Mutations in the 3′ untranslated region of *NOTCH1* are associated with low CD20 expression levels in chronic lymphocytic leukemia

In chronic lymphocytic leukemia (CLL), mutations in the *NOTCH1* coding region (coding *NOTCH1* mutations) have been associated with impaired degradation of NOTCH1 protein[1,2] and, clinically, with shorter time to first treatment, shorter overall survival,[1-3] and resistance to anti-CD20 immunotherapy in the fludarabine plus cyclophosphamide plus rituximab combination.[4,5] In this context, we recently provided evidence that coding *NOTCH1* mutations in CLL are associated with reduced CD20 expression, due to a *NOTCH1* mutation-driven epigenetic dysregulation involving histone deacetylases.[6] More recently, novel recurrent mutations have been identified in the 3′ untranslated region (3′ UTR) of *NOTCH1* (3′ UTR *NOTCH1* mutations), determining an alternative splicing event within the last *NOTCH1* exon,[7] again leading to impaired degradation of NOTCH1 protein through a mechanism similar to that occurring in CLL cells bearing coding *NOTCH1* mutations. CLL with 3′ UTR *NOTCH1* mutations show features of adverse prognosis

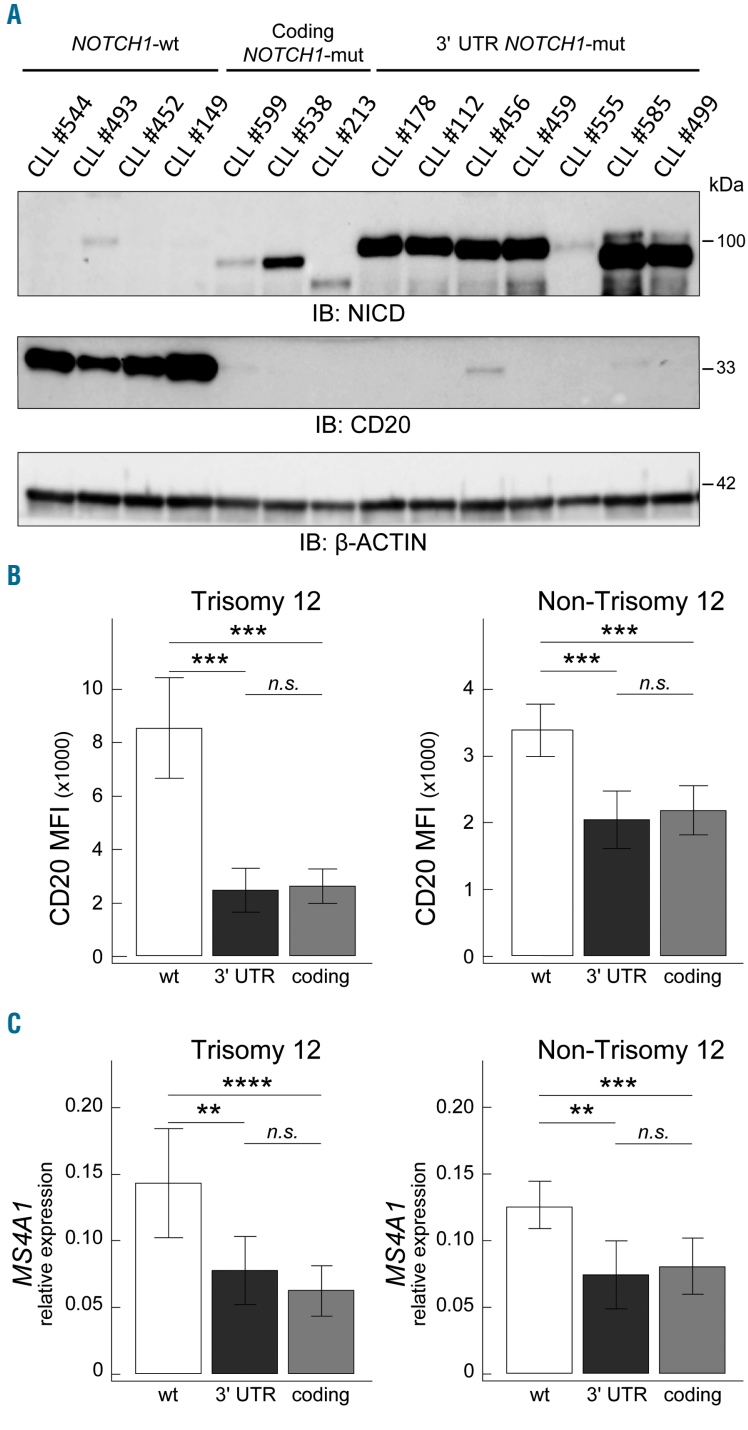

Figure 1. Correlation between 3′ UTR *NOTCH1* mutations and CD20 expression. (A) Western blot showing NICD (upper panel) and CD20 (L26 epitope, with short exposure, lower panel) protein expression in representative CLL cases, i.e. four *NOTCH1*-wt cases, three coding *NOTCH1*-mut cases (2 cases with the g.139390648CAG>C, c.7541-7542delCT, p.P2514Rfs*4; 1 case with the g.139390929AC>A, c.7261delG, p.V2421*), and seven 3′ UTR *NOTCH1*-mut cases (5 cases with the g.139390152T>C, c.*7668+371A>G; 2 cases with the g.139390145T>C, c.