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· 基础研究 ·

Aurora-B 诱导着丝粒蛋白 U 磷酸化促进胆管癌细胞增殖的作用研究

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摘要

背景与目的: 有丝分裂激酶 Aurora-B 是胆管癌原癌基因, 着丝粒蛋白 U (CENPU) 参与有丝分裂受 Aurora-B 激酶磷酸化调控, 笔者团队前期在胆管癌中筛选出 CENPU 并证实 CENPU 表达水平明显高于其相应的癌旁组织, 从而推测 Aurora-B 可能通过调控 CENPU 的磷酸化参与胆管癌的肿瘤生物学过程。因此, 本研究探讨 Aurora-B 与 CENPU 在胆管癌中的关系及作用。

方法: 采用免疫组化检测 10 对胆管癌组织和癌旁组织中 Aurora-B 和 CENPU 以及磷酸化 CENPU (p-CENPU) 的表达。采用 TCGA 数据库的数据分析胆管癌中 Aurora-B 和 CENPU 的表达及相关性。构建 Aurora-B 基因敲减的胆管癌 QBC939 细胞系, 用 Western blot 检测 CENPU 和 p-CENPU 的表达, CCK8 法检测细胞增殖活性。构建 CENPU 磷酸化位点突变的胆管癌 QBC939 细胞系, 用 DMSO 或 Aurora-B 抑制剂处理后, 观察 Aurora-B、CENPU 和 p-CENPU 表达以及细胞增殖活性的变化。

结果: 免疫组化结果显示, 胆管癌组织中 Aurora-B 和 CENPU 表达阳性率分别为 22.61% 和 12.34%, 而两者在癌旁组织中几乎无表达, 同时胆管癌组织中 p-CENPU 的表达也明显高于癌旁组织。TCGA 数据显示, 胆管癌中 Aurora-B 和 CENPU 的上调 (均 $P < 0.05$), 且 Aurora-B 和 CENPU 的表达水平呈正相关 ($r = 0.7322$, $P < 0.05$)。敲减 Aurora-B 表达后, QBC939 细胞 CENPU 表达无明显变化, 但 p-CENPU 表达降低、增殖活性明显减弱 ($P < 0.05$)。QBC939 细胞在突变 CENPU 磷酸化位点或加入 Aurora-B 抑制剂后, 前者 Aurora-B 表达无明显变化, 后者明显降低, 两者 CENPU 表达均无明显变化, 但 p-CENPU 表达明显降低; 突变 CENPU 磷酸化位点后, Aurora-B 介导的 QBC939 细胞增殖活性较野生型明显下降 ($P < 0.05$)。

结论: 在胆管癌中, CENPU 是 Aurora-B 的磷酸化底物, Aurora-B 可能通过磷酸化 CENPU 促进胆管癌细胞的增殖。

关键词

胆管肿瘤; 极光激酶 B; 着丝粒蛋白 U; 细胞增殖

中图分类号: R735.8

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Aurora-B promoting the proliferation of cholangiocarcinoma cells by inducing phosphorylation of centrosome protein U

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Abstract

Background and Aims: The mitotic kinase Aurora-B is considered to be a proto-oncogene of cholangiocarcinoma. The action of centrosome protein U (CENPU) in mitosis is governed by phosphorylation regulation of Aurora-B kinase. The previous study conducted by the authors' group has identified CENPU in cholangiocarcinoma and confirmed that the expression level of CENPU is significantly higher in cholangiocarcinoma tissue than that in its corresponding adjacent tissue. So, it is speculated that Aurora-B probably participates the tumor biological process of cholangiocarcinoma through regulation of the CENPU phosphorylation. Therefore, this study was performed to investigate the relationship between Aurora-B and CENPU and their effects in cholangiocarcinoma.

Methods: The expressions of Aurora-B and CENPU as well as the phosphorylated CENPU (p-CENPU) in 10 pairs of specimens of cholangiocarcinoma and para-carcinoma tissue were determined by immunohistochemical staining. The expressions of Aurora-B and CENPU and their correlation in cholangiocarcinoma were analyzed in TCGA database. The cholangiocarcinoma QBC939 cells with Aurora-B gene knockout were constructed, and then, the expressions of CENPU and p-CENPU were determined by Western blot analysis and the proliferative activity was detected by CCK8 assay. QBC939 cells with mutated phosphorylation site of CENPU were constructed, in which, the changes in expressions of Aurora-B, CENPU and p-CENPU as well as the proliferation activity were observed.

