

*SIRT6*基因沉默对裸鼠异位移植人肝癌细胞增殖和凋亡的影响

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摘要 该文旨在探讨沉默信息调节因子6(silent information regulator 6, *SIRT6*)基因沉默对裸鼠异位移植人肝癌细胞增殖和凋亡的影响。构建稳定细胞系*SIRT6*-shRNA-SK-Hep-1和shCont-SK-Hep-1, 并应用定量逆转录PCR(qRT-PCR)和Western blot检测*SIRT6*基因的表达水平; 将稳定转染细胞注入裸鼠皮下, 实时监测裸鼠移植瘤的生长, 7周后剥离出裸鼠移植瘤并称重; 免疫组织化学分析*SIRT6*、Ki-67的蛋白质水平。进一步应用qRT-PCR检测*SIRT6*基因沉默对下游靶向分子凋亡抑制蛋白(inhibitor of apoptosis proteins, IAPs)家族的影响; Western blot检测X连锁凋亡抑制蛋白(X-linked inhibitor of apoptosis protein gene, XIAP)和多腺苷二磷酸核糖聚合酶(poly ADP-ribose polymerase, PARP)蛋白质水平。结果显示, 成功构建了沉默*SIRT6*基因的SK-Hep-1稳定细胞系; *SIRT6*-shRNA-SK-Hep-1组裸鼠移植瘤体积和质量较shCont-SK-Hep-1组均减少($P<0.01$); 免疫组织化学发现, *SIRT6*-shRNA-SK-Hep-1组Ki-67水平下调; 沉默*SIRT6*基因下调XIAP mRNA和蛋白质水平, 并增加PARP蛋白质的剪切水平。该研究结果提示, *SIRT6*基因沉默可能通过下调XIAP的表达抑制裸鼠异位移植人肝癌细胞的生长。

关键词 肝细胞癌; *SIRT6*; XIAP; 移植瘤; 生长

Effects of *SIRT6* Gene Silencing on Human Hepatocellular Carcinoma Cell Proliferation and Apoptosis in Ectopic Xenograft Nude Mouse Model

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Abstract This study investigated the effects of *SIRT6* gene silencing on human hepatocellular carcinoma cell proliferation and apoptosis in ectopic xenograft nude mouse model. *SIRT6*-shRNA-SK-Hep-1 and shCont-SK-Hep-1 stable cell lines were established. The mRNA and protein levels of *SIRT6* gene were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot, respectively. The stable cell lines were injected into the subcutaneous of nude mouse and the growth of ectopic xenograft nude mouse were monitored at regular intervals. The ectopic xenograft nude mouse were stripped out and weighted after injection for 7 weeks. The protein levels of *SIRT6*, Ki-67 were detected by immunohistochemical staining. Then, effects of *SIRT6* gene silencing on downstream target molecules inhibitor of apoptosis proteins (IAPs) family were determined by qRT-PCR. The

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protein level of X-linked inhibitor of apoptosis protein gene (XIAP) and poly ADP-ribose polymerase (PARP) were determined by Western blot. Our results showed that *SIRT6*-depleted SK-Hep-1 stable cell lines were established successfully. Both the size and weight of ectopic xenograft nude mouse were lower in *SIRT6*-shRNA-SK-Hep-1 group than that in shCont-SK-Hep-1 group ($P < 0.01$). The protein level of Ki-67 was down-regulated in *SIRT6*-shRNA-SK-Hep-1 group detected by immunohistochemical staining. Knockdown of *SIRT6* gene down-regulated the mRNA and protein levels of XIAP, and raised the protein cleavage of PARP. These data indicated that *SIRT6* gene silencing might inhibit human hepatocellular carcinoma cell proliferation and apoptosis in ectopic xenograft nude mouse model by down-regulating XIAP protein level.

