SEARCH FOR NON-CODING RNA AS NEW BIOMARKERS AND TARGETS TO IMPROVE THE RESPONSE TO IBRUTINIB TREATMENT CHRONIC IN LYMPHOCYTIC LEUKEMIA

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1. Summary

Chronic lymphocytic leukemia (CLL) is a very common lymphoid neoplasm and patients show diverse biological features, clinical behaviors and treatment responses. We wanted to address which genes are particularly relevant for predicting response to newly developed drugs against CLL such as ibrutinib. In particular, we wanted to study a special gene family named non-coding RNAs that are very important in the regulation of cellular functions through contributing to the expression of other genes that are translated into proteins, some of which are related with CLL aggressiveness and treatment response. Moreover, we have previously described some non-coding RNAs related with stimuli from the tumoral microenvironment on CLL cells, boosting their proliferation and helping to prevent cell death. Therefore, the identification of more such non-coding RNAs would represent new advances for prediction of ibrutinib response among CLL patients and of new targets for complementary treatments based on their expression downregulation in order to overcome the resistance to this treatment that has been observed in some CLL patients.

We approached this goal in a collaborative work involving two teams (Barcelona (IDIBAPS) and Aviano (CRO)) working in parallel on screening for such RNAs, but focusing on different families: lncRNA and microRNAs respectively. In a first screening phase the expression of all RNA families was studied in different CLL sample cohorts (with or without molecular features such as TP53 gene alterations or trisomy 12), in an ex vivo model emulating some microenvironmental effects relevant for the ibrutinib treatment (CpG-ODN, CD40L, IL10, BCR stimulation). LncRNAs found overexpressed compared to controls in response to such stimuli were further screened for clinical impact using expression and clinical data of an independent series of 266 CLL patients. Moreover, pathway enrichment analyses were also used to narrow the number of clinically relevant lncRNAs involved in the molecular pathways stimulated by the microenvironmental factors. One of those lncRNAs (MALAT1) was particularly noticeable regarding its clinical impact, being independent of all other known prognostic factors in CLL. We also showed its potential as an interesting clinical non-invasive biomarker of the microenvironmental stimuli occurring in the patients' lymph nodes. On top of that, we were able to select 3 other lncRNA candidates of particular interest both biologically and clinically. Further analyses made it possible to refine to the level of transcriptional variant the amplicons to be used to monitor their expression

in the functional experiments. We analyzed the functional relevance of these candidates in silencing experiments in the MEC1 CLL cell line and we were able to demonstrate their effective involvement in regulating the expression of genes involved in similar pathways to those previously observed in association in the experimental model of CLL primary cells treated ex vivo. Noticeably, among the genes modulated by these lncRNAs we found a precursor of the miR-155 microRNA. Interestingly the CRO team also studied the microRNA modulated by the same microenvironmental factors, and miR-155 was found among the upregulated microRNAs.

Altogether, our results suggest that part of the identified lncRNA oncogenic effect is mediated through indirect control of the levels of this microRNA. In conclusion, we raised evidence for the potential synergic effects of using ibrutinib with synergic therapies focusing on silencing the lncRNA here characterized as the blocking of miR-155.

2. Results

Sample Selection

A total of 16 CLL samples were selected based on: (a) unmutated IGHV status, (b) genetic profile from groups with 11q13, trisomy 12 or TP53mut/del17p, and another group without any of the previous alterations (WT), (c) treatment-naive (particularly with respect to ibrutinib). Based on the capacity of anti-IgM beads to activate BCR signaling with an increase of p-BTK and p-ERK levels, we selected for further experiments 6 WT CLL cases, and 6 TP53 deleted/mutated CLL cases. In the other cohorts we found more cases not clearly activated by IgM beads, so in the end we could include only 3 cases with trisomy 12 (studied for lncRNA only).

