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NOTCH1 mutations associate with low CD20 level in chronic lymphocytic leukemia: evidence for a _NOTCH1_ mutation-driven epigenetic dysregulation

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Running title: ***NOTCH1* mutations and CD20 expression in CLL**

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Abstract

In chronic lymphocytic leukemia (CLL), *NOTCH1* mutations have been associated with clinical resistance to the anti-CD20 rituximab, although the mechanisms behind this peculiar behavior remain to be clarified. In a wide CLL series (n=692), we demonstrated that CLL cells from *NOTCH1* mutated cases (87/692) were characterized by lower CD20 expression, and lower relative lysis induced by anti-CD20 exposure in-vitro. Consistently, CD20 expression by CLL cells was up-regulated in-vitro by γ-secretase inhibitors or NOTCH1-specific siRNA, and the stable transfection of a mutated (c.7541-7542delCT) NOTCH1 intracellular domain (NICD-mut) into CLL-like cells resulted in a strong downregulation of both CD20 protein and transcript. By using these NICD-mut transfectants, we investigated protein interactions of RBPJ, a transcription factor acting either as activator or repressor of NOTCH1 pathway when respectively bound to NICD or hystone deacetylases (HDACs). Compared to controls, NICD-mut transfectants had RBPJ preferentially complexed to NICD, and showed higher levels of HDACs interacting with the promoter of the CD20 gene. Finally, treatment with the HDAC inhibitor valproic acid upregulated CD20 in both NICD-mut transfectants and primary CLL cells. In conclusion, *NOTCH1* mutations are associated with low CD20 levels in CLL and are responsible for a dysregulation of HDAC-mediated epigenetic repression of CD20 expression.

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Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with highly variable clinical courses and survivals ranging from months to decades. In particular, a subset of CLL patients is known to experience a progressive symptomatic disease poorly responsive to the common immuno-chemotherapeutic regimens.[1,2] A fraction of these high risk CLL, overall accounting for 5-10% of cases, can be identified by screening for *TP53* mutation/deletion,[1,2] while an additional fraction of cases has been recently shown to bear mutations involving the *NOTCH1*, *SF3B1* and *BIRC3* genes. Overall, alterations of these genes occur in approximately 20% of CLL patients at diagnosis and have significant correlations with survival in consecutive series from independent institutions.[3-7]

Mutations of *NOTCH1* are found in about 10% of CLL cases at diagnosis, with frequency increasing in advanced disease phases, in chemorefractory patients, and during transformation to Richter Syndrome.[3-5,7,8] Moreover, *NOTCH1* mutations are enriched in CLL patient subgroups defined by trisomy 12 and an unmutated *IGHV* gene status.[9,10] *NOTCH1* encodes for a transmembrane receptor acting as a ligand-activated transcription factor.[11,12] In particular, NOTCH1 signaling initiates when the ligand, from either the JAGGED or DELTA families, binds to the receptor and induces successive proteolytic cleavages, resulting in the release and nuclear translocation of the NOTCH1 intra-cellular domain (NICD). In the nucleus, the NICD becomes part of an activation complex along with the transcription factor RBPJ, that leads to the de-repression/activation of specific target genes, including genes of the HES family.[13-20] At variance with normal B cells, CLL cells constitutively express the NOTCH1 receptor as well as its ligands JAGGED1 and JAGGED2, suggesting autocrine/paracrine loops for NOTCH1 signaling activation.[21] In CLL, virtually all NOTCH1 mutations are frameshift or non-sense events clustering within exon 34, including a highly recurrent c.7541-7542delCT frameshift deletion, represented in 80% of cases.[3,4,10] These mutations result in the truncation of the C-PEST regulatory domain of the protein and the subsequent impaired degradation of the NICD,[3,4,22-24] which in turn determines to an intense and sustained activation of the NOTCH1 pathway.[25]

Recently, the presence of *NOTCH1* mutations has been associated with a relative resistance to anti-CD20 immunotherapy in a prospective clinical study comparing the effectiveness of the fludarabine plus cyclophosphamide (FC) regimen versus the FC plus rituximab (FCR) regimen[26], although the biological mechanisms underlying the differential activity of rituximab in relation to *NOTCH1* mutational status is still to be elucidated.

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Materials and Methods

Primary cells from CLL patients and healthy donors

The study was approved by the Internal Review Board of the Aviano Centro di Riferimento Oncologico (Approval n. IRB-05-2010), and included peripheral blood samples from 692 patients with CLL.[27] Informed consent was obtained in accordance with the declaration of Helsinki. CLL cases were characterized for *IGHV* mutational status, the main cytogenetic abnormalities, CD38, CD49d, ZAP70 expression, as described.[28]

Primary CLL cells and normal B cells from healthy donors (n=3) were obtained from peripheral blood samples by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and used either directly or cryopreserved until use. All studies were performed on highly purified cells (>95% pure), as results of negative selection by immunomagnetic beads when required.[29] In-vitro studies were performed in CLL cells from *NOTCH1* mutated cases with relevant *NOTCH1* mutational burden, i.e >25% of total DNA, or in *NOTCH1* wild type cases, as control.

