LETTER TO THE EDITOR

NON-CODING NOTCH1 MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA; THEIR CLINICAL IMPACT IN THE UK CLL4 TRIAL

In chronic lymphocytic leukemia (CLL), ‘coding’ NOTCH1 mutations were initially detected in exon 34, where they result in truncation of the C-PEST regulatory protein sequence, with consequent impaired degradation of the Notch1 intracellular domain (NCID), constitutive activation of Notch signalling and increased cell survival and resistance to apoptosis.\(^1\) Mutations occur in 6-10% of cases at diagnosis, with increasing prevalence in advanced disease stages, treatment-refractory disease, and after transformation to Richter syndrome.\(^4,5\) In diagnostic and clinical trial cohorts, patients with NOTCH1 mutations exhibited reduced survival.\(^5,6\) In 2015, Puente and colleagues identified recurrent ‘non-coding’ mutations clustered to the 3’ UTR of NOTCH1 in 2% (11/506) previously untreated patients with CLL or monochlonal B-cell lymphocytosis.\(^7\) The presence of these 3’UTR mutations cause a novel splicing event, preferentially between a cryptic donor site located in the last exon and a newly created acceptor site in the 3’UTR of exon 34, resulting in the removal of the PEST sequence and constitutive activation of downstream signalling.\(^7\) Patients with non-coding NOTCH1 mutations had similar outcomes to those with coding mutations, with shorter time to first treatment and shorter overall survival than wild type cases.\(^7,8\)

Given the highly variable natural history of CLL and the often-serendipitous date of initial diagnosis, we aimed to establish the clinical significance of non-coding NOTCH1 mutations in DNA samples available from 489 patients at enrolment to the United Kingdom Leukemia Research Fund Chronic Lymphocytic Leukemia 4 (UK LRF CLL4) chemotherapy trial.\(^9\) NOTCH1 3’ UTR mutations were identified by High Resolution Melt (HRM) analysis in whole genome amplified DNA (F: TGCTCGTTCAACTTCCCTTC; R: CAAGCAAGTTCTGAGAGCCA) and confirmed by Sanger sequencing of genomic DNA (F: CCTAACAGGCAGGTAGCT; R: ATCTGGCCCCAGGTAGAAAC) The results were combined with the data pertaining to coding NOTCH1 mutations in the same patient cohort from our previous publication.\(^5\) 53 patients with wild-type HRM traces were sequenced, and no additional non-coding mutations were identified. It was not possible to differentiate between clonal and sub-clonal NOTCH1 mutations using our HRM/Sanger approach. We defined associations between the presence of NOTCH1 coding and non-coding mutation and a comprehensive panel of clinical and biological features reported in previous CLL4 papers,\(^10-13\) by univariate logistic regression. Kaplan-Meier, log-rank test and Cox regression analysis were used to assess the impact of NOTCH1 status on survival using Stata, where overall (OS) and progression-free (PFS) survival were defined as time from randomization to death from any cause and to relapse needing treatment, progression or death from any cause at last follow-up, respectively.

In addition to exon 34 coding mutations observed in 47/489 (9.6%) CLL4 patients, we detected an additional 11/489 (2.2%) patients harbouring the non-coding mutations 139390152 A>G (n=7) and 139390145 A>G (n=4) (Figure 1A), both previously reported to result in aberrant NOTCH1 splicing.\(^7\) Importantly, the non-coding variants were mutually...
exclusive to coding variants, constituting 19% of the total NOTCH1 mutational burden of CLL4 cases, with 11.8% of patients carrying either type of NOTCH1 mutation. NOTCH1 non-coding mutations were not identified in cases with mutations of TP53, BIRC3, BRAF (V660E), MYD88 (L265P), NFKBIE and RPS15 mutations, but did co-occur with SF3B1 [n=2] and ATM [n=2] mutations (Figure 1B). Next, we evaluated the association between NOTCH1 mutations and the main clinico-biological characteristics in CLL (Table S1). As expected, when all 58 mutations were considered together, NOTCH1 mutations were significantly more prevalent in CLL4 cases with unmutated IGHV genes (OR: 2.9, 95% CI: 1.4-6.2, P=0.005), CD38 (OR: 4.5, 95% CI: 2.3-8.7, P<0.001) and ZAP70 positivity (OR: 3.1, 95% CI: 1.5-6.4, P=0.002), high expression of CLLU1 (OR: 2.33, 95% CI: 1.2-4.4, P=0.01), trisomy 12 (OR: 4.0, 95% CI: 2.2-7.4, P<0.001) and ≥15x10^9/l absolute pro-lymphocytes (OR: 3.12, 95% CI: 2.0-7.9, P<0.001). However, for non-coding mutations on its own only the association with Trisomy 12 remained significant (OR: 5.6, 95% CI: 1.6-18.8, P=0.006), in spite of the limited number of cases with these mutations. Of the 364 deaths in CLL4 patients with NOTCH1 data, 14 (4%) were due to Richter’s syndrome (RS). With non-coding NOTCH1 mutations included, four of fourteen (29%) Richter’s deaths occurred in patients with NOTCH1 mutation, an association that was non-significant (P=0.062).

