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Differential expression of long non-coding RNAs are related to proliferation and histological diversity in follicular lymphomas

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Summary

Long non-coding RNAs (lncRNAs) comprise a family of non-coding transcripts that are emerging as relevant gene expression regulators of different processes, including tumour development. To determine the possible contribution of lncRNA to the pathogenesis of follicular lymphoma (FL) we performed RNA-sequencing at high depth sequencing in primary FL samples ranging from grade 1-3A to aggressive grade 3B variants using unpurified (n = 16) and purified (n = 12) tumour cell suspensions from nodal samples. FL grade 3B had a significantly higher number of differentially expressed lncRNAs (dif-lncRNAs) with potential target coding genes related to cell cycle regulation. Nine out of the 18 selected dif-lncRNAs were validated by quantitative real time polymerase chain reaction in an independent series (n = 43) of FL. RP4-694A7.2 was identified as the top deregulated lncRNA potentially involved in cell proliferation. RP4-694A7.2 silencing in the WSU-FSCCL FL cell line reduced cell proliferation due to a block in the G1/S phase. The relationship between RP4-694A7.2 and proliferation was confirmed in primary samples as its expression levels positively related to the Ki-67 proliferation index. In summary, lncRNAs are differentially expressed across the clinico-biological spectrum of FL and a subset of them, related to cell cycle, may participate in cell proliferation regulation in these tumours.

Keywords: LncRNA, RNA-seq, follicular lymphoma, cell proliferation.

Follicular lymphoma (FL) is one of the most common lymphoid neoplasms in Western countries (*Jaffe et al*, 2017). The clinical course is usually indolent but some patients may follow a more aggressive evolution with an adverse outcome in the first years whereas others may survive for decades after the initial diagnosis (*Jaffe et al*, 2017). Similarly to the clinical heterogeneity, FL may display a spectrum of pathological features that are related in part to the biological behaviour of the tumour (Leich *et al*, 2011). Most FL are composed of a variable number of centrocytes and centroblasts (FL grade 1-3A) that maintain a follicular growth pattern whereas a subset of tumours are composed exclusively of centroblasts (FL grade 3B) and are frequently associated with a diffuse component. FL grade 1-3A are considered a histological spectrum of the same entity whereas grade 3B FL have phenotypic and genetic alterations that are closer to those of diffuse large B-cell lymphoma (DLBCL) (Ott *et al*, 2002; Bosga-Bouwer *et al*, 2003; Horn *et al*, 2011). Clinically, some studies have reported that patients with FL grades 1-3A have a more indolent behaviour than patients with FL3B (Wahlin *et al*, 2012) but in others the outcome of FL3A and FL3B is similar (Koch *et al*, 2016). Some cases may also transform into a DLBCL (tDLBCL), an event associated with a dismal outcome (Ginè *et al*, 2006; Wong & Dickinson, 2012).

The molecular features underlying the morphological and biological spectrum of FL are not fully understood. A previous transcriptomic study observed a relatively homogeneous gene expression profiling in the different histological grades of FL, but also identified differences between FL1-3A and FL3B (Piccaluga *et al*, 2008). Although some differences in coding gene expression profiles have also been described between FL1-2 and FL3A, these FL subtypes are also closely related, as also shown at the genetic and immunohistochemical level (Horn *et al*, 2018). Non-coding RNA expression profiling studies in FL have basically analysed expression and sequence alterations of microRNAs (Musilova & Mraz, 2015; Hezaveh *et al*, 2016). A recent pilot study comparing three FL3A cases to reactive lymph nodes identified four lncRNAs overexpressed in the tumour samples, suggesting that these transcripts may be relevant in these tumours (Pan *et al*, 2016). LncRNAs comprise a family of non-coding transcripts ranging from 200 bp to 100 kbp and represent the largest fraction of the mammalian non-coding transcriptome in terms of diversity. Compared to coding genes, lncRNAs are expressed at lower levels and show more tissue-specific expression patterns (Mercer *et al*, 2009; Derrien *et al*, 2012). An increasing number of different cellular process are being recognized as regulated by these non-coding RNAs, such as imprinting, dosage compensation, proliferation, differentiation and development (Huang *et al*, 2012; Melissari & Grote, 2016). The mechanisms used by lncRNAs to regulate these functions are poorly understood, partly due to the difficulties in identifying their regulated target genes (Mercer *et al*, 2009). Interestingly, altered expression of several lncRNA has been increasingly found in different neoplasms and their modulated target genes include those known to be involved in oncogenesis (Huang *et al*, 2012; Bhan *et al*, 2017). The differential expression of lncRNA in lymphoid neoplasms has been less studied, but some lncRNAs have been identified with significant relationship with prognosis or treatment response, such as *MINCR* in Burkitt Lymphoma or *MALAT1* and others in DLBCL (Doose *et al*, 2015; Li *et al*, 2017; Zhao *et al*, 2017; Zhou *et al*, 2017).

Therefore, in this study we used RNA sequencing (RNA-seq) analysis at high depth sequencing aiming to characterize the lncRNA expression landscape in the spectrum of FL. We also performed further *in silico* and experimental studies to identify potentially relevant target genes of these lncRNAs in the pathogenesis and progression of this lymphoid neoplasm.

