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索拉非尼与重组腺病毒 H101 对肝癌细胞株 HepG2 的作用及机制研究 *

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摘要 目的：研究索拉非尼与重组腺病毒 H101 对肝癌细胞株 HepG2 的作用并分析其具体机制。**方法：**选择索拉非尼、重组腺病毒 H101 分别组成重组腺病毒 H101 组、索拉非尼组、两药联合组以及空白对照组，并分别作用于购自中国科学院上海细胞生物研究所细胞库的肝癌细胞株 HepG2。采用流式细胞技术检测 HepG2 细胞的凋亡情况；采用 Western blot 法检测细胞外信号调节激酶 1/2（ERK1/2）、磷酸化 ERK1/2（p-ERK1/2）以及髓样细胞白血病 -1（Mcl-1）蛋白相对表达量；采用酶联免疫吸附法检测不同组别细胞培养上清液中的血管内皮生长因子（VEGF）水平。**结果：**重组腺病毒 H101 组、索拉非尼组、两药联合组的 G_0-G_1 期与 G2-M 期细胞均明显低于空白对照组，S 期细胞均明显高于空白对照组，且两药联合组较重组腺病毒 H101 组与索拉非尼组更明显，差异均有统计学意义（均 P<0.05）；重组腺病毒 H101 组、索拉非尼组、两药联合组的 HepG2 细胞凋亡率明显高于空白对照组，而两药联合组又明显高于重组腺病毒 H101 组与索拉非尼组，差异均有统计学意义（均 P<0.05）。重组腺病毒 H101 组、索拉非尼组、两药联合组的 p-ERK1/2、Mcl-1 蛋白相对表达量和 VEGF 表达均明显低于空白对照组，而两药联合组又明显低于重组腺病毒 H101 组与索拉非尼组，差异均有统计学意义（均 P<0.05）。**结论：**索拉非尼、重组腺病毒 H101 均可抑制肝癌细胞株 HepG2 的增殖，并诱导其凋亡，控制 VEGF 表达，联合应用具有更明显的效果，其主要机制可能与二者协同作用有效抑制 p-ERK1/2、Mcl-1 蛋白表达有关。

关键词：肝癌细胞株 HepG2；细胞凋亡；索拉非尼；重组腺病毒 H101；作用机制

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Effect and Mechanism of Sorafenib and Recombinant Adenovirus H101 on Hepatocellular Carcinoma Cell Line HepG2*

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ABSTRACT Objective: To study the effect of sorafenib and recombinant adenovirus H101 on hepatocellular carcinoma cell line HepG2 and to analyze its specific mechanism. **Methods:** Sorafenib, recombinant adenovirus H101 were used to form recombinant adenovirus H101 group, sorafenib group, two drug combination group and blank control group, and they were respectively acted on hepatocellular carcinoma cell line HepG2 which were purchased from cell bank of Shanghai Institute of cell biology, Chinese Academy of Sciences. The apoptosis of HepG2 cells was detected by flow cytometry, the protein expression of extracellular signal regulated kinase 1/2 (ERK1/2), phosphorylated-ERK1/2 (p-ERK1/2), myeloid leukemia-1(Mcl-1) were detected using Western blot method, the levels of vascular endothelial growth factor (VEGF) in cell culture supernatant of different groups were detected by enzyme linked immunosorbent assay. **Results:** The cells in G_0-G_1 and G2-M phase in the recombinant adenovirus H101 group, sorafenib group and two drug combination group were significantly lower than those in the blank control group, the cells in S phase were significantly higher than those in the blank control group, compared with the recombinant adenovirus H101 group and sorafenib group, the two drug combination group was more significantly, the differences were statistically significant (P<0.05). The apoptosis rate of HepG2 cells in recombinant adenovirus H101 group, sorafenib group and two drug combination group was significantly higher than that in the blank control group, the two drug combination group was significantly higher than the recombinant adenovirus H101 group and sorafenib group, the differences were statistically significant (P<0.05). The relative expression of p-ERK1/2 and Mcl-1 protein and the expression of VEGF in recombinant adenovirus H101 group, sorafenib group and two drug combination group were significantly lower than those in blank control group, the two drug combination group was significantly lower than the recombinant adenovirus H101 group and sorafenib group, the differences were statistically significant (P<0.05). **Conclusion:** Both sorafenib and recombinant adenovirus H101 can inhibit the proliferation of hepatoma cell line HepG2, induce apoptosis and control the expression of VEGF, the combined application has more obvious effect, and the main mechanism may be related to the synergistic effect of the two factors, which can effectively inhibit the expression of p-ERK1/2 and Mcl-1

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proteins.

