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Computational Analysis of Epstein-Barr Virus *Bam*HI A Rightward Transcript (BART) MicroRNAs (miRNAs) Regulation on Messenger RNAs and Long Non-Coding RNAs in Nasopharyngeal Cancer

DAPHNE OLIVIA JAWAI ANAK SADAI & EDMUND UI HANG SIM*

Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
*Corresponding author: uhsim@unimas.my
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ABSTRACT

To date, the regulatory framework mediated by Epstein-Barr virus (EBV) *Bam*HI A rightward transcript (BART) microRNAs (miRNAs) via their interaction with long non-coding RNAs (lncRNAs) in the context of nasopharyngeal cancer (NPC) pathogenesis remains partially understood. To derive a more complete insight into this phenomenon, we embarked on a computational study to identify BART miRNAs, mRNAs, lncRNAs, and all associated factors relevant to NPC tumourigenesis and to characterise their interactions. *In silico* integration of multi-level RNA expression and construction of regulatory networks were performed. We found six EBV BART miRNAs (ebv-miR-BART21-3p, ebv-miR-BART19-3p, ebv-miR-BART15, ebv-miR-BART2-5p, ebv-miR-BART20-3p and ebv-miR-BART11-5p) that could interact with four mRNAs (EYA4, EYA1, EBF1 and MACROD2) associated with NPC pathogenesis. These mRNAs can interact with six non-EBV miRNAs (hsa-miR-1246, hsa-miR-93-5p, hsa-miR-16-5p, hsa-miR-135b-5p, hsa-miR-211-5p and hsa-miR-1305), which in turn, could interact with three lncRNAs (CASC2, TPTE2P1 and ARHGEF26-AS1). These findings could shed light on the roles of dysregulated competing endogenous RNA (ceRNA) network in NPC oncogenesis. In addition, we have also predicted the oncogenic and tumour suppressive functions of BART miRNAs and lncRNAs, and more precisely, the involvement of BART miRNAs in DNA repair regulation and apoptosis.

Keywords: BART miRNAs, bioinformatics, EBV, lncRNA, nasopharyngeal cancer

INTRODUCTION

Nasopharyngeal cancer (NPC) is a rare head and neck cancer on a global scale, yet it disproportionately affects populations in southern China and Southeast Asia, including Malaysia (Chang & Adami, 2006). Originating from the fossa of Rosenmüller and lining the nasopharynx epithelial (Tabuchi *et al.,* 2011), NPC is ranked as the sixth most common cancer in Malaysia, where Malaysian males are primarily affected by it, ranking fifth most common cancer affecting males in Malaysia (Global Cancer Observatory, 2022). The correlation of NPC with Epstein-Barr virus (EBV) infection has been well-documented, leading to aberrant RNA regulation within the host (Nakanishi *et al.,* 2017; Tsao *et al.,* 2017). This association is particularly prominent in type II (differentiated non-keratinizing carcinoma) and type III (undifferentiated non-keratinizing carcinoma) NPC (Su *et al.,* 2023), prevalent among Asian populations (Wang *et al.,* 2013).

Epstein-Barr virus (EBV) is a linear, double-stranded DNA virus classified in the family of *Herpesviridae*, a subfamily of *Gammaherpesviridae* (Sarwari *et al.,* 2016). The EBV genome is capable of expressing miRNAs that act similarly to mammalian miRNAs by binding to the 3'UTR of host mRNAs, albeit via imperfect complementary binding mediated at the 5' seed region of EBV miRNAs (Skalsky & Cullen, 2010). The miRNAs clusters encoded by EBV are the *Bam*HI A rightward transcript (BARTs) and *Bam*HI H rightward open reading frame 1 (BHRF1) clusters (Cai *et al.*, 2006).

In terms of disease association, EBV infection has been correlated with NPC pathogenesis (Nakanishi *et al.,* 2017; Tsao *et al.,* 2017). The post-infection period involving latency pattern type II is characterised by the presence of the BART cluster and the absence of the BHRF1 cluster, where the BART miRNAs function as oncogenes via down-regulating the apoptotic host mRNAs by binding to their

3'UTR (Kang *et al.,* 2015). Incidentally, lncRNA levels are also affected during EBV infection (Zhang *et al.,* 2020), with a significant negative correlation between lncRNAs and BART miRNAs levels as observed in the down-regulation of LOC553103 following the up-regulation of EBV-miR-BART6-3p (He *et al.,* 2016). Thus far, the intricacies underlying the regulatory network concerning BART miRNAs and lncRNAs in NPC pathogenesis have not been fully and definitively established, although it is apparent that the dysregulation of lncRNAs (He *et al.,* 2017) and the competing endogenous RNAs (ceRNA) network (Xu *et al.,* 2020) may play a role in the disease.

The exploration and understanding of regulatory mechanisms between BART miRNAs and the ceRNA network could unveil potential biomarkers on a preliminary level for early diagnosis, improve survival rates, and develop therapeutic targets for highly metastatic EBV-positive NPC. The deep-seated location of the nasopharynx, coupled with the absence of noticeable clinical symptoms in the early stage, contributes to the high mortality in NPC owing to its invasive and metastatic nature (Tabuchi *et al.,* 2011; Wang *et al.,* 2017; Dwijayanti *et al.,* 2020; Tan *et al.,* 2022).The ceRNA regulatory network involves the binding of miRNAs to both mRNAs and lncRNAs and is based on the hypothesis that lncRNA and mRNAs regulate each other by competing for binding sites on shared miRNAs through partially complementary sequences known as the miRNA recognition elements (MREs) (Salmena *et al.,* 2011). Anomalies in this regulatory network are commonly linked to tumorigenesis (Lee & Young, 2013).

To unravel the interaction among EBV-BART miRNAs, mRNAs, and lncRNAs in NPC pathogenesis, we performed a computational analysis involving constructing and analysing a regulatory network that combines the ceRNA and EBV-miRNAs-mRNAs networks. The outcomes were complemented by the curative and integrative analysis of the expression data of lncRNA, miRNA and mRNA extracted from the Gene Expression Omnibus (GEO) database. Our findings reveal novel conceptual insights into the regulation of lncRNAs by EBV-BART miRNAs with the uncovering of potential biomarkers for therapeutic design and the plausible effects on the modulation of DNA repair and apoptosis in the context of NPC pathogenesis.

MATERIALS AND METHODS

Retrieval of Microarray Data Pertaining to MicroRNAs (miRNAs), Long Non-Coding RNAs (lncRNAs) and Messenger RNAs (mRNAs)

The microarray gene expression profiles of messenger RNAs (mRNAs), microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) from nasopharyngeal cancer (NPC) were retrieved from the Gene Expression Omnibus (GEO) database hosted on the National Centre for Biotechnology Information (NCBI) website (Karagkouni *et al.,* 2020). Specifically, the access year of the datasets was between 2020 and 2023. The keyword "nasopharyngeal carcinoma" was used, and the organism filter was set to *Homo sapiens*. Excluded datasets were from those that are: (i) of cell line samples, (ii) without normal nasopharyngeal controls, and (iii) those submitted before 2014. This filtering is to ensure that the expression datasets follow updated microarray guidelines and protocols. Subsequently, all the datasets were checked for quantile normalization, and quantile normalization was done using an R software called Limma R package in R studio (version 4.0.1).

Identification of Differentially Expressed mRNA (DEmRNA), Differentially Expressed lncRNA (DElncRNA), and Differentially Expressed miRNA (DEmiRNA)

The DEmRNAs, DEmiRNAs, and DElncRNAs were identified using GEO2R (Chen *et al.,* 2015). The cut-off criteria were p-value < 0.05 (Ye *et al.,* 2015) and $|\log2 \text{ fold-change}| \geq 1$ (Liu *et al.,* 2019) to ensure statistically significant. Subsequently, the overlaps of differentially expressed RNAs between two or more datasets were identified and visualised using Venn diagrams via the Venny Tool resource.

Identification of DEmiRNA-Target mRNA Interaction

Gene targets of DEmiRNAs were identified following inverse expression correlation using computationally-predicted functions in miRwalk 2.0 and an experimentally validated database of miRTarBase (Zhu *et al.,* 2020). For miRWalk 2.0,

the cut-off criteria were p-value < 0.05, and those are common in at least five databases integrated into miRWalk 2.0, while for miRTarBase, the predicted target mRNAs with both "less strong" and "strong evidence" validation methods were selected. Then, all the target mRNAs from all the databases were combined. The overlapping datasets of predicted target mRNAs and DEmRNAs were examined and visualised using Venn diagrams.

Identification of DEmiRNA-Target lncRNA Interaction

The lncRNAs targeted by DEmiRNAs were identified using experimentally supported interaction databases, namely DIANA-LncBase V3 and StarBase V2 (Hu *et al.,* 2020). The parameters for DIANA-LncBase V3 were set to *Homo sapiens* for species and high miRNA confidence level to ensure accuracy. Meanwhile, for StarBase V2, the parameters were set to strict stringency (>=5) for the CLIP Data and low stringency (>=1) for the Degradome Data parameter (Liang & Sun, 2019). For the computational prediction, miRcode and DIANA-LncBase V2 databases were utilized. The parameters for miRcode were set as gene class for all lncRNAs, site conservation for most mammals, and transcript region for any/ncRNA. Conversely, the parameter for the DIANA-LncBase V2 database was set to a default threshold ≥ 0.07 for high sensitivity and precision (Liang & Sun, 2019). All the target lncRNAs from all the databases were combined. The comparison of predicted target lncRNAs with DElncRNAs was examined and visualised using Venn diagrams.

