Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia

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Short title: iAMP21 as a primary event in BCP-ALL

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Abstract

Intrachromosomal amplification of chromosome 21 (iAMP21) defines a distinct subgroup of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) with a dismal outcome on standard therapy. For improved diagnosis and risk stratification, the initiating genetic events need to be elucidated. To investigate the genetic basis, genomes of 94 iAMP21 patients were interrogated by arrays, fluorescence in situ hybridization and Multiplex Ligation-dependent Probe Amplification. Most copy number alterations targeted chromosome 21, reinforcing the complexity of this chromosome. The common region of amplification on chromosome 21 was refined to a 5.1 Mb region; including RUNX1, miR-802 and genes mapping to the Down Syndrome Critical Region. Recurrent abnormalities affecting genes in key pathways were identified; IKZF1 (22%), CDKN2A/B (17%), PAX5 (8%), ETV6 (19%) and RB1 (37%). Investigation of clonal architecture provided evidence that these abnormalities, and P2RY8-CRLF2, were secondary to chromosome 21 rearrangements. Patient outcome was uniformly poor on standard therapy irrespective of the presence or absence of these changes. This study has provided evidence that chromosome 21 instability is the only anomaly amongst those so far investigated that is common to all iAMP21 patients. Thus, the initiating event is likely to be found among the complex structural rearrangements of this abnormal chromosome.
Introduction

Chromosomal changes are important in diagnosis and prediction of outcome in acute lymphoblastic leukemia (ALL). A number of clinically relevant abnormalities are used in risk stratification for treatment within the context of clinical trials. One such abnormality is iAMP21 (intrachromosomal amplification of chromosome 21) that occurs at an incidence of approximately 2% in older children with B-cell precursor ALL (BCP-ALL). iAMP21 was originally identified as a consequence of routine screening for the presence of the ETV6-RUNX1 (TEL-AML1) fusion by fluorescence in situ hybridization (FISH) and is defined by multiple copies of the RUNX1 gene. In interphase nuclei, patients exhibit 3 or more additional RUNX1 FISH signals, which are duplicated along the arms of an abnormal chromosome 21. Importantly, iAMP21 has been shown to be linked to a dismal outcome on standard therapy, associated with an increased risk of both early and late relapses.

Currently, FISH with probes directed to the RUNX1 gene provides the only reliable detection method. Thus, improved diagnostic approaches are required for accurate risk stratification of these patients.

Using cytogenetics, FISH and BAC array-based comparative genomic hybridization (aCGH), we have previously demonstrated the complex and heterogeneous nature of the chromosome 21 structure in patients with iAMP21. A 6.6 Mb common region of amplification (CRA) on chromosome 21 containing RUNX1 and a 3.3 Mb common region of deletion (CRD) at the telomere were identified in 100% and 77% of patients, respectively. We also showed that microarray-based global gene expression profiling of 8 iAMP21 patients did not detect differential expression of any biologically relevant genes on chromosome 21, or specifically within the CRA or CRD, when compared with other ALL subtypes. Although these findings assisted in the genomic characterization of iAMP21, the relevant genetic mechanisms leading to the development of ALL in these patients remain unknown.

We have recently reported a genomic alteration involving deletion within the pseudoautosomal region (PAR1) of the sex chromosomes. It leads to a P2RY8-CRLF2
fusion, resulting in overexpression of the type 1 cytokine receptor, \textit{CRLF2} \textsuperscript{10-12}. This abnormality has been found at a high incidence of 38\% in iAMP21 patients \textsuperscript{10}. High-resolution array-based whole-genome studies have identified recurrent deletions in childhood BCP-ALL, which disrupt a number of genes with important roles in B-cell differentiation and cell cycle regulation. These deletions contribute directly to the pathogenesis of BCP-ALL \textsuperscript{13-15}. Of particular note was the observation that focal deletions involving the \textit{IKZF1} (Ikaros) gene were associated with a poor outcome in high-risk childhood BCP-ALL \textsuperscript{16}. However, the incidence and involvement of \textit{IKZF1}, as well as the other recurrent abnormalities, have not been fully investigated within individual ALL subtypes, including iAMP21.