Key words: Hepatocellular carcinoma cell line HepG2; Apoptosis; Sorafenib; Recombinant adenovirus H101; Mechanism of action
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前言

原发性肝癌属于临床上最为常见的消化系统恶性肿瘤之一,其发病率在全球范围内的常见恶性肿瘤中位居第五[1]。我国属于肝癌的高发国家,发病率居全球首位。由于该病发病早期具有极强的隐匿性,大部分患者一经发现便已是中晚期,错失了手术根治的时机,而射频消融、放疗、化疗以及分子靶向治疗等治疗方式的效果并不十分理想,且预后较差[2,3]。因此,寻找一种有效提高肝癌治疗效果的方式显得尤为重要。近年来,随着相关研究报道的不断深入,生物靶向治疗成为治疗原发性肝癌的研究热点[4]。有研究报道显示,新生血管的形成与Raf/MEK/ERK级联的信号传导可能在肝细胞癌的进展过程中发挥着至关重要的作用[5]。索拉非尼属于Raf激酶与其受体络氨酸激酶抑制剂,具有抑制肿瘤细胞增殖以及血管生成的双重功效[6,7]。鉴于此,本文通过研究索拉非尼与重组腺病毒H101肝癌细胞株HepG2的作用并分析其具体机制,旨在为临床有效治疗原发性肝癌提供参考方式,以改善患者预后,现作如下报道。

1 材料及方法

1.1 细胞培养

取HepG2细胞株(购自中国科学院上海细胞生物研究所细胞库)放置于37℃、二氧化碳浓度为5%的孵育箱中进行培养。培养基的组成成分为含10%灭活小牛血清的RPMI1640以及含1%的青霉素与链霉素双抗。细胞为上皮样细胞,传代频率为2~3d/次,取对数生长期的细胞作为研究样本。

1.2 采用流式细胞技术检测HepG2细胞的凋亡情况

分别取对数生长期的HepG2细胞接种在6孔板上,在培养24 h后加入相关药物:重组腺病毒H101组加入重组腺病毒H101(上海三维生物技术有限公司,国药准字:S20060027,规格:5.0× 10^{11}vp/0.5 mL/支)4 μmol/L;索拉非尼组加入索拉非尼(Bayer Pharma AG,注册证号:H20160201,规格:0.2 g)4 μmol/L;两药联合组分别加入重组腺病毒H101与索拉非尼组各4 μmol/L;空白对照组不加药物。在加药后72 h收集细胞,调节细胞浓度为5× 10^5/mL,随后进行细胞周期与凋亡情况的检测。(1)细胞周期检测:采用浓度为75%的乙醇于-20℃条件下进行固定并放置过夜,随后进行洗涤、离心,加入含有0.1%的核糖核酸酶、磷酸盐缓冲液以及500 μL的碘化丙啶(propidium iodide,PI)10 μg/mL,在室温避光染色30 min后采用流式细胞仪(购自美国BD公司)进行检测。(2)细胞凋亡检测:加入5 μL的PI以及10 μL的Annexin V-FITC,于室温避光条件下反应15 min,随后上机检测,数据均采用ModFit 2.0软件系统进行分析。凋亡率=凋亡细胞数/视野总细胞数× 100%。

1.3 采用Western blot法检测细胞外信号调节激酶1/2(extracellular signal-regulated kinase 1/2,ERK1/2)、磷酸化ERK1/2(phosphorylation ERK1/2,p-ERK1/2)以及髓样细胞白血病-1(myeloid leukemia-1,Mcl-1)蛋白表达

采集各组细胞加入裂解液,并放置于冰上静置15 min,以10000 r/min离心10 min,取上清液采用紫外分光度计测定蛋白表达水平。加入2× SDS上样缓冲液,煮沸5min,离心后保存于-20℃冰箱中待检。配置SDS-PAGE胶,分别取四组样本各20 μg蛋白行常规电泳以及转膜。其中膜与一抗比例为1:1000,放置于摇床室温条件下摇2 h,并与4℃孵育箱中过夜;膜与二抗比例为1:7000,孵育1 h,以X线胶片曝光、显影、定影,最后分析图片。

