European Review for Medical and Pharmacological Sciences 2020; 24: 6858-6863

CDCA2 promotes proliferation and migration of melanoma by upregulating CCAD1

W.-H. JIN[1,2], A.-T. ZHOU[3], J.-J. CHEN[1], Y. CEN[1]

[1]Department of Burn and Plastic Surgery, West China Hospital of Sichuan University, Chengdu, China
[2]Department of Burn and Plastic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, China
[3]Department of Reproductive Center, Affiliated Hospital of Zunyi Medical University, Zunyi, China

Abstract. – OBJECTIVE: This study aims at investigating the functional role of CDCA2 (cell division cycle associated 2) in enhancing proliferative and migratory abilities in melanoma by upregulating CCAD1, thus aggravating the progression of melanoma.

PATIENTS AND METHODS: CDCA2 levels in melanoma tissues and cell lines were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. Regulatory effects of CDCA2 on proliferative and migratory abilities in melanoma cells were assessed by Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU), and wound healing assay, respectively. At last, rescue experiments were conducted to explore the involvement of CCAD1 in CDCA2-regulated progression of melanoma.

RESULTS: CDCA2 was upregulated in melanoma tissues, especially in those with metastasis. Identically, in vitro level of CDCA2 was upregulated in melanoma cell lines. The knockdown of CDCA2 in A375 and sk-mel-110 cells inhibited the proliferative and migratory abilities. The overexpression of CCAD1 could partially abolish the inhibitory effects of silenced CDCA2 on proliferative and migratory abilities in melanoma.

CONCLUSIONS: CDCA2 stimulates proliferative and migratory abilities in melanoma cells by upregulating CCAD1, thus aggravating the malignant progression of melanoma.

Key Words:
Melanoma, CDCA2, CCAD1.

Introduction

Melanoma is a type of skin malignancy, which is deteriorated from nevi or black spots formed by melanocytes[1]. In recent years, melanoma has become a malignancy with the fastest growing incidence. Once melanoma develops into the rapid growth phase, it leads to an extremely poor prognosis with a very high mortality[2]. It is urgent to search for hallmarks identifying progression of melanoma and develop effective target strategies.

CDCA2 (cell division cycle associated 2) is a cell cycle protein[3,4]. CDCA2 prepares mitotic chromatin for interphase transition and control protein phosphatase 1γ (PP1γ)-dependent DNA damage response (DDR)[5,6]. CDCA2 is abnormally expressed in many types of tumors, which is closely linked to tumor progression[7-9]. Ryu et al[10] analyzed gene microarrays in a series of melanoma cell lines, and they found that CDCA2 is upregulated.

CCAD1 is a primary medium for cell adhesion in Xenopus gastrulation[11]. Guilford et al[12] and Perl et al[13] supported the findings that CCAD1 is closely linked to tumor progression. Serum level of soluble CCAD1 is a vital indicator for preventing, diagnosing, and treating gastrointestinal cancer[14,15]. Shields et al[16] demonstrated that CCAD1 deficiency inhibits the anti-tumor activity of CD103 and reduces checkpoint response of melanoma. In this paper, we mainly uncovered the potential influences of CDCA2 on phenotype changes of melanoma cells and the underlying mechanism.

Patients and Methods

Sample Collection

Melanoma tissues (n=66) were surgically resected from melanoma patients treated in our hospital from December 2016 to December 2018. Patients were included according to the pathological diagnostic criteria of melanoma. Patients with recurrence, metastasis, or non-first-diagnosed patients who have been diagnosed in other hospitals were excluded, and cases with incomplete clinical data and pathological diagnosis with doubt

Corresponding Authors: Ying Cen, MD; e-mail: 79701617@qq.com

or unknown diagnosis were excluded. Normal skin tissues (n=60) were collected from patients undergoing skin transplantation. Tissues were stored at -80°C. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of West China Hospital of Sichuan University. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture and Transfection

The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 μg/mL penicillin, and 100 μg/mL streptomycin. After cell reached 80% confluence, those in good condition were inoculated and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 24 h later.

