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Long Non-coding RNAs As Novel Therapeutic Agents and Biomarkers for Treating Epstein-Barr Virus Associated Diffuse Large B-cell Lymphoma

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BACKGROUND INFORMATION

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of the Non-Hodgkin Lymphomas, accounting for about 30% of all diagnosed cases [1]. It usually presents in the seventh-decade of life as a highly proliferative disease with basophilic cytoplasm and multiple nucleoli, although traditional forms may also occur in children [1]. DLBCL can be distinguished from other lymphomas using classical immunohistochemical markers such as MYC, BCL2, BCL6, and Ki67 [2]. This highly aggressive and potentially fatal disease can be cured in approximately 50% of cases using multi-drug regimens [3]. However, up to one third of patients develop refractory disease which can be either difficult to treat or incurable [4]. Ultimately, identifying putative therapeutic and prognostic biomarkers of DLBCL is essential for potentially treating both primary and relapsed/refractory DLBCL cases.

The Epstein-Barr virus (EBV) is a known oncovirus that has been implicated in DLBCL and multiple other lymphoproliferative disorders [5]. EBV is a prominent double-stranded DNA human herpesvirus well-known for its role in causing infectious mononucleosis. It is primarily spread via saliva and genital secretions which likely accounts for its ubiquitous presence in over 90% of the world's population [6]. EBV infects humans via $CD21^+$ B lymphocytes residing in host tonsillar lymph nodes using its viral glycoprotein gp350 [7]. Studies suggest that EBV hijacks B cell differentiation pathways and modifies host and viral DNA methylation patterns to promote long-term latency (viral persistence) and oncogenic potential [8]. Interestingly, EBV infection drives B cells to differentiate into memory B cells where they virus stays latent in episomal form [9]. Among other things, these latency transcripts of EBV are believed to play key roles in B cell transformation [10]. In this sense, EBV hijacks host machinery to transcribe latency products which perturb B lymphocyte developmental pathways leading to DLBCL.

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B cells derive from hematopoietic stem cells and mature by proliferating within secondary lymphoid tissues upon T-cell antigen presentation. The germinal centre (GC) is an important region of the lymph nodes where proliferative centroblasts undergo two important reactions, somatic hypermutation (SHM) and class switch recombination (CSR) [11]. Here SHM and CSR promote favorable genetic manipulations in IgV genes to produce high affinity antibodies, and confer antibody class respectively. Both these events require the enzyme activation-induced cytidine deaminase (AID) which has been linked to genetic alternations like chromosomal translocation(s) and aberrant SHM seen in DLBCL [12]. As a result, this stage of B cell development represents a critical point for neoplastic transformation. This article aims to discuss the role EBV may play in hallmark events of DLBCL onset and progression. It will focus on the molecular mechanisms that implicate the virus in B cell transformation, and discuss the how non-coding RNAs may represent a novel therapeutic targets and prognostic biomarkers that should be explored.

> “Ultimately, identifying putative therapeutic and prognostic biomarkers of DLBCL is essential for potentially treating both primary and relapsed/refractory DLBCL cases”

RESEARCH QUESTIONS

Understanding the complex interplay between EBV and DLBCL may uncover novel therapies moving forward. In addition to primary and refractory cases, recent evidence suggests that EBV$^+$ DLBCL is becoming a unique concern among elderly patients (>50 years of age) [13] and in younger adults [14]. Notably, this suggests EBV may play a role in more aggressive forms of this disease as its presence predicts worse clinical and survival outcomes. Moreover, EBV-associated DLBCL may not only occur in immunocompromised or immunosuppressed individuals. Thus, the target population of this study would include both elderly and young adults (<50 years of age) showing signs of early lymphoma. Any novel treatments or biomarkers discovered would be characterized across primary, refractory, and EBV$^+$ cases in order to broaden the scope of this research. In this sense, it may be possible to mitigate increases in EBV$^+$ DLBCL by examining targeted therapies across all three of these DLBCL forms. Ultimately, EBV may simply play a role in initiating the neoplastic transformation of B cells, or it may infect cancerous cells to confer survival advantages.

