Screening and Validation of Key Genes of Autophagy in Acute Myocardial Infarction Based on Bioinformatics

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ABSTRACT

AIMS: Autophagy plays a significant role in the development of acute myocardial infarction (AMI), and cardiomyocyte autophagy is of major importance in maintaining cardiac function. We aimed to identify key genes associated with autophagy in AMI through bioinformatics analysis and verify them through clinical validation.

MATERIALS AND METHODS: We downloaded an AMI expression profile dataset GSE166780 from Gene Expression Omnibus (GEO). Autophagy-associated genes potentially differentially expressed in AMI were screened using R software. Then, to identify key autophagy-related genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, protein-protein interaction (PPI) analysis, Receiver Operating Characteristic (ROC) curve analysis, and correlation analysis were performed on the differentially expressed autophagy-related genes in AMI. Finally, we used quantificational real-time polymerase chain reaction (qRT-PCR) to verify the RNA expression of the screened key genes.

RESULTS: TSC2, HSPA8, and HIF1A were screened out as key autophagy-related genes. qRT-PCR results showed that the expression levels of HSPA8 and TSC2 in AMI blood samples were lower, while the expression level of HIF1A was higher than that in the healthy controls.

CONCLUSIONS: TSC2, HSPA8, and HIF1A were identified as key autophagy-related genes in this study. They may influence the development of AMI through autophagy. These findings may help deepen our understanding of AMI and may be useful for the treatment of AMI.

KEYWORDS: Acute myocardial infarction, autophagy, bioinformatics, key genes

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Introduction

Acute myocardial infarction (AMI) is myocardial necrosis caused by acute and persistent ischemia and hypoxia of the coronary artery.[1] It is one of the most serious cardiovascular diseases and remains a major threat to global health, leading to more than 7 million deaths each year.[2,3] The prognosis of patients with AMI can be greatly affected by the development of complications such as septal rupture, left ventricular free wall rupture, pseudoaneurysm, papillary muscle rupture, and true aneurysm. Therefore, early diagnosis and treatment of AMI are particularly important in improving its prognosis.[4] There is increasing evidence that multiple biological functions such as pyroptosis, ferroptosis, and autophagy are involved in the development and pathogenesis of AMI,[5-7] among which, autophagy plays an important role in the occurrence and progression of AMI.

Autophagy is a conserved mechanism by which cytoplasmic substances are degraded by lysosomes, and its levels are known to fluctuate in the pathological process of AMI.[8] LncRNA 2810403D21Rik/Mirf can regulate autophagy and promote ischemic myocardial injury by targeting Mir26a.[9] Angiotensin IV prevents AMI by inhibiting autophagy.[10] Besides, autophagy is also implicated in the protective effects of 1,25-dihydroxyvitamin D3 against AMI through the PI3K/AKT/mTOR pathway.[11] Studies have shown that autophagy-related genes, including WDFY3 and TP53INP2, presents novel prospects for the diagnosis of AMI.[12] However, there is still need for further investigation into the key autophagy-related genes in AMI, by which it is expected that potential therapeutic targets can be identified for AMI.

Wang et al[13] completed a coronary artery disease (CAD)-related dataset (GSE166780), in which a total of 169 genes differentially expressed in patients with different severity of CAD were analyzed. In the present study, the Differentially Expressed Genes (DEGs) in AMI were identified based on the sample data of 8 AMI patients and 8 healthy controls from the GSE166780 dataset in the GEO database, and subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, protein-protein interaction (PPI) analysis, and Receiver Operating Characteristic (ROC) analysis. Finally, a clinical validation was conducted by detecting the expression levels of the key genes in both AMI patients and healthy controls using qRT-PCR.

Materials and Methods

GEO datasets and autophagy-related genes

The AMI expression dataset GSE166780 was downloaded from the GEO database, containing data that were collected from the peripheral blood mononuclear cells from 8 control samples and 8 AMI samples on the microarray platform GPL20795.[13] A collection of 232 autophagy-related genes was obtained from the Human Autophagy Database,[14] and their detailed information can be found in Additional File 1: Table S1.

Data preprocessing

According to the count expression files, data preprocessing and standardization were performed using the edgeR package. Genes were filtered according to count expression >1, and then standardization was carried out using the TMM method.

Differential expression analysis

Using the R software (version: 4.0.3), 210 autophagy-related genes were identified in GSE166780.[15] Using the edgeR package, DEGs were screened out based on $|\log FC| > 1$, adj. P-value $< .05$. The fdr method was used for multiple correction for P-values. All the DEGs were then visualized using volcano plots, and those showing significant differences were selected for plotting heat map.

GO and KEGG enrichment analysis

We performed GO and KEGG enrichment analysis on the DEGs using R's clusterProfiler package in order to investigate their potential biological functions.[16] GO analyses included biological process (BP), cellular component (CC) and molecular function (MF), and adj. P-value $< .05$ indicated significant enrichment. Benjamini-Hochberg (BH) method was applied for the correction of the p-values of our statistical results. We used the DAVID web tool for enrichment analysis and bubble map display. GO secondary classification selected the top 10 channels for each category, and KEGG selected the top 20 channels.

