Epigallocatechin gallate attenuates uric acid-induced injury in rat renal interstitial fibroblasts NRK-49F by up-regulation of miR-9

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Abstract. – OBJECTIVE: Hyperuricemia is a common symptom in chronic kidney disease (CKD) and uric acid (UA) was observed to be elevated in CKD. Epigallocatechin gallate (EGCG) was reported to have multiple protective functions in inflammatory process. In this study, we investigated the effects of EGCG in UA-treated NRK-49F cells.

PATIENTS AND METHODS: NRK-49F cells induced by UA were treated with EGCG and/or transfected with microRNA-9 (miR-9) inhibitor or its negative control (NC). Cell viability and cell apoptosis were detected by Cell Counting Kit-8 (CCK-8) assay and flow cytometry, respectively. In addition, the expression of cell survival- and fibrosis-related factors was measured by qRT-PCR and Western blot, respectively. Concentrations of inflammatory cytokines were detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Increasing cell viability (p < 0.01) and decreasing cell apoptosis (p < 0.01), cell fibrosis (p < 0.01) and inflammatory cytokines (p < 0.05) were observed by administration of EGCG in UA-treated cells. In addition, miR-9 was significantly up-regulated by EGCG (p < 0.05 or p < 0.001). Furthermore, transfection with miR-9 inhibitor impaired the protective functions of EGCG in UA-treated NRK-49F cells (p < 0.05 or p < 0.01). EGCG significantly down-regulated expression of IkBa, p65, janus kinase (JAK) 2 and signal transducers and activator of transcription (STAT) 3 (all p < 0.05).

CONCLUSIONS: EGCG attenuates UA-induced injury in NRK-49F cells by up-regulation of miR-9 and might by inactivation of NF-κB and JAK-STAT signal pathways.

Key Words: Hyperuricemia, EGCG, miR-9, NF-κB, JAK-STAT.

Introduction

Hyperuricemia is a common symptom in chronic kidney disease (CKD)1,2, which is defined according to a serum uric acid (UA) level greater than 7.0 mg/dL, as measured by the automated enzymatic (uricase) method 3. The major complications of hyperuricemia include gout, urolithiasis, and acute uric acid nephropathy 3. UA was observed to be elevated in CKD 4. In addition, abnormal UA expression, both below and above the reference range, can be treated as a marker independent risk factor, or both for metabolic and cardiovascular morbidity and mortality for etiology of various systemic diseases 5. Although accumulating researches have been done in this field, well understanding of the pathogenetic mechanisms of CKD remains uncertain and this hampers the development of effective therapeutic strategies. Epigallocatechin gallate (EGCG), a major constituent purified from green tea, has been widely demonstrated to have a variety of physiological activities. Feng et al6 reported that
EGCG was observed to have higher antioxidant and anti-tumor activities. In addition, EGCG was demonstrated to have antiviral effects in vitro through cell culture and in vivo in broiler chickens against the challenge of fowl adenovirus type 4 (FAdV-4). Furthermore, EGCG showed the effects of inhibiting the proliferation and inducing apoptosis of multiple myeloma cells. Also, EGCG revealed a protective effect on high glucose-induced H9C2 cell damage. Importantly, EGCG was reported to prevent the UA-induced inflammatory effect of human umbilical vein endothelial cells (HUVEC), microRNAs (miRNAs), small evolutionarily conserved noncoding RNAs, with 19 to 25 nucleotides, are reported to be important in gene regulation and play critical roles in diverse biologic processes. Important ly, EGCG was observed to be closely correlated with miRNAs. For example, miR-16 was involved in mediating the apoptotic effect of EGCG in human cancer cells; EGCG suppressed lung cancer cell growth through up-regulation of miR-210 expression. Meanwhile, dysregulation of miRNAs was also found in hyperuricemia, and the concentration of UA was also observed to be related to miRNA expression. Among all these identified miRNAs, miR-9 was demonstrated to have functions in suppressing pro-fibrogenic transformation of fibroblasts and in preventing organ fibrosis. In addition, miR-9 regulated cardiac fibrosis in rats and significantly inhibited the gene expression of CLDN14 in the thick ascending limb of the human kidney, which is a key regulator for renal Ca2+ homeostasis. Furthermore, miR-9 expression was inhibited by up-regulation of claudin-14 in mice, which was related to calcium nephrolithiasis. Therefore, we suggested that miR-9 was involved in the effects of EGCG on hyperuricemia. The current study was performed to investigate the effects of EGCG on UA-induced injury in rat renal interstitial fibroblasts NRK-49F cells and the underlying mechanisms were also investigated. Our findings may provide a better understanding about hyperuricemia and a potential new therapeutic target for hyperuricemia-induced kidney diseases.