In order to understand more about this malignancy, its association with EBV latent infection, and long non-coding RNAs (lncRNAs) as therapeutic or prognostic biomarkers, a few concerns must be addressed. Firstly, EBV is a known risk factor of lymphoma. However its ubiquitous presence in the human population is a barrier for viral-directed therapies as its co-existence suggests an evolutionary relationship. Thus, exploring more about the role of this virus in relation to known oncogenic hallmarks is critical for elucidating host B cell pathways perturbed by EBV. Once this is clarified, differential analysis between healthy and diseased samples may identify new therapies for DLBCL. Recent advancements in transcriptomic studies have identified a novel portion of the cellular network not heavily studied, regulatory non-coding RNAs. Interestingly, it is believed that around 90% of the genome is actively transcribed, but only approximately 2% of it encodes for proteins [15]. The remaining transcriptional events were thought to give rise to transcriptional “noise” that played no functional role [16]. However, mounting evidence suggests that these ncRNAs may represent actual molecules that influence cellular events [17]. Within this category of regulatory ncRNAs are lncRNAs which have been identified as players in DLBCL and other cancers [18]. Studying lncRNAs in DLBCL may open new therapeutic and biomarker-based alternatives to conventional protein-coding genes. In this proposal, lncRNAs will be examined as potential host targets and as exosome-secreted biomarkers in DLBCL patients.

PROJECT NARRATIVE

How does EBV infection of B lymphocytes act as a driver in the development of DLBCL?

All cancers exhibit a diverse range of genetic and epigenetic modifications allowing classification of malignancies. Hanahan and Weinberg used this to create a now remodeled interpretation describing the key hallmark events of cancer [19]. These include

sustained proliferative signaling, growth suppression evasion, cell death resistance, replicative immortality, angiogenesis induction, and activation of invasion and metastasis programs. Underlying these changes is genome instability. If EBV latent programs deregulate traditional B cell developmental programs, it may foster these hallmark events leading to DLBCL onset. In order address this question it is pertinent to first evaluate previous research elucidating the functions of the suggested EBV latency genes.

In the beginning, studies on EBV-induced primary B cell transformation started by collecting EBV-infected peripheral lymphocytes from seropositive individuals. Interestingly, this led to the discovery of the first transformed B cells called lymphoblastoid cell lines (LCLs) [20]. This suggested an underlying mechanism for lymphomagenesis *in vitro*. Characterization of these B cell LCLs (B-LCLs) by means of PCR and antibody-based experiments showed multiple episomal copies of the EBV genome and a set of EBV proteins including nuclear antigens (EBNA-1,-2,-3A/B/C,-LP) and latent membrane proteins (LMP-1,-2A,2B) [21]. *In vitro* expression of these nine proteins and other viral products suggest EBV infection supplements cell proliferation, survival, and differentiation signals in developing B cells [22]. Here, examples of how these EBV latency products potentially disturb B cell development will be discussed.

Naïve B cells undergo CSR and SHM mediated by the critical enzyme AID. EBV infection of B-LCLs is known to promote AID expression and SHM via EBNA-3C, an event associated with p53 and BCL6 mutations [23,24]. Given BCL6 is a key transcription factor of the GC reaction and p53 is a master regulator of apoptosis, angiogenesis, and cell cycle progression, this evidence suggests EBV-infected B cells may accumulate driver mutations while continuously undergoing SHM. Alternately, EBV LMP-1 may induce B cell CSR, another key event regulated by AID [25]. These findings link EBV latency and AID deregulation which likely disrupts CSR and SHM. Unregulated B cell developmental programs may then give rise to phenotypic changes characteristic of oncogenesis. Even more concerning is the notion that LMP-1 and LMP-2 are known to induce aberrant GC reactions [9]. In brief, AID instability likely represents one reason for lymphoma onset.

The EBV-encoded EBNA proteins also play a variety of roles in B cells. EBNA-1 is one of the major EBV-encoded proteins expressed during latency. It plays a role in replication, persistence, and transcription of the EBV genome [26]. In addition to this, EBNA-1 evades lytic reactivation by inducing the let-7 microRNA (miRNA) family in infected cells [26]. This likely prolongs EBV latency and may account for changes in metastasis programs, a key hallmark event in cancer [27]. The viral EBNA-1 protein has also been implicated in p53 deregulation and the induction of survival pathways [26]. This suggests EBV EBNA-1 may modulate proliferative programs and promote cancer-associated immortality, two more hallmark events.

