·基础研究·

急性髓系白血病新型预后分子标志物*NKX2-3*的发现

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摘　要：【目的】通过分析生物信息数据库中影响急性髓系白血病(AML)患者的预后的分子标志物的表达，为进一步探索AML预后的新型分子标志物提供实验基础。【方法】从癌症基因组图谱(TCGA)生物信息数据库中的179例AML患者的预后数据进行差异性基因分析及生存分析；利用基因表达集锦(GEO)数据库中的GSE13159数据集的74例健康人(HI)骨髓标本与542例AML初发患者骨髓标本进行分析，检测差异性目的基因表达水平在AML初发患者与健康人中的差异性；收集初发18例AML患者的外周血及骨髓样本，同时收集年龄和性别匹配的20例健康志愿者的样本作为对照，采用实时荧光定量PCR验证差异基因在AML患者体内的表达水平。【结果】生物信息数据分析显示，根据R语言计算出的NK2转录因子相关基因位点3(*NKX2-3*)的最佳截断值0.051进行生存分析，发现与低表达*NKX2-3*的AML初发患者相比，高表达*NKX2-3*的AML初发患者总体生存率较差($P = 0.003\ 6$)；与HI相比，*NKX2-3*在AML初发组患者中显著高表达($P < 0.001$)；实时荧光定量PCR的验证结果也证实*NKX2-3-1*和*NKX2-3-2*在AML初发组患者中高表达，且与健康人组相比有显著相关性($P = 0.000$，$P = 0.000$)。【结论】*NKX2-3*在AML初发组患者中高表达，且高表达*NKX2-3*的AML患者总体生存较差；*NKX2-3*可能与AML临床转归与预后密切相关。

关键词：急性髓系白血病；*NKX2-3*；生物信息学；预后；基因表达；相关性
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Discovery of A New Prognostic Molecular Marker *NKX2-3* for Acute Myeloid Leukemia

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Abstract:【Objective】To analyze the expression of molecular marker affecting the prognosis of acute myeloid leukemia (AML) patients from bioinformatics database, thus providing an experimental basis for further exploration of a novel molecular marker for the prognosis of AML.【Methods】The prognostic data of 179 AML patients from The Cancer Genome Atlas (TCGA) database were examined for differential gene analysis and survival analysis. The bone marrow samples of 74 healthy individuals (HI) and 542 de novo AML patients in the dataset GSE13159 downloaded from the Gene Expression Omnibus (GEO) database were analyzed to detect the difference in the expression levels of differential target genes. Periph-

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eral blood and bone marrow samples were collected from 18 de novo AML patients and 20 age- and gender-matched healthy controls, and real-time fluorescent quantitative PCR was used to validate the expression levels of the differential genes in the AML patients.【Results】Bioinformatics data analysis showed that the optimal cut-off value of Homo sapiens NK2 homeobox 3 (*NKX2-3*) calculated by R language was 0.051. Survival analysis revealed a statistically poorer overall survival in de novo AML patients with high *NKX2-3* expression than in those with low *NKX2-3* expression (P = 0.0036). *NKX2-3* was highly expressed in patients with de novo AML than in HI and the difference was statistically significant (P < 0.001). Real-time fluorescence quantitative PCR verified the expression levels of the *NKX2-3* gene in AML patients and confirmed that compared with those in HI, in the de novo AML patients, *NKX2-3-1* and *NKX2-3-2* were highly expressed and were significantly correlated (P = 0.000, P = 0.000).【Conclusion】*NKX2-3* is highly expressed in de novo AML patients, and the AML patients with high *NKX2-3* expression have poor overal survival. *NKX2-3* may be closely related to the clinical outcome and prognosis of AML.

Key words: acute myeloid leukemia (AML); *NKX2-3*; bioinformatics; prognosis; gene expression; correlation

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急性髓系白血病(acute myeloid leukemia, AML)是起源于髓系造血细胞的恶性克隆性疾病,其病情发展迅速,自然病程仅数月[1-3]。AML属于最常见的成人急性白血病类型,其发病率为3.7/100 000人;并且,该疾病的发生风险随年龄增长而增加,死亡率近2.7-18/100 000人[4-5]。虽然近年来AML的诊疗水平有显著提高,预后有所改善;但AML总体生存率仍偏低,60岁以上老年患者及高危患者5年生存率甚至不到15%[6-8]。因此,深入研究AML发生发展相关机制,寻找新的临床诊疗靶点及预后相关标志物是血液学领域的重要研究课题,具有重要的科学意义。NK转录因子相关基因位点3(homo sapiens NK2 homeobox 3, *NKX2-3*)家族是一类属于孤儿同源蛋白的转录因子,参与调节多种细胞发育过程,在肿瘤发生发展过程中也可能起着重要的作用。既往研究发现*NKX2-1*、*NKX2-2*和*NKX2-8*在实体瘤中具致癌作用[9-10];*NKX2-1*、*NKX2-3*和*NKX2-5*在急性T淋巴细胞性白血病(T-ALL)中低表达并参与发病机制[11-13];*NKX2-3*也参与了部分淋巴瘤和急性B淋巴细胞性白血病(B-ALL)的发病进程[14-15]。然而,*NKX2*家族在AML中的表达情况及其与临床转归和预后的相关性尚无报道。癌症基因组图谱(the cancer genome atlas, TCGA)数据库主要储存包括RNAseq, miRNAseq, DNA甲基化, CNV, SNP等各类癌症基因组相关信息,是肿瘤研究领域的一个功能强大且数据全面的分子数据类型存储库,该数据集是肿瘤领域最大和最常用的公共资源,能提供数千个肿瘤样本的体细胞突变、基因表达、基因甲基化和拷贝数变异等数据[16-20]。利用TCGA数据库筛选影响AML临床转归和预后的有效分子标志物,可为AML早期诊疗和预后评价提供新的思路和策略。本研究通过分析数据库中AML患者预后数据,筛选出*NKX2*家族与AML患者临床转归和预后密切相关的差异性基因;应用实时荧光定量PCR验证差异基因在AML患者体内的表达水平,初步筛选出了AML预后的新型分子标志物。

1 材料与方法

1.1 通过生物信息学方法对AML患者的预后数据进行分析

首先通过TCGA数据库(https://portal.gdc.cancer.gov)下载AML的数据,纳入179例样本,纳入标准:①样本被诊断为AML;②样本有完整的临床信息。使用基因集富集分析(gene set enrichment analysis, GSEA, https://www.gsea-msigdb.org/gsea/msigdb/cards/WP_FERROP-TOSIS.html)数据库筛选出*NKX2*家族与AML患者临床转归和预后密切相关的差异性基因;通过R语言计算得出最佳截断值,再利用最佳截断值把基因分为高表达和低表达两组,结合AML患者的临床标本信息进行生存分析。

1.2　分析结果的差异性

利用基因表达集锦（gene expression omnibus，GEO，https：//www.ncbi.nlm.nih.gov）数据库中的GSE13159数据集进行分析，其中包含2 022例血液肿瘤的病人（包括AML、MDS、T-ALL、B-ALL、CLL、CML）和74例健康人（healthy individual，HI）（GSE13159数据集中的编号为：Non-leukemia and healthy bone marrow）的骨髓标本，其中在2 022例血液肿瘤中，包含542例AML骨髓样本，其纳入标准与上述在TCGA数据库中的相同。检测上述目的基因在AML初发患者骨髓表达与健康人骨髓表达是否存在差异性。用Prism 9.0软件将计算结果绘制成阶梯图及小提琴图。

1.3　样本的收集

收集18例初发AML患者的外周血或骨髓样本，收集AML患者临床疗效等相关临床资料；同时，收集7例完全缓解的AML患者和20例健康人外周血样本作为对照。在18例AML初发患者中，男12例，女6例，年龄21~85岁，中位年龄为58岁；在7例完全缓解对照组中，男3例，女4例，年龄在32~72岁，中位年龄为54岁；在20例健康对照组中，男9例，女11例，年龄在28~63岁，中位年龄为41岁。所有样本采集已通过暨南大学医学伦理委员会的伦理审查，患者均已签署知情同意书。

1.4　外周血或骨髓单个核细胞的分离

① 将人淋巴细胞分离液（Ficoll）4 mL加至15 mL尖底离心管管底，用1×PBS缓冲液1∶1稀释外周血或者骨髓样本后，混匀充分，将其沿离心管管壁缓慢平铺于Ficoll液面，在500×*g*转速下离心15 min。② 离心后，轻轻吸取中间白色云雾状细胞层，加至装有已准备好的8 mL 1×PBS缓冲液的15 mL尖底离心管，混匀充分，在350×*g*转速下离心10 min。③ 弃掉上清后，用1×PBS缓冲液重悬细胞并计数。

1.5　RNA的提取和cDNA的合成

① 按照RNA提取Trizol试剂盒的说明书操作方法，依据每10^7细胞加Trizol 1 mL至1.5 mL无菌Ep离心管以提取RNA。将已混合有Trizol的每管分别加入0.2 mL氯仿，缓慢上下翻转充分混匀30 s，在室温下静置5 min；利用低温高速离心机以4 ℃、13 400×*g*转速离心30 min。② 小心吸取上清转移至1.5 mL新Ep管后加入0.5 mL异丙醇混匀充分，低温静置10 min，以4 ℃、13 400×*g*转速离心15 min。③ 吸取上清，加入-20 ℃的75 %乙醇1 mL，充分混匀30 s，以4 ℃、10 000×*g*离心10 min。④ 尽量去除乙醇后在冰上进行干燥。⑤ 根据所得RNA的量不同，分别将其溶解于20-30 μL超纯水，于-20 ℃保存。⑥取2 μL RNA溶液，用0.8 %（w/v）的琼脂糖凝胶电泳检测提取RNA的质量。⑦ 按照反转录酶试剂盒操作说明反转录合成cDNA第一链，反应条件为25 ℃，10 min；37 ℃，60 min；85 ℃，5 min，反应结束后产物于-20 ℃贮存备用。⑧ 以合成后的cDNA作为模板，经PCR检测管家基因β_2-微球蛋白（β_2M）（上游引物β_2M-f：5′-TACACTGAATTCACCCCCAC-3′；下游引物β_2M-b：5′-CATCCAATCCAAATGCGGCA-3′）的扩增情况，用以检验样本的质量。

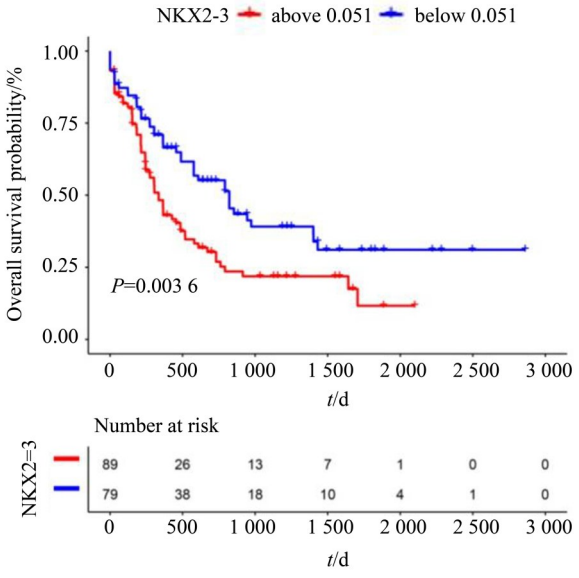
1.6　实时荧光定量PCR检测目的基因表达水平

①按照SuperReal PreMix Plus试剂盒的操作指南，采用SYBR Green I染料检测各cDNA样本中的目的基因表达水平。②反应体系：总体系为20 μL，分别包括2×SuperReal PreMix Plus 10 μL，0.5 mmol/L的上下游引物各0.6 μL，RNase-free ddH_2O 7.8 μL和cDNA 1 μL。每一样本均设置2个复孔；同时，设置无模板的阴性对照用以排除假阳性结果。③采用比较Ct值的相对定量PCR法，以β_2M基因作为内参基因，分析各样本中目的基因mRNA的相对表达水平，经扩增效率一致性检测后，利用所得Ct值，应用公式计算相对mRNA表达量=$2^{-\Delta Ct}$×100%，ΔCt= Ct（目的基因）- Ct（β2M）

2　结　果

通过对TCGA生物信息数据库中179例AML患者的RNA-seq数据及预后数据进行分析，在*NKX*家族的17个基因中筛选与AML患者临床转归和预后密切相关的差异性基因，发现只有*NKX2-3*在AML中高表达并与不良预后有关。将危险分层对*NKX2-3*进行矫正（多因素COX回归分析）并得到矫正的*P*值（$P = 0.192$），说明*NKX2-3*不是独立于危险分层的预后影响因子，其对预后的影响可能受危险分层的影响。并且我们发现与低表达

*NKX2-3*的AML患者相比,高表达*NKX2-3*的患者的总体生存率较差,差异具有统计学意义(log-rank test: $P = 0.003\ 6$;图1)。



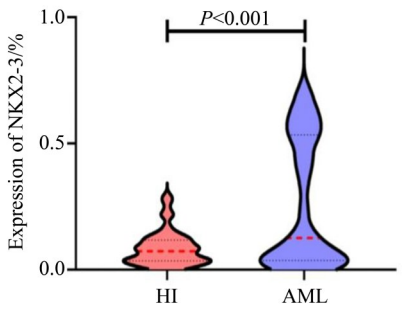
Blue lines represent low expression and red lines represent high expression. Compared with AML patients with low expression of *NKX2-3*, the overall survival rate of patients with high expression of *NKX2-3* was poorer, with statistical difference (sample: 79 cases; log-rank test: $P = 0.003\ 6$).

图1 高表达和低表达*NKX2-3*的AML患者的生存曲线分析

Fig. 1 Survival curves of AML patients with high and low expression of *NKX2-3*

为探索*NKX2-3*在AML初发患者骨髓表达与HI骨髓表达是否存在差异性,对GEO数据库中的GSE13159数据集的74例健康人骨髓标本与542例AML初发患者骨髓标本进行了分析。结果发现,与HI组相比,*NKX2-3*在AML初发组患者中高表达,且差异具有统计学意义(如图2所示,$Z = -4.063$, $P < 0.001$)。

为了探索*NKX2-3*在AML初发患者骨髓中表达与患者预后的相关性,对TCGA数据库中179例AML患者的骨髓样本的转录组测序结果结合临床数据进行了分析,通过R语言计算得出最佳截断值,随后,利用最佳截断值把基因分为高表达(high expression)和低表达(low expression)两组,结合患者的临床标本信息进行生存分析。结果显示,*NKX2-3*在R语言计算得出的最佳截断值为0.051;生存分析发现,与低表达*NKX2-3*的AML初发患者相比,高表达*NKX2-3*的AML初发患者总体生存率较差,具有统计学差异($P = 0.003\ 6$)。高表达*NKX2-3*的AML初发患者3年生存率为24%,低表达*NKX2-3*的AML初发患者生存率为41%。高表达的*NKX2-3*基因可能为影响AML患者预后的相关因素。



Compared with the HI group, *NKX2-3* was highly expressed in the AML-onset group, and the difference was statistically significant (74 bone marrow cases from healthy individuals and 542 bone marrow cases from AML-onset patients; $P < 0.001$).

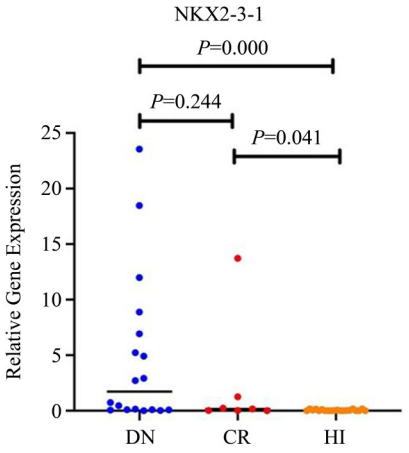
图2 GSE13159数据集中AML患者与健康人*NKX2-3*基因的表达情况

Fig. 2 The expression of *NKX2-3* gene in AML patients and healthy people in the GSE13159 dataset

进一步应用实时荧光定量PCR验证了*NKX2-3*基因在AML患者体内的表达水平(图3-4)。结果显示,18例AML初发组患者中*NKX2-3-1*和*NKX2-3-2*的基因表达水平均显著高于20例健康人对照组的基因表达水平($P < 0.001$, $P < 0.001$);7例AML完全缓解组患者中*NKX2-3-1*和*NKX2-3-2*基因表达水平亦显著高于健康对照组中的表达水平($Z = -2.047$, $P = 0.041$; $Z = -2.988$, $P = 0.002$)。但AML完全缓解组的*NKX2-3-1*和*NKX2-3-2*基因表达水平与AML初发组无显著差异性($Z = -1.210$, $P = 0.244$; $Z = -1.997$, $P = 0.047$)。

3 讨 论

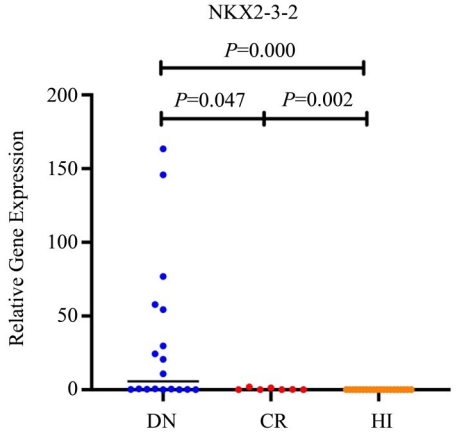
AML总体生存率迄今仍偏低,60岁以上老年患者及高危患者更低,因此,寻找新的临床诊疗靶点及预后相关标志物具有非常重要的意义。我们开展了γδ T细胞上共抑制分子TIGIT及其竞争性共刺激受体CD226表达模式的前期研究,证实在初发AML患者中,$CD226^+$ γδ T细胞比例减少,$TIGIT^+$ γδ T细胞比例增加,且具有较高比例$TIGIT^+CD226^-$



The gene expression levels of NKX2-3-1 in the 18 AML-DN patients were significantly higher than those in the 20 HI(P < 0.001), The expression level of NKX2-3-1 gene in 7 cases of AML-CR group was also significantly higher than that in HI group (P = 0.041), and the NKX2-3-1 expression levels in the AML-CR groups were not significantly different from those in the AML-DN groups (P = 0.244).DN: de novo patient; CR: complete remission patient; HI: healthy individual.

图3　荧光定量PCR检测AML患者与健康人*NKX2-3-1*基因的表达情况

Fig. 3　Fluorescent quantitative PCR detection of *NKX2-3-1* gene expression in AML patients and healthy people



The gene expression levels of NKX2-3-2 in the 18 AML-DN patients group were significantly higher than those in the 20 cases of HI groups (P< 0.001); the NKX2-3-2 gene expression levels in the 7 AML-CR group were significantly higher than those in the HI group (P=0.002). However, there was no significant difference in the expression level of NKX2-3-2 gene between the AML-CR group and the AML-DN group (P = 0.047).

图4　荧光定量PCR检测AML患者与健康人*NKX2-3-2*基因的表达情况

Fig. 4　Fluorescent quantitative PCR detection of *NKX2-3-2* gene expression in AML patients and healthy people

γδ T细胞的非M3-AML患者显示出较低的总体生存率[21]。此外，基于PD-1和Foxp3基因表达与AML患者预后关联性分析发现，与健康人相比，初发AML患者体内PD-1$^+$ γδ、Foxp3$^+$ γδ和PD-1$^+$Foxp3+ γδ T细胞比例显著增高，且含有较高比例PD-1$^+$Foxp3+ γδ T细胞的AML患者的总体生存率较低[22]。因此，深入分析白血病肿瘤微环境中差异性分子表达，筛选出高敏感的分子标志物，不仅能够帮助精准诊断，还可以协助白血病的疗效分析和预后评价[23]，亟待寻找更多的与AML临床预后相关的分子标志物。

转录因子*NKX2*家族参与调节多种基本的细胞发育过程，包括头部模式、心脏和肺的发育以及神经细胞的特异性发育等[24-25]，还可控制唾液腺、牙齿和小肠发育，并已被确定为脾的形态形成和血管结构形成的重要管理蛋白[9]。*NKX2*家族在肿瘤发生发展过程中也可能起着重要的作用。既往研究发现*NKX2-1*、*NKX2-2*和*NKX2-8*在实体瘤中起着致癌作用[9-10]；*NKX2-1*、*NKX2-3*和*NKX2-5*还可以使基因组发生重排，在急性T淋巴细胞白血病（T-ALL）中低表达并参与发病机制[11-13]；*NKX2-3*在部分淋巴瘤患者的边缘区肿瘤细胞中存在过表达，并且，参与ETV6/RUNX1阳性的急性B淋巴细胞性白血病（B-ALL）和T-ALL的发病进程[14-15]。但关于*NKX2*家族在AML中的表达情况及其与临床转归和预后的相关性尚无报道。

本研究通过生物信息学方法对TCGA生物信息数据库中AML患者的预后数据进行分析，筛选出了*NKX2*家族与AML患者临床转归和预后的相关性密切相关的差异性基因*NKX2-3*；利用GEO数据库中的GSE13159数据集对健康人与AML初发患者样本进行分析，证实*NKX2-3*在AML初发组患者中高表达；应用实时荧光定量PCR验证*NKX2-3*基因在AML患者体内的表达水平，进一步证实*NKX2-3*在AML初发组患者中高表达。本研究初步证实了*NKX2-3*作为预后新分子标志的可能性，后期还可以将*NKX2*分子与其他分子标志物联合，检测其在AML细胞或T细胞上的表达情况，为进一步探索*NKX2-3*作为AML预后的新型分子标志物奠定实验基础，帮助制定AML早期诊疗方案和预后评价，为AML患者预后改善带来新的希望。

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