

AZU1 (HBP/CAP37) and PRKCG (PKC-gamma) may be candidate genes affecting the severity of Acute Mountain Sickness.

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Research Article

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Abstract

Background

Acute Mountain Sickness (AMS) is one of the diseases that predispose to sudden ascent to high altitudes above 2500 meters. Among the many studies on the occurrence and development of AMS, there are few studies on the severity of AMS. Some unidentified phenotypes or genes that determine the severity of AMS may be vital to elucidating the mechanisms of AMS. This study aimed to explore essential candidate genes and phenotypes for moderate-to-severe AMS to provide evidence for a better understanding of the mechanisms of AMS.

Methods

Nineteen subjects from GSE103927 dataset (Downloaded from the Gene Expression Omnibus database) were classified as having moderate to severe AMS (MS-AMS) and mild or no AMS (NM-AMS) based on the Lake Louise score (LLS). Various bioinformatics analyses, such as weighted gene co-expression network analysis (WGCNA), differentially expressed genes screening, and ROC curves were used to compare the differences between the two groups. Another dataset in the GEO database and Real-time quantitative PCR (RT-qPCR) was used to validate the analysis results.

Result

No statistically significant differences in phenotypic and clinical data existed between the MS-AMS and NM-AMS groups. WGCNA revealed 530 genes associated with LLS. Three hundred sixty-eight differentially expressed genes between the two groups. Eight differential genes are associated with LLS, and their biological functions are related regulating of the apoptotic process and programmed cell death. The ROC curves showed that FAM86B3P, AZU1, and PRKCG had a better predictive performance for MS-AMS. The transcript levels of AZU1 and PRKCG were significantly higher in the MS-AMS group compared to the NM-AMS group, a result validated by another dataset. RT-qPCR showed that the transcript levels of AZU1 and PRKCG were elevated in BEAS-2B cells after hypobaric hypoxia treatment. AZU1 and PRKCG were enriched in the Neutrophil extracellular trap formation pathway, suggesting the importance of this pathway in influencing the severity of AMS.

Conclusion

AZU1 and PRKCG were finally identified as the candidate genes affecting AMS severity. AZU1 and PRKCG may affect the severity of AMS by influencing lung endothelial cell permeability through the Neutrophil extracellular trap formation pathway. Our study provides a new perspective to explore the molecular

mechanism of AMS, and inhibiting or targeting these genes may help high altitude acclimation and reduce the occurrence of AMS or moderate to severe AMS.

1 Background

Acute Mountain Sickness (AMS) has defined as a syndrome involving headache, dizziness, gastrointestinal symptoms, insomnia and fatigue after arrival at a high altitude (> 2500 m)[1]. The disease occurs in people who have lived on the plains for a long time and have been exposed to high-altitude areas for a short period. Many factors contribute to AMS, and acute hypobaric hypoxia may serve as a principal etiological factor for Acute Mountain Sickness (AMS) [2]. In China, about 15 million people go to high-altitude for work or travel every year, and the incidence rate of AMS is almost 43%~69%[3, 4]. Besides, AMS may progress to high altitude pulmonary edema, high altitude cerebral edema, and even death[5, 6]. Therefore, AMS has become a public health problem of increasing concern[7].

Multiple factors influence the occurrence of AMS. In addition to environmental factors, for the population, women are less likely to develop AMS than men[8]. Being overweight or obese is another factor contributing to AMS[9]. In terms of individuals, many clinical phenotypes were changed at high altitudes. The partial pressure of arterial oxygen decreases for a short period, resulting in lower arterial oxygen saturation[10]. Slowly, the bioavailability of NO decreases, leading to pulmonary vasoconstriction and consequent high-altitude pulmonary edema[11]. Under prolonged hypoxic conditions, hemoglobin production increases, increasing blood viscosity and leading to altitude erythrocytosis[12]. While some changes benefit athletes in terms of improved athletic performance, they are detrimental to most of the general population exposed to high altitudes for short periods[13].

The underlying cause of phenotypic alterations is genetic alterations[14]. Previous studies have shown that the hypoxia-inducible factor (HIF) pathway plays a significant role in hypoxic adaptation[15]. Under hypoxic conditions, hydroxylation and degradation of HIF are inhibited, and HIF induces transcription of downstream genes by binding to hypoxia response elements (HREs)[16]. For example, HIF regulates vascular endothelial growth factor (VEGF)[17]. VEGF promotes endothelial cells to add value and form new blood vessels, which facilitates the improvement of the ventilation-perfusion ratio (V/Q) to adapt to the hypoxic environment[18, 19]. Erythropoietin (EPO) is regulated by HIF and promotes erythropoiesis[20]. Many related genes, such as PPAR, NF-kb, p53, and P13K, are part of pathways that function together to adapt to the hypoxic environment[21].

However, few studies in this field of research have focused on the severity of the disease. In recent years, with the deepening of understanding of AMS and the widespread use of drugs such as Rhodiola Rosea, the severity of AMS may be partially masked by the effects of drugs[22, 23]. The discovery of more phenotypes or genes associated with the severity of AMS may lead to a better understanding of the mechanisms of AMS occurrence and treatment. Currently, the diagnosis and severity evaluation of AMS relies on the Lake Louise Score (LLS). Since its inception in 1993, LLS has been increasingly used for the diagnosis of AMS in the course of its continuous development[24]. LLS is a subjective judgment by the

study participants of their symptoms of headache, gastrointestinal symptoms of fatigue, and dizziness[25]. People rate the severity of each symptom (0–3) based on how they feel, and the sum of the symptom scores is the total LLS score. Based on previous studies and the 2018 edition of the LLS, in the present study, study subjects with LLS scores ≥ 6 were considered to have moderate-to-severe AMS, and the rest were in another group[26].

Although many genes have been found to be associated with AMS, the gene-phenotype interactions have not been explained in detail. Linking candidate genes to phenotypes is one of the challenges of studying AMS. Therefore, more key genes need to be discovered because the process of gene annotation overlooks an entire class of potentially essential genes making this work more complex[27, 28]. Nowadays, more publicly available datasets are submitted to Gene Expression Omnibus (GEO) database with the development of technologies[29]. Transcriptome and microarray analysis have been used in various diseases, including tumors, AMS, and high altitude pulmonary edema (HAPE). Through bioinformatics analysis methods, such as weighted gene co-expression network analysis (WGCNA), the genes most associated with AMS can be identified to discover potential biomarkers and pathways associated with AMS, which are critical for disease diagnosis, treatment, and prevention.

Therefore, this study aimed to explore essential candidate genes and phenotypes for moderate-to-severe AMS to provide evidence for a better understanding of the mechanisms of AMS. The dataset GSE103927 and its original study in the GEO database contains general characteristics of the study subjects, clinical data, LLS, and transcriptome information. This dataset meets the needs of this study. We analyzed this dataset by bioinformatics methods such as WGCNA and ROC curves. Four genes were finally identified that were associated with AMS severity.

2 Methods

2.1 Data Collection and Study Design

The datasets analyzed during the current study are available in the GEO repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103927>. Dataset GSE103927 was downloaded from GEO database (<https://www.ncbi.nlm.nih.gov/gds>) by R software (version 4.0.4) on March 25, 2022. One hundred twelve total samples representing 21 subjects at 7-time points (not all subjects represented at all time points) were taken. Nineteen samples exposed to the high altitude at noon on the first day (H1) were included in this study.

General characteristics and clinical data of subjects were obtained from the supplementary material of *Andrew's* study[30]. Clinical data at other time points were excluded except for those measured at H1 and Sea Level (SL). Multiple imputation, an approach used to generate synthetic data that accurately represents group-level results, was used to impute missing values. This process was implemented through the “mice” package in R software (version 4.0.4)[31, 32]. Since all data used for analysis in this

study were obtained from a publicly available database, Ethics Committee approval was not required. Figure 1 briefly illustrates our study design.

2.2 Definition of Acute Mountain Sickness and Grouping

Based on the 2018 Lake Louise Score (LLS) criteria, AMS cases must have headache symptoms (headache score > 0), then a total score of 3–5 was defined as mild AMS, moderate AMS as 6–9 points, and severe AMS as 10–12 points[24]. Based on the latest LLS and previous study, subjects were divided into two groups, namely moderate to severe AMS (MS-AMS, subjects' LLS $\geq$ 6) and mild or no AMS (NM-AMS, subjects' LLS < 6)[26].

2.3 Difference Analysis of Clinical Data and Differentially Expressed Genes (DEGs)

SPSS software (version 26.0) was used to compare general characteristics and clinical data between different groups to understand phenotypic changes better. The 'limma' package in R language software (version 4.0.4) based on a generalized linear model was used to analyze the DEGs[33]. DEGs with |Fold change|>1 and P value < 0.05 were screened in this research[34]. Use SangerBox (<http://vip.sangerbox.com/>) for graphing and visualizing the results using volcano and heatmap[35].

2.4 Weighted gene co-expression network analysis (WGCNA)

The Median Absolute Deviation (MAD) of each gene was calculated, genes with MAD less than the median were excluded, outlier genes and samples were removed by the 'goodSamplesGenes' method of the R package 'WGCNA', and finally, a co-expression network was created for the genes in the MS-AMS group[36]. LLS was selected as a representative to identify modules and associated genes. Genes in modules associated with LLS are considered to be co-expressed LLS-related genes.

