RESEARCH COMMUNICATION

Gene Silencing of ß-catenin by RNAi Inhibits Proliferation of Human Esophageal Cancer Cells by Inducing G0/G1 Cell Cycle Arrest

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Abstract

  Objectives: The aim of the present study was to explore mechanisms underlying the effects of down-regulating ß-catenin expression on esophageal carcinoma (EC) cells. **Methods:** Cell cycle distribution and apoptosis were determined using flow cytometry and annexin V apoptosis assay, respectively. Transmission electron microscopy (TEM) was used to examine changes in ultrastructure, while expression of cyclin D1 protein and mRNA was detected by western blot and real-time PCR. Proliferating cell nuclear antigen (PCNA) and extracellular signal-regulated kinase (ERK) 1⁄2 were evaluated by Western blot analysis. PCNA labeling index (LI) was determined by immunocytochemistry. **Results:** Compared with pGen-3-con transfected and Eca-109 cells, the percentage of G0/G1-phase pGen-3-CTNNB1 transfected cells was obviously increased ($P<0.05$), with no significant difference among the three groups with regard to apoptosis ($P>0.05$). pGen-3-CTNNB1 transfected cells exhibited obvious decrease in cyclin D1 mRNA and protein expression ($P<0.05$) and the ultrastructure of Eca-109 cells underwent a significant change after being transfected with pGen-3-CTNNB1, suggesting that down-regulating β-catenin expression can promote the differentiation and maturation. The expression of PCNA and the ERKI/2 phosphorylation state were also down-regulated in pGen-3-CTNNB1 transfected cells ($P<0.05$). At the same time, the PCNA labeling index was decreased accordingly ($P<0.05$). **Conclusion:** Inhibition of EC Eca-109 cellproliferation by down-regulating ß-catenin expression could improve cell ultrastructure by mediating blockade in G0/G1 through inhibiting cyclin D1, PCNA and the MAPK pathway (p-ERK1/2).

  Keywords: ß-catenin - esophageal carcinoma - cell cycle - cyclin D1 - extracellular signal - regulated kinase1/2

Introduction

  Esophageal carcinoma (EC) is prevalent in some regions of the world, and occurs at a very high frequency in certain parts of China and the mortality rate ranked the fourth among cancer-related death (Zou et al., 2002; Jemal et al., 2011) . Esophageal squamous cell carcinoma (ESCC) is the most common subtype of EC. The previous studies have indicated that ß-catenin functioned as an oncogene in the cancerization process of ESCC, and accumulation of ß-catenin in cytoplasm and nucleus was frequent events during the carcinogenesis of the squamous epithelium of the esophagus (Kimura et al., 1999; de Castro et al., 2000; Zhou et al., 2002). Abnormal cytoplasmic/nuclear accumulation of ß-catenin was significantly associated with growth, invasion and lymphatic node metastasis in ESCC (Ji et al., 2007; Wang et al., 2011).

  Studies by us and other researchers have indicated that silencing of β-catenin gene could inhibit the proliferation

and growth of human EC Eca-109 cells in vitro and in nude mice (Veeramachaneni et al., 2004; Wang et al., 2009), but the exact mechanisms remained unclear. Recently, we conducted a comparative proteomic analysis to investigate the differentially expressed proteins between the established cell lines of pGen-3-con (Eca-109 cells transfected by control vector) and pGen-3-CTNNB1 (Eca-109 cells transfected by ß-catenin shRNA), and identified 13 differential proteins between the two cell lines by matrix-assisted laser desorption/ionization time-offlight mass spectrometry (MALDI-TOF-MS) (Ren et al., 2010). Though great improvement has been achieved in this domain, the current knowledge of the components of Wnt/ß-catenin pathway is still not sufficient to fully explain many aspects of ß-catenin function in ESCC, and it is still unclear what mechanisms and molecules are involved in the suppression of proliferation and growth of EC cells mediated by shRNA aginst ß-catenin.

  In this report, we used the established cell lines of

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pGen-3-con and pGen-3-CTNNB1 as cell models, and explored the preliminary mechanisms underlying the inhibitory effect of down-regulating ß-catenin expression on EC cells.

