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·基础研究·

环状RNA在口腔白斑和口腔扁平苔藓中的共同表达

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【摘要】 目的　探讨环状RNA在口腔白斑（oral leukoplakia，OLK）和口腔扁平苔藓（oral lichen planus，OLP）疾病发生发展中的共同表达，为研究OLK与OLP的发病机制及癌变机制提供基础。**方法**　本研究获得医院伦理审批。采用高通量测序技术检测OLK、OLP、口腔鳞状细胞癌和正常口腔黏膜组织中差异表达的circRNA，采用qRT-PCR、酶耐受实验和sanger测序筛选并验证OLK与OLP中共同显著差异表达circRNA，并对关键circRNA扩大样本量验证；采用GO和KEGG富集分析差异circRNA可能涉及的生物学过程及信号通路；采用TargetScan与MiRanda预测circRNA下游的miRNA并绘制ceRNA网络。**结果**　OLK与OLP共同差异表达的circRNA共49个，包括30个上调和19个下调的circRNA；经验证，筛选的3个上调circRNA（circHLA-C，circTTN，circRNF13）和2个下调circRNA（circSEPN2，circALDH3A2）表达趋势与测序结果一致。其中circHLA-C是具有反式剪接位点的关键circRNA，经扩大样本量验证，circHLA-C在OLK与OLP中显著上调。ROC曲线分析显示，OLK中circHLA-C曲线下面积为0.955，OLP中circHLA-C曲线下面积为0.988。GO功能富集显示与免疫功能生物学过程密切相关，KEGG通路分析中富集分数最高的信号通路是“自然杀伤细胞介导的细胞毒性”，HLA-C在这些通路中显著富集，ceRNA网络分析表明circHLA-C可能与多种癌症相关miRNA相互作用，如hsa-miR-26a- 5p、hsa-miR-129-5p和hsa-miR-29a-3p。**结论**　OLK与OLP存在相同差异表达的circRNA，circHLA-C是OLK与OLP共有的最显著上调的circRNA；circHLA-C可能会成为OLP与OLK诊断和预后的潜在生物标志物。

【关键词】 环状RNA；　circHLA-C；　口腔白斑病；　口腔扁平苔藓；　口腔黏膜潜在恶性疾病；　癌变；　生物标志物；　高通量测序
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Co-expression of circRNA in oral leukoplakia and oral lichen planus

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【Abstract】 Objective　To find any differentially expressed circRNAs in oral leukoplakia (OLK) and oral lichen planus (OLP), to investigate the possible role of circRNAs in the pathogenesis of these two diseases. **Methods**　This study obtained hospital ethical approval. High-throughput sequencing was used to detect differentially expressed circRNAs in OLK, OLP, oral squamous cell carcinoma and normal oral mucosal tissues. CircRNAs were verified by qRT-PCR, enzyme tolerance assays and Sanger sequencing. GO functional analysis and KEGG pathway analysis were performed to predict

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the functions of circRNAs in OLP. TargetScan and miRanda were applied to predict targeted miRNAs and mRNAs of circRNAs, and ceRNA networks were mapped. **Results** A total of 49 circRNAs were differentially expressed in OLK and OLP together, including 30 upregulated and 19 downregulated circRNAs. The five circRNAs confirmed with RT-qPCR, including circHLA-C, circRNF13, circTTN, circSEPN2 and circALDH3A2, were all abnormally expressed in OLK and OLP, among which circHLA-C was a key circRNA with trans splice sites, which was validated by expanding the sample size. ROC curve analysis showed that the area under the circHLA-C curve for predicting OLK was 0.955, and the area under the circHLA-C curve for predicting OLP was 0.988. GO functional analysis showed enrichment of many biological processes related to the immune process. The KEGG pathway with the highest enrichment score was "Natural killer cell mediated cytotoxicity". HLA-C was significantly enriched in these processes/pathways. CeRNA network analysis showed that circHLA-C interacted with a variety of miRNAs, such as hsa-miR-26a-5p, hsa-miR-129-5p, and hsa-miR-29a-3p. **Conclusion** Many circRNAs were differentially expressed in both OLK and OLP, circHLA-C being the most elevated. CircHLA-C is valuable for the early diagnosis of OLK and OLP and may serve as a potential biomarker for the diagnosis and prognosis of OLK and OLP.

【Key words】 circRNA; circHLA-C; oral leukoplakia; oral lichen planus; oral potentially malignant disorders; malignant transformation; biomarkers; high-throughput sequencing

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口腔黏膜潜在恶性疾病(oral potentially malignant disorders, OPMD)指发生在口腔黏膜组织的一种形态改变,与正常口腔黏膜(normal oral mucosal, NOM)相比更容易发生口腔癌,包括口腔白斑(oral leukoplakia, OLK)、红斑、口腔黏膜下纤维化、口腔扁平苔藓(oral lichen planus, OLP)、盘状红斑狼疮等[1-2]。OLK的癌变率最高,为1.1%～40.8%[3];而OLP是临床发病率最高的口腔黏膜病之一,其癌变率为1.14%[4]。环状RNA(circular RNA, circRNA)是一类具有共价闭合环状的单链RNA分子,大量研究发现circRNA在胃癌[5]、结直肠癌[6]、乳腺癌和肺癌等癌症中异常表达。在口腔黏膜下纤维化和口腔鳞状细胞癌(oral squamous carcinoma cell, OSCC)中circRNA可作为有效的诊断生物标志物和治疗靶点[7-8],目前尚无可靠的生物学标志物对OLK与OLP行早期诊断与治疗。本研究通过高通量测序描绘OLK、OLP、OSCC和NOM组织中circRNA的表达谱,对显著差异表达的circRNA进行验证分析,初步探索circRNA在OLK与OLP发生发展中的共同表达及作用机制。

1　资料和方法

1.1　患者及样本来源

患者来自上海交通大学医学院附属第九人民医院口腔黏膜病科及口腔颌面头颈肿瘤外科,所有样本在获取后立即置于液氮或-80 ℃冰箱中保存。纳入标准:病理诊断为"口腔白斑"、"口腔扁平苔藓"、"口腔鳞状细胞癌"及正常口腔黏膜组织各26例。排除标准:年龄小于18岁或大于80岁的患者;放化疗史以及合并其他肿瘤病史者;凝血功能异常或有出血性倾向者;重要脏器功能受损或存在严重的系统性疾病者;妊娠或哺乳期妇女。参与者均在术前签署研究样本采集知情同意书,本研究经上海市第九人民医院伦理委员会批准[审批号:89(2012)21]。选取以上组织各6例进行高通量测序,对测序结果分组对比分析。PN组:OLP(P)与NOM(N)差异表达circRNA;KN组:OLK(K)与NOM(N)差异表达circRNA ;SP组:OSCC(S)与OLP(P)差异表达circRNA;SK组:OSCC(S)与OLK(K)差异表达circRNA。