CD20 expression

CD20 expression was evaluated by flow cytometry at the Clinical and Experimental Onco-Hematology Unit (CRO, Aviano), in the 692 CLL cases entering this study, as part of the routine diagnostic procedures for CLL assessment. In particular, 495 cases were evaluated by a FITC-conjugated anti-CD20 antibody, while, in the remaining 197 cases, a PE-Cy7-conjugated antibody was employed (clone L27, in both cases, BD Biosciences, Milan, Italy), due to a modification of the flow cytometry diagnostic panel. For CD20 expression analyses, these two cohorts were kept separated. All experiments were performed on FACSCanto II (BD Biosciences, Milan, Italy).[28,29]

NOTCH1 mutational status

The presence of c.7541-7542delCT *NOTCH1* mutation was investigated by amplification refractory mutation system (ARMS) PCR, as described.[3,8,10] The load of c.7541-7542delCT *NOTCH1* mutation was evaluated by next generation sequencing (NGS) using a MiSeq sequencer (Illumina, San Diego, CA), with a ~1000X coverage-fold.

The presence of *NOTCH1* mutations other than the c.7541-7542delCT was investigated by Sanger sequencing in the entire *NOTCH1* PEST domain, as reported.[30] The mutational load was roughly determined (about 50%, 25-50%, about 25%, <25% of mutated DNA) by visual inspection of sequence electropherograms, as reported.[31]

Cell Sorting

CLL cells from selected *NOTCH1* mutated cases were sorted according to CD20 expression by using the PE-conjugated anti-CD20 antibody (BD Biosciences). The CD20^{low} or CD20^{high} fractions were selected below the 25th percentile or above the 75th percentile of CD20 expression, respectively. After CDC assay, CLL cells from selected *NOTCH1* mutated cases were sorted according to 7-aminoactinomycin (7-AAD, BD Biosciences) expression. Viable cell fraction was identified as 7-AAD-negative. Sorting was performed utilizing a FACSAriaIII cell sorter (BD Biosciences), as described.[29]

NICD plasmids and transfection

NICD Plasmids were engineered cloning the NICD coding sequence in a pcDNA3.1-NT-GFP-TOPO vector (Life Technologies, Monza, Italy). The c.7541-7542delCT mutation (NICD-mut) or c.5304G>A (NICD-null) mutation were inserted with the Quikchange II XL Mutagenesis kit

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(Agilent, Milan, Italy). MEC-1 cells were transfected with the Amaxa Nucleofector (Lonza, Basel, Switzerland).

Primary CLL cells were transfected with siRNA for NOTCH1 (TriFECTa, RNAi kit, IDT, Leuven, Belgium) using the Amaxa Nucleofector, as reported.[32] NOTCH1 protein expression was evaluated by flow cytometry using the PE-conjugated anti-NOTCH1 antibody (clone MHN1-519, BD Biosciences).

Co-immunoprecipitation experiments

Nuclear extracts were obtained as reported.[33] Co-immunoprecipitation was performed using anti-RBPJ (clone ab25949, Abcam) and isotype (Millipore, Milan, Italy) antibodies. WB was performed using anti-NOTCH1 (D1E11, CST), anti-HDAC1 (10E2, Abcam), anti-HDAC2 (HDAC2-62, Abcam), anti-RBPJ (D10A4, CST) antibodies. Anti-ERK 1/2 (BD Biosciences) and anti-BRG1 (Santa Cruz Biotechnology, Heidelberg, Germany) were used as loading controls for cytoplasmic and nuclear lysates.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with SimpleChIP enzymatic Chromatin IP kit (CST), according to standard manufacturer’s protocol, using anti-HDAC1 (10E2, Abcam), anti-HDAC2 (HDAC2-62, Abcam), anti-Hystone H3 (kit provided) or control isotype (kit provided) antibodies. Qualitative PCR amplification of *MS4A1* promoter was performed as reported.[34] Quantification of *MS4A1* promoter DNA was determined by QRT-PCR.

Further details regarding the methods and the statistical approaches are provided as Supplementary Information.

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Results

NOTCH1 mutational status and NOTCH1 protein expression in CLL

The presence of the c.7541-7542delCT *NOTCH1* mutation was investigated by ARMS PCR in 692 CLL cases. With this approach, the c.7541-7542delCT was detected in 81 cases (Table S1). Additional 6 cases with a *NOTCH1* mutation other than the c.7541-7542delCT were detected by Sanger sequencing (Table S1). Overall considered, *NOTCH1* mutated (*NOTCH1*-mut) cases represented about the 12% (i.e. 87/692 cases) of the cohort, in keeping with previous studies.[3-5] A quantitative detection of the c.7541-7542delCT was performed by NGS. As shown in Table S2, the *NOTCH1* mutational load ranged from 1% to 50% of total DNA, in agreement with the heterozygous nature of *NOTCH1* mutations and with its subclonal representation in some instances.[3-5]

NOTCH1 protein expression was evaluated by WB in *NOTCH1*-mut cases, chosen among those with high mutational load (i.e. > 25% of *NOTCH1* mutated DNA) and, for comparison, in *NOTCH1* wild type (*NOTCH1*-wt) CLL. In keeping with the presence of the c.7541-7542delCT that generates truncated protein with impaired degradation,[35] *NOTCH1*-mut cases showed high transmembrane NOTCH1 and NICD levels, both with molecular weights consistent with the truncation of the *NOTCH1* mutated protein (Figure S1).[4,21,25] Conversely, *NOTCH1*-wt CLL, although expressing discrete amount of transmembrane NOTCH1 in some instances, usually expressed less NICD protein than *NOTCH1*-mut cases (Figure S1).[4,21,25]