Material and methods

Samples

In this study we used cryopreserved cell suspensions, obtained from 28 patients with nodal FL, for RNA-seq analysis. Tumour cells were purified prior to RNA extraction in 12 of these samples using negative immunomagnetic selection. The final tumour cell content was over 95% in all samples (Data S1). These FL cases included 8 grade 1-3A and 4 grade 3B, 2 of them with focal areas of DLBCL (Table SI). RNA was extracted from the whole tumour cell suspensions in the remaining 16 cases, 12 FL grade 1-3A and 4 FL grade 3B/DLBCL. These cases had a median tumour cell content examined by flow cytometry of 89%, range: 71·3–94·9% (Table SI). Furthermore, we used a validation series of 43 formalin-fixed paraffin-embedded (FFPE) samples that included 23 FL grade 1-3A, 16 FL grade 3B-DLBCL, and 4 DLBCL transformed from a previous FL (tDLBCL) (Table SII). All cases were diagnosed according to the WHO criteria (*Jaffe et al*, 2017). Ki-67

immunohistochemical staining and quantification was performed as previously described (Giné *et al*, 2010). All samples were obtained from the Haematopathology collection of the Biobank of the Hospital Clínic de Barcelona-IDIBAPS (Spain) and the Institute of Pathology of the University of Würzburg (Germany). Details on cryopreserved FL sample collection, processing and RNA extraction could be found in Data S1.

RNA-seq and expression data analysis

RNA-seq was performed according to standard protocols in the National Centre for Genomic Analysis (CNAG, Barcelona, Spain). Details regarding library preparation and RNA sequencing and the bioinformatic tools used to analyse the data (including differential expression, correlation and pathway enrichment analyses) are detailed in the Data S1. The expression data has been deposited at the European Gen- omephenome Archive (EGA) which is hosted at the Euro- pean Bioinformatics Institute and the Centre for Genomic Regulation, under accession number EGAS00001002980.

Quantitative real time polymerase chain reaction (qRT-PCR) expression studies

Total RNA obtained from the FFPE FL validation series was used for expression studies of selected lncRNAs using a preamplification protocol (Fluidigm, South San Francisco, CA, USA). Detailed qRT-PCR design, protocol and analysis are shown in Data S1. qPCR primers are detailed in Table SIII.

RP4-694A7.2 silencing in WSU-FSCCL FL cell line

The t(14;18) positive WSU-FSCCL cell line was selected for functional experiments as a representative model of FL tumour cells based on their phenotype, genetic alterations and behaviour in co-culture models with follicular dendritic cells. (Mohammad *et al*, 1993; Matas-Céspedes *et al*, 2014). To silence the expression of the lncRNA RP4-694A7.2 in this cell line we used locked nucleic acid (LNA™) oligos named Gapmers (Exiqon, Woburn, MA, USA) that are spontaneously incorporated into the cells (gymnosis) (Stein *et al*, 2010). Details of RP4-694A7.2 silencing as well as proliferation and apoptosis assays performed upon silencing are detailed in Data S1.

Results

RNA-seq of purified FL cell samples and differential gene expression analysis

We initially performed RNA-seq in a FL series of unpurified cryopreserved samples (N = 16) from lymph nodes grouped according to the histological grade (FL1-3A and FL3B/DLBCL) (Table SI). An initial unsupervised principal component analysis showed a great degree of variability among samples that was not related to the histological grade or treatment status, untreated or relapsed (Figure S1). Accordingly, these samples had a relatively similar expression profile with a low number of differentially expressed genes between low- and high-grade FL subgroups (84 coding and 33 non-coding differentially expressed transcripts) (Tables SI and SIII). Nevertheless, FL3B/DLBCL had a subset of significantly overexpressed genes that were related to cell proliferation (i.e. *MCM6*, *PBK*, *KIF2C*) and different cell types such as T-regulatory cells, dendritic cells and monocyte-derived macrophages, suggesting that they may reflect different compositions of the tumour microenvironment (Table SIV; Figures S2 and S3).

Therefore, to improve the sensitivity in the detection of differentially expressed lncRNAs by tumour cells, we performed RNA-seq analysis using purified tumour cells from 12 FL cell suspension samples. An initial unsupervised principal component analysis revealed that part of the transcriptional variability observed in this dataset was associated with the two histological subgroups under study (FL1-3A and FL3B/DLBCL) (Fig 1A). Subsequently, the supervised differential expression analysis comparing these two FL subgroups detected 1864 coding and 1297 non-coding differentially expressed genes (Fig 1B and Table SIV). Remarkably, approximately 80% of these transcripts were upregulated in FL1-3A, a percentage that was consistent for both the coding and non-coding fraction (Fig 1B and Table SIII). The majority (90%) of the differentially expressed lncRNAs (dif-lncRNAs) belonged to three families, i.e. antisense lncRNAs, long intergenic noncoding RNAs (lincRNAs) and pseudogenes, previously shown to include cancer-deregulated lncRNAs (Tsai *et al*, 2011; Vitiello *et al*, 2015; Fang & Fullwood, 2016) (Fig 1C). Antisense lncRNAs were found in a higher proportion of lncRNAs overexpressed in FL3B/DLBCL (42%) than in FL1-3A (10%). On the contrary, lincRNA and pseudogenes were found in a higher proportion of lncRNAs overexpressed in FL1-3A (35% and 45%, respectively) than in FL3B/DLBCL (17% and 32%) ($P < 0{\cdot}0001$) (Fig 1C). Altogether, these results suggested widespread differences in the lncRNA expression landscape between FL3B/DLBCL and FL1-3A groups.

