·论著·

基于机器学习筛选阿霉素诱导的心肌病中铁死亡的关键基因与验证

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摘要 **目的** 基于生物信息学分析铁死亡在阿霉素诱导的心肌病(DIC)中的关键基因,结合体外实验验证,探讨铁死亡在DIC中的作用。**方法** 二价铁荧光染色佐证DIC中心肌细胞发生铁死亡。检索基因表达综合数据库(GEO)得到GSE207737数据集,与检索FerrDb数据库得到的铁死亡相关基因作交集,对交集基因进行基因本体(GO)以及京都基因和基因组数据库(KEGG)富集分析。将最小绝对值选择与收缩算子(LASSO)回归算法和支持向量机递归特征消除(SVM-RFE)2种机器学习方法得到的基因取交集获得DIC的铁死亡关键基因,并通过实时PCR在正常和DIC模型的H9C2细胞中进行验证,对于生物信息学和实时PCR结果不符者采用Western blotting进一步验证。**结果** 共获得38个DIC的铁死亡相关基因,GO和KEGG分析结果表明这些基因主要参与细胞代谢,通过机器学习方法获得DIC的铁死亡关键基因为*Mpc1*、*Prdx1*、*Kdm4a*、*Alox12b*和*Tfrc*。体外实验结果表明,与正常组相比,DIC模型组*Mpc1*、*Prdx1*和*Kdm4a* mRNA表达显著下调($P < 0.001$),*Alox12b* mRNA表达显著上调($P < 0.001$),而*Tfrc* mRNA和蛋白的表达水平均无统计学差异($P > 0.05$)。**结论** *Mpc1*、*Prdx1*、*Kdm4a*和*Alox12b*为DIC的铁死亡关键基因,可能成为从铁死亡角度防治DIC的靶点。

关键词 阿霉素诱导的心肌病;铁死亡;生物信息学;基因
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Screening and validation of key genes for ferroptosis in doxorubicin-induced cardiomyopathy on machine learning

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Abstract **Objective** To explore the role of ferroptosis in DIC through bioinformatics analysis of hub genes involved in ferroptosis in doxorubicin-induced cardiomyopathy (DIC), combined with in vitro experimental validation. **Methods** Divalent iron fluorescence staining confirms the occurrence of ferroptosis in myocardial cells of DIC. The GSE207737 dataset was retrieved from the Gene Expression Comprehensive Database (GEO) and intersected with the FerrDb database to identify ferroptosis-related genes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the intersected genes and intersecting the genes obtained from LASSO regression analysis and SVM-SFR machine learning methods were used to obtain ferroptosis hub genes for DIC. Real-time PCR was used to validate H9C2 cells in the control and DIC model groups, and Western blotting was used to further validate those whose bioinformatics and real-time PCR results that did not match. **Results** Thirty-eight ferroptosis-related genes in DIC were identified, and GO and KEGG analyses showed that these genes mainly participate in cell metabolism. Five hub genes for ferroptosis in DIC were obtained using machine learning methods: *Mpc1*, *Prdx1*, *Kdm4a*, *Alox12b*, and *Tfrc*. Through in vitro experiments, the mRNA expression levels of *Mpc1*, *Prdx1*, and *Kdm4a* were downregulated in the DIC model group compared to those in the control group ($P < 0.001$), whereas the mRNA expression level of *Alox12b* was upregulated ($P < 0.001$). There were no significant differences in the mRNA or protein expression levels of Tfrc ($P > 0.05$). **Conclusion** *Mpc1*, *Prdx1*, *Kdm4a*, and *Alox12b* are key genes involved in ferroptosis in doxorubicin-induced cardiomyopathy and potential targets for the prevention and treatment of doxorubicin-induced cardiomyopathy in ferroptosis.

Keywords doxorubicin-induced cardiomyopathy; ferroptosis; bioinformatics; gene

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阿霉素（doxorubicin，DOX）是一种抗癌化合物，具有抑制DNA合成的作用，可有效作用于大多数肿瘤细胞。但DOX的有效治疗需要高剂量用药，会产生心脏毒性等不良反应，极大地限制了DOX在临床的使用[1]。DOX对心脏造成的损伤不可逆，DOX诱导的心肌病（DOX-induced cardiomyopathy，DIC）包括心肌损伤、心律失常和心力衰竭等，是癌症患者死亡的重要原因[2]。

铁死亡在DIC中发挥关键作用，DOX会增加细胞内游离的Fe^{2+}，通过形成DOX-Fe^{2+}复合物及抑制GPX4等方式诱导过度脂质过氧化，进而导致铁死亡[3]。目前，DOX通过铁死亡途径引起心肌细胞死亡并诱发心肌病的具体机制尚不明确。本研究基于生物信息学方法筛选出DIC铁死亡的关键基因，并进行初步实验验证，为从铁死亡角度防治DIC提供参考。

1　材料与方法

1.1　获取DIC基因集与差异基因

采用基因表达综合（Gene Expression Omnibus，GEO）数据库（http://www.ncbi.nlm.nih.gov/geol）的GSE207737数据集，GSE207737包含3例健康小鼠心脏和4例DOX心脏毒性小鼠心脏，设定调整$P<0.05$，以$|\log_2FC|>1.5$作为筛选条件，筛选出差异表达基因。

1.2　获取铁死亡基因集

在FerrDb（http://zhounan.org/ferrdb/）数据库检索铁死亡相关基因，获取该数据库目前收录的484个铁死亡相关基因。

1.3　DIC与铁死亡相关的交集基因获取及基因本体论（Gene Ontology，GO）、京都基因和基因组数据库（Kyoto Encyclopedia of Genes and Genomes，KEGG）富集分析

将上述获得的差异表达基因和铁死亡相关基因进行交集，得出DIC与铁死亡相关的交集基因。对交集基因进行GO和KEGG分析。

1.4　筛选DIC的铁死亡关键基因

采用最小绝对值选择与收缩算子（least absolute selection and shrinkage operator，LASSO）回归算法和支持向量机递归特征消除（support vector machines-recursive feature elimination，SVM-RFE）2种机器学习方式分别筛选出特征基因，2组特征基因取交集，得出DIC的铁死亡关键基因。

1.5　细胞培养与处理

将大鼠心肌细胞（H9C2，武汉普诺赛生命科技有限公司）分为正常组和DIC模型组，DIC模型组使用含1 μmol/L DOX溶液和15%胎牛血清的DMEM培养基处理，正常组使用含15%胎牛血清的DMEM培养基培养，在37 ℃ 5%CO_2孵箱中孵育24 h。

1.6　Fe^{2+}荧光染色

将2组细胞弃去培养上清，PBS洗涤1遍，按照Fe^{2+}荧光探针试剂盒（上海懋康生物科技有限公司）说明书操作，荧光显微镜（美国伯腾仪器有限公司）下观察拍摄。

1.7　实时PCR

采用RNA提取试剂盒（湖南艾科瑞生物工程有限公司）提取细胞中的总RNA，根据反转录试剂盒（湖南艾科瑞生物工程有限公司）逆转录为cDNA，以cDNA为模板，根据PCR试剂盒（湖南艾科瑞生物工程有限公司）进行实时PCR，各引物序列见表1，引物均购自广州天一辉远基因科技有限公司。采用$2^{-\Delta\Delta Ct}$法计算相对表达量。

1.8　Western blotting

RIPA裂解和提取缓冲液（美国ThermoFisher Scientific公司）裂解H9C2细胞并提取蛋白质，蛋白质经定量、电泳、转膜、封闭后，加入一抗Tfrc（1 ∶ 5 000）、β-actin（1 ∶ 10 000），4 ℃孵育过夜，加入二抗（1 ∶ 10 000），室温孵育1 h，TBST洗涤3次，观察蛋白质条带，采用ImageJ分析蛋白灰度值。抗体均购自英国abcam公司。

1.9　统计学分析

采用SPSS 19.0软件进行统计分析，所有实验重复3次，数据以$\bar{x}\pm s$表示，采用独立样本t检验进行组间差异比较，$P<0.05$为差异有统计学意义。

2　结果

2.1　铁染色

Fe^{2+}染色结果表明，与正常心肌细胞相比，DOX处理后的心肌细胞荧光强度显著增加，DOX处理后的心肌细胞Fe^{2+}数量显著增多，见图1。

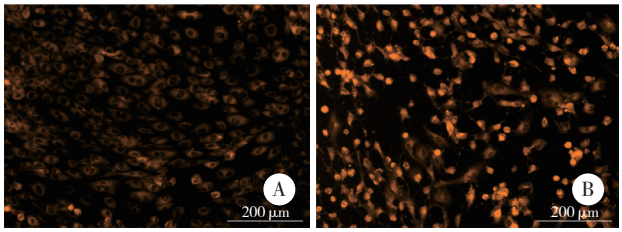
2.2　DIC基因集获取

将GSE207737数据集进行矫正和标准化处理，在处理后的数据集中筛选出4 492个差异基因，其中上调2 696个，下调1 796个，根据结果绘制火山图，见图2。

表1　实时PCR引物序列

Tab.1　Real-time PCR primer sequence

Gene	Primer sequences (5'–3')	Product length (bp)
β-actin	Forward: TGCTCTCCCTCACGCCATCC	154
	Reverse: GTCACGCACGATTTCCCTCTCAG	
Mpc1	Forward: TGAGGGGAAAACACGGAAGACTATC	98
	Reverse: TAGCATTGATAAGGCAGCCGAGAG	
Prdx1	Forward: CCCACGGAGATCATTGCTTTCAG	101
	Reverse: GCCAGATGACAGAAGTGAGAATCC	
Kdm4a	Forward: CAGAAGGTCATCAGCAAGCACAAG	81
	Reverse: TTCGTAGAAGGTTTCGGTGGTGAG	
Alox12b	Forward: GCTCCCAACTGTCGTGTCTACC	148
	Reverse: TGGCTCTGATCTCTTCTTGTCTGTG	
Tfrc	Forward: GATCGGCTACCTGGGCTATTGTAAG	82
	Reverse: CTTGTCCGCCTCTTCCGCTTC	



A, control group; B, DIC model group.

图1　Fe^{2+}荧光染色

Fig.1　Fe^{2+} fluorescence staining

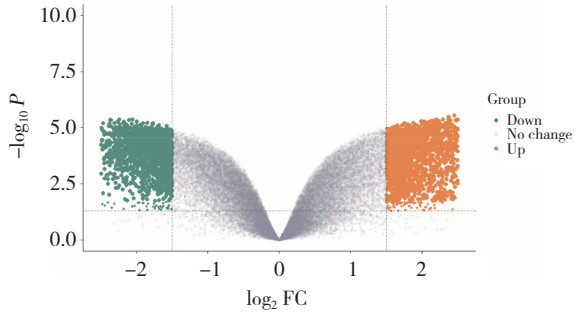


图2　DIC的差异表达基因的火山图

Fig.2　Volcanic map of differentially expressed genes in DIC

2.3　铁死亡基因集获取

通过FerrDb数据库获得的484个铁死亡相关基因，与上述差异基因取交集后共获得38个DIC与铁死亡交集基因。

2.4　GO和KEGG富集分析

对筛选出的38个DIC与铁死亡交集基因进行功能注释（图3），发现其在生物过程（biological process，BP）中主要参与核糖核苷酸代谢过程、核糖磷酸代谢过程、核苷酸生物合成过程、核苷酸磷酸生物合成过程、含硫化合物代谢过程等；细胞组分（cellular component，CC）显示其主要存在于过氧化物酶体、微体、线粒体基质、细胞器膜的组成部分、细胞器膜的内在成分等；在分子功能（molecular function，MF）上，其主要在铁离子结合、花生四烯酸辅酶A连接酶活性、长链脂肪酸辅酶A连接酶活性、脂肪酸连接酶活性、辅酶A连接酶活性中发挥作用。KEGG富集分析显示其参与过氧化物酶体增殖物激活受体信号通路、过氧化物酶体、铁死亡、脂肪酸生物合成、柠檬酸循环等信号通路。

