·论　著·

ARHGAP4通过调控HK2表达促进肝癌细胞生长的研究

欧阳晓春[1]，邹叶青[2]，李玉梅[3]，丁小兵[3]

1. 中国人民解放军联勤保障部队第 908 医院神经内科，江西 南昌 330006；
2. 南昌大学第二附属医院江西省重点分子实验室，江西 南昌 330006；
3. 井冈山大学医学部，江西 吉安 343000

［摘要］　**背景与目的：**Rho GTP酶活化蛋白4（Rho GTPase activating protein 4，ARHGAP4）与肿瘤的进展密切相关，且对肿瘤细胞恶性增殖具有重要的调节作用。探讨ARHGAP4在肝癌组织中的表达及其对肝癌细胞生长的影响。**方法：**采用实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）、蛋白质印迹法（Western blot）及免疫组织化学法分析肝癌组织中ARHGAP4的表达水平，并分析其表达与肝癌临床病理学特征及预后之间的关系。沉默ARHGAP4的表达，采用细胞计数试剂盒（cell counting kit-8，CCK-8）及EdU实验观测肝癌细胞的增殖情况，并检测肝癌细胞中己糖激酶2（hexokinase 2，HK2）的表达水平，进一步分析肝癌组织中HK2的表达及与ARHGAP4的相关性。在稳定低表达ARHGAP4的肝癌细胞中上调HK2的表达，在稳定高表达ARHGAP4的肝癌细胞中沉默HK2的表达，采用Western blot分析HK2的表达情况，采用EdU分析肝癌细胞的增殖能力。**结果：**肝癌组织中ARHGAP4的表达水平显著高于癌旁组织（P＜0.01），且高表达的ARHGAP4与患者肿瘤体积大小及TNM分期密切相关。沉默肝癌细胞中ARHGAP4的表达，肝癌细胞的增殖能力及HK2的表达水平明显减弱（P＜0.05）；反之过表达ARHGAP4可显著增强HK2的表达及肝癌细胞的增殖能力（P＜0.05），且肝癌细胞中ARHGAP4与HK2的表达呈正相关。**结论：**ARHGAP4通过正向调控HK2的表达进而促进肝癌细胞的生长。

［关键词］ Rho GTP酶活化蛋白4；己糖激酶2；肝癌；增殖；凋亡
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ARHGAP4 promotes growth of hepatocellular carcinoma cells by regulating HK2 expression

OUYANG Xiaochun[1], ZOU Yeqing[2], LI Yumei[3], DING Xiaobing[3] (1. Department of Neurology, the 908th Hospital of the Chinese People’s Liberation Army Joint Logistic Support Force, Nanchang 330006, Jiangxi Province, China; 2. Key Molecular Laboratory of Jiangxi Province, Second Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China; 3. School of Medicine, Jinggangshan University, Ji’an 343000, Jiangxi Province, China)
Correspondence to: DING Xiaobing E-mail: 563109451@qq.com

［**Abstract**］ **Background and purpose:** Rho GTPase activating protein 4 (ARHGAP4) is closely related to tumor progression, and has important regulatory effects on tumor cell malignant proliferation. This study aimed to investigate the expression of ARHGAP4 in hepatocellular carcinoma and its effect on the growth of hepatocellular carcinoma cells. **Methods:** The expression of ARHGAP4 in hepatocellular carcinoma tissues was analyzed by real-time fluorescence quantitative polymerase chain reaction (RTFQ-PCR), Western blot and immunohistochemistry. The relationship between the expression of ARHGAP4 and clinicopathological characteristics and prognosis of hepatocellular carcinoma was analyzed. Then, the expression of ARHGAP4 was silenced. Cell counting kit-8 (CCK-8) and EdU experiments were used to observe the proliferation of hepatocellular carcinoma cells, and the expression level of hexokinase 2 (HK2) in hepatocellular carcinoma cells was detected. The expression of HK2 in hepatocellular carcinoma tissues and its correlation with ARHGAP4 were further analyzed. Finally, HK2 expression was up-regulated in hepatocellular carcinoma cells stably expressing low levels of ARHGAP4, while HK2 expression was silenced in hepatocellular

