



***RNY4* in Circulating Exosomes of Patients With Pediatric Anaplastic Large Cell Lymphoma: An Active Player?**

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Emerging evidence indicates that extracellular vesicles, particularly exosomes, play a role in several biological processes and actively contribute to cancer development and progression, by carrying and delivering proteins, transcripts and small RNAs (sRNAs). There is high interest in studying exosomes of cancer patients both to develop non-invasive liquid biopsy tests for risk stratification and to elucidate their possible involvement in disease mechanisms. We profiled by RNA-seq the sRNA content of circulating exosomes of 20 pediatric patients with Anaplastic Large Cell Lymphoma (ALCL) and five healthy controls. Our analysis disclosed that non-miRNA derived sRNAs constitute the prominent fraction of sRNA loaded in exosomes and identified 180 sRNAs significantly more abundant in exosomes of ALCL patients compared to controls. YRNA fragments, accounting for most of exosomal content and being significantly increased in ALCL patients, were prioritized for further investigation by qRT-PCR. Quantification of *RNY4* fragments and full-length sequences disclosed that the latter are massively loaded into exosomes of ALCL patients with more advanced and aggressive disease. These results are discussed in light of recent findings on the role of *RNY4* in the modulation of tumor microenvironment.

Keywords: ALCL, liquidbiopsy, exosomes, YRNA, RNA-seq, small RNA

INTRODUCTION

Extracellular vesicles (EVs) are cell-derived membrane particles secreted from many cell types and circulating in body fluids, including plasma. Among different classes of EVs of different size and intracellular origin, exosomes are 40–150 nm endosome-derived EV originating from the inward budding of the limiting membrane of multivesicular bodies (1). In this process, exosomes are packed with proteins, lipids, DNAs, messenger RNAs (mRNAs) and non-coding RNAs, which can be transferred to recipient cells, and function both as paracrine and endocrine factors (2).

A large body of evidence collected in the last years proved the functional involvement of exosomes in cancer progression and spreading, induction of angiogenesis, as well as in

chemoresistance and immune response evasion during tumor development (3). In this scenario, defining the peculiarities of exosomal cargo in cancer patients is a hot topic in biomedical research. The characterization of small non-coding RNAs (sRNAs) in plasmatic exosomes of cancer patients attracted interest for the identification of non-invasive disease biomarkers and, notably, in consideration of sRNA regulatory functions and their direct involvement in cancer mechanisms (4, 5).

Most functional studies on circulating sRNAs carried by tumor-derived exosomes were focused on microRNAs (miRNAs) because of their well-characterized regulatory roles in key signaling axes: exosome-delivered miRNAs have been shown to promote epithelial-mesenchymal transition (6, 7), induce angiogenesis and increase vascular leakage (8–10), prepare pre-metastatic niches to promote metastasis (11, 12) or induce tumor resistance to immune responses (13, 14).

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of lymphoid malignancies and the fourth most common malignancy across the pediatric age spectrum. Considerable progress has been achieved in developing curative therapy for pediatric NHL, with an overall survival rate now exceeding 80% (15). There are three major categories of NHL: mature B-cell neoplasms, Lymphoblastic Lymphoma, and Anaplastic Large Cell Lymphoma (ALCL). Other NHL subtypes, including peripheral T-cell lymphomas, follicular lymphomas, and rare entities, represent <3% of the cases (16).

ALCL accounts for 10–15% of pediatric and adolescent NHL. Differently from ALCL in adults, ALCL in children is nearly universally ALK-positive and, in almost all of the cases, it is characterized by the *t*(2;5)(p23;q35) translocation, which leads to the constitutive expression of the NPM-ALK fusion protein (17). Although current treatment strategies achieve an event-free survival (EFS) of ~75% after 5 years, about 30% of the patients are resistant to therapy or experience a relapse (18). In this clinical context, new disease biomarkers are needed to enable the early identification of high-risk patients and a better tailoring of treatment. In ALCL, the identification of new active players in lymphomagenesis and in cancer cells dissemination mechanisms would have high potential for the design of innovative therapeutic interventions.

