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Genistein inhibited the proliferation of kidney cancer cells via *CDKN2a* hypomethylation: role of abnormal apoptosis

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Abstract

Introduction Genistein is recognized as a potent anti-oxidant in soybean-enriched foods, which is a kind of phytoestrogen involved in anticancer activity in various cancers.

Objective The objective of this study was to investigate the molecular mechanism of *CDKN2a* hypomethylation involved in the anti-tumor effect of genistein on kidney cancer.

Methods The *CDKN2a* expression was measured using qRT-PCR. The level of *CDKN2a* methylation was detected using methylation-specific PCR. The apoptosis was detected via flow-cytometric analysis. The cell viability was detected using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results Our results indicated that genistein induced cell apoptosis and inhibited the cell proliferation of kidney cancer cells. Moreover, genistein increased the expression of *CDKN2a* and decreased *CDKN2a* methylation.

Conclusions Our results demonstrated that the anti-tumor effect of genistein might induce cell apoptosis and inhibit the proliferation of kidney cancer cells via regulating *CDKN2a* methylation.

Keywords Genistein · Kidney cancer cells · *CDKN2a* hypomethylation · Proliferation · Apoptosis

Introduction

Genistein is a potent anti-oxidant present in dietary plants and is considered to be a predominant kind of isoflavone [1–3]. Increasingly experimental and epidemiological evidences demonstrated that genistein is involved in biological anticancer activity in various cancer types, such as prostate cancer, breast cancer, colon cancer, gastric cancer, lung cancer, pancreatic cancer, and lymphoma [4]. Although genistein has positive effects on cancer prevention, the underlying mechanisms were far from clear, especially in kidney cancer. It is urgent need to conduct a detailed investigation on the anti-tumor effect of Genistein.

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Genistein-induced apoptosis has been well recognized and confirmed [5]. Many studies reported that genistein has dual effects of estrogen-like and anti-proliferative activities [6]. It has proposed that genistein could enhance radiosensitivity by promoting DNA damage-induced apoptosis [5]. Due to the dissociation of Bcl-xL and Beclin-1, genistein promoted Beclin-1-mediated autophagy [5]. The relationship between Genistein-induced DNA methylation and apoptosis was still unknown. Recently, epigenetic events were confirmed to play an important role in carcinogenesis progress [7]. DNA methylation is the crucial epigenetic mechanism that occurs at the stages of carcinogenesis [8]. DNA methylation is an epigenetic marker which regulated a variety of biological processes, such as cell growth, proliferation, and apoptosis [9]. In previous studies, hypomethylation of the *CDKN2a* was linked to *CDKN2a* regulating cell proliferation [10].

CDKN2a is well regarded as a tumor suppressor gene, and known to decrease the risk of cancer [11]. *CDKN2a* has a crucial function in cell apoptosis and was known to be inactivated in various types of cancer. Epigenetic inactivation of *CDKN2a* is considered as a common hallmark of most cancers, and *CDKN2a* loss was negative regulators of

cell cycle G1 progression [12–14]. In several types of malignancy, studies has been reported that *CDKN2a* hypomethylation resulted in its activation [15], including head and neck [16], hepatocellular [17], lung [18], breast [19], and esophageal [20] cancers. However, the molecular mechanisms of *CDKN2a* methylation in kidney cancer remain to be elucidated.

Due to the facts that the role of DNA methylation on the anti-tumor effects of genistein was not clear, our goal was to explore whether the occurrence of *CDKN2a* hypomethylation leads to Genistein-induced abnormal apoptosis and proliferation in kidney cancer. Our study found that genistein inhibited the proliferation of kidney cancer cells via apoptosis. Furthermore, genistein increased the expression of *CDKN2a* and decreased *CDKN2a* methylation. Our results demonstrated that the anti-tumor effect of genistein might induced cell apoptosis and inhibited the proliferation of kidney cancer cells via regulating *CDKN2a* methylation.

Materials and methods

Cell culture and genistein treatment

HEK293, HK-2, 786-O, CAKI-1, 769-P, and CAKI-2 cell lines were obtained from American Type culture collection (ATCC) and used according to their reported protocol. DMEM medium containing with 10% FBS in a humidified incubator with 5% at 37 °C CO2 was used to culture cells. Genistein (Sigma, St Louis, MO, USA) was prepared into 10 mM genistein using DMSO and stored at − 20 °C. Various concentrations of genistein (0, 25, 50, 100 μM) were made in a complete medium and used as the working concentration. For experiments, cells were treated with a dose of genistein and cultured in plates.

