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Article

Drug Repurposing for Japanese Encephalitis Virus Infection by Systems Biology Methods

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Abstract: Japanese encephalitis is a zoonotic disease caused by Japanese encephalitis virus (JEV). It is mainly epidemic in Asia with an estimated 69000 cases occurring per year. However, no approved agents are available for the treatment of JEV infection, and existing vaccines cannot resist various types of JEV strains. Drug repurposing is a new concept for finding new indication of existing drugs, and recently, it has been used to discover new antiviral agents. Identifying host proteins involved in the progress of JEV infection and using these proteins as targets are the center of drug repurposing for JEV infection. In this study, based on the gene expression data of JEV infection and the phenome-wide association study (PheWAS) data, we identified 286 genes participating in the progress of JEV infection using the systems biology methods. The enrichment analysis of these genes suggested that the genes identified by our methods were predominantly related to viral infection pathways and immune response-related pathways. We found that bortezomib which can target these genes may have potential effect on the treatment of JEV infection. Subsequently, we evaluated the antiviral activity of bortezomib using the JEV-infected mice model. The results showed that bortezomib can lower JEV-induced lethality in mice, alleviate suffering in JEV-infected mice and reduce the damage in brains caused by JEV infection. This work provides a new method for the development of antiviral agents.

Keywords: Japanese encephalitis virus; drug repurposing; systems biology; antiviral agents

1. Introduction

Japanese encephalitis virus (JEV) is the main pathogen that cause severe encephalitis in humans. JEV belongs to the genus of *Flavivirus* which also includes other arbovirus, such as Dengue virus (DENV), West Nile virus (WNV) and Zika virus (ZIKV) [1]. JEV is a positive-sense single-stranded RNA virus. The genome of JEV is about 11kb in length, containing a single open reading frame (ORF) flanked by the 5′- and 3′-untranslated regions (UTRs). The ORF encodes a long polyprotein which is cleaved into three structure proteins (capsid [C], pre-membrane [prM], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [2]. The structural proteins make up the infectious viral particle and the nonstructural proteins participate in multiple steps of viral life cycle including viral replication, virion assembly and immune evasion [2].

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Since the first record in the late 1800s, JEV has posed a significant threat to global health [3]. It is reported that there are 69000 cases of JEV infection per year [4]. The average mortality rate caused by JEV can be as high as 30% in the past 30 years, and the proportion of survival with permanent neurological or psychiatric sequelae is about 44% [1]. With its epidemic area expansion, JEV affects about 25 countries in Asian, and about 60% of population lives in the risk of JEV infection [2]. At present, vaccination is the most effective way to prevent JEV infection. The common vaccines include inactivated mouse brain-derived vaccine (JE-VAX), inactivated BHK-21 cell-derived vaccine, live-attenuated vaccine (SA14-14-2), inactivated Vero cell-derived vaccine, and chimeric attenuated vaccine [5]. However, about 80% cases of JEV infection occur in areas covered by JEV vaccination program due to the failures of immunization strategies or the limitation of vaccines themselves [1]. To date, no clinically approved antiviral agents have been available for the treatment of JEV infection. What's more, few randomized clinical trials have tested treatments for JEV. In the past 30 years, only six agents for the treatment of JEV infection have been tested by clinical trials, but none of them have been found effective [1]. Therefore, it is essential and urgent to find a safety and effective treatment.

Drug repurposing has recently become a very popular method for drug discovery, which provides old drugs (including approved drugs, under research drugs, and withdrawn drugs) with new indications by exploring new molecular pathways and targets [6, 7]. With this strategy, finding an alternative agent to treatment JEV infection will be fast and safe. During the past decades, the traditional method for drug repurposing depends on high-throughput screening of small-molecule libraries consisting of approved and developing drugs [8]. However, the success rate of high-throughput screening for effective repurposed drugs has dropped dramatically [9]. With the development of computational methods, the high-throughput omics data, virtual screening and text mining have been used for drug repurposing [9, 10]. One of the computational methods for antiviral drug repurposing is to target pathogen to block its lifecycle. Using the crystal structure of the E protein and the strategy of structural-based virtual screening (SBVS), Leal et al. identified a compound exhibiting marked antiviral activity against DENV with its EC50 being 3.1μM [11]. The other methods for antiviral drug repurposing are targeting host genes to inhibit pathogen infection. Identifying the proteins participating in the pathogen infection process is the basis of host-targeted drug repurposing approaches [9]. Quan et al. identified 170 *Mycobacterium tuberculosis* (Mtb) infection-associated genes by theoretical genetic analysis, and obtained high potential anti-Mtb drugs by targeting these genes [12]. Therefore, it is possible to rapidly identify effective therapeutics for JEV infection using the method of drug repurposing through targeting JEV-susceptible genes.

