建立稳定敲减TNIK表达的A549细胞系及对其初步分析

孙雪花[1,2] 尹讯[1,2] 王茹[1,2] 张涛[1,2] 陈全[3] 张春冬[1,2]*

([1]重庆医科大学生物化学与分子生物学教研室, 重庆 400016; [2]重庆医科大学分子医学与肿瘤研究中心, 重庆 400016; [3]重庆医科大学免疫学教研室, 重庆 400016)

摘要 TNIK(TRAF2 and NCK interacting kinase)属丝氨酸/苏氨酸激酶家族成员, 在多种生理及病理过程中起关键作用。研究发现, TNIK在肺鳞癌组织中高表达且可驱动癌细胞增殖等恶性表型, 但其在肺腺癌中的作用仍未知。该研究在构建稳定敲减TNIK表达的肺腺癌A549细胞后, 流式细胞术检测发现稳定敲减TNIK阻滞细胞周期进程并诱发凋亡, 细胞增殖及运动实验证明肺癌细胞增殖与迁移能力被显著抑制。进一步通过免疫荧光染色分析发现, 稳定敲减TNIK表达后会诱导细胞微丝骨架排列紊乱并抑制黏着斑动态周转(组装/解聚)。综上研究结果表明, 肺腺癌细胞中TNIK可能通过调节微丝骨架排列, 从而影响黏着斑动态周转最终控制细胞增殖及迁移运动; 推测肺腺癌细胞中高表达的TNIK可能通过调控细胞微丝骨架系统以维持癌细胞恶性表型。

关键词 肺腺癌细胞; 细胞增殖; 细胞运动; 微丝骨架; TNIK

Establishment and Preliminary Analysis of Cell Line A549 with Stable Knockdown of TNIK Expression

SUN Xuehua[1,2], YIN Xun[1,2], WANG Ru[1,2], ZHANG Tao[1,2], CHEN Quan[3], ZHANG Chundong[1,2]*

([1]*Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing 400016, China*; [2]*Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing 400016, China*; [3]*Department of Immunology, Chongqing Medical University, Chongqing 400016, China*)

Abstract TNIK (TRAF2 and NCK interacting kinase), a member of the family of serine/threonine kinase, has played a key role in many physiological and pathological process. Recent studies have found that TNIK is highly expressed in lung squamous cell carcinoma tissues and drives cancer cell proliferation and other malignant phenotypes, but its role in lung adenocarcinoma is still poorly understood. In this study, a lung adenocarcinoma A549 cell line with stable knockdown TNIK expression were constructed. Flow cytometry showed that the cell cycle was arrested and apoptosis was induced. Cell proliferation and movement experiments proved that the proliferation and migration of lung cancer cells were significantly inhibited. Further immunofluorescence staining analysis showed that stable knockdown of TNIK expression could induce disorder of cell actin cytoskeleton and inhibit dynamic turnover (assembly/depolymerization) of focal adhesions. In conclusion, TNIK in lung adenocarcinoma cells may influence the dynamic turnover of focal adhesions and ultimately control cell proliferation and migration by regulating the arrangement of actin cytoskeleton. It is speculated that the highly expressed TNIK in lung adenocarcinoma cells can maintain the malignant phenotype of cancer cells by regulating the actin cytoskeleton system.

Keywords lung adenocarcinoma cells; cell proliferation; cell motility; actin cytoskeleton; TNIK

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*通讯作者。Tel: 13635420731, E-mail: zhangcd@cqmu.edu.cn

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*Corresponding author. Tel: +86-13635420731, E-mail: zhangcd@cqmu.edu.cn

肺腺癌是肺癌主要亚型之一, 约占所有肺癌病例的40%[1]。虽然近年来治疗手段有所进步, 但其5年生存率仍然较低[2-5]。肺腺癌的恶性表型是多种因素共同作用的结果, 其具体分子机制尚不完全清楚, 导致肺腺癌缺乏早期诊断、易复发、易转移和预后差[6]。因此, 探索发掘新的潜在治疗靶点对于改善肺腺癌的低生存率尤为迫切。

肿瘤坏死因子受体相关因子2和Nck相互作用蛋白激酶(TRAF2 and Nck Interacting Kinase, TNIK)在鉴定TRAF2与Nck互作蛋白时被发现。TNIK包括一个N-端激酶结构域、一个C-端的Citron同源结构域(CNH)以及一个中间可变区域[7-8]。TNIK是生发中心激酶(germinal center kinase, GCK)家族的成员之一, 其激酶结构域具有丝氨酸/苏氨酸磷酸激酶活性[9]。文献报道显示, 结直肠癌中TNIK通过与T细胞因子4(T cell factor 4, TCF4)和β-连环蛋(β-catenin)相互作用, 使TCF4在丝氨酸154位点磷酸化, 从而通过WNT/β-catenin信号通路调控结直肠癌发生发展[10-11]。另外, TNIK激酶活性与血液系统等恶性癌变密切相关[12]。但迄今为止, 关于TNIK在肺癌尤其是非小细胞肺腺癌中的具体作用及潜在分子机制仍不明确。

