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GENOMIC ANALYSIS OF THE CLONAL ORIGINS OF RELAPSED ACUTE LYMPHOBLASTIC LEUKEMIA

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Abstract

Most children with acute lymphoblastic leukemia (ALL) can be cured, but for the subset of patients who undergo relapse prognosis is dismal. To explore the genetic basis of relapse, we performed genome-wide DNA copy number analyses on matched diagnosis and relapse samples from 61 patients with ALL. In the majority of cases, the diagnosis and relapse samples showed different patterns of genomic copy number abnormalities (CNAs), with the abnormalities acquired at relapse preferentially affecting genes involved in cell cycle regulation and B cell development. Although the diagnosis and relapse samples were genetically related, most relapse samples lacked some of the CNAs present at diagnosis, suggesting that the cells responsible for relapse are ancestral to the primary leukemia cells. Backtracking studies demonstrated that cells corresponding to relapse clone were often present as minor sub-populations at diagnosis. These data suggest that genomic abnormalities contributing to ALL relapse are selected for during treatment and that the signaling pathways affected by these acquired alterations may be rational targets for therapeutic intervention.

Despite cure rates for pediatric acute lymphoblastic leukemia (ALL) exceeding 80% (1), treatment failure remains a significant problem. Relapsed ALL ranks as the fourth most common childhood malignancy and has an overall survival rate of only 30% (2,3). Important biological and clinical differences have been identified between diagnostic and relapsed leukemic cells including the acquisition of new chromosomal abnormalities, gene mutations, and reduced responsiveness to chemotherapeutic agents (4-7). However, many questions remain about the molecular abnormalities responsible for relapse, as well as the relationship between the cells giving rise to the primary and recurrent leukemias in individual patients.

Genome-wide analyses of DNA copy number abnormalities (CNAs) and loss-of-heterozygosity (LOH) using single nucleotide polymorphism (SNP) arrays have provided important insights into the pathogenesis of newly diagnosed ALL. We have previously reported multiple recurring somatic CNAs in genes encoding transcription factors, cell cycle regulators, apoptosis mediators, lymphoid signaling molecules and drug receptors in B-progenitor and T-lineage ALL(8,9). To gain insights into the molecular lesions responsible for ALL relapse, we have now performed genome-wide CNA and LOH analyses on matched diagnostic and relapse bone marrow samples from 61 pediatric ALL patients (table S1). These samples included 47 B-progenitor and 14 T-lineage ALL (T-ALL) cases (10). Samples were flow sorted to ensure at least 80% tumor cell purity prior to DNA extraction (fig. S1). DNA copy number and LOH data were obtained using Affymetrix SNP 6.0 (47 diagnosis-relapse pairs) or 500K arrays (14 pairs). Remission bone marrow samples were also analyzed for 48 patients (table S1).

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These analyses identified a mean of 10.8 somatic CNAs per B-ALL case at diagnosis, and 7.1 CNAs per T-ALL case (table S4, fig. S2 and S4). 48.9% of B-ALL cases at diagnosis had CNAs in genes known to regulate B-lymphoid development, including *PAX5* (N=12), *IKZF1* (N=12), *EBF1* (N=2), and *RAG1/2* (N=2) (tables S5, S6 and S9). Deletion of *CDKN2A/B* was present in 36.2% of B-ALL and 71.4% T-ALL cases, and deletion of *ETV6* in 11 B-ALL cases. We also identified novel CNAs involving *ARID2*, which encodes a member of a chromatin remodeling complex (11), the cyclic AMP regulated phosphoprotein *ARPP-21*, the *IL3RA* and *CSF2RA* cytokine receptor genes (fig. S3), and the Wnt/β-catenin pathway genes *CTNNB1*, *WNT9B* and *CREBBP* (tables S5-S6).

Although evidence for clonal evolution and/or selection at relapse has been previously reported (4,6,7,12-21), we observed a striking degree of change in the number, extent, and nature of CNAs between diagnosis and relapse in paired samples of ALL. A significant increase in the mean number of CNAs per case were observed in relapse B-ALL samples (10.8 at diagnosis versus 14.0 at relapse, P=0.0005) with the majority being additional regions of deletion (6.8 deletions/case at diagnosis versus 9.2/case at relapse, P=0.0006; and 4.0 gains/case at diagnosis versus 4.8 gains/case at relapse, P=0.03; table S4 and fig. S4). By contrast, no significant changes in lesion frequency were observed in T-ALL (table S4).