In this study we have expanded our previous investigations to include a more detailed analysis of the iAMP21 genome in a cohort of 94 patients and demonstrate that chromosomal instability of chromosome 21 is the likely primary genetic event.

\section*{Patients and Methods}

\textbf{Patients}

All patients had a diagnosis of BCP-ALL confirmed by morphology and immunophenotyping. Informed consent was obtained in accordance with the Declaration of Helsinki, and approval was granted by the institutional review boards of all participating institutions. Diagnostic cytogenetics were performed in regional cytogenetics laboratories and data were collected centrally by the Leukaemia Research Cytogenetics Group (LRCG) as previously described \textsuperscript{17}. A total of 94 patients were investigated; 78 at diagnosis and 15 at both diagnosis and relapse (Supplementary Table 1).

\textit{Fluorescence in-situ hybridization (FISH) and Multiplex Ligation-dependent Probe Amplification (MLPA)}

These patients with iAMP21 were identified using the dual color probe kit, LSI TEL/AML1 ES (Abbott Diagnostics, UK), designed to detect the translocation,
t(12;21)(p13;q22) or specific probes targeting the RUNX1 gene, as previously described\(^2,6\). A cut off value of 3 or more additional RUNX1 signals was used to define iAMP21. In those cases with a normal karyotype or failed cytogenetic result, with no metaphases available to confirm the presence of the abnormal chromosome 21, FISH using a probe specific for the subtelomeric region of chromosome 21 (TelVysion21q, Abbott Diagnostic, Maidenhead, UK) was used to determine that the extra RUNX1 signals had not arisen from the gain of intact copies of chromosome 21 as might be seen in high hyperdiploid karyotypes. FISH was also used to exclude the presence of the established chromosomal abnormalities, BCR-ABL1, ETV6-RUNX1 and MLL rearrangements\(^5\). Copy number of chromosome X was determined using specific centromere probes (Cytocell, Banbury, UK). Deletions of CDKN2A, IKZF1, PAX5 and the PAR1 region, resulting in P2RY8-CRLF2, were tested for by FISH. All FISH probes were grown, hybridized and analyzed as previously described\(^18\). Deletions within these genes and others were also detected using the SALSA MLPA kit P335-A1 (MRC Holland, Amsterdam, Netherlands) as previously described\(^19\). Deletions of exons 1-4 of ETV6 were also detected using the LSI TEL/AML1 ES probe. Evidence of clonal heterogeneity was investigated using FISH probes targeting RUNX1 to identify those cells with the abnormal chromosome 21 characteristic of iAMP21, together with IKZF1, PAX5 or P2RY8-CRLF2. The FISH probes used in this study are listed in Supplementary Table 2.

**Genomic oligonucleotide arrays (aCGH)**

DNA was extracted from diagnostic bone marrow samples of 17 of the 94 diagnostic iAMP21 patients and 1 relapse sample using the DNeasy blood and tissue kit (Qiagen, USA). Array CGH was performed on an Agilent Human Genome CGH Microarray kit 185A (Agilent Technologies, UK) as per the manufacturer’s instructions. Data were processed and analyzed as previously described\(^10\) and further analysis were performed using custom software SNPView2 (Ilixa, UK) and Partek Genomics Suite (Partek, USA). Data is available to download from Gene Expression Omnibus (GSE26192).
**RUNX1 and JAK mutational analysis**

Primers designed to cover the *RUNX1* coding exons (exons 2-7) and the *JAK* gene mutational hotspots: *JAK1* (exons 13 and 14), *JAK2* (exons 14, 18 and 19) and *JAK3* (exon 18) were used to amplify genomic DNA sequences from 14 diagnostic and 1 relapse samples (Supplementary Table 2). The amplicons were screened for mutations by denaturing high-performance liquid chromatography (DHPLC) using a Transgenomic Wave system. Amplicons with potential mutations were validated by direct Sanger sequencing using BigDye Terminator sequencing chemistry (Applied Biosystems, UK).

