Original Article

Detection and Clinical Significance of Circulating Microvesicles in Severe Fever with Thrombocytopenia Syndrome

Li-ping Sun¹, Hui-yu Li², Xiao-ling Yi¹, Aime Niyonsaba¹, Ning-fang Wang² and Sheng-hua Jie¹

Objective Microvesicles (MV) released from blood cells play an important role in the progress of diseases. The purpose of this study is to detect circulating MV level of patients suffering from severe fever with thrombocytopenia syndrome (SFTS).

Methods The plasma samples of SFTS patients and healthy controls were collected. And MV from samples were isolated and MV levels were detected quantitatively.

Results The results showed that the level of the circulating MV in SFTS patients was obviously higher than that of the healthy control, with statistically significant difference. Further analysis revealed that MV level was relevant to the severity of SFTS patients, namely the higher the concentration of MV, the more severity of the disease. Linear correlation analysis showed that the circulating MV level in SFTS was correlated positively with leukocyte count ($r = 0.243$, $P < 0.05$), but no correlation with the platelet count ($r = 0.193$, $P > 0.05$).

Conclusions We demonstrated that there existed high level of circulating MV in SFTS patients, and the level of circulating MV had a close relationship with the severity of the disease and leukocyte count. Therefore, monitoring the level of circulating MV may provide a new indicator for predicting the prognosis of SFTS patients.

Key words: Microvesicles; SFTS bunyavirus; Leukocytes; Blood platelets

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease in rural areas of some provinces in central China. Recently, it was reported that the pathogenic mechanism in patients with SFTS is caused by severe fever with thrombocytopenia bunyavirus (SFTSV).¹ SFTSV can damage a variety of cells and organs,² and the major clinical symptoms included fever, thrombocytopenia, and multiple organ dysfunction. The tick sting is mainly responsible for SFTS spreading, but infection can also occur with the blood of SFTS patients when the virus load is high.³ Initially, the patients with SFTS was misdiagnosed because the clinical manifestations were similar to the hemorrhagic fever with renal syndrome, so the mortality rate was up to 30%.¹

Microvesicles (MV) are secreted as particles of

0.05-1 μm in diameter, which are generated by the outward budding and fission of membrane vesicles from the cell surface.⁴ MV was once regarded as “cellular dust”, but this view has been subjected to debates and discussions. Recent studies have found that MV, as an important carrier of biological information, can carry membrane receptor, protein, mRNA, miRNA, etc., and can be used to reprogram for target cell.⁵ MV mainly bind membrane surface ligand and target cell surface receptor to form a complex signal, and then to stimulate the target cells. They can also transport protein, mRNA and biologically active lipids for target cells to affect the biological function. The function of MV depends on the cargo they carry. In turn, it is dependent on the cell type from which they originate. In the steady state, the order of abundance is platelet-derived MV (80%), endothelium-derived MV (10%), and leukocyte-derived MV (10%).⁶

It had been reported that pathogenic microorganisms induced the host cell to release the corresponding cell-derived MV after infection. When activated and infected, cell could secrete highly specific MV.⁷ MV released by the infected cells with the characteristics

¹Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province 430022, China

²Center for Stem Cell Research and Application, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province 430022, China

Correspondence: Sheng-hua Jie, Tel: +86-27-85726135. Fax: +86-27-85729267. E-mail: abeyed@126.com

of cells and virus, including the physicochemical properties and the capacity of the biological activity of transporting macromolecules between cells.⁸ Cells which are sensitive to virus can be induced to make infection by MV in the circulating blood, while virus may make use of a variety of MV or secretory pathways of MV to complete transcription leading to the spread of virus. There is no report on SFTS and related diseases of MV. The aim of study is to detect the level of MV in the circulating blood of SFTS and analyze the relationship between the level of MV and the progress of SFTS.

PATIENTS AND METHODS

Patients

A total of 32 SFTS patients aged 35-55 years who were admitted to Department of Infectious Diseases of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between May 2012 and September 2012 were included in this study. All patients had a clinical diagnosis. Plasma of 7 patients in the beginning of the epidemic in May were collected and viral RNA were extracted for sequencing, and finally bunyavirus infection was confirmed. Other specimens were collected by the Center for Disease Control and Prevention of Wuhan City, and SFTS virus genes were detected by RT-PCR from the patient's blood. And control samples from 21 healthy volunteers were also collected. All patients and volunteers signed informed consents. The study was also approved by the ethics committee of the hospital.

Samples collection and preparation

After an overnight fast, 3 ml fresh peripheral blood sample was drawn from all patients and the controls in the fasting state. Blood samples were collected in EDTA containing tubes, which were immediately sent to the laboratory and prepared for the next steps. The whole blood was centrifuged at 2 000 g for 10 min to isolate blood plasma, and then centrifuged at 2 500 g for 20 min at 4°C and the precipitate was discarded prior to further ultracentrifugation at 16 000 g for 1 hour at 4°C with collected supernatants. The precipitation contained MV, which were resuspended in lysate and then stored at -80°C preparing for use.