Immunophenotypical characterization of stimulated CLL cells

A cell cytometry panel of 6-colors (assessing CD19, CD5, CD69, MCL1, cleaved PARP, live/dead staining) was performed to assess the phenotype changes obtained using the 4 treatments proposed: (A) Cells only treated with polyclonal Ig-coated beads, (B) Cells treated with IgM-coated beads + ibrutinib, (C) Same treatment as A + agonist factor mix (CpGODN, Mega CD40L and IL-10), (D) Same treatment as B + agonist factor mix. In keeping with the proliferative effect of the agonist mix WT cases showed

an increase of CD69 and MCL1 expression levels (condition A vs C and B vs D). On the other hand TP53-disrupted cases showed no differences between the two conditions possibly due to a relative high expression of both CD69 and MCL1 in the context of TP53 disrupted compared with WT cases. Ibrutinib was able to block the CD69 and MCL1 activation induced by anti-IgM stimulation while the addition of microenvironmental stimuli seemed to counteract CD69/MCL1 inhibition at least in part. TP53-disrupted samples showed a slight decrease of CD69 and MCL1 following ibrutinib treatment mostly related to the low effect of anti-IgM stimulation in this set of cases.

Non-coding RNA expression profiling and functional studies

MicroRNAs. We searched for those differentially expressed between experimental conditions with dedicated microarrays (Agilent). Among WT and TP53-disrupted cases we identified 32 microRNA clearly splitting WT cases from TP53-disrupted cases. As previously reported in other tumors, miR-34 was significantly up-regulated in WT cases. These data could explain the dismal outcome of TP53-disrupted cases, miR-34 being a negative regulator of c-Myc, and BCL2, and involved in a positive feedback loop with TP53 extending its inhibitory effect on the tumor.

Comparing condition A versus C we identified an overexpression of the microRNA belonging to the miR-17-92 cluster family following microenvironmental stimulation in keeping with our previous studies. This upregulation is clear in the WT cases, while in the context of TP53-disrupted cases members of the miR-17-92 family showed no differences in terms of expression, pointing to a possible interference of TP53 mutated protein in the expression of these microRNAs. In addition, miR-155, one of the best characterized oncomiRs in hematological malignancies, was overexpressed after stimulation. On the other hand, with respect to miR-17-92 microRNAs, miR-155 upregulation turned out to be independent of the TP53 status. In fact significant miR-155 overexpression was reported both in WT and in TP53-disrupted cases upon stimulation. Interestingly, even in the context of samples stimulated with anti-IgM and treated with ibrutinib we observed a similar microRNA profile. WT samples upregulated microRNA belonging to the miR-17-92 family, indicating that as expected, ibrutinib was not able to block these microRNA. These results are in keeping with the capacity of ibrutinib to block the signaling from BCR rather than other stimuli that do not rely on BTK and also with the notion that ibrutinib does not usually induce apoptosis. It should

be noted that miR-132, a well known upregulated microRNA following anti-IgM stimulation, was downregulated only in condition D (combination of ibrutinib and other microenvironmental stimuli), probably supporting a BTK-independent regulation. As reported above for canonical microenvironmental stimulation even in this context miR-155 was the only microRNA differentially expressed in common between WT and mutated samples. We also performed expression profiling analysis of mRNA by arrays to investigate associated mRNA modulation which was carried out by using the RNAseq technique. By analyzing the differential gene expression profiling (GEP) of condition A vs C we identified 1614 genes differentially expressed (1306 upregulated and 308 downregulated in the condition). In keeping with the upregulation of miR-17-92 family members and miR-155, gene set enriched analysis (GSEA) identified a significant downregulation of the targets of these microRNAs in condition C, again underlining the importance of these microRNAs. The same results were obtained comparing the differential GEP of conditions B and D. Again, GSEA revealed an upregulation of miR-17-92 family members and miR-155 in condition D.