**miR-802 expression analysis**

Total RNA was isolated using the TRIzol method (Invitrogen, UK) from patients with: iAMP21 (n=2), gain of chromosome 21 (n=6), *ETV6-RUNX1* fusion (n=5), unclassified karyotypes with no known established abnormalities (n=6) and a normal karyotype (n=1). MicroRNA expression levels were assessed by quantitative real-time PCR (qPCR) using Taqman MicroRNA assays (Applied Biosystems, UK) and the 7900HT Real-Time PCR instrument (Applied Biosystems, UK). Each sample was analyzed in triplicate in 3 independent replicates. The comparative Ct (ΔΔCt) method was used to assess the expression level of *mir-802* relative to 2 endogenous controls, *RNU6B* and *RNU43*, and each test sample was normalized to the mirVana miRNA Reference Panel v 9.1 (Applied Biosystems, UK).

**Statistical analysis**

Event-free survival (EFS) was measured as the time from diagnosis to first adverse event (death or relapse). An event was described as early if it occurred whilst the patient was on treatment (2 years for girls and 3 years for boys) or 6 months after the end of treatment. A late event refers to any time after this 6 month period. EFS was estimated using the Kaplan-Meier method, as previously described. Hazard ratios comparing outcome between subgroups were estimated using univariate Cox models. All tests were conducted
at the 1% significance level. All analyses were done using Intercooled Stata 11.0 for Windows.

Results

Demographic, clinical and cytogenetic details of patients with iAMP21

This study included 94 BCP-ALL patients defined as iAMP21 using our specified criteria of 3 or more additional RUNX1 signals (Supplementary Table 1). The cohort consisted of 44 females and 50 males with a median age of 9.5 years (range 2-30 years). Their median white blood cell count was 4.6 x 10^9/L (range 0.5-226 x 10^9/L). Cytogenetic analysis was attempted on all 94 diagnostic samples. In 17 patients (18%) analysis failed, while 8 (8.5%) had a normal karyotype, including one with a constitutional translocation (8987). Apart from 1 patient with 52 chromosomes, all abnormal karyotypes were near-diploid (45-48 chromosomes) with visible abnormalities of chromosome 21 or loss of one copy of chromosome 21 plus an unidentified marker chromosome. The abnormal chromosome 21 was described according to its morphology, as duplicated (dup) or ring (r) etc, and the karyotypes were written in accordance with the International System for Human Cytogenetic Nomenclature (ISCN, 2009) 21. In 12 patients the abnormal chromosome 21 was the sole visible cytogenetic abnormality, while 15 patients had a simple karyotype comprising 1 abnormality in addition to the abnormal chromosome 21. The remaining 42 patients showed karyotypes with 3 or more abnormalities. The most frequently observed additional abnormality was gain of an X chromosome in 13 patients.

Genomic analysis of chromosome 21

To identify a common abnormality which may be the initiating event giving rise to iAMP21, we initially focused our investigations on chromosome 21. High-resolution aCGH analysis of 17 iAMP21 diagnostic and 1 relapse sample identified a mean of 4.8 (range 2-10) copy number abnormalities (CNA) involving chromosome 21 per patient (Supplementary Table 3). Further detailed analysis of the probes mapping to chromosome 21 indicated a
high level of complexity indicative of chromosomal instability of this chromosome (Figure 1a). The aCGH profiles of 2 patients showed amplification in a single step with no evidence of a telomeric deletion, while the majority showed a step-wise increase in copy number from the centromere towards the telomere followed by a sharp decrease at the telomere (Figure 1b). In 4 patients the profile was highly complex with 9 or 10 abnormalities mapping to chromosome 21. The CRA, as defined by patient 8983, was refined from 6.6 Mb to 5.1 Mb spanning from 32,813,553 to 37,941,425 bp (Figures 1a and b). This region included 47 known protein-coding genes, including RUNX1 and genes located within the Down Syndrome Critical Region (DSCR), 20 pseudogenes, 18 non-coding RNAs and a single microRNA, miR-802 (Supplementary Table 4). Expression levels of miR-802, 1 of 5 microRNAs mapping to chromosome 21, were determined in 2 iAMP21 patients and compared to 18 ALL patients from different cytogenetic subtypes. Despite an obvious increase in copy number in the iAMP21 patients, this did not translate into a relative increase in expression in iAMP21 patients when compared with the other subtypes. In fact the expression of miR-802 was low among all patients tested, although no statistical analysis could be reliably performed owing to low numbers (Supplementary Figure 1).

