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IGFBP3 基因启动子高甲基化所致表达下降促进胃癌化疗耐药*

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摘要 目的:研究胰岛素样生长因子结合蛋白 3(IGFBP3)在胃癌化疗敏感及化疗耐药细胞和组织中的表达情况,初步探讨其在胃癌化疗耐药中的作用及表达异常机制。**方法:**采用荧光实时定量 PCR (qRT-PCR) 及蛋白质免疫印迹实验 (WB) 的方法检测 IGFBP3 在化疗药物敏感的胃癌细胞 AZ521、SC-M1, 对应顺铂耐药细胞 AZ521/cisplatin、SC-M1/cisplatin 和胃癌组织中的表达水平;在降低和增加 IGFBP3 表达量后,采用细胞计数试剂盒-8(CCK-8)试剂及流式细胞术(FCM)检测胃癌细胞对化疗药物的敏感性的变化;甲基化特异性 PCR(MSP)检测 IGFBP3 基因启动子区甲基化水平。**结果:**IGFBP3 在化疗耐受的胃癌细胞及组织中表达低于化疗敏感的胃癌细胞及组织 ($P<0.05$);在敏感细胞中干扰 IGFBP3 表达可促进胃癌细胞的耐药性,而在耐药细胞中恢复 IGFBP3 表达可显著逆转耐药性;MSP 结果显示,IGFBP3 的表达受 DNA 甲基化调控,耐药细胞中 IGFBP3 启动子高甲基化导致其表达下降($P<0.05$);甲基转移酶抑制剂地西他滨(DAC)处理耐药的胃癌细胞,可恢复 IGFBP3 表达,并提高其对化疗药物的敏感性($P<0.05$)。**结论:**IGFBP3 启动子区发生 DNA 高甲基化导致其表达下降,使得化疗药物诱导胃癌细胞发生凋亡的能力下降,最终导致胃癌细胞的化疗耐药。

关键词:IGFBP3; 胃癌; 化疗耐药; 甲基化**中图分类号:**R-33; R735.2 **文献标识码:**A **文章编号:**1673-6273(2019)08-1441-05

Decreased Expression of IGFBP3 Gene Promoter Induced by Hypermethylation Promotes Chemoresistance in Gastric Cancer*

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ABSTRACT Objective: To study the expression of insulin-like growth factor binding protein 3 (IGFBP3) in chemosensitivity and chemoresistance cells and tissues of gastric cancer, and to explore its function and mechanism of abnormal expression in the chemoresistance of gastric cancer. **Methods:** The expression of IGFBP3 in chemotherapeutic drug-sensitive gastric cancer cells AZ521 and SC-M1, expressions of cisplatin-resistant cells AZ521/cisplatin, SC-M1/cisplatin and gastric cancer were detected by real-time quantitative fluorescence polymerase chain reaction (qRT-PCR) and Western blot assay (WB). After decreasing and increasing the expression of IGFBP3, the sensitivity of gastric cancer cells to chemotherapeutic drugs was detected by cell counting kit-8 (CCK-8) reagent and flow cytometry (FCM). The promoter methylation level of IGFBP3 was determined by the methylation specific PCR (MSP) assay. **Results:** The expression of IGFBP3 in chemo-tolerant gastric cancer cells and tissues was lower than that in chemo-sensitive gastric cancer cells and tissues ($P<0.05$). Interference of IGFBP3 expression in sensitive cells can promote drug resistance of gastric cancer cells. While recovery of IGFBP3 expression in drug-resistant cells can significantly reversed drug resistance. MSP results showed that the expression of IGFBP3 was regulated by DNA methylation. Higher methylation of IGFBP3 promoter in drug-resistant cells resulted in decreased expression of IGFBP3 ($P<0.05$). The drug-resistant gastric cancer cells were treatment with methyltransferase inhibitor dicetabine (DAC), the expression of IGFBP3 was restored, its sensitivity to chemotherapeutic drugs were increased ($P<0.05$). **Conclusion:** DNA hypermethylation in the promoter region of IGFBP3 leads to a decrease in its expression, which leads to a decrease in the ability of chemotherapeutic drugs to induce apoptosis of gastric cancer cells, and ultimately leads to chemotherapeutic resistance of gastric cancer cells.

