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在线全文

外泌体来源的circRNA_051778在肺腺癌性恶性胸腔积液和结核性胸腔积液中的表达及作用研究*

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【摘要】 目的 分析circRNA_051778在肺腺癌性恶性胸腔积液（LA-MPE）和结核性胸腔积液（TPE）样本中的临床意义。**方法** 本研究为横断面研究。2018年10月–2019年9月间于江西省胸科医院共募集212例患者，收集患者入院第1天胸腔积液和/或血浆。使用circRNA微阵列分析LA-MPE和TPE样本中的外泌体环状RNA（circRNAs），通过微滴式数字PCR验证差异表达环状RNA（DECs）。此外，构建可能的circRNA-miRNA-mRNA网络，并进行了GO（Gene Ontology）分析和KEGG（Kyoto Encyclopedia of Genes and Genomes）通路分析，以预测DECs的功能。通过二分类逻辑回归和受试者工作特征曲线评估circRNA_051778的诊断价值。**结果** circRNA_051778的表达水平在LA-MPE样本中为（3.92±0.48）拷贝数/100 ng cDNA，在TPE样本中为（21.53±2.22）拷贝数/100 ng cDNA。与TPE相比，LA-MPE样本中的circRNA_051778下调（P<0.001）。circRNA_051778的潜在靶标富集于GTPase活性正调控、细胞质、蛋白结合和癌症相关通路中。circRNA_051778与液基薄层细胞学检查（TCT）、红细胞沉降率（ESR）和结核抗体（TBA）联合检测的曲线下面积为0.98（95%置信区间：0.97~ 1.00），敏感性为88.0%，特异性为100.0%。**结论** 外泌体中的circRNA_051778在LA-MPE中下调，GO和KEGG分析结果显示其可能在癌症的发展中发挥作用，与TCT、ESR、TBA联合有望作为LA-MPE和TPE鉴别诊断标志物。

【关键词】 环状RNA 外泌体 胸腔积液 肺结核 肺腺癌

Expression of circRNA_051778 in Lung Adenocarcinoma-Associated Malignant and Tuberculous Pleural Effusions and Its Clinical Significance

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【Abstract】 Objective To investigate the expression and clinical significance of circular RNA (circRNA) 051778 in lung adenocarcinoma-malignant pleural effusion (LA-MPE) and tuberculous pleural effusion (TPE). **Methods** This is a cross-sectional study. A total of 212 patients were recruited from the Jiangxi Chest Hospital between October 2018 and September 2019, and their pleural effusion samples and/or plasma samples were collected. The exosomal circRNA profile was sketched by circRNA microarray. Differentially expressed circRNAs (DECs) were verified by droplet digital PCR. In addition, a putative circRNA-miRNA-mRNA network was constructed, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to predict the functions of the DECs. The diagnostic value of circRNA_051778 was evaluated by binary logistic regression and receiver operating characteristic curve. **Results** The expression level of circRNA_051778 in the LA-MPE samples was (3.92±0.48) copies/100 ng cDNA, while that in the TPE samples was (21.53±2.22) copies/100 ng cDNA. Compared to that in the TPE samples, circRNA_051778 was significantly downregulated in the LA-MPE samples (P<0.001). The potential targets of circRNA_051778 were enriched in positive regulation of GTPase activity, cytoplasm, protein binding, and cancer-related pathways. The area under the curve (AUC) for the combined assessment of circRNA_051778 with liquid-based thin-layer cytology (TCT), erythrocyte sedimentation rate (ESR), and tuberculosis antibody (TBA) was 0.98 (95% confidence interval: 0.97-1.00), with the sensitivity being 88.0% and the specificity being 100.0%. **Conclusion** Exosomal circRNA_051778 is downregulated in LA-MPE. According to the findings from the GO and KEGG analyses, exosomal circRNA_051778 may play a role in cancer development and has the potential to serve as a marker for differential

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diagnostic of LA-MPE and TPE when it is used in combination with TCT, ESR, and TBA.

【Key words】 Circular RNA　Exosome　Pleural effusion　Tuberculosis　Lung adenocarcinoma

正常机体胸膜腔内有3 ~ 15 mL液体, 在呼吸运动时起润滑作用, 腔内液体的产生和重吸收始终保持着动态平衡, 这种状态有利于呼吸。然而, 当胸膜毛细血管的静水压和渗透性增加, 以及胶体渗透压降低、淋巴回流障碍或胸部损伤时, 都会对胸膜液的再吸收产生不利影响, 导致胸腔积液[1]。胸腔积液(pleural effusion, PE)是指胸膜层之间多余液体的积聚, 可在肺结核和恶性肿瘤等各种病理条件下观察到。我国结核性胸腔积液(tuberculous pleural effusion, TPE)和恶性胸腔积液(malignant pleural effusion, MPE)的发病率在过去十年中显著增加[2], 而与其他肺癌相比, 肺腺癌与胸腔积液的相关性更高。肺腺癌性恶性胸腔积液(lung adenocarcinoma-malignant pleural effusion, LA-MPE)和TPE可以分别用于诊断腺癌和肺结核。胸腔积液诊断的敏感度差异较大, 从50%~90%不等[3]。目前, LA-MPE和TPE的主要诊断依据是生化指标、肿瘤标志物或抗结核抗体以及胸腔镜细胞学分析[4], 然而, 在某些情况下, 即使将上述方法联合应用也不能保证诊断的准确性[5], 因此, 亟需更准确的诊断方法[6]。