A decrease in copy number at the telomere of chromosome 21 occurred in 88% (16/18) of iAMP21 patients resulting in a region of deletion or normal copy number at the telomere, with the breakpoint locations spanning a 9 Mb region from within the KCNJ6 gene, at position 37,941,425 bp, to the telomere (Figure 1c). The region common to the 16 patients (termed CRD) was defined by patient 7045, with the smallest telomeric deletion of 283.8 kb. The breakpoint mapped to a 13.75 kb region (46,646,724 to 46,660,477 bp) and occurred within the PCNT gene (Supplementary Table 3). Notably, identical breakpoints were identified within 2 genes in 2 patients each, PDE9A (4279, 6111) and COL6A2 (7583, 9028). Breakpoints were also identified in the DSCAM and PCNT gene in 3 (4414, 6996, 7255) and 2 (7045, 7732) patients, respectively, although at different genomic positions within both genes.
No evidence of RUNX1 mutations in patients with iAMP21

As the RUNX1 gene was always found to be present in the CRA of patients with iAMP21 and abnormalities of this gene are common in leukemia, we searched for mutations in the coding exons 2-7 of RUNX1. No mutations were found in 15 patients (14 diagnostic and 1 relapse sample).

Investigation of recurrent events throughout the genome of patients with iAMP21

A search for other recurrent events elsewhere within the genome of patients with iAMP21 was carried out on the 18 patients analyzed by aCGH (Figure 2a). In addition to the 4.8 CNA involving chromosome 21, a mean of 8.7 (range 1-18) CNA were identified per patient (excluding common CNVs including those involving the antigen receptor loci) (Supplementary Table 3). Overall, deletions were more frequent than gains, with 64 regions involving focal deletions (<1 Mb) among the autosomes compared to only 11 regions of focal gain. The gain of chromosome X was the only whole chromosome gain observed by aCGH, in agreement with the finding from cytogenetic analysis. Recurrent deletions involving genes known to regulate B-cell differentiation and cell cycle were detected, including deletions of CDKN2A/B (9p21.3) (n=4), IKZF1 (7p13) (n=4) and PAX5 (n=2). The IKZF1 and PAX5 deletions were heterozygous, while CDKN2A deletions were more complex with some patients having sub-clones with either heterozygous or homozygous deletions as confirmed by FISH and/or MLPA (Supplementary Figure 2). Deletions were also detected by aCGH in other genes previously associated with leukemia, namely RB1 (n=4) and ETV6 (n=4), but also in regions containing poorly characterized genes, such as TP53TG3 on chromosome 16 (n=5).

As our global analysis identified recurrent abnormalities affecting genes in key pathways, we screened available samples from the full cohort of 94 iAMP21 patients using a combination of FISH and MLPA to determine their frequency. As shown in Table 1, RB1 deletions were present at the highest incidence of 37%, whereas PAX5 deletions occurred at the lowest incidence of 8%. We also determined that the incidence of P2RY8-CRLF2 by
FISH and MLPA was 22%. Gain of chromosome X was confirmed in 24% of patients; 6 also had $P2RY8$-$CRLF2$. We showed that these abnormalities were not mutually exclusive (Supplementary Table 1).