EBNA-1 is assisted by the EBNA-3 family which acts on a variety of cellular pathways including those involved in transcriptional control, cell cycle dysregulation, inhibition of apoptotic machineries, and metastasis programs [28]. EBNA-3A and -3C specifically have been studied in detail. EBNA-3A has been shown to interact with RBP-Jκ and modulate Notch signaling, which controls a known oncogene, *MYC* [29]. Myc regulates cell proliferation by controlling around 15% of all cellular genes [30]. Given *MYC* is genetically altered in a notable number of DLBCL cases [30], EBNA-3A upregulation of Notch signaling may lead to *MYC* overexpression and downstream oncogenic events. Alternately, Viral EBNA-3C is known to block p53 expression and apoptotic processes, and degrade tumor suppressors like Rb to deregulate cell cycle progression [26]. Notably, EBNA-3A/-3C also induces oncogenic miRNA-221/-222 (oncomiR) cluster expression [31]. The previous miRNA cluster targets a cyclin-dependent kinase, $p57^{KIP2}$, which is commonly repressed in multiple cancers. Inability to regulate cell cycle progression through loss of p53, Rb, and p57 represent ways by which EBV promotes cancerous hallmark features. Together, these mechanisms represent only a fraction of all the possible roles EBV latency plays in lymphoproliferative disorders like DLBCL.

Based on the previous information, most EBV latency gene products have been well-studied with regards to neoplastic B cell development. However, areas of study not yet exhausted are the EBV-encoded RNAs (EBERs) and EBV-miRNAs [26]. A series of *in vitro* experiments employing EBER or EBV-miRNA mimics and inhibitors may identify correlations between these viral latency products and host regulatory mechanisms. An example of how EBV-miRNAs may induce oncogenesis could be through repression of a pro-apoptotic factor like caspase 3, thereby preventing cell death. Thus, B-LCLs could be used to study how viral RNAs interact with host factors such as proteins, lncRNAs, or miRNAs. This could be accomplished using RNA-sequencing experiments on silenced-infected cells or those overexpressing certain EBV genes. Deregulated proteins, miRNAs, and

lncRNAs could be examined, and further RNA-immunoprecipitations or related mechanistic studies may provide novel insight into the functions of EBV RNAs in lymphoma. Moreover, simple *in vitro* functional assays assessing proliferation, apoptosis, metastatic potential, and rescue phenotypes among others may also work to clarify the complexity of this viral-host interaction. It is hypothesized these experiments may elucidate previously unknown associations that drive lymphomagenesis and poor clinical outcomes.

Ultimately, there is mounting evidence to suggest EBV may be involved in the development of lymphomas. Notably, EBV latency genes including EBNAs, LMPs, and EBERs or EBV-miRNAs may slowly promote the accumulation of genetic alternations leading to cellular phenotypic changes (Fig. 1). Aberrant AID expression may underlie many of these critical genetic alterations that drive initiation of DLBCL programs. Here, RNA-sequencing and *in vitro* studies investigating proliferative and anti-apoptotic programs, metastasis promoting pathways, and mechanistic roles were hypothesized as means to highlight the roles of the EBV latency genes. Specifically, omics technologies improved our ability to probe global changes in cells upon infection or gene transfection. In this regard, transcriptome sequencing can also be utilized to explore novel therapeutic avenues not well-examined in DLBCL. In hopes of widening the scope of targetable molecules for this disease, lncRNAs will be studied.

Do long non-coding RNAs represent a novel therapeutic avenue for DLBCL?

The spectrum of targetable cancer therapeutics is changing. LncRNAs are defined as non-protein coding transcripts of >200 nucleotides in length. Similarly to protein coding genes, many identified lncRNAs are transcribed by RNA polymerase II, are polyadenylated and epigenetically modified, and are transcriptionally controlled by regulators like p53 [32]. Importantly, lncRNAs have been studied in cancer and represent a therapeutic avenue that is only now being explored. Their functions are numerous including the ability to act as sponge molecules to sequester host miRNAs [33]. Cytoplasmic localization of lncRNA-sponges, herein referred to as competitive endogenous RNAs (ceRNAs), is essential. This mechanism is one of many but it is highlighted here to indicate the complex processes of lncRNAs. Methods to first analyze these unique players will be explored followed by an example of a well-studied lncRNA in DLBCL. Hence, previously unannotated lncRNAs may represent a novel therapeutic avenue.