The siRNA sequences of CDCA2 were as follows: siRNA1: 5′-CACCUGCCUUUCUAAAUAUTT-3′; siRNA2: 5′-GGGCAAAGGAUCAAGUGAUTT-3′; siRNA3: 5′-CUGCCUUGGAAAGGAUUGATT-3′.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for qRT-PCR detection by SYBR Green method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The primer sequences were listed as follows: CDCA2: forward: 5′-TGCCGAATTACCTCCTAATCCT-3′ and reverse: 5′-TGCTCTACGGTTACTGTGGAAA-3′; CCAD1: forward: 5’-ATTGCTCACATTTCCCAACTC-3’ and reverse: 5’-GTCACCTTCAGCCATCCT-3’; GAPDH: forward: 5′-GGAGCGAGATCCCTCCAAAAT-3′ and reverse: 5′-GGCTGTTGTCATACTTCTCATGG-3′.

Western Blot

The cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes reacted with the primary antibodies, including GAPDH (Sigma Aldrich, St. Louis, MO, USA), CDCA2 (1:200, Abcam, Cambridge, MA, USA) and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Cell Counting Kit-8 (CCK-8)

The cells were inoculated in a 96-well plate with 2×10^3 cells/well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were inoculated in a 96-well plate with 1×10^3 cells per well. They were labeled with EdU solution in the dark for 30 min, and stained with Hoechst 33342 for another 30 min. Images of EdU-labeled cells, 4’,6-diamidino-2-phenylindole (DAPI)-labeled nuclei and the merged one were taken under a fluorescence microscopy.

Wound Healing Assay

The cells were inoculated in a 6-well plate and cultured for 24 h. An artificial wound was created using the 200 μL pipette tip. Wound healing was observed at 0 and 24 h, respectively.

Statistical Analyses

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing differences between two groups. $p<0.05$ indicated the significant difference.

Results

CDCA2 Was Upregulated in Melanoma

Compared with 60 normal skin tissues, CDCA2 was upregulated in 66 melanoma tissues (Figure 1A). In particular, CDCA2 level was higher in melanoma patients with metastasis (Figure 1B). In melanoma cell lines, *in vitro* level of CDCA2 remained higher at both mRNA and protein levels, especially A375 and sk-mel-110 cells among the four tested cell lines (Figure 1C, 1D).

Silence of CDCA2 Suppressed Proliferative Ability in Melanoma

We constructed three CDCA2 siRNAs and tested their transfection efficacy in A375 and sk-mel-110 cells by qRT-PCR, and finally, siRNA-2#