1.2　主要试剂和仪器

Trizol裂解液(Invitrogen life technologies,美国);Ribo-Zero rRNA Removal Kits(Illumina,美国);TruSeq Stranded Total RNA Library Prep Kit(Illumina,美国);SuperScript Ⅲ逆转录试剂盒(Invitrogen,美国);qRT-PCR引物(上海云序生物工程技术有限公司,中国);qPCR SYBR Green master mix(上海云序生物科技有限公司,中国);RNaseR(Epicen-

tre，美国）；RNase inhibitor（Millipore，美国）；NanoDrop ND-1000仪器（Thermo Fisher Scientific，Waltham，MA，美国）；BioAnalyzer 2100仪器（Agilent Technologies，美国）；Illumina HiSeq 4000测序平台（Illumina，美国）；实时荧光定量PCR仪（Thermo Fisher，美国）；GeneAmp PCR System（Applied Biosystems，美国）。

1.3　RNA提取和质控

使用Trizol试剂盒提取总RNA，使用NanoDrop ND-1000测定分离的总RNA的纯度和数量。以吸光度OD_{260}/OD_{280}值用作RNA纯度指数，OD_{260}/OD_{280}值为1.8～2.1作为合格范围。

1.4　RNA测序

使用Ribo-Zero rRNA Removal Kits移除总RNA中的rRNAs，使用TruSeq Stranded Total RNA Library Prep Kit预处理RNA，构建测序文库。使用BioAnalyzer 2100仪器进行文库质控和定量，由上海云序生物科技有限公司提供高通量测序。根据Illumina测序说明在Illumina HiSeq测序仪上采用双端模式进行150 cycle测序。

1.5　测序分析

测序使用Illumina HiSeq 4000测序仪并收获双端reads，使用cutadapt（v1.9.3）与STAR（v2.5.1b）软件，去低质量reads，将高质量reads比对到参考基因组或转录组上，使用DCC软件（v0.4.4）、circBase数据库和circ2Traits，进行circRNA检测鉴定与注释。

使用edgeR软件（v3.16.5）进行数据标准化和差异表达circRNA筛选。选择参考倍数变化（fold change，FC）≥2.0，$P<0.05$作为筛选差异circRNA的阈值。circRNA高通量测序由上海云序生物有限公司完成检测及数据初步分析。

1.6　qRT-PCR、酶耐受、Sanger测序

使用SuperScript Ⅲ逆转录试剂盒将提取的RNA逆转录为cDNA，GAPDH作为内参基因，通过QuantStudio 5 Real-Time PCR System进行qRT-PCR反应，本研究所用引物序列由上海生工提供（表1）。根据PCR结果选取差异表达显著的circRNA，使用RNase处理提取的RNA，37 ℃水浴20 min进行酶耐受实验，结果合格者进行Sanger测序验证。对经过验证的circRNA，在OLK、OLP、OSCC及NOM各20例组织中扩大样本量验证。采用$2^{-\Delta\Delta Ct}$法对目的基因的相对表达水平进行计算，得到各目的基因差异表达情况，通过Graphpad Prism进行ROC曲线分析。

表1　qRT-PCR引物信息

Table 1　The primers used for qRT-PCR experiments

circRNA ID	Primer type	Primer sequence (5′-3′)
circHLA-C	Forward	CGGCAAGGATTACATCGC
(chr6:31238920-31324013-)	Reverse	CCTTCCCGTTCTCCAGGT
circTTN	Forward	CCTGCTAAAGTGCCTGAAGTT
(chr2:179516028-179516243-)	Reverse	AACCACCAGAGGCACCTTC
circRNF13	Forward	GACATAGAGCTAGAAGAAACAGACT
(chr3:149613260-149639014+)	Reverse	GCCTTGTATCCTGCTCTCTG
circALDH3A2	Forward	CTGTTGCTCACTTTCCTGGG
(chr17:19554860-19575269+)	Reverse	TGACTTCCTGACTGTACACATTG
circSENP2	Forward	ACAGCTGAATGGGAGTGATTG
(chr3:185293003-185344181+)	Reverse	GTGGCAGCACAGAACCTTC

1.7　ceRNA网络的构建与生物信息学分析

采用TargetScan、MiRanda与Cytoscape（v2.8.0）软件进行circRNA的潜在靶miRNA预测及其下游miRNAs和mRNA网络构建。通过基因本体（Gene Ontology，GO）富集和京都基因和基因组百科全书（Kyoto Encyclopedia of Genes and Gnomes，KEGG）分析组间共同差异表达的circRNA源基因的功能通路，GO包括3个领域：生物过程（biological process，BP）、细胞成分（cellular component，CC）和分子功能（molecular function，MF）。