Construction of mRNA-miRNA-lncRNA ceRNA Network

The mRNA-miRNA-lncRNA competing endogenous RNA (ceRNA) network was constructed using Cytoscape software (Version 3.8.2) (Yang *et al.,* 2011) to merely visualize mRNA-miRNA-lncRNA interaction according to the parameters mentioned during the curation of target mRNAs and lncRNAs for DEmiRNAs. Within the network, the lncRNAs served as the target lncRNAs of DEmiRNA, while the mRNAs acted as the target genes for DEmiRNAs. The mRNAs and lncRNAs, regulated by common miRNAs, were selected for constructing the ceRNA network by merging DEmiRNA-target mRNA and DEmiRNA-target lncRNA interactions.

Construction of EBV miRNA-mRNA Interaction Network

The predicted EBV miRNAs-mRNAs interaction was identified by using ViRBase (Zhou *et al.,* 2019), and the keyword search for Virus Type was set to *Human gammaherpesvirus 4* (Jing *et al.,* 2018). The cut-off criterion was set to a confidence score of 0.5 to 1.00. Only EBV miRNAs from the *Bam*H1 fragment A transcript (BART) cluster were selected due to its overexpression in NPC tumor samples and cell lines (Zhang *et al.,* 2022). Next, the mRNAs interacting with the EBV miRNAs overlapped with the ceRNA DEmRNA using a Venn diagram. Subsequently, the EBV miRNA-mRNA interaction network was constructed and visualised using Cytoscape (Zhou *et al.,* 2019).

Construction of Protein-Protein Interaction (PPI) Network

The physical protein-protein interaction (PPI) among the expressed proteins of DEmRNAs was identified using the Search Tool for the Retrieval of Interacting Gene (STRING) database (Jing *et al.,* 2018) to visualise the indirect and direct interaction types of the PPI**.** A cut-off criterion of a combined score > 0.4, representing a medium or high confidence level, was applied (Tang *et al.,* 2018). Then, the PPI network was constructed, visualised and analysed in Cytoscape software using the STRING database plug-in; with a combined score of more than 0.55, a medium or high confidence was set for stronger interaction evidence. Following this, the cytoHubba plug-in of the Cytoscape was used to filter out the top 20 hub genes, which are strongly connected to each other, using the degree algorithm (Xu *et al.,* 2020). Here, the hub genes refer to the genes expressing the proteins in the PPI network. Additionally, the Network Analyzer plug-in of the Cytoscape was utilised to further filter out hub genes among the top 20 hub genes by calculating the node degree (Karagkouni *et al.*, 2020). An undirected network parameter was applied to further filter out the hub genes. Genes with a connectivity degree greater than five were considered hub genes level genes due to being deemed as contributor causes of biological processes (Han *et al.,* 2004; Ye *et al.,* 2018). Lastly, the

Molecular Complex Detection (MCODE) plug-in of the Cytoscape was used to analyse and screen for the gene modules of the PPI network (Chen *et al.,* 2015). The parameters used were degree cut-off = 2 for network scoring, a node score cut-off = 0.2 for cluster finding, k-core = 2, and max. Depth = 100 (Yan *et al.,* 2019). Self-edges nodes were excluded by turning off Loops, the Haircut option was included to remove singly connected nodes from clusters, and the Fluff option was excluded to prevent the expansion of the cluster core (Bader *et al.,* 2020). The gene modules with scores ≥ 4 and nodes ≥ 4 were considered significant.

Construction of Transcription Factors (TF)-mRNAs Network

The predicted transcription factors (TFs) among the DEmRNAs were identified using the TFcheckpoint database (Zhong *et al.,* 2019), focusing solely on TFs supported by experimental evidence. A comparison of TFs with the DEmRNAs was made using Venn diagrams. Subsequently, the TFs-DEmRNAs network was constructed and visualized using Cytoscape (Zhou *et al.,* 2019). Any of the TFs observed as hub genes in the PPI network were considered as hub TFs (Jing *et al.,* 2018).

Construction of Cross-Regulatory Networks (Transcription Factor (TF)-DmRNAs, Competing Endogenous Network (ceRNA), EBV miRNAs-DEmRNAs)

All three networks (TF-DEmRNAs, ceRNA, EBV miRNAs-DEmRNAs) were examined using Cytoscape to identify overlapping RNAs. These shared RNAs were then used to construct a cross-regulatory network to visualise the regulatory interactions between the RNAs with EBV BART miRNAs using Cytoscape (Zhou *et al.,* 2019).

Functional and Pathway Enrichment Analysis of DEmRNAs Affected by EBV miRNAs

Database for Annotation, Visualisation and Integrated Discovery (DAVID) database was utilised to identify the cellular functions and pathways affected by EBV BART miRNAs in NPC tumorigenesis (Yan *et al.,* 2019). The database provides Gene Ontology (GO) terms, including Biological Processes (BP), Molecular Function (MF) and Cellular Component (CC), to dissect the affected cellular functions of the DEmRNAs targeted by EBV BART miRNAs (Zhang *et al.,* 2019). Besides this, DAVID provided KEGG pathway enrichment analysis used to explore the affected pathway by EBV BART miRNAs (Yang *et al.,* 2019). Both GO and KEGG pathway analyses were subjected to a cut-off criterion of p-value < 0.05 (Zhou *et al.,* 2019). The results of the enrichment analyses were visually represented using bubble plots in GraphPad Prism (Version 9.1.0).

Statistical Analysis and Network Visualization

The differentially expressed RNAs between groups in this study were estimated by using a student t-test generated by the GEO2R, while the Benjamini–Hochberg false discovery rate was used to generate p-value adjustment to minimise false positive results (National Center for Biotechnology Information, 2024). For Gene Ontology (GO) enrichment analysis, DAVID utilises Fisher's exact test to avoid counting duplicated genes (DAVID Bioinformatics Resources, 2021). Notably no statistical analysis was done on all the networks constructed using Cytoscape software, as its primary function is only to link and visualise interactions between EBV BART miRNAs and all the RNAs. The interactions between the EBV BART miRNAs and RNAs retrieved from the databases used were identified by using algorithms tailored to each database, especially computationally predicted.

RESULTS

Microarray Data of MicroRNAs (miRNAs), Long Non-Coding RNAs (lncRNAs) and Messenger RNAs (mRNAs)

Five datasets were extracted from the microarray gene expression profiles curated from Gene Expression Omnibus (GEO) database. These include one dataset for miRNAs (GSE70970), three datasets for lncRNA (GSE95166, GSE126683 and GSE61218), and two datasets for mRNA (GSE64634 and GSE126683). The information about these datasets is provided in Supplementary Table 1. GSE126683 and GSE61218 each contain a combination of lncRNA and mRNA expression data.

Identification of Differentially Expressed mRNA (DEmRNA), miRNA (DEmiRNA) and lncRNA (DElncRNA)

The DEmRNA, DEmiRNA and DElncRNA data, assayed via Gene Expression Omnibus 2 R (GEO2R), is provided in Supplementary Table 2. Comparative analysis of each type of differentially expressed RNAs is illustrated in Supplementary Figures 1 and 2. Regarding the lncRNA datasets from GSE95166, GSE126683 and GSE61218, no overlap was observed among the up-regulated lncRNAs (Supplementary Figure 1a). However, for down-regulated lncRNAs, overlapping data showing three common/shared lncRNAs among the datasets was evident (Supplementary Figure 1b). These were Cancer Susceptibility Candidate 2 (CASC2), Transmembrane Phosphoinositide 3-phosphatase and Tensin homolog 2 pseudogene 1 (TPTE2P1), and Rho Guanine Nucleotide Exchange Factor 26 Antisense RNA 1 (ARHGEF26-AS1). For the overlapping data of the three mRNA datasets of GSE64634, GSE126683 and GSE61218 they revealed 65 commonly up-regulated (Supplementary Figure 2a) and 123 down-regulated mRNAs (Supplementary Figure 2b). No comparison was made for the miRNA dataset (GSE70970) because only one such dataset was available from 2014 onwards until 2023.

Identification of Interaction Between DEmiRNA and mRNA or lncRNA

The target mRNAs interacting with DEmiRNAs (in the GSE70970 dataset) were obtained from miRwalk 2.0 and miRTarBase databases. The results of the target mRNAs for both up-regulated and down-regulated miRNAs from the combined databases were compared with the DEmRNAs and illustrated using a Venn diagram (Supplementary Figure 3 and 4, Supplementary Tables 3 and 4). Specifically, the Venn diagram illustrates the number of target mRNAs of the up-regulated miRNAs when overlapped with the 123 down-regulated DEmRNAs. For the target lncRNAs interacting with the DEmiRNAs, they were obtained from the DIANA-LncBase V3, StarBase V2, miRcode and DIANA-LncBase V2 databases. Results of the target lncRNAs for the up-regulated miRNAs from the combined databases were compared with the DElncRNAs and illustrated using a Venn diagram (Supplementary Figure 5 and Supplementary Table 5). The Venn diagram demonstrates the target lncRNAs of the up-regulated miRNAs when they overlapped with the three down-regulated lncRNAs.

lncRNA-miRNA-mRNA ceRNA Competing Endogenous RNA (ceRNA) Network Construction

The constructed lncRNA-miRNA-mRNA competing endogenous RNA **(**ceRNA) network comprises of 17 up-regulated DEmiRNAs, interacting with 58 down-regulated DEmRNAs, and 3 down-regulated DElncRNAs (Figure 1 and Supplementary Table 6). The ceRNA network illustrates the intricate interactions and interplay among all the RNAs, both directly and indirectly, shedding light on how the dysregulation of one RNA could affect the expression level of other RNAs in the context of NPC tumorigenesis. However, it is worth noting that only one ceRNA was constructed due to no common up-regulated lncRNAs in the three GEO datasets (GSE95155, GSE126683 and GSE61218).

