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Genetic Characterization of *SF3B1* Mutations in Single Chronic Lymphocytic Leukemia Cells

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LETTER TO THE EDITOR,

Like other cancers, chronic lymphocytic leukemia (CLL) is initiated and/or progresses as a consequence of concurrent chromosomal abnormalities and recurrent somatic mutations. Using next-generation sequencing, a short list of recurrent mutations in various genes has been identified in CLL.[1,2] One of these genes is *SF3B1* that encodes a key component of the mRNA splicing machinery. *SF3B1* mutations were initially discovered in myelodysplastic syndromes (MDS) and in some other solid tumors suggesting it is playing an important role in cancer biology. In CLL, *SF3B1* mutations are associated with disease subtype,[1] progression,[3,4] chemotherapy resistance,[4,5] and overall patient survival.[6] However, the biological consequences of *SF3B1* mutations in CLL pathogenesis are largely unknown. Here we find that acquisition of mutations in *SF3B1* eventually leads to the loss of the wild-type copy of this gene suggesting the mutant *SF3B1* gene plays a dominant role in clonal evolution. We also provide evidence that *SF3B1* mutations are potentially oncogenic, supporting the possibility that mutant *SF3B1* is an attractive druggable therapeutic target.

Wang et al reported that *SF3B1* mutations are more prevalent in CLL patients with 11q deletion.[1] We randomly picked 73 cryopreserved PBMC samples with 11q deletion from our CLL patient cohort, and screened for *SF3B1* mutations. We identified 8 patients with various missense mutations including 5 patients with K700E (2098A>G), 1 with K649E (1945A>G), 1 with K622E (1866G>T), and 1 with K666E (1996A>G). These mutations have been observed by others in CLL, MDS, and other cancers.[1,2,7] We also found that *SF3B1* mutations are only present in a sub-allelic-fraction (ranging from 10% to 45%) of bulk DNA samples (Figure 1A).

Cancer progression is typically characterized by the emergence and outgrowth of newly evolved subclones. By analyzing the allelic burden of *SF3B1* mutations in CLL using

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Sanger sequencing in serial patient samples, Schwaederle et al[3] showed that the weight of mutant *SF3B1* increases as the disease progresses. However, the size of *SF3B1* DNA allelic fractions does not necessarily reflect the size of the subclone, since it remains unknown if the observed mutant *SF3B1* allelic increase at the bulk cell population level reflects a change in size of the mutant *SF3B1* subclone or instead if there is a change in zygosity of *SF3B1* mutations of the subclone. In fact, it has been postulated that SF3B1 mutations are heterozygous in MDS and CLL[7-9] largely based on the observation that allelic burdens of mutant *SF3B1* are typically <50%. To ascertain the zygosity of *SF3B1* mutations in CLL, we analyzed *SF3B1* mutations at the single cell level by DNA-based PCR (Figure 1B). As expected, many single cells exhibited either wild-type only (wt/wt), or wild-type plus mutant *SF3B1* sequences (heterozygous, wt/mu). To our surprise owing to previous predictions, in all 4 CLL samples we detected multiple single cells possessing solely *SF3B1* mutant sequences resembling “homozygous” genotypes (mu/mu-like). This observation suggests that a prominent CLL subclone in these patients exclusively carries mutant *SF3B1*. Figures 1C and 1D depict representative Sanger sequencing data of single cells from patient 35 and the summary of all 4 patients, respectively. Similar data were also obtained when single cells were collected directly into 96 well PCR plates using FACS (results not shown).

It is known that allelic drop out (ADO) is an artifact that can occur in DNA-based single cell PCR due to the fact that only one copy of DNA from each allele is present for testing. However, potential ADO can be largely overcome by analyzing 8 or more single cells.[10,11] Because we performed single cell PCR analysis on 11 to 43 single cells from each patient, we believe it is unlikely that ADO underlies our observations that some CLL cells express only mutant *SF3B1*. In an added measure to rule out possible ADO in our DNA-based single cell PCR, we also performed RNA-based single cell RT-PCR on 18 single CLL cells from patient 35. Thus, the rate of ADO using this method is vastly reduced by the presence of many more copies of *SF3B1* mRNA transcripts (wildtype or mutant) in a single cell as compared to DNA. Indeed, we also observed that a similar subset of CLL cells carry solely mutant *SF3B1* transcripts (Figure 1C and D), confirming the reliability of our DNA-based single cell PCR.

