Predicting diagnostic gene Biomarkers associated with N6-methyladenosine and ferroptosis in patients with acute myocardial infarction

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Research

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Abstract

Background

This study aimed to provide an early potential diagnosis of acute myocardial infarction (AMI) and determine its correlation with ferroptosis, immune checkpoints, and N6-methyladenosine (m6A).

Methods

We downloaded microarray data from NCBI (GSE61144, GSE60993, and GSE42148) and identified differentially expressed genes (DEGs) in samples from healthy subjects and patients with AMI. We also performed systematic gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses and used STRING to predict the interactions between proteins. Then we proceeded with the identification of the first ten DEGs by plotting the receiver operating characteristic curve using the multi-scale curvature classification algorithm, and verification of their diagnostic significance. Next, we investigated the relationship between these target genes and immune checkpoints, ferroptosis, and m6A.

Results

Through screening, we identified 253 DEGs, including 181 downregulated and 72 upregulated genes. According to GO, KEGG, and key gene screening results, *GZMB*, *GZMA*, *PRF1*, *KLRB1*, *CD2*, *KLRD1*, *IL2RB*, *NKG7*, *GZMK*, and *CCL5* could be used for early prediction of AMI. Moreover, we found that AMI was associated with ferroptosis, immune checkpoints, and m6A.

Conclusion

GZMB, *GZMA*, *PRF1*, *KLRB1*, *CD2*, *KLRD1*, *IL2RB*, *NKG7*, *GZMK*, and *CCL5* are effective markers for the diagnosis of AMI, which can provide new prospects for future studies on the pathogenesis of AMI.

Background

Coronary heart disease affects 17.1 million people worldwide and results in a considerable number of fatalities, making it a global health concern[1]. Acute myocardial infarction (AMI) is the most serious ischaemic heart disease caused by the rupture of atherosclerotic plaque[2-4]. Early and correct diagnosis may be of great benefit in treatment[5]. Therefore, it is necessary to explore new biomarkers with high sensitivity and high specificity for the diagnosis of cardiovascular disease[6].

Previous studies have identified several risk factors associated with the onset of AMI, including age, gender, hypertension, diabetes, smoking, alcohol consumption, and physical labour[7-9]. There is growing evidence that genetic factors contribute to the development of AMI. In recent years, various therapeutic

targets for AMI have been identified through the study of genetic factors such as, the mRNA loci assigned to AMI in genome association studies (GWAs) [10-12]. Non-coding RNA (ncRNA) is also one of such genetic factors. Previous studies have extensively investigated the regulatory effects of micro-large RNA (miRNA) on several pathological aspects of AMI, including myocardial apoptosis, inflammation, angiogenesis, and fibrosis. Further study of miRNAs, long ncRNAs (LncRNAs), and circular RNAs (CircRNAs) can facilitate the regulation of these processes through various interesting mechanisms.

In recent years, the development of microarray technology has allowed the identification of biomarkers for diagnosis and prognosis through differentially expressed genes (DEGs)[12, 13]. Some ncRNAs serve as biomarkers for many cardiovascular diseases[14-16], including AMI (mir-1, mir-133, mir-208, and mir-499)[17, 18], acute coronary syndrome (mir-208a, mir-34a, mir-133a, and mir-499)[19], and heart failure (mir-499, mir-133, mir-423-5p, and mir-126) [20-22]. Thus, these stable, conserved, and specific RNAs may provide a novel approach.

Herein, we downloaded the NCBI microarray data, identified DEGs in AMI samples, and compared them with normal controls[23]. The identification of DEGs was followed by systematic GO and KEGG analyses[24-27]. Protein-protein interactions (PPIs) among the products of DEGs were studied using STRING[28, 29]. Finally, the key genes were identified and examined to determine if the genes associated with AMI were associated with immune checkpoints, ferroptosis, and N6-methyladenosine (m6A) modification[27, 30, 31]. In conclusion, this study provides new insights into the molecular mechanisms responsible for the occurrence of AMI.

