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Original article

Reactivation of Epstein-Barr virus in SFTSV infected patients

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ABSTRACT

Background: Severe fever with thrombocytopenia syndrome (SFTS) is an emerging hemorrhagic fever caused by a tick-borne bunyavirus SFTSV with case fatality up to 30%. The reactivation of Epstein-Barr virus (EBV) has been proven to occur in individuals with various immune suppression conditions.

Methods: Here, we diagnosed 22 SFTSV infected patients with PCR in a hospital in Shandong Province, China in 2020. To understand the consequences of SFTSV infection leading to EBV reactivation, we examined EBV reactivation in SFTSV-infected patients with PCR and RT-PCR.

Results: We found that EBV was reactivated in 18.2% (4/22) of SFTS patients, suggesting that EBV reactivation is common in SFTS patients. Compared with SFTS patients without EBV reactivation, SFTS patients with EBV-reactivation had a significantly lower median level of serum albumin (32.45 g/L vs. 26.95 g/L, $p = 0.03$) and a significantly higher median number of urine red blood cells (0 cells/μL vs. 9 cells/μL, $p = 0.04$).

Conclusion: SFTS infection can reactivate EBV in patients, which may make the clinical condition of patients worsen.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne hemorrhagic fever with a high case fatality of up to 30% [1]. SFTS is caused by SFTSV, a tick-borne bunyavirus, which was first isolated in 2009 from a patient in China and subsequently had been reported in other East and Southeast Asia countries [1–4]. Though SFTSV is mainly transmitted by tick bites, person-to-person transmission has also been frequently reported [5–9]. Person-to-person transmission was most likely caused by the spread of the virus through the conjunctiva or oral mucosa [10]. Effective therapies or vaccines are unavailable and the mechanisms of SFTS pathogenesis are poorly understood.

Epstein-Barr virus (EBV) is a ubiquitous gamma herpesvirus that infects humans across the world [11]. EBV is one of the most common virus, infecting more than 90% of humans and persisting for the lifetime of an individual following the acute phase of infection [12]. Primary EBV infection usually occurs in childhood without any symptoms, but usually causes infectious mononucleosis when primary infection occurs in adults [12]. B lymphocytes are the target cells of EBV. Upon infection, EBV stimulates naive B lymphocyte differentiation to memory B cells, which become the viral reservoir. In latent infection, the EBV genomic DNA exists as a closed circular plasmid and behaves like host chromosomal DNA [13]. Impairment of the cellular immune response can cause EBV reactivation, in which the EBV genome is replicated by the viral replication machinery [13,14]. EBV has been reported to

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be reactivated by psychological stress [15,16] and is associated with some viral infections such as dengue virus, SARS-CoV-2, cytomegalovirus, hepatitis A virus, hepatitis C virus, hepatitis E virus, and human immunodeficiency virus (HIV) [17–26]. The symptoms of EBV reactivation are generally mild and easily overlooked, and may include fevers, cervical lymphadenitis, pharyngotonsillitis, periorbital edema, hepatomegaly, and splenomegaly [27]. EBV reactivation by other viruses is rarely studied. It is not clear whether SFTSV infection can trigger EBV reactivation and what are the consequences of EBV reactivation in SFTSV patients. In this study we analyzed EBV reactivation in SFTSV patients from a hospital in Qingdao City, Shandong Province, China.

2. Materials and methods

2.1. Samples of suspected SFTS patients collection

From March to September in 2020, suspected SFTS patients were identified in a hospital in Qingdao City, Shandong Province, China. Suspected SFTS patients were patients presenting with fever (≥38 ˚C), vomiting, diarrhea, dizziness, fatigue, thrombocytopenia, or leukocytopenia, living in rural hilly areas, or had previous exposure to ticks. Suspected SFTS patients were admitted into the hospital within 14 days from diseases onset and the blood samples of the patients were collected upon administration into the hospital and were frozen immediately at −80 ˚C for further PCR analysis.

