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Original Article

ShRNA lentivirus mediated knockdown of RECQL5 enhances the tumor malignancy of Hep-2 laryngeal squamous carcinoma cells

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Abstract: RECQL5 is closely associated with the maintenance of DNA stability and the replication and repair of DNA. Our previous study has indicated that RECQL5 genetic polymorphism and haplotypes were associated with larynx cancer risk in China. This study is to explore the potential role of RECQL5 in the progression of laryngeal squamous cell carcinoma using RECQL5 shRNA lentivirus. In this study, RECQL5 in Hep-2 cells were knocked down using RECQL5 shRNA lentivirus. Before and after the downregulation of RECQL5, the proliferation, metastasis and apoptosis of the cells were measured using CCK-8 assay, transwell invasion assay and flow cytometry, respectively. Meanwhile, some classical indicators in the malignancy-related pathways of laryngeal squamous cell carcinoma were detected using western blotting and qRT-PCR as well, including p53, Erk, and Wnt/β-catenin signaling pathways. The expression of RECQL5 was decreased in Hep-2 cells by transfecting with RECQL5 shRNA lentivirus. Knockdown of RECQL5 enhanced Hep-2 cells growth and metastasis and depressed the Hep-2 cells apoptosis. Knockdown of RECQL5 reduced the p53 expression, but improved the phosphorylation of Erk1/2 and the expressions of Wnt1 and β-catenin in Hep-2 cells. In conclusion, RECQL5 inhibits the tumor development related signaling transduction in tumor cells. Knockdown of RECQL5 in laryngeal squamous cell carcinoma using RECQL5 shRNA lentivirus will relieve the suppression and enhance its tumor malignancy.

Keywords: RECQL5, laryngeal squamous cell carcinoma, Hep-2 cells, tumor malignancy, signaling pathway

Introduction

RecQ helicase as a DNA helicase plays an important role in maintaining the DNA stability. RECQL1, WRN, BLM, RECQL4 and RECQL5 are the members of human RecQ helicase family and the mutation of BLM, WRN and RECQL4 has a relationship with Bloom Syndrome, Werner Syndrome and Rothmund-Thomsom Syndrome respectively, but no diseases have been found to be associated with RECQL1 or RECQL5 [1]. Early studies have showed the functions of RECQL5 in maintaining DNA stability, such as inhibition of sister chromatid exchange [2], homologous recombination repair and DNA double-stranded breaks [3], synergy between RECQL5 and Topoisomerase II alpha, and regulation of RNAP II transcription process [4, 5]. Chromosome instability is the common characteristic for many tumor cells and the foundation of malignant tumor formation. What's more, RECQL5 has been showed a low expression in osteosarcoma tissues and cells, overexpression of RECQL5 inhibits proliferation and promotes apoptosis in MG-63 cells [6]. RECQL5 knockout mice show a cancer prone phenotype [7]. Colorectal cancer cells show a significant enhancement in camptothecins sensitivity when treated with deletion of RECQL5 [8].

P53 is a negative regulator in cell cycle and has a relationship with cell cycle control, DNA repair, cell differentiation, cell apoptosis and other crucial biological functions. So p53 also is crucial for cancer cell growth [9]. Erk1/2 as a protein kinase regulates the cell growth and differentiation via activating a variety of extracellular signals (cytokines, tumor factor and so on) and nuclear transcription factors [10]. Wnt/β-catenin pathway contains GSK3β/APC/Axin complex, β-catenin and other transcription factors.

Abnormal activation of Wnt/β-catenin pathway will induce epithelial mesenchymal transition (EMT) in tumor tissues and promotes tumor cells invasion and migration, so abnormal activation of Wnt/β-catenin pathway is related with the development, stage and migration of tumor [11].

Laryngeal carcinoma is an important malignancy and its incidence is increasing in recent years. Laryngeal squamous cell carcinoma (LSCC) is a major type of laryngeal carcinomas, and there is little known about the relationship between RECQL5 and LSCC, especially the functional mechanism of RECQL5 in LSCC cells development, proliferation, migration and apoptosis. Therefore, we investigated the roles and potential mechanisms of RECQL5 on the growth, migration and apoptosis of Hep-2 cells through RNA interference, qRT-PCR, Western blot, Transwell assay and Flow cytometry. Our study showed that RECQL5 inhibition enhanced the Hep-2 cells proliferation and migration but decrease the Hep-2 cells apoptosis, and this might be caused by downregulation of p53, increased phosphorylation of Erk1/2 and activation of Wnt/β-catenin signaling.