EBV miRNA-mRNA Interaction Network Construction

Utilising the ViRBase database, it revealed 5,411 interactions between Epstein-Barr Virus (EBV) *Bam*H1 A transcript (BART) miRNA and mRNA. Results of overlapping this data with the down-regulated DEmRNAs in the competing endogenous RNA **(**ceRNA) network using the Venn diagram and Cytoscape revealed four common down-regulated DEmRNAs, namely Early B-cell factor transcription factor 1 (EBF1), Mono-ADP Ribosylhydrolase 2 (MACROD2), Eye Absent transcriptional coactivator And phosphatase 1 (EYA1) and Eye Absent transcriptional coactivator And phosphatase 4 (EYA4) (Supplementary Figure 6 and Figure 2). These DEmRNAs were used to construct the EBV BART miRNA-mRNA network and visualised using Cytoscape (Figure 3). We demonstrated the regulatory relationship between the EBV BART miRNA and the DEmRNAs, whereby the former could down-regulate the latter. Specifically, we inferred that EYA4 interacts with ebv-miR-BART19-3p and ebv-miR-BART15; EYA1 interacts with ebv-miR-BART21-3p and ebv-miR-BART12-5p; MACROD2 interacts with ebv-miR-BART20-3p; and EBF1 interacts with ebv-miR-BART11-5p (Figure 3).

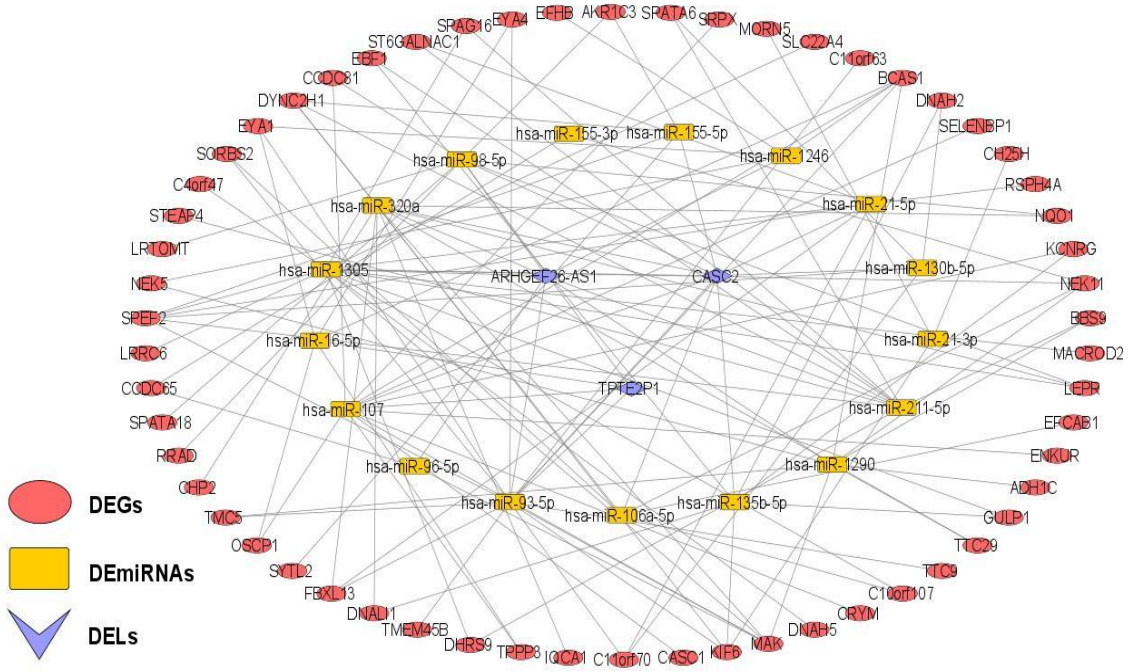

Figure 1. The lncRNA-miRNA-mRNA ceRNA network with 17 up-regulated DEmiRNAs (yellow rectangle), interacting with 58 down-regulated DEGs (red eclipse), and- 3 down-regulated DELs (purple diamond)

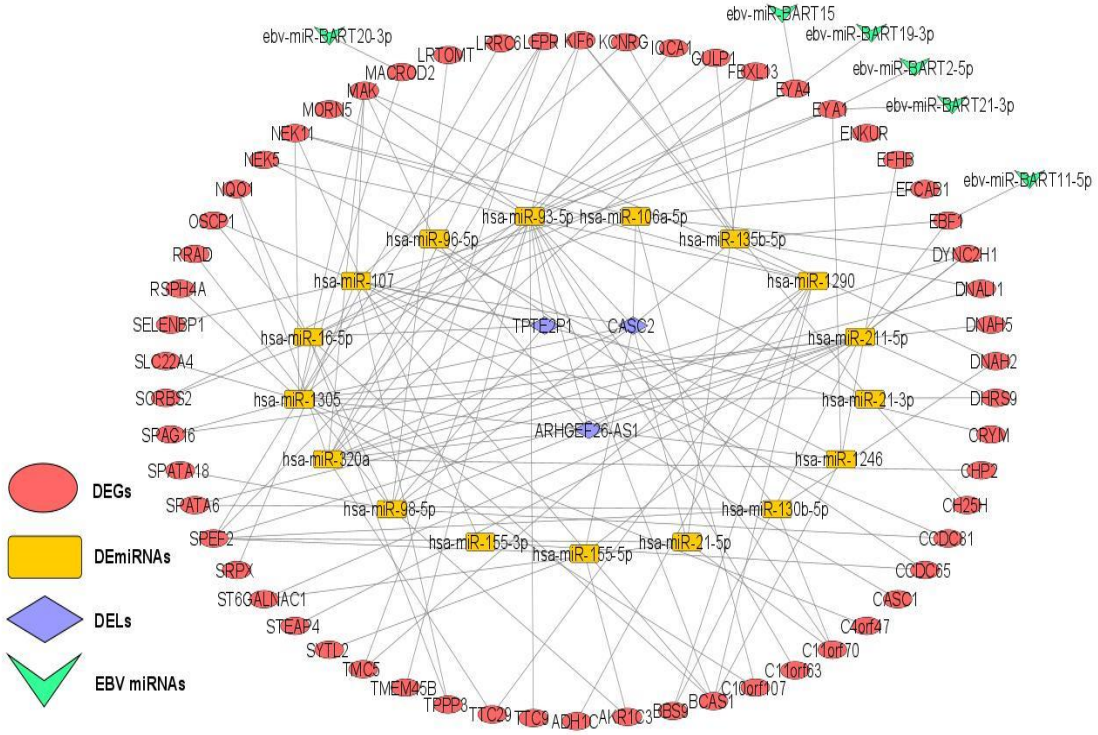

Figure 2. Overlapped down-regulated DEmRNAs in the ceRNA network with target mRNAs for BART miRNAs in Cytoscape revealed 4 common down-regulated DEmRNAs, namely EBF1, MACROD2, EYA1 and EYA4. The EBV BART miRNAs are in green V

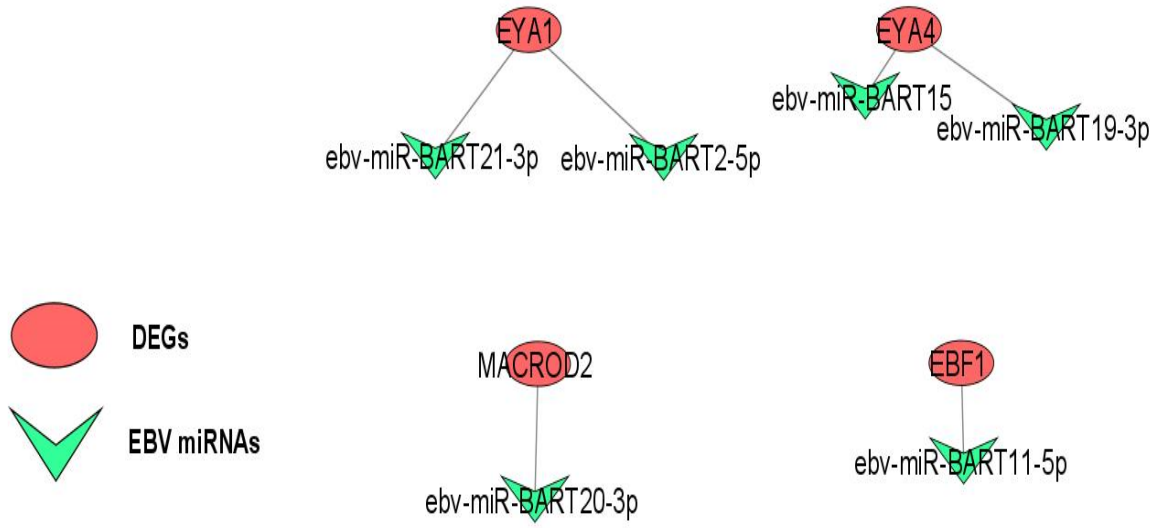

Figure 3. The EBV miRNAs-mRNAs network illustrating the interactions of the four down-regulated ceRNA DEGs (red eclipse) with six EBV BART miRNAs (green V)

Protein-Protein Interaction (PPI) Network Construction

As indicated by the Search Tool for the Retrieval of Interacting Gene (STRING) database of the Cytoscape plug-in, the protein-protein interaction (PPI) network of the down-regulated common mRNAs in three of the GEO datasets (GSE64634, GSE126683 and GSE61218) exhibited 123 nodes and 179 edges. Notably, seventy-seven of the proteins expressed interacted with each other (Figure 4), hinting at shared similar biological functions. Following CytoHubba filtration of the top 20 DEmRNAs, DNAH5 was ranked first with 21 degrees of connectivity, while DNAAF1 exhibited the lowest connectivity degree with 7 degrees (Figure 5). Subsequently, eighteen of the DEmRNAs have a connectivity degree of greater than five based on Network Analyzer analysis. Among them, DNAH5 interacted with 18 DEmRNAs, exhibiting the highest connectivity degree whereas both DNAAF1 and TEKT1 exhibited the lowest connectivity by interacting with 6 DEmRNAs (Figure 6, Supplementary Table 7). These DEmRNAs are considered hub genes and potential tumour suppressors due to their ability to control the expression of other multiple genes. Furthermore, 2 gene modules or protein sets in the PPI network (Module 1 and 2) were generated via MCODE (Table 1, Figure 7). The genes in Module 1 consisted of hub genes, unlike Module 2. Notably, the genes within both modules are neighbouring genes packed closely together in the PPI network and are highly interconnected with each other compared to other genes in the PPI network. Meaning these DEmRNAs or proteins are closely related and functionally similar in each module. The functions primarily influence proper cilia functions and motility. This suggests that the down-regulation of genes in these modules may cause nasopharyngeal carcinoma (NPC) by affecting the nasal ciliary motor's function, impairing fluid and particle clearance in the nasopharynx (Ringers *et al*., 2020).