Materials and Methods

Cell Culture

Human ESCC cell line Eca-109 (Institute of Biochemistry and Cell Biology, Shanghai, China), which has a high expression of β-catenin, was stably transfected with pGen-3-con or pGen-3-CTNNB1 vector as described previously (Wang et al., 2009; Wang et al., 2010), and maintained in RPMI1640 medium (Gibco Biocult, Paisley, UK) supplemented with 10% calf bovine serum (Sijiqing Biotechnology, Hangzhou, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (North China pharmaceutical Group Corporation, Shijiazhuang, China) at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. For G0/G1 synchronization, when cells grew to 70% confluence, the routine medium was removed and replaced by free-serum medium for 24 h.

RNA Isolation and QRT-PCR Analysis

Total RNA was extracted from specimens using Trizol reagent (Invitrogen) and treated with DNaseI (Tiangen Biotechnology, Beijing, China). The quantity and purity of RNA were determined by UV absorbance spectroscopy. cDNA was synthesized from 2 µg of total RNA according to the manufacturer's instruction in a total volume of 25 µL (Fermentas, Maryland, USA), and negative control reactions were run without reverse transcriptase (RT). Quantitative real-time PCR was carried out using FTC2000 real-time PCR system (Funglyn, Toronto, Canada) and Shine Sybr Real Time qPCR Kit (Shine-Gene) according to the manufacturers' protocol. Sequences for the primers and PCR condition for each gene are shown in Table 1 and experiments were performed three times in the same reaction. To quantify difference in gene expression, the analysis of the relative gene expression of cyclin D1 was performed as described by Livak and Schmittgen (Livak and Schmittgen, 2001) who used the 2-DDC(t) method. Using this method, the data are presented as the fold change in the target genes (cyclin D1) in pGen-3-CTNNB1 normalized to β-actin and relative to pGen-3-con cell line.

Western Blot Analysis

Cells were washed twice with ice-cold phosphatebuffered saline (PBS), collected by adding 0.25%

Table 1. Primers and Real-time PCR Conditions for Each Gene

Gene name	Primer	Annealing (°C)	Product (bp)
CyclinD1			
sense	5'GCAATGACCCCGCACGAT3'	55	110
antisens	5'GCACAGAGGGCAACGAAGG3'		
β-actin			
sense	5'TGACGTGGACATCCGCAAAG3'	60	205
antisens	5'CTGGAAGGTGGACAGCGAGG3'		

trypsin and lysed in buffer (50 mMTris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% TritonX-100, 0.1% sodium dodecyl sulfate [SDS], 1 mM NaF, 1 mM Na3VO4), and protease inhibitors (10 mg/L aprotinin and 1 mM phenylmethylsulfonyl fluoride) were added to obtain whole cell protein. Equal amounts of cell protein, quantified by Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA), were subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk in Tris Buffered Saline with Tween 20 (TBST, 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) for 2 h at room temperature, and subsequently incubated with primary antibody (anti-cyclin D1, 1:200, ß-actin, 1:2000, anti-PCNA, 1:200, anti-ERK, 1:500, anti-p-ERK, 1:500, were purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA) in blocking buffer at 4 °C overnight. Following a wash with TBST, the membranes were incubated with horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (1:1000, Dako, Glostrup, Denmark) for 2 h at room temperature. The membranes were washed with TBST, and protein bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (KPL, Gaithersburg, USA). The ß-actin bands were taken as loading control. The protein quantity was analyzed by UTHSCSAImage Tool 3.0. The target protein expression was evaluated by the relative intensity ratio of target protein/loading control.

Flow Cytometry Assay

Cell cycle distribution was determined by staining DNA with PI (Nunez 2001). In brief, According to the routine method, 1×10^6 cells from three groups were harvested by trypsinization and centrifugation, respectively, washed twice with ice-cold PBS and resuspended in PBS containing 10 mg/L propidium iodide (Sigma) and 100 mg/L RNase A (Huamei Biotechnology, Wuhan, China) and then incubated at 25 °C in the dark for at least 30 min. The percentage of cell population in each phase of the cell cycle was measured using FACStar and the results were analyzed with the software CELLQUEST (Becton Dickinson and Company, New jersey, USA).

