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研究论文

环状RNA在胃癌细胞系中的差异表达

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摘 要：本文旨在探讨环状RNA (circular RNA, circRNA)在不同分化程度的胃癌细胞MGC-803、SGC-7901、NCI-N87与正常胃上皮细胞GES-1中的表达谱变化。体外培养低分化胃癌细胞MGC-803、中分化胃癌细胞SGC-7901、高分化胃癌细胞NCI-N87和正常胃上皮细胞GES-1，利用高通量circRNA芯片技术检测三种不同分化程度胃癌细胞和正常胃上皮细胞中差异表达的circRNA，借助生物信息学软件预测可能与circRNA相互作用的微小RNA (microRNA, miRNA)，结合文献检索初步选定可能在胃癌中具有重要意义的circRNA。结果显示，与正常胃上皮细胞相比，在不同分化程度胃癌细胞中均表达上调的circRNA有79条，均表达下调的有229条。生物信息学分析和文献检索结果显示，hsa_circ_0001897与胃癌分期、转移相关，与其结合的miR-150-5p与多种消化道肿瘤密切相关，如结肠癌、肝癌、胆管癌；与hsa_circ_0008106、hsa_circ_0060456存在结合位点的miR-16-5p已被证实参与胃癌的发生和发展。上述结果提示，hsa_circ_0001897、hsa_circ_0008106、hsa_circ_0060456可能通过与miRNA相互作用参与胃癌的发生和发展。

关键词：环状RNA；胃癌；基因芯片
中图分类号：R735.2

Differentially expressed circular RNAs in human gastric cancer cells

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Abstract: The purpose of this study was to investigate the expression profile of circular RNA (circRNA) in gastric cancer cells MGC-803, SGC-7901 and NCI-N87 with different degrees of differentiation and normal gastric epithelial cells GES-1. High throughput circRNA microarray technique was used to detect the differential expression of circRNA between three kinds of differentiated gastric cancer cells and normal gastric epithelial cells. The interaction of microRNAs (miRNAs) with circRNAs was predicted by bioinformatics software, and circRNA, which might have great significance in gastric cancer, was identified by literature search. The results showed that there were 79 up-regulated circRNAs and 229 down-regulated circRNAs in gastric cancer cells with different degrees of differentiation compared with those in normal gastric epithelial cells. Through bioinformatics software analysis and literature retrieval, it was found that hsa_circ_0001897 was related to the staging and metastasis of gastric cancer, while the miR-150-5p, which combined with it, was closely related to many kinds of digestive tract tumors, such as colon cancer, liver cancer, and cholangiocarcinoma. The miR-16-5p, which has binding sites with hsa_circ_0008106 and hsa_circ_0060456, has been confirmed to be involved in the development of gastric cancer. The above results suggest that hsa_circ_0001897, hsa_circ_0008106 and hsa_circ_0060456 may be closely related to the occurrence and development of gastric cancer by interacting with miRNA.

Key words: circRNA; gastric cancer; microarray

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胃癌是世界上最常见的癌症之一，我国胃癌发病例数和死亡例数分别占全球胃癌发病和死亡的42.6% 和 45.0%，在全球 183 个国家中位于发病率第 5 位、死亡率第 6 位 [1]。目前，虽然癌症基因、抑癌基因和与胃癌相关的肿瘤信号通路已经被发现和证实，但是由于我们对多基因参与肿瘤的调控步骤的认识有限，并且缺乏有效的干预手段，胃癌的诊治及预后仍不乐观。因此，寻找新的生物靶点来监测和控制胃癌的发生和发展具有重要的科学和实用价值。

