

IL-4/IL-4R对肝细胞癌生物学行为的影响及潜在的作用机制

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Effect of IL-4/IL-4R on Biological Behavior of Hepatocellular Carcinoma and Its Underlying Mechanism

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Abstract: Objective To investigate the effect of Interleukin-4/Interleukin-4 receptor (IL-4/IL-4R) on the biological behavior of hepatocellular carcinoma (HCC) and its underlying mechanism. **Methods** Huh7 cells were cultured *in vitro* and then transfected with small interfering RNAs (siRNAs). Cell proliferation was assessed by CCK8. Cell cycle and apoptosis were analyzed by flow cytometry. The possible signaling proteins were measured by Western blot. **Results** The proliferation of Huh7 cells was increased by IL-4 ($P=0.01$), however the proliferation was suppressed by silencing IL-4R ($P=0.00033$). After knocking down IL-4R, the early apoptosis of Huh7 cells ($P=0.014$) and the expression of apoptosis-related proteins Bax ($P=0.016$), Caspase-3 ($P=0.029$) were enhanced, and the expression of anti-apoptosis-related protein Bcl-2 was decreased ($P=0.003$). However the late apoptosis rate ($P=0.108$) and the cell cycle (G_0/G_1 phase, $P=0.677$; S phase, $P=0.139$; G_2/M phase, $P=0.855$) were not changed. Furthermore, IL-4 up-regulated the phosphorylation of JAK1 ($P=0.01$) and STAT6 ($P=0.005$). The phosphorylation levels of JAK1 ($P=0.016$) and STAT6 ($P=0.019$) in Huh7 cells were down-regulated by IL-4R depletion. **Conclusion** IL-4/IL-4R can activate JAK1/STAT6 signaling pathway and enhance the proliferation of HCC. Furthermore, IL-4/IL-4R can inhibit the apoptosis of HCC through regulating the expression of apoptotic proteins and anti-apoptotic proteins.

Key words: Hepatocellular carcinoma; Interleukin-4; Interleukin-4 receptor; Proliferation; Apoptosis; Cell cycle

摘要: 目的 检测白细胞介素-4/白细胞介素-4受体 (Interleukin-4/Interleukin-4 receptor, IL-4/IL-4R) 对肝细胞癌 (hepatocellular carcinoma, HCC) 生物学行为的影响及潜在的作用机制。**方法** 体外培养 Huh7 细胞, 并进行转染, 沉默细胞中 IL-4R 基因的表达。CCK8 法检测细胞增殖。流式细胞术检测细胞凋亡和细胞周期。Western blot 法探索可能的作用机制。**结果** IL-4 促进 Huh7 细胞的增殖 ($P=0.01$), 沉默 IL-4R 可抑制 Huh7 细胞的增殖 ($P=0.00033$), 且细胞早期凋亡增加 ($P=0.014$), 凋亡蛋白 Bax ($P=0.016$) 和 Caspase-3 ($P=0.029$) 表达增加, 抗凋亡蛋白 Bcl-2 ($P=0.003$) 表达减少。沉默 IL-4R 并不影响细胞晚期凋亡 ($P=0.108$) 及细胞周期 (G_0/G_1 期: $P=0.677$; S 期: $P=0.139$; G_2/M 期: $P=0.855$) 变化。IL-4 促进 JAK1 ($P=0.01$) 和 STAT6 ($P=0.005$) 的磷酸化。沉默 IL-4R 后细胞内 JAK1 ($P=0.016$) 和 STAT6 ($P=0.019$) 的磷酸化水平均降低。**结论** IL-4/IL-4R 可以激活 HCC 内 JAK1/STAT6 信号通路、促进 HCC 的增殖。此外, 还通过调节凋亡蛋白和抗凋亡蛋白的表达, 最终抑制细胞凋亡。

