Saudi Journal of Biological Sciences 27 (2020) 2215–2220

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Differential proteomics analysis of serum exosomein burn patients

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ARTICLE INFO

Article history:
Received 16 April 2020
Revised 26 May 2020
Accepted 12 June 2020
Available online 26 June 2020

Keywords:
Bioinformatics
Burns
Exosomes
Label free quantification
Proteomics

ABSTRACT

The exosome is an emerging concepts biomarkers due to their abnormal expression in various diseases. Research on exosome has already shifted from the laboratory to clinical application. This study uses bioinformatics technology to identify functional changes in proteins of serum exosomes from burn patients. A total of 231 quantifiable differentially-expressed proteins were screened out, 31 of them had statistically significant changes in expression levels. In the test group, expression of2 proteins had downregulated, whereas that of 29 proteins upregulated. Gene Ontology analysis demonstrates that differentially-expressed proteins were primarily identified in extracellular vesicles and platelet α granules, which can alter enzyme inhibitor activities, heparin-binding, coagulation, and lipid transport. Kyoto Encyclopedia of Genes and Genomes pathway analysis demonstrates that ITGA2B and ITGB3 proteins, which were significantly upregulated in the burn group, were primarily involved in the PI3K/AKT signaling pathway. Western blotting confirmed that the expressions of ITGA2B and ITGB3 in burn patient tissue samples were higher than those in the control group; conversely, the expression of CD9 was lower than that in the control group. In burn patients, the upregulated proteins ITGA2B and ITGB3 of serum exosomes likely participate in injury detection and repair via PI3K/AKT signaling pathways.

1. Introduction

Since the publication of the human proteome in 2014, proteomics research has been expanding day-by-day and has been widely used in basic scientific research, clinical trials, and other fields. Proteomics combines laboratory results with bioinformatics. By mapping the protein fingerprint of a given sample, a large number of disease-related protein markers can be identified, in recent years, with the research on exosomes heating up, many researchers have turned their attention to analyzing the function of specific exosome proteins and other exosome biomarkers (Cobelli et al., 2017).

Exosomes are 40–150 nm vesicles that exist in most humoral environments. Lipids and proteins are the main components of exosome membranes (Mathivanan et al., 2010; Simons and Raposo, 2009; J. Zhang et al., 2015). Exosomes carry a variety of bioactive substances and participate in multiple signaling pathways (Gross et al., 2012; Li et al., 2016). Exosomes are secreted to the external environment from cells through an "intake-integration-excretion" mechanism (Y. Zhang et al., 2015). Exosomes are rich in proteins, nucleic acids, microRNAs, and other non-coding RNAs (Sato-kuwabara et al., 2015), and they involve in angiogenesis, cell proliferation, differentiation, and other biological processes. However, whether the number of exosomes in body fluids as well as the quantity and categories of protein cargo they carry changes in response to serious injuries (e.g., severe burns), is yet to be elucidated (Karami et al., 2019). Therefore, we performed proteomic analyses for serum exosomes collected from both burn patients and healthy adults using a label-free quantification (LFQ) combined with computational tools to screen differentially-expressed proteins in exosomes, which were then verified. The mechanism of post-burn injury repair was interpreted and speculated from the protein level, and this may provide new targets for the treatment of burn injuries.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.06.024
1319-562X/

2. Materials and methods

2.1. Patient enrollment in test and control groups

A total of 30 burn patients (total burn area ≥30%) from the Plastic and Burn Department (Tangdu Hospital of The Fourth Military Medical University from October 2018 to May 2019) were selected as the test group. Another 30 healthy adults who received circumcisions in the same period were chosen as the control group. All patients and healthy volunteers signed an appropriate informed consent form. This study was approved by the Ethics Committee of the Tangdu Hospital of The Fourth Military Medical University.

2.2. Clinical samples

Blood samples were extracted to collect exosomes. Venous blood (5 mL) was collected from each subject into containing tubes without procoagulant, and centrifuged (3500 rpm × 8 min) to extract serum at room temperature. All serum samples were stored at −80 °C. Tissues of debridement of burn patients and prepuce of circumcision were collected,and were stored at −80 °C until protein extraction.