外泌体是直径为30~ 100 nm的膜性囊泡, 可由多种类型的细胞分泌, 分泌后进入血液、尿液和胸膜液等体液中[7]。外泌体内含与亲本细胞相关的特定蛋白质、RNA和DNA序列[8]。环状RNA(circRNA)是有闭环结构的一类非编码RNA[9], 因对RNA外切酶和核糖核酸酶R具有抗性而高度稳定, 因此circRNA有望作为生物标志用于各种疾病的诊断[10-12]。本研究分析了从TPE和LA-MPE中分离得到的外泌体的circRNAs表达, 并预测了差异表达的circRNAs的潜在功能和诊断价值。

1　资料与方法

1.1　研究对象

随机选取2018年10月–2019年9月在江西省胸科医院就诊, 且诊断为TPE或LA-MPE的患者共212例, 其中TPE患者112例, LA-MPE患者100例, 并收集PE和/或血浆样本。

TPE诊断依据: PE涂片、培养或活检中查见结核分枝杆菌; 或胸膜活检组织中查有肉芽肿伴干酪样坏死, 且抗酸染色阳性, 并除外其他可以引起肉芽肿病变的疾病[13]。排除标准: ①接受免疫抑制治疗或抗结核治疗的患者; ②HIV抗体阳性或有任何恶性肿瘤的患者。LA-MPE诊断依据: PE的细胞沉积物或胸膜活检组织中发现肺腺癌细胞, 排除鳞癌、小细胞肺癌和其他器官来源的恶性肿瘤等[4]。排除标准: ①接受免疫抑制治疗的患者; ②HIV抗体阳性; ③干扰素γ释放试验(IGRA)阳性的患者。该研究获得江西省胸科医院伦理委员会批准(赣胸伦初审字【2021】2号), 所有受试者均知情并签署知情同意书。

1.2　样本收集及处理

本研究共纳入212例患者, 均于入院第一天收集样本, 共收集212份PE样本和30份血浆样本(LA-MPE和TPE患者各15份)。将15 mL PE样本收集于15 mL离心管中, 4 ℃(5810R, Eppendorf, 德国)1 600 r/min离心30 min。弃沉淀, 用0.22 μm膜过滤上清, –20 ℃保存。使用EDTA涂层管收集2 mL外周血样本, 4 ℃ 1 600 r/min离心10 min。0.22 μm膜过滤血浆, 分装, 每份500 μL, 保存于–80 ℃冰箱中。

1.3　外泌体鉴定

将预过滤的样本(PE样本7 mL, 血浆样本500 μL)加入exoEasy离心柱中, 根据exoRNeasy Serum/Plasma Maxi Kit(Qiagen, 德国)说明书进行处理。用100 μL PBS代替试剂盒中的QIAzol从离心柱中洗脱外泌体。使用透射电镜(TEM)(HT-7700, Hitachi, 日本)观察外泌体形态; 采用纳米颗粒跟踪分析(NTA)技术(ZetaVIEW, Particle Metrix, 德国)检测外泌体直径。使用Western blot检测4种外泌体标志物(Calnexin、TSG101、CD81和CD63), 并以此评估分离的外泌体质量。

1.4　外泌体RNA提取及逆转录

根据exoRNeasy Serum/Plasma Maxi Kit (Qiagen, 德国)说明书提取外泌体RNA。使用超微分光光度计(NanoPhotomete®N60, Implen, 德国)测定外泌体RNA的浓度和纯度。按照制造商的说明, 使用随机引物混合物和iScript cDNA Synthesis Kit(Bio-Rad, 美国)进行逆转录, 模板为1 μg(PE)或200 ng(血浆)RNA。反应参数为: 25 ℃ 5 min, 46 ℃ 30 min, 95 ℃ 1 min。

1.5　circRNAs微阵列

通过Human circRNA Array V2(Arraystar, 美国)分析6个外泌体RNA样本(TPE和LA-MPE各3个)的circRNA表达谱。首先, 用Rnase R(Epicentre, Inc., 美国)消化每个样本的全部的RNA, 并根据Arraystar Super RNA Labeling kit (Arraystar, Inc., 美国)的说明, 用荧光标记的随机引物进行扩增。使用RNeasy Mini Kit(Qiagen, 德国)纯化标记的cRNA, 并通过NanoDrop ND-1000 (ThermoFisher Scientific, 美国)测量其浓度和比活性(pmol Cy3/μg

cRNA)。随后使用5 μL 10× Blocking Agent和1 μL 25× Fragmentation Buffer在60 ℃下将1 μg RNA片段化30 min。用25 μL 2× Hybridization Buffer稀释反应混合物后,将50 μL溶液分装到垫圈载玻片中,并组装到circRNA表达微阵列载玻片中。在65 ℃的Agilent Hybridization Oven中孵育17 h后,清洗、固定杂交阵列,使用Agilent Scanner G2505C扫描。将扫描的图像导入Agilent Feature Extraction software,对原始数据进行分位数归一化并过滤低强度信号。计算两组之间每个circRNA的倍数变化,使用*t*检验进行差异性检验,倍数变化≥2且*P*值≤0.05的circRNAs被定义为差异表达circRNAs(DECs)。

