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动脉粥样硬化中差异表达铁死亡相关基因的鉴定：生物信息学分析

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摘　要

目的：铁死亡在动脉粥样硬化的发生和发展中起着至关重要的作用。本研究旨在已有研究的基础上，通过生物信息学分析进一步筛选鉴定与动脉粥样硬化中铁死亡相关的差异表达基因。**材料方法：**首先，mRNA表达谱数据集GSE100927从基因表达综合数据库中筛选，并使用R软件(4.0.0版)分析与动脉粥样硬化和铁死亡相关的潜在差异表达基因。随后，对选定的候选基因进行蛋白质 – 蛋白质相互作用分析、基因本体富集分析和京都基因与基因组百科全书通路富集分析等研究，最终构建受试者工作曲线，来预测其功能。**结果：**我们通过差异性分析确定了23个可能的与动脉粥样硬化铁死亡相关的潜在靶点。进一步通过基因本体富集分析发现这些候选基因的功能可能主要与氧化应激有关。蛋白质 – 蛋白质相互作用网络展示出绝大多数候选靶点存在密切的相互作用。最终我们再次从23个候选的潜在靶点中筛选了2个基因作为关键基因，即HMOX1和NOX4。**结论：**生物信息学分析鉴定了23个潜在的动脉粥样硬化铁死亡相关差异表达基因，而HMOX1和NOX4这2个基因可能作为关键基因在动脉粥样硬化过程中发挥更关键的作用。本研究结果可能有助于进一步了解动脉粥样硬化的发病机制，并为进一步指导临床治疗提供重要依据。

关键词

动脉粥样硬化，铁死亡，NOX4，生物信息学分析，氧化应激

Identification of Differentially Expressed Ferroptosis-Related Genes in Atherosclerosis: Bioinformatics Analysis

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Abstract

Purpose: Ferroptosis plays a crucial role in the development and progression of atherosclerosis. The aim of this study was to identify differentially expressed genes associated with ferroptosis in atherosclerosis through bioinformatics analysis. Materials and Methods: First, the mRNA expression profile dataset GSE100927 was screened from the comprehensive gene expression database and analyzed using R software (version 4.0.0) for potential differentially expressed genes associated with atherosclerosis and iron death. Subsequently, protein-protein interaction analysis, gene ontology enrichment analysis and Kyoto Gene and Genome Encyclopedia pathway enrichment analysis were performed on the selected candidate genes, and finally the subject working curve was constructed to predict their functions. Results: We identified 23 possible potential targets associated with ferroptosis through differential analysis. The function of these candidate genes may be mainly related to oxidative stress through gene ontology enrichment analysis. Protein-protein interaction networks show close interactions at most candidate targets. Finally, we selected two key genes, namely HMOX1 and NOX4, from the 23 candidate potential targets. Conclusion: Bioinformatics analysis identified 23 potential differentially expressed genes associated with ferroptosis in atherosclerosis, two genes, HMOX1 and NOX4, may play a more critical role as key genes in the process of atherosclerosis. The results of this study may help to further understand the pathogenesis of atherosclerosis and provide an important basis for further clinical treatment.

Keywords

Atherosclerosis, Ferroptosis, NOX4, Bioinformatics Analysis, Oxidative Stress

1. 介绍

动脉粥样硬化是一种慢性炎症状态，其过程是动脉粥样硬化斑块在动脉壁内膜积聚，进一步发展为动脉粥样硬化破裂、慢性管腔狭窄和血栓形成，最终危及生命[1]。心血管疾病是全球死亡的主要原因，主要由动脉粥样硬化引起[2]。目前，动脉粥样硬化发生趋向于年轻化，并且开始被认为其与全球的心血管相关死亡有关[3]。迄今为止，动脉粥样硬化没有特效的治疗方法[4]，亟待寻找其治疗靶点。多项研究指出，包括铁死亡、自噬和炎症在内的各种生物学过程都参与了动脉粥样硬化的发生和发展。例如在动脉粥样硬化进展过程中，伴侣介导的自噬功能受损，增加 NLRP3 炎症小体活化和 IL-1β 分泌，促进血管炎症和动脉粥样硬化进展[5]。BACH1 的缺失通过减少内皮炎症来减轻动脉粥样硬化[6]。红系 Jak2V617F 表达通过参与巨噬细胞铁死亡促进动脉粥样硬化[7]。铁死亡在动脉粥样硬化的发病机制中起到关键作用[8]-[14]。

铁死亡是指由不受控制的脂质过氧化引起的细胞死亡，与多种心血管疾病密切相关[15] [16]。例如，

激活转录因子 3 (ATF3)可能通过铁死亡参与动脉粥样硬化斑块的形成[17]。此外，BRD4770 通过抑制铁死亡来预防主动脉夹层[18]。因此鉴定参与动脉粥样硬化的铁死亡相关基因可能为该病的进一步研究提供有意义的生物标志物和靶点。目前已有一些相关研究取得了成果[13] [19] [20] [21]。李等人发表的关于动脉粥样硬化中巨噬细胞相关的铁死亡的研究引起了我们的关注[13]。由于铁死亡对于动脉粥样硬化的影响不仅仅限于巨噬细胞，也发生在血管平滑肌细胞和血管内皮细胞等与动脉粥样硬化发生发展密切相关的细胞中[17]。慢性铁死亡通过 ROS 和环氧合酶通路导致内皮功能障碍，而内皮功能障碍是动脉粥样硬化的初始阶段[17] [22]。香烟烟雾提取物可以显著诱导血管平滑肌细胞的铁死亡并进而参与动脉粥样硬化的发生发展，但不诱导细胞凋亡或坏死性凋亡[23]。因此我们希望在李等人研究成果的基础上进一步分析动脉粥样硬化中铁死亡相关的靶向分子。

公开的基因表达数据集 GSE100927 和 GSE43292 可在基因表达综合数据库(GEO)获取。我们分析发现了 7 个可能的与动脉粥样硬化铁死亡相关的潜在生物学靶点。并通过对候选基因进行基因本体(GO)富集分析和蛋白质－蛋白质相互作用(PPI)对我们筛选的结果进一步研究。