环状 RNA (circular RNA, circRNA) 是一类由特殊的前体 RNA (pre-RNA) 通过反向剪接的方式产生的新型非编码 RNA[2]。研究人员已经证实 circRNA 能够与微小 RNA (microRNA, miRNA) 相互作用，从而参与结肠癌、肝癌、肺癌等肿瘤的发生和发展 [3–5]。本研究在不同分化程度胃癌细胞与正常胃上皮细胞中，利用高通量 circRNA 芯片技术筛选出差异表达的 circRNA，借助生物信息学软件预测可能与 circRNA 相互作用的 miRNA，为深入探讨 circRNA 在胃癌发生和发展中的作用机制奠定基础，同时也为胃癌的诊治提供新的思路。

1 材料和方法

1.1 主要试剂 Trizol 试剂购自美国 Invitrogen 公司；RNase R 购自美国 Epicentre 公司；Rneasy Mini Kit 购自德国 QIAGEN 公司；Cy3-dCTP 购自美国 GE Healthcare 公司；SuperScriptTM III Reverse Transcriptase 购自美国 Invitrogen 公司；RPMI-1640 培养基、无支原体胎牛血清购自美国 GIBCO 公司；0.25% Trypsin-EDTA 胰酶购自美国 Hyclone 公司；DMSO 购自美国 Sigma 公司；Arraystar circRNA 芯片 (8 × 15K) 由上海康成生物公司提供。

1.2 细胞培养 正常胃上皮细胞 GES-1 购自中南大学湘雅医学院细胞中心，人低分化胃癌细胞株 MGC-803 购自上海吉玛制药技术有限公司，人中分化胃癌细胞株 SGC-7901 购自上海吉凯基因公司，人高分化胃癌细胞株 NCI-N87 购自中国科学院干细胞库，用 RPMI-1640 培养基 (含 10% 胎牛血清)，在培养箱内于 5% CO_2、37 °C 条件下培养。

1.3 样品总 RNA 的提取和质控 待细胞完全贴壁后，弃去培养液，按照 Trizol 试剂说明书，从不同分化程度的胃癌细胞株 MGC-803、SGC-7901、NCI-N87 和正常胃上皮细胞 GES-1 中提取总 RNA，应用 Nanodrop-1000 分光光度计分别检测各个样本在 230 nm、260 nm 及 280 nm 的吸光度值，计算 RNA 浓度和质量。

1.4 circRNA 芯片数据采集 将上述获得的总 RNA 用 RNase R 处理以去除线性 RNA 分子，按照 Arraystar Super RNA Labeling protocol 对 circRNA 进行扩增并转录成荧光 cRNA，用 Rneasy Mini Kit 对标记的 cRNA 进行纯化，然后放在 circRNA 芯片 (8 × 15K) 上杂交，于杂交炉中 65 °C 孵育 17 h，杂交反应结束后，取出芯片，首先在 42 °C 含 0.2% SDS 的 2 × SSC 的洗液中洗涤 5 min，然后在室温下，将芯片放入 0.2 × SSC 的洗液中洗涤 5 min，取出芯片甩干，将洗片固定在 Agilent Scanner G2505C 扫描仪上，检测芯片的荧光强度。将芯片所扫描的图片导入 Agilent Feature Extraction (版本 11.0.1.1) 软件以提取数据，然后运用 R 软件包对所得数据进行标准化并进一步分析，样本间差异表达的 circRNA 通过倍数变化 (fold change, FC, FC > 1.5) 和 P 值 (< 0.05) 进行筛选，实验重复 3 次。两样本间比较采用 t 检验，当 $P < 0.05$ 时，差异具有统计学意义。circRNA 芯片数据的采集、以及火山图与聚类图的绘制和分析由上海康成生物公司完成。

1.5 生物信息学分析 根据差异表达 FC 和 P 值筛选 circRNA 分子，利用生物信息学软件 Targetscan 和 Miranda 预测与 circRNA 存在结合位点的 miRNA。

