Original Article

Bioinformatics analysis combined with experiments to verify potential autophagy genes in wound healing

Yong-Fang Wu[1], Da-Lang Fang[2], Jie Wei[3]

[1]Department of Burn and Plastic Surgery, the People's Hospital of Baise, Baise, China; [2]Department of Breast and Thyroid Surgery, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China; [3]Department of Hematology, the People's Hospital of Baise, Baise, China

Contributions: (I) Conception and design: YF Wu, J Wei; (II) Administrative support: DL Fang; (III) Provision of study materials or patients: YF Wu; (IV) Collection and assembly of data: YF Wu; (V) Data analysis and interpretation: YF Wu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Da-Lang Fang. Department of Breast and Thyroid Surgery, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China, 18 Zhongshan 2nd Road, Youjiang District, Baise 533000, China. Email: 604925698@qq.com; Jie Wei. Department of Hematology, the People's Hospital of Baise, 8 Chengcheng Road, Baise 533000, China. Email: 654680956@qq.com.

Background: The skin is the most exposed tissue and has multiple functions. Wound healing is a major medical problem due to trauma and pathophysiological alterations suffered by patients. The aim of the present study was to search for potential autophagy genes associated with wound healing.

Methods: The GSE168760 dataset was obtained from the Gene Expression Omnibus (GEO) database, and sequencing results were obtained for 14 patient traumas at different time periods. Differentially expressed gene (DEG) analysis, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed. Immune cell and correlation analysis were performed for autophagy genes and DEGs. Peripheral blood was collected from patients at different time periods and Western blot (WB) assay was performed to verify autophagy genes.

Results: A total of 226 DEGs were screened on days 0, 7, and 14, of which 162 genes were upregulated and 64 genes were downregulated. Of these, eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*) and retinoblastoma 1 (*RB1*) were autophagy-associated genes. The DEGs were mainly involved in response to virus, cellular response to type I interferon Epstein-Barr virus infection, human papillomavirus infection, ribosome, hepatitis B and RIG-I-like. *EIF2AK2* and *RB1* showed positive correlation with some of the immune cells, and WB showed that *EIF2AK2* and *RB1* proteins were significantly increased with wound healing.

Conclusions: The comprehensive analysis of GEO data in the present study provides a new theoretical basis for the molecular pathogenesis of trauma healing and potential autophagy-related therapeutic targets.

Keywords: Bioinformatics; wound healing; autophagy; immunity

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Introduction

The skin is the most exposed, susceptible, and largest tissue in humans, and is the main barrier that protects the internal tissues of the organism from damage caused by external harmful substances (1). Trauma is damage to normal skin tissue caused by external injurious factors, as well as intrinsic factors, of the body. Large skin defects and a variety of pathological states or diseases due to diabetes, vascular diseases, infections, and cancer, can lead to delayed or non-healing of skin wounds (2). Poor and delayed wound healing not only seriously affect patients' physical and mental health and treatment but can also cause significant economic burden to patients and society. With increasing

trauma cases, wounds have become an important medical problem (3). Therefore, how to effectively control wound development and improve healing has become a key issue in clinical research.

Wound healing is a dynamic process involving a complex set of biologic behaviors. A number of cells and cytokines play an important role in the process of trauma healing. It has been demonstrated that autophagy is involved in the regulation of mesenchymal stem cell therapy related to wound repair (4). In addition, autophagy also plays an important role in regulating the function of cells involved in various stages of trauma healing. Autophagy is the process by which lysosomes fuse with intracellular components, degrade them, and ultimately achieve the metabolic needs of the cell itself and the renewal of certain organelles (5). Studies have shown that autophagy is involved in the regulation of a number of cellular functions and is closely related to the development of diseases, such as cancer, aging, and trauma, and is currently a hotspot in research (6,7). Autophagy can reduce the production of inflammatory factors, induce wound angiogenesis, reepithelialization and scar formation in the process of wound healing, and can also improve the impaired cell activity in high glucose environment (8-11). However, the mechanism of the role of autophagy in trauma healing is still unclear.

High-throughput sequencing technologies provide genome-wide analyses of gene expression, helping researchers to study dynamic changes in gene expression and explore changes in molecular and cellular states over time (12). Researchers now use DNA microarrays to study the physiological and pathophysiological transcriptional responses of wound healing (12,13). However, these studies have not been fully elucidated. With the help of bioinformatics and *in vitro* experiments, we paid more attention to the role of autophagy related genes in wound repair and carried out experimental verification, the aim of the present study was to further elucidate the mechanisms of action associated with the trauma healing process, providing a theoretical basis and practical foundation for improving trauma healing. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2033/rc).