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通信作者：丁小兵　E-mail: 563109451@qq.com

carcinoma cells stably expressing high levels of ARHGAP4. Western blot was used to analyze the expression of HK2, and EdU was used to analyze the proliferation of hepatocellular carcinoma cells. **Results:** The expression levels of ARHGAP4 mRNA and protein in hepatocellular carcinoma tissues were significantly higher compared with adjacent tissues (*P*<0.01), and the high expression of ARHGAP4 was closely related to the tumor size and TNM stage of patients. Silencing the expression of ARHGAP4 significantly reduced the proliferative ability and HK2 expression level in hepatocellular carcinoma cells (*P*<0.05). Conversely, overexpression of ARHGAP4 significantly enhanced HK2 expression and cell proliferative ability (*P*<0.05), and expressions of ARHGAP4 and HK2 in hepatocellular carcinoma were positively correlated. Mechanistically, ARHGAP4 positively regulated the expression of HK2 to affect the growth of hepatocellular carcinoma. **Conclusion:** ARHGAP4 promotes the growth of hepatocellular carcinoma cells by positively regulating the expression of HK2.

［**Key words**］ Rho GTPase activating protein 4; Hexokinase 2; Hepatocellular carcinoma; Proliferation; Apoptosis

原发性肝癌（hepatocellular carcinoma，HCC）是世界上较为常见的肿瘤之一，在中国，其发病率一直居高不下[1-2]。尽管近年来医学临床检验技术及HCC治疗方法取得了巨大进展，然而HCC患者的5年生存率却一直徘徊不前，很多根治性HCC切除术后复发率依旧很高[3]，究其原因主要是HCC细胞生长迅速，易发生转移[4]，因此，探索HCC细胞生长迅速的机制对于HCC的治疗尤为重要。

Rho GTP酶活化蛋白4（Rho GTPase activating protein 4，ARHGAP4）属于Rho家族蛋白的负调节因子，它可以水解将GTP纳入非活跃GDP并进行负调控[5]。有研究[6]报道，ARHGAP4通过哺乳动物雷帕霉素靶蛋白（mammalian target of rapamycin，mTOR）和低氧诱导因子-1α（hypoxia inducible factor-1α，HIF-1α）信号通路调节胰腺癌代谢并进一步促进胰腺癌的生长，另外有研究[7-9]也证实了ARHGAP4在头部和颈部鳞状细胞癌、胶质母细胞瘤、乳腺癌、肺癌、结肠癌和前列腺癌的表达情况，然而在HCC细胞中ARHGAP4的表达及其是否影响HCC生长的情况并不清楚。

糖代谢异常是肿瘤细胞的一个重要特征，赋予肿瘤特异增殖和迁移的能力，糖酵解为细胞提供充足的能量，可显著增强肿瘤细胞对缺血及缺氧的耐受性，能够避免由于氧化磷酸化受抑制而导致细胞的凋亡[10-11]。有研究[12-14]证实，肿瘤细胞中糖酵解增强与糖酵解限速酶活性增强有关，己糖激酶2（hexokinase 2，HK2）作为糖酵解过程中的限速酶，在肿瘤的生长过程中发挥着重要作用，HK2在结肠癌、膀胱癌和多形性胶质母细胞瘤中的表达量均明显升高，且与肿瘤的恶性程度成正比，同时过表达HK2能明显促进肿瘤细胞的增殖、耐药和体内肿瘤的生长。虽然目前已有研究证实了HCC组织中HK2的表达升高[15]，但其机制仍有待进一步研究。因此，本研究旨在探讨ARHGAP4及HK2在HCC组织中的表达及对HCC细胞生长的影响并进一步了解两者间的相互作用关系。

