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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 YANG Jingwen, SONG Yuhuan, XU siming, GE Shuyun, ZHOU Haiwen. Department of Oral Mucosal Diseases, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, College of Stomatology, Shanghai Jiao Tong University National center for Stomatology, National Clinical Research Center for oral diseases, Shanghai Key Laboratory of Stomatology, Shanghai Research Institute of Stomatology, Shanghai 200011, China

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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 °C 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	GCCTTGATCTCTGCTCTCTG
circALDH3A2	Forward	CTGTTGCTCACTTTCCTGGG
(chr17:19554860-19575269+)	Reverse	TGACTTCCTGACTGTACACATTG
circSENP2	Forward	ACAGCTGAATGGAGTGATTG
(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 Genomes, 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)。

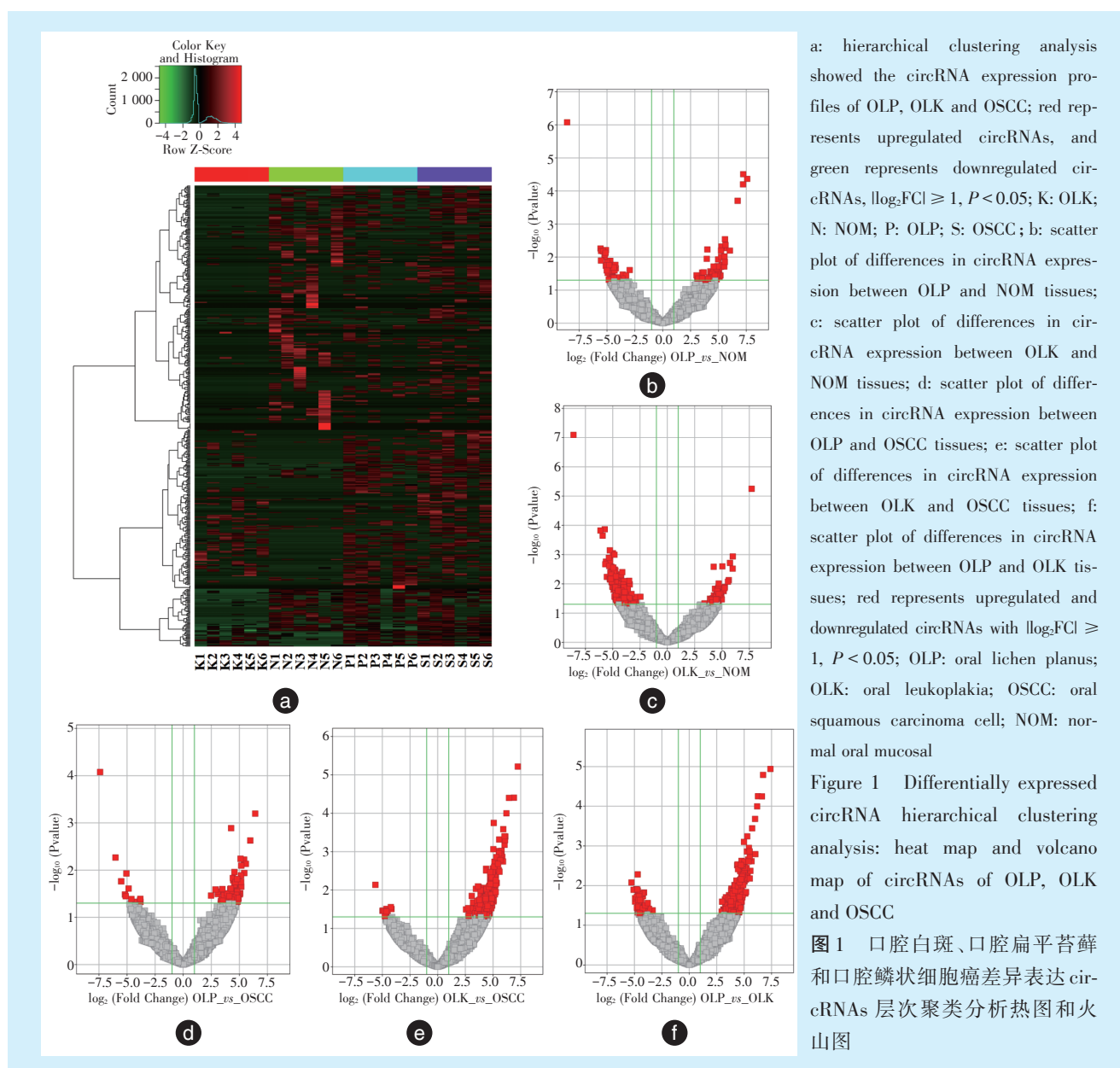
1.8 统计学分析

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 $\pm$ 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, $|\log_2FC| \geq 1$, $P < 0.05$; 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 $|\log_2FC| \geq 1$, $P < 0.05$; 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 circ-

