A specific JAK2 mutation (JAK2R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukaemia

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**Running title:** Activating JAK2 mutation in Down syndrome ALL

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Abstract

Children with Down syndrome (DS) have a greatly increased risk of acute megakaryoblastic leukaemia (AMKL) and acute lymphoblastic leukaemia (ALL). Both DS-AMKL and the related transient myeloproliferative disorder (TMD) have GATA1 mutations as obligatory, early events. To identify mutations contributing to leukaemogenesis in DS-ALL we undertook sequencing of candidate genes including FLT3, RAS, PTPN11, BRAF and JAK2. Sequencing of the JAK2 pseudokinase domain identified a specific, acquired mutation, JAK2R683, in 12/42 (28%) DS-ALL cases. Functional studies of the common JAK2R683G mutation in murine Ba/F3 cells demonstrated growth factor independence and constitutive activation of the JAK/STAT signalling pathway. High resolution SNP array analysis of nine DS-ALL cases identified additional submicroscopic deletions in key genes including ETV6, CDKN2A, and PAX5. These results infer a complex molecular pathogenesis for DS-ALL leukaemogenesis, with trisomy 21 as an initiating or first hit and chromosome aneuploidy, gene deletions and activating JAK2 mutations as complementary genetic events.
Introduction

Children with Down syndrome (DS), characterised by constitutional trisomy 21, have a 50 fold increased risk of developing acute leukaemia in the first few years of life\(^1\). This comprises the normally extremely rare subtype acute megakaryoblastic leukaemia (AMKL) as well as the common variety (B-cell precursor) of acute lymphoblastic leukaemia (ALL)\(^2\). Both DS-AMKL and the transient myeloproliferative disorder (TMD) that often precedes it are consistently associated with acquired mutations in the \(GATA1\) gene\(^5\). Concordant \(GATA1\) mutations in the blast cells of identical twins with TMD\(^6\) and mutations in the neonatal blood spots of DS newborns\(^7\) indicate that this is an early/prenatal event in DS leukaemogenesis.

Much less is known about the genetic events predisposing to DS-ALL. Candidate genes for activating mutations in DS-ALL include \(FLT3\) and \(RAS\), both mutated in high hyperdiploid ALL (with acquired trisomy 21)\(^8\), \(PTPN11\) and \(BRAF\), mutated in B-cell precursor ALL\(^10\). An additional candidate is the \(JAK2\) pseudokinase domain mutation \(JAK2\DeltaIREED\), reported in a single case of DS-ALL\(^12\). A different \(JAK2\) pseudokinase domain mutation, \(JAK2\)V617F, is frequently found in the myeloproliferative disorders (MPD) and believed to be an initiating event\(^13-15\). Submicroscopic deletions involving genes linked functionally to deregulation of cell cycling or B cell differentiation have also been implicated in the molecular pathogenesis of ALL\(^16-19\). In this study we undertook both sequencing of candidate genes and high resolution SNP array analysis to identify genetic events associated with ALL in DS.

Materials and Methods

Patients
Patients’ samples (Table 1, S1) were obtained with informed consent in accordance with the Declaration of Helsinki and the study was carried out with ethical review committee approval from all participating institutions. DNA was extracted from archival (frozen cell or cytogenetic fixed pellet) leukaemic samples.

**Sequencing**

Primers were designed to amplify the following candidate genes: F1T3 ITD, F1T3 kinase domain, KIT kinase domain, PTPN11, BRAF, NRAS, and KRAS (Table S2A). Exons 12-23 of the JAK2 gene were PCR-amplified and sequenced using previously described primers13. All samples found to be mutated were PCR-amplified and sequenced in a second, independent experiment. Pyrosequencing was carried out in accordance to manufacturer’s instructions (Biotage AB, Upsala, Sweden) using the primers shown in Table S2B.

**Ba/F3 proliferation assay**

MSCV-neo-IRES-GFP murine wild type JAK2 and JAK2R683G constructs were transfected into GP2 packaging cells to produce retroviruses as previously described13. Ba/F3 cells were co-transduced with MSCV-puro-TpoR retrovirus and selected in media containing 2 mg/ml G418 and 0.5μg/ml puromycin, and further cultured in the presence or absence of IL-3 with or without 10μM tyrosine kinase inhibitor AG490 (Invitrogen).