The majority (88.5%) of relapse samples harbored at least some of the CNAs present in the matched diagnosis sample, indicating a common clonal origin (table S5 and fig. S5); however, 91.8% exhibited a change in the pattern of CNAs from diagnosis to relapse (table S7). 34% acquired new CNAs, 12% showed loss of lesions present at diagnosis, and 46% both acquired new lesions and lost lesions present at diagnosis. In 11% of relapsed samples (three B-ALL and four T-ALL cases) all CNAs present at diagnosis were lost at relapse, raising the possibility that the relapse represents the emergence of a second unrelated leukemia. One case (BCR-ABL-SNP-#15) retained the same translocation at relapse, indicating a common clonal origin. In four, lack of similarity of the patterns of deletion at immunoglobulin (Ig) and T-cell antigen receptor (TcR) gene loci, or lack of deletions at these loci, suggested that relapse represented emergence of a distinct leukemia (see below and fig. S6 and S7). For all other relapse cases (86%), analysis of Ig/TCR deletions demonstrated a clonal relationship between diagnostic and relapse samples (table S21 and fig. S6).

The genes most frequently affected by CNAs acquired at relapse were *CDKN2A/B*, *ETV6*, and regulators of B-cell development (Table 1, Fig. 1, tables S8-18 and fig. S8). Sixteen B- and two T-ALL cases acquired new CNAs of *CDKN2A/B*, 10 of which lacked *CDKN2A/B* deletions at diagnosis (Fig. 1A-B, tables S17 and S18). The *CDKN2A/B* deletions acquired at relapse were bi-allelic in 70% of cases, resulting in a complete loss of expression of all three encoded proteins: INK4A (p16), ARF (p14), and INK4B (p15). Deletion of *ETV6*, a frequent abnormality at diagnosis in *ETV6-RUNX1* B-ALL (8,19), was also common in relapsed ALL, being identified in 11 cases (10 B-ALLs and one T-ALL), with only one case *ETV6-RUNX1* positive (fig. S8). Mutations of genes regulating B cell development are common at diagnosis in B-ALL (8), and additional lesions in this pathway were observed at relapse, with a number of cases acquiring multiple hits within the pathway (table S9). Four cases lacked CNAs in this pathway at diagnosis but acquired deletions in *PAX5* (N=1), *IKZF1* (N=2), or *TCF3* (N=1) at relapse. Eleven cases with CNAs in this pathway at diagnosis acquired additional lesions at relapse, most commonly *IKZF1* (5 cases), *IKZF2* (two cases) and *IKZF3* (one case) (fig. S9-10). New CNAs were also observed in *PAX5* (N=3), *TCF3* (N=3), *RAG1/2* (N=2; fig. S9-10) and *EBF1* (N=1, fig. S9). CNAs involving genes encoding regulators of lymphoid development were also observed in four T-ALL relapse samples but involved the early lymphoid regulators *IKZF1* (N=2), *IKZF2* (N=1) and *LEF1* (N=2; table S9), rather than B lineage specific genes such as *PAX5* and *EBF1*.

A number of other less frequent CNAs previously detected in diagnostic ALL samples (8) were also observed as new lesions at relapse, including CNAs of *ADD3*, *ARPP-21*, *ATM*, *BTG1*, *CD200/BTLA*, *FHIT*, *KRAS*, *IL3RA/CSF2RA*, *NF1*, *PTCH*, *TBL1XR1*, *TOX*, *WT1*, *NR3C1* and *DMD* (table S8 and fig. S11); and progression of intrachromosomal amplification of chromosome 21, a poor prognostic marker in childhood ALL (22) (fig. S12). In addition, relapsed T-ALL was remarkable for the loss and acquisition of sentinel lesions in T-ALL, including the loss of *NUP214-ABL1* in one case, and the acquisition of *NUP214-ABL1*, *LMO2*, and *MYB* amplification (one case each) at relapse (8,23-25) (table S8 and fig. S13).