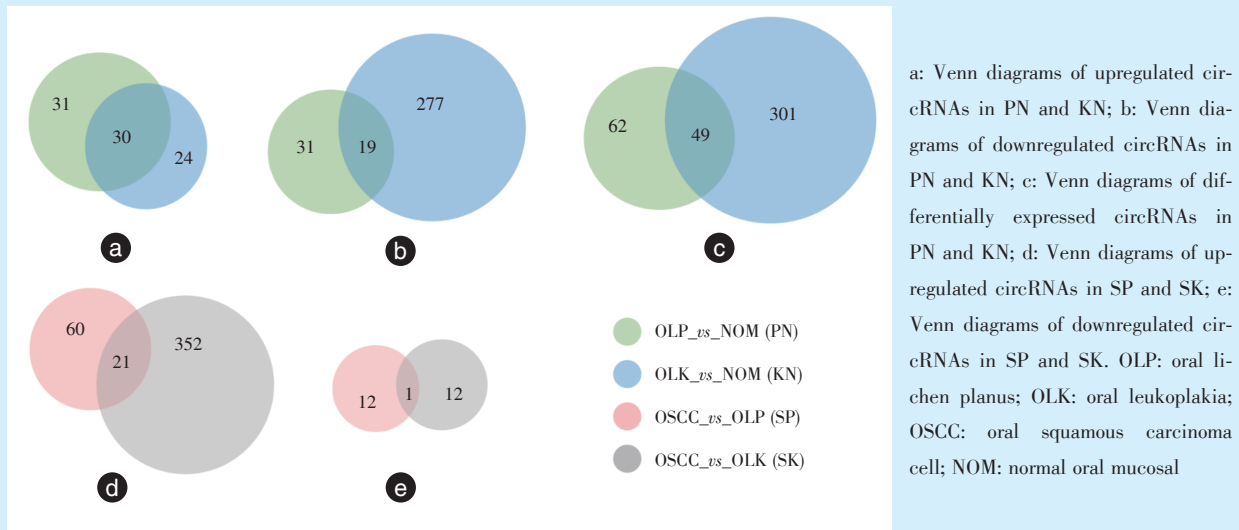


Figure 2 Venn diagrams of differentially expressed circRNAs of OLP, OLK and OSCC

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

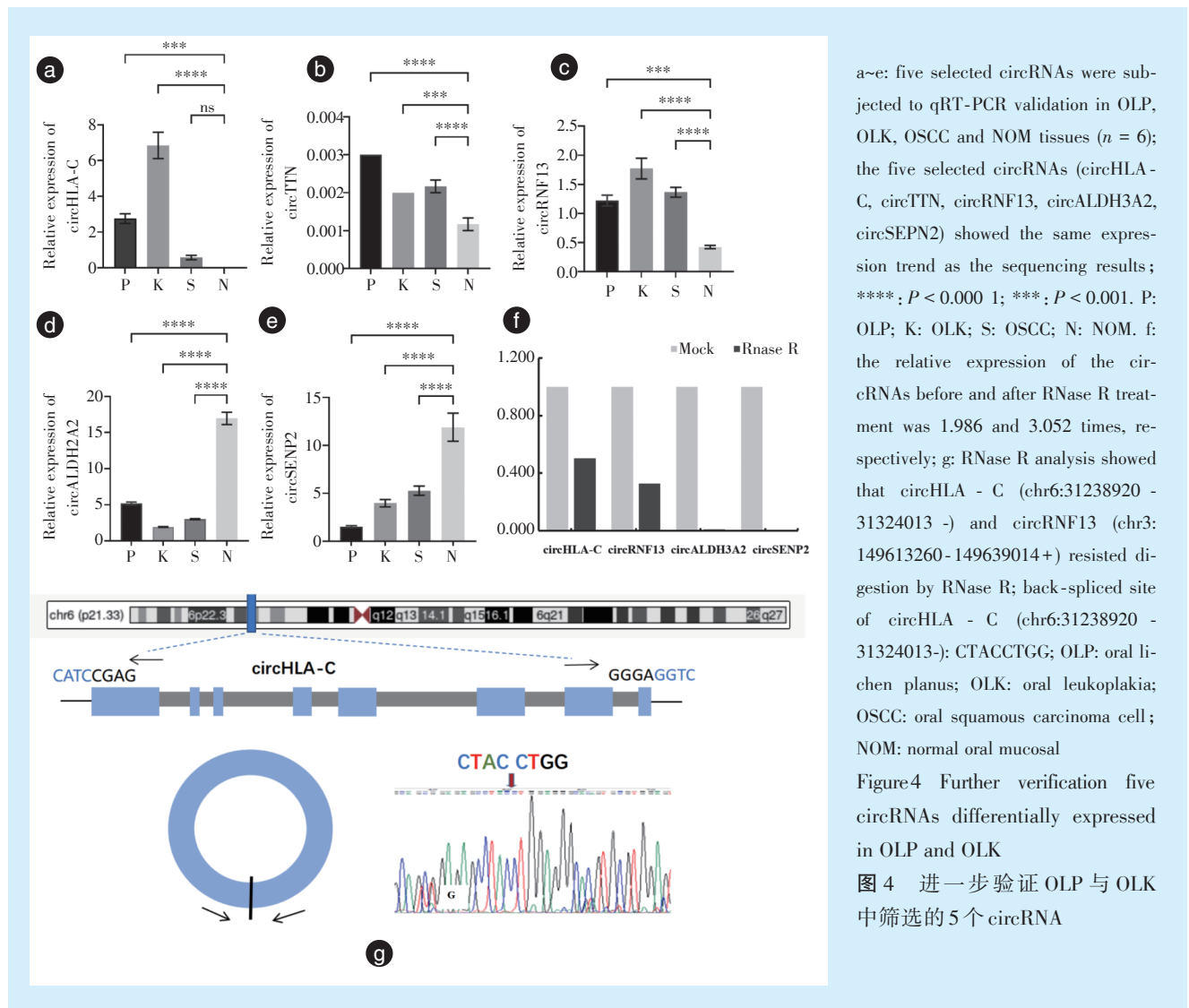