**SNP arrays**

Leukaemic DNA from the patient’s bone marrow at presentation of ALL and remission DNA (as germline control) was genotyped with Affymetrix Human GeneChip Mapping 250K NspI and StyI arrays as previously described19,20. Array hybridization data were analyzed for loss of heterozygosity and signal intensity using the GOLF software program (for more details see
Results and Discussion

Sequencing of selected exons of the FLT3, KIT, PTPN11, BRAF, NRAS and KRAS genes in a series of ten DS-ALL cases did not identify any mutations (Table S1). Sequencing of JAK2 exon 14 in 42 cases of DS-ALL identified a point mutation at the conserved arginine (R683) affected by the IREED deletion\textsuperscript{12} in 12/42 (28\%) cases. Ten of these were an identical arginine to glycine (JAK2R683G) and two an arginine to serine (JAK2R683S) substitution. The mutation was acquired in the leukaemic blasts (Figure 1A). Pyrosequencing confirmed the mutations and allowed quantitation of the mutant allele. Sequencing of exon 14 in 41 non-DS ALL cases, including 23 of high hyperdiploidy (with acquired trisomy 21), did not identify any mutations in this group. Similarly, we did not identify any JAK2R683 mutations in 13 DS AMKL cases (Table 1). Seven DS-ALL cases without JAK2R683 mutations were also screened for mutations in the JAK2 kinase domain (exons 15 - 23). None were observed.

Preliminary structural modelling of the JAK2 pseudokinase domain suggests that the JAK2R683G/S mutations most likely alter the inter-domain interactions with the C-terminal kinase domain (Supplementary data S4. Functional studies indicate that the JAK2R683G mutation results in an activated kinase. Western blotting of Ba/F3 cells co-transduced with TpoR and JAK2R683G mutant showed constitutive phosphorylation of JAK2 and STAT5 proteins, whereas the wild type JAK2 did not (Figure 1B). The R683G mutation allowed IL-3 dependent Ba/F3 cells to survive and proliferate in the absence of IL-3 and this effect was abrogated in the presence of the JAK2 kinase inhibitor AG490 (Figure 1C). These data parallel the impact of the JAK2V617F mutation in MPD\textsuperscript{13-15}.
Cytogenetic analyses of our cases of DS-ALL, with or without the JAK2R683 mutation, indicated the presence of additional chromosomal lesions (Table S1). In common with other reports, our series had a lower than usual incidence of common fusion genes (e.g. ETV6-RUNX1) and high hyperdiploidy. There do not appear to be any cytogenetic features that distinguish the DS-ALL cases with the JAK2R683 mutation from those without.

High resolution SNP array analysis of nine DS-ALL cases identified between 1 and 13 regions of loss or gain per case (Table S3). Small focal deletions, often involving a single gene, were identified in many cases. The most common deletion (4/9 cases) involved the ETV6 gene (Figure S1). Focal deletions of other genes implicated in ALL pathogenesis included the CDKN2A (3/9 cases) PAX5 (2/9 cases) BTLA, EBF1, and RB1 genes (one case each). The pattern and number of microdeletions in this series, in particular the high incidence of ETV6 deletions, more closely resembled ETV6-RUNX1 positive ALL than other subgroups of ALL. There were no microdeletions unique to the JAK2 mutated cases.

Our data indicates that multiple genetic events including cytogenetically detectable chromosome aneuploidy, submicroscopic deletions of genes including ETV6, CDKN2A and PAX5, as well as activating JAK2 mutations can occur, collectively complementing constitutional trisomy 21 in DS-ALL. The functional studies of the JAK2R683G mutation indicate that this is most likely a ‘driver’ mutation in a subset of patients with DS-ALL but the sequence of acquired genetic events, in utero and post-natally, remains to be established. In one case in the present study (Patient 9, Table S1), the JAK2R683G mutation was acquired only at relapse. In another (Patient 10, Table S1) the mutation appeared to be subclonal, as pyrosequencing found 32% mutant compared with 99% blasts. These data indicate that the mutation can, in some cases, be a late or secondary event.