Materials and Methods

Cell Culture

NRK-49F cell line is a type of rat renal interstitial fibroblasts, which was obtained from Shanghai Institute for Biological Science (Shanghai, China). NRK-49F cells were maintained at 37°C under 5% CO2 in Dulbecco’s modified Eagle medium (DMEM, high-glucose medium, HyClone, South Logan, UT, USA) adding 100 IU/mL penicillin and 10% fetal bovine serum (FBS, Life Science, South Logan, UT, USA).

Cell Treatment

NRK-49F cells were starved with 0.5% FBS (Life Science, UT, USA) for 12 h and then exposed to 0.4 mM UA (Sigma-Aldrich, St. Louis, MO, USA) with or without the pretreatment of EGCG. EGCG was also obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in distilled water to a stock concentration of 10 mM and stored in 4°C.

Cell Counting Kit-8 (CCK-8) Assay

To investigate the cytotoxicity of EGCG and/or UA and to explore UA-treated cells by transfection with miR-9 inhibitor and its negative control (NC), CCK-8 assay was performed to analyze cell viability. For cell cytotoxicity, cells were treated with increasing EGCG (0, 2.5, 5, 7.5 and 10 mM) and UA concentrations (0, 0.1, 0.2, 0.4 and 0.8 mM), respectively for 72 h. Briefly, for CCK-8 assay, cells were seeded in 96-well plate with 5000 cells/well. After stimulation, removed culture medium and cells were washed twice by PBS. CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD) was added to the new supplementary culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis Assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Beijing Biossea Biotechnology, Beijing, China). The cells (100,000 cells/well) were seeded in 6 well-plate. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer’s instruction and measured with flow cytometer (Beckman Coulter, Brea, CA, USA) to differentiate apoptotic cells (Annexin-V po-
sitive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

**The Enzyme-Linked Immunosorbent Assay (ELISA) Assay**

Culture supernatant was collected from 24-well plates and concentrations of inflammatory cytokines monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) were measured by ELISA using protocols supplied by the manufacturer (R&D Systems, Abingdon, UK) and normalized to cell protein concentrations.

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

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. The sequences of miR-9 primer were as below:

**Sense**: GCGCGTCTTTGGTTATCTAGC - GTATG.

**Antisense**: GCTGTCAACGATACGCTACG.

The TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-9 in cells. U6 was the internal control. For mRNA expression, a quantity of 1 μg RNA was reverse-transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcription system (Promega, Madison, WI, USA). Real-time RT-PCR was performed by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green fluorescence signals, as described previously. Relative MCP-1, TNF-α, IL-6 expression levels compared with control, were calculated after normalization to GAPDH.

**Transfection**

miR-9 inhibitor and the NC were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol.

**Western Blot**

The protein used for Western blot was extracted using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions. Primary antibodies included: anti-CyclinD1 antibody (ab134175), anti-cyclin-dependent kinases 4 (CDK4) antibody (ab199728), anti-p21 antibody (ab109199), anti-β-actin antibody (ab227387), anti-pro-Caspase-3 antibody (ab32499), anti-cleaved-Caspase-3 antibody (ab49822), anti-Bcl-2-associated X protein (Bax), antibody (ab32503), anti-B-cell lymphoma 2 (Bcl-2) antibody (ab196495), anti-Mcl-1 antibody (ab32087), anti-a-smooth muscle actin (α-SMA) antibody (ab108424), anti-Collagen 1 antibody (ab34710), all from Abcam (Cambridge, MA, USA); anti-t-NF-kB inhibitor (IκBα) antibody (4812), anti-p-IκBα antibody (2859), anti-t-p65 antibody (8242), anti-p-p65 antibody (3033), anti-t-janus kinase (JAK) 2 antibody (3230), anti-p-JAK 2 antibody (3776), anti-t-signal transducers and activator of transcription (STAT) 3 antibody (12640), anti-p-STAT3 antibody (52075), all from Cell Signaling Technology (Danvers, MA, USA). Next, these primary antibodies were prepared in 5% blocking buffer and diluted according to the product instructions. Primary antibodies were incubated with the polyvinylidene difluoride (PVDF) membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase (HRP) for 1 h at room temperature. After rinsing, the membrane carried blots and antibodies was transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Graphpad Prism 5 software (GraphPad, La Jolla, CA, USA). The p-values were calculated using a one-way analysis of variance (ANOVA). Multiple comparisons
between the groups were performed by Bonferroni’s Post-Hoc test. A p-value of < 0.05 was considered as statistically significant.