2. 材料方法

2.1. 铁死亡相关基因数据集和微阵列数据

GSE100927 数据集在 GPL17077 平台(Agilent-039494 SurePrint G3 人 GE v2 8x60K 微阵列芯片 039381)主要包含动脉粥样硬化病变和无动脉粥样硬化病变(来自己故器官供体)的临床样本。对照动脉是从颈动脉、股动脉和腘下动脉获得的。GSE43292 来自于平台 GPL6244。以上数据集均是基因表达综合数据库(GEO)的公开数据库。GSE100927 和 GSE43292 分别作为训练集和外部验证集。开放的人类铁死亡数据库中含有 259 个基因 http://www.ncbi.nlm.nih.gov/geo/。

2.2. 差异表达铁死亡相关基因的分析

为了获得微阵列数据的标准化表达矩阵，我们从数据集中下载信息，并根据注释文件注释探针。使用 R 软件(版本 4.0.0)中的"Limma"工具包对数据进行标准化。|log2(fold change)| > 0.5 和 P 值 < 0.05 作为差异基因表达的标准。热图和火山图是使用 R 软件(版本 2.4.0)中的"热图"和"ggplot0"包绘制的。

2.3. 重点基因的筛选

我们使用最小绝对收缩和选择算子(LASSO)逻辑回归进行关键基因筛选。数据集 GSE100927 作为独立矩阵数据进行分析。使用"glmnet"包对 LASSO 算法进行了分析。LASSO Cox 回归选择了模型中的铁死亡特征。LASSO Cox 回归模型基于每位患者 RPI 评分中的最佳 lambda 值。我们通过使用 LASSO Cox 回归模型按最低标准找到了 7 个关键基因。P < 0.05 被认为具有统计学意义。

2.4. 差异表达铁死亡相关基因的蛋白质－蛋白质相互作用及相关性分析

Cytoscape 软件(版本 3.8.1)和用于检索相互作用基因(STRING)数据库的搜索工具：https://string-db.org/ 用于对差异表达的铁死亡相关基因进行 PPI 分析。首先，利用 STRING 数据库构建包含差异表达的铁死亡相关基因的 PPI 网络；然后将 PPI 文件导入 Cytoscape (版本 3.8.1)并映射到 PPI 上。

2.5. 基因本体论和京都百科全书的基因和基因组通路富集铁死亡相关基因的分析

使用 R 软件中的 cluster Profiler 工具包进行基因本体和 KEGG 通路富集分析。GO 分析的主要领域是生物过程(BP)和分子功能。

2.6. 统计分析

使用 R 软件(4.0.0 版)对生物信息学数据进行统计分析。学生 t 检验用于评估临床样本中的基因表达水平。P 值 < 0.05 表示差异具有统计学意义。

3. 结果

3.1. 动脉粥样硬化中铁死亡相关基因差异表达的回顾性分析

我们以 P 值 < 0.05 and |log2| > 0.5 为标准从 259 个基因的铁死亡相关数据库中筛选出 23 个可能为动脉粥样硬化铁死亡相关的候选基因。其中 14 个上调的候选基因为溶质载体家族 40 成员 1 (SLC40A1)、丝裂原和应激激活激酶信号的关键调节因子(MAP3K11)、白细胞介素 1β (IL1B)、细胞周期蛋白依赖性激酶抑制剂 2A (CDKN2A)、突触核蛋白(SNCA)、血红素加氧酶 1 (HMOX1)、细胞色素 B-245β 链(CYBB)、异柠檬酸脱氢酶 1 (IDH1)、铁蛋白轻链(FTL)、BH3 相互作用结构域死亡激动剂(BID)、中性粒细胞胞质因子 2 (NCF2)、巨噬细胞加帽蛋白(CAPG)、肌因子组织蛋白酶 B (CTSB)和巨噬细胞中 5-脂氧合酶(ALOX5)。而 9 个下调的候选基因为核受体亚家族 1 组 d 成员 1 (NR1D1)、NADPH 氧化酶 4 (NOX4)、雄激素受体(AR)、脂肪细胞增强结合蛋白 2 (AEBP2)、极低密度脂蛋白受体(VLDLR)、周脂蛋白 4 (PLIN4)、烟酰胺 N-甲基转移酶(NNMT)、缺氧诱导脂滴相关蛋白(HILPDA)和 DNA 损伤诱导的转录本 4 (DDIT4)。GSE100927 数据库中动脉粥样硬化组和正常组之间差异表达的 23 个铁死亡相关基因分别展示在热图(图 1(a))和火山图中(图 1(b))。

(a)　　　　　　　　(b)
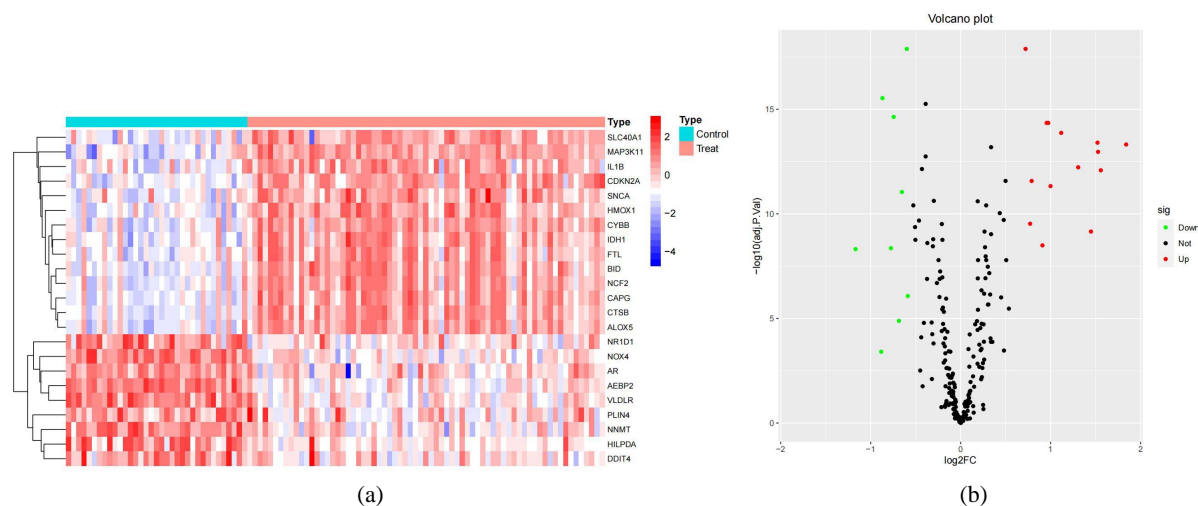
注：显著上调和下调的基因分别用红点和蓝点表示。用于识别差异的标准：P < 0.05 和|logFC| > 0.5。Control：健康样本 Treat：动脉粥样硬化样本。