Key words: IGFBP3; Gastric cancer; Chemoresistance; Methylation**Chinese Library Classification(CLC):** R-33; R735.2 **Document code:** A**Article ID:** 1673-6273(2019)08-1441-05

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前言

胃癌是中国最常见的恶性肿瘤之一,其发病率呈逐年上升趋势^[1-3]。化疗是胃癌治疗的重要手段之一,但在化疗过程中癌细胞常对化疗药物产生耐药,导致治疗失败。肿瘤耐药性产生原因复杂多样,针对这类患者目前尚无有效的解决办法,因此深入探讨胃癌耐药机制,寻找新的潜在治疗靶点,将为改进胃癌化疗策略提供新的思路。胰岛素样生长因子结合蛋白3(Insulin-like growth factor binding protein3, IGFBP3)是胰岛素样生长因子的重要调节分子,与细胞分化、增殖及个体生长发育密切相关^[4-6]。研究发现,IGFBP3与肿瘤的发生密切相关,在多种肿瘤中常常表达缺失,发挥着抑癌基因的功能,如结肠癌^[7]、胶质母细胞瘤^[8]、前列腺癌^[9]等。目前对IGFBP3在肿瘤发生中的作用研究较多,但其在耐药中的作用却一直不太清楚。凋亡能力下降是肿瘤细胞的十大特征之一,由于大多数化疗药物最终都是通过激活凋亡通路来杀死肿瘤细胞^[10],因而凋亡能力的下降也是肿瘤细胞对化疗药物耐受的重要原因。研究发现,IGFBP3在凋亡通路发挥着重要调控作用。如IGFBP3的高表达可诱导肝癌细胞发生凋亡^[11]。而IGFBP3在胃癌细胞中能否通过参与细胞凋亡反应,进而导致胃癌化疗耐药的产生,尚有待探讨。本研究通过探讨IGFBP3在胃癌化疗耐药性产生过程中的作用及IGFBP3在耐药的胃癌细胞中表达异常的机制,为逆转已产生耐药的胃癌患者的治疗提供参考。

1 材料与方法

1.1 细胞培养及试剂

由中国上海科学院细胞库提供的人胃癌细胞AZ521、SC-M1及对应顺铂耐药细胞AZ521/cisplatin、SC-M1/cisplatin(由中南大学湘雅医院实验室诱导获得并保存)于37℃,5%CO₂的条件下无菌培养。培养液为含有10%胎牛血清的RPMI1640(美国Invitrogen公司)细胞培养基(含200 U/mL青霉素,100 μg/mL链霉素)。细胞传代时用0.25%胰蛋白酶消化。

1.2 胃癌组织的收集

新鲜胃癌组织标本来自本院手术切除病例,其中初发胃癌组织20例,复发胃癌组织20例,所有标本均置于液氮中保存。

1.3 荧光实时定量PCR(Real-time quantitative PCR, qRT-PCR)用Trizol试剂(美国Invitrogen公司)提取细胞总RNA。取1 μg RNA,用逆转录试剂盒(瑞士Roche公司)进行逆转录。IGFBP3定量引物(上海生工生物工程股份有限公司)序列如下:F:AGAGCACAGATACCCAGAACT;R:GGTGATTCAGTGTGTCTTCCATT。内参引物β-Actin序列如下:F:TCATGAAGTGTGACGTGGACAT;R:CTCAGGAGGAGCAATGATCTTG。目的基因的相对变化用2^{-ΔΔCt}法分析,反应采用两步法,在实时荧光定量PCR仪(Light Cycler480 II,瑞士Roche公司)上进行,反应程序为:95℃,10 min;95℃,30 s;60℃,1 min。重复第二步,循环40次。mRNA相对表达量由2^{-ΔΔCt}方法表示。