Potential functions of differentially expressed lncRNAs in FL

To identify the potential biological functions of the dif-lncRNAs found between FL1-3A and FL3B/DLBCL, we defined the set of coding genes whose expression was highly correlated with each dif-lncRNA using a Treelet-based algorithm (Data S1). For each FL histological subgroup (FL1-3A and FL3B/DLBCL) we defined a set of correlated genes. Then, we performed a parallel functional enrichment analysis of the highly correlated coding gene sets associated with lncRNAs overexpressed in FL1-3A or FL3B/DLBCL (Tables SV and SVI). Noticeably, 12 different biological

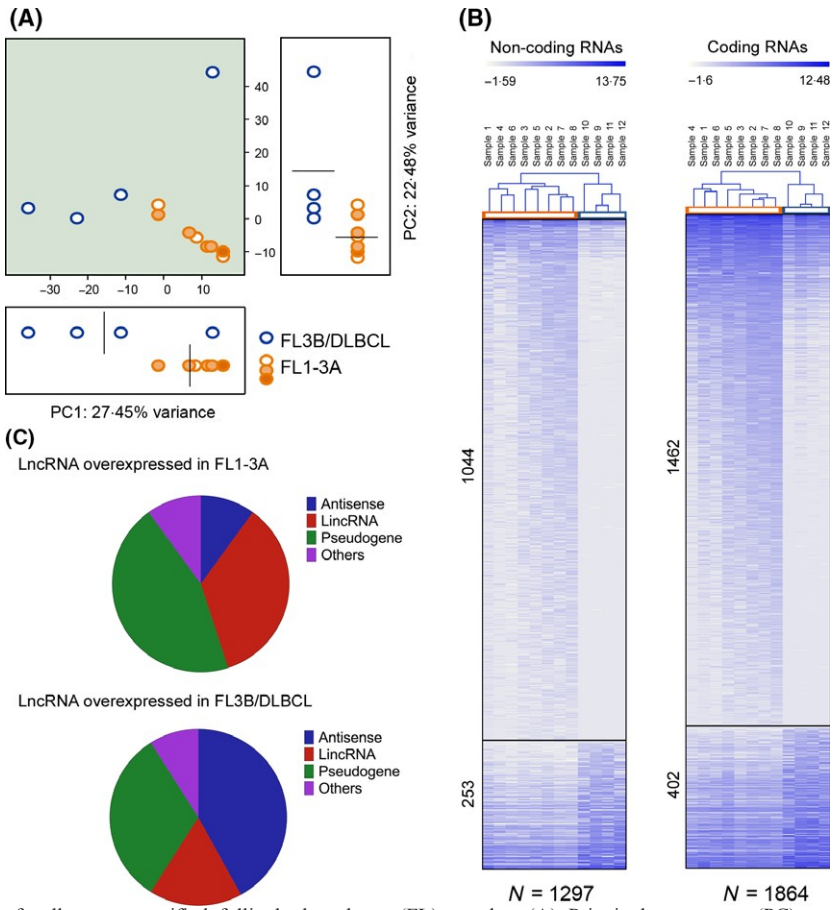

Fig 1. RNA-seq analysis of cell tumour purified follicular lymphoma (FL) samples. (A) Principal component (PC) analysis of RNA sequencing (RNA-seq) data obtained from purified tumour cells samples shows expression differences between FL1-3A *versus* FL3B/DLBCL. The scatter plot of samples across the two components is depicted along with corresponding projections for each one of them. No differences could be observed between samples obtained in untreated (empty circle) or previously treated (light filled circle) patients in FL1-3A (cases with no information on treatment are displayed as a dark filled circle). All FL3B/DLBCL samples were obtained before patient treatment. Lines indicate mean values per group. (B) Clustering of the supervised analysis output comparing the two major histological groups of samples (FL3B/DLBCL in blue and FL1-3A group in orange) considering coding and non-coding genes independently. Higher numbers of genes upregulated in FL1-3A compared to FL3B/DLBCL were similarly found either in coding (78·4%) and non-coding (80·5%) genes. (C) Pie chart depicting the proportional representation of the subsets of long non-coding families of transcripts found differentially expressed between FL1-3A *versus* FL3B/DLBCL purified samples. Antisense lncRNAs were found in a higher proportion of lncRNAs overexpressed in FL3B/DLBCL (42%) than in FL1-3A (10%), whereas lincRNA and pseudogenes were found in a higher proportion of lncRNAs overexpressed in FL1-3A (35% and 45%, respectively) than in FL3B/DLBCL (17% and 32%) ($P < 0{\cdot}0001$). DLBCL, diffuse large B-cell lymphoma; FL1-3A, follicular lymphoma grades 1-3A; FL3B, follicular lymphomas grade 3B; lincRNA, long intergenic noncoding RNA; lncRNA, long non-coding RNA.

processes were significantly different between the studied FL groups, with cell cycle (higher in FL3B/DLBCL) and signal transduction (higher in FL1-3A) reaching the highest statistical significance (Fig 2) (Table SVII). Interestingly, cell proliferation is a relevant deregulated process with clinical impact in FL (Janikova *et al*, 2011; Samols *et al*, 2013; Kedmi *et al*, 2014). Our results suggested that changes in lncRNA expression between FL histological subgroups could contribute in part to the differential biological and clinical features by modulating the expression of proliferation-related genes. In line with this finding, we also observed that a fraction of these cell cycle-related target genes (34/224, 15%) were also differentially expressed between FL1-3A and FL3B/DLBCL groups (Figure S4).