1 材料和方法

1.1 主要试剂

胎牛血清（fetal bovine serum，FBS）及DMEM培养基购自美国Gibco公司，胰蛋白酶酶、磷酸盐缓冲液（phosphate-buffered saline，PBS）、青霉素-链霉素（双抗）均购自美国BI公司，RIPA裂解液、二喹啉甲酸（bicinchoninic acid disodium，BCA）及蛋白抽提试剂盒均购自上海碧云天生物技术有限公司，ECL发光试剂购自北京全式金生物技术有限公司，凋亡试剂盒购自美国BD公司，ARHGAP4、HK2、GAPDH等主要抗体购自英国Abcam公司，Lipofectamine™3000购自美国Thermo Fisher公司，细胞培养所需耗材购自美国Next公司。

1.2 细胞培养及转染

HCC细胞系MHCC97H、HCCLM3均购自中国科学院典型培养物保藏委员会细胞库，所有细胞均置于含有10%FBS的完全培养基中，隔天换液，待细胞生长至80%~90%时给予1∶3传代，培养条件为：37 ℃、CO_2体积分数为5%及湿度约为

95%的细胞培养箱。

取对数生长的HCC细胞，将细胞以1×10^6接种于6孔培养板中，待细胞生长至60%~70%时予以无血清的培养基每孔2 mL，以每孔7.5 μL Lipofectamine™3000及shRNA加入含2 mL无血清的培养皿中，于6 h后更换成含有10%FBS的完全培养基，继续于细胞培养箱中培养，24~36 h后提取细胞RNA，48~72 h后提取细胞总蛋白。

1.3 组织标本

经南昌大学医学伦理审查委员会批准，并经过全部患者知情同意后，收集了南昌大学第二附属医院及中国人民解放军联勤保障部队第908医院的62例经HCC切除术标本及其癌旁组织，每例标本都经病理学检查确诊为HCC标本及癌旁组织。

1.4 实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）

采用TRIzol法提取细胞及组织RNA，通过紫外分光光度计检测各组的RNA浓度，按照宝生物工程（大连）有限公司的反转录试剂盒说明要求进行反转录制备cDNA，并进一步进行RTFQ-PCR，每个样品至少设置3个复孔，测定其*Ct*值并进行计算。

1.5 蛋白质印迹法（Western blot）

收集细胞及组织蛋白质，经RIPA裂解液及蛋白质抽提试剂盒分别提取细胞及肿瘤组织总蛋白，经BCA定量后将蛋白质样品于沸水中煮10 min，于10%十二烷基硫酸钠聚丙烯酰胺凝胶电泳（sodium dodecyl sulfate polyacrylamide gel electrophoresis，SDS-PAGE）分离，分离的蛋白质条带通过电印迹转移至聚偏二氟乙烯（polyvinylidene fluoride，PVDF）膜中，经脱脂牛奶封闭后，与对应的一抗进行杂交温育（4 ℃过夜），并与对应来源性的二抗进行杂交温育（1 h），经ECL发光试剂显影后拍照分析。

1.6 细胞计数试剂盒（cell counting kit-8，CCK-8）

取对数生长的HCC细胞MHCC97H及HCC-LM3细胞，经胰蛋白酶消化计数后，接种1×10^4个细胞于96孔板中，给予对应的处理24 h后，每孔加入CCK-8试剂10 μL，于细胞培养箱中培养2 h，最后置于酶标仪于450 nm波长处测量其吸光度（*D*）值并分析。

1.7 EdU

细胞处理方法同上，每孔加入100 μL EdU培养基于细胞培养箱中继续培养2 h，随后加入100 μL 4%多聚甲醛溶液固定30 min，每孔加入100 μL甘氨酸，加入100 μL Apollo和100 μL 0.5%Triton-X，100 μL PBS洗两遍，100 μL Hoechst33342避光、室温下脱色摇床30 min，100 μL PBS洗两遍，采用荧光显微镜观察细胞增殖情况并拍照记录分析。

1.8 流式细胞术测定细胞凋亡实验

按照shNC、shARHGAP4进行分组，经胰蛋白酶消化后收集细胞沉淀，2 mL PBS清洗细胞两遍，根据细胞凋亡试剂盒步骤说明，分别加入碘化丙啶（propidium iodide，PI）及异硫氰酸荧光素（fluorescein isothiocyanate，FITC）染料，上流式细胞仪检测。

1.9 统计学处理

所有实验数据均采用SPSS 19.0和GraphPad Prism7数据软件进行分析。当两组比较时，采用*t*检验分析两组间的差异，采用单因素方差分析比较两组间的差异。$P<0.05$为差异有统计学意义。

2 结　果

2.1 ARHGAP4在HCC中的表达及其与临床预后的关系

为了检测HCC组织中ARHGAP4的表达情况，我们收集了62例患者HCC组织及其相应的癌旁组织，首先通过RTFQ-PCR分析了HCC组织和癌旁组织中ARHGAP4的mRNA表达，结果显示，HCC组织标本中ARHGAP4的mRNA表达显著提升（$P<0.01$，图1A），进一步通过Western blot及免疫组织化学法分析了ARHGAP4的蛋白质水平，结果表明，HCC组织标本中ARHGAP4的蛋白质水平显著高于癌旁组织（图1B~C），进一步分析了ARHGAP4的表达与临床病理学特征的关系，结果表明，ARHGAP4高表达与肿瘤大小及TNM分期密切相关（$P<0.05$，表1），最后通

过TCGA数据库分析了ARHGAP4与预后之间的关系，结果表明，高表达ARHGAP4的HCC患者预后差（图1D）。以上结果表明ARHGAP4在HCC组织中高表达且与患者预后密切相关。