1.4 采用酶联免疫吸附法检测不同组别细胞培养上清液中血管内皮生长因子(vascular endothelial growth factor,VEGF)水平

首先采用无血清的RPMI1640培养液进行细胞培养,在细胞对数期分别加入索拉非尼、重组腺病毒H101、两药联合以及不加入任何药物,继续培养48 h后提取细胞培养液,根据酶联免疫吸附试剂盒说明书进行相关操作。VEGF酶联免疫吸附法试剂盒购自武汉博士德生物公司。

1.5 统计学方法

本研究数据均采用SPSS20.0软件进行检测分析,ERK1/2、p-ERK1/2以及Mcl-1蛋白相对表达量及VEGF表达水平等计量资料采用均数± 标准差($\bar{x}$± s)描述,采用t检验,多组间对比采用单因素方差分析,计算F值,检验标准设置为α=0.05。

2 结果

2.1 不同组别处理72 h后的HepG2细胞周期比例以及凋亡率对比

重组腺病毒H101组、索拉非尼组、两药联合组的G0-G1期与G2-M期细胞均明显低于空白对照组,而两药联合组又明显低于重组腺病毒H101组与索拉非尼组,差异均有统计学意义(均P<0.05);重组腺病毒H101组、索拉非尼组、两药联合组的S期细胞均明显高于空白对照组,而两药联合组又明显高于重组腺病毒H101组与索拉非尼组,差异均有统计学意义(均P<0.05);重组腺病毒H101组、索拉非尼组、两药联合组的HepG2细胞凋亡率均明显高于空白对照组,而两药联合组又明显高于重组腺病毒H101组与索拉非尼组,差异有统计学意义(均P<0.05),见表1。

2.2 四组ERK1/2、p-ERK1/2以及Mcl-1蛋白相对表达量对比

四组ERK1/2的蛋白相对表达量比较,差异无统计学意义(P>0.05);重组腺病毒H101组、索拉非尼组、两药联合组的p-ERK1/2、Mcl-1蛋白相对表达量均明显低于空白对照组,且两药联合组又明显低于重组腺病毒H101组与索拉非尼组,差异均有统计学意义(均P<0.05),见表2。

2.3 四组细胞培养上清液中VEGF表达水平对比

重组腺病毒H101组、索拉非尼组、两药联合组的VEGF表达水平分别为(22.66± 5.89)pg/mL、(23.14± 6.02)pg/mL、(10.28± 4.27)pg/ml,均明显低于空白对照组的(56.98± 6.34)pg/ml,而两药联合组又明显低于重组腺病毒H101组与索拉非尼组,差异均有统计学意义(均P<0.05)。

表 1 不同组别处理 72 h 后的 HepG2 细胞周期比例以及凋亡率对比($\bar{x}\pm s$)

Table 1 Comparison of HepG2 cell cycle ratio and apoptosis rate in different groups after treatment of 72 h($\bar{x}\pm s$)

Groups	Sample size	Cell cycle(h)			Apoptosis rate(%)
		G0-G1	G2-M	S	
Blank control group	3	65.01± 1.33	10.57± 0.19	24.02± 1.26	3.29± 0.58
Recombinant adenovirus H101 group	3	56.77± 0.88*#	4.41± 0.13*#	40.82± 0.86*#	18.02± 1.12*#
Sorafenib group	3	53.52± 1.09*#	3.42± 0.31*#	44.84± 1.30*#	14.57± 3.68*#
Two drug combination group	3	27.02± 1.04*	2.33± 0.30*	70.98± 0.70*	41.06± 1.37*

Note: compared with the blank control group, *P<0.05; compared with the two drug combination group, #P<0.05.

表 2 四组 ERK1/2、p-ERK1/2 以及 Mcl-1 蛋白相对表达量对比($\bar{x}\pm s$)

Table 2 Comparison of relative expression levels of ERK1/2, p-ERK1/2 and Mcl-1 protein in four groups($\bar{x}\pm s$)

Groups	Sample size	ERK1/2	p-ERK1/2	Mcl-1
Blank control group	3	2.60± 0.49	3.01± 0.48	1.65± 0.33
Recombinant adenovirus H101 group	3	2.81± 0.47	1.44± 0.62*#	1.10± 0.55*#
Sorafenib group	3	2.84± 0.46	1.40± 0.61*#	1.07± 0.52*#
Two drug combination group	3	2.77± 0.46	0.84± 0.31*	0.72± 0.35*

Note: compared with the blank control group, *P<0.05; compared with the two drug combination group, #P<0.05.