Validation of selected lncRNAs in an independent FL series

To validate dif-lncRNAs between FL1-3A *versus* FL3B we used an independent series of 43 FL samples. Given that these samples were FFPE FL and therefore contain both tumour and microenvironment cells, we selected for validation lncRNA transcripts that were found differentially expressed both in unpurifed and purified FL samples. Among the 33 dif-lncRNAs between FL1-3A and FL3B/DLBCL in unpurified samples, 18 were also differentially expressed in purified FL samples, including 7 related with cell cycle regulation (Table SVIII and Figure S5). The validation series included 3 FL1, 10 FL2, 10 FL3A, 10 FL3B, 4 FL3B/DLBCL

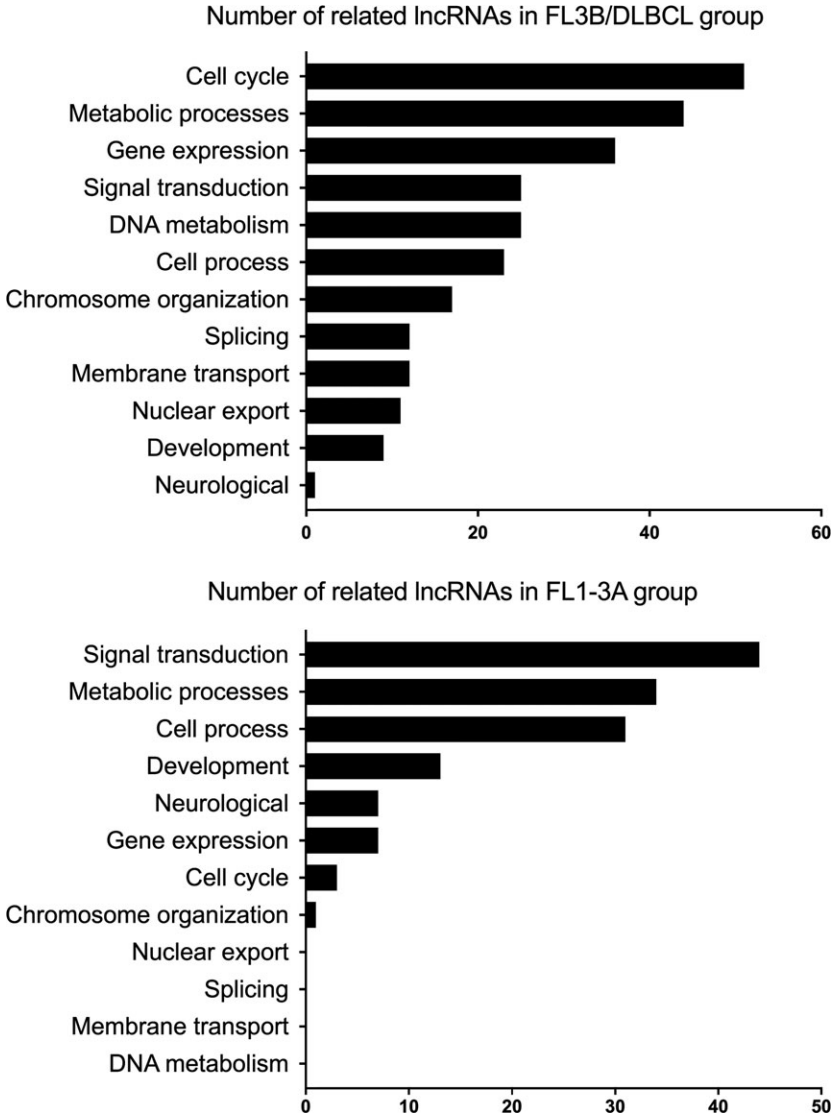

Fig 2. Plots of significantly enriched pathways found among the coding genes highly correlated in their expression with differentially expressed (dif) lncRNAs between FL3B/DLBCL and FL1-3A. Different pathways related to upregulated lncRNAs were found in separated analysis by FL histology group. The pathways involving the higher number of lncRNAs were signal transduction for the FL1-3A group and cell cycle regulation in FL3B/DLBCL. DLBCL, diffuse large B-cell lymphoma; FL1-3A, follicular lymphoma grades 1-3A; FL3B, follicular lymphomas grade 3B; lncRNA, long non-coding RNA.

and 4 tDLBCL. The expression levels of the 18 lncRNA were similar among FL1/2 and FL3A subsets and also among the FL3B, FL3B/DLBCL and tDLBCL subsets and therefore these cases were pulled together for further analysis (Figure S6). Using this approach, confirmed that the expression levels of 9 of these 18 dif-lncRNAs were significantly higher in the aggressive subset of FL than in FL1-3A (Table SVIII and Figure S7).

Five of the 9 validated dif-lncRNAs were predicted to be involved in cell proliferation by the guilt-by-association analysis (Tables SV and SVIII). Among them, RP4-694A7.2 had been previously described as upregulated in several solid cancers compared to their respective normal tissues (Yan *et al*, 2015). This lncRNA was also found overexpressed in highly proliferative stages of normal B cell differentiation, such as precursor cells and centroblasts (Petri *et al*, 2015). Therefore, we selected RP4-694A7.2 for further functional analyses, as it may be related to the increased tumour cell proliferation in aggressive FL subtypes.

