Loss of Heterozygosity in Childhood Acute Lymphoblastic Leukemia Detected by Genome-Wide Microarray Single Nucleotide Polymorphism Analysis

Julie A.E. Irving, Lisa Bloodworth, Nick P. Bown, Marian C. Case, Linda A. Hogarth, and Andrew G. Hall

Northern Institute for Cancer Research and Institute for Human Genetics, Newcastle University, Newcastle, United Kingdom

Abstract

Loss of heterozygosity (LOH) is detectable in many forms of malignancy, including leukemia, using techniques such as microsatellite analysis and comparative genomic hybridization. However, these techniques are laborious and require the use of relatively large amounts of DNA if the whole genome is to be examined. Here we describe the use of oligonucleotide microarrays to characterize single nucleotide polymorphisms (SNPs) in lymphoblasts isolated from children with acute lymphoblastic leukemia for the pan-genomic mapping of LOH with a resolution of 100 to 200 kb. Results were compared with DNA obtained during remission and on relapse. Abnormalities were seen in 8 of 10 cases. The two cases with no abnormalities and one case that showed identical changes at relapse and presentation remain in remission 1 to 9 years following retreatment. The remaining seven patients died following relapse. In four cases, LOH was only detectable at relapse suggesting that progressive LOH may be a cause of disease progression and/or drug resistance. This was supported by detailed analysis of one case in which LOH involving the glucocorticoid receptor was associated with mutation of the remaining allele. The most frequent abnormality detected involved chromosome 9p. In each of the four cases where this was observed LOH included the INK4 locus. In three of the four cases, INK4 loss was only observed at relapse, suggesting that this abnormality may be commonly associated with treatment failure. These observations show that SNP array analysis is a powerful new tool for the analysis of allelic imbalance in leukemic blasts. (Cancer Res 2005; 65(8): 3053-8)

Introduction

Loss of heterozygosity (LOH) is frequently observed in malignant cells and contributes to the deregulation of cell division and apoptosis through the deletion of tumor suppressor genes (1). Allelic imbalance may be detected by a variety of methods including karyotyping, comparative genomic hybridization, and microsatellite analysis, but these are either of low resolution or laborious to conduct on a genome-wide scale. The recent introduction of oligonucleotide microarrays designed for the genome-wide typing of single nucleotide polymorphisms (SNPs) facilitates detailed mapping for LOH using small amounts of DNA (2, 3). In this study, we describe the use of this technique to characterize progressive LOH in samples from children with acute lymphoblastic leukemia (ALL) who relapse after chemotherapy.

Materials and Methods

Samples. Ten patients were selected for study from our local tumor bank on the basis of the availability of cells obtained at presentation, during remission, and at relapse. Samples were collected and used according to local ethical guidelines (reference no. 2002/111). Mononuclear cells were purified by differential centrifugation before storage at −80°C. Samples obtained at presentation and relapse were shown to have >95% blasts by morphologic examination of stained cytopsins. In those obtained during remission, the blast count was <5%.

Single nucleotide polymorphism microarray analysis. DNA was extracted from thawed cell pellets using either a standard phenol chloroform method or QIamp DNA Minikit (Qiagen Ltd., Crawley, Sussex) and digested using XbaI before ligation to adaptors which recognize the cohesive four basepair overhangs. The PCR was used to amplify adapter ligated DNA fragments, using primers that recognize the adaptor sequence. The amplified DNA was fragmented, end-labeled with a fluorescent tag and hybridized to a GeneChip Mapping 10 K array (Affymetrix UK Ltd., High Wycombe, United Kingdom).

Each array allows analysis of 11,555 SNPs, distributed evenly across the genome with a mean interval of 105 kb and median interval of 210 kb. Each SNP on the array is represented by 40 different 25-bp oligonucleotides, each with slight variations that allow accurate genotyping. Hybridization to each probe was assessed using a GeneChip Scanner (Affymetrix) and results scored using proprietary software (GDAS, Affymetrix). Results were exported to Excel spreadsheets (Microsoft) for further analysis. Sample analysis was done by Medical Research Council Geneservice (Hinxton, Cambridge, United Kingdom).

The result spreadsheets contained information about the identity of each SNP and its chromosomal location. Each SNP was scored as AA, BB (homozygous), or AB (heterozygous), depending on the genotype or as “no call” in the event of equivocal results. To compare samples obtained at presentation and relapse with normal DNA, data was imported into a single spreadsheet. SNPs were sorted according to chromosome number and location. Using the filter function in Excel “no-call” results were excluded and informative loci selected for the normal samples (i.e., those scoring as AB). Results from the presentation or relapse samples were then filtered to contain only those samples that scored AA or BB at loci that scored AB in the normal samples. Areas showing consecutive loci with differing scores were highlighted and reexamined for the presence of heterozygous loci between them, which had failed in the normal samples. In cases where this was not seen, it was concluded that there was LOH between the loci examined.