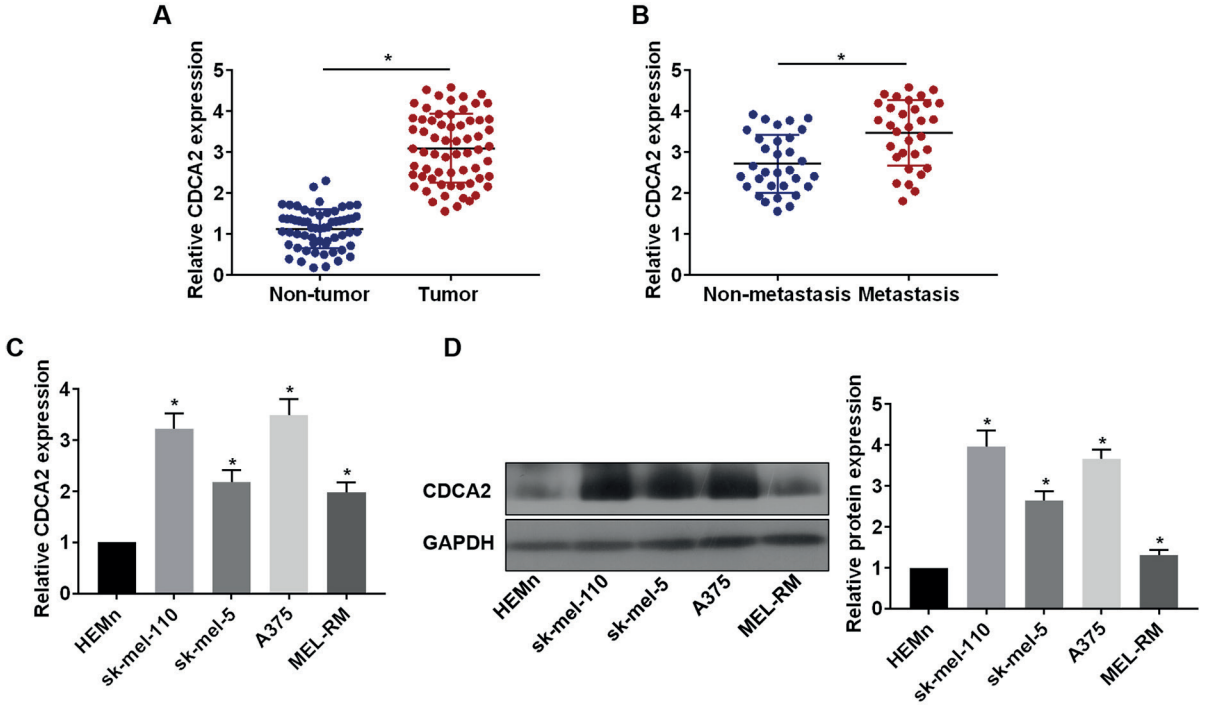

Figure 1. CDCA2 was upregulated in melanoma. **A,** CDCA2 levels in melanoma tissues (n=66) and normal tissues (n=60). **B,** CDCA2 levels in melanoma patients either with metastasis or not. **C-D,** The mRNA (**C**) and protein levels (**D**) of CDCA2 in melanoma cell lines.

was verified to be the optimal one and was utilized in the following experiments (Figure 2A). Protein level of CDCA2 was remarkably downregulated in melanoma cells transfected with si-CDCA2 (Figure 2B). Later, both CCK-8 and EdU assay demonstrated that viability and EdU-positive ratio declined in A375 and sk-mel-110 cells transfected with si-CDCA2, indicating the suppressed proliferative ability caused by silencing CDCA2 (Figure 2C, 2D).

Silence of CDCA2 Suppressed Migratory Ability in Melanoma

Wound healing assay was conducted to assess the influence of CDCA2 on migratory ability in melanoma. It is shown that silence of CDCA2 markedly decreased percentage of wound healing (Figure 3).

Overexpression of CCAD1 Partially Reversed the Regulatory Effect of CDCA2 on Proliferative Ability in Melanoma

Transfection of pcDNA-CCAD1 remarkably upregulated CCAD1 in sk-mel-110 cells, verifying the great transfection efficacy (Figure 4A). Furthermore, the decreased viability in sk-mel-110 cells transfected with si-CDCA2 was partially reversed by overexpression of CCAD1 (Figure 4B). Identically, inhibitory effect of silenced CCAD1 on EdU-positive ratio was abolished by co-transfection of pcDNA-CCAD1 (Figure 4C). It is demonstrated that CCAD1 was responsible for CDCA2-regulated phenotypes of melanoma cells.

Discussion

Melanoma is a strongly invasive skin cancer[17]. Melanoma has been well concerned because of strong invasiveness, high rate of metastases and resistance to chemotherapy or radiotherapy[18]. Great strides have been made on finding effective and sensitive hallmarks for melanoma[19,20].

CDCA2 is a PP1-binding protein[21]. Qian et al[22] reported that CDCA2 is a vital regulator for chromatin remodeling, which mediates cell cycle progression through dephosphorylation of Histone H3 *via* targeting PP1. In oral squamous cell carcinoma, protein level of CDCA2 is correlated to tumor volume and TNM staging. Knockdown of CDCA2 leads to cell cycle arrest, proliferation inhibition, and apoptosis induction[23,24]. CDCA2