Materials and methods

Cell culture and RECQL5 shRNA lentiviral particles transduction

The Hep-2 cells were obtained from Shanghai institutes for biological sciences in the Chinese Academy of Sciences and cultured in complete RPMI-1640 (HyClone, Logan, Utah, USA) contained 10% FBS (HyClone, Logan, Utah, USA) at 37°C with 5% CO_2. RECQL5 shRNA lentiviral particles and control shRNA lentiviral particles (as a negative scrambled shRNA) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and were transduced into Hep-2 cells in accordance with the specifications. Stable clones expressing the shRNA were continued incubating and treated with TRAIL (0.4 μg/ml) for 12 h.

Real time PCR

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was applied to total RNA extraction from Hep-2 cells with different treatments per the instruction. PrimeScript RT Reagent kit (Takara Bio, Japan) was used to obtain the cDNA from total RNA (2 μg) in accordance with the instruction. PCR was completed by SYBR Premix Ex Taq kit (Takara Bio) and ABI 7300 system (Applied Biosystem, Foster, CA, USA); PCR assay: initial denaturation at 95°C for 30 sec, PCR under 40 cycles of 95°C for 5 sec and 60°C for 30 sec. GAPDH mRNA level was detected as the internal control and the relative mRNA levels were analyzed using $2^{-\Delta\Delta Ct}$ method [12]. The PCR primers were as below: RECQL5 forward, 5'-CTTTCTACAAGGAGGGCAAG -3'; RECQL5 reverse, 5'- GAAGAAGTGCCTGATGAGGT -3'; p53 forward, 5'- AAGGAAATTTGCGTGTGGAGT -3'; p53 reverse, 5'- AAAGCTGTTCCGTCCCAGTA -3'; Wnt1 forward, 5'- CTCATGAACCTTCACAACAACGA -3'; Wnt1 reverse, 5'- ATCCCGTGGCACTTGCA -3'; GAPDH forward, 5'- CACCCACTCCTCCACCTTTG -3'; GAPDH reverse, 5'- CCACCACCCTGTTGCTGTAG -3'.

Western blotting

Cytosolic protein and nucleoprotein was extracted from Hep-2 cells with different treatments using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) following the product manual. The proteins were quantified by bicinchoninc acid kit (Thermo Fisher, Rockford, IL, USA). Destined proteins were isolated from the total protein using SDS-PAGE and then were transferred to NC membranes (Millipore, Massachusetts, USA). Membranes with interest proteins were blocked with 5% nonfat milk for 1 h followed by overnight incubating with the primary antibodies against RECQL5 (ab91422), p53 (ab62376), p-Erk1/2 (sc-23759-R), Wnt1 (ab15251), β-catenin (ab32572), β-actin (ab8227) and α-tubulin (ab6046) at 4°C respectively. HRP-conjugated goat ant-rabbit IgG were used as secondary antibodies. Signals were detected using chemiluminescenct substrate (Bio-Rad Laboratories) and analyzed by Image J software (Bethesda, MD, USA). Except p-Erk1/2 (sc-23759-R) was obtained from Santa Cruz, the other primary antibodies and secondary antibodies were purchased from Abcam (Cambridge, MA, USA) and used in accordance with the manufacturer' protocols.

Hep-2 cell proliferation assay

CCK-8 kit (Dojindo Laboratories, Japan) was used in accordance with the instruction to analyze the Hep-2 cells proliferation that express