Our results support a subclonal evolutionary pathway of *SF3B1* mutations in CLL proceeding from wt/wt→wt/mu→mu/mu-like. The true *SF3B1* genotype of the mutant *SF3B1*-only subclone is unknown, but it should fall into one of the following three possibilities: 1) *bona fide* homozygous *SF3B1* mutation with an identical mutation on both alleles; 2) *SF3B1* mutation on one allele with simultaneous loss of the wild-type copy on the other allele, i.e., loss of heterozygosity (LOH); or 3) copy-neutral LOH or uniparental disomy, where cells have gained a duplicated mutant copy of *SF3B1* but lost the wild-type copy of the gene. Accurate identification of the precise genotype of cells with mutant *SF3B1* at the single cell level, however, requires techniques that are yet to be developed. The emergence of mu/mu-like *SF3B1* mutant subclones suggests they have a selection advantage over their heterozygous and wild-type precursor subclones. However, it is also conceivable that patients with a similar bulk SF3B1 mutation weight but different sizes and genotypes of the subclones may exhibit differences in clinical outcome. We believe that our single cell analysis approach will enable us to distinguish the two when analyzing serial patient

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samples (studies in progress). In addition, our approach also provides a proof-of-concept means to analyze true clonal and subclonal mutations in other cancer genes.

To address the biological functions of *SF3B1*, Isono, et al demonstrated that *SF3B1* knockout in mice led to an early embryonic lethality.[12] *SF3B1* null embryos die around 2 days after conception (16-32 cell stage of development), the time point at which parental supplies of SF3B1 protein and mRNA are about to be exhausted. This observation suggests *SF3B1* is an essential gene for cell survival, at least in mouse embryonic cells. To further validate this finding in human cells, we performed shRNA knockdown of *SF3B1* in HEK293T cells and observed that knockdown of *SF3B1* severely inhibits the formation of cell clones (Figure 2A), consistent with the mouse knockout data that *SF3B1* is absolutely necessary for cell survival.

The association of *SF3B1* mutations with CLL progression[3,4] suggests these mutations may confer a faster rate of leukemia cell proliferation. This prediction is in opposition to the phenotypes of *SF3B1* knockout in mice and of shRNA knockdown in human cells. We, therefore, hypothesize that *SF3B1* mutations in CLL are in fact oncogenic gain-of-function mutations, rather than tumor suppressive loss-of-function mutations. Due to technical hurdles in the cloning of full-length *SF3B1* cDNA, we are unable to experimentally demonstrate the oncogenic activity of *SF3B1* mutations at this moment. To overcome this difficulty, we took an *in silico* approach using the Catalog of Somatic Mutations in Cancer (COSMIC)[13] database. It is well known that cancer develops upon mutational inactivation of tumor suppressor genes or mutational hyperactivation of oncogenes. We profiled all of the mutation data for the top 35 known cancer genes in this database including 18 tumor suppressor genes and 17 proto oncogenes for their frequencies of mutations that lead to ultimate protein truncation, namely frameshifting, and nonsense mutations. As anticipated, a significant portion of the mutations in 18 tumor suppressor genes are truncation mutations (varying from 97.9% in *NPM1* to 13.6% in *EZH2*), while very few truncation mutations (0.03% in *JAK2* to 2.5% in *ERBB2*) were found in all 17 known proto-oncogenes. This analysis suggests that the frequency of truncation mutations can accurately predict if an unknown gene is a tumor suppressor or an oncogene. We next profiled all 637 entries of the *SF3B1* gene mutations deposited in the COSMIC database (v63 release). We found that only 2 (0.3%) were protein truncation mutations and that most of the mutations are in the hotspot sites (K700, K666, H662, R625 and E622), strongly suggesting that *SF3B1* is a proto-oncogene that disfavors protein inactivating truncation mutations (Figure 2B). The results emerging from gene targeting in mice, gene knockdown in human cells, and *in silico* mutational analysis collectively suggest that *SF3B1* is a proto-oncogene, and is consistent with its dominant role in clonal evolution as we suggested above. Our results are also in agreement with the observation that *SF3B1* is often overexpressed in CLL cells,[4] and inhibitors to wild-type SF3B1 protein exhibit potent antitumor activity.[14] In fact, the splicing factor *SRSF1* has long been recognized as a potent oncogene.[15] Armed with this information, we suggest that specific targeting of *SF3B1* mutations such as K700E may be of therapeutic benefit for patients with CLL and other cancers housing *SF3B1* mutations.

