Acta virologica **66**: 216 – 227, 2022 doi: 10.4149/av_2022_303

A gene expression signature that correlates with CD8+T cell expansion in acute Epstein-Barr virus infection

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Received February 16, 2022; accepted May 31, 2022

Summary. – Acute infectious mononucleosis (AIM) is associated with Epstein-Barr virus (EBV) infection. We explored molecular mechanisms regarding the expression of CD8+T cells in convalescence stage (CONV). Differentially expressed genes (DEGs) were identified by analyzing GEO expression profiles. Subsequently, Gene Set Enrichment Analysis (GSEA), Protein-Protein Interactions (PPI) network, and gene-micro RNAs networks were used to identify hub genes and associated pathways. GSEA provided evidence that the top 3 gene sets in GSEA were all related to integrins. We identified ten hub genes in the PPI network and DGIdb was applied to predict potential targets that might reverse the expression of hub genes. Our study enhances a mechanistic understanding of the CD8+T cells expansion in acute EBV infection and provides potential treatment targets for further research.

Keywords: acute infectious mononucleosis; bioinformatics; CD8+T cells; differentially expressed genes; EBV

Introduction

Epstein-Barr virus (EBV) is arguably the most ubiquitous of human viruses, infecting at least 90% of adults worldwide (de-Thé *et al.*, 1975). Infectious mononucleosis is the disease named after an acute infectious disease consisting of fever, cervical lymphadenopathy and pharyngitis accompanied by atypical large peripheral blood lymphocytes. Its major cause is EBV (Balfour *et al.*, 2015). Prospective studies have determined that 75% of young adults between the ages of 18 and 22 develop typical infectious mononucleosis after primary EBV infection. Approximately 15% of adults have atypical symptoms and 10% are completely asymptomatic (Balfour *et al.*, 2013). Acute infectious mononucleosis (AIM) is associated with a massive CD8+ T-cell expansion, while symptoms can vary greatly in severity from a mild short influenza-like illness to a more severe syndrome with sore throat, lymphadenopathy, splenomegaly, hepatomegaly, and debilitating fatigue lasting months (Taylor *et al.*, 2015; Luzuriaga *et al.*, 2010). Virus-specific CD8+ T cells expand dramatically during acute EBV infection, and their persistence is important for lifelong control of viral replication (Catalina *et al.*, 2001; Moss *et al.*, 2001; Callan *et al.*, 1998). The short half-life of EBV load, together with the strong correlation between the number of EBV-specific CD8+ T cells and the rate of change of viral load indicates an active role for EBV-specific CD8+ T cells in elimination of EBV in AIM (Hoshino *et al.*, 2011) {Hoshino, 2011 #823}{Hoshino, 2011 #823}. With the rapid progress and widespread application of high-throughput system (HTS) technologies, integrated bioinformatics analysis has emerged as a promising approach to explore gene expression signature that correlates with CD8+T cell expansion in acute Epstein-Barr virus infection.

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Abbreviations: AIM = acute infectious mononucleosis; CDK = cyclin-dependent kinase; CCNA2 = cyclin A2; DEGs = differentially expressed genes; DGIdb = The Drug Gene Interaction Database; EBV = Epstein-Barr virus; GO = Gene Ontology; GSEA = Gene Set Enrichment Analysis; KEGG = Kyoto Encyclopedia of Genes and Genomes; LPAM-1 = α4β7 integrin; PLK = polo-like kinase; PPI = Protein-Protein Interactions

In this study, we analyzed the RNA expression profiles downloaded from the GEO database and found out that patients infected with acute infectious mononucleosis have EBV-specific CD8+ T cells in the acute and convalescent phases. We screened out differentially expressed genes. Subsequently, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) were used to study the molecular mechanisms related to regulation, and a Protein-Protein Interactions (PPI) network was constructed. Cytohubba was used to identify genes and hub genes. Finally, the Network Analyst database was used to analyze miRNAs, and the Drug Gene Interaction database (DGIdb) was used to predict possible interacting drugs or molecular compounds. Identifying viral proteins that can induce the strongest T cell response is crucial for vaccine design and the development of immunotherapy for EBV-related diseases.

Materials and Methods

Microarray data. We used GEO (http://www.ncbi.nlm.nih.gov/geo), a public functional database containing array- and sequence-based data for our study. In GSE71045, Affymetrix HuGene ST 1.0 microarrays were used to study and compare gene expression in peripheral blood CD8+ T cells of human patients with acute infectious mononucleosis (AIM; acute EBV infection) and during convalescences (CONV; 6–12 months after AIM). Blood samples were drawn from ten human patients infected with AIM and again in their CONV. Peripheral blood mononuclear cells were dissociated and frozen. Paired AIM and CONV samples were thawed and CD8+ T cells were purified with magnetic beads. RNA was extracted and processed according to the Affymetrix protocol.

Identification of differentially expressed genes. We downloaded the GSE71045 gene expression data set and divided it into two groups by expression status, AIM and CONV. We used the GEO2R platform to analyze DEGs, and constructed volcano maps using Sangerbox software. $p < 0.05$ and $|\log FC| \geq 1$ were set as the standards. The genes that fulfill the requirements of $p < 0.05$ and $|\log FC| \geq 1$ were screened out as up-regulated DEGs and genes that fulfill the requirements of $p < 0.05$ and $\log FC \leq -1$ were screened out as down-regulated DEGs. Heatmap was constructed by http://www.bioinformatics.com.cn.

