·基础研究·

# 肝细胞癌患者血清外泌体 mRNA 表达谱及临床意义分析

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**摘　要**　**背景与目的：**外泌体是包含了复杂 RNA 和蛋白质的小膜泡，其中肿瘤细胞分泌的外泌体中的 mRNA 携带了大量肿瘤细胞遗传信息，因此对外泌体中特异性 mRNA 的分析，有望找到肿瘤诊断的新型分子标志物和治疗靶点。本研究通过高通量筛选与生物信息学方法探讨肝细胞癌（HCC）患者血清外泌体 mRNA 的表达特征及其潜在功能。

**方法：**采集 3 例 HCC 患者及 3 例正常人的静脉血，用外泌体提取试剂盒提取血清外泌体，用 Magen 试剂盒提取外泌体 RNA，对血清外泌体 mRNA 进行纯化、行反转录形成 cDNA、PCR 扩增、测序，最后将对所得数据质量进行评价后与参考数据进行比对，分析得到差异表达外泌体 mRNA，并采用 GO 和 KEGG 通路富集分析对差异基因进行功能与通路注释。

**结果：**与正常人比较，HCC 患者有 397 个外泌体 mRNA 表达上调，192 个外泌体 mRNA 表达下调；其中，NRGN、PF4、RGS18 等 17 个基因表达明显上调，CXCL8、MORF4L2、SYCP1 等 14 个基因显明显下调。GO 富集分析显示，表达上调的外泌体 mRNA 的靶基因与蛋白质结合、蛋白质异二聚化活性、免疫系统过程的调节、胞外囊泡、细胞外细胞器、应激反应等有关，表达下调的外泌体 mRNA 的靶基因与嗅觉受体活性、细胞因子活性、CXCR 趋化因子受体结合、中间丝、中间丝状细胞骨架等有关；KEGG 通路分析显示，在上调的外泌体 mRNA 中，30 条通路被显著富集，在下调的外泌体 mRNA 中 9 条通路被显著富集，其中，在上调的外泌体 mRNA 中，血小板激活、Rap 1 信号通路、吞噬体、病毒致癌、肌动蛋白细胞骨架的调节与抗原处理和呈递等是最丰富和最有意义的通路；在下调的外泌体 mRNA 中，基础转录因子和细胞因子–细胞因子受体相互作用分别是最丰富和最有意义的通路。

**结论：**HCC 患者与正常人的血清外泌体 mRNA 表达特征存在较大差异，其与 HCC 发生、发展、转移可能密切相关，这为寻找新的诊断标志物及治疗靶点提供了依据。

**关键词**　癌，肝细胞；外泌体；RNA，信使；生物标记，肿瘤

**中图分类号：**R735.7

# Analysis of expression profile of serum exosomal mRNAs in patients with hepatocellular carcinoma and the clinical significance

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**Abstract**　**Background and Aims:** Exosomes are small vesicles containing diverse RNAs and proteins, and those secreted

from tumor cells carry considerable genetic information of the tumor cells. So, investigations of the specific mRNAs in the exosomes may provide chances for finding new molecular markers and therapeutic targets of tumors. This study was undertaken to investigate the expression profiles of the exosomal RNAs in hepatocellular carcinoma (HCC) patients and their potential functions by high-throughput screening and bioinformatics method.

**Methods:** The venous blood samples were collected from 3 HCC patients and 3 healthy subjects, the serum exosomes were extracted by using exosome isolation kit, and exosomal RNAs were extracted by Magen kit. Then, the serum exosomal mRNAs were purified, reverse-transcribed into cDNAs, amplified by PCR, and identified by sequencing. Finally, the obtained data were compared with the reference data after quality assessment (BAM files) and the differentially expressed exosomal mRNAs were identified and evaluated, and the GO and KEGG Pathway enrichment analysis were used to annotate the function and pathway of the differentially expressed genes.

