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TGF-β_1 immunohistochemistry and promoter methylation in chronic renal failure rats treated with uremic clearance granules

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Abstract: The aim of the study was the explain the mechanism related to therapeutic effects of Uremic Clearance Granules (Niaoduqing Keli in Chinese) on adenine-induced Chronic Renal Failure in rats. Thirty 8-week-old male Wistar rats were selected and randomly divided in to 3 groups: Normal Control Group (NCG)consisted of 10 rats, Chronic Renal Failure Pathological Control Group (PCG) 10 rats, and Uremic Clearance Granules Treatment Group (UCG) 10 rats. Each rat in PCG and UCG was fed with adenine-enriched diets, containing 10 g adenine per kg food for 6 weeks. After fed with adenine, each rat in UCG was administered orally with 2 ml solution of Uremic Clearance Granules for 6 weeks. The concentration of Uremic Clearance Granules solution was 0.42 g/ml which was 10 times of human. On days 42 and 84, the serum levels of creatinine, Blood Urea Nitrogen and homocysteine were determined. The methylation of *TGF-β_1* promoter was tested by methylation-specific PCR. *TGF-β_1* mRNA and protein expression in rat renal cortex were analyzed by real-time RT-PCR and Immunohistochemistry. (1) Experimented on model of Chronic Renal Failure in rats, the preparation was proved to be able to reduce serum creatinine, Blood Urea Nitrogen, and homocysteine ($p<0.05$), improve renal function. (2) The expression of *TGF-β_1* in mRNA and protein level were down-regulated. (3) *TGF-β_1* promoter was demethylated at some loci in PCG, and was recovered in UCG. After treatment with Uremic Clearance Granules, the Chronic Renal Failure Wistar rat's kidney function was recovered. The recovery may be result of the remethylation of *TGF-β_1* promoter and then lead to *TGF-β_1* be transcripted and translated normally. The experimental study explain the molecular mechanism by which Uremic Clearance Granules treat Chronic Renal Failure.

Key words: granules, immunohistochemistry, promoter methylation

Introduction

The Traditional Chinese Herbal Medicine were useful for some diseases, however, essential compounds have not been identified in most formulae, whereas precise mechanisms of formulae remain to be addressed by using cellular and molecular approaches.

Uremic Clearance Granule (Niaoduqing Keli in Chinese) (Guangzhou Consun Pharmaceuticcal Co., Ltd, China) was one of traditional Chinese herbal medicine having been used for treatment of Chronic Renal Failure (CRF) in clinic for many years. CRF was characterized by progressive loss of nephrons caused by increased intraglomerular pressure and hyperfiltration. The loss of autoregulatory ability exposes the glomeruli to the systemic blood pressure leading to glomerular hypertrophy and sclerosis. Uremic Clearance Granule attenuated the progression of renal insufficiency in both human and experimental animal models of CRF. In order to address the molecular mechanism of it, we had done some researches using the Wister rats as the CRF model.

Functions of Uremic Clearance Granule included not only discharging poisonous substances by catharsis, invigorating the spleen and eliminating eczema, but also promoting blood circulation via removing blood stasis. Indication included the patients with azotemia or early stage of uraemia, which were diagnosed to be hypospleen and hygrotoxin syndrome or hypospleen and blood stasis syndrome according to the Traditional Chinese Medicine (TCM). The preparation could

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decrease both Blood Urea Nitrogen (BUN) and serum Creatinine (sCr), protect the residual renal function, and postpone the beginning of dialysis therapy. It also had some effects on improving the anaemia of renal disease and increasing blood calcium and decreasing blood phosphate.

Hyperhomocysteinaemia (HHcy) occurred in several genetically determined and acquired disorders and was highly prevalent in patients with uraemia [1]. In these disorders, homocysteine precursor S-adenosy lhomocysteine (SAH), a powerful competitive inhibitor of S-adenosylmethionine (SAM) -dependent methyltransferases, was increased, suggesting unbalanced methylation. Clinical and experimental studies suggested that HHcy is also a risk factor for CRF [2,3]. The prevalence of HHcy in CRF patients was 82.5%, which was 2 to 3 times of normal level. The accumulation of Hcy *in vivo* would lead to abnormal methylation of DNA, proteins and other components [4,5].

The most common assay to detect DNA methylation changes was methylation-specific PCR (MSP) [6]. To study the mechanism relates to therapeutic effects of Uremic Clearance Granules, the adenine-induced CRF rats were treated with this TCM. In this study, the serum levels of sCr, BUN and Hcy were determined by Automatic Biochemical Analyzer and High Performance Liquid Chromatography (HPLC). The methylation of *TGF-β_1* promoter was tested by MSP. *TGF-β_1* mRNA and protein expression in rat renal cortex were analyzed by real-time RT-PCR and Immunohistochemistry (IHC).