Annexin V apoptosis assay

Apoptosis was also determined through Annexin V-APC Apoptosis Detection Kit (eBioscience, San Diego, USA) as described by the manufacture's instruction. Briefly, cells were trypsinized, washed with PBS, and centrifuged. The cells were washed with 1×binding buffer, centrifuged and resuspended in 1 ml 1×staining buffer. One hundred microlitre cell suspension prepared as above were added 5 µl Annexin V-APC, gently vortexed and incubated for 10 min at room temperature in the dark. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using dedicated software. The experiment was repeated in triplicate. Annexin V-APC binds to phospholipids that are inaccessible in viable cells, since they are located in the inner leaflet of the plasma membrane, but become

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accessible early in apoptosis when they are translocated to the outer leaflet of the plasma membrane or late when membrane integrity is lost (Van et al., 1998).

Immunocytochemistry

Cells were cultured on the coverslips placed in six well plates and then fixed with 4% paraformaldehyde for 15 min at room temperature. Next, cells were treated with 1 g/L TritonX-100 for 30 min and subsequently incubated with a blocking solution (10% normal goat serum in PBS) for 20 min, anti-PCNA monoclonal antibody in PBS (1 : 100, Santa Cruz, California, USA) overnight at 4 °C and followed by horseradish peroxidase-labeled goat anti-mouse IgG (Dako) for 1h at room temperature. The negative control was incubated with PBS instead of primary antibody under the same conditions. Finally, the cells were visualized and photographed with an Olympus fluorescence microscope (Tokyo, Japan). With regard to the PCNA-LI, only cells showing nuclear staining were considered to be positive. We randomly selected 10 high-power (400×) microscopic fields and counted the positive cells per field and total cell number, and then determined the average PCNA-LI. PCNA-LI = number of positive cells/total number of cells in each field×100% (15).

Transmission Electron Microscopy

TEM was used to examine the changes of ultrastructure in two different cells, 1×10^7 cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for 30 min. Fixed cells were subsequently washed with PBS and post-fixed with 1% osmium tetraoxide for 1h at pH 7.4. Samples were dehydrated in an ascending series of ethanol and infiltrated with absolute ethanol and resin, followed by final embedding in resin and allowed to polymerize and embed at 60 °C overnight. Samples were cut by an ultramicrotome, mounted on nickel grids and doubly stained with uranyl acetate and lead citrate. The grids were viewed in a Philips CM120 BioTWIN TEM (FEI, OR, USA).

Statistical Analysis

All measurements were carried out with the same instrument under the same experimental condition. Data were expressed as means ± standard deviation. All data were analyzed statistically by Student's t-test, with P<0.05 considered to be significant.

Results

Cell Cycle Kinetics of pGen-3-CTNNB1 Cell Line

Compared with that of pGen-3-con transfected and Eca-109 cells, the percentage of G0/G1-phase in pGen-3-CTNNB1 transfected cells obviously increased (P<0.05) (Figure 1). However, as to apoptosis, there is no significant difference in three groups (P>0.05) (Table2). Therefore, the growth inhibitory effect mediated by silencing of ß-catenin gene might be due to the G0/G1 cell cycle arrest but not apoptosis in Eca-109 cells.

Expression of Cyclin D1 Regulated by β-catenin

The down-regulation of ß-catenin by RNA interference

Table 2. Comparison of Apoptosis in Three Group Cells

| Group	pGen-3-CTNNB1	pGen-3-con	Eca-109
Apoptosis (%)	1.39±0.12*	1.51±0.28	1.42±0.20

*P<0.05, compared with Eca-109 cells

Table 3. Comparison of the Relative Abundance of Cyclin D1 mRNA in Three Group Cells

| Group	β-actin Ct Value	Relative copy number	CyclinD1 Ct Value	Relative copy number	Relative ratio CyclinD1/β-actin
pGen-3-con	11.065	2.22E+10	15.542	1.09E+09	0.497
Eca-109	15.5	1.12E+09	19.528	7.40E+07	0.66
pGen-3-CTNNB1	11.403	1.76E+10	18.404	1.58E+08	0.09*

