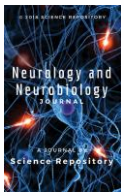

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Research Article

Bell's Palsy May Associate with Increased *IL1R1*: A Pilot Novel Gene and lncRNA Study

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ABSTRACT

Background: Bell's palsy is a widespread disease of the peripheral nervous system which causes not only physical disorders but also mental suffering as well. However, the etiological factor of Bell's palsy is still unclear. The present study aimed to search for potential influencing factors by identifying the key genes and long non-coding RNAs (lncRNAs) involved in patients with Bell's palsy using RNA-Seq data based on bioinformatics tools.

Methods: Differentially expressed genes (DEGs) and differentially expressed lncRNAs (DELs) in patients before and after therapy, and that in normal control group were identified. The competing endogenous RNAs (ceRNAs) regulatory network was constructed by integrating lncRNA-mRNA pairs, miRNA-mRNA regulatory pairs, and miRNA-lncRNA pairs using Cytoscape. The Gene Ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses of DEGs and DELs were evaluated to explore their functions. The targeted corresponding genes and pathogens were verified by ELISA and q-PCR.

Results: In the present study, hub proteins such as CXCR2 and IL1R1 in PPI network and 1039 lncRNA-mRNA co-expression pairs (eg., CYYR1-AS1-MDM2) were identified. 739 miRNA-mRNA pairs (eg., hsa-miR-147a-IL1R1), 255 miRNA-lncRNA pairs, and 363 mRNA-lncRNA co-expression pairs were included in ceRNA regulatory network. Meanwhile, CYYR1-AS1 was enriched into most pathways, including Epstein-Barr virus (EBV) infection. Subsequently, validation of neuroinflammation relevant IL1R1 and EBV showed that IL1R1 was upregulated in the serum of patients with Bell's palsy before therapy, while EBV was not found among them.

Conclusion: We hypothesized that etiological factor of Bell's palsy correlate to complex miRNA-lncRNA-mRNA interacting networks and IL1 might be involved in inflammation and immune regulation in the onset of Bell's palsy.

Introduction

Bell's palsy (BP) is a type of peripheral facial paralysis, which leads to the weak or paralyzed facial muscles on the affected side [1]. Epidemiological report suggests a high annual incidence of 23-37 per 100,000 per year in UK, and most commonly affects people of 15-45 years old [2, 3]. Diabetes and viral infection are determined as risk factors for Bell's palsy development [4, 5]. So far, anti-inflammatory or antivirus drugs such as corticosteroids and aciclovir have been used to improve outcomes [6]. However, development of further effective treatment has been limited due to unclear pathogenesis of the disease. This study aimed to search for potential influencing factors by identifying the key genes and long non-coding RNAs (lncRNAs) and their relevant networks involved in patients with Bell's palsy using

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RNA-Seq data based on bioinformatics tools, which will be helpful to reveal the underlying mechanism of the onset of BP.

Methods

I RNA Extraction and Illumina Sequencing

Serum samples of total 15 patients with Bell's palsy were collected at the timepoint of disease onset (before therapy group) and complete recovery (after therapy group) were collected in this study. And another 15 normal controls were set as normal control group. The total RNA from each sample was extracted using Trizol reagent (Invitrogen, 15596-018, USA). After the quality control of RNA, mRNA was enriched by oligo (dT) magnetic beads, and then broke into shot fragments by fragmentation buffer. Then, the RNA fragments were reverse transcribed into the first strand cDNA, and the second strand cDNA was compounded. The final cDNA library was constructed after double strands cDNA were purified and repaired. Then, the cDNA libraries were sequenced on an Illumina HiSeq™ 3500. The raw sequencing data have been uploaded to the public database NCBI. The reads with N content exceeding 10% of total base number and more than 50% low quality base of total base number ($Q \leq 5$) were deleted.

II Data Comparison and Transcriptional Annotation

Following sample sequencing and data filtration, the obtained clean reads were matched to the human reference genome derived from GENCODE (Release 25, GRCh38) using Tophat (v2.1.0) software [7]. Then, based on the annotation information, the read counts of each matched gene were obtained using featureCounts software (v1.6.0) [8].

III Identification of DEGs and DELs

The DEGs and DELs between after therapy group vs before therapy group, before therapy group vs normal control group, and after therapy group vs normal control group were identified using the edgeR package (version 3.4, (Link 1)), respectively [9, 10]. Thresholds of DEGs and DELs were defined as Log fold-change (FC)> 0.585 and P value < 0.05. Then, the overlapping DEGs and DELs with opposite expression trend in the two comparisons (after therapy vs before therapy and before therapy vs normal control) were selected. After removing the DEGs and DELs identified between after therapy group and normal control groups from above genes, the remaining shared DEGs and DELs were screened for further analyses. Subsequently, heatmaps of these DEGs and DELs in three groups were drawn using pheatmap (version 1.0.8, (Link 2)) in R software.

