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 cytospins. 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 *Xba*I 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 adapter 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. Details of losses of heterozygosity seen at presentation and relapse

Cytogenetics			11 q	11 p	10 q	10 p	9 q	9 p	8 q	8 p	7 q	7 p	6 q	6 p	5 q	5 p	4 q	4 p	3 q	3 p	2 q	2 p	1 q	1 p
46,XY	15*	P																						
46,XY		R																						
46,XY,add(7)(q3?),der(9)t(9;14)(p13;q1?)	83*	P						■																
same as presentation		R						■																
46,XY,del(13)(q14q22),r(16)(p?q?)	121*	P											■											
same as presentation		R											■											
52,XY,+X,+Y,+14,+14,+21,+22	151	P											▨	▨			▨	▨						
same as presentation		R											▨	▨			▨	▨						
56,XY,+X,add(1)(q),+6,+9,+12,+14,+17,+21,inc	152*	P																						
56,idem,−9,+10,+22,+der(?)t(?;17),inc		R							■	■			■								■	■		
45,XY,−20	169*	P						■																
45,XY,−20 / 45,idem,del(9)(q12q22)		R					■	■																
46,XY,t(8;14)(q24;q11)	173*	P																						
same as presentation		R						■									■							
Fail	181	P																						
Fail		R																						
51,X,−Y,+X,+4,+6,+14,+17,+21	184*	P																						
51,idem,del(4p)		R						■																
46,XY. TEL/AML+ve	423	P																						
same as presentation		R																						

(Continued on the following page)

Table 1. Details of losses of heterozygosity seen at presentation and relapse (Cont'd)

Cytogenetics	Patient	P/R	12p	12q	13p	13q	14p	14q	15p	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	21p	21q	22p	22q
46,XY	15*	P																						
46,XY		R		■																				
46,XY,add(7)(q3?),der(9)t(9;14)(p13;q1?)	83*	P																						
same as presentation		R																						
46,XY,del(13)(q14q22),r(16)(p?q?)	121*	P				■					■	■												
same as presentation		R				■					■	■												
52,XY,+X,+Y,+14,+14,+21,+22	151	P	▒	▒															▒	▒				
same as presentation		R	▒	▒															▒	▒				
56,XY,+X,add(1)(q),+6,+9,+12,+14,+17,+21,inc	152*	P																						
56,idem,−9,+10,+22,+der(?)t(?;17),inc		R																						
45,XY,−20	169*	P																		■				
45,XY,−20 / 45,idem,del(9)(q12q22)		R														■				■				
46,XY,t(8;14)(q24;q11)	173*	P																						
same as presentation		R														■								
Fail	181	P																						
Fail		R																						
51,X,−Y,+X,+4,+6,+14,+17,+21	184*	P																						
51,idem,del(4p)		R																						
46,XY. TEL/AML+ve	423	P																						
same as presentation		R																						

Table 1. Details of losses of heterozygosity seen at presentation and relapse (Cont'd)

		15*		83*		121*		151		152*		169*		173*		181		184*		423	
		P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R
X	p																				
	q																				
Cytogenetics		46,XY	46,XY	46,XY,add(7)(q3?),der(9)t(9;14)(p13;q1?)	same as presentation	46,XY,del(13)(q14q22),r(16)(p?q?)	same as presentation	52,XY,+X,+Y,+14,+14,+21,+22	same as presentation	56,XY,+X,add(1)(q),+6,+9,+12,+14,+17,+21,inc	56,idem,−9,+10,+22,+der(?)t(?;17),inc	45,XY,−20	45,XY,−20 / 45,idem,del(9)(q12q22)	46,XY,t(8;14)(q24;q11)	same as presentation	Fail	Fail	51,X,−Y,+X,+4,+6,+14,+17,+21	51,idem,del(4p)	46,XY. TEL/AML+ve	same as presentation

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.

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/INK4α* locus, a tumor suppressor gene previously reported by others to be deleted in ALL and to have adverse prognostic significance (5–8).