图3 显著差异表达的 circRNA 的分布特征

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



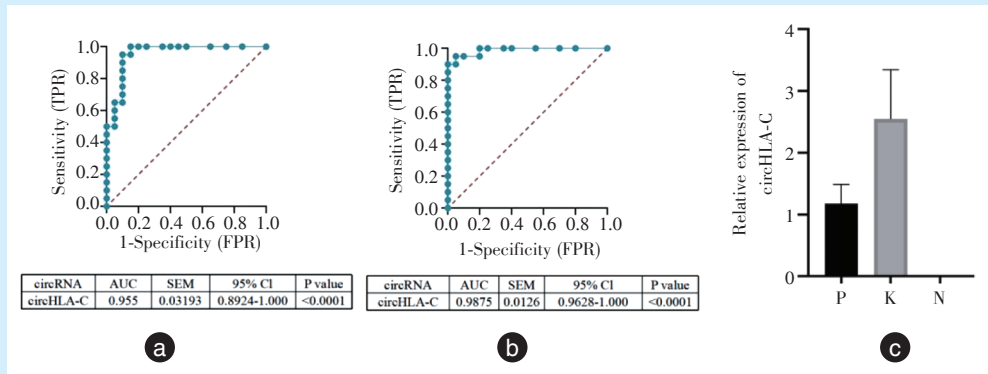
cRNF13 进行 Sanger 测序, 仅在 circHLA-C 上检测到了反向剪切位点 CTACCTGG (图 4g)。

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

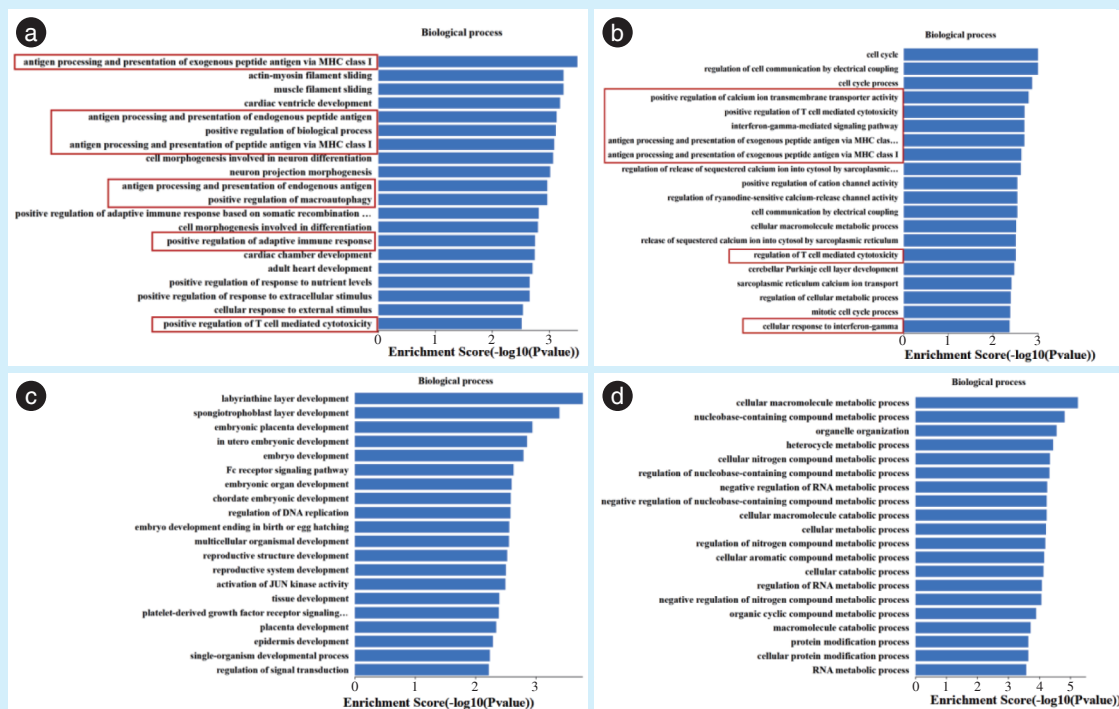
PN 组与 KN 组上调 circRNA 源基因的 GO 功能富集分析显示, PN 与 KN 组富集结果中相同生物学过程共 126 条, 其中上调通路占 114 条, 且与免疫



a: the ROC curves showed that the expression of circHLA-C has potential value in the diagnosis of OLP; b: the ROC curves showed that the expression of circHLA-C has potential value in the diagnosis of OLK; 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