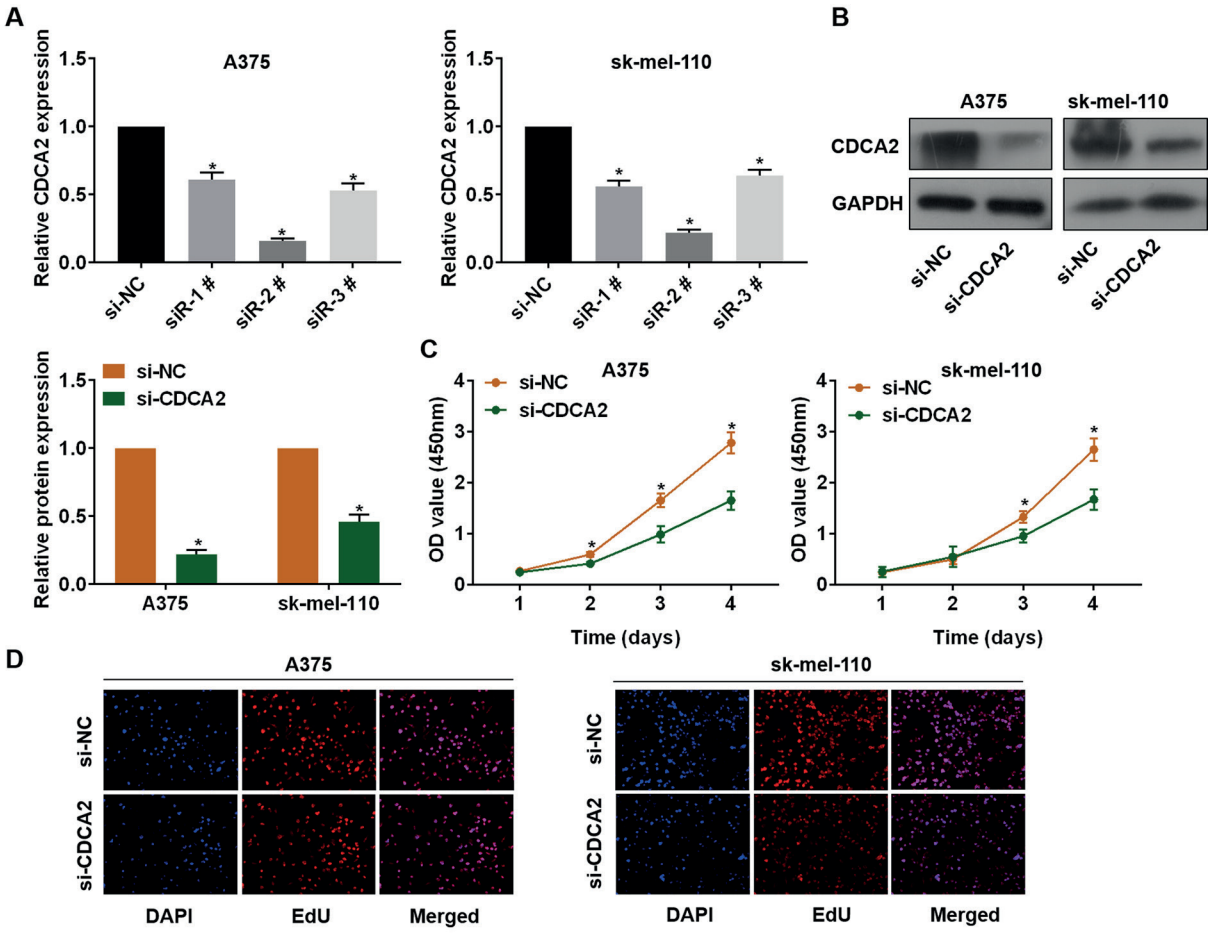

Figure 2. Silence of CDCA2 suppressed proliferative ability in melanoma. **A,** Transfection efficacy of three siRNA CDCA2 in A375 and sk-mel-110 cells. **B,** Protein level of CDCA2 in A375 and sk-mel-110 cells transfected with si-NC or si-CDCA2. **C,** CCK-8 assay showed cell viability in A375 and sk-mel-110 cells transfected with si-NC or si-CDCA2. **D,** EdU-assay showed images of EdU-positive cells, DAPI-labeled nuclei and merged one in A375 and sk-mel-110 cells transfected with si-NC or si-CDCA2 (magnification 40×).

is reported to be highly expressed in melanoma[10]. Consistently, our findings uncovered that CDCA2 was upregulated in melanoma tissues and cell lines. The knockdown of CDCA2 markedly suppressed proliferative and migratory abilities in melanoma cells.

Feng et al[25] demonstrated that the overexpression of CDCA2 triggers colorectal cancer (CRC) cells to proliferate. It is reported that CDCA2 stimulates the progression of cell cycle by upregulating CCND1 in CRC. Here, our findings uncovered that the overexpression of CCAD1 could partially abolish the inhibitory effects of silenced CDCA2 on proliferative and migratory abilities in melanoma. It is indicated that CCAD1 was responsible for CDCA2-regulated phenotypes of melanoma cells. Our findings provide a novel target for clinical treatment of melanoma.

In this study, we explored the expression of CDCA2 in melanoma and demonstrated that it promotes the occurrence and development of melanoma, and its mechanism occurs through the regulation of CCAD1. This research is helpful to promote our understanding of melanoma and has potential value for its diagnosis and treatment.

Conclusions

Shortly, CDCA2 stimulates proliferative and migratory abilities in melanoma cells by upregulating CCAD1, thus aggravating the malignant progression of melanoma.