Relationship between RP4-694A7.2 expression and cell proliferation in vitro and in primary follicular lymphoma samples

To further study the potential involvement of RP4-694A7.2 in FL pathogenesis, we analysed the impact of its *in vitro* downregulation in the FL representative cell line WSU-

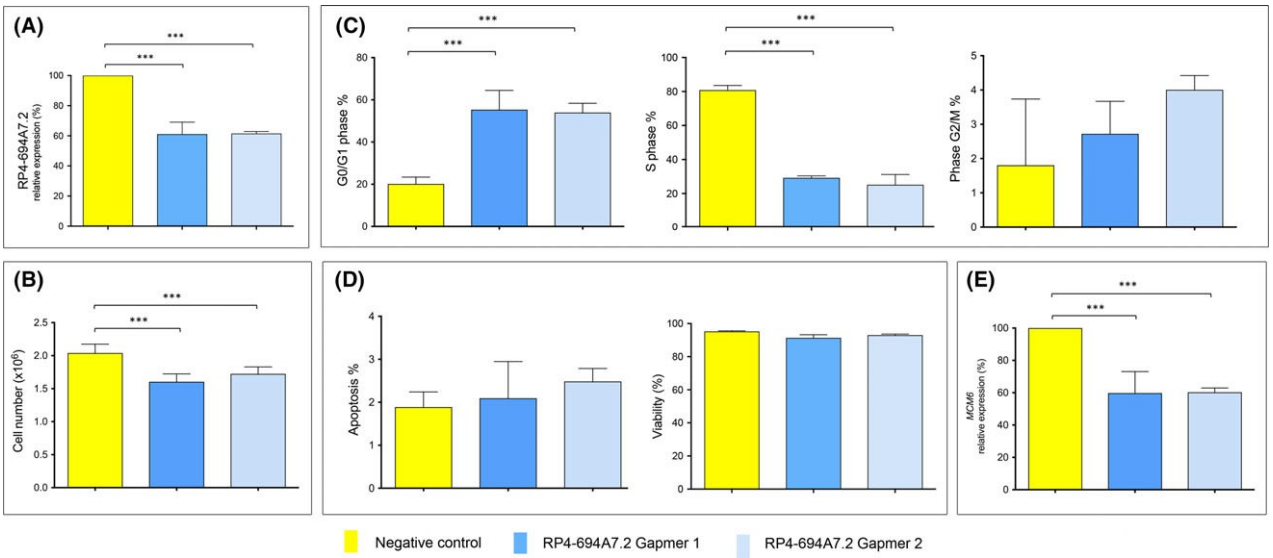

Fig 3. RP4-694A7.2 downregulation experiments in WSU-FSCCL FL cell line. (A) Level of downregulation of RP4-694A7.2 expression achieved using two different specific gapmers against this long non-coding RNA (lncRNA) in the WSU-FSCCL follicular lymphoma (FL) cell line. (B) RP4-694A7.2 silencing in WSU-FSCCL cell line with two different gapmers showed a significant reduction in cell number. (C) Noticeably, cell cycle analysis using 5-ethynyl-2-deoxyuridine (EdU)/propidium iodide (PI) cell cytometry quantification supported that the cell number reduc- tion was due to a cell cycle alteration, characterized by a significantly reduced proportion of cells in S-phase associated with an increase in G0/ G1-phase. G2/M-phase was not affected by the silencing of this lncRNA compared to the negative control. (D) Changes in cell number upon RP4-694A7.2 silencing were not significantly affected by changes in levels of apoptosis and total viability. (E) *MCM6* expression was also signifi- cantly downregulated in RP4-694A7.2 silenced cells, to a similar degree of the achieved downregulation of this lncRNA.

FSCCL using two different locked nucleic acid (LNA) oligonucleotides (gapmers, Exiqon) targeting its two exons. We measured the efficiency of the transcript downregulation and the impact in cell proliferation and viability. Gapmers 1 and 2 achieved a 42·6% and 43·8% reduction in RP4-694A7.2 expression, respectively (Fig 3A). Concordantly, we observed a significant reduction in the total number of cells upon RP4-694A7.2 silencing with both gapmers (Fig 3B). Moreover, RP4-694A7.2 silencing induced a significant decrease of the S-phase cell fraction and an increase of the G0/G1- with both gapmers (Fig 3C and Figure S8). Down-regulation of RP4-694A7.2 did not result in significant differences in apoptosis or percentage of viable cells (Fig 3D and Figure S9). All these findings support the involvement of RP4-694A7.2 in the regulation of cell proliferation in these cells.

Next, we searched for cell cycle-related coding genes potentially regulated by RP4-694A7.2. A total of 14 genes possibly regulated by this lncRNA were found differentially expressed in the purified samples, 12 overexpressed in FL3B and 2 downregulated in this subset. Four of these coding genes were also detected in unpurified cases (Tables SIV and SIX). Among them, *MCM6* was considered of particular interest because of its functional involvement in S-phase and its expression peaking at G1/S boundary (Ohtani *et al*, 1999), which is concordant with the type of cell cycle alteration observed upon RP4-694A7.2 silencing in WSU-FSCCL. To confirm this potential relationship, we performed a qRT-PCR analysis of *MCM6* before and after silencing RP4-694A7.2. Treatment of the cell line with the specific gapmers (1 and 2) induced a reduction of 40·3% and 39·7% of its expression, respectively (Fig 3E). These findings reinforced the role of RP4-694A7.2 in cell proliferation in FL that may be mediated in part by its regulatory effect on *MCM6* expression.