Construction of Transcription Factors (TF)-mRNAs Network

The TFcheckpoint database yielded 1,021 transcription factors (TFs), of which two, EBF1 and NR2F2, were found to overlap with the down-regulated DEmRNAs identified in the three GEO datasets (GSE64634, GSE126683 and GSE61218) by using Venn diagram (Supplementary Figure 7). However, upon visualization in Cytoscape, neither of these TFs showed links with other DEmRNAs in the protein-protein interaction (PPI) network (Figure 8). As such, no TF-mRNAs network was constructed.

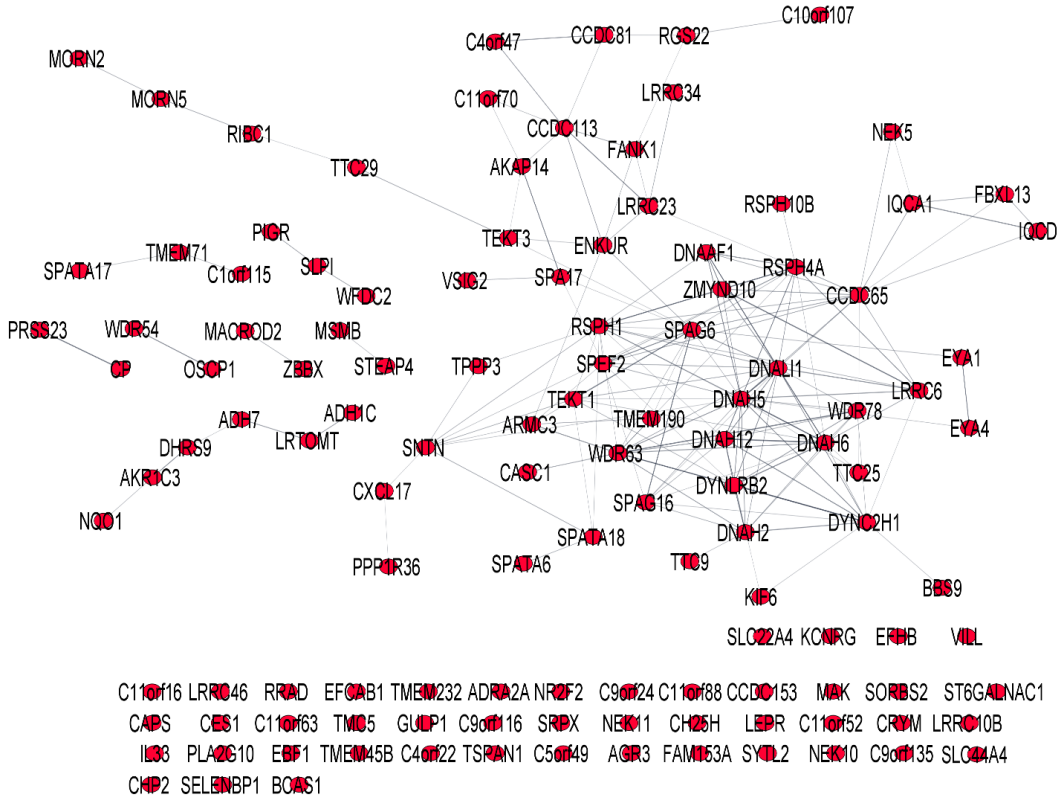

Figure 4. The protein-protein interaction (PPI) between all the down-regulated DEGs retrieved from the STRING database shows 123 nodes and 179 edges, with 77 of the DEGs interacting with each other out of all the 123 DEGs

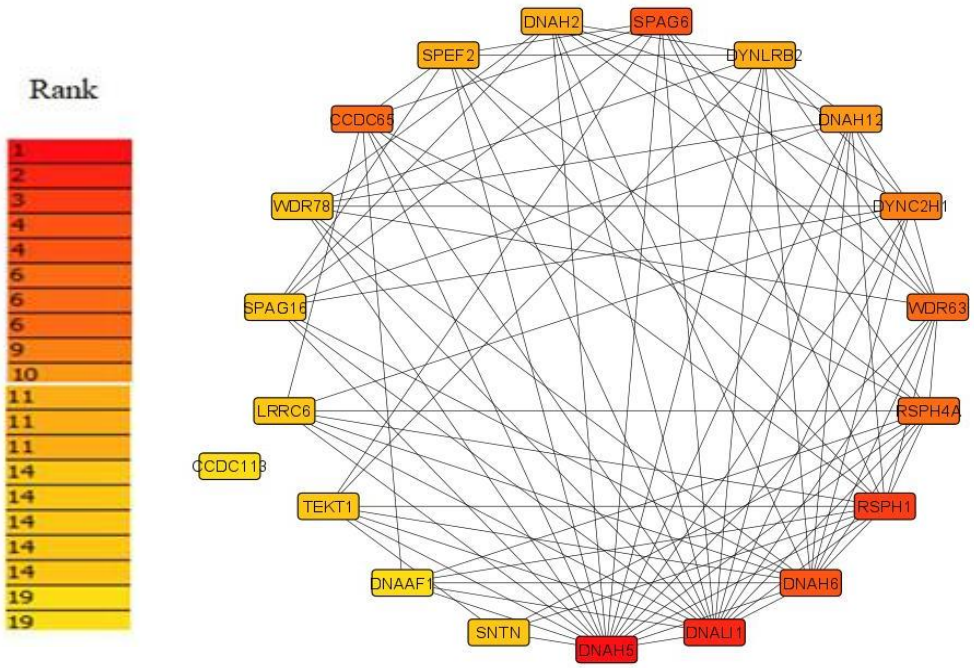

Figure 5. The top 20 DEGs from the PPI ranked according to connectivity degree. Red represents DEG with high connectivity degree and the colour scheme changes to orange and yellow as the connectivity degree decreases. The yellow represents the lowest connectivity degree

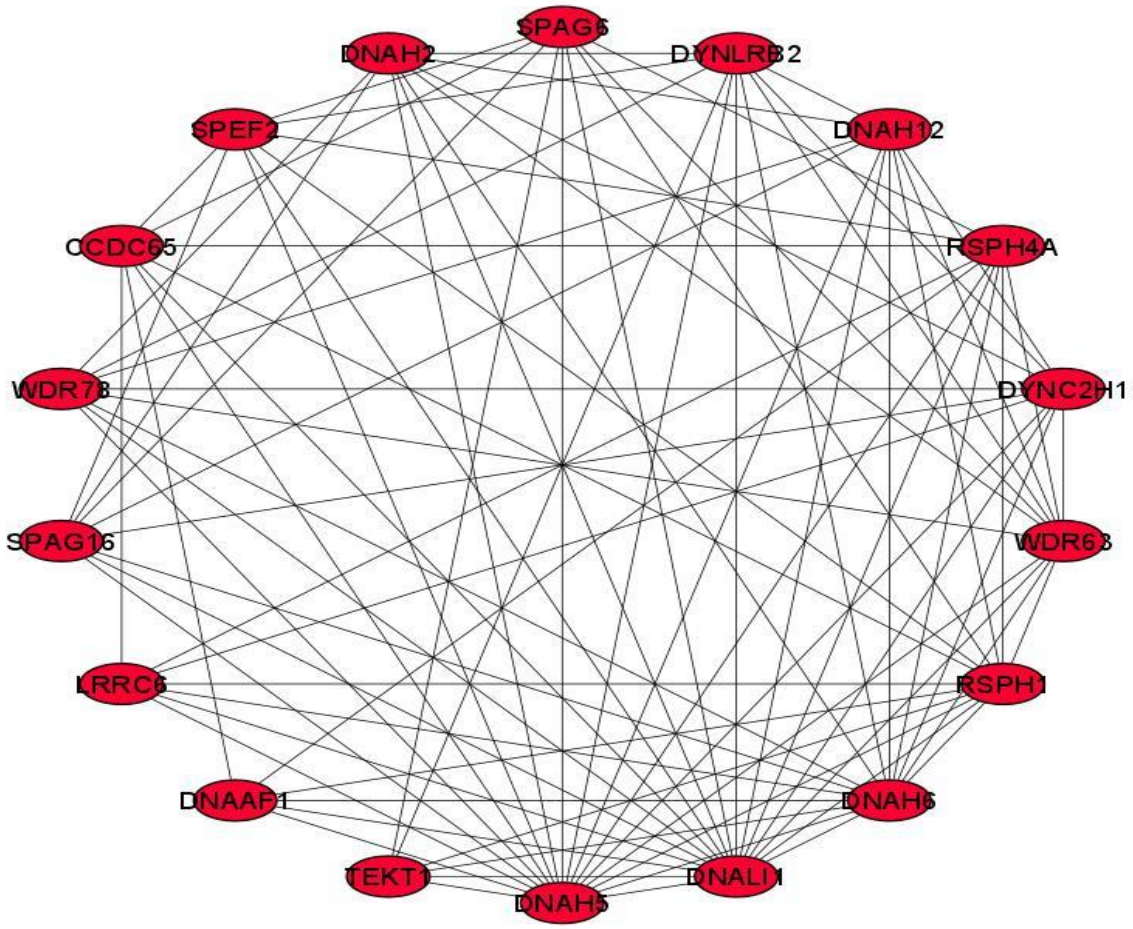

Figure 6. Eighteen hub genes or proteins with connectivity degree greater than 5 among the top 20 hub genes analysed using Network Analyzer analysis. DNAH5 has the highest connectivity degree where it interacts with 18 other DEGs and, gradually the interaction decreases in a counterclockwise from DNAH5 to TEKT1, that has the lowest connectivity degrees, similar to DNAAF1 where they interact with 6 other DEGs among themselves