Figure 1. (a) Heat maps of 23 differentially expressed genes associated with ferroptosis in atherosclerotic and healthy samples; (b) Volcanic map of differentially expressed genes related to ferroptosis

图 1. (a) 动脉粥样硬化样本和健康样本中 23 个差异表达铁死亡相关基因的热图；(b) 差异表达铁死亡相关基因的火山图

3.2. 候选铁死亡相关基因的基因本体富集分析

基因本体富集分析的结果显示候选基因的功能主要集中于前体产生的能量代谢和活性氧的代谢过程(图 2)。而在细胞构成方面则主要参与 NADPH 氧化酶复合物的构成(图 2)。在分子功能方面，候选基因则主要与血红素结合和 NADPH 相关的氧化过程有关(图 2)。另一方面，我们同时也进行了京都基因与基因组百科全书(KEGG)基因富集分析，但是遗憾的是，没有有价值的结果。
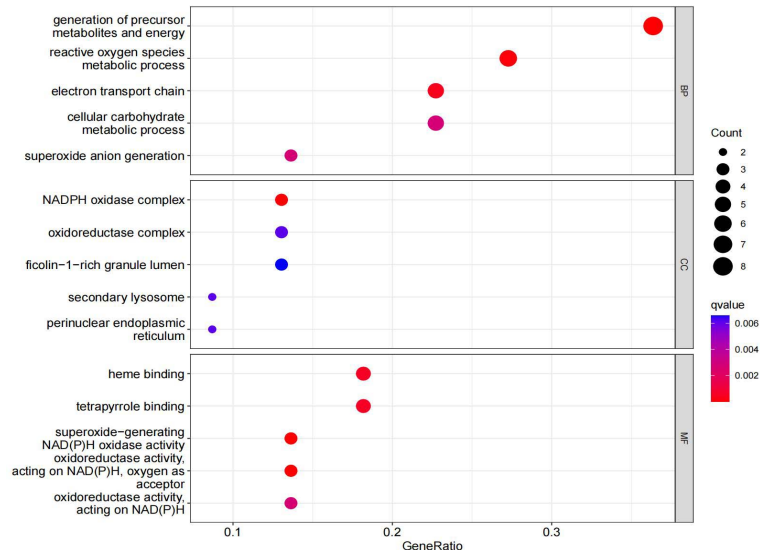
Figure 2. Gene ontology (GO) enrichment analysis bubble map of 23 differentially expressed genes related to ferroptosis. Count: Number of enriched genes
图 2. 23 个差异表达铁死亡相关基因的基因本体(GO)富集分析气泡图。Count：富集基因数量

3.3. 筛选重点基因

我们通过构建最小绝对收缩和选择算子(LASSO) Cox 回归模型从 23 个候选基因中筛选出 7 个更重要的基因并将其称为重点基因：CDKN2A、DDIT4、HMOX1、MAP3K11、NNMT、NOX4 和 NR1D1 (图 3(a)和图 3(b))。并收集了 7 个关键基因在数据集中的差异表达的箱式图，其差异表达情况均有统计学意义(P < 0.05) (图 4)。
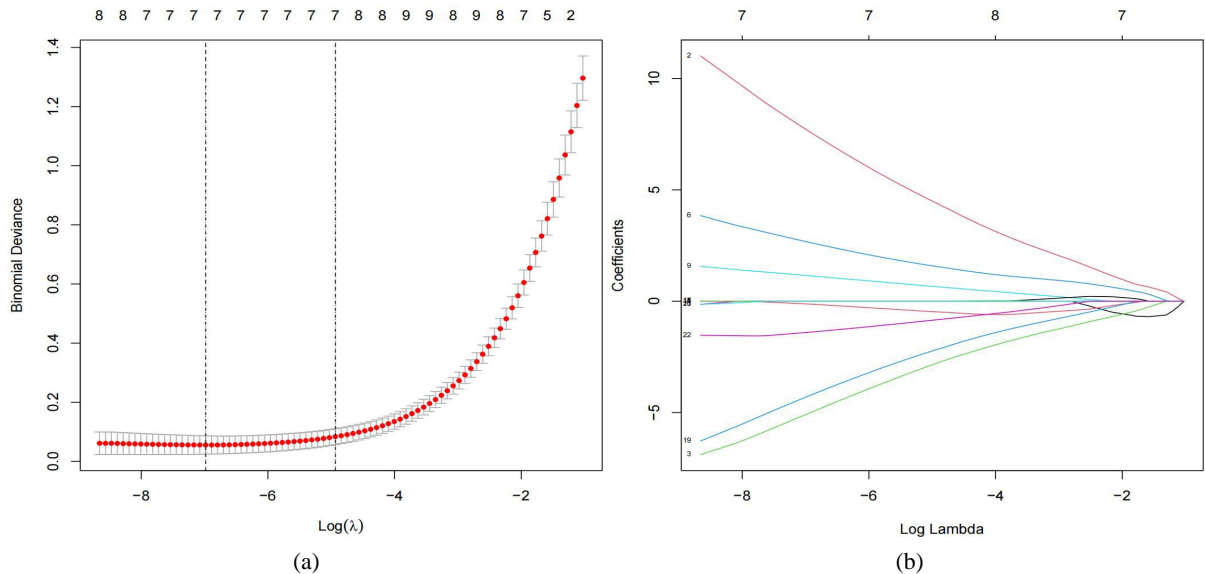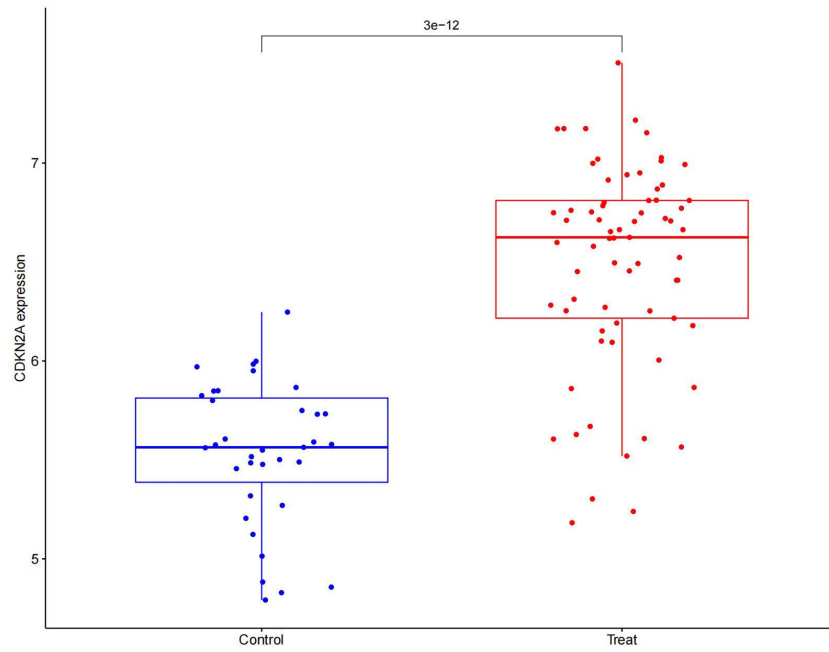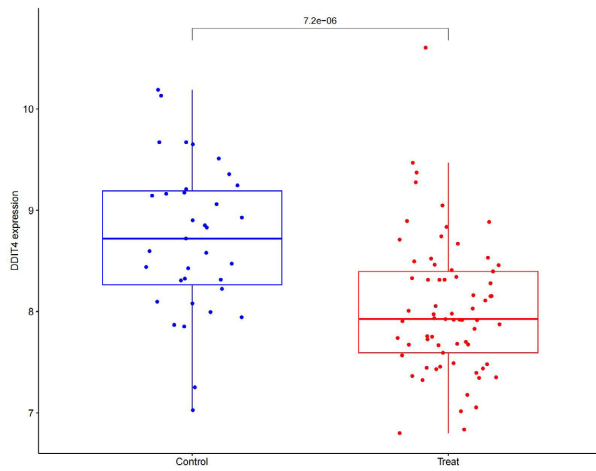

