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## OPEN

# Non-coding *NOTCH1* mutations in chronic lymphocytic leukemia; their clinical impact in the UK CLL4 trial

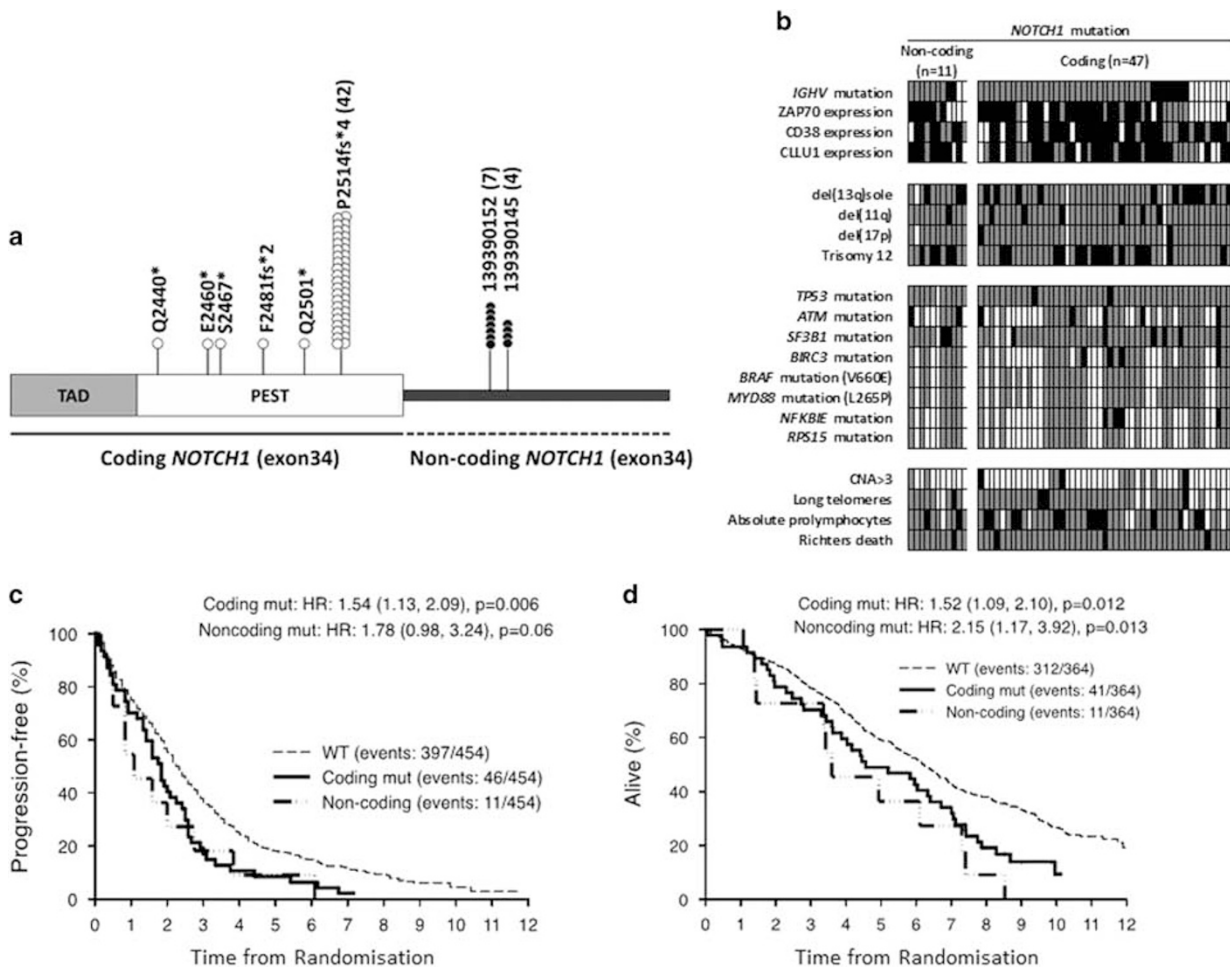
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In chronic lymphocytic leukemia (CLL), 'coding' *NOTCH1* mutations were initially detected in exon 34 where they result in truncation of the C-PEST regulatory protein sequence with consequent impaired degradation of the Notch1 intracellular domain (NICD), constitutive activation of Notch signalling and increased cell survival and resistance to apoptosis.<sup>1–3</sup> 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.<sup>4,5</sup> In diagnostic and clinical trial cohorts, patients with *NOTCH1* mutations exhibited reduced survival.<sup>5,6</sup> 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 monoclonal B-cell lymphocytosis.<sup>7</sup> 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 signaling.<sup>7</sup> 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.<sup>7,8</sup>