*7668+378A>G). β-actin was used as loading control. Identification (ID) number according to *Online Supplementary Table S1* is also reported. (B) Bar graphs showing CD20 protein expression levels evaluated by flow cytometry in 112 trisomy 12 CLL cases (9 3′ UTR *NOTCH1*-mut cases, 35 coding *NOTCH1*-mut cases, 68 *NOTCH1*-wt cases) and 550 non-trisomy 12 CLL cases (17 3′ UTR *NOTCH1*-mut cases, 55 coding *NOTCH1*-mut cases, 478 *NOTCH1*-wt cases). Data were analyzed using the *t* test. *: $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s.= not significant. Bar graphs represent mean values, error bars represent the 95% confidence interval (CI). Abbreviations: *NOTCH1*-wt: wt; 3′ UTR *NOTCH1*-mut: 3′ UTR; coding *NOTCH1*-mut: coding. (C) Bar graphs showing *MS4A1* transcript expression levels, as evaluated by quantitative real-time polymerase chain reaction, in 662 CLL cases subdivided according to *NOTCH1* mutational status as reported in (B). Data were analyzed using the *t* test. *$P<0.05$; **$P<0.01$; ***$P<0.001$; n.s.= not significant. Bar graphs represent mean values, error bars represent the 95% confidence interval. *NOTCH1*-wt: wt; 3′ UTR *NOTCH1*-mut: 3′ UTR; coding *NOTCH1*-mut: coding.

similar to CLL with coding *NOTCH1* mutations in terms of both time to first treatment and overall survival.[7] On the other hand, evidence is still lacking regarding the levels of CD20 expression in CLL cases carrying 3′ UTR *NOTCH1* mutations. In this study, we provide evidence that 3′ UTR *NOTCH1* mutations are associated with low CD20 expression and with relative resistance to anti-CD20 immunotherapy *in vitro*, thus indicating the need to expand the *NOTCH1* mutational analysis to 3′ UTR as a tool to identify anti-CD20 resistant cases.

