Inhibition of Isorhamnetin on β-Catenin/Tcf Signaling and β-Catenin-Activated Melanogenesis

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Abstract: Activated β-catenin/T-cell factor (Tcf) signaling plays various roles in human cell mechanisms. We identified the inhibitory effect of isorhamnetin against β-catenin/Tcf signaling in β-catenin activated cells. Isorhamnetin inhibited the transcriptional activity of β-catenin/Tcf in HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene. Also, isorhamnetin inhibited the β-catenin-activated melanogenesis in B16F1 cells. Regarding the inhibitory mechanism, the shift assay showed that the binding of Tcf complexes with its specific DNA-binding sites was suppressed by isorhamnetin. Western blot analysis showed a decreased level of β-catenin in nucleus caused by isorhamnetin. These results suggest that the isorhamnetin acts as a negative regulator of β-catenin/Tcf signaling and its inhibitory mechanism is related to the decreased binding of β-catenin/Tcf complexes to consensus DNA.

Keywords: β-catenin/Tcf signaling; inhibitor; isorhamnetin, melanogenesis.

1. INTRODUCTION

The Wnt/β-catenin pathway, which is activated by the interaction of Wnt1, Wnt3a, and Wnt8 with Frizzled (Fz) receptors controls cell differentiation, cell proliferation, and cell motility [1-4]. More than 20 target genes of the β-catenin signaling pathway have been identified. Among them, axin-2, Tcf, c-myc, cyclin D1, metalloproteinases, and vascular endothelial growth factor are regulators of cell fate, proliferation, migration, polarity, developmental control, and carcinogenesis [5, 6]. β-Catenin is ordinarily involved in cell-cell adhesion in cooperation with the cytoplasmic domain of E-cadherin [7]. Cytosolic β-catenin is phosphorylated by Axin–adenomatous polyposis coli (APC)–glycogen synthase kinase (GSK)-3β complex and recognized by an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by an ubiquitin–proteasome system [8]. In normal cells, β-catenin in the cytoplasm is rapidly degraded by the proteasome [9]. However, β-catenin accumulation may result from the mutational inactivation of the APC gene or β-catenin mutations at regulatory amino-terminal serine residues that stabilizes cytosolic β-catenin protein by blocking degradation [7]. When the failure of degradation in cells occurs, increased amount of β-catenin compared with normal cells accumulates in the cytoplasm and translocates into the nucleus [10]. A link between Wnt/β-catenin signaling and melanocyte differentiation has been suggested by the finding that the β-catenin accumulation with activation of Wnt/β-catenin signaling, forms a complex with lymphocyte enhancer factor-1 to up-regulate expression of the microphthalmia-associated transcription factor (MITF) gene [11, 12]. Enhanced expression of MITF as a master regulator of melanin synthesis resulted in melanocyte differentiation [13, 14]. MITF regulated the expression of tyrosinase and tyrosinase-related protein that catalyze the conversion of tyrosine into melanin pigments [12, 15-17].

β-Catenin has transcriptional activity in cooperation with T-cell factor (Tcf)/lymphoid enhancer factor (Lef) transcription factor in the nucleus. Since the APC gene or the GSK-3β phosphorylation site within the β-catenin gene are mutated in many cancer cells including colorectal cancer, melanoma, hepatocellular carcinoma and gastric carcinoma, the transcriptional activity of β-catenin is up-regulated in these cancer cells [18, 19]. Studies have reported that β-catenin nuclear localization occurs in one third of gastric cancers and that β-catenin mutations occur in both diffuse- and intestinal-type gastric cancers at a higher rate [2]. This suggests a possibility that the activated β-catenin/Tcf signaling by the accumulation of β-catenin in the nucleus is related to human carcinogenesis. Altogether, if β-catenin's transcriptional activity can be markedly downregulated, tumor growth or melanogenesis may be suppressed in β-catenin activated type of cells. Isorhamnetin is methylated quercetin, which is a flavonoid and occurs naturally in plants such as red turnip, goldenrod, mustard leaf and ginkgo biloba [20]. The dietary flavonoid quercetin reduces blood pressure and improves endothelial function in several rat models of hypertension [21]. Quercetin and isorhamnetin prevented endothelial dysfunction, superoxide production, and overexpression of p47phox induced by

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angiotensin II in rat aorta [21]. Isorhamnetin inhibits proliferation of various kinds of cancer cells in culture and suppresses the tumors of Lewis lung carcinoma cell allografts in mice and the skin tumor in an *in vivo* mouse xenograft using A431 cells [22-26].

