Molecular mechanisms underlying CLL biology and response to treatment

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MULTIPLE MECHANISMS OF KRAS ACTIVATION IN TRISOMY 12 CHRONIC LYMPHOCYTIC LEUKEMIA

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Background: Trisomy 12 (Tri12) chronic lymphocytic leukemia (CLL) identifies a cytogenetic subset with a peculiar clinical behavior (Bulian *et al*, Haematologica, 2017) and specific biological features, including high frequency of stabilizing *NOTCH1* mutations (*NOTCH1-mut*) (Rossi et al, Blood, 2013). Moreover, *KRAS* and its transcriptional regulator *HNRNPA1*, key components of Ras-Raf-MEK-ERK pathway are both hosted in the trisomic chromosome 12.
Aims: To investigate the role of KRAS expression and/or activation in Tri12 and *NOTCH1-mut* CLL.
Methods: Tri12 was assessed by FISH. *NOTCH1* and *KRAS* mutations were assessed by either Sanger or NGS. QRT-PCR and western blot were employed to evaluate HNRNPA1 and KRAS expression and down-stream signaling in primary CLL or CLL cell line models. Active GTP-bound RAS pull-down was performed by Raf1 RBD agarose beads assay. CI and MEC-1 cell lines were used as human *in vitro* model of Tri12 *NOTCH1-mut* CLL and non-Tri12 *NOTCH1-wild type* (*wt*) CLL, respectively. Mann-Whitney test, unpaired t-test or Chi-Square test were used to compare differences between groups.

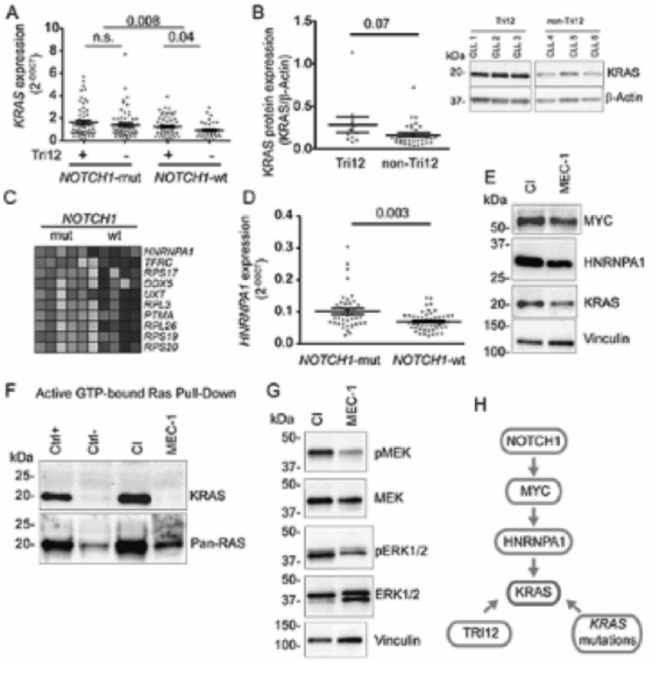

Figure 1.