2.3. Extraction of exosomes

Serum exosomes were extracted by differential centrifugation of serum samples with 10,000*g* for 30 min at 4 °C. Supernatants were then transferred to 5 mL ultrahigh-speed centrifugal tubes (supplemented with 1 × PBS) and centrifuged twice at 17,000*g* for another 2 h at 4 °C, after which supernatants were removed. The supernatants were then centrifuged again under the same conditions. The pellets were resuspended in the corresponding 1 × PBS, filtered with a 0.22 μm filter, and stored at −80 °C until further analysis.

2.4. Identification and confirmation of exosomes

Specific proteins of exosomes were confirmed by Wester Blot. Transmission Electron Microscope (TEM) was used for the identification of exosome morphology by JEM-1010 electron microscope (JEOL Ltd., Tokyo, Japan). Exosome nanoparticle size distribution was confirmed by Nanoparticle Tracking Analysis (NTA).

2.5. Extraction of differential protein in exosomes based on label free quantification (LFQ)

For the follow-up study, three cases were randomly selected from each group. Samples were processed by computer for mass spectrometry after preprocessing, Filter Aided Proteome Preparation (FASP) enzymolysis, ZipTip desalination, and LC-MS analysis. Samples were then scanned using data-dependent analysis (DDA). A maximum of 20 fragment spectra could be collected after every full scanning. The full-scanning resolution, MS/MS resolution, parention scanning range, and collision energy were set at 70,000, 17,500, 350–1600 *m*/*z*, and 28% HCD, respectively.

2.6. Bioinformatics analysis

Max Quant software was used for library searching. A statistical analysis of searching results was performed by eliminating Reverse and Potential Contaminant proteins. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on the differential proteins.

2.7. Verification of differentially expressed proteins in exosomes

Expressions of differentially expressed proteins in tissues of burn patients and the prepuce of healthy adults were verified by real-time PCR and Western Blot. For details see the sections of Real-time PCR and Western Blot.

2.8. Nanoparticle tracing assay (NTA)

Frozen exosome samples were selected and thawed in a water bath under 25 °C. After dilution and filtration, the mixture was tracked and detected by NTA.NTA 3.0 (build 0064) software was used to record two consecutive 60-second videos with 30 frames per second for each sample. The above steps were performed using a NanoSight NS300 with a monochromatic 488 nm laser, sCMOS camera, and a syringe pump (software and instruments are from Malvern Instruments, Malvern, UK).

2.9. Transmission Electron Microscopy (TEM)

Serum ultracentrifuged pellets were resuspended in 4% paraformaldehyde. Following this, 5 mL droplets were deposited on a TEM grid coated with formaldehyde and washed with PBS. Grids were incubated for 5 min in 1% glutaraldehyde and then washed seven times with water. Samples were contrasted and embedded with uranyl-oxalate and methyl cellulose-uranyl acetate. Finally, the grid was observed with a CM10 80 kV electron microscope (Thermo Scientific Metrios™, America).

2.10. Real-time PCR

Total RNA was isolated from cells using the Oligotex direct mRNA microkit (QIAGEN) and 1 μL of RNA was quantified. An equal amount of RNA was reverse transcribed for the synthesis of cDNA. Real-time PCR was performed with a real-time PCR system (ABI 7900 HT) using PCR mixture (SYBR Green I). In our study, the primers were synthesized using GENERAY Biotechnology Co., Ltd. (Shanghai, China). GAPDH was used as the internal control.

2.11. Western Blot

Samples in RIPA buffer (Solarbio) were cleaved and centrifuged for 20 min to extract the protein from the cell. The quality of the protein was measured using the Bradford method. An equal amount of protein was isolated using SDS-PAGE and the protein was transferred to the PVDF membrane at 4 °C with a primary antibody purchased from Sigma (America). Samples were then incubated for 24 h against CD9, ITGA2B, ITGB3, p-AKT, and AKT-detecting antibodies, and then re-incubated at room temperature with a secondary antibody for 1 h. β-actin was used as internal control.

2.12. Statistical analysis

SPSS software (version 23.0) was used to perform the statistical analyses. Data were presented as means ± SD. One-way ANOVA or Student *t*-test was used to examine for differences among groups, and p-value <0.05 was considered as statistically significant difference.

3. Results

3.1. Inspection and Confirmation of exosomes

The Western Blot results verified the extraction of exosomes successfully by showing the presence of CD9 and CD63 (Fig. 1A). NTA results showed that the particle sizes ranged between 30 and 180 nm, which met the standard criteria of exosomes. The results of three repeated experiments were consistent. The average concentrations of exosomes in the test group and control group were $(0.67 \pm 0.03) \times 10^{11}$/ml and $(1.12 \pm 0.03) \times 10^{11}$/ml, showing statistically significant differences ($p < 0.01$, Fig. 1B). The typical double-layer cup-shaped structure of exosomes was observed by TEM (Fig. 1C).