**Results:** In HCC patients compared with healthy subjects, there were 397 up-regulated exosomal mRNAs and 192 down-regulated was up-regulated exosomal mRNAs, in which 17 genes such as NRGN, PF4 and RGS18 were significantly up-regulated, and 14 genes such as CXCL8, MORF4L2 and SYCP1 were significantly down-regulated. GO enrichment analysis showed that the target genes of the up-regulated exosomal mRNAs were related to the protein binding, protein heterodimerization activity, regulation of the immune system process of adjusting and second extracellular vesicles, outside the cell organelles, stress reaction and so on, while the target genes of down-regulated exosomal mRNAs were related to olfactory receptor activity, cytokine activity, CXCR chemokine receptor binding, intermediate filaments, intermediate filamentous cytoskeleton. KEGG pathway analysis showed that and 30 pathways were significantly enriched in the up-regulated exosomal mRNAs and 9 pathways were significantly enriched in the down-regulated exosomal mRNAs, in which, the platelet activation, Rap 1 signaling pathway, phagocytosis, viral carcinogenesis, regulation of actin cytoskeleton and antigen processing and presentation were the most abundant and significant pathways in the upregulated exosome mRNA, while, the basal transcription factor and cytokine-cytokine receptor interaction were the most abundant and significant pathways in the down-regulated exosomal mRNAs.

**Conclusion:** There is a significant difference in expression profile in serum exosomal mRNAs between HCC patients and healthy individuals, which may be closely related to the occurrence, development and metastasis of HCC, and also provide a basis for finding new diagnostic markers and therapeutic targets.

**Key words**    Carcinoma, Hepatocellular; Exosomes; RNA, Messenger; Biomarkers, Tumor

**CLC number:** R735.7

肝细胞癌（hepatocellular carcinoma，HCC）恶性程度高，其发病率在所有肿瘤中高居第4位，致死率较高[1-3]，中国每年HCC死亡例数占约占全球总数的55%，现已成为严重威胁我国人民群众健康的重大恶性肿瘤之一[4-5]。目前临床医生对于HCC的诊断工具主要依靠彩超、CT、MR及AFP等检查、检验，但HCC起病隐匿，症状不明显，待发现时大多已属于中晚期，手术切除、射频消融、介入等治疗方法有效的提高了HCC患者生存率[6]，但总体预后难以令人满意，5年生存率约30%~40%[7]。因此，寻找新的HCC诊断分子标志物意义仍然重大。

目前，高通量测序技术能够从整个基因组或转录组水平探索疾病的发生、发展，已广泛用于一些疾病基因表达谱分析、基因克隆和寻找特异性分子标志物[8-9]。白文萱等[10]通过生物学信息分析的方法，对HCC基因芯片数据进行挖掘，发现磷脂酰肌醇蛋白聚糖（GPC3）在HCC中表达上调，可作为HCC特异性的免疫治疗靶点；Zhang等[11]通过高通量测序生物学分析的方法发现HCC差异表达的基因在抗原处理与呈递、补体级联和I型干扰素信号通路有协同或拮抗作用，而这些对HCC的发生与侵袭能力变化有重要影响，高通量测序技术是测序技术发展的里程碑，它为现代生

命技术研究提供了前所未有的机遇[12]。

外泌体是细胞分泌的功能性小体，直径为30~150 nm，在人体血液、唾液、尿液等体液中均能够被检测到[13]。其体积微小并且自身稳定，脂质双层膜结构可有效保护miRNA、mRNA和其它非编码RNA核酸分子不被降解[14-15]，被认为是肿瘤标志物的天然载体，近年在肿瘤诊断、发生发展机制、靶向治疗中越来越得到重视。Wang等[16]发现外泌体miR-122、miR-1246和miR-148a在HCC患者中呈显著性高表达，其中miR-148a诊断效能显著优于AFP（0.891 *vs.* 0.712），此外miR-122、AFP和miR-148a联合用于区分HCC和肝硬化患者的诊断效能高达0.931。有学者发现HCC患者血清外泌体中的miR-9-3P水平明显降低，成纤维细胞因子5（HBGF-5）在细胞增殖中具有重要作用，后者是miR-9-3P潜在的靶mRNA，miR-9-3P过表达显著下调了HBGF-5在mRNA和蛋白水平的表达，进而影响了HCC细胞的增殖[17]。

综上，笔者推测HCC患者与正常人之间血清外泌体mRNA存在较大差异，可能筛选出有意义的分子标志物，故拟通过高通量测序方法进行检测，以期寻找合适的HCC诊断标志物。

# 1　资料与方法

## 1.1　一般资料

收集2018年8月—2018年12月在新乡医学院第一附属医院就诊的HCC患者血液标本3例（Ca1、Ca2、Ca3），均有乙型肝炎及肝硬化病史，且肿瘤病理类型均经病理科医师确诊（其中Ca1为中分化HCC，部分侵及肝被膜，可见脉管内癌栓；Ca2为低分化HCC，紧邻被膜；Ca3为中分化HCC）。3例HCC患者中男2例、女1例。同时收集3例正常志愿者血液标本（N1、N2、N3）作对照，3例正常志愿者中男2例、女1例。采集样本前均未接受临床治疗。本研究经医院伦理委员会批准，且研究对象均知情同意并签署知情同意书。