**Results**

**EGCG Alleviated UA-Induced Cell Viability Inhibition and Apoptosis**

As shown in Figure 1A and 1B, cell viability was significantly decreased only when EGCG concentration was 10 mM (p < 0.05) and UA concentration was ≥ 0.2 mM (p < 0.05 or p < 0.01). In addition, under the treatment of UA 0.4 mM, cell viability was significantly increased when EGCG concentration was ≥ 5 mM (p < 0.01, Figure 1C). Subsequently, we chose the EGCG concentration of 5 mM and UA concentration of 0.4 mM in the following studies. We found that cell apoptosis was significantly increased by UA (p < 0.001), while was statistically alleviated by EGCG compared with UA treatment (p < 0.01, Figure 1D). Meanwhile, the expression of cell cycle-related proteins revealed that Cyclin D1 and CDK4 were down-regulated (both p < 0.05) while p21 was up-regulated by UA (p < 0.01), while the results were reversed by EGCG (all p < 0.05, Figure 1E). In addition, Western blot result of apoptosis-related proteins demonstrated that myeloid leukemia–1(Mcl-1) and Bcl-2 expression was significantly down-regulated (both p < 0.05) while the expression of Bax and cleaved-Caspase-3 were statistically up-regulated by UA (both p < 0.001) while EGCG treatment led the opposite results in UA-induced cells (p < 0.05 or p < 0.01, Figure 1F). This result demonstrated that EGCG promoted cell growth in UA-induced cells.

**EGCG Alleviated UA-Induced Cell Fibrosis and Inflammation Injury**

CKD are characterized by progressive fibrosis as a final common pathway, eventually affecting all the substructures of the kidney, leading to a final consequence of end-stage renal disease and dysregulation of α-SMA and Collagen 1 was found in fibrosis process. In addition, MCP-1, TNF-α and IL-6 are important inflammatory cytokines. Therefore, fibrosis and inflammation are extremely important symptoms for CKD. From the results of WB, we found that the expression of Collagen 1 and α-SMA was up-regulated by UA (both p < 0.001) while was significantly down-regulated by EGCG in UA-induced cells (both p < 0.01 Figure 2A-2B). In addition, the results from qRT-PCR and ELISA showed that the expression of MCP-1, TNF-α and IL-6 were all significantly up-regulated in mRNA level and protein level (p < 0.01 or p < 0.001) while EGCG led the opposite results in UA-induced cells (all p < 0.05, Figure 2C-2D). This finding demonstrated that EGCG alleviated UA-induced cell fibrosis and inflammation injury.

**EGCG Up-Regulated the Expression of MiR-9**

EGCG was reported to modulate several miRNAs expression meanwhile miR-9 was observed to have anti-fibrosis and anti-inflammation effects. Therefore, we detected whether the effects of EGCG was through regulation of miR-9. As shown in Figure 3, the expression of miR-9 was down-regulated under UA concentration of 0.4 mM (p < 0.01), while was significantly up-regulated by EGCG (p < 0.05 or p < 0.001). This data demonstrated that miR-9 might be involved in the effects of EGCG on UA-induced cell injury.