Functional and pathway enrichment analysis. GO analysis was used to identify the biological characters of genes, including biological process (BP), cell components (CC), and molecular function (MF) (The Gene Ontology (GO) project 2006). KEGG analysis provided full biological interpretation to genomic sequences and protein-protein interactions (Kanehisa and Goto, 2000).

Gene set enrichment analysis. Further, GSEA was carried out for all genes that were detected by use of GSEA software (version 4.1.0) (Subramanian *et al.,* 2005), providing another method to find significant differential biological functions between AIM and CONV. Gene sets were considered to be significantly enriched with the standard of $p < 0.05$ and a false discovery rate (FDR) < 0.25.

Construction of PPI network and identification of hub genes. We used STRING (http://www.string-db.org/), an online platform to find the interactions between proteins, and to construct the PPI network of DEGs. Cytoscape software was used to visualize the PPI network for DEGs (Shannon *et al.,* 2003). A plug-in of Cytoscape and Cytohubba was used to screen the essential nodes in the network to explore the hub genes which were contained in the PPI network (Chin *et al.,* 2014).

Construction of the target gene-miRNA network and the target gene-TF network. We applied Network Analyst (https://www.networkanalyst.ca/) to integrate miRNA potential databases; we also used miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php) and Regnetwork (http://www.regnetworkweb.org/) to predict the interactions among DEGs and miRNAs (Xia *et al.,* 2015). We visualized target gene-miRNA networks by Cytoscape software.

Identification of the potential drugs. DGIdb version 4.2.0 (https://www.dgidb.org) is an effective approach to find the drug target and sensitive genome and drug–gene interaction. We used the DGIdb to identify and predict drugs or molecular compounds interacting with the DEGs.

Results

Identification of DEGS

The DEGs are shown in the volcano plots and the heatmaps show sequence clustering performed with Euclidean distance. There were 226 DEGs between AIM and CONV, of which 169 DEGs were upregulated and 57 were downregulated (Fig. 1).

Gene ontology and KEGG pathway enrichment analyses

We used DAVID Bioinformatics Resources to conduct GO analysis and KEGG analysis on DEGs. The result of GO enrichment of upregulated DEGs indicated that for biological process (BP), DEGs were significantly enriched in DNA replication, G1/S transition of mitotic cell cycle, mitotic nuclear division, cell division, DNA replication initiation, sister chromatid cohesion, G2/M transition of mitotic cell cycle and cell proliferation. Regarding cellular component (CC), DEGs were significantly enriched in nucleoplasm, nucleus, midbody. For molecular function (MF), genes were significantly enriched in protein binding, ATP binding, microtubule binding.

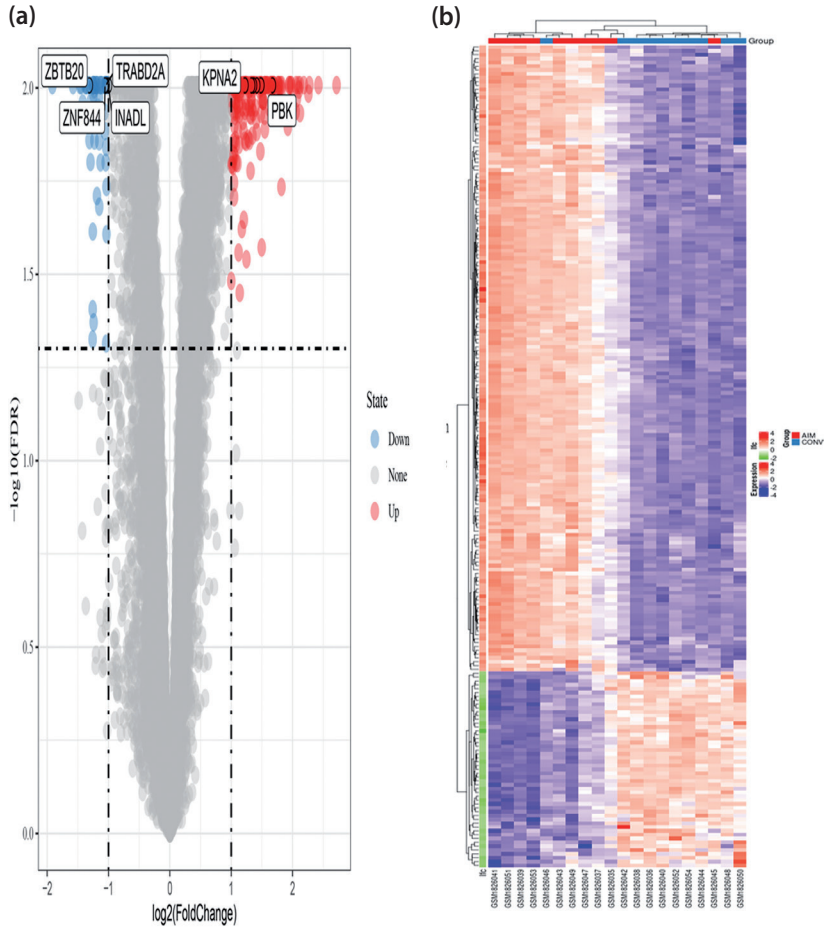

Fig. 1

Volcano map and heat map of DEGs

(a) Volcano map. Differentially expressed genes in AIM and CONV samples are shown in the volcano plot. The blue dots represent downregulated genes in AIM samples and the red dots represent upregulated genes. **(b)** Heatmap of differentially expressed genes. Data points in red represent upregulated, and blue represent downregulated genes.