本研究中, 通过敲低肺腺癌细胞中TNIK的表达, 检测细胞生长情况和对细胞侵袭和转移等恶性表型的影响, 探讨TNIK对人肺腺癌恶性表型的作用。研究结果表明, 在肺腺癌细胞中抑制TNIK表达会扰乱微丝骨架及黏着斑动态稳定从而抑制细胞迁移及细胞增殖, 推测在肺腺癌细胞中TNIK可能通过调控细胞骨架动态, 促进癌细胞恶性表型。

1 材料与方法

1.1 材料

人肺腺癌细胞株A549和人胚肾细胞株293T均由本实验室保存。胎牛血清(fetal bovine serum, FBS)、Opti-MED™培养基和转染试剂Lipofectamine 3000试剂购自美国Thermo Scientific公司; DMEM/F12培养基、DMEM高糖培养基和青霉素–链霉素溶液(内含10 kU/mL青霉素和10 mg/mL链霉素)购自Hyclone公司; RNA提取试剂盒、逆转录试剂盒和TB Green™ Premix Ex Taq™(Tli RNaseH Plus) RT-PCR试剂盒均购自TaKaRa公司; 引物由上海生物工程有限公司合成; BCA蛋白浓度测定试剂盒、RIPA裂解液、苯甲基磺酰氟(phenylmeth anesulfonyl-fluoride, PMSF)和PVDF膜(0.22 μm/0.45 μm)购自北京鼎国昌盛生物技术公司; SDS-PAGE凝胶试剂盒购自上海雅酶生物医药科技有限公司; 基质胶购自美国BD公司; Transwell小室(24孔板, 0.8 mm)购自Corning公司; 鬼笔环肽荧光染料和Vinculin抗体购自Sigma公司。TNIK抗体、p-FAK抗体和pHH3抗体均购自Cell Signaling Technology公司; GAPDH抗体购自Proteintech公司; 辣根过氧化物酶(horseradish peroxidase, HRP)标记的二抗均购自Abbkine公司; 免疫荧光二抗购自Thermo Scientific公司。

1.2 方法

1.2.1 细胞培养 人肺腺癌细胞A549和人胚肾细胞293T分别常规培养于添加10% FBS和1%双抗的DMEM/F12和DMEM高糖培养基中。细胞在含5% CO_2、温度为37 °C的无菌恒温培养箱中培养, 研究人员定期对细胞进行传代。

1.2.2 pLKO.1-Puro-shTNIK载体构建及慢病毒包装 *TNIK*基因两个shRNA靶向序列通过在线网站(www.sigmaaldrich.cn)设计, 1#: 5′-GCC TCA AGA ACA ACT TCT AT-3′; 2#: 5′-CCA TCT CAT ATT CAG GGC AAT-3′; shRNA引物序列由深圳华大基因科技有限公司合成。pLKO.1-Puro-shTNIK质粒经测序确认后分别被命名为shT-1#和shT-2#, 对照质粒为pLK0.1-Puro, 被命名为shCtrl。shT-1#、shT-2#和shCtrl质粒分别与包装质粒PMD2G和PSPAXL共同转染293T细胞。在48~72 h收集上清后, 对细胞进行感染或分装, 冻存于–80 °C冰箱。

1.2.3 稳定敲减TNIK细胞系构建 用本研究中包装的病毒感染A549细胞, 1 mg/mL嘌呤霉素筛选后获得阳性细胞。实时定量PCR和免疫印迹检测稳转细胞株中TNIK的表达。

1.2.4 细胞形态观察 密度在50%~60%时用光学显微镜观察细胞形态。

1.2.5 qRT-PCR法检测细胞中*TNIK* mRNA的转录水平 按RNA提取试剂盒说明书提取细胞总RNA, 以cDNA为模板进行实时定量PCR扩增, 测定各组细胞*TNIK* mRNA转录水平。qRT-PCR引物序列如下: *TNIK*上游引物为5′-TAA GGG TCG TCA TGT CAA AAC G-3′, 下游引物为5′-CCA TGC CTG GTG GGT TCT TT-3′; 内参基因*GAPDH*上游引物为5′-GGA GCG AGA TCC CTC CAA AAT-3′, 下游引物为5′-