2 结果

2.1 总RNA、cRNA的质量检测

分光光度计结果显示，胃癌细胞实验组和正常胃上皮细胞对照组 RNA OD_{260}/OD_{280} 均为 1.8~2.0，OD_{260}/OD_{230} 均 > 1.8，RNA 总量均 ≥ 0.5 μg，cRNA 总量均 ≥ 1.65 μg，Cy3 标记特异活性均 ≥ 9 pmol Cy3/μg，总 RNA 与 cRNA 质量检测均符合实验要求 (表 1)，可用于下一步芯片杂交实验。

2.2 胃癌中差异表达的circRNA

差异表达的 circRNA 筛选标准为：FC > 1.5，$P < 0.05$。芯片结果显示，与正常胃上皮细胞 GES-1 相比，在低分化胃癌细胞 MGC-803、中分化胃癌细胞 SGC-7901 和高分化胃癌细胞 NCI-N87 中均表达上调的 circRNA 有 79 条，均表达下调的有 229 条。表 2、表 3 列出了表达上调和下调的前 5 条 circRNA。

2.3 绘制火山图和聚类图分析表达谱差异

根据上述芯片差异表达谱绘制火山图和聚类图。火山图如图 1 所示，红点代表在胃癌细胞中差异表达倍数在 1.5 倍以上，*P* 值小于 0.05 的 circRNA。聚类图如图 2 所示，红色区域代表 circRNA 相对表达量高，绿色代表 circRNA 相对表达量低，差异表达的 circRNA 能有效区分胃癌细胞和正常胃上皮细胞。

2.4 初步确定在胃癌中具有重要意义的circRNA

运用生物信息学软件 Targetscan 和 Miranda 对 circRNA 与 miRNA 相互作用靶点进行预测，每条 circRNA 均预测到 5 个 miRNA 结合位点 (表 4、5、图 3)。在所有筛选出来的 circRNA 中，我们通过文献检索发现，hsa_circ_0001897 已被报导与胃癌分期、转移相关，与其结合的 miR-150-5p 已被证实与多种消化道肿瘤密切相关，如结肠癌、肝癌、胆

表1. 总RNA与cRNA的质控

Table 1. Quality assurance of total RNA and cRNA quantification

Sample	Total RNA					cRNA			
	OD_{260}/OD_{280}	OD_{260}/OD_{230}	Quantity (ng)	Volume (μL)	Concentration (ng/μL)	Cy3 positive (pmol Cy3/μg)	Quantity (μg)	Volume (μL)	Concentration (μg/μL)
GES-1_1	1.92	2.47	78 352.80	120	652.94	31.283 53	4.286 6	10	0.428 66
GES-1_2	1.94	2.46	56 376.80	80	704.71	29.639 95	4.666 0	10	0.466 60
GES-1_3	1.94	2.44	67 057.60	80	838.22	27.396 94	4.318 0	10	0.431 80
MGC-803_1	1.95	2.45	117 630.00	120	980.25	31.554 98	4.611 0	10	0.461 10
MGC-803_2	1.95	2.45	88 435.20	120	736.96	30.708 55	4.842 3	10	0.484 23
MGC-803_3	1.98	2.41	142 429.20	120	1 186.91	29.985 41	4.455 5	10	0.445 55
SGC-7901_1	1.97	2.38	111 660.00	120	930.50	30.671 96	4.460 1	10	0.446 01
SGC-7901_2	1.96	2.42	91 425.60	120	761.88	30.336 36	4.545 7	10	0.454 57
SGC-7901_3	1.96	2.44	151 070.40	160	944.19	28.344 02	4.007 9	10	0.400 79
NCI-N87_1	1.97	2.49	129 976.00	160	812.35	30.792 14	4.968 8	10	0.496 68
NCI-N87_2	1.98	2.42	152 438.40	160	952.74	29.049 60	4.093 0	10	0.409 30
NCI-N87_3	2.00	2.37	146 052.80	160	912.83	29.659 04	4.507 9	10	0.450 79

For spectrophotometer, the OD_{260}/OD_{280} ratio should be close to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable). The OD_{260}/OD_{230} ratio should be more than 1.8. If the yield is < 1.65 μg and the specific activity is < 9.0 pmol Cy3/μg, cRNA do not proceed to the hybridization step.

