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MEK/ERK 对 HT-29 结肠癌细胞分化、侵袭迁移及 NDRG1 基因表达的影响

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Effect of MEK/ERK on Differentiation, Invasion/Migration and NDRG1 Gene Expression of Colon Cancer Cell Line HT-29

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Abstract: Objective To characterize the mechanism of NDRG1-induced colon cancer aggressiveness including NDRG1-induced colon cancer cell migration and invasion. **Methods** HT-29 human colorectal cell, was employed to detect cell migration and invasion using transwell assays. The role of ERK in NDRG1-mediated cell migration and invasion was also evaluated by employing the MEK/ERK inhibitor. Change of cell morphology was microscopically examined. Transmission electron microscope was also used to examine the effect of ERK inhibitor on HT-29 ultra structure change. **Results** Treatment with ERK inhibitor changed HT-29 cell morphology. The size, appearance of the cells and nuclei were inclined with uniformity; the arrangement of the cells was glandule-like texture. There were more microvilli on the cell surface and the numbers of Golgi complexes, mitochondria were increasing; there were also more rough endoplasmic reticula; intracytoplasmic lumen was frequently seen; cells treated with ERK inhitors also induced cell apoptosis. Comparing with control groups, cell migratory ability was significantly inhibited by the inhibitors. Using an immunocytochemical staining approach, we demonstrated that increased NDRG1 protein was elevated in cells treated with the inhibitors, than that in control cells. Western blot confirmed this observation. **Conclusion** Our data indicate that blocking the MAPK pathway induces differentiation and apoptosis of the HT-29 colorectal cancer cells. In addition, inhibition of MAPK pathway also suppresses cancer cell invasive/migratory abilities through up-regulation of NDRG1 protein.

Key words: Neoplasm; HT-29; MAPK; NDRG1; Invasion

摘 要: 目的 探讨 MEK/ERK 在结肠癌细胞 NDRG1 基因表达调控和体外侵袭、迁移中的作用; 分析 ERK 通路、NDRG1 基因、肿瘤侵袭及迁移三者间的潜在联系。**方法** 将 MEK/ERK 抑制剂与 HT-29 结肠癌细胞共培养, 光学显微镜下观察细胞形态变化; 透射电子显微镜观察细胞超微结构变化; 24 孔-小室法检测癌细胞体外侵袭、迁移能力的改变; 免疫细胞化学染色、Western blot 检测 NDRG1 基因表达情况。**结果** 与抑制剂共培养后, HT-29 结肠癌细胞及胞核形态、大小趋于一致, 排列多呈腺样结构。细胞表面微绒毛、高尔基复合体、线粒体增多, 粗面内质网丰富, 胞质内微腺腔常见, 同时可见凋亡细胞。与对照组相比, 加抑制剂组在侵袭迁移实验中穿过微孔膜的细胞数减少, 差异有统计学意义($P<0.05$)。免疫细胞化学结果显示, HT-29 组与 DMSO 组 NDRG1 表达较少, 加抑制剂组表达显著增多; Western blot 结果显示, 加抑制剂各组均出现 NDRG1 蛋白表达条带, 而 HT-29 组与 DMSO 组则未见有 NDRG1 蛋白的表达。**结论** 阻断 ERK 通路可诱导 HT-29 结肠癌细胞发生分化且促进细胞凋亡, 同时可抑制其体外侵袭及迁移能力并明显上调其 NDRG1 蛋白的表达。

关键词: 肿瘤; HT-29; MAPK; NDRG1; 侵袭

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0 引言

大肠癌是最常见的恶性肿瘤之一, 同时也是世界范围内癌症死亡的主要原因。大肠癌的发生是一个复杂的多步骤过程, 包括肠上皮细胞增殖、分化、凋亡及幸存机制的进行性紊乱[1]。

ERK MAPK 通路是引起细胞增殖最为重要的信号通路之一[2]，其活性对肠上皮分化非常重要[3]。ERK MAPK 通路的激活与大肠癌发病机制、演进及其生物学行为关系密切。该通路相关的所有激酶均可作为大肠癌治疗中潜在的靶点。