Keywords hepatocellular carcinoma; *SIRT6*; XIAP; xenograft; growth

在癌症中, 肝细胞癌(hepatocellular carcinoma, HCC)的发病率和病死率分别位居世界第五和第二, 占原发性肝癌的85%~90%^[1]。在流行病学上, HCC具有极大的地域差异, 大部分HCC(70%~80%)发生于东方国家^[2], 而中国正是乙型肝炎感染、慢性肝炎、肝硬化的高发区, 虽然近年来医疗水平不断提高, 但HCC病死率仍然居高不下。目前, 外科手术切除是治疗HCC的常用手段, 但由于肝癌是一个由多因素参与、多途径形成的复杂病理过程的疾病, 其治疗总体效果仍不理想, 临床患者在手术后常出现复发或肝内转移^[3]。因此, 进一步探索HCC发生、发展的机制, 寻找新的治疗靶点, 对于提高肝癌的治疗效果具有重要的意义。

沉默信息调节因子(silent information regulation, Sirtuins)家族是一类依赖NAD⁺的去乙酰化酶, 不仅可使组蛋白底物去乙酰化, 还可使非组蛋白底物去乙酰化^[4-5]。哺乳动物Sirtuins家族共有7个成员, 即SIRT1~SIRT7, 其在细胞衰老、细胞代谢、细胞生长、细胞凋亡和昼夜调节等方面起着重要的调节作用^[6-8]。目前, Sirtuins家族的很多成员都成为了研究的热点, 特别是该家族的重要成员沉默信息调节因子6(silent information regulator 6, SIRT6), 主要分布于细胞核^[9]。人SIRT6蛋白由355个氨基酸组成, 相对分子质量达391 kDa^[10]。在生理学作用方面, *SIRT6*基因主要通过调节基因组的稳定性、衰老、炎症和糖代谢, 进而影响疾病的发生^[11]; 而在肿瘤中, *SIRT6*基因的功能越来越得到人们的重视。许多研究表明, *SIRT6*基因通过调节自身酶的活性, 从而影响参与肿瘤的发生、增殖和代谢等相关基因表达和蛋白质活性, 进而促进或抑制肿瘤的发生^[12-13]。目前, *SIRT6*基因在肝癌中发挥的作用尚不完全清楚。我们前期的研究发现, *SIRT6*基因沉默可以促

进入肝癌细胞凋亡, 并降低X连锁凋亡抑制蛋白(X-linked inhibitor of apoptosis protein gene, XIAP)的表达水平。*XIAP*是凋亡抑制蛋白(inhibitor of apoptosis proteins, IAPs)家族中抑制凋亡最具代表性的基因。近年来的研究发现, IAPs家族广泛存在于包括病毒、哺乳动物等多个物种中, 与肿瘤、神经细胞凋亡等疾病密切相关, 并有可能成为治疗肿瘤新的靶向分子。当细胞发生凋亡时, 聚ADP核糖聚合酶(poly ADP-ribose polymerase, PARP)被DNA缺口激活, 并对DNA进行修复, PARP剪切被认为是细胞凋亡的标志。本研究在裸鼠异位移植瘤模型上进一步探讨沉默*SIRT6*基因对肝癌细胞生长的影响, 并初步探讨其分子机制, 从而为肝癌基因治疗寻找新的途径。

1 材料与方法

1.1 材料

人肝癌细胞系SK-Hep-1购于ATCC(American Type Culture Collection), 靶向*SIRT6*慢病毒购于GeneChem公司(上海); shCont序列: 5'-GCA ACA AGA TGA AGA GCA CCA A-3'; sh*SIRT6*序列: 5'-GCT ACG TTG ACG AGG TCA TGA-3'; 嘌呤霉素(13884-50)购于Cayman公司; *SIRT6*抗体(NB-100-2522)购于Novus Biologicals公司; XIAP抗体(#2045)和PARP抗体(#9542)均购于Cell Signaling Technology公司; Ki-67抗体(BS1454)购于Bioworld公司; 免疫组织化学试剂盒购于Vector公司; 裸鼠购于重庆医科大学动物中心。