**No evidence of mutations in the JAK gene family members in patients with iAMP21**

Due to the previously reported strong association between $P2RY8$-$CRLF2$ and JAK2 mutations, we screened the mutation hotspots of the JAK gene family. No mutations were found in the 15 patients tested.

**Clonal heterogeneity of associated abnormalities indicates that iAMP21 is a primary genetic event**

The significance of the abnormalities associated with iAMP21 was further explored by comparing the diagnostic and relapse paired samples from 9 patients (Supplementary Table 1). The same FISH probes and MLPA kit used to determine the incidence of deletions of $IKZF1$, $PAX5$, $CDKN2A$ and the presence of $P2RY8$-$CRLF2$ at diagnosis were used to screen the matched relapse samples. Evidence of de novo deletions of these genes at relapse was detected in 2 patients; patient 4780 gained a deletion of $IKZF1$, while patient 10542 gained deletions of both $CDKN2A/B$ and $PAX5$ due to the formation of an isochromosome 9, as well as $P2RY8$-$CRLF2$. This isochromosome, i(9)(q10), is a known recurrent abnormality in ALL providing a mechanism of gene deletion, for this patient and two others in the series (3382, 19578) with corresponding deletions of $PAX5$ and $CDKN2A$. Patients 7255 and 4561 had deletion of $IKZF1$ and $P2RY8$-$CRLF2$ fusion, respectively, at diagnosis that were retained at relapse. In patient 4316 the $P2RY8$-$CRLF2$ fusion and $PAX5$ deletion seen at diagnosis were not observed at relapse; this finding was possibly due to a low blast count in the relapse sample. None of these abnormalities were present in the remaining patients tested.

Clonal architecture was investigated among 19 patients that had one of the abnormalities specified above in association with iAMP21. A dual color FISH approach was
designed to detect amplification of RUNX1, as an indication of the presence of iAMP21, and a second abnormality simultaneously (Supplementary Table 1). Examples of the populations detected using dual probes to detect RUNX1/iAMP21 and the presence of P2RY8-CRLF2 and IKZF1, respectively, are shown in Figures 3a and b. Of the patients tested, 3 of them showed evidence of mixed populations with and without the second abnormality (Figure 3c).

In a single sample, 7219, it was possible to predict the temporal order in which the genetic events occurred by studying the clonal architecture as determined by FISH. From sample 7219, we postulated that iAMP21 was the primary event with IKZF1 deletions and P2RY8-CRLF2 occurring later (Figure 3c). Although accounting for only small numbers of patients, these results indicate clonal heterogeneity within the iAMP21 samples, suggesting that these abnormalities occur as secondary events to iAMP21.

**No association between abnormalities and time to relapse**

In the childhood ALL treatment trial, ALL97, we had noted not only a high incidence of relapse among iAMP21 patients, but that these events occurred early (whilst the patients were on treatment and the first 6 months after) and late (6 months or more after the end of treatment) 20. Thus we examined the time to relapse within the cohort of 33 patients with follow-up data from this trial in relation to the associated abnormalities (Table 1). There was no difference in time to relapse relating to the presence of these abnormalities.

**Discussion**

Rearrangements targeting chromosome 21 are the hallmark of childhood BCP-ALL with iAMP21 and detailed analysis of chromosome 21 has demonstrated that they are highly complex and heterogeneous in structure. Over a quarter of copy number alterations detected by aCGH targeted chromosome 21 and included telomeric breakpoints in 88% of patients. The majority of patients showed a step-wise amplification of their chromosome 21 aCGH profile, frequently interspersed by additional regions of genomic gain and loss to varying levels of complexity, culminating in a decrease in copy number at the telomere. In this study,
we refined the CRA to 5.1 Mb; including the RUNX1 gene, miR-802 and genes mapping to the Down Syndrome Critical Region. Although RUNX1 is a well known leukemia related gene, it does not appear to be the target of iAMP21 for the following reasons: no breakpoints were detected within the gene by aCGH; no RUNX1 mutations were detected as also indicated by others; RUNX1 did not show significant over-expression compared to other BCP-ALL subtypes in our previous transcriptome analysis.