First, the Pearson correlation matrix and average linkage methods were performed for all pairs of genes. Then a weighted adjacency matrix is constructed using the power function $A_{mn} = |C_{mn}|^\beta$. β is a soft threshold parameter that emphasizes the strong linkage between genes and penalizes weak linkage. A suitable soft threshold of 18 was selected (**Figure S1**). The adjacency relationships are translated into a topological overlap matrix (TOM), which measures the network connectivity of a gene, and the corresponding dissimilarity (1-TOM) is calculated. To classify genes with similar expression profiles into gene modules, the average linkage hierarchical clustering was performed based on the TOM dissimilarity measure with a minimum gene dendrogram size (genome) of 30. The sensitivity is set to 3. To further analyze the modules, dissimilarities of module feature genes were calculated, a cut line was selected for the module dendrogram, and some modules were merged. Gene significance (GS) for LLS and modules membership (MM) were calculated. Five hundred eighty-seven genes with GS > 0.5 and MM > 0.8 of the modules significantly correlated with LLS were selected as key genes[35, 37].

2.5 Candidate Genes Selection, Correlation Analysis, and Differential Expression Analysis

Candidate genes were selected from the intersection of the DEGs in part 2.3 and the genes in part 2.4. The Wayne diagram visualizes the intersection. The 'corrplot' package in R software was used to explore correlations between candidate genes. The boxplot made by SangerBox (<http://vip.sangerbox.com/login.html>) was used to visualize the differences in the expression of candidate genes among different groups[35].

2.6 Functional Annotation and Pathway Enrichment Analysis

For gene set functional enrichment analysis, the 'org.Hs.eg.db' package and the 'clusterProfiler' package in R language software were used for GO annotation of genes[38]. A minimum gene set of 30 and a maximum gene set of 5000 were set, and the P value of < 0.05 or FDR of < 0.1 were considered statistically significant. KEGG pathway enrichment for key genes using online tools (<https://david.ncifcrf.gov/>). Visualize the results with a bar-plot, bubble-plot, and circle map[35].

2.7 Receiver Operating Characteristic(ROC) Curves Analysis

ROC curves were used to evaluate the predictive ability of candidate genes on the severity of AMS. The ROC curve analysis was completed by the 'ROCR' package and 'rms' package in R language software.

2.8 Cell culture and Hypoxia Treatment

BEAS-2B cells were obtained from laboratory passaged cultures. Cells were maintained in DMEM basic (Gibco, USA) with a mixture of 10% fetal bovine serum (BI, China) and 1% penicillin-streptomycin (Gibco, USA). All cells were cultured and passaged at 37°C, 5% CO₂ before treatment was applied. Control cells were cultured at 37°C and 5% CO₂ for 24 hours. Cells in the experimental group were treated with hypobaric hypoxia and cultured at 37°C in 1 ± 0.3% O₂, 5% CO₂, and 94% N₂ for 24 hours.

2.9 Real-Time quantitative PCR

RNA was isolated using the RNA-easy isolation Reagent (Vazyme, China). Then reversed transcribe the RNA to cDNA according to the reverse transcription kit instructions (GeneStar, China). Real-time quantitative PCR (qRT-PCR) was completed using SYBR Green (GeneStar, China). The primers were synthesized by Sangon Biotech (Shanghai, China) and are shown in Table 1. PCR amplification was performed for 40 cycles using the following conditions: denaturation 95°C for 15s, annealing 60°C for 30s and extension 72°C for 30s. The qRT-PCR results were evaluated using the $\Delta\Delta C_t$ method. Bar graphs present the results.

Table 1
Primers Sequence List

Genes	Forward primer 5'-3'	Reverse primer 5'-3'
AZU1	TGAGCGAGAATGGCTACGAC	GAGGCAGTGGCAGTATCGTC
PRKCG	AGCCACAAGTTCACCGCTC	GGACACTCGAAGGTCACAAAT
GAPDH	GCAGGGGGGAGCCAA AAGGG	TGCCAGCCCCAGCGTCAAAG

2.10 Statistical Analysis

The clinical data were statistically analyzed by IBM SPSS Statistics 26.0. Independent-Samples T-test was used to test statistically significant differences in the two groups. The filtering of DEGs is done through the 'limma' package in R language software (version 4.0.4). The 'corrplot' package in R was used to complete Pearson correlation analysis between key genes. ROC curve analysis was performed using SPSS 26.0 and plotted using the 'ROCR' package and the 'rms' package in R. P value < 0.05 means the difference is statistically significant.

3 Results

3.1 Difference of General Characteristics and Clinical Data

General characteristics and clinical data were obtained from previous research and its supplementary material. Missing values in these data were filled by multiple imputation[30, 32]. In general, 19 subjects (8 females) were included in this study.

By comparing the general characteristics at H1, no statistically significant difference in gender was found between the NM-AMS group and the MS-AMS group ($\chi^2 = 0.398$, $P = 0.528$). Besides, the differences between the two groups in other general characteristics were also not statistically significant (**Table S1**).

By comparing the clinical data at H1, the difference in HB between the NM-AMS group and MS-AMS group was close to statistical significance ($t = 2.103$, $P = 0.052$), and there was no statistically significant difference in general characteristics between the NS-AMS and MS-AMS groups (**Table S2**).

3.2 The LLS and DEGs between the two groups

There was a clear significant difference in LLS between the two groups, as shown in Fig. 2A. A total of 368 DEGs (252 were up-regulated and 116 were down-regulated) were identified between the NM-AMS group and the MS-AMS group, with the screening criteria of |Fold change|>1 and $P < 0.05$, the visualization result is shown in Fig. 2B, C[34].

3.3 Discovering the Most Significant LLS-Related Modules and Genes

After screening the DEGs, in order to find out the genes most related to LLS, a gene co-expression network was constructed through WGCNA. Each module contained a minimum of 30 genes, and the sensitivity was set to 3. Modules with a distance less than 0.25 were merged together, resulting in 44 modules, of which module blue4 ($R^2 = 0.90$, $P < 0.001$), module brown2 ($R^2 = 0.77$, $P = 0.020$), and module orange4 ($R^2 = 0.76$, $P = 0.020$) were the most relevant to LLS (Fig. 3A, B). A heat map showing the clustering of module feature vectors was made and can be seen in the attachment (**Figure S2**). All three modules were positively correlated with LLS.

The selection criteria for key genes were: the module in which the gene was located was significantly correlated with LLS, $GS > 0.5$ and $MM > 0.8$, and weighting threshold ≥ 0.1 . As shown in Figs. 3C, D, and E. According to this principle, 530 genes in the three modules were considered key genes when LLS was considered.

3.4 Candidate Genes Selection, Correlation analysis, and Differential Expression analysis

Genes in both DEGs and Key Genes obtained by WGCNA were selected as candidate genes. Figure 4A visualizes this result. The final 8 genes selected were CAAP1, ZNF45, FAM86B3P, PRKCG, RIPPLY3, PHLDA3, AZU1, and MYDGF. The expression differences of these eight genes among different groups are shown in Fig. 4B. Besides, Fig. 4C shows the correlation between the candidate genes.

3.5 Functional Annotation and Pathway Enrichment Analysis of Candidate Genes

The GO enrichment analysis included the following three portions: biological process (BP), cell component (CC), and molecular function (MF). The primary biological roles of candidate genes include regulation of apoptotic process (GO:0042981), regulation of programmed cell death (GO:0043067), regulation of cell death (GO:0010941), negative regulation of apoptotic process (GO:0043066), and calcium-dependent protein serine/threonine kinase activity (GO:0009931). As Figs. 5A, B, and C show. The candidate genes were primarily enriched in pathways associated with hsa04613: Neutrophil extracellular trap formation (Fig. 5D). Detailed information on these results is available in **Table S3 ~ 4**.

3.6 ROC Curves of Candidate Genes

To explore the performance of these eight genes in predicting MS-AMS and to identify more critical candidate genes, we made ROC curves for each of the eight genes. The ROC curves of FAM86B3P (AUC = 0.867), AZU1 (AUC = 0.800), and PRKCG (AUC = 0.822) were statistically significant ($P < 0.05$) and were better predictors of MA-AMS. It is worth noting that FAM86B3P is a pseudogene and is not discussed too much in this study, so the ROC curve of AZU1 together with PRKCG to predict the severity of AMS was

established (AUC = 0.833). The ROC curve is shown in Figs. 6A, B, C, and D. For more information on the ROC curves for other genes, see **Table S5**.

3.7 Verification by Other Dataset and Real-time Quantitative PCR

To validate the analysis results, we used GSE145935, another dataset from the GEO database, to verify the results of this study (Fig. 7A). We also performed real-time quantitative PCR to detect the expression of candidate genes under hypobaric hypoxic conditions. Since FAM86B3P is a pseudogene, we only tested AZU1 and PRKCG. The results showed that the transcript levels of AZU1 and PRKCG were increased under hypobaric hypoxic conditions (Fig. 7B and C).