## 1.2　仪器和主要试剂

仪器：illumina Hiseq Xten；模式：PE150；试剂：Agencourt Ampure XP beads，美国Beckman Coulter公司；NEBNext® Ultra™ RNA Library Prep Kit for Illumina，美国NEB公司；HiSeq Rapid SBS Kit V2（200 cycle），美国Illumina公司；HiSeq Rapid PE Cluster Kit V2，

美国Illumina公司。

## 1.3　血清外泌体的提取及鉴定

所有研究对象采集静脉血6 mL，EDTA-K2抗凝管收集静置，然后离心取血清。血清外泌体采用锐博外泌体提取试剂盒Ribo™ Exosome Isolation Reagent（for plasma or serum）提取，严格按照试剂说明进行操作。外泌体表面蛋白标志物采用Western blot进行检测，所用抗体包括CD9、CD63、TSGl01及羊抗兔二抗。

## 1.4　外泌体 RNA 提取

用Magen试剂盒提取外泌体RNA，操作步骤详见说明书。

## 1.5　外泌体 mRNA 高通量测序

首先对HCC患者血清外泌体mRNA进行纯化等前期处理，然后进行反转录形成cDNA、PCR扩增等实验操作，再经过文库质检后上机测序；再对测序质量进行评价，以取得高质量的数据，并对高质量的数据及参考数据进行比对，获得BAM文件。上机测序由广州市锐博生物公司测序，并进行差异表达外泌体mRNA进行分析，采用AUdics对差异表达mRNA进行分析。对差异表达的外泌体mRNA进行GO（Gene Ontology）和KEGG pathway分析。

# 2　结　果

## 2.1　差异外泌体 mRNA 筛选

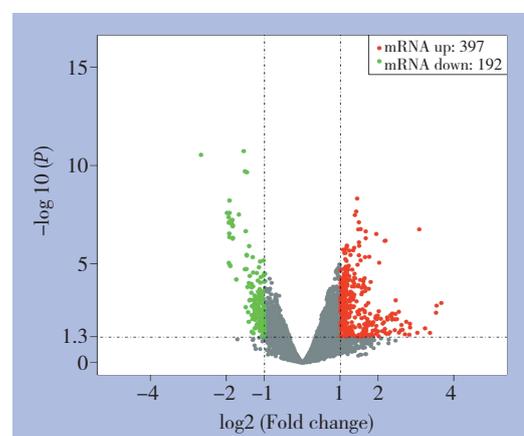筛选的差异表达外泌体mRNA统计结果如图1，其中HCC组相对正常人组上调397个外泌体mRNA，192个外泌体mRNA表达下调。



图 1　mRNA 差异表达火山图

**Figure 1　Volcanic map of the mRNAs with differential expression**

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## 2.2 显著差异表达的外泌体 mRNA

对差异外泌体mRNA进行表达差异性分析，采用HCC组相对正常人组外泌体mRNA表达fold change（log2，表达差异倍数）对差异蛋白进行筛选，挑选条件如下：Log2≤1.9或者Log2≥2.5；表达水平显著增加的外泌体mRNA17个，表达水平明显降低的外泌体mRNA14个（表1）。

<p align="center">表 1　两组表达差异显著的外泌体 mRNA</p>
<p align="center">Table 1　The exosomal mRNAs with significantly different expressions between the two group</p>