NDRG1 基因是与细胞分化及肿瘤转移有关的基因。研究表明，该基因与细胞生长、发育、分化、肿瘤的发生、转移和预后等均有关。

本研究初步探寻 MEK/ERK 在 NDRG1 基因表达调控和体外侵袭迁移中的作用；分析 ERK 通路、NDRG1 基因、肿瘤侵袭及迁移三者间的潜在联系。

1　材料与方法

1.1　实验材料

人结肠癌细胞株 HT-29 由中国医学科学院中国协和医科大学基础医学研究所细胞中心提供。MEK/ERK 抑制剂 PD98059、U0126 购自 Calbiochem 公司；兔抗人 NDRG1 多克隆抗体购自美国 Santa cruz Biotechnology 公司；DMEM/F12 培养液、胰蛋白酶、PBS 缓冲液购于 Hyclone 公司；标准胎牛血清购自 Tiangene 公司；DMSO 购自 Sigma 公司；Western 及 IP 细胞裂解液、BCA 蛋白测定试剂盒、SDS-PAGE 凝胶配制试剂盒购于碧云天(Beyotime)公司；24 孔-小室(24-transwell)购自 Costar；Matrigel 购于 BD 公司；即用型 EliVision™ plus 广谱试剂盒、DAB Kit 购自迈新生物技术有限公司。

1.2　实验方法

1.2.1　细胞培养及实验分组　传代培养的 HT-29 细胞在含 10％胎牛血清的 DMEM/F12 培养液中生长至 70％～80％融合时，根据文献及预实验结果分别加入浓度为 50 μmol/L 的 PD98059、10 μmol/L 的 U0126 共培养 48 h 后，收集细胞用于下列实验。

1.2.2　细胞爬片 HE 染色的普通光学显微镜观察　PBS 液洗涤后丙酮固定。常规 HE 染色，光学显微镜下观察。

1.2.3　透射电子显微镜观察　2％戊二醛、1％锇酸双固定，丙酮梯度脱水，环氧树脂浸透后包埋，超薄切片、饱和醋酸铀染色，透射电子显微镜观察细胞超微结构。

1.2.4　体外侵袭、迁移实验　按照文献[4]的方法，以人工基底膜包被 24 孔-小室滤膜，小室上室加入收获的无血清细胞悬液 100 μl(1×10^5 个/孔)，下室加入 700 μl 含 20％FBS 的 DMEM/F12 培养液，每组 4 个复孔；放入培养箱中 30 min 使细胞贴膜；取出，再加含两倍工作液浓度抑制剂的培养液 100 μl 于小室上室，37℃、5％CO_2 培养 24 h 以上。取出小室，PBS 洗涤后 95％酒精固定；结晶紫染色洗净后用棉签擦净小室滤膜的内表面，去掉未发生侵袭的细胞；显微镜下观察。选择中央及上、下、左、右 5 个 400 倍视野，计数穿过 8 μm 微孔膜的侵袭性细胞数目。取每个视野的平均数表示肿瘤细胞的体外侵袭能力。

迁移实验时不铺人工基底膜，其余步骤同侵袭实验。

1.2.5　NDRG1 基因表达情况的检测　(1)免疫细胞化学染色检测：细胞爬片 PBS 液洗涤后丙酮固定，常规 SP 法进行 NDRG1 表达的免疫细胞化学染色，以细胞质和细胞膜中出现棕黄色颗粒状产物为 NDRG1 免疫细胞化学染色阳性判定标准。(2)Western blot 检测：收集处理前后的各组细胞，裂解细胞提取总蛋白，根据蛋白质分子量制备 12％SDS-PAGE，每孔上样量 50 μg，常规电泳、转膜、封闭，一抗(稀释度为 1∶500)4℃孵育过夜，二抗(稀释度为 1∶1 000)37℃反应 1 h，采用 ECL 试剂盒进行化学显影。

1.3　统计学方法

应用 SPSS 13.0 软件进行统计学处理，数据以 $\bar{x}\pm s$ 表示，各组间比较采用单因素方差分析，$P<0.05$ 为差异有统计学意义。

2　结果

2.1　光学显微镜观察

对照组 HT-29 组及 DMSO 组细胞(抑制剂 PD98059、U0126 均以 DMSO 溶解)均呈梭形、多边形；排列紊乱、缺乏极向；细胞核大、深染，圆形、椭圆形，可见双核、多核，核浆比增大；(病理性)核分裂相易见，见图 1a、1b。PD98059 组细胞呈较规则的多边形，细胞及细胞核形态、大小趋于一致，见图 1c。U0126 组细胞形态较两对照组细胞规则，但不及 PD98059 组；可能是 U0126 更易分解的缘故，见图 1d。

2.2　透射电子显微镜观察

HT-29 细胞呈圆形、椭圆形，核大、核仁突出，核质比大，可见畸形核，细胞表面微绒毛少，粗面内质网偶见，可见固缩线粒体。与 PD98059 及 U0126 共培养后，细胞表面微绒毛丰富，线粒体增多、肿胀，见图 2a；胞质内微腺腔常见，见图 2b；粗面内质网丰富，偶见微丝束，见图 2c；凋亡细胞常见，见图 2d。

2.3　侵袭迁移实验

侵袭实验结果显示，加抑制剂组与 HT-29 组及 DMSO 组相比，相同时间内穿过人工基底膜的细胞数减少，差异有统计学意义($P<0.05$)；DMSO 组与 HT-29 组相比，相同时间内穿过人工基底膜的细胞数接近，差异无统计学意义($P>0.05$)，见图 3，表 1。

迁移实验结果显示：加抑制剂组与 HT-29 组及 DMSO 组相比，相同时间内穿过 24 孔-小室微孔膜的细胞数减少，差异有统计学意义($P<0.05$)；DMSO 组与 HT-29 组相比，相同时间内穿过 24 孔-小室微孔膜的细胞数接近，差异无统计学意义($P>0.05$)，见表 1。

表 1 四组细胞侵袭和迁移能力的比较($\bar{x} \pm s$)

Table 1 Comparison of the invasion and migration ability($\bar{x} \pm s$)

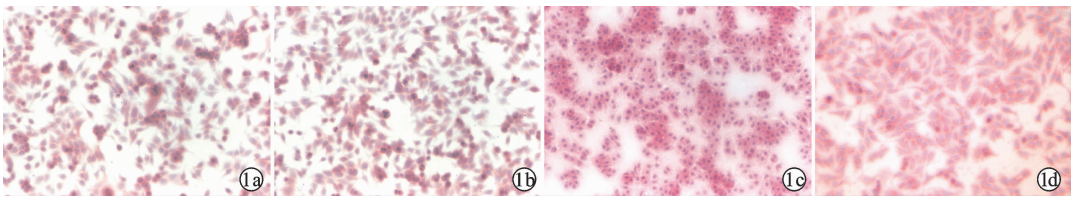
Groups	The number of invasion cells	The number migration of cells
HT-29	141.5 ± 11.3	150.0 ± 12.9
DMSO	144.5 ± 16.2[3]	147.6 ± 14.9
PD98059	121.6 ± 12.5[1]	128.9 ± 13.3
U0126	124.8 ± 9.9[2]	128.5 ± 11.0[2]

Note: treated with inhibitors group compared with DMSO group,[1,2]: $P < 0.05$; DMSO group compared with HT-29 group,[3]: $P > 0.05$

2.4 NDRG1 基因表达情况的检测

2.4.1 免疫细胞化学染色检测 NDRG1 蛋白的表达 结果显示:NDRG1 蛋白阳性着色主要定位于细胞膜与细胞质,呈黄色～棕黄色细颗粒;HT-29 组及 DMSO 组表达较少,加抑制剂组表达显著增多,见图 4。

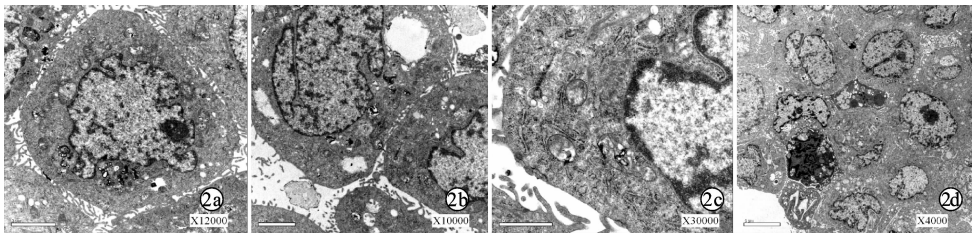
2.4.2 Western blot 检测 NDRG1 蛋白的表达 蛋白转移膜化学显影后,结果显示:加抑制剂组出现 NDRG1 蛋白表达条带;而 HT-29 组和 DMSO 组则未见有 NDRG1 蛋白的表达,见图 5。



1a: HT-29 group; 1b: DMSO group; 1c: PD98059 group; 1d: U0126 group

图 1 HT-29 细胞与 PD98059 及 U0126 共培养后细胞形态的变化(HE ×100)

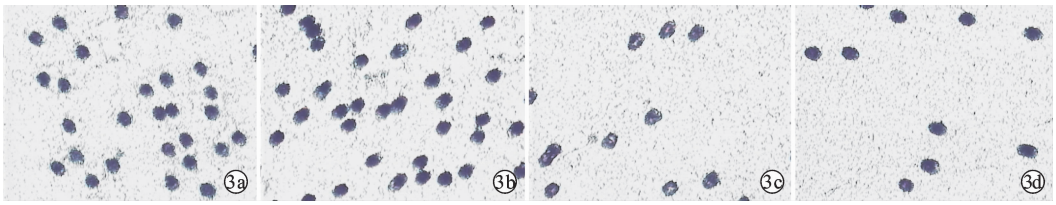
Figure 1 Changing of the cell morphology after treatment with the inhibitors(HE ×100)



2a: abundant microvilli on the cell surface; 2b: intracytoplasmic lumen was frequently seen; 2c: plentiful rough endoplasmic reticula; 2d: apoptosis cells can be usually seen

图 2 HT-29 细胞与 PD98059 及 U0126 共培养后细胞超微结构的变化

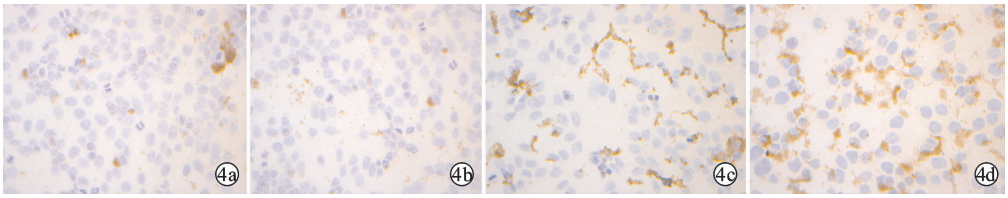
Figure 2 Changing of the cell ultra structure after treatment with the inhibitors



3a: HT-29 group; 3b: DMSO group: comparing with control groups, the number of cells was basicallysame transferring through the polycarbonate membrane with 8μm pore in the group co-cultured with inhibitor; 3c: PD98059 group: comparing with control groups, the number of cells was decreased transferring through the polycarbonate membrane with 8μm pore in the group co-cultured with inhibitor; 3d: U0126 group: comparing with control groups, the number of cells was decreased transferring through the polycarbonate membrane with 8μm pore in the group co-cultured with inhibitor

图 3 加入抑制剂后对 HT-29 细胞侵袭能力的影响(结晶紫染色 ×200)

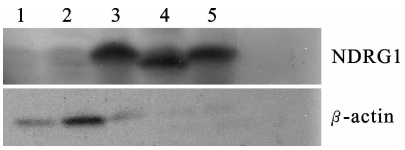
Figure 3 Influence of HT-29 cell invasion ability after treatment with the inhibitors(Crystal violet staining ×200)



4a: HT-29 group; 4b: DMSO group: expression of the NDRG1 protein was basically same with control groups; 4c: PD98059 group: expression of the NDRG1 protein was increased; 4d: U0126 group: expression of the NDRG1 protein was increased

图 4 HT-29 细胞与 PD98059 及 U0126 共培养后 NDRG1 蛋白表达的变化(SP ×200)

Figure 4 Expression of the NDRG1 protein after treatment with the inhibitors(SP ×200)



1:DMSO group;2:HT-29 group;
3:GW5074(Raf inhibitor)group;
4:PD98059 group;5:U0126 group

图 5　Western blot 检测加抑制剂前后各组 NDRG1 蛋白表达的变化

Figure 5　The variation of NDRG1 protein in each group before and after treatment with inhibitors by Western blot