1.2 确定嘌呤霉素最低筛选浓度

在24孔板中接种SK-Hep-1细胞, 5×10^4 /孔, 准备含不同浓度的嘌呤霉素筛选培养基(0、1.00、2.50、3.75、5.00 $\mu\text{mol/L}$), 24 h后加入筛选培养基, 约48~72 h更换新鲜的筛选培养基, 每日监测细胞存活的

比例,待全部细胞死亡的最低抗生素浓度为最低筛选浓度(2.50 μmol/L)。

1.3 构建沉默*SIRT6*基因的稳定细胞系

在6孔板中接种SK-Hep-1细胞,1×10⁵/孔,24 h后将表达shCont、sh*SIRT6*的慢病毒感染细胞,48 h后使用含2.50 μmol/L嘌呤霉素筛选培养基更换旧培养基,每天监测细胞存活比例,待细胞增殖至汇合度70%~80%时传代,持续筛选1周,并收集细胞检测沉默效率。

1.4 构建裸鼠肝癌异位移植瘤模型

将构建成功的稳定细胞系*SIRT6*-shRNA-SK-Hep-1和shCont-SK-Hep-1培养计数,分别取5×10⁶细胞注入裸鼠后腿根部区域皮下,每组各5只。注射后每周测量裸鼠移植瘤体积,并绘制裸鼠移植瘤生长曲线,7周后将裸鼠安乐死,尽量减轻裸鼠痛苦,剥离体内移植瘤并测量其体积和质量。

1.5 qRT-PCR检测mRNA水平

收集构建成功的稳定细胞系,加入Trizol(Invitrogen公司)裂解细胞,提取细胞总RNA后,利用iScriptTM cDNA Synthesis Kit(Bio-Rad公司)试剂盒将总RNA逆转录为cDNA,随后进行qRT-PCR,取1 μL cDNA、

5 μL SYBGreen(Roche公司)、上游引物和下游引物(10 μmol/L)各0.2 μL、3.6 μL ddH₂O,充分混匀后按照以下条件进行qRT-PCR: 94 °C 20 s、60 °C 20 s、72 °C 15 s,共35个循环。以β-actin为内参,每个样本重复3次,以2^{-ΔΔC_t}计算目的基因的相对表达水平。qRT-PCR引物序列见表1。

1.6 Western blot检测蛋白质水平

从裸鼠中剥离出移植瘤组织块后,切出黄豆大小的肿块,加入1 mL蛋白质裂解液RIPA(含PI),在冰上利用组织研磨器将其充分溶解,在低温离心机中16 000 ×g离心10 min以去除杂质,并将上清转移至新的EP管中;用BCA法测定蛋白质浓度,取30 μg蛋白质上样,120 V电泳1.5 h,90 V恒压湿转3.5 h,5%牛奶封闭1.5 h,一抗4 °C摇床过夜(*SIRT6*抗体1:4 000稀释,XIAP抗体1:2 000稀释,PARP抗体1:2 000稀释),第2 d用TBST(含Tween20)洗膜3次,每次5 min,在室温下孵育二抗2 h,TBST洗膜3次,每次5 min,ECL显影。