This study was part of a comprehensive CLL characterization approved by the Internal Review Board of the Aviano Cancer Referral Center (*approval n. IRB-05-2010, n. IRB-05-2015*) upon informed consent in accordance with the declaration of Helsinki. The study included peripheral blood samples from 662 patients affected by CLL.[8] All analyses, including evaluation of CD20 expression, and of *NOTCH1* mutational status, were performed on highly purified neoplastic cells (>95% pure). CLL case samples were subjected to purification for negative selection by immunomagnetic beads when required.[6,9,10] CD20 expression was evaluated by flow cytometry with a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody (clone L27, BD Biosciences, Milan, Italy), using a FACSCanto II (BD Biosciences).[6] *NOTCH1* mutational status was assessed by next-generation sequencing covering the whole *NOTCH1* exon 34 and part of the 3′ UTR.[7] Further details regarding the methods and statistical approaches are provided as *Online Supplementary Material* and in *Online Supplementary Tables S1-S5*.

NOTCH1 mutations were detected in 116/662 (17.52%) cases (*Online Supplementary Tables S6* and *S7*), overall accounting for 127 mutations (78 c.7541-7542delCT, 9 other frameshift, 14 nonsense, and 26 3′ UTR mutations) (*Online Supplementary Table S7*). No missense mutations were detected (*Online Supplementary Table S7*). Twenty-three of the 26 mutations at the 3′ UTR of *NOTCH1* were clonal, i.e. with a variant allele