3 讨 论

ERK MAPK 信号通路的激活对肠上皮分化非常重要[3-5]，并与人类大肠癌发病机制、演进及其生物学行为关系密切[6]。大肠癌中由 ERK 通路传递的信号增强，在过去的几年里，对这一现象作用的了解已越来越多[7-8]。研究表明，ERK 通路与大肠癌细胞凋亡、增殖及分化关系密切；其在大肠癌侵袭、转移中也有着重要作用。

NDRG1 基因是 N-myc 下游基因家族的一个成员，广泛参与多种生物学效应[9-20]：参与细胞生长、发育；抑制肿瘤细胞的生长；与肿瘤细胞的分化和转移有关；可被多种分化调节剂诱导表达。NDRG1 蛋白主要分布在上皮组织内[16]。NDRG1 在正常细胞和组织中有较高的表达，而肿瘤细胞中 NDRG1 的表达普遍降低；NDRG1 在高级别肿瘤和低分化肿瘤中的表达也较低[21-22]。

在对结肠癌[23-24]的研究中发现，从正常黏膜上皮向癌发展的过程中，NDRG1 mRNA 的表达逐步降低，且原发灶中的表达比转移灶中的表达要高。实验发现，NDRG1 可上调 E-钙黏连蛋白的表达[24]，而后者已证实为肿瘤黏附分子和转移抑制因子[22]。最近的研究也表明，NDRG1 低水平表达是进展期大肠癌独立的不良预后因子[25]。

信号通路抑制剂是研究信号转导通路的有力工具。PD98059 和 U0126 是抑制 MEK 活化与 MAPK 级联反应的高选择性抑制剂[26-27]。

本研究将 ERK 通路相关抑制剂与 HT-29 结肠癌细胞共培养后，光学显微镜和透射电子显微镜下细胞形态学变化、体外侵袭迁移实验结果、NDRG1 基因表达的免疫细胞化学染色、Western blot 检测结果提示，阻断 ERK 通路可诱导 HT-29 结肠癌细胞发生分化且促进细胞凋亡，同时可抑制其体外侵袭及迁移能力并明显上调其 NDRG1 蛋白的表达。

综合实验结果，我们推测在 HT-29 大肠癌细胞株中，ERK 通路活化后，可能通过下调 NDRG1 基因表达进而赋予癌细胞恶性表型；阻断 ERK 通路后，可能通过上调 NDRG1 基因表达从而促进细胞分化、凋亡并抑制肿瘤侵袭。在以后的研究中，我们将进一步检测与分化相关的分子 marker、敲除 NDRG1 基因后，再观察 MEK/ERK 抑制剂是否仍能促进 HT-29 细胞的凋亡、抑制 HT-29 细胞的迁移等功能。

参考文献：

[1] Calvert PM, Frucht H. The genetics of colorectal cancer[J]. Ann Intern Med, 2002, 137(7): 603-612.

[2] Troppmair J, Bruder JT, Munoz H, et al. Mitogen-activated protein kinase extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation[J]. J Biol Chem, 1994, 269(9): 7030-7035.

[3] Taupin D, Podolsky DK. Mitogen-activated protein kinase activation regulates intestinal epithelial differentiation[J]. Gastroenterology, 1999, 116(5): 1072-1080.

[4] Knutson JR, Lida J, Fields GB, et al. CD44/chondroitin sulfate proteoglycan and alpha 2 beta 1 integrin mediate human melanoma cell migration on type Ⅳ collagen and invasion of basement membranes[J]. Mol Biol Cell, 1996, 7(3): 383-396.

[5] Peláez IM, Kalogeropoulou M, Ferraro A, et al. Oncogenic RAS alters the global and gene-specific histone modification pattern during epithelial-mesenchymal transition in colorectal carcinoma cells[J]. Int J Biochem Cell Biol, 2010, 42(6): 911-920.

[6] Kress TR, Raabe T, Feller SM. High Erk activity suppresses expression of the cell cycle inhibitor p27Kip1 in colorectal cancer cells[J]. Cell Commun Signal, 2010, 8(1): 1.

[7] Watanabe M, Ishiwata T, Nishigai K, et al. Overexpression of keratinocyte growth factor in cancer cells and enterochromaffin cells in human colorectal cancer[J]. Pathol Int, 2000, 50(5): 363-372.