IV GO and KEGG Pathway Analyses for DEGs

The GO-biological process terms and KEGG pathway of DEGs were enriched by clusterProfiler package (version 3.8.1) in R software [11]. Benjamini and Hochberg (BH) method was utilized to adjust the enrichment statistic P values. The significant GO-biological process terms and KEGG pathways for DEGs were selected with cut off of P. adjust < 0.05.

V Constructing Protein-Protein Interaction (PPI) Network Based on DEGs

STRING (version: 10.0, (Link 3)) was used to analyse PPI between DEGs, and PPI with score (medium confidence) > 0.4 was used to construct network [12]. Then PPI network was visualized by Cytoscape software (Link 4). Subsequently, CytoNCA plug-in (version 2.1.6, (Link 4)) was applied for topological analysis of nodes in PPI network [13]. Parameter was set as 'without weight', and the result of Degree Centrality (DC) was output. At last, the hub protein was identified according to their DC ranking.

VI Co-Expression Analysis Between DEGs and DELs

Firstly, Pearson's correlation coefficient of each DEG and DEL was calculated. Then, thresholds of $P<0.05$ and correlation coefficient $(r)>0.8$ were used to identify significantly co-expressed lncRNA-mRNA pairs.

VII Function and Pathway Analyses for DELs

The function prediction of DELs was analysed using its corresponding DEGs in above screened significantly co-expressed lncRNA-mRNA pairs. Similarly, the GO and KEGG pathway terms of corresponding DEGs were enriched by clusterProfiler package (version 3.8.1). The enrichment statistic P values were adjusted by BH method, and significant results were obtained under $P<0.05$.

VIII Identification of miRNA-lncRNA and miRNA-mRNA Interaction Pairs

The miRNAs relative to lncRNA and mRNA in above screened co-expressed lncRNA-mRNA pairs were analysed, respectively. miRNA-lncRNA pairs were predicted using lnCeDB database based on miranda tool. In addition, miRWALK2.0 (Link 5) database, which integrated the information of 12 databases (including miRWalk, Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, Pictar2, PITA, RNA22, RNAhybrid and Targetscan), was used to identify upstream miRNA of mRNA (species: Homo). Finally, shared miRNA-mRNA interactions in at least 7 above databases were used.

IX Comprehensive Analysis of ceRNA Regulatory Network

ceRNA regulatory network was constructed by integrating the lncRNA-miRNA interaction pairs, miRNA-mRNA pairs, and lncRNA-mRNA co-expressed pairs ($r>0.8$) using Cytoscape. Subsequently, the degree of nodes in ceRNA network was analysed using CytoNCA plugin in Cytoscape. Parameter was set as without weight, and nodes with the higher degree were more important in the network.

X IL1R1 and EBV Specific IgG Antibody Detections by ELISA

Serum of 45 BP patients and 32 normal individuals were tested for the presence of IL1R1 and EBV using IL1R1 ELISA kit (eBioscience, USA) and Epstein-Barr Virus-Viral Capsid Antigen (EBV-VCA) IgM, Human, BioAssay ELISA Kit (USBiological, Beijing, China) according to the manufacturer's instruction. Cut-off was defined with positive and

negative control serum that were included in each assay, according to the manufacturer's instruction.

XI Quantification of Plasmatic EBV DNA and IL1R1 RNA

Plasma from 45 BP patients and 32 normal individuals were recovered and conserved at -80°C until use. A volume of 200 µl of plasma was used to extract DNA using QIAamp DNA Mini Kit (Qiagen, France). Circulating levels of EBV DNA were measured by real-time quantitative polymerase chain reaction (q-PCR). The q-PCR reaction was performed using the TaqMan PCR Kit-24 Reactions (Norgenbiotek, CA) on ABI Prism 7700 sequence detection system (Applied Biosystems). The thermal cycling conditions were 95°C for 10 min and 45 cycles of 95°C for 15 sec, 58°C for 1 min, 68°C for 30 sec. For each run, two negative controls without any DNA template were included. Primers of PCR for detection of EBV DNA are Forward: 5′-TGGAAAC CCGTCA CTCTC-3′, Reverse: 5′-TA-ATGGCATAGGTGGAATG-3′.

Total RNA was extracted using the Ultraspec Phenol Kit (Biotecx, USA) according to the manufacturer's instructions. Then, cDNA was synthesized from total RNA using the cDNA Synthesis Kit (Roche, Germany) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR detected the levels of IL1R1 using an SYBR-green detection system on an ABI-7500 Real-time PCR System (Applied Biosystems, USA). The mRNA expression was expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, while miRNA expression was expressed relative to U6 as an internal control. The relative expression levels of miRNAs were evaluated using the $2^{-\triangle\triangle ct}$ method and expression levels were normalized relative to those of U6. The PCR amplification reaction was carried out in a 20-µl system, including 1 µl cDNA, as well as 1 µl forward primer and 1 µl reverse primer. The primer sequences used were listed as followed: IL1R1(261 bps): Primer F 5' CTGTCACCAGCCACTAAG 3'; Primer R 5' TTCCCAAGCCCTCTACTC 3'; GAPDH (218bps): Primer F 5' AATCCCATCACCATCTTC 3', Primer R 5' AGGCTGTTGTCATACTTC 3'. All PCR assays were performed in triplicate.