2.5　机器学习

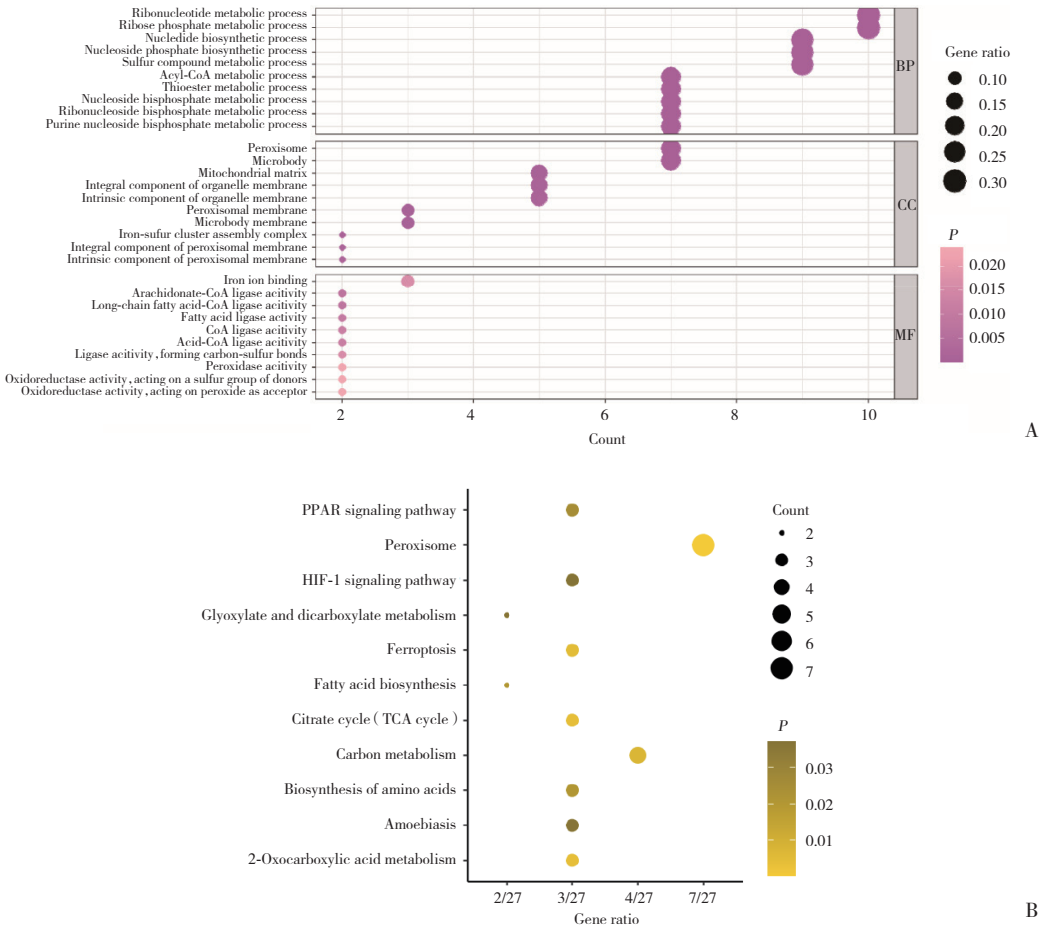
采用机器学习方法进一步对38个交集基因进行分析。利用SVM-RFE算法分析得到了34个特征基因（图4）。利用LASSO算法分析获得了5个特征基因（图5）。取2组特征基因交集，获得了5个DIC的铁死亡关键基因，分别为*Mpc1*、*Prdx1*、*Kdm4a*、*Alox12b*、*Tfrc*。

2.6　mRNA水平验证

实时PCR结果表明，与正常组相比，DIC模型组*Mpc1*、*Prdx1*和*Kdm4a*显著下调（$P < 0.001$），*Alox12b*显著上调（$P < 0.001$），而2组比较*Tfrc*无统计学差异（$P > 0.05$），见图6。

2.7　蛋白水平验证

通过Western blotting对生物信息学和实时PCR结果不一致的Tfrc进行进一步验证，结果表明，Tfrc表达2组无统计学差异（$P > 0.05$），见图7。



A, GO functional enrichment analysis; B, KEGG pathway enrichment analysis.