1.7 免疫组织化学

将剥离的移植瘤组织块放入4%多聚甲醛中进行固定后制成石蜡切片。将其在50 °C过夜后放入

表1 qRT-PCR引物序列
Table 1 Sequences of qRT-PCR primers

目的基因 Target genes	引物序列 Primer sequences
<i>HIAP-1</i>	F: 5'-TGG TCT TCT CCA GGT TCA AA-3' R: 5'-TGG TCT TCT CCA GGT TCA AA-3'
<i>HIAP-2</i>	F: 5'-CAA ATG GTT TCC AAG GTG TG-3' R: 5'-GGA CAA CAG CTG CTC AAG AA-3'
<i>XIAP</i>	F: 5'-GGA GGG CTA ACT GAT TGG AA-3' R: 5'-ATT TGC ACC CTG GAT ACC AT-3'
<i>ML-IAP</i>	F: 5'-CCA GCT GTC AGT TCC TGC T-3' R: 5'-CAG CTG GGA GTG AGT CTC C-3'
<i>Survivin</i>	F: 5'-CCC TGC CTG GCA GCC CTT TC-3' R: 5'-CTG GCT CCC AGC CTT CCA-3'
<i>ILP-2</i>	F: 5'-GAA GCC CGG CTC ATT ACT T-3' R: 5'-AGC TCT TGC AAG CTG CTC TT-3'
<i>NAIP</i>	F: 5'-CCA GAC AAC AAT GCC ACT TC-3' R: 5'-AAA TGC TCT GTC CGT CCT TT-3'
<i>BRUCE</i>	F: 5'-GAC ACT GCT CTG CAA ACT CC-3' R: 5'-AGA GCT GCT GTG CCT CTG TA-3'
<i>β-actin</i>	F: 5'-CTC TTC CAG CCT TCC TTC CT-3' R: 5'-AGC ACT GTG TTG GCG TAC AG-3'
<i>SIRT6</i>	F: 5'-GCA GTC TTC CAG TGT GGT GT-3' R: 5'-CCA TGG TCC AGA CTC CGT-3'

二甲苯I 45 s、二甲苯II 45 s, 立即依次放入无水乙醇I 30 s、无水乙醇II 30 s、90%乙醇30 s、80%乙醇30 s、70%乙醇30 s、双蒸水60 s进行脱蜡水化处理。100 mL 0.3% Triton X-100浸泡20 min, 随后1×TBS洗3次, 每次10 min。在修复盒中加入柠檬酸盐修复液, 放入微波炉中进行抗原修复, 20 min后放在室温中进行缓慢降温; 待冷却后加入100 mL 3%过氧化氢封闭内源性抗原, 10 min后用蒸馏水洗2次, 每次3 min, 1×TBS浸泡10 min。使用Vector试剂盒中的羊血清室温封闭2 h; 一抗4℃过夜(SIRT6: 1:200稀释; Ki-67: 1:100稀释; XIAP: 1:50稀释; PARP: 1:100稀释); 第2 d用TBST室温浸泡3次, 每次10 min; 二抗室温孵育1 h; TBST室温浸泡3次, 每次10 min; SABC室温孵育1 h; TBST室温浸泡3次, 每次10 min; DAB染色2 min; TBST室温浸泡3次, 每次10 min; 苏木素复染2 min后, 流水冲洗10 min, 蒸馏水浸泡10 min。再于70%乙醇、80%乙醇、90%乙醇、无水乙醇I、无水乙醇II中各浸泡30 s, 二甲苯I、II各浸泡45 s, 待完全干燥后用中性树脂进行封片, 显微镜拍照。

1.8 统计分析

采用SPSS 20.0软件进行统计分析, 两组间比较采用配对 t 检验, 以 $P<0.05$ 为差异具有统计学意义。

2 结果

2.1 构建沉默SIRT6基因的稳定细胞系

收集筛选出的SIRT6-shRNA-SK-Hep-1细胞系, 应用qRT-PCR检测细胞中SIRT6 mRNA水平。结果显示, 与shCont-SK-Hep-1相比, 其抑制率达70%, 差

异具有统计学意义($P<0.01$, 图1A)。应用Western blot检测细胞中SIRT6蛋白质水平, 发现shSIRT6成功抑制了肝癌细胞SK-Hep-1中SIRT6蛋白质水平(图1B), 提示沉默SIRT6基因的稳态细胞系SIRT6-shRNA-SK-Hep-1构建成功。