In our previous CLL4 study we confirmed the independent prognostic significance of a number of biomarkers including coding NOTCH1 mutations. In our current study, we determined the impact of coding and non-coding mutations on overall response rate (ORR), OS and PFS. Coding and non-coding mutations, inspected together or separately, were not associated with ORR in any of the three treatment arms (data not shown). Considered separately, univariate Cox regression analysis showed that patients with NOTCH1 non-coding or coding mutations exhibited a significantly shorter OS (median survival times: 43.2 and 54.8 months, respectively) than patients with wild-type NOTCH1 (median 74.6 months). Non-coding and coding NOTCH1 mutations were also associated with reduced PFS (median survival times: 22.0 and 13.0 months respectively) compared to the wild type NOTCH1 (28 months). In further support of their clinical importance, cases with non-coding NOTCH1 mutations showed a two-fold increase in the risk of mortality when compared to wild type (HR: 2.15, 95% CI: 1.17-3.92, P=0.013) and an 80% increase in the risk of progression or death (HR: 1.78, 95% CI: 0.98-3.24, P=0.05). The impact of coding and non-coding NOTCH1 mutations together on OS was sustained in a multivariable model where NOTCH1 status was controlled for gender, age, stage, IGHV and SF3B1 mutational status, 11q deletion, and TP53 mutation/deletion (adjusted HR: 1.5, 95% CI: 1.0-2.1, P=0.04, Table 1). On the contrary, the association between NOTCH1 mutational status and PFS was not significant when adjusted for the other variables listed above (adjusted HR: 1.3, 95% CI: 0.9-1.9, P=0.108) Taken together, we show that NOTCH1 status, based on the presence of either mutational type, is an independent risk factor for OS but not for PFS. The association between OS or PFS and the occurrence of non-coding mutations could not be estimated reliably in a multivariable analysis because of the small number of cases with such mutations in our series. Finally, we attempted to quantify the improved discriminatory power of including non-coding NOTCH1 mutations to coding mutations as a test to predict both the presence and
absence of PFS and OS events at last follow-up using sensitivity-specificity analysis. The
analysis was carried out on all 489 cases. NOTCH1 coding mutations correctly predicted
46/454 PFS (sensitivity of 10.1%) and 43/393 (sensitivity of 10.9%) OS events (Table S2A and
S3A). As expected, the sensitivity for OS and PFS was higher when both mutational types
were considered than when coding mutation alone was analysed: 13.7 versus 10.9% for OS
and 12.6 versus 10.1% for PFS events (Table S2A and S3A). This increase reflected the fact
that all 11 patients with non-coding NOTCH1 mutations exhibited an adverse OS and PFS
event resulting in 100% specificity for non-coding NOTCH1 mutation as a test. Accuracy
assesses the capability of a given biomarker to correctly predict both the presence and
absence of a survival event. Coding NOTCH1 mutations displayed 16.4 and 27.6% accuracy
for correctly predicting the presence or absence of a PFS and OS respectively. Accuracy was
increased to 18.6 and 29.9% for PFS and OS respectively, when non-coding mutations were
included in this analysis. The likelihood ratio, LR+, which adjusts sensitivity for false positives
and LR- which adjusts specificity for false negatives are prevalence-independent and their
ratio, LR+/LR- (diagnostic odds ratio), is an indicator of the predictive power of the
biomarker. A biomarker with a higher LR+/LR- value is a better predictor of the disease
outcomes. Consistent with the increased sensitivity and higher accuracy, we observe
increased LR+/LR- ratios for both PFS (3.81 versus 4.88) and OS (2.43 versus 3.66) when
both coding and non-coding mutations were considered together (Table S2A and S3A). In
addition, the positive predictive value (PPV) which is a measure of the proportion of true
positives out of all the outcomes predicted by the biomarker, is higher when non-coding
mutation was included in the test than when coding-mutation alone was used as the test
biomarker (98.3 versus 97.9% for PFS and 93.1 versus 91.5% for OS, Table S2B, S3B).