Results: *KRAS* transcript was analyzed in 215 cases purposely enriched in Tri12 (118) and *NOTCH1-mut* (121). As shown in Figure 1 A, Tri12 were characterized by higher *KRAS* level in the context of *NOTCH1-wt* CLL (p=0.04); conversely, no differences in KRAS expression were found between Tri12 and non-Tri12 in the context of *NOTCH1-mut* CLL cases, which however expressed higher *KRAS* transcript compared to *NOTCH1-wt* CLL (p=0.008). Consistently, KRAS protein level was higher in Tri12 vs. non-Tri12 in the context of *NOTCH1-wt* CLL (Figure 1 B). A gene expression profiling identified *HNRNPA1* as the top ranked gene among MYC target genes upregulated in *NOTCH1-mut* vs. *NOTCH1-wt* CLL. Accordingly, higher *HNRNPA1* levels were found by QRT-PCR in additional 41 *NOTCH1-mut* vs. 47 *NOTCH1-wt* CLL samples (p=0.003, Figure 1 C,D) without differences between Tri12 (42) and non-Tri12 (46) CLL. In line with a KRAS transcriptional activation mediated by NOTCH1-MYC-HNRNPA1 axis, higher MYC, HNRNPA1 and KRAS protein levels were found in the Tri12 *NOTCH1-mut* CI cell line when compared to the non-Tri12 *NOTCH1-wt* MEC-1 cells. Furthermore, higher levels of active GTP-bound KRAS and higher phosphorylation levels of MEK and ERK were found in CI cells (Figure 1 E,F,G), supporting the hypothesis of sustained Ras-Raf-MEK-ERK signaling in Tri12 and *NOTCH1-mut* CLL. Finally, analysis of *KRAS* genomic aberrations revealed higher mutations incidence in Tri12 CLL (10/77, 13%) when compared to non-Tri12 CLL (2/68, 2.9%, p=0.03). Altogether, these data foster the hypothesis of multiple mechanisms of KRAS overexpression/activation occurring in Tri12 CLL via the NOTCH1-MYC-HNRNPA1 axis in *NOTCH1-mut* cases, or due to a higher incidence of KRAS mutations or an overexpression of KRAS by the supernumerary chromosome 12 (Figure 1 H).
Summary/Conclusion: Our data, by describing a synergism between Tri12, *NOTCH1-mut* and *KRAS-mut* in boosting KRAS expression and activity in CLL, indicate the Tri12 subset as particularly addicted to the Ras-Raf-MEK-ERK signaling pathway and likely to benefit of ERK/MEK inhibitors, as recently emphasized (Dietrich *et al*, J Clin Invest, 2018).

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REGULATION OF HIF-1A IN TP53 DISRUPTED CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND ITS POTENTIAL ROLE AS A THERAPEUTIC TARGET

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Background: Treatment of high-risk chronic lymphocytic leukemia (CLL) patients remains an unmet clinical need. Disease aggressiveness can be ascribed to intrinsic features of the tumor cells (i.e. *TP53* disruption) and to the interaction of CLL cells with stromal cells (SC) of the microenvironment. HIF-1 is a transcription factor implicated in cell adaptation to hypoxia and is involved in the regulation of genes implicated in tumor progression. In CLL cells, the α subunit of HIF-1 (HIF-1α) is constitutively expressed even in normoxia and regulates the protective interactions that the leukemic cells establish with the microenvironment.
Aims: The aims of this study were to understand HIF-1α regulatory pathways in CLL cells from *TP53* disrupted ($TP53^{dis}$) and wild type ($TP53^{wt}$) patients, and to evaluate the ability of HIF-1α inhibition to exert synergistic cytotoxic effects in combination with fludarabine and ibrutinib.
Methods: Del(17p) in CLL cells was assessed by fluorescence in situ hybridization and the presence of *TP53* gene mutations was evaluated by Sanger sequencing. CLL patients with mutation of the *TP53* gene, or >40% del(17p) in the absence of *TP53* mutation, were included in the $TP53^{dis}$ subset. Patients with <10% del(17p) and without *TP53* mutation were considered $TP53^{wt}$. In selected experiments, CLL cells were cultured in the presence or absence of M2-10B4 SC, and exposed to PD98059, Y27632, LY249002, BAY87-2243, F-ara-A or ibrutinib. Culture conditions were 21% (normoxia) or 1% (hypoxia) O2, 5% CO2 at 37°C. Ras, ERK1-2, Akt, HIF-1α, Elk3 and pVHL expression was evaluated by Western Blot. RhoA and RhoA kinase acitivity was measured by specific immunoassays. *HIF-1A*, *p21* and *ENO1* gene expression was assessed by RT-PCR. Cell viability was analyzed by AnnexinV/propidium Iodide immunostaining.