RNA isolation and quantitative real-time polymerase chain reaction (qRT–PCR) for *CDKN2a* expression

TRIzol reagent (Thermo Fisher Scientific) was used to extract Total RNA. The mRNA levels of *CDKN2a* were determined using the synthesized RevertAid First strand cDNA (Thermo Fisher Scientific, USA) according to the instructions of manufacturer. Then, qRT-PCR was performed using SYBR Green (Thermo Fisher Scientific, USA). GAPDH was used as an internal control.

Primers used for RT-PCR: *CDKN2a*-F 5′-TTATTAGAGGGTGGGGTGGATTGT-3′, *CDKN2a*-R 5′-CAACCCCAAACCACAACCATAA-3′, GAPDH-F 5′-GCCTTCCGTGTCCCCACTGC-3′, GAPDH-R 5′-GGCTGGTGGTCCAGGGGTCT-3′.

DNA extraction and methylation-specific PCR (MSP) to measure the level of methylated *CDKN2a*

DNA was isolated using EZNA-DNA kit (Omego) according to the protocols. Total of 1 μg purified DNA was modified by CpGenome DNA Modification Kit (Chemicon International, USA) to detect the level of methylation and unmethylation [21]. The PCR products for *CDKN2a* were, respectively, measured by agarose gel electrophoresis. The level of *CDKN2a* methylation was visualized by a UV illuminator. All assays were performed for three times. The density of each band was applied to quantify using Image analysis software (GelPro 4.5). *CDKN2a* methylation in Genistein-treated group was expressed as a fold of the control band density.

The following primers were used: *CDKN2a* m-sense 5′-TTATTAGAGGGTGGGGCGGATCGC-3′, m-antisense 5′-GACCCCGAACCGCGACCGTAA-3′, u-sense 5′-TTATTAGAGGGTGGGGTGGATTGT-3′, and u-antisense 5′-CAACCCCAAACCACAACCATAA-3′.

Flow-cytometric analysis

Apoptosis was measured with the Annexin V-FITC/PI apoptosis kit (Annexin V-FITC Apoptosis Detection Kit I, BD). Cells were treated to genistein for 5 days, and then stained with Annexin V-FITC and PI at room temperature in the dark [22]. Total apoptotic cells were analyzed by flow cytometry (Becton Dickinson, USA). The results were calculated as the percentage of apoptotic cells.

Determination of cell proliferation

The MTT assay is widely used to investigate proliferation after exposing Genistein. Cells were plated in 96-well plates. The cells were then exposed to the varying concentrations of Genistein. After treatment, formazan crystals were dissolved after adding 15 ml of 5 mg/ml MTT into each well. The absorbance 492 nm was measured using a microplate reader.

Statistical analyses

Data were expressed as the mean ± S.D. and analyzed using SPSS 17.0. Normality and homogeneity of variances were evaluated prior to statistical analysis. Data were analyzed using multiple comparison and one-way analysis of variance was done between combined treated groups and control, otherwise followed by correlation analysis. $P < 0.05$ was considered to indicate a statistically difference.

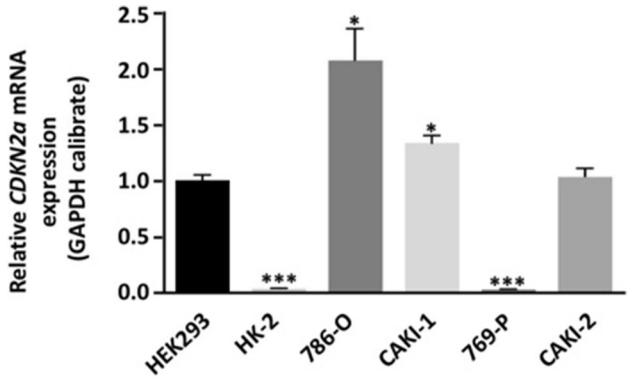

Fig. 1 *CDKN2a* expression was measured in multiple types of kidney cell lines. *CDKN2a* expression in multiple types of kidney cell lines was measured by qRT-PCR. $^{*}P<0.05$ and $^{**}P<0.01$, compared with control group. Data are expressed as mean ± S.D.; $n=3$