Currently, only a few, mainly descriptive, studies reported data regarding the sRNA cargo of lymphoma-derived exosomes and EVs. Moreover, only Diffuse Large B-cell Lymphoma (DLBCL), which is the most frequent histological subtype presenting in adults (19, 20), was investigated.

Studies aiming at the identification of clinically relevant sRNAs in plasmatic exosomes/EVs from lymphoma patients focused on miRNAs (21). The first evidence on EV miRNAs as a molecular diagnostic tool for disease monitoring in Hodgkin lymphoma (HL) patients was reported by Eijndhoven et al. (22). Specifically, lymphoma-associated miR-21-5p, miR-127-3p, miR-24-3p, let-7a-5p, and miR-155-5p were significantly increased in plasmatic EV from HL patients compared to healthy donors (HD). Exosome-derived miRNAs were also proposed as predictive biomarkers of chemotherapy resistance in DLBCL, where increased levels of miR-99a-5p and miR-125b-5p in patient plasmatic exosomes were associated with

reduced progression-free survival (23). Data on exosomal sRNAs in pediatric lymphomas setting are currently missing.

Further, our appreciation of the small transcriptome complexity largely increased in the last years. Alternative processing of miRNA precursors (24–26) and housekeeping non-coding RNAs can generate miRNA-like sRNAs that can be functional and play roles in malignancies (27, 28). It's worth noting that several RNA-seq studies on exosomes derived from both tumoral and non-tumoral cell lines revealed that, differently from secreting cells, miRNAs constitute a minor percentage of EV-enclosed RNA. Besides protein coding mRNA, the EV fractions contain vault RNAs, YRNAs, small nuclear and nucleolar RNAs (snRNAs and snoRNAs), transfer RNAs (tRNA), as well as fragments deriving from long non-coding RNAs and transcribed pseudogenes (29–31).

In this perspective article, we present original data about the characterization of exosomal sRNAs in pediatric ALCL aiming attention on non-miRNA derived sRNAs, and discuss them in the frame of current literature, providing an original viewpoint of the possible translational relevance of these findings.

RNY4 FRAGMENT ENRICHMENT IN CIRCULATING EXOSOMES OF ALCL PATIENTS DISCOVERED BY RNA-seq

To characterize the exosomal load of sRNAs in ALCL patients and disclose differences with healthy donors, sRNAs were examined by small RNA-seq of exosomes from 20 ALK-positive ALCL patients and five HD plasma samples, and from supernatant of five ALCL cell lines (Karpas299, SUDHL1, and SUP-M2, ALK-positive; FE-PD and MAC2A, ALK-negative). For patients, paired biopsy samples, all positive for the NPM-ALK fusion, were also sequenced. Exosomal RNA was extracted by using exoRNeasy Maxi/Midi kit (Qiagen) and assessed for proper amount and quality by Agilent 2100 Bioanalyzer (Agilent Technologies). RNA-seq libraries were prepared with NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs), as previously reported (32), and sequenced on an Illumina HiSeq 4000 platform with single-end reads and average depth of 15 and 30 M for exosomal and biopsy samples, respectively.

After a preprocessing phase for adapter trimming and selection of high-quality reads (Qphred \geq 30), data underwent analysis by miR&moRe software (24, 25). Briefly, miR&moRe maps filtered reads to genome assembly and the known hairpins sequences from miRBase extended in either directions by additional 30 bp and allows detection and prediction of miRNA hairpins and of the corresponding mature forms, as in Gaffo et al. (33). MiR&moRe allowed identification and quantification of 1,194 and 523 miRNA-derived sRNA species in biopsies and exosomal samples, including miRNAs and microRNA-offset RNAs derived from annotated and newly predicted miRNA precursors.