Materials and methods

Experimental animals. To get insight into the pharmacological action of Uremic Clearance Granule on CRF, Rat models were developed with CRF-like features induced by adenine-feeding.

After 7 days of acclimatization, thirty 8- to 10-week-old male Wistar rats (190 to 210 g; Laboratory Animal Center, The Academy of Military Medical Sciences, China) were randomized into 3 groups: 1 control group (Normal Control Group, NCG) of 10 animals, 1 pathological control group (CRF Pathological Control Group, PCG) of 10 animals, and 1 Uremic Clearance Granule Treatment Group (Uremic Clearance Group, UCG) of 10 animals, The PCG and UCG were fed with adenine-enriched diets containing 10 g adenine (Sigma) per kg food. Except for the added adenine, all diets were of identical composition. After 6-week adenine-feeding, each rat in the UCG was administered orally with 2 ml/d Uremic Clearance Granule solution containing 0.42 g Uremic Clearance Granule (Consun, China) per ml distilled water. The concentration of Uremic Clearance Granules solution was 0.42g/ml which was 10 times of human. The period of TCM treatment lasted 6 weeks. Animals were maintained at a constant humidity (ca. 60%) and temperature (ca. 23°C) with a light/dark cycle of 12 h in our institutional stable with free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of the Tianjin Medical University. We monitored body weight, food consumption, and fluid intake weekly.

We performed capillary blood sampling from the retrobulbar plexus at the end of the 6-week adenine-feeding period and 6-week Uremic Clearance Granule-treating period by use of heparin-coated capillaries. Immediately after blood sampling, serum was separated by centrifugation (10 min at 1500 g) and stored at -80°C until analysis. After the final blood sampling, animals were anesthetized using 10% Chloral Hydrate, and then kidneys were isolated, explanted, and rinsed thoroughly to remove adherent blood. Both kidneys of each rat were isolated, and the renal weights were measured using a Sartorius Genius scale (Sartorius AG). Left kidneys were fixed with 4% formalin and used for immunohistochemical analysis. Right kidneys were divided into 2 aliquots. One aliquot was shock-frozen with liquid nitrogen and stored at -80°C until extraction of RNA. One aliquot was used for the extraction of DNA.

sCr and BUN were detected by Hitachi 7020 Automatic Biochemical Analyzer. Hcy was detected by High Waters-600E Performance Liquid Chromatography.

Measurement of TGF-β_1 protein expression in renal cortex of CRF rats using immunohistochemical analysis. The method of streptavidin-biotin complex was used for immunolocalization of *TGF-β_1*. Formalin-fixed, paraffin-embedded rat's kidneys were sectioned at 4 mm on poly-L-lysine-coated slides. Immunostaining was carried out as previously reported with the following modifications. Briefly, sections were rehydrated and blocked for endogenous peroxidase activity in 3% H_2O_2 in methanol. Following microwave antigen retrieval using citrate buffer, samples were blocked with 5% normal goat serum. An additional avidin/biotin blocking step was used to eliminate further endogenous biotin. *TGF-β_1* primary antibody (Boster, China) was diluted 1 in 2000 in phosphate-buffered saline and was incubated at room temperature for 4 hours. The secondary antibody was biotinylated goat anti-rabbit followed by horseradish peroxidase streptavidin. Visualization was with the DAB-substrate chromogen system. Negative controls included substitution of PBS or IgG fractions from rabbit serum for the primary antibody. Brown staining in cytoplasm was regarded as the expression of *TGF-β_1* protein. Six fields of vision in renal section of each animal were randomly chosen for observing blindly the cortex at ×200 magnification under the Olympus light microscope equipped with a HPIAS-1000 image analysis system, and in each field of vision the ratio of the area stained positively to the field of vision was calculated, which is the mean area of positively stained TGF-β_1 protein (μm^2).

Measurement of *TGF-β_1* mRNA expression in renal cortex of CRF rats using real-time RT-PCR with SYBR Green I. Total RNA was extracted by the TRIzol® (Promega, USA) according to the manufacturer's instructions. The OD 260 and quality of the RNA were measured using the Nanodrop 1000. Total RNA (2 μg) was reversed transcribed in 20 μl using the ImRrom-II Reverse Transcriptase (Promega, USA) and Oligo dT primer according to the manufacturer's instructions.