In this report, we investigated the inhibitory effect of isorhamnetin using HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene and reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element. We demonstrate here that isorhamnetin acts as inhibitor of β-catenin/Tcf signaling and inhibits wnt3a-activated melanogenesis.

2. MATERIALS AND METHODS

2.1. Cell culture and Treatments

The HEK293 and B16F1 cell lines were purchased from Korean Cell Line Bank (Seoul, Korea). TOPflash and FOPflash were provided by Hans Clevers (Hubrecht Institute, Netherlands) and β-catenin mutant S33Y gene was provided by Eric R. Fearon (University of Michigan Medical School, Ann Arbor). Isorhamnetin and Wnt3a were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethylsulfoxide (DMSO) prior to use.

2.2. Transfection and Luciferase Assay

Transient transfection was performed using Lipofectamine Plus reagent (Invitrogen. Carlsbad, CA). Briefly, 1 x 10^4 HEK293 cells distributed in the medium were dispensed into wells of a 96-well plate. After 24 h, cells were transfected with 0.10 μg of the TOPflash or FOPflash luciferase reporter constructs, 0.04 μg of pcDNA β-catenin S33Y gene and 0.08 μg of pGL4.74 (*Renilla* luciferase) gene for normalization. After 24 h of transfection, inhibitor isorhamnetin was added to the medium. Cells were incubated for another 24 h, lysed and collected for assays of luciferase activity using Dual-Gio™ luciferase assay system (Promega, Madison, WI) with a luminescence multi reader (Zenyth 1100, Anthos, Austria). TOPflash luciferase activity was normalized to *Renilla* luciferase activity and expressed as relative value compared with control.

2.3. Determination of Melanogenesis in B16F1 Cells

1 x 10^5 B16F1 cells were plated in 100 mm plates and were allowed to attach overnight. Then the cells were incubated with Wnt3a with or without various concentration of isorhamnetin for 48 hours. The cells were then washed twice with ice-cold PBS, lysed with RIPA buffer and centrifuged at 10,000g for 10 min. Supernatants were analyzed for protein concentration, and pellets were solubilized in 200 μl of 1M NaOH. Following an incubation period of 2 h at 60 ℃, the absorbance was measured spectrophotometrically at 405 nm. Relative melanin production was normalized compared to the control without wnt3a.

2.4. Isolation of Nuclear Extracts

Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Fremont, CA) according to the manufacturer's instructions. HEK 293 cells transfected with β-catenin mutant gene were treated with lysis buffer (Panomics) for 10 min at room temperature with vigorous mixing and then scraping. After centrifugation, the pellet was resuspended in extraction buffer (Panomics) and incubated on ice for 1 h. Nuclear extracts were used as described in the following two subsections.

2.5. Electrophoresis Mobility Shift Assay

EMSA was performed using a gel shift kit according to the manufacturer's protocol (Panomics). Nuclear extract (4 μg) was incubated for 5 min at room temperature with poly dI-dC (1 μg), 5x binding buffer, and, if needed, inhibitors. Then, biotin-labeled β-catenin/Tcf binding probe (5'- CTTTGATCTTACC-3') was added and incubated for 30 min at 15^{o}C in a thermal cycler. The samples were analyzed by nondenaturing 6% polyacrylamide gel electrophoresis (PAGE) in 0.5x TBE buffer at 120 V for 55 min. Separated proteins were transferred to Biodyne B nylon membrane (Pall Life Science, Port Washington, NY) for 1 h at 300 mA. Target proteins were visualized by autoradiography using streptavidin-horse radish peroxidase and substrate solution.