The extent of the deletion seen in each of the affected cases is shown in Fig. 1. In two of these, L173 and L184, abnormalities were only seen at relapse. In one case, L169, the extent of loss was identical at presentation and on relapse. In the remaining case, loss
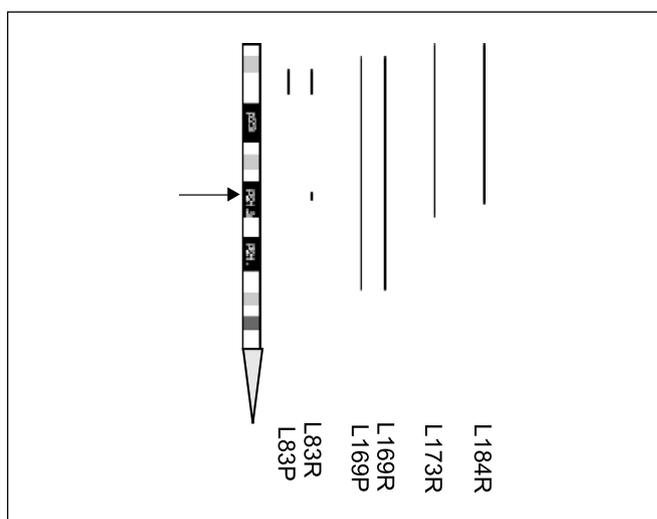

Figure 1. LOH affecting chromosome 9p. *Arrow,* position of p16^{ink4}.

initially involved a region distal to the *p16/INK4* locus, but at relapse, a second small interstitial loss of about 900 kb was seen which included 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.[1] 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 α, 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 cytogenetically. 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 cytogenetically. 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)t(9;20),−20 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,
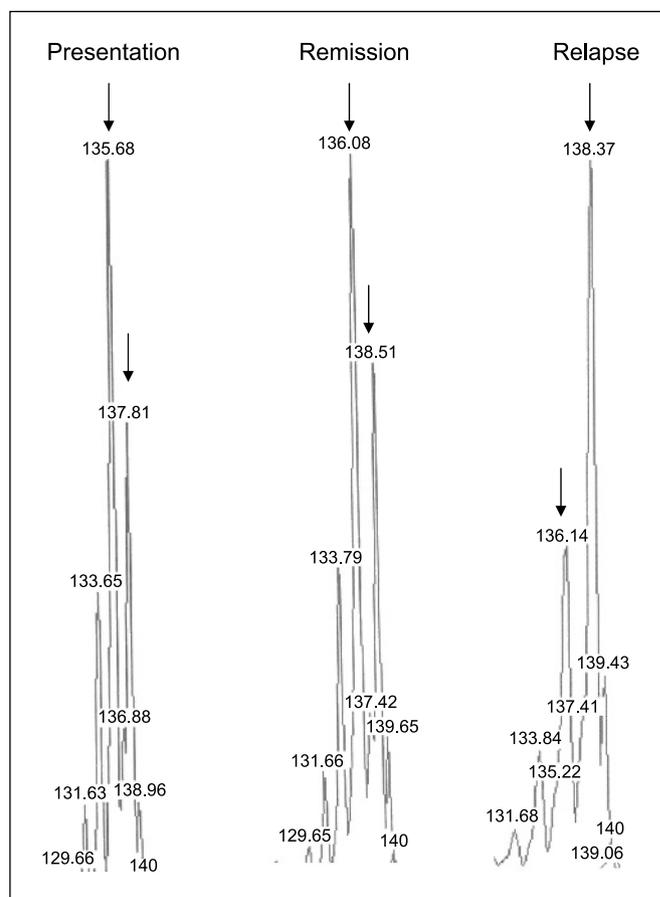

Figure 2. Microsatellite analyses of the dinucleotide repeat at the D5S207 microsatellite in samples of patient L173 showing the presence of two alleles (136 and 138 bp) in both remission (i.e., constitutive) and presentation samples but only one (138 bp) allele in the relapse sample, indicative of LOH.

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)(q12q22) 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

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[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

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