We showed that miR-802 expression was low in patients with iAMP21; with no relative difference in expression seen when compared with different BCP-ALL cytogenetic subtypes. This corresponds to our previous findings of no differential expression of genes within the CRA by gene expression profiling. The ratios of fold changes corresponding to genes within the CRA and CRD comparing iAMP21 patients with other ALL subtypes, those with ETV6-RUNX1 fusion (rearrangement and, in some cases, gain of whole or part of chromosome 21), high hyperdiploidy (one or more additional copies of chromosome 21) and unclassified patients (with no established chromosomal abnormalities) from this publication are summarised in Supplementary Table 4. Although patient numbers were low, it also appears that miR-802 is also not the target.

The overlap between the CRA and the DSCR at 21q22.3 is intriguing as 2 other abnormalities were also detected at high incidence in iAMP21 patients that mirror findings in DS-ALL; gain of the X chromosome and the presence of P2RY8-CRLF2 in 24% and 22% of patients, respectively. The presence of both abnormalities in association with iAMP21 was confirmed in this study and had been previously reported. Somatically acquired activating mutations in the Janus kinase, JAK2, have been identified in DS-ALL at a high incidence of 18%-28%, however we found no mutations in the hotspots of JAK1, JAK2 or JAK3 in patients with iAMP21. In view of the genetic changes in common with DS-ALL, other than JAK mutations, the eventual understanding of the underlying mechanism may emerge from more detailed analysis of similarities and differences between iAMP21 and DS-ALL.
In addition to the CRA, we have more accurately defined the CRD, previously described by FISH and BAC aCGH, to be a 283 kb region. Although this deleted region encompasses 3 coding genes, PRMT2, DIP2A and S100B, recognized in a number of different diseases and significant pathways, telomeric deletions were not detected in all patients with iAMP21, thus they are unlikely to be the target of this abnormality. Nevertheless, the higher resolution aCGH profiles of this study support our previous proposal: that iAMP21 arose from a series of breakage-fusion-bridge (BFB) cycles. Detailed mapping of the CRD breakpoints, the most likely site of the first BFB event, showed that they were variable; with only 2 genes, PDE9A and COL6A2, having identical, recurrent breakpoints in 2 cases each. Recently, the inverted repeat structure or the “fold back inversions” characteristic of BFB has been reported in pancreatic cancer. Thus it is interesting to speculate whether the telomeric deletion and the sequence architecture surrounding it represent the initiating event in iAMP21.

The number of CNA throughout the genome, excluding chromosome 21, was comparable to other ALL subtypes. Global analysis by genomic arrays identified recurrent abnormalities affecting genes in key pathways and the frequencies were determined in a larger cohort by FISH and/or MLPA; IKZF1 (22%), CDKN2A/B (17%), PAX5 (8%), ETV6 (19%) and RB1 (37%). The incidences of IKZF1, CDKN2A/B and PAX5 are comparable to other BCP-ALL subtypes, thus are unlikely to be the initiating events in iAMP21 patients. ETV6 and RB1 abnormalities have previously been associated with ALL, specifically; focal deletions of ETV6 were previously described in iAMP21 patients. In this study, we showed that RB1 deletions occurred at an incidence of 37%, compared to ~9% in BCP-ALL overall, highlighting a strong association with iAMP21.