(a)　(b)

Figure 3. (a) Select the lowest standard tuning parameter (λ) in the LASSO model. Display vertical dashed lines with optimal values using minimum criteria; (b) Seven important genes related to ferroptosis were selected by LASSO-Cox regression analysis
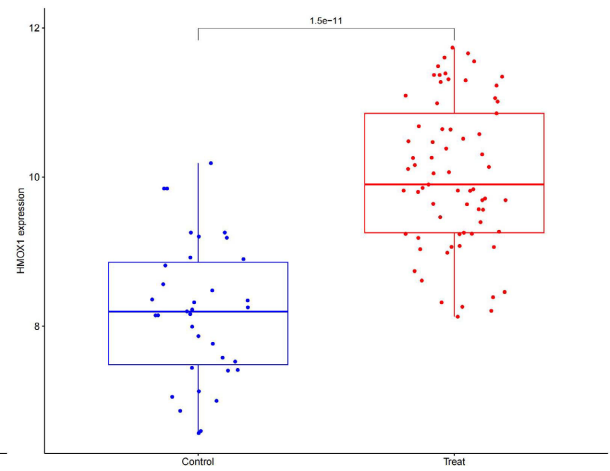**图 3.** (a) 在 LASSO 模型中选择最低标准的调谐参数(λ)。使用最小标准以最佳值显示垂直虚线；(b) 采用 LASSO-Cox 回归分析筛选出 7 个铁死亡相关重要基因
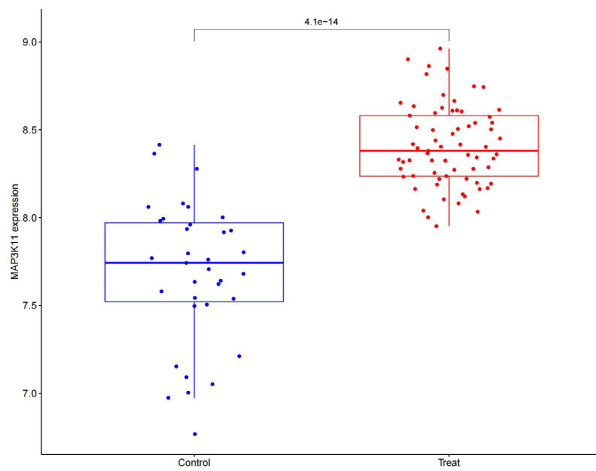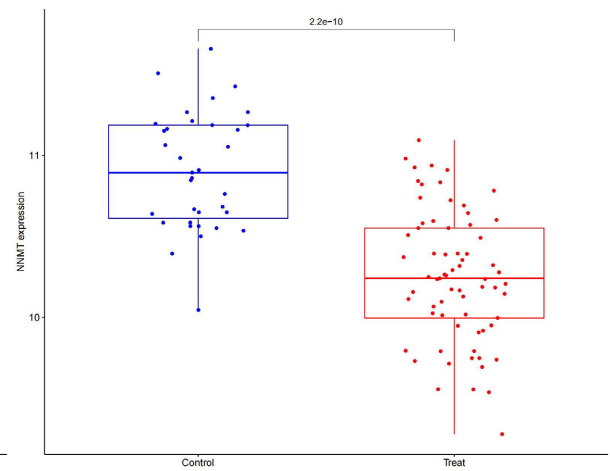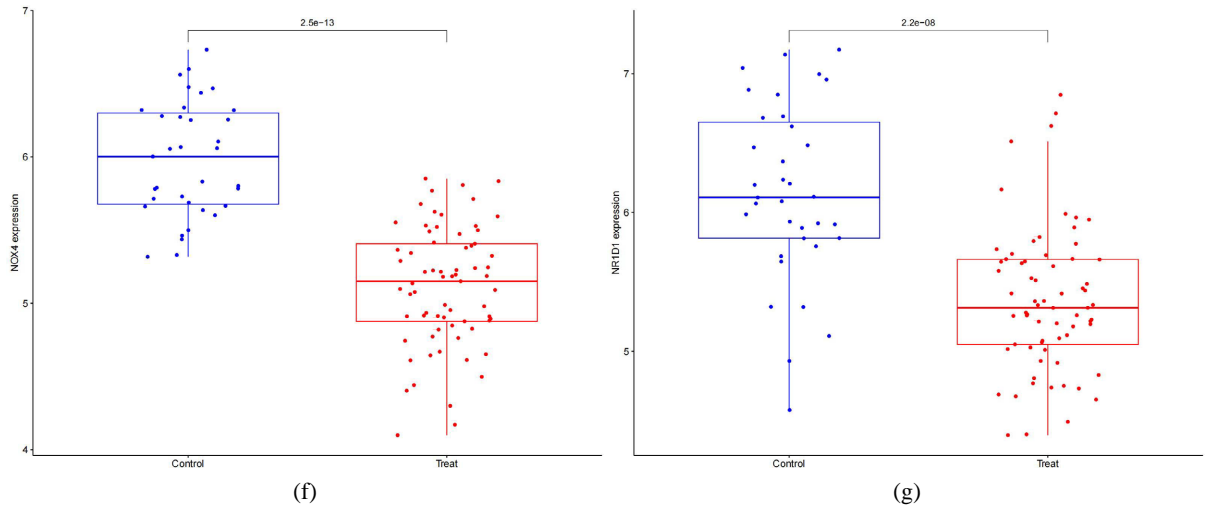
(a)