2.2. SFTS cases confirmed with RT-PCR

RNA was extracted from patients' peripheral blood with the AllPrep RNA Mini Kit (Qiagen, Hilden, Germany) and was amplified by a 2-step RT-PCR with SFTSV M segment primers (Table 1). The reverse transcriptase step was carried out as follows: 5 minutes at 25 ˚C, 60 minutes at 42 ˚C, and 15 minutes at 95 ˚C by the Reverse Transcription System Kit (Promega). Pure water was used as negative controls for PCR. The protocol of PCR was 1 cycle of 95 ˚C for 3 minutes, then 35 cycles of 95 ˚C for 1 minute, 50 ˚C for 1 minute, and 72 ˚C for 1 minute, and a final extension at 72 ˚C for 10 minute. PCR products were electrophoresed on and purified from 1.2% agarose gel with a gel extraction kit (Omega). Purified PCR product was cloned into pMD 19-T Vector (TAKARA Biotech). The recombinant plasmids were extracted from the positive bacterial clones and sequenced for the insert in both directions.

2.3. Detection of the EBV IgG and DNA in the SFTS patients

All SFTS patients' sera were tested for EBV antibody with the Human EBV IgG ELISA Kit (Jianglai Biotech); and serum DNA was tested for EBV with real-time quantitative PCR in the clinical laboratories. Total blood DNA was amplified by nested PCR with Taq Master Mix Kit (Vazyme) with primers derived from the first tandem internal repeats (IR1) of EBV (Table 1). EBV reactivation was defined as EBV DNA ≥ 500 copies/mL [28,29]. The transcript expression level of the human EBI2 gene is an indirect evidence for EBV reactivation [13,30]. The transcript expression level of EBV-induced 2 (EVI2) gene, and GAPDH in the peripheral blood mononuclear cell (PBMC) were determined with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules) with the SYBR qPCR Green Master Mix (Vazyme) and the primers are shown in Table 1. The mRNA transcript expression levels of EVI2, and GAPDH in PBMC of the reactivated EBV patients were compared with the levels in normal humans using the website (https://www.proteinatlas.org) with the following equation:

1) ΔCT-1 = CT value (EVI2, non-EBV group or EBV group) − CT value (GAPDH, non-EBV group or EBV group);
2) ΔCT-2 = nTPM (EVI2, normal human blood) / nTPM (GAPDH, normal human blood);
3) Ratio (EVI2, non-EBV group or EBV group) = 2^(−ΔCT-1) /ΔCT-2.

Note: The transcript expression value calculated as normalized transcripts per million (nTPM) values, which give

Table 1
Primers used for PCR amplification of SFTSV RNA and Epstein-Barr virus (EBV) DNA from patients.

Target gene	Primary/Nested	Primer names	Primer sequences	Amplicon size	Reference
SFTSV M segment	Primary	sm11-7wf	TGTTGCTTGTCAGCCTATGAC	480 bp	[31]
		sm11-7wr	CAACCAATGATCCTGAGTGGAAT		
	Nested	sm11-7nf	TTGCATGGATGGATGTTGGC		This study
		sm11-7nr	CCACTCGTGGCAGAACTACA		
EBV IR1	Primary	EBVF1	TTCATCACCGTCGCTGACT	122 bp	[32]
		EBVR1	ACCGCTTACCACCTCCTCT		
	Nested	EBVF2	CCAGAGGTAAGTGGACTT		
		EBVR2	GACCGGTGCCTTCTTAGG		
Human EVI1	Primary	EVI1F	AAGTCCTGGGTCTTCGGTGT	202 bp	This study
		EVI1R	GTGGCTAGTATGGCGCTG		
Human EVI2	Primary	EVI2F	CTGCCTGAGTATTGACCGCT	239 bp	This study
		EVI2R	TGAAACATGCCCCAAGCAGA		
Human GAPDH	Primary	GAPDHF	AAGGTGAAGGTCGGAGTCAA	108 bp	This study

a quantification of the gene abundance for the comparison between different genes and samples.

2.4. Phylogenetic analysis

DNA sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) to find sequences with homology. Phylogenetic tree was constructed by using MEGA7 with maximum-likelihood method. Bootstrap values were calculated with 1,000 replicates.

2.5. Statistical analysis

Continuous data or frequency variables were described as median, numbers, and/or percentages and compared by the Mann-Whitney test or Fisher's exact test, respectively. The clinical data of EBV-positive SFTS patients were compared with EBV-negative SFTS patients. The data were statistically analyzed with SPSS (version 17.0) and $p < 0.05$ was considered as significant difference.