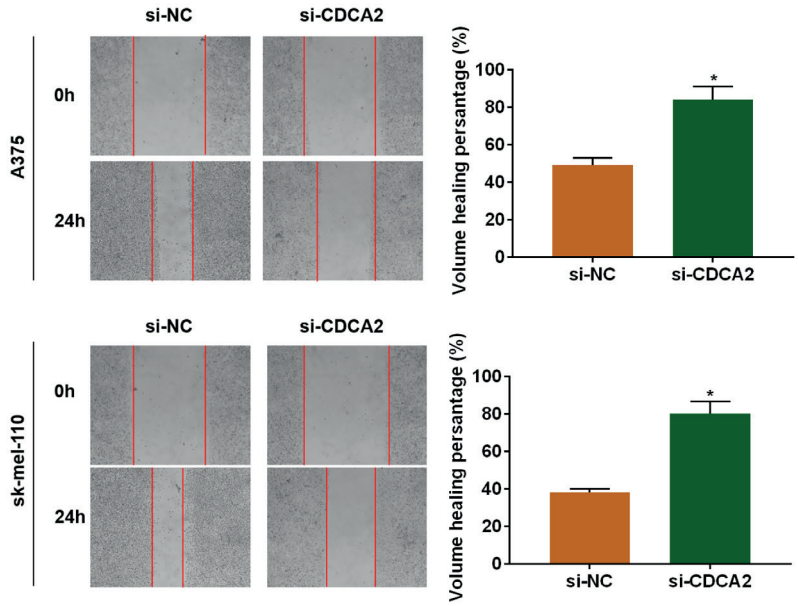

Figure 3. Silence of CDCA2 suppressed migratory ability in melanoma. Wound healing in A375 and sk-mel-110 cells transfected with si-NC or si-CDCA2 (magnification 40×).

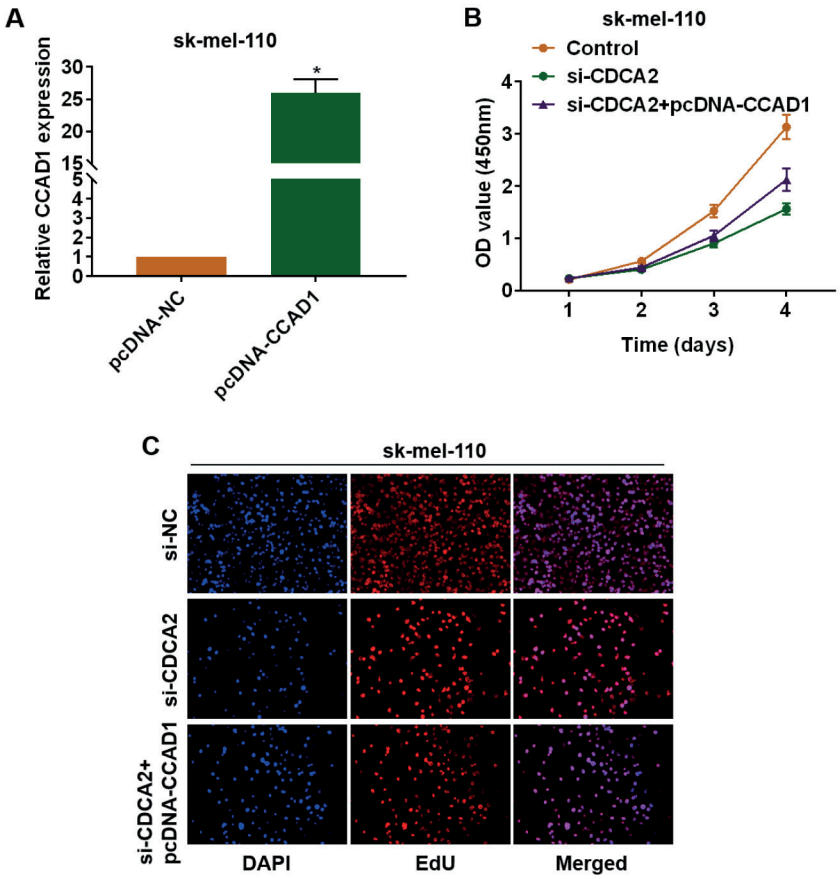

Figure 4. Overexpression of CCAD1 partially reversed the regulatory effect of CDCA2 on proliferative ability in melanoma. **A,** Transfection efficacy of pcDNA-CCAD1 in sk-mel-110 cells. Sk-mel-110 cells were transfected with NC, si-CDCA2 or si-CDCA2+pcDNA-CCAD1. **B,** Cell viability. **C,** Images of EdU-positive cells, DAPI-labeled nuclei and merged one (magnification 40×).