Results: The results of immunohistochemical staining showed that positive expression rates of Aurora-B and CENPU in cholangiocarcinoma tissue were 22.61% and 12.34%, respectively, but nearly none of them expressed in adjacent tissue. Meanwhile, the expression level of p-CENPU in cholangiocarcinoma tissue was remarkably higher than that in adjacent tissue. TCGA data showed that both expressions of Aurora-B and CENPU were up-regulated in cholangiocarcinoma (both $P < 0.05$), and there was a positive correlation between the expressions of Aurora-B and CENPU ($r = 0.7322$, $P < 0.05$). In QBC939 cells after knockdown of Aurora-B expression, there was no significant change in CENPU expression, but the p-CENPU expression was down-regulated, and the proliferation activity was significantly decreased ($P < 0.05$). In QBC939 cells after mutating the phosphorylation site of CENPU or adding Aurora-B inhibitor, Aurora-B expression showed no change in the former, but was significantly reduced in the latter, and in both conditions, the CENPU expressions showed no changes, but the p-CENPU expressions were decreased; after mutating the phosphorylation site of CENPU, the proliferation activity of QBC939 cells mediated by Aurora-B was significantly lower than that of the wild-type ($P < 0.05$).

Conclusion: CENPU is the phosphorylation substrate of Aurora-B in cholangiocarcinoma cells. Aurora-B promotes the proliferation of cholangiocarcinoma cells probably by phosphorylation of CENPU.

Key words

Bile Duct Neoplasms; Aurora Kinase B; Centrosome Protein U; Cell Proliferation

CLC number: R735.8

胆管癌的发病率约占消化系统的3%，是肝胆癌中第二常见的恶性肿瘤^[1-2]。目前，手术切除是

胆管癌的主要治疗策略^[3]。然而，胆管癌的术后复发率很高，5年生存率不到5%。因此，探索胆管

癌发生发展的分子机制,为胆管癌的治疗提供药物靶点对于胆管癌的防治研究有重要的临床意义。Aurora激酶是一类高度保守的丝氨酸/苏氨酸激酶,由Aurora-A、Aurora-B和Aurora-C 3个亚型组成。Aurora激酶参与细胞中心体的成熟和分离、纺锤体的组装和稳定性、染色体浓度和中板聚集等^[4-5]。在哺乳动物中,Aurora-B与INCENP、survivin和borealin等其他3种染色体乘客蛋白形成四聚体,在有丝分裂着丝粒和微管的正确定位中起重要作用^[6]。大量研究^[7-10]表明,Aurora激酶的突变增加了基因组的不稳定性,其过度表达和异常活性可导致纺锤体缺陷和染色体分离异常,导致细胞的非整倍体,从而导致细胞的恶性转化。在过去的几十年中,研究表明Aurora-B与肿瘤的发生发展和转移有关,并且已经成为一个重要的抗肿瘤靶点。尽管Aurora-B在胆管癌中的异常表达已被证实^[11],但Aurora-B对胆管癌的调控作用至今未见报道。

着丝粒蛋白U(CENPU)是着丝粒相关网络重要组成部分,研究证实CENPU在有丝分裂的染色体分离过程中发挥了重要的作用,CENPU已被鉴定为Aurora-B诱导的磷酸化作用的底物之一^[12]。本课题组在前期胆管癌中筛选出CENPU的基础上,通过临床病理标本检测首次发现CENPU在胆管癌中表达水平明显高于其相应的癌旁组织,采用siRNA技术将胆管癌QBC939细胞CENPU敲减,初步结果显示胆管癌细胞增殖受到抑制,提示CENPU可能为胆管癌发生发展的相关基因^[13]。因此,结合胆管癌中Aurora-B异常表达以及其可诱导CENPU磷酸化,推测Aurora-B可能通过CENPU的磷酸化来调控胆管癌的发生。