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.<sup>9</sup> *NOTCH1* 3'-UTR mutations were identified by High Resolution Melt (HRM) analysis in whole genome amplified DNA (F: TGCTCGTTCAACTCC

CTTC; R: CAAGCAAGTTCTGAGAGCCA) and confirmed by Sanger sequencing of genomic DNA (F: CCTAACAGGCAGGTGATGCT; R: ATCTGGCCCCAGGTAGAAAC). The results were combined with the data pertaining to coding *NOTCH1* mutations in the same patient cohort from our previous publication.<sup>5</sup> Fifty-three 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 subclonal *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,<sup>10–13</sup> 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 139390152A>G ( $n=7$ ) and 139390145A>G ( $n=4$ ; Figure 1a), both previously reported to result in aberrant *NOTCH1* splicing.<sup>7</sup> 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 the 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 the *NOTCH1* mutations and the main clinico-biological characteristics in CLL (Supplementary Table S1).



**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-Meier plots showing progression-free survival and overall survival, respectively.

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  $\geq 15 \times 10^9/l$  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 the *NOTCH1* data, 14 (4%) were due to Richter's syndrome (RS). With non-coding *NOTCH1* mutations included, 4 of 14 (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.<sup>5</sup> 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 with the wild-type *NOTCH1* (28 months, Figure 1c and d). 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 with 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 multi-variable 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

**Table 1.** Univariate and multivariate Cox proportional hazard analysis of OS and PFS in CLL4 patients

Variable	Overall survival						Progression-free survival						
	Univariate			Multivariate			Univariate			Multivariate			
	Total	Events	Median	95% CI	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
NOTCH1													
Wild type	431	312	74.6	67.8–81.5	1.6	1.2–2.2	0.001	1.5	1.0–2.1	0.04	1.6	1.2–2.1	0.001
	Mutated	58	52	53.4									
SF3B1	364	250	79.1	71.8–86.3	1.7	1.3–2.2	< 0.001	1.5	1.1–2.1	0.014	1.3	1.0–1.7	0.033
	Mutated	73	66	54.3									
Age					1.1	1.0–1.1	< 0.001	1.1	1.0–1.1	< 0.001	1	0.9–1.1	0.663
Sex													
Male	366	281	70.1	61.4–78.9	0.8	0.6–1.0	0.056	0.8	0.6–1.1	0.121	0.8	0.7–1.0	0.055
Female	129	86	79.6	66.5–93.0									
Binet stage													
A	112	76	80.6	63.4–97.7	1.3	1.0–1.7	0.049	1.5	1.1–2.1	0.013	0.9	0.8–1.3	0.995
B/C	383	291	71.5	64.6–78.3									
Del(11q)													
Undeleted	373	267	75	67.5–82.6	1.6	1.3–2.1	< 0.001	1.4	1.1–1.9	0.023	1.5	1.2–1.9	0.001
Deleted	92	79	57.7	42.4–73.0									
IGHV status													
Mutated	155	91	104.2	93.3–115.1	2.2	1.7–2.8	< 0.001	1.9	1.4–2.5	< 0.001	1.9	1.6–2.4	< 0.001
Unmutated	255	216	60.6	52–8–68.4									
TP53 status													
Normal	431	313	75.9	69.3–82.1	3.1	2.2–4.6	< 0.001	2.5	1.5–4.1	< 0.001	2.7	1.9–3.9	< 0.001
Del/Mut	32	31	26.1	4.9–47.4									
Treatment arm													
Chl	238	178	76.8	70.1–83.4	1.1	0.9–1.3	0.426	0.9	0.8–1.3	0.854	0.6	0.5–0.7	< 0.001
FDR/FC	257	189	68	57.9–78.1									
Abbreviations: Chl, chlorambucil; FC, fludarabine plus cyclophosphamide; FDR, fludarabine. OS multivariate, 342 cases with 252 events; 153 missing data. PFS multivariate, 342 cases with 315 events, 153 missing data.													