关键词: 肝细胞癌; 白细胞介素-4; 白细胞介素-4受体; 增殖; 凋亡; 细胞周期

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0 引言

据 GLOBOCAN 2012 统计, 肝癌在癌症死亡率中排名第二^[1]。虽然目前治疗肝癌的方法很多, 如手术、介入、射频消融、化疗、分子靶向治疗等, 但是对于晚期肝癌, 尤其是肝细胞肝癌 (hepatocellular carcinoma, HCC) 仍让很多临床工作者束手无策, 因此寻求新的治疗方法迫在眉睫。

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近年来许多研究显示白细胞介素-4 (Interleukin-4, IL-4) 可通过影响肿瘤细胞的凋亡, 促进肿瘤的进展和转移^[2-4]。此外肿瘤细胞表面上调的IL-4R和微环境中高表达的细胞因子还与肿瘤的分期、分级息息相关^[5]。这些都提示我们IL-4/IL-4R似乎参与癌症的发生、发展。

本研究前期发现芹菜素可以促进HCC细胞系 (Huh7) 的凋亡, 同时还将细胞阻滞于G₂/M期。通过基因芯片和Western blot发现, 芹菜素抑制HCC的生物学活性与下调白介素-4受体 (Interleukin-4 receptor, IL-4R) 有关^[6]。且目前就IL-4/IL-4R和HCC的关系鲜有报道, 因此本文主要探索IL-4/IL-4R对HCC细胞生物学活性的影响及潜在的分子机制。

1 材料与方法

1.1 细胞培养

实验中所用Huh7细胞株购于中国科学院细胞库 (上海, 中国)。细胞株用含11%胎牛血清 (ExCell Bio, 南美) 的DMEM培养液 (Solarbio, 上海) 培养, 除此之外加入30 ng/ml IL-4 (PeproTech, 美国) 于37℃、5%CO₂培养箱中。

1.2 细胞转染

小干扰RNA (small interfering RNA, siRNA) 设计并购买于上海吉玛公司, 包括用于阴性对照的空载片段: sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3', 三条干扰IL-4R基因的目的片段: IL-4R siRNA1, sense 5'-CAG UGC GGA UAA CUA UAC ATT-3', antisense 5'-UGU AUA GUU AUC CGC ACU GTT-3'; IL-4R siRNA2, sense 5'-CUG ACA AUU ACC UGU AUA ATT-3', antisense 5'-UUA UAC AGG UAA UUG UCA GTT-3'; IL-4R siRNA3, sense 5'-CAG UGC UAU AAC ACC ACC UTT-3', antisense 5'-AGG UGG UGU UAU AGC ACU GTT-3'。转染前一天将适量细胞接种至六孔板中, 转染时将Lipofectamine[®] 2000转染试剂 (Invitrogen, 美国)、三个目的片段和空载片段分别加至无血清无双抗的DMEM中, 然后将混有转染试剂和siRNA的培养液混合, 最后加入细胞中。

1.3 实时荧光定量PCR (real-time quantitative PCR, qPCR)

用TRIzol (全式金, 北京) 裂解细胞并抽提RNA, 并将反转录试剂盒 (TaKaRa, 日本) 反转录成的cDNA、IL-4R的上游引物: 5'-GGG

CGC GCA GAT AAT TAA AGA TT-3', 下游引物: 5'-AAG CCA CCC CAT TGG GAG AT-3' (Sangon Biotech, 上海) 与SYBR Premix Ex Taq[™] II (TaKaRa, 日本) 中的试剂配制成扩增体系, 使用Applied Biosystems 7900 Real-Time PCR System (ABI, 美国) 进行扩增。