表2. 胃癌细胞和正常胃上皮细胞中差异表达上调的前5条circRNA

Table 2. Top 5 up-regulated expressed circRNAs between gastric cancer cells and normal gastric epithelial cells

No.	circRNA	Fold change	*P*	circRNA type
1	hsa_circ_0003057	2.690 924 5	0.000 013 390 24	Exonic
2	hsa_circ_0005044	2.690 082 6	0.008 701 295 95	Intronic
3	hsa_circ_0001944	2.654 759 2	0.003 374 118 79	Exonic
4	hsa_circ_0091537	2.495 864 3	0.000 877 223 01	Exonic
5	hsa_circ_0001829	2.288 660 7	0.032 868 960 87	Exonic

circRNA type: The location of the circRNA in the chromosome.

表3. 胃癌细胞和正常胃上皮细胞差异表达下调的前5条circRNA

Table 3. Top 5 down-regulated expressed circRNAs between gastric cancer cells and normal gastric epithelial cells

No.	circRNA	Fold change	*P*	circRNA type
1	hsa_circ_0022382	12.279 568 4	0.000 273 211 7	Exonic
2	hsa_circ_0000994	10.698 419 1	0.000 002 434 35	Exonic
3	hsa_circ_0022383	10.386 687 1	0.000 454 694 93	Exonic
4	hsa_circ_0007444	8.968 459 1	0.002 682 606 23	Exonic
5	hsa_circ_0005232	8.295 784	0.000 001 892 63	Exonic

circRNA type: The location of the circRNA in the chromosome.

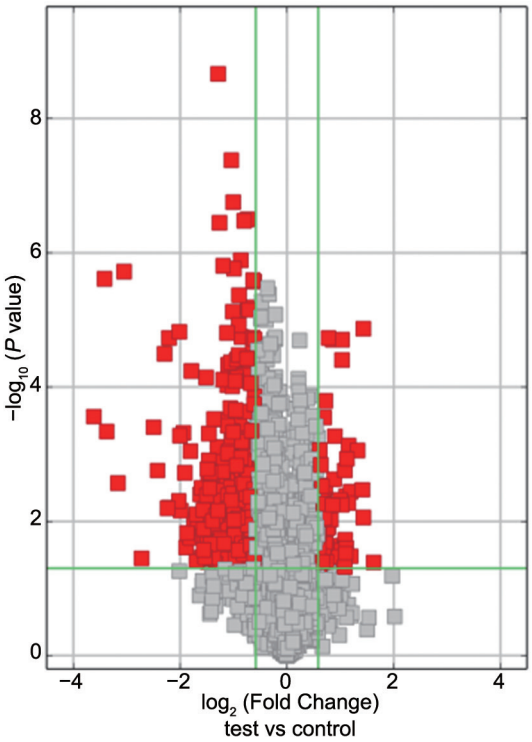

图 1. circRNA火山图

Fig. 1. Volcano plot of circRNAs. The red points in plot represent the differentially expressed circRNAs with statistical significance between gastric cancer cells and normal gastric epithelial cells. The left red points represent down-regulation, and the right red points represent up-regulation.