4 Discussion

In this study, dataset GSE103927 was downloaded from the GEO database. According to the 2018 edition of the LLS, the research subjects were divided into the NM-AMS group and the MS-AMS group. The differences in phenotypes and genes between the two groups at H1 were analyzed. The modules and key genes most associated with LLS were obtained by WGCNA analysis. The intersection of differentially expressed genes with key genes obtained by WGCNA was selected as a candidate gene for AMS. To explore their biological function, these candidate genes were enriched for GO and KEGG function. ROC curves were made to evaluate the predictive performance of the three candidate genes. FAM86B3P, AZU1, and PRKCG were selected as candidate genes. Finally, GSE145935, another dataset from the GEO database, and Real-Time quantitative PCR were used to verify the result.

In previous studies, the importance of hemoglobin in altitude-related diseases has been focused on [39–41]. In this study, the difference in HB between the MS-AMS group and the NM-AMS group at H1 was approaching significant. This result is consistent with previous research because it takes about 1 week for erythropoietin (EPO) to drive precursor erythroblasts to adult erythrocytes. Therefore the hemoglobin response to acute hypoxia is slow[42]. However, the differences in other variables were not significant. Does this evidence suggest that the severity of AMS cannot be distinguished from clinical data? However, we believe that this is because LLS is subjectively evaluated. The degree of tolerance for different symptoms in different people may not correspond to clinical data.

To gain insight into the biological roles of the eight key genes, we did an enrichment analysis. GO enrichment analysis suggested that these genes were associated with regulation of apoptotic process, regulation of programmed cell death, regulation of cell death, and calcium-dependent protein serine/threonine kinase activity. Besides, KEGG pathway enrichment analysis showed that AZU1 and PRKCG were enriched in the Neutrophil extracellular trap formation pathway. The results of GO and KEGG enrichment were surprising, significantly, since the candidate genes (AZU1 and PRKCG) derived from our analysis were enriched in the same pathway. In addition to providing a better understanding of AZU1 and PRKCG, these results also provide insight into their role in influencing AMS's severity.

Azurocidin 1 (AZU1), also known as heparin-binding protein (HBP) or cationic antimicrobial protein of 37 kDa (CAP37), is a neutrophil-derived granule protein[43]. Previous studies have shown that AZU1 expression levels in patients with acute lung injury (ALI) are higher than in patients without ALI. This phenomenon is also present in patients with acute respiratory distress syndrome (ARDS)[43–45]. Besides, the elevation of AZU1 is associated with a decrease in arterial oxygen partial pressure (PaO_2) [46]. Our study is consistent with previous studies. AZU1 transcript levels were higher in the MS-AMS group than in the NM-AMS group. This result was also verified in GSE145935.

This result is not difficult to explain. Low PaO_2 is one of the environmental characteristics of the highland region[47]. Acute hypoxia can cause damage to the lungs with an accompanying immune response[48]. In response to hypoxic stimulation, T-cells or monocytes release AZU1 chemokines, such as IL-8 (possibly via the HGF/cMet pathway)[49, 50]. Neutrophils, one of the first cells to respond to infection, release AZU1 pre-existing in secretory vesicles and sulfur cell granules after receiving stimulation from IL-8. The released AZU1 acts on glycosaminoglycans on the surface of endothelial cells, activating PKC and Rho kinases while allowing the entry of calcium ions. These alterations rearrange the endothelial cytoskeleton, leading to increased endothelial permeability and inducing the onset of edema while leaving the possibility for neutrophils to act on other tissues and cells[43, 51].

Interestingly, in the present study, we also observed elevated transcript levels of PRKCG in the MS-AMS group. Protein kinase C gamma (PRKCG, also known as PKC-gamma, PKC γ , and SCA14) is located on chromosome 19 and encodes the γ isoform of the PKC family[52, 53]. PKC is a family of protein serine/threonine kinases composed of multiple isoforms that play essential roles in cell mitosis and proliferation, apoptosis, and platelet activation[54]. As a pressure receptor, PRKCG is expressed mainly in brain and eye tissues[55]. It has been shown that PRKCG is associated with brain disorders caused by hypoxia. PRKCG is involved in the hypoxia-induced reduction of blood-brain barrier permeability[56]. In the oxygen-glucose deprivation model, PKC- γ expression was associated with hypoxia-induced mitochondrial depolarization, increased ROS, and elevated calcium ions[57]. However, some studies have shown that PRKCG has neuroprotective effects[58, 59]. Therefore, the mechanism of the neurological effects of PRKCG and whether such effects are related to headache symptoms in AMS needs further investigation.

It is known that the HIF pathway regulates oxygen homeostasis in the organism[15]. Studies have shown that the activation of HIF is associated with the PKC signaling pathway[60]. In the indirect hypoxia model, NADPH oxidase produces reactive oxygen species ROS, which elevates phosphorylated PLC γ (p-PLC γ) expression to activate IP3 receptors and activate IP3 receptors that regulates calcium ions. PLC γ is also involved with Ca^{2+} in the activation of PKC (detected by PKC-gamma) by indirect hypoxia, and these responses promote HIF α production[61]. Nevertheless, few studies on PRKCG and hypobaric hypoxia induced diseases such as AMS and high-altitude pulmonary edema. It is noteworthy that in a model of cardiac hypertrophy induced by hypobaric hypoxia, after short-term exposure to low pressure and hypoxia, mRNA expression of PKC- γ was significantly increased in the right ventricle but returned to

normal after that, whereas, in the left ventricle, PKC- γ expression was unchanged[62]. Therefore, the role of PRKCG in AMS deserves further investigation in the future.

High altitude pulmonary edema (HAPE) is a type of altitude sickness that is more severe than AMS and non-cardiogenic pulmonary edema[63]. Inflammation is not the culprit in the development of HAPE, but it is one of the factors that promote its development[63, 64]. Many studies have shown that the HBP (AZU1) and PKC pathways play a role in the development of pulmonary edema, as gates lead to enhanced endothelial cell permeability[46, 65]. The role of HBP and PKC in acute respiratory distress syndrome (ARDS, a non-cardiogenic pulmonary edema) and circulatory collapse has been explored[44, 45]. However, their role in HAPE has not been well explored. Whether targeted inhibition or treatment can reduce the severity of AMS or HAPE remains an open question.

The full name of FAM86B3P is a family with sequence similarity 86, member A pseudogene. There are few studies on this pseudogene. Pseudogenes are mutated copies of protein-coding genes that cannot be translated into proteins, and rarely can they be translated into proteins. Pseudogenes will not be discussed too much here.

Besides, the ROC curves showed good predictive performance of all three genes for the severity of AMS. The experimental results were also consistent with the analytical results. Based on the results and discussion above, we suggest that AZU1 (HBP) and PRKCG (PKC- γ) may be candidate genes influencing the severity of acute plateau illness. The effects of AZU1 and PRKCG alterations in a hypoxic environment appear to be systemic. However, the mechanism of their alterations induced by hypoxia still needs further study.

5 Conclusion

AZU1 (HBP or CAP37), PRKCG (PKC- γ), and FAM86B3P were identified as candidate genes affecting the severity of Acute Mountain Sickness by comparison of clinical data from GSE103927, differential expressed genes screening, WGCNA analysis, screening of candidate genes, ROC curve analysis and validation of other datasets and qRT-PCR. This study helps elucidate Acute Mountain Sickness's pathogenesis and the mechanisms affecting its severity. Our study provides a new perspective on it. Inhibition or targeting of these genes may improve the health effects of the high-altitude environment.

Abbreviations

AMS, acute mountain sickness, ARDS, acute respiratory distress syndrome, AUC, area under curve, BP, biological process, CC, cell component, cDNA, DNA complementary to RNA, DEGs , differentially expressed gene(s), EPO, erythropoietin, GEO, gene expression omnibus database, GP, gene ontology, GS, gene significance, H1, he first day on high altitude, HAPE, high altitude pulmonary edema, HB, hemoglobin, HREs, hypoxia response element(s), kDa, kilodalton(s), KEGG, kyoto encyclopedia of genes

and genomes, LLS, lake louise score, MAD, median absolute deviation, MF, molecular function, MM, modules membership, MS-AMS, moderate to severe AMS, NM-AM, mild or no AMS, ROC, receiver operating characteristic, qRT-PCR, real time quantitative PCR, SL, sea level, TOM, topological overlap matrix, V/Q, ventilation perfusion ratio, WGCNA, weighted gene co-expression network analysis, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103927>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed substantially to this study. Zhichao Xu was responsible for study design, data analysis, and manuscript writing. Qiong Li was responsible for collecting and organizing the data. Xiaobing Shen was responsible for reviewing the manuscript and providing guidance.