1.4 蛋白质免疫印迹实验(Western blotting, WB)

收取细胞,用冰浴的PBS洗涤两次,加入适量裂解液,冰上裂解20 min,4℃,12000 rpm,离心20 min,收集蛋白上清液,于-80℃保存,测定蛋白浓度后,加入SDS上样缓冲液,100℃

煮5 min变性样品,SDS-PAGE蛋白电泳,转膜,脱脂奶粉封闭1h,鼠抗人IGFBP3单克隆抗体(sc-135947,美国Santa公司,1:1000稀释),鼠抗人GAPDH单克隆抗体(sc-293335,美国Santa公司,1:2000稀释),4℃孵育过夜,TBST洗3遍,每遍10 min,HRP标记的羊抗鼠二抗(上海英基生物科技有限公司,1:10000稀释)室温孵育1h,TBST洗3遍,每遍10 min,孵育后ECL发光液(美国Millipore公司)显影,凝胶成像仪(GelDoc-It,美国UVP公司)对蛋白条带进行观察,获取图像,ImageLab软件对图像进行灰度分析,记录灰度值。

1.5 转染IGFBP3表达载体及干扰质粒

PCR扩增IGFBP3基因编码区(NM_000598.4),BamH I和EcoR I酶切后,插入pcDNA3.0真核表达载体,经酶切、PCR和测序鉴定,构建pcDNA3.0-IGFBP3表达载体。重组干扰质粒sh-IGFBP3,由武汉晶赛公司设计合成。取处于对数生长期的胃癌细胞4×10⁵个,培养24h后,采用脂质体分别将pcDNA3.0-IGFBP3及sh-IGFBP3转染至细胞中,操作步骤参照试剂说明书,qRT-PCR及WB实验检测转染效率。

1.6 流式细胞术(Flow Cytometry, FCM)检测细胞凋亡

采用FCM、Annexin V-FITC/PI双染法检测各组胃癌细胞凋亡比例,转染24h后,各组胃癌细胞中分别加入含有不同浓度顺铂的无血清培养液,继续培养48h,PBS洗涤2次,加入500 μL Binding Buffer重悬细胞,5 μL Annexin V-FITC及5 μL PI混匀,孵育10 min,流式细胞仪(FACSCanto II,美国BD公司)检测细胞凋亡比例。

1.7 细胞活力检测实验

处于对数生长期的细胞,接种于96孔板中,每孔接种(0.8-1)×10⁴个细胞,24h后,分别加入不同浓度的顺铂,设置3个复孔,继续培养72h。弃去上清,加入细胞计数试剂盒-8(cell counting kit-8, CCK-8)试剂,继续培养4h,测定450nm波长的吸光度值,SPSS软件计算IC₅₀值。

1.8 甲基化特异性PCR(Methylmion specific PCR, MSP)

用Axygen公司的基因组DNA抽提试剂盒提取基因组DNA,取1 μg DNA根据Millipore公司的亚硫酸氢盐修饰试剂盒说明书进行硫酸氢盐修饰。以经过亚硫酸氢盐修饰的DNA为模板进行MSP扩增,IGFBP3甲基化引物(上海生工生物工程股份有限公司)序列如下:F:GTGGGTTTTTGGGGATATAAATAGT;R:AATCACTCCTAACCAACTCAACAC。内参Actin引物序列:F:TGGTGATGGAGGAGGTTTAGTAAG;R:AACCAATAAAACCTACTCCTCCCTTAA。采用2^{-ΔΔCt}方法表示各基因相对表达量。