2.2 沉默SIRT6基因对裸鼠移植瘤生长的影响

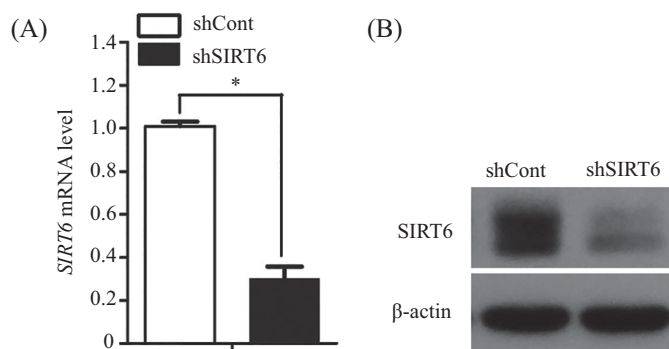
将已构建成功的SIRT6-shRNA-SK-Hep-1和shCont-SK-Hep-1稳定细胞系分别培养计数后注入裸鼠皮下后腿根部, 注射后每隔1周测量裸鼠移植瘤体积。结果发现, SIRT6-shRNA-SK-Hep-1组裸鼠移植瘤体积较shCont-SK-Hep-1组小, 差异具有统计学意义($P<0.01$, 图2)。随后将裸鼠安乐死, 将移植瘤从裸鼠体内剥离并称重。结果显示, SIRT6-shRNA-SK-Hep-1组移植瘤质量较shCont-SK-Hep-1减轻, 差异具有统计学意义($P<0.01$, 图3)。

2.3 免疫组织化学检测沉默SIRT6基因后肿瘤增殖标志物Ki-67蛋白质水平

将剥离出的两组裸鼠移植瘤制成石蜡切片, 应用免疫组织化学技术检测裸鼠移植瘤中SIRT6、肿瘤增殖标志物Ki-67的蛋白质水平。结果发现, 沉默SIRT6基因后, SIRT6-shRNA-SK-Hep-1组中SIRT6和肿瘤标志物Ki-67蛋白质水平较shCont-SK-Hep-1组降低(图4); 进一步提示沉默SIRT6基因可抑制肿瘤细胞生长。

2.4 沉默SIRT6基因下调裸鼠移植瘤中XIAP基因的表达

为了进一步阐述沉默SIRT6基因对裸鼠异位移植瘤的分子机制, 我们将剥离出的裸鼠移植瘤充分

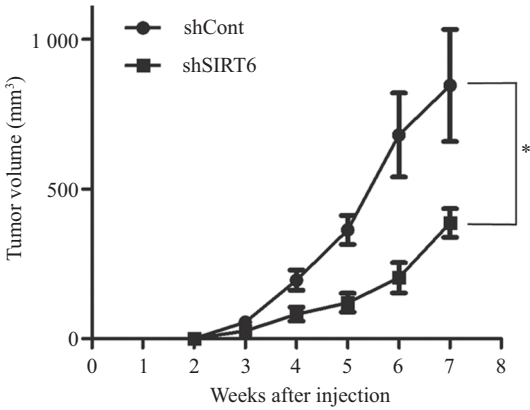


A: qRT-PCR检测稳定细胞系中SIRT6 mRNA水平, $*P<0.05$; B: Western blot检测稳定细胞系中SIRT6蛋白质水平。

A: the mRNA level of SIRT6 in stable cell lines was determined by qRT-PCR, $*P<0.05$; B: the protein level of SIRT6 in stable cell lines was determined by Western blot.