3. Results

3.1. SFTS patients

From April to September, 2020, we identified 40 suspected SFTS patients, and 22 of them were confirmed to be SFTSV positive by RT-PCR detection of the M segment from a hospital in Qingdao City, Shandong Province, China. Of the patients, 17 were from Qingdao City, and the rest were from adjacent areas including 2 from Rizhao City, 1 from Yantai City, and 1 from Weifang City. The ages of the patients ranged from 34 to 85 years with a median age of 66. Among the patients, 16 (73%) were male, 20 (91%) were farmers, and 4 (18.2%) died (Table S1).

3.2. Reactivation of EBV in the SFTS patients

ELISA assay for EBV IgG antibody were positive in 20 of 22 SFTS-patients with a median level of EBV IgG in acute serum of 25.02 ng/mL (Fig. 1A), indicating a high infection rate of EBV (90.9%) in the local population. PCR results showed that 4 of 22 (18%) SFTS patients had a high (≥500 copies/L) EBV DNA load in the peripheral blood, consistent with EBV reactivation. Based on the EBV DNA load, the SFTS patients were divided into the non-EBV group (n = 18) who were only infected with SFTSV and the EBV group (N = 4) who were PCR positive to both SFTSV and EBV. The median ratio of the relative expression of the EVI2 in the non-EBV group and EBV group were 14.61 and 86.96, respectively. The ratio of the EVI2 in the non-EBV group and EBV group was higher than that in normal human blood, but there was no significant difference between the non-EBV group and EBV group on the ratio of EVI2 (Fig. 1B).

3.3. Clinical characteristics of EBV-positive and EBV-negative SFTS patients

The major clinical manifestations of SFTS patients included fever, gastrointestinal symptoms, neural dysfunction, and hemorrhage (Table S2). The most common abnormalities of laboratory tests were thrombocytopenia (21/22, 95%) and leukocytopenia (18/22, 82%) (Table S3). Multiple organ failure developed rapidly in most patients, as shown by elevated levels of serum alanine aminotransferase, aspartate aminotransferase, creatine kinase, and lactate dehydrogenase. Nine (41%) patients had proteinuria and 10 patients had hematuria. Among the 22 confirmed SFTS cases, 3 patients died (13.6%).

The median level of plasma albumin in the EBV-positive SFTS patients (26.95 g/L) was significantly lower than that in the EBV-negative SFTS patients (32.45 g/L) ($p = 0.03$). Microscopic examination revealed that the median number of red blood cells in urine was significantly higher in the EBV-positive patients (9 cells/μL) than in the EBV-negative patients (0 cells/μL) ($p = 0.04$) (Fig. 2). The incidence of other clinical symptoms and laboratory findings were not significantly different between EBV-positive and EBV-negative SFTS patients ($p > 0.05$).

The case fatality rate was not significantly different between EBV-positive (25%, 1/4) and EBV-negative SFTS-patients (17%, 3/18). The median age of the EBV-positive SFTSV patients and EBV-negative SFTSV patients was 74 and 64, respectively, but the median age was not significantly different between the 2 groups of patients. There was also no significant difference in sex, occupation, and duration of hospitalization between the EBV-positive and the EBV-negative SFTS-patients ($p > 0.05$).

3.4. Genetic analysis

Phylogenetic trees indicated that SFTSV obtained in this study was homologous with SFTSV strains from Shandong, Liaoning, and Jiangsu provinces of China, and South Korea (Fig. 3). The EBV IR1 sequences obtained in this study were 100% homologous with the human gammaherpesvirus-4 (strain rMSHJ, MK973062.1) through BLAST analysis in the GenBank database.

3.5. GenBank deposition

DNA sequences from in this study were deposited in the GenBank with accession numbers: MW526368-MW526369 and MZ463043-MZ463058 for SFTSV and MZ439831 for EBV.

4. Discussion

In this study, we identified 22 SFTS patients between April and September, 2020 in a hospital in Qingdao City.