We observed that in biopsies ~59% of the reads derived from miRNAs, whereas the large majority of the reads from exosomal samples did not align to known nor predicted miRNA precursors,

concordantly in exosomes isolated from ALCL patients (2.5%), cell lines (2.6%), and healthy donors (5.3%) (**Figure 1A**). A similarly large fraction of non-miRNA sRNAs in EVs was described by other studies on lymphoma, melanoma, breast cancer and immune cells (19, 20, 29–31).

The non-miRNA sRNAs fraction in exosomes of ALCL patients was further characterized. First, reads not aligned to known or predicted miRNA precursors were mapped with Bowtie v1.1.2 (34) to the reference genome, allowing no mismatches and up to 15 multiple alignments. The alignments were then analyzed with *derfinder* software tool (35) to identify expressed RNAs and the corresponding genomic regions. Among the 9,181 regions supported by at least 10 reads we selected those of 13–50 contiguous bases, consistently with the size of sequencing library fragments. After expression normalization [DESeq2 (36)], 1,007 most abundant (top 5% of expression) putative non-miRNA-derived sRNAs were considered for further characterization. Principal component analysis of expression profiles of these 1,007 sRNAs clearly distinguished tissue from exosome samples, separating as well as exosomes from healthy donors, patients and cell lines (**Figure 1B**).

Next, 180 sRNAs were identified with significantly different abundance between exosomes of ALCL patients and HD (DESeq2 *p*.adj < 0.001). The heatmap in **Figure 1C** shows the expression of the nine sRNAs with highest abundance and increase in ALCL exosomes. Apparently, the sRNA present in greater supply in ALCL exosomes was defined by reads aligned to the *RNY4* gene, and to highly similar pseudogenes (*RNY4P7*, *RNY4P10*, and *RNY4P20*). Precisely, a fragment corresponding to the first 32 bases on the 5' end of *RNY4* (*RNY4-5'F*) (**Figure 1D**) accounted for at least 80% of the non-miRNA sRNA expression in exosomes, whereas it was less abundant in exosomes derived from the cell line supernatant, isolated with the same protocol (**Figure 1E**). Remarkably, *RNY4-5'F* was five times and significantly more abundant in ALCL than in HD exosomes (*p*.adj = 0.0003; **Figure 1F**). The expression of *RNY4-5'F* was assessed by quantitative real-time PCR (qRT-PCR; Custom TaqMan Small RNA assay designed on the 32 bases of the *RNY4* sequence; ThermoFisher Scientific, Life Technologies) in 25 independent samples (12 ALCL and 13 HD). No difference was observed comparing exosomes from ALCL and HD (**Figure 1G**) not confirming RNA-seq result (**Figure 1F**).

YRNAs were first discovered in 1981 as 83–112 nt RNA components of circulating ribonucleoproteins, complexed to Ro60 and La autoantigens, in serum of patients with autoimmune diseases (37, 38). Evolutionary conserved in vertebrates (39), YRNAs fold in characteristic stem-loop secondary structures, with lower and upper stem loop sequences being the most conserved (40). Four different human YRNAs (*RNY1*, *RNY3*, *RNY4*, and *RNY5*) are transcribed in the nucleus by RNA polymerase III from genes clustered together at a single locus on chromosome 7q36 (41). Intracellularly, binding of the lower YRNA stem to Ro60 was shown to be involved in the maintenance of RNA stability and in cellular response to stress (42), whereas the upper stem was proven to be essential for the initiation of chromosomal DNA replication (43). In addition, YRNAs were also linked to alternative splicing and regulation

of the translation of specific RNAs, since most YRNA-associated proteins are implicated in these processes (44).

