·基础研究·

Gab2-Akt-ARK5通路在胶质瘤侵袭中的研究*

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摘要 目的:探讨Gab2-Akt-ARK5通路在胶质瘤侵袭中的意义。**方法:**采用免疫组织化学SP法检测90例胶质瘤组织中ARK5及Gab2表达。采用小RNA干扰转染LN-229细胞株,Western Blot检测瞬时转染后ARK5及Gab2表达。体外侵袭实验检测转染后侵袭能力变化及Western Blot检测Gab2下降后Akt和ARK5的磷酸化。**结果:**胶质瘤组织中ARK5和Gab2免疫组织化学阳性结果呈正相关且在高级别胶质瘤(WHO分级为Ⅲ、Ⅳ级)中表达明显高于低级别胶质瘤(WHO分级为Ⅰ、Ⅱ级)。转染ARK5、Gab2、ARK5-Gab2及SCR质粒的LN-229细胞分别称siARK5/LN-229、siGab2/LN-229、siARK5-siGab2/LN-229和SCR/LN-229。其中siARK5干扰效率为70%,siGab2的干扰效率为75%。转染后,与SCR/LN-229相比,siARK5/LN-229中ARK5表达降低,siGab2/LN-229中Gab2表达降低,siARK5-siGab2/LN-229中ARK5和Gab2表达均降低。siARK5/LN-229和siGab2/LN-229侵袭并穿透Matrigel膜基质的细胞数均比对照组少($P<0.01$),且siARK5-siGab2/LN-229细胞数减少更显著($P<0.01$)。在IGF-1刺激下,siGab2/LN-229中Akt和ARK5的磷酸化减弱。**结论:**应用小RNA干扰技术降低ARK5或Gab2表达使LN-229细胞侵袭转移能力降低,同时Gab2表达降低抑制ARK5和Akt磷酸化,提示Gab2-Akt-ARK5通路参与胶质瘤细胞的侵袭。

关键词 胶质瘤 ARK5 Gab2 LN-229细胞 小RNA干扰 侵袭

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Gab2-Akt-ARK5 signaling pathway is associated with the invasion of glioma cells

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Abstract Objective: This study aimed to investigate the effect and significance of a binding protein-2 (Gab2)-Akt-ARK5 signaling pathway on the invasion of glioma cells. **Methods:** Immunohistochemical methods were used to detect the expressions of Gab2 and ARK5 in 45 cases of glioma tissue. siRNA plasmid was used to transfect LN-229 cells, and western blot was performed to analyze the protein expressions of Gab2 and ARK5. In vitro Matrigel invasion assay was conducted to detect variations in the invasiveness of transfected cells. Western blot was also conducted to analyze the protein phosphorylation of Akt and ARK5 in the cells transfected with Gab2 plasmid. **Results:** Immunohistochemical assay revealed that the expressions of ARK5 and Gab2 in glioma cells were positively correlated, and both expressions were higher in high-grade glioma (WHO grade Ⅲ, Ⅳ) than in low-grade glioma (WHO grade Ⅰ, Ⅱ). LN-229 cells transfected with ARK5 plasmid, Gab2 plasmid, ARK5 and Gab2 plasmid, and control plasmid were named siARK5/LN-229, siGab2/LN-229, siARK5 and siGab2/LN-229, and SCR/LN-229, respectively. After transfection was performed, the protein expressions of ARK5 and Gab2 were respectively decreased in siARK5/LN-229 and siGab2/LN-229. The protein expressions of ARK5 and Gab2 in siARK5 and siGab2/LN-229 were also respectively decreased. After ARK5 or Gab2 was downregulated, the number of glioma cells, which invaded and penetrated Matrigel, was decreased ($P<0.01$). The number of glioma cells also decreased significantly after ARK5 and Gab2 were downregulated. The phosphorylation of Akt and ARK5 in siGab2/LN-229 cells was decreased after these cells were stimulated by insulin-like growth factor-1. **Conclusion:** The silencing of ARK5 or Gab2 impaired glioma cell invasiveness. The decreased protein expression of Gab2 inhibited the phosphorylation of Akt and ARK5. These results suggested that the Gab2-Akt-ARK5 signaling pathway could be relevantly involved in glioma cell invasion.

