腺瘤性结肠息肉(APC)蛋白截短突变对MDCK细胞中细胞-基质和细胞-细胞间黏附的影响

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【摘要】 **目的** 探讨腺瘤性结肠息肉(adenomatous polyposis coli,APC)蛋白截短突变对MDCK细胞中细胞-细胞和细胞-基质之间黏附的影响及机制。**方法** 应用细胞黏附测定实验检验MDCK-N2-APC及MDCK-GFP稳定表达株系的黏附率。相对于对照细胞MDCK-GFP,MDCK-N2-APC细胞为稳定突变株,表达APC蛋白N端449-781氨基酸片段。免疫荧光染色、荧光定量PCR及Western blot测定在细胞黏附过程中发挥重要作用的黏附分子(CD29、E-cadherin)的表达情况。**结果** 与对照细胞相比,N2细胞-基质之间黏附率增加,平均升高约180%,而细胞-细胞间黏附率约下降30%。在MDCK-N2-APC细胞中CD29的表达水平增加,E-cadherin的表达水平降低。**结论** APC蛋白在细胞-基质及细胞-细胞间黏附中发挥重要作用。其截短突变片段N2可能通过改变CD29和E-cadherin等黏附分子的表达量来影响细胞黏附,进而影响细胞的侵袭和浸润。

【关键词】 腺瘤性结肠息肉(APC)蛋白; 细胞-基质黏附; 细胞-细胞黏附; 截短突变
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Effects of truncated mutation of adenomatous polyposis coli (APC) on cell-matrix and cell-cell adhesion of MDCK cells

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【**Abstract**】 **Objective** To explore the impact of mutational adenomatous polyposis coli (APC) on cell-matrix, cell-cell adhesion and the relative mechanism. **Methods** Cell-matrix and cell-cell adhesion assays were employed to determine the adhesion level of two stable cell lines MDCK-N2-APC and MDCK-GFP. The truncated APC of N2 fragment, which spans residues 449-781, was studied in comparison with control cells including GFP alone. Immunofluorescence staining, RT-PCR analysis and Western blot were applied to check several adhesion molecules which are key roles in cell contact process. **Results** In contract with control, cell-matrix adhesion was averagely increased to 180% in N2 cells, whereas cell-cell adhesion was reduced by about 30%. Our experiments accordingly indicated that CD29 expression level was enhanced and the expression level of E-cadherin was enervated in N2 cells. And these two molecules all have crucial roles in cell-matrix and cell-cell adhesion. **Conclusions** Full length APC plays a crucial role in cell-matrix and cell-cell adhesion. Truncated N2-APC may influence cell adhesion through changing the expression level of certain

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adhesion molecules, such as E-cadherin and CD29. The truncation mutation of APC fragment N2 restrained in the colon cancer cells will alter the cell invasion and migration by affecting cell adhesion and cell-matrix adhesion.

【Key words】 adenomatous polyposis coli (APC); cell-matrix adhesion; cell-cell adhesion; truncated mutation

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Introduction

Cell-cell and cell-matrix adhesive interactions play key roles in many different aspects of cell invasion and migration. These interactions are involved in the process of cell localization, effector recognition, and activation phenomena[1].

Cadherins are calcium-dependent cell adhesion receptors with well established roles in morphogenesis. Several studies have suggested that cadherin family members have important roles in the malignant progression of various human cancers. In addition to their established functions in modulating cell adhesion, aggregation, cell polarity, and morphogenesis, the classic aggregation molecules, E-cadherin, has also been implicated in the molecular pathogenesis of lung, breast, liver, and gastric cancers[2]. In epithelial cells, E-cadherin is very important for compact association of the cells in epithelial sheets, and in this capability, E-cadherin might function as a suppressor of invasiveness and metastasis of epithelial tumors[3]. CD29 (Integrin beta-1) is an integrin unit associated with very late antigen receptors. It is the beta subunit of an integrin family of molecules expressed on diverse cell types which function as the major receptors for extracellular matrix and as cell-cell adhesion molecules.