In addition to defining CNAs, we also performed an analysis of regions of copy-neutral LOH (CN-LOH) that can signify mutated, reduplicated genes. CN-LOH was only identified in 15 B- and 3 T-ALL cases (table S19). The most common region involved was chromosome 9p (N=8), which in each case contained homozygous *CDKN2A/B* deletion, consistent with reduplication of a hemizygous *CDKN2A/B* deletion.

To determine which biologic pathways were most frequently targeted by relapse-acquired CNAs, we categorized each gene contained within altered genomic regions into one or more of 148 biologic pathways. The pathways were then assessed for their frequency of involvement by CNAs across the dataset using Fisher‘s exact test (10). This analysis identified cell cycle regulation and B-cell development as the most common pathways targeted at relapse (table S20).

There was a clear clonal relationship between the diagnosis and relapse ALL samples in most cases (93.6% B-ALL and 71.4% T-ALL cases). This suggests that either (i) the relapse-associated CNAs were either present at low levels at diagnosis and selected for at relapse, or (ii) were acquired as new genomic alterations after initial therapy. To explore these possibilities, we mapped the genomic breakpoints of several CNAs acquired at relapse (*ADD3*, *C20orf94*, *DMD*, *ETV6*, *IKZF2*, and *IKZF3*) and developed lesion-specific PCR assays. Evidence of the relapse clone was detected in 7 of 10 diagnosis samples analyzed (figs. 1C-H, S14 and S15). Thus, the relapse clone is frequently present as a minor sub-population at diagnosis.

By carefully analyzing the changes in CNAs between matched diagnostic and relapse samples, we were able to map their evolutionary relationship (Fig. 2). In a minority of cases, “relapse” is a misnomer, as no CNAs were shared by the diagnostic and relapse clones. The recurrent disease in these cases either represents a secondary leukemia, or a leukemia arising from an ancestral clone that lacks any of the CNAs present in the diagnosis leukemia. In 8% of cases there were no differences in CNAs between the diagnostic and relapse clones, whereas in 34% of cases relapse represented clonal evolution of the diagnosis leukemic populations. Remarkably, however, in almost half of the cases the relapse clone was derived from an ancestral, pre-diagnosis leukemic precursor cell and not from the clone predominating at diagnosis. One illustrative case (Other-SNP-#29) had two relapse-acquired deletions (*ETV6* and *DMD*), only one of which was present in the diagnostic sample as a minor clone (*ETV6*, fig. S15), indicating that these lesions were acquired at different stages of evolution of the relapse clone. This case provides unequivocal evidence of a common ancestral clone that give rise to the major clone at diagnosis, and to a second clone that was present as a minor population at diagnosis but acquired different genetic alterations before emerging as the relapse clone.

These results extend previous studies examining individual genetic loci in relapsed ALL (6, 14,16,20,21,26-28), and provide important insights into the spectrum of genetic lesions that underlie this process. Although our data are limited to a single class of mutations (CNAs), they demonstrate that no single genetic lesion or alteration of a single pathway is responsible for relapse. Moreover, global genomic instability does not appear to be a prevalent mechanism.

Instead, a diversity of mutations appear to contribute to relapse with the most common alterations targeting key regulators of tumor suppression, cell cycle control, and lymphoid/B cell development. Notably, few lesions involved genes with roles in drug import, metabolism, export and/or response, (an exception being the glucocorticoid receptor gene *NR3C1*) suggesting that the mechanism of relapse is more complex than simple "drug resistance".