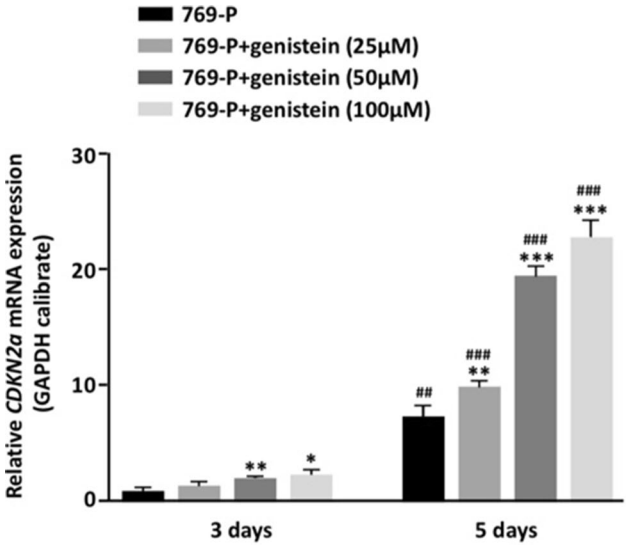

Fig. 2 Genistein up-regulated the level of *CDKN2a* expression in a dose- and time-independent manner. The concentrations of genistein (0, 25, 50, 100 μM) treated 769-P cells for 3 days and 5 days, respectively, and then, *CDKN2a* expression was detected using qRT-PCR. $^{*}P<0.05$ and $^{**}P<0.01$, compared with control group treated with 3 days; $^{\#\#}P<0.01$, compared with various concentrations of genistein group treated with 3 days. Data are expressed as mean ± S.D.; $n=3$

Results

CDKN2a expression in multiple types of kidney cells

To make a choice for the appropriate cell line, a variety of kidney cell lines were selected to detect *CDKN2a* expression in this study. As shown in Fig. 1, *CDKN2a* expression was obviously changed in HK-2, 786-O, CAKI-1, and 769-P compared with HEK293 cells. The HEK293 is an eternalized primary embryonic human kidney cell line, and we investigated the difference between normal cells and kidney cancer cells. Therefore, we did not choose HK-2 cells in our study. In these cells, the expression of *CDKN2a* was most significantly changed in 769-P cells, and the level of *CDKN2a* expression was significantly decreased.

Genistein up-regulated *CDKN2a* expression in a dose- and time-dependent manner

In the current study, various doses of genistein (0, 25, 50, 100 μM) treated 769-P cells for 3 days and 5 days, respectively, and then, the status of *CDKN2a* expression was detected. As shown in Fig. 2, *CDKN2a* expression increased in Genistein-treated 769-P cells in a dose-dependent manner. The expression of *CDKN2a* was higher at 5 days than at 3 days. The results implicated that genistein activated the expression level of *CDKN2a*.

Genistein-induced *CDKN2a* hypomethylation was detected using MSP method

The level of *CDKN2a* methylation was performed using MSP method. In left of 769-P lane, the result showed that there was difference between HEK293 cell line and 769-P cell line. In right of 769-P lane, we investigated whether various concentrations of genistein had anti-proliferative effects (Fig. 3). As shown in Fig. 3, the status of *CDKN2a* methylation decreased in Genistein-treated 769-P cells in a dose-dependent manner. The results implicated that genistein increased the expression level of *CDKN2a*. These results further confirm the relationship between decreased *CDKN2a* methylation and increased *CDKN2a* expression.

Genistein increased cell apoptosis

To confirm whether genistein could induce the cell apoptosis of 769-P cells, the apoptotic rate was measured by flow-cytometric analysis. The results showed that genistein increased total apoptotic rate in a dose-dependent manner (Fig. 4). In accordance with the MTT results, genistein decreased the viability of cells through the induction of apoptosis.

Genistein inhibited cell proliferation

The 769-P cells were treated with various doses of genistein, while control cells were cultured in medium. After treatment with genistein for 5 days, result of cells was evaluated using MTT assay. As shown in Fig. 5, the cell viability of treated groups decreased at all concentrations. The results demonstrated that genistein had an anti-proliferative effect with all of concentrations.