Adenomatous polyposis coli (APC), a tumor suppressor commonly mutated in cancer, is a cytoskeletal organizer for cell migration in the mammalian intestinal epithelium. Most human colorectal tumours carry mutations in the APC gene, which result in expression of truncated N-terminal APC fragments lacking sites required for the formation of the β-catenin targeting complex, so that intracellular β-catenin is not regulated properly and the normal genetic programme is altered. In addition, APC participates in several other cellular processes, including cytoskeletal regulation, so that loss or truncation of APC also directly affects cell migration and chromosome segregation[4]. Notably, APC truncation can be associated with these adherens junctions and evidence points to a role for APC in cellular adhesion. The association of truncated mutation of APC with the cytoskeleton and the plasma membrane at cell-cell junctions has led to suggestions of a role for APC in cell-cell adhesion[5], which could be relevant to its activity as a tumour suppressor. However, it is unknown whether there is a functional relationship between APC truncation and cell- cell contacts.

Here, we report that truncated APC affects cell-cell adhesion and cell-matrix adhesion, especially in MDCK-N2-APC, which contains N-terminal fragments 449－781 residues of APC. Our experiments showed that cell adhesion is increased and cell-matrix adhesion was reduced respectively by the over-expression of N2-APC in the cells. These results implicate the roles of truncated N2-APC fragment with the residues of 449－781 amino acids acting on cell-cell contacts and further effects on cancer cell invasion and migration.

Materials and Methods

Antibodies and cell culture Antibodies used in this study were: anti-E-cadherin (Bioworld), monoclonal anti-α-tubulin antibody (Sigma), anti-GFP (Cell Signaling), anti-CD29 (BD Biosciences). HRP-conjugated goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H + L) secondary antibodies were from

Invitrogen.

MDCK-GFP and MDCK-N2-APC stable cells (respectively expressing wild type and truncated APC spanning residues 449 - 781) were routinely maintained in Dulbecco's modified eagles medium (DMEM, HyClone) containing 250 μg/mL gentamycin (G418, Biosharp) at 37℃ in a 5% CO_2 incubator. The media contained 10% fetal calf serum (Boster), 1% penicillin/streptomycin (Solarbio).

Immunofluorescence staining Cells were grown on cover glasses in media at 37℃. Washed three times with phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100/PBS for 3 min, and pre-blocked in 3% BSA/PBS for 1 h. The slides were then incubated with a primary antibody (diluted 1∶1 000 in blocking solution) for 4 h at 4℃, washed three times with PBS, and incubated with a secondary antibody with a TRITC-conjugated anti-rabbit antibody (diluted 1∶100) for 1 h. After further washes, the slides were mounted with sealed tablet (Solarbio), and cells were examined with fluorescence optics on a confocal microscope.

Cell adhesion MDCK-GDP and MDCK-N2-APC cell adhesion was performed by a pre-coated 96-well plate with fibronectin (10 g/mL; Sigma) at 37℃ for 1 h. The cells were digested with 0.05% trypsin and resuspended at 5×10^5 cells/mL in DMEM media. Cells (100 μL) were added to each well and allowed to adhere for 0.5 or 2.5 h. At the end of the incubation period, cells were washed three times with PBS, and each well was added in 50 μL 4% for 10 min at room temperature. Then the wells were washed three times, stained with 0.5% crystal violet for 20 min at room temperature. Then, 50 μL of 1% sodium dodecyl sulfate was added to each well. The number of adherent cells was quantified by absorbance at 570 nm (D_{570}). Cell- matrix adhesion ratio was calculated by using the formula: experiment group D_{570}/control group D_{570}. Each experiment was repeated at least three times with identical results.

Cell aggregation assay Cells were washed with PBS twice and digested with 0.05% trypsin. Then were resuspended at 5×10^5 per 100 μL with DMEM media, and 100 μL of the cell suspension was seeded in each well of 96-well plates. The plates were placed in a 37℃ shaker and rotated at 80 r/min for 1 h. The wells were then gently stirred. The number of aggregates and single cells were counted with a hemacytometer. The rate of aggregation was calculated by the percentage of decrease in the number of single cells by using the formula $[(N_0 - N_i)/N_0]/100$. Where N_0 is the number of single cells at time 0, and N_i is the number of single cells detected in cultures at various time points after incubation. Each value is the average of at least three independent experiments.

RNA preparation and RT-PCR analysis To identify E-cadherin gene transcripts, quantitative real time PCR (qRT-PCR) was performed by using the Applied Biosystems. Total RNA isolation from cultured cells was performed by using Trizol on the basis of manufacturer's instructions (TAKARA). 1 μg of DNA-free total RNAs was reverse transcribed using PrimeScript RT Master Mix according to the manufacturer's instructions (TAKARA). For the qRT-PCR assay, 25 μL reaction containing 4 μL cDNA, 1 μL of each primer and 12.5 μL SYBR Premix Ex Taq™ (TAKARA) were used to monitor double-strand DNA synthesis. Primers used were 5′-AG-GACCAGGTGA-CCACCCTAGA-3′ (forward), 5′-ATGCCCA-AGATGGCAGGAAC-3′ (reverse) for E-cadherin and 5′-GCA-CCGTCAAGGCTGAGAAC-3′ (forward), 5′-TGGTGAAGACGCCAGT-GGA-3′ (reverse) for GAPDH.

Western blot Lysates were separated by 8% sodium dodecyl sulphatepolyacrylamide gels and transferred to nitrocellulose membranes. Anti-E-cadherin (1∶1 000; Bioworld), anti-α-tubulin (1∶1 000; Sigma), anti-CD29 (1∶1 000; BD Biosciences) antibodies were primary antibodies. After three times washing, membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody. Blots were developed with ECL plus Western blot detection system.

Statistical methods Statistical analysis was carried out using the SPSS software program.

Data, derived from three or four independent experiments, are presented as the means ± SD, and were analyzed with analysis of variance followed by the t test, with significance (P) set at 0.01.

Results

Expression of truncated N2-APC affected cell-cell adhesion in MDCK cells To verify whether N2 fragment of APC is involved in the regulation of cell-cell adhesion (cell aggregation), we examined the cell aggregation ratio of MDCK-N2-APC and the control MDCK-GFP cells. MDCK-N2-APC is stable cells which express residues 449-781 of APC. Fig 1A indicated clearly that cell aggregation ratio was decreased in N2-APC expressed cells. The followed calculation showed that aggregation ratio with N2-APC was reduced by about 30% compared with GFP control cells (P = 0.03, Fig 1B). These results suggested that N2-APC could affect cell-cell adhesion in MDCK cells.

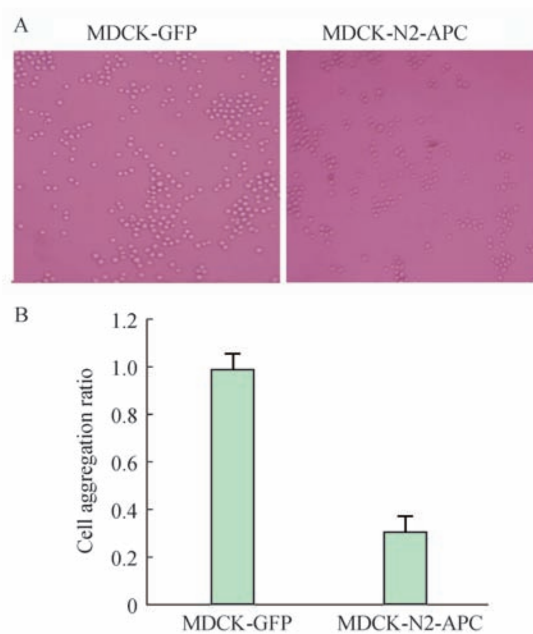

Fig 1 Cell-cell adhesion of MDCK stable cells was influenced by truncated APC

A: Photographs of MDCK-GFP and N2 cells at designated time points by microscope (×20). B: Cell-cell adhesion assay of MDCK-N2-APC cell and the control (P = 0.03). Bars represent the standard deviation of three independent experiments conducted in triplicate.