Microsatellite analysis. Microsatellite analyses were done using standard procedures for all samples showing LOH by SNP microarray. Sequences of the primers used and the PCR conditions are available on request. Briefly, forward primers were fluorescently labeled using a Beckman WellRed dye (D2, D3, or D4) and following PCR, 1 µl of each
### Table 1

<table>
<thead>
<tr>
<th>Cytogenetics</th>
<th>Follow-up</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>46,XY,TE/LAML+ve</td>
<td>same as presentation</td>
<td></td>
</tr>
<tr>
<td>45,X,Y-1,4,6,14,17,21</td>
<td>Fail</td>
<td></td>
</tr>
<tr>
<td>51,X,del(1)(p12),del(3)(q21)</td>
<td>same as presentation</td>
<td></td>
</tr>
<tr>
<td>46,XY,del(1)(p12),del(3)(q21)</td>
<td>same as presentation</td>
<td></td>
</tr>
<tr>
<td>46,XY,del(1)(p12),del(3)(q21)</td>
<td>same as presentation</td>
<td></td>
</tr>
<tr>
<td>45,X,Y-1,4,6,14,17,21</td>
<td>Fail</td>
<td></td>
</tr>
<tr>
<td>51,X,del(1)(p12),del(3)(q21)</td>
<td>same as presentation</td>
<td></td>
</tr>
</tbody>
</table>

*Continued on the following page*
Table 1. Details of losses of heterozygosity seen at presentation and relapse (cont'd)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46,XY: TEL/AML+ve</td>
</tr>
<tr>
<td>2</td>
<td>45,XX: idem, del(14)(q24.1)</td>
</tr>
<tr>
<td>3</td>
<td>45,XY: del(13)(q14q22), r(16)(p?q?)</td>
</tr>
<tr>
<td>4</td>
<td>52,XY: +X, +Y, +14, +21, +22</td>
</tr>
<tr>
<td>5</td>
<td>56,XY: +X, +Y, +14, +21, +22, +22</td>
</tr>
<tr>
<td>6</td>
<td>45,XY, -Y, +4, +6, +9, +12, +14, +17, +21</td>
</tr>
<tr>
<td>7</td>
<td>46,XY: t(8;14)(q24;q11)</td>
</tr>
<tr>
<td>8</td>
<td>51,XY: TEL/AML+ve</td>
</tr>
<tr>
<td>9</td>
<td>51,XX: del(17)(p?), +10, +22, +der(?)</td>
</tr>
<tr>
<td>10</td>
<td>51,XY: TEL/AML+ve</td>
</tr>
<tr>
<td>11</td>
<td>51,XX: +X, +Y, +14, +21, +22, +22</td>
</tr>
<tr>
<td>12</td>
<td>51,XX: +X, +Y, +14, +21, +22, +22</td>
</tr>
<tr>
<td>13</td>
<td>46,XY: del(13)(q14q22), +17(q17)</td>
</tr>
<tr>
<td>14</td>
<td>46,XY: del(13)(q14q22), +17(q17)</td>
</tr>
<tr>
<td>15</td>
<td>46,XY: del(13)(q14q22), +17(q17)</td>
</tr>
<tr>
<td>16</td>
<td>46,XY: del(13)(q14q22), +17(q17)</td>
</tr>
<tr>
<td>17</td>
<td>46,XY: del(13)(q14q22), +17(q17)</td>
</tr>
</tbody>
</table>

LOH Analysis Using SNP Microarray
reaction was combined with 40 µL of deionized formamide and 0.25 µL size standard-400 (Beckman Coulter UK, High Wycombe, Bucks, United Kingdom) labeled with Beckman WellRed dye D1. Automatic sizing of fluorescent PCR products was then done using a CEQ2000XL (Beckman Coulter UK) capillary system.

Results and Discussion

Matched samples were analyzed from 10 boys with ALL aged between 18 months and 7 years at the time of presentation. The abnormalities detected by SNP array analysis are summarized in Table 1. Microsatellite allelotype analysis was done at 19 of the 21 abnormal loci detected by the SNP microarray and in all cases confirmed LOH, thus validating this novel methodology (data not shown).