Keywords: glioma, ARK5, Gab2, LN-229 cell, siRNA, invasiveness

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胶质瘤是中枢神经系统常见的一类肿瘤，具有高侵袭性、难治愈、易复发等特点，深入研究胶质瘤的侵袭过程涉及的分子机制对有效治疗此病有重要作用。ARK5作为AMPK亚家族成员之一，是Akt下游信号分子[1]。ARK5被认为在肿瘤侵袭中起关键作用[2]。Grb2协同结合蛋白2（binding protein-2，Gab2），Gabs家族成员之一，可参与细胞内多种信号转导通路，在细胞增殖、分化、凋亡及迁移等生理过程发挥重要作用[3-5]。研究表明Gab2在人类白血病和卵巢癌中高表达[6-7]。本课题组前期实验已发现Gab2和ARK5均与胶质瘤的侵袭密切相关[8-9]。因此本实验用小RNA干扰技术降低ARK5和Gab2表达，通过体外实验检测转染后LN-229细胞侵袭能力变化及通过Western Blot检测Akt及ARK5的磷酸化，探讨Gab2-Akt-ARK5通路参与胶质瘤细胞侵袭的作用机制。

1 材料与方法

1.1 材料

1.1.1 病例资料 收集潍坊医学院病理学教研室2008年2月至2012年3月期间临床及病理资料齐全，且病理已证实为胶质瘤的90例蜡块标本为研究对象，其中低级别胶质瘤共计42例，高级别胶质瘤共计48例。所有患者术前均未经放疗、化疗。

1.1.2 主要试剂 免疫组织化学一抗ARK5和Gab2购于美国Santa Cruz公司；通用性二抗二步法检测试剂盒、PBS和枸橼酸钠均购自北京中杉金桥公司；1640培养液购自美国Hyclone公司；IGF-1购于美国R&D systems公司。胰蛋白酶、彩色预染蛋白、DOO18质粒中量抽提试剂盒均购自碧云天公司；胎牛血清为杭州四季青公司；侵袭实验所用24孔细胞培养板、Matrigel膜基质均购自美国Corning公司；24孔趋化小室、细胞转染试剂购自康为世纪公司。

1.2 方法

1.2.1 免疫组织化学 胶质瘤蜡块标本连续切片3张，每张5 μm厚。以PBS代替一抗做阴性对照，其余2张切片，滴加的一抗分别为ARK5和Gab2工作液，采用免疫组织化学SP法染色。高倍镜（×400）下每张切片至少5个随机视野中计数500个肿瘤细胞，阳性细胞占同类计数细胞的百分比为阳性细胞率。阳性结果的判定根据阳性细胞的百分率及显色深浅分级[14]。

1.2.2 细胞培养 胶质瘤细胞株LN-229购自美国ATCC细胞库。LN-229细胞常规培养于含10%胎牛血清中1 640液中。当细胞密度70%～85%时，分别转染插入ARK5目标片段5′-GAAGTTATGCTTTATTCAC-3′、Gab2目标片段5′-GTGAGAACGATGAGAAATA-3′的小RNA干扰质粒和SCR序列的小RNA干扰质粒，转染步骤参照转染试剂说明书。转染细胞株分别为实验组siARK5/LN-229、siGab2/LN-229，siARK5-siGab2/LN-229和对照组SCR/LN-229。

1.2.3 Western Blot实验 将转染后的实验组和对照组细胞培养72 h后提取蛋白，制备SDS-PAGE凝胶，蛋白质变性后电泳，转膜、封闭，分别滴加一抗为ARK5、Gab2进行孵育，二抗孵育后显影。当转染成功后进行侵袭实验。将所得结果分别进行分析。

1.2.4 体外癌细胞侵袭能力检测 按文献[6]操作，结果置400倍光镜下观察，5个高倍镜视野，计数Boyden小室下室面的细胞数即为穿透人工基膜的细胞数，每个实验重复3次，取平均数作为实验结果。

1.3 统计学分析

运用SPSS 16.0进行统计学处理。对所有定量资料数据结果用$\bar{x}\pm s$表示，进行χ^2检验、Spearman相关分析和两独立样本t检验。$P<0.05$为差异有统计学意义。