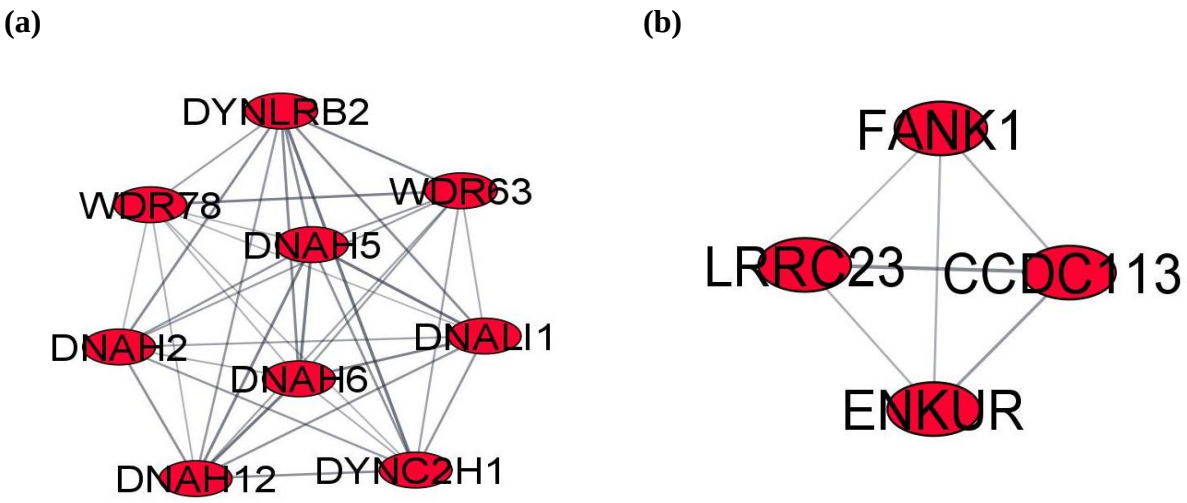

Figure 7. The two modules in the protein-protein interaction (PPI) network with scores ≥ 4 and nodes ≥ 4. Module 1 (a) consisting of all DEGs hub genes, and Module 2 (b) consisting of other DEGs in the PPI. Both modules are essential in proper cilia functions and motility

Table 1. The genes or expressed proteins by the genes consisted in gene modules 1 and 2 extracted from the protein-protein interaction (PPI) network.

Module	Score	Nodes	Edges	DEmRNAs or Proteins
1	9.000	9	36	WDR78, DNAH2, DNAH12, DYNLRB2, DNAH6, DNAH5, WDR63, DNALI1, DYNC2H1
2	4.000	4	6	FANK1, LRRC23, CCDC113, ENKUR

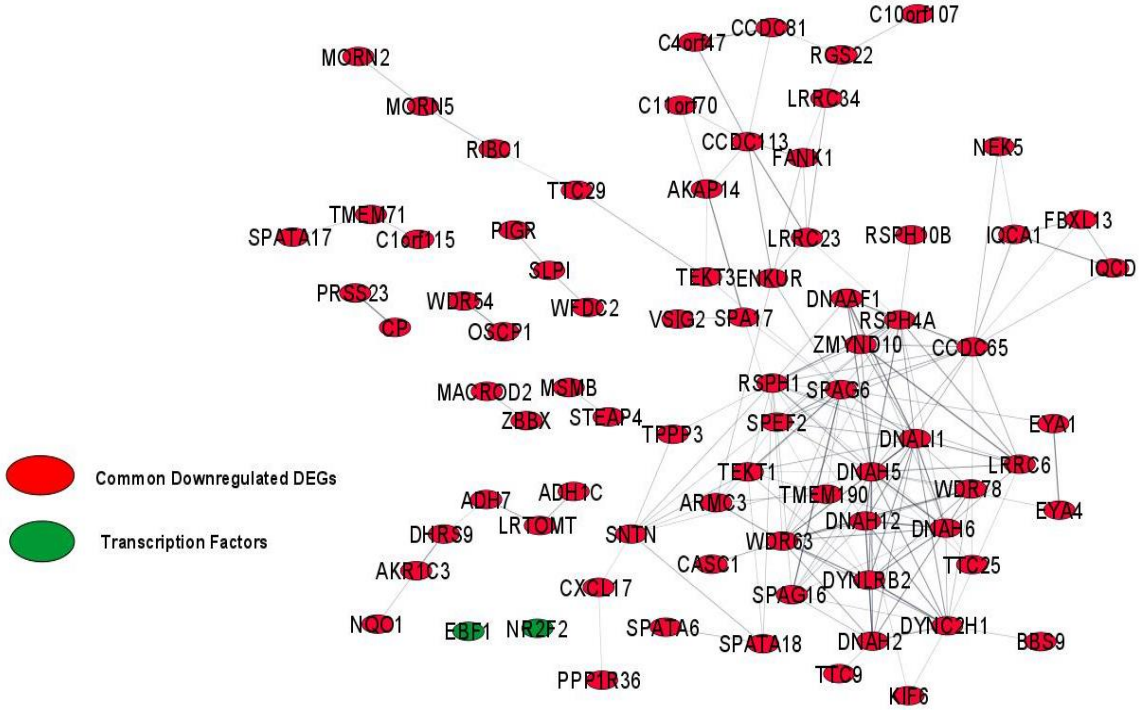

Figure 8. The transcription factors (TF)-mRNAs network model showing the two TFs, EBF1 and NR2F2 (green eclipse), that are not connected to the other down-regulated DEGs (red eclipse) in the protein-protein interaction (PPI) network

Construction of Cross-Regulatory Networks (Competing Endogenous RNA (ceRNA) and Epstein-Barr virus (EBV) *Bam*HI A rightward transcript (BART) miRNAs-DEmRNAs network)

The cross-regulatory network was constructed by merging the two networks, the competing endogenous RNA (ceRNA) network and Epstein-Barr virus (EBV) *Bam*HI A rightward transcript (BART) miRNAs-DEmRNAs network in Cytoscape. It unveils interactions among six EBV miRNAs (ebv-miR-BART21-3p, ebv-miR-BART19-3p, ebv-miR-BART15, ebv-miR-BART2-5p, ebv-miR-BART20-3p and ebv-miR-BART11-5p), four DEmRNAs (EYA4, EYA1, EBF1 and MACROD2), and six DEmiRNAs (hsa-miR-1246, hsa-miR-93-5p, hsa-miR-16-5p, hsa-miR-135b-5p, hsa-miR-211-5p and hsa-miR-1305). In addition, three DElncRNAs (CASC2, TPTE2P1 and ARHGEF26-AS1) are interacting with six of these DEmiRNAs (Figure 9 and Table 2). The cross-regulatory network reveals that EBV BART miRNAs down-regulate the four DEmRNAs, leading to the up-regulation of the six host miRNAs. This occurs due to the limited availability of DEmRNAs for repression of degradation, resulting in the down-regulation of the three DElncRNAs because no miRNA was sponged in EBV-positive nasopharyngeal cancer (NPC). Notably, the cross-regulatory network illustrates the interaction of the TF EBF1 with ebv-miR-BART11-5p. Nevertheless, EBF1 was not interacting with any other DEmRNAs in the network. Moreover, no hub genes are shown to interact with any of the EBV BART miRNAs.