There is increasing awareness of clonal heterogeneity among genetic changes in ALL. We studied the genetic architecture at the subclonal level in relation to iAMP21 and saw similar heterogeneity in these patients as that observed in ETV6-RUNX1 patients. From the examination of paired diagnostic and relapse samples, we demonstrated that P2RY8-CRLF2, IKZF1 or PAX5 together with CDKN2A present at relapse in 3 patients were
not observed at diagnosis, although cells with a high incidence of iAMP21 were observed in both samples. Secondly, we used a FISH approach to study iAMP21 and associated abnormalities within the same cells. Although only observed in a small number of cases, the finding of mixed populations of iAMP21 positive cells both with and without IKZF1 or PAX5 deletions or P2RY8-CRLF2 indicated the presence of clonal heterogeneity. Taken together, these observations provide conclusive evidence that, at least in some cases, deletions arise as secondary events to the formation of iAMP21. From the FISH analysis of one patient, 7219, we were able to demonstrate the clonal architecture of the diagnostic sample and generate a model predicting the temporal order of genetic events: iAMP21 as the primary abnormality followed by deletion of IKZF1, then thirdly the generation of P2RY8-CRLF2. Thus, this limited sequential FISH analysis has contributed to our knowledge of the sub-clonal, genetic architecture of leukemic cells.

This study has provided convincing evidence that chromosomal instability of chromosome 21 is the only recurrent abnormality amongst those so far investigated, which is common to all patients with iAMP21. The lack of any other consistent abnormality outside chromosome 21, detectable at the resolution of aCGH, reinforces the assertion that iAMP21 is the likely primary genetic event. Until whole genome screens of such cases become available, it cannot be excluded that some other, currently cryptic, abnormality precedes iAMP21. In addition, the observation that the frequency of secondary abnormalities (especially IKZF1 deletions) is broadly similar to other genetic subtypes and that, in this small study, their presence is not related to time to relapse strengthens our conclusion that the poor outcome of this subgroup is likely driven by iAMP21 itself. As no individual gene has yet emerged as a candidate, the initiation of iAMP21 is likely hidden within the complex structural rearrangements of the abnormal chromosome 21. While this conclusion may not be novel, the evidence is now more compelling. In the meantime focus will be on improved diagnosis of iAMP21 in order to identify those patients in which modified therapy may improve outcome and ensure that patients at reduced risk of relapse are not exposed to unnecessary intensive treatment.
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Authorship Contribution

VR and HP contributed equally to this work. CJH and JCS share senior authorship. VR and CJH designed the research and wrote the paper. HP, LJ, CS, HE, JI, LJ, DM, LM, HM, SR, HR, PS, AVM and JCS designed some aspects of the research, carried out the experiments and/or analyzed the data. All authors critically reviewed the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

References


Table 1: Incidence of recurrent abnormalities detected by FISH and/or MLPA in diagnostic samples of iAMP21 patients

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<td>N</td>
<td>22</td>
<td>62</td>
<td>6</td>
<td>5</td>
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<tr>
<td>D</td>
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<td>37</td>
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<td><strong>+X</strong></td>
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<tr>
<td>A</td>
<td>63</td>
<td>76</td>
<td>27</td>
<td>22</td>
<td>7</td>
<td>15</td>
<td>20%</td>
<td>1.22 (0.42,3.56)</td>
<td>0.72</td>
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<tr>
<td>P</td>
<td>20</td>
<td>24</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>20%</td>
<td></td>
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<tr>
<td><strong>P2RY8-CRLF2</strong></td>
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<tr>
<td>A</td>
<td>61</td>
<td>78</td>
<td>17</td>
<td>14</td>
<td>6</td>
<td>8</td>
<td>27%</td>
<td>0.52 (0.15,1.81)</td>
<td>0.3</td>
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<tr>
<td>P</td>
<td>17</td>
<td>22</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>40%</td>
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No., number of patient samples tested; %, percentage of patient samples tested; Early relapse, relapse whilst on treatment or up to 6 months after the end of treatment; Late relapse, 6 months or more after the end of treatment; EFS, event free survival; N, no evidence of deletion within gene; D, deletion within gene; A, abnormality is absent; P, abnormality is present; -, too few samples for statistical analysis.
Figure 1: Summary of chromosome 21 abnormalities in 18 iAMP21 patients