Systems biology has been used to identify the pathogenic mechanisms of complex human diseases by integrating genetic variation, genomics, pathways, and molecular networks [13]. The advent of systems biology provides a powerful method for facilitating drug development and drug repurposing [14]. The representative algorithms used in systems biology field include GeneRank and HotNet2 [15,16]. In this study, we applied the methods of HotNet2 and GeneRank to identify the genes essential in JEV infection. We also analyzed GO and KEGG pathway enrichment of these genes to validate our results. Using the information of drug-target, we obtained the agents which have potential treatment effect on JEV infection. We found that multiple targets of bortezomib play critical roles in the progress of JEV infection based on analysis of the PheWAS data of encephalitis and gene expression data of human microglial cells after JEV infection. Furthermore, we investigated the effect of bortezomib using JEV-infected mice model. Overall, our research established a new method for finding new antiviral agents.

2. Results and Discussion

2.1. Screening of genes associated with JEV infection by GeneRank algorithm

The gene expression data could reveal the relationship between genes and JEV infection. Therefore, we resorted to the GEO-contained gene expression datasets following JEV infection to identify the JEV-susceptible genes. The dataset GSE57330 includes 12 samples which were detected at three time points (6, 24, 48h) post JEV infection [17]. Taking the gene expression data detected at different time as a whole, we calculated the value of fold change using the mean of genes expression. Thus, we obtained the up-regulated genes and down-regulated genes after JEV infection of human microglial cells. Ordinarily, the genes whose fold change values are at least two folds above those of uninfected group and P value < 0.05 are defined as significantly associated with JEV infection. However, this approach may ignore those genes associated with JEV infection, but their expressions do not significantly altered. Therefore, we used the algorithm of GeneRank to identify genes assocated with JEV infection.

The algorithm of GeneRank was derived from the Google search engine PageRank [15]. It can take advantage of the biological network to identify key genes associated with diseases, regardless whether their expressions altered significantly or not. To find the genes associated with JEV infection, we ranked genes by GeneRank algorithm. Taking the value of fold change as the initial importance of gene, we obtained the order of functional genes participating in JEV infection. According to the result calculted by GeneRank, we defined the top 1% genes as significant genes involved in JEV infection process (Table S1). As indicated in Table S1, several genes have been reported to affect the process of JEV infection. For example, the expression of 2′,5′-oligoadenylate synthetases (OAS) family (OAS1, OAS2 and OASL) inhibited the replication of JEV in PK-15 cells in one previous study [18]. The members of tripartite-motif containing (TRIM) protein were reported to be a negative regulator of IFN-β during JEV infection and to inhibit JEV replication by degrading the viral protein in some other studies [19, 20]. The results suggested that the genes identified by GeneRank algorithm may play critical roles in the lifecycle of JEV.

To understand the biological functional genes ranked by GeneRank algorithm, the Gene Ontology (GO) enrichment analysis was conducted by using the clusterprofiler package in R [21]. The P-value < 0.05 was used as the cutoff criterion. The results showed that these genes were involved in different cellular functions including immune response, response to peptide, the regulation of DNA metabolic process, response to virus, response to interferon-γ, and the regulation of innate immune response (Figure 1). In addition, we also investigated the involvement of these genes in signal transduction pathways using clusterProfiler package. As shown in Figure 1, the most significant KEGG pathways in which the down-regulated genes were enriched included human cytomegalovirus infection, kaposi sarcoma-associated herpesvirus infection, proteoglycans in cancer. On the other hand, the up-regulated genes were enriched in viral infection pathways (including herpes simplex infection, influenza A, haposi sarcoma-associated herpesvirus, and human papillomavirus infection) and NOD-like receptor signaling pathway. The results suggested that the genes ranked by GeneRank algorithm were involved in viral infection pathways and immune response-related pathways.

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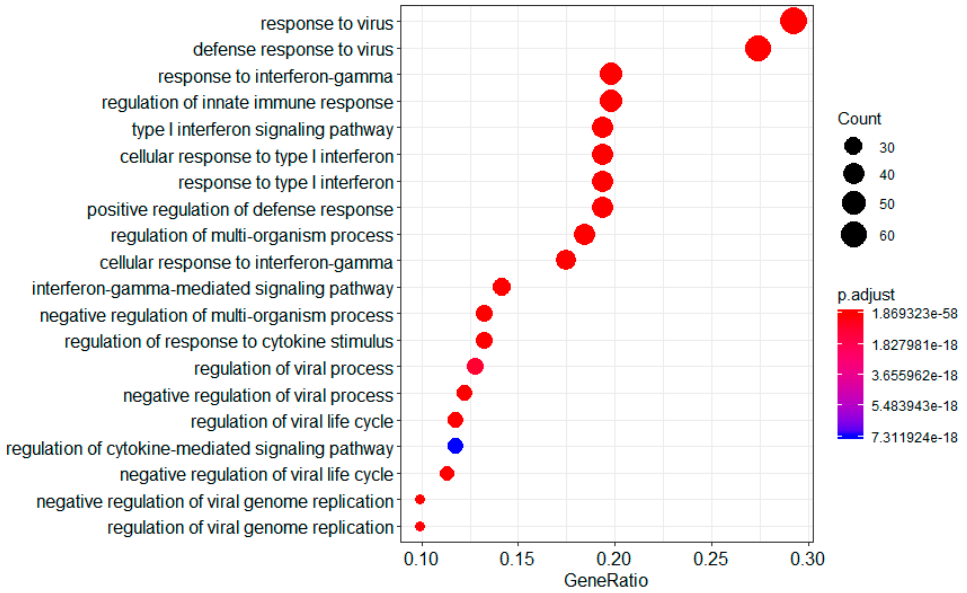