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References

1. Ding XH, Wang Y, Cui B, Qin J, Zhang JH, Rao RS, Yu SY, Zhao XH, Huang L: **Acute Mountain Sickness Is Associated With a High Ratio of Endogenous Testosterone to Estradiol After High-Altitude Exposure at 3,700 m in Young Chinese Men.** *Front Physiol* 2018, **9**:1949.
2. Liu C, Liu B, Liu L, Zhang EL, Sun BD, Xu G, Chen J, Gao YQ: **Arachidonic Acid Metabolism Pathway Is Not Only Dominant in Metabolic Modulation but Associated With Phenotypic Variation After Acute Hypoxia Exposure.** *Front Physiol* 2018, **9**:236.
3. Hackett PH, Roach RC: **High-altitude illness.** *N Engl J Med* 2001, **345**(2):107-114.
4. Gonggalanzi, Labasangzhu, Nafstad P, Stigum H, Wu T, Haldorsen OD, Ommundsen K, Bjertness E: **Acute mountain sickness among tourists visiting the high-altitude city of Lhasa at 3658 m above sea level: a cross-sectional study.** *Arch Public Health* 2016, **74**:23.
5. Johnson NJ, Luks AM: **High-Altitude Medicine.** *Med Clin North Am* 2016, **100**(2):357-369.
6. Sun K, Zhang Y, D'Alessandro A, Nemkov T, Song A, Wu H, Liu H, Adebiyi M, Huang A, Wen YE *et al*: **Sphingosine-1-phosphate promotes erythrocyte glycolysis and oxygen release for adaptation to high-altitude hypoxia.** *Nat Commun* 2016, **7**:12086.
7. Liu B, Huang H, Wu G, Xu G, Sun BD, Zhang EL, Chen J, Gao YQ: **A Signature of Circulating microRNAs Predicts the Susceptibility of Acute Mountain Sickness.** *Front Physiol* 2017, **8**:55.
8. Hanco I, Bailly S, Baillieu S, Doutreleau S, Germain M, Pepin JL, Verges S: **Excessive Erythrocytosis and Chronic Mountain Sickness in Dwellers of the Highest City in the World.** *Front Physiol* 2020, **11**:773.
9. San Martin R, Brito J, Siques P, Leon-Velarde F: **Obesity as a Conditioning Factor for High-Altitude Diseases.** *Obes Facts* 2017, **10**(4):363-372.
10. Hughes BH, Brinton JT, Ingram DG, Halbower AC: **The Impact of Altitude on Sleep-Disordered Breathing in Children Dwelling at High Altitude: A Crossover Study.** *Sleep* 2017, **40**(9).
11. Bourdillon N, Fan JL, Uva B, Muller H, Meyer P, Kayser B: **Effect of oral nitrate supplementation on pulmonary hemodynamics during exercise and time trial performance in normoxia and hypoxia: a randomized controlled trial.** *Front Physiol* 2015, **6**:288.
12. Fan X, Ma L, Zhang Z, Li Y, Hao M, Zhao Z, Zhao Y, Liu F, Liu L, Luo X *et al*: **Associations of high-altitude polycythemia with polymorphisms in PIK3CD and COL4A3 in Tibetan populations.** *Hum Genomics* 2018, **12**(1):37.
13. Sweeting AJ, Billaut F, Varley MC, Rodriguez RF, Hopkins WG, Aughey RJ: **Variations in Hypoxia Impairs Muscle Oxygenation and Performance during Simulated Team-Sport Running.** *Front Physiol* 2017, **8**:80.
14. Tulchinsky AY, Johnson NA, Porter AH: **Hybrid incompatibility despite pleiotropic constraint in a sequence-based bioenergetic model of transcription factor binding.** *Genetics* 2014, **198**(4):1645-1654.

15. Jiang X, Tian W, Tu AB, Pasupneti S, Shuffle E, Dahms P, Zhang P, Cai H, Dinh TT, Liu B *et al*: **Endothelial Hypoxia-Inducible Factor-2alpha Is Required for the Maintenance of Airway Microvasculature.** *Circulation* 2019, **139**(4):502-517.
16. Yue X, Lin X, Yang T, Yang X, Yi X, Jiang X, Li X, Li T, Guo J, Dai Y *et al*: **Rnd3/RhoE Modulates Hypoxia-Inducible Factor 1alpha/Vascular Endothelial Growth Factor Signaling by Stabilizing Hypoxia-Inducible Factor 1alpha and Regulates Responsive Cardiac Angiogenesis.** *Hypertension* 2016, **67**(3):597-605.
17. Wang T, Shi F, Wang J, Liu Z, Su J: **Kallistatin Suppresses Cell Proliferation and Invasion and Promotes Apoptosis in Cervical Cancer Through Blocking NF-kappaB Signaling.** *Oncol Res* 2017, **25**(5):809-817.
18. Huang J, Tang L, Zhao Y, Ding W: **TRIM11 promotes tumor angiogenesis via activation of STAT3/VEGFA signaling in lung adenocarcinoma.** *Am J Cancer Res* 2019, **9**(9):2019-2027.
19. Krauszman A, Mak TW, Szaszi K, Kuebler WM: **Role of phosphatase and tensin homolog in hypoxic pulmonary vasoconstriction.** *Cardiovasc Res* 2017, **113**(8):869-878.
20. Schodel J, Grampp S, Maher ER, Moch H, Ratcliffe PJ, Russo P, Mole DR: **Hypoxia, Hypoxia-inducible Transcription Factors, and Renal Cancer.** *Eur Urol* 2016, **69**(4):646-657.
21. Fidoamore A, Cristiano L, Antonosante A, d'Angelo M, Di Giacomo E, Astarita C, Giordano A, Ippoliti R, Benedetti E, Cimini A: **Glioblastoma Stem Cells Microenvironment: The Paracrine Roles of the Niche in Drug and Radioresistance.** *Stem Cells Int* 2016, **2016**:6809105.
22. Chiang HM, Chen HC, Wu CS, Wu PY, Wen KC: **Rhodiola plants: Chemistry and biological activity.** *J Food Drug Anal* 2015, **23**(3):359-369.
23. Lee SY, Li MH, Shi LS, Chu H, Ho CW, Chang TC: **Rhodiola crenulata Extract Alleviates Hypoxic Pulmonary Edema in Rats.** *Evid Based Complement Alternat Med* 2013, **2013**:718739.
24. Roach RC, Hackett PH, Oelz O, Bartsch P, Luks AM, MacInnis MJ, Baillie JK, Lake Louise AMSSCC: **The 2018 Lake Louise Acute Mountain Sickness Score.** *High Alt Med Biol* 2018, **19**(1):4-6.
25. Hufner K, Brugger H, Kuster E, Dunsser F, Stawinoga AE, Turner R, Tomazin I, Sperner-Unterweger B: **Isolated psychosis during exposure to very high and extreme altitude - characterisation of a new medical entity.** *Psychol Med* 2018, **48**(11):1872-1879.
26. Hunt JS, Jr., Theilmann RJ, Smith ZM, Scadeng M, Dubowitz DJ: **Cerebral diffusion and T(2): MRI predictors of acute mountain sickness during sustained high-altitude hypoxia.** *J Cereb Blood Flow Metab* 2013, **33**(3):372-380.
27. Romero IG, Ruvinsky I, Gilad Y: **Comparative studies of gene expression and the evolution of gene regulation.** *Nat Rev Genet* 2012, **13**(7):505-516.
28. Sberro H, Fremin BJ, Zlitni S, Edfors F, Greenfield N, Snyder MP, Pavlopoulos GA, Kyrpides NC, Bhatt AS: **Large-Scale Analyses of Human Microbiomes Reveal Thousands of Small, Novel Genes.** *Cell* 2019, **178**(5):1245-1259 e1214.
29. Zhang X, Sun Y, Wang P, Yang C, Li S: **Exploration of the molecular mechanism of prostate cancer based on mRNA and miRNA expression profiles.** *Onco Targets Ther* 2017, **10**:3225-3232.

30. Subudhi AW, Bourdillon N, Bucher J, Davis C, Elliott JE, Eutermoster M, Evero O, Fan JL, Jameson-Van Houten S, Julian CG *et al*: **AltitudeOmics: the integrative physiology of human acclimatization to hypobaric hypoxia and its retention upon reascent.** *PLoS One* 2014, **9**(3):e92191.
31. Vaden KI, Jr., Gebregziabher M, Dyslexia Data C, Eckert MA: **Fully synthetic neuroimaging data for replication and exploration.** *Neuroimage* 2020, **223**:117284.
32. Donders AR, van der Heijden GJ, Stijnen T, Moons KG: **Review: a gentle introduction to imputation of missing values.** *J Clin Epidemiol* 2006, **59**(10):1087-1091.
33. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK: **limma powers differential expression analyses for RNA-sequencing and microarray studies.** *Nucleic Acids Res* 2015, **43**(7):e47.
34. Xie F, Wang Q, Sun R, Zhang B: **Deep sequencing reveals important roles of microRNAs in response to drought and salinity stress in cotton.** *J Exp Bot* 2015, **66**(3):789-804.
35. Shen W, Song Z, Zhong X, Huang M, Shen D, Gao P, Qian X, Wang M, He X, Wang T *et al*: **Sangerbox: A comprehensive, interaction-friendly clinical bioinformatics analysis platform.** *iMeta*, n/a(n/a):e36.
36. Langfelder P, Horvath S: **WGCNA: an R package for weighted correlation network analysis.** *BMC Bioinformatics* 2008, **9**:559.
37. Tang J, Kong D, Cui Q, Wang K, Zhang D, Gong Y, Wu G: **Prognostic Genes of Breast Cancer Identified by Gene Co-expression Network Analysis.** *Front Oncol* 2018, **8**:374.
38. Yu G, Wang LG, Han Y, He QY: **clusterProfiler: an R package for comparing biological themes among gene clusters.** *OMICS* 2012, **16**(5):284-287.
39. Bhandari S, Cavalleri GL: **Population History and Altitude-Related Adaptation in the Sherpa.** *Front Physiol* 2019, **10**:1116.
40. Robinson JC, Graham BB, Rouault TC, Tudor RM: **The crossroads of iron with hypoxia and cellular metabolism. Implications in the pathobiology of pulmonary hypertension.** *Am J Respir Cell Mol Biol* 2014, **51**(6):721-729.
41. Wu TY, Ding SQ, Liu JL, Jia JH, Dai RC, Zhu DC, Liang BZ, Qi DT, Sun YF: **High-altitude gastrointestinal bleeding: an observation in Qinghai-Tibetan railroad construction workers on Mountain Tanggula.** *World J Gastroenterol* 2007, **13**(5):774-780.
42. Muckenthaler MU, Mairbaur H, Gassmann M: **Iron metabolism in high-altitude residents.** *J Appl Physiol (1985)* 2020, **129**(4):920-925.
43. Fisher J, Linder A: **Heparin-binding protein: a key player in the pathophysiology of organ dysfunction in sepsis.** *J Intern Med* 2017, **281**(6):562-574.
44. Johansson J, Brattstrom O, Sjoberg F, Lindbom L, Herwald H, Weitzberg E, Oldner A: **Heparin-binding protein (HBP): an early marker of respiratory failure after trauma?** *Acta Anaesthesiol Scand* 2013, **57**(5):580-586.
45. Lin Q, Shen J, Shen L, Zhang Z, Fu F: **Increased plasma levels of heparin-binding protein in patients with acute respiratory distress syndrome.** *Crit Care* 2013, **17**(4):R155.