Expression of truncated N2-APC enervated E-cadherin expression level in MDCK E-cadherin is an essential molecule which has functions on cell aggregation process. In order to further elucidate the effect of N2 fragment on cell aggregation, we examined mRNA level of E-cadherin in these two stable cell lines, MDCK-N2-APC and MDCK-GFP. As shown in Fig 2A, N2 exhibited lower mRNA level of E-cadherin compared with control cells. The decreased extent was about 60% (P = 0.04). Similar results were observed in our subsequent immunostaining experiments. In N2-APC expressed cells, E-cadherin staining was sharp weaker than GFP cells (Fig 2B). Moreover, we examined the protein expression level of E-cadherin by using Western analysis. Consistent with the above-mentioned results, N2-APC expressed cells had a lower level of E-cadherin (Fig 2C). Together, these results suggest that reduced aggregation of N2-APC expressed cell was possibly caused by diminishing E-cadherin expression level.

Truncated N2-APC promoted cell-matrix adhesion in MDCK cell line To determine the effects of N2-terminal APC on cell adhesion, we examined the cell adhesion ratio of MDCK-N2-APC to MDCK-GFP control. Compare with control cells of MDCK-GFP, we found that cells expressed N2-APC had relatively quick adhesion for various periods of time (Fig 3A). Then, we applied crystal violet to make further assay to the adhesion ratio. Consistent with the previous results, cell adhesion ratio was markedly increased in N2-APC stably expressed cells (P<0.01, Fig 3B). The rising extent was averagely 180%. Taking advantage of SPSS significance test, it demonstrated that these variations of cell adhesion ratio were pretty much behavior significant difference. At four different time points, cell-matrix adhesion ratios were 2.22 ± 0.045, 2.24 ± 0.024, 1.39 ± 0.015 and 1.36 ± 0.012 in N2 expressed cells respectively. Taken together, these results indicated that there is an intimate relationship between cell-matrix adhesion and N2-APC. Over-expression of N2-APC fragment of APC might affect the ability of MDCK cells to the matrix.

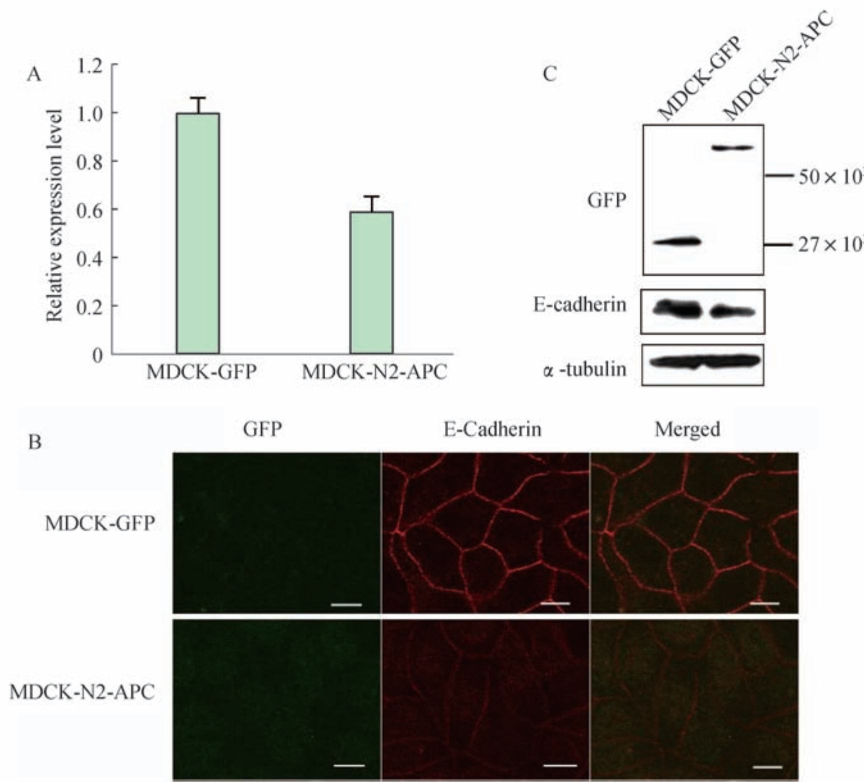

Fig 2 Cell-cell adhesion was reduced in MDCK-N2-APC cells

A: Quantitative RT-PCR analysis of E-cadherin in MDCK-N2-APC and MDCK-GFP cells ($P = 0.04$). Relative mRNA level was normalized with MDCK-GFP mRNA. The data were obtained from three independent experiments. The error bars represent the SD. B: Immunostaining of E-cadherin protein (red). Scale bars are 10 μm. C: Western blot analysis of E-cadherin in MDCK-N2-APC and MDCK-GFP cells. Western analysis for α-tubulin from the same blot is shown as a loading control.