To validate the relationship between RP4-694A7.2 and proliferation in primary FL samples, we compared the RP4-694A7.2 expression levels in the FFPE samples from the validation study with the Ki-67 index, a *bona fide* proliferation marker. A total of 28 primary FL samples could be analysed, as we excluded cases with no detectable expression of this lncRNA or with not evaluable Ki-67 staining. Overall, the proliferation index as a continuous variable (i.e. percentage of cells expressing Ki-67) showed a significant correlation with the expression levels of RP4-694A7.2 (Pearson r = 0·56, P = 0·0019) (Fig 4A). The Ki-67 proliferation index was very variable among cases in the different FL subgroups. To evaluate the relationship between RP4-694A7.2 and the proliferation index in the different FL groups we categorized the Ki-67 index as low (<40%) intermediate (40–70%) and high (>70%). FL1/2, FL3A and FL3B with higher Ki-67 had significantly higher RP4-694A7.2 expression levels than cases of the same subgroup with lower proliferative index (FL1-2, P = 0·030; FL3A, P = 0·026 and FL3B pure, P = 0·036) (Fig 4B). Cases of FL3B/DLBCL and tDLBCL both showed high proliferation and high RP4-694A7.2 levels.

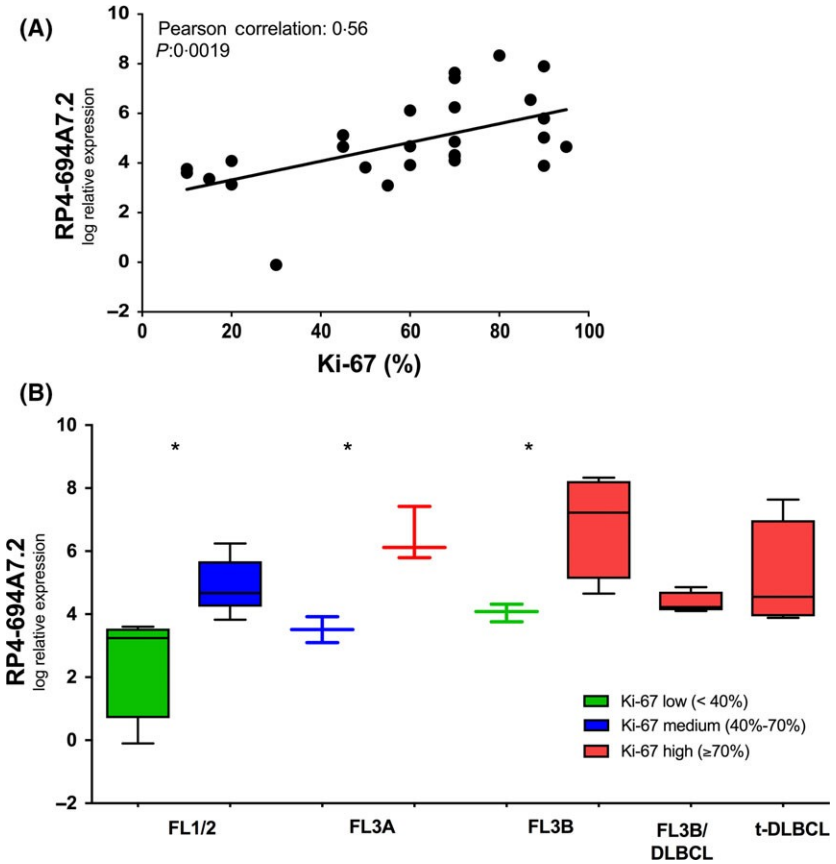

Fig 4. Relationship between RP4-694A7.2 expression levels detected by qRT-PCR and tumour cell proliferation in primary FL measured by the Ki-67 proliferative index. Panel A: A significant positive correlation between RP4-694A7.2 expression levels and Ki-67 proliferative index was observed in 28 FL (P = 0·0019). Panel B: Ki-67 index was categorized in three levels (low-medium-high) in the subsets of FL1/2, FL3A, FL3B and FL3B/DLBCL/tDLBCL. The RP4-694A7.2 expression levels were significantly higher in cases with higher Ki-67 index (FL1-2 P = 0·030, FL3A P = 0·026 and FL3B P = 0·036. All FL3B and FL3B-DLBCL/tDLBCL cases had high Ki-67 index and also high RP4-694A7.2 expression levels. DLBCL, diffuse large B-cell lymphoma; FL1-3A, follicular lymphoma grades 1-3A; FL3B, follicular lymphomas grade 3B; tDLBCL, DLBCL transformed from follicular lymphoma.

Discussion

LncRNAs are emerging as important actors in the regulation of genes involved in relevant processes for oncogenesis (Huang *et al*, 2012). Accordingly, expression deregulation of lncRNA is being increasingly described in relation to the pathogenesis and progression of many neoplasms (Bhan *et al*, 2017), including lymphoid tumours (Doose *et al*, 2015; Li *et al*, 2017; Zhao *et al*, 2017; Zhou *et al*, 2017). Nevertheless the lncRNA expression profiles in FL remain widely unexplored (Pan *et al*, 2016). In the present study, we performed a global analysis of the coding and non-coding expression landscape of FL using deep coverage RNA-seq, with special emphasis on the characterization of the lncRNA that may be related to the biological and clinical spectrum of this disease, particularly between indolent FL1-3A and aggressive FL3B/DLBCL.