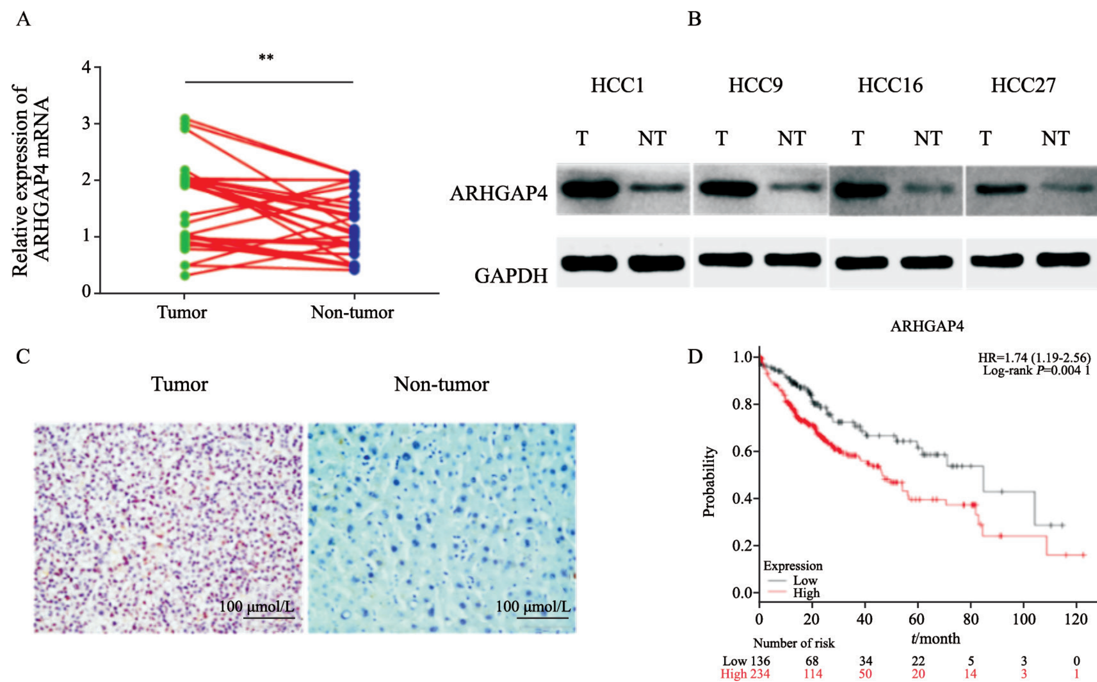

图 1 ARHGAP4在HCC中的表达及其与临床预后的关系

Fig. 1 Expression of ARHGAP4 in HCC and its relationship with clinical prognosis

A-B: Quantitative RTFQ-PCR and Western blot were used to analyze the expression level of ARHGAP4 in HCC and adjacent tissues; C: Western blot was used to analyze the expression level of ARHGAP4 in HCC and adjacent tissues; D: The TCGA database was used to analyze the relationship between ARHGAP4 expression and prognosis in HCC. **: P<0.01, compared with each other; T: Tumor; NT: Non-tumor

表 1 ARHGAP4的表达与HCC患者的临床数据分析

Tab. 1 Analysis of the relationship between ARHGAP4 expression and clinical data of HCC

Characteristics	*n*	ARHGAP4		*P* value
		Low (N=25)	High (N=37)	
Age/year				0.495
≤60	39	17	22	
＞60	23	8	15	
Gender				0.933
Male	45	18	27	
Female	17	7	10	
Tumor size D/cm				0.025
＜5	22	13	9	
≥5	40	12	28	
HBsAg				0.123
Negative	25	13	12	
Positive	37	12	25	
AFP ρ_B/(ng·mL^{-1})				0.233
＜400	34	16	18	
≥400	28	9	19	
TNM stage				0.011
Ⅰ/Ⅱ	35	19	16	
Ⅲ/Ⅳ	27	6	21	

2.2 抑制HCC细胞中ARHGAP4的表达显著抑制其生长

为了明确ARHGAP4参与HCC细胞的生长调节过程，首先在HCC细胞中沉默ARHGAP4的表达，利用RTFQ-PCR及Western blot证实ARHGAP4下调成功（$P<0.01$，图2A~B）。然后进行shNC、shARHGAP4分组，利用CCK-8及EdU实验检测了HCC细胞增殖情况。结果显示，沉默HCC细胞中ARHGAP4的表达后，HCC细胞增殖明显减弱（$P<0.01$，图2C~D）。进一步采用流式细胞术细胞凋亡实验分析下调ARHGAP4后HCC细胞的凋亡情况，结果表明，HCC细胞凋亡比例显著增加（$P<0.05$，图2E~F）。以上结果表明，沉默ARHGAPE能够显著抑制HCC细胞的生长并促进HCC细胞的凋亡。