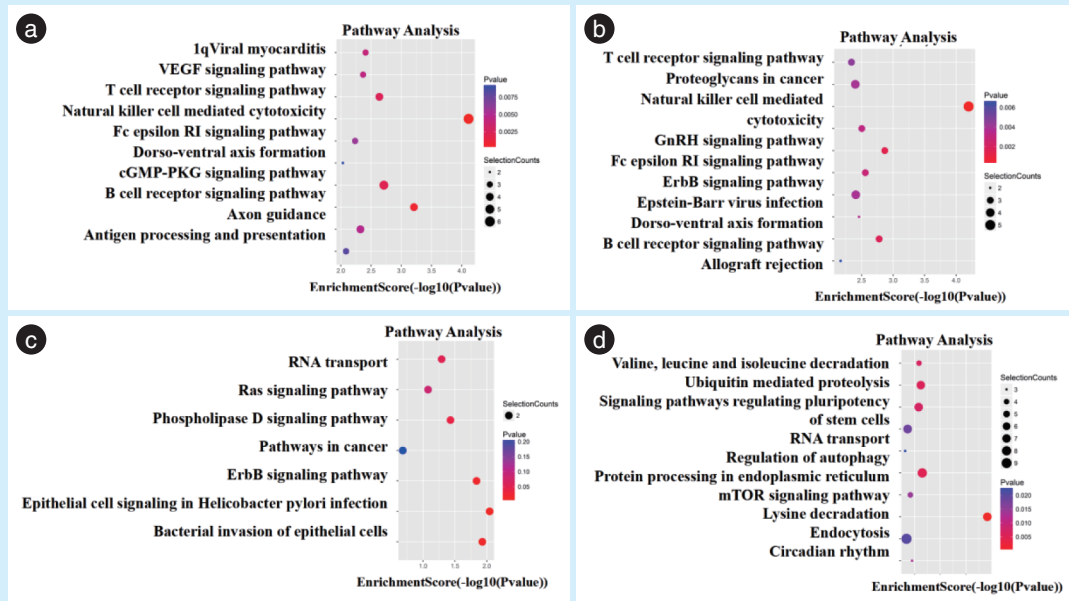
功能相关(通常认为上调富集的通路为激活), HLA-C在这些通路中显著富集(图6):OLK组富集的前20个上调BP功能条目中HLA-C参与7条, OLP组富集的前20个上调BP功能条目中HLA-C

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//FOXPI1//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)。