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Supplementary Material

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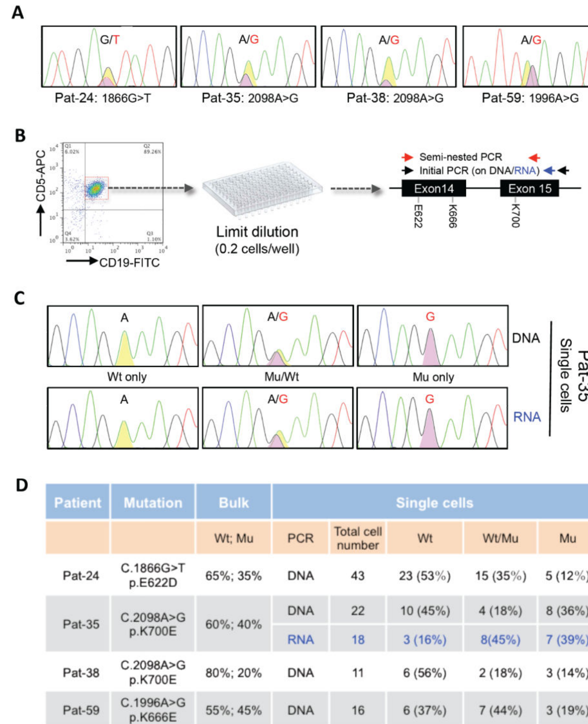

Patient	Mutation	Bulk	Single cells				
		Wt; Mu	PCR	Total cell number	Wt	Wt/Mu	Mu
Pat-24	C.1866G>T p.E622D	65%; 35%	DNA	43	23 (53%)	15 (35%)	5 (12%)
Pat-35	C.2098A>G p.K700E	60%; 40%	DNA	22	10 (45%)	4 (18%)	8 (36%)
			RNA	18	3 (16%)	8(45%)	7 (39%)
Pat-38	C.2098A>G p.K700E	80%; 20%	DNA	11	6 (56%)	2 (18%)	3 (14%)
Pat-59	C.1996A>G p.K666E	55%; 45%	DNA	16	6 (37%)	7 (44%)	3 (19%)

Figure 1. *SF3B1* genotyping in bulk and in single CLL cells

A. Sanger DNA sequencing chromatograms of *SF3B1* sequences amplified from four representative CLL patient samples. Purple and yellow color filled peaks show allelic burdens of mutant and wild-type nucleotide sequences, respectively. **B.** Schematic flowchart of single CLL cell preparation for *SF3B1* mutation analysis. $CD19^+/CD5^+$ leukemic cells are sorted from patient PBMCs, and plated by limiting dilution in 96-well PCR plates for two rounds of PCR amplification for *SF3B1* sequences. **C.** Representative chromatograms of Sanger DNA sequencing of *SF3B1* sequences amplified from single CLL cells from patient 35. Purple and yellow color filled peaks are mutant and wild-type nucleotide sequences, respectively. **D.** Summary table of all four patient samples analyzed using DNA or RNA at the single cell level using a limiting dilution approach.

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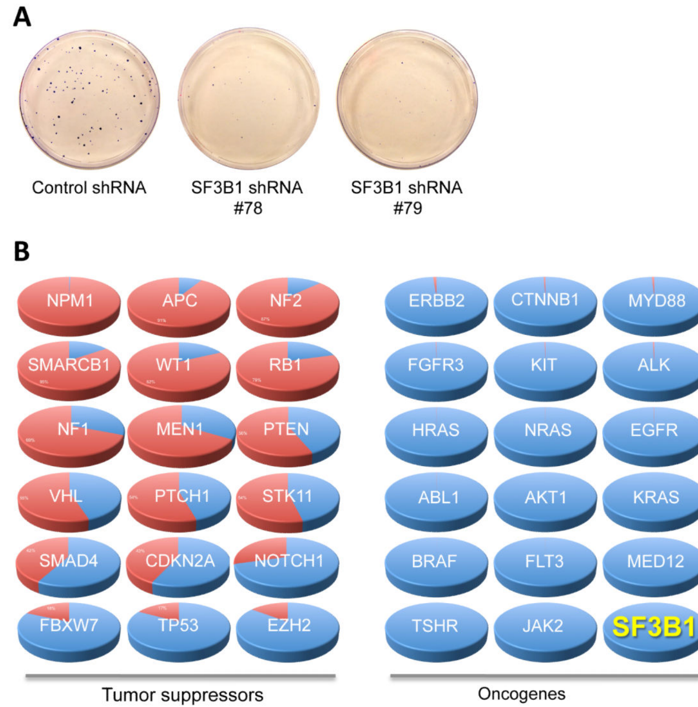

Figure 2. *In silico* profiling of *SF3B1* mutations in the COSMIC database

A. Clonogenic assay of HEK293 cells infected with control or *SF3B1* shRNAs. **B.** Systemic profiling of mutations of the top 35 cancer genes in the COSMIC database. The portions in red are the percentages of protein truncating (frameshifting and nonsense) mutations while the blue coloring reflects the percentages of non-protein truncating mutations. The mutation patterns of mutated *SF3B1* reported to date predict *SF3B1* is a proto-oncogene.