Based on our previous data and the results of these experiments that underlined the importance of miR-17 in the context of microenvironmental stimuli we had the chance to investigate the capacity of miR-17 expression to predict prognosis both in CLL, as well as in other primary lymphoproliferative disorders (e.g. mantle cell lymphoma, MCL, and diffuse large B cell lymphoma, DLBCL). Specifically, 399 CLL samples, 77 MCL samples and 78 DLBCL samples were analyzed for miR-17 expression levels. In CLL and MCL, it was possible to demonstrate that high levels of miR-17 were able to distinguish patients with a lower overall survival compared to patients who had low levels of miR-17. According to the data reported above we selected miR-17 and miR-155 as candidates for silencing in primary samples. Preliminary experiments demonstrated the ability of treatment with antagomiR-17 to reduce viability and increase apoptosis of tumor cell lines (MCL in this case, where the technique was originally set up). The data showed that following transfection with nanobubbles containing an miR17 antagonist (antagomiR-17) there is an increase in apoptosis. The transfection not only caused an increase in apoptosis but also a reduction in cell proliferation. In conclusion, here we provide preliminary data indicating that miR17 is involved in CLL cell proliferation upon microenvironmental interactions and that antagonizing miR17 may have a role in CLL cell survival and proliferation. Similar experiments are ongoing to investigate the role of miR155 in the same context.

lncRNAs. We profiled the above-mentioned samples using the Clariom D WT Pico microarray platform (Thermofisher). Preliminary analysis (PCA) demonstrated that groups of wild type CLL from Barcelona and Aviano as well as TP53mut/del17p group had 1 sample each that were considered as outliers according to the global expression profile. Therefore, we excluded these samples for the differential expression analysis. From the coding genes included in the array we were able to confirm the differential expression of genes/pathways that are concordant with the expected impact of the agonist mix treatment (i.e. EZH2 is dramatically upregulated by this treatment, which is an epigenetic regulator factor previously described as key in the microenvironmental stimulation of CLL cells in the lymph nodes. PMID: 31227476). We also obtained a list of lncRNA candidates that were modulated by such treatment in the different sample groups studied. Among them should be noted the upregulation of MALAT1 lncRNA by agonist mix only in some of the studied cohorts. In silico data analysis of expression data on previously studied CLL cases allowed us to identify MALAT1 as an lncRNA with robust clinical impact in CLL and potentially associated with pathways induced by the lymph node microenvironment, being a surrogate marker of the degree of microenvironmental stimulation in the lymph nodes. Therefore, these results pointed to MALAT1 as a potential lncRNA of interest in relation with our project.

To narrow down the number of lncRNA candidates we also performed transcriptome-wide analyses of clinical impact of lncRNA expression on time to treatment (TTT) of a large series of CLL with available expression data from ICGC project (N=266). We identified 1904 lncRNAs, from 1171 of which a higher expression was associated with a shorter TTT, which were the most interesting ones for our validation approach for aim 3. We looked at which of the candidate lncRNAs upregulated by agonist mix treatment were included in the subset of lncRNAs identified with a more aggressive clinical behavior. A total of 83 common lncRNA were identified between both analyses. We also applied different complementary criteria to narrow even more the number of lncRNA candidates of interest. We had previous data from the ICGC project regarding several CLL cell lines. The available MEC1 cell line was included so we could check the expression of the most interesting candidates and identify which were best for further functional analyses because higher basal expression levels would allow the monitoring of their expression upon silencing in cell lines such as MEC1. We selected three of the top highly expressed candidate lncRNAs in the MEC1 cell line also by qRT-PCR. Further pathogenetic evaluation using pathway enrichment analysis on the ranked list of coding

genes correlated with selected lncRNA candidates demonstrated their potential involvement as actual regulators of relevant pathogenetic pathways modulated by microenvironmental stimuli.