Results

I Identification of DEGs and DELs

In total, 587, 8339, and 8757 DEGs were identified between the comparison groups of after therapy vs before therapy, before therapy vs normal control, and after therapy vs normal control, respectively. In addition, a total of 214, 3252, and 3319 DELs were identified in the above comparisons (Table 1). Further, a set of 71 shared DELs and 200 shared DEGs with opposite expression trend in the comparisons of after therapy group vs before therapy and after therapy group vs control was obtained. After removing the DEGs and DELs identified between after therapy group and control groups from above genes, a total of 140 DEGs and 46 DELs related to treatment were finally identified. As shown in (Figure 1), the expression patterns of DEGs and DELs in after therapy group were basically consistent with control group, while were opposite with disease group, indicating that medicinal therapy intervention could obviously alter the expressions of mRNA and lncRNA.

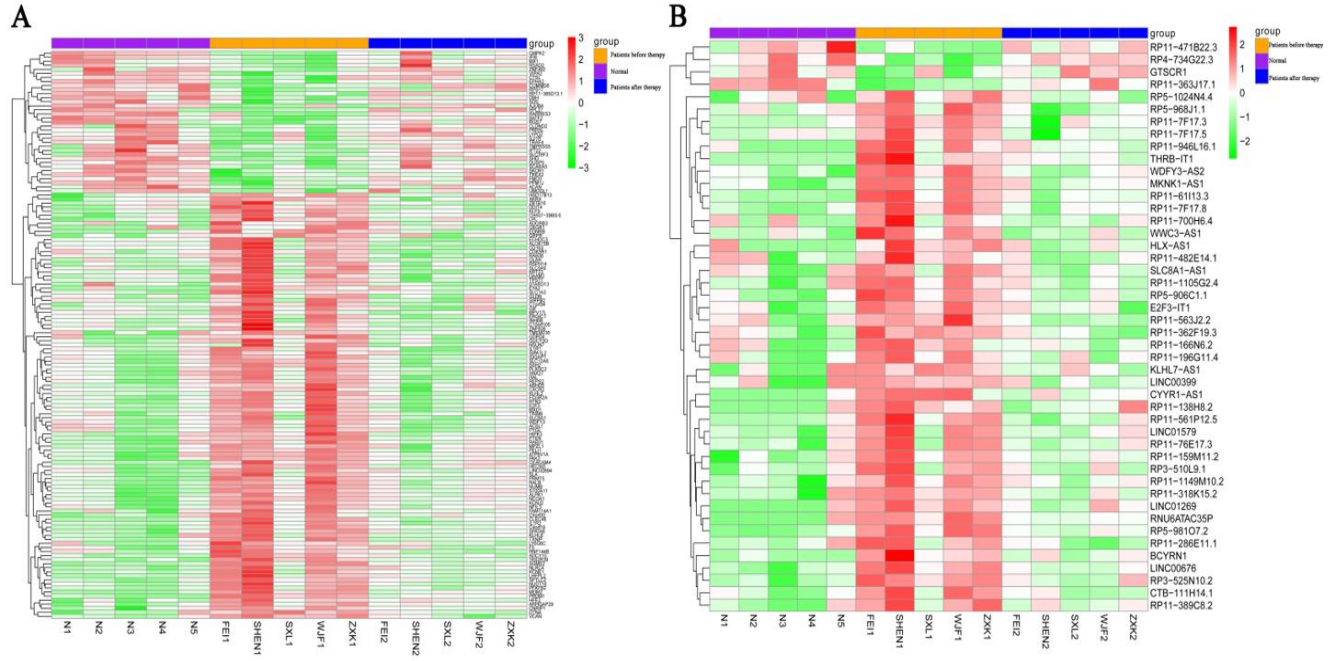

Figure 1: Heatmap of **A)** DEGs and **B)** DELs. Purple in the top indicates normal control samples; orange in the top indicates Bell's palsy samples without treatment; dark blue in the top indicates Bell's palsy samples with treatment. Green indicates down-regulated DEGs or DELs; Red indicates up-regulated DEGs or DELs.

DEGs: Differentially Expressed Genes; DELs: Differentially Expressed lncRNAs.

Table 1: The statistics of differently expressed genes and lncRNA identified by different group comparisons.

		before therapy vs normal control	after therapy vs before therapy	after therapy vs normal control
lncRNA	up	2424	56	2454
	down	828	158	865
	total	3252	214	3319
mRNA	up	4665	125	4799
	down	3674	462	3958
	total	8339	587	8757

II Functional Analysis of DEGs

Using pre-set threshold value, the DEGs were totally enriched in 111 GO BP and 4 pathways (Fatty acid biosynthesis, Prostate cancer, Melanoma and Glioma). Among the 111 GO BP terms, the DEGs were closely associated with the terms of "blood circulation" (e.g., *CXCR2*, *MDM* and *SLC8A1*), "negative regulation of phosphorylation", and "dephosphorylation" were closely associated with (Figure 2).