1.4 Western blot法检测Huh7细胞中相关蛋白表达

用裂解缓冲液裂解细胞, 并加入抗蛋白酶肽、磷酸酶抑制剂和苯甲基磺酰氟。所得到的蛋白均用BCA蛋白试剂盒 (Thermo Fisher Scientific, 美国) 测量蛋白浓度。加入相同浓度的各组细胞总蛋白至10% SDS-聚丙烯酰胺胶中电泳, 并转入聚偏氟乙烯 (polyvinylidene difluoride, PVDF) 膜中。接着封闭2 h, 将封闭后的PVDF膜放入一抗溶液中, 4℃过夜。然后再置入二抗中孵育1 h, 最后曝光, 并用Image J软件分析结果。实验中的抗体分别是: 抗β-actin和抗GAPDH单克隆抗体 (Proteintech, 武汉), 抗IL-4R多克隆抗体 (Abcam, 美国), 抗Bcl-2、抗Bax和抗Caspase-3多克隆抗体 (Proteintech, 武汉), 抗JAK1、抗p-JAK1、抗STAT6、抗p-STAT6多克隆抗体 (Cell Signaling Technology, 美国)。含辣根过氧化物酶山羊抗鼠IgG (H+L)、含辣根过氧化物酶山羊抗兔IgG (H+L) (全式金, 北京)。

1.5 CCK8法检测Huh7细胞增殖能力

将适量细胞种至96孔板中, 并设置5个复孔。第2天转染, 并设定转染后为0 h, 用10%的CCK8分别测定转染后0、24、48和72 h的吸光度。

1.6 流式细胞学检测细胞凋亡和细胞周期

每组收集适量细胞, 并用缓冲液重悬成单细胞悬液, 再加入Annexin V-FITC及PI, 通过流式细胞仪检测各实验组细胞凋亡率。

每组收集适量细胞, 提前用70%乙醇固定, 测量当天再加入DNA染色液和碘化丙锭溶液, 最后通过流式细胞仪检测各实验组细胞周期。

1.7 统计学方法

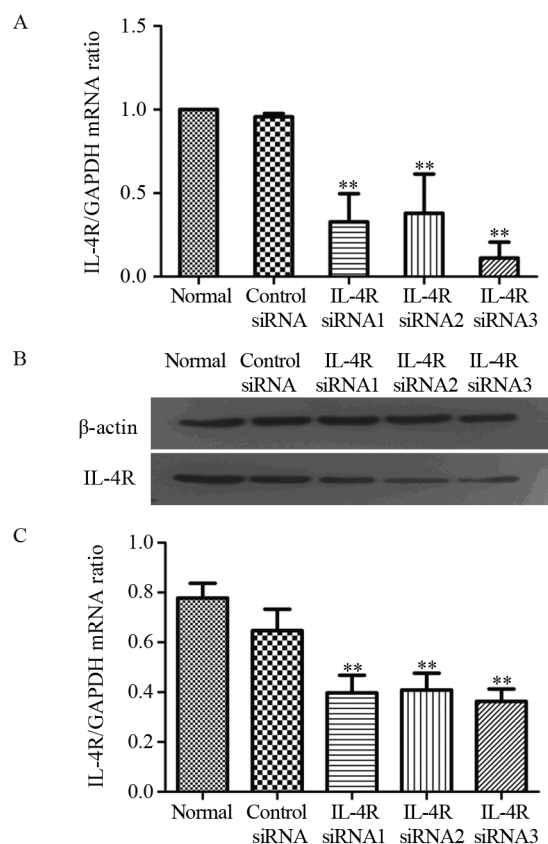
采用SPSS17.0统计软件处理数据。所有数据均为至少进行三次独立实验的平均值和标准差。通过单因素方差分析 (ANOVA) 和LSD-*t*检验的多重比较进行统计学分析。以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 siRNA干扰Huh7细胞中IL-4R的表达

设计三条siRNA干扰片段 (siRNA1、

siRNA2、siRNA3) 沉默IL-4R, 分别用qPCR和Western blot筛选出最佳沉默片段。qPCR结果显示siRNA1 ($P=0.00022$)、siRNA2 ($P=0.00041$)、siRNA3 ($P=0.00019$) 均有不同程度的干扰效果。Western blot也得到与qPCR相似的结果 (siRNA1: $P=0.01$ 、siRNA2: $P=0.01$ 、siRNA3: $P=0.00042$) , 其中siRNA3的干扰效果最显著, 见图1。



A: the relative ratio of IL-4R mRNA to GAPDH in each group; B: the protein expression of IL-4R after transfection in each group; C: the relative ratio of IL-4R protein to β -actin after transfection in each group; **: $P<0.01$, compared with Control siRNA group

