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◇临床医学◇

抑制血清应答因子表达影响转化生长因子β1介导的食管癌上皮细胞-间质转化的作用研究

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摘要:目的 研究血清应答因子(serum response factor,SRF)小干扰RNA(small interference RNA,siRNA)影响转化生长因子β1(transforming growth factor-β1,TGF-β1)介导的Eca-109食管癌细胞发生上皮间质转化(epithelial-mesenchymal transition,EMT)的作用机制。**方法** 体外培养Eca-109食管癌细胞,实验分组为阴性对照siRNA组、TGF-β1+阴性对照siRNA组、TGF-β1+SRF-siRNA组。划痕实验检测细胞迁移能力;免疫细胞化学染色法检测E-钙黏蛋白(E-cadherin)的表达;蛋白质印迹法检测E-钙黏蛋白、SRF、N-钙黏蛋白(N-cadherin)、α-平滑肌肌动蛋白(α-smooth muscle actin,α-SMA)蛋白的表达。**结果** 与阴性对照siRNA组细胞迁移百分比(10.00±2.00)%相比较,TGF-β1+阴性对照siRNA组细胞迁移百分比为(50.67±4.73)%,迁移能力增强;与TGF-β1+阴性对照siRNA组相比较,TGF-β1+SRF-siRNA组细胞迁移百分比为(29.00±3.00)%,迁移能力下降,均差异有统计学意义($P<0.001$)。与阴性对照siRNA组的E-钙黏蛋白(1.07±0.12)、N-钙黏蛋白(0.28±0.25)、SRF(0.25±0.06)、α-SMA(1.19±0.37)蛋白相比较,TGF-β1+阴性对照siRNA组E-钙黏蛋白(0.45±0.06)表达下调,而N-钙黏蛋白(3.27±0.67)、SRF(2.48±0.05)、α-SMA(4.23±0.53)蛋白表达上调(均$P<0.001$);与TGF-β1+阴性对照siRNA组相比较,TGF-β1+SRF-siRNA组E-钙黏蛋白(0.82±0.05)表达上调,N-钙黏蛋白(1.31±0.13)、SRF(1.46±0.16)、α-SMA(2.60±0.28)蛋白表达下调(均$P<0.001$)。**结论** 基因沉默SRF能够抑制TGF-β1介导的食管癌细胞发生EMT。

关键词:食管肿瘤/病因学; 血清反应因子; 钙黏着糖蛋白类; 肌动蛋白类; 上皮-间质转化; 小干扰RNA

The inhibitory effects of SRF-siRNA on EMT in Eca-109 cells

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Abstract: Objective To study the inhibitory effects of serum response factor (SRF)-small interference RNA (siRNA) on epithelial-mesenchymal transition (EMT) in Eca-109 cells induced by transforming growth factor-β1 (TGF-β1). **Methods** Eca-109 cells

were cultured and divided into 3 group,including negative control-siRNA (NC-siRNA),TGF-β1+NC-siRNA,and TGF-β1+SRF-siRNA.Cell migration was measured by cell scratch test.The expression of E-cadherin was observed by immunocytochemistry.The expression of E-cadherin,SRF,N-cadherin,and α-smooth muscle actin (α-SMA) were measured by western blotting.**Results** Compared with siRNA-NC group (10.00±2.00)%,cell migration was increased in TGF-β1+siRNA-NC group (50.67±4.73)%,and the migration ability was enhanced;compared with TGF-β1+ siRNA-NC group,cell migration was (29.00±3.00)% in TGF-β1+SRF-siRNA group,and the migration ability was decreased.Compared with the expressions of E-cadherin (1.07±0.12),N-cadherin (0.28±0.25),SRF (0.25±0.06),and α-SMA (1.19±0.37) in the negative control siRNA group,the expression of E-cadherin (0.45±0.06) in the TGF-β1+ negative control siRNA group was down-regulated,while the expression of N-cadherin (3.27±0.67),SRF (2.48±0.05),and α-SMA (4.23±0.53) were up-regulated ($P<0.001$).Compared with TGF-β1+ NC-siRNA group,the expression of E-cadherin (0.82±0.05) in the TGF-β1+ SRF-siRNA group was up-regulated,and the expression of N-cadherin (1.31±0.13),SRF (1.46±0.16),and α-SMA (2.60±0.28) were down-regulated ($P<0.001$).**Conclusion** Knock-down of SRF can inhibit the EMT induced by TGF-β1 in Eca-109 cells.