Correlation between CD20 expression and *NOTCH1* mutational status in CLL

CD20 expression was investigated by flow cytometry using either a FITC- or a PE-Cy7-conjugated antibody (Table S1), and separately analyzed (Figures S2a and S3a). In the cohort of 495 cases (60 *NOTCH1*-mut) in which CD20 expression was evaluated by the FITC-conjugated antibody, CD20 levels were generally lower in the CLL component than in the normal non-neoplastic residual B cell counterpart (Figure S2a), as reported.[27] Moreover, when CLL cases were stratified according to the classification of the main cytogenetic aberrations,[36] variable CD20 levels were found, the highest levels being detected in trisomy 12 CLL (Figure S2a).[37] When the CD20 expression was evaluated with respect to *NOTCH1* mutational status, *NOTCH1*-mut CLL expressed lower MFI values than *NOTCH1*-wt cases in both trisomy 12 CLL (mean MFI in 20 *NOTCH1*-mut cases = 1 893±196; mean MFI in 69 *NOTCH1*-wt cases = 7 051±819; p<0.0001) and non-trisomy 12 CLL (mean MFI in 40 *NOTCH1*-mut cases = 1 858±203; mean MFI in 366 *NOTCH1*-wt cases = 2 426±112; p=0.017, Figures 1a and S2b).

Superimposable results were obtained in the remaining 197 CLL (27 *NOTCH1*-mut and 170 *NOTCH1*-wt cases), in which the CD20 expression was evaluated with a PE-Cy7-conjugated antibody (Table S1), both in trisomy 12 CLL (mean MFI in 6 *NOTCH1*-mut cases = 12 926±3 676; mean MFI in 17 *NOTCH1*-wt cases = 28 216±5 228; p=0.027) and non-trisomy 12 CLL (mean MFI in 21 *NOTCH1*-mut cases = 10 207±1 310; mean MFI in 153 *NOTCH1*-wt cases = 15 208±1 578; p=0.017, Figure S3a,b).

In keeping with flow cytometry results, transcript levels of *MS4A1*, the gene encoding for CD20,[38] as evaluated in 275 cases (46 *NOTCH1*-mut), were lower in *NOTCH1*-mut than in *NOTCH1*-wt cases both in the trisomy 12 (p=0.006) and in the non-trisomy 12 (p=0.019) CLL categories (Figure 1b).

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To corroborate the correlation between CD20 expression and *NOTCH1* mutations, we performed cell sorting experiments to isolate the extreme CD20^{low} and CD20^{high} subpopulations in five CLL cases with different *NOTCH1* mutational load (Figure S4a), as determined by NGS, i.e. 3% (CLL#406), 8% (CLL#34), 27% (CLL#171), 35% (CLL#243) and 41% (CLL#266) of total DNA. As shown by NGS re-sequencing of the separated subpopulations, CD20^{low} sorted cells always had a relative enrichment in the *NOTCH1* mutational burden when compared to the CD20^{high} counterpart, i.e. 9% vs. 1% (CLL#406), 14% vs. 3% (CLL#34), 32% vs. 15% (CLL#171), 38% vs. 32% (CLL#243), 48% vs. 39% (CLL#266). Consistently, the amount of *MS4A1* transcripts was always significantly lower in the CD20^{low} than in the CD20^{high} subpopulation (Figure S4b).

NOTCH1 mutational status and susceptibility to anti-CD20 in CLL

Then we investigated if *NOTCH1* mutational status could effectively influence susceptibility to anti-CD20 immunotherapy. To evaluate the capability of rituximab to kill in-vitro CLL cells bearing or not *NOTCH1* mutations, CDC assay was performed utilizing purified CLL cells from 9 *NOTCH1*-mut and 9 *NOTCH1*-wt cases. *NOTCH1*-mut CLL cells showed significantly lower relative lysis induced by rituximab than *NOTCH1*-wt CLL cells (mean % of relative lysis = 2.5±0.8 vs. 26.3±8.9, p=0.021), and the killing capacity of rituximab directly correlated with CD20 levels (Figure 1c).

We further investigated the correlation between *NOTCH1* mutational status and susceptibility to rituximab by evaluating in three *NOTCH1*-mut cases the enrichment of *NOTCH1* mutational burden after CDC assay upon rituximab and subsequent cell sorting of the residual viable cell population. The *NOTCH1* mutational burden, as detected by NGS, resulted higher in the post-CDC sorted viable cells than in the pre-CDC unsorted counterpart in all the three tested cases (Figure S4c). Consistently, the amount of *MS4A1* transcripts, as detected by QRT-PCR, were lower in the viable cell populations than in the pre-CDC unsorted counterparts (Figure S4c).

We also evaluated the capability of the alternative anti-CD20 antibody ofatumumab to kill in-vitro CLL cells from 9 *NOTCH1*-mut and 9 *NOTCH1*-wt cases. Although the killing capacity of ofatumumab resulted generally higher than that of rituximab, *NOTCH1*-mut CLL cells showed significantly lower relative lysis than *NOTCH1*-wt CLL cells (mean % of relative lysis = 30.6±8.5 vs. 60.6±5.8, p=0.011), again consistently with CD20 expression levels (Figure 1d).