Methods

Raw data

The GSE168760 dataset was downloaded from the Gene Expression Omnibus (GEO) database and contained the wound sequencing data of 14 patients at different time periods. A list of autophagy-related genes was downloaded from the Human Autophagy Database.

Differentially expressed genes (DEGs) at different time periods

To find the DEGs that changed during the healing process, we compared the sequencing results of the wound on day 7 and day 0 and between day 14 and day 0. The filtering standard was log fold change >0 and false discovery rate <0.05. A heatmap and volcano plot of DEGs were obtained from ggplot2 (R package, https://www.r-project.org/).

DEG functional enrichment analysis

We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses through clusterProfiler (R package); the results were observed in ggplot2.

Screening of autophagy-related genes in early wound healing

We intersected DEGs and autophagy-related genes to obtain wound healing-related autophagy genes. The expression levels of autophagy-related genes at different time periods were further analyzed.

Immune cell infiltration analysis during early wound healing

We compared the immune infiltration of wounds on day 7 and day 0 via single-sample gene set enrichment analysis (ssGSEA), and compared the immune infiltration of wounds on day 14 and day 0. The relationship between autophagy-related genes and immune cell infiltration in wound healing was also analyzed.

Clinical sample collection and Western blot (WB) experiments

The dataset GSE168760 we analyzed was based on sequencing of peripheral blood. On the one hand, it was consistent with the dataset; on the other hand, peripheral blood was easier to collect and samples could still be collected after the patient's wound healed. A total of

5 mL peripheral venous blood was collected on days 0, 7, and 11 from 7:00 a.m. to 7:30 a.m. using an Ethylene Diamine Tetraacetic Acid (EDTA) vacuum blood collection tube. Whole blood proteins were extracted using a whole blood protein extraction kit (BB-3140; Bestbio, Shanghai, China). Protein concentration was determined using a BCA kit (P0012; Beyotime). Following sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transfer to Polyvinylidene Fluoride (PVDF) membranes, the membranes were closed at room temperature for 2 h, and then washed with Western wash buffer TBST and incubated with *EIF2AK2* (18244-1-AP, Proteintech Group) and *RB1* (10048-2-Ig; Proteintech Group) primary antibodies at 4 ℃ overnight. Electrochemiluminescence was used to develop the bands, and the grayscale values of the target bands were analyzed by Image J software (NIH). GAPDH (60004-1-Ig; Proteintech Group) was used as the internal reference to calculate the ratio of the target protein to the internal reference grayscale value and to compare the protein expression levels of each group. And all patients' samples were collected from the People's Hospital of Baise. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethic committee of People's Hospital of Baise (No. LLSC-2022-10). Patients provided signed informed consent prior to study commencement.

Statistical analysis

WB results were analyzed using SPSS version 23.0 (IBM, Armonk, NY, USA). All data were expressed as mean ± standard deviation (n=3); *t*-tests were used to compare differences between the experimental and control groups. $P<0.05$ was considered statistically significant.

Results

DEG results on day 7 and 14 compared with those on day 0

The difference analysis of day 7 compared with day 0 indicated that there were 248 downregulated genes and 474 upregulated genes. The difference analysis on day 14 compared with day 0 showed that there were 114 downregulated genes and 275 upregulated genes (*Figure 1*).

To find up regulated and down regulated genes, we obtained 64 co-downregulated genes and 162 co-upregulated genes by intersecting the DEGs on day 7 and day 14 compared with day 0, respectively (*Figure 2*).

Functional enrichment analysis of co-regulated DEGs in early wound healing

GO enrichment function analysis results indicated that response to virus, defense response to virus, type I interferon signaling pathway, cellular response to type I interferon, and response to type I interferon were the top 5 biologic processes. Collagen-containing extracellular matrix, focal adhesion, cell-substrate junction, cytosolic ribosome, and complex of collagen trimers were the top 5 cellular components. Extracellular matrix structural constituent, double-stranded RNA binding, integrin binding, RNA helicase activity, and extracellular matrix structural constituent conferring tensile strength were the top 5 molecular functions (*Figure 3A*,*3B*).