PPI analysis

PPI network for the DEGs was constructed using the STRING (version: 11.5) database and visualized using Cytoscape (version: 3.8.2).[17] We employed 2 algorithms, Maximal Clique Centrality (MCC) and Degree, both available in the CytoHubba plug-in of Cytoscape, to analyze the PPI network. Key genes were identified by intersecting the results obtained from these 2 algorithms.

ROC analysis

ROC analysis was performed using the pROC package based on group clinical features as response and gene expression as predictor. In the ROC context, the area under the curve (AUC) is used as a metric to evaluate classifier performance and is frequently applied for method comparison.[18] A higher AUC means a better classification. AUCs were calculated using the ROC curve for the evaluation of the diagnostic value of key DEGs in AMI. $P < .05$ was considered statistically significant.

Correlation analysis

We further screened the key genes with autophagy marker genes LC3 and P62[19] for correlation analysis based on the expression of key genes and LC3 & P62 $|\text{correlation}| > .4$, $P < .05$ for autophagy-related key genes.

AMI patients and healthy controls

Thirty patients with AMI and 30 healthy controls were collected as study samples from December 1, 2021 to April 5, 2022 in Zibo Central Hospital, Shandong Province, China. We collected the healthy controls with coronary artery stenosis <50% on coronary angiography. AMI were diagnosed according to the criteria set forth by the *2017 ESC STEMI and 2020 ESC NSTEMI*[20,21] based on the evaluation of patient symptoms, laboratory results and coronary angiography results by 2 interventionists. Patients with aortic coarctation, pulmonary embolism, malignancy, severe heart failure, severe renal insufficiency, myocarditis, or pericarditis were excluded. The study received approval from the Hospital Medical Ethics Committee (No. 202111004), and all participants provided informed consent prior to enrollment. Venous blood samples were collected from all the participating cases and the healthy controls for carrying out quantificational real-time polymerase chain reaction (qRT-PCR).

Clinical validation of qRT-PCR[22]

Ficoll solution (Solarbio Life Sciences, Beijing, China) and TRIzol reagent (Thermo Fisher Scientific, Shanghai, China) were used to isolate peripheral blood single nuclei and extract total RNA from blood samples collected within 24 hours of hospitalization. Then, Evo M-MLV RT Premix (Accurate Biology, Hunan, China) were adopted to conduct the reverse transcription. Real-time PCR analysis was performed using Hieff qPCR SYBR Green Master Mix (YEASEN, Shanghai, China). The internal control of mRNA was β-actin. The relative expression levels were calculated using 2-ΔΔCq method and the primers are shown in Table 1. The nucleotide sequences of the target genes were retrieved from the National Center for Biotechnology Information (NCBI). Sequence homology analysis and comparison were performed using DNAStar, and primer design was accomplished using Primer Premier 5.0.

Statistical analysis

GraphPad 9.0 was used for statistical analysis. Differences between the 2 groups were compared using the Student's

t-test, and $P < .05$ was considered a statistically significant difference.

Results

DEGs in AMI

Using the R software (version: 4.0.3), 210 autophagy-related genes were identified in GSE166780 and based on $|\log FC| > 1$, adj. *P*-value $< .05$, we screened the differentially expressed autophagy-related genes between groups as the autophagy-related DEGs of AMI. Finally, 34 DEGs related to autophagy were screened out, of which 14 were up-regulated and 20 were down-regulated, and they were displayed using volcano plots (Figure 1A) and heat maps (Figure 1B).

GO and KEGG functional analyses

We performed GO and KEGG enrichment analyses using the R software to analyze the functions of these DEGs. GO enrichment results were annotated to BP, CC and MF. The most significant enrichment terms for GO analysis involved macroautophagy, autophagosome assembly (BP); autophagosome, pigment granule (CC); heat shock protein binding, ubiquitin-like protein ligase binding (MF) (Figure 2A–C). The DEGs were mainly involved in autophagy-animal, lipid and atherosclerosis revealed by the KEGG enrichment analysis (Figure 2D). The top 5 enriched functions or pathways in GO and KEGG analyses are respectively shown in the circle diagrams (Figure 2E and F).

Table 1. Primer sequences used for qRT-PCR.

PRIMER	SEQUENCES (5′-3′)
TSC2-F	GGAGTACGGTAGGAACTGGAGAGG
TSC2-R	CACTGCTAAGGCTGCTGGCTTC
HSPA8-F	TCCCTTGGTATTGAAACTGCTGGTG
HSPA8-R	GTCTGTGTCTGCTTGGTAGGAATGG
HIF1A-F	CCATTAGAAAGCAGTTCCGCAAGC
HIF1A-R	GTGGTAGTGGTGGCATTAGCAGTAG

PPI network analysis

PPI networks for the DEGs were constructed using the STRING database (Figure 3A), and visualized using Cytoscape circles (Figure 3B). The algorithms of Degree (Figure 3C) and MCC (Figure 3D) were separately used for screening key genes, and the genes obtained were intersected using the Venn diagram (Figure 3E). As a result, a total of 9 key genes were obtained, namely CDKN1B, FOS, HIF1A, HSPA5, HSPA8, MAPK1, PINK1, SIRT1, and TSC2.