(a) Heatmap of chromosome 21 abnormalities detailing the regions of deletion (red), gain (green) and normal copy number (white) relative to genomic location. The CRA, as defined by sample 8983, is highlighted and (R) denotes that 4279 is a relapse sample. (b) Examples of chromosome 21 profiles from 3 iAMP21 patients that clearly demonstrate the complexity, and diversity of abnormalities targeting this chromosome. Sample 9864 has a single region of amplification and no telomeric deletion, sample 7583 has a characteristic step-wise profile with a telomeric deletion and sample 8983 demonstrates a complex step-wise profile with interspersed regions of deletion. The CRA was defined by sample 8983, which is highlighted by a green bar. (c) Heatmap showing the telomeric breakpoints (indicated by a change from dark to light red) on chromosome 21 for the 18 patients profiled by aCGH. The region spans ~9 Mb and the most centromeric and telomeric genes with breakpoints, their relative location to the heatmap and the corresponding samples are shown; KCNJ6 (black) and PCNT (orange). Recurrent breakpoints were identified in 4 genes: PDE9A (purple) and COL6A2 (blue) had identical breakpoints but the breakpoints in DSCAM (green) and PCNT (orange) occurred at different genomic positions.

Figure 2: Global abnormalities identified in the iAMP21 genome as detected by aCGH.

Idiograms indicating CNAs detected in 18 iAMP21 patients. Each vertical line represents a genomic abnormality identified in a single patient and the length indicates the extent and position of each CNA. Deletions are shown in red and gains in green.

Figure 3: FISH evidence that P2RY8-CRLF2, IKZF1 and PAX5 are secondary events to iAMP21 and a putative model showing the clonal architecture of sample 7219.

(a) Five interphase nuclei from patient 7219 hybridized with a probe targeting RUNX1 (red), which detects the multiple copies of this gene that defines iAMP21, and a probe targeting CSF2RA and IL3RA, centromeric to CRLF2, which when deleted indicates the presence of
P2RY8-CRLF2 (green). The example shows evidence of clonal heterogeneity; populations of iAMP21 positive cells both with and without P2RY8-CRLF2 are indicated. (b) Three interphase nuclei from patient 7219 hybridized using the same RUNX1 probe (red) and a probe for IKZF1 (green) showing iAMP21 positive cells both with and without the IKZF1 deletion. (c) Examples showing data which indicates subclonal architecture in 3 patients: 20684, 7219 and 4444. In patients 7219 and 20684, 9% and 6% of cells, respectively, had amplification of RUNX1 with no deletion of IKZF1, while 79% and 89%, respectively, showed an IKZF1 deletion. In patient 4444, 83% of cells had amplification of RUNX1 with no PAX5 deletion, while 10% of cells showed the deletion. The major clone (53%) detected in patient 7219 showed iAMP21 without P2RY8-CRLF2, while 39% showed the P2RY8-CRLF2 fusion. These observations provide evidence that these events are secondary to iAMP21. (c) A putative model predicting the temporal order of events in the diagnostic sample from patient 7219. Apparent linear architecture with 3 populations indicating that iAMP21 is the primary event followed by deletion of IKZF1 and the presence of P2RY8-CRLF2.
Figure 3

a. RUNX1/P2RY8-CRLF2
   iAMP21 P2RY8-CRLF2
   iAMP21 No P2RY8-CRLF2

b. RUNX1/IKZF1
   iAMP21 IKZF1 normal
   iAMP21 IKZF1 deletion

c. 20684
   iAMP21 6% → iAMP21 IKZF1 del 89%
   4444
   iAMP21 83% → iAMP21 PAX5 del 10%
   7219
   iAMP21 53% → iAMP21 P2RY8-CRLF2 39%
   7219
   iAMP21 9% → iAMP21 IKZF1 del 79%

d. 7219
   iAMP21 100%
   iAMP21 100%
   7219
   iAMP21 IKZF1 del
   iAMP21 100%
   iAMP21 100%
   iAMP21 IKZF1 79%
   iAMP21 IKZF1 79%
   iAMP21 P2RY8-CRLF2 39%
Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia