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鉴定主动脉夹层中的关键铁死亡基因

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摘　要

目的：生信分析结合组织验证以鉴定主动脉夹层(aortic dissection, AD)中的关键铁死亡基因。方法：Limma筛选正常主动脉和AD主动脉间的差异表达基因(differentially expressed genes, DEGs)。基因本体论(gene ontology, GO)和京都基因和基因组百科全书(Kyoto Encyclopedia of Genes and Genomes, KEGG)富集分析预测DEGs在AD中的功能和代谢过程。DEGs与铁死亡相关基因(ferroptosis-related genes, FRGs)数据库FerrDB取交集得到差异表达的铁死亡相关基因(differentially expressed ferroptosis-related genes, DEFRGs)。构建AD小鼠模型，验证前4个DEFRGs。结果：正常主动脉样本和AD主动脉样本间存在774个DEGs。DEGs与酶活性、细胞亚结构及糖脂代谢等相关。DEGs与FerrDB数据库交集出17个DEFRGs。通过构建AD小鼠模型，进一步鉴定了DEFRGs中的前4个关键基因，它们分别是雷帕霉素靶蛋白，Mammalian Target of Rapamycin (MTOR)、脂质运载蛋白2，Lipocalin-2 (LCN2)、DNA损伤诱导转录因子4，DNA damage-inducing transcription factor 4 (DDIT4)和淋巴特异性解旋酶，Lymphoid-specific helicase (HELLS)。其中MTOR、LCN2在AD小鼠主动脉中上调，DDIT4在AD小鼠主动脉中下调而HELLS在AD小鼠主动脉和正常主动脉间无变化。结论：铁死亡是AD发展中必不可少的病理过程之一，一些DEFRGs通过介导细胞铁死亡来影响AD的进展。这一发现更深入地认识了AD的致病机制和分子靶点。

关键词

主动脉夹层，铁死亡，生物信息学，关键基因

To Identify the Key Ferroptosis Genes in Aortic Dissection

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Abstract

Objective: To identify the key ferroptosis genes in aortic dissection (AD) by bioinformatics analysis combined with tissue validation. Methods: Limma screened differentially expressed genes (DEGs) between normal aorta and AD aorta. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were used to predict the potential functions and metabolic processes of DEGs in AD. Differentially expressed ferroptosis-related genes (DEFRGs) were determined by intersection of DEGs and FerrDB, which is a database of ferroptosis-related genes (FRGs). AD mouse models were constructed to validate the first four DEFRGs. Results: The analysis indicated that there were 774 DEGs between normal aortic samples and AD aortic samples. DEGs were related to enzyme activity, cell substructure and glucose and lipid metabolism. Seventeen DEFRGs were obtained by intersectional analysis of DEGs and FerrDB database. By constructing an AD mouse model, we further identified the top four key genes in DEFRGs. They are Mammalian Target of Rapamycin (MTOR), Lipocalin-2 (LCN2) and DNA damage inducing transcription factor 4 (DDIT4) and Lymphoid-specific helicase (HELLS). Among them, MTOR and LCN2 were up-regulated in AD mouse aorta, DDIT4 was down-regulated in AD mouse aorta, while HELLS had no change between AD mouse aorta and normal aorta. Conclusion: These results suggested that ferroptosis is one of the essential pathological processes in AD and that several DEFRGs affect the progression of AD by mediating cell ferroptosis. This finding provides deeper insights into the pathogenesis and molecular targets of AD.

Keywords

Aortic Dissection, Ferroptosis, Bioinformatics, Key Genes

1. 引言

AD 是最常见的急性主动脉综合征(acute aortic syndrome, AAS)，占所有 AAS 的 85%~95% [1]，由主动脉内膜撕裂伴血液流入中膜引起[2]。AD 的发病率约为每年 15 例/100,000 人[3]。近年来随着高血压及高血糖等代谢紊乱的增加，其发病率明显升高[4]。根据斯坦福系统，AD 可分为 A 型或 B 型。A 型 AD (Type A AD, TAAD)初始病死率最高，如果未被及时诊断通常是致命的[5]。而 B 型主动脉夹层(Type BAD, TBAD)的总体预后取决于复杂因素的存在，如发生器官缺血或破裂可大大增加其发病率[6]。总之，AD 病情危急，需要了解其发病机制以指导诊疗。

越来越多的证据表明包括铁死亡、自噬和焦亡在内的多种细胞生物学功能都参与了 AD 的发生发展。例如，李等人发现铁死亡抑制剂 liproxstatin-1 下调了 AD 小鼠主动脉中的铁死亡驱动物 4-羟基壬烯醛和转铁蛋白受体，同时其明显减轻了主动脉中膜变性和弹性纤维断裂[7]。Clément 等人证实平滑肌细胞(smooth muscle cells, SMCs)自噬相关蛋白 ATG5 的缺失会增加 AD 发生率和血管损伤程度[8]。同时段等人的研究表明抑制 SMC 焦亡可明显改善 AD [9]。其中，新近发现的铁死亡在 AD 发生中起关键作用[7] [10]。铁死亡是一种以铁毒性为特征的调节性坏死[11]。已有研究发现心血管疾病中存在广泛的铁死亡现象。例如，叶等人证实 SMC 铁死亡可促进血管钙化[12]。另一项研究发现 BRD4770 可通过抑制铁死亡

进而对 AD 发挥保护作用[10]。而李等人的研究进一步提出靶向人主动脉 SMCs (human aortic smooth muscle cells, HASMCs)的铁死亡可缓解 AD [7]。可见靶向铁死亡可成为治疗 AD 的有效策略。而鉴定 DEFRGs 可为 AD 提供诊断性生物标记物或干预靶点。

本研究通过结合基因表达综合数据库(Gene Expression Omnibus, GEO)中的数据集 GSE98770 和 FRGs 数据库 FerrDB 筛选出 774 个 DEGs 和 17 个 DEFRGs。随后，我们对候选的基因进行 GO 和 KEGG 富集分析。最后我们在 AD 小鼠主动脉和正常主动脉中验证了前 4 个 DEGs。

2. 材料与方法

2.1. 数据来源

GSE98770 的 mRNA 表达谱来自于 GPL14550，包含 6 例无家族性胸主动脉疾病的 TAAD 患者的夹层升主动脉内中膜和 5 例移植供体的非夹层升主动脉内中膜。

2.2. DEGs 和 DEFRGs 的鉴定

Limma 是一种基于广义线性模型的差异表达筛选方法[13]。本文采用 R 软件包 Limma (版本 3.40.6)进行 DEGs 分析，以获得正常主动脉样本与 AD 主动脉样本间的 DEGs。具体而言，以$|\log FC| \geq 2$ 和 P 值 < 0.05 作为界定差异基因表达的标准，使用 R 软件中的“ggplot2”包绘制火山图。从 FerrDB 数据库获得 FRGs，并与 DEGs 相交获得 DEFRGs。

2.3. 基因富集分析

为了研究 AD 主动脉和正常主动脉之间 DEGs 的潜在生物学功能，采用 R 包中的 clusterProfiler 进行了 GO 与 KEGG 分析。

2.4. AD 小鼠和健康小鼠主动脉的获取

购进满 3 周龄的 C57BL/6J 雄性小鼠(中国济南朋悦)。每天给予标准饮食及规律光照适应环境一周后，小鼠被随机分为两组(11 只/组)：正常组及 AD 组。AD 组小鼠腹腔注射血管紧张素 II (AngII, 6 mg/kg/12h, 中国上海吉尔)和 β-氨基丙腈(BAPN，0.33 g/kg/24h，中国上海皓鸿)，正常组小鼠腹腔注射等量的生理盐水。14 天后对小鼠进行安乐死，取主动脉拍照后储存在-80℃超低温冰箱以备分子学检测或固定于 4%多聚甲醛以备组织学分析。所有动物实验操作均经青岛大学附属医院动物伦理委员会审批。

2.5. RNA 提取和 qRT-PCR

使用 Kz-111-fp 高速低温研磨仪(中国青岛塞维尔)研磨组织并使用 TRIZOL 试剂(中国青岛思科捷)提取总 RNA。cDNA 的逆转录是根据制造商(中国南京诺唯赞)提供的说明进行的。使用通用 SYBR qPCR 混合物(中国南京诺唯赞)，反应在 95℃下运行 30 秒，95℃运行 5 秒，60℃运行 30 秒并≥40 个循环。所有实验数据均表示为从三个独立实验中获得的平均值，并使用 $2^{-\Delta\Delta}$ 循环阈值法进行统计学分析。甘油醛-3-磷酸脱氢酶(glyceraldehyde-3-phosphate dehydrogenase, GAPDH)用作内参。qRT-PCR 所用的引物如表 1 所示。

2.6. 苏木素&伊红(Hematoxylin & Eosin, H&E)染色

将固定的主动脉样本制成石蜡切片。用 H&E 染色试剂盒(中国大连美仑)按照制造商提供的方案进行染色。使用光学显微镜(日本东京 Olympus)拍摄图像。

Table 1. Primers used for qRT-PCR
表 1. qRT-PCR 所用的引物

引物	序列(5′→3′)
mus-MTOR-forward	GCTCACTGGTCGGGATTTCTCT
mus-MTOR-reverse	TGTAGCACTGGCAGAGGTTCTC
mus-LCN2-forward	AATGTCACCTCCATCCTGGTCA
mus-LCN2-reverse	GGCCACTTGCACATTGTAGCTC
mus-DDIT4-forward	CGGAGGAAGACTCCTCATACCTG
mus-DDIT4-reverse	AGGTTGGCACACAGGTGCTC
mus-Hells-forward	TGGTCAGACAAAGCCAGTTGT
mus-Hells-reverse	ACCCAGACTGACCACCTTTGA

2.7. 统计学分析

GraphPad Prism 8.3.0 软件分析所有采集的原始数据。计数数据以均数±标准差表示。两独立组间样本比较采用非配对 t 检验。$P < 0.05$ 有显著性差异。

3. 结果

3.1. 确定 DEGs

我们在 GSE98770 中发现了 AD 主动脉样本和正常主动脉样本间存在 774 个 DEGs，其中有 468 个上调基因，306 个下调基因。DEGs 的火山图如图 1 所示。

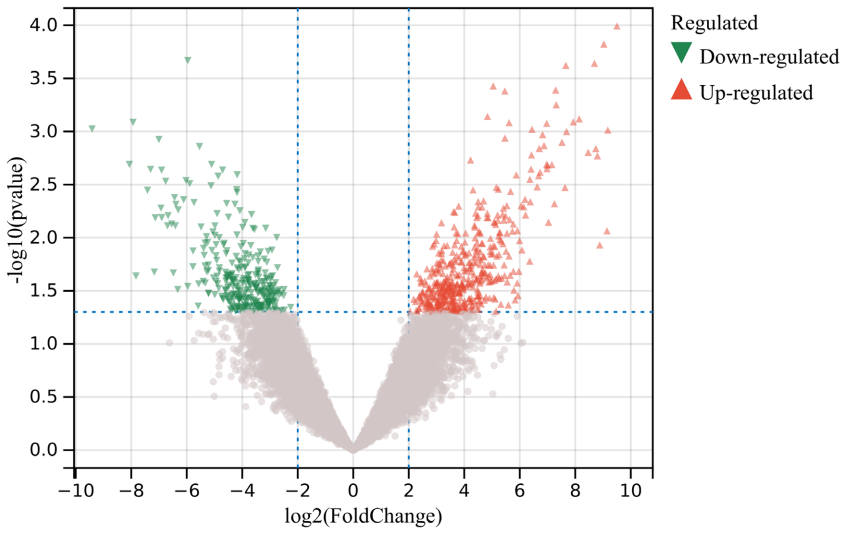

Figure 1. Volcano plot of DEGs between AD aorta and healthy aorta
图 1. AD 主动脉和健康主动脉间 DEGs 的火山图

3.2. 候选 DEGs 的基因富集概况

我们进行了 GO 和 KEGG 富集分析以了解 DEGs 的相关功能和参与的信号通路。GO 富集分析中，DEGs 在分子功能类别(molecular function, MF)主要富集于磷蛋白磷酸酶活性、NAD 结合、外肽酶活性(图 2(A))。DEGs 在细胞成分类别(cell component, CC)主要富集于细胞器亚区室、高尔基体和高尔基体子室(图