1.9 统计学分析

用SPSS23.0进行统计学分析。实验结果均为计量数据,以3次独立实验的($\bar{x} \pm s$)表示,组间差异用双尾t检验。检验水准α=0.05。

2 结果

2.1 耐药的胃癌细胞和组织中IGFBP3的表达

qRT-PCR结果显示,与敏感细胞相比,IGFBP3的转录水平在耐药细胞中降低($P < 0.05$),WB结果显示,IGFBP3的蛋白水平在耐药细胞中也降低($P < 0.05$);复发胃癌组织中IGFBP3

表达低于初发胃癌组织中的表达($P<0.05$)。见表 1。

表 1 IGFBP3 在耐药的胃癌细胞和组织中的表达($\bar{x}\pm s$)
Table 1 Expression of IGFBP3 in drug-resistant gastric cancer cells and tissues($\bar{x}\pm s$)

Project	Groups	n	Relative level	t	P
IGFBP3 mRNA	AZ521	3	0.993± 0.040	9.353	0.001
	AZ521/cisplatin	3	0.567± 0.068		
	SC-M1	3	1.003± 0.057	10.159	0.001
	SC-M1/cisplatin	3	0.427± 0.080		
IGFBP3 protein	AZ521	3	0.996± 0.062	12.086	0.000
	AZ521/cisplatin	3	0.317± 0.075		
	SC-M1	3	1.014± 0.07	12.112	0.000
	SC-M1/cisplatin	3	0.287± 0.08		
IGFBP3 mRNA	Initial cases	20	1.433± 0.743	3.436	0.002
	Relapsed cases	20	0.802± 0.350		

2.2 干扰 IGFBP3 促进胃癌细胞的化疗耐药

在 IGFBP3 高表达的 AZ521、SC-M1 胃癌细胞中转染干扰质粒 sh-IGFBP3, qRT-PCR 检测干扰效率, 结果显示 IGFBP3 被有效干扰($P<0.05$); CCK-8 结果显示, 干扰 IGFBP3 表达可

显著升高顺铂药物处理下胃癌细胞存活率($P<0.05$); FCM 结果显示, 干扰 IGFBP3 细胞在顺铂药物处理下调亡率较未干扰显著下降($P<0.05$)。见表 2。

表 2 干扰 IGFBP3 促进胃癌细胞的化疗耐药($\bar{x}\pm s$)
Table 2 Interferes with IGFBP3 to promote chemotherapeutic resistance in gastric cancer cells($\bar{x}\pm s$)

Project	Groups	n	Relative level	t	P
The relative mRNA level of IGFBP3	AZ521	3	0.998± 0.038	20.704	0.000
	AZ521 sh-IGFBP3	3	0.353± 0.035		
	SC-M1	3	1.012± 0.047	14.585	0.000
	SC-M1 sh-IGFBP3	3	0.400± 0.044		
The IC ₅₀ value (μg/mL)	AZ521	3	5.243± 0.438	9.364	0.001
	AZ521 sh-IGFBP3	3	9.732± 0.612		
	SC-M1	3	7.327± 0.659	11.386	0.000
	SC-M1 sh-IGFBP3	3	12.632± 0.937		
The percentage of apoptotic cells	AZ521	3	58.400± 5.231	12.288	0.000
	AZ521 sh-IGFBP3	3	20.033± 1.484		
	SC-M1	3	62.033± 5.229	14.297	0.000
	SC-M1 sh-IGFBP3	3	17.067± 1.527		

2.3 过表达 IGFBP3 显著逆转胃癌耐药细胞的耐药性

为进一步确认 IGFBP3 在胃癌化疗耐药中的作用, 本研究在 IGFBP3 低表达的胃癌顺铂耐药细胞 AZ521/cisplatin、SC-M1/cisplatin 中通过转染 IGFBP3 表达载体, 恢复其表达, 观察细胞对化疗药物的敏感性。CCK-8 结果显示, 过表达 IGFBP3 显著抑制了顺铂药物处理下 AZ521/cisplatin、SC-M1/cisplatin 细胞存活率($P<0.05$); FCM 结果显示, 过表达 IGFBP3 细胞在顺铂药物处理下调亡率较未转染 IGFBP3 的显著升高($P<0.05$)。见表 3。