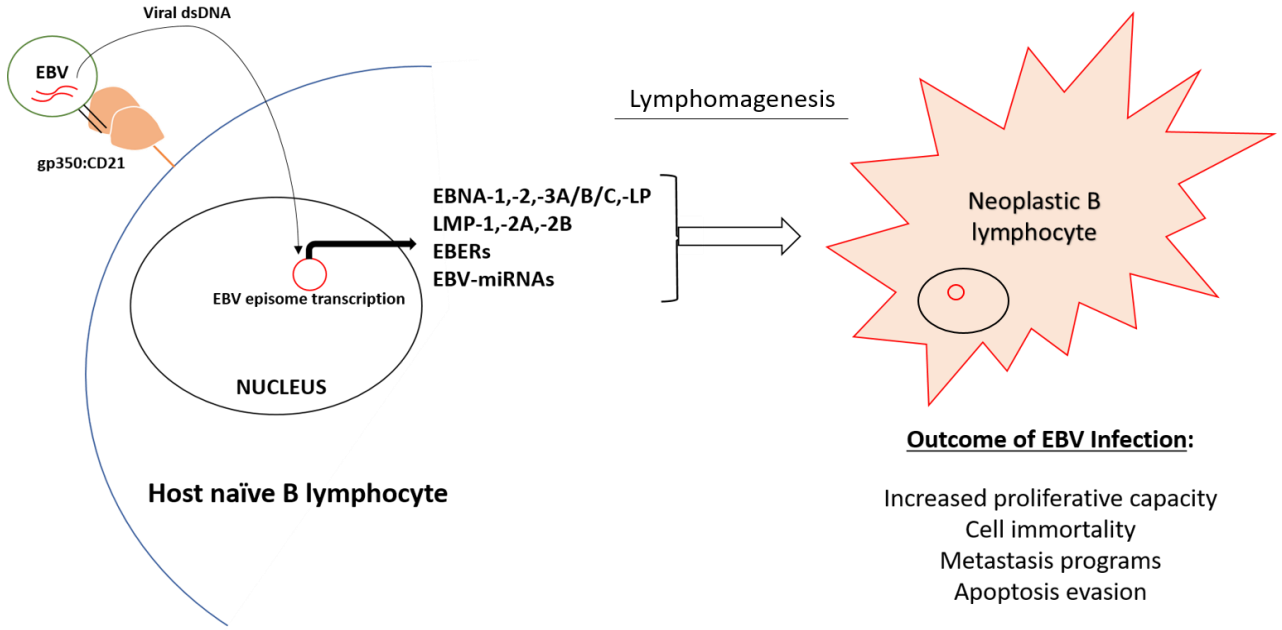

FIG. 1 Understanding Epstein-Barr virus (EBV) from primary infection to neoplastic B-cell transformation. Viral gp350:CD21 interactions on naïve B lymphocytes near sites of exposure promote viral entry, nuclear translocation, and latency establishment. Next, EBV dsDNA forms an extrachromosomal circular episome transcribed by host RNA polymerases. Viral gene products bolded above promote genetic alternations and lymphomagenesis leading to pre-DLBCL features. These include major cancer hallmarks such as cell immortality, excessive growth patterns, metastasis, and apoptosis evasion among others. Ultimately, this figure depicts hypothesized events that may occur during EBV latency, and how they might be implicated in the development of DLBCL.

LncRNA analysis requires whole transcriptome sequencing. Recent advancements in next-generation sequencing platforms have enabled faster, more accurate, higher-throughput collection of sample data. Bioinformatic analysis of post-experiment data enables cross-comparison between diseased and wild-type samples in hopes of establishing patterns amongst transcripts (eg. the protein-coding genes or ncRNAs). The end result in this case would be the identification of host lncRNAs differentially expressed between healthy controls and DLBCL models. This is now possible using a new DLBCL-like NOD/SCID murine model [34]. Target lncRNAs identified here can also be compared to patient-derived lncRNA datasets [18]. Once candidate targets have been selected, links to clinical outcomes can be drawn using resources such as Oncomine and cBioPortal. If pre-clinical *in vitro* and *in vivo* knockdown and overexpression studies succeed, one would expect this data to support the use of lncRNAs in the clinic. Most importantly, this research would pave the way to exploring non-coding genes as easily targetable players in host-directed cancer therapies. Figure 2 below depicts a generalized workflow to study lncRNAs in disease from initial screening to potential intellectual patent formulation. An example of a well-studied lncRNA in DLBCL is known. Here HOTAIR will be examined to understand the potential clinical significance of the lncRNAs.