The diagnostic value of key genes in AMI

The expression levels of the 9 key genes were analyzed in the original GSE166780 dataset, and box plots were used to show their expression differences between the AMI group and the healthy controls (Figure 4A–I). The analysis revealed that CDKN1B, FOS, HSPA8, MAPK1, PINK1, and TSC2 were down-regulated in the AMI group compared to the healthy controls, whereas HIF1A, HSPA5, and SIRT1 were up-regulated in the AMI group compared with the healthy controls. The 9 identified genes demonstrated good diagnostic value in distinguishing between AMI and control samples, with AUCs of 84.40% for CDKN1B, 84.40% for FOS, 93.80% for HIF1A, 92.20% for HSPA5, 92.20% for HSPA8, 92.20% for MAPK1, 85.20% for PINK1, 85.90% for SIRT1, and 75.80% for TSC2 (Figure 5A–I).

Correlation analysis

A number of studies have confirmed that LC3 and P62 are autophagy marker genes, and that LC3 II expression is upregulated and P62 expression is downregulated when increasing

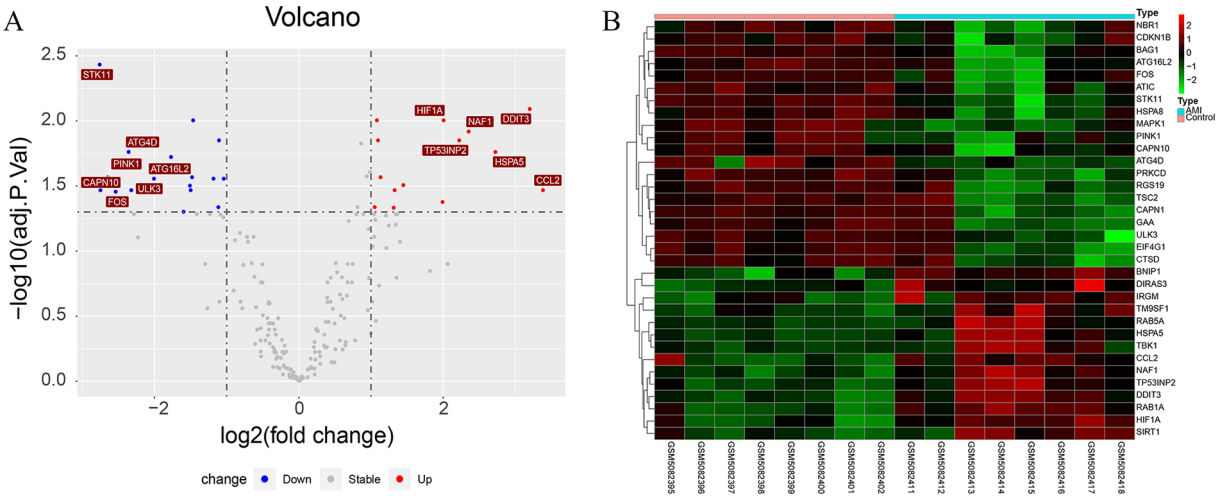

Figure 1. DEGs in GSE166780 dataset shown separately in volcano (A) and heat maps (B).