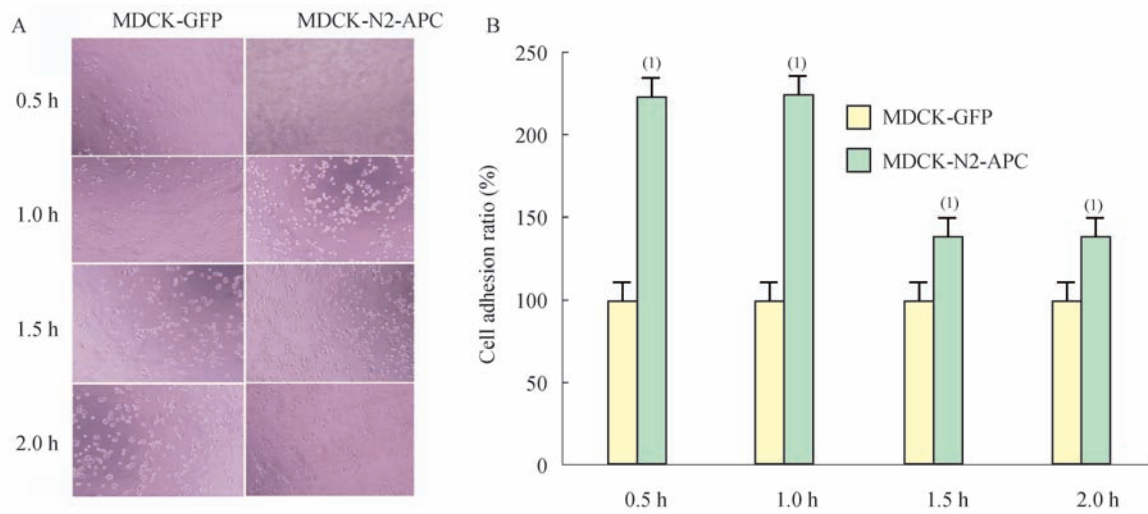

Fig 3 Truncated N2-APC increased cell-matrix adhesion in MDCK

A: Photographs of MDCK-N2-APC and MDCK-GFP cells at different time points by microscope (×20). B: Cell-matrix adhesion assay in MDCK-N2-APC and MDCK-GFP expressed stable cells. The data were obtained from three independent experiments. The data are expressed as the means of three independent experiments ± SD. (1) *vs.* MDCK-GFP, $P < 0.01$.

Truncated N2-APC increased the expression of integrin beta-1 in MDCK It has been known that CD29 is a key molecule in the process of cell-matrix adhesion. To further study how truncated APC affect cell adhesion, we examined the protein expression level of CD29 in these two stable cell lines. As shown in Fig 4, Western blot result illustrated that CD29 expression level was notably increased in MDCK-N2-APC cells, which suggested that N2 fragment could enhance cell adhesion by improving CD29 expression level.

On analysing CD29 protein in MDCK-GFP and MDCK-N2-APC cells, Western analysis for α-tubulin from the same blot was shown as a loading control.