图1 转染干扰片段对各组细胞中IL-4R mRNA和蛋白表达水平的影响

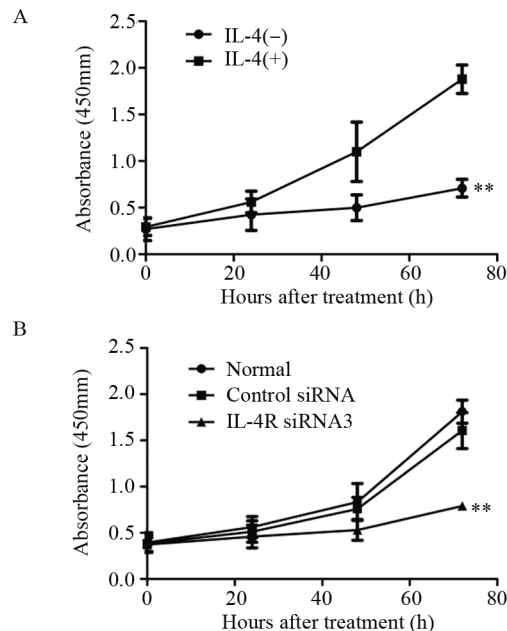
Figure1 Effect of siRNA interference on mRNA and protein level of IL-4R in each group

2.2 沉默IL-4R对Huh7细胞增殖的影响

用CCK8法检测IL-4/IL-4R对Huh7细胞增殖的影响, 发现加入IL-4后细胞增殖增加 ($P=0.01$) , 而同样加入IL-4, 转染siRNA3后的细胞从第48 h后其吸光度较正常组和空载组显著下降 ($P=0.00033$) , 见图2。提示沉默IL-4R可以抑制Huh7细胞的增殖。

2.3 沉默IL-4R对Huh7细胞凋亡和周期的影响

流式细胞学检测结果显示: 相对于正常组和



A: the impact of IL-4 on the proliferation of Huh7 cells was measured by CCK8; B: cell proliferation was detected by CCK8 assay from 0h to 72h after transfection; **: $P<0.01$, compared with Control siRNA group