1 材料与方法

1.1 免疫组化染色

用免疫组化法检测Aurora-B激酶、CENPU蛋白以及磷酸化CENPU(p-CENPU)蛋白的表达。收集10例胆管癌患者的肿瘤组织和配对的正常癌旁组织,所有参与的胆管癌患者均提供书面知情同意书。所有标本在65℃的烤箱中烘烤30 min,然后将载玻片在二甲苯中脱蜡30 min,并在100%至75%乙醇中再水合。将玻片样本架放入含1×乙二胺四乙酸的修复缸中100℃煮沸30 min,室温冷却,然后用3% H_2O_2 封闭5 min,再用5%血清封闭

15 min。Aurora-B(1:100, Affinity, Cat#AF5361), CENPU(1:50, Affinity, Cat#DF2320), p-CENPU(1:50, Abcam, Cat#ab117078)抗体孵育4℃过夜,然后加入相应的二抗染色。玻片在黑暗中用DAB染色5 min,苏木精复染,使用中性树胶进行封片。玻片在显微镜下观察并拍摄图像。IHC评分采用阳性细胞评分和染色强度评分总和。阳性细胞评分分为4级:1级(1%~24%),2级(25%~49%),3级(50%~74%),4级(75%~100%)。染色强度从0分(无信号色)到3分(浅黄色、棕色和深棕色)。

1.2 细胞培养

胆管癌细胞系QBC939细胞购买于ATCC,并培养于含10%FBS(GIBCO, Cat#10091-148)和青霉素链霉素(100×)(GIBCO, Cat#15140122)的DMEM(GIBCO, Cat#1868985)培养液中,置于含5% CO_2 和湿度为70%~80%的培养箱中(三洋, Cat#MCO-175),培养温度为37℃。培养基每3天更换1次。

1.3 慢病毒载体构建及细胞转染

Aurora-B的shRNA靶序列由上海懿贝瑞生物科技有限公司设计,用限制性内切酶Age I和EcoR I(Cat#R3552L和R3101L, NEB)线性化LV-013载体,并与设计序列连接。将连接产物转化到大肠杆菌受体细胞中,用PCR方法筛选阳性克隆。用EndoFree midi质粒试剂盒(Cat #DP118-2, TIANGEN)提取高纯度质粒。构建CENPU磷酸化位点(苏氨酸78)失活突变慢病毒载体。将突变和非突变CENPU克隆到表达载体LV-013中(LV-013载体元件包含:pCDH-CMV-MCS-3FLAG-EF1-copGFP-T2A-Puro),根据PCR测序结果构建重组表达载体LV-013-CENPU。在6孔板中培养QBC939细胞,直到细胞融合达到80%,使用Lipofectamine 2000(Thermo Fisher Scientific)将质粒转染到细胞中。培养72 h后,在倒置荧光显微镜下观察GFP绿色荧光表达情况,并计算转染效率,转染效率=绿色荧光蛋白(GFP)阳性细胞数/总细胞数×100%。

1.4 qRT-PCR

收集细胞并以2 000 r/min离心5 min,去除上清液,向细胞沉淀中添加1 mL TRIzol(Sigma),并根据TRIzol的说明提取总RNA。将RNA沉淀溶解在无RNase酶的水中,并使用Nanodrop100分光光

度计 (Thermo, America) 分析和测定提取 RNA 的浓度和质量。根据 hiscript QRT-super-mix (Vazyme) 的说明书将 RNA 逆转录合成 cDNA。按照 SYBR-Green-mastermix 试剂盒的要求制备 qRT-PCR 反应体系, 绘制熔融曲线。以 GAPDH 为内参照, 计算基因表达量, $2^{-\Delta\Delta Ct}$ 代表各靶基因的相对表达。每个实验至少重复 3 次。PCR 反应条件为: 95 °C 预变性 1 min, 94 °C 变性 10 s, 60 °C 退火 30 s, 55 °C 解离 1 min。Aurora-B 引物的上游序列: 5'-TGG CTC GGG AGA AGA AA-3', 下游序列: 5'-CGC AGG ATG TTG GGA TG-3'。