GGC TGT TGT CAT ACT TCT CAT GG-3′。每个反应设3个复孔, 扩增完毕后, 用$2^{-\Delta\Delta Ct}$法测定目的基因mRNA的表达量。

1.2.6 Western blot 实验 用含PMSF(100×)的RIPA裂解液提取细胞的总蛋白, BCA测定总蛋白浓度后, 按每孔40 mg蛋白进行7.5% SDS-PAGE分离蛋白, 恒流电转至PVDF膜后, 用5%的脱脂牛奶室温封闭2 h。加入相应一抗, 其稀释比例分别是Anti-TNIK为1:1 000, Anti-Vinculin为1:2 000, Anti-pFAK为1:1 000, Anti-GAPDH为1:5 000, 4 °C摇床过夜。PBST洗涤后, 选择对应的鼠二抗(1:5 000)或兔二抗(1:5 000)室温孵育2 h, 最后用ECL化学发光试剂盒显影, 使用Image J软件分析条带灰度值。

1.2.7 流式细胞术分析细胞周期和凋亡 各组细胞用胰酶消化后, 收集于离心管中, 用PBS洗涤2次后加入75%预冷乙醇固定, 染色前用PBS洗去。加入PI/RNase A染色工作液, 避光室温放置30 min, 上机检测并分析细胞周期。细胞用PBS洗涤细胞2次后, 加入300 mL 1× 结合缓冲液悬浮细胞, 用Annexin-VFITC标记, 上机前5 min加5 mL PI染色, 同时补加200 mL 1× 结合缓冲液, 上机检测细胞凋亡。

1.2.8 Transwell迁移和侵袭实验 将细胞接种于不含或含Matrigel小室的上层进行细胞的迁移(5×10^5个/孔)和侵袭(1×10^5个/孔)实验。在培育12 h或24 h后, 进行结晶紫染色及拍照分析。

1.2.9 平板集落形成实验 将细胞以500个/孔细胞接种于12孔孔板中, 放入培养箱中。6天后, PBS清洗后进行结晶紫染色及分析。

1.2.10 划痕实验检测细胞迁移 待各组细胞生长密度达到95%左右时, 在培养板上划出细线并标志出要观察的位置。在48 h时拍照观察划痕愈合情况。

1.2.11 免疫荧光检测 各组细胞生长汇合至70%左右, PBS洗涤细胞2次, 用4%多聚甲醛固定细胞。30 min后, 加入500 mL 0.5%的TritonX-100, 室温通透10 min, PBS清洗后, 5% BSA 37 °C封闭1 h。用PBS稀释一抗, 4 °C封闭孵育过夜。按说明书稀释鬼笔环肽染料。PBS稀释荧光二抗, 37 °C孵育1 h。DAPI进行核染色。最后, 用共聚焦显微镜拍照分析。

1.2.12 数据统计 所有实验均独立重复3次, 数据采用GraphPad Prism 8.0软件分析作图。结果采用$\bar{x}\pm s$表示, 两组间比较采用t检验分析, $P<0.05$为差异具有统计学意义。

2 结果

2.1 检测稳定敲减细胞中TNIK表达情况

获得稳定敲减TNIK肺腺癌A549细胞系后, 通过qRT-PCR和Western blot检测TNIK的表达水平。如图1A和1B所示, 稳定敲减组细胞中TNIK mRNA及蛋白水平均显著低于对照组($P<0.05$)。这表明, TNIK在肺癌A549细胞中实现稳定敲减, 可以用于后续实验。光学显微镜观察稳定敲减TNIK细胞的形态, 结果表明细胞体积明显变大(图1C)。

2.2 稳定敲减TNIK阻滞细胞周期进程并诱发凋亡

流式细胞术检测敲减TNIK后细胞周期变化情况。结果显示, 与对照组相比, 敲减TNIK导致G_1期细胞比例增高, 而S期和G_2/M期的细胞比例降低, 表明敲减TNIK后会导致肺腺癌细胞周期阻滞(图2A)。同时, 细胞凋亡检测结果显示, 敲减TNIK后A549细胞凋亡显著增加(图2B)。表明肺腺癌细胞中TNIK表达对维持细胞周期进程及抑制细胞凋亡具有重要作用。

为进一步探讨TNIK表达对A549细胞增殖的影响。首先采用平板集落形成实验, 发现TNIK敲减组的克隆形成率明显低于对照组(图2C)。随后利用免疫荧光pHH3染色发现, 敲减TNIK表达的A549细胞中, pHH3阳性细胞数目明显减少(图2D)。