NOTCH1 signaling and CD20 expression in CLL

To evaluate if *NOTCH1* signaling could influence CD20 expression in primary CLL cases, CLL cells from 5 *NOTCH1*-mut and 6 *NOTCH1*-wt cases were treated at different time points with the GSI L-685,458, able to block the proteolytic generation of NICD.[21] Upon GSI treatment, NOTCH1 signaling was consistently impaired, as defined by a reduction of *HES1* expression (at 6 hours) in both *NOTCH1*-wt and *NOTCH1*-mut CLL, although decreases were lower in the *NOTCH1*-mut category (p=0.005), according to the presence of higher levels of NICD in the latter cases (Figure S5a and Figure S1). More important, both *MS4A1* transcripts (at 6 hours) and CD20 expression levels (at 24 hours) were significantly upregulated by GSI in *NOTCH1*-wt and, to a lesser extent, in *NOTCH1*-mut cases (Figure S5b). No effect on CD20 expression was observed in purified normal B cells from healthy donors exposed in-vitro to GSI, in keeping with the notion of a lack of NOTCH1 expression in these cells (not shown).[21]

To further confirm the association between NOTCH1 signaling and CD20 expression, CLL cells from 6 *NOTCH1*-mut and 5 *NOTCH1*-wt cases were transiently transfected with siRNA for NOTCH1. In both *NOTCH1*-mut and *NOTCH1*-wt cases, siRNA transfection effectively reduced

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NOTCH1 transcript at 6 hours (p=0.001, not shown) and protein at 24 hours (*NOTCH1*-mut cases, mean MFI = 538±119 vs. 184±32, p=0.011; *NOTCH1*-wt cases, mean MFI = 524±64 vs. 204±17, p=0.003). Consistently, CD20 expression resulted augmented both at transcript level (at 6 hours, *NOTCH1*-mut, p=0.034, *NOTCH1*-wt, p= 0.012, not shown) and protein level (at 24 hours, *NOTCH1*-mut cases, mean MFI = 2685±887 vs. 3035±916, p=0.001; *NOTCH1*-wt cases, mean MFI = 1707±434 vs. 1923±434, p=0.003, Figure S5c).

Establishment of an in-vitro model of mutated NICD-transfected CLL-like cells
To investigate the mechanism(s) through which *NOTCH1* mutations may affect CD20 expression in CLL, we established an in-vitro model of NICD transfected cells by taking advantage of the CLL-like MEC-1 cell line. MEC-1 cells, constitutively expressing a wild-type *NOTCH1* form, were stably transfected with vectors encoding for: i) a modified NICD with the c.7541-7542delCT (NICD-mut); ii) a modified NICD with a nonsense mutation inserted after the beginning of the coding sequence, as a null control (NICD-null). NICD-mut cells showed higher constitutive NOTCH1 protein levels than NICD-null cells (Figure 2a). Consistently, *HES1* and *HES5* transcript levels were higher in NICD-mut than in NICD-null cells (Figure S6a).

When CD20 expression was tested, NICD-mut cells showed constitutive lower CD20 expression at both protein and transcript level than NICD-null cells (Figures 2a,b and S6b), and, consistently, lower relative lysis induced by rituximab and ofatumumab by CDC assay (p=0.043, p=0.025, respectively, Figure 2c). Moreover, upon GSI treatment, CD20 protein and transcript expression was significantly up-regulated in both NICD-null cells and NICD-mut cells (Figure 2d and not shown).

According to these validations, we assumed the NICD-mut cells as in-vitro model of *NOTCH1*-mut CLL, in which the increased NICD accumulation, due to a decreased degradation of truncated form,[15] is mimicked by the enforced expression of an exogenously transfected mutated NICD.

Immunoprecipitation of the RBPJ transcription factor in NICD transfectants
When released by proteolytic cleavages and translocated into the nucleus upon activation of the NOTCH1 pathway, NICD interacts with the RBPJ transcription factor and converts its function from repressor to activator of gene transcription.[13,15,35] In fact, NICD is able to displace RBPJ from a HDAC-containing repression complex, thus forming, with RBPJ itself and other co-activators, the major gene transcriptional activation complex of the NOTCH1 pathway.[13,15,35]

To evaluate whether NICD accumulation, as it occurs upon *NOTCH1* mutations, could alter the balancing of the two functions of RBPJ, i.e. transcriptional activator (complexed with NICD) or transcriptional repressor (complexed with HDACs),[13,15] we performed co-immunoprecipitation experiments aimed at investigating the alternative presence of NICD or HDACs (namely HDAC1 and HDAC2) bound to RBPJ in NICD transfectants. As shown in Figure 3a, co-immunoprecipitation experiments revealed that NICD-mut cells had higher levels of NICD bound with RBPJ than NICD-null cells. On the contrary, NICD-mut cells showed lower levels of HDAC1 or HDAC2 co-immunoprecipitated with RBPJ than NICD-null cells (Figure 3a). Notably, no difference was found by comparing NICD transfectants regarding the levels of immunoprecipitated RBPJ, and the nuclear and cytoplasmic levels of RBPJ, HDAC1 and HDAC2, as evidenced by control WB experiments (Figure S7a,b,c). Consistently, comparable constitutive *HDAC1/HDAC2* expression levels were found in *NOTCH1*-mut versus *NOTCH1*-wt primary CLL (Figure S8).

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The un-balancing of the transcriptional activation/repression equilibrium of RBPJ turned in favor of the activation of NOTCH1 signaling detected in NICD-mut cells was also in keeping with the higher *HES1* and *HES5* transcript levels detected in these cells (Figure S6a).