1.5 Western blot

细胞中加入细胞裂解液, 置于 4 °C 冰上至细胞完全溶解。细胞裂解样品在 12 000 r/min 下离心 10 min。提取总蛋白加入 1×SDS 上样缓冲液。蛋白质 (20 μg/lane) 经 10%SDS-PAGE 分离后转移到 PVDF 膜上。用封闭液含 5% 脱脂牛奶的 1×TBST 溶液, 置于室温摇床。然后去除液体, 添加抗体, 包括 CENPU (1:1 000, Affinity, Cat. #DF2320)、p-CENPU (1:1 000, Abcam, Cat. #ab117078) 以及 GAPDH (1:3 000, Bioworld, Cat. #AP0063), 在 4°C 冰箱孵育过夜。用 1×TBST 清洗 3 次, 用 Goat Anti-Rabbit 二抗 (1:3 000, Beyotime, Cat. #A0208) 4 °C 孵育 4 h。最后洗膜 3 次, 用 Immobilon Western Chemiluminescent HRP Substrate 试剂盒显色, 得到蛋白条带, 拍摄照片。

1.6 CCK8 实验

CCK8 法检测 QBC939 细胞增殖活性。在 96 孔板上接种细胞, 每孔 2 000 个, 孵育 72 h。每孔加入 10 μL 1×10 比例 Kit-8 溶液, 再孵育 4 h。在 0、6、12、24、48 h 候用酶标仪测定 450 nm 波长处的吸光度值, 计算细胞存活率。

1.7 统计学处理

细胞实验数据取自至少 3 次独立的重复实验, 数据以平均数 ± 标准差 ($\bar{x} \pm s$) 表示。用 SPSS 20.0 和 GraphPad-Prism 软件 7.0 进行统计分析。*t* 检验比较差异, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 Aurora-B 和 CENPU 在胆管癌中的表达

为了初步探讨 Aurora-B 和 CENPU 在胆管癌中的作用, 首先采用免疫组化方法检测了它们在胆

管癌及癌旁组织中的表达。结果显示, Aurora-B 和 CENPU 在胆管癌组织中均有表达, 阳性率分别为 22.61% 和 12.34%, 而在癌旁组织中几乎没有表达 (图 1)。从 TCGA 数据库^[4]收集的数据也表明了胆管癌中 Aurora-B 和 CENPU 的上调 (图 2A-B)。此外, 相关性分析显示, 在胆管癌中, Aurora-B 和 CENPU 的表达水平呈正相关 ($r=0.7322$, $P < 0.05$) (图 2C)。进一步的免疫组化分析表明, 与癌旁组织比较, 胆管癌肿瘤组织中 p-CENPU 表达上调, 提示 Aurora-B 与 CENPU 磷酸化之间存在潜在的联系。