The GO enrichment result of downregulated DEGs indicated that for BP, DEGs were significantly enriched in cell division, DNA repair and cell proliferation. Regarding CC, DEGs were significantly enriched in nucleoplasm, cytosol and nucleus. For MF, DEGs were significantly enriched in protein binding and ATP binding.

KEGG pathway-enrichment indicated that upregulated DEGs were mainly enriched in DNA replication, cell cycle, progesterone-mediated oocyte maturation and oocyte meiosis, while downregulated genes were mainly enriched in pyrimidine metabolism (Fig. 2).

GSEA analysis

GSEA was performed to identify the possible mechanism on CD8+ T cell expansion. Samples were divided into AIM group and CONV group. 4163/4616 gene sets were upregulated in phenotype CONV and 453/4616 gene sets were upregulated in phenotype AIM. GSEA plots showed the most enriched gene sets of all detected genes between AIM and CONV in the GSE71045 dataset (Fig. 3, Table 1).

PPI network analysis and hub gene selection

The top 10 hub genes selected by the Degree method in the Cytohubba plug-in included CDK1, CCNA2, CDC20, CCNB2, BUB1, PLK1, KIF11, TOP2A, NDC80, CDCA8. The PPI analysis results suggested that CDK1, CCNA2 and CDC20 were the most influential factors (Fig. 4).

Construction of the target gene-miRNA network

The top 3 targeted DEGs for miRNAs were BUB1 that was modulated by 67 miRNAs, CDK1 that was modu-

Table 1. The top gene sets in GSEA

Gene set	Size	ES	NES	NOM *p*-value	FDR *q*-value	Rank at max	Leading edge
REACTOME_GRB2_SOS_PROVIDES_LINKAGE_TO_MAPK_SIGNALING_FOR_INTEGRINS_	15	0.711	3.253	0	0	3,693	tags=53%, list=16%, signal=64%
REACTOME_INTEGRIN_SIGNALING	27	0.603	3.144	0	0	4,096	tags=37%, list=18%, signal=45%
REACTOME_P130CAS_LINKAGE_TO_MAPK_SIGNALING_FOR_INTEGRINS	15	0.667	2.855	0	0	3,693	tags=40%, list=16%, signal=48%
REACTOME_ION_TRANSPORT_BY_P_TYPE_ATPASES	55	0.484	2.837	0	0	3,708	tags=31%, list=16%, signal=37%
PID_ARF6_TRAFFICKING_PATHWAY	48	0.574	2.743	0	0	4,099	tags=42%, list=18%, signal=51%