(b)

(c)

(d)

(e)

Figure 4. Box plots of seven differentially expressed important genes associated with ferroptosis in atherosclerotic and healthy samples. "Expression" on the *Y*-axis represents the gene expression. The blue and red boxes represent Control (healthy sample) and Treat (atherosclerosis sample), respectively

图 4. 动脉粥样硬化样本和健康样本中 7 个差异表达铁死亡相关的重要基因的箱线图。*Y* 轴上的"expression"表示相对基因表达。蓝色和红色框分别代表 Control (健康样本)和 Treat (动脉粥样硬化样本)

3.4. 蛋白质–蛋白质相互作用网络分析

蛋白质–蛋白质相互作用网络(Protein-Protein Interaction, PPI)分析显示大部分候选基因中存在着密切的相互作用。在已经进一步筛选的关键基因中 NOX4、HMOX1 和 CDKN2A 同时也处在蛋白质–蛋白质相互作用网络的枢纽地位(图 5)。
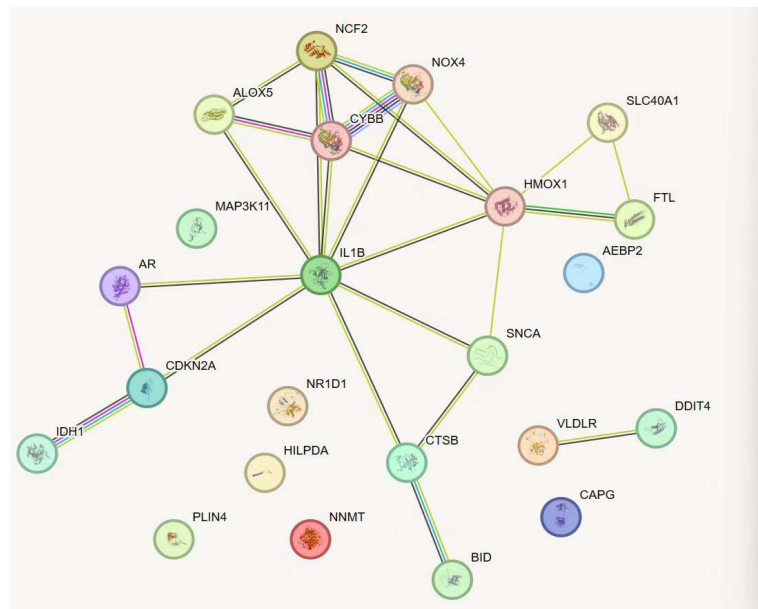
Figure 5. PPI analysis of candidate differentially expressed genes associated with ferroptosis. PPI networks of all 23 candidate genes associated with ferroptosis in atherosclerosis

图 5. 候选差异表达铁死亡相关基因的 PPI 分析。所有共 23 个动脉粥样硬化中铁死亡相关候选基因的 PPI 网络

3.5. 通过受试者工作特征曲线检测重要基因的功能并使用外部验证集进行验证

为了检测每个重要基因的功能，我们对所有重要基因都构建了逻辑回归模型并绘制了受试者工作特征曲线。除去 DDIT4 的曲线下面积(Area Under Curve, AUC)为 0.77 外，其他关键基因得分均较高，尤其是 CDKN2A、HMOX1、MAP3K11 和 NOX4 的得分均为 0.9 以上(图 6)。但通过使用外部检验集通过受试者工作曲线进行验证后发现候选基因中只有 NOX4 与 HMOX1 的得分为 0.8 以上(图 7)。证明与 NOX4 和 HMOX1 相关的逻辑回归模型对于动脉粥样硬化的发生发展具有较准确的预测能力。最终我们预测 NOX4 和 HMOX1 可能为动脉粥样硬化中与铁死亡相关的关键基因。

(a)

(b)