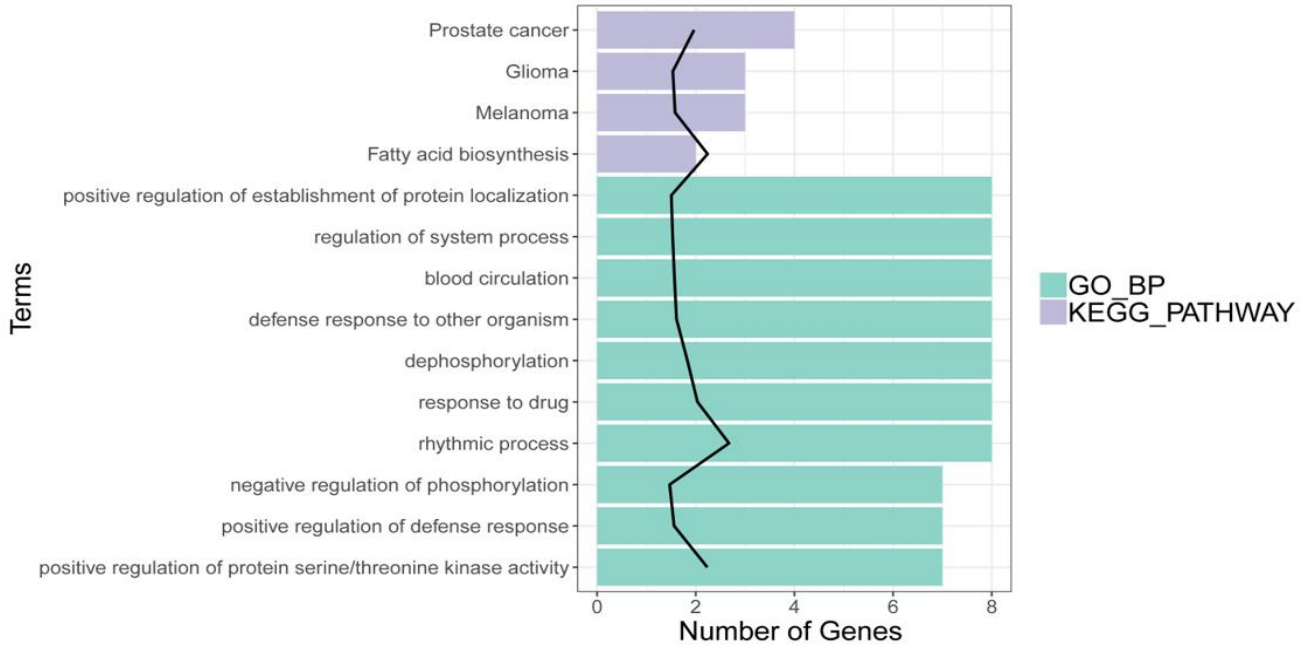

Figure 2: Results of GO function and KEGG pathway for DEGs. X-axis indicates the count of genes in the function terms and pathways; Y-axis indicates the GO terms and pathways.

III PPI Network Analysis

In PPI network, there were 78 nodes and 85 interaction pairs, including 59 up-regulated DEGs and 19 down-regulated DEGs (Figure 3). Based on the topological properties analysis, the top 10 hub genes were *PTEN* (degree=10), *ZBTB16* (degree=8), *KLHL2* (degree=7), *CXCR2* (degree=6) and *PRMT5* (degree=5), *IL1R1* (degree=4), *TXNIP* (degree=4), *TRIM9* (degree=4), and *LPL* (degree=4), *RNF144B* (degree=4), respectively.

IV lncRNA-mRNA Co-Expression Pairs and lncRNA Functional Prediction

Overall, 1039 lncRNA-mRNA pairs (e.g.) were identified after the lncRNA-mRNA co-expression analysis, including 105 DEGs and 43 DELs. In addition, 15 lncRNAs were enriched to 59 pathways such as Cytokine-cytokine receptor interaction (hsa04060), Sphingolipid metabolism (hsa00600), Arachidonic acid metabolism (hsa00590), and Th17 cell differentiation (hsa04659) (Figure 4). In addition, CYYR1-AS1(cysteine and tyrosine rich 1 antisense RNA 1) was enriched into most pathways, including Epstein-Barr virus infection (MDM2).

V miRNA Prediction and ceRNA Regulatory Network

Totally, 3107 miRNA-lncRNA interaction pairs were identified after InCeDB (Link 6), including 844 miRNAs and 35 lncRNAs. Additionally, 10698 miRNA-mRNA interactions were obtained, including 1487 miRNAs and 122 DEGs. Then, the miRNAs (degree ≥ 10) that had both regulatory pairs with co-expressed lncRNA and mRNA were selected, and corresponding ceRNA regulatory network was comprised of 1257 interaction pairs (involving 739 miRNA-mRNA pairs, 255 lncRNA-miRNA pairs and 363 lncRNA-mRNA co-expression pairs) and 174 nodes (involving 29 lncRNA, 73 miRNA and 72 mRNA) (Figure 5). In ceRNA regulatory network, *CTB-111H14.1*, *RP11-61I13.3*, *PFKFB2*, *SPTLC2* and *PAK2* were the top 5 nodes with higher degree values.