46. Bentzer P, Fisher J, Kong HJ, Morgelin M, Boyd JH, Walley KR, Russell JA, Linder A: **Heparin-binding protein is important for vascular leak in sepsis.** *Intensive Care Med Exp* 2016, **4**(1):33.
47. Song S, Yao N, Yang M, Liu X, Dong K, Zhao Q, Pu Y, He X, Guan W, Yang N *et al*: **Exome sequencing reveals genetic differentiation due to high-altitude adaptation in the Tibetan cashmere goat (*Capra hircus*).** *BMC Genomics* 2016, **17**:122.
48. Huertas A, Das SR, Emin M, Sun L, Rifkind JM, Bhattacharya J, Bhattacharya S: **Erythrocytes induce proinflammatory endothelial activation in hypoxia.** *Am J Respir Cell Mol Biol* 2013, **48**(1):78-86.
49. Le QT, Fisher R, Oliner KS, Young RJ, Cao H, Kong C, Graves E, Hicks RJ, McArthur GA, Peters L *et al*: **Prognostic and predictive significance of plasma HGF and IL-8 in a phase III trial of chemoradiation with or without tirapazamine in locoregionally advanced head and neck cancer.** *Clin Cancer Res* 2012, **18**(6):1798-1807.
50. Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, Longo DL, Taub DD, Oppenheim JJ: **Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils.** *J Biol Chem* 1996, **271**(6):2935-2940.
51. Kuhn M: **Endothelial actions of atrial and B-type natriuretic peptides.** *Br J Pharmacol* 2012, **166**(2):522-531.
52. Sailer A, Scholz SW, Gibbs JR, Tucci A, Johnson JO, Wood NW, Plagnol V, Hummerich H, Ding J, Hernandez D *et al*: **Exome sequencing in an SCA14 family demonstrates its utility in diagnosing heterogeneous diseases.** *Neurology* 2012, **79**(2):127-131.
53. Zhang Y, Xu J, Zhu X: **A 63 signature genes prediction system is effective for glioblastoma prognosis.** *Int J Mol Med* 2018, **41**(4):2070-2078.
54. Gorman D, Lin HY, Williams C: **Early evidence of a regulated response to hypoxaemia in sheep that preserves the brain cortex.** *Neurosci Lett* 2006, **394**(3):174-178.
55. Barnett ME, Madgwick DK, Takemoto DJ: **Protein kinase C as a stress sensor.** *Cell Signal* 2007, **19**(9):1820-1829.
56. Fleegal MA, Hom S, Borg LK, Davis TP: **Activation of PKC modulates blood-brain barrier endothelial cell permeability changes induced by hypoxia and posthypoxic reoxygenation.** *Am J Physiol Heart Circ Physiol* 2005, **289**(5):H2012-2019.
57. Surendran D: **PKCgamma and PKCepsilon are Differentially Activated and Modulate Neurotoxic Signaling Pathways During Oxygen Glucose Deprivation in Rat Cortical Slices.** *Neurochem Res* 2019, **44**(11):2577-2589.
58. Zhang N, Yin Y, Han S, Jiang J, Yang W, Bu X, Li J: **Hypoxic preconditioning induced neuroprotection against cerebral ischemic injuries and its cPKCgamma-mediated molecular mechanism.** *Neurochem Int* 2011, **58**(6):684-692.
59. Menard C, Bastianetto S, Quirion R: **Neuroprotective effects of resveratrol and epigallocatechin gallate polyphenols are mediated by the activation of protein kinase C gamma.** *Front Cell Neurosci* 2013, **7**:281.

60. Gonzalez-Perez RR, Xu Y, Guo S, Watters A, Zhou W, Leibovich SJ: **Leptin upregulates VEGF in breast cancer via canonic and non-canonical signalling pathways and NFkappaB/HIF-1alpha activation.** *Cell Signal* 2010, **22**(9):1350-1362.
61. Yuan G, Nanduri J, Khan S, Semenza GL, Prabhakar NR: **Induction of HIF-1alpha expression by intermittent hypoxia: involvement of NADPH oxidase, Ca²⁺ signaling, prolyl hydroxylases, and mTOR.** *J Cell Physiol* 2008, **217**(3):674-685.
62. Uenoyama M, Ogata S, Nakanishi K, Kanazawa F, Hiroi S, Tominaga S, Seo A, Matsui T, Kawai T, Suzuki S: **Protein kinase C mRNA and protein expressions in hypobaric hypoxia-induced cardiac hypertrophy in rats.** *Acta Physiol (Oxf)* 2010, **198**(4):431-440.
63. Hackett P, Rennie D: **High-altitude pulmonary edema.** *JAMA* 2002, **287**(17):2275-2278.
64. Swenson ER, Maggiorini M, Mongovin S, Gibbs JS, Greve I, Mairbaur H, Bartsch P: **Pathogenesis of high-altitude pulmonary edema: inflammation is not an etiologic factor.** *JAMA* 2002, **287**(17):2228-2235.
65. Gautam N, Olofsson AM, Herwald H, Iversen LF, Lundgren-Akerlund E, Hedqvist P, Arfors KE, Flodgaard H, Lindbom L: **Heparin-binding protein (HBP/CAP37): a missing link in neutrophil-evoked alteration of vascular permeability.** *Nat Med* 2001, **7**(10):1123-1127.

Figures

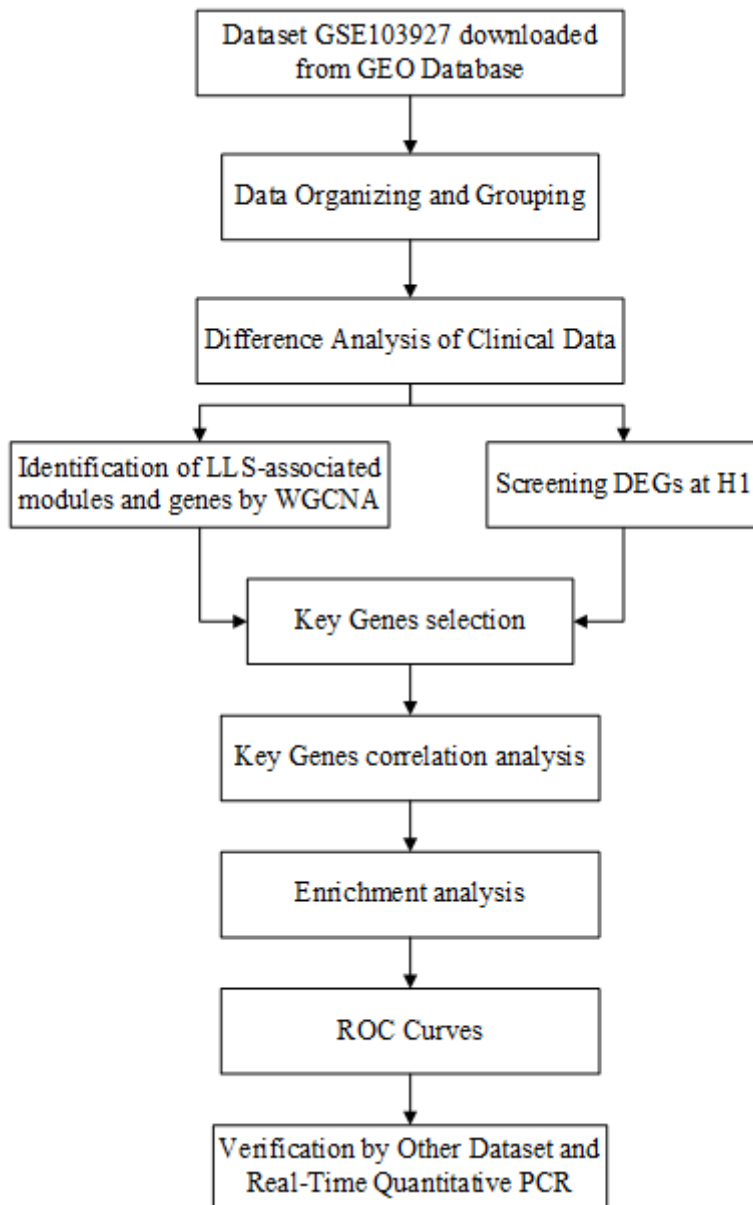


Figure 1

Flow chart of the study design.