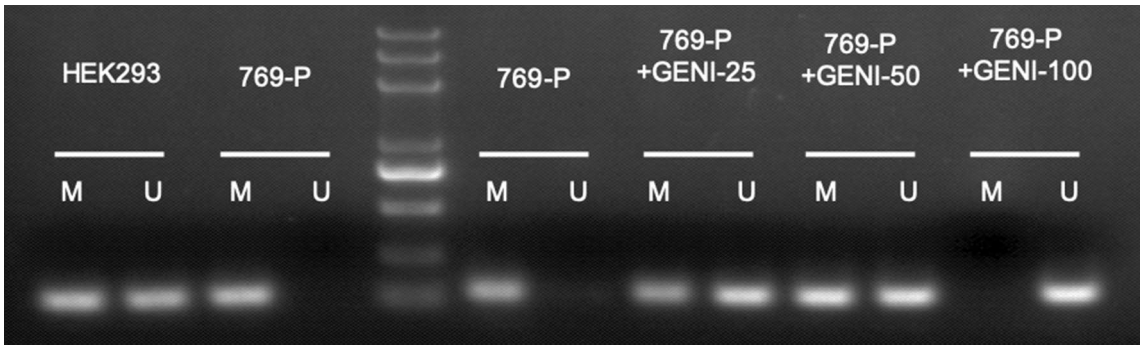

Fig. 3 Genistein induced *CDKN2a* hypomethylation in 769-P cells. Treating concentrations of genistein (0, 25, 50, 100 μM) for 5 days, and then, DNA was isolated and *CDKN2a* methylation status was measured via using methylation-specific PCR method. *M* methylated *CDKN2a, U* unmethylated *CDKN2a*.**P* < 0.05, comparing with control group

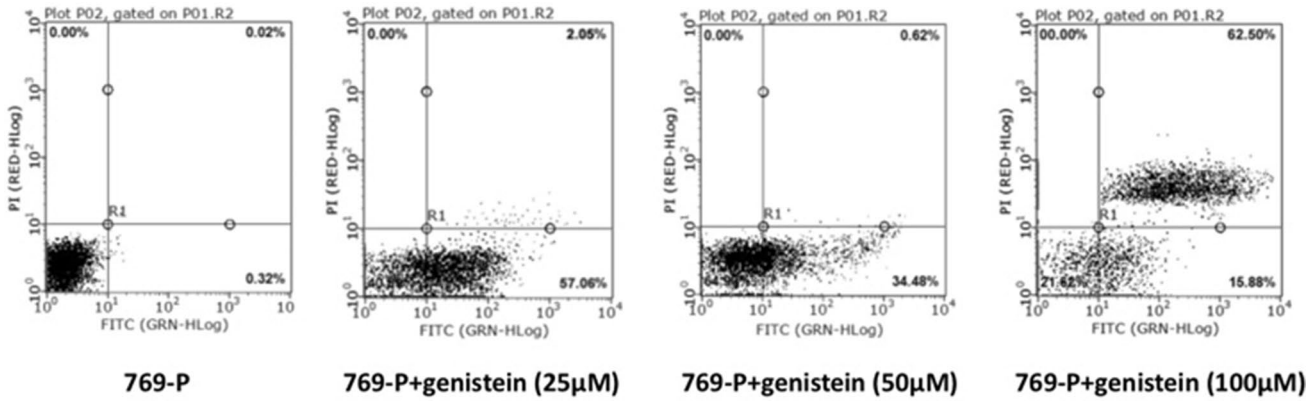

Fig. 4 Genistein promoted cell apoptosis. Apoptosis of 769-P cells was analyzed after treating genistein (0, 25, 50, 100 μM). Annexin V-FITC/PI was used to stain cells, and then investigated by flow cytometry to detect the cells apoptosis in control and Genistein-treated groups. Data are expressed as means ± S.D. From three independent experiments

CDKN2a methylation mediated Genistein-induced apoptosis and proliferation

To further determine whether *CDKN2a* methylation was associated with Genistein-induced apoptosis, the correlation was analyzed using Pearson's correlation analysis. Figure 6a shows that the decrease of *CDKN2a* methylation was negatively correlated with genistein-induced apoptosis. Therefore, the correlation between the level of *CDKN2a* methylation and cell viability was also analyzed by correlation analysis. The cell viability increased when the level of *CDKN2a* methylation increased (Fig. 6b). Besides, results further confirm the relationship between the increased apoptosis and the decreased cell viability. The results indicated that *CDKN2a* hypomethylation regulated kidney cancer via genistein-induced apoptosis and cell proliferation.