(c)
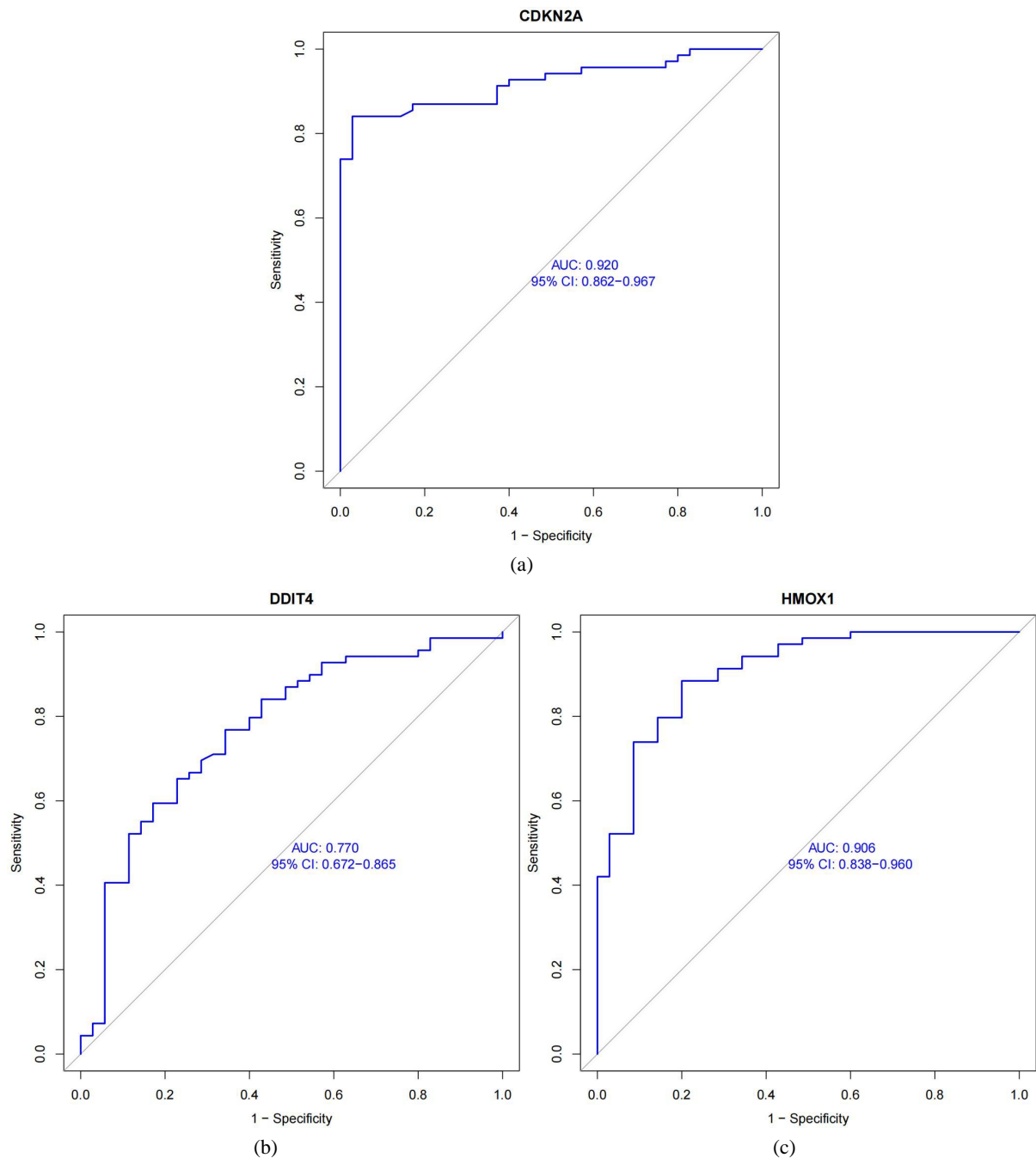
Figure 6. When GSE100927 was used as a training set, the function of 7 important candidate genes was detected, and the receiver operating characteristic curve of them

图 6. 以 GSE100927 为训练集时，检测 7 个重要候选基因的功能，并绘制了与其相关的受试者工作特征曲线

4. 讨论

动脉粥样硬化是一种慢性动脉疾病，其特征是脂质和纤维成分在动脉内膜中积聚，与沉积到内膜的低密度脂蛋白修饰有关[24]。尽管通常被定义为一种慢性疾病，但是其所致的动脉粥样硬化斑块的成和破裂是全球主要临床心血管事件的罪魁祸首[25] [26]。炎症被认为是参与动脉粥样硬化发生发展的重要生物学过程[11] [27] [28] [29]。动脉粥样斑块的形成过程是多种异常生物学过程交织产生的[10] [11] [12]。铁死亡被认为是其中非常重要的一环[12] [13] [14]。例如，刘等人的研究证实斑块巨噬细胞对红细胞的吞噬

CDKN2A

AUC: 0.658
95% CI: 0.529~0.784

(a)

DDIT4

AUC: 0.798
95% CI: 0.673~0.898

(b)

HMOX1

AUC: 0.848
95% CI: 0.740~0.934

(c)

MAP3K11

AUC: 0.760
95% CI: 0.633~0.874
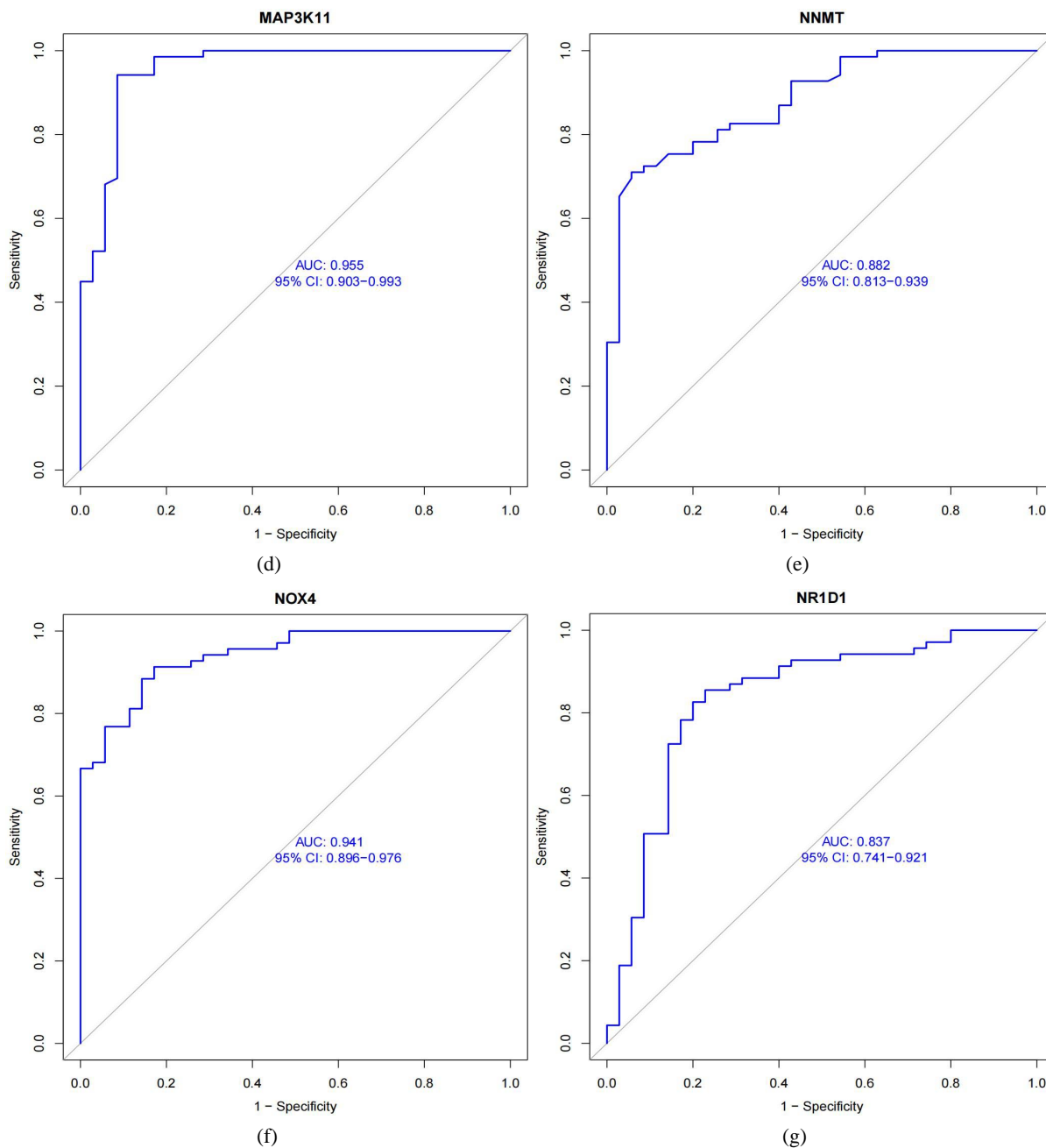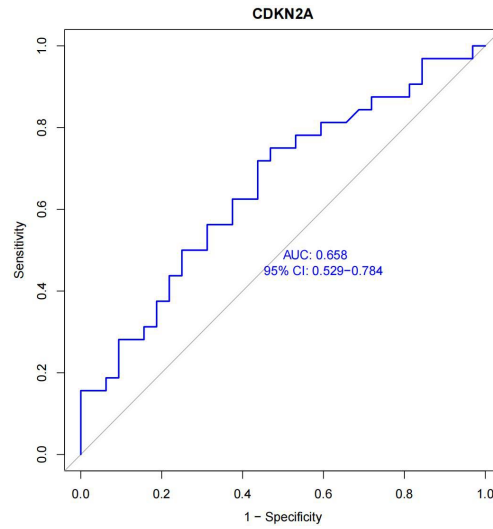
(d)

