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Tyrosine phosphorylation of β-catenin affects its subcellular localization and transcriptional activity of β-catenin in Hela and Bcap-37 cells

He-Ya Qian [a], Ding-Guo Zhang [a], Hong-Wei Wang [a], Dong-Sheng Pei [a,*], Jun-Nian Zheng [a,b,*]

[a] Jiangsu Key Laboratory of Biological Cancer Therapy, Xuzhou Medical College, 84 West Huai-hai Road, Xuzhou 221002, Jiangsu, China
[b] Center of Clinical Oncology, Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, China

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ABSTRACT

In order to investigate the relationship between tyrosine phosphorylation of β-catenin and transcriptional activity of β-catenin in Hela and Bcap-37 cells, genistein (a tyrosine kinase inhibitor) was used to inhibit tyrosine phosphorylation in cells. Our results showed the total β-catenin protein levels were mainly equal in Hela, Bcap-37 and HK-2 cells, β-catenin was mainly present in nucleus in Hela and Bcap-37cells, while in HK-2 cell β-catenin was mainly located in cytoplasm. Genistein could inhibit tyrosine phosphorylation of β-catenin and downregulate nuclear β-catenin expression in Hela and Bcap-37 cells. In addition, genistein suppressed Ki-67 promoter activity and Ki-67 protein level, thus promoted cell apoptosis. Furthermore, β-catenin could increase the Ki-67 promoter activity in Hela and Bcap-37 cells. From these findings we conclude that tyrosine phosphorylation of β-catenin can regulate the cellular distribution of β-catenin and affect the transcriptional activity of β-catenin.

The Wnt signaling transduction pathway plays an important role in a number of developmental processes, including body axis formation and mouse mammary gland development,[1,2] and also known to be involved in tumorigenesis.[1,3,4] The Wnt family of proteins consists of more than 15 closely related secreted glycoproteins. Receptors for the Wnt proteins are members of the frizzled family of *trans*-membrane proteins, and the Wnt signal is transduced to a cytoplasmic protein, Dishevelled (Dvl). Upon activation by the Wnt signal, Dvl inhibits the activity of glycogen synthase kinase-3β (GSK-3β). In the absence of the Wnt signal, GSK-3β is thought to phosphorylate and consequently induce the degradation of β-catenin. Therefore, the Wnt signal stabilizes and causes the accumulation of β-catenin, which in turn associates with TCF/LEF family transcription factors, ultimately altering the expression of Wnt signaling target genes.[5–9]

β-Catenin consists of an N-terminal region of approximately 130 amino acids, a central region of 550 amino acids, and a C-terminal region of 100 amino acids.[1,2] The N-terminal region contains consensus phosphorylation sites for GSK-β, while the C-terminal region possesses the *trans*-activator function required for activation of target genes. The central region contains 12 imperfect sequence repeats of 42 amino acids known as armadillo repeats, which are required for the interaction with various proteins, including cadherins, APC and TCF/LEF.[10] β-Catenin is a multifunctional protein that plays important roles in both cell–cell interactions and transcriptional regulation. As a transcriptional regulator, β-catenin is the critical effector of the canonical Wnt signaling pathway, in which nuclear β-catenin co-activates transcription in association with T-cell factor/lymphoid enhancer factor (TCF/LEF) family members.[5,11]

Ki-67 protein encoding by Ki-67 gene is a DNA-binding protein, which is obligatory for the carcinoma cells proliferation.[12] In addition, Ki-67 was also measured in order to evaluate the interplay of β-catenin with proliferation and elucidate their possible interrelation in Wnt-signaling pathway.[13] Tyrosine phosphorylation of β-catenin has been reported to play important roles in cadherin complex and transcriptional regulation.[14] A number of oncogenic tyrosine kinases have been reported to directly promote tyrosine phosphorylation of β-catenin in melanoma, breast and pancreatic cancer in chronic myelogenous leukemia,[15,16] however the relationship between tyrosine phosphorylation of β-catenin and Ki-67 transcriptional activity has not been fully elucidated.

In the current study, we evaluated the correlation between tyrosine phosphorylation of β-catenin and transcriptional activity of β-catenin in Hela and Bcap-37 cells. Our data demonstrated that tyrosine phosphorylation of β-catenin enhanced β-catenin

* Corresponding authors. Tel.: +86 0516 85582513 (D.-S. Pei).
E-mail addresses: dspei@xzmc.edu.cn (D.-S. Pei), jnzheng@xzmc.edu.cn (J.-N. Zheng).

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translocation from cytoplasm into nucleus in Hela and Bcap-37 cells. Furthermore, we also identified that nuclear accumulation of β-catenin upregulated Ki-67 promoter activity in Hela and Bcap-37 cells. Taken together, our study provides evidence that tyrosine phosphorylation of β-catenin affects its subcellular localization and transcriptional activity of β-catenin in Hela and Bcap-37 cells.

The HK-2 (the human proximal tubular epithelial cells), Hela (the human cervical cancer cells) and Bcap-37 (the human breast cancer cells) cells lines were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Hela cells were cultured in DMEM, HK-2 cells were grown in DMEM/F12 = 12:1 and Bcap-37 cells were maintained in RPMI 1640. All these mediums were supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C.

Firefly luciferase expression plasmid pGLBK235 (contains the Ki-67 gene core promoter sequence (−223 to +12) which was introduced into pGL3-Basic), was generated by our laboratory previously. pcDNA3.1 (empty control vector) and β-catenin expression plasmid were purchased by genecopoeia Inc (Guangzhou FulenGen Co, Ltd). The basic control plasmid pGL3-Basic was products of Promega Corporation. β-Catenin si-RNA and nonspecific si-RNA were purchased from santacruz.

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were plated at a density of 1×10^5 cells per well in 24-well dishes and allowed to settle overnight. The following morning, pGL3-BK235 plasmid was co-transfected with β-catenin expression plasmid and/or different concentrations of genistein (0, 5, 10, 20, 40 mM). Cell extracts were prepared for luciferase activity assays. Firefly and Renilla luciferase activities in the cell lysates were measured using the Dual-Luciferase® Reporter Assay System (Promega, USA) according to the manufacturer's instructions. The firefly luciferase activity was normalized by the Renilla luciferase activity. Results were presented as the relative luciferase activity.

Cells seeded in 6-well plates were cultured with different concentrations of genistein in the presence of 5% CO_2 at 37 °C. After 24 h immunocytochemistry was performed according to the avidin biotinylated-HRP complex (ABC) method using a standard ABC kit (Zhongshan biotech, China). Cells were washed in cold phosphate-buffered saline (PBS) for 3 times, fixed in paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for another 15 min at room temperature, and incubated with primary antibody Ki-67 (1:200) (Santa Cruz Biotechnology, USA) overnight at 4 °C. The cells were then incubated with the secondary antibody for 30 min at room temperature. Diaminobenzidine (DAB) (Zhongshan Biotech, China) was used to produce a brown precipitate, and then cells were observed under invert microscopy (Nikon CX21FS1, Japan). Negative control was performed by replacing the primary antibody with PBS. The nuclei that appeared brown were positive cells. The grayscale analysis of the immunohistochemistry was analyzed by image pro plus software.

Hela and Bcap-37 cell lines were analyzed for apoptosis, using the Annexin V-FITC kit. Briefly, cells (1 ml, 2×10^5/ml per well) were plated in 24 well plates. When cells were grown to 70–80% confluence, cells were treated with genistein for 24 h. Then, cells were washed twice with medium, then incubated with Annexin V-FITC, and then visualized under a fluorescence microscope. Under microscopy, 6 fields were randomly selected from every sample and independent observers performed cell counting in a blind fashion. The apoptotic rate = (number of total apoptotic cells/total number of cells) × 100%.

β-Catenin proteins were extracted from cells and quantified with nuclear and cytoplasmic Protein Extraction Kit. Protein concentrations were determined using the BCA Kit (Pierce, USA) according to the manufacturer's instructions. Equal amounts of protein (100 mg/lane) were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking for 3 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies in TBST for 2 h and developed using NBT/BCIP colour substrate (Promega, Madison, WI, USA). The densities of the bands on the membrane were scanned and analysed with an image analyser (LabWorks Software, UVP Upland, CA, USA).

Equal amounts of proteins (400 mg/lane) were diluted 4-fold with 50 mM HEPES buffer, (pH 7.4), containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40 and 1 mM each of EDTA, EGTA, PMSF and Na3VO4. Proteins were incubated with p-Tyr antibody (Santa Cruz Biotechnology, USA) immobilized onto protein A-Sepharose for 1 h at 4 °C, and then centrifuged to remove proteins adhered non-specifically to protein A. The supernatants were incubated with 1–2 mg primary antibodies for 4 h or overnight at 4 °C. Protein A was added to the tube for another 2 h incubation. Samples were centrifuged at 10,000*g* for 2 min at 4 °C and the pellets were washed with immunoprecipitation (IP) buffer for three times. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS–PAGE loading buffer and then isolated by centrifuge. The supernatants were used for immunoblot analysis. We select immunoblotting bands of β-catenin as a loading control.

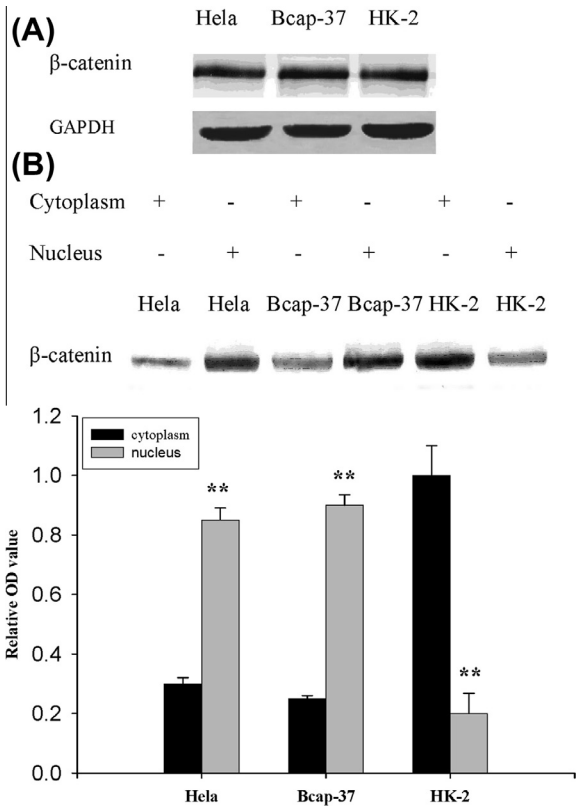

Figure 1. Subcelluar localization of β-catenin in cells. Cells were grown to 90–95% confluence and the protein levels were determined by Western blot assays. (A) Immunoblotting analysis of the total protein levels of β-catenin with anti-β-catenin antibody. GAPDH was used as a control for protein loading. (B) Immunoblotting analysis of the nucleus and cytoplasm protein levels of β-catenin with anti-β-catenin antibody. Bands corresponding to β-catenin were scanned and the intensity was determined by optical density (O.D.) measurements. Data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus respective β-catenin protein level in cytoplasm.