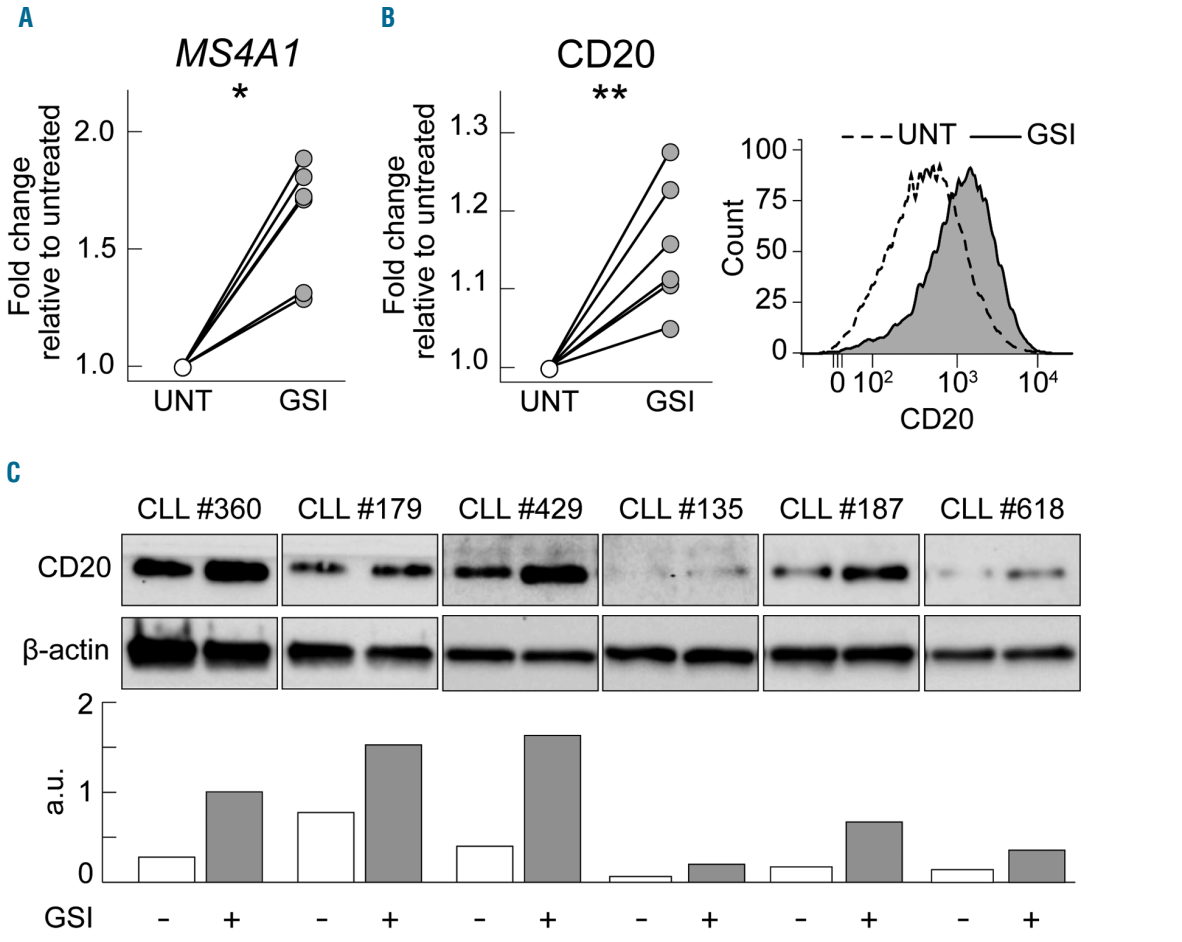

Figure 2. Induction of CD20 expression by NOTCH1 signaling inhibition. (A) Dot-and-line plots showing fold change increases of *MS4A1* transcript expression levels between untreated (UNT) samples and samples treated with a gamma-secretase inhibitor (GSI) for 6 h, of six 3′ UTR *NOTCH1*-mut CLL cases, as evaluated by quantitative real-time polymerase chain reaction. Data were analyzed using a paired *t*-test. *: $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s.= not significant. (B) Dot-and-line plots showing fold-change increases of CD20 protein expression levels between untreated (UNT) samples and samples treated with GSI (GSI) for 24 h, of six 3′ UTR *NOTCH1*-mut CLL cases, as evaluated by flow cytometry; a representative overlay histogram of CD20 expression by flow cytometry of CLL cell samples left untreated or GSI treated of a 3′ UTR *NOTCH1*-mut case is also shown. Data were analyzed using a paired *t*-test. *: $P<0.05$; **$P<0.01$; *** $P<0.001$; n.s.= not significant. (C) Upper panel: western blot showing CD20 protein expression of CLL cell samples left untreated (-) or GSI treated (+) of six 3′ UTR *NOTCH1*-mut cases. Lower panel: bar graphs representing the relative densitometric analysis of the same western blot. β-actin was used as loading control. For evaluation of CD20 expression high sensitivity conditions were applied (*Online Supplementary Table S5*).

frequency ≥12%,[7,11] whereas 55/101 mutations in the *NOTCH1* coding region were clonal mutations (*Online Supplementary Figure S1A-E*).

For the purpose of our analysis, the 116 mutated cases were subdivided into cases with coding *NOTCH1* mutations (coding *NOTCH1*-mut, 90 cases) and cases with 3′ UTR *NOTCH1* mutations (3′ UTR *NOTCH1*-mut, 26 cases). Five cases with concomitant 3′ UTR *NOTCH1* mutation and coding *NOTCH1* mutation were assigned to the 3′ UTR *NOTCH1*-mut group according to the substantially higher variant allele frequency detected for the 3′ UTR *NOTCH1* mutation.

NOTCH1 protein expression was evaluated by western blot in CLL cases carrying either 3′ UTR *NOTCH1* mutations or coding *NOTCH1* mutations, and, for comparison, in *NOTCH1* wild-type (*NOTCH1*-wt) cases. As shown in Figure 1A, 3′ UTR *NOTCH1*-mut cases showed high *NOTCH1* intracellular domain (NICD) levels, consistent with the presence of an alternative splicing event resulting in a large deletion that disrupts the C-PEST domain causing the subsequent impaired degradation of the NICD.[7] In keeping with the presence of coding *NOTCH1* mutations that generate a truncated protein with impaired degradation,[12] coding *NOTCH1*-mut cases also showed accumulation of NICD, with molecular weights consistent with the presence of a mutated protein (Figure 1A), both in cases with the c.7541-7542delCT mutation and in cases with other coding *NOTCH1* mutations.[2,6] In this context, NICD levels were generally consistent with *NOTCH1* mutational burden both in 3′ UTR *NOTCH1*-mut cases and coding *NOTCH1*-mut cases (*Online Supplementary Table S7* and