1.6 circRNAs验证

选择小样本量中错误发现率(FDR)< 0.05的前10个circRNAs,用QX200 Droplet Digital PCR (ddPCR) System (Bio-Rad,美国)进行扩增。使用Circinteractome网络工具为每个circRNA设计不同引物(https://circinteractome.nia.nih.gov/),使用Primer BLAST(https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi)设计*GAPDH*的引物,引物序列如附表1所示。每份Mix中含有10 μL 2× QX200 ddPCR Eva Green Supermix(Bio-Rad, USA),100 nmol/L od引物,100 ng(PE)或50 ng(血浆)cDNA。使用QX200 Droplet Generator(Bio-Rad,美国)生成液滴,液滴中含有20 μL mix和70 μL Droplet Generation Oil for EvaGreen(Bio-Rad,美国),将微滴转移至96孔板中,用铝箔封板,并在C1000 TouchTouch thermal cycler(Bio-Rad,美国)中进行PCR,反应条件为:95 ℃ 5 min,随后95 ℃ 30 s、60 ℃ 1 min,共40个循环,4 ℃ 5 min,90 ℃ 5 min以稳定信号。在室温下读取Bio-Rad QX200液滴读取器上信号,并通过Quanta Soft软件进行分析。将每个circRNA阳性的样本分别合并、扩增、纯化并进行Sanger测序。基于这些结果,采用dd-PCR分析所有PE和血浆样本中DECs的拷贝数。常规PCR的体系总体积为25 μL,体系中含有12.5 μL 2× Taq PCR master mix(Vazyme,中国),正反引物各1 μL和10.5 μL cDNA。PCR条件为95 ℃ 3 min,然后在95 ℃ 15 s、60 ℃ 15 s和72 ℃ 30 s,循环35次,最后72 ℃延伸5 min,并对扩增产物进行Sanger测序。

1.7 生物信息学分析

用聚类分析方法和箱线图比较两组circRNAs的分布,并用散点图和火山图对DECs进行可视化。使用基于target Scan和miRanda的Arraystar's miRNA target prediction software预测circRNA/miRNA相互作用。使用miRDIP(http://ophid.utoronto.ca/mirDIP/)和miRWalk(http://mirwalk.umm.uni-heidelberg.de/)预测每个miRNA的靶点mRNAs。使用Veen diagram webtool(http://bioinformatics.psb.ugent.be/webtools/Venn/)可视化上述靶点mRNAs交集。最后,通过DAVID数据库(Database for Annotation, Visualization and Integrated Discovery)对mRNAs进行GO(Gene Ontology)和KEGG(Kyoto Encyclopedia of Genes and Genomes)分析。

1.8 统计学方法

连续变量以$\bar{x}\pm s$表示,使用Windows SPSS 13.0版(SPSS Inc.,美国)中的单样本Kolmogorov-Smirnov test进行正态性检验。通过Graph Pad Prism 9(Graph Pad Software Inc.,加拿大)进行统计分析,正态分布数据通过独立样本*t*检验或配对样本*t*检验进行比较,非正态分布变量通过Mann-Whitney检验进行比较。使用SPSS 13.0版(SPSS Inc.,美国)中二元逻辑回归和受试者操作特征(ROC)曲线分析方法,对circRNA_051778和其他区分TPE和LA-MPE的方法的诊断价值进行研究,诊断价值的高低主要通过ROC曲线下的面积(AUC)来判断:AUC越接近1,表示该诊断方法的准确性越高,即诊断价值越大。*P*< 0.05时,差异有统计学意义。本研究使用和分析的数据集可从基因表达综合数据库获得(GEO, https://www.ncbi.nlm.nih.gov/geo/),GEO编号为GSE171540。

2 结果

2.1 患者临床信息和病理特征

LA-MPE 患者与TPE 患者年龄差异有统计学意义(*P*< 0.001);LA-MPE-P组与TPE-P组患者年龄及性别差异均无统计学意义(表1)。

表 1 受试者临床信息

Table 1 The clinical information of the subjects

Index	LA-MPE (*n*=100)	TPE (*n*=112)	*P*	LA-MPE-P (*n*=15)	TPE-P (*n*=15)	*P*
Age/yr., $\bar{x}\pm s$	62.16±15.86	48.05±21.10	<0.001	63.20±2.63	54.67±3.84	0.08
Sex/case (%)			0.16			0.43
Male	58 (58.0)	80 (71.0)		9	12	
Female	42 (42.0)	32 (29.0)		6	3	

LA-MPE: lung adenocarcinoma-malignant pleural effusion; TPE: tuberculous pleural effusion; LA-MPE-P refers to the LA-MPE patients who provided plasma samples; TPE-P refers to the TPE patients who provided plasma samples.

LA-MPE组(100例)中, T4期最为常见(50.0%), N2和N3阶段的淋巴结转移较多, 远处转移主要为M1b(70.0%), 肿瘤分级以Ⅳ级为主(98.0%)。LA-MPE-P组(15例)中, T2期、T4期较多(均为5/15), N2阶段的淋巴结转移较多(10/15), M1a远处转移的比例较高(8/15), 肿瘤分级以Ⅳ级为主(13/15), Ⅲ级相对较少(2/15)(表2)。

表 2　受试者病理特征

Table 2　The pathological features of the subjects

Index	LA-MPE (*n*=100)	LA-MPE-P (*n*=15)
Topography/case (%)		
TI	20 (20.0)	1
T2	16 (16.0)	5
T3	14 (14.0)	4
T4	50 (50.0)	5
Lymph node metastasis/case (%)		
N0	8 (8.0)	1
N1	20 (20.0)	1
N2	28 (28.0)	10
N3	26 (26.0)	3
NX	18 (18.0)	0
Metastasis/case (%)		
M0	2 (2.0)	2
M1a	22 (22.0)	8
M1b	70 (70.0)	2
M2a	6 (6.0)	3
Grade/case (%)		
Ⅰ	0	0
Ⅱ	0	0
Ⅲ	2 (2.0)	2
Ⅳ	98 (98.0)	13

LA-MPE: lung adenocarcinoma-malignant pleural effusion; TPE: tuberculous pleural effusion; LA-MPE-P refers to the LA-MPE patients who provided plasma samples; TPE-P refers to the TPE patients who provided plasma sample.

2.2　外泌体特征

TEM下, 分离出来的外泌体为中央凹陷的圆饼状。TPE和LA-MPE的外泌体直径平均大小为165.7~ 182.0 nm。Western blot检测外泌体蛋白提取物中外泌体标志蛋白CD9和TSG101阳性(附图S1)。所有附图见网络附件。

2.3　外泌体RNA质量浓度

从LA-MPE和TPE样本中提取的全部外泌体RNA质量浓度为(130.70±14.26) ng/μL和(151.30±21.67) ng/μL, $OD_{260}/_{280}$比值分别为1.88±0.02和1.90±0.02。从肺腺癌和肺结核患者的血浆样本中提取的全部外泌体RNA质量浓度为(8.48±2.91) ng/μL和(7.12±4.16) ng/μL, $OD_{260}/_{280}$比值分别为1.44±0.17和1.51±0.17, 两组之间差异无统计学意义。

2.4　cricRNAs表达谱

LA-MPE组中标记的cRNAs的总量和比活性分别为(7.43±0.08) μg和(22.13±0.24) pmol Cy3/μg cRNA, TPE组分别为(7.30±0.13) μg和(22.62±0.48) pmol Cy3/μg cRNA。共分析13 617个circRNAs的表达谱, 7 633个circRNAs的分层聚类显示了6个样本的不同表达模式(附图S2)。此外, 相对于TPE, LA-MPE中有236个circRNAs上调, 214个下调(附图S3)。circRNA_406246、circRNA_100759、circRNA_025016、circRNA_012889和circRNA_102101的上调最为显著, 分别上调21.38倍、13.32倍、10.38倍、8.75倍和8.69倍, 而circRNA_ 007850、circRNA_400019、circRNA _051778、circRNA_004121和circRNA_403658的下调最为明显, 分别下调约93.87%、93.69%、92.93%、92.83%和92.95%。

2.5　差异表达circRNAs的验证

通过dd-PCR和Sanger测序, 在13个LA-MPE和TPE样本中验证了上述10个DECs的拷贝数。如附图S4所示, 与TPE相比, 只有circRNA_051778在LA-MPE中显著下调约86.6%(P< 0.001)。进一步在212个(100个LA-MPE和112个TPE)样本中进行验证, 结果显示, circRNA_051778的表达水平在LA-MPE样本中为(3.92±0.48)拷贝数/100 ng cDNA, 在TPE样本中为(21.53±2.22)拷贝数/100 ng cDNA (经年龄调整后P< 0.001)(图1)。此外, circRNA_051778在肺腺癌患者血浆中表达水平为(0.96±1.16)拷贝数/50 ng cDNA, 在肺结核患者血浆中为(1.71±1.80)拷贝数/50 ng cDNA。肺腺癌患者的血浆和PE中的外泌体circRNA _051778水平相似(图2A), 肺结核患者的PE样本中circRNA_051778的表达比其在血浆样本的表达高4倍

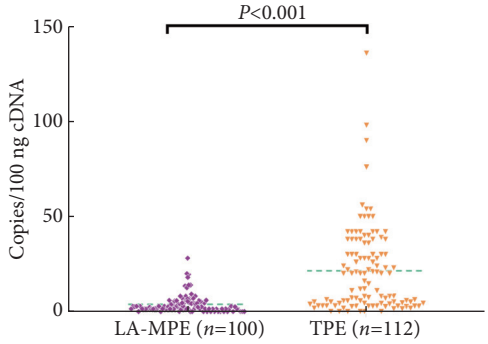

图 1　100个肺腺癌性恶性胸腔积液样本和112个结核性胸腔积液样本中circRNA_051775表达情况

Fig 1　The expression of circRNA_051778 in 100 LA-MPE samples and 112 TPE samples

In the scatter plots, the straight line represents the mean. The abbreviations are explained in the note to Tab. 1.

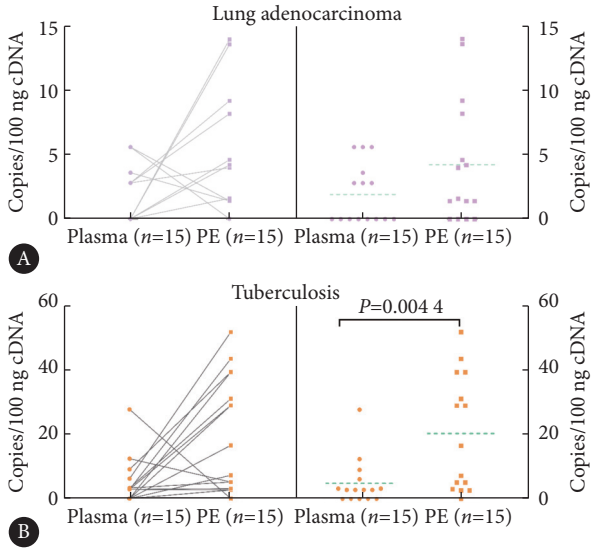

图 2 circRNA_051778在肺腺癌和肺结核配对的血浆和PE样本中的表达

Fig 2 The expression of circRNA_051778 in matched plasma and PE samples from the lung adenocarcinoma and tuberculosis groups

A, Exosomal circRNA_051778 level in the malignant PE and plasma samples of lung adenocarcinoma patients. B, Exosomal circRNA_051778 level in the PE and plasma samples of tuberculosis patients. In the scatter plots, the straight line represents the mean. PE: pleural effusion.

(图2B)。

2.6 circRNA_051778靶基因的功能注释

研究表明, circRNAs上有miRNA应答元件(MERs), 可以作为miRNA海绵螯合特定的miRNA, 调节>60%的mRNA表达。因此, 本研究接下来分析了circRNA_051778可能的circRNA-miRNA-mRNA相互作用网络。根据MERs的互补序列推定, circRNA_051778的可能靶标miRNA为miR-6762-5p、miR-762、miR-4697-5p、iR-4739和miR-4640-5p。circRNA-mi-RNA相互作用网络如附图S5所示。接下来, 本研究使用miRDIP和miRwalk数据库预测上述miRNA的靶mRNA,数据库预测结果显示, miR-6762-5p有6 758个mRNA靶标、miR-762有11 653个靶标、miR-4697-5p有8 882个靶标、miR-4739有12 545个靶标、miR-4640-5p有9891个靶标。对上述mRNA取交集后得到2 852个靶mRNA (附图S6)。进一步对交集靶mRNA进行GO分析, 分析结果表明生物过程(BP)主要富集于GTP酶活性的正调控、信号转导和细胞内信号转导、细胞质、胞质溶胶和膜的细胞组分[14], 分子功能(MF)主要富集于蛋白质结合、钙调蛋白结合和丝氨酸/苏氨酸蛋白激酶活性。大多数靶mRNA富集于信号转导, 922个靶mRNA富集在细胞质中, 超过一半的靶mRNA富集于蛋白质结合。富集前十的BP、细胞组分(CC)和MF如表3所示。KEGG分析显示, 上述交集mRNA对应基因富集于83个通路中, 其中23个是信号转导通路, 包括心肌细胞肾上腺素能信号、催产素信号、甲状腺激素信号、AMPK信号和雌激素信号通路; 还有13种癌症相关通路, 包括小细胞肺癌和非小细胞肺癌; 结核相关通路也位于富集通路中。前五个显著富集的通路是心肌细胞的肾上腺素能信号通路、胆碱能突触、催产素信号通路、甲状腺激素信号通路和癌症通路, 大多数基因富集在癌症相关的通路中, 前15个显著富集的通路如图3所示。

2.7 诊断价值评估

本研究评估了circRNA_051778、红细胞沉降率(ESR)、液基薄层细胞学检查(TCT)、结核抗体(TBA)、腺苷脱氨酶(ADA)、乳酸脱氢酶(LDH)、5种肿瘤生物标志物(CA199、CA153、CA125、AFP和CEA)以及上述方法组合的诊断价值。如表4所示, LDH的诊断价值不高, 而TCT的AUC为0.88 (95%CI: 0.08 ~ 0.93), 这表明其具有较高诊断价值。此外, circRNA_051778的AUC为0.82 (95%CI: 0.76 ~ 0.87), 与ESR、TBA、ADA、血清或胸腔积液肿瘤生物标志物相比, 有更好的诊断价值。当阈值为1.7拷贝数/100 ng cDNA时, circRNA_051778的特异性和敏感性分别为92.9%和43.0%。当阈值增加到20.4拷贝数/100 ng cDNA时, 敏感性达到99.0%, 但特异性仅为44.6%。与circRNA _051778联合应用时, TCT、ESR、TBA各自的诊断价值分别显著提高。TCT、ESR和TBA组合后AUC高达0.96(95%CI: 0.94 ~ 0.99), 与circRNA_051778组合后, 无论其他指标是否存在, AUC进一步增加至0.98 (95%CI: 0.97 ~ 1.00), 四者联合诊断的敏感性和特异性分别为88.0%和100.0%, 预测概率为0.250。当预测概率为0.962时, 特异性达到100.0%, 但敏感性仅为40.2%。circRNA_051778, TCT, TCT、ESR和TBA联合, TCT、ESR、TBA和circRNA_05778联合的ROC曲线如图4所示。

3 讨论

PE是各种病理条件下常见的临床症状, 常根据身体外观、pH、细胞计数、LDH和葡萄糖水平进行诊断。此外, PE中肿瘤生物标志物、抗TBA抗体、恶性细胞、结核杆菌和ESR的检测可以补充对癌症和结核病的诊断。然而, 这些检测的阳性率在临床上仍不够用[15]。尽管PE涂片或培养物的抗酸反应阳性可以证实肺结核, 但PE培养物和痰液的最高阳性率仅为63%和55%[16]。胸膜液外泌体[17-19]中特异性肽[20-21]、蛋白质[22]、miRNA[23-25]和mRNA[26-27]的存在和水平可以更准确地识别某些疾病[28]。然而, 关于胸膜液中外泌体circRNAs的诊断意义知之甚少。