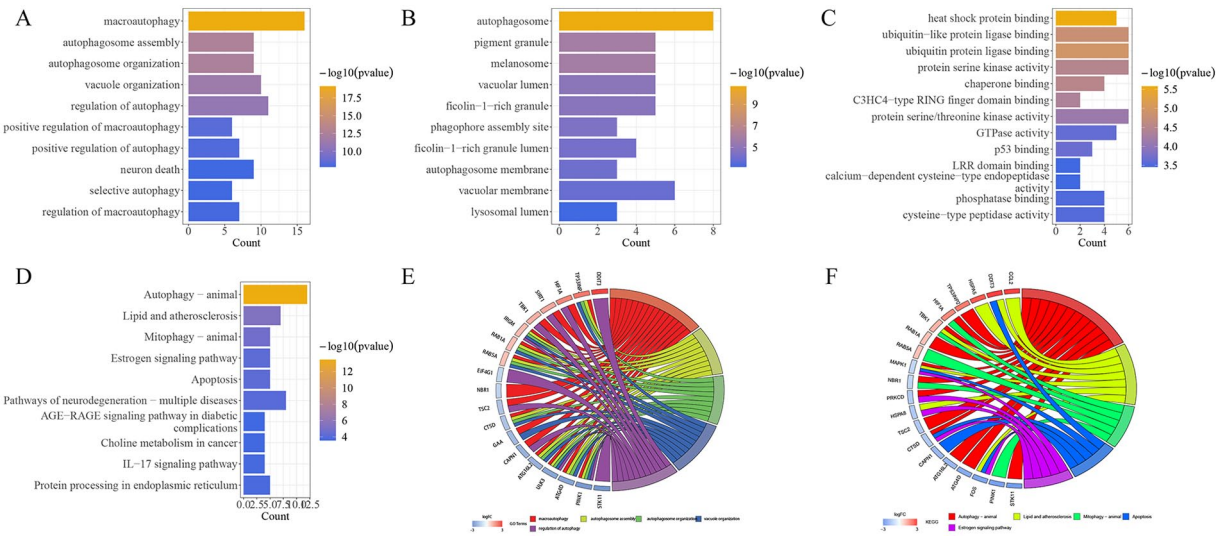

Figure 2. GO and KEGG functional analyses: (A) GO-BP enrichment analysis, (B) GO-CC enrichment analysis, (C) GO-MF enrichment analysis, (D) KEGG pathway enrichment analysis, (E) the top 5 GO enrichment functions, and (F) the top 5 KEGG pathway enrichment functions.

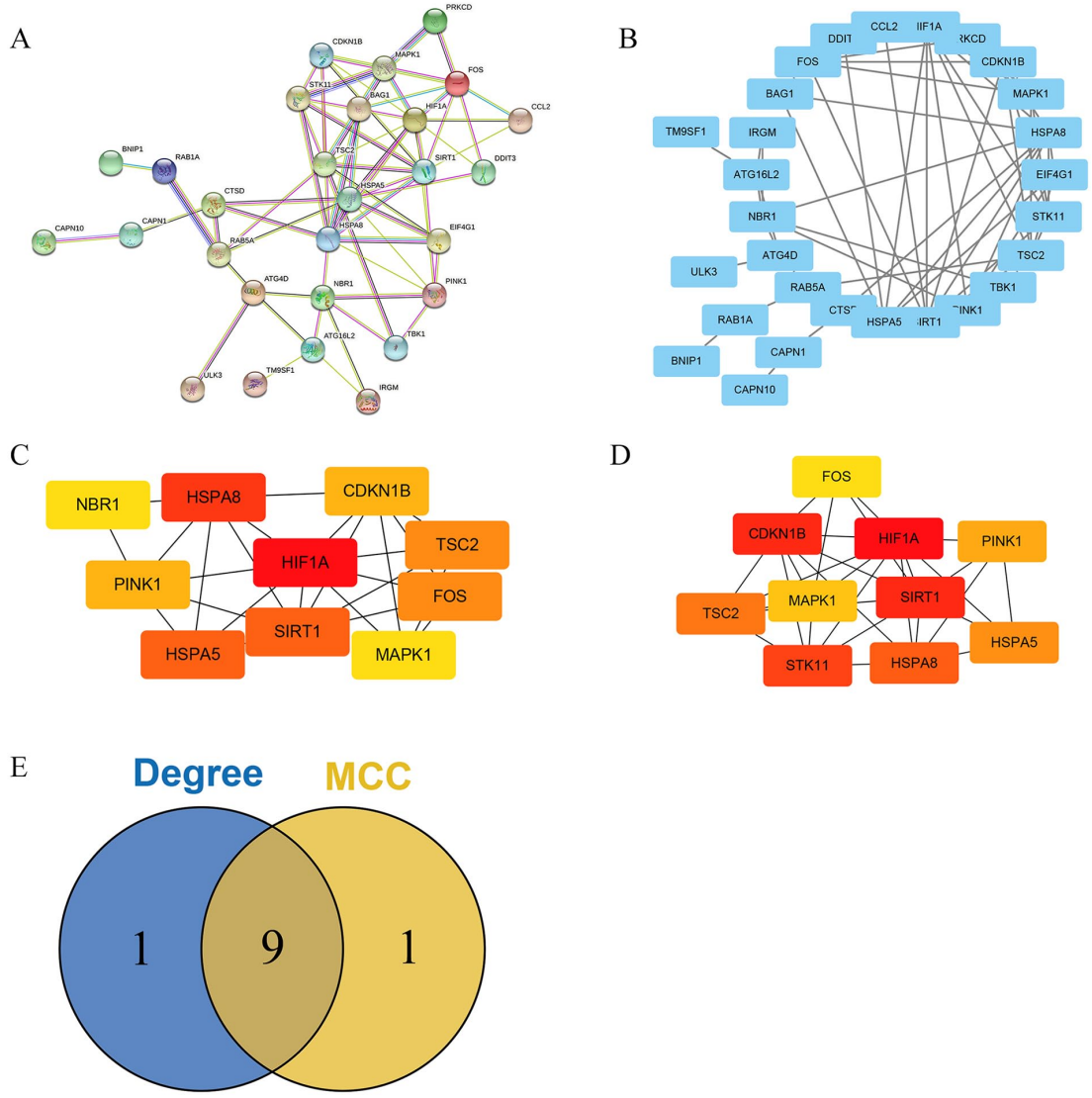

Figure 3. PPI networks constructed for the DEGs (A) and displayed in circles (B). The Degree algorithm (C) and the MCC algorithm (D) were separately used for the screening of key genes, and the genes obtained were intersected using the Venn diagram (E).