2 结果

2.1 胶质瘤组织中ARK5、Gab2蛋白的表达

在显微镜下观察，结果显示：ARK5和Gab2在高级别胶质瘤中阳性表达高于低级别胶质瘤，差异具有统计学意义（$P<0.05$，图1，表1）。90例胶质瘤患者中ARK5和Gab2表达呈正相关（$r=0.418$，$P<0.05$）。

2.2 siARK5 /LN-229、siGab2/LN-229、siARK5、siGab2/LN-229和SCR/LN-229细胞中ARK5及Gab2的表达

应用质粒转染细胞，转染成功后siARK5/LN-229、siARK5-siGab2/LN-229细胞中ARK5表达减少。siGab2/LN-229、siARK5-siGab2/LN-229细胞中Gab2表达降低，灰度与SCR/LN-229相比：siARK5/LN-229为0.3，siGab2/ LN-229为0.25（图2）。

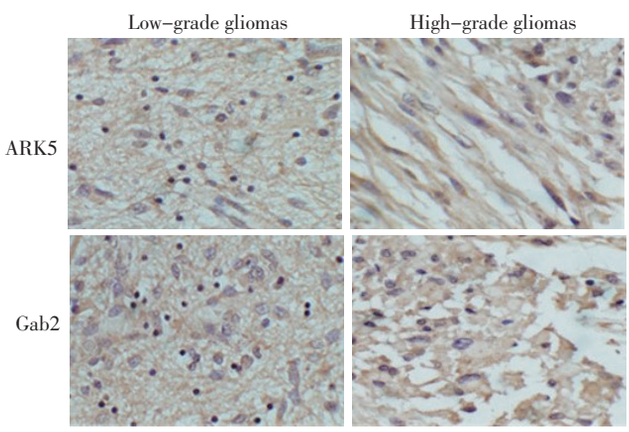

图1 ARK5和Gab2在低级别胶质瘤及高级别胶质瘤中的表达（SP×400）

Figure 1 Expression of ARK5 and Gab2 proteins in low-grade and high-grade gliomas (SP×400)

表1 ARK5和Gab2的表达与临床病理分型的关系

Table 1 Relationship between ARK5 and Gab2 expressions and clinicopathological types

WHO grade	ARK5 expression		*P*	Gab2 expression		*P*
	Low or none	High		Low or one	High	
Low-grade Ⅰ and Ⅱ	22	20	0.002	24	18	0.002
High-grade Ⅲ and Ⅳ	10	38	<0.05	8	40	<0.05

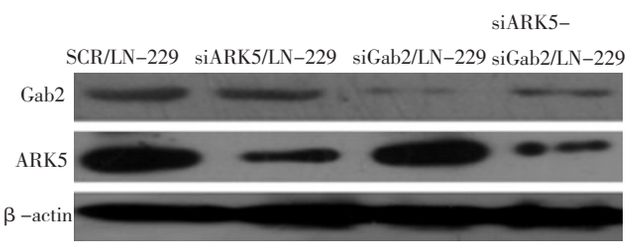

图2 Western Blot法检测实验组和对照组细胞中ARK5及Gab2蛋白的表达

Figure 2 Western blot results of ARK5 and Gab2 protein expression in experimental group cells and SCR/LN-229 cells (control group)

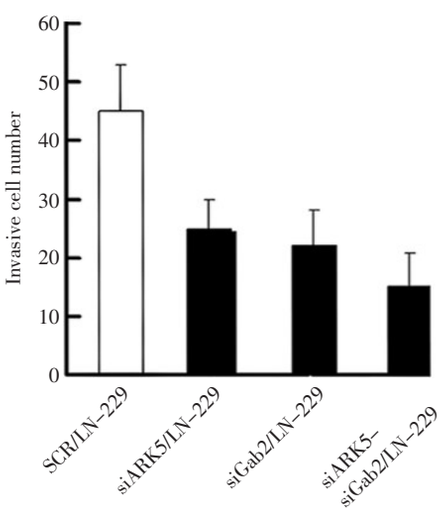

图3 ARK5和Gab2降低表达对LN-229细胞侵袭性的影响

Figure 3 Effect of decreased ARK5 and Gab2 expressions on the invasiveness in LN-229 cells