KEGG enrichment function analysis indicated that coronavirus disease 2019, herpes simplex virus 1 infection, hepatitis C, Epstein-Barr virus infection, measles, influenza A, human papillomavirus infection, ribosome, hepatitis B, and the retinoic acid-inducible gene I-(RIG-I)-like receptor signaling pathway were the top 10 enrichment signaling pathways (*Figure 3C*,*3D*).

Expression levels of autophagy-related genes in early wound healing

Through the intersection of co-regulated DEGs and autophagy genes, we obtained the following 2 autophagy genes related to wound healing: *EIF2AK2* and *RB1* (*Figure 4A*). The findings indicated that the expression levels of *EIF2AK2* and *RB1* on the day 7 and day 14 were higher than those on the day (*Figure 4B-4E*).

ssGSEA immune cell infiltration analysis during early wound healing

Compared with day 0, the infiltration level of central memory CD8 T cells, effector memory CD8 T cells, activated CD4 T cells, and central memory CD4 T cells on day 7 and day 14 were higher (*Figure 5A*,*5B*). Compared with day 0, the expression levels of *EIF2AK2* and *RB1* were positively correlated with central memory CD8 T cells, effector memory CD8 T cells, activated CD4 T cells, and central memory CD4 T cells (*Figure 5C*,*5D*).