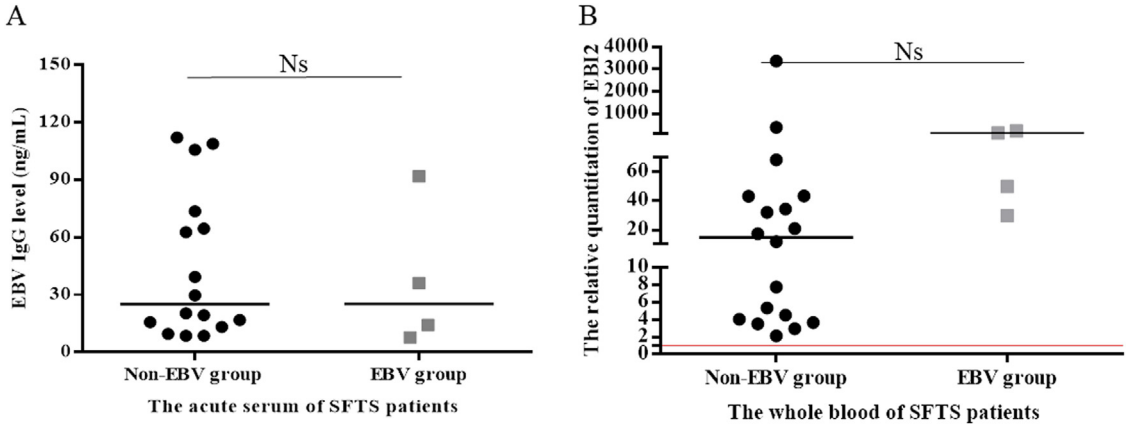

Fig. 1. Reactivation of EBV in the SFTS patients. (A) 2 blood samples in the Non-EBV group were insufficient for analysis, thus the total sample numbers of the non-EBV group and EBV group were 16 and 4, respectively. The median level of EBV IgG was 25.02 ng/mL and 25.14 ng/mL, respectively and the difference between the 2 groups was analyzed with the Mann-Whitney test ($p = 0.60$). The total sample number of the non-EBV group and EBV group were 18 and 4, respectively and the difference between the 2 groups on EVI2 was analyzed with the Mann-Whitney test ($p = 0.06$) in (B) Ns indicated there was no significant difference between the 2 groups. A red line in Figure 1B indicated value 1.

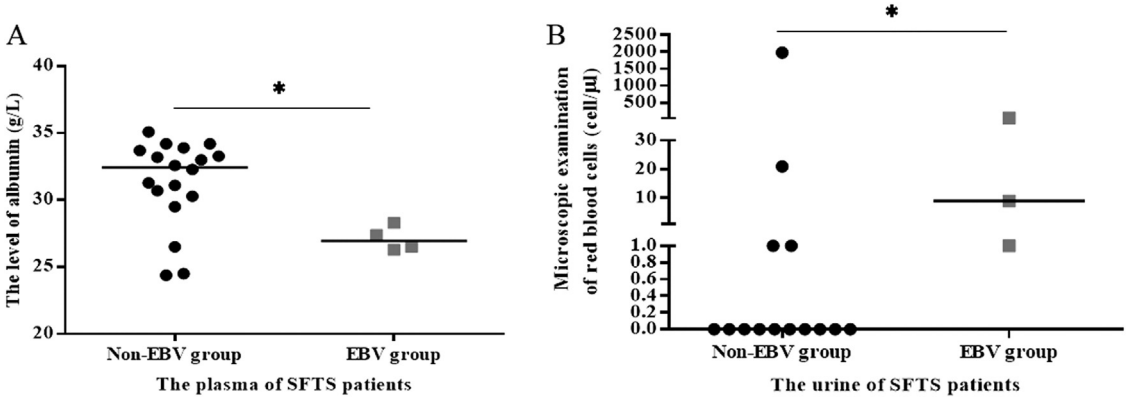

Fig. 2. The level of plasma albumin and microscopic examination of urine red blood cell count. (A) The total sample number of EBV-negative and EBV-positive SFTS patients was 18 and 4, respectively and the difference between the 2 groups was performed with the Mann-Whitney test ($p = 0.03$). (B) The total sample number of EBV-negative and EBV-positive SFTS patients was 14 and 3, respectively and the difference between the 2 groups was performed with the Mann-Whitney test ($p = 0.04$).