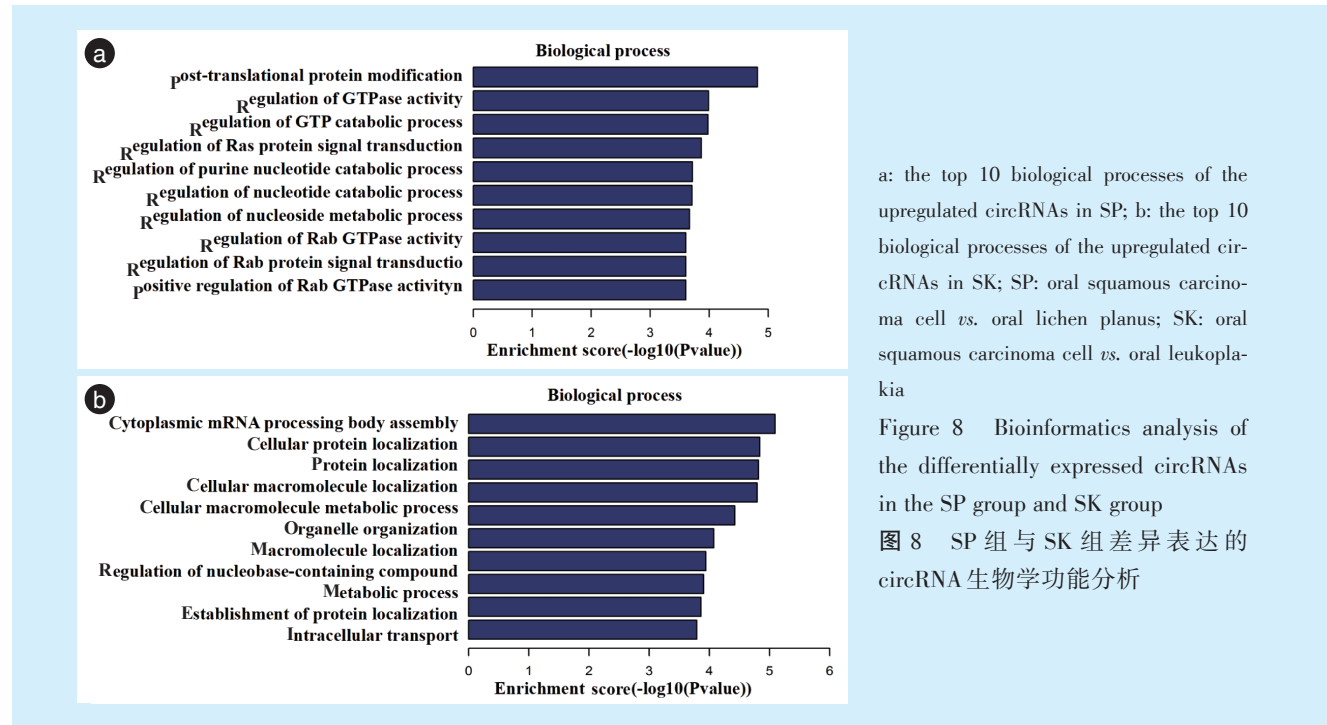


表4 SP与SK差异表达circRNA共同参与的KEGG通路

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

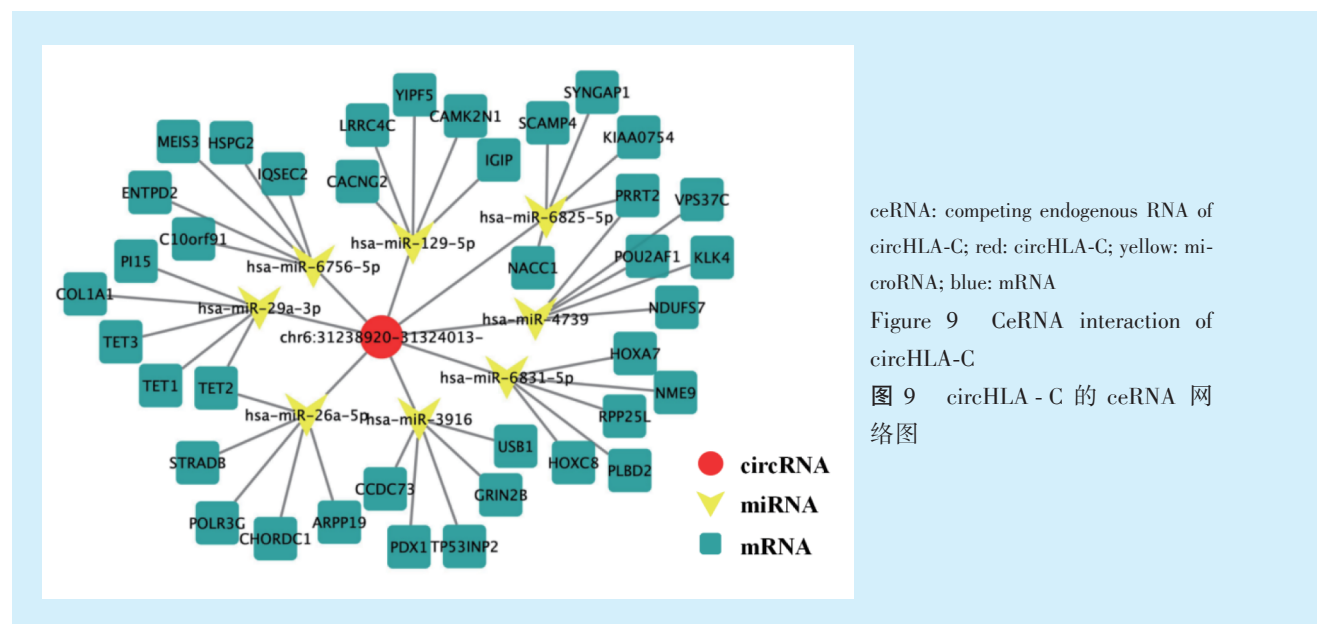
PathwayID	Definition	Genes
hsa05166	HTLV-I infection	AKT3//ATM//CANX//CREBBP//HLA-B//HLA-C
hsa05169	Epstein-Barr virus infection	AKT3//CREBBP//HLA-B//HLA-C
hsa04612	Antigen processing and presentation	CANX//HLA-B//HLA-C//HLA-DQB1
hsa05416	Viral myocarditis	HLA-B//HLA-C//HLA-DQB1//HLA-DRB5//ITGAL
hsa05330	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//DYNC1L1//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//RPGD1
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。



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仍为白斑^[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物物质的基础,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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