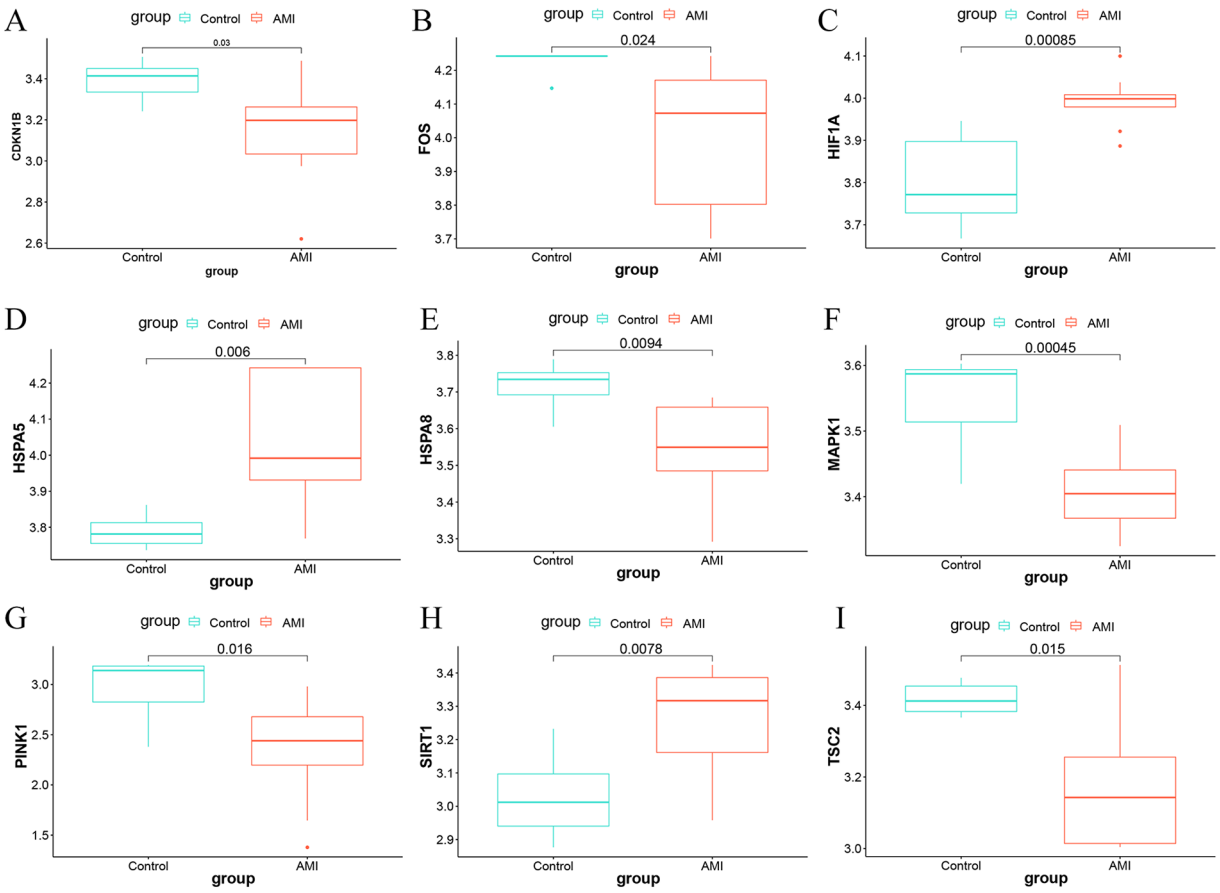

Figure 4. (A–I) Box plots showing the expression of the 9 key genes between AMI and healthy controls in GSE166780 dataset.

the autophagy levels.[23,24] We further obtained 3 genes (TSC2, HSPA8, and HIF1A) based on LC3 & P62 |correlation| > 0.4, $P < .05$. The lollipop plot showed that TSC2 and HSPA8 were negatively correlated with autophagy levels while HIF1A was positively correlated with autophagy levels (Figure 6).

Validation of the key DEGs in AMI patients

We further examined the expression levels of the above 3 key autophagy-related genes in our clinical samples by qRT- PCR. Clinicopathological variables for the AMI and control groups were summarized in Table 2. The basic patient information between groups in this study were detailed in Additional File 2: Table S2. Bar chart showed that the expression levels of HSPA8 and TSC2 in AMI blood samples were lower while the expression level of HIF1A was higher than that of the healthy controls, which was consistent with the results of the GSE166780 dataset (Figure 7A–C).