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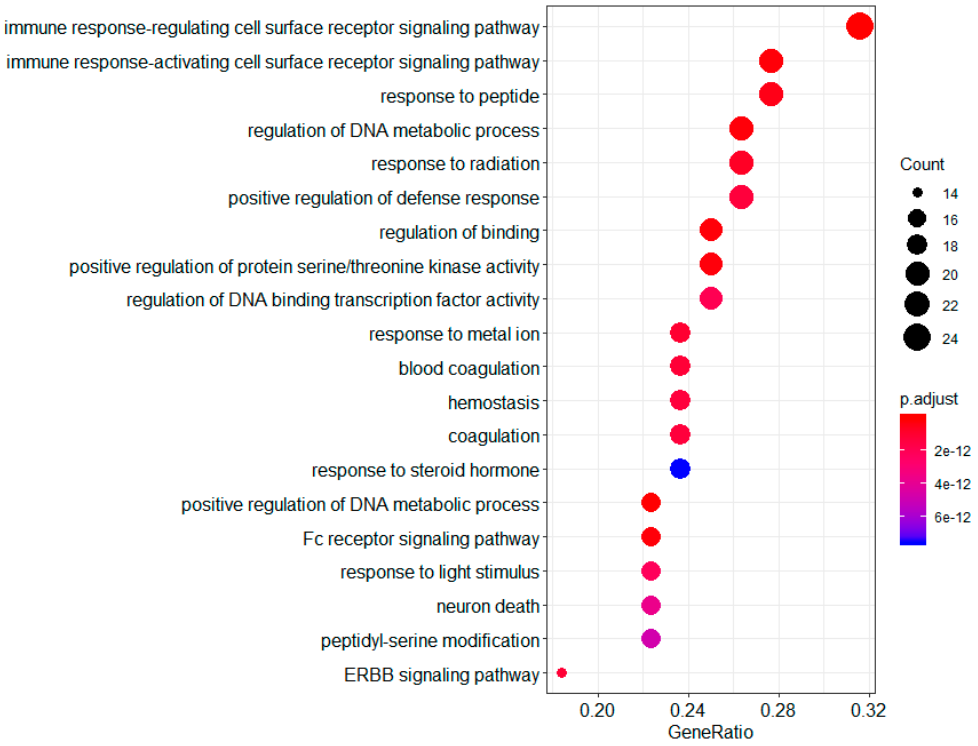

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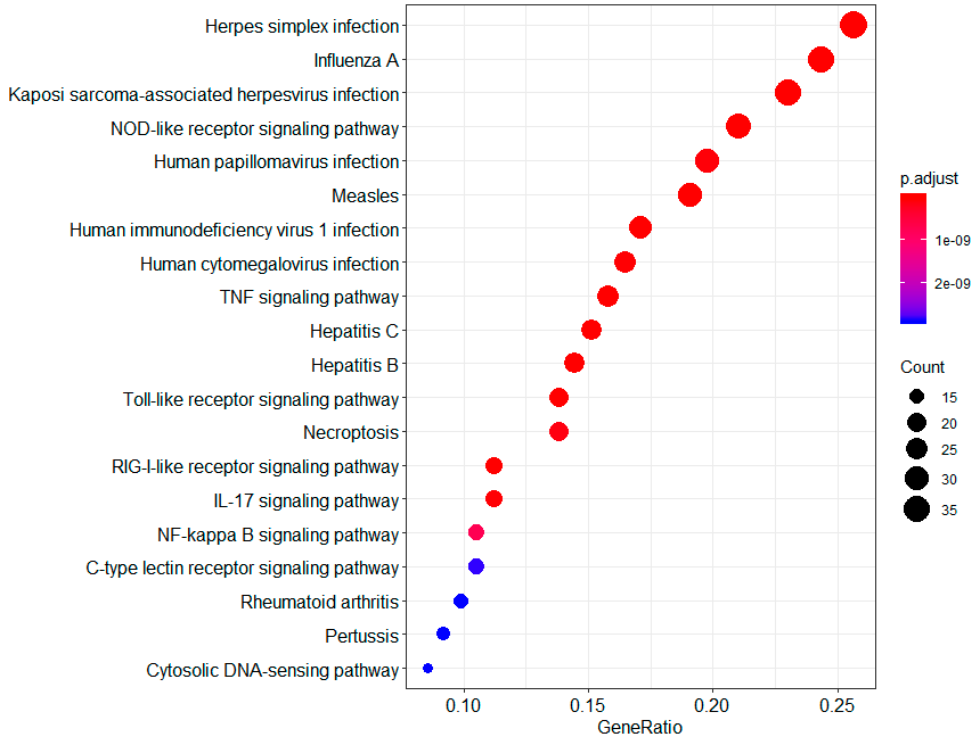