HOTAIR is a spliced and polyadenylated lncRNA encoded by the *HoxC* gene cluster on chromosome 12 [35]. It is implicated in many cancers and functions largely as a scaffold for the epigenetic complex, PRC2, which is known to silence gene transcription through H3K27 trimethylation [35]. Additionally, HOTAIR is also known to interact with another complex of proteins involved in demethylation of H3K4me2 [35]. In this sense, HOTAIR is able to indirectly regulate DNA methylation status and target gene expression. Many lncRNAs exhibit either cytoplasmic or nuclear localization, but HOTAIR is known to show dual-localization [36]. This is relevant when considering its ability to function as a sponge to modulate miRNA activity as well. While in the nucleus, HOTAIR can act as a scaffold molecule PRC2 and LSD1 to illicit epigenetic control over a variety of genes. Alternatively, when HOTAIR exits the nucleus and resides in the cytoplasm, it can act as a ceRNA [37]. In order to better understand this complex method of miRNA regulation, it is important to understand miRNA function and known examples of HOTAIR sequestration.

MiRNAs are short transcripts of approximately 22 nucleotides, and are known to be produced by two

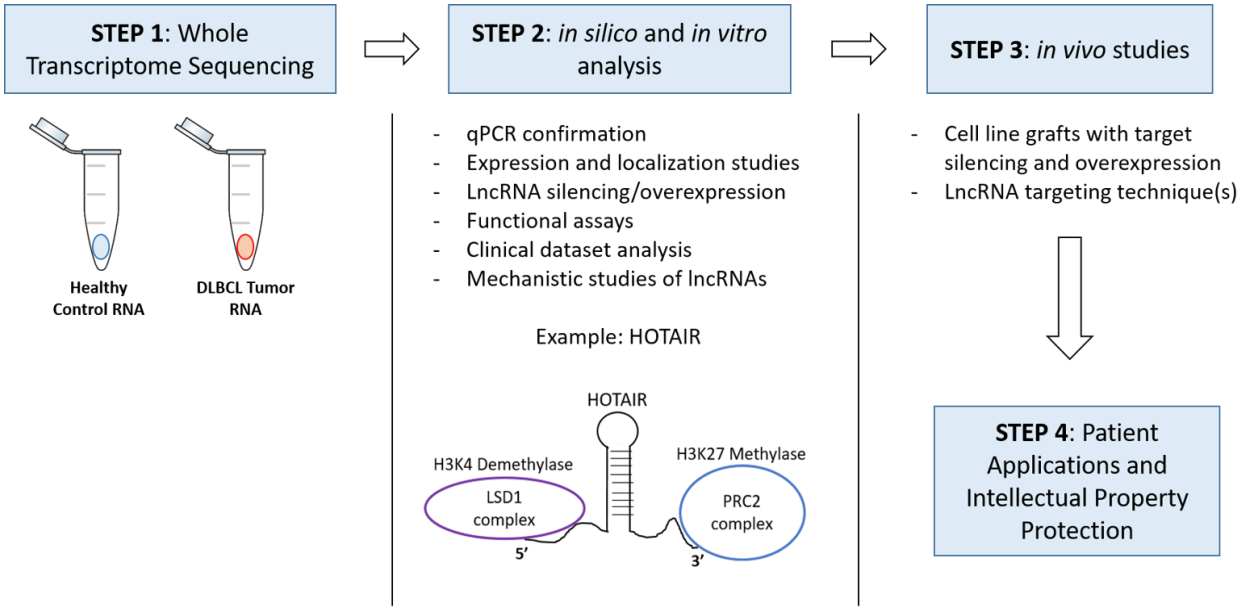

FIG. 2 A generalized workflow to describe how novel putative lncRNA targets for DLBCL can be identified using next-generation whole RNA-sequencing analysis. Tumor samples are first harvested from murine models (wild-type NOD/SCID vs. DLBCL-like NOD/SCID), patient samples, or B-LCLs. Using one or many of these, differentially expressed lncRNAs may be identified and cross-referenced for improved significance. Next, *in vitro* studies may pave the way for the necessary pre-clinical research including understanding how the target lncRNA functions in neoplastic B cells. Lastly, *in vivo* trials employing cell line grafts or xenograft technologies may underpin feasible lncRNA targeting mechanisms (ASOs vs. siRNAs) and further support the clinical potential of the chosen candidate lncRNA. Once these studies are completed, patent protection of the novel therapeutic agent is required.