图3　GO和KEGG富集分析

Fig.3　GO and KEGG enrichment analysis

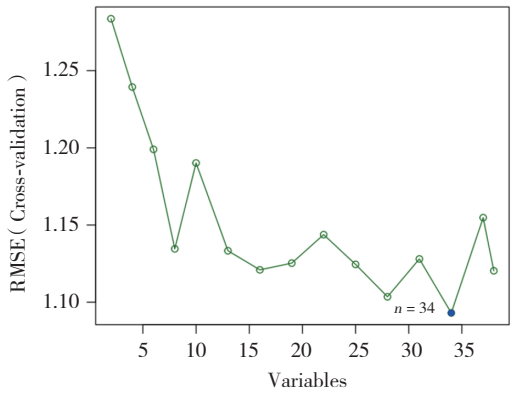
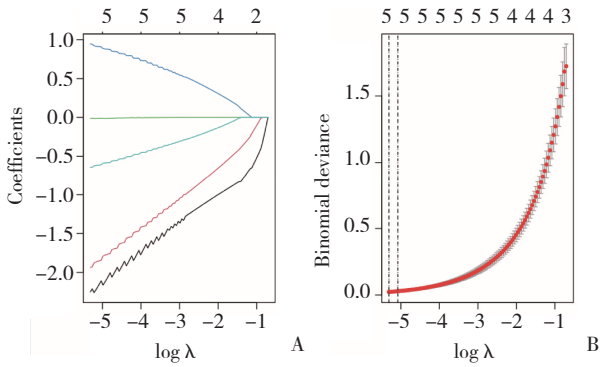


图4　对差异基因的SVM-RFE算法分析

Fig.4　Analysis of the SVM-RFE algorithm for differential genes

3　讨论

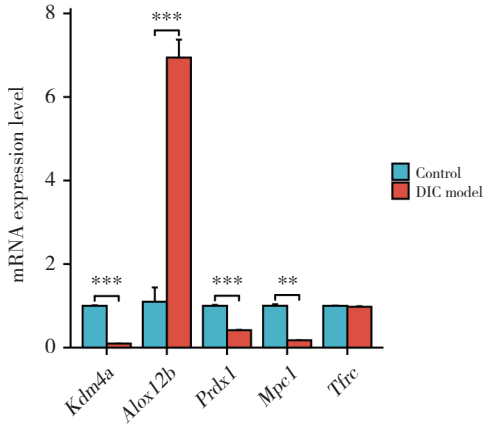
DOX是蒽醌类广谱抗肿瘤药物，由于较强的心脏毒性，其治疗潜力受到限制[1]。DIC的主要病理特征为心肌细胞凋亡、能量代谢紊乱和活性氧聚集[4]。铁死亡是以脂质过氧化物和铁依赖性积累为特征的细胞死亡形式[5]，在心力衰竭、肥厚型心肌病等心血管疾病的发展中扮演重要角色[6-7]。DOX可以下调*GPX4*，并通过线粒体中的DOX-Fe^{2+}复合物诱导过度的脂质过氧化，导致心肌细胞铁死亡[3]。



A, the variation characteristics of the variable coefficients; B, the selection process of the optimum value of the parameter λ in the LASSO regression model by cross-validation method.