2.6. Western Blot Analyses

Nuclear extracts were used to measure β-catenin level. The extracts were subjected to 12% sodium dodecyl sulfate (SDS)-PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the membrane was saturated by incubation, at 4°C 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS)–0.1% Tween-20 (TBST) and then incubated with antibody overnight at 4 °C. After washing with TBST, the membrane was incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz

Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membrane was washed three times with TBST and the blots were developed using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Normalization was performed using tubulin antibody (Santa Cruz Biotechnology).

2.7. RNA Isolation and Polymerase Chain Reaction (PCR)

HEK 293 cells transfected with β-catenin mutant gene were treated with or without inhibitors for 24 h and total RNA was extracted by homogenization in Trizol reagent (Invitrogen) according to the manufacturer's instructions. Synthesis of cDNA was performed as previously described [27]. Briefly, 50 µg of total RNA was reverse-transcribed to double stranded cDNA using oligo(dT) primers (iNtRON, Gyeonggi-Do, Korea) and reverse transcriptase (iNtRON). For real-time PCR, 40 ng of cDNA was mixed with 5 µM of each primer in IQ SYBR Green Super-Mix (Bio-Rad). Real-time PCR was performed using a CFX 96 Real-time system (Bio-Rad) with the following thermal profile: 95 °C, 3 min; (95 °C, 10 s; 56 °C, 10 s; 72 °C, 20 s) x 39 cycles; 95 °C, 10 s; 65 °C, 20 min; 95 °C, 15 s. All amplifications were run in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the endogenous control. The conditions were optimized to show single peaks in the melting curve. ΔC_T was computed by subtracting the C_T (the number of cycles to reach the threshold) for GAPDH from the C_T value for each gene. The expression level for each gene expressed in units of GAPDH was then taken as $2^{-\Delta C}{}_T$. These units were normalized so that the GAPDH mRNA level was 10,000. Primer pairs were designed for c-myc, cyclin D1, axin2 and GAPDH. The information for each probe was obtained from the Stanford Online Universal Resource for Clones and ESTs (SOURCE; http://www.source.stanford.edu), which compiles information from several publicly accessible databases including UniGene, dbEST, Swiss-Prot, GeneMap99, RHdb, GeneCards and LocusLink.

2.8. Quantitative Analysis

Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad). In all experiments, statistical significance was calculated for the data from three or four independent experiments using one-way ANOVA or student t-test (Sigma Plot, LaJolla, CA). The error bars represent the standard deviations of the mean.

3. RESULTS

3.1. Inhibition of β-catenin/Tcf Signaling by Isorhamnetin

HEK293 cells have low transcriptional activity of β-catenin/Tcf for the low endogenous amounts of β-catenin protein. However, when they were co-transfected with β-catenin mutant gene, the Tcf signaling luciferase activity was elevated about 10-fold. Isorhamnetin (Figure **1**) modulated β-catenin/Tcf signaling in HEK293 cells transiently transfected with constitutively activated mutant β-catenin gene. Cells were also transiently transfected with the reporter plasmids containing either an optimal Tcf binding site (TOPflash) or mutant binding site (FOPflash) and *Renilla* luciferase (pGL4.74) to normalize for transfection efficiency. Isorhamnetin was treated with successively increasing doses for 24 h. Figure **2** shows that isorhamnetin suppressed β-catenin/Tcf transcriptional activity in these cells in a concentration-dependent manner. Isorhamnetin of 1 µM inhibited β-catenin/Tcf signaling to 45–55% compared with the control.

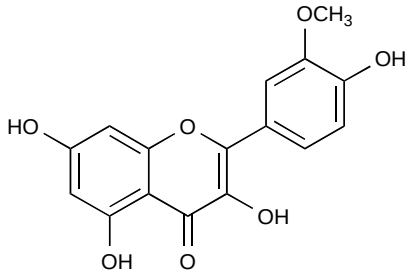

Figure 1: Structure of isorhamnetin.