In recent years, YRNAs and YRNA fragments derived from site-specific cleavage by RNase L were reported to be enriched in different types of EV compared to secreting cancer cells (19, 31, 45–47). Noteworthy, RNA fragments corresponding to the 5' region of the *RNY4*, almost exactly corresponding to the *RNY4-5'F* detected in the present study, were shown to be the most abundant sRNA species in plasma samples from HD (30, 48–50) and melanoma patients (51), as well as in breast cancer patients' exosomes and plasma (30, 49), and plasmatic exosomes from non-small cell lung cancer (NSCLC) and chronic lymphocytic leukemia (CLL) patients (52, 53).

Since YRNA fragments derive from conserved ends of the YRNA hairpin, it was initially hypothesized that YRNAs could “conceal miRNAs” and be processed in miRNA-sized YRNA fragments that could function as miRNAs (54). However, this hypothesis was not supported by later studies. Since YRNA fragment biogenesis resulted to be Dicer-independent, they were found in complexes different from those associated with microRNAs and they did not co-immunoprecipitate with Ago2 (55). Moreover, they did not regulate targets tested by Thomson and colleagues in a miRNA-like manner (56).

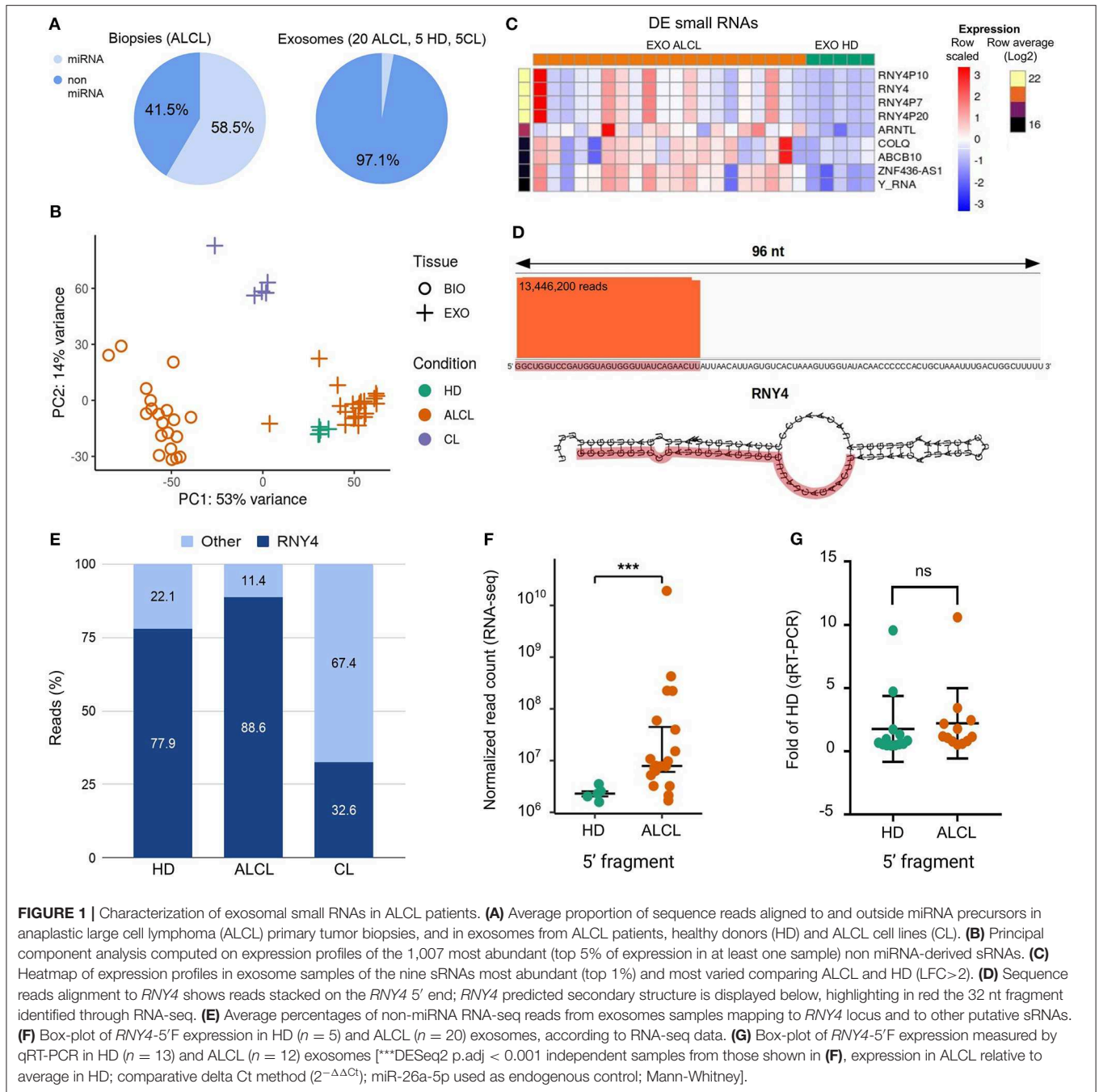
Recently, since *RNY5* fragments administration to human primary fibroblasts was shown to induce cell death (46) a role for *RNY3* in enhancing “cleavage and polyadenylation specificity factor” (CPSF) recruitment to histone locus bodies has been proposed (57), thus associating YRNA fragments to functional activities different from those typical of miRNAs.