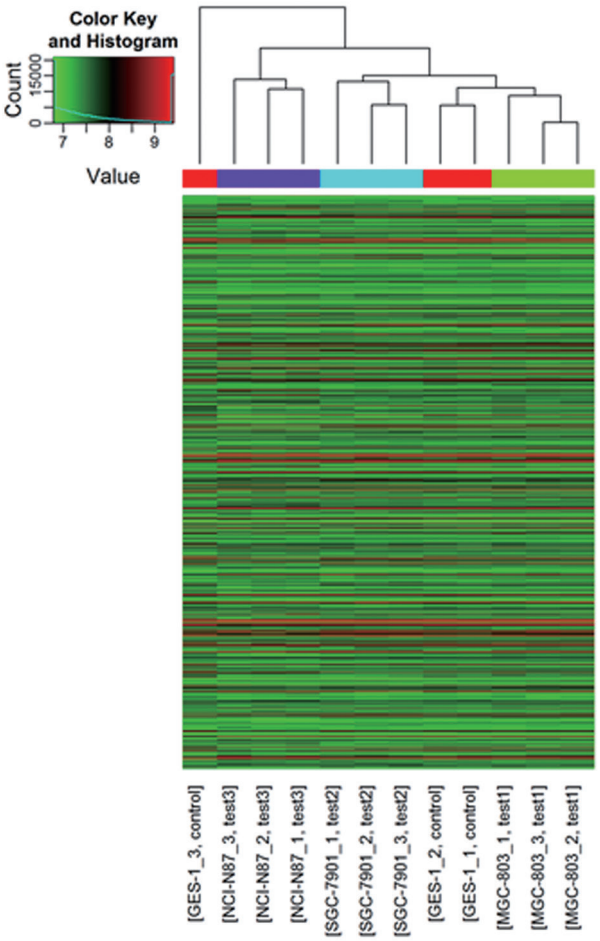

图 2. 差异circRNA聚类图

Fig. 2. Clustering plot of the differentially expressed circRNAs between gastric cancer cells and normal gastric epithelial cells. The result from unsupervised hierarchical clustering analysis shows distinguishable circRNA expression profiling among samples. Each column represents the expression profile of a cell sample, and each row corresponds to a circRNA. "Red" indicates higher expression level, and "green" indicates lower expression level.

管癌 [6–8]；与 hsa_circ_0008106、hsa_circ_0060456 存在结合位点的 miR-16-5p 已被证实参与胃癌的发生和发展，并且与肿瘤恶性程度相关 [9, 10]。

3 讨论

CircRNA 作为非编码 RNA 家族的新成员，正吸引着越来越多人的注意。生物信息技术的飞速发展将人们带入 circRNA 的世界，人们对 circRNA 的认识正在不断深入，其许多生物学特性已被发现，其中一些重要的特征包括广泛性、高度稳定性及保守性等 [11–14]。目前，研究已经证实 circRNA 的差异表达与包括胃癌在内的恶性肿瘤密切相关。CircRNA 广泛性、高度稳定性等生物学特征及在肿瘤中的表达特点提示我们：它可能作为新型肿瘤的生物标志物并对肿瘤的诊断起到支持作用。CircRNA 在肿瘤中的分子作用机制有待进一步研究。

最近，有学者提出竞争性内源 RNA (competing endogenous RNA, ceRNA) 假说，认为具有相同 miRNA 应答元件 (miRNA response element, MRE) 的 RNA 分子，可以通过竞争性地结合相同的 miRNA，实现对 miRNA 的靶向调控 [15]。CircRNA 作为 ceRNA 家族新发现的重要成员，本身富含 miRNA 结合位点，可发挥"海绵作用"吸附 miRNA，降低 miRNA 对靶基因的抑制作用，增加靶基因的表达水平，进而起到调控生物学过程的作用 [11, 16]。Cao 等在研究肝癌时观察到，circMTO1 能够靶向结合 miR-9 来调控 p21 的表达，从而抑制肿瘤细胞的增殖和侵袭 [17]。Chen 等在研究口腔癌时观察到，circRNA_ 100290 可通过靶向结合 miR-29，从而解除 miR-29 对 CDK6 的抑制，促进口腔鳞状细胞癌的发生和发展 [18]。