Methods

Microarray data

From the GEO database (<http://www.ncbi.nih.gov/geo>) we used the MiniML microarray dataset (GSE42148, GSE60993, and GSE61144). GSE42148 was based on a GPL13607 platform Agilent-028004 SurePrint G3 Human GE 8x60K Microarray (Feature Number Version). GSE60993 was based on Illumina HumanWG-6 V3.0 Expression Beadchip of the GPL6884 platform. GSE61144 was based on Sentrix Human-6 V2 Expression BeadChip of the GPL6106 platform. The GSE42148 dataset included peripheral blood samples from six patients with AMI and 11 healthy controls. The GSE60993 dataset included seven patients with ST-segment elevation myocardial infarction (STEMI), ten patients with non-STEMI (NSTEMI), and seven healthy controls. The GSE61144 dataset included STEMI (n=7) peripheral blood samples. We log2 transformed and normalised transcriptome data employing the pre-process Core package in R Foundation for Statistical Computing version 4.0.3 (3027). Based on platform annotation information, we converted the probes into gene symbols through the Strawberry Perl language (version 5.32.1.1), and excluded probes containing multiple genes. Additionally, we calculated the mean values of the genes corresponding to multiple probes. Furthermore, we controlled the initial quality using ANOVA and removed the batch effect using sva packing in R[1-3].

Filtering DEGs

We identified differential expression of RNAs using the "Limma" package in R. There were 30 AMI cases and 28 healthy controls. We then analysed the adjusted P values to correct the false positive results in the GEO dataset. The adjusted P-value < 0.05 and | log2 fold-change (FC) | > 1.5 represented statistical standards for RNA expression screening.

We obtained a box graph using the R package GGplot2. The R packages ggord and pheatmap were used to draw the PCA diagram and heatmap, respectively. The above analysis methods and R packages were implemented using R Foundation of Statistical Calculation (2020) version 4.0.3[32].

Functional enrichment analysis

We used GO for functional gene annotation, particularly in molecular function (MF), biological pathways (BP), and cellular components (CC). The KEGG enrichment analysis provided a good reference for gene function research and the correlating high level genomic functional information. To have a better understanding of the effect caused by mRNAs, we applied the ClusterProfiler package (version: 3.18.0) in R to analyse GO functions of potential targets and the KEGG pathway enrichment.

Screening of candidate diagnostic biomarkers

The Interactive Gene Retrieval Tool, STRING, is an online biological database that provides gene analysis and builds gene interaction networks at the protein level [33]. In this study, we constructed the protein-protein interaction network of DEGs using STRING (Version 11.0) [27, 34]. We then visualised the PPI network using Cytoscape version 3.8.2(3330). MCC and MCODE algorithms in cytoHubba plug-in were used to screen key genes in the network.

Diagnostic Value of Characteristic Biomarkers in AMI

In order to test the predictive value of identified biomarkers, we used the GLM function in R (version 3.6.3) package to build logistics model, and used the GGplot2 package to visualize the results. Receiver operating characteristic (ROC) curves were generated using the mRNA expression data from the GSE42148, GSE60993, and GSE61144 dataset. There were 30 patients with AMI and 28 patients without AMI. The diagnostic value of the identified hub genes was evaluated using the area under the ROC curve (AUC), which was between 0.5 and 1. The closer the AUC is to 1, the better is the diagnostic effect. The accuracy of AUC ranged from 0.5 to 0.7, while that of AUC ranged from 0.7 to 0.9. The accuracy of AUC was higher than 0.9.

Effect of the immune checkpoint-, m6A-, and ferroptosis-related gene expression in AMI.