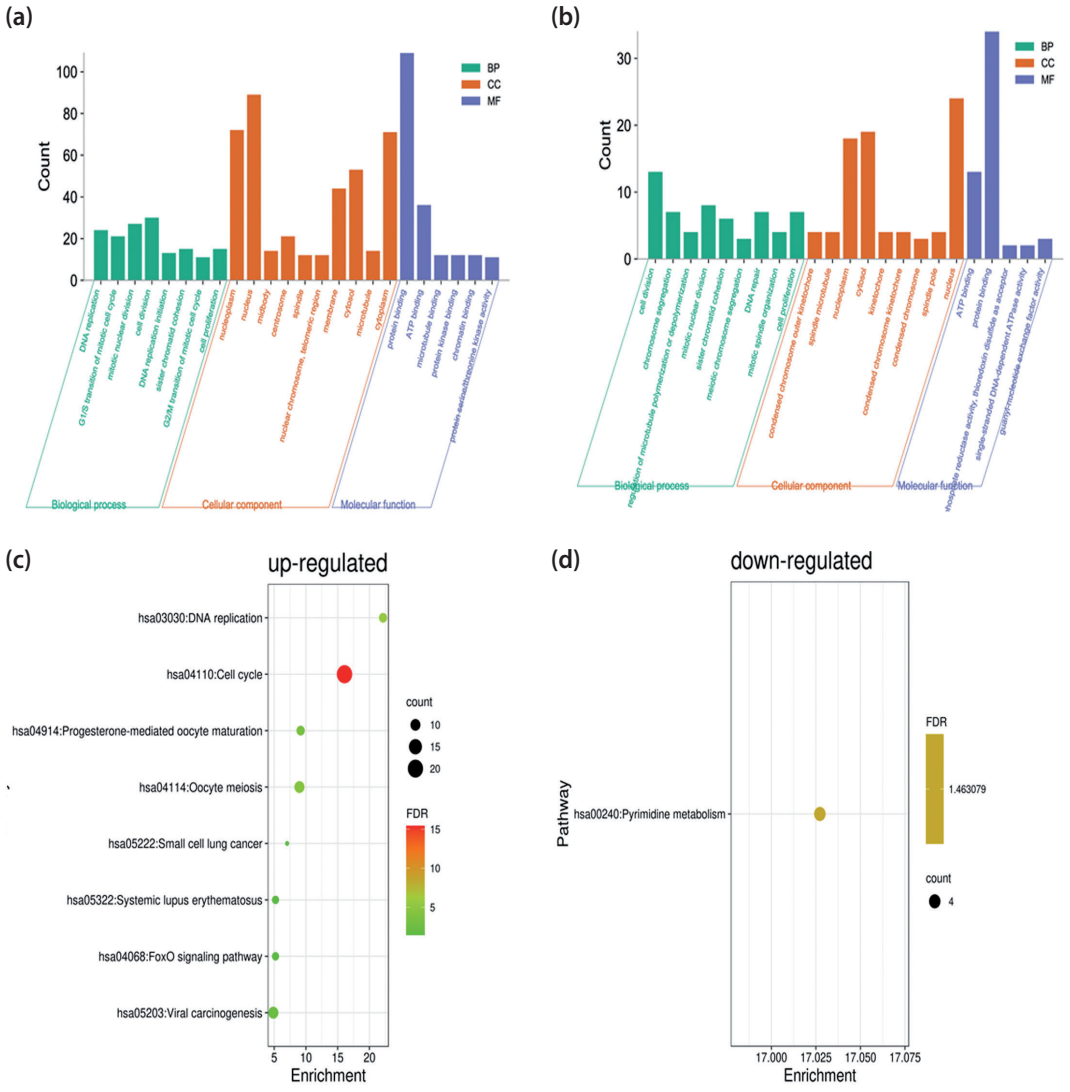

Fig. 2

GO analysis and KEGG analysis of DEGs

(a) GO analysis of upregulated DEGs. **(b)** GO analysis of downregulated DEGs. Different colors stand for different GO classifications. **(c)** KEGG analysis of upregulated DEGs. **(d)** KEGG analysis of downregulated DEGs.

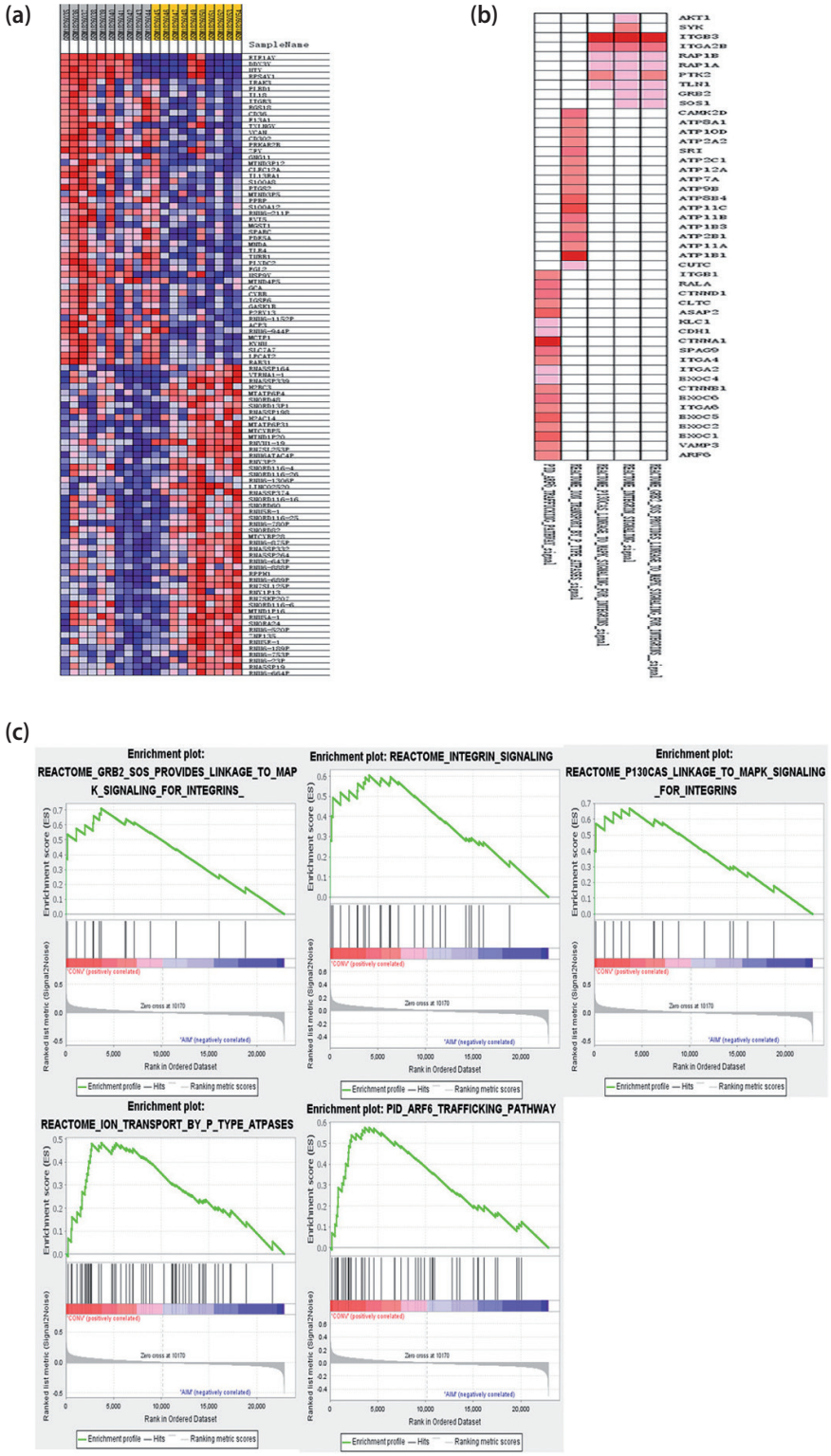

Fig. 3

GSEA analysis revealed substantial enrichment between AIM and CONV in GSE71045 dataset

(a) Heat Map of the top 50 features for each phenotype. **(b)** The graph uses color intensity to show the overlap between subsets. **(c)** The top 5 gene sets in GSEA. According to the order of the normalized enrichment scores (NES) from high to low, the top five pathways of GSEA were screened out.

lated by 52 miRNAs, and PLK1 that was modulated by 39 miRNAs. The miRNA that may control the largest number of DEGs (7 genes) was hsa-mir-193b-3p (Fig. 5, Table 2).