Transmission electron microscopy

MV were processed by 2.5% glutaraldehyde before dehydrating, embedding, slicing and staining. Then Jeol

1210 transmission electron microscope was applied to examine the sections and take pictures.

MV detection

The concentration of MV was detected with BCA protein quantification. Firstly, working solution was prepared: the tender green standard working solution is composed of the mixture of 50 volumes of BCA alkaline solution and 1 volume of copper sulfate solution. Secondly, standard protein solution was prepared: 2 mg/ml standard bovine serum albumin was diluted with PBS to make a final concentration of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025, 0 mg/ml. Thirdly, the mixture of 5 µl sample and 100 µl working liquid was bathed in water at 37°C for 30 min. Fourthly, the optical density (A) values at 562 nm were measured.

Statistical analysis

Data were analyzed by SPSS 17.0 statistical analysis software and were shown as means ± SD. Comparison of various groups were performed using the Student's *t* test. And *P* values < 0.05 were considered statistically significant.

RESULTS

Morphology of MV under the transmission electron microscope

The morphology of acquired MV were furtherly observed by electron microscope. Under the electron microscope, the diameter of MV from SFTS patients was less than 1 µm, which were around cells in Figure 1A as well as for the normal controls in Figure 1B. Meanwhile, our results also confirmed that there was no significant difference between morphology of MV derived from the peripheral blood of SFTS patients and MV derived from normal controls (Figure 1).

Level of MV in circulating peripheral blood of SFTS patients

Our results showed that the level of MV in 32 SFTS patients was 4.86 ± 2.18 µg/ml, and 3.0 ± 1.28 µg/ml for the normal control, with significantly difference (*P* < 0.05, Figure 2). It demonstrated the presence of high levels of MV in circulating peripheral blood of SFTS patients.

The relationship between the severity of SFTS and the level of MV

According to the condition severity of the SFTS patients, samples were divided into three groups: mild

Figure 1. The MV under the transmission electron microscope.

Representative photographs of MV derived from the peripheral blood of SFTS patients (Figure 1A) and normal control (Figure 1B). Single arrow pointed to MV.

Figure 2. The concentrations of MV in peripheral blood of SFTS patients and the normal control.

Notes: * $P < 0.05$ compared with the normal control using Student t test. Data expressed as means $\pm$ SEM.

Figure 3. The level of MV in patients with SFTS in mild group, moderate group and severe group.

Notes: * $P < 0.05$ compared with mild group; ** $P < 0.05$ compared with moderate group. Data expressed as means $\pm$ SEM.

group, moderate group and severe group. The standards of mild group were leucocyte count $(3-4) \times 10^9/L$, platelet count $(70-100) \times 10^9/L$ and the temperature $< 38^\circ C$, with no significant organ dysfunction. The standards of moderate group were leucocyte count $(2-3) \times 10^9/L$, platelet count $(30-70) \times 10^9/L$, the temperature ranging from $38^\circ C$ to $39^\circ C$ and with two organs dysfunction. The standards for severe group were leucocyte count $< 2 \times 10^9/L$, platelet count $< 30 \times 10^9/L$, the temperature $> 39^\circ C$ and multiple organ dysfunction, even systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulation (DIC) tendency. The concentration of MV for mild SFTS patients was $3.42 \pm 1.54 \mu g/ml$, $5.04 \pm 2.52 \mu g/ml$ for moderate SFTS patients, and $5.84 \pm 2.23 \mu g/ml$ for severe SFTS patients (Figure 3). The level of MV was closely related with the severity of SFTS, namely the higher the concentration of MV, the

severer the disease was.

The correlation between the level of MV in SFTS patients and leucocyte count

If SFTS patients were in the early stages of the disease, namely in the onset of the first week, the leucocyte count was less than $4 \times 10^9/L$, with the average expression level of MV as $3.06 \pm 1.04 \mu g/ml$. On the condition that SFTS patients with secondary infection (positive results of blood culture and sputum culture), the leucocyte count was higher than $10 \times 10^9/L$, and the level of MV was $5.33 \pm 2.08 \mu g/ml$. During the recovery period, the leucocyte count gradually returned to normal, and the levels of MV was $5.54 \pm 2.29 \mu g/ml$ (Figure 4). Statistical analysis showed that leucocyte count was positively correlated ($P < 0.05$) with the MV level (the correlation coefficient $r = 0.243$).

Figure 4. The level of MV in SFTS patients in acute phase, recovery phase and secondary infection phase.

* $P < 0.05$ compared with acute phase; ** $P < 0.05$ compared with acute phase. Data expressed as means $\pm$ SEM by Student t test.

The correlation between the level of MV in SFTS patients and platelet count

According to the levels of thrombocytopenia, SFTS patients were divided into three groups: mild group, moderate group and severe group. In the mild group, platelet count was higher than $70 \times 10^9/L$, and the level of MV was $3.73 \pm 1.83 \mu g/ml$. In the moderate group, platelets were $(30 - 70) \times 10^9/L$, and the MV level was $4.15 \pm 1.42 \mu g/ml$. In the severe group, platelet count was lower than $30 \times 10^9/L$, and MV level was $6.48 \pm 2.77 \mu g/ml$ (Figure 5). Bleeding did not occur in SFTS patients. Even with equal platelet count, the MV level in SFTS patients with subcutaneous ecchymosis was lower than that in SFTS patients without ecchymosis. Meanwhile, the correlation analysis showed that there was no significant relationship between the MV level and platelet count, with correlation coefficient r as 0.193, $P > 0.05$.