GENUINE miRNA-LIKE *RNY4* FRAGMENTS OR FULL-LENGTH *RNY4*?

The increased levels of *RNY4*-derived fragments or full-length transcripts circulating in plasma or exosomes of cancer patients compared to HD (30, 49, 53) triggered interest in the potential use of *RNY4* as a cancer biomarker. However, whether *RNY4*-derived fragments detected by small RNA-seq are genuine fragments or reflect the presence of the full-length *RNY4* is still a matter of debate.

By Northern blotting, Dhahbi et al. confirmed 5' *RNY4* fragments in plasma of HD (48), Haderk et al. validated the presence of both *RNY4* full-length and 5' fragments in CLL exosomes (53), whereas Driedonks et al. showed that EV released from dendritic cells mostly contain full-length *RNY1* and only small amounts of 19–35 nt *RNY1* fragments (58). In this regard, Driedonks and Nolte-t Hoen suggested that YRNA secondary structures might impede full-length cDNA synthesis, leading to overestimation of fragmented non-coding RNA in sequencing data (59). Indeed, in a RNA-seq study, Godoy et al. detected fragments derived from both the 5' and 3' arms (60) whereas other works reported mostly full-length YRNAs (61, 62).

To verify if full-length *RNY4* might be responsible for the differential expression detected by small RNA-seq, not validated by RT-PCR specific from *RNY4-5'F*, we quantified the full-length *RNY4* in 12 ALCL and 12 HD plasmatic exosomes by qRT-PCR



using primers from Tolkach et al. (63). The full-length *RNY4* was significantly more abundant in ALCL than in HD (Mann Whitney, $p = 0.017$) (Figure 2A).

Further, we considered that full-length YRNAs were mainly reported as contained in exosomes (59), whereas fragments were previously validated by Northern blotting in total plasma and in exosomes (48, 53). We thus investigated the amount of full-length *RNY4* and the *RNY4*-5'F, also distinguishing RNA loaded into exosomes and freely circulating in plasma (RNA of six HD and six ALCL from both exosomes and the column flow-through

after the exosome binding step). Of note, most of the full-length *RNY4* was inside exosomes (5.6 times more abundant inside, on average) of both HD (Mann-Whitney $p = 0.009$) and ALCL patient ($p = 0.002$) samples, whereas the fragment was present at a similar level in exosomes and as free circulating RNA (Figure 2B). Taken together, these results suggested that *bona fide* *RNY4* fragments circulate in plasma both enclosed in membranes and as free RNAs, in amounts not discriminating ALCL and HD, whereas full-length *RNY4* is mainly enclosed in exosomes, where it is significantly enriched in ALCL patients.