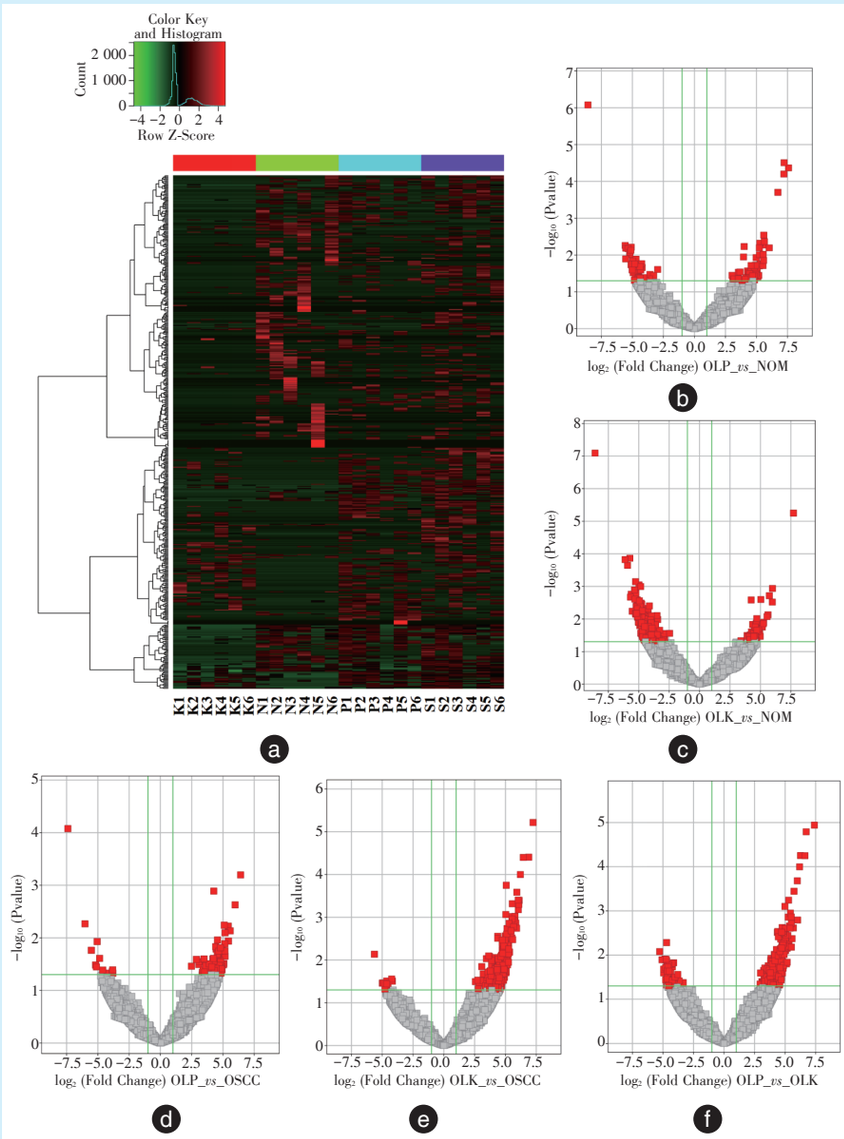
1.8　统计学分析

使用SPSS 19.0和Graphpad Prism 8.0软件进行数据分析。计量资料采用平均数±标准差表示，采用独立t检验进行组间比较。GO项富集的显著性差异采用Fisher检验。$P<0.05$为差异具有统计学意义。

2　结　果

2.1　差异表达circRNA的表达谱和特征

PN组差异表达circRNA共135个，包括83个上调和52个下调circRNA；KN组差异表达circRNA共366个，其中65个circRNA上调，301个circRNA下调；SP组差异表达circRNA共94个，其中上调81个，下调13个；SK组差异表达circRNA共389个，包括376个上调和13个下调circRNA；此外，P组和K组差异表达circRNA共329个，275个circRNA上调，54个circRNA下调；利用层次聚类分析热图和火山图分析circRNA在OLK（K）、OLP（P）、OSCC（S）和NOM（N）中的表达情况（图1）。原始数据已



a: hierarchical clustering analysis showed the circRNA expression profiles of OLP, OLK and OSCC; red represents upregulated circRNAs, and green represents downregulated circRNAs, $|\log_2 FC| \geq 1$, $P < 0.05$; K: OLK; N: NOM; P: OLP; S: OSCC; b: scatter plot of differences in circRNA expression between OLP and NOM tissues; c: scatter plot of differences in circRNA expression between OLK and NOM tissues; d: scatter plot of differences in circRNA expression between OLP and OSCC tissues; e: scatter plot of differences in circRNA expression between OLK and OSCC tissues; f: scatter plot of differences in circRNA expression between OLP and OLK tissues; red represents upregulated and downregulated circRNAs with $|\log_2 FC| \geq 1$, $P < 0.05$; OLP: oral lichen planus; OLK: oral leukoplakia; OSCC: oral squamous carcinoma cell; NOM: normal oral mucosal

Figure 1　Differentially expressed circRNA hierarchical clustering analysis: heat map and volcano map of circRNAs of OLP, OLK and OSCC

图1　口腔白斑、口腔扁平苔藓和口腔鳞状细胞癌差异表达circRNAs层次聚类分析热图和火山图

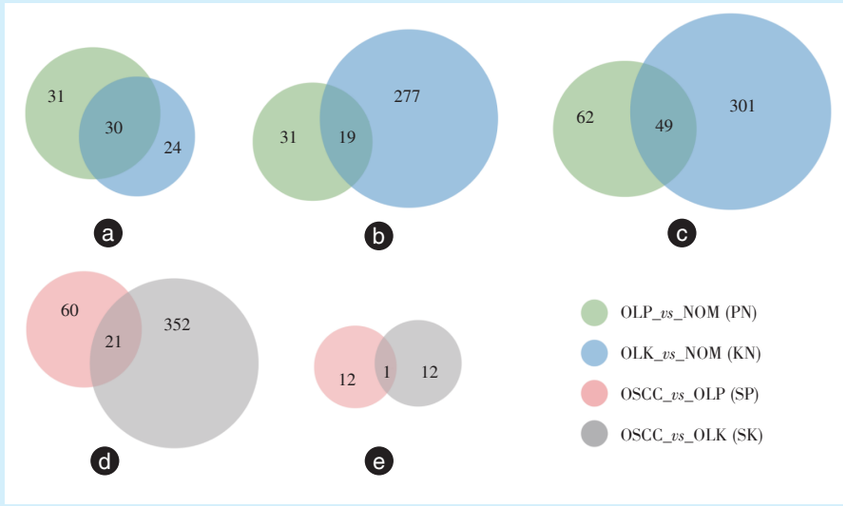
上传至GEO网站（https://www.ncbi.nlm.nih.gov/geo/），GEO序列号为GSE131567、GSE131568和GSE131182。

对PN组与KN组差异表达的circRNA取交集，共有的circRNA共49个（约36.3%），即在P组中显著上调的circRNA有30个在K组中同样显著上调，在P组中显著下调的circRNA有19个在K组中同样显著下调（图2a~2c）。类似的，对SP组与SK组取交集，在SP组中显著上调的circRNA有21个在SK组中同样显著上调，在SP组中显著下调的circRNA有1个在SK组中同样显著下调（图2d、2e）。

PN组与KN组中共有的49个差异表达的circRNA多数为外显子来源，并且已纳入circbase，其中7个新的circRNA尚未纳入circbase，它们长度集中在两组：<500和500~2 000个核苷酸，几乎位于所有人类染色体上，包括21条常染色体和X染色体（图3）。根据差异倍数及P值，与这两种疾病显著相关的circRNA为circHLA-C、circRNF13（hsa_circ_0006801）、circTTN、circSEPN2和circALDH3A2（hsa_circ_0008603）见表2。

2.2　进一步验证目的circRNA

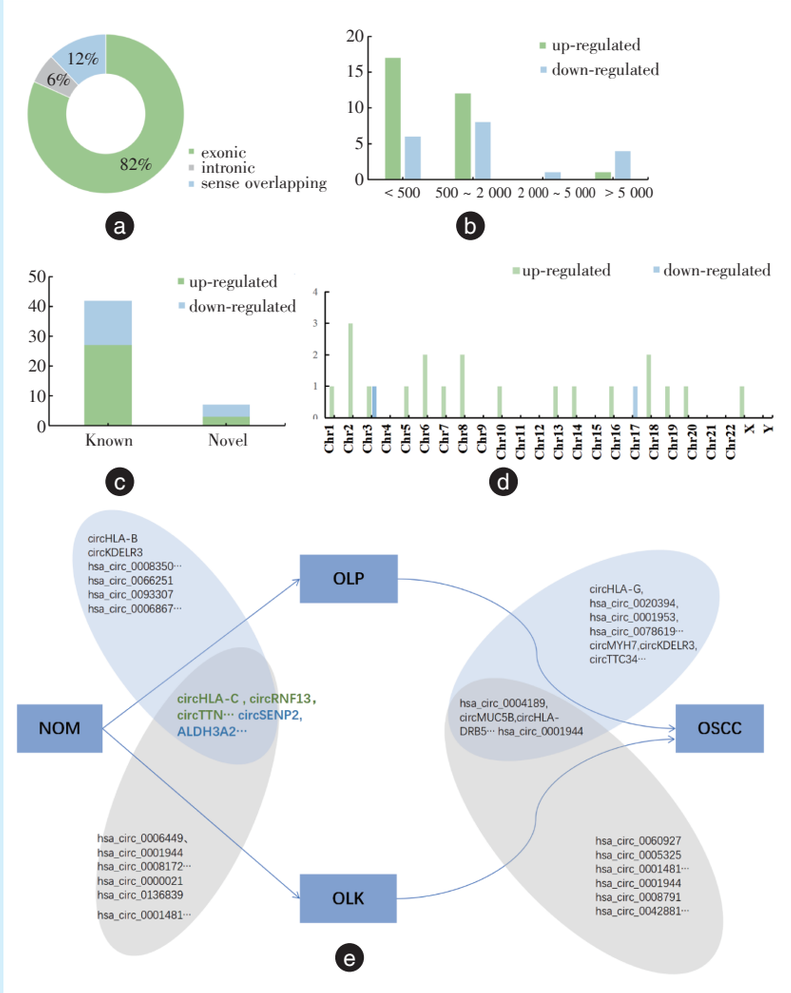
对筛选的circRNA行qRT-PCR验证，结果显示circRNA的表达趋势均与测序结果相同（图4a~4e）。对circRNA进行酶耐受试验（剔除长度小于300 nt的环状RNA），其中circHLA-C和circRNF13的变化倍数分别为1.986和3.052（图4f），表明这两种circRNA可以抵抗核糖核酸酶（RNase）的水解，在组织中稳定表达；对circHLA-C和cir-



a: Venn diagrams of upregulated circRNAs in PN and KN; b: Venn diagrams of downregulated circRNAs in PN and KN; c: Venn diagrams of differentially expressed circRNAs in PN and KN; d: Venn diagrams of upregulated circRNAs in SP and SK; e: Venn diagrams of downregulated circRNAs in SP and SK. OLP: oral lichen planus; OLK: oral leukoplakia; OSCC: oral squamous carcinoma cell; NOM: normal oral mucosal

Figure 2　Venn diagrams of differentially expressed circRNAs of OLP, OLK and OSCC

图2　口腔白斑、口腔扁平苔藓和口腔鳞状细胞癌差异表达circRNAs Venn图



a: differentially expressed circRNAs in PN and KN were divided into 4 types according to the host gene structure (exon, intron and sense overlapping circRNA); b: classification according to circRNA length; c: seven of the differentially expressed circRNAs were newly discovered; d: chromosome distribution of significantly upregulated and downregulated differentially expressed circRNAs. e: coexpression and differentially expressed circRNAs in the PN group and KN group, SP group and SK group. PN: oral lichen planus *vs*. normal oral mucosa; KN: oral leukoplakia *vs*. normal oral mucosa; SP: oral squamous carcinoma cell *vs*. oral lichen planus; SK: oral squamous carcinoma cell *vs*. oral leukoplakia

Figure 3　Distribution of the characteristics of significantly dysregulated circRNAs

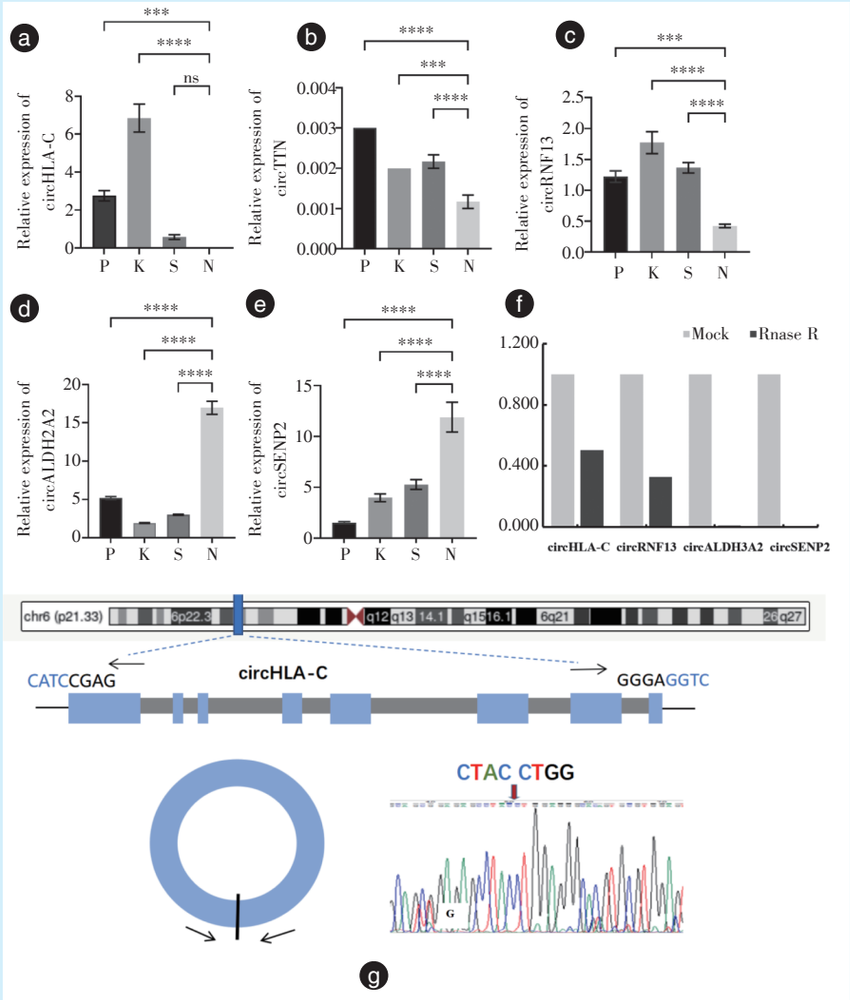
图3　显著差异表达的circRNA的分布特征

表2　口腔白斑、口腔扁平苔藓中筛选的5个差异表达circRNA

Table 2　Five circRNAs differentially expressed in oral leukoplakia and oral lichen planus

circRNA ID	Genename	circbase ID	Category	Length	Regulation	OLP *vs.* NOM		OLK *vs.* NOM		OSCC *vs.* NOM	
						logFC	*P*	logFC	*P*	logFC	*P*
chr6:31238920-31324013-	HLA-C	novel	sense overlapping	85 094	up	7.229	< 0.001	7.719	< 0.001	6.584	< 0.001
chr2:179516028-179516243-	TTN	novel	intronic	216	up	7.213	< 0.001	5.034	0.003	6.946	< 0.001
chr3:149613260-149639014+	RNF13	hsa_circ_0006801	exonic	379	up	5.271	0.005	5.735	0.002	5.269	0.005
chr17:19554860-19575269+	ALDH3A2	hsa_circ_0008603	exonic	1 290	down	-5.533	0.012	-5.532	0.006	-5.532	0.017
chr3:185293003-185344181+	SENP2	novel	sense overlapping	51 179	down	-8.583	< 0.001	-8.583	< 0.001	-5.502	0.017