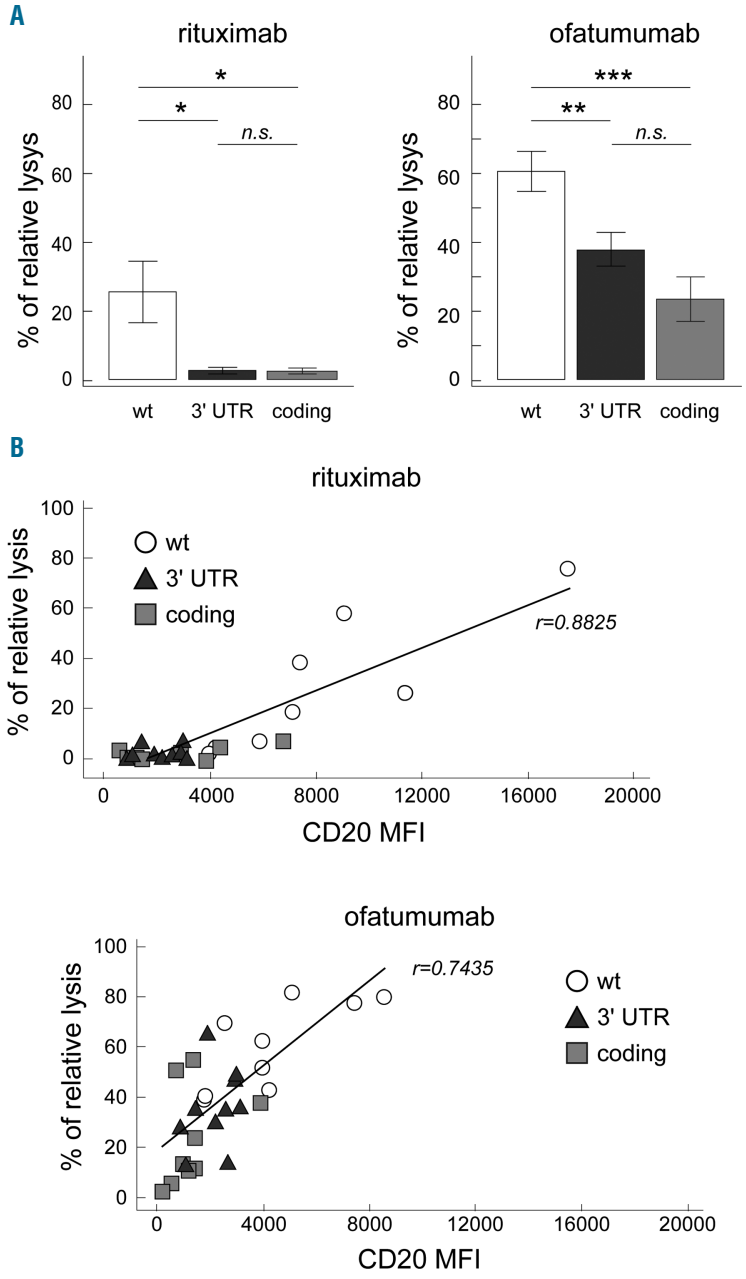

Figure 3. 3′ UTR *NOTCH1* mutations and susceptibility to anti-CD20 antibodies in CLL. (A) Left panel: bar graphs showing the percentage of relative lysis of CLL cells, from nine 3′ UTR *NOTCH1*-mut, nine coding *NOTCH1*-mut and nine *NOTCH1*-wt CLL cases, treated with rituximab in a standard complement-dependent cytotoxicity assay. Right panel: bar graph showing the percentage of relative lysis of CLL cells, from nine 3′ UTR *NOTCH1*-mut, nine coding *NOTCH1*-mut and nine *NOTCH1*-wt CLL cases, treated with ofatumumab in a standard complement-dependent cytotoxicity assay. Data were analyzed using a *t* test. *: $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s.= not significant. Bars represent mean values, error bars represent standard error of the mean (SEM). (B) Correlation plots showing CD20 expression *versus* percentage of relative lysis (r= Pearson correlation coefficient) in 3′ UTR *NOTCH1*-mut, coding *NOTCH1*-mut and *NOTCH1*-wt CLL cases, as evaluated by a complement-dependent cytotoxicity assay using rituximab (upper panel) or ofatumumab (lower panel). *NOTCH1*-wt: wt; 3′ UTR *NOTCH1*-mut: 3′ UTR; coding *NOTCH1*-mut: coding.

Figure 1A). Conversely, *NOTCH1*-wt CLL, although expressing discrete amounts of transmembrane *NOTCH1* in some instances, usually expressed negligible NICD levels (Figure 1A and *Online Supplementary Figure S2A*).[2,6]

Using flow cytometry to evaluate CD20 expression by mean fluorescence intensity (MFI) in the 662 cases classified according to the main cytogenetic aberrations,[13] variable CD20 levels were found, the highest levels being detected in trisomy 12 CLL (*Online Supplementary Figure S3A*).