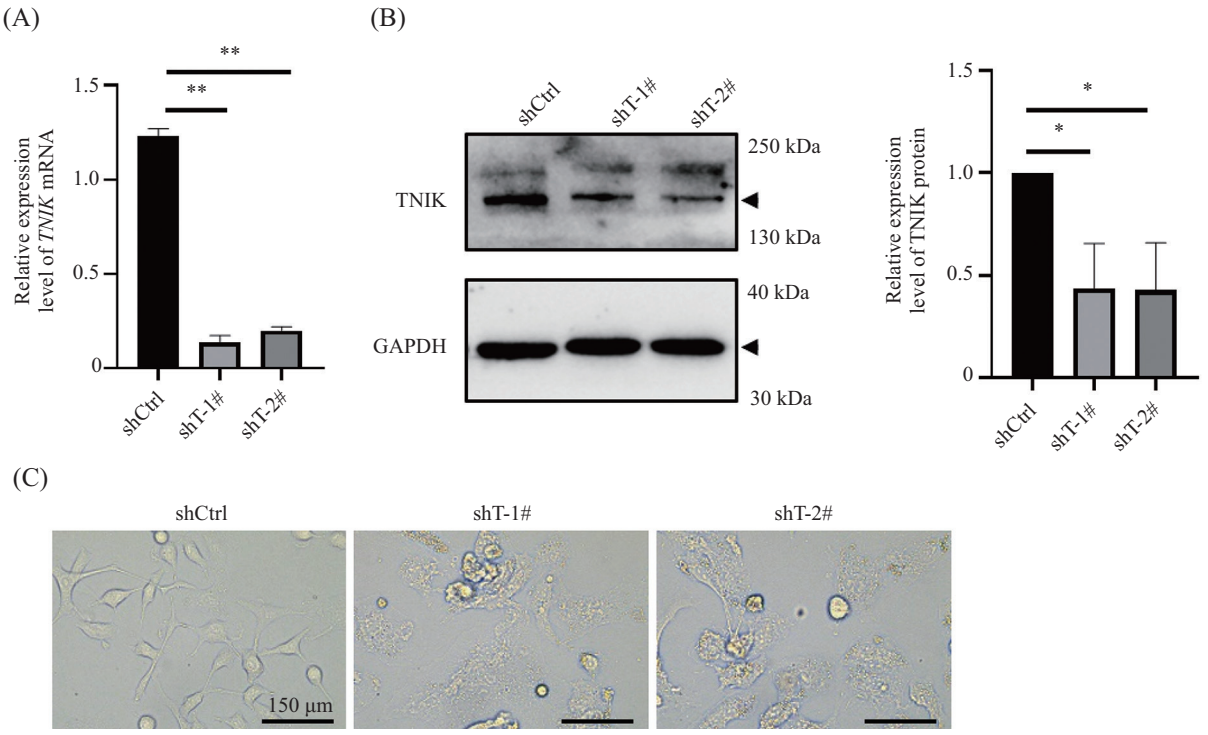
2.3 肺腺癌细胞中敲减TNIK抑制细胞迁移运动

细胞划痕和Transwell实验检测敲减TNIK对细胞迁移和侵袭能力的影响。细胞划痕实验结果显示, TNIK敲减组的迁移区域明显小于对照组(图3A)。Transwell迁移及侵袭实验显示, 在A549细胞中, 敲减TNIK表达后迁移及侵袭细胞数量明显低于对照组(图3B)。这些结果表明敲减TNIK可抑制肿瘤细胞的迁移能力。

2.4 肺腺癌细胞中敲减TNIK扰乱细胞微丝骨架及黏着斑动态

细胞骨架与生理功能密切相关, 且肌动蛋白是细胞产生动力的重要结构[13-14]。已有文献报道证明, TNIK的CNH结构域调节微丝骨架系统稳定[15]。为探究肺腺癌细胞中敲低TNIK是否影响微丝骨架聚合。利用免疫荧光染色发现, 稳定敲减TNIK表达后, 细胞应力纤维的数量增加但排列紊乱(图4A)。

微丝骨架聚合紊乱抑制黏着斑组装/解聚, 从而抑制细胞迁移运动等过程[16-18]。随后, 我们采用免疫荧光染色及免疫印迹检测黏着斑组装/解聚情况。



A: 荧光定量PCR检测稳定细胞株中*TNIK*的mRNA水平(**P<0.01); B: Western blot检测稳定细胞株中TNIK的蛋白表达水平(*P<0.05); C: 光学显微镜下观察细胞的形态。

A: mRNA level of *TNIK* in stable cell line was detected by qRT-PCR (**P<0.01); B: the level of TNIK protein in stable cell line was detected by Western blot (*P<0.05); C: observe cell morphology with an optical microscope.