Based on the results of previous studies, we identified immune checkpoint-, ferroptosis-, and m6A-related genes. The dataset we downloaded was from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), and the data format was MiniML. We extracted the expression of immune checkpoint-related genes. To derive ferroptosis-related genes, we used the systematic analysis of the aberrances and functional implications of ferroptosis in cancer published by Ze-Xian Liu et al. Moreover, we used the molecular

characterisation and clinical significance of m6A modulators across 33 cancer types published by Juan Xu to derive the m6A-related genes. We used the R package ggord to draw PCA graphs; and implemented a box plot using the R package ggplot2. In addition, we established a heatmap using the R package pheatmap. All the above analysis methods and R package were implemented using R foundation for statistical computing (2020) version 4.0.3. [34-37].

Results

Identification of differentially expressed genes

DEGs in GSE42148, GSE60993 and GSE61144 were identified using Limma quartile normalisation and background correction methods. Limma screening identified 253 DEGs, including 181 down-regulated and 72 up-regulated genes (Figure 1).

Functional correlation analysis

Using the "clusterProfiler" package in Bioconductor and the gene function spectrum obtained through enrichment analysis of GO and KEGG pathways, we found that DEGs were mainly concentrated in the following functional categories: allograft rejection, cell adhesion molecules (CAMs), and graft versus host disease, Th1 and Th2 cell differentiation natural killer cells mediated cytotoxicity, leukocyte cell-cell adhesion, immune response-activating signal transduction, immune response-activating cell surface, and T cell activation (Fig. 2).

Identification and validation of biomarkers for diagnostic characteristics.

The Search Tool for the Retrieval of Interacting Genes (STRING) is an online biological database that provides gene analysis and constructs networks of gene interactions at the protein level[28]. In this study, we used STRING (version 11.0) to construct the protein-protein interaction (PPI) network of the DEGs[30]. In order to further explore central genes related to restenosis and their mechanism of action, 72 genes with up-regulated expression in the 253 differentially expressed genes in the restenosis group were found and uploaded to STRING online database to build a PPI network, and a PPI network with 72 genes as nodes and 72 edges was realized (as shown in figure). Nodes represent differentially expressed genes enriched in STRING database, while edges reflect the interactions between differentially expressed genes. Since genes with high binding degree and high clustering coefficient are more important in maintaining the stability of the entire network, this paper searched genes with high binding degree and clustering coefficient greater than 0.4 through PPI network. The average node degree was 7.36, the average local clustering coefficient was 0.526, and the P-value of PPI enrichment was $1.0e-16$. Among the 72 nodes, the top 10 genes with high binding degree were found by Cytoscape(version 3.8.2) MCODE and MCC calculation methods, which were as follows: GZMB, GZMA, PRF1, KLRB1, CD2, KLRD1, IL2RB, NKG7, GZMK, CCL5 (Fig. 3,4) were identified as central genes that play key roles in AMI.

Diagnostic effect of characteristic biomarkers on acute myocardial infarction

Seven biomarkers were used to distinguish AMI from control samples demonstrating highly diagnostic predictive results (Figure 5). The AUC value of *CCL5* was 0.864 (95% CI =0.770-0.959), *GZMA* was 0.836 (95% CI =0.731-0.942), *IL2RB* was 0.785 (95% CI =0.654-0.915), and *GZMA* was 0.836 (95% CI =0.731-0.942). The AUC value of *PRF1* was 0.751 (95% CI =0.622-0.881), *GZMB* was 0.799 (95% CI =0.681-0.916), and *NKG7* was 0.883 (95% CI =0.794-0.972). The AUC value of *KLRB1* was 0.856 (95% CI =0.758-0.954), *CD2* was 0.791 (95% CI =0.665-0.917), and *GZMK* was 0.832 (95% CI =0.730-0.934). The AUC value of *KLRD1* was 0.823 (95% CI =0.712-0.934). Logistic regression showed that the combined diagnostic ability of 10 biomarkers was 0.929 (95% CI =0.866-0.991).

Acute myocardial infarction is associated with immune checkpoints, m6A, and iron death.