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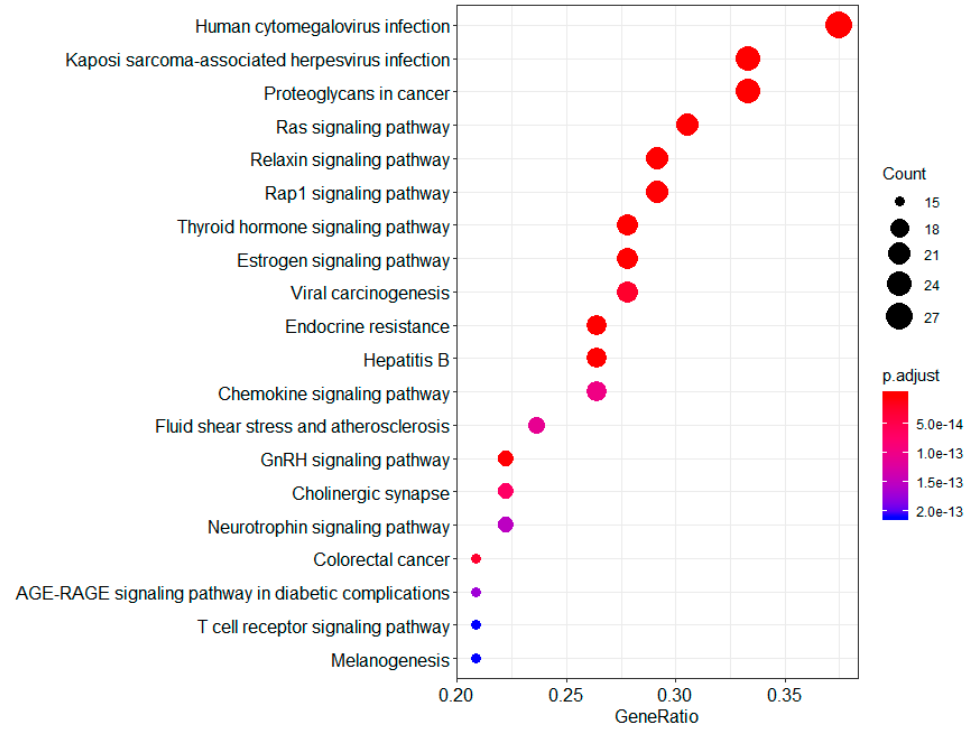

(d)

Figure 1. Functional characterization of the genes ranked by GeneRank algorithm. Down-regulated and up-regulated genes which were ranked by GeneRank algorithm were subjected to GO enrichment analysis (biological processes) and KEGG pathway enrichment analysis using the clusterprofiler package in R. Top 20 of the GO and pathways in which the up- and down-regulated genes were significantly enriched, respectively (P-value < E-10). (a) GO enrichment analysis of up-regulated genes; (b) GO enrichment analysis of down-

regulated genes; (c) KEGG pathway enrichment analysis of up-regulated genes; (d) KEGG pathway enrichment analysis of down-regulated genes.

2.2. Drug repurposing for JEV infection by targeting GeneRank-derived genes

To identify approved drugs for the treatment of JEV infection, we collected the information about the association between chemical agents and its targets from Drug-Gene Interaction database (DGIdb, http://dgidb.genome.wustl.edu/), Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/) and DrugBank (http://www.drugbank.ca/) [22-24]. By targeting the top 1% genes derived from GeneRank calculation, we obtained 103 FDA-approved drugs which might have potential effect on the treatment of JEV infection (Table S2). It should be noted that among these agents we found bortezomib which was reported to have the ability to inhibit DENV and ZIKV infection [25, 26]. Given the fact that both DENV, ZIKV, and JEV belong to the genus of *flavivirus*, we speculated that bortezomib may have the potential ability to treat JEV infection. In addition to bortezomib, other agents such as aspirin, curcumin, etanercept, and minocycline, were also found to have effects on the inhibition of JEV infection (Table 1) [27-35]. Furthermore, according to the research of Chen et al., tumor necrosis factor-α (TNF-α) plays a key role in JEV-induced neuronal death [36]. The inhibitors of TNF (such as lenalidomide and adalimumab) may also have potential effect on the treatment of JEV infection, which were consistent with the mechanism underlying the treatment of etanercept against JEV infection. Interestingly, these inhibitors were also found in our study. The results suggested that the drugs identified by targeting the top 1% genes after GeneRank calculation may be effective in the treatment of JEV infection.