3.2. Inhibition of β-Catenin-Activated Melanogenesis by Isorhamnetin

As shown in Figure **3A**, Wnt3a induced melanogenesis to 143% within 48hr and isorhamnetin suppressed Wnt3a-induced melanogenesis to 76% of activated level in B16F1 cells. Additionally, the expression of MITF that was up-regulated by activation of the Wnt/β-catenin signaling was repressed by isorhamnetin in these cells (Figure **3B**).

3.3. Inhibition of Tcf Complex Binding to DNA by Isorhamnetin

Nuclear extracts of mutant β-catenin gene-transfected cells were prepared and incubated with β-catenin/Tcf binding consensus DNA and inhibitors *in vitro*. Because nuclear extract was prepared without isorhamnetin treatment and the isorhamnetin was

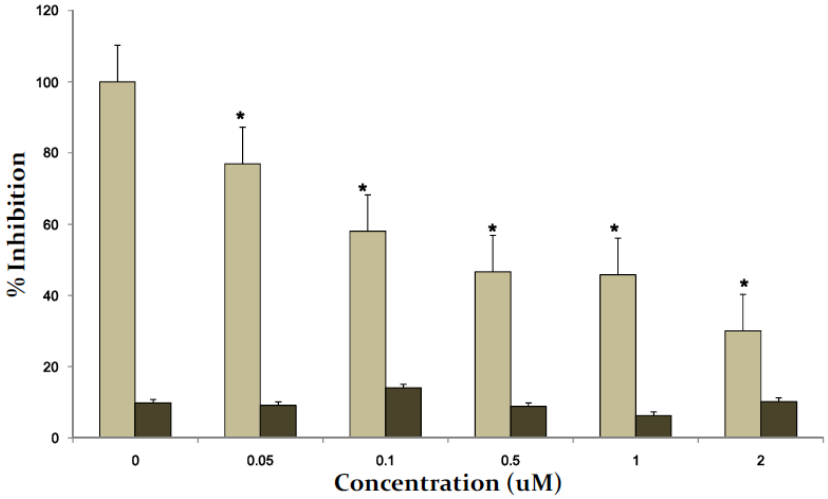

Figure 2: Inhibition of β-catenin/Tcf signaling by isorhamnetin in HEK293 cells transfected with constitutively active mutant β-catenin gene. HEK293 cells were cotransfected with β-catenin mutant gene, Renilla gene and reporter genes harboring Tcf-4 binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively. Twenty-four hours after transfection, increasing amounts of isorhamnetin as indicated were added to the cells. Luciferase activity was determined after 24 h of treatment and normalized against values for the corresponding renilla activity. An equivalent volume of DMSO substituted for isorhamnetin was used as a vehicle control. Values represent means ± SD of four independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. **P < 0.01