| mRNA | RNA 类型 | 基因 | 基因类型 | Log2（fold change） | P |
|---|---|---|---|---|---|
| NM_001126181.1 | mRNA | NRGN | 蛋白质编码 | 3.657617454 | 0.000922185 |
| NM_006176.2 | mRNA | NRGN | 蛋白质编码 | 3.532022736 | 0.001263149 |
| NM_002619.3 | mRNA | PF4 | 蛋白质编码 | 3.517962473 | 0.002894941 |
| NM_130782.2 | mRNA | RGS18 | 蛋白质编码 | 3.358151332 | 0.030458394 |
| NM_002704.3 | mRNA | PPBP | 蛋白质编码 | 3.228366412 | 0.017874175 |
| NM_014380.2 | mRNA | BEX3 | 蛋白质编码 | 3.075346704 | 1.69E-07 |
| NM_021109.3 | mRNA | TMSB4X | 蛋白质编码 | 3.025417657 | 0.030113849 |
| NM_002727.3 | mRNA | SRGN | 蛋白质编码 | 2.834783891 | 0.016251181 |
| NM_000129.3 | mRNA | F13A1 | 蛋白质编码 | 2.829778398 | 0.010467425 |
| NM_004048.2 | mRNA | B2M | 蛋白质编码 | 2.814042874 | 0.037556495 |
| NM_030773.3 | mRNA | TUBB1 | 蛋白质编码 | 2.733704502 | 0.008312526 |
| NM_001101.3 | mRNA | ACTB | 蛋白质编码 | 2.698418532 | 0.035903098 |
| NM_002970.3 | mRNA | SAT1 | 蛋白质编码 | 2.636033419 | 0.021323284 |
| NM_005514.7 | mRNA | HLA-B | 蛋白质编码 | 2.607465232 | 0.012074514 |
| NM_001321053.1 | mRNA | SRGN | 蛋白质编码 | 2.589231164 | 0.02241961 |
| NM_002736.2 | mRNA | PRKAR2B | 蛋白质编码 | 2.541893658 | 0.029694094 |
| NM_002620.3 | mRNA | PF4V1 | 蛋白质编码 | 2.537698144 | 0.002589005 |
| NM_001282541.1 | mRNA | SYCP1 | 蛋白质编码 | −1.901671799 | 1.23E-05 |
| NM_001282542.1 | mRNA | SYCP1 | 蛋白质编码 | −1.909400883 | 1.09E-05 |
| NM_001142427.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.917381194 | 6.98E-08 |
| NM_001142418.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.921683395 | 2.48E-08 |
| NM_001136025.4 | mRNA | PLS3 | 蛋白质编码 | −1.923888886 | 5.84E-09 |
| NM_001142426.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.924537387 | 4.23E-07 |
| NM_001142421.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.924749837 | 7.07E-08 |
| NM_001142420.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.929036325 | 2.79E-07 |
| NM_001142425.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.933151847 | 7.50E-08 |
| NM_001142423.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.940263429 | 7.69E-08 |
| NM_003176.3 | mRNA | SYCP1 | 蛋白质编码 | −1.941506196 | 8.64E-06 |
| NM_001142419.1 | mRNA | MORF4L2 | 蛋白质编码 | −1.946314003 | 4.07E-08 |
| NM_012286.2 | mRNA | MORF4L2 | 蛋白质编码 | −1.988287748 | 2.47E-08 |
| NM_000584.3 | mRNA | CXCL8 | 蛋白质编码 | −2.673415581 | 2.81E-11 |

## 2.3 层次聚类分析

根据差异外泌体mRNA检测结果进行层次聚类分析，分析结果如图2，其中X轴代表进行聚类分析的差异比较组，Y轴代表样本差异外泌体mRNA。颜色代表差异倍数，越绿表示下调倍数越大，越红则表示上调倍数越大。

## 2.4 GO 显著性富集分析

分别对表达下调与表达上调的外泌体mRNA的靶基因做GO显著性富集分析，如图3–4，其中横坐标为差异外泌体mRNA的靶基因数，纵坐标表示GO terms。GO terms总共有3类，分别以不同颜色标注（橙色：分子功能，红色：细胞成分，绿色：生物过程）。结果显示表达下调的外泌体mRNA的靶基因与嗅觉受体活性、细胞因子活性、CXCR趋化因子受体结合、中间丝、中间丝状细胞骨架等有关；表达上调的外泌体mRNA的靶基因与蛋白质结合、蛋白质异二聚化活性、免疫系统过程的调节、胞外囊泡、细胞外细胞器、应激反应等有关。

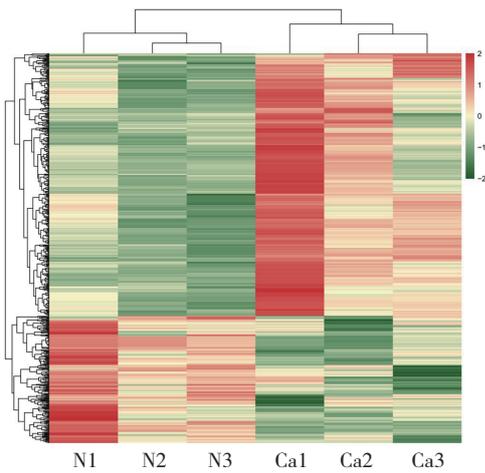　　　　　　　　　　　　　　　　　　　　　　　　　　　　　http://www.zpwz.net