Key words: Esophageal neoplasms/etiology; Serum response factor; Cadherins; Actins; Epithelial interstitial transformation; Small interference RNA

上皮间质转化(epithelial interstitial transformation,EMT)在肿瘤的发生、发展中起到了重要的调节作用,与肿瘤的增殖、迁移和侵袭,复发和预后不良关系密切,也是肿瘤放化疗耐药的重要因素[1]。体内外研究均发现,EMT的发生多提示食管癌预后不良,促进肿瘤进展,使食管癌细胞迁移和侵袭能力增加[2-3]。研究表明,血清应答因子(serum response factor,SRF)在肿瘤中的高表达与EMT关系密切,其作为转录因子能够下调E-钙黏蛋白(E-cadherin)的表达[4],同时亦能够调节下游靶蛋白α-平滑肌肌动蛋白(α-smooth muscle actin,α-SMA)的表达并促进EMT的形成[5]。本课题组前期研究也发现,SRF在食管癌组织中的异常高表达,采用基因沉默方法降低SRF的表达,则能够有效的在体外抑制食管癌细胞的增殖[6]。因此本研究于2019年1—12月拟采用转化生长因子β1(transforming growth factorβ1,TGF-β1)在体外诱导Eca-109食管癌细胞发生EMT,并观察基因沉默SRF对其的干预效应,为开发食管癌新的基因治疗提供初步的实验依据。

1 材料与方法

1.1 主要试剂 Eca-109食管癌细胞购置于中科院上海细胞库;DMEM培养基、胎牛血清购置于美国GIBIO公司;TGF-β1购置于美国peprotech公司;SRF、E-钙黏蛋白、N-钙黏蛋白(N-cadherin)、GAPDH购置于美国santa cruz公司;α-SMA购置于英国abcam公司;小干扰RNA(small interference RNA,siRNA)产品购置于广州锐博生物科技有限公司。

1.2 方法

1.2.1 *细胞培养及转染* Eca-109细胞用含体积分数为10%胎牛血清的1640培养基,置于37 ℃,5%二氧化碳孵育箱中培养。待细胞次融合状态后,实验分组为:(1)阴性对照siRNA组:阴性对照siRNA转染6 h,更换无血清培养基24 h;(2)TGF-β1+阴性对照siRNA组:阴性对照siRNA转染6 h,更换含5 ng/mL TGF-β1诱导24 h;(3)TGF-β1+SRF-siRNA组:SRF-siRNA转染6 h后,更换含5 ng/mL TGF-β1诱导+SRF-siRNA。按照转染手册步骤进行,每25平方厘米细胞培养瓶加入50 nmol/mL脂质体转染试剂和10 μL siRNA阴性对照或SRF-siRNA。siRNA阴性对照序列:正向序列:5′-UUCUCCGAACGUGUCACGUTT-3′,反向序列:5′-ACGUGACACGUUCGGA GAATT-3′;SRF-siRNA序列:正向序列:5′-GCAAGGCACUGAUUCAGACTT-3′,反向序列:5′-GUCUGAAUCAGUGCCU UGCTT-3[6]。

1.2.2 *细胞划痕实验* 将Eca-109细胞按照6000个/孔种植于24孔板,待细胞贴壁密度为80%时用200 μL枪头划痕,按照实验分组诱导48 h,分别于0 h和48 h在相同位置拍照,采用IPP 6.0图像分析软件测量划痕区域面积,并计算迁移百分比,即(0 h面积−48 h面积)/0 h面积×100%。

1.2.3 *免疫细胞化学染色* 常规制备细胞玻片,高压修复及封闭内源性过氧化物酶后,一抗E-钙黏蛋白(1∶200)4 ℃过夜,二抗37 ℃孵育20 min,DAB显色,镜下控制,苏木素轻度复染,中性树胶封片。

1.2.4 *蛋白质印迹法* 采用RIPA裂解液提取蛋白,BCA法测定蛋白浓度。按照20微克/泳道上样,13%聚丙烯酰胺凝胶电泳,转膜;E-钙黏蛋白、SRF、N-钙黏蛋白、α-SMA、GAPDH一抗(1∶1 000)4 ℃过夜,二抗(1∶3 000)37 ℃孵育45 min,ECL发光。采用IPP6.0图像分析软件测定条带光密度值,以相应内参(GAPDH)的比值作为该蛋白的相对表达量。