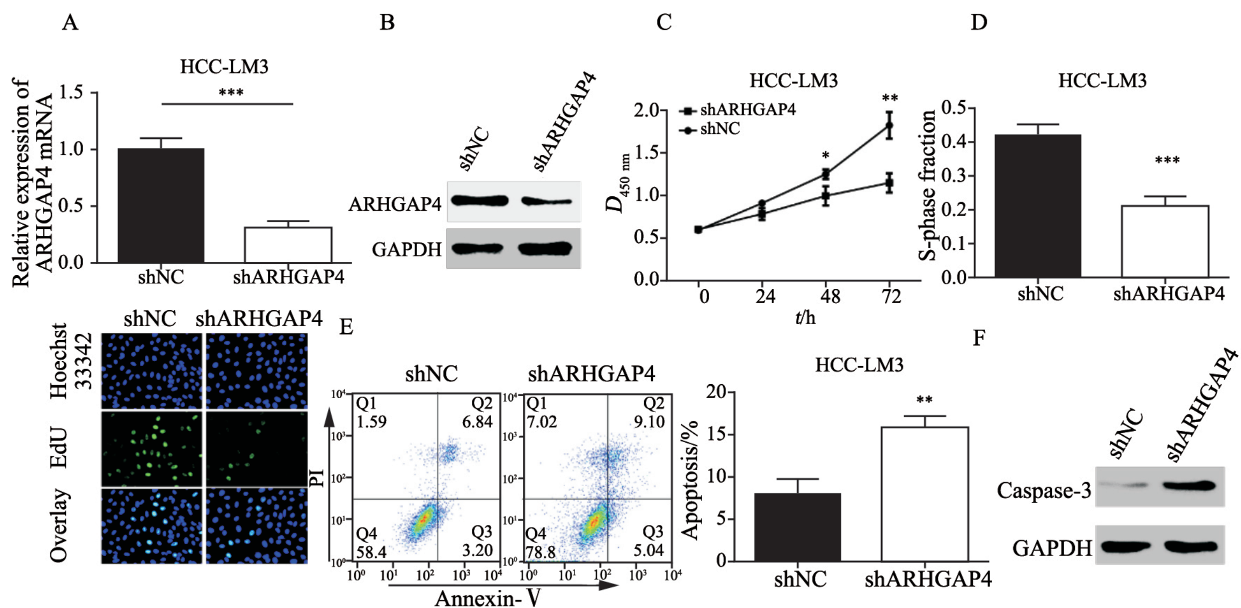

图 2 抑制HCC细胞中ARHGAP4的表达显著抑制其生长

Fig. 2 Suppression of ARHGAP4 expression in HCC cells significantly inhibited proliferation of HCC cells

A: RTFQ-PCR detected ARHGAP4 silencing effect; B: Western blot was used to verify ARHGAP4 silencing effect; C: CCK-8 detection after ARHGAP4 down-regulation of cell proliferation; D: EdU cell proliferation was detected after silencing ARHGAP4; E: Flow cytometry was used to detect the apoptotic rate of ARHGAP4 after silencing; F: The expression of apoptosis-related protein caspase-3 was detected by immunoblotting after silencing of ARHGAP4. *: $P<0.05$, compared with the other group; **: $P<0.01$, compared with the other group; ***: $P<0.001$, compared with the other group

2.3 ARHGAP4调控HK2的表达且两者表达呈正相关

首先在HCC细胞中沉默ARHGAP4的表达，结果发现HK2的表达也随之下降，相反过表达ARHGAP4后HK2的表达显著上升（图3A~B，$P<0.01$）。接下来通过RTFQ-PCR及Western blot证实了HK2在HCC中高表达（图3C~D，$P<0.01$）。进一步研究发现，ARHGAP4的表达与HK2呈正相关（图3E，$P<0.01$）。

2.4 ARHGAP4调控HCC细胞的生长依赖于HK2的表达

为了进一步明确ARHGAP4调控HCC细胞生长的具体机制，在稳定低表达ARHGAP4的HCC细胞中同时过表达HK2，Western blot结果显示，ARHGAP4表达降低，而HK2表达抑制在过表达HK2后恢复（图4A），同样，HCC细胞的增殖能力同样恢复（图4B）。接下来上调ARHGAP4的表达，HCC的生长能力及HK2的表达显著提升。进一步在过表达ARHGAP4的同时沉默HK2的表达，结果表明，ARHGAP4的蛋白质水平上升，而HK2的表达及HCC细胞生长能力在HK2沉默后被抑制（图4C~D）。以上结果表明，ARHGAP4调控HCC细胞的生长能力依赖于HK2的表达。