From the 24 genes associated with iron death that were collected changes were observed in gene expression between patients with AMI, stable angina, and healthy controls. Among the patients, we observed that *ACSL4*, *CARS*, *CISD1*, *CS*, *GPX4*, *NFE2L2*, *RPL8*, and *SAT1* iron death-related genes were closely associated with AMI. Studies have shown that high levels of the antioxidant enzyme glutathione peroxidase (GPx) are associated with improved prognosis after acute coronary syndrome (ACS) and have a protective effect[38]. Many regulators are involved in RNA methylation, including methyltransferase (Writer), RNA-binding protein (Reader), and demethylase (Erasers)[34, 36, 37]. Therefore, we collected genes associated with these three regulatory types and investigated their association with AMI. We found that the expression of *METTL3*, *WTAP*, *YTHDC1*, and *YTHDF2* was significantly increased in patients with AMI ($P \leq 0.01$). During the verification of immune checkpoint, *LAG3*, *SIGLEC15*, and *TIGIT* were found to be closely related to AMI ($P < 0.01$) (Figure 6,7,8).

Discussion

In this study, we first used the GEO gene expression dataset to detect differential gene expression associated with AMI and identify DEGs via functional analysis. Thereafter, we used MCC and MCODE to screen out ten genes as potential diagnostic markers. We also analysed the independent prediction and the joint prediction ROC curve. Subsequently, we validated the association of AMI with immune checkpoints, ferroptosis, and m6A. This study will contribute to the timely diagnosis and improved treatment of AMI.

Myocardial infarction is a leading cause of morbidity and mortality worldwide. Studies show that in 2015 alone, 15.9 million patients suffered from AMI[39]. Despite significant improvements in the early diagnosis and treatment of AMI in the past decade, it remains a leading cause of death and disability. Therefore, the identification of new biomarkers for the early diagnosis of AMI requires further investigation. In recent years, RNA has emerged as a particular primary biomarker for cardiovascular disease. For example, *SOCS3* can be used as a biomarker to predict the risk of AMI, and high expression of *SOCS3* is an independent risk factor for AMI[40]. Small RNAs, such as mir-34, have significant regulatory effects after heart failure and provide important information about heart failure[41, 42]. *PTGS2*

is associated with reduced risk of stroke and myocardial infarction[43]. With the development of gene chip technology, microarrays have been widely used in heart disease research[44, 45].

On comparing the expression levels of patient target genes that earlier predicted STEMI development, we found significant differences in *GZMB*, *GZMA*, *GZMK*, *PRF1*, *KLRB1*, *CD2*, *KLRD1*, *IL2RB*, *NKG7*, and *CCL5* expression. The expression of these genes was significantly increased after STEMI. Granase B (*GZMB*), a member of the serine protease family of granase, promotes apoptosis and is currently the most widely studied granase in the field of health and disease[46]. Studies have shown that mir-518a-5p can target *GZMB* to reduce hypoxia-reoxygenation-induced vascular endothelial cell injury, thereby improving coronary artery disease[47]. *GZMK*, belonging to the granase serine protein family, also plays a key role in myocardial ischaemia. For example, microRNA-145 can protect mice from myocardial ischaemia reperfusion injury by regulating the expression of *GZMK* under sevoflurane treatment[48]. *CD2* is an immunoglobulin superfamily transmembrane glycoprotein expressed on the surface of T cells, NK cells, thymocytes, and dendritic cells[49, 50]. Due to the increased expression of *CD2* on activated and memory T cells and its importance for spontaneous NK cell toxicity, *CD2*-targeted therapy may be an effective tool for regulating the activation of these cell types in transplant patients or patients with autoimmune diseases [51]. It is not difficult to predict that it may also play a key role in the occurrence of AMI. *IL2RB* mutations can lead to immune dysregulation driven by T and NK cells [52]. In addition, *PRF1* mutations can also alter immune system activation, inflammation, and autoimmune risk[53]. Therefore, we believe that immune dysregulation or immune inflammation is significantly associated with AMI. However, further experiments are warranted to verify this hypothesis.