[6,14] According to *NOTCH1* mutational status, 3′ UTR *NOTCH1*-mut cases expressed lower levels of CD20 than did *NOTCH1*-wt cases in both trisomy 12 CLL (mean MFI in 9 3′ UTR *NOTCH1*-mut cases = 2446 *versus* mean MFI in 68 *NOTCH1*-wt cases = 8504; $P<0.0001$) and non-trisomy 12 CLL (mean MFI in 17 3′ UTR *NOTCH1*-mut cases = 2049 *versus* mean MFI in 478 *NOTCH1*-wt cases = 3389; $P<0.0001$) (Figure 1B and *Online Supplementary Figure S3B*). The CD20 levels found in 3′ UTR *NOTCH1*-mut cases were similar to those detected in coding *NOTCH1*-mut cases (trisomy 12 CLL: mean MFI in 35 coding *NOTCH1*-mut cases = 2601; $P=0.7470$; non-trisomy 12 CLL: mean MFI in 55 coding *NOTCH1*-mut cases = 2181; $P=0.6275$). As expected,[6] coding *NOTCH1*-mut cases had lower levels of CD20 expression than *NOTCH1*-wt cases in both trisomy 12 CLL ($P<0.0001$) and non-trisomy 12 CLL ($P<0.0001$) (Figure 1B and *Online Supplementary Figure S3B*).

When CD20 expression was investigated by western blotting, both 3′ UTR *NOTCH1*-mut and coding *NOTCH1*-mut cases showed negligible CD20 levels (Figure 1A) that, in the majority of cases, became detectable only with very high antibody concentrations and long exposure time (*Online Supplementary Figure S2B*). On the other hand, *NOTCH1*-wt cases had relevant amounts of CD20 protein (Figure 1A and *Online Supplementary Figure S2B*). Western blotting also confirmed the inverse correlation between NICD and CD20 levels in both 3′ UTR *NOTCH1*-mut and coding *NOTCH1*-mut cases (Figure 1A). Thus, although with discrepancies allegedly due to the different detection method (western blotting *versus* flow cytometry) and anti-CD20 clone employed (clone L26 in western blotting *versus* clone L27 in flow cytometry), the western blot experiments corroborated the observation of lower CD20 protein expression in both 3′ UTR *NOTCH1*-mut and coding *NOTCH1*-mut cases compared to *NOTCH1*-wt cases, as determined by flow cytometry.

