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N-terminal truncation mutations of adenomatous polyposis coli are associated with primary cilia defects

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ABSTRACT

Adenomatous polyposis coli (APC) gene is a tumor suppressor gene and its truncated mutations cause a few cilia-related diseases such as Gardner's syndrome. However, little is known about the mechanism that links APC mutations and cilia disorder. APC mutations lead to the expression of N-terminal fragments, which have dominant effects in tumors owing to loss of the C-terminal region or a gain of function. The present study investigated the impact of tumor-associated N-terminal APC fragments on primary cilia assembly and the possible molecular mechanism involved. We discovered that expression of tumor-associated N-terminal APC fragments (APC-N, APC-N1, APC-N2, and APC-N3, which contain amino acids 1-1018, 1-448, 449-781, and 782-1018 respectively), resulted in primary cilia defects. We found that a β-catenin/PI3K/AKT/GSK-3β feedback signal cascade is responsible for causing N-terminal APC fragment-induced cilia defects. In this cascade, dysfunctions of both β-catenin and GSK-3β were involved in the up-regulation of HDAC6 and subsequent decreased acetylated tubulin levels, which thereby led to cilia defects. These data suggest a mechanism for linking N-terminal APC fragments and cilia loss, thus accelerating our understanding of human cilia-related diseases such as Gardner's syndrome and their cause due to APC mutations.

1. Introduction

Adenomatous polyposis coli (APC) gene is a tumor suppressor gene and was first identified and isolated in familial adenomatous polyposis (FAP) (Groden et al., 1991; Kinzler et al., 1991). The APC gene encodes a multifunctional protein participating in several fundamental cellular processes. APC inhibits classical Wnt signaling through forming complexes with both GSK3β and axin to promote β-catenin degradation (Cobas et al., 2004). APC is also involved in other vital processes including cellular adhesion and migration, organization of actin and microtubule skeleton network, spindle formation, and chromosome segregation (Aoki and Taketo, 2007). Mutations in APC gene are commonly responsible for sporadic colon cancer, and FAP, an autosomal dominantly inherited disease (Groden, Thliveris, 1991). APC mutations result in loss of C-terminal regions and the expression of N-terminal fragments. N-terminal APC fragments have been suggested to not only alter its tumor suppressor function but may also have oncogenic properties (Tighe et al., 2004). Truncating APC mutations can also exert dominant functions such as causing chromosome instability (Green and Kaplan, 2003; Tighe et al., 2004).

Cilium is a conserved microtubule-based organelle projecting from surface of cells. The axoneme of cilium arises from a basal body, which differentiates from a mother centriole. Based on the differences of microtubules that the axoneme is consisted of, cilia are classified into "9 + 2" motile cilia and "9 + 0" primary cilia. Primary cilia are found on most human cell types and considered as a kind of degenerative organelle (Bisgrove and Yost, 2006; Fliegauf et al., 2007). Recent works have showed that primary cilia are not only involved in diverse sensory function but also participate in controlling cell proliferation and are involved in several developmental pathways including Hedgehog, Wnt, and PDGFα signaling pathways (Michaud and Yoder, 2006). Because of the roles of cilia in development and physiology, cilia defects have been

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associated with a range of diseases such as cystic kidney disease, liver fibrosis, and brain malformations, which collectively are termed ciliopathies (Bisgrove and Yost, 2006; Fliegauf et al., 2007; Quinlan et al., 2008).

Interestingly, truncating APC mutations also exist in some cilia-related diseases, implying a connection between APC mutations and cilia disorder (Gomez Garcia and Knoers, 2009; Li et al., 2012). Gardner's syndrome is a variant of FAP and used to describe extracolonic manifestations of FAP patients, such as osteomas, skin tumors, congenital hypertrophy of the retinal pigment epithelium (CHRPE), and desmoid tumors. Gardner's syndrome and cilia-related diseases share common clinical manifestations, while truncating APC mutations are the pathogenic molecular basis of Gardner's syndrome. These data provide a clue for APC mutations and their involvement in cilia disorder. However, no direct connections between them have been reported. In this study we found that tumor-associated N-terminal APC fragments can exert dominant effects which can cause cilia defects. We suggest that a feedback loop of β-catenin/PI3K/AKT/GSK-3β regulates N-terminal APC fragment-induced cilia defects. In this loop, both β-catenin and GSK-3β acted to up-regulate HDAC6 which led to decreased acetylated tubulin levels, thereby causing cilia defects. The present study provides a new insight into the relationship between APC mutations and disease caused by primary cilia abnormality.