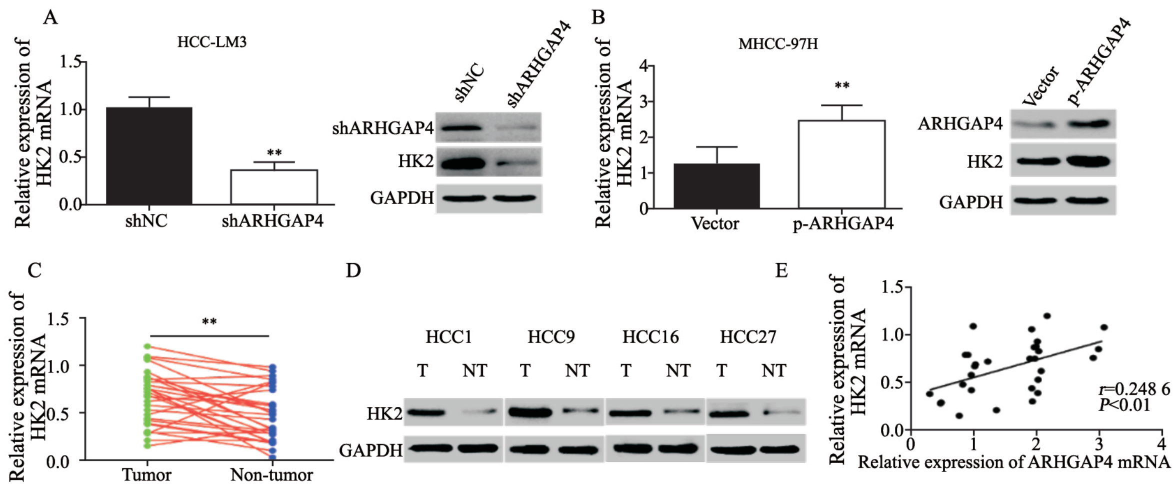

图 3　ARHGAP4调控HK2的表达且两者表达呈正相关

Fig. 3　ARHGAP4 regulates HK2 expression and expressions of ARHGAP4 and HK2 are positively correlated

A-B: After silencing and overexpressing ARHGAP4, the expression levels of HK2 mRNA and protein in HCC cells were detected by RTFQ-PCR and Western blot; C-D: The expression levels of HK2 in HCC tissues and their adjacent tissues were detected by RTFQ-PCR and Western blot; E: Correlation analysis between ARHGAP4 and HK2 in HCC. **: P<0.01, compared with each other; T: Tumor; NT: Non-tumor