图1 稳定细胞株A549中TNIK表达水平分析

Fig.1 Determination of TNIK expression levels in stable cell line A549

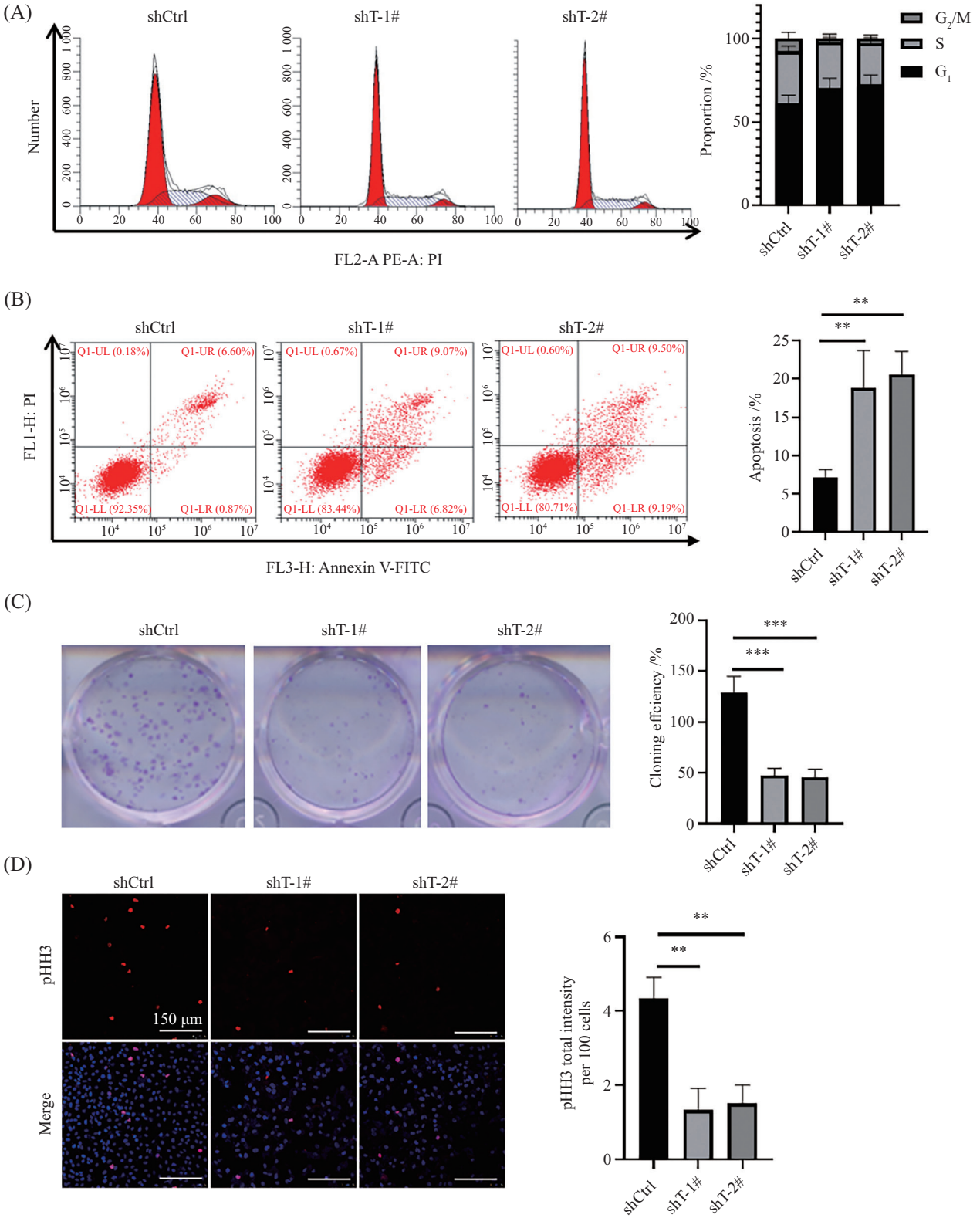
黏着斑标记物染色结果显示, 稳定敲低TNIK表达导致黏着斑蛋白(vinculin)的数量和大小明显增加(图4B和图4C)。

FAK是黏着斑的主要成分之一, 参与黏着斑形成和调节[19]。在大多数肿瘤中, FAK的高磷酸化水平促进了肿瘤侵袭和转移[20]。在本研究中, 我们分析磷酸化FAK^{Y397}活化情况。免疫印迹及免疫荧光分析发现(图4C), 稳定敲低TNIK导致FAK^{Y397}的磷酸化水平降低。这些研究结果表明, 敲低TNIK可能部分通过紊乱微丝骨架系统排列, 影响FAK活性从而调控黏着斑动态周期, 最终抑制肺腺癌细胞增殖及迁移侵袭。