Identification of the potential drugs

DGIdb was applied to determine the potential drug or molecular compounds that could reverse the expression of DEGs between AIM and CONV. As shown in the Table 3, 4 drugs or molecular compounds included CHEMBL1082552, aruncin B, riviciclib, flianesib which differentially regulated the expression of CDK1. In addition, 3 drugs or molecular compounds, for instance, CHEMBL481931, AZD-4877, NMS-1286937, were found to interact with KIF11. Further, three drugs or molecular compounds that included GSK-461364, TAK-960, AMONAFIDE regulated PLK1 and 3 drugs or molecular compounds that included amrubicin, valrunicin and CHEMBL319467 regulated TOP2A (Table 3).

Table 3. The potential drug/molecular compounds

Gene	Drug	Interaction type and directionality	Query score	Interaction score
	CHEMBL1082552	n/a	8.71	1.93
CDK1	ARUNCIN B	n/a	8.71	1.93
	RIVICICLIB	inhibitor	4.36	0.96
	FLIANESIB	n/a	13.07	10.02
KIF11	CHEMBL481931	n/a	13.07	10.02
	AZD-4877	inhibitor	8.71	6.68
	NMS-1286937	inhibitor	13.07	0.95
PLK1	GSK-461364	inhibitor	10.89	0.79
	TAK-960	inhibitor	8.71	0.63
	AMONAFIDE	n/a	17.42	2.34
TOP2A	AMRUBICIN	inhibitor	13.07	1.76
	VALRUBICIN	inhibitor	8.71	1.17
	CHEMBL319467	n/a	4.36	1.83
CCNA2	MERIOLIN 3	n/a	4.36	1.83
	CHEMBL341273	n/a	4.36	1.83

Discussion

Most people have an asymptomatic herpes virus infection; however the virus is life-threatening for people with immunodeficiency. This reflects the key role of T cells, especially CD8+ T cells that play an important role in the control of the herpes virus infection. EBV is a pathogenic human herpes virus. Primary EBV infection produces a severe flu-like disease called infectious mononucleosis (Taylor *et al.,* 2015), while persistent infections are associated with a series of EBV-related cancers like Burkitt's lymphoma (Epstein *et al.,* 1964, 1966), Hodgkin lymphoma (Hjalgrim *et al.,* 2003), nasopharyngeal carcinoma (Old *et al.,* 1966; zur Hausen *et al.,* 1970), and gastric cancer (Shibata *et al.,* 1991). In the course of acute EBV infection, the number of virus-specific CD8+ T cells increased largely, and their persistence is significant to the life-long control of EBV-related diseases. To determine the generation and maintenance of these effective CD8+ T cell responses, microarray technology was used to analyze the gene expression and EBV-specific CD8+ T cells in the peripheral blood of 10 patients with acute infectious mononucleosis AIM. The expression of T cells was characterized in both AIM and CONV stages.

In this study, comprehensive bioinformatics methods were used to analyze the changes in the expression of key genes. According to the GEO dataset (GSE 71045), we identified 226 DEGs, including 57 down-regulated and 169 up-regulated DEGs ($p < 0.05$). Further functional enrichment analyses were used to identify the role of upregulated and downregulated DEGs.

GSEA plots showed the most enriched gene sets of all detected genes in the subjects between AIM and CONV

Table 2. miRNAs and its target genes

miRNA	Genes targeted by miRNA	Gene count
has-mir-495-3p	CDK1, TOP2A	2
has-mir-5688	CDK1, TOP2A	2
has-mir-24-3p	CDK1, CCNA2	2
has-mir-6507-5p	CDK1, KIF11	2
has-mir-548aa	CDK1, BUB1	2
has-mir-186-3p	CDK1, BUB1	2
has-mir-548t-3p	CDK1, BUB1	2
has-mir-548ap-3p	CDK1, BUB1	2
has-mir-340-5p	KIF11, BUB1	2
has-mir-186-5p	TOP2A, KIF11, BUB1	3
has-mir-34a-5p	KIF11, CDC20	2
has-mir-30a-5p	KIF11, CDC20	2
has-mir-92a-3p	CDK1, CDC20	2
has-mir-193b-3p	TOP2A, KIF11, BUB1, CDC20, NDC80	5
has-mir-22-3p	CCNA2, PLK1	2
has-let-7b-5p	CCNA2, PLK1, CDCA8, CCNB2	4
has-mir-4779	PLK1, CDCA8	2
has-mir-23b-3p	CDC20, CCNB2, CDCA8	3
has-mir-10b-3p	BUB1, PLK1, CCNA2	3
has-mir-16-5p	CDC20, PLK1, CDK1, CDCA8	4