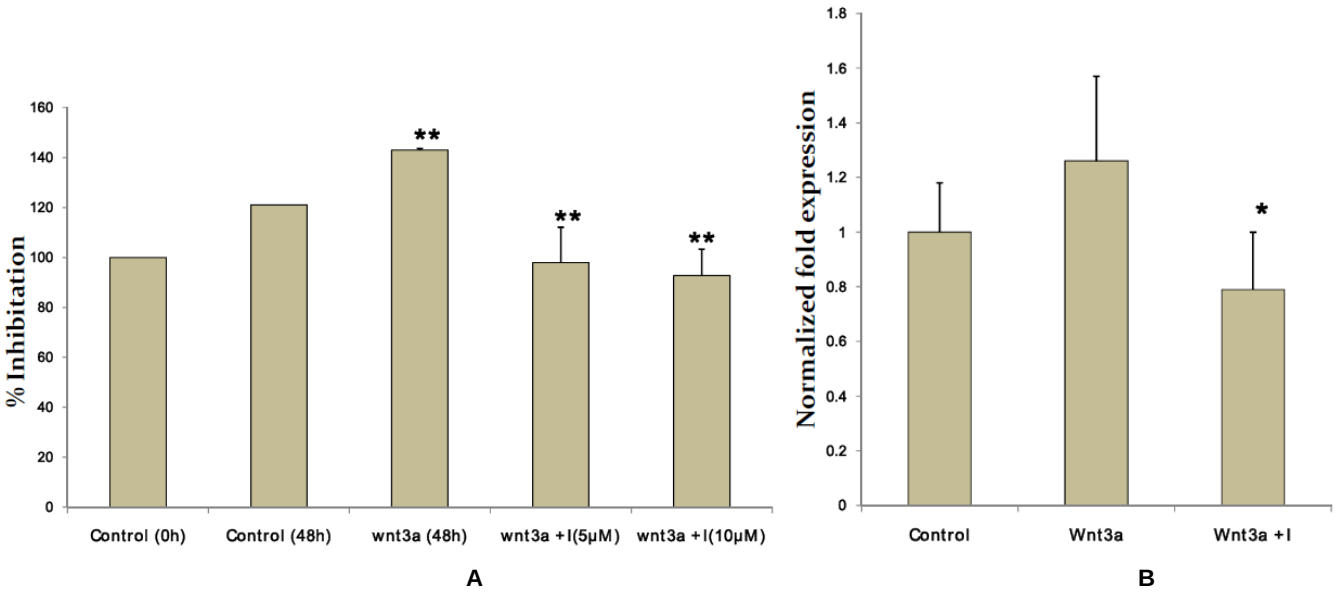

Figure 3: Inhibition of isorhamnetin on Wnt3a-induced melanogenesis in B16F1 melanoma cells. **A**. Cell extracts were prepared from melanoma cells treated with either vehicle (DMSO, lane 1) or isorhamnetin (5 and 10 μM, lane3 and 4) in the presence of Wnt3a (50 nM) for 48 h and the contents of melanin were measured. **B**. Effect of isorhamnetin on the expression of MITF. Cells were treated with isorhamnetin (10 and 20 μM) for 48 h and were trypsinized to isolate RNA and real time PCR was performed to study the change of amount of MITF mRNA by isorhamnetin. Results were obtained using triplicate experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. **P < 0.05, *P < 0.01 by Student's t-test as compared to the control.

added during incubation in EMSA, the effect of inhibition was likely to be ascribed, not to an indirect effect to diminish β-catenin/Tcf-DNA complex formation, but to a direct blocking of complex formation. When excess unlabeled Tcf-binding oligonucleotide was competitive with labeled Tcf-binding oligonucleotide, the retarded bands disappeared (Figure **4A**). This result suggested that the shifted DNA protein complex was specific for Tcf complexes binding to the consensus DNA. As represented in Figure **4A**, isorhamnetin showed a direct inhibitory effect against the β-catenin/Tcf-DNA complex formation in an *in vitro* assay with 10 μM. To investigate whether the effect of inhibition was ascribed to a direct blocking of complex