Two microliters of first strand cDNA samples were subjected to PCR in a volume of 20 μl using 2 μM forward and reverse primer [7] (Table 1), 2 μl dNTPs (each 10 mM dATP, dCTP, dGTP, dTTP), 1×PCR reaction buffer, and 2.5 U Taq DNA polymerase (Takara, Japan). All PCR-assays were conducted in capillaries (20 μl-reaction volume) on an Applied Biosystems 7300 Real-Time PCR System (ABI, USA) using the LC-FastStart DNA Master SYBR Green I kit (Takara, Japan). Conditions were: 95°C for 10 min, 95°C for 45 s, annealing temperatures (Table 1) for 5 s, 72°C for 15 s (40 cycles) and 72°C for 1 min. The melting curve analysis was carried out by monitoring fluorescence signals over the range from 65°C to 95°C in steps of 0.1°C/s. PCP-specific products were determined as a clear single peak at the melting curves above 80°C. The *TGF-β_1* RNA level was acquired from the value of the threshold cycle (Ct) of the real-time PCR as related to that of GAPDH through

Equation 1: $\Delta Ct = Ct\ (GAPDH) - Ct\ (TGF\text{-}\beta_1)$

Table 1. Primer sequences, annealing temperatures, and sizes of generated fragment.

Genes	Forward primer (5'→3')	Reverse primers (5'→3')	Annealing temperature	Size (bp)
GAPDH	ACCACAGTCCTAGCCATAAC	TCCACCACCCTGTTGCTGTA	65°C	452bp
TGF-β_1	TATAGCAACAATTCCTGGCG	CAGAAGTTGGCATGGTAGCC	62 °C	446bp

Table 2. Comparison of Cr, BUN and Hcy concentrations in studied groups.

Groups	n	sCr (μmol/L)	BUN (mmol/L)	Hcy (μmol/L)	*TGF-β_1* mRNA	TGF-β_1 protein (μm^2)
NCG	10	29.33±6.45	7.73±1.27	10.56±1.20	1.1905±0.0032	6.1±0.5
PCG	10	149.67±24.95	33.18±2.95	15.42±1.02	6.4480±0.0079	15.9±1.07
UCG	10	85.00±13.89*	19.50±3.20*	11.37±1.31*	2.6720±0.0047*	9.9±1.22*

* $p<0.05$ *vs.* PCG

Final results expressed at N-fold differences in *TGF-β_1* gene expression relative to *GAPDH*, termed "$N_{TGF\text{-}\beta1}$" were determined as

Equation 2: $N_{TGF\text{-}\beta1}=2^{\Delta Ct\ (TGF\text{-}\beta1)\ -\ \Delta Ct\ (GAPDH)}$

TGF-β_1 promoter methylation status tested by MSP. Genomic DNA (gDNA) was isolated from renal cortex using standard procedures involving the use of proteinase K and phenol extraction. The DNA was checked for quality and quantity by agarose gel electrophoresis and the Nanodrop 1000. Genomic DNA was stored in -20°C.

Bisulfate modification of gDNA has been described previously [6]. In brief, 700 ng (up to 2 μg) DNA was denatured in 50 μl of 0.2 mM NaOH at 37°C for 20 min; 30 μl of freshly prepared 10 mM hydroquinone (Sigma, USA) and 520 μl of 3 M sodium bisulphate (Sigma) at pH 5.0 were added. Incubation was under a layer of mineral oil at 55°C for 14~16 hours. The DNA was purified using the Gene Clean kit (Tiandz, China) and eluted with 100 μl water. Finally, the DNA was desulphonated by a 20 min treatment in 0.3 M NaOH at 37°C, followed by an ethanol precipitation. The DNA pellet was resuspended in 1 mM Tris-HCl, pH 8.0, and stored at -20°C.

MSP was used for the detection of methylation in the promoter regions of the *TGF-β_1* gene. Two CpG islands were found in promoter sequence of *TGF-β_1* gene (presented by shadows).