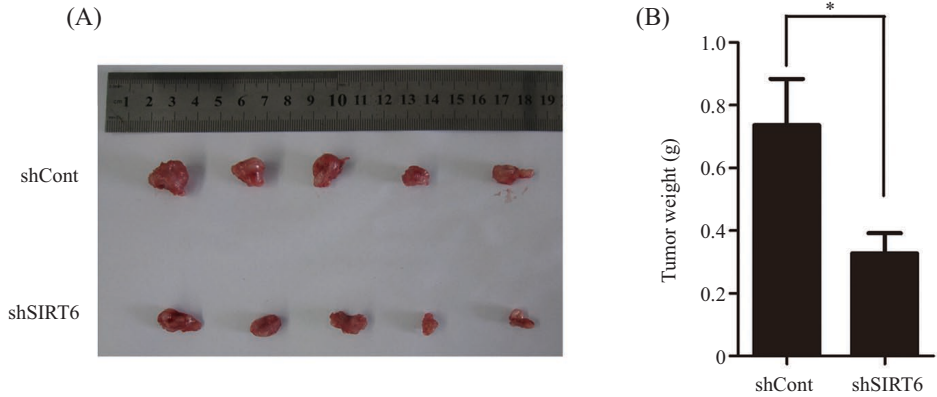
图1 qRT-PCR和Western blot检测稳定细胞系中SIRT6 mRNA和蛋白质水平

Fig.1 The mRNA and protein level of SIRT6 was determined by qRT-PCR and Western blot in stable cell lines



裸鼠移植瘤体积=长×宽×高。**P*<0.05, 与shCont相比较。
The volume of ectopic xenograft nude mouse is equal to L×W×H. **P*<0.05 vs shCont.

图2 *SIRT6*基因沉默对裸鼠移植瘤生长的影响
Fig.2 Effects of *SIRT6* gene silencing on the growth of ectopic xenograft nude mouse



A: 注射7周后裸鼠移植瘤的实体图; B: 裸鼠移植瘤的质量, **P*<0.05。
A: the entity graph of ectopic xenograft nude mouse at 7 weeks after injection. B: the tumor weight of ectopic xenograft nude mouse. **P*<0.05.

图3 *SIRT6*基因沉默对裸鼠移植瘤质量的影响
Fig.3 Effects of *SIRT6* gene silencing on the tumor weight of ectopic xenograft nude mouse

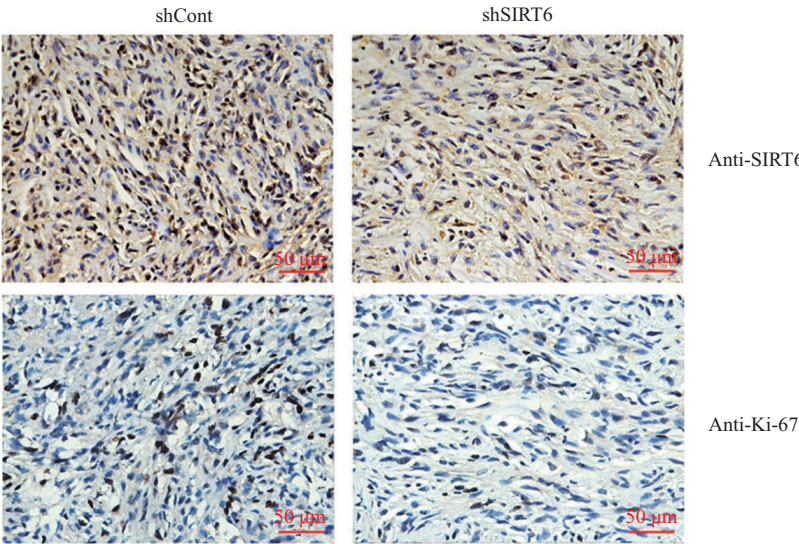
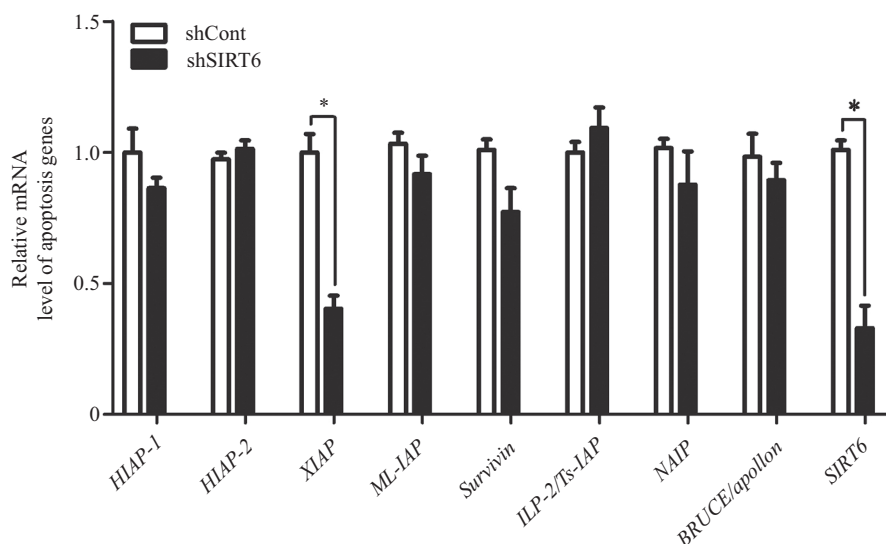