*P<0.05, compared with Eca-109 cells
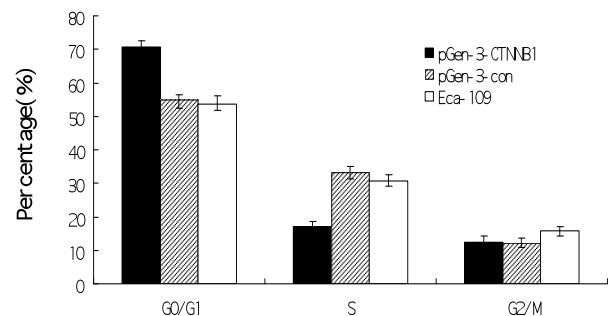
Figure 1. Effect of Down-regulation of ß-catenin Expression on Cell Cycle Progression. Cell cycle distribution determined by flow cytometry in three group cells. Data represent the mean±S.D. of three independent experiments. *P<0.05, as compared with Eca-109 cells
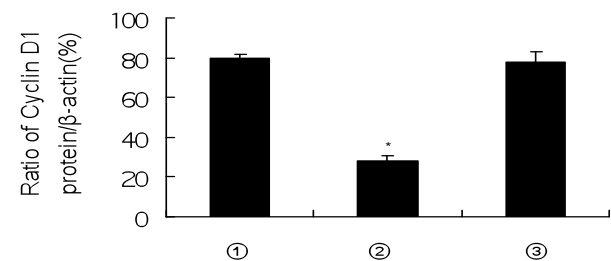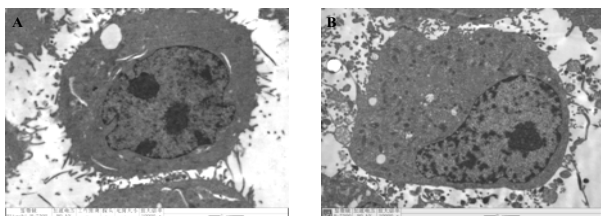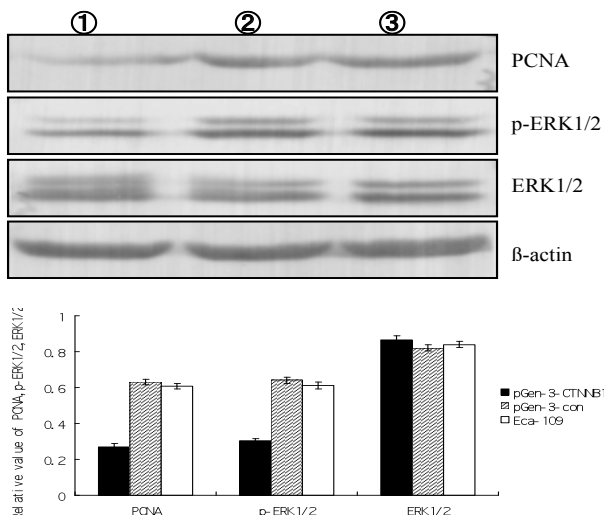

Figure 2. Western Blot Analysis of Cyclin D1 Protein Levels in Three Group Cells. ß-actin was employed as a loading control. The results shown are representative of three independent experiments: 1. Eca-109; 2. pGen-3-CTNNB1; 3. pGen-3-con. *P<0.05, as compared with Eca-109 cells

(RNAi) can be confirmed by measuring cyclin D1 expression, a known Tcf/Lef target gene (Tetsu and McCormick, 1999). The difference in cyclin D1 mRNA among these cells was analyzed by quantitive RT-PCR. As shown in Table 3, compared with the pGen-3-con transfected cells, the pGen-3-CTNNB1 transfected cells revealed an obvious decreasing of cyclin D1 mRNA (P<0.05). The down-regulated efficiency of cyclin D1 protein was analyzed by western blot. The expression intensities of cyclin D1 protein in pGen-3-CTNNB1 transfected cells, pGen-3-con transfected cells and Eca-109 cells were 24.6±2.3%, 79.7±2.6%, and 78.3±2.1%, respectively (Figure 2), and a markedly decreasing of expression of cyclin D1 protein in pGen-3-CTNNB1 transfected cells, which was consisted with the result