2.2 Aurora-B 敲减对胆管癌细胞增殖和 CENPU 磷酸化的影响

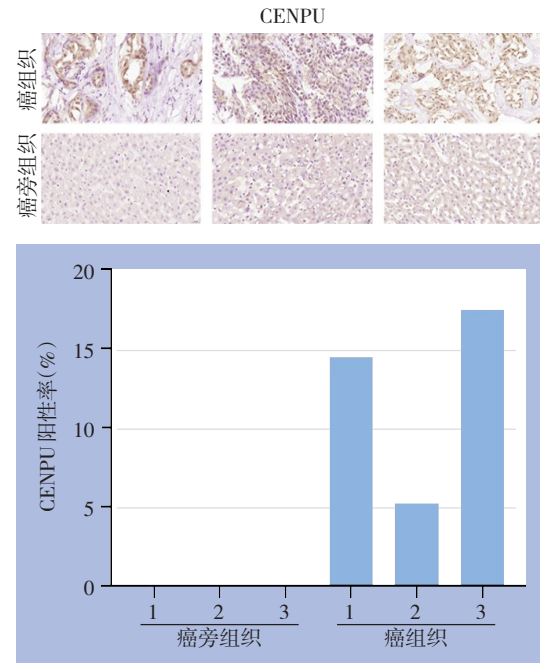
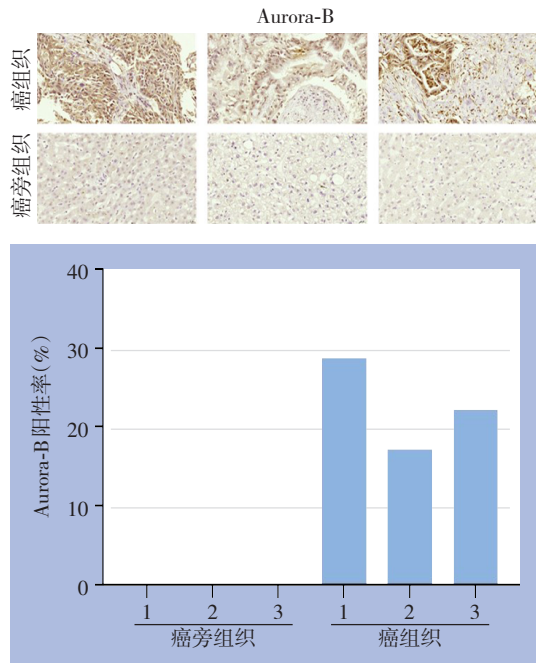
为了验证 Aurora-B 在胆管癌中的作用及其机制, 采用转染表达靶向 Aurora-B 的 shRNA 的慢病毒载体构建了 Aurora-B 基因敲减的胆管癌 QBC939 细胞模型。首先采用 qPCR 评估了 3 个靶向 Aurora-B 的 shRNA 对 Aurora-B 的敲减效率, 其结果显示 shAurora-B-2 的效果最佳。因此, 后续实验全部采用 shAurora-B-2 (简称为 shAurora-B 组) 进行细胞构建 (图 3A)。细胞增殖实验的结果显示, 敲减 Aurora-B 的表达可明显抑制 QBC939 细胞的增殖率 (图 3B)。此外, Western blot 的结果显示, shAurora-B 组中 CENPU 的磷酸化水平明显低于转染阴性 shRNA 序列对照组 (图 3C)。这些结果证明 Aurora-B 不仅对胆管癌的细胞增殖具有调控作用, 同时这一作用可能与诱导胆管癌中 CENPU 的磷酸化有关。

2.3 CENPU 磷酸化位点变异对胆管癌细胞增殖和 CENPU 磷酸化的影响

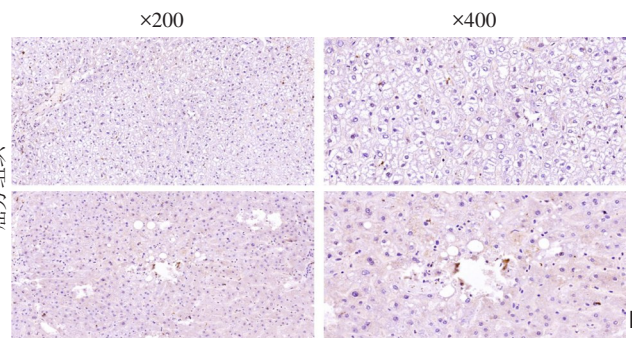
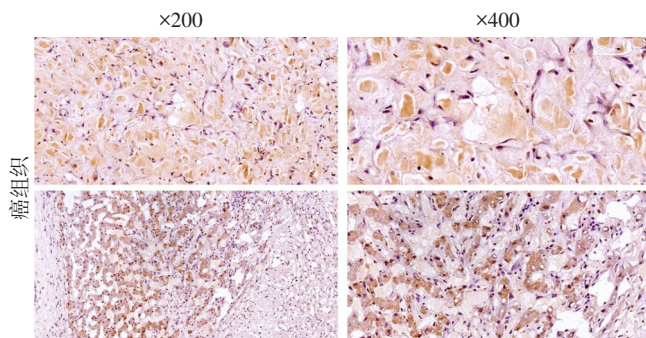
为了揭示 Aurora-B 诱导的 CENPU 磷酸化在胆管癌细胞增殖中的调控作用, 进一步构建了 CENPU 磷酸化位点 (Thr78) 突变体, 并用相应的慢病毒载体转染 QBC939 细胞, 同时以 CENPU 野生型 QBC939 细胞作为阴性对照。通过观察慢病毒载体上 GFP 绿色荧光蛋白的表达情况, 计算并确认对照组 (空载慢病毒)、CENPU-WT (野生型) 和 CENPU-MUT (磷酸化位点突变体) 在 QBC939 细胞中的转染效率 (图 4A)。结果显示, CENPU 的磷酸化缺陷减弱了 Aurora-B 诱导的 CENPU 磷酸化, 其具有与 Aurora-B 抑制剂相似的作用, 但 Aurora-B 抑制剂能明显抑制 Aurora-B 的表达, CENPU 磷酸化位点突变对 Aurora-B 的表达无影响 (图 4B)。CCK8 的结