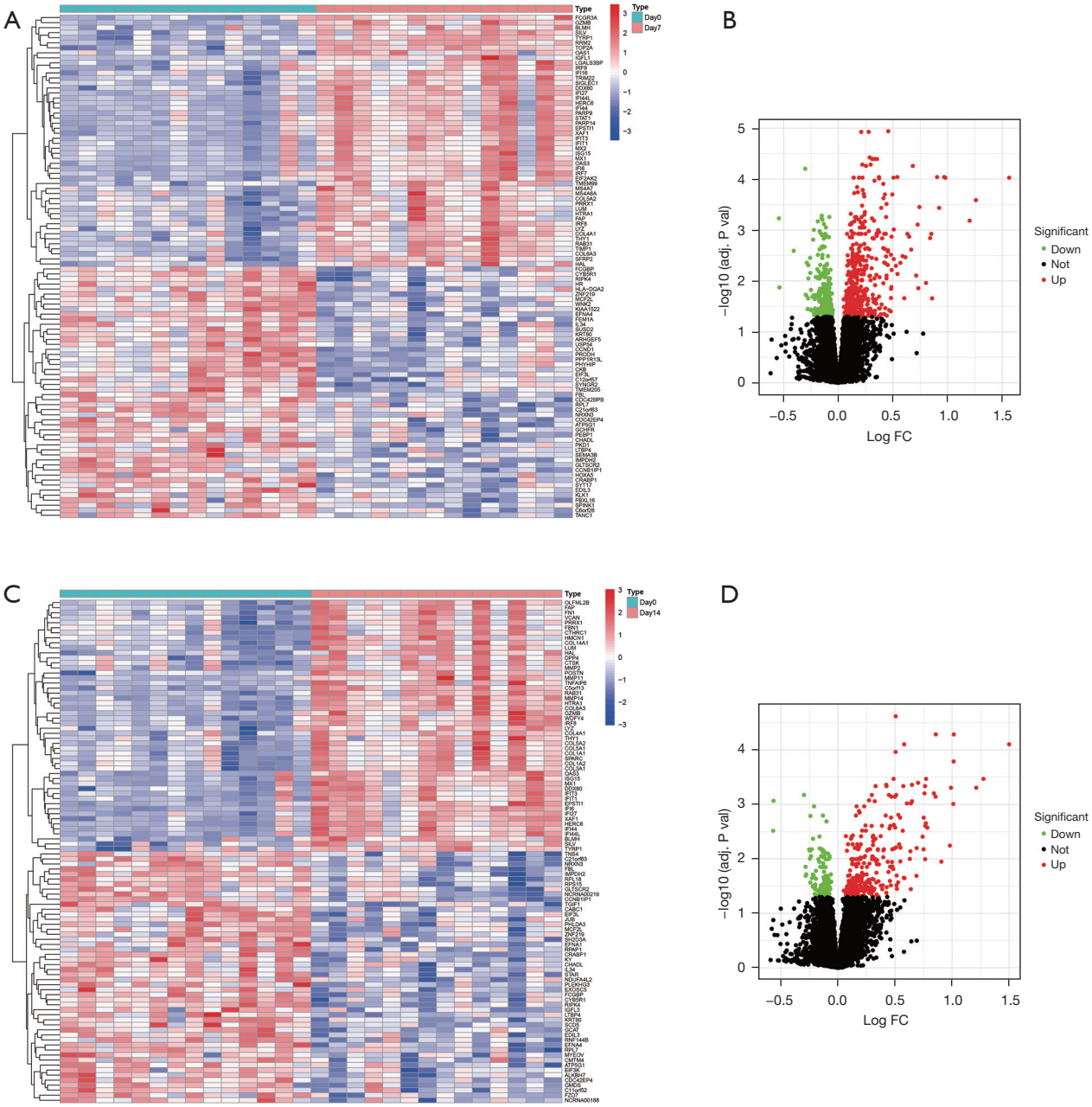

Figure 1 DEG in wound healing. (A,B) DEGs heatmap and volcano map for day 0 and 7; (C,D) DEGs heatmap and volcano map for day 0 and 14. DEG, differentially expressed genes; FC, fold change; adj. P val, adjusted P value.

Expression of autophagic proteins at different time points of wound healing

WB analysis results showed that, compared with day 0, EIF2AK2 and RB1 protein expressions were significantly increased on day 7 and day 11 by 1-way analysis of variance (*Figure 6*).

Discussion

Wound healing is a complex pathophysiological process that consists of the following 4 main stages: coagulation, verification, proliferation, and remodeling (14). Although a large number of studies have been conducted, the molecular mechanisms associated with each stage remain unclear.

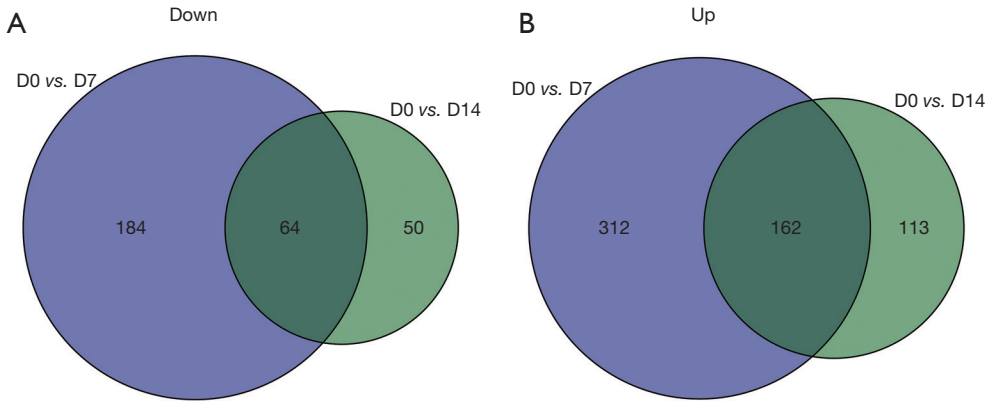

Figure 2 Up regulated and down regulated DEGs on day 7 and day 14 compared with day 0. Venn diagrams of co-downregulated DEGs (A) and co-upregulated DEGs (B). DEGs, differentially expressed genes.

Therefore, the key to research is to identify key molecules in the developmental mechanisms of trauma healing for in-depth studies and the development of potential therapeutic targets. Nuutila *et al.* collected biopsy samples from skin graft donors at different time points and subsequently performed transcriptome sequencing, which revealed ploidy changes in gene transcription, but they did not evaluate and analyze the function of DEGs (13). In recent years, bioinformatics technology has rapidly changed, and comprehensive analyses of multicenter data from large samples are becoming the focus of research. In the present study, we analyzed transcriptomic data of patient trauma from different time points in a representative manner. The results showed that a large number of DEGs were present in the trabeculae at 7 and 14 days after treatment, with a total of 226 genes undergoing significant upregulation or downregulation. DEG enrichment analysis revealed that the main function of these genes was defense against the virus. Therefore, we speculate that immune cells and immune response play an important role in trauma healing.