OLP: oral lichen planus; OLK: oral leukoplakia; OSCC: oral squamous carcinoma cell; NOM: normal oral mucosal



a~e: five selected circRNAs were subjected to qRT-PCR validation in OLP, OLK, OSCC and NOM tissues (n = 6); the five selected circRNAs (circHLA-C, circTTN, circRNF13, circALDH3A2, circSEPN2) showed the same expression trend as the sequencing results; ****: P < 0.000 1; ***: P < 0.001. P: OLP; K: OLK; S: OSCC; N: NOM. f: the relative expression of the circRNAs before and after RNase R treatment was 1.986 and 3.052 times, respectively; g: RNase R analysis showed that circHLA-C (chr6:31238920-31324013 -) and circRNF13 (chr3:149613260-149639014+) resisted digestion by RNase R; back-spliced site of circHLA-C (chr6:31238920-31324013-): CTACCTGG; OLP: oral lichen planus; OLK: oral leukoplakia; OSCC: oral squamous carcinoma cell; NOM: normal oral mucosal

Figure4　Further verification five circRNAs differentially expressed in OLP and OLK

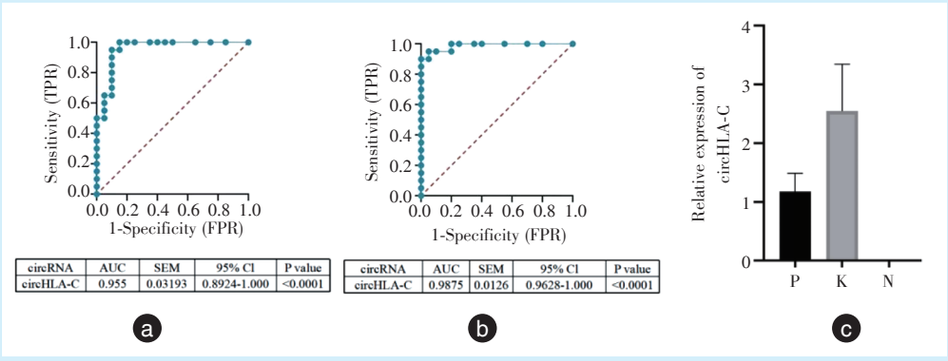
图4　进一步验证OLP与OLK中筛选的5个circRNA

cRNF13进行Sanger测序，仅在circHLA-C上检测到了反向剪切位点CTACCTGG（图4g）。

对20例OLK、OLP组织中的circHLA-C行qRT-PCR扩大样本量验证，NOM组织20例作为对照组，ROC曲线分析显示OLK中circHLA-C曲线下面积为0.955（95% *CI*：0.892～1.00，P < 0.001），OLP中circHLA-C曲线下面积为0.988（95% *CI*：0.988～1.00，P < 0.001），ROC分析显示circHLA-C是诊断OLK、OLP的潜在生物标志物（图5）。

2.3　GO功能和KEGG功能通路富集分析

PN组与KN组上调circRNA源基因的GO功能富集分析显示，PN与KN组富集结果中相同生物学过程共126条，其中上调通路占114条，且与免疫



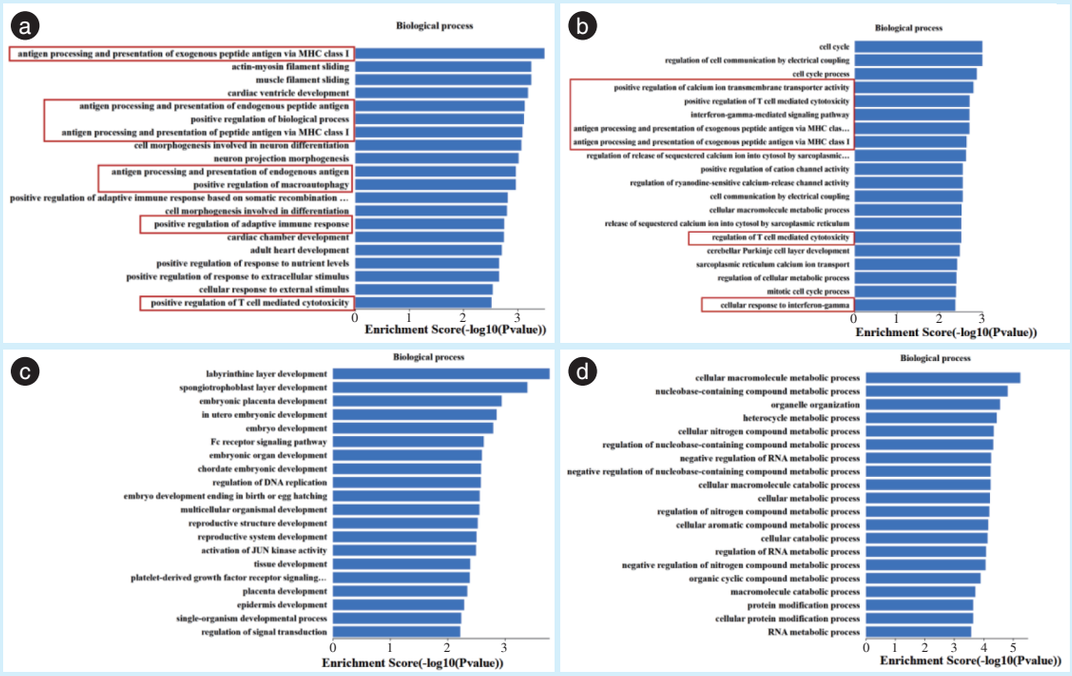
circRNA	AUC	SEM	95% CI	P value
circHLA-C	0.955	0.03193	0.8924-1.000	<0.0001

circRNA	AUC	SEM	95% CI	P value
circHLA-C	0.9875	0.0126	0.9628-1.000	<0.0001

a: the ROC curves showed that the expression of circHLA-C has potential value in the diagnosis of OLK; b: the ROC curves showed that the expression of circHLA-C has potential value in the diagnosis of OLP; c: circHLA-C was subjected to qRT-PCR validation in OLP and OLK (n = 20). P: OLP, oral lichen planus; K: OLK, oral leukoplakia; N: NOM, normal oral mucosal