3 讨论

最初TNIK被发现参与调控肌动蛋白细胞骨架和神经数突细胞的伸展过程[7,21]。随后, 越来越多研究证明, TNIK激酶活性通过作用于Wnt信号通路、c-Jun氨基末端激酶(c-Jun N-terminal protein kinase, JNK)、核因子NF-κB(nuclear fator kappa B, NF-κB)、磷脂酰肌醇3激酶(phosphatidy-linositol 3 kinase, PI3K)/蛋白激酶B(prot-ein kinase B, PKB)等信号通路, 最终调节机体免疫反应、细胞增殖、分化、细胞周期进程及凋亡等重要生命活动[15,22-26], 且相关研究表明, TNIK是一个潜在的肿瘤治疗靶点[10]。

已有研究表明, 胃癌细胞中通过siRNA介导的TNIK表达沉默显著抑制细胞增殖并诱发细胞凋亡[27]。且TNIK在乳腺癌中可通过Wnt/β-catenin信号通路控制乳腺癌细胞的恶性增殖及侵袭转移[28]。最近研究人员利用肺鳞癌患者来源的异种移植肿瘤模型研究发现, 抑制TNIK表达可显著抑制癌细胞增殖活性并诱发细胞凋亡[29]。而在人肺腺癌细胞中通过TNIK小分子抑制剂NCB-0005或KY-05009可抑制由转化生长因子β1(transforming growth factor-β1, TGF-β1)诱导的上皮–间质转化(epithelial–mesenchymal transition, EMT)[30]。本研究中, 我们通过构建稳定敲减TNIK表达的非小细胞肺癌细胞系, 发现敲减TNIK表达可明显阻滞细胞周期进程并诱发细胞凋亡。综上所述, 这些结果提示TNIK在肺癌发生发展中起着