Figure 3. Effect of Silencing of β-catenin Gene on Eca-109 Cells Ultrastructure. A: pGen-3-con; B: pGen-3-CTNNB1. (Original magnification × 10 000)

Figure 4. Western Blot Analysis of PCNA, p-ERK1/2 and ERK1/2 Protein Levels in Three Group Cells. ß-actin was employed as a loading control. The results shown are representative of three independent experiments: 1. pGen-3-CTNNB1; 2. pGen-3-con; 3. Eca-109. *P<0.05, as compared with Eca-109 cells

of the mRNA analysis. The statistical analysis showed that the expression of cyclin D1 mRNA and protein in pGen-3-CTNNB1 transfected cells was down-regulated significantly, compared with that in pGen-3-con transfected and Eca-109 cells (P<0.05), and there is no significant difference between the pGen-3-con transfected cells and Eca-109 cells (P>0.05).

Transmission Electron Microscope Observation

To test whether silencing of β-catenin gene can inhibit the malignant phenotype of tumor cells, we observed by means of TEM the ultrastructure changes. The nucleo-cytoplasmic ratio of Eca-109 cells was relatively larger, the cells showed giant irregular nuclei with scanty and irregularly clumped chromatin and scanty cytoplasm (Figure 3A). However, the ultrastructure of Eca-109 cells had undergone a significant change after being transfected with pGen-3-CTNNB1, the volume of nucleolus and the nucleo-cytoplasmic ratio lessened, the nuclear shape became regular, euchromatin in nucleus decreased while heterochromatin increased, and more cell organelle appeared (Figure 3B).

Expressions of PCNA and ERK1/2

To validate the potential molecular mechanism of the growth inhibitory effect of silencing of ß-catenin gene in Eca-109 cells, we examined the expression alterations of PCNA and ERK1/2 by Western blot analysis. The
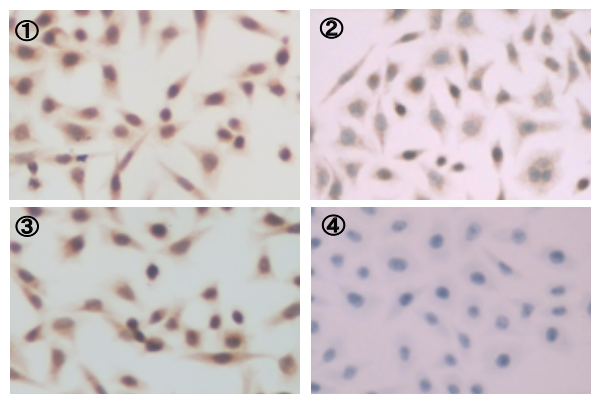
Figure 5. Immunocytochemistry Examination of Cellular Proliferation Labeling Index Labeled by PCNA Immunoreactivity. 1. Eca-109; 2. pGen-3-CTNNB1; 3. pGen-3-con; 4. Negative control. SP×200

Table 4. Comparison of PCNA-LI in Three Group Cells

Group	pGen-3-CTNNB1	pGen-3-con	Eca-109
PCNA-LI (%)	31±9*	64±15	67±10

*P<0.05, compared with Eca-109 cells

expression of PCNA and ERK1/2 phosphorylation state was decreased in pGen-3-CTNNB1 transfected cells, compared with that in pGen-3-con transfected and Eca-109 cells (P<0.05) (Figure 4).

pGen-3-CTNNB1 Transfected Cells were With a Decreased Proliferative Activity

The proliferative activity in three group cells was evaluated with PCNA immunocytochemistry. The results showed that the cellular proliferation rate was decreased in pGen-3-CTNNB1 transfected cells as compared to that in pGen-3-con transfected and Eca-109 cells (P<0.05) (Figure 5, Table 4).