Discussion

Despite advancements in surgical interventions and pharmacological treatments, AMI as well as its associated severe complications remain the leading causes of deaths in developed countries in the field of cardiovascular disease.[3,4] More and more studies have been conducted to explore the key targets of AMI in order to develop precise and targeted treatments for AMI, improve its prognosis and reduce associated risks of death. However, the clinical significance of these targets remains to be further elucidated.[25-27]

In recent years, autophagy has been proved to be closely related to cardiac diseases, especially myocardial infarction. Autophagy is involved in cardiac metabolism and function, and plays an extremely important role in the pathogenesis of AMI.[28] There is no denying that autophagy has become a new perspective for investigating AMI. However, autophagy-related genes in AMI remain to be explored. Therefore, this study aimed to identify key autophagy-related DEGs in AMI based on a cohort from the GSE166780 dataset. After screening, a total of 9 autophagy-related key genes (CDKN1B, FOS, HIF1A, HSPA5, HSPA8, MAPK1, PINK1, SIRT1, and TSC2) were identified in the AMI group versus the control group through bioinformatics analysis. Numerous studies have confirmed LC3 and P62 as autophagy markers.[23] P62 acts as an autophagy-specific substrate that interacts with LC3 to infiltrate into the autophagosome and is efficiently degraded by autophagic lysosomes. Therefore, changes in P62 expression can also be used as an indicator for monitoring changes in autophagy.[29] LC3 is also involved in autophagy formation, and when autophagy is formed, cytoplasmic LC3 (LC3-I) enzymatically cleaves off a small segment of the polypeptide and transforms into membrane LC3 (LC3-II). Elevated LC3II therefore represents the initiation of autophagy.[30] They were

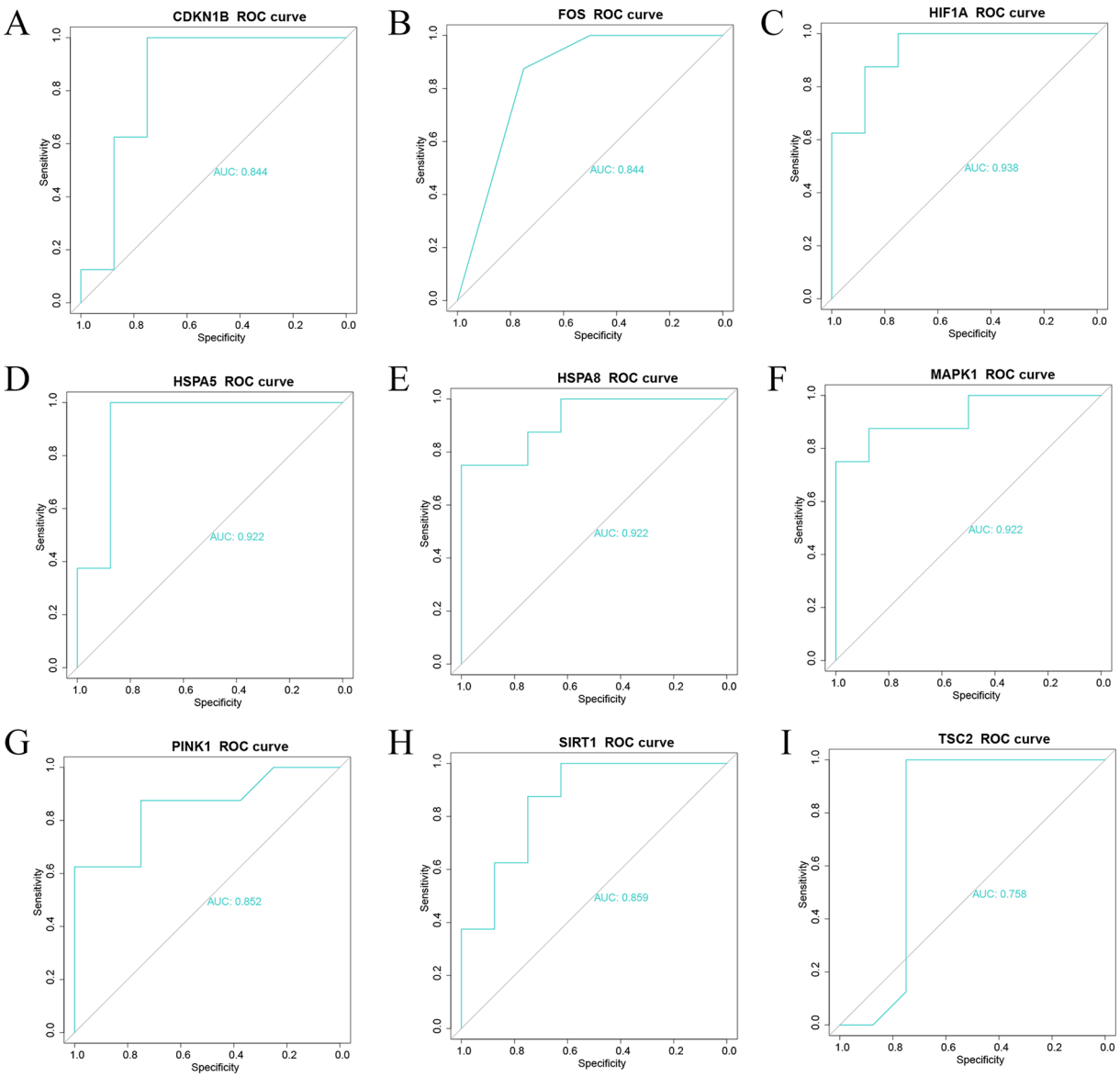

Figure 5. (A–I) ROC curves for the 9 key genes in GSE166780 dataset.