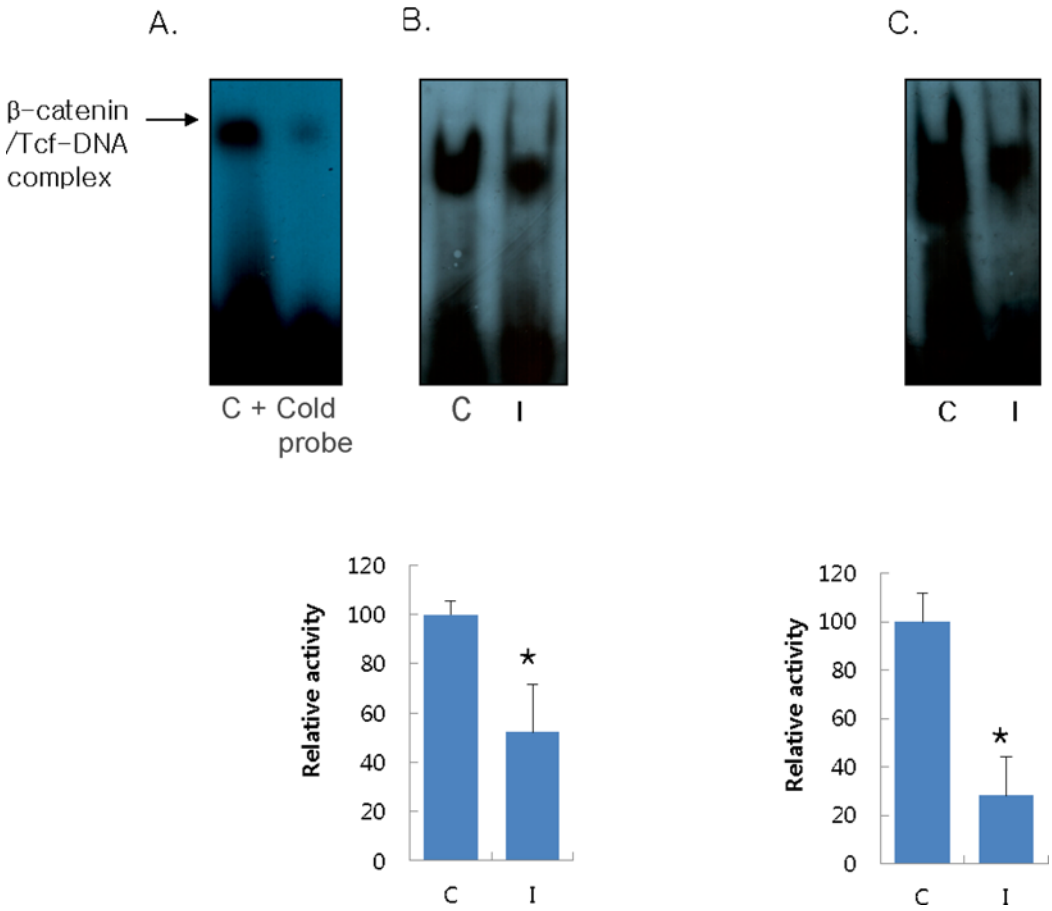

Figure 4: Effect of isorhamnetin on the binding of Tcf complexes with DNA. HEK 293 cells were transfected with constitutively active mutant β-catenin and nuclear extracts were isolated. EMSA was performed with 4 μg nuclear extracts with or without isorhamnetin. **A**. Lane 2 shows that 10-fold excess of unlabeled Tcf-binding oligonucleotide used as a competitor inhibits the binding of the β-catenin/Tcf to oligonucleotide containing Tcf-binding region. **B**. The cells were treated with isorhamnetin before preparing the nuclear extract used in EMSA (lane 1: control, lane 2: isorhamnetin 10 μM). **C**. To investigate whether the effect of inhibition was ascribed to a direct blocking of complex formation, nuclear extract was prepared and isorhamnetin was added during incubation in EMSA (lane 1: control, lane 2: isorhamnetin 10 μM). Quantitative analysis was performed using densitometry and results are expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. *P < 0.05 by Student's t-test as compared to the control.

formation, nuclear extract was prepared and isorhamnetin was added during incubation in EMSA (Figure **4A**). When the cells were treated with the inhibitor before preparing the nuclear extract used in EMSA, isorhamnetin also showed an inhibitory effect against the β-catenin/Tcf-DNA complex formation as shown in Figure **4B**. However, when the cells were treated with the inhibitor before preparing the nuclear extract used in EMSA, it could not be explained whether the effect of inhibition was ascribed to an indirect effect to diminish β-catenin/Tcf-DNA complex formation or to a direct blocking of complex formation. The IC_{50} value of 10 μM was not exactly identical to that of 1 μM obtained in the luciferase activity assay shown in Figure **3**, which was determined in a cell-based system. This result represented that the inhibition of β-catenin/Tcf signaling in the cell-based luciferase activity assay was not totally due to the direct interference of interaction between dimer and DNA.