DISCUSSION

SFTS is an emerging infectious disease in recent years in China. In this study, we observed the molecular characteristics of the MV in the circulating peripheral blood of SFTS patients to explore the relationship between MV and SFTS. We found that the expression level of MV in SFTS patients was obviously higher than that in the control group. The results demonstrated that there existed a higher level MV in the circulating peripheral blood of SFTS patients.

It showed that the expression level of MV was correlated with the severity of SFTS. Namely, the severer the disease changed, the higher the expression

Figure 5. The relation between the level of MV and platelet count in SFTS patients.

* $P < 0.05$ compared with mild group; ** $P < 0.05$ compared with moderate group. Data expressed as means $\pm$ SEM by Student t test.

level of MV was. Previous studies have shown that MV widely existed in the surrounding of the host cells as an information carrier, which greatly helped to increase the opportunity of infection with host cells. MV can transport virulence factors and other pathogenic ingredients to the host cells.^{9,10} Simultaneously, the specific MV excreted by pathogens with antigen component can stimulate leucocyte, platelet, endothelial cells and fully activate inflammation, coagulation system and the immune response of complex network systems. Thereby, serious consequences include SIRS, multiple organ dysfunction syndrome (MODS), DIC and so on may occur. We speculated that MV might play a pro-thrombotic and pro-inflammatory roles through stimulating platelets, leukocytes to aggravate the conditions. On the other hand, they could also raise immune cells such as monocytes, T lymphocytes, B lymphocytes, NK cells to stimulate immune responses and cause immune pathological damages, even multiple organ dysfunction syndromes.¹¹ So MV may be one of the reasons which make SFTS more serious.

Our study also found that the level of MV was positively correlated with leukocyte count. Taking the occurrence mechanism of MV into account, we speculated that SFTS pathogens firstly bind to leucocytes through related receptors, such as P-selectin glycoprotein ligand-1 after infection, and then encroach upon their cytoplasm to entice TF (tissue factor, TF) expression and produce MV containing TF. After the destruction of cell structure, the SFTS pathogens released into circulating blood decreased the level of leukocytes, but enhanced the level of MV. On the condition that SFTS patients were in secondary infection, their levels of leukocytes and MV were

higher than those in the normal control. The level of MV was positively correlated with leucocyte count. It indicated that SFTS patients with the secondary infection were more serious than SFTS patients without the secondary infection. Otherwise, the level of MV of SFTS patients in recovery phase was higher than that of SFTS patients in acute phase, which may be related to MV metabolic cycle. As a large number of MV derived from leukocytes of SFTS patients still remain in the initial stage, and MV continued to be produced when the leucocyte count was normal in this stage, so MV were at a high concentration compared with the initial stage. Therefore, MV derived from leukocytes in SFTS may be effective indicators to assess the severity of SFTS.

The results also showed that MV level from SFTS patients with platelet count lower than $30 \times 10^9/L$ was higher than that of patients with platelet count higher than $30 \times 10^9/L$. MV level of SFTS patients without bleeding was distinctly higher than that of SFTS patients with bleeding. It is reported that cells released various MV in different states, different types, different stimulation conditions, and with different functions. MV content was lower than 3% when the platelets were in resting state. But once they were activated, MV increased significantly. Therefore, MV derived from platelets were the products of platelet activation, also a type of potent coagulant. As GP- I b, GP II b - III a, P-selectin, Ca^{2+} dependent protease and pro-coagulant phospholipid(s) were rich in platelet-derived MV membranes, which could furtherly activate platelets and provide greater phospholipids catalytic surface for coagulation cascade.¹² As far as promotion of clotting effect is concerned, platelet-derived MV are 50-100 times stronger than the activated platelets.¹³ Therefore, platelet-derived MV may play an important role on the coagulation in SFTS. But we have not found that the SFTS patients with the high level of MV had thrombosis. We also found that platelet number had no significant correlation with MV level in SFTS patients.

Numerous studies have also showed that the pathogenic microorganism can make host cells release corresponding MV originated from cells after contraction of the host and it can also release the pathogen-specific MV.^{9,10} These are the reasons for MV level increasing. Therefore, we speculated that increased MV level in SFTS patients might partly come from the fragments of SFTSV which were formed by the destruction and decomposition. These fragments were MV-specific components, and might play a special

role in the occurrence and development of SFTS.

In conclusion, our study has explored the relationship between MV and SFTS for the first time. In SFTS patients, MV increased significantly and might play important roles in the development of SFTS. MV level was positively correlated with the severity of SFTS and the level of white blood cells. Platelet-derived MV were also important in the procession of SFTS. MV level in SFTS patients may become an indicator which can be used for diagnosis and prognosis of SFTS patients.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose the conflict of interest with this manuscript.

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