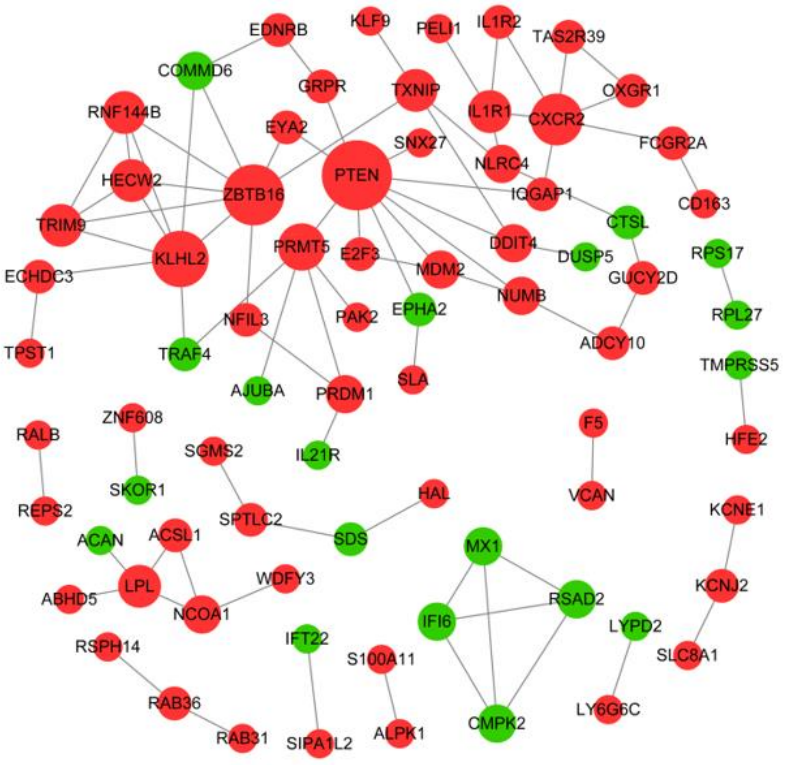

Figure 3: Protein-protein interaction network. Nodes in red circle indicate up-regulated DEGs, while node in green circle indicates down-regulated DEGs. Node size is proportional to its degree value.