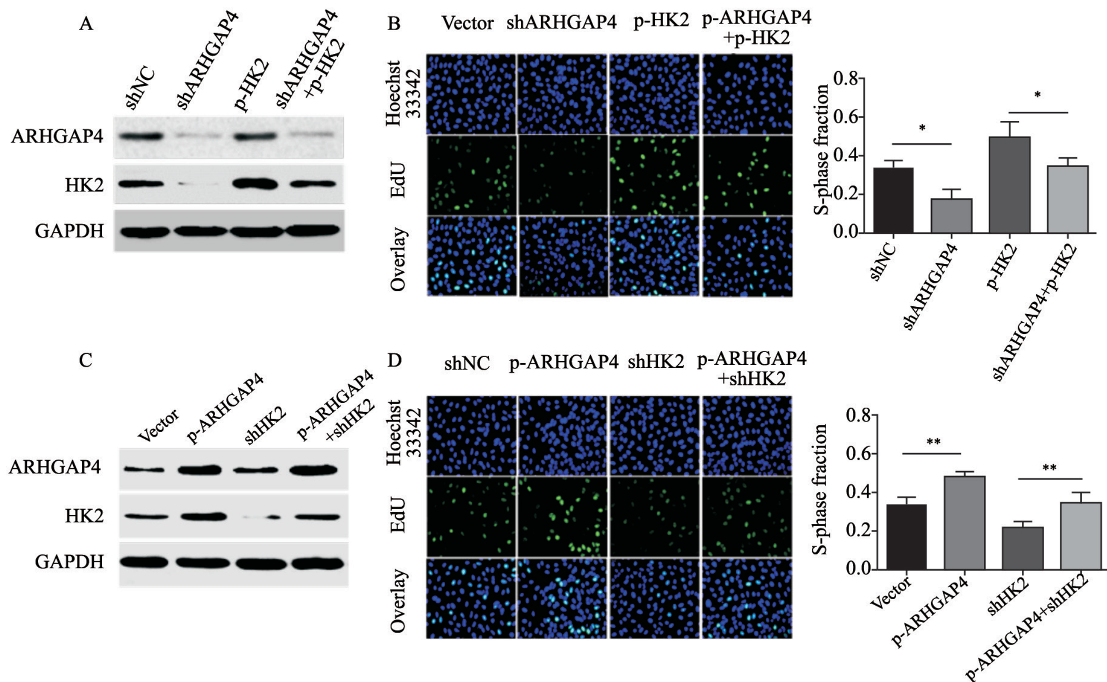

图 4　ARHGAP4调控HCC细胞的生长依赖于HK2的表达

Fig. 4　ARHGAP4 regulates the growth of HCC cells via HK2 expression

A: Western blot analysis of the expressions of ARHGAP4 and HK2 in HCC cells. Overexpression of HK2 could restore the inhibition of HK2 by shARHGAP4. B: EdU results showed that overexpression of HK2 could restore the inhibition of proliferation of shARHGAP4 by HCC. C: Western blot analysis of the expressions of ARHGAP4 and HK2 in HCC cells. Silencing HK2 could inhibit the up-regulation of p-ARHGAP4 HCC cells on HK2. D: EdU results confirmed that silencing HK2 could inhibit p-ARHGAP4 to promote the growth of HCC cells. *: P<0.05, compared with the other group; **: P<0.01, compared with the other group

3 讨论

HCC严重影响人类健康，许多研究者密切关注HCC的发生、发展，目前普遍认为细胞的恶性生长是癌症的必要环节[16]。越来越多的证据表明，HCC细胞的增殖和凋亡与原癌基因和抑癌基因异常表达密切相关[17]。Rho GTP酶活化蛋白基因与肿瘤的发生、发展相关，其中以ARHGAP4尤为重要，目前有研究[18]证实，ARHGAP4在胰腺癌等多种肿瘤中高表达，沉默肿瘤细胞中ARHGAP4的表达后显著抑制肿瘤细胞的增殖能力并诱导细胞周期阻滞，进而促进肿瘤细胞凋亡。另外有研究[18]也报道了ARHGAP4的表达与胰腺癌细胞的糖酵解水平相关。然而在HCC中尚未见报道，本研究检测了ARHGAP4在HCC组织中的表达，并探讨了ARHGAP4的表达与HCC细胞生长的关系。

糖酵解作为肿瘤细胞中大部分的能量来源，已经有越来越多的研究[19-21]证实在不同的肿瘤细胞中存在着糖酵解增强的现象。Lee等[19]研究发现，在HCC中糖酵解增强，且主要与其关键酶HK2的活化相关。Wang等[20]也发现，在膀胱癌中同样存在着HK2高表达，并发现HK2作为独立影响因子影响着膀胱癌的进展。Lv等[21]研究发现，在肺癌的组织标本中同样存在着HK2表达升高，并且显著影响其生长与转移。本研究数据表明，HK2在HCC组织中高表达，降低HCC细胞中ARHGAP4的表达后HK2表达被抑制，且两者呈正相关。总之，这些数据表明，ARHGAP4可能作为致癌基因发挥重要作用，并可能在HCC的发生、发展中发挥重要作用。虽然本研究观察了ARHGAP4对HCC生长的影响，但并未揭示其调控增殖的具体机制。

综上所述，ARHGAP4能够正向调控HK2的表达进而促进HCC的生长，可望为HCC的靶向治疗提供新的线索。

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《抗癌》杂志征稿启事

《抗癌》杂志于1988年创刊，主管单位为上海市科学技术协会，主办单位为上海市抗癌协会，杂志刊号：CN31-1664/R ISSN 1008-3065。征稿栏目及内容如下。

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通信地址：上海市东安路270号10号楼4楼《抗癌》杂志社 邮 编：200032

电 话：021-64188274；021-64175590转83574 E-mail：anti-cancer@163.com

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