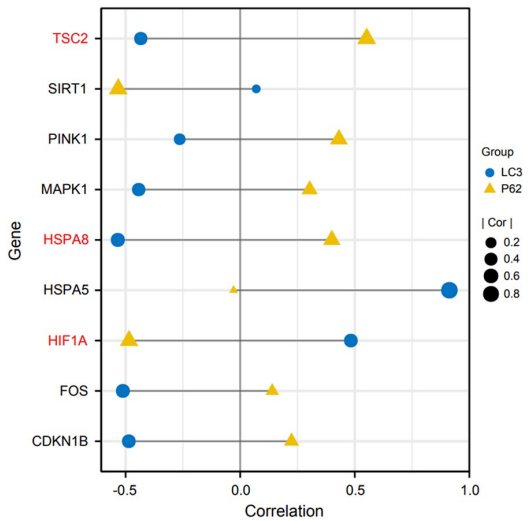

Figure 6. Correlation between the 9 key genes, LC3 and P62.

shown to be closely related to autophagosome development and maturation, and are now widely used to monitor autophagic activity.[24] By analyzing the correlation between the 9 identified key genes and the autophagy markers LC3 and P62, a total of 3 autophagy-related key genes (HIF1A, HSPA8, and TSC2) were screened out. TSC2 and HSPA8 were found to be negatively correlated with autophagy levels while HIF1A was shown to be positively correlated with autophagy levels. Then, we used qRT-PCR to verify the expression of the above 3 key genes. The results showed that the expression levels of HSPA8 and TSC2 in AMI blood samples were lower than those in the healthy controls, while the expression level of HIF1A was higher than that in the healthy controls. The qRT-PCR verification results were basically similar to the results obtained through bioinformatics analysis.

Hypoxia-inducible factor 1α (HIF1A) is localized on human chromosome 14, regions q21-24, and is regulated by

Table 2. Clinicopathological variables of AMI and controls in this study.

VARIABLES	AMI (N = 30)	CONTROL (N = 30)
Age (y)	64.77 ± 13.49	60.00 ± 12.32
Gender (male/female)	20/10	19/11
BMI (kg/m^2)	26.50 ± 4.26	26.17 ± 5.02
Smoking history (yes/no)	9/21	7/23
Hypertension (yes/no)	17/13	12/18
Diabetes (yes/no)	12/18	1/29
Total cholesterol	4.92 ± 0.72	3.58 ± 0.62
Low density lipoprotein	3.38 ± 0.59	2.32 ± 0.49
Ejection fraction	55.00 ± 6.70	63.50 ± 4.22

Data are presented as mean ± SD.

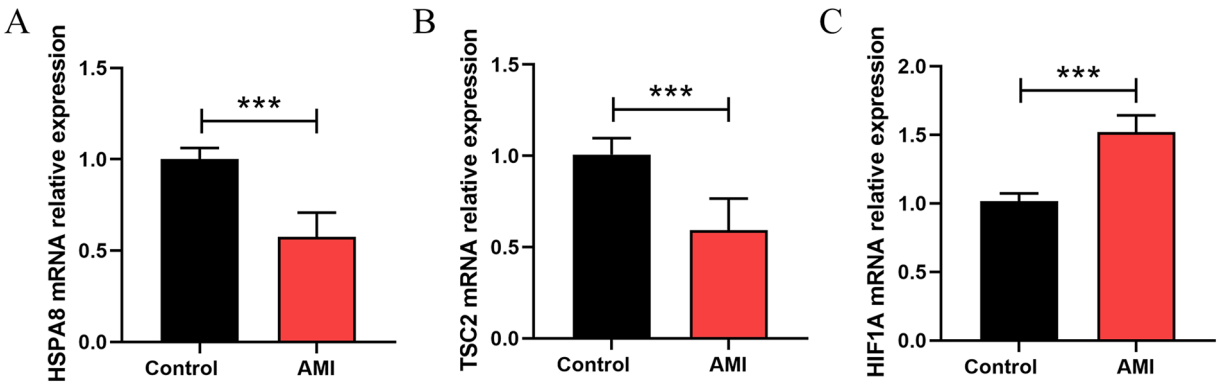

Figure 7. (A–C) qRT-PCR results demonstrated differential gene expressions of HSPA8, TSC2, and HIF1A in the clinical samples.