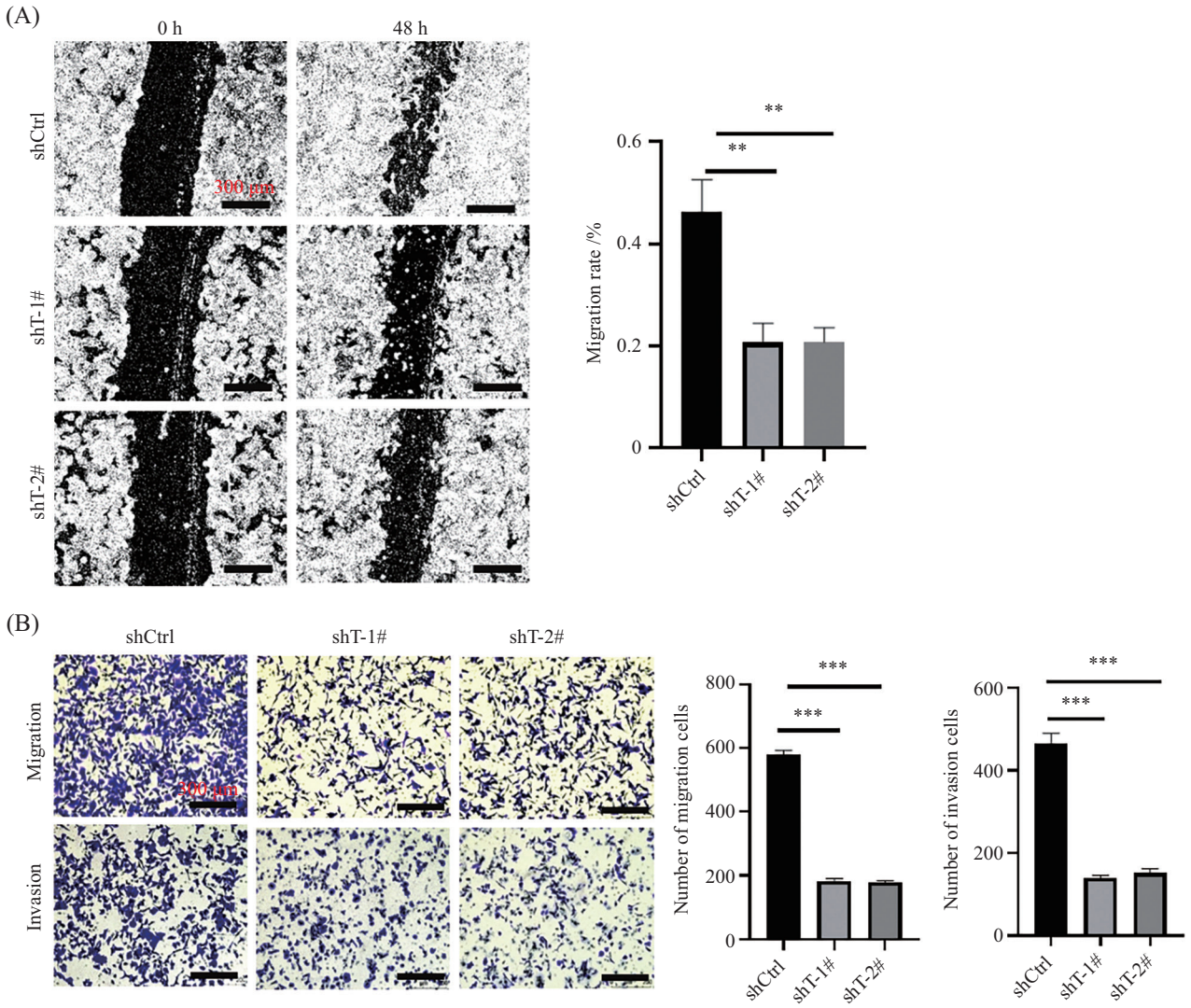


A、B: 流式细胞术检测各组细胞的周期和凋亡(**P<0.01); C: 克隆增殖实验评估各组细胞的生长情况(***P<0.001); D: 将转染shCtrl、shT-1#和shT-2#的细胞固定, 用抗pHH3抗体染色(红色)检测细胞增殖, 细胞核DAPI染色(蓝色)(**P<0.01)。

A,B: cell cycle and apoptosis were detected by flow cytometry (*P<0.05, **P<0.01); C: the colony formation assay was used to evaluate the growth of cells in each group (***P<0.001); D: cells transfected shCtrl, shT-1# and shT-2# were fixed, and cell proliferation was detected by anti-pHH3 antibody staining (red), and nuclear DAPI staining (blue) (**P<0.01).

图2 A549细胞中敲低TNIK阻滞细胞周期进程并诱发凋亡

Fig.2 TNIK knockdown in A549 cells blocks cell cycle progression and induces apoptosis



A: 划痕实验检测各组细胞的迁移能力(**P<0.01); B: Transwell检测各组细胞的迁移和侵袭能力(***P<0.001)。

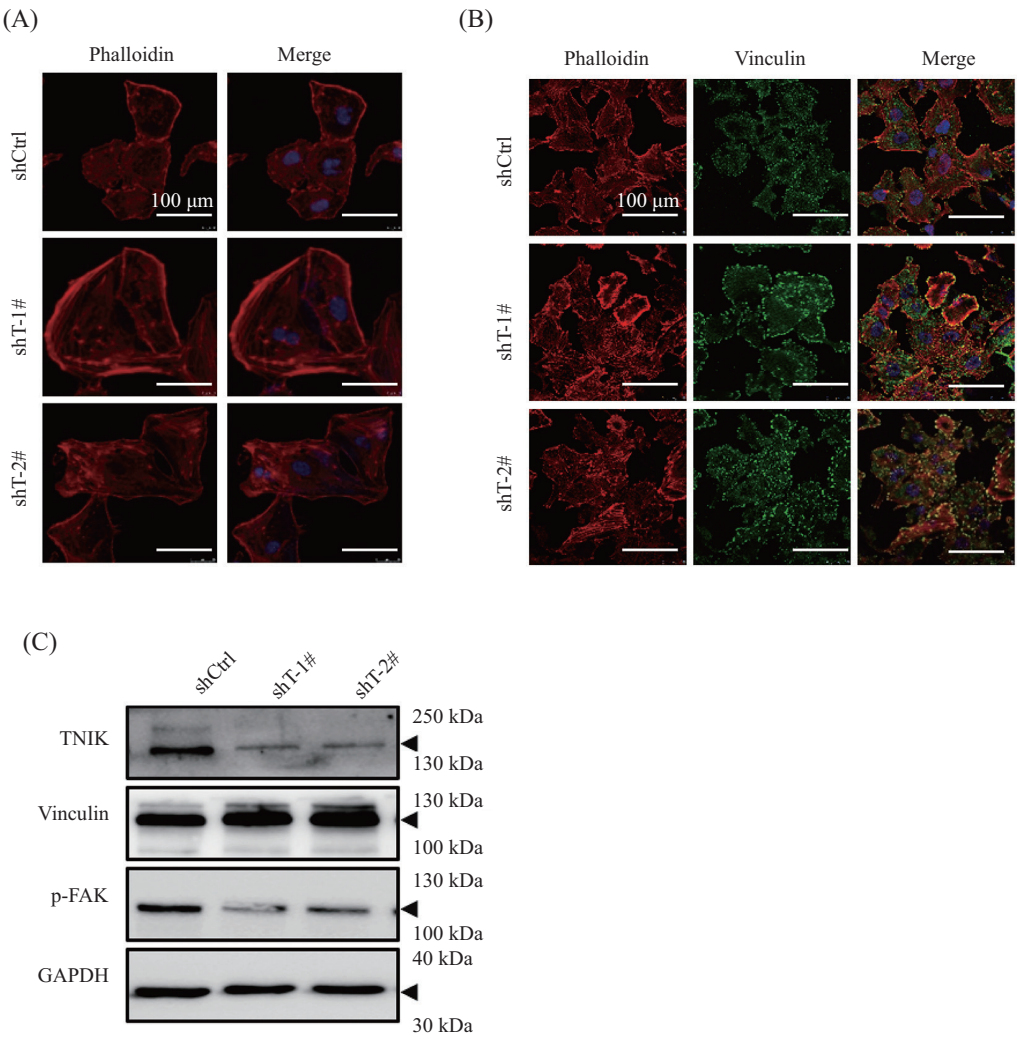
A: results of the migration ability in each cell group detected by wound-healing assay (**P<0.01); B: results of the abilities including migration and invasion in each cell group detected by Transwell (***P<0.001).