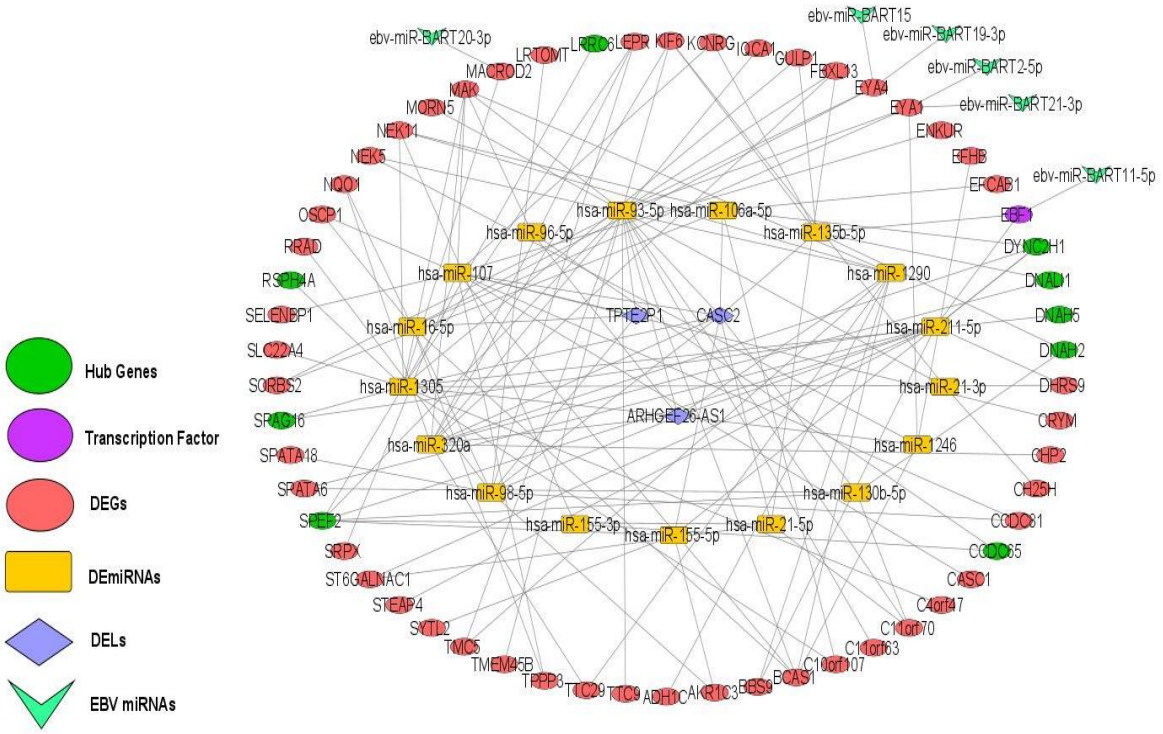

Figure 9. The cross-regulatory network for the DEL-DEmiRNAs-DEGs and EBV miRNAs-DEGs networks, showing four of the EBV miRNAs (ebv-miR-BART21-3p, ebv-miR-BART19-3p, ebv-miR-BART15, ebv-miR-BART2-5p, ebv-miR-BART20-3p and ebv-miR-BART11-5p) interacting with four of the DEGs (EYA4, EYA1, EBF1 and MACROD2). Moreover, these DEGs were interacting with six miRNAs (hsa-miR-1246, hsa-miR-93-5p, hsa-miR-16-5p, hsa-miR-135b-5p, hsa-miR-211-5p and hsa-miR-1305), and these miRNAs are interacting with three lncRNAs (CASC2, TPTE2P1 and ARHGEF26-AS1)

Table 2. The intricate interactions between Epstein-Barr virus (EBV) *Bam*HI A rightward transcript (BART) miRNAs, DEmRNAs, DEmiRNAs and DElncRNAs in the cross-regulatory network

Up-regulated EBV BART miRNAs	Down-regulated DEmRNAs	Up-regulated DEmiRNAs	Down-regulated DElncRNAs
EBV-miR-BART11-5p	EBF1	hsa-miR-211-5p	ARHGEF26-AS1
		hsa-miR-135b-5p	CASC2
EBV-miR-BART20-3p	MACROD2	hsa-miR-1305	CASC2 and ARHGEF26-AS1
EBV-miR-BART21-3p and EBV-miR-BART2-5p	EYA1	hsa-miR-1246	ARHGEF26-AS1
		hsa-miR-93-5p	ARHGEF26-AS1, CASC2 and TPTE2P1
EBV-miR-BART15 and EBV-miR-BART19-3p	EYA4	hsa-miR-93-5p	ARHGEF26-AS1, CASC2 and TPTE2P1
		hsa-miR-16-5p	TPTE2P1

Functional and Pathway Enrichment Analysis of DEmRNAs

The Gene Ontology (GO) enrichment results (from DAVID analysis), for the four DEmRNAs (EYA1, EYA4, EBF1 and MACROD2) targeted by the six Epstein-Barr virus (EBV) *Bam*HI A rightward transcript (BART) miRNAs (ebv-miR-BART21-3p, ebv-miR-BART19-3p, ebv-miR- BART15, ebv-miR-BART2-5p, ebv-miR-BART20-3p and ebv-miR-BART11-5p) in the cross-regulatory network revealed enrichment in eleven GO terms at the cut-off P-value < 0.05. These were eight GO terms for Biological Processes (BP), two GO terms in Molecular Function (BF) and one GO term for Cellular

Component (CC) (Figure 10). The Biological Processes (BP) revealed the involvement of the DEmRNAs in regulating DNA repair and apoptosis. Due to down-regulation by EBV BART miRNAs, these DEmRNAs were predicted to be unable to perform their functions, potentially leading to tumour cell proliferation and NPC tumorgenesis. Besides, the Molecular Function (BF) analysis exhibited that the proteins encoded by these DEmRNAs require metal ion binding for DNA repair and immune systems surveillance. Additionally, all these DEmRNAs were presumed to be located in the nucleus of the cells when targeted by EBV BART miRNAs, as indicated by the Cellular Component (CC) term. However, the KEGG pathway enrichment analysis for the four DEmRNAs targeted by the six EBV BART miRNAs in the cross-regulatory network did not yield any significant results.

DISCUSSION

In the present study, we constructed a comprehensive cross-regulatory network by integrating the competing endogenous RNA (ceRNA) regulatory and EBV miRNA-mRNA interaction networks to unravel the intricate molecular mechanisms mediated by EBV BART miRNAs during NPC tumorigenesis. We demonstrated that EBV BART miRNAs target and dysregulate mRNA expression, thereby disrupting the intricate balance of gene regulation. This dysregulation extends to microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), leading to the oncogenesis of NPC. We also infer that the candidate EBV BART miRNAs and lncRNAs could potentially have oncogenic and tumour-suppressive functions. These insights shed light on the complex interplay between EBV BART miRNAs, mRNAs, miRNAs and lncRNAs, providing an understanding of NPC tumorgenesis and the development of novel therapeutic strategies.

The competing endogenous RNA (ceRNA) network exhibits 58 mRNAs that are indirectly dysregulated by 3 lncRNAs. This is through 18 miRNAs sponged/targeted by the 3 lncRNAs. The lncRNAs are suggested to be down-regulated, which consequently caused the down-regulation of the mRNAs, independent of the presence of EBV BART miRNAs. In a previous study, a ceRNA network showed 2,654 mRNAs were regulated by 132 lncRNAs via 565 corresponding miRNAs in NPC using datasets that were submitted even before 2014 (Xu *et al.,* 2020). When considering the presence of EBV BART miRNAs, the four DEmRNAs targeted by BART miRNAs are assumed to be down-regulated by these EBV BART miRNAs. This causes the up-regulation of host miRNAs and subsequent down-regulation of the lncRNAs. In this scenario, the BART miRNAs mimic the function of the host's miRNAs, sponging/targeting the host's mRNAs. In other words, the BART miRNAs competing with the host's miRNAs for mRNAs and incidentally down-regulated the lncRNAs. This intricate interplay is believed to contribute to the development of EBV-associated NPC.

In this study, we uncovered 14 up-regulated miRNAs similar to those in a previous study (Xu *et al.,* 2020), but this is not the case for lncRNAs and mRNAs, which are novel discoveries. Therefore, 58 mRNA, 17 miRNA and 3 lncRNA are new non-coding RNAs potentially associated with NPC tumorigenesis. For the cross-regulatory network, six of the miRNAs are up-regulated in NPC tumour samples, cell lines and serum, excluding hsa-miR-211-5p and hsa-miR-16-5p (Zhu *et al.,* 2009; Plieskatt *et al.,* 2014; Tang *et al.,* 2014), and our results here are the first to connect them with the tumorigenesis of NPC. Interestingly, hsa-miR-16-5p, hsa-miR-211-5p and hsa-miR-1305 are observed to demonstrate opposite effects in various cancers, including non-small cell lung cancer (NSCLC) and hepatocellular carcinoma (HCC), where they are down-regulated and elicit the up-regulation of their target mRNAs, including XIST, MDM2 and ACSL4 (Cai *et al.,* 2019; Qin *et al.,* 2020; Du *et al.,* 2021).

In addition, to identify interactive relationships among down-regulated mRNAs, the protein-protein interaction (PPI) network was constructed (Tang *et al.,* 2018) and it unveils 18 hub genes with connectivity degree greater than 5. These genes, interacting with more than 5 other genes in the PPI network, possess the capacity to regulate the expression of other multiple genes. Notably, these hub genes are potential new candidate NPC-associated hub genes compared to a previous study by Xu *et al.* (2020). However, the cross-regulatory network analysis did not reveal the targeting of these hub genes by six of the BART miRNAs