1.3 统计学方法 采用SPSS 13.0统计学软件,数

据以$\bar{x}\pm s$表示。多组间采用完全随机设计的单因素方差分析，多重比较方差齐采用LSD-t检验的方法，$P<0.05$为差异有统计学意义。

2　结果

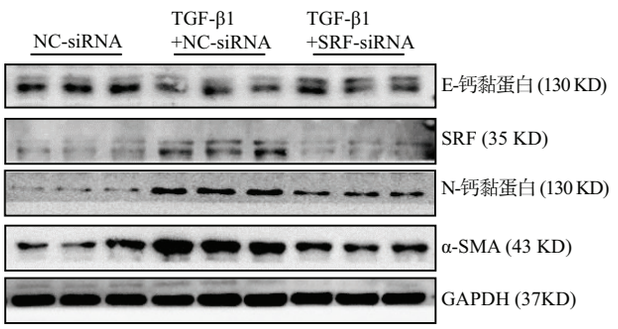
2.1　SRF基因沉默抑制TGF-β1介导的Eca-109食管癌细胞的迁移能力　如图1所示，方差分析结果显示（$F=105.462$，$P<0.001$），方差齐（$F=1.519$，$P=0.293$）采用LSD-t检验，与阴性对照siRNA组（10.00±2.00）%相比较，TGF-β1+阴性对照siRNA组的迁移百分比为（50.67±4.73）%，差异有统计学意义（$P<0.001$）；与TGF-β1+阴性对照siRNA相比较，TGF-β1+SRF-siRNA组细胞迁移百分比为（29.00±3.00）%，差异有统计学意义（$P<0.001$）。

2.2　SRF基因沉默抑制TGF-β1介导的Eca-109食管癌细胞的EMT进程　如图2所示，免疫细胞化学染色结果显示，E-钙黏蛋白阳性表达于细胞膜，阴性对照siRNA组可见明显的E-钙黏蛋白膜阳性表达，TGF-β1+阴性对照siRNA组E-钙黏蛋白膜阳性表达减少，与TGF-β1+阴性对照siRNA组相比较，TGF-β1+SRF-siRNA组E-钙黏蛋白膜阳性表达增多。

如图3、表1所示，蛋白质印迹法结果显示，与阴性对照siRNA组相比较，TGF-β1+阴性对照siRNA组E-钙黏蛋白表达下调（$P<0.001$），而N-钙黏蛋白、SRF、α-SMA蛋白表达上调（均$P<0.001$）；与TGF-β1+阴性对照siRNA组相比较，TGF-β1+SRF-siRNA组E-钙黏蛋白表达上调（$P=0.001$），而SRF、N-钙黏蛋白、α-SMA蛋白表达下调（$P<0.001$；$P=0.001$；$P=0.003$），经方差分析（LSD-t检验），两两比较均差异有统计学意义（$P<0.05$）。

3　讨论

目前研究发现，SRF在多种肿瘤中，包括甲状腺癌[7]、肝癌[8]、胃癌[9]等多种肿瘤中异常高表达，并与肿瘤细胞的增殖、迁移和侵袭能力有关[10-12]。SRF能够与miR-199a-5p启动子结合并促进其转录，而miR-199a-5p则能够与E-钙黏蛋白mRNA结合从而抑制其表达[4]。采用TGF-β1诱导Eca-109食管癌细胞，能够显著促进其迁移和侵袭能力，下调E-钙黏蛋白的表达，上调间质表型包括N-钙黏蛋白、波形蛋白（Vimentin）、Slug等蛋白的表达，同时通过上调细胞周期蛋白D1的表达从而促进了Eca-109细胞的增殖[13]。