Figure 5　ROC curve analysis of circHLA-C in OLP and OLK

图5　OLP与OLK中circHLA-C的ROC曲线分析



a: the top 20 biological processes of upregulated circRNAs in OLP; b: the top 20 biological processes of upregulated circRNAs in OLK; c: the top 20 biological processes of the downregulated circRNAs in OLP; d: the top 20 biological processes of the downregulated circRNAs in OLK. OLP: oral lichen planus; OLK: oral leukoplakia. PN: oral lichen planus *vs.* normal oral mucosa; KN: oral leukoplakia *vs.* normal oral mucosa. red box: the biological processes involved in HLA-C

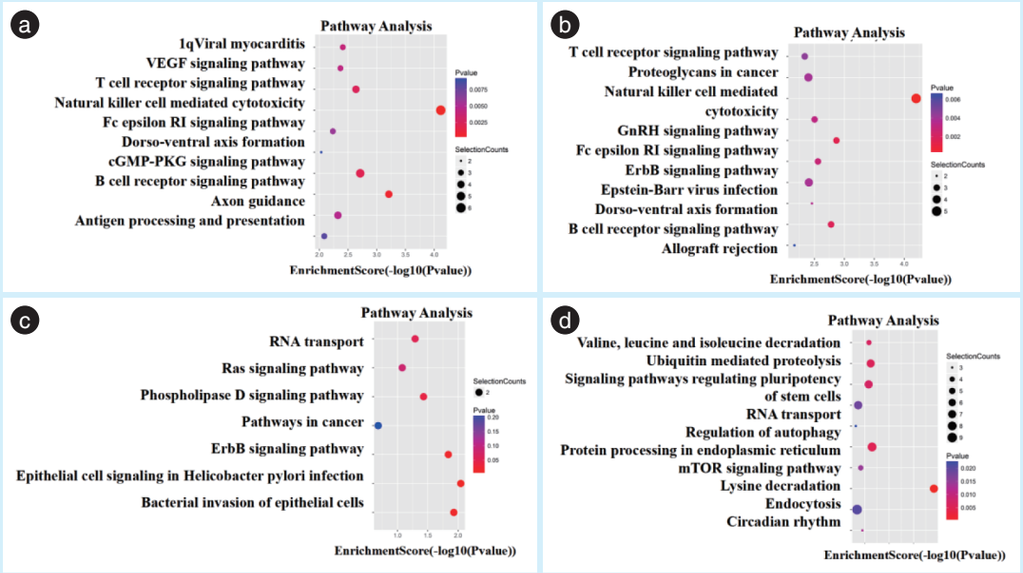
Figure 6　Bioinformatics analysis of significantly dysregulated expression of circRNAs of PN group and KN group

图6　PN组与KN组差异表达的circRNA生物学功能分析

功能相关(通常认为上调富集的通路为激活),HLA-C在这些通路中显著富集(图6):OLK组富集的前20个上调BP功能条目中HLA-C参与7条,OLP组富集的前20个上调BP功能条目中HLA-C参与8条,这些生物学过程均与免疫相关。两组共有的两个生物学功能条目为“外源肽抗原通过MHC Ⅰ类进行的抗原处理和呈递”与“T细胞介导的细胞毒性的正调控”,由HLA-B、HLA-C与HLA-

G共同参与。

KEGG分析显示PN与KN组差异表达circRNA的源基因涉及的共同通路共60条，其中上调通路占57条，HLA-C参与13条，且前20条显著富集的通路中有8条为HLA-C参与，分别是自然杀伤细胞介导的细胞毒性、病毒性心肌炎、抗原处理和呈递、HTLV-Ⅰ感染、同种异体移植排斥反应、移植物抗宿主病、Ⅰ型糖尿病、自身免疫性甲状腺疾病



a: top 10 KEGG pathways of the upregulated circRNAs in OLP; b: top 10 KEGG pathways of upregulated circRNAs in OLK; c: all of the KEGG pathways of downregulated circRNAs in OLP; d: top 10 KEGG pathways of downregulated circRNAs in OLK; OLP: oral lichen planus; OLK: oral leukoplakia. PN: oral lichen planus *vs.* normal oral mucosa; KN: oral leukoplakia *vs.* normal oral mucosa

Figure 7　KEGG analysis of the differential expression of circRNA in PN group and KN group

图7　PN组与KN组差异表达的circRNA源基因KEGG通路分析

表3　PN与KN差异表达circRNA共同参与的KEGG通路

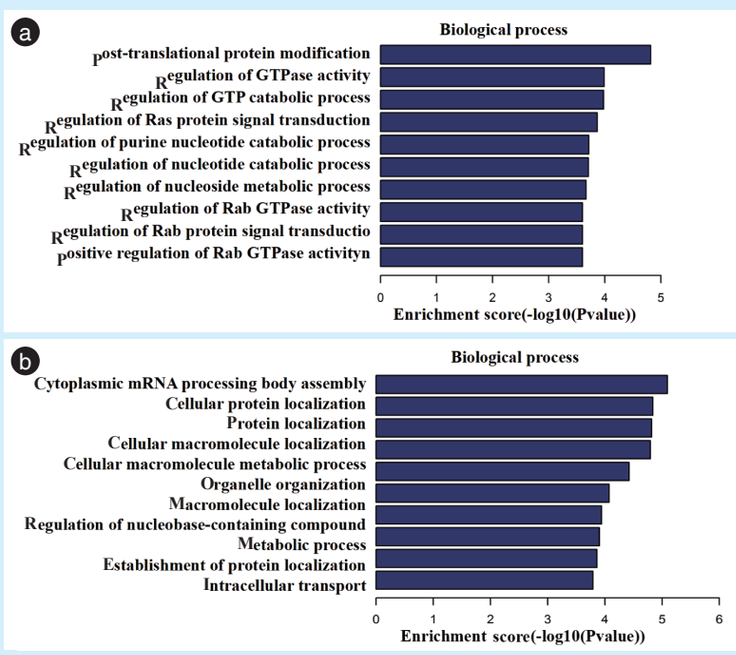
Table 3　circRNAs differentially expressed in PN and KN participate in KEGG