First, we analyzed the expression profiles of 16 unpurified FL samples with a wide range of tumour cell content. This study showed relatively low numbers of differentially expressed coding and non-coding transcripts. The subset of coding genes more expressed in FL3B/DLBCL were related to the stromal cell component, indicating that the heterogeneous tumour and microenvironment cell content in unpurified samples hampers the identification of specific transcripts coming from FL tumour cells (Amé-Thomas & Tarte, 2014). This may be particularly important for the detection of low-expressed lncRNAs. Therefore, we further performed RNA-seq in 12 cases in which we could purify the tumour cells. This study revealed a large number of differentially expressed coding and lncRNAs (1864 and 1297, respectively) between indolent FL1-3A and FL3B/DLBCL. Moreover, we observed a different spectrum of lncRNA subtypes (antisense, lincRNA and pseudogenes) in those found differentially expressed, suggesting possible functional differences in the studied FL groups. These observations are in agreement with the previously described specificity of lncRNAs regarding the different subtypes found during B-cell differentiation, and that in FL

it could be also associated with the histological heterogeneity of these lymphoid neoplasms (Winkle *et al*, 2017).

Most of the lncRNAs identified had not been functionally annotated, probably due in part to the difficulties to perform a direct identification of their target genes. To determine the putative target genes of the differentially expressed lncRNA in FL we used an indirect widely used approach that estimates the lncRNA function by a so-called guilt-by-association analysis (Rinn & Chang, 2012). This kind of analysis assumes that the coding genes whose expression is highly correlated with that of lncRNAs may be regulated by them and therefore, such coding genes can be used as proxy to perform pathway enrichment analysis and infer the function of lncRNAs of interest (Signal *et al*, 2016). This approach also included a Treelet-based algorithm for correlation measurement, which has previously shown to have a high-performance in data sets with small sample sizes and low-expressed transcripts (Bosio *et al*, 2011, 2012a,b; Tapia *et al*, 2011). In this way, we selected subsets of highly correlated coding genes for each dif-lncRNA in separated analyses from FL1-3A and FL3B/DLBCL expression data. Downstream pathway enrichment analysis revealed differences in pathways potentially related to dif-lncRNAs in both FL histological subgroups. The two pathways with the highest significant differences in the number of related dif-lncRNA were signal transduction for those overexpressed in FL1-3A and cell cycle for lncRNA significantly overexpressed in FL3B/DLBCL.

Regarding the potential pathogenetic and clinical impact of the lncRNA expression deregulation found between FL histological groups, we noticed that cell cycle-related lncRNAs, with increased expression in FL3B/DLBCL compared to FL1-3A, were the largest subset found among dif-lncRNAs. This finding suggests that lncRNA deregulation in FL3B/DLBCL may represent a molecular mechanism associated with increased proliferation in FL. This is noticeable as FL3B and DLBCL have higher proliferation than the low grade tumours and proliferation may have prognostic impact in FL independently of the histological grade (Janikova *et al*, 2011; Samols *et al*, 2013; Kedmi *et al*, 2014). Interestingly, a number of lncRNA have been also related to cell proliferation in the context of normal B-cell differentiation, where a subset of lncRNAs were associated with the expression of cell cycle regulatory genes in pre B1/B2 cells and centroblasts, two highly proliferative compartments of the B-cell differentiation process (Petri *et al*, 2015).

To confirm these results we selected 18 lncRNA that were studied in an independent series of 43 FL, including 23 FL1-3A and 20 FL3B-DLBCL/tDLBCL. The validation series was composed only of FFPE routine samples and for this reason we selected lncRNA that could be found as differentially expressed both in purified and unpurified FL samples. We could confirm that 9 of these lncRNA showed higher expression in FL3B/DLBCL. Interestingly, 5 of the 9 validated lncRNA were related to cell cycle regulation, including RP4-694A7.2 that was previously found over-expressed in different types of solid cancers compared to their respective normal tissues (Yan *et al*, 2015). RP4-694A7.2 was also previously identified to be one of the lncRNA overexpressed in the highly proliferative stages of normal B-cell differentiation (Petri *et al*, 2015). Our functional analyses of RP4-694A7.2 in the FL cell line (WSU-FSCCL) confirmed its potential involvement in cell cycle control, as its downregulation reduced the S-phase fraction together with an increase of the G0/G1 cell fraction without inducing apoptosis. We also identified *MCM6* as a potential candidate gene regulated by RP4-694A7.2. MCM6 is involved in DNA replication and its expression peaks at the G1/S boundary (Ohtani *et al*, 1999). We observed that *MCM6* expression was reduced upon RP4-694A7.2 silencing supporting the idea that it may be a target of this lncRNA in FL.

In line with this functional evidence linking RP4-694A7.2 to cell cycle regulation, we observed that increased levels of this lncRNA in primary FL tumours were globally associated with a higher proliferation index measured by Ki-67 immunostaining. The proliferation index was very variable among cases in the different histological FL subgroups. Interestingly, the categorization of Ki-67 index in low (<40%), intermediate (40–70%) and high (>70%) subgroups revealed that increased RP4-694A7.2 expression levels were associated with higher Ki- 67 expression even within FL1/2, FL3A and FL3B subgroups.

In summary, this study provides novel information on the contribution of lncRNA to understand the molecular differences between FL from different histological subtypes. We observed a link between lncRNAs that are potentially involved in cell cycle regulation and proliferative FL cases, and validated functionally the lncRNA RP4-694A7.2, using *in vitro* experiments. We identified 9 potential lncRNA, including RP4-694A7.2, that could be potential biomarkers to stratify FL cases into prognostic groups in the clinical setting. Further studies in extended patient cohorts are warranted.