NNMT

AUC: 0.594
95% CI: 0.454~0.734
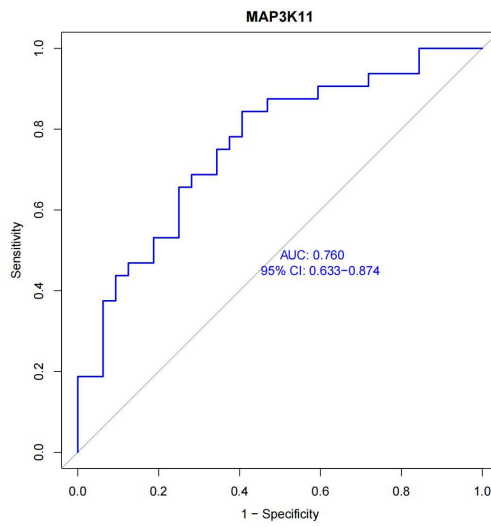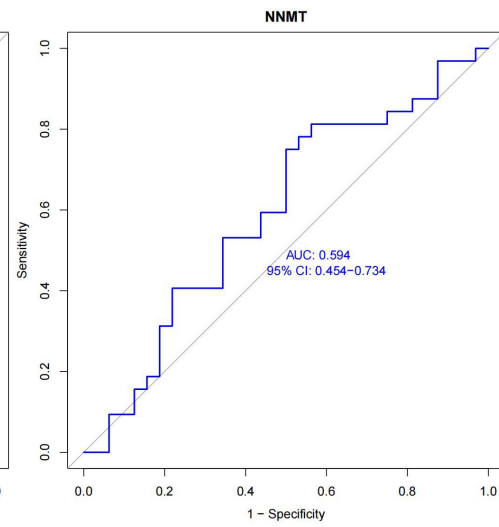
(e)
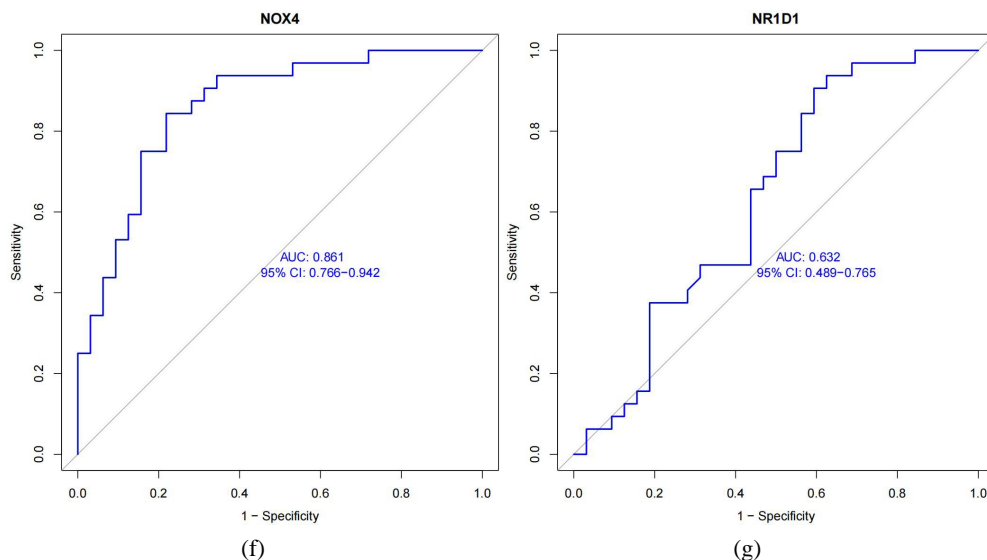
Figure 7. With GSE43292 as the external test set, the function of 7 important candidate genes was detected, and the receiver operating characteristic curve of them
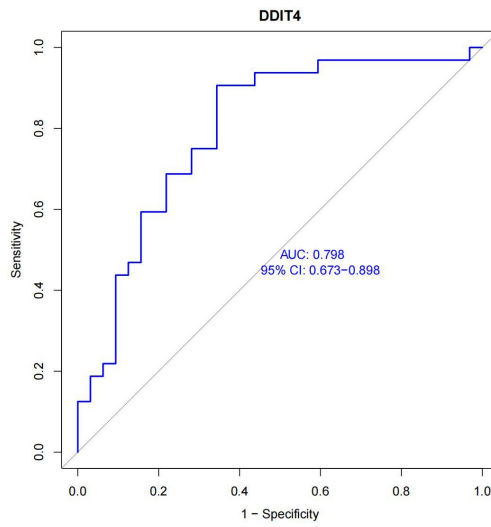
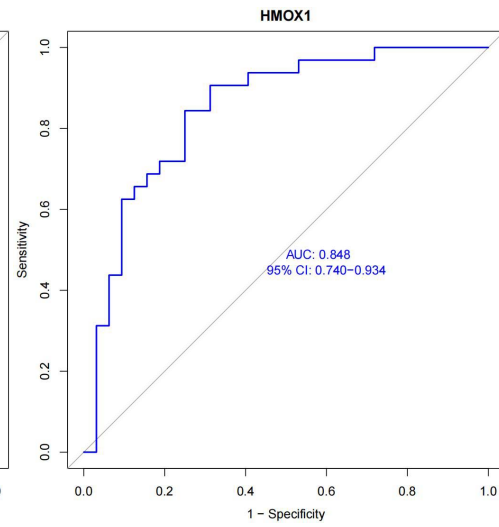图 7. 以 GSE43292 为外部检验集时，检测 7 个重要候选基因的功能，并绘制了与其相关的受试者工作特征曲线