Autophagy is present throughout the trauma healing process and plays a role in the inflammatory phase, revascularization phase, re-epithelialization phase, and scar phase to varying degrees (15). The inflammatory phase is the first stage after trauma, during which a large number of inflammatory cells are activated and inflammatory cytokines are released. During this stage, the trauma inflammatory response is severe. In contrast, promoting autophagy inhibits the production of inflammatory cytokines and contributes to the regression of inflammation. Reducing inflammation is essential to prevent tissue damage and subsequent fibrosis (16). Thymosin β4 (Tβ4) has anti-inflammatory activity, and it was found that Tβ4 can significantly reduce inflammatory cytokines by activating the LAP/DAPK1 signaling pathway to downregulate nuclear factor kappa-B (NF-κB), which in turn inhibits inflammation (17). This suggests that Tβ4 can reduce inflammation by regulating autophagy and reducing inflammatory factor production associated with the inflammatory phase, thereby reducing inflammation. Traumatic injuries are often accompanied by vascular deficits, resulting in traumatic hypoxia. It was recently shown that hypoxia can activate autophagy-related proteins, which stimulate endothelial cell proliferation and differentiation and induce angiogenesis (18). In addition, it was found that the trabecular hypoxic environment can downregulate Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) to induce an increase in mammalian target of rapamycin 1 (mTORC1) downstream target expression, promoting cell motility and trabecular re-epithelialization (19). Scarring is the result of skin connective tissue healing after trauma, with fibroblast proliferation and collagen deposition as the main features. The increase in LC3 autophagy protein in fibroblasts during scar formation on the trauma surface the edge of the wound is higher than the center also suggest that autophagy is associated with fibroblast function (20). EIF2AK2 is a serine/threonine protein kinase that is activated by autophosphorylation after binding to dsRNA. A previous study have shown that EIF2AK2 can activate the pro-inflammatory pathway and trigger anti-inflammatory activity, which plays a dual role in inflammation and tumorigenesis (21). RB1 is a negative regulator of the cell