The absence (or rarity) of JAK2 mutations in non-DS B-cell precursor ALL, including cases with acquired trisomy 21, and the high risk of ALL in DS syndrome indicates that
constitutive trisomy 21 provides selective pressure within the early B cell lineage for the specific \textit{JAK2R683} mutation which is different from the \textit{JAK2V617F} activating mutation in a stem cell resulting in MPD\textsuperscript{14}. That this is less consistent than the obligatory \textit{GATA1} mutations in myeloid/erythroid progenitors in TMD and AMKL suggests alternative genetic changes may activate the JAK-STAT pathway in DS-ALL. This would be in accord with the essential role this pathway plays in early B cell development\textsuperscript{23}.

Children with DS and standard risk ALL have an inferior clinical outcome to non-DS patients\textsuperscript{24}. The occurrence of mutational activation of \textit{JAK2} in a substantial fraction of DS-ALL therefore may have potential therapeutic implications, as in the MPD with the \textit{JAK2V617F} mutation\textsuperscript{15}.

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\textbf{Author contributions}

L.K. and M.G. designed the research and wrote the paper. A.M.F. supervised the research, analyzed the data and wrote the paper. D.G.D.C, J.Y, J.P, M.E-I, S.W.H, C.M.B, K.A and T.C performed the research. L.K, D.G.D.C and S.W.H analyzed the data. B.D.Y, C.J.H,
H.K. and C.W.E.S. provided expertise and/or vital analytical tools or reagents. All authors critically reviewed the manuscript.

**Conflict of interest statement**

The authors have no relevant financial conflict of interest to disclose.
References


Figures and Tables:

**Table 1.** Childhood leukaemia cases sequenced for exon 14 *JAK2* mutations

**Figure 1.** *JAK2*R683G is an activating mutation in DS-ALL. (A) Sequence analysis showing the *JAK2*R683G mutation in a DS-ALL patient. At the top, sequence obtained from bone marrow DNA at presentation and at the bottom, complete remission. The A-G substitution is shown by an arrow. During remission the sequence was identical to wild type, demonstrating that the mutation is acquired. (B) Western blotting analysis of JAK2 and STAT5 protein in IL-3-dependent Ba/F3 cells. Extraction and Western blotting analyses were performed using standard protocols. Antibodies against JAK2 and phospho-JAK2 (p-JAK2) and phospho-STAT5 (p-STAT5) were from Cell Signalling Technology (New England Biolabs, Hitchin, UK). Lane 1 = untransformed Ba/F3 cells, lane 2 = empty vector control (v) (with neo and puro), lane 3 = empty vector + TpoR, lane 4 = wild type (wt) *JAK2* transformed Ba/F3 cells, lane 5 = *JAK2*R683G mutant transformed Ba/F3 cells. (C) Ba/F3 proliferation after IL-3 withdrawal. The *JAK2*R683G mutation was generated in the IMAGE clone 6838318 containing murine *JAK2* cDNA using site-directed mutagenesis (Quikchange-XL, Stratagene) and confirmed by full-length DNA sequencing. Ba/F3 cells were co-transduced with thrombopoietin receptor (TpoR) and wild type or mutant (R683G) *JAK2*. Cells were washed three times in PBS and cultured at $1 \times 10^5$/ml in the absence of IL-3 for 6 days with or without the *JAK2* inhibitor AG490. Cell numbers and viability were assessed in duplicate after Trypan Blue exclusion staining.
Table 1. Childhood leukaemia cases sequenced for exon 14 JAK2 mutations

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number</th>
<th>JAK2R683G</th>
<th>JAK2R683S</th>
<th>JAK2 WT</th>
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<tr>
<td>DS-ALL†</td>
<td>42</td>
<td>10</td>
<td>2</td>
<td>30</td>
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<tr>
<td>BCP-ALL (HeH)</td>
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<td>0</td>
<td>0</td>
<td>41 (23)</td>
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<tr>
<td>DS-AMKL</td>
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<td>0</td>
<td>0</td>
<td>13</td>
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</tbody>
</table>

†GATA1 mutation status unknown; 4 cases with matched remission DNA for sequencing, 7 with matched remission DNA for SNP arrays (see Table S1, S3 for details). DS-ALL = Down syndrome ALL; HeH = high hyperdiploidy; BCP-ALL = B cell precursor / common ALL (cALL); DS-AMKL = Down syndrome acute megakaryoblastic leukaemia; WT = wild type
Figure 1

A

Presentation

Remission

B

p-JAK2

JAK2

p-STAT5

STAT5

C

Viable cells per mL

Days after IL-3 withdrawal
A specific JAK2 mutation (JAK2R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukaemia

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