图3 TNIK表达下调对A549细胞迁移和侵袭能力影响

Fig.3 The cells migration and invasion abilities of A549 cells affected by down-regulated TNIK expression

关键作用, 但具体机制仍有待深入研究。

已有文献报道显示, TNIK在其C-端包含一个与微丝骨架调控密切相关的Citron同源结构域, 且在NIH3T3细胞中通过此结构域与Rap2相互作用, 影响细胞中微丝骨架聚合与排列从而控制细胞迁移运动[8]。在本研究中, 我们通过shRNA敲减体系在非小细胞肺癌A549细胞中稳定敲减TNIK表达细胞。检测结果显示, 稳定敲减TNIK表达可诱导细胞的体积变大, 细胞迁移运动被显著抑制。进一步分析发现, 敲减TNIK表达细胞中微丝骨架聚合紊乱, 黏着斑动态周(组装及解聚)被抑制而导致黏着斑数目及黏附区域增加。与此一致的是, 稳定敲减TNIK表达后, 细胞中活化型斑激酶(focal adhesion kinase, FAK)即pFAK^{Y397}显著下调。这些研究结果表明, 非小细胞肺癌细胞A549细胞中稳定沉默TNIK表达可能通过扰乱微丝骨架系统组装与排列影响黏着斑激酶活化, 从而抑制黏着斑动态周转, 进而抑制细胞迁移运动与增殖。报道证明, FAK是黏着斑组装及解聚的关键激酶, Tyr397位点的自磷酸化是FAK活化的标志, 而FAK信号通路活化受微丝骨架调控, 与黏着斑动态周转密切相关。研究发现, FAK在结肠癌、肝癌、肺癌、胃癌、乳腺癌和卵巢癌中的表达水平显著升高, 且其活性与癌细胞增殖、迁移运动与侵袭、存活及癌细胞自我更新等过程密切相关[20,31-37]。小鼠



A: 将各组细胞固定, F-actin偶联Phalloidin(红色)染色, DAPI (蓝色)标记细胞核。B: 各组细胞中局灶黏连标记物Vinculin的免疫标记。细胞固定后用Vinculin抗体染色(绿色)。C: Western blot检测Vinculin和p-FAK在各组细胞中的表达水平。

A: the cells of each group were fixed, stained with F-actin-binding Phalloidin (red), and the nuclei were stained with DAPI (blue). B: Immunolabeling of focal adhesion marker Vinculin in each group. Cells were fixed and stained with Vinculin antibody (green). C: the expression levels of Vinculin and p-FAK were detected by Western blot in each group.

图4 TNIK扰乱细胞微丝骨架及黏着斑动态

Fig.4 TNIK disturbs actin cytoskeleton and focal adhesion turnover

模型研究发现, FAK在皮肤癌、乳腺癌侵袭及肺转移中也发挥重要作用[38-39]。我们的研究证明非小细胞肺腺癌细胞中TNIK可能通过微丝骨架系统调控FAK活性从而控制细胞增殖、凋亡及迁移运动。

本研究表明, 丝氨酸/苏氨酸磷酸激酶TNIK与肺腺癌的增殖和迁移密切相关, 并初步探讨TNIK通过微丝骨架系统促进肺腺癌增殖迁移的分子机制, 为后续研究奠定了基础。

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