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

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 (Supplementary 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 (Supplementary 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 (Supplementary 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, Supplementary Table S2B and 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 subclonal *NOTCH1* mutations.<sup>8,14,15</sup> Most recently, Nadeu and colleagues demonstrated that the clonal mutations predicted for short OS, while subclonal mutations predicted for short time to first treatment.<sup>9</sup> It will be important to employ these same deep sequencing approaches to ascertain the clinical significance of subclonal *NOTCH1* mutations in the clinical trials setting. The UK CLL4 trial benefits from long-term clinical follow-up and the 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.<sup>6</sup> Mutant *NOTCH1* currently represents a therapeutic target in T-ALL, with several mechanistic approaches

under clinical development, including  $\gamma$ -secretase and metallo-proteinases 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.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

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.

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## OPEN

# A novel t(3;13)(q13;q12) translocation fusing FLT3 with GOLGB1: toward myeloid/lymphoid neoplasms with eosinophilia and rearrangement of FLT3?

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According to the 2016 World Health Organization classification, myeloid neoplasms with eosinophilia (MPN-Eo) are associated with genetic abnormalities of genes coding for type III tyrosine kinase (TK) receptors, mainly PDGFRA, PDGFRB and FGFR1, but also JAK2.<sup>1</sup> Beside these translocations, very rare FLT3 gene rearrangements have been reported, which raises the double question of its association with myeloid neoplasms and of its specific targeted therapy.<sup>2–7</sup>

A new t(3;13)(q13;q12) was found from a case of atypical mixed lymphoid/myeloid neoplasm. This case, diagnosed MPN-Eo, was characterized by the coexistence of bone marrow myeloproliferation with circulating hypereosinophilia and T-cell lymphoblastic lymphoma in lymph node (Supplementary Results for detailed description). The patient could not benefit from new tyrosine kinase inhibitors. Evolution was fatal in 3 months despite conventional CHOP chemotherapy (Cyclophosphamide, Hydroxydaunorubicin, Oncovin and Prednisolone).

Karyotype of tumor cells from lymph nodes and bone marrow revealed a single clonal t(3;13)(q13;q12) translocation (Figure 1a, left panel). Absence of *FGFR1* gene rearrangement was checked by fluorescence *in situ* hybridization (FISH) and RT-PCR according to methods described by others.<sup>8</sup> BCR-ABL gene translocation, FLT3-ITD and D835 mutation were also absent. FISH walking on both chromosomes 3 and 13 with BAC and fosmid probes showed that the breakpoint was located in a 58.6 kb region encompassing *HCLS1* and *GOLGB1* on chromosome 3 and in a 65.5 kb region containing the *FLT3* locus on chromosome 13 (Figure 1a, right panel).

*FLT3* maps to band q12 of chromosome 13 and *GOLGB1* to chromosome band 3q13. We hypothesized that this translocation would lead to a fusion transcript. Since the breakpoint region covered 15 out of the 23 exons of the *GOLGB1* gene, we hypothesized that *GOLGB1* gene could be a fusion partner. *FLT3* gene was the only candidate on chromosome 13. A multiplex PCR amplified a specific product located between exons 13 and 15 of *GOLGB1* and *FLT3* respectively (Figure 1b). Direct sequencing showed that this 2000 bp PCR product was specific. The rearrangement fused exons 14 of both *GOLGB1* and *FLT3* genes. Moreover, 36 bp of intron 14 of *GOLGB1* were inserted between the two exons 14 of *GOLGB1* and *FLT3* (Figure 1c). The genomic fragment corresponding to the der(3) contains the 5' sequence of *GOLGB1* fused in frame to the 3' sequence of *FLT3* at nucleotide 8841 which corresponds to the beginning of exon 14. Genomic DNA sequencing showed that breakpoints were within *GOLGB1* intron 14 and *FLT3* exon 14 (not shown).

This t(3;13)(q13;q12) translocation identifies *GOLGB1* as a new partner of *FLT3*. *GOLGB1* encodes for giantin, a golgin subfamily B member 1 and the largest golgi complex-associated protein (372 kD), with numerous coiled-coil regions. *GOLGB1*-*FLT3* protein fused together the three coiled-coil *GOLGB1* domains with the split kinase TK domain of *FLT3*, that could lead to a constitutively multimerized active protein. Alternatively, constitutive TK activation could be due to the loss of the inhibitory juxtamembrane domain of *FLT3*, as reported for FIP1L1-PDGFRα gene rearrangement.<sup>9</sup> *GOLGB1* has been recently reported as a fusion partner with PDGFRB in a t(3;5)(q13;q33) translocation in a male patient with MLN-Eos.<sup>10</sup> PDGFRB has also been reported to be fused with another golgin subfamily member, *GOLGA4*.<sup>11</sup> The other published *FLT3* partners,