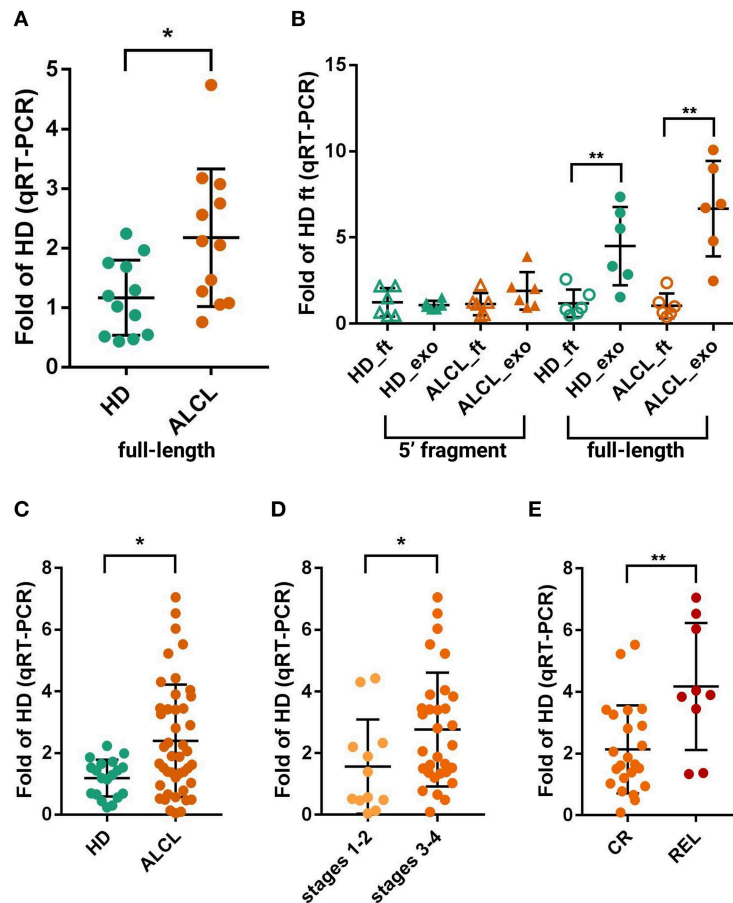


FIGURE 2 | Quantification of *RNY4*-5'F and full-length *RNY4* by qRT-PCR. **(A)** Expression of full-length *RNY4* in 12 HD and 12 ALCL plasmatic exosomes measures by qRT-PCR. **(B)** Expression of *RNY4*-5'F (5' fragment) and full-length *RNY4* loaded into exosomes (exo) and freely circulating in plasma (flow-through, ft); expression in ALCL relative to average in HD_ft. Full-length *RNY4* was significantly more abundant in both HD and ALCL exosomes than as free circulating RNA (6 HD, 6 ALCL), whereas the *RNY4*-5'F was almost equally distributed inside exosomes and as free circulating sRNA in both HD and ALCL. **(C)** Evaluation of full-length *RNY4* in exosomes from an extended cohort of 44 ALCL and 19 HD confirmed a significantly increased expression of the full-length form in ALCL compared to HD. Full-length *RNY4* was significantly more abundant in patients with stage 3–4 disease compared to those in stages 1–2 **(D)** and also among stage 3–4, in relapsed patients (REL) compared to those in stable complete remission (CR) **(E)**. For panels **(A,C,D,E)**, expression in ALCL has been calculated relative to average in HD; comparative delta Ct method ($2^{-\Delta\Delta Ct}$), miR-26a-5p as endogenous control, Mann-Whitney test for sample comparison were used for all panels. (*0.01 < p < 0.05, ** p < 0.01).

