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Systematic Genomic Screen for Tyrosine Kinase Mutations in CLL

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Increasing data suggest that activating kinase mutations play a significant causative role in chronic and acute hematologic malignancies. The prototypical example is the BCR-ABL kinase that is activated in CML and Philadelphia chromosome positive ALL, but activating kinase mutations have also been identified in chronic myelomonocytic leukemia, chronic eosinophilic leukemia, and systemic mastocytosis. Circumstantial evidence suggests that gain-of-function tyrosine kinase alleles could also contribute to the pathogenesis of chronic lymphocytic leukemia (CLL). Microarray studies of gene expression differences between steadily progressive CLLs that lack somatic hypermutation of the immunoglobulin heavy chain variable gene region (IgVH unmutated) and more indolent IgVH mutated CLLs have found that many of the differentially expressed genes are involved in B cell receptor signaling, most notably the ZAP-70 tyrosine kinase. Other kinases found to be overexpressed in CLL by gene expression studies include BLK, JAK2, LYN, and NTRK3, although mutational analysis of these genes in CLL has been limited. The goal of this project was therefore to search systematically for activating mutations in protein tyrosine kinases in CLL using high-throughput DNA resequencing.

A total of 178 exons were sequenced from each of 95 patients. The patients were selected to encompass the range of prognostic groups defined by IgVH homology, ZAP-70 expression, and FISH cytogenetics. IgVH homology (unmutated defined as greater than or equal to 98% homology to the closest germline match) and ZAP-70 expression (positive defined as >20%) were determined by the CLL Research Consortium tissue core as previously described[1], and cytogenetics were evaluated by FISH for the most common abnormalities (del 13q, trisomy 12, del 11q, del 17p, chromosome 14 rearrangements) at DFCI[2]. Although the CLL cases studied showed some bias toward lower risk features (IgVH mutated 56%, unmutated 28%, ND 16%; ZAP70 negative 52%, positive 24%, ND 24%; CD38 negative (<30%) 70%, positive 24%, ND 6%), nonetheless both high and low risk patients of each type were included in the study. Similarly, the common cytogenetic features of CLL were well represented, including 38% with del 13q only, 7% normal, 17% with del 11q, 21% with del 17p and 8% with complex cytogenetics (see Supplementary Table 1 for a complete list). 15.6% of these patients reported a family history of CLL, non-Hodgkin's lymphoma or Hodgkin's disease, which is comparable to the rate in our general clinic population[3]. Thus the patient population studied encompassed the full spectrum of biologic variability in CLL.

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The tissue banking protocol (#99-224) was approved by the Dana-Farber Cancer Institute Institutional Review Board, and all patients signed informed consent prior to participation. Any patient 18 years or older with CLL/SLL seen at DFCI was eligible. Two 10 ml sodium heparin tubes of peripheral blood were drawn and peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated by density centrifugation through Ficoll. DNA was isolated from PBMCs and granulocytes using the QIAamp DNA Blood Maxi Kit (Qiagen). For some patients buccal swabs were obtained and DNA isolated using the QIAamp DNA Mini Kit (Qiagen); for others, saliva samples were obtained and DNA isolated using the Oragene DNA Self-Collection Kit (Oragene).

70 of the 95 known tyrosine kinases were selected for resequencing based on their involvement in lymphocyte biology, known altered gene expression in CLL, or location at a genomic site of copy number gain or loss in CLL (see Supplementary Table 2 for a complete list). Exons encoding the activation loops and juxtamembrane domains of receptor tyrosine kinases, and activation loops of non-receptor tyrosine kinases, were resequenced. In addition, all coding exons of ZAP-70 were resequenced. Nested M13 tailed primers were designed to amplify and sequence the selected exons as previously described[4,5], and bidirectional sequence analysis was performed using Mutation Surveyor version 2.28 (SoftGenetics).

All putative mutations were reviewed manually. Any candidates previously reported to be single nucleotide polymorphisms (SNPs) in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) were excluded from further analysis. Candidate mutations not identified as SNPs were then amplified and resequenced from the original CLL DNA sample for verification; in parallel the same exon was amplified and resequenced from the paired germline sample. All germline analyses were performed using granulocyte, buccal or saliva DNA and in many cases included at least two of the above (the results of which were concordant in all cases tested). The allele locations reported in Table 1 are derived from the annotations in Ensembl and are generally given in relation to the coding sequence for clarity and uniformity.

Our attention was initially focused on the identification of somatic non-synonymous mutations. Surprisingly, we identified only eight such candidate alleles (Table 1), all of which were present in the cognate germline sample, suggesting that they represent previously undescribed germline polymorphisms. Although these SNPs could conceivably alter kinase function and thereby contribute to the pathogenesis of CLL, their rarity among these samples suggests that they would play at most a minor role in the overall pathogenesis of the disease. Only one of the patients with a nonsynonymous allele reported a family history of CLL, so familial CLL was not enriched in this group of patients.