3.2. LFQ results

A total of 231 quantifiable proteins were tested. Among them, 31 proteins had significantly differential expressions, including 29 with upregulated expressions and two with downregulated expressions. Proteins with significantly upregulated expression included ITGA2B, ITGB3, YWHAZ, MMRN1, S100A9, IGFALS, MPO, FCN2, and CAT. The two proteins with significantly downregulated expressions were CD9 and IGLV3–1. Details are shown in Fig. 2A and B.

3.3. GO enrichment analysis

Three gene ontologies (GO) were selected: molecular function (MF), cellular component (CC), and biological process (BP). Results demonstrated that differentially expressed proteins are located mainly in extracellular vesicae and platelet α granules. These proteins can influence the activity of many enzyme inhibitors and the binding force of heparins (Page, 2013). Moreover, they participate in coagulation and lipid transfer (Fig. 2C). KEGG pathway analysis reflected that proteins ITGA2B and ITGB3, whose expressions were upregulated, mainly participate in the PI3K–Akt signaling pathway (Fig. 2D).

3.4. Verification of differential proteins

Real-time PCR showed that the expression of ITGA2B and ITGB3 was significantly higher in the test group (n = 30; tissues of burn patients) than that of the control group (n = 30; tissues of foreskin from healthy adults; about 2.5-fold, 2.3-fold; Fig. 3A). Moreover, expression of p-AKT and AKT was also upregulated by 1.85-fold and 1.62-fold in the tissues of burn patients (n = 30; Fig. 3B). The protein level of CD9 expression was significantly decreased in the tissues of burn patients (n = 30; Fig. 3C).Western Blot results showed that ITGA2B, ITGB3, p-AKT, and AKT expressions in the test group were higher than those in the control group, meanwhile the expression of CD9 was lower than that in the control group (Fig. 3A–C).

3.5. Discussions

Exosomes are primary components for the normal function of paracrine of cells (Gu et al., 2018; Kanlikilicer et al., n.d.; Nakamura et al., 2019; Raeven et al., 2018). Clinical studies on exosomes have shown the following advantages: (1) Exosomes possess relatively low levels of immune responses and high safety (Chen et al., 2017; Y. Zhang et al., 2015). (2) Exosomes can be used as carriers and have high biopotency. (3) Exosomes have an extensive number of sources and can be extracted from various fluids. Concentration, particle size, and categories of the proteins carried in exosomes may vary in different tumors; influencing disease development. (4) Exosomes have a double-layered membrane structure, which can protect proteins and RNA from enzyme degradation and destruction by the humoral environment. Therefore, the detection of exosomal biological markers can provide increased accuracy than the direct measurements of biological markers in body fluids. Previous studies have confirmed that changes in exosome concentration, particle size, and protein type can be observed in all types of tumors and immune diseases and can alter the disease course (Zhang et al., 2019). However, changes to exosomes after trauma, especially burns, have not been studied.

This study found that CD9 expression in the test group was significantly down regulated. These results are consistent with decrease in exosome concentration in the test group, as indicated by NTA results, and it confirms that decrease in exosome concentration after burns might partly account for the body dysfunction. The most significant upregulated proteins were ITGA2B and ITGB3, confirming that burn damage may promote the synthesis and secretion of ITGA2B and ITGB3 in serum exosomes. ITGA2B and ITGB3 belong to the integrin family, and are involved in many signal transduction pathways that alter cellular signaling, including exercise, growth, and even cell survival. It has also been reported that they are associated with tumor-cell invasion, cell–cell