RNase III proteins known as Drosha and Dicer [38]. They are known to repress gene expression by binding the 3′ UTR of target cellular mRNAs in an RNA-induced silencing complex [38]. Hence, miRNA-directed gene expression is essential for cellular homeostasis. Thus, aberrant miRNA activity can lead to oncogenic changes. CeRNAs function by sequestering these miRNAs thereby mitigating their effects. HOTAIR has been shown to regulate a few miRNAs including miR-331 via this method [37]. As a result, differential HOTAIR expression may be one driver of downstream miRNA changes in DLBCL tumorigenic cells. HOTAIR expression was also shown to be controlled by c-Myc [39]. Given *MYC* is commonly overexpressed in DLBCL, it is theorized that HOTAIR is also upregulated. Another example highlighting HOTAIR as a ceRNA is in its ability to sponge miR-373, an oncomiR implicated in tumor invasion and metastasis [40]. Although metastasis is not common in DLBCL, this once again shows how lncRNAs can contribute to cancer hallmark events via oncomiR dysregulation. Online miRNA-interaction software like Mircode can be explored to further support this interesting lncRNA function.

Mircode is an online bioinformatics software that utilizes curated databases to examine potential RNA-miRNA interactions. HOTAIR was predicted to bind mir-34a, a miRNA that was identified in a study employing miRNA predictive biomarkers for lymphoma [41]. Notably, mir-34a is known to target BCL6 and p53, and reduced expression of it is associated with poorer prognosis in DLBCL [42]. One could investigate this lncRNA as a ceRNA for mir-34a to further support the lncRNA sponge effect in lymphomagenesis. In short, HOTAIR may bind mir-34a leading to upregulation of altered BCL6 and p53. This may contribute to host genomic instability and the onset of hallmark oncogenic changes.

Epigenetic scaffolding and miRNA-sequestration represent a couple mechanisms by which lncRNAs contribute to cancer development. HOTAIR was used as an example to suggest putative roles of dysregulated lncRNAs. Differential transcriptomic analyses conducted on murine models of DLBCL or B-LCLs may identify more of these lncRNAs to study in primary, refractory, and EBV$^+$ cases. In this sense, lncRNAs like HOTAIR represent a therapeutic gold mine that has not been fully investigated. Not only that, lncRNAs can be targeted easily using antisense oligonucleotides (ASOs) or small-interfering RNA molecules (siRNAs). These may act to repress aberrant lncRNA expression and attenuate disease progression. Alternatively, biomarker-based diagnoses also require many of the same proof-of-concept *in vitro/vivo* studies as pre-clinical therapeutic evaluation (Fig. 2). It can be theorized that similar lncRNAs can also act as prognostic biomarkers to predict clinical cases of DLBCL. Here, exosome-based avenues will be evaluated.

Are circulating lncRNAs an alternative to invasive biopsy-based DLBCL diagnoses?

Diagnosis of DLBCL is complex and involves multiple steps including surgical excisional biopsy [2]. This invasive procedure is time-consuming and harmful to the patient. Tissue biopsies may be replaceable by less-invasive biomarkers found in patient fluids. Liquid biopsies now enable exosomal isolation easily and safely. Hence, serum exosome-derived lncRNAs may be evaluated for DLBCL diagnosis. This research avenue may work to distinguish primary, refractory, and EBV$^+$ forms of DLBCL. To investigate this hypothesis, exosomal-RNA isolation and characterization will be explored.

Exosomes are small vesicular compartments secreted by many types of cells. They have been shown to act as intercellular signaling molecules that cargo various host factors including proteins, miRNAs, and mRNAs [43]. Interestingly, exosome isolation has become a popular field of research in cancer. Several larger companies including Qiagen now produce kits that can be used to isolate exosomal-RNAs via simple column-based methods (eg. exoRNeasy Serum Plasma Kits). This is highly relevant for DLBCL diagnosis as lncRNAs such as HOTAIR have been shown to be cargoed in exosomes [43,44]. In addition, B cells have been implicated as major *in vivo* sources of exosomes [45]. EBV infection of B cells may drive malignant transformation (Fig. 1) and lead to the accumulation and secretion of multiple host factors including DLBCL-associated lncRNAs. Thus, lncRNAs packaged into serum exosomes may be evaluated as a means to circumvent an invasive biopsy (Fig. 3).