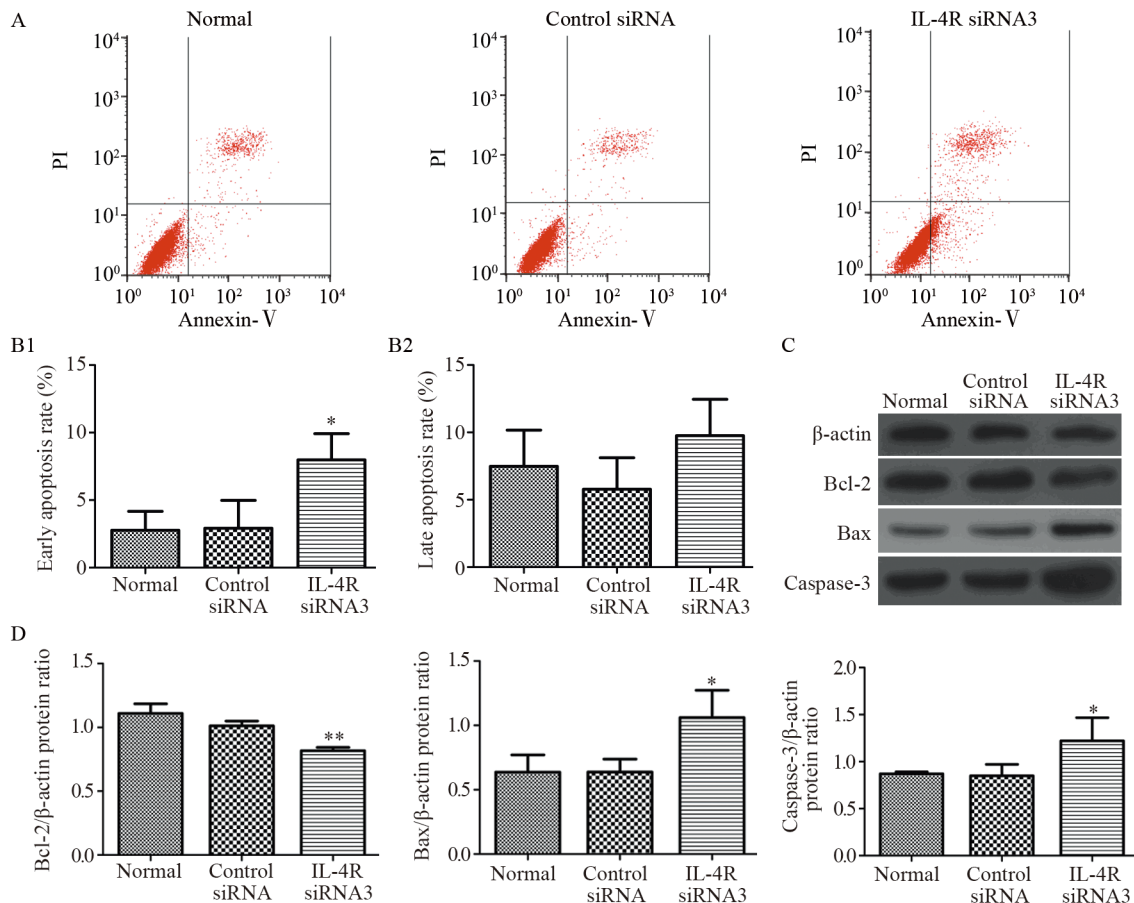
图2 IL-4/IL-4R对Huh7细胞增殖的影响

Figure2 Impact of IL-4/IL-4R on proliferation of Huh7 cells

阴性对照组, 沉默IL-4R后细胞早期凋亡显著升高 ($P=0.014$) , 而细胞晚期凋亡各组之间差异并无统计学意义 ($P=0.108$) , 见图3。Western blot提示沉默IL-4R后凋亡蛋白Bax ($P=0.016$)、Caspase-3 ($P=0.029$) 的表达上调, 抗凋亡蛋白Bcl-2 ($P=0.003$) 的表达量下降, 见图3。提示沉默Huh7细胞中的IL-4R表达可以调控凋亡蛋白和抗凋亡蛋白的表达, 进而促进肿瘤细胞凋亡。此外, 实验证实各组细胞周期分布的差异均无统计学意义 (G_0/G_1 期: $P=0.677$ 、S期: $P=0.139$ 、 G_2/M 期: $P=0.855$) , 即IL-4/IL-4R不影响Huh7细胞的周期变化, 见图4。

2.4 IL-4/IL-4R激活Huh7细胞内的JAK1/STAT6 信号通路

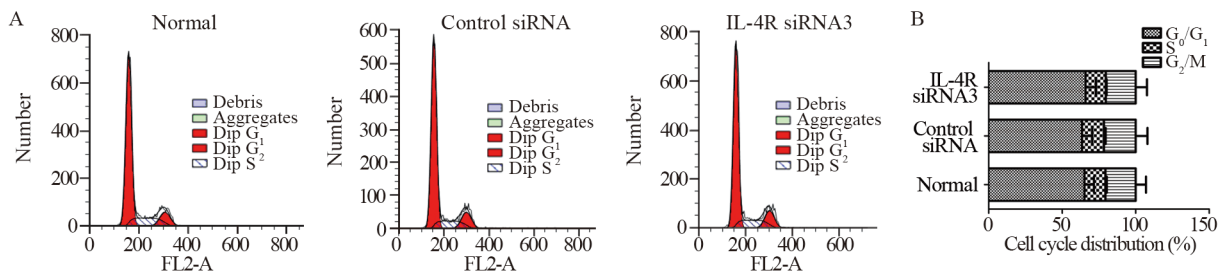
Western blot检测可能的分子机制, 发现加入IL-4后并不影响JAK1 ($P=0.737$)、STAT6 ($P=0.206$) 总蛋白的变化, 却显著上调JAK1 ($P=0.01$)、STAT6 ($P=0.005$) 磷酸化蛋白的表达。同样, 在各组细胞中加入IL-4时, 进一步下调其中一组细胞的IL-4R表达, 各组细胞中JAK1 ($P=0.475$)、STAT6 ($P=0.279$) 总蛋白表达的差异并无统计学意义, 而相对于正常组和阴性对照组, 转染siRNA3的细胞中JAK1 ($P=0.016$)、STAT6