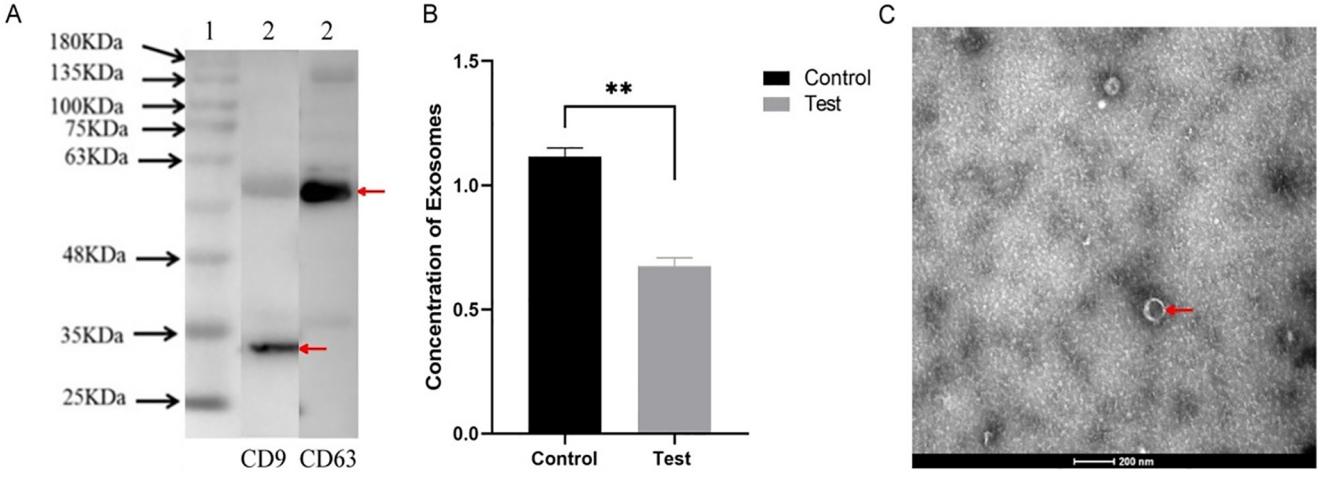

Fig. 1. Confirmation of exosomes. Exosome specific marker proteins CD9 and CD63 were positive (A). 1 is lysate and 2 is exosome sample in this figure. Red arrows indicate CD9 and CD63. Concentration of Test and Control group of exosomes were measured by NTA, and the difference between the two groups was statistically significant (B) (*p<0.05, **p < 0.01). Typical exosome structures were observed by TEM (7500×): double capsule, cup-shaped structure. Red arrow indicates exosome (C).