HDAC-mediated ChIP in NICD transfectants

Previous studies identified epigenetic silencing of CD20 expression via HDACs as a mechanism conferring resistance to rituximab in lymphomas.[34,39,40] To evaluate whether the preferential interaction of RBPJ with NICD could result in higher levels of HDAC1/HDAC2 available for the transcriptional repression of *MS4A1*,[13,15] ChIP assays were performed on nuclear lysates from NICD transfectants. As shown in Figure 3b, higher levels of DNA corresponding to the *MS4A1* promoter were found in HDAC1 and HDAC2 chromatin immunoprecipitates from NICD-mut compared to NICD-null cells. Of note, a higher involvement of HDAC2 with respect to HDAC1 was evidenced ChIP experiments, in keeping with the higher levels of HDAC2 expressed by NICD transfectants (Figure S7c). On the other hand, lower levels of DNA corresponding to the *HES1* promoter were found by ChIP of NICD-mut cells compared to NICD-null cells (not shown).

These results suggest that higher NICD levels, as occurring in NICD-mut cells, may cause a NICD-dependent dislodgement of RBPJ from the HDAC-containing repression complexes. This phenomenon is associated with an increased availability of HDACs to repress transcription of the *MS4A1* gene.

HDAC inhibition and CD20 expression

To further evaluate if the higher levels of HDACs bound to the *MS4A1* promoter could effectively affect CD20 expression, NICD transfected cells were treated with the HDAC inhibitor VPA for 48 hours. In both NICD-mut and NICD-null cells, VPA treatment was able to significantly increase *MS4A1* transcript levels (NICD-mut, mean fold increase =1.7, p=0.001; NICD-null, mean fold increase =1.5 p=0.003, Figure S9a) and CD20 protein expression (NICD-mut, mean fold increase =1.3, p=0.041; NICD-null, mean fold increase =1.4, p=0.029, Figures 4a and S9b).

Similar results were obtained by treating with VPA primary CLL cells of 7 *NOTCH1*-mut and 6 *NOTCH1*-wt cases. In both categories, VPA treatment was able to significantly increase *MS4A1* transcripts (*NOTCH1*-mut, mean fold increase =1.5, p=0.05; *NOTCH1*-wt, mean fold increase = 1.8, p=0.02, Figure S9c) and CD20 protein (*NOTCH1*-mut, mean fold increase = 1.3, p=0.05; *NOTCH1*-wt, mean fold increase = 1.3, p=0.005, Figures 4b and S9d). These increments were not associated with significant increases of relative lysis by in-vitro CDC assays (not shown).

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Discussion

The FCR immuno-chemotherapy combination still represents the frontline regimen for treatment of patients in good physical conditions.[1,2] In particular, the addition of rituximab to the FC combination has been definitely proved to improve the clinical outcome of CLL patients, despite the relative low levels of CD20 usually expressed on the surface of CLL cells.[26,27] Recently, however, it has been clearly demonstrated that such a benefit does not include patients affected by CLL bearing *NOTCH1* mutations,[26,41] although the reason for this different clinical behaviour remains to be elucidated.

In the present study, we demonstrated that *NOTCH1* mutations identify a CLL subset characterized by particularly low levels of CD20, both in non-trisomy 12 CLL, and in the trisomy 12 CLL category, that usually has relatively higher CD20 levels and a higher frequency of *NOTCH1* mutations.[9,10,37] Conversely, Stilgenbauer *et al* did not find any difference in CD20 expression between *NOTCH1*-mut and *NOTCH1*-wt CLL although in this study CD20 levels were checked exclusively by flow cytometry in a minority of cases.[26] Here, the lower CD20 expression by *NOTCH1*-mut cases was corroborated by the parallel finding of lower *MS4A1* transcript levels. Moreover, in cell sorting experiments of CLL cases with different *NOTCH1* mutation levels, higher percentages of *NOTCH1* mutated DNA were found in the sorted CD20^{low} component compared to the CD20^{high} counterpart. Finally, the dramatic downregulation of CD20 expression levels obtained by stably transfecting the CLL-like MEC-1 cells with a mutated NICD definitely confirmed this inverse correlation.

The low CD20 expression by *NOTCH1*-mut CLL cells is consistent with their lower sensitivity to rituximab and ofatumumab exposure in-vitro, as shown here, in agreement with previous reports.[42] Results of the present study also indicate that the residual CLL cells surviving upon CDC assay with rituximab, usually expressed lower CD20 levels and a greater *NOTCH1* mutational load. In keeping, *NOTCH1* mutations have been demonstrated to impact on rituximab sensitivity of CLL patients also when present at subclonal level.[26,41,43]

These data may also suggest that, in CLL, the constitutive expression of NOTCH1, in its mutated configuration but also in the wild type form,[21] could be related with the generally lower CD20 levels observed in neoplastic versus normal B cells, in which NOTCH1 is not expressed at all.[21] In keeping, we demonstrated here that GSI treatment in-vitro was able to substantially augment CD20 expression both in *NOTCH1*-wt and *NOTCH1*-mut CLL cells, although in the latter the accumulation of NICD due to truncating mutations makes these cells relatively less susceptible to NOTCH1 signaling perturbation. Since theoretically GSI may have off-target genes,[44] NOTCH1 was also inhibited by specific siRNA. Again, transfection with siRNA increased CD20 expression both in *NOTCH1*-wt and *NOTCH1*-mut CLL cells.[35]

In humans, the balance of hystone acetylation/deacetylation, respectively induced by hystone acetyl transferases and HDACs, represents one of the main epigenetic mechanisms of modification of chromatin conformation and regulation of gene expression.[45,46] In particular, the transcriptional activity due to the triggering of the NOTCH1 pathway is known to be greatly sensitive to chromatin modifications and hystone rearrangements.[35] In this context, the main effector of the NOTCH1 pathway at nuclear level is a DNA-binding protein named RBPJ.[13,15,35] This protein, in association with NICD and other co-activators forms an activation complex that is essential for NICD-dependent transcription and target gene expression. Such an activation complex is degraded via NICD phosphorylation, and its subsequent ubiquitination, these molecular reactions requiring an

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intact C-terminal PEST region of the NICD protein.[13,15,35] The specific degradation of NICD results in the dissociation among RBPJ and the other co-activators. In the absence of NICD, RBPJ is free to associate with specific co-repressors, which in turn recruit HDAC1 and HDAC2; this newly obtained repression complex represses NOTCH1 signaling.[13,15,35]. A simplified scheme of these multi-protein interactions is reported in Figure 5a.