In keeping with western blot and flow cytometry results, transcript levels of the *MS4A1* gene, encoding the CD20 protein, were lower in 3′ UTR *NOTCH1*-mut cases than in *NOTCH1*-wt cases in both trisomy 12 and non-trisomy 12 categories ($P=0.0053$ and $P=0.0013$, respectively), and similar to those of coding *NOTCH1*-mut cases ($P=0.3294$ and $P=0.6990$, respectively) (Figure 1C). Again, coding *NOTCH1*-mut cases showed lower *MS4A1* transcript levels than *NOTCH1*-wt cases in both trisomy 12 CLL ($P=0.0004$) and non-trisomy 12 CLL ($P=0.0009$) (Figure 1C).[6]

To confirm the direct correlation between *NOTCH1* signaling and CD20 expression levels also in 3′ UTR *NOTCH1*-mut cases,[6] CLL cells from 3′ UTR *NOTCH1*-mut cases were exposed to a gamma-secretase inhibitor and evaluated for CD20 expression.[6] Treatment with a gamma-secretase inhibitor, performed in six 3′ UTR *NOTCH1*-mut cases, increased both *MS4A1* transcript levels, at 6 h ($P=0.0138$) (Figure 2A), and CD20 protein levels, at 24 h, as shown by flow cytometry (mean MFI in untreated samples = 1939 *versus* mean MFI in gamma-secretase inhibitor-treated samples = 2147; $P=0.0011$) (Figure 2B) and by western blotting (Figure 2C).[6]

Finally, we investigated whether the presence of 3′ UTR *NOTCH1* mutations could effectively influence susceptibility to anti-CD20 immunotherapy,[6] by evaluating the capability of rituximab and ofatumumab to kill *in vitro* CLL cells in a standard complement-dependent cytotoxicity assay.[15] Consistent with CD20 expression levels, 3′ UTR *NOTCH1*-mut cases showed lower relative lysis induced by rituximab than did *NOTCH1*-wt cases (9 3′ UTR *NOTCH1*-mut cases, mean relative lysis upon rituximab: 2.09% *versus* 9 *NOTCH1*-wt cases, mean relative lysis upon rituximab: 25.57%; $P=0.0314$) (Figure 3A), and similar to those of coding *NOTCH1*-mut cases (9 coding *NOTCH1*-mut cases; mean relative lysis upon rituximab: 2.36%; $P=0.8159$). In the same manner, 3′ UTR *NOTCH1*-mut cases showed lower relative lysis induced by ofatumumab than did *NOTCH1*-wt cases (9 3′ UTR *NOTCH1*-mut cases, mean relative lysis upon ofatumumab: 37.97% *versus* 9 *NOTCH1*-wt cases, mean relative lysis upon ofatumumab: 60.64%; $P=0.0095$), and again similar to those of coding *NOTCH1*-mut cases (9 coding *NOTCH1*-mut cases; mean relative lysis upon ofatumumab: 23.40%; $P=0.0970$). As expected,[6] coding *NOTCH1*-mut cases showed lower relative lysis induced by rituximab and ofatumumab than did *NOTCH1*-wt cases ($P=0.0330$ and $P=0.0006$, respectively) (Figure 3A). Moreover, CD20 levels directly correlated with the killing capacity of both rituximab and ofatumumab, as expressed by percentage of relative lysis ($r=0.8825$ and $r=0.7435$, respectively) (Figure 3B). In this context, ofatumumab appeared more efficient than rituximab (Figure 3B).[6,15] These results remain to be confirmed by considering another effector function such as antibody-dependent cell-mediated cytotoxicity in a specific *in vitro* assay.

In conclusion, we showed here that 3′ UTR *NOTCH1* mutations are associated with low CD20 expression and with relative resistance to anti-CD20 immunotherapy *in vitro*, as previously demonstrated for CLL carrying coding *NOTCH1* mutations.[6] This suggests that it would be useful to introduce a comprehensive diagnostic evaluation of *NOTCH1* mutational status, including 3′ UTR *NOTCH1* mutations, in CLL patients undergoing anti-CD20 immuno-chemotherapy.

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