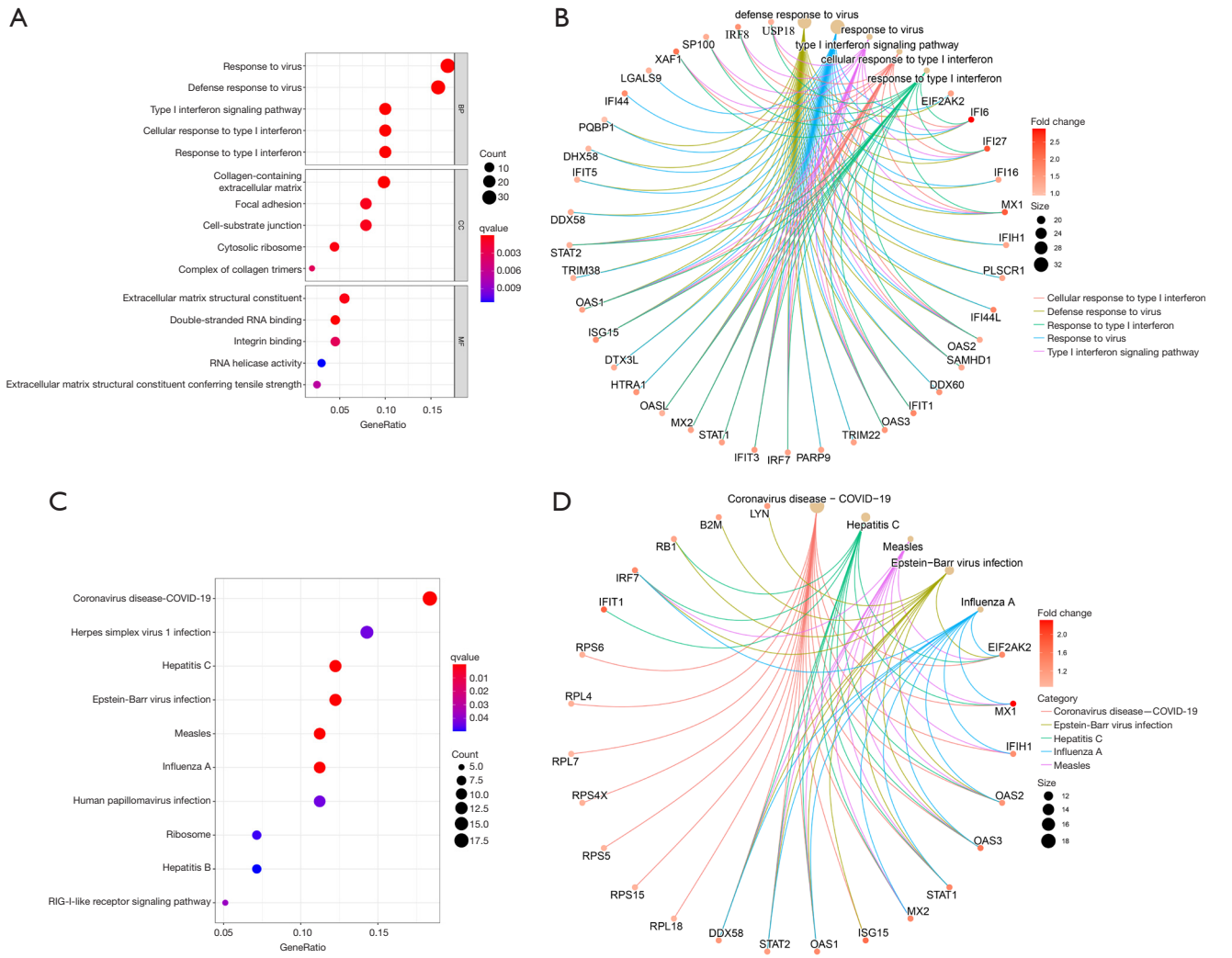

Figure 3 Functional enrichment analysis of DEGs in wound healing. (A) GO enrichment function analysis; (B) specific enrichment genes of the biologic process; (C) top 10 KEGG pathways; (D) specific enrichment genes of the top 5 signaling pathways. Size of the bubble represents the number of enrichment genes, and the color represents the Q-value and P value. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

cycle and was the first tumor suppressor gene found (22). Recent evidence suggests that RB1 regulates innate immune responses and determines the fate of immune progenitor cells (23). We intersected DEGs with autophagy genes and found that *EIF2AK2* and *RB1* could be key genes involved in autophagy in trauma healing. WB validation of blood samples from patients at different time points of wound healing revealed that EIF2AK2 and RB1 were gradually increased with wound healing, suggesting that these can both positively regulate wound healing.

The trabecular microenvironment consists of the following 2 parts: the external microenvironment on the outside of the trabeculae and the internal microenvironment on the perimeter of the trabeculae filled with cells and extracellular matrix. Together, they influence the trabecular microenvironment, which also includes temperature, humidity and pH (24). Regulatory T cells (Tregs) play an important role in maintaining skin homeostasis by secreting arginase and anti-inflammatory factors [interleukin (IL)-10 and transforming growth factor-β1 (TGF-β1)],