Results of this study suggest that NOTCH1 with C-terminal truncations, as those determined by the c.7541-7542delCT, may influence the epigenetic downregulation of CD20 by HDACs allegedly via an impaired ubiquitination and degradation of the truncated NICD. In fact, as defined by co-immunoprecipitation experiments, in the condition of NICD accumulation due to the c.7541-7542delCT, RBPJ showed a preferential binding to NICD, in the context of the activation complex, rather than to HDACs, in the context of the repression complex. In NICD-mut cells, in turn, HDACs were mainly associated to the *MS4A1* promoter, as defined by ChIP experiments. A necessary prerequisite is the persistence of the activation complex due to the lack of degradation of the truncated NICD (Figure 5b).[13,15,35] In keeping, the rare *NOTCH1*-mut CLL carrying truncating mutations other than the c.7541-7542delCT (6 cases in our cohort) were all characterized by low CD20 levels, comparable with those of *NOTCH1*-mut CLL carrying the c.7541-7542delCT. Conversely, three cases (not included in this cohort) carrying *NOTCH1* missense mutations (e.g. p.G2292R, p.V2214M and p.T2484M) expressed CD20 levels comparable with those of *NOTCH1*-wt CLL (F.P., personal communication).

To restore epigenetic regulation, a wide range of compounds inhibiting HDAC functionality have been identified, some of them employed in anticancer therapies.[45,46] In addition, HDAC inhibitors are known to augment the cytotoxic activity of rituximab by increasing CD20 expression in lymphoma cells.[34,39,40] In this study, treatment with the HDAC inhibitor VPA was capable to up-regulate both *MS4A1* transcript and CD20 protein either in NICD transfected cells or in primary CLL cells from *NOTCH1*-mut and *NOTCH1*-wt cases.

In conclusion, we provided evidence that truncating *NOTCH1* mutations in CLL are associated with low CD20 expression, and with a relative resistance to anti-CD20 immunotherapy in-vitro. The low CD20 expression in *NOTCH1*- mut CLL can be ascribed to a *NOTCH1* mutation-driven epigenetic dysregulation of a transcriptional repression mechanism involving HDACs. Clinically, drugs interfering with the NOTCH1 pathway and/or inhibiting HDACs might have a role to increase CD20 expression in-vivo, thus overcoming the relative resistance of *NOTCH1*-mut CLL to rituximab-containing therapies.

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Authorship Contributions

F.P. contributed to write the manuscript, analyzed the data and performed the research, T.B. performed the research, F.A., P.B., P.M., E.T., B.G., F.M.R., R.B., A.Z., D.B., M.D., contributed to perform the research, G.D.A., A.C., F.Z., G.P., D.R., G.G., G.D.P., S.D. provided well characterized biological samples and contributed to write the manuscript, V.G. and M.D.B. designed the study, interpreted data, and wrote the manuscript.

Conflict of Interest

The Authors declare no competing financial interests.

Supplementary information is available at Leukemia's website.

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Figure legends

Figure 1. Correlation between *NOTCH1* mutations, CD20 expression, and susceptibility to anti-CD20 antibodies in CLL (a) Box-and-whiskers plots showing CD20 protein expression levels, evaluated as above, in 89 trisomy 12 CLL cases (20 *NOTCH1*-mut cases, 69 *NOTCH1*-wt cases) and 406 non-trisomy 12 CLL cases (40 *NOTCH1*-mut cases, 366 *NOTCH1*-wt cases). The corresponding p values are reported. **(b)** Box-and-whiskers plots showing *MS4A1* transcript expression levels, as evaluated by QRT-PCR, in 52 trisomy 12 CLL cases (15 *NOTCH1*-mut cases, 37 *NOTCH1*-wt cases) and 223 non-trisomy 12 CLL cases (31 *NOTCH1*-mut cases, 192 *NOTCH1*-wt cases). The corresponding p values are reported. **(c)** Box-and-whiskers plots showing the percentage of relative lysis of CLL cells, from *NOTCH1*-mut and *NOTCH1*-wt CLL cases, treated with rituximab in a standard CDC assay. The corresponding p value is reported (left panel). Correlation graph showing CD20 expression versus percentage of relative lysis in *NOTCH1*-mut and *NOTCH1*-wt CLL cases, as evaluated by CDC assay (r= Pearson correlation coefficient, right panel). **(d)** Box-and-whiskers plots showing the percentage of relative lysis of CLL cells, from *NOTCH1*-mut and *NOTCH1*-wt CLL cases, treated with ofatumumab in a standard CDC assay. The corresponding p value is reported (left panel). Correlation graph showing CD20 expression versus percentage of relative lysis in *NOTCH1*-mut and *NOTCH1*-wt CLL cases, as evaluated by CDC assay (r= Pearson correlation coefficient, right panel).