Given this apparently low frequency of somatic mutation, we decided to define the somatic mutation rate in CLL by determining whether each nucleotide alteration in our samples, including synonymous coding and noncoding alleles, was present in matched germline DNA. We identified 10 synonymous coding alleles and 6 alleles in the 3′ untranslated region or intronic sequences immediately flanking the exons (Table 1). Analysis of matched germline DNA demonstrated that all 16 alterations were also present in the germline, consistent with previously unidentified rare SNPs.

In this analysis of 70 tyrosine kinases in 95 CLLs, we have detected 24 novel SNPs that have not been previously described, but no somatic mutations. Our data are consistent with a somatic mutation rate in CLL of less than one mutation per 6.21 megabases DNA (< 0.16 mutation per Mb DNA). A previous study has found that the somatic mutation rate varies significantly among different cancers, from 0.12 to 32.29 mutations per Mb DNA[6]. The highest mutation rates were found in cancers arising from highly proliferative epithelia, namely colon and

lung[6]. The only leukemia studied was acute lymphoblastic leukemia (ALL), in which 2 mutations were found in 8 cases, for a somatic mutation rate of 0.57 mutations per Mb DNA[6].

The observation that the somatic mutation rate in CLL is lower than that observed in common epithelial tumors is not surprising, given the degree of genomic instability in epithelial neoplasms. CLL is a relatively indolent neoplasm with a lower proliferative rate than many solid tumors. However the complete absence of somatic alterations is surprising, particularly given the long natural history of CLL and its known tendency to accumulate multiple chromosomal aberrations over time[2]. These data suggest that the mechanisms of genomic alteration that are responsible for the pathogenesis and progression of CLL may be those that result in large-scale chromosomal alterations or translocations, or perhaps epigenetic modifications, rather than point mutations.

In this study we chose to resequence selected exons, including those encoding the kinase domain with P loops and juxtamembrane domains of receptor tyrosine kinases, based on previous work indicating that these most frequently harbor activating mutations. Indeed, although activating mutations have been reported in other conserved domains[6], the available data suggests that for any given kinase that is mutated in hematologic malignancy, at least a fraction of patients will have mutations in either the juxtamembrane domain or the activation loop, in addition to other alleles that may be identified outside these domains. Furthermore, the very low somatic mutation rate we observe in CLL argues against the possibility that epidemiologically significant activating mutations were missed. This study was powered such that if the true (but unknown) probability of a tyrosine kinase mutation in a given patient's CLL is at least 2.4%, we would expect to find at least one patient in 95 with such a mutation. This study thus indicates that activating mutations in the tyrosine kinome are unlikely to play a significant role in CLL, and therefore suggests that additional in-depth resequencing in CLL should focus on other potential disease alleles. In this regard, these data are similar in character to those recently obtained from kinome resequencing in AML[7,8], in which few potential disease alleles were identified.

This study also provides a cautionary note about the need for germline comparison when assessing human cancer samples for possible somatic mutations. Many SNPs have not yet been catalogued, and nonsynonymous coding region alleles are often undescribed SNPs, as we have observed. Thus, even nonsynonymous coding region alleles cannot be assumed to be somatic in origin unless matched germline DNA is assessed for each candidate alteration.

In conclusion, this systematic assessment of the tyrosine kinome for somatic mutations in CLL has found no evidence for activating mutations, including in the entire ZAP-70 coding region, and suggests that the rate of acquired somatic mutation in CLL is low. The frequency of previously undescribed polymorphisms is significant and mandates the use of matched germline DNA in human cancer screens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Alleles Identified

Gene	Exon	Mutation	# With Mutn	Present in Germline?
		Coding Nonsynonymous		
BLK	8	G-A Arg 238 Gln	2/95	Yes
BLK	12	G-A Val 434 Met	1/95	Yes
EPHA7	10	G-A Ala 625 Thr	1/95	Yes
EPHA8	10	C-A Pro 607 His	1/95	Yes
FLT4	23	G-A Asp 1049 Asn	1/95	Yes
MET	14	C-T Thr 992 Ile	1/95	Yes
MUSK	15	A-G Asp 578 Ser	3/95	Yes
ZAP-70	7	C-T Pro 307 Leu	1/95	Yes
		Coding Synonymous		
CSF1R	19	C-G 3rd pos Codon 845	3/95	Yes
EPHA2	14	C-T 3rd pos Codon 784	1/95	Yes
FGFR1	14	C-T Homozygous 3rd pos Codon 651	1/95	Yes
FGFR2	10	G-A 3rd pos Codon 454	1/95	Yes
KIT	17	C-T 3rd pos Codon 798	1/95	Yes
NTRK1	16	G-A 3rd pos Codon 698	1/95	Yes
PTK7	19	C-G 3rd pos Codon 975	1/95	Yes
TEK	15	G-C 3rd pos Codon 818	2/95	Yes
TEK	18	T-C 3rd pos Codon 988	1/95	Yes
ZAP70	9	C-T 3rd pos Codon 489	2/95	Yes
		Noncoding		
AKT2	13	C-T 3′UTR Nt1715	1/95	Yes
AKT2	13	G-A 3′UTR Nt1722	2/95	Yes
EPHA8	13	C-T intron	1/95	Yes
FER	18	A-G intron	1/95	Yes
FGFR4	14	G-A intron	2/95	Yes
FYN	9	C-T intron	1/95	Yes