图 2　差异表达外泌体 mRNA 层次聚类分析
**Figure 2　Hierarchical clustering analysis of differentially expressed exosomal mRNAs**
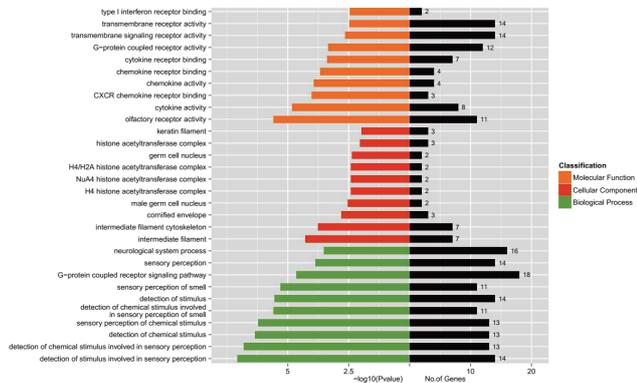


图 3　表达下调 mRNA 的 GO 分析
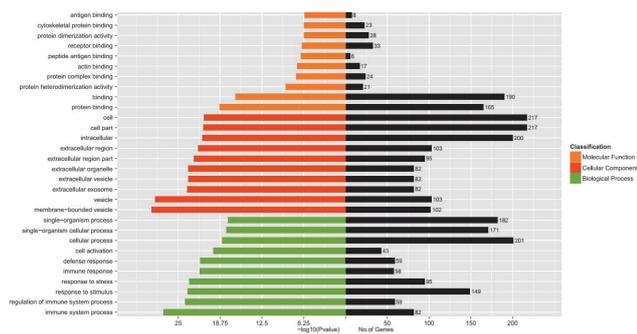**Figure 3　GO analysis of the down-regulated mRNAs**



图 4　表达上调 mRNA 的 GO 分析
**Figure 4　GO analysis of the up-regulated mRNAs**

## 2.5　KEGG 通路显著性富集分析

　　分别对表达上调及下调的血清外泌体 mRNA 进行 KEGG 富集分析，结果详见图 5 与图 6 所示，横坐标表示差异外泌体 mRNA 的靶基因数量，纵坐标表示二级 KEGG 通路，图中用不同颜色表示一级

通路类别。KEGG 通路分析显示，在下调的外泌体 mRNA 中 9 条通路被显著富集（图 5），并且在上调的外泌体 mRNA 中，30 条通路被显著富集（图 6）。其中，在下调的外泌体 mRNA 中，"基础转录因子"和"细胞因子–细胞因子受体相互作用"分别是最丰富和最有意义的通路。在上调的外泌体 mRNA 中，"血小板激活"、"Rap 1 信号通路"、"吞噬体"、"病毒致癌"、"肌动蛋白细胞骨架的调节"与"抗原处理和呈递"等是最丰富和最有意义的通路。
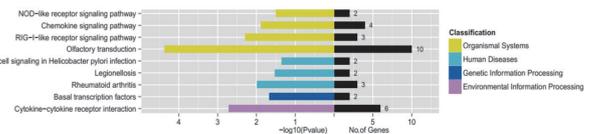


图 5　表达下调 mRNA 的 KEGG 通路
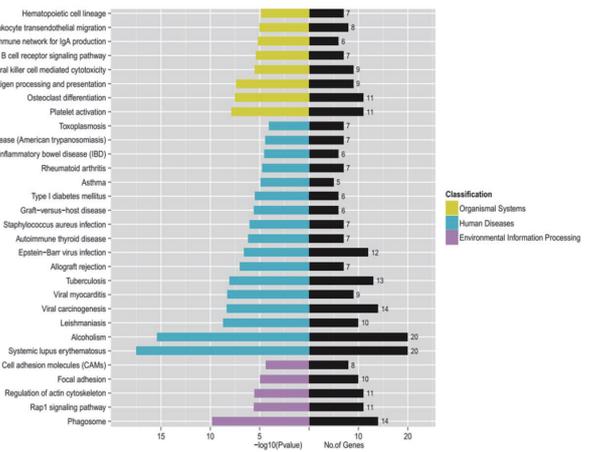**Figure 5　KEGG pathway analysis of the down-regulated mRNAs**



图 6　表达上调 mRNA 的 KEGG 通路
**Figure 6　KEGG pathway analysis of the up-regulated mRNAs**