hypoxic signaling.[31] It has been reported to improve cardiac function in a rat model of AMI,[32] and regulate the autophagy of cardiomyocytes after hypoxia by transferring miR-30a. In addition, KEGG enrichment results showed that autophagy-related DEGs was significantly enriched in the IL-17 signaling pathway and other signaling pathways. Interestingly, it has been found that the abundance of HIF subtypes is closely related to the increase of IL-17 levels, and the IL-17-STAT3-HIF-1α axis can participate in autophagy defects, and targeting this axis can effectively alleviate chronic inflammation in skin diseases.[33] Therefore, it is reasonable to speculate that HIF1A may affect the level of autophagy by mediating the IL-17 signaling pathway, thus playing an important role in the occurrence and development of AMI. HSPA8, also known as the heat shock homolog HSC70, is a member of the family encoding heat shock protein 70. Extracellular HSC70 plays a key role in the regulation of natural immune response and cardiac function after myocardial ischemia-reperfusion,[34] and it has been shown to decisively regulate the autophagic process.[35] New research has highlighted the involvement of chaperone-mediated eNOS autophagy in myocardial ischemia and reperfusion injury, and HSC70 has been shown to play an important part in this process.[36] Additionally, studies have demonstrated that BAG-1 can be regulated by HSC70 to induce autophagy, thus affecting the survival of cardiomyocytes.[37,38] As shown by the GO enrichment analysis, autophagy-related DEGs were significantly enriched in molecular functions such as heat shock protein binding, confirming that heat shock family genes and pathways were largely involved in the process of autophagy during AMI. Therefore, targeting heat shock genes and pathways to mediate autophagy levels may be a new way to improve AMI therapy in the future. Mutations in the TSC2 gene cause tuberous sclerosis and its gene product is thought to be a tumor suppressor that stimulates specific GTPases, which in turn control cell growth and survival.[39] Increasing evidence suggests that the TSC2-mTOR signaling pathway is associated with autophagy and is involved in various processes such as cardiomyocyte apoptosis, affecting myocardial remodeling, and myocardial contraction. Studies have shown that lncRNA MALAT1 can inhibit autophagy by regulating the TSC2-mTOR signaling pathway, thereby enhancing myocardial cell apoptosis in AMI mice.[40,41] Moreover, TSC2-mTOR signaling pathway has been validated in murine models of cardiac-specific TSC2 deficiency (TSC2-/-).[42] Therefore, TSC2 may

participate in the TSC2-mTOR signaling pathway, thereby affecting the autophagy level of AMI. The AUCs from ROC analysis indicate that HIF1A, HSPA8 and TSC2 were 3 genes that can well distinguish AMI from the control samples, with values of 93.80% for HIF1A, 92.20% for HSPA8, and 75.80% for TSC2. This suggests that these 3 key genes may be potential biomarkers for AMI, and their expression levels could be analyzed to predict the occurrence and development of AMI in clinical settings. Moreover, the identification of these 3 key autophagy-related genes may offer insights into the development of new targeted drugs for AMI treatment.

As revealed by GO and KEGG enrichment analysis, autophagy-related DEGs are mainly involved in signaling pathways such as apoptosis. Balancing the transition between apoptotic and autophagic responses has become a new concept in the treatment of coronary artery disease.[43,44] To a certain extent, the induction of autophagy, as a catabolic process, can provide a first line of protection against major injuries such as apoptosis and necrosis in cardiac myocytes.[45] However, basic autophagy can be beneficial for maintaining cell homeostasis, while excessive autophagy may lead to a series of adverse effects such as apoptosis, which may further harm the cardiovascular system.[46] In conclusion, the above findings may provide potential biomarkers and therapeutic targets for the diagnosis and treatment of AMI. However, there are still many deficiencies in this study. One limitation of our study is that we only experimentally validated the expression of the identified autophagy-related genes in the peripheral venous blood samples from AMI patients and healthy controls, but the conditions for taking myocardial tissues for validation are insufficient. Next, the limited clinical sample size is another limitation, due to which there were differences in total cholesterol level, low density lipoprotein level and diabetes prevalence between the AMI and control groups in this study. It is not possible to completely eliminate the potential impact of factors related to the occurrence of AMI or autophagy, including total cholesterol level, low density lipoprotein level and diabetes on the expression of key genes. Therefore, additional studies in with larger sample sizes are necessary. In addition, it is imperative to delve deeper into the potential mechanisms of action of these key autophagy-related genes in AMI cells and animal models.

Conclusion

In our study, 3 key autophagy-related genes (TSC2, HSPA8, and HIF1A) were identified through bioinformatics analysis, of which TSC2 and HSPA8 were found to be negatively associated with autophagy levels while HIF1A was shown to be positively associated with autophagy levels. This suggests that these 3 key genes may be potential biomarkers for AMI, and their expression levels could be analyzed to predict the occurrence and development of AMI in clinical settings. Moreover, the identification of these 3 key autophagy-related genes may offer insights into the development of new targeted drugs for AMI treatment. The results of this study facilitate our understanding of AMI, and provide potential targets for targeted interventions to treat AMI and improve its prognosis.

Author Contributions

Yingjie Geng participated in the study design, analyzed the data and was a major contributor in writing the manuscript. Yu'e Han and Shujuan Wang collected the data. Jia Qi validated the results. Xiaoli Bi was mainly in charge of the manuscript modification. All authors read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

The study was reviewed and permitted by the Medical Ethics Committee of Zibo Central Hospital of Shandong Province (No. 202111004). All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all participants.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplemental Material

Supplemental material for this article is available online.

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