Abnormalities were detected either at relapse or at both presentation and relapse in eight cases. In one case, L151, allelic imbalances involving whole chromosomes (4, 6, 12, and 20) were identical at presentation and relapse. In the remaining cases LOH was either only apparent at relapse or involved losses in addition to those seen at presentation. In no cases were there losses seen at presentation which were not apparent at relapse, suggesting that relapse was associated with clonal evolution. Although the number of cases studied is too small to give a definite indication of the prognostic significance of LOH, it is striking that all the patients with progressive LOH at relapse have progressed and died from their disease, whereas those with either no abnormalities or whole chromosome loss responded well to salvage therapy and are disease free 1 to 9 years after relapse.

The most common abnormality detected, occurring in four cases, involved loss of portions of the short arm of chromosome 9. In the only comprehensive allelotype analysis of relapsed ALL reported to date, Takeuchi et al. (4) also showed that 9p was the most frequent site for LOH when leukemia recurs, occurring in 15 of 38 informative cases. The region found to be deleted in this study, and in the cases reported here, includes the p16/INK4a locus, a tumor suppressor gene previously reported by others to be deleted in ALL and to have adverse prognostic significance (5–8).

NOTE: *, patients who have died following relapse. Solid cells, LOH restricted to partial chromosome arms. Checkered cells, LOH throughout the entire length of a chromosome.
initially involved a region distal to the \( p16\) locus, but at relapse, a second small interstitial loss of about 900 kb was seen which includes this area.

These observations provide valuable "proof of principle" that analysis of LOH by SNP microarrays can indicate biologically significant areas of allelic imbalance. Further proof is provided by case L173 in which LOH occurred in chromosome 5q at relapse. The area of loss included the glucocorticoid receptor gene at 5q31.3. To confirm LOH at this locus, microsatellite analysis was done using a marker 200 kb from the \( GR \) gene. The results (Fig. 2) confirmed the findings of the SNP array analysis. LOH was associated with partial deletion of the partner allele. Complete loss of glucocorticoid receptor function would be associated with marked steroid resistance.

Further confirmation of the possible functional significance of progressive LOH at relapse was provided by case L169 in which relapse was associated with loss of a portion of chromosome 17q21.2 which included the \( TOP2A \) gene. Microsatellite analysis confirmed LOH at three of the four loci studied. Minor inconsistencies between results obtained by mapping arrays and microsatellites have been noted before (3) and may indicate errors due to miscalling ("noise") of the SNP array or mapping of the SNP loci which may not be completely stable. Topoisomerase II \( \alpha \), encoded by \( TOP2A \), is an important target for chemotherapy and loss of \( TOP2A \) gene has been associated with the development of resistance to agents used in the treatment of children with leukemia (e.g., anthracyclins and etoposide; ref. 9). As steroids and anthracyclins are an important component of antileukemic therapy, it may be inferred that these genetic alterations present at relapse are likely to have functional significance, although drug sensitivity of the leukemic blasts has not been formally tested in these patients.

Results of bone marrow cytogenetic analyses at presentation were available in all cases apart from L181, which failed to produce metaphases for conventional karyotyping. Case L423 showed a normal karyotype by cytogenetics and no LOH by microarray analysis. Three cases (152, 173, and 184) showed karyotype abnormalities but no LOH. Two of these involved hyperdiploidy typically seen in ALL and one involved a balanced translocation; in none of these three cases were regions of chromosome loss evident cyogenetically. Case L83 showed complete concordance between LOH findings (9p LOH) and classic cytogenetics (unbalanced 9p translocation resulting in visible segment loss). Three cases showed partial concordance between LOH and visible chromosomal changes. L121 had visible deletions of 13q and of chromosome 16 (ring) corresponding to LOH findings for these regions, but the LOH detected on 6q was not apparent cyogenetically. Case L169 was reported as showing monosomy 20 as the sole cytogenetic aberration in bone marrow cells, but LOH analysis identified 9p LOH and loss of 20q, with retention of heterozygosity for 20p. These findings strongly implicate unbalanced translocation der(9)(q22)x2 as described by Clark et al. (10). This cryptic rearrangement was recognized as a recurrent abnormality in ALL in 2000 and results in partial monosomies for 9p and 20q. The most discrepant results for the comparison of cytogenetics and mapping array analysis were for case L151, which showed a hyperdiploid karyotype with no regions of loss evident by chromosome analysis, but LOH events affecting the entire length of chromosomes 4, 6, 12, and 20. It is possible that this apparent loss is due to uniparental disomy, as has previously been shown for chromosome 6 (11).

Hence, in presentation bone marrows, microarray analysis identified six regions of LOH (from three cases) which were undetected by routine cytogenetics.