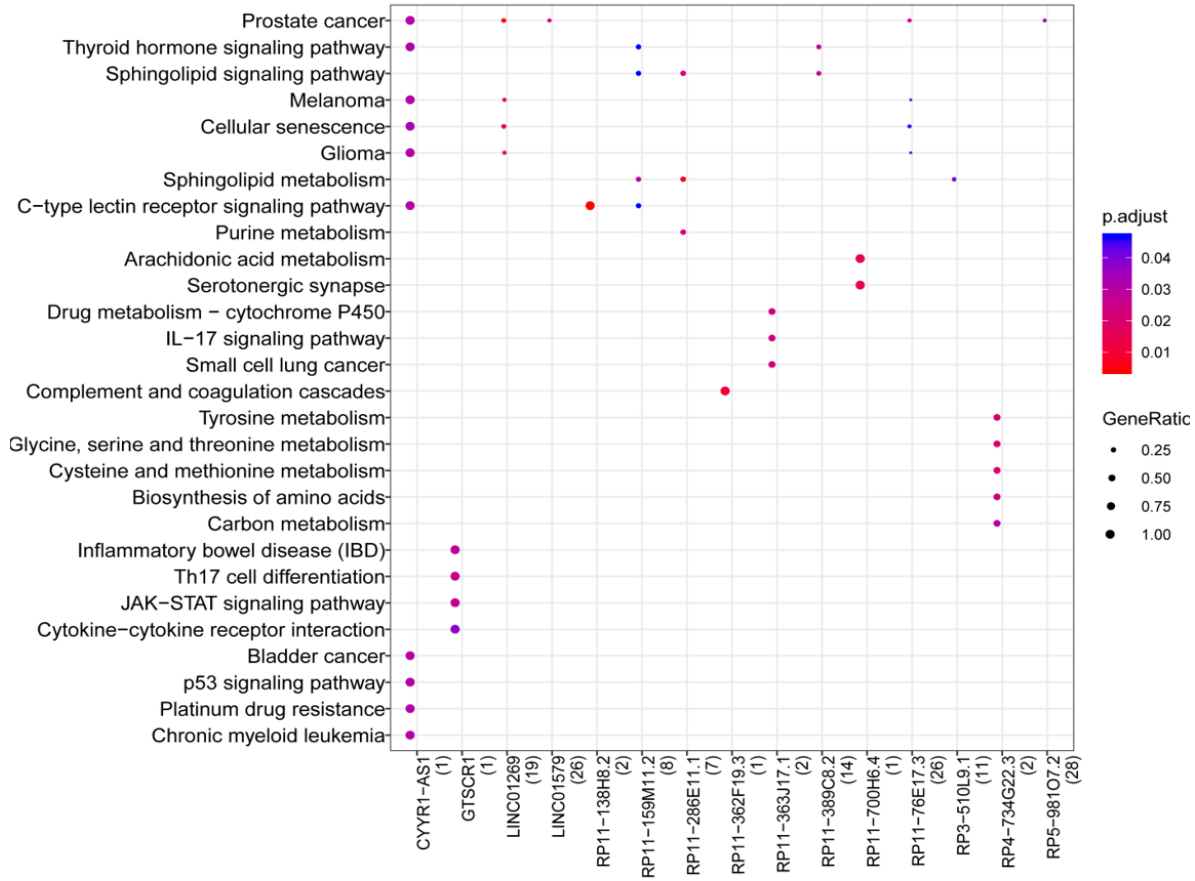

Figure 4: Results of pathway prediction for LncRNA. The color changes from red to blue represent the p values from small to large, while the bubble size indicates the proportion of gene counts involved in one term to all genes.

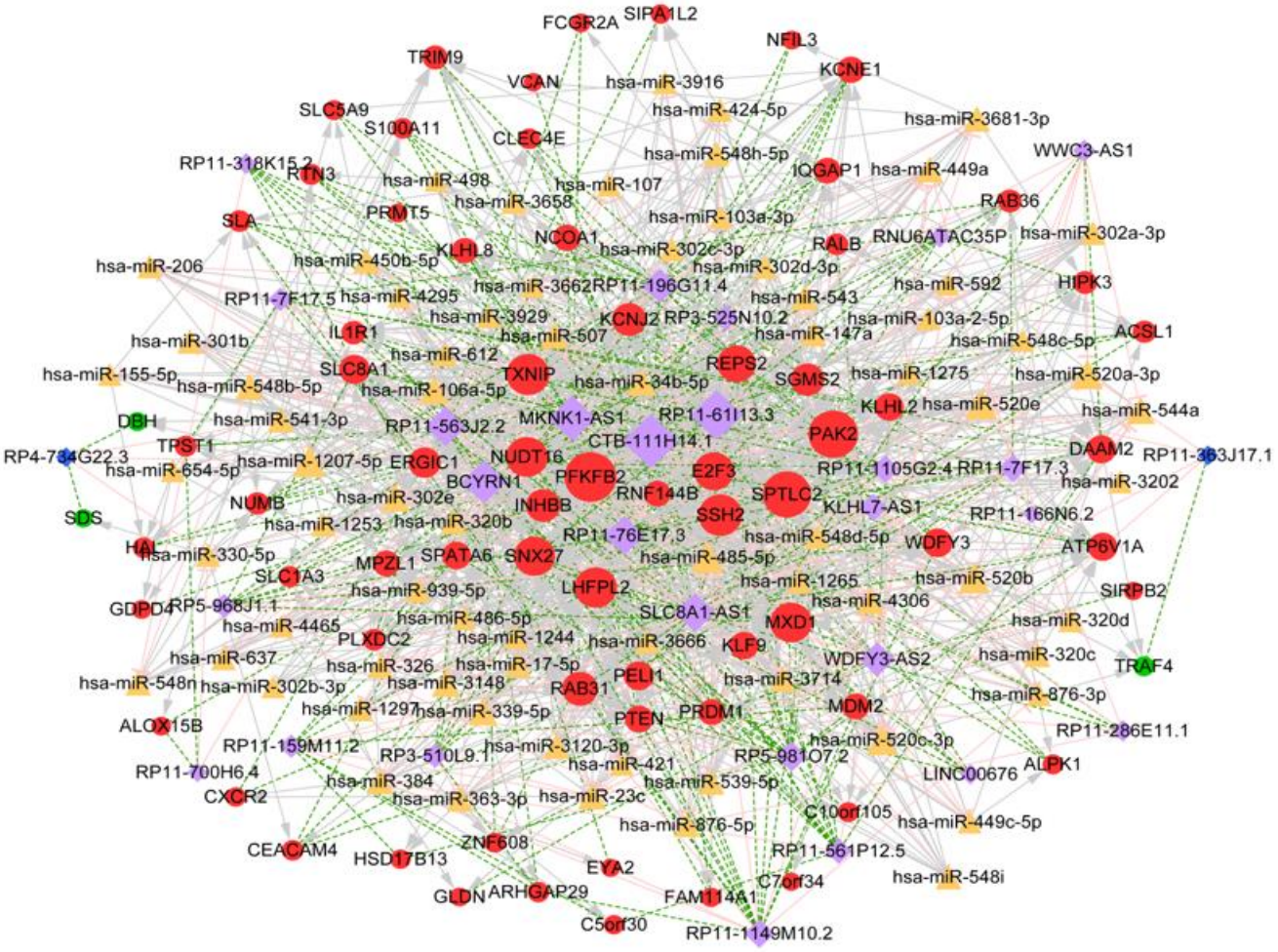

Figure 5: ceRNA regulatory network. Yellow triangle indicates miRNA; light purple rhombus indicates up-regulated lncRNA; dark blue rhombus indicates down-regulated lncRNA red circle indicates up-regulated genes, green circle indicates down-regulated genes. The higher the degree value, the more important the node is.