## 3　讨　论

　　外泌体作为理想的肿瘤标志物的载体，广泛分布于人体各种体液中。大量研究[18-22]已经证实其携带的蛋白质、mRNA、miRNA 与 lncRNA 还有 rRNA、tRNA、vtRNA、Y-RNA、circRNA 等在细胞间进行信息交换和调节受体细胞活动，而肿瘤来源的外泌体可以调节宿主微环境，增强侵袭能力，最终导致疾病进展。有研究[23]显示，来自 HCC 细胞的外泌体 miRNA 可调节转化生长因子 β 活化激酶-1 的表达和信号传导，促进受体细胞的生长侵袭。有学者[24]发现，HCC 患者血清外泌体

miR-665水平明显高于健康受试者，其升高水平与肿瘤大小、局部浸润、临床分期及生存时间密切相关，推测血清外泌体miR-665可以用于HCC诊断和判断预后。另有研究[25]结果显示，HCC患者血清外泌体miR-18a、miR-221、miR-222、miR-224水平显著高于慢性乙型肝炎（CHB）和肝硬化患者。Liu等[26]发现大鼠血清外泌体中的miRNA-10b在肝硬化即表达增加，在HCC阶段明显增加可达正常水平的10倍以上。Hoshion等[27]发现肿瘤的器官亲嗜性转移与外泌体转运整联蛋白激活受体细胞的Src基因和S100基因表达密切相关。肿瘤生长依赖于血液供应的大量营养物质，大量研究发现外泌体参与的内皮细胞迁移及血管再生对HCC的发展转移至关重要，Huang等[28]发现HCC细胞可分泌Ⅰ型跨膜蛋白VASN（vasorin）至外泌体，通过硫酸肝素蛋白聚糖（HSOGs）介导的胞吞作用转运至人脐静脉内皮细胞中促进其迁移。综上，HCC血清外泌体携带的核酸类物质是肝癌发生、发展中的重要因素，近年来对HCC外泌体miRNA研究较多[29]，而mRNA研究较少，本研究通过高通量测序技术对HCC患者血清外泌体mRNA进行检测，进一步证实了外泌体内携带的肿瘤来源物质可用于HCC的早期诊断、耐药性检测、预后评估及治疗[30]。

He等[31]发现外泌体mRNA直接参与HCC的转移活动，并且还发现由外泌体激活了PI3K/ART通路，从而导致了肿瘤进展。Akiba等[32]曾发现HCC组织mRNA、蛋白质水平中的CXCL8基因表达均升高，其可增强HCC细胞和内皮细胞的运动能力，使肿瘤细胞进一步转移。CXCL8促进肿瘤血管生成的作用可能与其氨基端的ELR（Glu-Leu-Arg，谷氨酸-亮氨酸-精氨酸）序列有关[33]。BEX蛋白（brain expressed X-linked protein）的基因属于X染色体连锁基因家族[34]，Braeuning等[35]发现肝癌组织中BEX1的mRNA水平比正常组织中高400倍，其推测BEX1是HCC的可靠的肿瘤标志物。趋化因子PF4(又称为血小板因子4)属于ELR-CXC趋化因子亚家族，其编码基因位于4号染色体长臂，有研究表明趋化因子PF4能够通过干扰血管内皮生长因子、成纤维细胞生长因子及其受体的相互作用，抑制血管新生[36]。另有研究[37]表明PF4与其受体相互作用后，可通过cAMP/PKA通路抑制m-钙蛋白酶的活化，从而阻止细胞迁移。此外RGS18基因与G蛋白偶联受

体（GPCR）通路有关，而GPCR通路异常与肿瘤的发生和转移密切相关[38]。GPCR家族趋化因子受体在多种肿瘤的转移中具有重要作用，其异常高表达可增强肿瘤细胞的迁移能力，趋化因子在肿瘤微环境中局部释放后能通过自分泌和旁分泌途径增强癌细胞的运动和存活能力[39]，笔者推测这两种基因在HCC中同样具有重要作用。

根据上述CXCL8及BEX、RGS18等基因在HCC发生发展中的作用来看，本实验所筛选出来的关键基因在一定程度上是可靠的，有进一步研究的价值。我们有理由相信有可靠的外泌体mRNA会成为HCC诊断的"金标准"，甚至未来在靶向基因治疗方面有立足之地。但本研究样本量较小，下一步拟对差异显著的mRNA在扩大样本量的血清及HCC组织中进行进一步实验验证。

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