3.4. Effect of Isorhamnetin on β-Catenin Upstream Components

Tcf signaling activation results from accumulation of nuclear β-catenin [6]. We examined the possibility that the decreased formation of the β-catenin/Tcf complex and the suppressed binding to DNA result from the decreased distribution of nuclear β-catenin products by inhibitors. Western blot analysis was performed to determine the change in the amount of β-catenin in nuclear fractions. Compared with controls, the isorhamnetin decreased β-catenin levels in the nucleus (Figure **5**). A reduction of 31% and 52% in nuclear β-catenin levels were observed with addition of 1 μM and 5 μM, respectively, of isorhamnetin for 24 h.

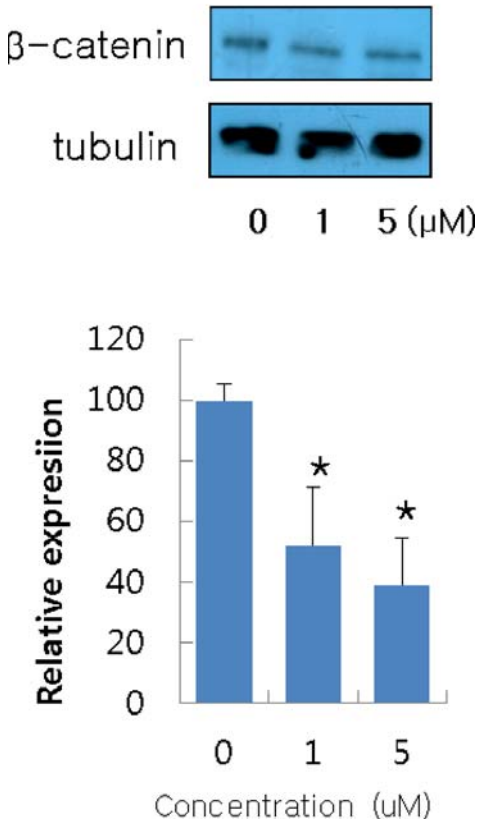

Figure 5. Effect of isorhamnetin on β-catenin distribution in nucleus. HEK293 cells were transfected with β-catenin mutant gene for 24 h and then treated with isorhamnetin (1 μM and 5 μM) for 24 h and were trypsinized for preparing nucleus extracts. To demonstrate equivalent loading of the lines, anti-tubulin was used. Each cell fraction was used for Western blot with anti β-catenin antibody. All pictures are representative of three independent experiments. Quantitative analysis was performed using densitometry and results are expressed as relative activity to untreated control. Results are shown as mean ±SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. * P < 0.05 by Two-way ANOVA as compared to the control.

3.5. Effect of Isorhamnetin on the Expression of β-Catenin Target Genes such as c-myc, axin 2 and Cyclin D

c-Myc, axin 2 and cyclin D1 are β-catenin target genes [8-10]. Therefore, we investigated whether isorhamnetin would downregulate the c-myc, axin 2 and cyclin D1 gene transcription by inhibiting β-catenin/Tcf signaling. In HEK293 cells transfected with β-catenin, c-myc, axin 2 and cyclin D1 were downregulated by 10 μM ofisorhamnetin (Figure **6**). As a control, non-target gene β-actin expression was investigated and not affected by isorhamnetin. These results indicate that the functioning of β-catenin as a transcription activator is rendered inoperative by inhibitors.

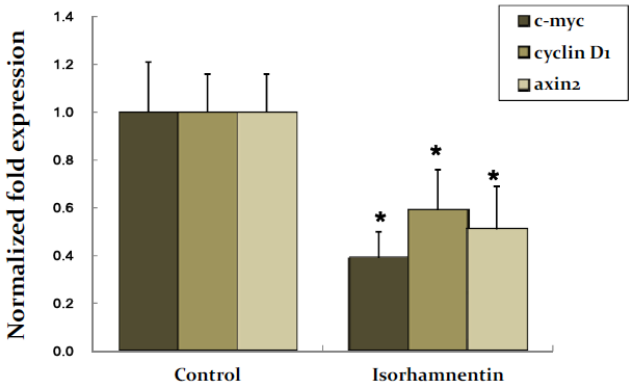

Figure 6: Effect of isorhamnetin on the expression of c-myc, axin 2, cyclin D1 and β-actin in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. Cells were treated with isorhamnetin (10 μM) for 24 h and were trypsinized to isolate RNA and real time PCR was performed to study the change of amount of mRNA by inhibitor. Results were obtained using triplicate experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. *P < 0.01 by Student's t-test as compared to the control.