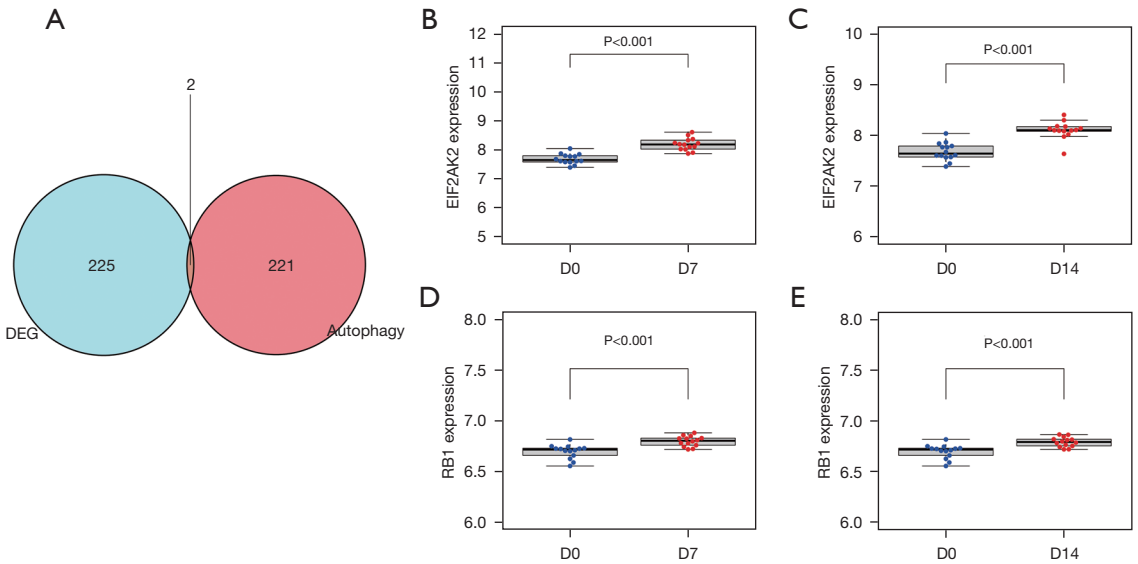

Figure 4 Expression levels of autophagy-related genes in early wound healing. (A) Venn diagrams of co-regulated DEGs and autophagy genes; (B) expression levels of *EIF2AK2* between day 7 and 0; (C) expression levels of *EIF2AK2* between day 14 and 0; (D) expression levels of *RB1* between day 7 and 0; (E) expression levels of *RB1* between day 14 and 0. DEGs, differentially expressed genes; *EIF2AK2*, eukaryotic translation initiation factor 2 alpha kinase 2; *RB1*, RB transcriptional corepressor 1.

promoting anti-inflammatory macrophage polarization and suppressing inflammatory responses, as well as promoting stromal formation by secreting IL-4, IL-5, and IL-21 (25). In addition, Treg-depleted mice have slower wound healing compared with wild-type mice, which is in contrast to natural killer T cells that have an inhibitory effect on wound healing (26,27). T cells play an important role in shaping the early wound microenvironment by secreting different cytokines and inflammatory factors, and their mechanisms of regulating wound healing are related to those of recognizing antigens and producing various cytokines (26,28). Studies have reported that B cells can act as powerful regulators of tissue regeneration, and that the extracellular matrix of the trauma surface can induce CD19-dependent Toll-like receptor signaling in trauma B cells, which in turn induces cytokine IL-10 and TGF-β production, directly reducing the inflammatory response (29,30). In addition, during the proliferative phase of the wound healing process, mast cells stimulate fibroblast proliferation by secreting IL-4, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor. With the assistance of numerous mediators, activated mast cells regulate key components of inflammation, therefore participating in the regulation of the local immune response (31,32). In the present study, we found a positive correlation between EIF2AK2 and RB1 and immune cells, such as CD4 and CD8, through comparison and comparison at different time points, the PATIENTS 'T cells and B cells maintained a high level with the wound healing. Therefore, we speculated that EIF2AK2 and RB1 autophagy genes promote wound healing by upregulating the level of immune cells.

The findings of the present study elucidated the possible mechanisms involved in wound healing, mainly through bioinformatics analysis, focusing on the role of immune cells involved in the regulation of autophagy in wound healing. The lack of large clinical data and experimental validation is a shortcoming of the study, and further systematic studies of these pivotal and key functional genes to explore their detailed mechanisms are warranted.

Conclusions

In the present study, multiple transcriptomic data were systematically investigated, and EIF2AK2 and RB1 autophagy genes were found to be closely associated with the wound healing process. However, subsequent experiments are needed to further confirm the biologic functions of autophagy genes in wound healing.

