypes. One interpretation of this specificity is that the two parts of the domain might interface with different crucial lineage-specific signalling molecules. Clues might be provided by structural analysis of the JAK2 protein. As shown by our structural model, the R683 and V617 residues lie in two different exposed patches consisting of solvent accessible aminoacids that are evolutionarily conserved and located close to each other. Another

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structural model of the whole JAK2 protein, with fewer details of the pseudokinase domain (see webappendix 2 for comparisons with our model), has been reported.<sup>27</sup> Although V617 is predicted to be located at the interface between the pseudokinase and the kinase domains, R683 is predicted to be located on the opposite side, at the pseudokinase to Src homology 2 domain interface.<sup>27</sup> Even though the validity of these theoretical models remains to be proven, the distinct locations of V617 and R683 predicted by both theoretical models suggest that different protein-to-protein interactions might underlie the unusual genotype-phenotype association.

The involvement of JAK2 in myeloproliferative disorders has led to the development of new JAK2 inhibitors, which are being assessed in phase I clinical trials.<sup>28</sup> Children with Down's syndrome-associated acute lymphoblastic leukaemia are very sensitive to the toxic effects of conventional chemotherapy and hence fare worse than those with the sporadic form of this leukaemia.<sup>7</sup> The patients with the acquired R683 mutation could, therefore, be considered for inclusion in clinical trials of JAK2 inhibitors. The mutated *JAK2* gene could be used as a molecular marker for minimal residual disease detection during treatment. Moreover, our findings that *JAK2* is mutated in acute lymphoblastic leukaemia that arises in Down's syndrome and a previous report that *JAK1* is mutated in 18% of adults with T-cell acute lymphoblastic leukaemia,<sup>29</sup> suggest that the JAK/STAT pathway might be implicated through other mechanisms in other malignant diseases of the lymphoid system.

These results suggest that the R683 mutations define a distinct acute lymphoblastic leukaemia subgroup that arises exclusively in the context of trisomy 21. The sensitivity of pro-B cells transduced with the R683S *Jak2* to pharmacological inhibition of the JAK/STAT pathway is of a potential therapeutic value.

#### Contributors

DB participated in mutation screening, design and analysis, and wrote parts of the report. IG participated in doing the BaF3, western blot, inhibitor, and colony-forming and replating assays; RT-PCR and sequence analysis of RNA samples; clinical data gathering and analysis, and writing parts of the report. LMS was involved in the BaF3 proliferation assays, sequence analysis of 96 V617F-negative patients with essential thrombocythaemia, and writing the report. GW and NBT were involved in modelling the JAK2 pseudokinase domain, and did the structural and evolutionary analysis of the mutations and model. YB was involved in doing the colony-forming and replating assays. AE participated in mutation screening and PCR subcloning. CS screened mutations and did the PCR subcloning. GCaz participated in the analysis of recurrent molecular genetic lesions and the minimal residual disease monitoring in Italian patients with Down's syndrome-associated acute lymphoblastic leukaemia. ABon was responsible for the genetic and minimal residual disease monitoring of all Italian childhood acute lymphoblastic leukaemia patients, and collected clinical data from Italian patients with Down's syndrome-associated acute lymphoblastic leukaemia. GB participated in the identification and characterisation of some patients, discussion of results, and critical revision of the report. GCar participated in collection and provision of patient bone marrow samples, and biological and clinical data, and contributed to data analysis. MSC mainly contributed to the data analysis and discussion of results. MSt participated in patient sample acquisition, data collection, and proofreading of the report. SS provided patient DNA and RNA

samples, participated in the analysis of cytogenetic and molecular genetic data, and critically reviewed the report. OAH provided DNA and RNA samples, and cytogenetic and clinical data. GM provided clinical data and critically read the report. VB and ABor allocated patient material and clinical information and data. HK participated in collection of bone marrow, DNA, and RNA samples, and clinical data. JT participated in sample and data collection, and processing of samples. BB provided bone marrow samples from patients and clinical data. SA provided the DNA samples. BS contributed bone marrow samples, and participated in cytogenetic and fluorescent *in situ* hybridisation analysis. OS provided patient bone marrow samples and assisted with writing of the report. ND supplied material that corresponded to the study criteria and did the cytogenetic analysis. JPB provided biological and clinical data, samples from patients with Down's syndrome-associated acute lymphoblastic leukaemia, and had a major contribution in writing the report. ARG supervised experimental work, provided reagents, and assisted with the writing of the report. SI designed and organised the study, and participated in data analysis and writing most of the report. All authors have seen and approved the final version of the report to be published.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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