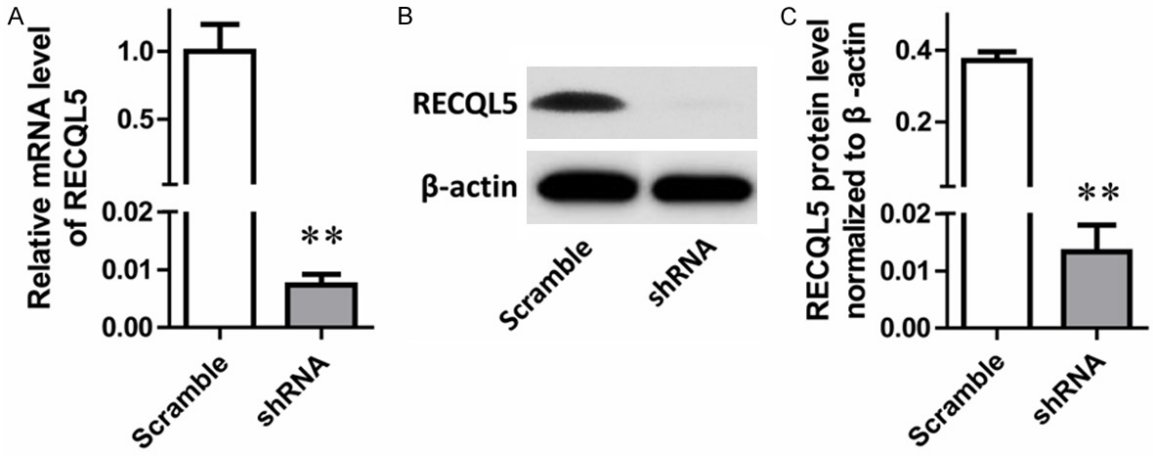

Figure 1. Knockdown of RECQL5 in Hep-2 cells. A: The RECQL5 mRNA level was declined considerably in Hep-2 cells transduced with RECQL5 shRNA, the value was showed as mean ± SD, $^{**}P < 0.01$ versus Scramble group; B: The RECQL5 protein level in Hep-2 cells expressing RECQL5 shRNA was dropped sharply; C: Relative protein level of RECQL5 to β-actin, the value was showed as mean ± SD, $^{**}P < 0.01$ versus Scramble group.

shRNA at 24 h, 48 h and 72 h respectively. The values of absorbance at 450 nm were recorded.

Hep-2 cell migration assay

Cell migration was assayed using Transwell with 8-μm-pore filters (Corning; New York, NY, USA). Hep-2 cells expressing shRNA (1×10^5 in 1 ml RPMI-1640 with 10% FBS) were seeded in the upper chamber (12-well plate format) with serum-free RPMI-1640 medium in the lower chamber. After incubating for 6 h, the medium in the upper and lower chambers was replaced with 5% and 20% serum-containing medium respectively. Cells in the upper chamber were considered as no migration and completely removed at 48 h, then migrated cells at the lower surface of upper chamber were fixed with formaldehyde followed by staining using crystal violet, and cell number of migrated Hep-2 cells was counted.

Hep-2 cell apoptosis assay

Cell apoptosis were assayed using Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Biotechnology, Haimen, China) and flow cytometry in accordance with the instruction. Hep-2 cells transduced with RECQL5 shRNA lentiviral particles/control shRNA lentiviral particles in the presence of TRAIL or not were all detected.

Statistical analysis

Each experiment was performed in triplicate. All data was analyzed using Graphpad Prism (Graphpad Software, San Diego, CA, USA) and displayed as the mean ± SD. The statistical significance between different groups was detected by one-way analysis of variance (ANOVA) with Tukey's adjustment for pairwise comparisons. If $P < 0.05$, there is a statistical difference.

Results

Downregulation of RECQL5 expression in Hep-2 cells transduced with RECQL5 shRNA lentiviral particles

Hep-2 cells expressing RECQL5 shRNA not only significantly suppressed the mRNA level of RECQL5 compared with Hep-2 cells transduced with Scramble shRNA ($P < 0.01$, **Figure 1A**), but also reduced the protein level of RECQL5 ($P < 0.01$, **Figure 1A**, **1B**). This indicates that RECQL5 shRNA can effectively inhibit RECQL5 expression in Hep-2 cells *in vitro*.

Knock-down of RECQL5 promotes the proliferation and metastasis of Hep-2 cells and suppresses the Hep-2 cells apoptosis

Then we determined the influence of knockdown of RECQL5 on Hep-2 cells proliferation, migration and apoptosis by CCK-8 assay, Transwell assay and flow cytometry. Proliferation of Hep-2 cells expressing RECQL5 shRNA increased with the time compared to Scramble group ($P < 0.01$, **Figure 2A**). Metastasis of Hep-2 cells with RECQL5 inhibition was obviously enhanced compared with Scramble group