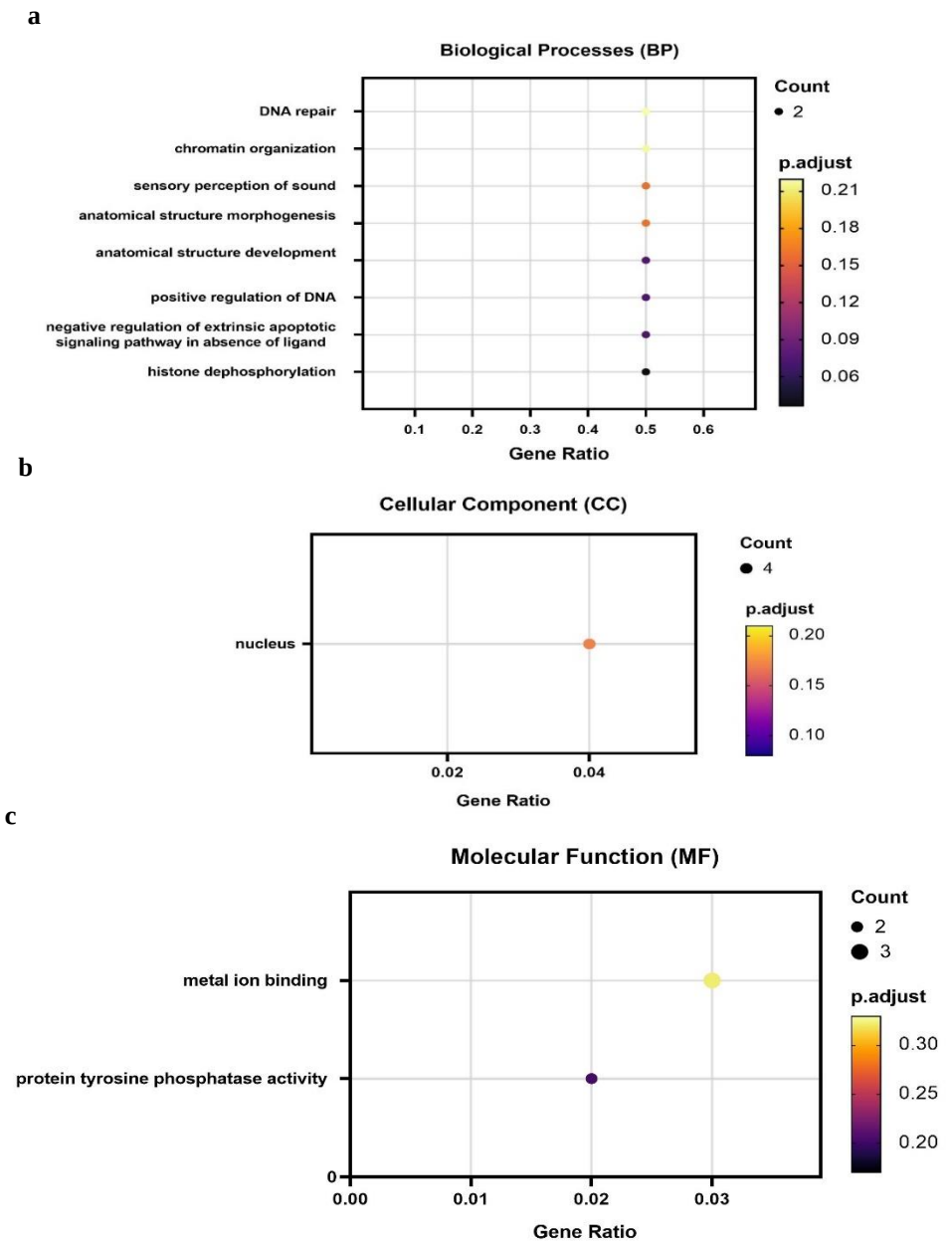

Figure 10. The Gene Ontology (GO) enrichment results for the DEGs targeted by EBV BART miRNAs in the cross-regulatory network showing (a) 8 GO terms for Biological Processes (BP), (b) one GO term for Cellular Component (CC), and (c) one GO terms for Molecular Function (BF)

Furthermore, these dysregulated hub genes co-expressed together in a module, termed module 1, affecting similar biological functions essential for healthy nasopharynx functions. Both module 1 and module 2, are pivotal for normal motile cilia functions (Rashid *et al.,* 2006; Johnson *et al.,* 2018; Zhu *et al.,* 2019; Vig *et al.,* 2020; Bazan *et al.,* 2021; Braschi *et al.,* 2022). Thus, hypothetically the down-regulation of these modules genes contributes to NPC by affecting the nasal ciliary motor. Motile cilia lining the nasopharynx is crucial for coordinated beating, facilitating proper flow and clearance of fluids and particles (Ringers *et al.,* 2020; Lee *et al.,* 2021).

Therefore, when EBV infection occurs, individuals with mucociliary clearance defect are predisposed to EBV-positive NPC tumorigenesis resulting from the ineffective sweeping of mucus covering the nasal epithelium towards the nasopharynx by coughing, impeding the removal of particles and pathogens from the respiratory tract (Marttin *et al.,* 1997; Gizurarson, 2015; Kamiya *et al.,* 2020). Consequently, EBV gains the opportunity to colonize epithelial cells and B-cells in the nasopharynx. Our data from the analysis of this

cross-regulatory network highlights the interaction between six EBV miRNAs (ebv-miR-BART21-3p, ebv-miR-BART19-3p, ebv-miR-BART15, ebv-miR-BART2-5p, ebv-miR-BART20-3p and ebv-miR-BART11-5p) and four DEmRNAs (EYA4, EYA1, EBF1 and MACROD2), resulting in their down-regulation. Subsequently, this causes the up-regulation of six miRNAs (hsa-miR-1246, hsa-miR-93-5p, hsa-miR-16-5p, hsa-miR-135b-5p, hsa-miR-211-5p, and hsa-miR-1305), leading to the up-regulation of three lncRNAs (CASC2, TPTE2P1, and ARHGEF26-AS1). Based on this, the association of EBV with NPC can be deduced through the regulatory mechanisms of EBV BART miRNAs targeting the host's mRNAs, thereby affecting both miRNAs and lncRNAs that interact with them. Currently, the interplay interactions between EBV miRNAs and lncRNAs in NPC have not yet been updated, apart from our study, as the interest has shifted towards understanding the regulatory mechanisms of EBV miRNA/lncRNA on circular RNA (circRNA) in NPC.

Previous studies have consistently reported high expression levels of all six BART miRNAs in NPC tissue samples, cell lines and serum samples (Cosmopoulos *et al.,* 2009; Song *et al.,* 2016; Lung *et al.,* 2018; Jiang *et al.,* 2020; Zhou *et al.,* 2022). However, conflicting findings were noted for BART15 expression in NPC cell lines, with Amoroso *et al.* (2011) revealing it was barely expressed, and Lung *et al.* (2018) revealing it is undetected, while Cosmopoulos *et al.* (2009) observed it is highly expressed in both NPC cell line and tumour biopsy. In terms of the interaction of these six BART miRNAs in NPC, only BART2-5p was found to down-regulate the gene RND3 expressions in NPC pathogenesis (Jiang *et al.,* 2020). In other cancers, only BART11-5p was observed interacting with EBF1 in the EBV-transformed lymphoblastoid cell line (LCL) (Ross *et al.*, 2013), which is consistent with our result. However, the information regarding the interactions of BART19-3p, BART15, BART21-3p, and BART20 in cancer is currently unavailable.

With reference to the Biological Processes (BP) from the Gene Ontology (GO) term, two of the DEmRNAs targeted by the BART miRNAs, which are EYA1 and EYA4, are associated with DNA repair, chromatin organization, histone dephosphorylation, apoptosis, anatomical structure morphogenesis and development and sensory perception of sound. These DEmRNAs are also metalloproteins based on the Molecular Function (MF) of the GO term as metal ion binding (MIB). Our cross-regulatory network data revealed that EYA1 is down-regulated by ebv-miR-BART21-3p and ebv-miR-BART2-5p, while EYA4 is down-regulated by ebv-miR-BART19-3p and ebv-miR-BART15. Despite this finding, there is no information on these interactions in NPC to our knowledge.

The *EYA* gene family encodes proteins tyrosine phosphatase that respond to DNA damage when bound by Mg^{2+} ion, based on the Molecular Function (MF) of the GO term as metal ion binding (MIB) **(**Tadjuidje *et al.,* 2012; Sadatomi *et al.,* 2013; Lung *et al.*, 2018). This protein works in combination with the protein tyrosine kinases, and their structural/functional aberrancies usually lead to no chromatin organization and histone dephosphorylation, consequently inhibiting DNA repair and eventually diseases, including cancers (Tadjuidje *et al*., 2012; Sadatomi *et al*., 2013; Kong *et al.,* 2019). In fact, EYA4 is known to be involved in the promotion of DNA repair and inhibition of apoptosis in oesophagal squamous cell carcinoma (Xu & Fisher, 2012), while EYA1 is down-regulated in gastric cancer tissues and correlated with tumour size and metastasis (Nikpour *et al*., 2014). We suspected that, in NPC tumorigenesis, EBV utilizes its BART21-3p, BART2-5p, BART19-3p, and BART15 miRNAs to hijack these biological processes by down-regulating EYA1 and EYA4.

Similarly, we suspected that EBV utilizes these BART miRNAs to inhibit apoptosis. Typically, when histone dephosphorylation is defective during DNA damage, JNK (c-Jun N-terminal protein kinase) is recruited for extrinsic apoptosis pathway activation (Dhanasekaran & Reddy, 2008; Nowsheen *et al.,* 2018). The suggestion of EBV using BART21-3p, BART2-5p, BART19-3p and BART15 miRNAs in down-regulating the major histocompatibility complex class I chain-related peptide A (MICA) and peptide B (MICB) ligands, expressed upon viral infection and DNA damage by targeting EYA1 and EYA4 (Zingoni *et al.,* 2018; Png *et al.,* 2021), is plausible**.** The absence of these ligands facilitates EBV to escape natural killer (NK) cell-mediated immune surveillance by inhibiting JNK signalling pathway activation and avoiding

apoptosis (Wong *et al.,* 2018).

Without apoptosis, infected cells proliferate uncontrollably, leading to anatomical structure morphogenesis and development, including angiogenesis and vasculogenic mimicry (VM), commonly observed in cancerous cells, including NPC (Luo *et al.,* 2021; Tian *et al.,* 2021). The proliferation and growth of the tumour tissues and cells require more oxygen and nutrients in the tumour microenvironment, thus the formation of angiogenesis and VM to sustain the tumour growth (Zuazo-Gaztelu & Casanovas, 2018; Xiang *et al.,* 2018; Fernández-Cortés *et al.,* 2019; Teleanu *et al.,* 2019). As the size of tumour cells increases, it covers and impairs the adjacent Eustachian tube, causing middle ear effusion or Otitis media with effusion (OME), tinnitus, and consequently, hearing loss (Utama *et al.,* 2022). Interestingly, OME, tinnitus, and hearing loss are the symptoms of NPC (Ho *et al.,* 2008; Tsunoda *et al.,* 2021), where a history of OME is prevalent among NPC patients (Huang *et al.,* 2012).

The buildup of fluid in the middle ear, known as middle ear effusion or OME, puts pressure on the tympanic membrane and impedes its proper vibration, leading to hearing loss (Searight *et al.,* 2022). A decrease in the cilia density of the mucociliary epithelium and the increase in goblet cells lining the Eustachian tube obstructs the ear secretion drainage from the middle ear into the nasopharynx (Matsune *et al.,* 1992; Depreux *et al.,* 2008; Casale *et al.,* 2023). From this, tinnitus occurs subsequently, combined with an increase in the size of tumour cells covering the Eustachian tube, stems from negative pressure in the middle ear due to no pressure equalization in the middle ear with atmospheric pressure (Bal & Deshmukh, 2022; Mayo Clinic, 2022; Casale *et al.,* 2023). Middle ear ventilation is essential for proper eardrum vibration and sound transmission (Casale *et al.,* 2023). Our findings have provided molecular insights into the causes of hearing impairment among NPC patients, mediated by non-coding EBV RNAs (BART miRNAs) and EYA genes. The verification and detailed mechanisms for this must be further investigated via relevant functional studies.