[8] Chao C, Han X, Ives K, et al. CCK2 receptor expression transforms non-tumorigenic human NCM356 colonic epithelial cells into tumor forming cells[J]. Int J Cancer, 2010, 126(4): 864-875.

[9] van Belzen N, Dinjens WN, Diesveld MP, et al. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms[J]. Lab Invest, 1997, 77(1): 85-92.

[10] Wang Z, Wang GY, Wang F. N-myc downstream regulatecl gene 1 and tumor[J]. Zhonghua Bing Li Xue Za Zhi, 2003, 3(2): 162-164. [王震，王国英，王芳. 分化相关基因 NDRG1 与肿瘤[J]. 中华病理学杂志，2003，32(2)：162-164.]

[11] Agarwala KL, Kokame K, Kato H, et al. Phosphorylation of

RTP, an ER stress-responsive cytoplasmic protein [J]. Biochem Biophys Res Commun, 2000, 272(3): 641-647.

[12] Taketomi Y, Sunaga K, Tanaka S, et al. Impaired mast cell maturation and degranulation and attenuated allergic responses in ndrg1-deficient mice [J]. J Immunol, 2007, 178 (11): 7042-7053.

[13] Guan RJ, Ford HL, Fu Y, et al. Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer [J]. Cancer Res, 2000, 60(3): 749-755.

[14] Nishio S, Ushijima K, Tsuda N, et al. Cap43/NDRG1/Drg-1 is a molecular target for angiogenesis and a prognostic indicator in cervical adenocarcinoma [J]. Cancer Lett, 2008, 264 (1): 36-43.

[15] Park H, Adams MA, Lachat P, et al. Hypoxia induces the expression of a 43-kDa protein (PROXY-1) in normal and malignant cells[J]. Biochem Biophys Res Commun, 2000, 276(1): 321-328.

[16] Lachat P, Shaw P, Gebhard S, et al. Expression of NDRG1, a differentiation-related gene, in human tissues [J]. Histochem Cell Biol, 2002, 118(5): 399-408.

[17] Kalaydjieva L, Gresham D, Gooding R, et al. N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom[J]. Am J Hum Genet, 2000, 67(1): 47-58.

[18] Sonja S, Roh V, Keogh A, et al. Hypoxia increases cytoplasmic expression of NDRG1, but is insufficient for its membrane localization in human hepatocellular carcinoma[J]. FEBS Lett, 2007, 581(5): 989-994.

[19] Ellen TP, Ke Q, Zhang P, et al. NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states[J]. Carcinogenesis, 2008, 29 (1): 2-8.

[20] Bandyopadhyay S, Pai SK, Gross SC, et al. The Drg-1 gene suppressur tumor metastasis in prostate cancer[J]. Cancer Res, 2003, 63(8): 1731-1736.

[21] Stein S, Thomas EK, Herzog B, et al. NDRG1 is necessary for p53-dependent apoptosis [J]. J Biol Chem, 2004, 279 (47): 48930-48940.

[22] Ahmed N, Oliva K, Wang Y, et al. Downregulation of urokinase plasminogen activator receptor expression inhibits Erk signalling with concomitant suppression of invasiveness due to loss of uPAR-beta1 integrin complex in colon cancer cells[J]. Br J Cancer, 2003, 89(2): 374-384.

[23] Folkman J. Tumor angiogensis: role in regulation of tumor growth[J]. Symp Soc Dev Biol, 1974, 30(0): 43-52.

[24] Strzelczyk B, Szulc A, Rzepko R, et al. Identification of high-Risk Stage Ⅱ colorectal tumors by combined analysis of the NDRG1 gene expression and the depth of tumor invasion[J]. Ann Surg Oncol, 2009, 16(5): 1287-1294.

[25] Crews CM, Alessandrini A, Erikson RL. The primary structure of MEK a protein kinase that phosphorylates the ERK gene product (extracellular signal-regulated kinases) [J]. Science, 1992, 258(5081): 478-480.

[26] Alessi DR, Saito Y, Campbell DG, et al. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1[J]. EMBO J, 1994, 13(7): 1610-1619.

[27] Rosen LB, Ginty DD, Weber MJ, et al. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras[J]. Neuron, 1994, 12(6): 1207-1221.

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