Table 1. Agents that reported have effect on the treatment of JEV infection. Agents with the evidence of anti-JEV activity are shown in the table. Among these agents, the effect of minocycline and ribavirin on the treatment for JEV has been tested by randomized clinical trials [37, 38]. Etanercept and minocycline inhibited JEV replication both in vitro and in vivo.

Agent	Anti-JEV potential	Reference
Aspirin	Aspirin suppressed JEV propagation in neuronal and non-neuronal cells	27
Chlorpromazine	Chlorpromazine reduced the positive rate of JEV infection by 50% in vitro	28
Curcumin	Curcumin inhibited the production of infective JEV particle in vitro	29
Etanercept	Etanercept significantly relieved clinical symptoms and reduces mortality in JEV-infected mice	30
Genistein	Genistein protected neurons from JEV-induced decrease in the number of visible neurons	31
Minocycline	Minocycline protected 70% of mice from JEV-induced death, and inhibited JEV replication in vitro	32
Quercetin	Quercetin inhibited JEV replication in vitro	33
Ribavirin	Ribavirin inhibited JEV replication in vitro	34
Valproic acid	Valproic acid reduced the cytopathic effects caused by JEV	35

2.3. Screening of genes and drugs associated with JEV infection by HotNet2 algorithm

HotNet2 (HotNet diffusion-oriented sub-networks) algorithm is based on a heat diffusion kernel algorithm that considers the heats of individual genes as well as the topology of gene-gene interactions. Because HotNet2 algorithm can reduce the false positive rate, identify subnetworks with high biological relevance, and be sensitive to both real and simulated data, it was used to find significant sub-networks associated with various diseases [16].

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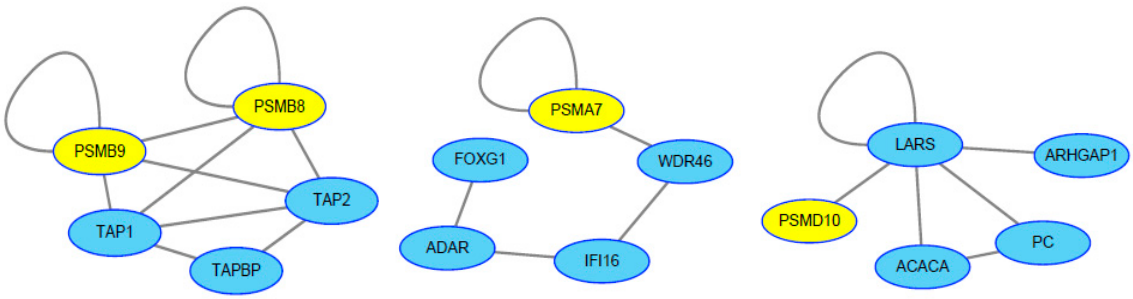
To further screen agents for JEV infection, we applied the HotNet2 algorithm to identify the genes which may contribute to JEV infection. According to the SNP-to-gene mapping method, we mapped the single nucleotide polymorphisms (SNPs) in the phenome-wide association study (PheWAS) data to genes to identify potential genes associated with encephalitis, which exhibits the similar symptom to JEV infection [39, 40]. To recognize the gene-interaction networks related to encephalitis, we used the P-values derived from PheWAS data and the HotNet2 algorithm to calculate the sub-network. We obtained 16 sub-networks which involved 64 genes associated with encephalitis (Table S3). By targeting the genes identifited by HotNet2 algorithm, we obtained 20 agents which might have potential effect on the treatment of JEV infection (Table S4). Interestingly, we also found bortezomib among these agents, which was consistent with the agents obtained by GeneRank calculation. In addition, it should be noted that four genes among three sub-networks belong to the ubiquitin proteasome system (UPS) (Figure 2), which agrees with the results that encephalitis-related virus, including JEV, West Nile Virus (WNV) and Venezuelan equine encephalitis virus (VEEV), could utilize the UPS to promote viral entry, replication, and release [41, 42, 43]. What's more, the genes belonging to UPS were the target of bortezomib, which confirmed our findings that bortezomib may have the ability to treat JEV infection. These results further demonstrated that the agents obtained by GeneRank algorithm have great potentials for treating JEV infection.



(a) (b) (c)

Figure 2. Significant sub-networks associated with encephalitis. (a), (b) and (c) represent different sub-networks related to encephalitis. The genes marked by yellow belong to the ubiquitin proteasome system (UPS).