We have observed BART11-5p targeting the transcription factor (TF) EBF1 in the cross-regulatory network. EBF1 is a transcription factor that works with E2A and PAX5 in B cell development (Poh *et al.,* 2016). However, our results did not reveal EBF1 interacting with the DEmRNAs in the ceRNA network; thus, no TF-mRNA network was constructed. We attribute this to the limited NPC microarray dataset available in the time frame chosen for our study. Based on the literature, EBF1 requires metal ion binding (MIB) for structural conformational, regulatory, and enzymatic activity, specifically divalent zinc ion (Zn^{2+}) (Hagman *et al.,* 2011; Vilagos *et al.,* 2012). This is also shown by the Molecular Function (MF) of the GO term. It has been shown that the targeting of EBF1 by BART11-5p causes abnormal germinal centre reactions in the lymphoid tissues, thereby inhibiting mature B-cells development (Permyakov, 2021). We suspect that when BART11-5p targets EBF1, it inhibits MIB-EBF1 interaction, leading to the deterioration of B-cell production. This hypothesis is yet to be experimentally tested. More importantly, this could be one of the mechanisms EBV uses for immune evasion in the context of EBV-related carcinogenesis, including NPC – a research prospect that warrants exploration.

The final DEmRNA targeted by BART miRNA in our data is MACROD2, which is targeted by ebv-miR-BART20-3p. MACROD2 plays a crucial role in reversible ADP-ribosylation, acting as a hydrolase (Žaja *et al.,* 2020), and is associated with critical cellular pathways of DNA repair and apoptosis in eukaryotes (Golia *et al.,* 2017). It acts as a negative feedback loop and restricts the recruitment repair factors and effector proteins to the DNA break site when phosphorylates by ATM and exported out of the nucleus (Golia *et al.,* 2017). Following DNA repair, MACROD2 facilitates the removal of terminal autoinhibitory mono-ADP-ribose from the transferase Poly(ADP-ribose) polymerases 1 (PARP1), a process known as dePARylation or histone dephosphorylation to prevent entrapment of proteins involved in DNA repair at the damage site and causing hypersensitivity to DNA damage (Sakthianandeswaren *et al.,* 2018; Kassab *et al.,* 2020). Its dysregulation is associated with various cancers (Linnebacher *et al.,* 2013; Mohseni *et al.,* 2014; Briffa *et al.,* 2015; van den Broek *et al.,* 2015; Hu *et al.,* 2016; Cohen & Chang, 2018). In fact, its down-regulation in colorectal cancer has been linked to the increase in PARP1 mono-ADP-ribosylation

that reduces its transferase activity, leading to DNA repair impairment while heightening sensitivity to DNA damage (Hu *et al.,* 2016). Prior to our findings, the link between non-coding EBV RNAs (BART miRNA per se) and MACROD2 with respect to NPC oncogenesis has never been implied. As such, the ebv-miR-BART20-3p/MACROD2 regulatory network is an important and potent mechanism for deeper investigation.

In our current study, we have elucidated the interaction of three lncRNAs (CASC2, TPTE2P1, and ARHGEF26-AS1) with six miRNAs (hsa-miR-1246, hsa-miR-93-5p, hsa-miR-16-5p, hsa-miR-135b-5p, hsa-miR-211-5p, and hsa-miR-1305). Our data also showed that the three lncRNAs were down-regulated, thus implying their roles as tumour suppressors. Indeed, it is not uncommon for lncRNAs to act as tumour suppressors (Sakthianandeswaren *et al.,* 2018). However, only CASC2 and ARHGEF26-AS1 were hinted by previous studies could act as tumor suppressors. Specifically, in the context of nasopharyngeal carcinoma (NPC), only CASC2 was observed to be down-regulated in NPC tissue samples and cell lines, where it promotes cell proliferation and inhibits apoptosis by down-regulating RBBP8 through up-regulation of miR-18a-5p (Miao *et al.,* 2019). Nevertheless, studies on the down-regulation of CASC2 in EBV-positive NPC remain limited.

Our findings on the down-regulation of CASC2 and ARHGEF26-AS1 in NPC are consistent with previous studies in cancers, such as lung adenocarcinoma (LUAD) and esophageal carcinoma (Chen *et al.,* 2021; Yao *et al.,* 2021), but not for TPTE2P1. Based on previous studies, TPTE2P1 is typically up-regulated in other cancers, including colorectal, gallbladder, and hepatocellular carcinomas (Liu *et al.,* 2019), suggesting its potential dual role as both a tumour suppressor and an oncogene. Nevertheless, there are limited studies conducted on TPTE2P1 and ARHGEF26-AS1 expression in NPC, particularly in EBV-positive NPC. Moreover, we are the first ones to demonstrate the interaction of ARHGEF26-AS1 and CASC2 with six of the miRNAs, as well as the interaction of TPTE2P1 with four of the miRNAs in NPC. Notably, the interaction of these lncRNAs with the miRNAs is also limited in other cancer contexts. One limitation of our study is its reliance on curated microarray datasets rather than RNA sequencing datasets. Consequently, the limited dataset, especially for the miRNA dataset, is not amenable for deriving pathway(s) affected by EBV miRNAs.

In addition, the exclusion of constructing an EBV-negative ceRNA counterpart to compare to the result may introduce the potential for false positives. However, it is noteworthy that the mRNA and lncRNA microarray datasets curated for this study comprise samples from NPC patients residing in endemic regions, including China and Hong Kong, which are highly associated with EBV-positive NPC. This context increases the credibility of our results, reducing the likelihood of false positives. Although this rationale may not apply to the miRNA dataset, the presence of some significantly up-regulated EBV BART miRNAs, with p-value < 0.05, insinuating that all the NPC patients within this dataset might be EBV-positive NPC.

Moreover, the mRNA and lncRNA targeted by miRNAs, as well as the EBV miRNAs targeting the mRNAs, are curated from up-to-date databases that amalgamate experimental evidence with computational prediction. For example, the miRTarBase uses high-throughput techniques, including cross-linking and immunoprecipitation and high-throughput sequencing (CLIP-seq), cross-linking, ligation, and sequencing of hybrids (CLASH-seq) and DEmRNA radome-seq which are validated by using the miRNA-target gene expression profiles sourced from prominent repositories such as GEO and The Cancer Genome Atlas (TCGA) databases, for identification of miRNA-target mRNA (Moore *et al.,* 2015; Miao *et al.,* 2019).

Likewise, the DIANA-LncBase V3 and StarBase V2 also curate interactions between miRNA and target lncRNA that are validated by using CLIP-seq, including HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation), PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation) and CLEAR-CLIP (covalent ligation and endogenous Argonaute-bound RNA) which enable the identification of Argonaute (AGO):miRNA binding sites on targeted RNAs (Huang *et al.,* 2020). Next, the EBV BART interactions with target mRNAs curated from VirBase undergo experimental validation using CLIP-seq, quantitative real-time PCR, Western

Blot and immunohistochemistry analysis techniques. A high confidence score was set to curate EBV miRNAs-mRNAs interactions as this is commensurate to more evidence resources supporting these interactions. Nevertheless, the results of this study need to be validated experimentally in the future.

A comprehensive study in the future would necessitate the incorporation of RNA-sequence datasets as microarray datasets have become less favored in recent years, contributing to the limited datasets in our study. The preference for RNA-sequencing stems from its ability to provide extensive quantification through whole transcriptome sequencing, enabling the detection of more differentially expressed genes with higher fold changes, thereby enhancing result accuracy. Regardless, data processing and analysis are standardized and well-established for microarray datasets compared to RNA-sequence datasets (Rao *et al.,* 2019). From this arises the complexity of data processing and analysis due to the multitude of tool options available, which may overwhelm beginners and potentially lead to false positives compared to microarray datasets (Corchete *et al.,* 2020). Besides, different expression analysis methods in RNA-seq may result in under- or overestimated read counts, introducing variability in the data interpretation process (Christelle & Watson, 2015). Furthermore, the physiological veracity of our findings will require molecular experimentations that include quantitative real-time PCR, Western Blot, and immunohistochemistry analyses. These experiments will enable the verification of EBV miRNA mechanisms in NPC pathogenesis through expression level and the roles of the predicted DEmRNAs targeted by the EBV miRNAs.

CONCLUSION

Our constructed cross-regulatory network has uncovered novel insights into the interplay between EBV miRNAs and lncRNAs presumptively associated with NPC pathogenesis. The cross-regulatory network illuminates the intricate interactions and the muti-level regulation between EBV BART miRNAs, mRNA, lncRNA, and miRNAs further upon EBV infection. We identified that BART miRNAs shift the ceRNA activity by indirectly regulating lncRNAs in EBV-associated NPC. Furthermore, from the cross-regulatory network, the biological, cellular, and molecular processes caused by the dysregulation of the four DEmRNAs, namely EYA1, EYA4, MACROD2, and EBF1, targeted by the BART miRNAs, were also inferred. These four DEmRNAs involve those responsible for DNA repair regulation and apoptosis induced by DNA damage. Additionally, we provided hypothetical scenarios wherein lncRNAs were indirectly targeted by BART miRNAs and implied both factors as potential prognostic biomarkers and therapeutic targets of NPC to mitigate the adverse effects of current treatments. Lastly, our analysis hints at a potential tumor suppressor-like role of BART miRNAs and lncRNAs linked to specific DEmRNAs, albeit in a preliminary capacity, as inferred from the cross-regulatory network.

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