As previously discussed, lncRNAs have emerged as novel players in cancer research. Their functions are well-elucidated, and certain kingpins like HOTAIR have even been shown as prognostic or therapeutic targets. LncRNAs identified as having therapeutic potential can also act as biomarkers. Comparisons between different cases of DLBCL is possible by first collecting blood samples from various patients. Clustering these samples by DLBCL type and subtype may then enable us to distinguish between forms of the cancer that harbour certain lncRNA signatures. Through the use of exosomal-RNA purification kits and

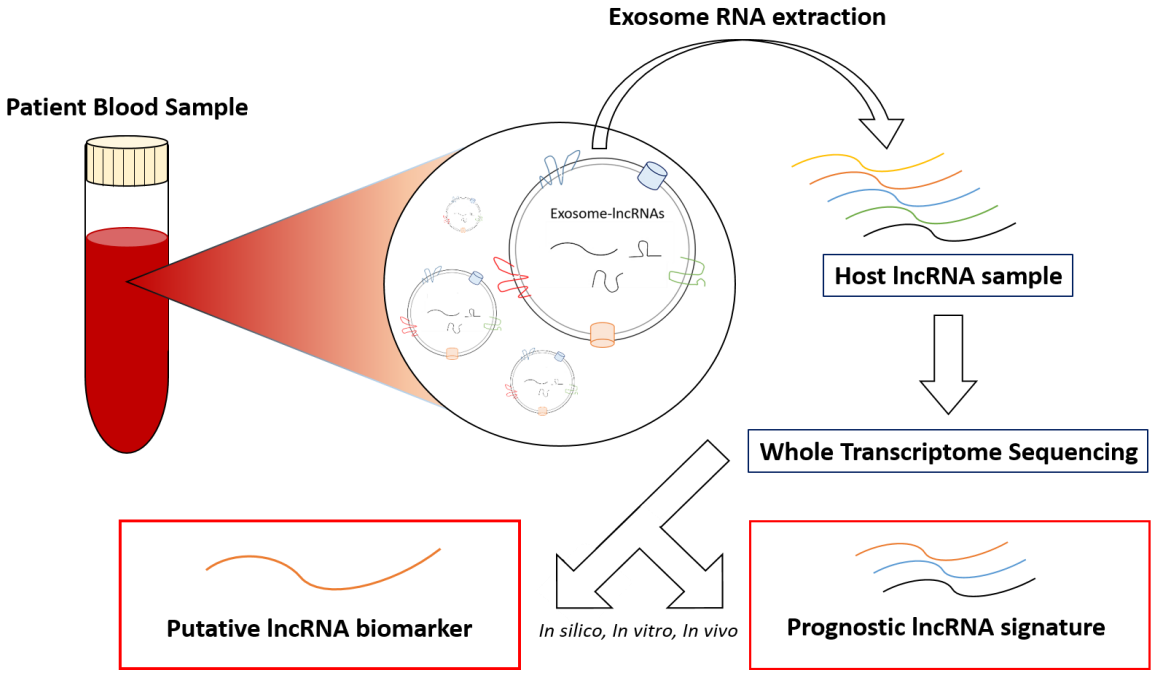

FIG. 3 Identifying putative lncRNA biomarkers using patient-derived serum as a non-invasive technique to alleviate the need for tissue biopsy diagnosis. Here, B cell-secreted exosomes cargoing lncRNAs deregulated in DLBCL can be first extracted using Qiagen exoRNeasy kits, followed by whole transcriptome sequencing. After un/annotated lncRNAs are found, further *in vitro, in silico,* and *in vivo* studies like those proposed in Figure 2 can examine the prognostic potential of candidate lncRNAs as biomarkers to diagnose primary, refractory, or EBV-positive DLBCL.

whole transcriptome sequencing, many annotated and unannotated host lncRNAs may be identified. Alternatively, lncRNAs that may have been previously identified can be screened in patient exosomal extracts using probe-based qPCR allowing the detection of both novel and characterized targets.