Discussion

In our previous study, MTT, clone formation assay, and xenograft tumor model were employed to investigate the impact of down-regulating ß-catenin expression on EC Eca-109 cells growth, and results indicated that knocking down ß-catenin expression might inhibit cell proliferation (Wang et al., 2009), but the exact mechanism remained unclear. To determine whether the inhibition of proliferation in pGen-3-CTNNB1 transfected cells was due to altered cell cycle regulation and/or apoptosis, cell cycle profiles were monitored by flow cytometric analysis of DNA content. We found that there was an increased fraction of cells in the G0/Gl-phase and decreased in the S-phase, but failed to reveal a significant differences in apoptosis. Therefore, the inhibition of proliferation in pGen-3-CTNNB1 transfected cells might be attributed to cell cycle G0/G1 arrest but not induction of apoptosis. Veeramachaneni's report implied that the decreased cell proliferation of EC cells mediated by ß-catenin down-regulation depended on the increased apoptosis but not cell cycle G0/G1 arrest (Veeramachaneni et al., 2004). Although the specific mechanism remains to be elucidated,

different cell lines used in Veeramachaneni's and our experiment are supposed to be one of the explanations.

The cyclin D1 gene is a target of the ß-catenin/LEF-1 pathway, which is also a regulatory kinase critical for progression through the G1 to S phase transition of the cell cycle (Shtutman et al., 1999; Coqueret, 2002). When quiescent cells reenter the cell cycle and divide, cyclin D1 is the first cyclin to be activated (Takahashi et al., 2007). Overexpression of cyclin D1 may stimulate the cells to overcome the cell cycle checkpoints and enhance cell proliferation (Shiina et al., 2002). Down-regulation of ß-catenin induced a decrease of cyclin D1 mRNA and protein abundance and an increase in the number of cells in the G0/G1-phase.

Mitogen-activated protein kinase (MAPK) is an important player in the early intracellular mitogenic signal transduction for cell growth. Among the MAPK family, ERK1⁄2 plays a central role in the control of cell proliferation. In normal cells, sustained activation of ERK1⁄2 is necessary for G1 to S phase progression and is associated with the induction of positive regulators of the cell cycle and inactivation of antiproliferative genes (Xu et al., 2011). For example, activation of ERK1⁄2 is required for the continued expression of cyclin D1 in the G1 phase and is involved in the mitogen-induced down-regulation of p27 and upregulation of p21 (Meloche and Pouyssegur, 2007). Phosphorylated ERK1⁄2 was examined as an indicator of ERK1⁄2 activation. In the present study, phosphorylated ERK1⁄2 was evaluated to elucidate the possible upstream mechanism by which ß-catenin down-regulation affects cell cycle regulators. Results indicated down-regulation of ß-catenin significantly decreased phosphorylation of ERK1⁄2. This suggests that ß-catenin down-regulation may be able to arrest cell cycle progression, at least in part, in association with the suppression of ERK1/2 activation. Further experiments are necessary to identify the detailed mechanisms underlying the inhibitory effect of down-regulation of ß-catenin on ERK1⁄2 activation in EC cells. In addition, it remains to be investigated whether the effects of silencing of ß-catenin gene on EC Eca-109 cells proliferation are due to interference with the other members of the MAPK family, such as c-jun N-terminal kinase and p38, and phosphatidylinositol 3-kinase/AKT, which are also involved in cell growth (Sears and Nevins, 2002; Krishna and Narang, 2008). At the same time, the expression of PCNA and PCNA-LI decreased accordingly, which is associated with the G1/S transition of mitotic cell cycle, DNA replication, cell proliferation et al.

TEM detection revealed that the ultrastructure of Eca-109 cells had undergone a significant change after being transfected with pGen-3-CTNNB1, which suggests that down-regulating β-catenin expression can promote the differentiation and maturation and reverse partly malignant phenotype of Eca-109 cells.

In conclusion, inhibition of EC Eca-109 cells proliferation by down-regulating ß-catenin expression could improve cell ultrastructure by mediating blockade in the G0/G1 through inhibiting cyclin D1, PCNA and MAPK pathway (p-ERK1/2), and the results might provide some clues to further advance our understanding of the carcinogenesis mechanisms of ß-catenin in EC.

Acknowledgements

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