The patients came from Qingdao City and the adjacent areas such as Yantai City, Rizhao City, and Weifang City. All patients lived in rural hilly areas in these regions where our current and previous studies indicated are regions endemic to SFTSV [33,34]. The case fatality of SFTS patients was high (18%, 4/22) in this study, which was similar to the case fatality in other places of China [35]. With a high case fatality of SFTS and no effective therapy for SFTS, public health education is needed in SFTS endemic areas in China to prevent SFTSV infection and promote physician's ability to diagnose SFTSV infection.

We demonstrated that EBV was frequently reactivated rather than primary infected in SFTS patients, which was because there was no significant difference in EBV IgG level between the EBV-positive and EBV-negative patients, suggesting that these patients had already been chronically infected with EBV. More than 90% of SFTSV pateitns in this study were previously infected with EBV, indicating a high infection rate of EBV in the local area of Qingdiao City. Indirect evidences for EBV reactivation are the detection with IFN-γ release from T lymphocytes against EBV latent and lytic viral proteins, IgA antibody to EBV early antigen (EA), neutralising IgG to EBV dUTPase and DNA polymerase, and EBI2 gene and protein expression [13,36–39]. EBI 2 is expressed in B-lymphocyte cell lines but not in peripheral blood T lymphocytes and is more likely to be mediator of EBV effects on B lymphocytes than the ohter genes known to be up-regulated by EBV infection [30]. Our data showed that EBV reactivation in the SFTS-patients was the upregulation of human EVI2, which was consistent with others' conclusion [31].

We found that the urine red blood cells count was significantly higher in EBV-positive SFTS-patients than in EBV-negative SFTS-patients. Blood in urine may be caused by several conditions including urinary tract infections, medicines, cancer, kidney injury. Apparently the patients with hemoturia did not have any of these condations. The hemoturia in the patietns associated with EBV reactivation may be caused by glomerular injury resulting from EBV and SFTSV co-infection of kidney. A pre-

Fig. 3. Phylogenetic analysis of SFTSV sequences from patients from Qingdao City and adjacent areas, Shandong Province, China from April to September 2020. The tree was generated using the Maximum Likelihood method, the Kimura 2-parameter model and 1,000 replicates for bootstrap testing in MEGA 7.0 software. Only bootstrap values > 50% were shown. Each SFTSV strain from this study was shown with a dot. Scale bar indicated nucleotide substitutions per site. The SFTSV strains from this study were numbered from 1 to 18 and the GenBank accession numbers of reference sequences were shown in each line.

vious study reported that EBV can damage the glomerular mesangium mediated by immunoglobulin in patients with various chronic glomerulonephritides [32]. We also found hypoalbuminemia in EBV-positive SFTS patients. Albumin is exclusively synthesized in the liver and the synthesis rate is about 10 to 15 grams per day [32]. Hypoalbuminemia may be a result of decreased production of albumin by the liver or increased loss of albumin via the kidneys. Judging from the perspective of renal leakage of red blood cells, the serum albumin of EBV-positive SFTS patients may have also leaked out from the kidneys resulting in hypoalbuminemia. Proteinuria occurred in half of the EBV-positive patients, indicating loss of albumin via the kidney. Due to extreme difficulty in obtaining biopsy tissues or autopsy tissues in China, one limitation of our study was the lack of histochemical staining of kidney tissues from patients. Another limitation of our study is the small number of samples, which may affect the accuracy of the statistics.

We concluded that SFTSV infected patients frequently reactivated EBV, which was associated with glomerular inflammation.

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Author contributions

L.-Z. Fang and X.-J. Yu: conceptualization and design, data curation, analysis, investigation, methodology, validation, writing-original draft preparation, writing–

review and editing. Y.-H. Dong, Z.-J. Yan and C.-M. Zhou: data collection, supervision and software. X.-R. Qin: Writing-review and editing, final approval.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data available statement

Due to data privacy regulations and the sources of data involved multiple departments that are subject to third party restrictions, the raw data on both individual level and institutional level of this study cannot be shared.

Ethics statement

The study was conducted with the approval of the Ethics Committee of Wuhan University (2020YF0051).

Informed consent

Informed consent was obtained from all participants.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2023.04.005.

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