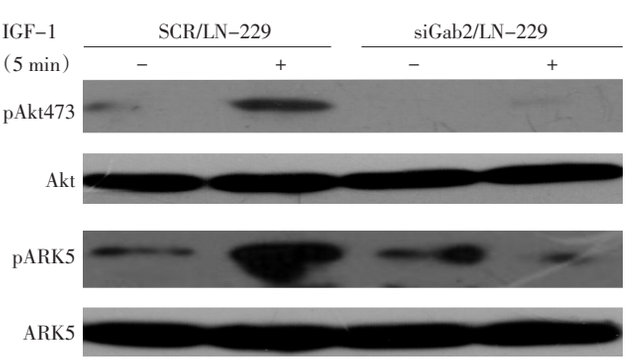

图4 Western Blot检测siGab2/LN-229细胞和SCR/LN-229细胞中Akt和ARK5的磷酸化

Figure 4 Western Blot results of phosphorylation of Akt and ARK5 in siGab2/LN-229 cells and SCR/LN-229 cells

2.3 体外侵袭实验

转染成功后，进行侵袭实验。转染对LN-229细胞的影响：与SCR/LN-229相比，siARK5/LN-229、siGab2/LN-229、siARK5-siGab2/LN-229穿过8μm微孔滤膜细胞数减少，且siARK5-siGab2/LN-229细胞数目减少更甚，差异有统计学意义（$P<0.01$，图3）。

2.4 降低Gab2的LN-229细胞Akt和ARK5磷酸化的变化

为了研究ARK5、Gab2表达降低引起胶质瘤侵袭能力下调的原因及ARK5和Gab2间的相互关系，再次用Western Blot检测，结果显示：在IGF-1刺激下，与SCR/ LN-229相比，siGab2/LN-229中Akt和ARK5的磷酸化减弱（图4）。

3 讨论

胶质瘤是中枢神经系统常见的一类肿瘤。其平均生存时间12～15个月[10]，被称为人类最具危害性的肿瘤。而肿瘤恶性程度与肿瘤细胞的侵袭有关，因此研究胶质瘤细胞的侵袭对提高治疗功效具有重要的作用[11]。已有研究表明：ARK5在胶质瘤及人类乳腺癌的侵袭中起到不同程度的作用[12-13]。Gab2具有潜在的原癌基因特性，在人类卵巢癌和乳腺癌中高表达[14-15]但鲜见ARK5和Gab2与胶质瘤侵袭相关研究。因此明确ARK5和Gab2在胶质瘤侵袭中的作用及ARK5与Gab2在胶质瘤侵袭相关的信号转导通路之间的相互关系，对抑制胶质瘤的侵袭具有重要作用。

本文通过免疫组织化学表明：ARK5与Gab2在高级胶质瘤中的表达明显高于在低级胶质瘤中的表达。这一结果与本课题组前期研究结果相一致[8,12]。同时实验还证实，ARK5与Gab2在胶质瘤中的表达呈正相关。此外，通过小RNA干扰技术将相关质粒转染进胶质瘤LN-229细胞株，培养72 h后，Western Blot检测显示ARK5和Gab2蛋白量降低。随后的体外癌细胞侵袭实验也证实ARK5和Gab2参与了癌细胞侵袭过程且ARK5和Gab2同时存在时癌细胞侵袭更明显。为了进一步研究ARK5和Gab2表达降低后引起胶质瘤侵袭下调的原因并明确ARK5与Gab2在胶质瘤侵袭相关的信号转导通路之间的相互关系，再次做Western Blot检测。结果发现：在IGF-1刺激下，与

SCR/ LN-229相比，siGab2/LN-229中Akt和ARK5的磷酸化减弱。说明ARK5和Gab2表达降低后引起胶质瘤侵袭下调的原因是由于降低了ARK5和Akt的磷酸化，同时也验证ARK5和Akt在信号转导通路中位于Gab2下游，结合相关研究证明的ARK5作为Akt的下游信号分子参与细胞的侵袭[12]，最终得出Gab2和ARK5通过Gab2-Akt-ARK5通路参与胶质瘤侵袭。

综上所述，本研究证实Gab2-Akt-ARK5通路是抑制胶质瘤侵袭的重要信号通路，纠正胶质瘤细胞中Gab2和ARK5蛋白高表达，可有效阻断其侵袭过程，为胶质瘤的治疗有望提供新的靶点。

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“国家基金研究进展综述”栏目介绍

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