Five cases showed LOH events at relapse, which were not apparent at presentation and these were compared with karyotype data. L15 developed a 12q LOH that was not detected by cytogenetics, which indicated normal bone marrow karyotype at both diagnosis and relapse. L152 acquired LOH for 1q, 2p, 5p, 8p, and 8q. These losses cannot be correlated with the hyperdiploid karyotypes observed by chromosome analysis. L169 acquired 9q LOH consistent with the appearance of del(9)(q12)(q22) in the relapse karyotype. L173 developed LOH at 5q, 9p, and 18p; chromosome analysis at relapse did not identify segment losses at these locations. L184 developed 9p LOH: this was not visible in the relapse karyotype.

Hence, on relapse, 10 regions of loss were identified (from four cases) which were not detected by cytogenetics.

These results suggest that SNP array analysis is capable of detecting abnormalities not apparent by routine karyotyping. This may be because the losses are too small, because allelic loss occurs as a consequence of chromosome duplication or mitotic recombination with LOH distal to the recombination site (12) or because subclones containing these aberrations fail to divide in culture

---

1 Irving et al., submitted for publication.
before karyotype analysis (13). In a study using high-resolution
comparative genomic hybridization (HRCGH), Kristensen et al. (13)
also found significantly more aberrations than G-band karyotyping
in childhood ALL studied at presentation. Twenty-one percent of
cases had 9p losses seen by HRCGH, whereas only 5% had losses in
the same region detectable by cytogenetics.

The amount of DNA required to perform SNP array analysis, 250
ng per sample, was less than the amount used by Takeuchi for the
genome-wide microsatellite analysis (5-25 ng per PCR for 71
markers, equivalent to between 355 and 1,775 ng) and gave data at
162 times more loci. The resolution of the technique allowed the
determination of the extent of LOH in a single assay. Although the 3-
to 5-Mb resolution of HRCGH was greater than that of micro-
satellite analysis (14), this is still considerably less than the 210-kb
resolution of SNP arrays and the amount of DNA required is much
higher (5 μg), limiting the number of cases which can be studied.

In summary, SNP array analysis is a technique which facilitates
the analysis of LOH in a small sample of DNA from patients with
leukemia. Unlike cytogenetics it is not dependent on the ability of
cells to undergo division in culture and can be used for the
retrospective analysis of cell pellets and purified DNA. It offers
better resolution than either microsatellite analysis or HRCGH and
uses less starting material than either.

Acknowledgments

Received 7/26/2004; revised 12/16/2004; accepted 2/9/2005.

Grant support: Leukaemia Research Fund, Tyneside Leukaemia Research
Association, and North of England Children's Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page
charges. This article must therefore be hereby marked advertisement in accordance
with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Lynne Minto for expert technical assistance and Christine Harrison for
her advice.

References

1. Lengauer C, Kinzler KW, Vogelstein B. Genetic
over 10,000 SNPs using a one-primer assay on a high-
analysis by high-density single-nucleotide polymorphic
allele (SNP) array with whole genome amplified DNA.
analysis in relapsed childhood acute lymphoblastic
Deletion of the Ink4 locus (the p16ink4a, p14ARF and
p15ink4b genes) predicts relapse in children with ALL
treated according to the Nordic protocols NOPHO-86
outcome after intensified therapy of childhood lymphoblastic
leukaemia: results from Medical Research Council United Kingdom acute lymphoblastic leukaemia
genomic hybridization in childhood acute lymphoblast-
ic leukemia: correlation with interphase cytogenetics
and loss of heterozygosity analysis. Cancer Genet
8. Heerema NA, Sather HN, Sensel MG, et al. Associa-
tion of chromosome arm 9p abnormalities with
adverse risk in childhood acute lymphoblastic leuke-
emia: a report from the Children’s Cancer Group. Blood
1999;94:1537–44.
numbers of genes as a mechanism for acquired drug
a pointer to dicentric (9;20) in acute lymphoblastic
whole chromosome 6 loss and duplication of the
remaining chromosome in acute lymphoblastic leuke-
12. Takeuchi S, Tsukasaki K, Bartram CR, et al. Long-
term study of the clinical significance of loss of heterozygosity in childhood acute lymphoblastic leuke-
yields a high detection rate of chromosomal aber-
rations in childhood acute lymphoblastic leukaemia. Eur
10 megabasepairs are detected in comparative genomic
hybridization by standard reference intervals. Genes
Loss of Heterozygosity in Childhood Acute Lymphoblastic Leukemia Detected by Genome-Wide Microarray Single Nucleotide Polymorphism Analysis


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/65/8/3053

Cited articles  This article cites 14 articles, 3 of which you can access for free at: http://cancerres.aacrjournals.org/content/65/8/3053.full#ref-list-1

Citing articles  This article has been cited by 14 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/65/8/3053.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.