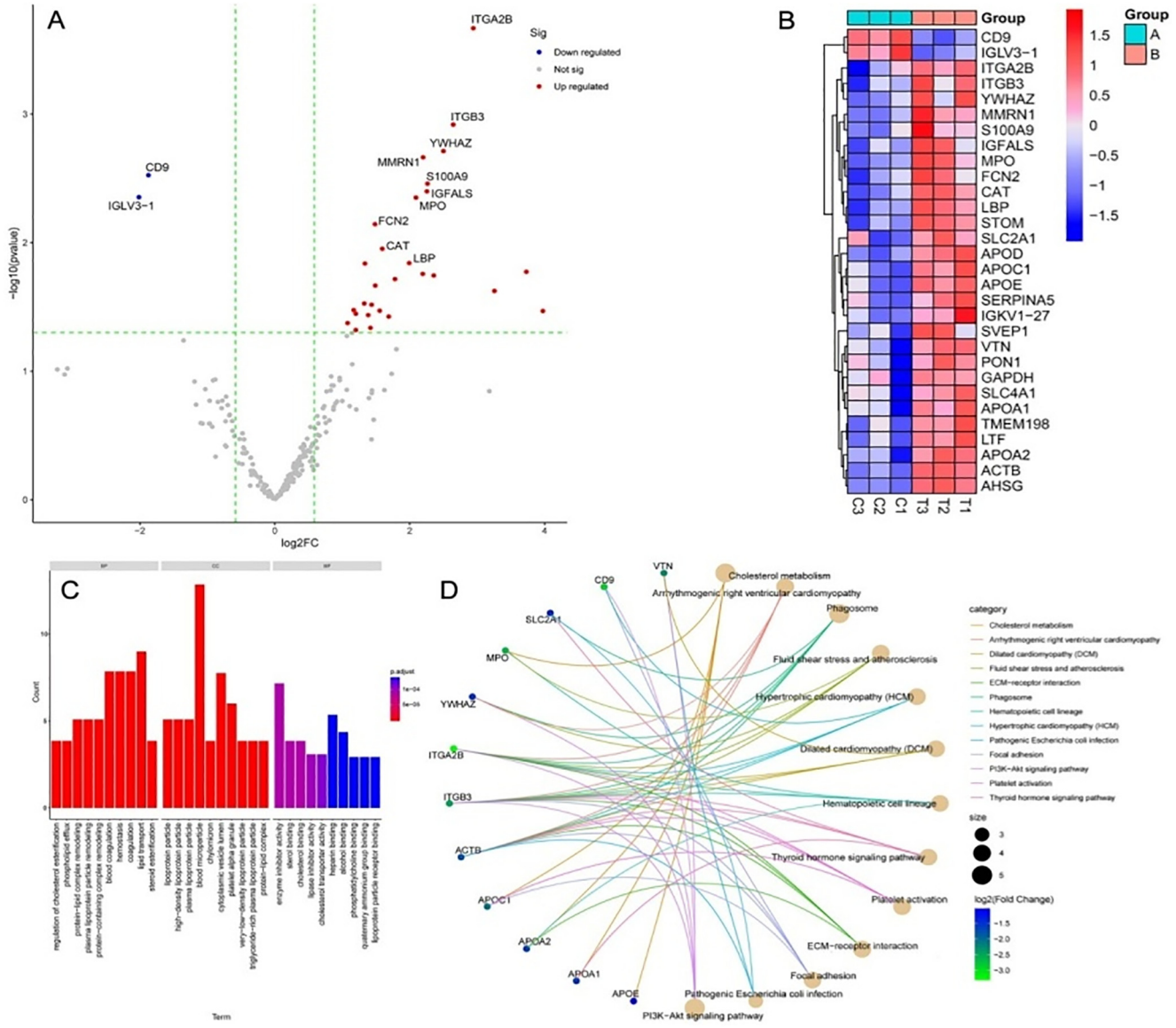

Fig. 2. Bioinformatics techniques for screening differential proteins. Differential Protein Volcano Map. X-axis: logarithmic value of fold change; logarithmic value of p value. Gray dots represent proteins that are not significantly different, red dots represent upregulated proteins, and blue dots represent downregulated proteins. According to the fold change from high to low, each top10 protein is selected to display the protein name, and less than 10 are all displayed (A). Differential protein clustering heat map. Each column is a sample and each row is a protein. The shorter the distance between clusters, the higher the similarity of the proteins. Test group compared to Control group: Red represents upregulated protein and blue represents downregulated protein. The darker the color, the greater the change (B). GO enrichment analysis of differential proteins. The analysis results of GO were clustered according to the coincidence percentage of differential proteins. When the shared differential proteins exceed 50%, they are classified into the same cluster. Different clusters are displayed in different colors. Each cluster uses the name of the most significant group. The redder the color, the smaller the P value (C). The blue and green dots in the left half of the figure represent differential proteins; the closer to green, the more obvious the protein differences between the two groups; the brownish yellow dots in the right half are the pathway names, and the larger the dots, the higher the enrichment of the pathway. Different colored lines indicate the correlation between proteins and pathways. One protein can correspond to multiple pathways, and one pathway can also be related to multiple proteins (D).

adhesion, and neovascularization. ITGA2B and ITGB3 are also known as the platelet glycoprotein IIb of the IIb/IIIa complex, and platelet glycoprotein IIIa. Studies have shown that the aforementioned proteins are functionally related to cell-to-cell matrix attachment, platelet development and function, and coagulation mechanisms, which are consistent with GO enrichment analyses: Differential proteins primarily exist in extracellular vesicles and platelet alpha particles. Exosomes are extracellular vesicles, and these results confirm that the proteins they carry or secrete have been altered after burns (Ludwig et al., 2019). The platelet α granules contain Fg, vWF, and β-TG. They are related to coagulation, thrombosis, angiogenesis, and platelet release, consistent with blood coagulation, percutaneous wound thrombosis, and active angiogenesis after burns, further confirming the reliability of this study. According to previous reports, ITGA2B can be combined with Fg to alter its activity. When ITGA2B expression is upregulated, wound blood clots retract, platelet release is improved, and wound healing process is positively regulated. ITGB3 is closely related to vWF, which can surround blood clots. When ITGB3 expression is upregulated, it can quickly inhibit wound bleeding and reduce the risk of infection. Therefore, we have reason to believe that the upregulation of ITGA2B and ITGB3 expression may improve post-burn wound repair.