果显示,转染CENPU-WT的细胞显示出增强的增殖活性,这一作用能够被CENPU磷酸化缺陷所抑制

($P<0.05$) (图4C)。以上结果均表明CENPU磷酸化在胆管癌增殖中起关键作用。



A



B

图1 Aurora-B与CENPU的免疫组化检测 A: Aurora-B与CENPU在胆管癌组织与癌旁组织中的表达 ($\times 400$); B: p-CENPU在胆管癌组织与癌旁组织中的表达

Figure 1 Immunohistochemical staining for Aurora-B and CENPU A: Expressions of Aurora-B and CENPU in cholangiocarcinoma and adjacent tissues ($\times 400$); B: Expressions of p-CENPU in cholangiocarcinoma and adjacent tissues

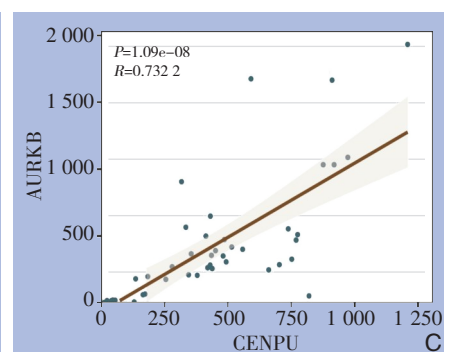
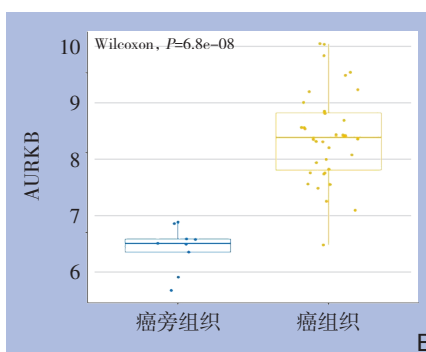
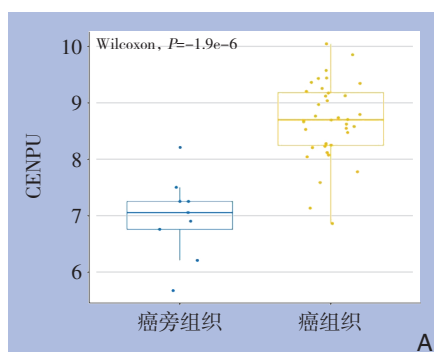


图2 TCGA 数据库中分析 A-B: Aurora-B (AURKB) 与CENPU在胆管癌组织中的表达高于癌旁正常组织; C: 胆管癌组织中CENPU与Aurora-B的表达呈正相关

Figure 2 TCGA database analysis A-B: Higher expressions of Aurora-B (AURKB) and CENPU in cholangiocarcinoma tissue than normal adjacent tissue; C: A positive correlation between CENPU and Aurora-B expressions in cholangiocarcinoma