PathwayID	Definition	Genes
hsa04650	Natural killer cell mediated cytotoxicity	HLA-B//HLA-C//MAPK1//NFATC2//PPP3CC//SOS2
hsa05416	Viral myocarditis	HLA-B//HLA-C//MYH7
hsa04612	Antigen processing and presentation	HLA-B//HLA-C//IFI30
hsa05166	HTLV-I infection	HLA-B//HLA-C//MAP2K4//NFATC2//PPP3CC
hsa05330	Allograft rejection	HLA-B//HLA-C
hsa05332	Graft-versus-host disease	HLA-B//HLA-C
hsa04940	Type I diabetes mellitus	HLA-B//HLA-C
hsa05320	Autoimmune thyroid disease	HLA-B//HLA-C
hsa04010	MAPK signaling pathway	MAP2K4//MAPK1//PPP3CC//SOS2
hsa05206	MicroRNAs in cancer	CYP24A1//FOXP1//MAPK1//SOS2
hsa05169	Epstein-Barr virus infection	HLA-B//HLA-C//MAP2K4
hsa05205	Proteoglycans in cancer	ANK1//MAPK1//SOS2
hsa05203	Viral carcinogenesis	HLA-B//HLA-C//MAPK1
hsa04514	Cell adhesion molecules (CAMs)	HLA-B//HLA-C
hsa04145	Phagosome	HLA-B//HLA-C
hsa04141	Protein processing in endoplasmic reticulum	AMFR//PDIA4
hsa05164	Influenza A	MAP2K4//MAPK1
hsa05168	Herpes simplex infection	HLA-B//HLA-C
hsa04015	Rap1 signaling pathway	LPAR3//MAPK1
hsa05200	Pathways in cancer	LPAR3//MAPK1//SOS2

PN: oral lichen planus vs. normal oral mucosal; KN: oral leukoplakia *vs.* normal oral mucosal

（图7，表3）。另外富集通路中“MicroRNAs in cancer”“ Proteoglycans in cancer”“ Pathways in cancer”均为癌症相关（表3）。

此外，GO分析显示SP组与SK组共同生物学过程74条，上调通路占71条，包括翻译后的蛋白质修饰、调节Ras蛋白的信号转导、调节Ras GTP酶的活性、调节小GTP酶介导的信号转导、对Ras GTP酶活性的正向调节等（图8）。KEGG结果显示SP组富集到的21条信号通路中，20条上调通路是与SK组相同，值得注意的是，HLA-C在这些通路中也显著富集，除上述PN组与KN组交集KEGG通路8条通路外，还包括单纯疱疹感染、细胞粘附分子、吞噬体、Epstein-Barr病毒感染、病毒致癌作用、内吞作用（表4）。



a: the top 10 biological processes of the upregulated circRNAs in SP; b: the top 10 biological processes of the upregulated circRNAs in SK; SP: oral squamous carcinoma cell *vs.* oral lichen planus; SK: oral squamous carcinoma cell *vs.* oral leukoplakia

Figure 8　Bioinformatics analysis of the differentially expressed circRNAs in the SP group and SK group

图8　SP组与SK组差异表达的circRNA生物学功能分析

表4　SP与SK差异表达circRNA共同参与的KEGG通路

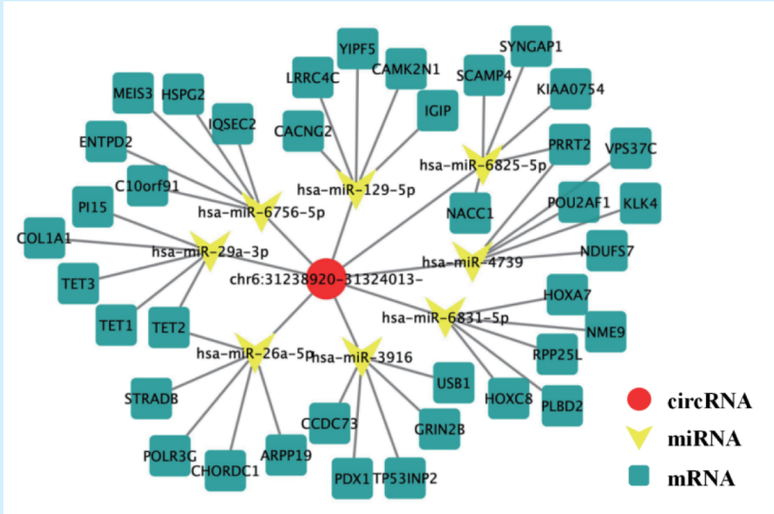
Table 4　circRNAs differentially expressed in SP and SK participate in KEGG

PathwayID	Definition	Genes
hsa05166	HTLV-I infection	AKT3//ATM//CANX//CREBBP//HLA-B//HLA-C
hsa05169	Epstein-Barr virus infection	AKT3//CREBBP//HLA-B//HLA-C
hsa04612	Antigen processing and presentation	CANX//HLA-B//HLA-C//HLA-DQB1
hsa05416	Viral myocarditis	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5//ITGAL
hsa05330	Allograft rejection	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5
hsa05332	Graft-versus-host disease	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5
hsa04145	Phagosome	CANX//DYNC1H1//DYNC1LI1//HLA-B//HLA-C
hsa04144	Endocytosis	AP2A2//CBLB//DNAJC6//EPS15//FGFR2//HLA-B//HLA-C
hsa04940	Type I diabetes mellitus	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5
hsa03013	RNA transport	CLNS1A//KPNB1//NUP98//PABPC1//RGPD1
hsa05320	Autoimmune thyroid disease	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5
hsa04514	Cell adhesion molecules (CAMs)	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5
hsa04810	Regulation of actin cytoskeleton	ARHGEF7//FGFR2//ITGAL//ITGB6//PIK3CA
hsa05164	Influenza A	AKT3//CREBBP//HLA-DQB1//HLA-DRB5//NUP98//PIK3CA
hsa05168	Herpes simplex infection	CREBBP//HLA-B//HLA-C//HLA-DQB1
hsa05203	Viral carcinogenesis	CREBBP//HLA-B//HLA-C//PIK3CA
hsa05202	Transcriptional misregulation in cancer	ATM//ETV6//PTK2//WHSC1//ZEB1
hsa04650	Natural killer cell mediated cytotoxicity	HLA-B//HLA-C//ITGAL//PIK3CA
hsa04141	Protein processing in endoplasmic reticulum	CANX//SEC31A//UBQLN1//UGGT2
hsa04510	Focal adhesion	AKT3//ITGB6//PIK3CA//PTK2

SP: oral squamous carcinoma cell *vs.* oral lichen planus; SK: oral squamous carcinoma cell *vs.* oral leukoplakia

2.4　circHLA-C的ceRNA 网络的预测和可视化

通过TargetScan和miRanda生信分析预测circHLA-C与miRNA之间可能的相互作用关系。根据已发表的与癌变相关的mRNA及miRNA，通过cytoscape软件构建了相互作用关系的ceRNA网络图（图9）。circHLA-C可与hsa-miR-4739、hsa-miR-3916、hsa-miR-6756-5p、hsa-miR-6825-5p、hsa-miR-6831-5p等发挥“海绵”机制作用。



ceRNA: competing endogenous RNA of circHLA-C; red: circHLA-C; yellow: microRNA; blue: mRNA