图4 免疫组织化学检测移植瘤中SIRT6、Ki-67蛋白质水平
Fig.4 The protein level of SIRT6 and Ki-67 in ectopic xenograft determined by immunohistochemistry



* $P < 0.05$.

图5 qRT-PCR检测SIRT6基因沉默对XIAP家族mRNA水平的影响

Fig.5 Effects of *SIRT6* gene silencing on mRNA levels of XIAP family determined by qRT-PCR

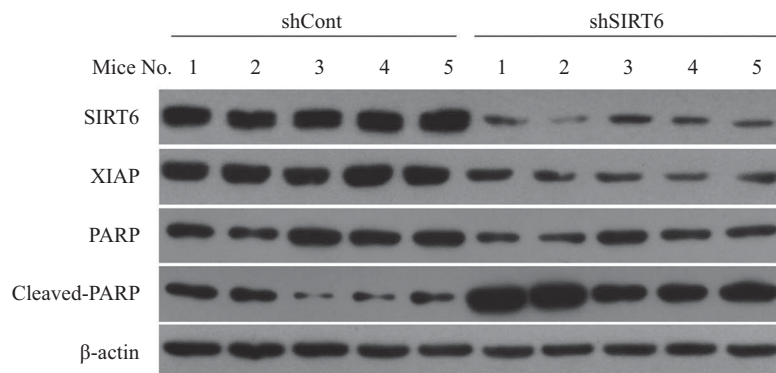


图6 Western blot检测沉默SIRT6基因对XIAP、PARP和Cleaved-PARP蛋白质水平的影响

Fig.6 Effects of *SIRT6* gene silencing on protein levels of XIAP, PARP and Cleaved-PARP determined by Western blot

研磨后提取总RNA,应用qRT-PCR检测IAPs家族成员mRNA水平。结果显示, SIRT6-shRNA-SK-Hep-1组中XIAP mRNA水平显著下降,差异具有统计学意义(图5, $P < 0.01$)。同时, Western blot检测发现,沉默SIRT6后XIAP表达水平下降, Cleaved-PARP表达水平升高,而总PARP表达水平明显降低,提示沉默SIRT6基因可通过下调XIAP蛋白质水平,进而诱导裸鼠异位移植人肝癌细胞的凋亡(图6)。

3 讨论

近年来, SIRT6基因在肿瘤细胞生物学中的作用及在肿瘤诊断治疗中的潜在价值越来越得到人们的重视。一方面, SIRT6基因可作为潜在的抑癌基因。Lu等^[14]发现,过表达SIRT6通过激活自噬抑制异丙肾上腺素诱导的心肌肥厚。在动脉粥样硬化患者中, SIRT6表达下调,并发现该类患者更易导致心