The diversity of genes that are targeted by relapse-associated CNAs, coupled with the presence of the relapse clone as a minor sub-population at diagnosis that escapes drug-induced killing, represent formidable challenges to the development of effective therapy for relapsed ALL. Nevertheless, our study has identified several common pathways that may contain rational targets against which novel therapeutic agents can be developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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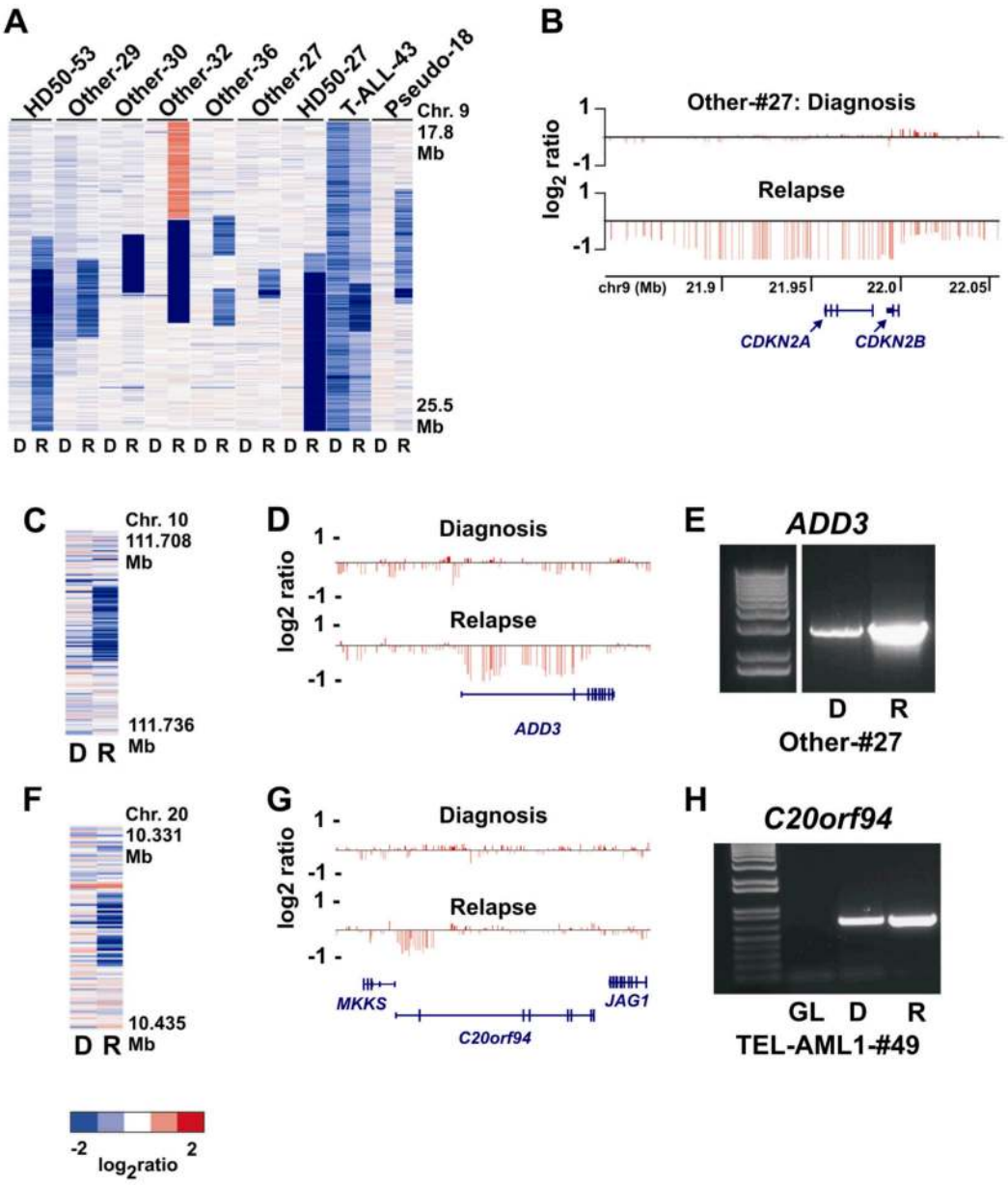

Figure 1. Deletions of *CDKN2A/B* and *ETV6* in relapsed ALL

A, Log_2 ratio SNP 6.0 copy number data (median smoothed with a window of five markers; blue is deletion and red is gain) of chromosome 9p flanking *CDKN2A/B* for 9 representative cases showing new or more extensive deletions at this locus at relapse. Deletions in selected cases were confirmed by quantitative genomic PCR (table S17). **B** coverage of the locus for one case. Each vertical red line represents the genomic position and log_2 ratio copy number of an individual marker. This case has acquired a homozygous deletion extending involving exon 2 of *CDKN2B* and all of *CDKN2A* at relapse. **C-H** Backtracking of CNAs using lesion-specific genomic PCR assays in ALL. **C-E** *ADD3*, **E-H** *C20orf94*. Log_2 ratio copy number heatmaps for diagnosis and relapse samples are shown in panels **C** and **F;** the genomic location of SNP probes and regions of deletion are shown in **D** and **G; E** and **H:** PCR for each lesion was performed for diagnosis, relapse, and where available, germline DNA samples. In each case,

a CNA-specific PCR product was observed at diagnosis as well as relapse, indicating that each CNA was present at diagnosis.