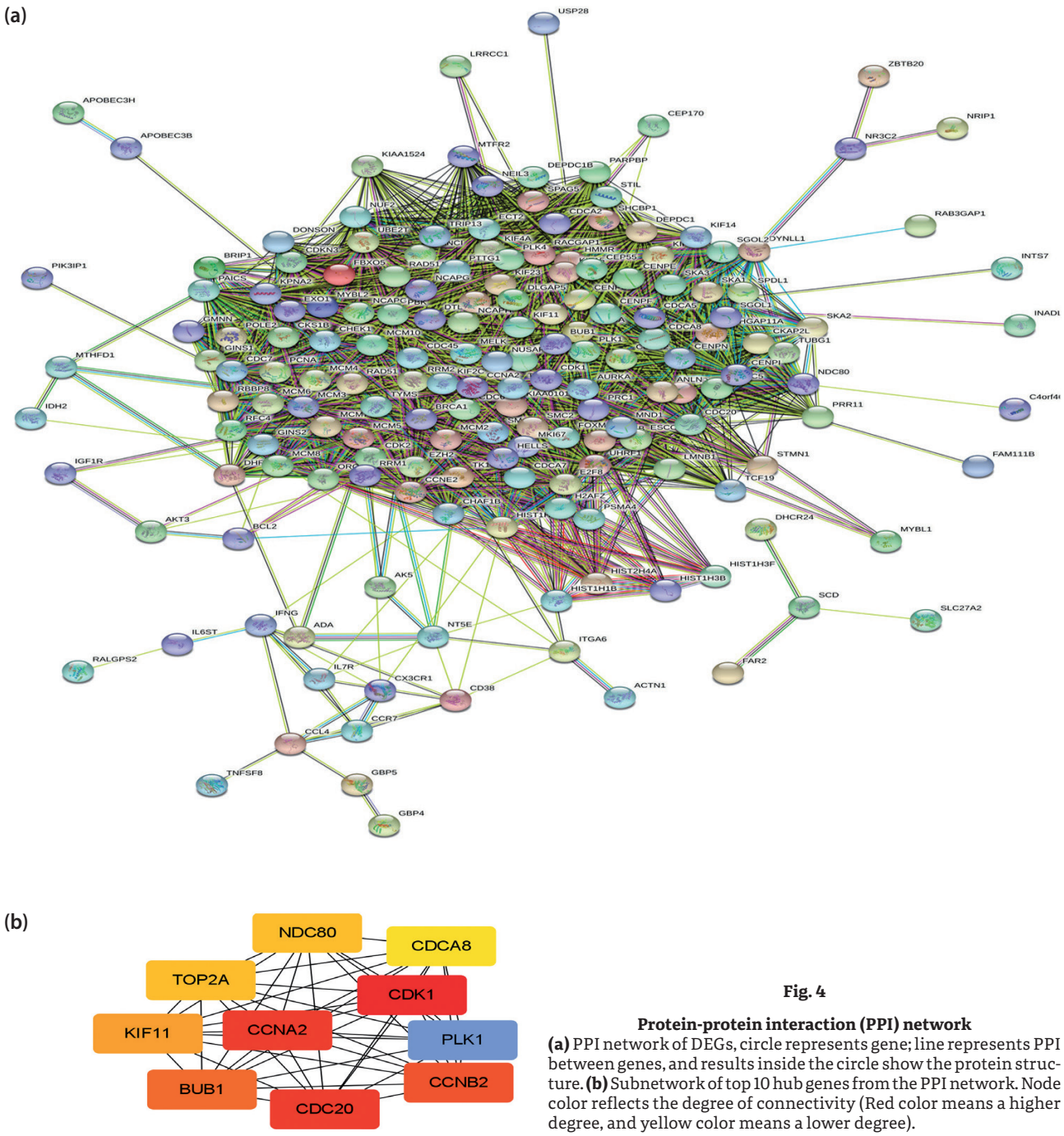

Fig. 4

Protein-protein interaction (PPI) network

(a) PPI network of DEGs, circle represents gene; line represents PPI between genes, and results inside the circle show the protein structure. **(b)** Subnetwork of top 10 hub genes from the PPI network. Node color reflects the degree of connectivity (Red color means a higher degree, and yellow color means a lower degree).

in the GSE71045 dataset. GSEA suggested that most of the genes mainly enriched in GRB2 SOS provide linkage to MAPK signaling for integrins, integrin signaling pathway, P130CAS linkage to MAPK signaling for integrins, ion transport by p type ATPases, and ARF6 trafficking pathway. It was found that ITGB3, ITGA2B, RAP1B, RAP1A, PTK2 and TLN1 genes appeared frequently in the above pathways.

By constructing a PPI network and analyzing by Cytohubba, 10 genes were identified: CDK1, CCNA2, CDC20, CCNB2, BUB1, PLK1, KIF11, TOP2A, NDC80, and CDCA8. Among these hub genes, only TOP2A was downregulated in AIM.

miRNAs are endogenous non-coding RNA molecules targeting the 3'UTR region of genes with a length of 18–22 nt and can regulate gene expression at a post-tran-

scriptional level to degrade or inhibit the translation of target genes. Thus, we predicted the network between miRNAs and the hub genes. miR-22-3p was revealed to control SIRT1 in periodontal ligament stem cell (PDLSC) and to regulate the proliferation and differentiation of PDLSC by SIRT1 silencing (Zheng *et al.*, 2020). The expression of Fas and miRNA hsa-let-7b-5p in addition to traditional risk factors could also increase the dis-