**EGCG Alleviated UA-Induced Cell Injury Through Up-Regulation of MiR-9**

To determine the effects of miR-9 in the process of EGCG on UA-induced cells, miR-9 inhibitor or NC was transfected into NRK-49F cells. Down-regulation of miR-9 by transfection with miR-9 inhibitor indicated high transfection efficiency (p < 0.01, Figure 4A). Results demonstrated that cell viability was significantly decreased by transfection with miR-9 inhibitor compared with NC in UA-induced NRK-49F cells (p < 0.05, Figure 4B). Cell cycle-related proteins Cyclin D1 and CDK4 were down-regulated (both p < 0.05) while p21 was up-regulated (p < 0.01) by transfection with miR-9 inhibitor compared with NC (Figure 4C-4D). Furthermore, the apoptosis-related proteins revealed that the expression of Mcl-1, Bcl-2 was down-regulated (both p < 0.05) while Bax and cleaved-Caspase-3 were up-regulated (p < 0.01 Figure 4F and 4G), consistently with the results in Figure 4E showing that cell apoptosis was significantly increased by transfection with miR-9 inhibitor compared with NC (p < 0.05). These findings demonstrated that EGCG alleviated UA-induced cell injury through up-regulation of miR-9.

**EGCG Alleviated UA-Induced Cell Fibrosis and Inflammation Injury Through Down-Regulation of mir-9**

In order to identify whether miR-9 was also participated in EGCG-mediated cell fibrosis and
Figure 1. Epigallocatechin gallate (EGCG) promoted cell growth in uric acid (UA)-induced NRK-49F cells. (A-C) Cell viability with administered with EGCG and/or UA was detected by Cell Counting kit-8 (CCK-8) assay. (D) Cell apoptosis was detected by flow cytometry assay. (E-F) Cell cycle-related proteins and apoptosis-related proteins were both measured by Western blot. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times *p < 0.05, **p < 0.01, ***p < 0.001.
inflammatory injury, further experiments were performed. As shown in Figure 5A and 5B, the expression of Collagen 1 and α-SMA was significantly up-regulated by transfection of miR-9 inhibitor compared with NC in UA-induced NRK-49F cells (both \( p < 0.05 \)). In addition, the expression of MCP-1, TNF-1, IL-6 was significantly up-regulated by transfection with miR-9 inhibitor compared with NC in UA-induced NRK-49F cells (both \( p < 0.05 \), Figure 5C and 5D). This data demonstrated that EGCG alleviated UA-induced cell fibrosis and inflammation injury through down-regulation of miR-9.

**EGCG Inactivated NF-κB and JAK2/STAT3 Signal Pathways Through Up-Regulation of miR-9**

Previous researches\(^{27,28}\) once reported that miR-9 was involved in the JAK-STAT signal pathway. Results shown that the expression of IκBα, p65, JAK2 and STAT3 was significantly up-regulated by UA (\( p < 0.01 \) or \( p < 0.001 \)), while was down-regulated after supplement with EGCG (both \( p < 0.05 \), Figure 6A and 6B). On the other hand, transfection with miR-9 inhibitor reversed the results under EGCG treatment in UA-induced cells compared with NC (all \( p < 0.05 \), Figure 6A and 6B). This result revealed that EGCG inhibited NF-κB and JAK2/STAT3 signal pathways through up-regulation of miR-9.