VI Validation of *IL1R1* and EBV

Serum IL1R1 of patients with Bell's palsy and health individuals as control were detected by ELISA and q-PCR analysis. As showed in (Figure 6), IL1R1 was upregulated in patients with Bell's palsy before therapy compared to that in Bell's palsy before therapy($P<0.01$) or health control ($P<0.01$). While serum EBV was not found in any group by ELISA or q-PCR analysis.

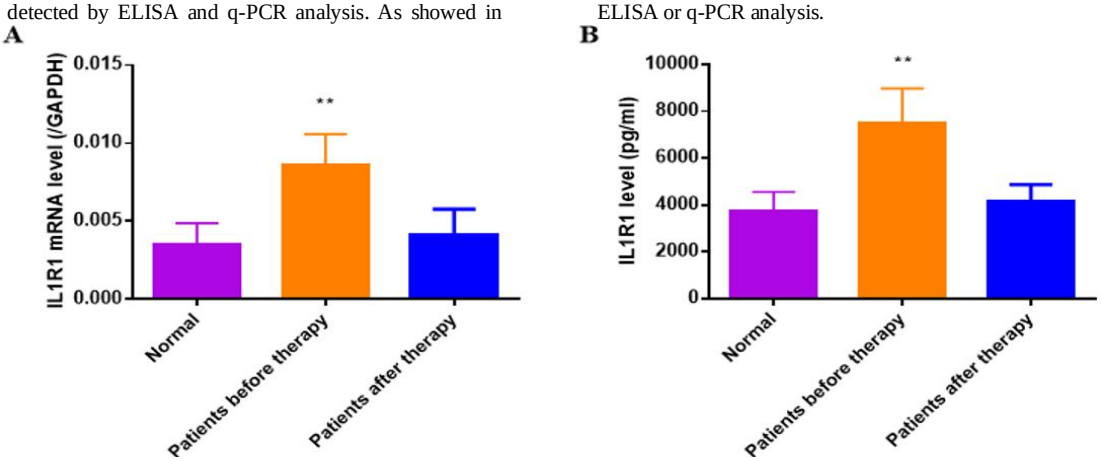

Figure 6: The IL1R1 mRNA and protein expression had been analysed by q-PCR and ELISA. **A)** The serum IL1R1 mRNA expression was up-regulated at the onset of BP compared to that of normal controls($P<0.01$), which decreased when the disease completely recovered ($P<0.01$). **B)** The serum IL1R1 protein expression of BP patient before therapy was detected higher than normal controls ($P<0.01$) and that of after therapy ($P<0.01$).

Discussion

In this study, a total of 140 DEGs and 46 DELs related to medicinal treatment were identified and several hub proteins such as CXCR2 (C-X-C Motif Chemokine Receptor 2) and IL1R1 (Interleukin 1 Receptor Type 1) were identified in PPI network. Subsequently, 1039 lncRNA-mRNA co-expression pairs (eg., CYYR1-AS1-MDM2), 3107 miRNA-lncRNA interaction pairs, and 10698 miRNA-mRNA interactions were obtained. Finally, 739 miRNA-mRNA pairs (eg., hsa-miR-147a-IL1R1), 255 miRNA-lncRNA pairs, and 363 mRNA-lncRNA co-expression pairs were included in ceRNA regulatory network. In addition, among DELs, *CYYR1-AS1* was enriched into most pathways, including Epstein-Barr virus infection.

CXCR2, (Link 7) an interleukin 8 (IL-8) receptor, plays a crucial role in neutrophil-driven inflammation and immune functions, motor-neuron degeneration [14, 15]. But CXCR2/IL-8 is more closely associated with tumor-related diseases, such as oesophageal squamous cell carcinoma, colon cancer, and mammary carcinoma, so it was not further verified and discussed in this study [16-19]. But interestingly, another cytokine had come into view which is IL1R1. IL1R1 is a receptor for interleukin-1 (IL-1) alpha and IL-1 beta, which has key roles in many cytokines induced immune and inflammatory responses [20]. A previous study confirms that IL-1, IL-6, and TNF-α levels are increased in serum samples from patients with Bell's palsy compared with that of healthy populations. We predicted that miR-147a was an upstream regulator of *IL1R1*. It has been reported that miR-147a as an anti-inflammatory factor, is upregulated by *IL-1b* stimulation in mesenchymal stem cells [21]. Similarly, upregulation of miR147a and miR147b may decrease the expression of pro-inflammatory factors IL-6 and COX-2 after IL-1β stimulation in astrocyte cells [22]. In this study, *IL1R1* was detected highly expressed in patients with Bell's palsy which indicated that IL1 might be involved in inflammation and immune regulation in the onset of Bell's palsy.