In summary, our data confirm the prognostic importance of non-coding NOTCH1 mutations
in patients requiring first-line treatment with chemotherapy as part of the UK CLL4 trial.
Importantly, restricted analysis of exon 34 neglected to identify 19% of patients with
pathogenic NOTCH1 mutations in its 3’ UTR region. In addition, we show that the
discriminatory power of NOTCH1 mutation status to predict outcomes is improved with the
inclusion of non-coding mutations. Taken together, our study supports the analysis of the 3’
UTR region of the NOTCH1 gene to identify additional patients with reduced survival.
Several recent studies have provided conflicting data on the clinical significance of clonal
and sub-clonal NOTCH1 mutations. Most recently, Nadeu and colleagues
demonstrated that clonal mutations predicted for short OS while subclonal mutations
predicted for short time to first treatment. It will be important to employ these same deep
sequencing approaches to ascertain the clinical significance of sub-clonal NOTCH1 mutations
in the clinical trials setting. The UK CLL4 trial benefits from long-term clinical follow-up and
expansive associated clinico-biological data but only assessed the utility of traditional
chemotherapy. Therefore, it will be necessary to establish the impact of non-coding
NOTCH1 mutations in patients treated with chemo-immunotherapy, where they are likely to
identify a significant number of additional patients destined to respond poorly to rituximab-
containing treatment regimens. Mutant NOTCH1 currently represents a therapeutic target
in T-ALL, with several mechanistic approaches under clinical development, including γ-
secretase and metalloproteinases inhibitors, antibodies directed against the extracellular
domain of Notch1, and antagonists that act by directly targeting the Notch transactivation domain. Screening for non-coding NOTCH1 mutations identifies additional CLL patients with Notch1 activation, offering motivation for clinical trials development. Assuming these approaches are ultimately approved for the treatment of CLL, it will be critical to identify all patients that will benefit from these treatments, as there will be important clinical and cost implications. These studies will help establish a stratified and individualized approach to clinical management, including the more accurate selection of patients for targeted therapy.

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Authorship Contributions

Contribution: ML, MJJR-Z, HP, SB, JF and ZD performed the experimental work; ML, MJJR-Z, LK, AC and ME conducted the statistical analysis; DGO, ME and DC contributed patient samples and data; JCS designed the study; ML, LK, DGO and JCS wrote the paper; all the authors critically reviewed the final paper.

Conflicts of Interest

The authors declare no conflict of interest.

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References


Figure 1. The genomic and clinical characteristics of NOTCH1 non-coding and coding mutations in the LRF CLL4 trial. (A) The distribution of mutations in NOTCH1. The NOTCH1 gene contains 34 exons and encodes a protein with a C-terminal TAD-PEST domain, which is a hotspot for mutation in CLL. Part of exon 34 and the 3' UTR are magnified and the location of each mutation is shown; coding (white) and non-coding mutations (black) are indicated. Each dot represent a single mutation. (B) The mutual relationship between coding and non-coding NOTCH1 mutations and other clinico-biological characteristics in CLL. Rows correspond to specific clinical and biological features and columns represent individual patients (only patients with a NOTCH1 mutation are shown). Boxes colored black and grey show the presence or absence of a parameter. A white box denotes that no data were available. (C) and (D) Kaplan-Meir plots showing progression-free survival and overall survival, respectively.
Table 1. Univariate and multivariate Cox proportional hazard analysis of OS and PFS in CLL4 patients.

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Footnote. Chl: chlorambucil, FDR: fludarabine, FC: fludarabine plus cyclophosphamide. OS multivariate: 342 cases with 252 events, 153 missing data. PFS multivariate: 342 cases with 315 events, 153 missing data