3 讨论

原发性肝癌属于临床常见的恶性肿瘤,其在全球发病率有逐渐上升的趋势。有研究表明,我国肝癌病例占全球一半以上,疾病带来的身心痛苦以及负担对人们生活质量有着极大的影响[8,9]。当前,临床上低于肝癌的主要治疗手段是对患者进行肝切除治疗,但由于肝细胞癌具有一定的隐秘性,发病早期诊断较困难,再加上其病情进展较快,因此临床上能接受及时手术治疗的患者占比较少,且手术治疗预后效果也不理想[10-12]。近年来,溶瘤病毒作为一种安全有效的抗肿瘤治疗方式开始被临床所关注,其主要是指采用可自主复制病毒,利用肿瘤细胞自身功能性基因缺失或失活的特性予以靶向性感染并杀伤肿瘤细胞,最终达到治疗目的[13,14]。而重组腺病毒 H101 主要是由通过基因工程技术敲除了人 2 型腺病毒的部分基因片段所获取的溶瘤腺病毒,目前已被广泛应用于临床头颈部多种恶性肿瘤治疗中,效果明显[15,16]。另有研究报道显示[17,18],Raf/MEK/ERK 级联信号传导在原发性肝癌的进展过程中起着至关重要的作用。而索拉非尼属于 Raf-1 激酶抑制剂之一,可通过靶向作用于肿瘤细胞以及肿瘤血管上的受体络氨酸激酶, 从而有效抑制 Raf/MEK/ERK 过程中的丝氨酸 / 苏氨酸激酶的水平,进一步达到抑制肿瘤细胞增殖的目的[19,20]。

本研究结果发现:重组腺病毒 H101 组、索拉非尼组、两药联合组的 HepG2 细胞凋亡率均明显高于空白对照组, 而两药联合组又明显高于重组腺病毒 H101 组与索拉非尼组（P＜0.05), 这说明了重组腺病毒 H101 与索拉非尼均可有效促进 HepG2 细胞凋亡,而两药联合的效果更加明显。提示了重组腺病毒 H101 与索拉非尼应用于原发性肝癌的治疗中具有一定的协同作用。分析原因, 作者认为可能是索拉非尼通过对 Raf/MEK/ERK 信号传导通路产生抑制作用, 从而上调肿瘤细胞表面的柯萨奇 - 腺病毒受体表达,进一步提高了肿瘤细胞对腺病毒的敏感性, 继而促使溶瘤腺病毒在肿瘤细胞中增殖,最终达到提高临床治疗效果的目的[21,22]。而重组腺病毒 H101 主要是利用肿瘤细胞自身功能性基因缺失或失活的特性予以靶向性感染并杀伤肿瘤细胞,以达到治疗的目的。因此两者联合应用具有一定的协同作用。此外,重组腺病毒 H101 组、索拉非尼组、两药联合组的 p-ERK1/2、Mcl-1 蛋白相对表达量均明显低于空白对照组,而两药联合组又明显低于重组腺病毒 H101 组与索拉非尼组(P<0.05),这和李璐璐等人的研究相一致[23],说明了重组腺病毒 H101 与索拉非尼治疗原发性肝癌的主要机制可能与影响 Raf/MEK/ERK 信号通路有关。索拉非尼可对 Raf/MEK/ERK 信号传导通路起到阻断作用, 从而抑制血管形成,同时下调抗凋亡蛋白 Mcl-1 的表达水平,进一步诱导肿瘤细胞的凋亡[24]。与此同时,索拉非尼可提高肿瘤细胞表面的柯萨奇 - 腺病毒受体,从而增强重组腺病毒 H101 的溶瘤作用。另外,重组腺病毒 H101 组、索拉非尼组、两药联合组的 VEGF 表达水平均明显低于空白对照组,而两药联合组又明显低于重组腺病毒 H101 组与索拉非尼组(P<0.05),这表明了重组腺病毒 H101 联合索拉非尼可有效抑制 VEGF 的表达。有研究报道显示,通过下调 VEGF 的表达以及转录,可有效抑制肿瘤细胞的增殖[25],因此,可认为重组腺病毒 H101 联合索拉非尼治疗原发性肝癌的主要机制可能与抑制 VEGF 表达有关。这为今后的研究提供了发现以及新的思路,在临床中采用 VEGF 抑制剂可能有效达到治疗原发性肝癌的目的。

综上所述,联合应用索拉非尼和重组腺病毒 H101 可有效抑制肝癌细胞株 HepG2 的增殖, 其主要机制可能与二者协同作用有效抑制 Raf/MEK/ERK 信号传导通路有关, 而且有效控

制 VEGF 表达,可能是临床治疗原发性肝癌的有效手段,值得深入探究。

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