2(B))。DEGs 生物过程类别(biological process, BP)主要富集于骨骼系统形态发生、脂多糖代谢过程和糖脂生物合成过程(图 2(C))。在 KEGG 富集分析中，大部分 DEGs 与人 t 细胞白血病病毒 1 感染通路和产热作用通路有关(图 2(D))，该结果提示 AD 中可能有这些通路的激活。

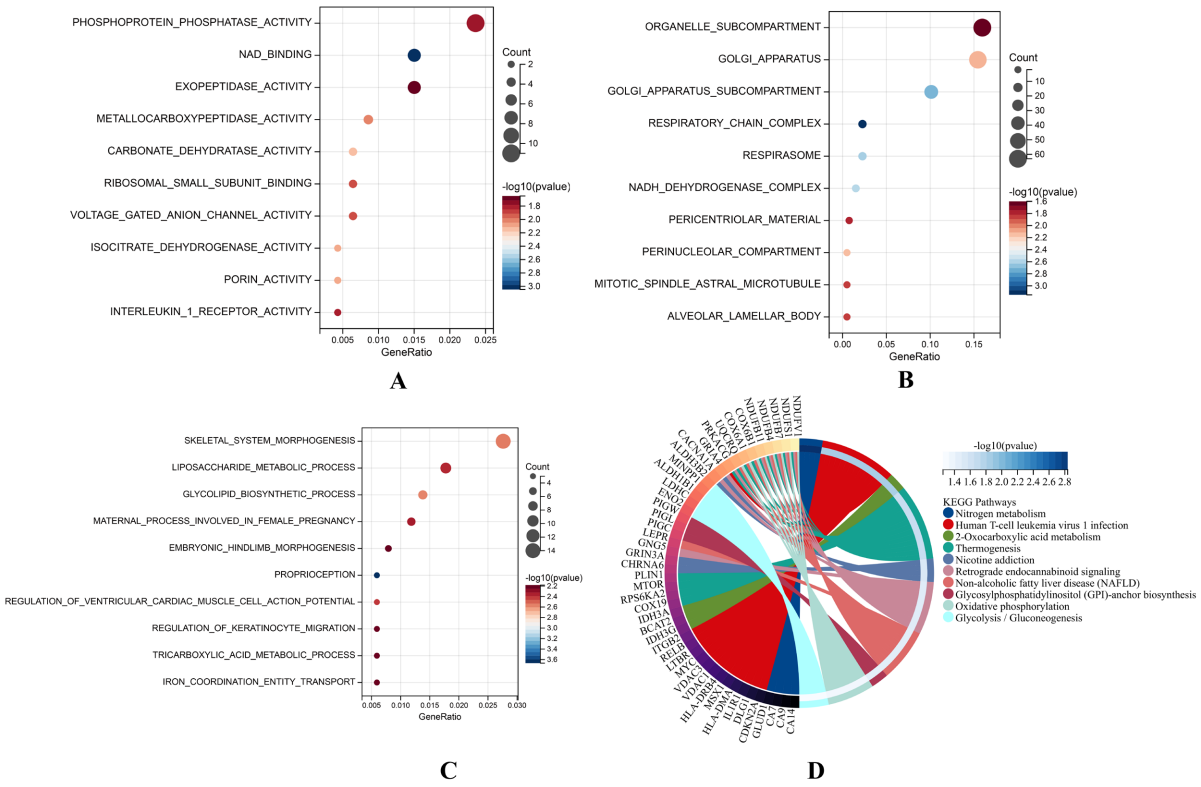

Figure 2. GO and KEGG enrichment analysis of 774 DEGs. (A) Bubble plot of GO enriched MF categories; (B) Bubble plot of GO enriched CC categories; (C) Bubble plot of GO-enriched BP categories; (D) Chordograms of KEGG enrichment
图 2. 774 个 DEGs 的 GO 和 KEGG 富集分析。(A) GO 富集 MF 类别的气泡图；(B) GO 富集 CC 类别的气泡图；(C) GO 富集 BP 类别的气泡图；(D) KEGG 富集的弦图

3.3. 确定 DEFRGs

既往研究表明铁死亡促进了 AD 的发生发展。为了分析哪些 FRGs 参与了 AD 的病理进程，我们对 DEGs 与 FRGs 取交集获得 17 个 DEFRGs，其中有 9 个上调 DEFRGs，8 个下调 DEFRGs 如图 3，表 2 所示。