通过对肺腺癌和肺结核患者的PE中circRNAs表达谱进行分析发现,相对于TPE样本, LA-MPE中circRNA_

表 3　circRNA_051778的2852个预测 mRNA 靶标的前10富集的生物过程（BP）、细胞组分（CC）和分子功能（MF）GO术语

Table 3　The top 10 enriched biological process (BP), cellular component, and molecular function (MF) Gene Ontology terms for the 2852 predicted mRNA targets of circRNA_051778

Classification	GO ID	Term	Number of genes	Number of select	−lg (*P*)
Biological process					
	GO: 0007165	Signal transduction	2490	230	(5,10]
	GO: 0043547	Positive regulation of GTPase activity	2490	135	(5,10]
	GO: 0006468	Protein phosphorylation	2490	100	(2,5]
	GO: 0035556	Intracellular signal transduction	2490	95	(5,10]
	GO: 0007411	Axon guidance	2490	41	(2,5]
	GO: 0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	2490	28	(2,5]
	GO: 0060291	Long-term synaptic potentiation	2490	15	(2,5]
	GO: 0042177	Negative regulation of protein catabolic process	2490	14	(2,5]
	GO: 0051968	Positive regulation of synaptic transmission, glutamatergic	2490	10	(2,5]
	GO: 0021795	Cerebral cortex cell migration	2490	7	(2,5]
Cellular component					
	GO: 0005737	Cytoplasm	2638	922	(15,20]
	GO: 0005886	Plasma membrane	2638	673	(2,5]
	GO: 0005829	Cytosol	2638	587	(5,10]
	GO: 0005654	Nucleoplasm	2638	478	(2,5]
	GO: 0016020	Membrane	2638	395	(5,10]
	GO: 0005789	Endoplasmic reticulum membrane	2638	163	(2,5]
	GO: 0000139	Golgi membrane	2638	119	(2,5]
	GO: 0016324	Apical plasma membrane	2638	68	(2,5]
	GO: 0014069	Postsynaptic density	2638	47	(2,5]
	GO: 0030027	Lamellipodium	2638	46	(5,10]
Molecular function					
	GO: 0005515	Protein binding	2492	1476	(15,20]
	GO: 0005524	ATP binding	2492	276	(2,5]
	GO: 0004674	Protein serine/threonine kinase activity	2492	86	(2,5]
	GO: 0004672	Protein kinase activity	2492	78	(2,5]
	GO: 005096	GTPase activator activity	2492	65	(2,5]
	GO: 005516	Calmodulin binding	2492	52	(10,15]
	GO: 0005089	Rho guanyl-nucleotide exchange factor activity	2492	23	(2,5]
	GO: 0003707	Steroid hormone receptor activity	2492	20	(2,5]
	GO: 0017112	Rab guanyl-nucleotide exchange factor activity	2492	13	(2,5]
	GO: 0008331	High voltage-gated calcium channel activity	2492	7	(2,5]

051778显著下调。此外, 与血浆样本相比, 肺结核患者PE样本中circRNA_051778的水平显著更高。研究表明, 肺部恶性肿瘤或肺结核使通透性增加, 从而促进血浆在毛细血管壁和胸膜间皮细胞的渗透, 最终导致液体积聚[29]。此外, 感染结核杆菌的胸膜间皮细胞产生高水平的MMP-1、MMP-9、白细胞介素(IL)-8和IL-18, 这些因子会进一步破坏胸膜。笔者推测在结核性PE中检测到的circRNA_051778主要源自受感染的胸膜间皮细胞[30-32]。circRNA_051778来源于支链氨基转移酶2基因(*BCAT2*)的外显子(chr19: 49298318-49303095)。BCAT2的编码蛋白在支链氨基酸(BCAA)分解代谢途径中发挥重要作用, 进而调节脂肪生成和胰岛素分泌[33]。

circRNAs上有多个miRNA结合位点, 可作为miRNA海绵(miRNA sponge)或竞争性内源RNA(ceRNA)与mRNA竞争结合miRNA, 从而调节mRNA的稳定性、转录和翻译, 影响基因表达[34]。根据与miRNA种子序列互补的8mer、7mer-m8和6mer位点鉴定, miR-6762-5p、miR-762、miR-4697-5p、miR-4739和miR-4640-5p为