Figure 2. Establishment of an in-vitro model of mutated NICD-transfected CLL-like cells. (a) NOTCH1 and CD20 protein expression levels of NICD-null and NICD-mut cells, as evaluated by WB. β-actin was used as loading control. Exogenous transfected mutated NICD is indicated as GFP-NICD, endogenous NICD is indicated as NICD. **(b)** Histograms (left panel) and box-and-whiskers plots (right panel) showing constitutive *MS4A1* transcript and CD20 protein expression levels of NICD-null and NICD-mut cells, as evaluated by QRT-PCR and flow cytometry, respectively. The corresponding p values are reported. **(c)** Box-and-whiskers plots showing the percentage of relative lysis of NICD-null (empty histogram) and NICD-mut cells (grey histogram), upon rituximab or ofatumumab, as evaluated by CDC assay. The corresponding p value are reported. Results of three independent experiments are reported. **(d)** Box-and-whiskers plots showing CD20 protein expression levels of NICD-null and NICD-mut cells, untreated (UNT) and upon GSI treatment (GSI) for 24 hours, as evaluated by flow cytometry. The corresponding p values are reported. Results of three independent experiments are reported.

Figure 3. Characterization of a HDAC dependent epigenetic repression mechanism of CD20 expression in NICD transfected cells. (a) Immunoblotting with antibodies recognizing the total NOTCH1 (upper panel), HDAC1 (middle panel), and HDAC2 (lower panel) in whole nuclear lysates (WNL), immunoprecipitates with isotypic control (ISO) and immunoprecipitated with RBPJ (RBPJ) derived from NICD-mut and NICD-null cells. Exogenous transfected mutated NICD is indicated as GFP-NICD, endogenous NICD is indicated as NICD. **(b)** Analysis of the *MS4A1* promoter in total chromatin preparation (INPUT), and ChIP with isotypic control (ISO), antibodies recognizing HDAC1 and HDAC2, as evaluated by qualitative PCR (upper panel). Results from a representative experiment out of three experiments is reported. Analysis of the *MS4A1* promoter in ChIP with isotypic control (ISO), antibodies recognizing HDAC1 and HDAC2, as evaluated by QRT-PCR (lower panel). Results of three independent experiments are reported.

Figure 4. Induction of CD20 expression by HDAC inhibition in NICD transfectants and in primary CLL cells. (a) Box-and-whiskers plots showing CD20 protein expression levels of NICD-mut and NICD-null cells, untreated (UNT) and VPA treated (VPA) for 48 hours, as evaluated by

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flow cytometry. The corresponding p values are reported. Results of three independent experiments are showed. **(b)** Dot-and-line diagrams showing CD20 expression levels in primary CLL cells, untreated (UNT) and VPA treated (VPA) for 48 hours, from *NOTCH1*-mut and *NOTCH1*-wt cases, as evaluated by flow cytometry. The corresponding p values are reported.

Figure 5. Putative model of a *NOTCH1* mutation-dependent mechanism of CD20 down-regulation via HDAC1/HDAC2 epigenetic repression in CLL. (a) Regulated balancing in *NOTCH1*-wt CLL (phospho, phosphorylation; ub, ubiquitination; Co-A, co-activators; Co-R, co-repressors). **(b)** Dysregulated balancing in *NOTCH1*-mut CLL. See text for further details.

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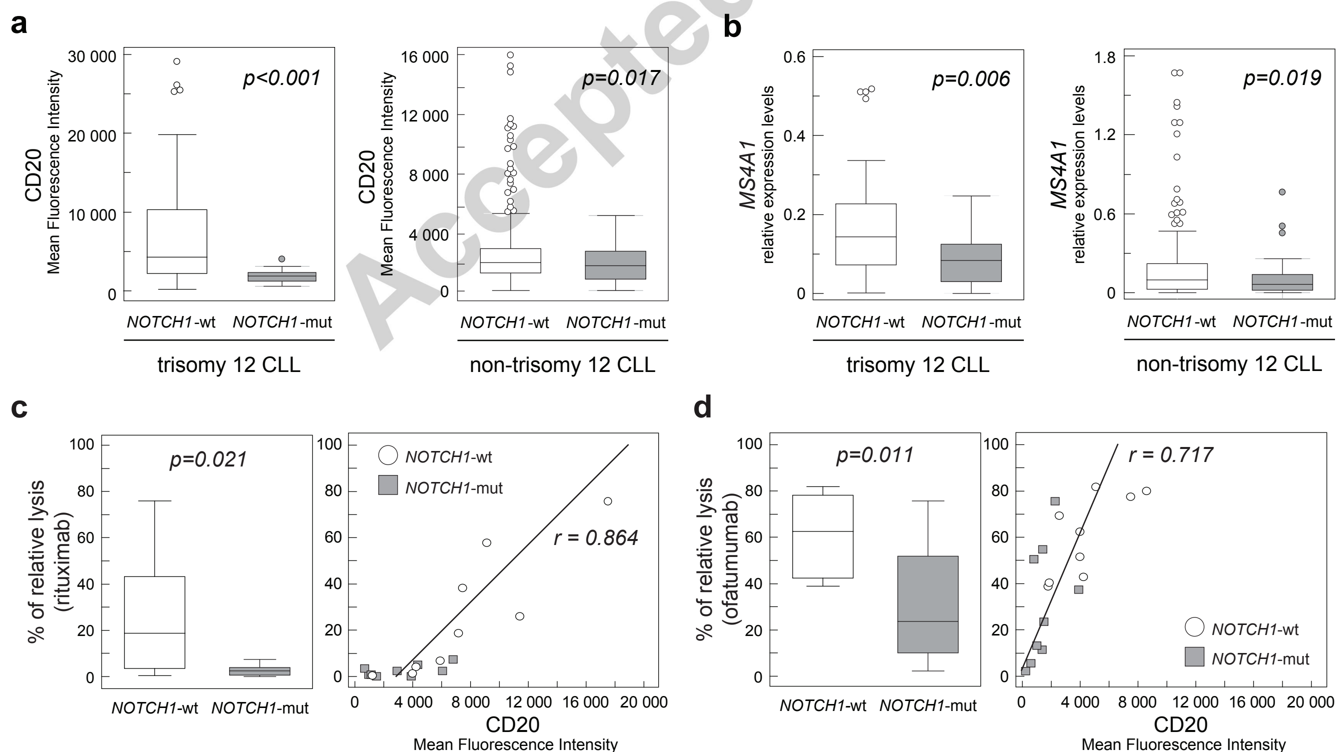