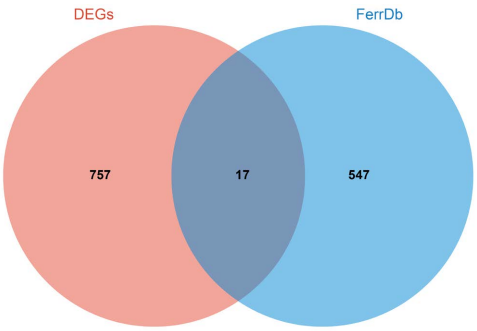

Figure 3. Venn diagram showing the intersection of genes between DEGs from GSE98770 and FRGs from FerrDB
图 3. 维恩图显示了 GSE98770 的 DEGs 与 FerrDB 的 FRGs 之间基因的交集

Table 2. Seventeen DEFRGs identified in AD aortic samples compared to healthy aortic samples
表 2. 与健康主动脉样本相比，AD 主动脉样本中鉴定出的 17 个 DEFRGs

基因	Log FC	变化	P 值	调整后的 P 值
ACVR1B	2.850375592	上调	0.019440488	0.965717469
BCAT2	−2.608543047	下调	0.048619016	0.965717469
CA9	−3.929346336	下调	0.045761926	0.965717469
CDKN2A	−4.321904937	下调	0.025526545	0.965717469
CYP4F8	3.141281932	上调	0.029120956	0.965717469
DAZAP1	2.510730852	上调	0.034071267	0.965717469
DDIT4	−6.389734203	下调	0.004856257	0.965717469
DDR2	−3.347429545	下调	0.013636601	0.965717469
GABARAPL2	−3.416143016	下调	0.020171116	0.965717469
GPX4	3.906003637	上调	0.018881765	0.965717469
HELLS	7.034464167	上调	0.007149613	0.965717469
KDM6B	3.775296694	上调	0.046501325	0.965717469
LCN2	4.941833404	上调	0.012162798	0.965717469
MTOR	5.616498739	上调	0.000826739	0.965717469
NGB	4.817942138	上调	0.006465372	0.965717469
PDK4	−3.086370556	下调	0.036464164	0.965717469
TFAM	3.018739388	上调	0.027871524	0.965717469

3.4. 构建 AD 小鼠模型

为了进一步在 AD 主动脉组织中确认获得的 DEFRGs，我们构建了 AD 损伤小鼠模型。主动脉大体图片显示与正常主动脉相比，AD 主动脉明显增粗(图 4(A))。H&E 染色显示 AD 主动脉内径明显增宽，主动脉各层结构紊乱，弹性纤维断裂(图 4(B))。这些数据表明 AD 模型构建完成。

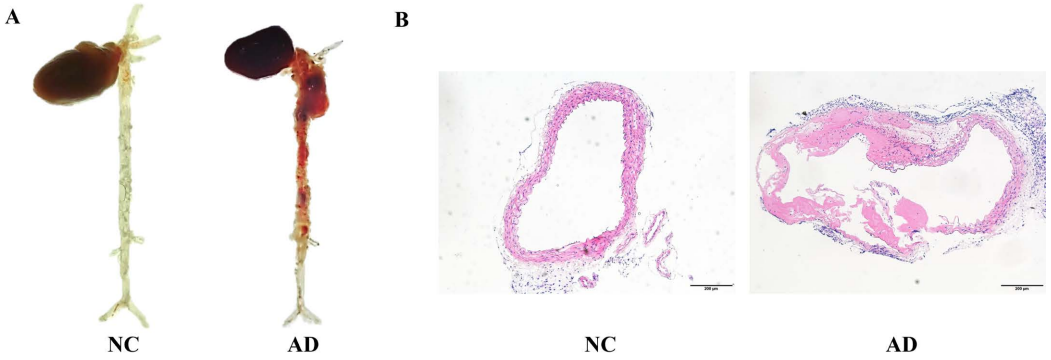

Figure 4. (A) Gross photographs of the aorta of mice between the two groups; (B) Aortic H&E staining of mice between different treatment groups, bar = 200 μm
图 4. (A) 两组间小鼠的主动脉大体照片；(B) 不同处理组间小鼠的主动脉 H&E 染色，比例尺 = 200 μm