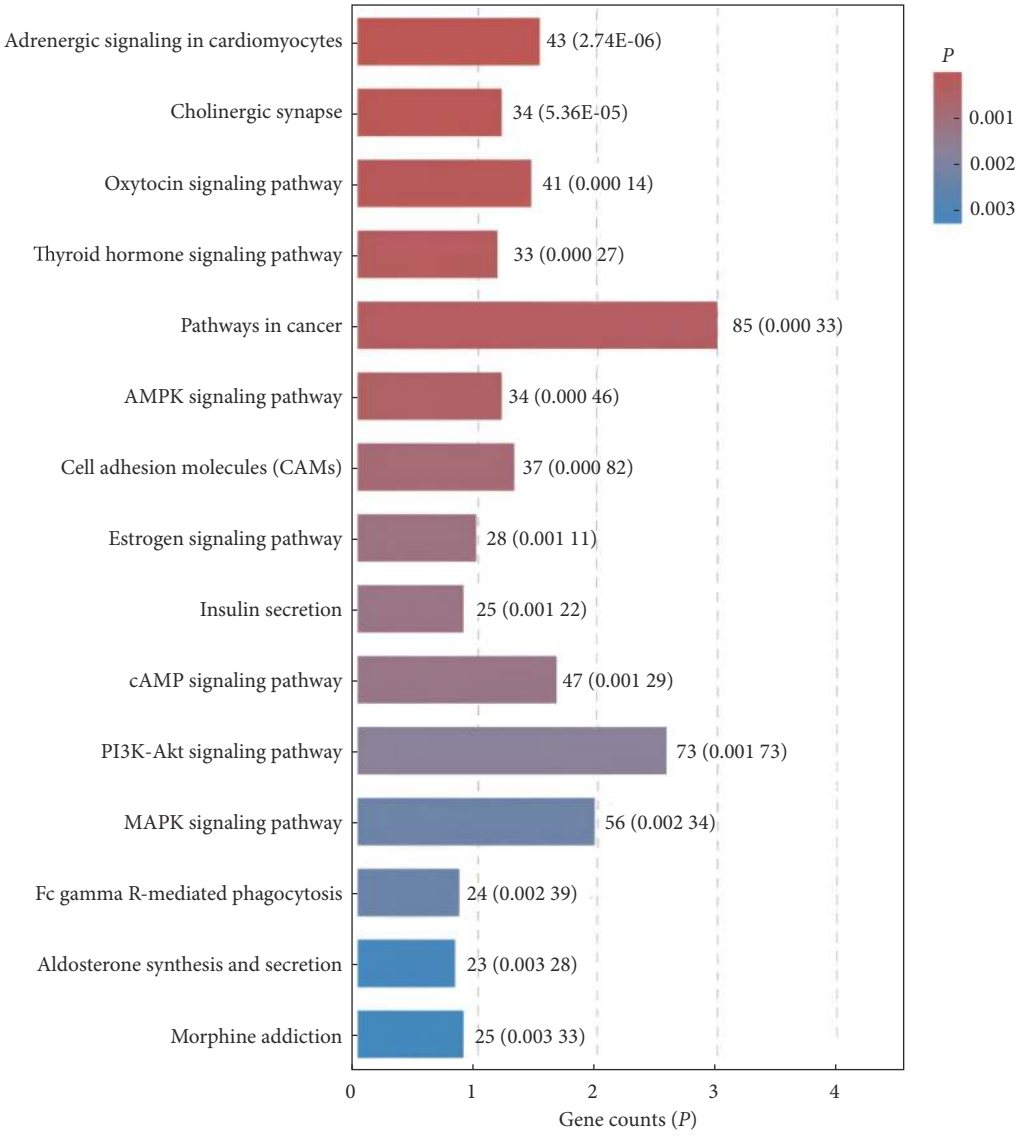

图 3 circRNA_051778的2 852个预测mRNA靶点的前15个显著富集的KEGG通路

Fig 3 The top 15 significantly enriched KEGG pathways for the 2 852 predicted mRNA targets of circRNA_051778

cirRNA_051778的靶标。研究表明，miR-6762-5p通过激活RhoA促使细胞骨架发生改变，从而在宿主中成功传播细菌，在结核感染中也可能发挥了类似的作用[35]；上调的miR-762表明癌症预后不良[36-37]，较低的miR-762水平可下调非小细胞肺癌癌细胞的增殖能力[36]；miR-4697-5p在癌症中高表达，有作为肿瘤生物标志物的潜力[38]；miR-4739上调可能与某些癌症预后不良相关[39]。

miRNA是内源性小型非编码RNA，在转录后水平调节基因表达，通过降解mRNA或抑制翻译发挥作用[40]。本研究鉴定了2 852个mRNA作为上述5种miRNA的共同假定靶点。GO分析表明，GTP酶活性相关基因*FAM13A*和*RGS4*生物过程和分子功能均有参与，这表明circRNA_051778具有巨大的潜在生物功能。KEGG通路富集于信号传导和癌症相关通路，这说明了circRNA _051778在癌症发展发挥了作用。

与TCT以外的其他方法相比，外泌体circRNA_051778对LA-MPE和TPE的鉴别诊断准确性更高。无论是否包含ADA、LDH或肿瘤生物标志物，TCT、ESR和TBA联合使用均可提高诊断准确性，这与以往的研究结果相矛盾[41]。将circRNA_051778纳入上述组合中，使用不同的阈值时，AUC将增加到0.98（95%CI: 0.97 ~ 1.00），敏感性或特异性增加到100.0%。添加其他指标并没有进一步改善结果。因此，临床医生应考虑对疑似LA-MPE或TPE病例进行这些检测的必要性。与TCT相比，circRNA_051778在外泌体中的稳定性较高，不容易被降解，适合长期储存和运输，提高了检测的灵敏性和特异性，而TCT的检测结果可能受到细胞样本质量和处理方法的影响。

本研究存在几个局限性。尽管一些原发性或转移性

表 4　常用诊断方法及其与circRNA_051778结合的曲线下面积

Table 4　Area under the curve of commonly used diagnostic methods and their combined use with circRNA_051778