表4. hsa_circ_0001897、hsa_circ_0008106和hsa_circ_0060456的详细信息

Table 4. Details of hsa_circ_0001897, hsa_circ_0008106 and hsa_circ_0060456

circRNA	Fold change (down-regulated)	*P*	circRNA type	miRNA binding site
hsa_circ_0001897	1.846 206 2	0.000 065 301 39	Exonic	hsa-miR-150-5p
				hsa-miR-29a-3p
				hsa-miR-29c-3p
				hsa-miR-29b-3p
				hsa-miR-767-5p
hsa_circ_0008106	2.055 149 4	0.001 133 053 95	Exonic	hsa-miR-29b-2-5p
				hsa-miR-29a-5p
				hsa-miR-518c-5p
				hsa-miR-214-3p
				hsa-miR-16-5p
hsa_circ_0060456	1.861 731 1	0.001 182 505 61	Exonic	hsa-miR-647
				hsa-miR-412-3p
				hsa-miR-637
				hsa-miR-660-3p
				hsa-miR-16-5p

circRNA type: The location of the circRNA in the chromosome. For each circRNA, we identified 5 miRNAs with the highest mirSVR score to establish a "Top-5" circRNA-miRNA network (1 circRNA connecting to 5 miRNAs)

表5. hsa_circ_0001897、hsa_circ_0008106和hsa_circ_0060456在胃癌细胞中的表达

Table 5. The expression of hsa_circ_0001897, hsa_circ_0008106 and hsa_circ_0060456 in gastric cancer cells

	Fold change		
circRNA	MGC-803 *vs* GES-1	SGC-7901 *vs* GES-1	NCI-N87 *vs* GES-1
hsa_circ_0001897	1.950 956 1	1.756 716 1	1.836 079 7
hsa_circ_0008106	1.834 483 0	2.222 134 8	2.129 345 3
hsa_circ_0060456	1.505 752 4	1.804 972 4	2.374 251 7

GES-1 cells were normal gastric epithelial cells and used as the control group.