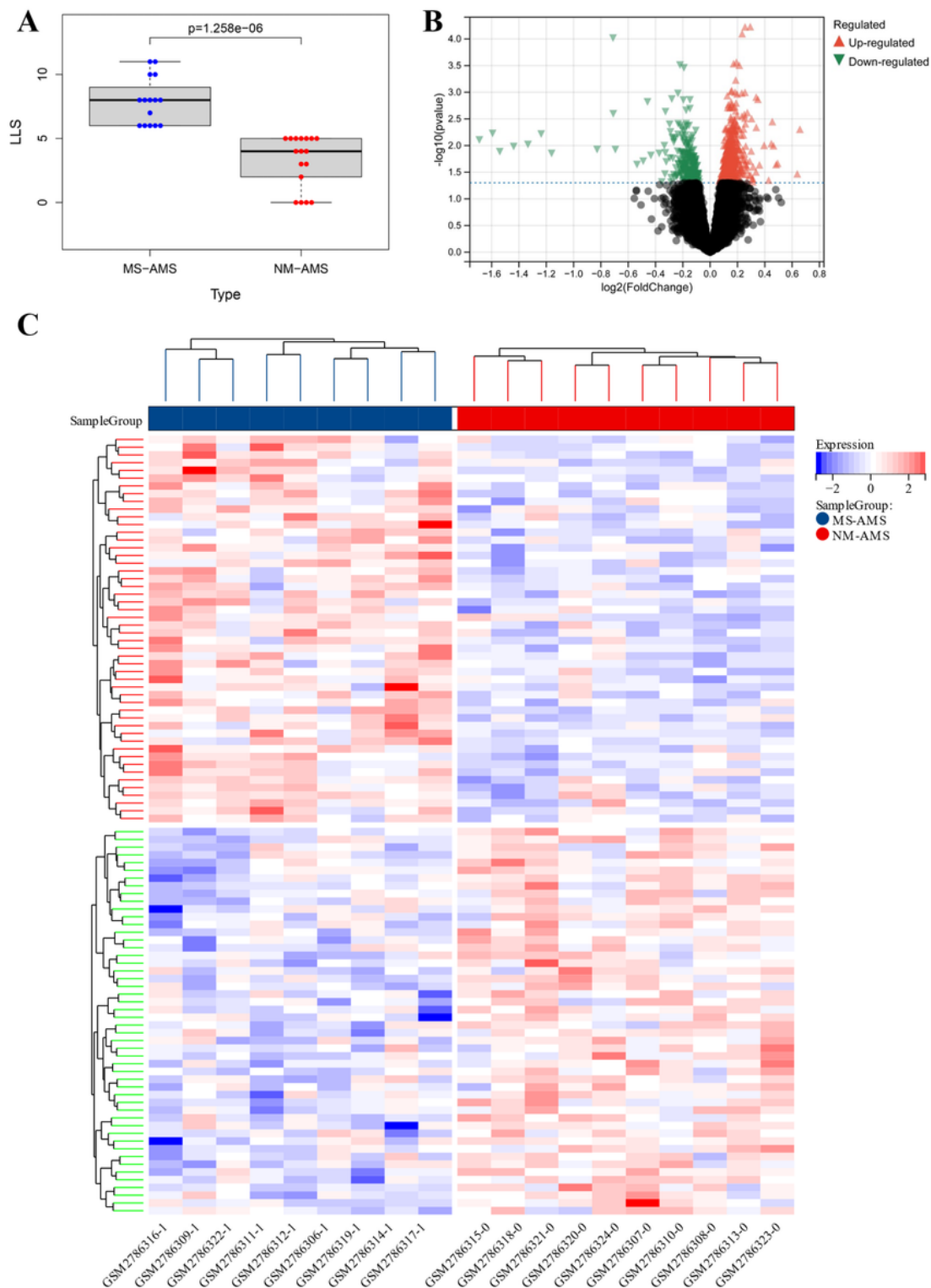


Figure 2

The LLS and DEGs between the two groups. (A) Differences in LLS between the MS-AMS group and the NM-AMS group. (B)(C) The volcano plot and heatmap show DEGs in the MS-AMS group vs. the NM-AMS group. In total, 368 DEGs between the NM-AMS group and the MS-AMS group were identified, of which 252 were up-regulated, and 116 were down-regulated. LLS, lake louis score; DEGs, differentially expressed genes.

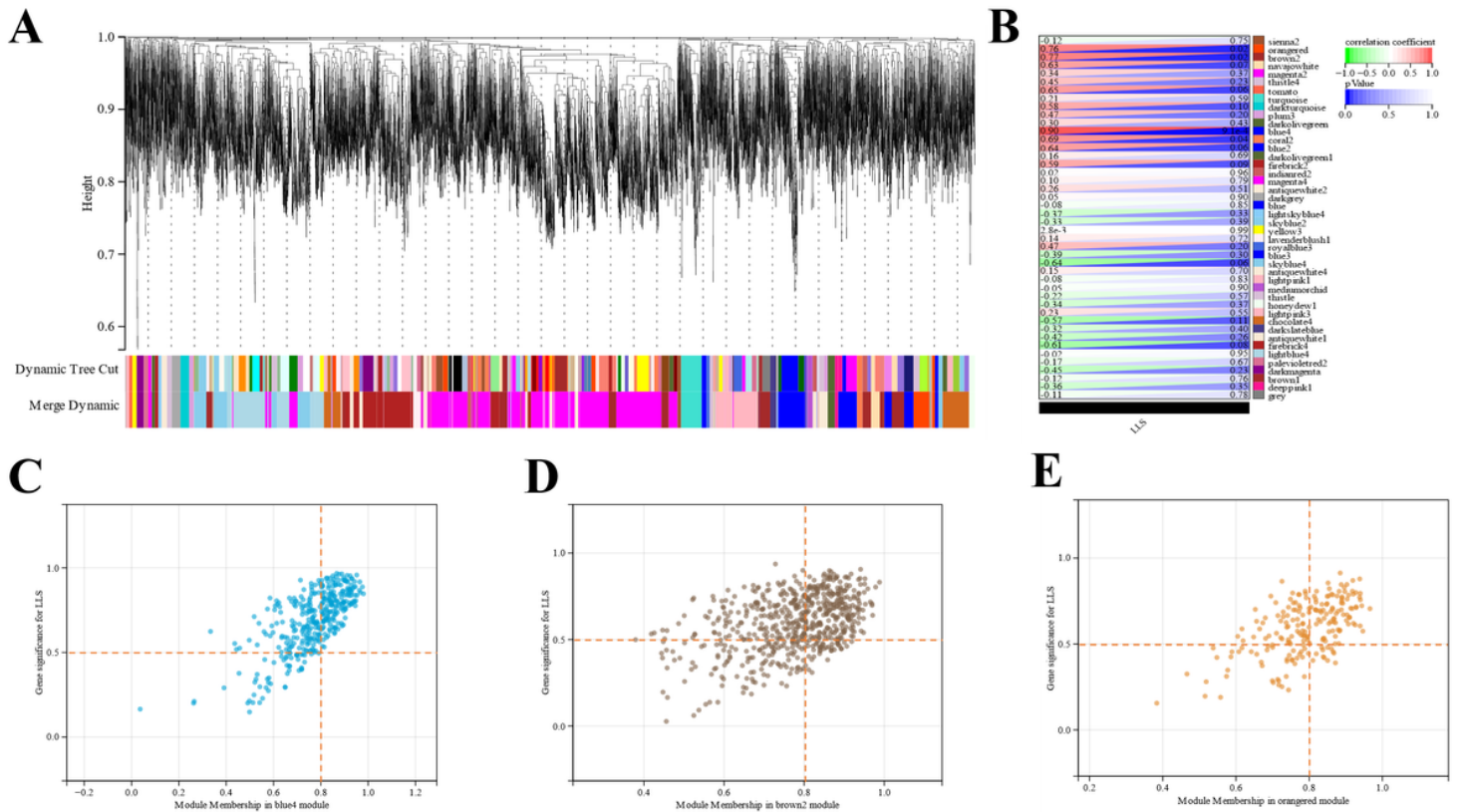


Figure 3

The Most Significant LLS-Related Modules and Genes. (A) Co-expression module identification in MS-AMS group. The branches of the cluster dendrogram represent the 44 different gene modules. Each module denoted a collection of co-related genes and was given a unique color. Each piece of the leaves on the cluster dendrogram represents a gene. (B) A heatmap shows the correlations and significant differences between the gene modules and LLS. The upper left corner of each cell displays R^2 , between 0 and 1. The P -value is displayed in the lower right corner of each cell. Significantly associated modules are blue4 (C), brown2 (D), and orangered (E), and the scatter plot of module characteristic genes is shown in Figure. Each circle represents a gene, and the genes in the upper right corner represent key genes for that module. LLS, lake louis score.