Diagnostic index	Area	Std. Error	95% confidence interval	
			Lower bound	Upper bound
ESR	0.76	0.03	0.70	0.83
STB	0.69	0.04	0.61	0.76
PTB	0.63	0.04	0.55	0.70
TBA	0.75	0.04	0.68	0.81
TCT	0.88	0.03	0.08	0.93
ADA	0.68	0.04	0.61	0.75
LDH	0.59	0.04	0.51	0.66
circRNA_051778	0.82	0.03	0.76	0.87
ESR+circRNA_051778	0.90	0.02	0.86	0.97
TCT+circRNA_051778	0.96	0.01	0.94	0.99
TBA+circRNA_051778	0.89	0.02	0.85	0.93
STB+circRNA_051778	0.85	0.03	0.80	0.90
PTB+circRNA_051778	0.82	0.03	0.77	0.88
ADA+circRNA_051778	0.84	0.03	0.78	0.89
LDH+circRNA_051778	0.82	0.03	0.76	0.87
ESR+TCT	0.94	0.02	0.90	0.97
ESR+TBA+TCT	0.96	0.01	0.94	0.99
ESR+STB+PTB+TBA+TCT+ADA+LDH	0.96	0.01	0.94	0.99
ESR+TCT+circRNA_051778	0.98	0.01	0.96	0.99
ESR+TBA+TCT+circRNA_051778	0.98	0.01	0.97	1.00
ESR+STB+PTB+TBA+TCT+ADA+LDH+ circRNA_051778	0.98	0.01	0.97	1.00

ESR: erythrocyte sedimentation rate; STB: serum tumor biomarkers consisting of CA199, CA153, CA125, AFP, and CEA; PTB: pleural tumor biomarkers consisting of CA199, CA153, CA125, AFP, and CEA; TBA: tuberculosis antibody; TCT: thin-prep cytology test; ADA: adenosine deaminase; LDH: lactate dehydrogenase.

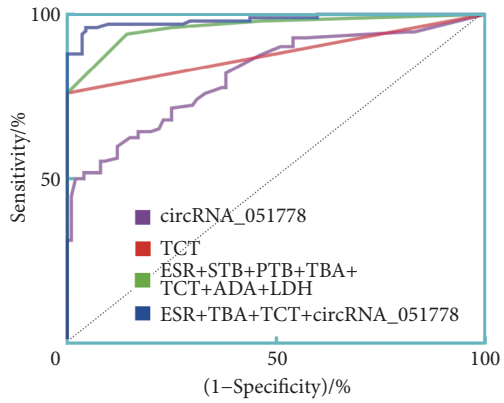

图 4 circRNA_051778，TCT，TCT、ESR和TBA联合，TCT、ESR、TBA和circRNA_051778联合的ROC曲线

Fig 4 The ROC curves of circRNA_051778, TCT, the combination of TCT, ESR and TBA, and the combination of TCT, ESR, TBA, and circRNA_051778

恶性肿瘤均可导致恶性PE, 但本研究仅分析了肺腺癌性PE。这是由于肺癌在引起恶性PE的各种来源的肿瘤中占比最高[41-42], 而在引起恶性PE的原发性肿瘤中只有约6%与肺癌无关, 其中又以肺腺癌最为常见, 约63%[43]。课题组目前正在招募符合入选标准的其他类型癌症患者以进行进一步的分析。此外, 由已确诊的心脏、肝脏和肾脏疾病引起的PE患者未纳入本研究, 根据Light's criteria, 这些患者的PE类型诊断为渗出液, 因此可能没有任何诊断价值, 但未纳入这些病例可能导致对PE中外泌体circRNA_051778的生物学功能的理解不完整。

本研究虽与WEN等[44]的研究类似, 但结果不同。WEN等的研究关注点在于LA-MPE和TPE细胞沉淀中circRNAs的表达谱, 研究表明了circRNAs在红细胞、白细胞、淋巴细胞、间皮细胞等中的表达, 而本研究更关注的是circRNAs在无细胞上清中的表达。尽管本研究和WEN等的研究从不同的样本类型中筛选出不同的circRNAs, 但本研究结论均证明了circRNA在癌症中的重要作用。

本研究进一步了解了肺腺癌性恶性胸腔积液和结核性胸腔积液外泌体中circRNAs的表达, 从DECs中筛选出了外泌体circRNA_051778, 并构建了可能的circRNA-miRNA-mRNA网络。circRNA_051778可能在信号转导和癌症发展中发挥重要作用, 与TCT、ESR、TBA联合有作为LA-MPE和TPE鉴别诊断标志物的潜在价值。

*　　*　　*

作者贡献声明　叶芷杉和农雪萍负责论文构思、数据审编、正式分析、调查研究、研究方法和初稿写作, 王艳云负责论文构思、数据审编、可视化、初稿写作和审读与编辑写作, 车光璐负责数据审编、正式分析和审读与编辑写作, 周斌负责研究项目管理和经费获取, 黄建华负责提供资源和监督指导, 张林负责提供资源、监督指导和经费获取。所有作者已经同意将文章提交给本刊, 且对将要发表的版本进行最终定稿, 并同意对工作的所有方面负责。

Author Contribution YE Zhishan and NONG Xueping are responsible for conceptualization, data curation, formal analysis, investigation, methodology, and writing--original draft. WANG Yanyun is responsible for conceptualization, data curation, writing--original draft, and writing--review and editing. CHE Guanglu is responsible for data curation, formal analysis, and writing--review and editing. ZHOU Bin is responsible for project administration and funding acquisition. HUANG Jianhua is responsible for resources and supervision. ZHANG Lin is responsible for resources, supervision, and funding acquisition. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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Declaration of Conflicting Interests ZHANG Lin is the Editor-in-Chief of the journal. All processes involved in the editing and reviewing of this article were carried out in strict compliance with the journal's policies and there was no inappropriate personal involvement by the author. Other than this, all authors declare no competing interests.

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