肌肥厚和心力衰竭^[15]。Zhong等^[16]和Sebastian等^[17]提出, SIRT6基因可以阻碍细胞向有氧糖酵解转化,从而抑制肿瘤的发生。在患有肺癌的雄性小鼠中, Kanfi等^[18]发现, SIRT6基因过表达比野生型小鼠寿命延长了11.7%,提示SIRT6基因在癌症中发挥抑癌作用。另一方面, SIRT6基因也可以作为潜在的癌基因。许多研究表明, SIRT6基因在慢性淋巴细胞白血病^[19]、胰腺癌^[20]、鳞状细胞癌^[21]中表达上调,提示SIRT6基因是一个潜在的肿瘤促进因子。以上研究说明, SIRT6在不同肿瘤组织中表达不同,并通过多种途径在肿瘤发生、发展中发挥不同的作用,但其具体机制仍有待进一步研究。本研究首先构建了沉默SIRT6基因的稳态细胞系SIRT6-shRNA-SK-Hep-1和shCont-SK-Hep-1,并将其注入裸鼠皮下,构建裸鼠肝细胞癌异位移植瘤模型,发现沉默SIRT6基因抑制了肝细胞癌皮下移植瘤的生长,提示SIRT6高表达

可能与肝癌细胞的恶性生长密切相关。

目前,引起肿瘤细胞生长的因素有:凋亡抑制、增殖信号、逃避生长抑制、拮抗细胞死亡、基因启动子永久性复制、血管生成增加、活化侵袭和转移等^[22],其中细胞凋亡机制受阻或调节异常被认为是一种潜在的肝癌发生机制^[16]。近年的研究表明,*SIRT6*参与了多种与凋亡密切相关的信号通路。Feng等^[23]发现,*SIRT6*通过抑制氧化应激和JAK2(Janus kinase 2)/STAT3(signal transducer and activator of transcription 3)信号通路的激活,从而诱导神经胶质瘤凋亡。Kok等^[24]发现,*SIRT6*通过抑制成骨细胞的糖酵解,从而抑制低氧诱导的凋亡。Min等^[25]发现,在肝癌中,*SIRT6*基因表达上调可减少肝癌的发生,这可能是由于c-fos减少了*SIRT6*基因的转录,从而减弱凋亡抑制蛋白存活蛋白(survivin)的活性以及抑制NF- κ B(nuclear factor- κ B)信号通路;此外,沉默*SIRT6*后还可通过去乙酰化H3K9位点并激活Bcl-2/Bax信号通路,从而诱导肝癌细胞凋亡^[26]。我们前期研究发现,沉默*SIRT6*基因可能通过调控XIAP的表达影响人肝癌细胞凋亡。我们进一步在裸鼠异位移植瘤上发现,沉默*SIRT6*基因下调XIAP的表达水平,并上调PARP的剪切水平,而总PARP表达水平下降,提示*SIRT6*基因沉默可以调控XIAP的表达从而影响裸鼠肝细胞癌移植瘤的生长。有研究表明,XIAP高表达与HCC肿瘤细胞的凋亡指数下降密切相关^[27],并可作为HCC患者无癌生存率和肝细胞癌术后总体生存率的独立预测因素^[28]。此外,XIAP mRNA、蛋白质水平在肝癌细胞系和肝癌组织中明显升高,这可能是促进肝细胞发生癌变的重要因素^[29-30]。XIAP高表达患者3年总体生存率明显低于XIAP低表患者^[27]。沉默XIAP的表达降低了肝癌细胞的生存能力并促进其凋亡水平,增加肝癌细胞对甲氨喋呤的敏感性^[31]。综上所述,本研究发现,沉默*SIRT6*基因可能通过调控XIAP的表达抑制裸鼠肝细胞癌移植瘤的生长,为HCC的靶向治疗提供了新的方向。

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