**Discussion**

In the present work, we investigated whether EGCG had potential effects on the UA-induced NRK-49F cells, as well as the underlying mechanisms. We found that EGCG promoted cell
growth and alleviated cell fibrosis and inflammation injury through up-regulation of miR-9. These effects might be by inactivation of NF-κB and JAK2/STAT3 signal pathways. Hyperuricemia was found to impair the kidney through the obstruction of renal tubules with urate crystal deposition, which resulted in the renal tubular cell injury and subsequent interstitial fibrosis. In our study, we used UA to stimulate NRK-49F cells to establish hyperuricemia cell model in vitro. We found that UA significantly inhibited cell growth and promoted cell inflammation and fibrosis, which was consistent with the previous report that UA induced cell injury. In contrary, EGCG was demonstrated effects in alleviating UA-induced injury through increasing cell viability, decreasing cell apoptosis, indicating that EGCG could attenuate UA-induced cell loss. Xiao et al. demonstrated that EGCG attenuated fibrosis and inflammation in non-alcoholic fatty liver disease rat model. In addition, EGCG treatment significantly ameliorated liver inflammation and fibrosis through suppressing expression of the genes associated with inflammation and fibrogenesis, including TNF-α, IL-1β, TGF-β1, α-SMA and EGCG attenuated collagen production in Bleomycin-induced rats. Notably, similar results were also found in our study that EGCG revealed functions in anti-fibrosis and anti-inflammation effects through decreasing the expression of Collagen 1, α-SMA and MCP-1, TNF-α and IL-6 in UA-induced cells. Taken together, our results revealed that EGCG promoted cell growth and attenuated cell fibrosis and inflammation in UA-induced cells. The underlying mechanism about how EGCG affected UA-induced cells was investigated in this study. Accumulating studies revealed that EGCG functions in cells through modulations of miRNAs expression. Importantly, miR-9 was observed to play an important role in the anti-inflammatory regulation of lipo-polysaccharides (LPS)-activated microglia cells and participated in inflammatory processes in human monocytes. In addition, up-regulation of miR-9 attenuated cardiac fibrosis. Anti-fibrosis and anti-inflammation effects of miR-9 promoted us to perform further experiment to investigate whether miR-9 was also involved in the anti-fibrosis and anti-inflammation effects of EGCG in UA-induced NRK-49F cells. Data revealed that the expression of miR-9 was downregulated by UA while up-regulated as the administration of EGCG in UA-induced cells. This result showed that miR-9 might play an important role in EGCG alleviated UA-induced cell injury. In order to clarify whether miR-9 was involved in the process of EGCG alleviated UA-induced cell injury, we transfected with miR-9 inhibitor and its NC. Of note, transfection of miR-9 inhibitor impaired the protective functions of EGCG in UA-induced cells compared with NC through decreasing cell viability and inducing cell apoptosis. Meanwhile, the expression of fibrosis-related protein Collagen 1 and α-SMA and inflammation-related protein MCP-1, TNF-α and IL-6 expression were all up-regulated by transfection of miR-9 inhibitor. This result was consistent with the report that down-regulation of miR-9 induced the up-regulation of proinflammatory cytokines IL-1β, TNF-α and MCP-1. All these findings indicated that miR-9 was modulated by EGCG and participated in the effects of protective function of EGCG in UA-treated cells. These data were consistent with the previous studies that miR-9 had anti-fibrosis and anti-inflammation effects and NF-κB and JAK-STAT signal pathway was reported to be involved in kidney dysfunction. In our study, we found that EGCG inactivated NF-κB and JAK-STAT signal pathways, which were activa-
Figure 4. Epigallocatechin gallate (EGCG) promoted uric acid (UA)-induced cell growth by up-regulation of microRNA-9 (miR-9). (A) miRNA expression was detected by quantitative Real-time polymerase chain reaction (qRT-PCR) for cells transfection with miR-9 and its negative control (NC). (B) Cell viability was detected by Cell Counting kit-8 (CCK-8) assay. (C-D) Cell cycle-related proteins Cyclin D1, cyclin-dependent kinases (CDK) 4 and p21 were measured by Western blot. (E-G) Cell apoptosis and its related proteins were detected by flow cytometry and Western blot, respectively. Each experiment was performed in three times *p < 0.05, **p < 0.01, ***p < 0.001.
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However, transfection with miR-9 led activation of NF-κB and JAK-STAT signal pathways. These results were consistent with the results that EGCG exhibit chemo-preventive effects in cholangiocarcinoma cells via suppression of JAK/STAT signaling pathway and down-regulation of miR-9 activated JAK/STAT signaling pathway and NF-κB pathway.

Figure 5. Epigallocatechin gallate (EGCG) inhibited uric acid (UA)-induced cell fibrosis and inflammatory cytokines by up-regulation of microRNA-9 (miR-9). (A-B) Fibrosis-related protein Collagen 1 and α-SMA was detected by Western blot. (C-D) The expression of inflammatory cytokines in RNA and protein level was detected by quantitative Real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Each point represented the mean ±standard deviation (SD) of triplicates. Each experiment was performed in three times *p < 0.05, ** p < 0.01, *** p < 0.001.
Conclusions

We showed that EGCG had functions in alleviating UA-induced NRK-49F cell injury through promoting cell growth, decreasing cell fibrosis and inflammation by up-regulation of miR-9. These effects might through inactivation of NF-κB and JAK-STAT signal pathways. Our study might provide a foundation for the further researches and treatment of kidney dysfunction caused by hyperuricemia.

Conflict of Interest
The Authors declare that they have no conflict of interest.

References
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