In the analysis of immune checkpoint-related genes in patients, we found that *LAG3*, *SIGLEC15*, and *TIGIT* immune checkpoint proteins were closely related to the occurrence of AMI. *LAG3*, a member of Ig superfamily protein, binds with MHC Class II with high affinity and is a strong immune checkpoint inhibitor for activating T cells[54, 55]. Studies have shown that plasma *LAG3* is a potential independent predictor of HDL-C levels and the risk of coronary heart disease[56]. In recent years, ferroptosis has been a hot topic in investigations of atherosclerotic lesions, and frequent and long-term whole blood donation can reduce iron content in the body, which may be related to the reduced risk of atherosclerotic cardiovascular events[57]. In our study on the relationship between DEGs and ferroptosis in AMI, we found that eight genes related to ferroptosis were closely related to the occurrence of AMI. However, the specific mechanism of the relationship between AMI and iron death has not been explained via basic relevant research. Similarly, in the analysis of m6A-related genes in patients with AMI, we found that methylation of *METTL3*, *WTAP*, *YTHDC1*, and *YTHDF2* was closely related to the occurrence of AMI. Studies have shown that cytoplasmic proteins *YTHDF1* and *YTHDF2* mediate methylation-dependent translation promotion and degradation of target genes, respectively[58, 59]. However, the mechanism of its association with AMI remains to be further studied. Therefore, we believe that the occurrence of AMI is related to the immune checkpoint, ferroptosis, and m6A, but this hypothesis needs to be verified.

This study has some limitations. For example, there are several studies on the differential expression of *ACS* genes. However, the results of those studies are different to this study. This could be due to the

following reasons: (1) different batches of microarray analyses have different results to some extent; (2) compared with other studies, this study adopted three AMI data sets, providing a comprehensive analysis method for bioinformatics for AMI. Therefore, the results of this study are reliable. It is also important to verify the results in subsequent experiments. In addition, the reproducibility of immune checkpoint-, ferroptosis-, and m6A-related genes obtained from the dataset needs to be further validated. Further large-scale basic studies can be carried out to verify the conclusions of this study.

Conclusions

The timely diagnosis and treatment of AMI can help improve global health. Considering this, our study aimed to identify new genetic markers associated with AMI. We found ten genes related to the occurrence of AMI. Furthermore, we believe that the occurrence of AMI is related to immune checkpoint, ferroptosis, and m6A; however, since the mechanisms of association for these phenomena with AMI remains unclear, our hypothesis needs further verification.

Abbreviations

AMI - Acute myocardial infarction

NCBI - National Centre for Biotechnology Information

STRING - Search Tool for the Retrieval of Interacting Genes/Proteins

DEGs - Differentially expressed genes

GO - Gene ontology

KEGG - Kyoto Encyclopedia of Genes and Genomes

ncRNA - Non-coding RNA

miRNA - micro-large RNA

LncRNAs - long ncRNAs

CircRNAs - round RNAs

GEO - Gene Expression Omnibus

PCA - Principal Component Analysis

STEMI - ST-segment elevation myocardial infarction

NSTEMI - non-ST-segment elevation myocardial infarction

MF - Molecular function

BP - Biological pathways

CC - Cellular components

ROC - Receiver operating characteristic

GZMB - Granase B

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets analysed and generated in this study can be at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42148>, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61144>, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60993>.

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: Xiao Tong conceived and designed the study, conducted the experiments, analysed the data, interpreted the results, prepared the charts, drafted the manuscript, and edited it. All authors contributed to the article and approved the submitted version.

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Figures

Figure 1

A: Box plot after data standardisation. B: PCA results before batch removal for multiple data sets. C: PCA results after batch removal. D: Volcano plots were constructed using fold-change values and adjusted P. The red point in the plot represents the upregulated mRNAs and the blue point indicates the downregulated mRNAs with statistical significance. E: Hierarchical clustering analysis of mRNAs, which were differentially expressed between patients with AMI and healthy controls.