Figure 9　CeRNA interaction of circHLA-C

图9　circHLA-C的ceRNA网络图

3　讨　论

OLK和OLP在临床上均以白色损害为主要表现，二者都有癌变风险，是重要的口腔黏膜潜在恶性疾病。这两种疾病有共性也有差异，有些病例为典型斑块型OLK表现，但病理诊断为“口腔扁平苔藓”，有些病例临床为典型网状型OLP表现，其病理诊断却为“口腔白斑”；此外，不乏两种疾病共存于同一患者的情况——即在口腔内同时存在OLK与OLP，或在不同时间段先后出现两种疾病。一些学者研究发现，部分损害发生从OLP到OLK的演化，Garcia-Pola对515例患者随访14.5年发现14例（2.7%）OLP患者演变为增殖性疣状口腔白斑[9]；Chainani-Wu等[10]在首次病理诊断为扁平苔藓的患者临床随访中（1.5～6.5年）发现9例患者口内出现局部白斑。在病理诊断方面也有文献报道OLP与OLK有时难以区分，高岩等[11]发现OLP癌变过程中出现OLK样病理改变：在19例OLP中，发现伴有白斑样病理表现的有9例；其中有1例初次活检时诊断为OLP，第2次活检时诊断为白斑伴上皮异常增生，第3次活检时证实为癌变；在19例OLP癌变组织中发现，其癌变区周围组织病变的表现为白斑[11]。

由此笔者推测这两种疾病存在内在联系，在这两种疾病的发生发展中可能存在一些相同的功能通路。circRNA通过转录和转录后修饰参与各类疾病发生，近年研究表明在口腔癌发生过程中circRNA发挥重要作用[12]，circ_0001874和circ_0001971是诊断口腔鳞状细胞癌的潜在生物标志物，对口腔白斑与口腔鳞状细胞癌患者唾液中的circRNA研究表明，hsa_circ_0001971/miR-186和hsa_circ_0001874/miR-296信号通路可通过协同激活SHP2/PLK1信号促进口腔鳞状细胞癌细胞的增殖[13]。研究OLP与OLK发生发展中circRNA调控机制的文献极少，课题组前期研究表明，circRNA在OLP与OLK组织中存在显著差异[14-15]，本文进一步深入研究发现这两种疾病的发生发展存在以下共同之处。

3.1　OLK、OLP与癌变组织中差异表达circRNA存在部分重合

PN组差异表达circRNA约36.3%与KN组相同，即PN组与KN组中均上调的circRNA有30个，PN组与KN组中均下调的circRNA有19个，如circHLA-C、circTTN、circRNF13、circUGGT2等；SP组差异表达circRNA约23.2%与SN组相同，其中仅1个circRNA下调，其余21个均为上调circRNA，如hsa_circ_0004189、circMUC5B等。现有文献表明

circHLA-C通过海绵miR-150在狼疮性肾炎中发挥重要作用[16]；circTTN通过IGF2/PI3K/AKT信号通路作为miR-432海绵促进成肌细胞的增殖和分化[17]；circRNF13通过SUMO2抑制鼻咽癌的增殖和转移[18]。

3.2　circHLA-C是OLK与OLP共同上调的circRNA中差异表达倍数最高的circRNA

circHLA-C在NOM中低表达，在OLK组织和OLP组织中显著增高。ROC分析表明circHLA-C对OLK与OLP的诊断具有较高的特异性和灵敏性；此外，circHLA-C表达与OLK发育异常呈正相关，circHLA-C的表达在OLK到OSCC的进展中起重要作用[15]。

3.3　OLP与OLK的发生过程中可能存在许多相同功能通路

OLP与OLK共有的BP功能条目“T细胞介导的细胞毒性的正调控”由HLA-B与HLA-C共同参与。人类白细胞抗原（human leucocyte antigen，HLA）由HLA基因控制并产生，是人类的主要组织相容性复合体，它存在于细胞的表面，调控人体特异性免疫应答，是免疫系统区分本身和异体物质的基础，包括HLA-A、HLA-B和HLA-C[19-20]。最新研究发现，通过输注自体T细胞在体内特异性表达HLA-C限制性T细胞受体可以靶向治疗胰腺癌[21]。近年研究发现，炎症介质和浸润性免疫细胞在OLK和OLP进展中具有重要作用，$CD4^+$ T淋巴细胞、$Foxp3^+$调节性T细胞、$CD68^+$巨噬细胞和IL-4在OLP与OLK中共同表达[22]，推测调控特异性免疫的基因表达是OLK与OLP发生发展的主要原因之一。

3.4　PN与KN组共同通路中HLA-C显著富集

KEGG功能通路分析结果显示，PN与KN组共同通路中HLA-C显著富集，HLA-C的功能富集通路显示与T细胞免疫显著相关，如T细胞介导的细胞毒性的正调控、调节T细胞介导的细胞毒性和T细胞介导免疫的正向调节等，这一结果提示OLK与OLP的发生与HLA-C显著相关，且与感染和免疫抑制因素有关，circHLA-C可能是一个关键因素。OLP与OLK的发展与癌变中也可能存在许多相同功能通路，SP与SK组的GO功能富集分析显示这两种疾病发展与Ras信号转导、细胞分化增殖和凋亡显著相关；KEGG功能通路分析显示SP组20个上调通路均与SK组共有。值得注意的是，HLA-C在这些通路中也显著富集，其中就包括PN组与KN组交集的8条KEGG通路，即自然杀伤细胞介导的细胞毒性、病毒性心肌炎、抗原处理和呈递等，提示这些通路可能在OLK与OLP发生发展中起到重要作用。

3.5　circHLA-C可能与多种癌症相关miRNA相互作用

circRNA能够作为“海绵”吸附microRNA[23]。本研究发现circHLA-C可通过互补配对与hsa-miR-26a-5p、hsa-miR-129-5p、hsa-miR-29a-3p、hsa-miR-4739等miRNA结合作用于细胞调控网络。研究表明，在OLK和癌组织中，miR-26a和miR-29a的表达显著下调，但在OLP中表达上调[24]；miR-129-5p在OLK-OSCC组织中表达水平显著下调[25]。circHLA-C可能具有多个不同miRNA的结合位点，通过“海绵”吸附这些miRNAs来调控OLK与OLP的发生发展，这些途径对OLP与OLK发展具有重要意义。

综上所述，circHLA-C是OLK与OLP中共有的最显著上调的circRNA，GO功能分析和KEGG通路富集发现circHLA-C在OLK与OLP细胞免疫中起关键作用，circHLA-C可能是OLK、OLP诊断中有价值的生物标志物，为未来OLK与OLP的诊断和预后带来了新的启示。

【Author contributions】 Yang JW designed the study, collected and analyzed the data, drafted the article. Song YH, Xu SM, Ge SY designed the study, collected and analyzed the data, revised the article. Zhou HW designed the study, guided and critically, reviewed the article structures. All authors read and approved the final manuscript as submitted.

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