A: the apoptosis of Huh7 cells in each group was tested by flow cytometry; B1: the early apoptosis rates in three groups were depicted in histogram; B2: the late apoptosis rates in three groups were calculated in histogram; C: the protein expression of Bcl-2, Bax, Caspase-3 were tested in each group; D: the graph of relative ratio of tested proteins to β-actin in each group; *: $P < 0.05$, **: $P < 0.01$, compared with Control siRNA group

图3 沉默IL-4R对Huh7细胞凋亡和凋亡蛋白的影响

Figure3 Influence of IL-4R silencing on apoptosis of Huh7 cells and expression of apoptosis-related proteins



A: cell cycle distribution was monitored by flow cytometry; B: there was no difference in cell cycle distribution among these groups; *: $P < 0.05$, **: $P < 0.01$, compared with Control siRNA group

图4 沉默IL-4R对Huh7细胞周期的影响

Figure4 Effect of IL-4R silencing on cell cycle of Huh7 cells

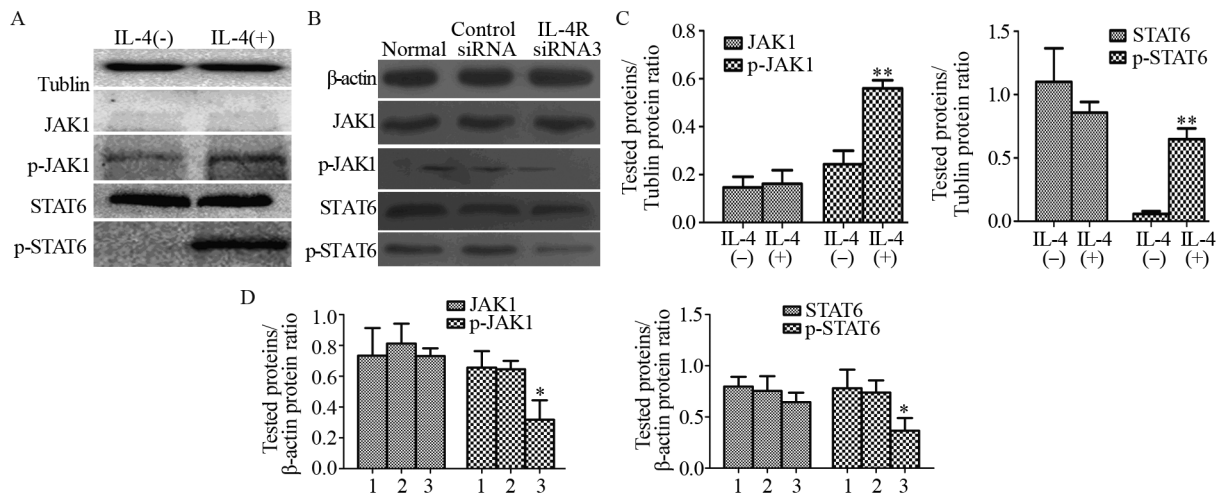
($P = 0.019$) 的磷酸化水平显著下降, 见图5。

3 讨论

细胞表面的IL-4R有两种类型: I型主要由IL-4Rα链和γ_c链组成, II型受体由IL-4Rα链和IL-13Rα1链构成。其中与IL-4特异性结合的I型受体主要参与机体的免疫调节, 例如参与抗寄生虫反应、引发超敏反应等。IL-4/IL-4R II型复合物常见