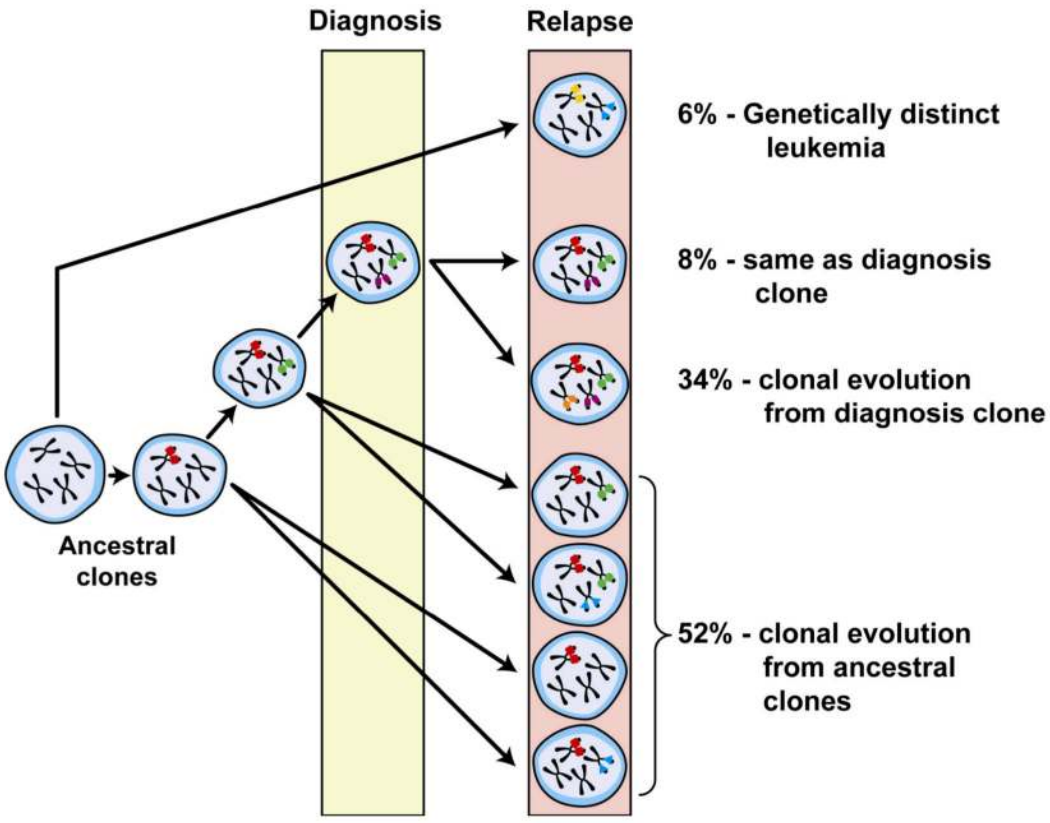

Figure 2.

Clonal relationship of diagnosis and relapse samples in ALL. The majority of relapse cases have a clear relationship to the presenting diagnosis leukemic clone, either arising through the acquisition of additional genetic lesions, or more commonly, arising from a ancestral (pre-diagnosis) clone. In the latter scenario, the relapse clone retains some but not all of the lesions found in the diagnostic sample, while acquiring new lesions. Lesion specific backtracking studies revealed that in most cases the relapse clone exists as a minor sub-clone within the diagnostic sample prior to the initiation of therapy. In only a minority of ALL cases does the relapse clone represent the emergence of a genetically distinct and thus unrelated second leukemia.

Table 1

Targets of relapse-acquired CNA in ALL, ranked in order of frequency

Lesion		B-progenitor ALL	T-lineage ALL
Deletion	*CDKN2A*	16	2
	ETV6	10	1
	IKZF1	5	2
	NR3C1	4	0
	TCF3	3	0
	DMD	2	0
	ARPP-21	2	0
	CD200	2	0
	RAG1/2	2	0
	IKZF2	1	1
	BTLA	1	1
	ADD3	1	0
	C20orf94	1	0
	TBL1XR1	1	0
	IKZF3	1	0
Gain	*MYB*	0	2
	DMD	1	0