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Author contributions

A.R., A.N., M.A. and L.H., performed the research. A.R., G.C., B.G-F., P.P-G., A.E-C., M.D., S.H., M.G., M.B., P.B.,

P.S., A.O., I.S., L.M., H.H., A.R., G.O., and L.H. analyzed data. P.J., J.I.M-S, E.C., and L.H. wrote the manuscript. A.L-G., E.C. and L.H. designed and supervised the research study. All authors reviewed the manuscript. We are indebted to the HCB-IDIBAPS Biobank-Tumour Bank and Haematopathology Collection for sample procurement. We are indebted to the Genomics core facilities of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for their technical help. We are grateful for the excellent technical assistance of Jennifer Modamio, Miriam Prieto, Concepción Muñoz and Noelia García. We are also grateful to Guillem Clot, Ana Belén Larque, María Adela Saco, Eva Giné, Antonio Martinez and Marina Narbaitz for their help and advice in this work. This work was partially developed at the Centro Esther Koplowitz (CEK), Barcelona, Spain.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Principal component analysis of RNA-seq data obtained from unpurified tumor cells samples does not show major differences between FL1-3A *versus* FL3B/DLBCL. The two main principal components summarizing the information of the two FL histological subgroups are depicted along with corresponding projections for each one of them. No differences could be observed between samples obtained from untreated (empty circle) or previously treated (light filled circle) patients in the FL1-3A group (cases with no information on treatment are displayed as a dark filled circle). All FL3B/DLBCL samples with information available were obtained before patient treatment. Lines indicate mean values per group.

Fig S2. Coding gene differentially expressed (dif) in unpurified FL samples and its relationship to non B-cell types. Expression heatmap showing the degree of specific expression related to several subtypes of normal microenvironment cells in dif-coding genes found by RNA-seq in the unpurified FL samples according to RNA-seq data from public databases (GENvestigator software). Genes showing different degrees of expression specificity were identified regarding macrophages and dendritic cells (bottom left). Several genes related to different normal T-cell subsets were also identified although with less specificity (right).

Fig S3. Pathway enrichment analysis of the dif-coding genes found in unpurified FL samples using Metascape web tool either considering general GO terms and pathways (A) as specific immunological signatures (B). Cell proliferation-related pathways and some processes involving typical normal cell types of FL microenvironment were found significantly enriched. Similar results were found from dif-coding genes overexpressed only in FL3B/DLBCL (data not shown).

Fig S4. A fraction of the cell cycle related genes found highly correlated with lncRNAs differentially expressed between FL1-3A and FL3B/DLBCL purified samples were also found differentially expressed in these samples (34/224; 15%).

Fig S5. LncRNA selection for validation of their differential expression between FL histological groups in FFPE samples was based in the previously observed viability of differential expression detection even in the presence of a non-tumoral component in unpurified criopreserved FL samples. Venn diagrams showed these 18 lncRNAs and including 7 of them that we identified potentially related to cell cycle regulation.

Fig S6. Expression levels of 9 dif-lncRNAs comparing FL1/2 and FL3A(Panel A) as well as in comparison of the FL3B, FL3B/DLBCL and tDLBCL subsets (Panel B). No significant differences were found in the expression of these lncRNAs between the compared subsets of cases supporting their consolidation as unique groups for comparison (FL1-3A *versus* FL3B/FL3B/DLBCL/tDLBCL). Similar results were found for the remaining lncRNAs analyzed by qRT-PCR in these samples.

Fig S7. Panel A: Expression summary of validated dif-lncRNAs in FFPE FL samples. These lncRNAs were found significantly expressed at higher level in FL3B/DLBCL group (adjusted $P < 0.05$) as previously found in purified FL cells. Panel B: Expression summary of non-validated dif-lncRNAs including those without significant expression differences found (adjusted $P > 0.05$). The AP000462.1 lncRNA was included in this group although a significant expression difference was found because its expression was found increased in the FL1-3A group whereas was found significantly decreased in the same group in the purified samples.

Fig S8. Reduction of S-phase and increasing of G1 is visually noticeable using Click-iT Edu flow citometry assay with additional PI staining upon RP4-694A7.2 silencing in the WSU-FSCCL cell line.

Fig S9. Examples ofAnnexin V/PI apoptosis analysis. No significant differences were found upon RP4-694A7.2 silencing in the WSU-FSCCL cell line.

Table SI. Basic features of criopreserved FL samples analyzed by RNAseq.

Table SII. Basic features of FFPE FL samples.

Table SIII. qRT-PCR primer list.

Table SIV. Differential expression in criopreserved FL samples (FL3B/DLBCL *versus* FL1-3A).

Table SV. Significant functional enrichments found regarding coding genes highly correlated to dif-lncRNAs in FL1-3A samples.

Table SVI. Significant functional enrichments found regarding coding genes highly correlated to dif-lncRNAs in FL3B/DLBCL samples.

Table SVII. Statistics of enriched pathways among genes highly correlated to dif-lncRNAs between FL3B/DLBCL and FL1-3A groups.

Table SVIII. Statistical analysis of differential expression validation of selected lncRNAs in FFPE FL samples and additional related data.

Table SIX. Differentially expressed cell cycle-related coding genes (FL3B/DLBCL *versus* FL1-3A) also found highly correlated with dif-lncRNA.

Data S1. Supplementary Material and Methods

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