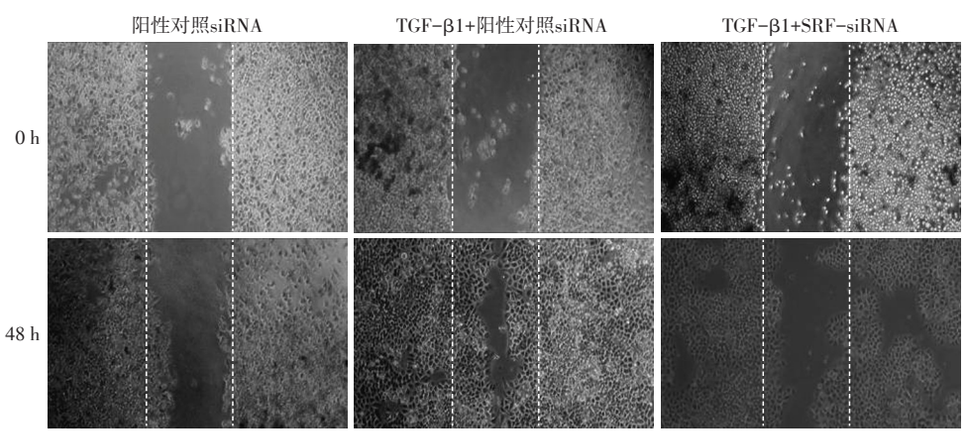
注：NC为阴性对照，siRNA为小干扰RNA，TGF-β1为转化生长因子β1

图3　血清应答因子（SRF）基因沉默对E-钙黏蛋白、SRF、N-钙黏蛋白、α-平滑肌肌动蛋白（α-SMA）蛋白表达的影响（蛋白质印迹法）

表1　血清应答因子（SRF）基因沉默对E-钙黏蛋白、SRF、N-钙黏蛋白、α-平滑肌肌动蛋白（α-SMA）蛋白表达的影响/$\bar{x}\pm s$

组别	重复次数	E-钙黏蛋白	SRF	N-钙黏蛋白	α-SMA
阴性对照siRNA	3	1.07±0.12	0.25±0.06	0.28±0.25	1.19±0.37
TGF-β1+阴性对照siRNA	3	0.45±0.06[a]	2.48±0.05[a]	3.27±0.67[a]	4.23±0.53[a]
TGF-β1+SRF-siRNA	3	0.82±0.05[b]	1.46±0.16[b]	1.31±0.13[b]	2.60±0.28[b]
F值		44.814	353.936	37.350	40.648
P值		<0.001	<0.001	<0.001	<0.001

注：与阴性对照小干扰RNA（siRNA）组相比较，[a]$P<0.05$；与转化生长因子β1（TGF-β1）+阴性对照siRNA组相比较，[b]$P<0.05$



注：siRNA为小干扰RNA

图1　血清应答因子（SRF）基因沉默对转化生长因子β1（TGF-β1）介导的Eca-109食管癌细胞迁移的影响（细胞划痕实验）

本课题组前期研究也发现,基因沉默SRF能够下调β-连环蛋白、细胞周期蛋白D1的表达,同时上调E-钙黏蛋白的表达,从而抑制了Eca-109食管癌细胞的增殖和侵袭能力[6]。在本研究中也发现,TGF-β1能够显著上调SRF的表达,同时促进Eca-109食管癌细胞的迁移能力的提高,同时减少细胞间紧密连接蛋白(E-钙黏蛋白)的表达,并促进间质表型(N-钙黏蛋白、α-SMA)的改变,诱导了食管癌细胞发生EMT。基因沉默SRF则能够显著抑制TGF-β1诱导的Eca-109食管癌细胞发生EMT,并降低了其迁移能力。

SRF及其转录辅因子心肌相关转录因子(Myocardin-related transcription factors,MRTF)是调节肌动蛋白的重要转录因子,在细胞黏附、细胞收缩、细胞骨架蛋白、细胞外基质等方面发挥了重要的调控作用,能够与α-SMA基因启动子结合并促进其转录[14-17]。在肿瘤研究中,α-SMA常作为肿瘤相关成纤维细胞或EMT标记物,与肿瘤进展和转移有关[18]。本研究结果也显示,在TGF-β1诱导的Eca-109食管癌细胞EMT过程中,伴随了α-SMA表达的上调,而予以基因沉默SRF,能够显著抑制Eca-109食管癌细胞α-SMA的表达。其他研究也显示,在体外沉默SRF,能够显著抑制肿瘤细胞的增殖和侵袭,并能够抑制EMT进程[19]。综上所述,SRF在食管癌发生、发展中起到了较为重要的调节作用,可能是基因靶向治疗的一个潜在靶点。

(本文图2见插图9-4)

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