After lncRNA isolation and initial *in vitro* and *in silico* analyses similar to Figure 2, predictive biomarkers for DLBCL can be compared with clinical outcomes in patient samples. Correlations between variables such as age, gender, tumor stage, metastases can be analyzed with exosomal lncRNA expression or presence. Research similar to this has been previously conducted [44]. Even more, patient-derived exosomes can be used to identify a panel of lncRNAs that may predict clinical outcomes for DLBCL specifically. Cross-comparison of differentially expressed lncRNAs using clinical datasets may identify correlations between stage, progression, aggressiveness and more. Additionally, validation of these lncRNA biomarkers may differentiate primary DLBCL from refractory or EBV$^+$ cases. Moreover, EBV$^+$ DLBCL cases can be examined in more detail by comparing exosomal profiles in patients prior to and after EBV spread is confirmed. This research may work to replace the need to take tissue samples directly. By means of exosomes, combinations of lncRNA biomarkers (panels or single entities) with other prognostic markers may distinguish DLBCL early, and throughout its progression.

Notably, no research is without difficulties. The practicality of this replacement technology relies on our ability to qualify representative molecular biomarkers. Similar to biopsies, exosomal biomarker isolation requires time. However, multiple patient blood samples may be analyzed simultaneously at low cost which may increase feasibility. In short, exosomal-lncRNAs represent a novel research avenue that may be useful for future diagnosis of DLBCL. In order to shift from tissue-based examination to biofluid-derived patient signatures, combinations of various lncRNAs, miRNAs, and proteins may be needed. The feasibility of this technology is high, and it may even lead to more tailored patient treatment regimens in the near future. Given today's push to personalized medicines, these larger scale biomarker studies may end up revealing more about an individual's disease than simple biopsies could.

SUMMARY AND CONCLUSION

There is an apparent link between EBV and lymphomagenesis. Many EBV latency gene products

have been evaluated *in vitro* and *in vivo* contributing to our understanding of diseases like DLBCL. Advancements RNA-sequencing technology now enable the investigation of global cellular changes following EBER and EBV-miRNA expression. Although these transcripts have been studied with respect to B cell transformation [26], they have not likely been scrutinized to the extent of EBV proteins. As a result, EBV *in vitro* and *in vivo* studies investigating mechanistic or clinically-relevant phenomena can be utilized to draw correlations with disease progression. Unfortunately, treatment of EBV-associated DLBCL may be our only choice.

Current therapies for primary and refractory DLBCL use drug combinations such as R-CHOP. This treatment regimen has proven to improve patient survival largely due to rituximab (R), an anti-CD20 monoclonal antibody [46]. Its mechanism has been evaluated in detail, but resistance to this general therapy is common [47]. Thus, combination regimens targeting specific host factors dysregulated in cancer may prove more effective. Interestingly, lncRNAs like HOTAIR may be targeted using approaches including ASOs or siRNAs packaged into nanoparticle-based delivery systems. ASOs for many diseases like cancer are currently being investigated in clinical trials [48]. Chemical modifications to interfering RNAs to improve bioavailability and stability *in vivo* enable "naked" delivery of ASOs as well. Hence, this proposal examines the possibility of utilizing RNA-sequencing to characterize lncRNAs as therapeutic targets and biomarkers for DLBCL. Although there are no lncRNA-directed therapies for cancer today, continual research into this field may enable their use alongside protein-directed inhibitors. Not only that, transcriptomic analysis of various DLBCL samples may reveal additional mutations and genetic alterations not yet discovered. This is critical for establishing a more individualized approach to patient treatment options moving forward.

There are some concerns for lncRNAs in the clinic. Since RNA-sequencing is so new, we only have a limited breadth of knowledge on how lncRNAs function in disease. Consequently, we may not know if lncRNAs act as key contributors to disease development, or whether they are simply altered during disease progression [49]. This is attributable to the short time frame we have had to describe these molecules and their underlying biological functions. Conversely, protein-coding genes have long been studied, and many therapies targeting deregulated cellular pathways utilize small molecule inhibitors. Thus, lack of knowledge is one of the major challenges faced by researchers today. Alternative concerns involve lncRNA targeting. The limiting factor here is current delivery technologies such as liposomal nanoparticles. Without first understanding how the body reacts to these formulated transporters, we cannot expect to create a "bullet" cure for cancer. In addition, we are unable to reliably overexpress targets like tumor suppressors in the clinic which is another limitation to cancer therapies. Based on our current knowledge, we know many cellular pathways are also suppressed in diseased states. Without the ability to return these pathways to "normal" function, we limit ourselves to targeted repression of other overactive circuits. In the end, lncRNAs can be investigated in the therapeutic or biomarker setting. At the forefront of this research is RNA-sequencing.

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