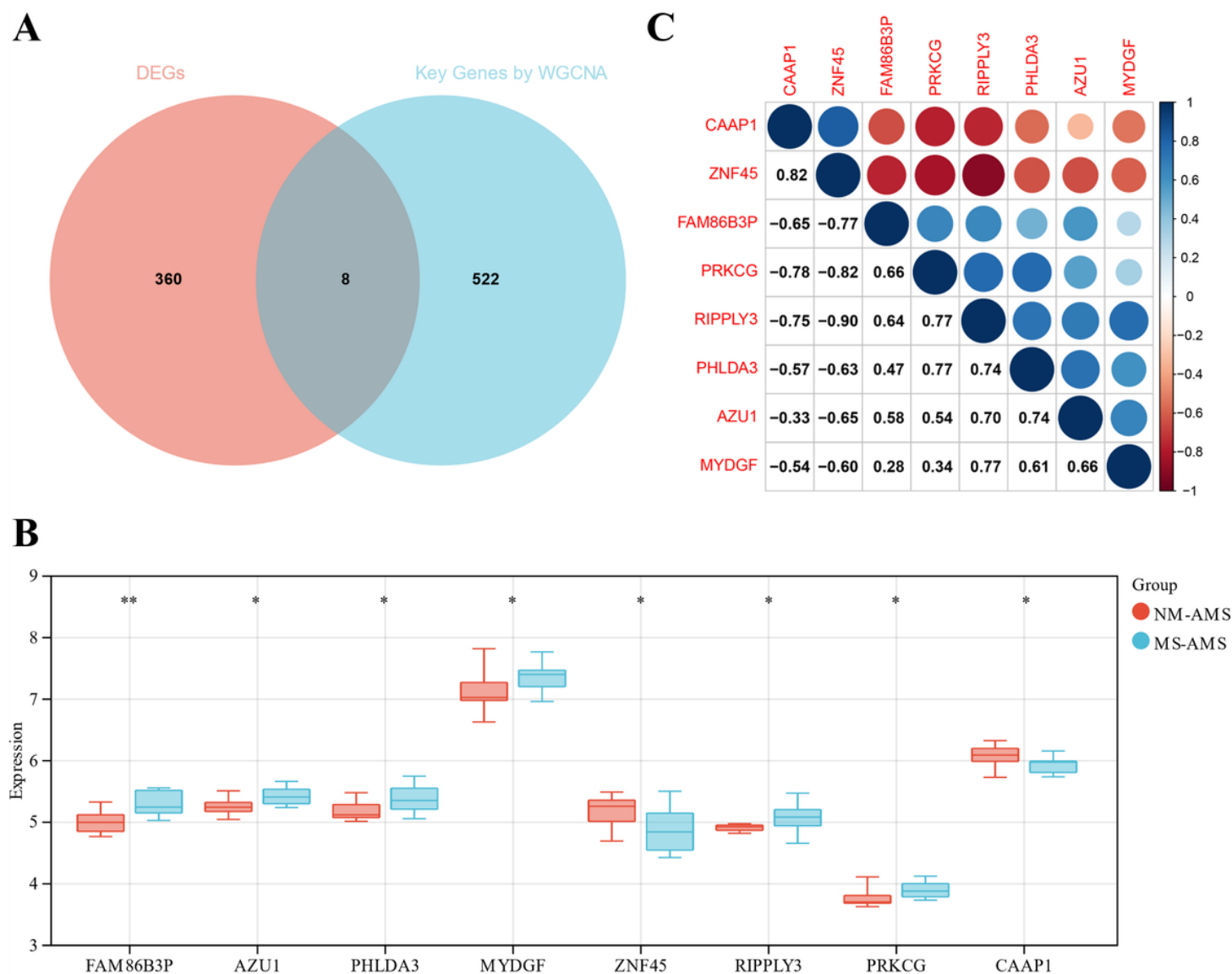


Figure 4

Candidate Genes Selection, Correlation analysis, and Differential Expression analysis. (A) A Venn diagram showing the number of DEGs versus the number of Key Genes obtained by WGCNA repeats. (B) Differential expression of candidate genes among different groups. (C) Candidate gene correlation. The lower left part shows the correlation coefficient, and the size of the circle in the upper right part is consistent with the size of the absolute value of the correlation coefficient; the more significant the absolute value of the correlation coefficient, the larger the graph is, and the darker the color. Red represents negative correlation, and blue represents positive correlation, as shown in the legend on the right. DEGs, differentially expressed genes; WGCNA, weighted gene co-expression network analysis. *, $P < 0.05$; **, $P < 0.01$.

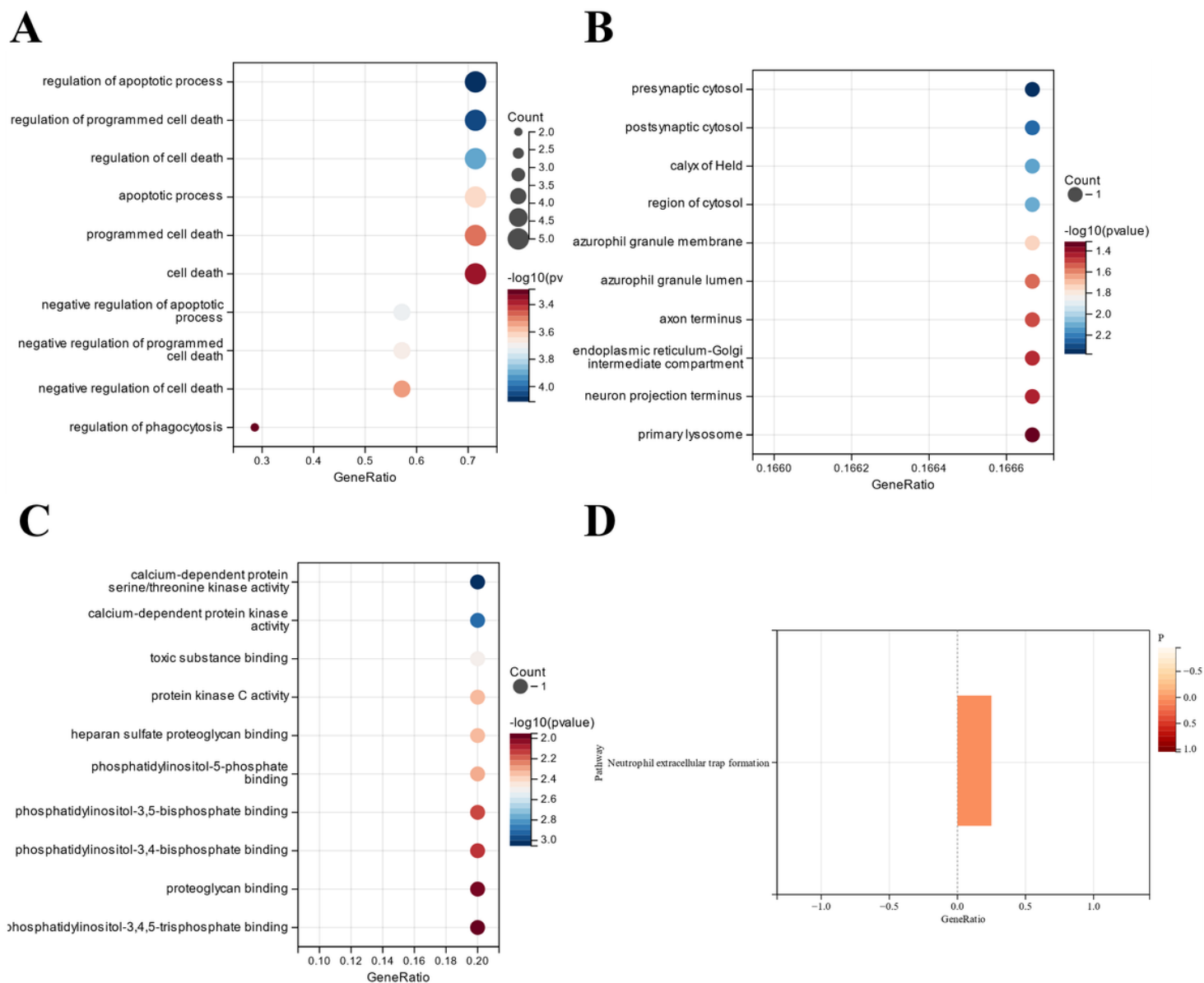


Figure 5

Functional Annotation and Pathway Enrichment Analysis of Candidate Genes. (A, B, C) The GO enrichment analysis of the candidate genes. Bubble chart showing the top terms in BP, CC, and MF groups. (D) The term for KEGG pathway analysis of candidate genes is shown with bar graphs. GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; BP, biological process; CC, cell component; MF, molecular function.