ITGA2B and ITGB3 activate the PI3K/Akt signal pathway. As a cell survival signal pathway, it can protect cells, especially the nerves (Xu et al., 2008; Yuan et al., 2011). The PI3K/Akt pathway participates in cell proliferation, differentiation, and apoptosis (Chen et al., 2014; Wang et al., 2011). When this pathway is highly

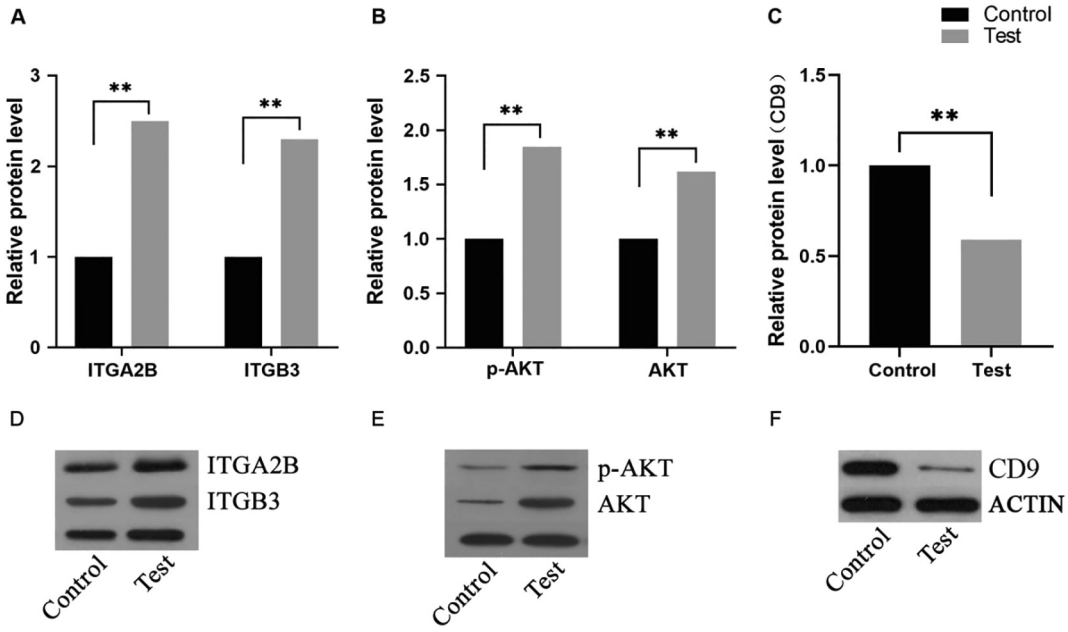

Fig. 3. Confirmation of differential proteins. The expression of ITGA2B(A), ITGB3(A), p-AKT(B), AKT(B) and CD9(C) in the tissues of debridement of burn patients (n = 30) and were detected using qRT-PCR, foreskin from circumcision of healthy adults (n = 30) as the control (* $p < 0.05$, ** $p < 0.01$). The protein expressions of ITGA2B(D), ITGB3(D), p-AKT(E), AKT(E) and CD9(F) in the burn wound tissues and foreskin from circumcision of healthy adults were measured by western blot assay.

activated, it can strongly inhibit inflammatory responses, reduce atherosclerosis, and promote angiogenesis. Activation of the PI3K/Akt signal pathway can also induce inositol phosphorylation on cell membranes, producing PI-3P, PI-3,4-P2, and PI3,4,5-P 3. PI-3,4-P2, and PI3,4,5-P 3 can bind to the PH-domain of Akt to for its partial activation Akt (Huang et al., 2018). This pathway plays an important role in injury through the mediation of activated Akt on insulin, a variety of cytokines, thrombin, etc. The specific mechanism is as follows: First, the activation of the PI3K-Akt signaling pathway changes the cell cycle and inhibits apoptosis. Second, it induces macrophage migration, accelerating the elimination of metabolic waste and necrotic tissue, reducing tissue damage. Third, it regulates the cell membrane permeability, further promoting the elimination of metabolic waste, reducing ischemia and hypoxia, accelerating the rate of angiogenesis, and wound healing.

In summary, ITGA2B and ITGB3 expressions are upregulated in the serum exosome of burn patients and participate in wound healing after burn injuries, making serum exosomes a new target for burn treatment. In addition, ITGA2B and ITGB3 can be negatively regulated by miRNAs, which is the focus of our follow-up studies.

4. Conclusion

In burn patients, the upregulated proteins ITGA2B and ITGB3 of serum exosomes likely participate in injury detection and repair via PI3K/AKT signaling pathways.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

This work was part of the National Natural Science Foundation of China, No: 31570986.

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