图5　对差异基因的LASSO算法分析

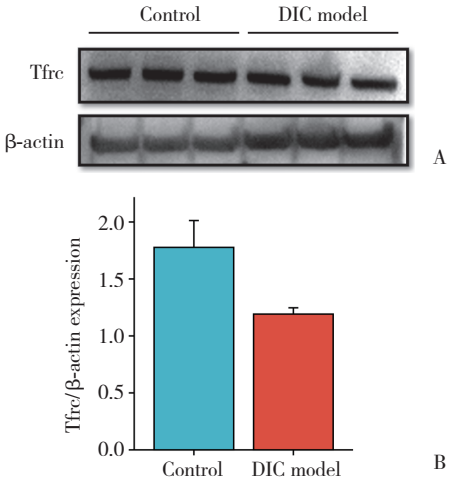
Fig.5　LASSO algorithm analysis of the DEGs



$^{**}P < 0.01$, $^{***}P < 0.01$.

图6　实时PCR检测各组中*Kdm4a*、*Alox12b*、*Prdx1*、*Mpc1*和*Tfrc* mRNA表达

Fig.6　Real-time PCR detection of *Kdm4a*, *Alox12b*, *Prdx1*, *Mpc1* and *Tfrc* mRNA in each group



A, band diagrams of Tfrc protein; B, comparison of Tfrc/β-actin expression.

图7　Western blotting检测各组中Tfrc蛋白的表达

Fig.7　Western blotting analysis of Tfrc protein expression in each group

Kdm4a是一种赖氨酸特异性的组蛋白去甲基化酶，可识别甲基化赖氨酸使之去甲基化[8-9]。Kdm4a对转录的调节作用影响细胞的铁死亡，Kdm4a在宫颈癌和骨肉瘤中皆通过介导组蛋白去甲基化抑制细胞铁死亡[10-11]。在本研究中，*Kdm4a*在DIC中显著下调，*Kdm4a*的下调可能导致组蛋白去甲基化减少，增加了铁死亡的发生。

Mpc1编码一种线粒体丙酮酸盐的载体蛋白，可将丙酮酸转运至线粒体，加快丙酮酸–乳酸轴中丙酮酸和乳酸盐的积累，使活性氧减少[12-13]。Mpc1表达的下调增加了耐受厄洛替尼头颈部癌细胞的铁死亡敏感性[14]。在心力衰竭患者和小鼠的心脏中*Mpc1*表达下调，其缺乏会诱使线粒体衰竭、心脏肥大甚至心力衰竭[15]。*Mpc1*的研究发现DIC中*Mpc1*也显著下调，*Mpc1*的抑制可能增加了心肌细胞对铁死亡的敏感性。

Prdx1编码一种过氧化物还原酶，具有抗氧化的能力，在抑制线粒体破碎和细胞凋亡中发挥重要的作用[16]。在人肝癌细胞中发现Prdx1的抑制可以促进铁死亡的发生，角膜内皮细胞中氧化应激的增加也会导致Prdx1表达丧失[17]。本研究显示在DIC中Prdx1的表达下调，导致细胞抗氧化能力减弱，细胞的铁死亡抵抗被抑制。

Alox12b编码一种脂氧合酶，负责将花生四烯酸转化为12R羟基二十碳四烯酸[18]。Alox转化不饱和脂肪酸的同时促进脂质过氧化，从而诱导细胞发生铁死亡[19]。DOX可以诱导人结肠癌细胞和非小细胞肺癌细胞中发生P53依赖性的Alox12b转录水平升高[20]。本研究结果显示，在DIC中心肌细胞*Alox12b*表达上调。

Tfrc编码的转铁蛋白受体1是发挥主要作用的铁转运蛋白，由Tfrc转运至细胞内的Fe^{2+}可通过Fenton反应促进脂质过氧化物的形成[21]。在心脏缺血/再灌注模型中敲除*Tfrc*可抑制心肌细胞铁死亡，而过表达*Tfrc*可增加神经母细胞瘤细胞的铁死亡敏感性[21-22]。本研究生物信息学技术分析结果显示，DIC中*Tfrc*的表达下调，但在实验结果中却发现其表达无明显变化，有待今后进一步验证。

综上所述，本研究结合生物信息学及体外实验分析，发现铁死亡在DIC中的发生可能与*Mpc1*、*Prdx1*、*Kdm4a*和*Alox12b*基因相关。

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