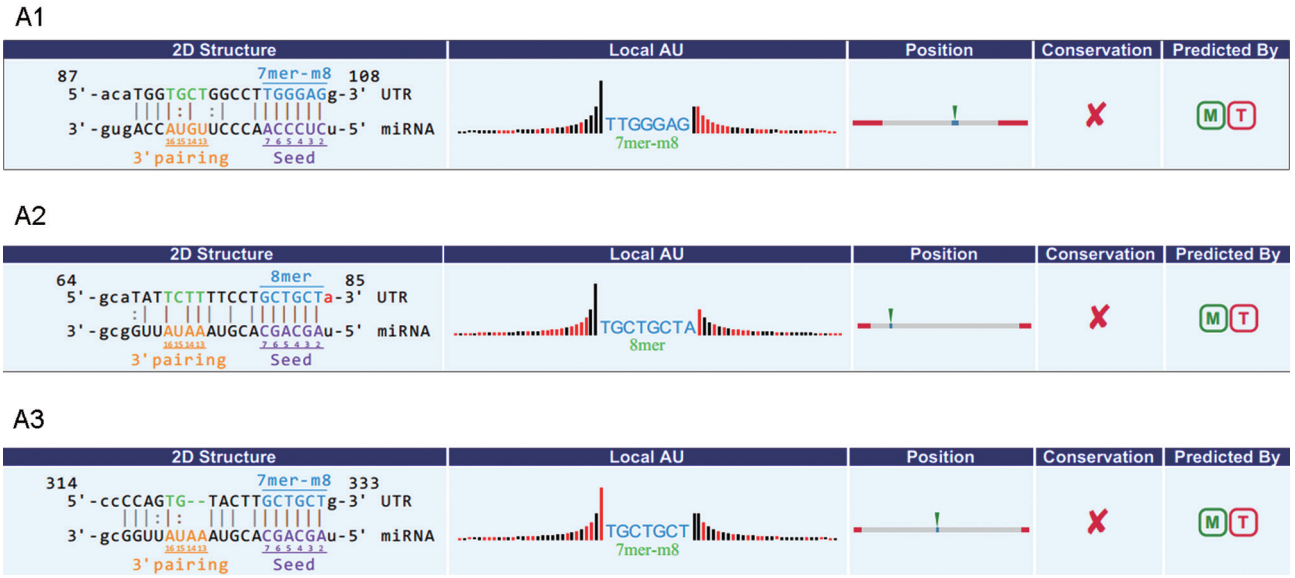

图 3. circRNA与miRNA相互作用位点的详细注释图

Fig. 3. The detailed annotation for circRNA-miRNA interactions. *A1*: Complementary situations of hsa_circ_0001897 and miR-150-5p. *A2*: Complementary situations of hsa_circ_0008106 and miR-16-5p. *A3*: Complementary situations of hsa_circ_ 0060456 and miR-16-5p.

在胃癌相关 circRNA 的研究方面，Zhu 等利用高通量 RNA 测序技术观察到，与癌旁组织对比，circLARP4 在胃癌组织中明显低表达；通过生物信息学技术观察到 circLARP4 与 miR-424-5p 存在结合位点，LATS1 为其下游靶基因，随后借助双荧光素酶实验和 RNA 免疫共沉淀技术验证三者之间的结合位点，最后通过 MTT、克隆增殖等实验观察到 circLARP4 通过结合 miR-424-5p 来调控 LATS1 的表达，进而抑制胃癌细胞的增殖与侵袭 [19]。Li 等利用 circRNA 基因芯片技术观察到 46 条表达差异显著的 circRNA，随后选定低表达的 circRNA-100269 为研究对象，利用 RT-PCR 验证了芯片结果，随后通过生物信息学软件预测 circRNA-100269 与 miR-630 之间可能存在结合位点，在后续的分子生物学功能实验中，通过过表达实验观察到 circRNA-100269 能够调控 miR-630 的表达，从而抑制胃癌细胞的增殖，并且发现 miR-630 高表达能够阻止 circRNA-100269 对胃癌细胞的抑制效应 [20]。Guo 等亦认为 hsa_circ_0000190 可能作为新的胃癌诊断和治疗标志物，相比传统的胃癌肿瘤标志物 CEA、CA19-9，hsa_circ_0000190 敏感性和特异性均较高 [21]。

本研究借助 Arraystar 公司 circRNA 芯片筛选不同分化程度胃癌细胞 MGC-803、SGC-7901、NCI-N87 与正常胃上皮细胞 GES-1 间差异表达的 circRNA。结果显示，与正常胃上皮细胞相比，在三种不同分化程度的胃癌细胞中均表达上调的 circRNA 有 79 条，均表达下调的有 229 条。在这些差异表达的 circRNA 中，我们通过文献检索并挑选了具有研究价值的 circRNA：hsa_circ_0001897 在胃癌细胞中表达下调，已有文献报道其与胃癌分期、转移相关 [22]，但它在胃癌中的分子作用机制还未被阐明，通过生物信息学软件 Targetscan 和 Miranda 预测，hsa_circ_0001897 与 miR-150-5p 存在结合位点，进一步检索发现，miR-150-5p 与多种消化道肿瘤密切相关，如结肠癌、肝癌、胆管癌 [6–8]，由此，我们推测 hsa_circ_0001897 可能通过调控 miR-150-5p 抑制胃癌细胞的侵袭与转移；hsa_circ_0008106、hsa_circ_006045 在胃癌细胞中表达下调，与其存在结合位点的 miR-16-5p 已被证实参与胃癌的发生和发展 [9,10]，这些结果提示我们，hsa_circ_ 0008106、hsa_circ_0060456 可能通过与 miR-16-5p 相互作用，从而抑制胃癌细胞的侵袭与转移，目前，hsa_circ_0008106、hsa_circ_0060456 还未曾被报道。后续的研究应深入探讨 hsa_circ_0001897、hsa_circ_0008106 和 hsa_circ_0060456 在胃癌中的分子作用机制，以期为寻找胃癌新的诊治方法和药物治疗靶点提供理论基础。

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