于肿瘤中。然而在肿瘤的发生、发展中IL-4/IL-4R所扮演的角色尚无统一论, 在不同类型的肿瘤中其作用可能完全相反。

早在1992年Morisaki等就发现了IL-4/IL-4R可以将胃癌细胞阻滞在G₀/G₁期, 进而抑制细胞增殖^[7]。然而近年来有研究显示, 在乳腺癌和前列腺癌中, IL-4/IL-4R却可以促进肿瘤细胞的增殖^[8-10]。不仅如此, IL-4/IL-4R还可以通过上调抗凋亡蛋白从而



A: the effect of IL-4 on signal proteins of JAK1/STAT6 signaling pathway was measured by Western blot; B: after transfecting siRNA3, the expression of JAK1, STAT6, p-JAK1 and p-STAT6 were tested by Western blot; C: after adding IL-4, the relative ratio of tested proteins to tubulin were calculated in histogram; D: after transfecting siRNA3, the relative ratio of tested proteins were depicted in histogram; *: $P < 0.05$, **: $P < 0.01$, compared with Control siRNA group; 1: Normal; 2: Control siRNA; 3: IL-4R siRNA3

图5 Western blot法检测JAK1、STAT6、p-JAK1、p-STAT6蛋白的表达

Figure5 Protein levels of JAK1, STAT6, p-JAK1 and p-STAT6 detected by Western blot

抑制乳腺癌和结肠癌细胞的凋亡^[11]。通过CCK8、流式细胞学及对凋亡相关蛋白的检测,结果提示IL-4/IL-4R除了可以促进HCC的增殖,还可以通过调节凋亡蛋白Bax、Caspase-3和抗凋亡蛋白Bcl-2的表达,从而抑制HCC的凋亡。但并不影响HCC细胞周期的改变。

JAK/STAT信号通路是常见的可以将胞外化学信号和核内DNA的转录联系在一起,调控基因表达,从而影响细胞免疫、增殖、分化、凋亡的纽带通路。由于活化的STATs可以促进下游基因的转录,如c-MYC、CCND1和VEGF,因此参与正常细胞的增殖和生存^[12-13]。同样异常激活的JAK/STAT信号通路也参与肿瘤细胞过度生长。有研究显示无论是体内还是体外实验,药物抑制STAT蛋白,或负性调控JAK/STAT信号通路可以抑制肝细胞肝癌的生长^[14-16]。Hosoyam和Burt分别在横纹肌肉瘤和恶性间皮瘤中证实,IL4/IL-4R/STAT6轴可以促进肿瘤细胞的增殖^[14-16]。同样Bankaitis等也发现,IL-4/IL-4R可以通过调节JAK1/STAT6信号通路,促进上皮细胞性癌细胞的生长^[17]。

2012年一项关于霍奇金淋巴瘤(Hodgkin's lymphoma, HL)的研究显示,阻滞IL-4/IL-4R/STAT6轴,可以上调Bcl-x_L的表达,从而协同化疗促进HL细胞凋亡^[18]。另一项关于HCC的研究也证实了,在体外抑制JAK/STAT信号通路可以抑制HCC细胞增殖及促进HCC细胞凋亡^[19]。同样在结肠癌中,也发现了IL-4/IL-4R/STAT6参与肿瘤的凋

亡和周期^[20]。本实验中,我们发现IL-4/IL-4R也参与调节Huh7细胞中JAK1/STAT6信号通路的激活,提示IL-4/IL-4R对HCC的生物学行为可能与JAK1/STAT6密切相关。

综上所述,IL-4/IL-4R可以活化HCC中的JAK1/STAT6信号通路,调节细胞内凋亡、抗凋亡蛋白的表达,最终促进HCC细胞增殖和抑制细胞凋亡。提示IL-4/IL-4R有望成为治疗HCC的新靶点,均有进一步探索的价值。在今后的研究中,我们也将进一步揭示IL-4/IL-4R/JAK1/STAT6信号轴参与调控HCC生物学行为的具体分子机制,并探索是否在体内实验中也得到相同的结果。

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