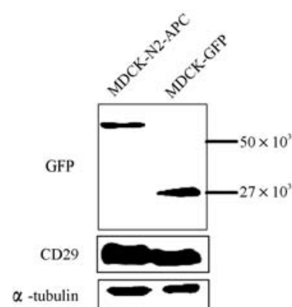

Fig 4 Integrin beta-1 expression level was increased in MDCK-N2-APC

Discussion

APC is a cytoskeletal organizer for cell migration and a scaffold, and it affects GSK3β/CKI-mediated phosphorylation and degradation of the Wnt key molecular β-catenin. A majority of colorectal cancers exhibit inactivating mutations in APC, and loss of APC function is an early event during tumorigenesis[6]. Abrogation of cell-adhesive properties is a hallmark of invasive carcinomas. Full length APC has more recently shown to play a role in cell-cell adhesion. It has been detected at lateral plasma membrane in some cells, and loss of APC correlated with defective intercellular adhesion[7]. Previous studies implicated APC in cell adhesion because expression of full-length APC in colon carcinoma cells restored cell-cell adhesion[8]. It was reported in another study, however, that loss of both *APC*1 and *APC*2 did not impair cadherin-based cell-cell adhesion in Drosophila[9]. Our results found that APC had a crucial role in cell adhesion. Truncated APC could result in significant variation of cell adhesion and aggregation in MDCK cells. The experiments implicated that N2-APC fragments has distinct effects on cell adhesion.

The specific mechanism of APC regulates cell adhesion and aggregation has not been completely clear. Harris[10] *et al* considered it unlikely that APC regulates cell-cell adhesion directly, as the interaction of APC with V/E-cadherin is minimal. However, Faux[8] *et al* also observed changes in the post-transcriptional modification of E-cadherin in APC truncated cells, which could be linked to its APC-induced redistribution to the plasma membrane. Kamal[11] and colleague testified that APC could dominate cell adhesion indirectly by regulating the validity of β-catenin for connecting into adherens junctions or by regulating vesicle transporting to and from the plasma membrane by tethering microtubules to cell-cell contacts.

In keeping with recent reported studies, we also found that truncated APC has powerful influences on cell adhesion and aggregation. N2 fragment probably affected cell adhesion by regulating certain molecules which participate in cell-cell contacts process. In other words, intracellular distribution and expression level of these adhesion molecules were changed owing to loss of APC function. Consistent with previous results, the pathways of truncated APC that affects expression of these adhesion molecules may associate with alteration of β-catenin. Specifically, the basics of canonical Wnt signaling are well known: In the absence of Wnt signaling, cytoplasmic β-catenin is recruited to a complex containing axin, APC and GSK-3β, which is then able to phosphorylate β-catenin, triggering its ubiquitin-dependent degradation. When the signaling pathway is aberrant activated, such as truncated mutation of APC, APC-β-catenin-GSK-3β complex disaggregated, which can result in accumulation of β-catenin and its subsequent translocation into the

nucleus[12]. Because of the interaction between β-catenin and E-cadherin, redistribution of β-catenin can also lead to similar changes in E-cadherin, and can reduce its expression level in cell membrane. Integrin α3 is a transmembrane integrin receptor subunit which forms heterodimers with integrin β1 (CD29) in MDCK cells[13]. α3β1 integrin is a laminin receptor that localizes to sites of cell-cell contacts through its interaction with the E-cadherin/β-catenin complex[14]. Therefore, when APC mutated, β-catenin began to accumulate in cytoplasm. In the meantime it affected the expression level of CD29.

This study demonstrated that there is an intimate relationship between N2-APC and cell adhesion. In MDCK cells, N2 may enhance cell adhesion via ascend of CD29, and reduce cell aggregation through lowering E-cadherin expression level. Evidence is beginning to emerge that APC proteins may be another example of a dual-function protein with dividable roles in Wnt signaling pathway and cell adhesion[5]. However, up to now, it has not been shown that APC proteins participate directly in the two processes. There are multiple comprehensions to explain the mechanism how does APC control cell adhesion. Our data support the standpoint of Kamal[11], that truncated APC may influence cell adhesion by regulating β-catenin, which has interactions with certain adhesion molecules such as E-cadherin and CD29. This work concretely focuses on the N2 fragment of APC, which affects cell adhesion and aggregation. Our experiments demon-strated that N-terminal of APC, especially N2 fragment, has an important role in the process of cell adhesion. Our data provided novel evidence for studying the effects of APC truncation on cell adhesion in colon cancer cells. Further understanding on the mechanism of APC and cell adhesion will have a significant impact on promoting the fundamental study in the areas pertaining to APC and carcinomas, which in turn will hold great potential for clinical therapy of patients with tumors.

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