Discussion

Genistein is a demethylating agent extracted from soy, which can suppress the initiation and development of various types of tumors. It can act as a chemotherapeutic agent against various types of cancers, such as breast, prostate, liver, lung, colorectal, and gastric [23–25]. Genistein had been reported to have estrogenic and anticancer activities. Genistein has dual effects of estrogen-like and anti-proliferative activities. In line with our results, genistein has anticancer effect by inhibiting proliferation. However, we mainly investigate the role of anti-proliferative activity in kidney cancer cell. However, the underlying molecular mechanism of genistein on kidney cancer is still unclear.

The alteration in tumor suppressor gene is associated with tumorigenesis. *CDKN2a* is one of the tumor suppressor genes and is expressed in many cancers especially in the kidney cancer [26]. In addition, previous studies also found that

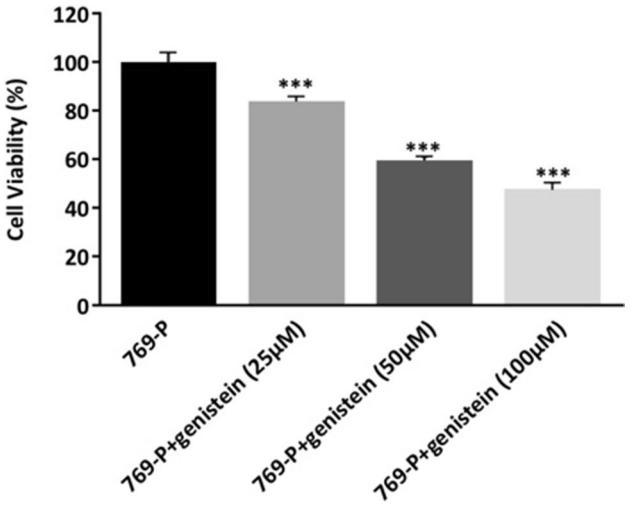

Fig. 5 Genistein induced suppression of cell proliferation in 769-P cells. The concentrations of genistein (0, 25, 50, 100 μM) treated with 769-P cell lines for 5 days. The change of cell viability induced by genistein was detected via using MTT assays. *$P < 0.05$, comparing with control group

the cell proliferation of 769-P was closely related to kidney cancer [10, 27]. We also selected 786-O, 769-P, and CAKI-1 to measure the level of *CDKN2a*. 786-O cells derived from such metastatic tumors can be cultured in vitro in 3D systems that retain bone metastasis characteristics [28]. CAKI-1 is a widespread model line of metastatic renal cell cancer. Most stick to widely known cell lines, such as: 786-O, 769-P, and CAKI-1 [29]. However, there was no obvious changes 786-O and CAKI-1 cells. Thus, this studies showed that the expression of *CDKN2a* was markedly changed in kidney cancer cell (769-P), suggesting that *CDKN2a* might regulate the effect of genistein on the kidney cancer. The molecular mechanism of *CDKN2a* on the anti-tumor effect of genistein is not yet clear.

Abnormal DNA methylation was found in the early stages of tumor and is of great significance in tumor early diagnosis [30]. Recent studies have shown that some drugs do not cause mutations of DNA, but can lead to certain chemical modifications of the genetic material, such as acetylation, phosphorylation, methylation, and the like [31]. Methylation was speculated involved in kidney cancer [32], and thus, we investigated anti-tumor effect and potential molecular mechanism of *CDKN2a* methylation induced by genistein in human kidney cancer in vitro. The result of *CDKN2a*