作用促进了铁死亡，并认为这是降低红细胞介导的心血管风险的治疗靶点[7]。高等人的研究指出 Sestrins 1 (SESN1)在动脉粥样硬化的发生发展中起着重要的调控作用，而其功能可能主要为参与调控内皮细胞的铁死亡。但到目前为止，关于动脉粥样硬化中铁死亡的更多潜在机制仍然是未知的。

我们在对动脉粥样硬化中铁死亡相关的潜在候选基因进行基因本体富集分析时，发现其功能主要聚焦于氧化应激。这一发现似乎并不令人感到意外，因为氧化应激被认为是铁死亡发展的一个可能的关键因素[30] [31] [32]。铁死亡涉及铁稳态异常和脂质过氧化代谢：铁催化的细胞代谢紊乱破坏了氧化还原平衡，最终导致细胞死亡[13] [33] [34]。而铁死亡已被认定为许多心血管疾病的关键因素[35]。例如在主动脉夹层中 BRD4770 作为一种新型铁死亡抑制剂，通过抑制血管平滑肌细胞的流失来对抗血管壁结构的改变[18]。而在心肌病中，AMPK/NRF2 途径可以拮抗铁死亡的发生，并保护心肌细胞[36]。本研究通过生物信息学分析，确定了 23 个可能在动脉粥样硬化铁死亡中发挥作用的基因，基因本体富集分析表明，这些基因主要参与氧化应激反应，铁稳态与氧化应激相关，异常的有氧环境控制铁的毒性并导致细胞铁死亡，而氧化应激参则被认为参与了动脉粥样硬化的发生和发展[30] [37] [38]。基于此，本研究可为动脉粥样硬化中过氧化物应激反应诱导的铁死亡提供参考。

NOX4 来自于参与氧化应激的 NADPH 氧化酶家族[39]。NADPH 家族参与许多关键的生理过程，包括宿主防御、蛋白质的翻译后加工、细胞信号传导、基因表达调节和细胞分化等[40] [41] [42]。在已发表的研究中，NOX4 已经被证明在维持内皮细胞功能稳定方面发挥重要作用[42] [43] [44]。例如王等人发现芦丁通过干扰 NOX4 和来维持内皮功能稳定[45]。而动脉血管系统病变中的内皮功能障碍已被定义为动脉粥样硬化性心血管疾病病理生物学的重要因素[46]。动脉粥样硬化最初的异常变化是以血管内皮功能障碍为开端的：由于内皮功能障碍导致循环脂蛋白颗粒在内皮下空间的局灶性渗透和物理化学修饰，促使血液中的循环单核细胞被选择性募集到内膜，进而分化为巨噬细胞并将修饰的脂蛋白内化为泡沫细胞，最终诱导血管平滑肌细胞产生纤维肌斑块，导致纤维帽的形成[47]-[52]。另一方面，NOX4 在活性氧产生的过程中也有重要地位[41]。NOX4 可以将电子从胞质电子供体跨膜传输到细胞外的电子受体，而 NADPH 在绝大多数情况下作为电子供体，氧作为电子受体，最终导致产生活性氧[53]。而如上所述氧化应激是铁

死亡发生发展的关键。由此可见 NOX4 在动脉粥样硬化的发生发展过程中通过调控活性氧的产生进而参与内皮细胞铁死亡并不令人觉得意外。奇怪的是，到目前为止并没有研究声称发现 NOX4 在动脉粥样硬化中调控铁死亡。但是已有研究表明 NOX4 可以通过调控活性氧参与动脉粥样硬化的发生发展中[44] [54] [55] [56]。例如，胡等人的研究表明内皮 NOX4 通过可溶性环氧化物水解酶调节动脉粥样硬化。而在糖尿病相关的动脉粥样硬化中，NOX4 衍生的活性氧限制纤维化并抑制血管平滑肌细胞的增殖[55]。尽管我们相信在不久的将来，关于 NOX4 在动脉粥样硬化铁死亡相关进程中发挥的作用就将被揭开。但是目前我们不得不承认这方面的机制研究几乎依旧是一个空白。HMOX1 是构成血红素加氧酶重要同工酶，后者可以催化血红素降解为一氧化碳、亚铁和胆绿素，并最终转化为胆红素[57]。HMOX1 的表达与多种内源性和外源性刺激诱导有关，其中氧化应激是非常重要的一条[57]。而氧化应激是铁死亡发生的基础。实际上在许多疾病中已经发现 HMOX1 参与铁死亡进程的证据[58] [59] [60] [61]。在肝细胞癌中，多纳非尼和葛兰素史克-J4 被证明通过上调 HMOX1 表达协同诱导铁死亡[62]。而异甘草素则可以诱导 HMOX1 介导的胆囊癌细胞铁死亡[58]。吴等人的研究证明 HMOX1 抑制剂可以保护血管平滑肌细胞免受铁死亡的损害[20]。这一发现意义重大。众所周知，血管平滑肌的功能异常在动脉粥样硬化的发生发展中起到了关键作用。但是，HMOX1 是否在动脉粥样硬化中促进血管平滑肌细胞的铁死亡进程依旧需要更多的潜在机制相关研究来佐证。总之，我们的研究最终确定了两个可能与动脉粥样硬化铁死亡相关的候选靶向基因，据我们所致，类似的研究很少。

本研究存在一些局限性。首先，我们缺乏临床验证与动物实验建模的支持，其次，我们缺乏对于关键基因潜在机制的探索。这些需要后续的研究来进一步支持。

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