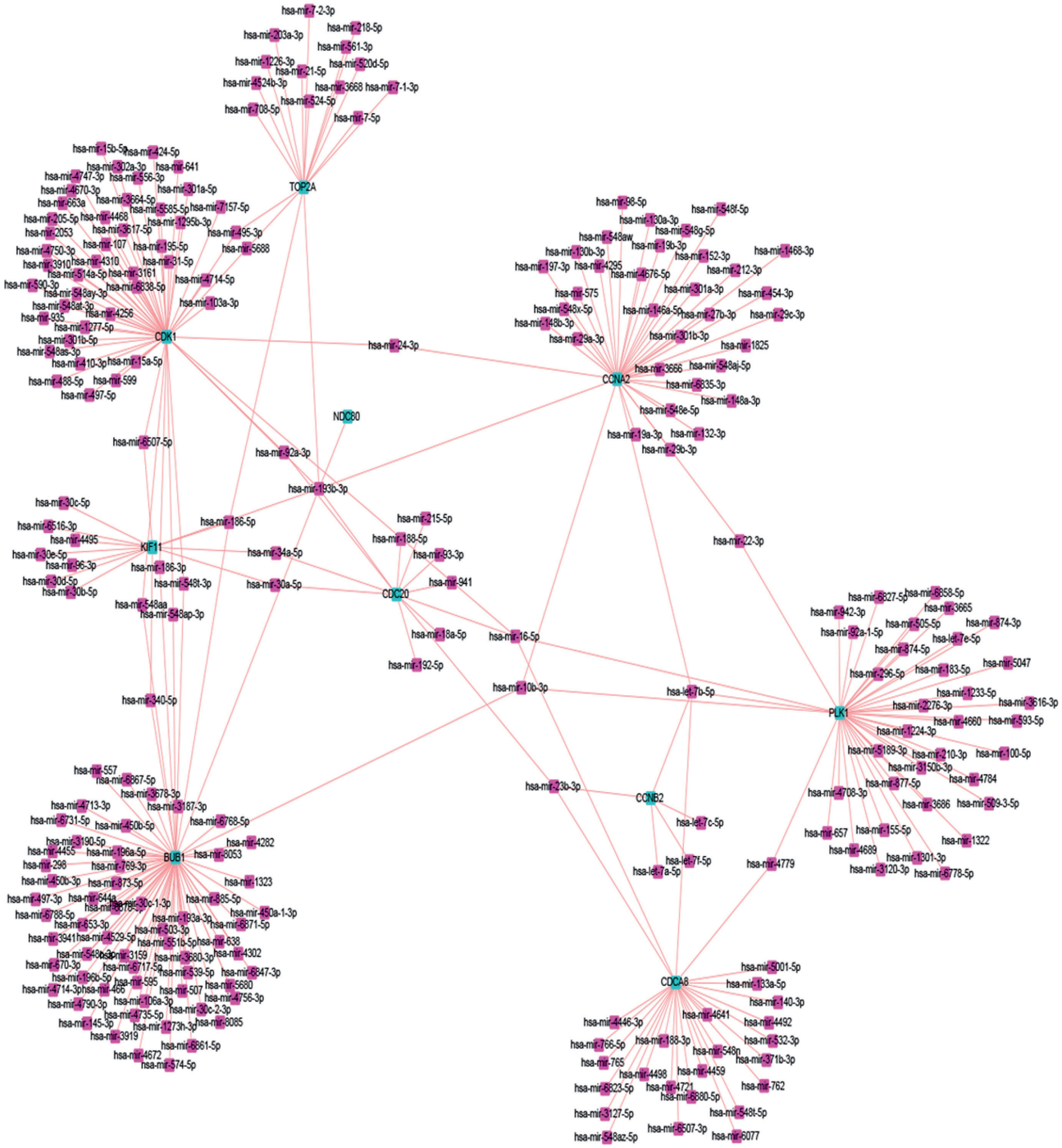

Fig. 5

Target gene-miRNA network
The blue nodes represent the 10 hub genes, and the pink nodes represent miRNAs.

crimination and predictive ability for poor prognosis (Chi *et al.,* 2020).