2. Materials and methods

2.1. Plasmids and antibodies

Plasmids for expression of GFP-APC-N, GFP-APC-N1, GFP-APC-N2, and GFP-APC-N3 (gifts from Prof. Inke S. Nathke) have been described previously (Li et al., 2008b; Li and Nathke, 2005). Mouse monoclonal anti-acetylated tubulin and anti-γ-tubulin were from Sigma. Rabbit polyclonal anti-pericentrin and anti-GSK3β were from Abcam. Rabbit polyclonal anti-P-Rb (Ser807/811), P-GSK3β (Ser9), anti-P-AKT (Thr308), AKT, and anti-GFP were from Cell Signaling Technology. Rat anti-α-tubulin was a generous gift from Prof. Inke S. Nathke (University of Dundee, UK). Mouse monoclonal anti-β-catenin was from Abmart. Rabbit polyclonal anti-HDAC6 was from Bioword Technology. Secondary antibodies for immunoflourescence microscopy (IFM) were TRITC-conjugated goat-anti-mouse IgG and goat-anti-rabbit IgG (all from Invitrogen), and Cy5-conjugated goat-anti-rabbit IgG (CW Biotech). Secondary antibodies for western blotting were HRP-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG and goat-anti-rat IgG (all from Invitrogen).

2.2. Cell culture, transfection, and stable cell lines

Swiss NIH3T3 mouse fibroblasts and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum and 10 ml/L penicillin–streptomycin at 37 °C in 5% CO_2. 70–80% confluent NIH3T3 fibroblasts were serum-starved for 24 h to induce growth arrest. NIH3T3 fibroblast cultures were passaged every 3–4 days by trypsinization (0.5%) and only passage numbers 10–30 were used for experiments. NIH3T3 and MDCK cells were transfected using Lipofectamine 2000 (Invitrogen, UK) according to the manufacturer's instructions. MDCK cells stably expressing GFP, GFP-APC-N1, GFP-APC-N2, and GFP-APC-N3 were generous gifts from Prof. Inke S. Nathke (University of Dundee, UK). About 90% confluent MDCK cells transiently expressing GFP-APC-N and stably expressing GFP-APC-N1, GFP-APC-N2, and GFP-APC-N3 were examined after further culturing for 3 days to grow cilia.

2.3. Drug treatment experiments

The GSK3β inhibitor SB216763 (Sigma), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Beyotime Institute of Biotechnology), and HDAC inhibitor trichostatin A (TSA) (Sigma) were added to MDCK cells expressing N-terminal APC fragments during 3 days of further culturing. On the basis of initial experiments to establish effective range, SB216763, LY294002, and TSA were used at 0.05 μM, 40 nM, and 30 μM, respectively.

2.4. Immunofluorescence microscopy

Cells grown on glass coverslips were fixed in methanol for 5 min, permeabilized in 0.2% Triton X-100, quenched in PBS with 3% BSA, and then incubated with primary antibodies at 4 °C for 3 h. After washing in PBS, cells were incubated with secondary antibodies for 1 h and DAPI for 20 min at room temperature. Antibodies were used at the following dilutions: anti-acetylated tubulin: 1:1000; anti-γ-tubulin: 1:1000; anti-pericentrin: 1:1000. Fluorescent secondary antibodies and DAPI were diluted at 1:100. Cells were visualized using a Delta Vision restoration microscope (Applied Precision, Issaquah, WA) or an IX70 confocal laser scanning microscope (Olympus, Tokyo, Japan). Digital images were processed with Adobe Photoshop CS version 8.0.1. Approximately 100 cells from three independent experiments were counted. Data are presented as the mean ± standard deviation.

2.5. RNA interference

For knockdown of β-catenin, 1×10^6 MDCK cells expressing N-terminal APC fragments were transfected with 40 nM of siRNA (Dharmacon) and Lipofectamine 2000 according to the manufacturer's instructions. After transfection, cells were further cultured for 3 days to grow cilia.

2.6. Protein extraction, SDS-PAGE, and western blotting

Cells grown in petri dishes were treated with western lysis buffer for 10 min and then centrifuged at 13,000 × g to precipitate insoluble material. Protein concentrations of the supernatants were measurement using a BCA protein kit (Beyotime Institute of Biotechnology). Total cell lysates (40 μg) were separated by 4–12% or 12% SDS-PAGE and transferred to nitrocellulose membranes using BIO-RAD Mini Trans-Blot system. The membranes were blocked in 5% milk in TBST (10 mM Tris/HCL (PH 7.5), 120 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with primary antibodies diluted in block solution overnight at 4 °C. Primary antibodies were diluted as follow: anti-acetylated tubulin; 1:1000, anti-GSK3β; 1:1000, anti P-GSK3β (Ser9); 1:1000 anti-β-catenin; 1:1000, anti-GFP; 1:1000, anti-P-AKT (Thr308); 1:500, anti-HDAC6; 1:500, anti-P-Rb (Ser807/811); 1:500, and anti-α-tubulin; 1:100. Detection was performed with HRP-conjugated anti-rabbit, anti-rat or anti-mouse antibodies (1:1000) followed by enhanced chemiluminescence (Pierce, Tottenhall, United Kingdom).

2.7. Quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Takara Biotechnology, Japan), and cDNA was generated using PrimeScript RT Master Mix. (Takara Biotechnology, Japan). For quantitative real-time PCR assay, 25 μL reaction mixture containing 4 μL cDNA, 1 μL primers and 12.5 μL SYBR Premix Ex TaqTM were used to detect double-stand DNA synthesis. The expression of *HDAC6* gene was normalized to GAPDH mRNA content. The primer