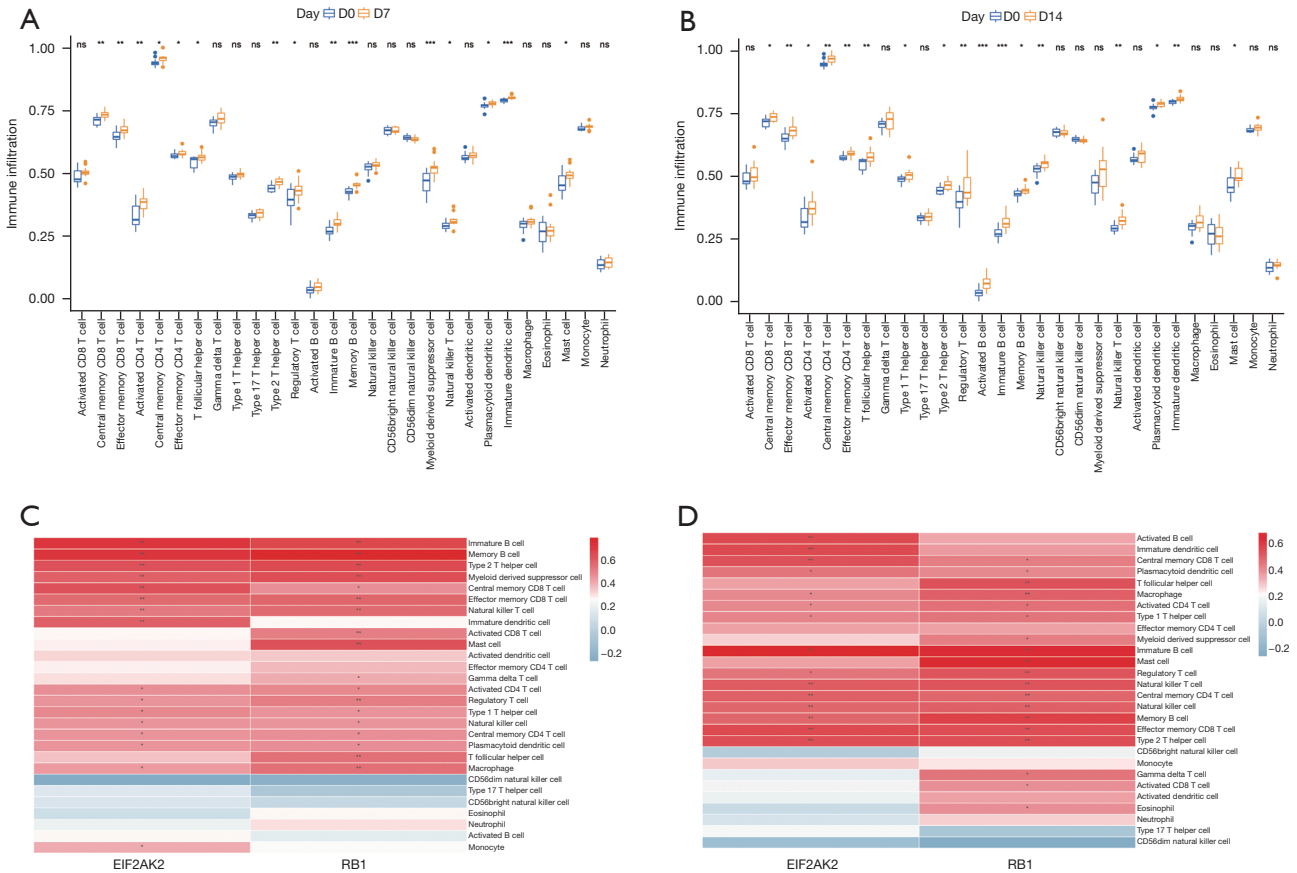

Figure 5 Single-sample gene set enrichment analysis immune cell infiltration analysis during early wound healing. (A) Box plot of the level of immune cell infiltration in the wound on day 0 and day 7; (B) box plot of the level of immune cell infiltration in the wound on day 0 and day 14; (C) correlation analysis heatmap of the expression level of *EIF2AK2* and *RB1* on immune cell infiltration in the wound on day 0 and day 7; (D) correlation analysis heatmap of the expression level of *EIF2AK2* and *RB1* and immune cell in the wound on day 0 and day 14. *P<0.05, **P<0.01, ***P<0.001. *EIF2AK2*, eukaryotic translation initiation factor 2 alpha kinase 2; *RB1*, RB transcriptional corepressor 1; ns, not significant.

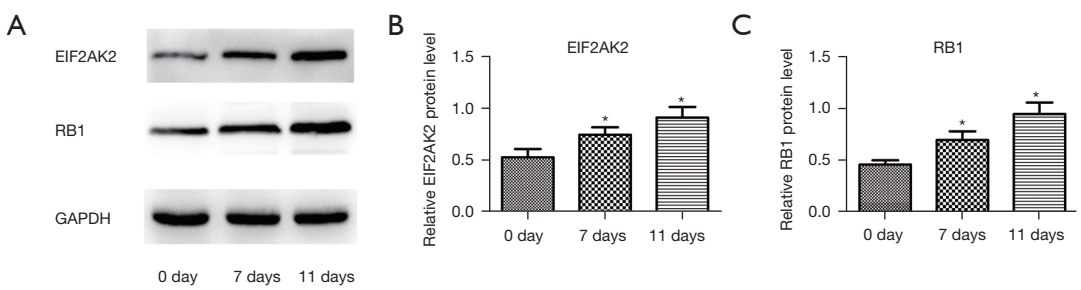

Figure 6 Expression of autophagic protein at different time points of wound healing. (A) Western blot analysis was used to detect related protein expression; (B) expression of EIF2AK2 protein; (C) expression of RB1 protein. *P<0.05. EIF2AK2, eukaryotic translation initiation factor 2 alpha kinase 2; RB1, RB transcriptional corepressor 1.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2033/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2033/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2033/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethic committee of People's Hospital of Baise (No. LLSC-2022-10). Patients provided signed informed consent prior to study commencement.

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