Cyclin-dependent kinases (CDKs) are a family of protein kinases that drives the major events of cell cycle in eukaryotic cells (Holt *et al.,* 2009). A large number of articles have illustrated that the dysregulation of CDKs induces not only tumor growth, but also continued or spontaneous proliferation of cancer cells (Malumbres *et al.,* 2009). Consistent with our result, CDK1 and PLK1 were upregulated in AIM. In addition, long-term results are linked to Hodgkin lymphoma and multiple sclerosis (Balfour *et al.,* 2015). CDK1 played an important role in AIM. Emerging evidence showed that tumor cells might require specific CDKs interphase for proliferation. Thus, selective CDK inhibition might provide therapeutic benefits against specific human tumors (Malumbres *et al.,* 2009). In a murine xenograft model, the Sp1 inhibitor could downregulate the expression of CDK1 and significantly suppressed the growth of established NNKTL (nasal natural killer/T-cell lymphoma), also an EBV-related tumor (Nagato *et al.,* 2019). It was shown that targeting CDK1 could specifically sensitize tumor cells to DNA-damaging agents without affecting the sensitivity of normal epithelial cells (Johnson *et al.,* 2009). PLK1 interference could inhibit the proliferation and invasion of Raji cells, and induced G2 arrest and apoptosis, which provided novel therapeutic targets for EBV-associated lymphomas (Tang *et al.,* 2019). The study of Duane H. Hamilton showed that high expression of brachyury drove the loss of the cyclin-dependent kinase inhibitor 1 resulting in decreased tumor susceptibility to immune-mediated lysis, and the reconstitution of CDK inhibitor 1 expression increased the lysis of brachyury-high tumor cells mediated by antigen-specific CD8+ T cells (Hamilton *et al.,* 2018). Several cyclin genes have been shown to be overexpressed in cancer and cell cycle regulators have been suggested as potential targets of antitumor immune intervention (Hunter and Pines, 1991). Cyclin A2 (CCNA2) has been implicated in the pathogenesis of cancer and it is overexpressed in acute and chronic leukemia and lymphoma as well as several solid tumors (Paterlini *et al.,* 1993). Cyclin A2 is an attractive candidate for immune intervention in a significant number of cancer patients and high avidity T cells can be readily generated using CD40-B cells as antigen-presenting cells (Kondo *et al.,* 2009). CCNA2 could explain a part of cell cycle's role in AIM. Cells with CDC20 mutants blocked cell division and stopped cell cycle progression toward anaphase and chromosome segregation (Hartwell *et al.,* 1970). The study of Chen Xiong demonstrated that CDC20 might be an immune-associated therapeutic target in HCC because of its correlation with immune infiltration (Xiong *et al.,* 2021). It was suggested that topoisomerase II is required for expression of the EBV genome and that both topoisomerases I and II are involved in replication of the EBV genome during the lytic phase of the life cycle (Kawanishi, 1993). Etoposide and camptothecin, two topoisomerase inhibitors directed against topoisomerases II, induced apoptosis of mitogen-activated but not resting CD4+ and CD8+ T lymphocytes (Ferraro, 2000). This could explain the effect of DNA replication in CD8+T cells in AIM. EBV could potentially encode an extensive pool of T cell epitopes that activate other cross-reactive memory T cells. It was said that cross-reactive memory CD8+ T cells activated by EBV contributed to the characteristic lymphoproliferation of IM (Clute *et al.,* 2005).

Above all, these 10 hub genes play a vital role at the molecular level of CD8+T cells, providing us with some new potential targets for transforming AIM into CONV.

The top 3 gene sets in GSEA were all related to integrins. Integrin-related signaling pathways were linked with CD8+ T cells. A study showed that the disruption of CD8+ T cells blocks the tumor-promoting effects of integrin β3 antagonism (Su *et al.,* 2016). Susanne Delecluse found that EBV infection induced the expression of integrin beta 7 (ITGB7), an integrin that is associated with integrin alpha 4 to form the LPAM-1 dimer. LPAM-1 was key for homing of B cells to the gastrointestinal tract, suggesting that induction of this molecule was the mechanism through which EBV-infected cells entered this organ (Delecluse *et al.,* 2019). Stéphanie Corgnac found that integrin triggers bidirectional signaling events that cooperated with TCR signals to enable T-cell migration and optimal cytokine production (Corgnac *et al.,* 2018). The above studies were consistent with our research results that EBV might regulate CD8+T cells by integrin-related signaling pathways.

To predict the potential effective therapy for AIM, we used the DGIdb database to determine therapeutic agents that might reverse the expression of hub genes. S-trityl-L-cysteine is a reversible, tight binding inhibitor of the human kinesin Eg5 that specifically blocks mitotic progression (Skoufias *et al.,* 2006). It may regulate KIF11 and change the expression of CD8+T cells. Meriolins 3 exhibited antiproliferative properties with nanomolar IC50 and induced cell-cycle arrest and CDK inhibition associated with apoptotic events in human glioma cell lines (Jarry *et al.,* 2014). Meriolins thus constitute a new CDK inhibitory scaffold, with promising antitumor activity (Bettayeb *et al.,* 2007). They may react to CCNA2. Exposure of human Jurkat T cells to aruncin B, purified from Aruncus dioicus, caused apoptosis along with microtubule damage, G(2)/M-arrest, Bcl-2 phosphorylation, Bak activation, mitochondrial membrane potential ($\Delta\psi m$) loss, cytochrome c release, activation of multiple caspases, and PARP degradation (Han *et al.,* 2012). Amonafide is a novel topoisomerase II (Topo II) inhibitor and DNA

intercalator that induces apoptotic signaling by blocking the binding of Topo II to DNA (Allen SL and Lundberg AS 2011). The roles of the drugs or molecular compounds above in EBV-related disease still need to be further explored as potential therapeutic targets.

Conclusions

In the present study, we identified differentially expressed genes in EBV-specific CD8+ T cells in AIM and CONV to gain insight into the evolving virus-specific response. We identified 10 hub genes that were validated, uncovered the possible pathways, analyzed the target genes for miRNA, and predicted potential therapeutic agents to explore the critical potential mechanisms that might plausibly be involved. This study could empower the discovery of novel potential therapeutic targets to treat AIM and other EBV-related diseases.

Acknowledgments. This work was supported by Natural Science Foundation of Shandong Province [BS2014YY052]. Yijing Chen and Hanqing Wang designed research; performed research and wrote the paper; Yijing Chen analyzed data; Yijing Chen agreed to be accountable for all aspects of the work. Xia Liu provided financial support. Thanks all those who have helped this research. Authors affirm that the study obtained the required ethical clearance and respected all ethical considerations. This study was approved by the local ethics committee (No. 20,170,810) and informed consent was obtained.

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