4. DISCUSSION

As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on β-catenin inhibitors and their inhibitory mechanisms are being conducted [28]. Recently, it was reported that blocking β-catenin/Wnt signaling by extracellular Wnt inhibitors (secreted frizzled-related protein-like molecules) suppressed *in vivo* cell proliferation and tumor growth in cells carrying active β-catenin [29]. Additionally, elevated β-catenin/Tcf signaling by accumulation of β-catenin in the nucleus is involved in a number of human carcinogenesis [2, 5, 6]. Recent studies have demonstrated that Dickkopf 1 (DKK1; a secreted protein antagonist of Wnt/b-catenin signaling), inhibits the function and proliferation of melanocytes *via* suppression of β-catenin, MITF, and tyrosinase [12, 30, 31]. Moreover, a natural product, cardamonin suppressed melanogenesis by inhibition of Wnt/b-catenin signaling [12]. Activation of an abnormal APC/β-catenin/Tcf signaling pathway and alterations in cellular adhesion mediated through changes in β-catenin homeostasis within the colonic epithelium are crucial factors in the development of the majority of colorectal cancers [18]. As the importance of β-catenin as aregulator of celluar signaling, many more studies on β-catenin inhibitors and their inhibitory mechanisms are being conducted [32]. Non-steroidal anti-inflammatory drugs and nitric oxide-generating aspirin were reported to inhibit the β-catenin/Tcf activity [33, 34]. Curcumin was also reported to inhibit the β-

catenin/Tcf transcriptional activity and induce apoptosis in HCT116 colon cancer cells [35]. We have previously reported the inhibitory effects of curcumin, curcumin analogs, quercetin and naringenin against β-catenin/Tcf signaling [35-37]. In our previous reports, various kinds of flavonoids such as qercetin and naringenin inhibited β-catenin/Tcf transcription activity [35, 37, 38]. Our experiment using luciferase reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element showed that β-catenin/Tcf-driven transcription was suppressed by isorhamnetin in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. Moreover, isorhamnetin suppressed Wnt3a-activated melanogenesis in melanocyte cell lines. We conclude that the inhibitory mechanism of isorhamnetin is related to the direct inhibition of the β-catenin/Tcf-DNA complex formation. To transcribe target genes, transcription factors, including Tcf complexes, must bind to consensus DNA. The decrease in nuclear localization of β-catenin induced by the suppression of GSK-3β was observed by the treatment with isorhamnetin. Therefore, we suggest a possible inhibitory mechanism by isorhamnetin. Inhibitor isorhamnetin directly blocks the complex formation of β-catenin/Tcf with DNA and the mechanism might be responsible for the inhibitory mechanism of β-catenin/Tcf signaling by isorhamnetin. Given their function in inhibiting β-catenin/Tcf signaling, isorhamnetin may be valuable as a chemotherapeutic agent against tumorigenesis or melanogenesis in β-catenin-activated type of cells.

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ABBREVIATIONS

APC = adenomatous polyposis coli

DMSO = dimethyl sulfoxide

Dsh = disheveled

EMSA = electrophoretic mobility shift assay

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

GSK = glycogen synthase kinase

IC_{50} = half maximal inhibitory concentration

Lef = lymphoid enhancer factor

NF-κB = nuclear factor-κB

PCR = polymerase chain reaction

SDS = sodium dodecyl sulfate

TBE = Tris base boric acid EDTA

TBS = Tris buffered saline

TBST = Tris buffered saline and Tween-20

Tcf = T-cell factor

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