Results: We found that primary CLL cells from patients carrying *TP53* abnormalities (*TP53*^{dis} CLL cells) had constitutively higher transcriptional activity and expression levels of the α subunit of HIF-1 compared to CLL cells isolated from *TP53*^{wt} samples (*TP53*^{wt} CLL cells). HIF-1α upregulation detected in the *TP53*^{dis}subset was due to a reduced expression of the HIF-1α ubiquitin ligase von Hippel-Lindau protein (pVHL) and more active PI3K/Akt and Ras/ERK1-2 signalling pathways. Hypoxia and SC further enhanced HIF-1α accumulation in both *TP53*^{dis} and TP53^{wt} CLL cells. Hypoxia-mediated HIF-1α upregulation was due to a decreased pVHL expression and to the activation of PI3K/Akt and Ras/ERK1-2 signalling pathways. SC did not affect pVHL expression, but induced an increased activity of Ras/ERK1-2, RhoA/RhoA kinase and PI3K/Akt pathways, leading to HIF-1α accumulation. Interestingly, *in vitro fludarabine-resistant* CLL cells were mostly *TP53*^{dis} and expressed significantly higher levels of *HIF-1A* mRNA compared to *fludarabine-sensitive* cells. The HIF-1α inhibitor BAY87-2243 reversed the constitutive fludarabine resistance of leukemic cells isolated from patients carrying *TP53* abnormalities, and counteracted the fludarabine resistance induced by SC. BAY87-2243 also elicited a strongly synergistic cytotoxic effect in combination with ibrutinib.
Summary/Conclusion: Overall, our data indicate that HIF-1α is overexpressed in CLL cells, especially in the presence of *TP53* abnormalities, and is susceptible of positive regulation by hypoxia and SC. From the translational standpoint, HIF-1α can be regarded as a crucial target whose inhibition warrants further evaluation, also in combination with currently available therapies.

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IBRUTINIB DOES NOT SUPPRESS CLONAL EVOLUTION IN HIGH RISK CHRONIC LYMPHOCYTIC LEUKEMIA

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Background: The B-cell receptor (BCR) is one of the most important surface molecules that CLL cells use to gain oncogenic signals from the microenvironment. The critical role of BCR signaling for the pathogenesis of CLL is supported by the therapeutic success of ibrutinib, a targeted agent that disrupts the BCR pathway. Beside microenvironment-promoted oncogenic signals, the biology of CLL is also driven by molecular lesions and clonal evolution, that mark CLL progression and treatment resistance. The interconnection between microenvironment-promoted oncogenic signals and clonal evolution has been postulated in CLL but never proven because of the lack of suitable *ex vivo* models.
Aims: Ibrutinib allows the unprecedented opportunity of assessing the contribution of BCR to cancer clonal evolution directly *in vivo* in patients.
Methods: The IOSI-EMA-001 study (NCT02827617) is an observational, non-interventional, multicenter study consisting in the prospective and longitudinal collection of peripheral blood samples and clinical data from high risk CLL patients treated with ibrutinib monotherapy. Tumor DNA derived from sorted CLL cells (purity >99%) and germline DNA derived from sorted T cells were used for somatic mutation identification by CAPP-seq targeted deep next generation. A gene panel including 133 genes recurrently mutated in mature B-cell tumors or targeted by the aberrant somatic hypermutation process, was used. The number of the libraries loaded in the NexSeq500 sequencer (Illumina) was tailored at obtaining at least a coverage >2000x in >80% of the region of interest. A stringent bioinformatic pipeline was applied to suppress the background noise allowing to call variants with a sensitivity of $3x10^{-3}$. To track clonal trajectories across serial samples, we first measured the variant allele fraction (VAF) of all mutations identified across the different timepoints per patient. VAFs were transformed to cancer cell fractions (CCFs) using the ABSOLUTE tool. For each patient, we compared the clonal composition of the baseline sample with all the available longitudinal samples (up to 72 weeks of therapy).
Results: The study cohort comprised 31 high risk CLL patients, including 15 treatment naïve, 16 relapsed, 80% IGHV unmutated, 42% 17p deleted and 55% TP53 mutated (Figure 1A). Median duration of ibrutinib treatment was 45 weeks (range 24-72 weeks). Overall, 285 individual mutations were longitudinally discovered and monitored across a total of 119 sequential timepoints collected during ibrutinib treatment. Significant changes in CCF over time, defined as a FDR adjusted p value of <0.1 for change in CCF >0.1 in the largest rising or falling clone was observed in 21/31 (67.7%) cases (Figure 1B), a proportion that is superimposable to the clonal evolution rate previously documented in CLL treated with chemoimmunotherapy. Clonal evolution appeared to be puzzled and involved different genes without a stereotypic targeting (Figure 1C). Consistently, none of the main driver gene mutations was homogeneously selected or suppressed by ibrutinib. Clonal evolution rate neither associated with IGHV or TP53 mutation status (Figure 1D), nor changed over time (Figure 1E).