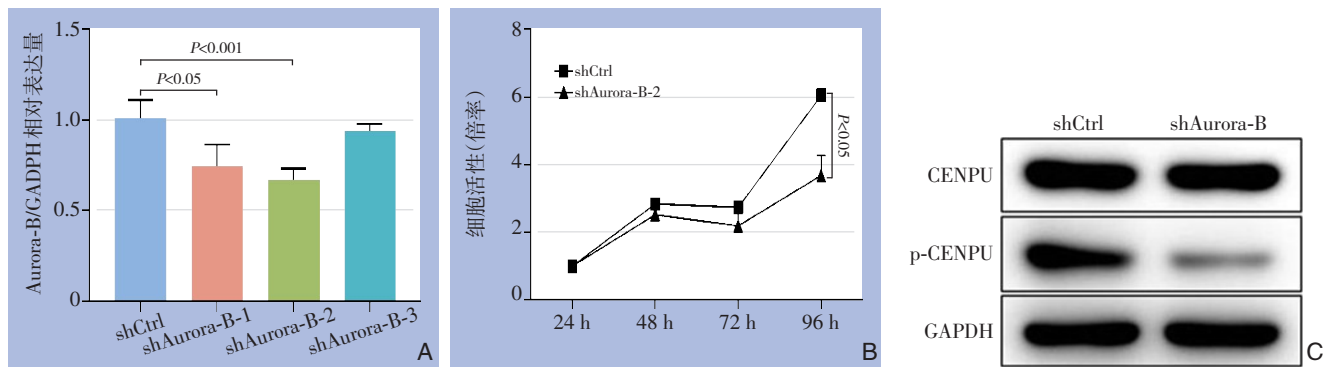


图3 QBC939细胞Aurora-B敲减实验 A: qRT-PCR检测3个shRNA的敲除效率; B: CCK8法检测Aurora-B基因敲减对QBC939细胞增殖的影响; C: Western blot法检测Aurora-B基因敲减对QBC939细胞CENPU和p-CENPU表达的影响

Figure 3 Experiment of Aurora-B knockdown in QBC939 cells A: The knockdown efficiencies of 3 shRNAs evaluated by qRT-PCR; B: Influence of Aurora-B knockdown on proliferation of QBC939 cells detected by CCK8 assay; C: Influence of Aurora-B knockdown on expressions of CENPU and p-CENPU of QBC939 cells determined by Western blot analysis

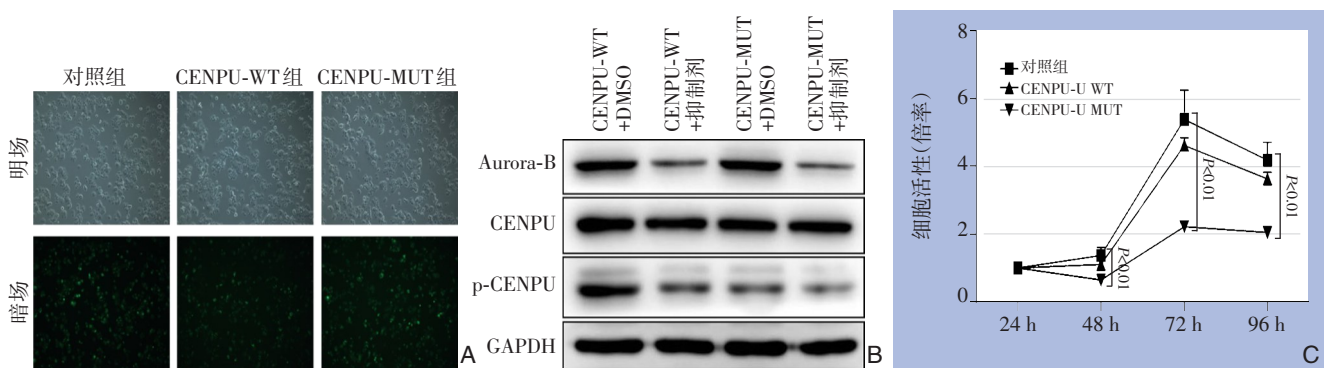


图4 QBC939细胞CENPU磷酸化位点突变与Aurora-B抑制剂作用实验 A: 荧光成像技术检测对照组、CENPU-WT组和CENPU-MUT组慢病毒转染效率($\times 200$); B: DMSO或Aurora-B抑制剂处理后,在有或无CENPU磷酸化位点突变的QBC939细胞中Aurora-B、CENPU和p-CENPU的表达; C: CCK8检测有或无CENPU磷酸化位点突变的QBC939细胞的增殖

Figure 4 Experiments of phosphorylation site mutation of CENPU and Aurora-B inhibitor treatment in QBC939 cells A: Fluorescence imaging assessment of the lentivirus transfection efficiencies in control, CENPU-WT and CENPU-MUT groups ($\times 200$); B: The expressions of Aurora-B, CENPU and p-CENPU in QBC939 cells with or without phosphorylation site mutation of CENPU treated with DMSO or Aurora-B inhibitor; C: CCK8 assay for proliferation abilities of QBC939 cells with or without phosphorylation site mutation of CENPU