2.4. Therapeutic effects of bortezomib on JEV-infected mice

In order to further evaluate the above findings that bortezomib has potential ability to inhibit JEV infection, we established a mouse model of JEV infection. Four-week-old BALB/c mice were randomly divided into 4 groups: a PBS group; a JEV-infected group; a bortezomib-treated group; a JEV-infected and bortezomib-treated group. The mice in infected groups were intraperitoneally injected with 10^6 PFU of JEV P3 strain. We administered bortezomib intravenously once every day for the first two days and then administered every two days (Figure 3a). As anticipated, most mice in untreated infected group died of JEV infection with the mortality rate of 90%. On the contrast, the mortality rate of bortezomib-treated infected group was 40% (Figure 3b). All of the mice in bortezomib and PBS groups survived until the end of the experiment, indicating that bortezomib has the ability to protect mice from death caused by JEV infection.

To verify the effects of bortezomib on clinical symptoms, we scored the clinical behavior of mice during the experiment [30, 45]. The JEV-infected mice showed different behavior of movement limitation, frequent blinking, body stiffening and hind limb paralysis. The clinical behavior of bortezomib-treated infected group was alleviated comparing with the untreated infected group

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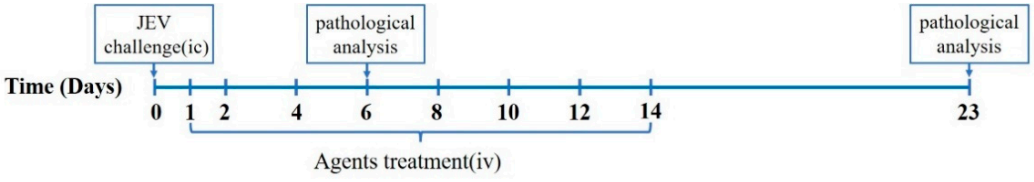
(Figure 3c), indicating that bortezomib treatment prevented the JEV-infected mice from pain. The mice in bortezomib and PBS groups did not show any alterations in behavior, suggesting that bortezomib has potential to alleviate the suffering caused by JEV infection.

Moreover, to further explore the protection of bortezomib against JEV infection in brains, we collected the brain tissues for H&E staining on day 6 and day 23 post-infection. As is shown in Figure 3d, the mice in JEV-infection group suffer from significant meningitis, vacuolar degeneration and glial nodules. While the symptoms of mice in bortezomib-treated group were remarkably alleviated. The mice without JEV infection did not show any histological changes, regardless of whether the mice were treated with bortezomib or not. The mice in all groups showed no evidence of meningitis on day 23 post-infection. This result indicated that bortezomib could significantly reduce the damage in brains caused by JEV infection. These results further suggested the clinical importance of bortezomib in the treatment of flavivirus infection and confirmed the crucial role of UPS in the lifecycle of flavivirus.



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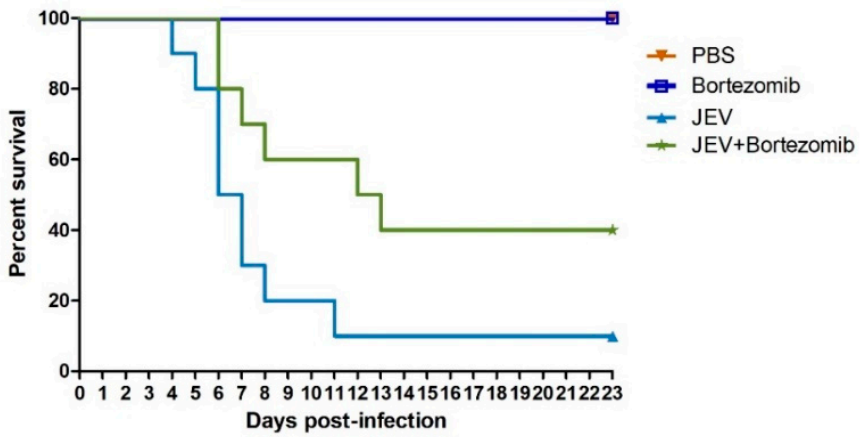

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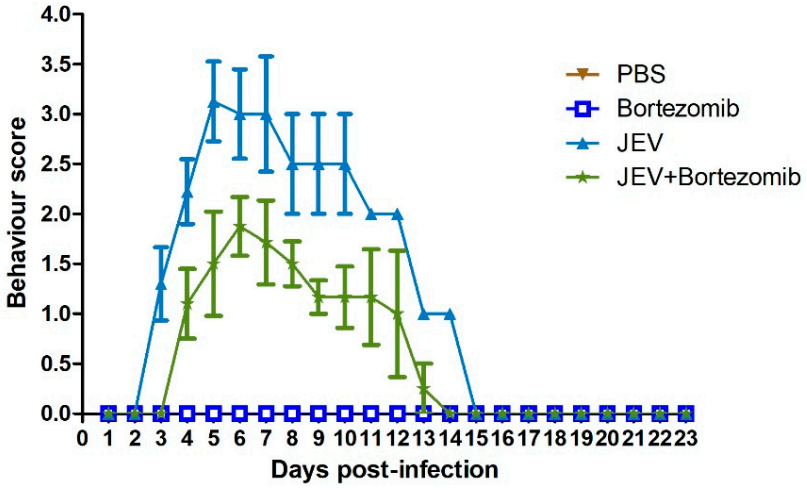