3.5. 验证 DEFRGs

为了进一步验证差异表达的 DEFRGs 在小鼠主动脉中的差异表达，采用 qRT-PCR 检测了前 4 个候选 DEFRGs 的表达。结果表明，MTOR 和 LCN2 在 AD 小鼠主动脉中上调(图 5(A)、图 5(B))，而 DDIT4 在

AD 小鼠主动脉中下调同时 HELLS 在 AD 小鼠主动脉和正常主动脉间无变化。

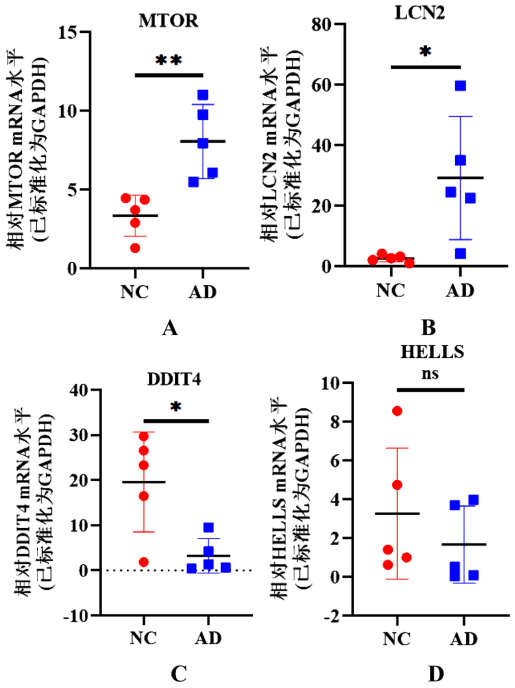

Figure 5. (A) qRT-PCR detection of MTOR in mouse aortas among groups; (B) qRT-PCR was used to detect LCN2 in the aorta of mice among groups; (C) qRT-PCR was used to detect LCN2 in the aorta of mice among groups; (D) qRT-PCR detection of HELLS in mouse aortae across groups. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$

图 5. (A) qRT-PCR 检测各组间小鼠主动脉中的 MTOR；(B) qRT-PCR 检测各组间小鼠主动脉中的 LCN2；(C) qRT-PCR 检测各组间小鼠主动脉中的 LCN2；(D) qRT-PCR 检测各组间小鼠主动脉中的 HELLS。$^{ns}P > 0.05$，$^{*}P < 0.05$，$^{**}P < 0.01$

4. 讨论

AD 与炎症、细胞外基质(extracellular matrix, ECM)降解、氧化应激以及 SMCs 的表型转换和凋亡相关。这些病理机制相互作用以促进 AD 的发生[14]。比如，炎症细胞分泌的炎症因子和蛋白酶通过损伤内皮细胞[15]、降解 SMCs 收缩蛋白[16]以及诱导主动脉中膜细胞凋亡[17]引起 AD。此外，氧化应激可引起主动脉功能障碍[18]。实验研究发现，硫化氢(H_2S)可通过减少氧化应激产物生成来抑制 AD 进展[19]。具体来说，与对照组相比，H_2S 升高了 AD 组小鼠主动脉中的超氧化物歧化酶活性并降低了丙二醛和一氧化氮生成量。SMCs 从静止的收缩型转变为活跃的增殖迁移型称为 SMCs 的表型转换或去分化[20]。张等人证实低表达的多囊蛋白-1 通过激活 mTOR/S6K/S6 信号通路调节 SMCs 表型转换和 ECM 重塑而导致胸主动脉夹层(thoracic aortic dissection, TAD) [21]。虽然已有研究证实与氧化应激和炎症密切相关的铁死亡参与了 AD 的进展[10] [22]，但我们对铁死亡引发 AD 的机制认识仍然有限。

本研究的 MF 富集分析显示 AD 与白细胞介素-1 (interleukin-1, IL-1)受体活性有关。IL-1 主要由巨噬细胞分泌，是促炎细胞因子家族中的重要一员[23] [24]。郭等人发现 IL-1β 通过升高 MMP-2 和 MMP-9 而引起弹性纤维破裂及主动脉壁应力改变，最终促进了 TAD 形成[25]。而抗 IL-1β 治疗可减轻 TAD。这一发现与蒋等人的研究结果一致[26]。此外铁死亡可参与 IL-1β 介导的炎症反应。例如，徐等人证实谷胱甘肽过氧化物酶(glutathione peroxidase, GPX4)缺失的 T 淋巴细胞发生了铁死亡且 IL-1β 生成增加[27]。事实上，多种 IL 家族成员可广泛参与 AD 的进展且与铁死亡密切相关。例如，临床研究发现 AD 患者的 IL-6 水平升高[28]。且 IL-6 可作为诊断 AD 及评估 AD 治疗及预后的可靠生物标记物。而降低 IL-6 的抗炎治