3 讨论

Aurora-B是Aurora激酶家族的3个成员之一。其位于细胞核中央,属于功能性激酶,参与有丝分裂的调控。近年来,Aurora激酶家族在肿瘤发生发展中的作用越来越受到人们的关注。许多研究发现,Aurora-B在多种的人类癌症中都有表达升高的现象,这显示出其与恶性肿瘤的发生发展的潜在相关性。例如,Shen等^[11]研究了有丝分裂调节蛋白(包括Aurora激酶、survivin和p53)在胆管癌中的表达和生物学功能。他们的研究成果显示37.8%

的肝内胆管癌患者的癌细胞内存在着Aurora-B的过度表达,同时Aurora-B的表达与肿瘤分级之间存在着统计学正相关的关系。Al-Khafaji等^[15]认为Aurora-B的活性是非小细胞肺癌细胞对紫杉醇的敏感性的一个重要表征指标和调节剂。Ding等的研究^[16]揭示了CREPT/RPRD1B和Aurora-B之间的相互作用,其结果说明Aurora-B能够促进胃癌细胞的G2/M周期转换,进而促进胃癌的进展。Nie等^[7]还发现Aurora-B在胃癌中具有类似于癌基因的功能,其能够通过激活细胞周期相关蛋白cyclin D1的表达发挥肿瘤启动子的作用。另一方面,越来越多

的研究表明, Aurora-B诱导的磷酸化在人类癌症的发展和进展中起着关键作用。一项最新研究表明, Aurora-B可以通过直接诱导MYC的磷酸化稳定其表达,从而与myc形成一个调节回路,促进T细胞白血病的发生^[17]。Xu等^[18]认为Aurora-B诱导的Ser27位点的H1.4磷酸化在胃癌的发生发展中起到了重要的作用,而这一功能可被Ras-ERK1/2信号通路抑制。尽管已经有前期的研究显示了Aurora-B在胆管癌中的异常表达^[13, 19],但其在胆管癌发生发展中的具体作用仍不清楚。

2006年, Mellone等^[20-21]首次发现了CENPU,其又被称为KLIP1、PBIP1或CENPU/50。其后的研究显示, CENPU对正常细胞来说不是必需的,但在纺锤体损伤恢复的情况下,对于黏附和防止姐妹染色单体分离是必需的^[22-24]。此外,近年来研究人员还发现了CENPU在多种人类癌症中的重要作用,包括卵巢癌^[25]、非小细胞肺癌^[26-27]、膀胱癌^[28]、三阴性乳腺癌^[29]、肺腺癌^[22]和肝细胞癌^[30]。

在本研究中发现,与癌旁组织相比, Aurora-B和CENPU在胆管癌组织中的表达水平均有上调的趋势。敲低胆管癌细胞中Aurora-B的表达水平可显著抑制胆管癌细胞CENPU的磷酸化。研究显示CENPU与Aurora-B激酶均可影响着丝粒与微管的连接,其中CENP-U通过与Hec1交互作用调控染色体着丝粒与微管的连接^[12],而Aurora-B激酶负责修正着丝粒与微管的错误结合^[31]; CENPU与Aurora-B激酶的过表达均能使染色体滞后于分裂中期,导致染色体分离错误,引起基因的不稳定性以及非整倍体细胞数目的增加,直至发生癌变^[32]。最近Hua等^[12]通过体外激酶实验证实CENP-U上存在有丝分裂调节蛋白Aurora-B激酶的磷酸化位点Ser³⁴⁹和Ser³⁵⁰。本研究结果证实胆管癌细胞中Aurora-B激酶可以调控CENPU磷酸化,初步的机制研究表明Aurora-B对胆管癌细胞增殖水平的调控作用可能是通过其对CENPU的磷酸化作用来实现的,当CENPU的磷酸化位点发生突变时这一调控作用也受到了抑制。

综上所述,本研究证实了Aurora-B对胆管癌细胞增殖水平的促进作用,并且显示这一作用可能是通过对CENPU的磷酸化修饰来进行的。因此,本项研究针对Aurora-B调控胆管癌进展的作用进行了功能层面的验证以及机制层面的研究,为胆管癌分子机制研究以及靶向药物研究提供了理论

依据以及潜在的分子靶点。

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