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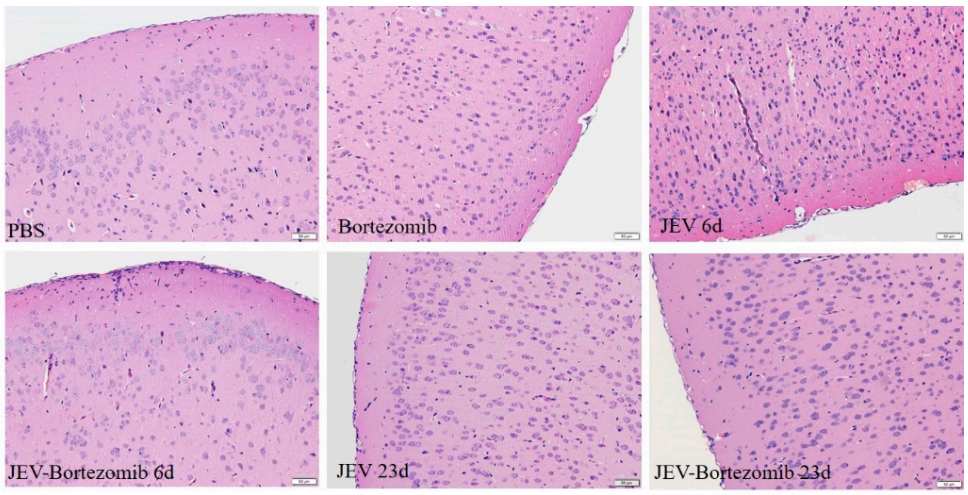

(d)

Figure 3. Therapeutic effects of bortezomib on JEV-infected mice. (a) Flow chart of animal studies. Mice infected with JEV were treated with PBS or bortezomib (0.5 mg/kg). Brain samples were analyzed on day 6 and day 23 of post-infection. (b) Survival of mice in each group during 23 days after JEV infection. Data are shown as Kaplan-Meier survival curves (n = 10 for each group). (c) Behavior score of mice in each group during 23 days after JEV infection. 0 = no restriction of movement; no blink frequently; no body stiffening; no hind limb paralysis. 1 = no restriction of movement; blink frequently; no body stiffening; no hind limb paralysis. 2 = restriction of movement; blink frequently; no body stiffening; no hind limb paralysis. 3 = restriction of movement; body stiffening; no hind limb paralysis. 4 = restriction of movement; eyes closed; body stiffening; hind limb paralysis, sometimes tremor. (d) Bortezomib reduces the damage in brains caused by JEV infection. Hematoxylin-eosin staining of brain coronal sections was performed to observe the pathological changes.

3. Conclusion

At present, the treatment of JEV infection mainly depends on symptomatic therapy and supportive therapy. Unfortunately, the effect of existing treatment is far from perfect. About 30% - 50% survivors were reported to experience serious sequelae [2]. Although, many drugs have been found to have anti-JEV activity, the evaluation of these drugs mainly focused on animal models and cellular levels with few clinical trials reported. Therefore, it is it is necessary to rapid identify effective therapeutics for JEV infection using the method of drug repurposing. Furthermore, since JEV belongs to the same genus with DENV and ZIKV, identifying the agents may provide treatment strategies for those viruses as well.

Identifying the functional genes in JEV infection is essential not only for finding new antiviral agents, but also for understanding the virus replication and pathogenesis. This study utilized the algorithm of HotNet2 and GeneRank to identify host genes participating in the progress of JEV infection. We combined the gene expression data with PPI database to rank JEV infection related genes which could be used as the targets to find new antiviral agents. The results showed that host proteins involved in JEV infection include viral infection pathways and immune response related pathways, which was consistent with the infection mechanism of JEV. Afterwards, we found that bortezomib might be a potential agent for the treatment of JEV infection by targeting these genes. In addition, we identified genetic interaction networks related to encephalitis by HotNet2 algorithm. Using these genes as the targets to screen drugs, we also found that bortezomib could be used for JEV treatment.

Based on the above results, we confirmed the effect of bortezomib on treatment of JEV infection by mice model. Mice treated with bortezomib show a significant alleviation in histopathological symptoms and clinical symptoms, and 30% reduction in mortality caused by JEV was observed, compared with mortality of untreated JEV-infected mice (Figure 3). These results further support the application of host-targeted approaches for new antiviral agents.

Above all, our results provided new insights into the molecular mechanism of JEV infection, and offered a novel and promising therapeutic method for the treatment of JEV infection.

4. Materials and Methods

4.1 Data resources

In this study, the PheWAS data were derived from the work by Denny et al., which included 3,144 phenotype-associated single nucleotide polymorphisms (SNPs) [40]. The JEV infection datasets (GEO accession No. GSE57330) came from GEO (www.ncbi.nlm.nih.gov/geo/) [18]. The protein-protein interaction (PPI) network used in HotNet2 algorithm was obtained from HINT, iRefIndex, and Multinet, which included about 390,000 interactions [16]. The protein-protein interaction (PPI) network used in GeneRank algorithm was derived from STRING database (Version: 10.5).