疗可明显缓解 AD。同时，张等人发现 elabela 通过调节 IL-6/信号转导因子 3 (signal transducer and activator of transcription 3, STAT3)/GPX4 信号通路而拮抗铁死亡[29]。此外，本研究的 BP 富集分析发现 AD 与铁配位体运输有关。而铁运输中任一环节障碍一方面会导致病理性铁积累，由此引发细胞氧化应激损伤而致铁死亡[22]。例如，介导铁摄取的转铁蛋白受体缺失可抑制铁死亡[30] [31]。另一方面铁运输障碍也会导致铁缺乏而影响 SMCs 功能。比如，钟等人揭示了铁缺乏通过破坏 SMCs 的细胞骨架而促进主动脉中膜变性[32]。上述铁缺乏所致的主动脉结构性损伤最终增加了 AD 的患病风险[33]。

在小鼠主动脉样品中，我们验证了 MTOR、DDIT4 和 LCN2 与微阵列测序结果一致。虽然 MTOR 是肿瘤发生的关键驱动因子[34]，但 MTOR 也与 AD 有关[35]。比如，李等人发现激活 MTOR 复合物 1 (MTOR complex 1, MTORC1)会促进 SMCs 增生而导致进行性主动脉中膜变性和 TAAD [36]。另一项研究显示雷帕霉素可能通过抑制 MTOR 信号转导减少炎细胞浸润和 MMP-9 的产生，从而抑制 TAAD 的形成[37]。研究人员首先证明小鼠 TAAD 组织中介导 MTORC1 信号级联反应的主要活化靶蛋白 p-S6K 和 p-S6 上调。而雷帕霉素治疗的 TAAD 小鼠中 p-S6K 和 p-S6 下调，同时 TTAD 形成及主动脉扩张减少、弹性纤维断裂减少、破坏弹性纤维的 MMP-9 下调以及促进 ECM 降解的中性粒细胞和巨噬细胞浸润减少。这进一步佐证了 MTOR 的促 AD 作用。而何等人的研究与上述发现相反。他们发现抑制 MTOR 信号传导可防止主动脉破裂，但促进 AD。因此应谨慎探索抑制 MTOR 对主动脉疾病的作用[38]。同时，研究表明 MTOR 可促进铁死亡。韩等人发现第 3 类去乙酰化酶(type 3 sirtuins, SIRT3)通过激活腺苷酸活化蛋白激酶(adenosine 5’-monophosphate-activated protein kinase, AMPK)-MTOR 通路参与了滋养细胞的铁死亡[39]。综上，MTOR 是否通过铁死亡途径促进 AD 值得进一步探索。DDIT4 是一种高度保守的应激反应蛋白和铁死亡相关蛋白[40]。罗等人已证明下调 DDIT4 可抑制铁死亡[41]。但目前没有关于 DDIT4 调控 AD 的报道。作为一种铁螯合细胞因子，范等人发现下调 LCN2 可增强铁死亡诱导剂的致铁死亡作用[42]。同时作为一类分泌型脂肪因子，LCN2 是心衰病理生理学中氧化应激和免疫反应的重要调节剂[43]。但目前尚无研究表明 LCN2 参与调控 AD。鉴于 LCN2 在调节铁死亡及心血管疾病中的强大作用，未来有必要阐明其在 AD 中的作用。

综上，我们利用小鼠 AD 主动脉样本中鉴定了一些关键的 DEFRGs。但限于临床取样的难度，目前无法在临床主动脉样本中验证这些基因，因此未来仍需收集临床样本以进一步鉴定这些 DEFRGs。此外，更多 AD 数据集的开发及更多 FRGs 的鉴定也会为改善 AD 提供更多的诊断标记物及治疗靶点。

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