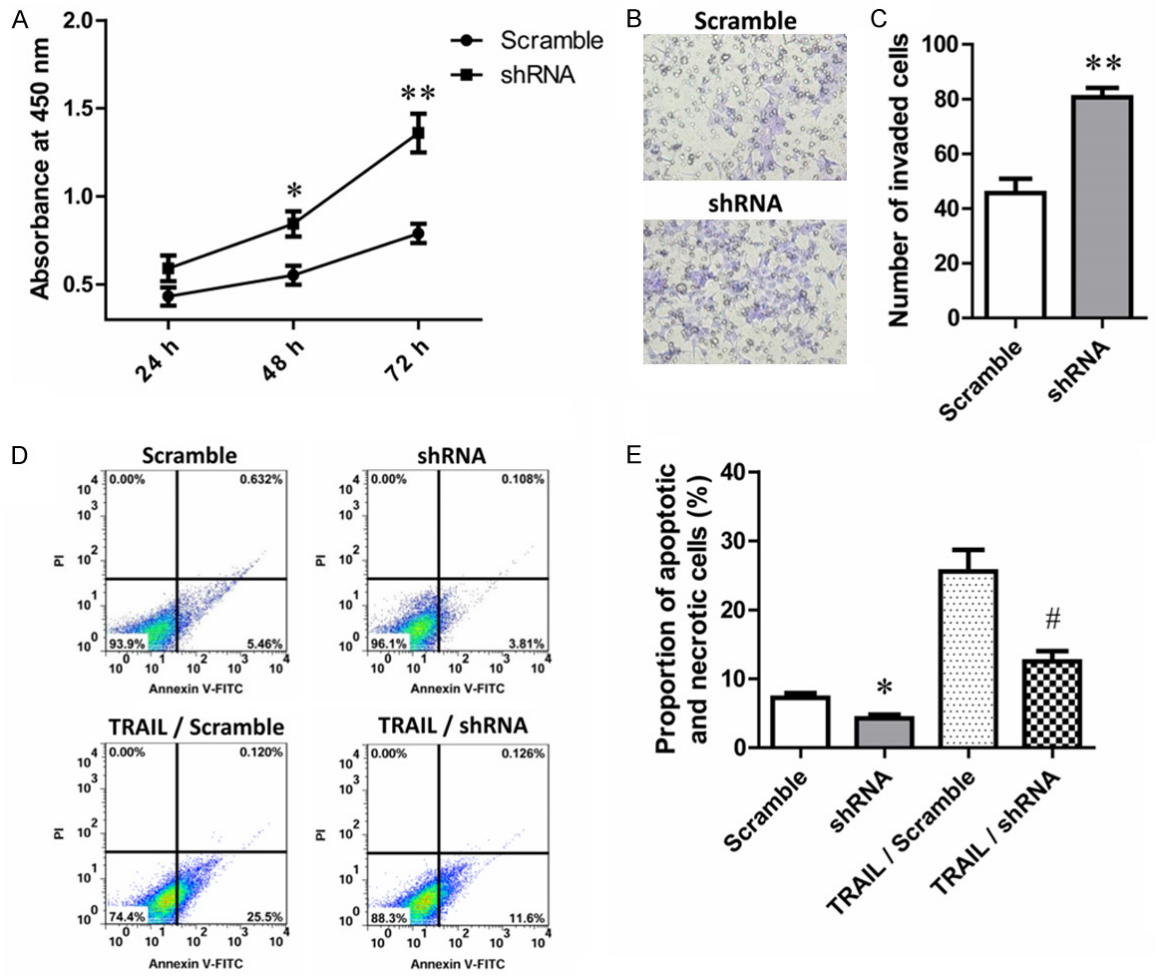

Figure 2. Knockdown of RECQL5 promoted the Hep-2 cells proliferation and migration and inhibited apoptosis. A: Hep-2 cells proliferation was detected by CCK-8 assay, the value was showed as mean ± SD, $*P < 0.05$, $**P < 0.01$ versus Scramble group; B, C: Hep-2 cells migration was detected by Transwell assay, B: Migrated Hep-2 cells under 100×, C: Average cell number in each sight, the value was showed as mean ± SD, $**P < 0.01$ versus Scramble group; D, E: Hep-2 cells apoptosis was detected by flow cytometry, E: The value was showed as mean ± SD, $*P < 0.05$ versus Scramble group, $^{\#}P < 0.05$ versus TRAIL/Scramble group.

($P < 0.01$, **Figure 2B**, **2C**). Knock-down of RECQL5 inhibited the Hep-2 cells apoptosis *in vitro* ($P < 0.05$, **Figure 2D**, **2E**). Together, the results demonstrate that inhibition of RECQL5 promotes the Hep-2 cells growth and metastasis and suppresses apoptosis.

Knock-down of RECQL5 in Hep-2 cells suppresses the p53 expression and increases the phosphorylation level of Erk1/2

We detected the expression of p53 and p-Erk1/2 using qRT-PCR and western blotting. The p53 expression was reduced significantly in Hep-2 cells with knock-down of RECQL5. But p-Erk1/2 was enhanced dramatically in Hep-2 cells with knock-down of RECQL5 compared to cells transduced with Scramble shRNA ($P < 0.05$) (**Figure 3**).

Knock-down of RECQL5 increases the expression of Wnt1 and β-catenin in Hep-2 cells

We detected the expression of Wnt1 and β-catenin using qRT-PCR and western blotting. Knock-down of RECQL5 not only increased the mRNA level and protein level of Wnt1 in Hep-2 cells, but also increased β-catenin in Hep-2 cell's nucleus (**Figure 4**). This indicates that RECQL5 gene silencing activates Wnt/β-catenin signaling pathway components in Hep-2 cells.

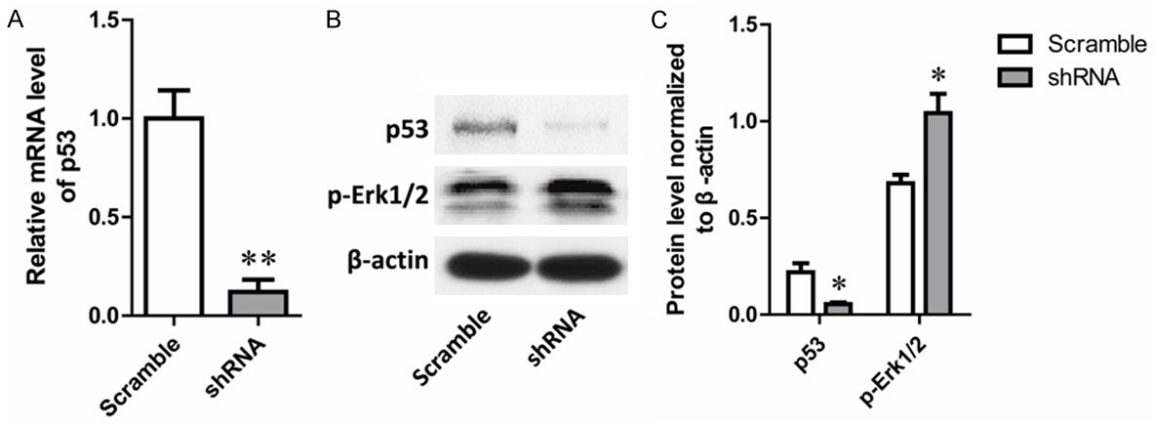

Figure 3. Downregulation of p53 and increased phosphorylation of Erk1/2 in Hep-2 cells with knockdown of RECQL5. A: mRNA expression of p53 was detected by qRT-PCR, error bars represent ± SD, $**P < 0.01$ versus Scramble group; B: p53 protein and p-Erk1/2 were detected using western blot; C: Relative protein level of p53 and p-Erk1/2 to β-actin, the value was showed as mean ± SD, $*P < 0.05$ versus Scramble group.