Actually, virus can indeed invade the central nervous system and cause paralysis of the facial nerve, including some cases who were diagnosed Bell's palsy that lack rigorous etiology tests. Among them, the most common viruses are the varicella-zoster virus, human herpesvirus 6, herpes simplex virus, enterovirus, and Epstein-Barr virus. In some opinions, virus infection is considered as one main etiology of peripheral facial paralysis [23-25]. Reportedly, Epstein-Barr virus infection is associated with bilateral facial nerve palsy, or even COVID-19 co-infection, including adults and infants [26, 27]. Additionally, in the study of Ramos *et al.*, it shows 70.5% Bell's palsy cases are positive to Epstein-Barr virus antibodies [19]. It has revealed that viruses may lead to axon degeneration via regulating abnormal expressions of *p53* to upregulate apoptosis modulator [28-30].

A possible explanation of Epstein-Barr virus mediated neural dysfunction is the activation of apoptotic pathways and intra-axonal degradation. For example, *IL-1* and *IL1R* were found increased after EBV infection or even in nasopharyngeal carcinoma cell growth [31-33]. IL-1 signaling plays an important role in inflammation and early activation of host innate immune responses following virus infection [34]. But it was both expected and unexpected that no Epstein-Barr virus was found in serum from neither Bell's palsy patients nor normal controls. Here we set a hypothesis that EBV invades the human body under specific conditions, it leads to a significant increase of *IL-1* and the damage of facial nerve function. However, EBV might colonize at specific sites but not in peripheral blood, such as facial ganglion, so it had not been detected in serum. Of course, this must be verified by further scientific experiments.

On the other hand, it was predicted in this study that *CYYR1-AS1* was enriched in "Epstein-Barr virus infection", and *MDM2* was a co-expressed target of "*CYYR1-AS1*". It has been suggested that Epstein-Barr virus nuclear antigen 3C enhances *p53* ubiquitination and degradation via deubiquitinating *MDM2* [35]. Cysteine and tyrosine-rich 1 (*CYYR1*)-*AS1*, located on human chromosome 21, play as a lncRNA to regulate the *CYYR1* locus [36]. Although no direct studies have reported a connection of *CYYR1-AS1* and Epstein-Barr virus infection, study has suggested that *CYYR1* is upregulated in patients with influenza under febrile convulsion, indicating the abnormal expression of *CYYR1* or *CYYR1-AS1* may be response to virus infection [37].

However, there were some limitations in this study at present. Serum samples rather than tissue samples were detected in this study, so it can only indirectly explain the situation of pathogen infection of nerve tissue, but to harvest a tissue sample in a living individual is really a very difficult task. And the key DELs and DEGs expression in Bell's palsy patients had not been verified through experiment due to a longtime span in numbers of cases. Based on the current exploratory research with insufficient samples meeting this need, the experiment was not performed in this study. In the future, large scale investigations for the causes of the disease are in need and further confirmation is needed to determine whether there is a viable EB virus in the ganglion. Last but not least, how does Epstein Barr virus invade facial nerve and increase *IL1R1* should be put into consideration.

Conclusion

In conclusion, the occurrence and development of Bell facial paralysis is quite complex and may involve related to complex miRNA-lncRNA-mRNA interacting networks and *IL1* might be involved in inflammation and immune regulation in the onset of Bell's palsy. Results of the present study indicated the direction for further exploration in the future.

Ethical Approval

The Institutional Ethics Committee, Baoshan Hospital of Integrated Traditional Chinese Medicine and Western Medicine, Shanghai approved the study (Approval No. 201809-03). This study has been registered at the Chinese Clinical Trials Registry (ChiCTR1800018972).

Consent

All participants had signed informed consent and agreed to supply their blood samples for research use and publication.

Data Availability

The data and material in this study is available from the corresponding author on reasonable request. (Link 8)

Competing Interests

None.

Funding

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Author Contributions

Zhidan Liu: Conception, Design and Administrative Support; Xiaoyan Li: Provision of Study Materials or Patients; Chuang Zhao and Chunlan Chen: Collection and Assembly of Data; Xiaoyan Li, Ying Zhao, Chuang Zhao, Zunyuan Li, Wenge Huo: Data analysis and Interpretation; All authors: Manuscript Writing; All authors: Final Approval of Manuscript.

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Not applicable.

Abbreviation

DEGs: Differentially Expressed Genes
DELs: Differentially Expressed lncRNAs
ceRNAs: Competing Endogenous RNAs
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
EBV: Epstein-Barr Virus
PPI: Protein-Protein Interaction
q-PCR: quantitative Polymerase Chain Reaction
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase
CYYR1-AS1: Cysteine and Tyrosine Rich 1 Antisense RNA 1
MDM2: Murine Double Minute2
CXCR2: C-X-C Motif Chemokine Receptor 2
IL1R1: Interleukin 1 Receptor Type 1

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