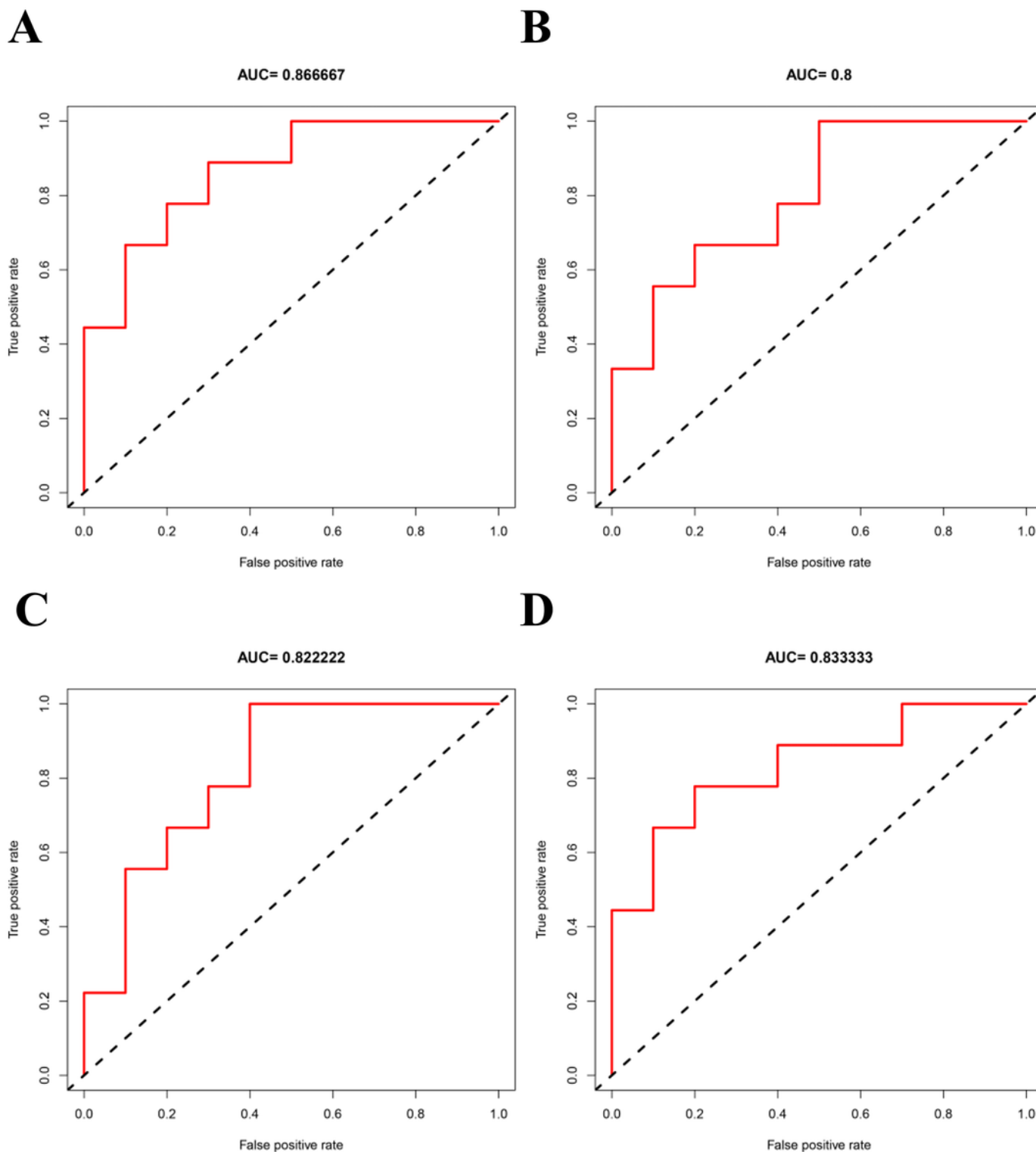
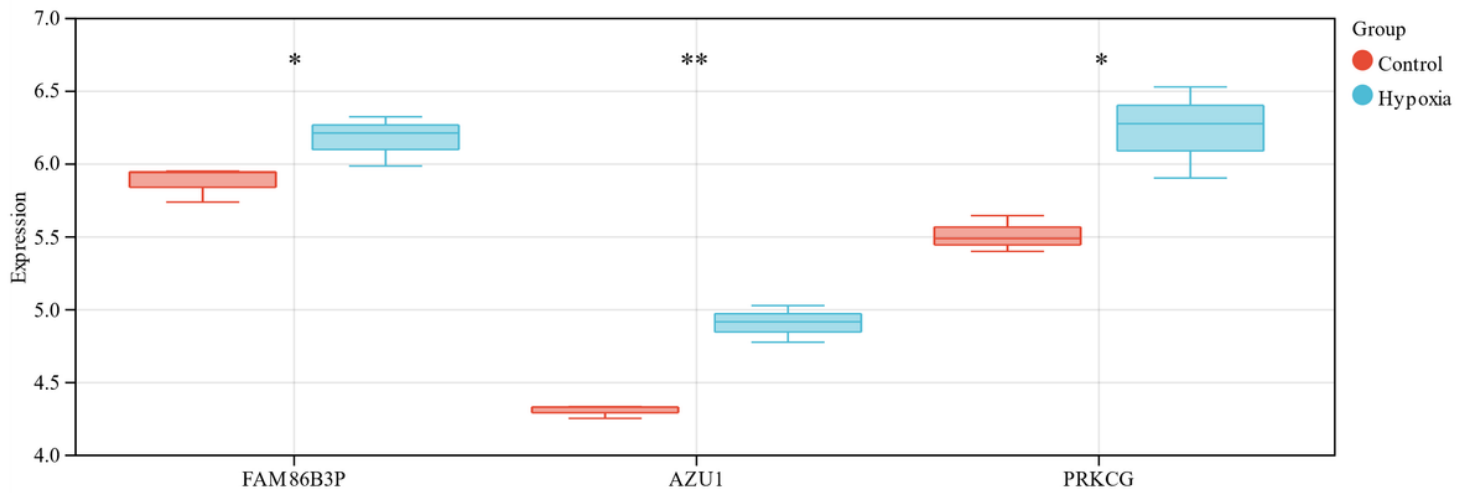
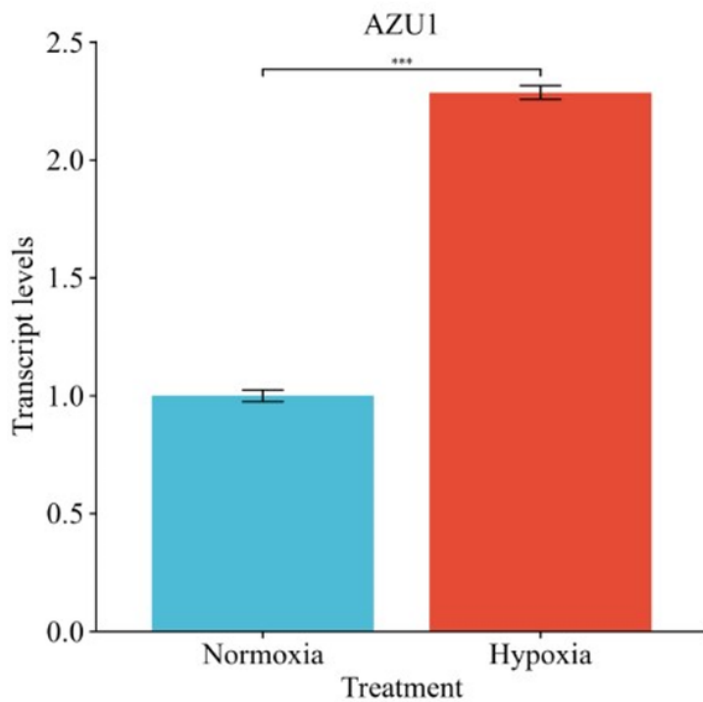
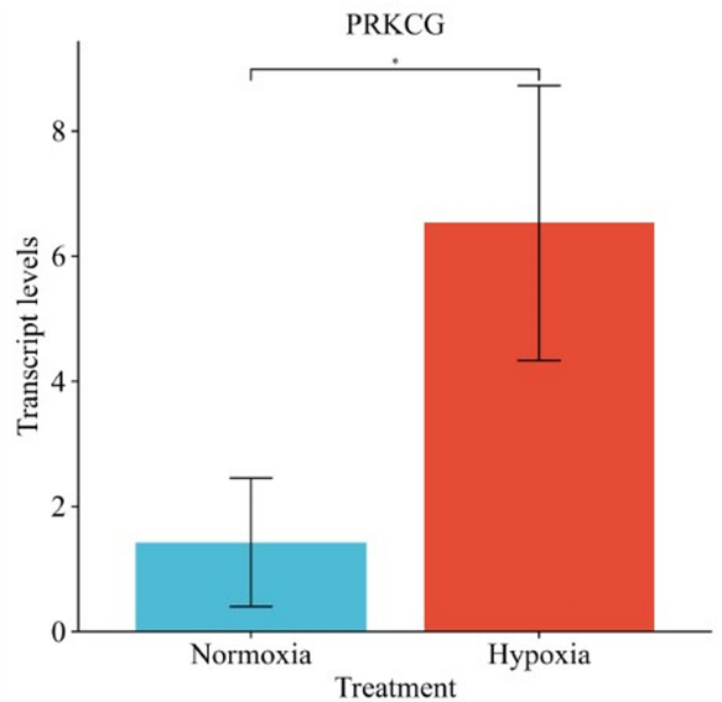


Figure 6

ROC Curves of Candidate Genes (A) ROC curve of FAM86B3P to predict MS-AMS. (B) ROC curve of AZU1 to predict MS-AMS. (C) ROC curve of PRKCG to predict MS-AMS. (D) ROC curve of AZU1 together with PRKCG. ROC curve, receiver operating characteristic curve.

A**GSE145935****B****C****Figure 7**

Transcript levels of AZU1 and PRKCG in GSE145935 and hypobaric hypoxia environment. (A) Transcript levels of candidate genes in GSE145935. (B) Transcript levels of AZU1 under hypobaric hypoxia treatment. (C) Transcript levels of PRKCG under hypobaric hypoxia treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Files

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