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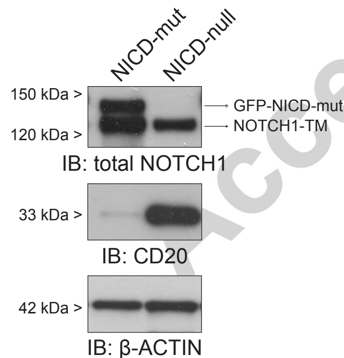
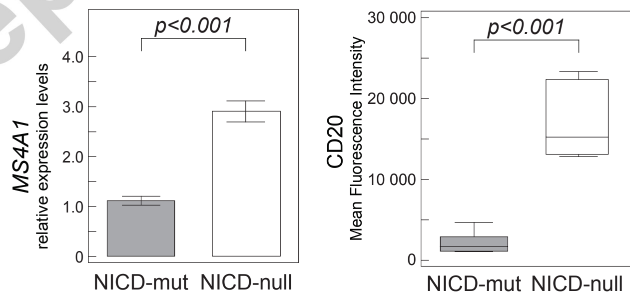
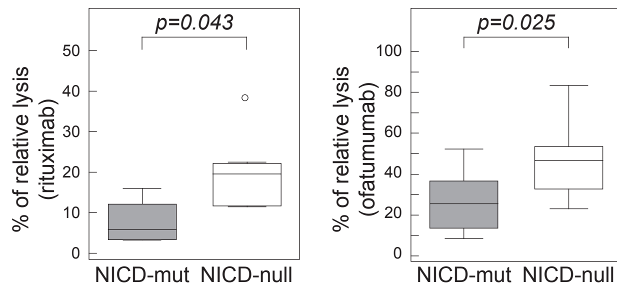
Figure 1







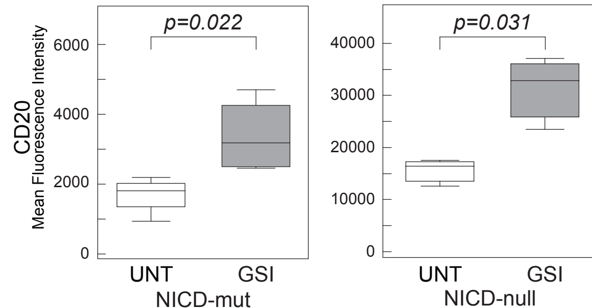


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Figure 2

a

NICD-mut NICD-null

WNL IP: ISO IP: RBPJ WNL IP: ISO IP: RBPJ

150 kDa >

100 kDa >

IB: total NOTCH1

GFP-NICD NICD

50 kDa >

IB: HDAC1

50 kDa >

IB: HDAC2

b

NICD-mut NICD-null

INPUT ISO HDAC1 HDAC2 INPUT ISO HDAC1 HDAC2

300bp >

NICD-mut

NICD-null

% of input

2.0

1.5

1.0

0.5

0

ISO HDAC1 HDAC2

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Figure 3

a

p=0.041

CD20 Mean Fluorescence Intensity

4 000
3 000
2 000
1 000
0

UNT VPA

NICD-mut

p=0.029

CD20 Mean Fluorescence Intensity

20 000
16 000
12 000
8 000
4 000
0

UNT VPA

NICD-null

b

p=0.050

CD20 Mean Fluorescence Intensity

4 000
3 000
2 000
1 000
0

UNT VPA

NOTCH1-mut

p=0.005

CD20 Mean Fluorescence Intensity

12 000
10 000
8 000
6 000
4 000
2 000
0

UNT VPA

NOTCH1-wt

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Figure 4

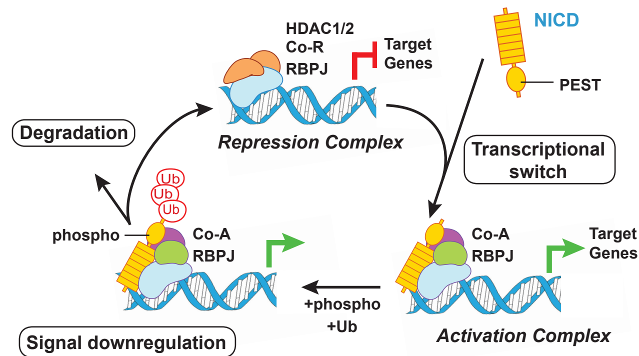

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Figure 5