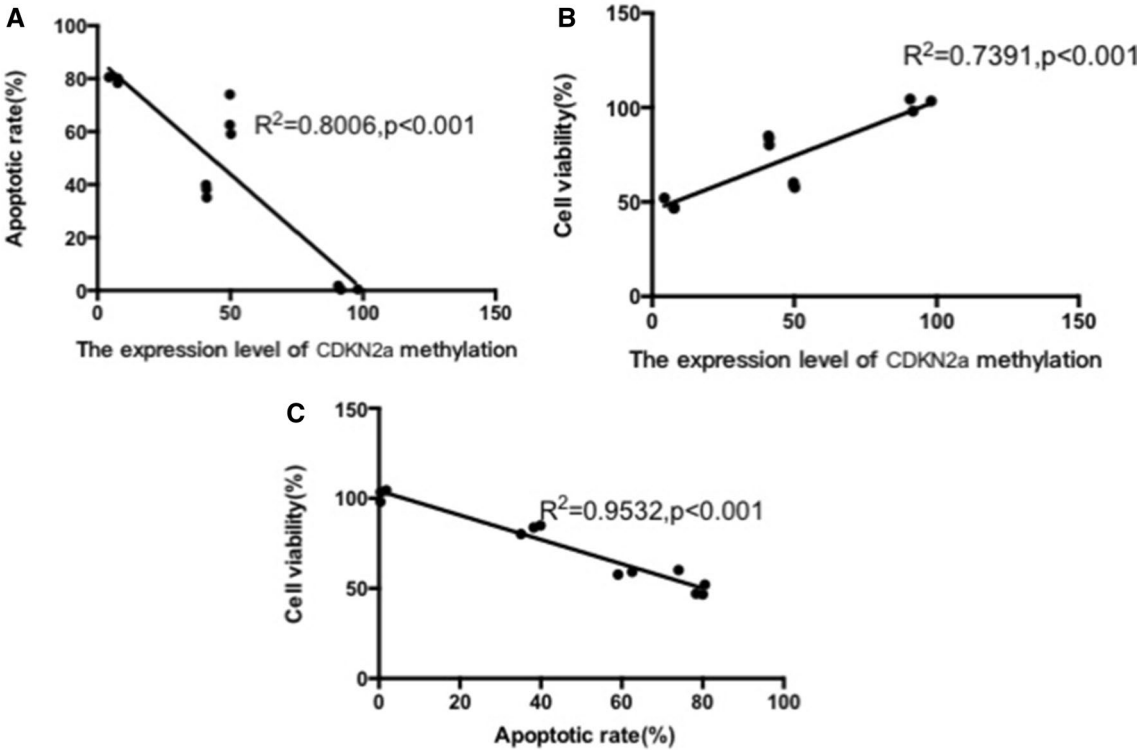

Fig. 6 *CDKN2a* methylation mediated genistein-induced apoptosis and proliferation. The correlation analysis among *CDKN2a* methylation status, apoptotic rate, and cell viability was analyzed. **a** The correlation between *CDKN2a* methylation status and apoptotic rate was analyzed by the correlation analysis. **b** The relationship between *CDKN2a* methylation status and cell proliferation was analyzed by the correlation analysis. **c** The correlation between apoptotic rate and cell proliferation was measured by the method of correlation analysis. (*Statistically significant correlation between two variables, $P < 0.05$)

hypomethylation induced by genistein is consistent with that of kidney cancer, which implicates that the decreasing *CDKN2a* methylation levels plays an important role in human kidney cancer. Previously, *CDKN2a* was reported to be closely related to apoptosis, and *CDKN2a* mediates the development of the disease by regulating apoptosis [33, 34]. We further speculated that *CDKN2a* hypomethylation might mediate the anti-tumor effect of genistein via apoptosis.

The role of apoptosis in the anti-tumor effect of genistein has been reported by many researchers. Previous studies have shown that genistein induced cell apoptosis [35], thus resulting in suppression of cell proliferation [36, 37]. However, limited data suggest that apoptosis and cell proliferation can be regulated by gene methylation status and influence the disease occurrence. Our results found that genistein was associated with gene hypomethylation, cell apoptosis, and cell proliferation, and can reduce the gene methylation level and increase apoptosis to inhibit cell proliferation. Previously, genistein was confirmed to induce apoptosis and inhibit cell proliferation in cervical cancer cells [38], but the apoptosis and cell proliferation triggered by genistein in the kidney cancer is still unclear. We found that genistein could induce apoptosis and inhibit cell proliferation in kidney cancer. More importantly, for the first time, we demonstrated that genistein enhanced the 769-P cells by simultaneously stimulating apoptosis and cell proliferation via decreased *CDKN2a* methylation. The patients with kidney cancers harbored *CDKN2a* hypermethylation had poor survival [39]. Our research may contribute to the prevention and treatment of kidney cancer.

Conclusions

In this study, genistein inhibited the proliferation of kidney cancer cells via regulating cell apoptosis. Furthermore, genistein increased the expression of *CDKN2a* and decreased *CDKN2a* methylation. Our results demonstrated that the anti-tumor effect of genistein might induce cell apoptosis and inhibit the proliferation of kidney cancer cells via regulating *CDKN2a* methylation.

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Compliance with ethical standards

Conflict of interest None of the authors has any commercial or other associations that might pose a conflicts of interest.

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