In this regard we presented a poster in the 28th Congress of the European Hematology Association (EHA). June 8-15, 2023. Frankfurt, Germany. These results correspond to one of these selected candidates (LINC00152-CYTOR) because previous published data was pointing to lack of clinical value of this lncRNA in CLL. On the contrary, our results clearly show that it is a relevant lncRNA in this neoplasm and its expression is related with microenvironmental stimuli. The functional validation of the three candidates was successful, as we achieved a significant degree of silencing of the three candidates in MEC1 cell line, according to expression levels measured in the microarray platform (see below) as well as by qRT-PCR. We performed an analysis to identify those genes significantly modulated by the silencing for each lncRNA assayed in the MEC1 CLL cell line. We also performed pathway enrichment analysis on the modulated gene lists. Noticeably, these results showed some relevant genes (including MCL1) modulated by the microenvironmental stimuli. Moreover, among the differentially expressed genes we also saw the involvement of the three candidates in the expression of miR-155 precursor (MIR155HG). This is concordant with the upregulation found by the CRO team, involving the mature form of this microRNA and suggesting that part of the oncogenic function of those lncRNAs in CLL would be mediated by their influence in miR-155 expression, also linking this phenomenon with microenvironmental stimulation on CLL cells. Finally, the enriched pathways found included several previously known to be pathogenetically deregulated in CLL, involving elements in the response and signal transduction from microenvironmental stimuli (Toll-like receptors, cytokines, interleukins, among others). Complementary experiments are still ongoing to demonstrate the potential benefits of combining in vitro silencing of these lncRNAs with ibrutinib treatment.

3. Relevance and possible clinical applicability of the results

As lncRNA expression could be reduced in vitro (as shown in this project), but as it is also known that it could also be done in vivo using the same LNA-oligos (gapmers), the identification of lncRNAs involved in the control of pathogenetic pathways boosted by

microenvironmental factors gave us new targets for the potential use of gapmers as a tool in cotreatments with ibrutinib in order to improve the low sensitivity that some CLL patients show to such treatment. As we also determined that our 3 microenvironment-related lncRNAs are regulators of a miR-155 precursor, our results suggest that part of their oncogenic effect is mediated through the indirect control of the levels of this microRNA. Relevantly, the clinical application of all these findings is quite evident as there is an already developed drug (MRG-106; cobomarsen) that is an miR-155 inhibitor synthesized as a locked nucleic acid (LNA oligo). A phase 1 clinical trial with this agent in several lymphoma types (other than CLL, though) was very successful, and may be promising for clinical applications in the future, including the improvement of ibrutinib response in CLL.

4. Derived scientific publications

1- Scientific article: MALAT1 Expression is Associated with Aggressive Behavior in Indolent B-Cell Neoplasms: an investigation based on multiple transcriptomic approaches. Elena María Fernández-Garnacho, Ferran Nadeu, Silvia Martín, Pablo Mozas, Andrea Rivero, Eva Gine, Armando López-Guillermo, Martí Duran-Ferrer, Itziar Salaverria, Cristina López, Silvia Beà, Santiago Demajo, Pedro Jares, Jose Ignacio Martín-Subero, Xose Puente, Elias Campo, Lluís Hernández. SciRep 2023 Oct 6;13(1):16839. doi: 10.1038/s41598-023-44174-8. PMID: 37803049.

2- Scientific article: ENDOG Impacts on Tumor Cell Proliferation and Tumor Prognosis in the Context of PI3K/PTEN Pathway Status. Barés G, Beà A, Hernández L, Navaridas R, Felip I, Megino C, Blasco N, Nadeu F, Campo E, Llovera M, Dolcet X, Sanchis D. Cancers (Basel). 2021 Jul 28;13(15):3803. doi: 10.3390/cancers13153803. PMID: 34359707.

3- Congress poster: Hernández Ll.; Nadeu F.; Delgado J.; et al.; Gattei V. The expression of CYTOR LncRNA has poor prognostic value in CLL patients and is associated with microenvironmental stimuli. EHA2023 Hybrid Congress - 28th Congress of the European Hematology Association (EHA). June 8-15, 2023. Frankfurt, Germany. PMC10429169.