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Ahlqvist E, Van Zuydam NR, Groop LC, McCarthy MI. The genetics of diabetic complications. Nat Rev Nephrol 2015; 11: 277-287.
2) Donate-Correa J, Martín-Núñez E, Muros-de-Fuentes M, Mora-Fernández C, Navarro-González JF. Inflammatory cytokines in diabetic nephropathy. J Diabetes Res 2015; 2015: 948417.
3) Wang HY. Third edition of nephrology. People's Publishing House 2008: 1321-1322.
4) Jiang C, Li X, Zhao H, Liu H. Long non-coding RNAs: potential new biomarkers for predicting tumor invasion and metastasis. Mol Cancer 2016; 15: 62.
5) Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. Cancer Cell 2016; 29: 452-463.
6) Tan J, Qiu K, Li M, Liang Y. Double-negative feedback loop between long non-coding RNA TUG1 and miR-145 promotes epithelial to mesenchymal transition and radioresistance in human bladder cancer cells. FEBS Lett 2015; 589: 3175-3181.
7) Duan LJ, Ding M, Hou LJ, Cui YT, Li CJ, Yu DM. Long noncoding RNA TUG1 alleviates extracellular matrix accumulation via mediating microRNA-377 targeting of PPARγ in diabetic nephropathy. Biochem Biophys Res Commun 2017; 484: 598-604.
8) Zhang H, Li H, Ge A, Guo E, Liu S, Zhang L. Long non-coding RNA TUG1 inhibits apoptosis and inflammatory response in LPS-treated H9c2 cells by down-regulation of miR-29b. Biomed Pharmacother 2018; 101: 663-669.
9) Meng SH, Rong W, Xiao BL, Ming HF, Jian GL, Jun HZ, Li QC, Zhi ML. LncRNA MALAT1 is dysregulated in diabetic nephropathy and involved in high glucose-induced podocyte injury via its interplay with b-catenin glucose-induced podocyte injury via its interplay with b-catenin. J Cell Mol Med 2017; 21: 2732-2747.
10) Jiao HY, Xie DL, Qiao YH. LncRNA PRINS is involved in the development of nephropathy in patients with diabetes via interaction with Smad7. Exp Ther Med 2019; 17: 3203-3208.
11) Han J, Zhou W, Jia M, Wen J, Jiang J, Shi J, Zhang K, Ma H, Liu J, Ren J, Dai M, Hu Z, Hang D, Li N, Shen H. Expression quantitative trait loci in long non-coding RNA PAX8-AS1 are associated with decreased risk of cervical cancer. Mol Genet Genomics 2016; 291: 1743-1748.
12) Yu XL, Cao Y, Tang L, Xia JY, Yang YC, Chen F. Baicalein inhibits breast cancer growth via activating a novel isoform of the long noncoding RNA PAX8-AS1-N. J Cell Biochem 2018; 119: 6842-6856.
13) Vega-Warner V, Ransom RF, Vincent AM, Brosius FC, Smoyer WE. Induction of antioxidant enzymes in murine podocytes precedes in jury by puromycin aminonucleoside. Kidney Int 2004; 66: 1881-1889.
14) De-Vriese AS, Titton RG, Stephan CC, Lameire NH. Vascular endothelial growth factor is essential for hyperglycemia induced structural and functional alterations of the peritoneal membrane. J Am Soc Nephrol 2001; 12: 1734-1741.
15) Morishita Y, Hanawa S, Miki T, Sugase T, Sugaya Y, Chinda J, Iimura O, Tsunematsu S, Ishibashi K, Kusano E. The association of plasma prorenin level with an oxidative stress marker, 8-OHdG, in nondiabetic hemodialysis patients. Clin Exp Nephrol 2011; 15: 398-404.
16) Tervaert TW, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, Ferrario F, Fogo AB, Haas M, de Heer E, Joh K, Noël LH, Radhakrishnan J, Seshan SV, Bajema IM, Bruijn JA; Renal Pathology Society. Pathologic classification of diabetic nephropathy. J Am Soc Nephrol 2010; 21: 556-563.
17) Jiang Z, Seo JY, Ha H, Lee EA, Kim YS, Han DC, Uh ST, Park CS, Lee HB. Reactive oxygen species mediate TGF-beta1-induced plasminogen activator inhibitor-1 upregulation in mesangial cells. Biochem Biophys Res Commun 2003; 309: 961-966.
18) Wang M, Wang S, Yao D, Yan Q, Lu W. A novel long non-coding RNA CYP4B1-PS1-001 regulates proliferation and fibrosis in diabetic nephropathy. Mol Cell Endocrinol 2016; 426: 136-145.