Figure 2

The enriched KEGG signalling pathways were selected to demonstrate the primary biological actions of major potential mRNAs. The abscissa indicates gene ratio and the enriched pathways were presented in the ordinate. Gene ontology (GO) analysis of potential mRNA targets. The biological pathways (BP), cellular component (CC), and molecular function (MF) of potential targets were clustered based on the

ClusterProfiler R package (version: 3.18.0). In the enrichment result, $p < 0.05$ or FDR < 0.05 were considered meaningful.

Figure 3

In the PPI network, nodes with fillers inside indicate that the 3D structure of the protein was known or predicted, and empty nodes indicate that the 3D structure was unknown. The connections between proteins represent the predicted functional associations that are specific and meaningful. There are seven

differently-colored lines: 1. Light blue for database auxiliary evidence; 2. Purple for experimental proof; 3. Red for gene fusion; 4. Yellow-green for evidence mined from the article; 5. Green for gene close; 6. Blue for gene co-generation; and 7. Black for gene co-expression.

Figure 4

(A) Figure represents the 10 key genes calculated by MCC algorithm in cytoHubba; the darker the color, the more critical the gene. (B) Figure denotes the gene related to the module with the highest score in

MCODE calculation methods using Cytoscape. (C) The intersection of the key genes calculated by MCC and MCODE is visualized using Venn diagram.

Figure 5

ROC curve of differentially expressed genes related to AMI, independence, and union TPR: true positive rate, the ratio of positive samples to all positive samples predicted by classifier, i.e., $TP/(TP+FN)$; FPR: False negative rate, the ratio of positive classes to all negative classes in the sample predicted by the

classifier, i.e., $FP/(FP+TN)$. By changing different thresholds, a pair of TPR and FPR will be obtained. ROC curve is a curve drawn with FPR as abscissa and TPR as ordinate. As shown in the figure, each point on the curve corresponds to FPR and TPR at different thresholds. (The meaning of TPRate is the proportion of all samples of true category 1 that are predicted to be category 1. The meaning of FPRate is the proportion of all samples with true category 0 that are predicted to be category 1. AUC means that a positive sample and a negative sample are randomly selected from the sample. The probability that the classifier predicts the positive sample to be positive is P_1 , and the probability that the negative sample is positive is P_2 . AUC means the probability that $P_1 > P_2$).

Figure 6

A: Box plot after data standardisation. B: PCA results before batch removal for multiple data sets. C: PCA results after batch removal. D: Immune checkpoint-related gene analysis results. G1 represents the healthy control group and G2 represents the AML group. The significance of the two groups was statistically analyzed by Wilcoxon test. The horizontal axis represents different Immune checkpoint-related mRNAs, the vertical axis represents the expression distribution of related genes, where different

colors represent different groups, and the upper left corner represents the significance with asterisks indicating the significance levels *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

A: Box plot after data standardisation. B: PCA results before batch removal for multiple data sets. C: PCA results after batch removal. D: Ferroptosis-related gene analysis results. G1 represents the healthy control group and G2 represents the restenosis group. The significance of the two groups was statistically

analyzed by Wilcoxon test. The horizontal axis represents different ferroptosis-related mRNAs, the vertical axis represents the expression distribution of related genes, where different colors represent different groups, and the upper left corner represents the significance with asterisks indicating the significance levels *P < 0.05, **P < 0.01, ***P < 0.001

Figure 8

A: Box plot after data standardisation. B: PCA results before batch removal for multiple data sets. C: PCA results after batch removal. D: m6A-related gene analysis results. The figure represents the expression distribution of m6A molecule, in which the horizontal axis represents different m6A molecules, the vertical axis represents the expression distribution of related genes. G1 represents the healthy control group and G2 represents the AMI group. The significance of the two groups was tested by Wilcoxon test. The asterisk represents the degree of significance (* P). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Files

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