A further examination of an extended cohort of 44 ALCL and 19 HD plasmatic exosomes confirmed the upregulation of the full-length form in ALCL samples (Mann Whitney, $p = 0.017$) (Figure 2C).

FULL-LENGTH *RNY4* LOAD IN EXOSOMES OF ALCL PATIENTS CORRELATES WITH DISEASE AGGRESSIVENESS

Next, the extended cohort was further examined considering clinical data. Of importance, *RNY4* abundance correlated with ALCL patients' clinical characteristics. The full-length *RNY4* was more abundant in exosomes of ALCL patients with advanced disease stages (32 ALCL in 3–4 stage vs. 12 ALCL in 1–2 stage; Mann Whitney, $p = 0.049$) (Figure 2D). Since most (9/10) of the relapsed patients were diagnosed in stages 3–4

(Figure 2D), we analyzed *RNY4* amount at diagnosis in relation to relapse, considering only advanced stages. Compared to cases in stable complete remission ($N = 23$), relapsed patients ($N = 9$) presented at diagnosis with increased levels of exosomal full-length *RNY4* (Mann Whitney, $p = 0.0065$) (Figure 2E).

These findings indicate exosomal *RNY4* as a promising biomarker of disease aggressiveness in ALCL, to be quantified with a simple and non-invasive liquid biopsy. Moreover, our data and literature evidence collectively encourage further investigation to ascertain a possible functional role of *RNY4* in ALCL disease aggressiveness, as well as in other lymphoproliferative diseases or different malignancies. Indeed, *RNY4* delivery by CLL exosomes has been recently shown to induce key leukemia-associated phenotypes in monocytes, such as the release of pro-tumorigenic cytokines (CCL2, CCL4, and IL-6) and the expression of the immunosuppressive protein PD-L1, thus generating a tumor-supporting microenvironment

(53). ALCL tumors are characterized by variable histological patterns, mostly depending on tumor cell size and the presence of a large number of reactive histiocytes in the background (64). The biological functions of YRNAs could be multidirectional and we speculate that the association between ALCL and changes in these circulating YRNA reflects some aspects of either the biology of the tumor or the immunosystem reaction of the individual to the tumor. In particular, our results pave the way for investigating the role of *RNY4* as mediator of immunoescape in lymphoma patients. The treatment of monocytes *ex vivo* with tumor exosomes, the uptake as well as exosome-mediated responses by flow cytometry, or cytokine quantification can be used in the next future to elucidate this intriguing aspect.

In conclusion, *RNY4* is a massively loaded molecule in exosomes of ALCL patients, with *RNY4* significantly increased in patients compared to controls. Notably, significantly higher *RNY4* levels were observed in patients diagnosed at advanced stages, and among them, in those that later relapsed. These findings, in the light of available functional data on exosomal *RNY4*, encourage further study of *RNY4* involvement in ALCL tumor microenvironment and disease aggressiveness.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study, these can be found in the NCBI Gene Expression Omnibus (GSE144781).

ETHICS STATEMENT

The study was approved by the ethics committee for clinical experimentation of the Padova hospital CESC (Comitato Etico per la sperimentazione clinica azienda ospedaliera di Padova). Written informed consent was obtained from patients and/or their legal guardians in accordance with the institution's ethical review boards.

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AUTHOR CONTRIBUTIONS

FL designed the RNA-seq experiment and the experimental work, analyzed data, and wrote the manuscript. PD performed RNA-seq data analysis and qRT-PCR, and contributed to write the manuscript. EG contributed to RNA-seq data analysis and revised the manuscript. PD, FL, and EG prepared figures. CD, AG, and IG processed clinical samples and performed qRT-PCR. EC and MP collected clinical data and commented on manuscript. AB revised the manuscript. SB conceived the study, supervised the bioinformatics work, contributed to experimental results interpretation, and wrote the manuscript. LM conceived the study, supervised the experimental work, and revised the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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