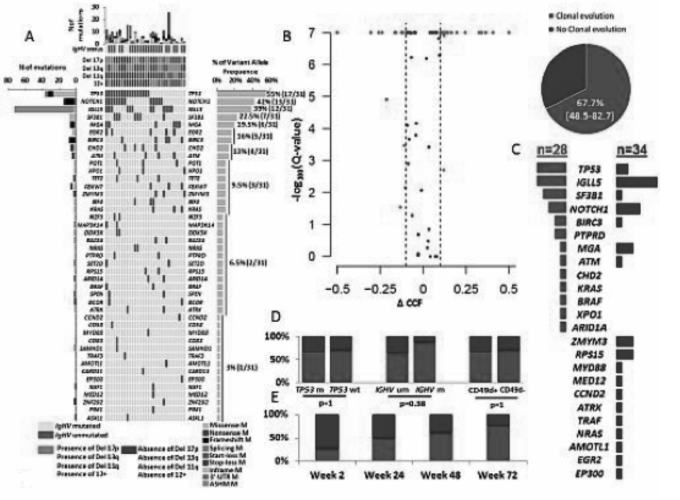

Figure 1.

Summary/Conclusion: Our results suggest that clonal evolution, a known pathogenic mechanism of progressive CLL: i) is not abrogated by ibrutinib; and ii) is quantitatively similar, but qualitatively different, than clonal evolution under chemoimmunotherapy, without specific pathways being targeted.

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RITUXIMAB INDUCES A PRO-INFLAMMATORY MICROENVIRONMENT THAT INCREASES NOTCH1 SIGNALLING IN CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS

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Background: The addition of the anti-CD20 monoclonal antibody (mAb) rituximab to chemotherapy improves responses in almost all chronic lymphocytic leukaemia (CLL) patients, with the exception of those having *NOTCH1* mutations. NOTCH1 is a cell surface receptor releasing its intracellular domain (NICD1) after two ligand-induced cleavage steps performed by metalloproteases and γ-secretase. NICD1 acts as transcription factor and *NOTCH1* mutations in CLL lead to longer lasting transcription factor activity.
Aims: To understand the relationship between rituximab and NOTCH1, we studied the effects rituximab treatment has on NOTCH1 signalling.
Methods: Freshly isolated peripheral blood mononuclear cells (PBMCs) from CLL patients attending St. Bartholomew's Hospital, London were enriched for CD19+ cells and treated with rituximab. Whole protein lysates were obtained after 15, 30 and 60 min of mAb treatment; RNA was isolated after 150 min. NICD1 was semi-quantitatively assessed by western blot. Expression of *HES1*, the best established NICD1 target gene, and *CCL2* was quantified by TaqMan-probe-based quantitative PCR. Rituximab