Information about the association between chemical agents and its targets was obtained from the Drug-Gene Interaction database (DGIdb, http://dgidb.genome.wustl.edu/), Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/), and DrugBank (http://www.drugbank.ca/).

4.2 GeneRank algorithm

Genes can be ranked by GeneRank method, based on their expression values and interaction information. GeneRank algorithm was derived from PageRank [16]. The algorithm is described as follows:

$$r_j^n = (1-d)ex_j + d\sum_{i=1}^{N}\frac{w_{ij}r_i^{n-1}}{\deg_i}$$

Where, r_j^n and r_i^{n-1} are the importance of gene j and i after n or n-1 iterations, respectively; ex_j is the initial importance of gene j, and ex_j is defined as the fold change value in this work ; w_{ij} represents the relationship between gene j and gene i in PPI network, if gene i interacts with gene j, then $w_{ij} = 1$, otherwise $w_{ij} = 0$; deg_i is the out-degree of gene i, which means the number of genes interacting with gene i; N is the total number of genes in PPI network; the parameter d ($0 \le d < 1$) is a constant representing the proportion of PPI network in calculation. The greater d is, the more important PPI network is. In this study, we set the value of d as 0.5.

4.3 HotNet2 algorithm

HotNet diffusion-oriented subnetworks (HotNet2) algorithm is a topology-based method for finding significant subnetworks associated with disease. Originally, HotNet2 algorithm was used to analyze somatic mutation data from cancer datasets [16].

The initial input in HotNet2 algorithm is a heat vector containing the fraction of each gene and a network of protein interactions. At each step, the heat of nodes was passed to and received from adjacent nodes, but also a fraction β ($0 \le \beta \le 1$) of heat was retained. This process runs until equilibrium. Therefore, the heat of each node at equilibrium depends on its initial heat, the local topology of the network around the nodes, and the value β. The process is described as follows:

$$F = \beta(I-(1-\beta)\,W) - 1$$

where

$$W_{ij} = \begin{cases} \frac{1}{\deg(j)} & if\ noede\ i\ interacts\ with\ j, \\ 0 & otherwise. \end{cases}$$

where deg(i) is the number of neighbors (i.e., the degree) of protein in the interaction network.

In this study, we used the P-values of encephalitis derived from PheWAS data as heat scores in HotNet2 algorithm.

4.4 Agents and virus

Bortezomib (PS-341) was purchased from Selleck Chemicals (Houston, TX, USA). DMSO and PEG300 were purchased from Sigma-Aldrich (St. Louis, MO). JEV P3 strains were kindly provided by Yun-Feng Song, State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China.

4.5 Animal studies

All female BALB/c mice (4-week-old) were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). The mice were randomly divided into 4 groups: a PBS group; a JEV-infected group; a bortezomib-treated group; a JEV-infected and bortezomib-treated group. For JEV-infected group, the mice were intraperitoneally injected with 10^6 PFU of JEV P3 strain in 100μl PBS. For the PBS group, mice were intraperitoneally injected with 100 μl PBS. For the bortezomib-treated and vehicle-treated group, mice were intravenously injected with 0.5 mg/kg bortezomib, or PBS with 2% DMSO and 30% PEG 300.

After JEV infection, the mice were treated with Bortezomib once every day for the first two days and then treated once every two days. On day 6 and day 23 post-infection, five mice from each group were euthanized and the brains was used for subsequent H&E staining. Ten remaining mice were monitored daily to assess behavior and mortality. Behavioral scoring was performed basing on the presence of symptoms [30, 45]. This experiment was approved by the Huazhong Agricultural University's Research Ethics Committee of the College of Veterinary Medicine.

4.6 H&E staining

For the histology analysis, brain tissues were fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin for pathological analysis.

4.7 Data analysis

All statistical analyses were conducted using GraphPad Prism v5.0 (GraphPad Software Inc.). Cytoscape 3.6.1 was used to visualize the sub-networks.

Supplementary Materials: The following are available online. Table S1: the functional genes participating in JEV infection; Table S2: the potential anti-JEV agents discovered by GeneRank algorithm; Table S3: the significant sub-networks associated with encephalitis; Table S4: The potential anti-JEV agents discovered by HotNet2 algorithm.

Author Contributions: Hong-Yu Zhang conceived and designed the project; Bo-Min Lv, Xin-Yu Tong, Yuan Quan and Meng-Yuan Liu performed and analyzed the data; Qing-Ye Zhang and Yun-Feng Song designed the experiments; Bo-Min Lv and Xin-Yu Tong performed the experiments; Bo-Min Lv wrote the manuscript; and Hong-Yu Zhang revised the manuscript.

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