2.4 IGFBP3 启动子区高甲基化导致其表达沉默

耐药细胞中 IGFBP3 启动子区甲基化显著高于敏感细胞($P<0.05$); 地西他滨(5-aza-2 deoxycytidine, DAC)是一种甲基转移酶抑制剂, 可降低 DNA 甲基化水平, DAC 处理 AZ521/cisplatin、SC-M1/cisplatin 细胞 72h 后, MSP 结果显示 IGFBP3 启动子区甲基化显著下降, 且 qRT-PCR 结果显示其表达水平得到恢复($P<0.05$)。见表 4。

2.5 DAC 可逆转胃癌耐药细胞的耐药性

本研究用 DAC 预处理 AZ521/cisplatin、SC-M1/cisplatin 细胞 24h 后, 再给予不同浓度的顺铂处理 48h, 结果显示, DAC 降低了耐药细胞的存活率($P<0.05$)。见表 5。

表 3 过表达 IGFBP3 显著逆转胃癌耐药细胞的耐药性($\bar{x} \pm s$)

Table 3 Overexpression of IGFBP3 significantly reversed the drug resistance of drug-resistant gastric cancer cells ($\bar{x} \pm s$)

Project	Groups	n	Relative level	t	P
The IC ₅₀ value (μg/mL)	AZ521/cisplatin	3	26.181± 2.140	3.528	0.024
	AZ521/cisplatin IGFBP3	3	20.462± 1.818		
	SC-M1/cisplatin	3	27.971± 3.716	3.115	0.036
	SC-M1/cisplatin IGFBP3	3	20.796± 1.452		
The percentage of apoptotic cells	AZ521/cisplatin	3	15.133± 1.935	8.792	0.001
	AZ521/cisplatin IGFBP3	3	26.837± 1.253		
	SC-M1/cisplatin	3	14.469± 1.716	6.111	0.003
	SC-M1/cisplatin IGFBP3	3	33.503± 5.116		

表 4 IGFBP3 启动子区高甲基化导致其表达沉默($\bar{x} \pm s$)

Table 4 Hypermethylation of IGFBP3 promoter region results in silencing of its expression($\bar{x} \pm s$)

Project	Groups	n	Relative level	t	P
The methylation level of IGFBP3 promoter	AZ521	3	0.997± 0.041	8.131	0.001
	AZ521/cisplatin	3	1.440± 0.085		
	SC-M1	3	1.014± 0.053	14.838	0.000
	SC-M1/cisplatin	3	1.773± 0.071		
	AZ521/cisplatin	3	0.987± 0.052	10.194	0.000
	AZ521/cisplatin DAC	3	0.550± 0.053		
	SC-M1/cisplatin	3	1.012± 0.046	20.671	0.000
	SC-M1/cisplatin DAC	3	0.350± 0.031		
The mRNA relative expression level of IGFBP3	AZ521/cisplatin	3	0.997± 0.047	12.892	0.000
	AZ521/cisplatin DAC	3	2.167± 0.150		
	SC-M1/cisplatin	3	1.021± 0.043	28.790	0.000
	SC-M1/cisplatin DAC	3	2.907± 0.105		

表 5 DAC 可逆转胃癌耐药细胞的耐药性($\bar{x} \pm s$)

Table 5 DAC reverses drug resistance of gastric cancer drug-resistant cells($\bar{x} \pm s$)

Groups	n	The IC ₅₀ value(μg/mL)	t	P
AZ521/cisplatin	3	26.181± 2.140	3.955	0.017
AZ521/cisplatin DAC	3	19.953± 1.691		
SC-M1/cisplatin	3	27.971± 3.716	5.265	0.006
SC-M1/cisplatin DAC	3	15.697± 1.579		