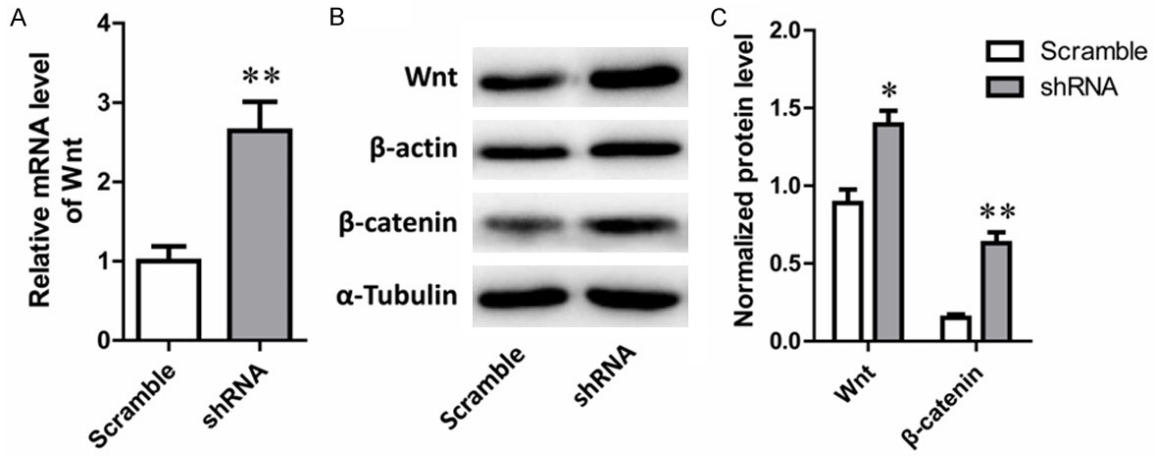

Figure 4. Upregulation of Wnt1 and β-catenin in Hep-2 cells with knockdown of RECQL5. A: mRNA expression of Wnt1 was detected by qRT-PCR, the value was showed as mean ± SD, $**P < 0.01$ versus Scramble group; B: Wnt1 and β-catenin proteins were detected using western blot; C: Relative protein level of Wnt1 and β-catenin, the value was showed as mean ± SD, $*P < 0.05$, $**P < 0.01$ versus Scramble group.

Discussion

The mutation of RECQL4 can induce tumorigenesis, and the overexpression of RECQL4 also can induce osteosarcoma, prostatic cancer, breast cancer and cervical cancer [13-16]. RECQL5 is one of the RECQ helicase family members as well as RECQL4, participates in the DNA replication, repair, recombination and transcription. Knockout of RECQL5 in mouse will cause chromosome instability (CIN) and add the tumor susceptibility of some tissues and organs [3]. Our previous study has showed that a relationship between RECQL5 gene polymorphism and haplotypes and larynx cancer risk is existed in China [17]. In this study, we found that knockdown of RECQL5 promoted the Hep-2 cells proliferation and migration, but inhibited the Hep-2 cells apoptosis; which suggests that RECQL5 may participate in suppressing the Hep-2 cells malignancy.

P53 is an important cancer suppressor genes and one of the most crucial genes in the cell signaling transduction, the production of *p53* gene is a nuclear transcription factor. The deletion or mutation of p53 can induce the malignant cell transformation [18]. Mutation of *p53*

is existed in more than 50% of malignancies, and p53 is also overexpressed in Hep-2 cells and participates in Hep-2 cells growth and metastasis [19]. In the present study, knockdown of RECQL5 suppressed the p53 expression; this suggests that there is some correlation between RECQL5 and p53 in Hep-2 cells development.

P-Erk1/2 as the activation of Erk1/2 can act on Elk-1, c-myc, c-fos, NF-Kb and other transcription factors in the cell nucleus to participate in cell growth and cell development and cells malignant transformation. Previous researches showed that Erk1/2 was aberrantly expressed or activity enhanced in liver cancer, LSCC, prostatic cancer and other tumor tissues [20-22]. Furthermore, Erk1/2 is involved in regulating LHSCC cell invasion [23]. In this study, phosphorylation of Erk1/2 was increased in Hep-2 cells with knockdown of RECQL5 indicates that RECQL5 can depress the Hep-2 cell migration via negatively regulating the activation of Erk1/2.

Increasing evidences have proved that abnormal activation of Wnt pathway elements play key role in tumorgenesis, such as overexpression of Wnt1 and cyclinD1, ectopic expression of β-catenin, and inactivation of APC [24-26]. Galera-Ruiz et al. detected the Wnt-1 and β-catenin in LSCC tissues and the results showed the classical Wnt pathway was inactive in LSCC [27]. And in our study, knockdown of RECQL5 increased the expressions of Wnt1 and β-catenin, this result suggests that RECQL5 may participate in blocking the Wnt/β-catenin signaling.

In conclusion, in this study, we showed RECQL5 not only suppressed the Hep-2 cells proliferation and migration, promoted the Hep-2 cells apoptosis, but also increased the p53 expression and decreased the expression of Wnt1, translocation of β-catenin into nucleus and phosphorylation of Erk1/2. And our study indicates RECQL5 suppress Hep-2 cells malignancy via enhancing the p53 expression and inhibiting Wnt/β-catenin signaling and activation of Erk1/2. So RECQL5 may be a potential target for inhibiting the LSCC development.

Disclosure of conflict of interest

None.

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