3 讨论

化疗是目前治疗恶性肿瘤最有效的手段之一,但化疗过程中,肿瘤细胞耐药性的产生往往导致治疗失败,是目前临床肿瘤治疗的主要障碍。肿瘤耐药机制复杂多样^[12-14]。凋亡能力下降是肿瘤细胞对化疗药物耐受的重要机制之一^[15-17]。早期研究证明 IGFBP3 在介导细胞凋亡反应过程中发挥重要调控作用^[18-20],本研究发现,在胃癌细胞中干扰 IGFBP3 可抵抗顺铂诱导的细胞凋亡,而在耐药细胞中过表达 IGFBP3 则可增强顺铂诱导的凋亡反应,证明 IGFBP3 可通过改变凋亡反应来改变胃

癌细胞对化疗药物的耐受性。肿瘤耐药机制复杂多样,是多因素共同参与的结果,除下降的凋亡反应外,如增强的损伤修复能力、高表达的药物转运泵、药物代谢改变等。异常表达的 IGFBP3 是否可通过其他机制介导胃癌化疗耐药的发生,值得进一步探讨。

IGFBP3 是胰岛素样生长因子结合蛋白(IGFBPs)家族的一个成员,该家族包含 6 个与 IGF 有高亲和力的蛋白,IGFBPs 除了在体内调节 IGF 的生物利用度,还具有其他作用^[21-23]。目前,除了 IGFBP3 在肿瘤中的作用研究较透彻,其他成员在肿瘤中的作用还不是很清楚。本研究的结果证实 IGFBP3 可通过细胞凋亡途径发挥在胃癌耐药中的作用,IGFBPs 家族其他成员是

否也有类似作用有待进一步探讨。

肿瘤发生发展过程中常常伴随着 DNA 异常甲基化^[24-26],本研究中首先对 IGFBP3 启动子区序列进行了分析,发现其启动子区存在 CpG 岛,进一步实验发现耐药细胞中 IGFBP3 启动子区的甲基化水平较敏感细胞高,且抑制 IGFBP3 启动子区的甲基化可升高其表达,说明耐药细胞中下降的 IGFBP3 是由 DNA 高甲基化所致。近年来,越来越多的研究发现 DNA 异常甲基化参与了肿瘤耐药,DNA 异常甲基化是一种表观遗传的改变,是可逆的,这为临床上治疗耐药的肿瘤提供了新的可能,即开发、寻找能改变 DNA 甲基化状态的小分子化合物,将它们与传统化疗药物联用,为临床耐药肿瘤的治疗带来希望^[27]。研究发现,胃癌细胞中多个抑癌基因启动子均呈高甲基化,且肿瘤细胞化疗耐药与抑癌基因的高甲基化密切相关^[28-30]。本研究发现胃癌细胞中 IGFBP3 基因的高甲基化跟胃癌化疗耐药密切相关,由于 DAC 可重新激活那些由于 DNA 过度甲基化而失活的基因,因而本研究进一步探讨了在 DAC 处理对胃癌化疗耐药表型的影响,结果发现 DAC 处理可降低耐药胃癌细胞中 IGFBP3 的启动子甲基化并激活其表达,逆转耐药细胞的耐药表型。本研究结果提示,用 DAC 对胃癌细胞行去甲基化治疗有望成为解决化疗耐药的新突破点。

综上所述,IGFBP3 在耐药的胃癌细胞及组织中表达下降,IGFBP3 的低表达量会降低胃癌细胞对化疗药物的凋亡反应和对药物的敏感性,证明了其在胃癌化疗耐药性产生过程中的促进作用。此外,IGFBP3 在耐药的胃癌细胞中异常低表达是由于其启动子区发生了高甲基化所致,甲基转移酶抑制剂的应用可降低其甲基化并恢复其表达水平,这为逆转临床已产生耐药的胃癌患者的治疗提供新的治疗策略和理论依据。

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