.....G C C A T (-629) G T G C C T A G C A G C C C A G G C A C T
C A C C A G C T G G A C T G C C C T A C A T G G A G G C C C C T
G G G T A G T T G G A G G G A G C A G C T A G C A C G G G C T
T T C G T G G G T G G C G G G C C A C A G C T G C T G C A C G
C A G A C (-509) A C C A T C T A C A G C G G G G C C G A C C G
C T A C C G C C T G C A C A C G G C C G C G G G T G G C A C A
G T G C A C C T T G G T A T C G G T C T G C T G C T G C G C C A
C T T T G A T C C T C C A G A C A G C T A G G C C C C C G G C C
G G G G C A G G G G G G A C G C C C C T T C G G G G C A C C C
C C G G C T C T G A G C C G C C C C G C G G A A C C G G C C T C
C G C T G G G A G C C G G C A A G G G A G C A G C G G A G G A
G C C G T C C G A G G C C C C A G A G T C T G A G A C C A G C
C G C C G C C G C C G C A G G G A G G A G G G G G A G G A G
G A G T G G G A G G A G G G G A C G A G C T G G T T G G G G A G A
A G A G G A A A A A G T T T T G A G A C T T T T C C G C T G C T
A C T G C A A G T C A G A G A C G G G G G G G A C T (-149) G C T
T.....

U primer pairs and M primer pairs were designed according this sequence.

About 100 ng of bisulphate-modified DNA was amplified with primer pairs specific to unmethylated [U primer pairs: d (G T T G A T T G T T A T T G T T T G T A T A T G G T T G) , d(AAAACCAATTCCACAAACAACTC)] or methylated DNA sequences [M primer pairs: d(TAGCGGGGTCGATCGTTATCG), d(AACCGATTCCGCGAACGAC)], respectively. After an initial denaturation at 95°C for 3 min, DNA was amplified in 30 cycles of 95°C for 30 s, 60°C (U primer pair) or 65°C (M primer pair) for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 5 min.

The PCR products were separated by electrophoresis through a 1% agarose gel containing Ethidium Bromide. DNA bands were visualized by ultraviolet light. PCR products were gel purified with an agarose gel DNA fragment recovery kit (Tiandz, China) according to the manufacturer's instructions and were sequenced by Invitrogen (USA).

Data analysis. The data were analyzed using the software SPSS 12.0 for Windows. Data were presented as mean±SD. For comparison between multiple groups, quantitative data were analyzed using one-way ANOVA and additional analysis was carried out using Student's test for multiple comparisons within treatment groups, or student's t-test between two groups. $P<0.05$ was considered significant.

Results

At the beginning of this study, all rats behaved normally. After six weeks, all rats feed with adenine developed a series of reactions. They shows decreased food intake, dishevelled hair, lost weight, and tended to crouch. Meanwhile, they presented diuresis and an increase in water intake. The reaction severity in the rats of PCG was significantly different from those in NCG and UCG, while the rats in NCG behaved normally during the experiment.

sCr, BUN and Hcy decreased in UCG

The sCr and BUN levels were diagnostic indices of renal failure. After 12 weeks of treatment, PCG animals exhibited significant high Cr and BUN concentrations (Table 2). The concentration decreased in the UCG group.

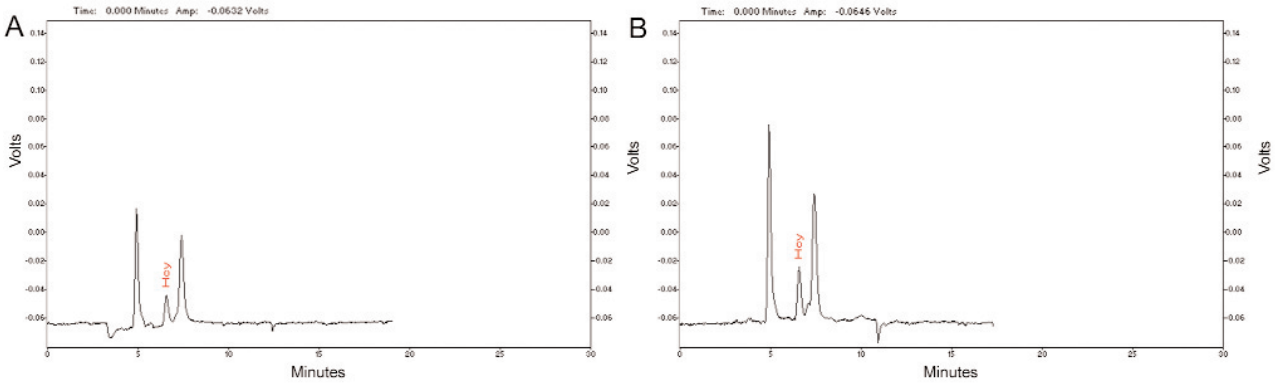

Fig. 1. Hcy concentrations by HPLC. (**A**): Hcy concentration was 16.461 μmol/L, result of a rat in PCG; (**B**): Hcy concentration was 10.779 μmol/L, result of a rat in UCG.

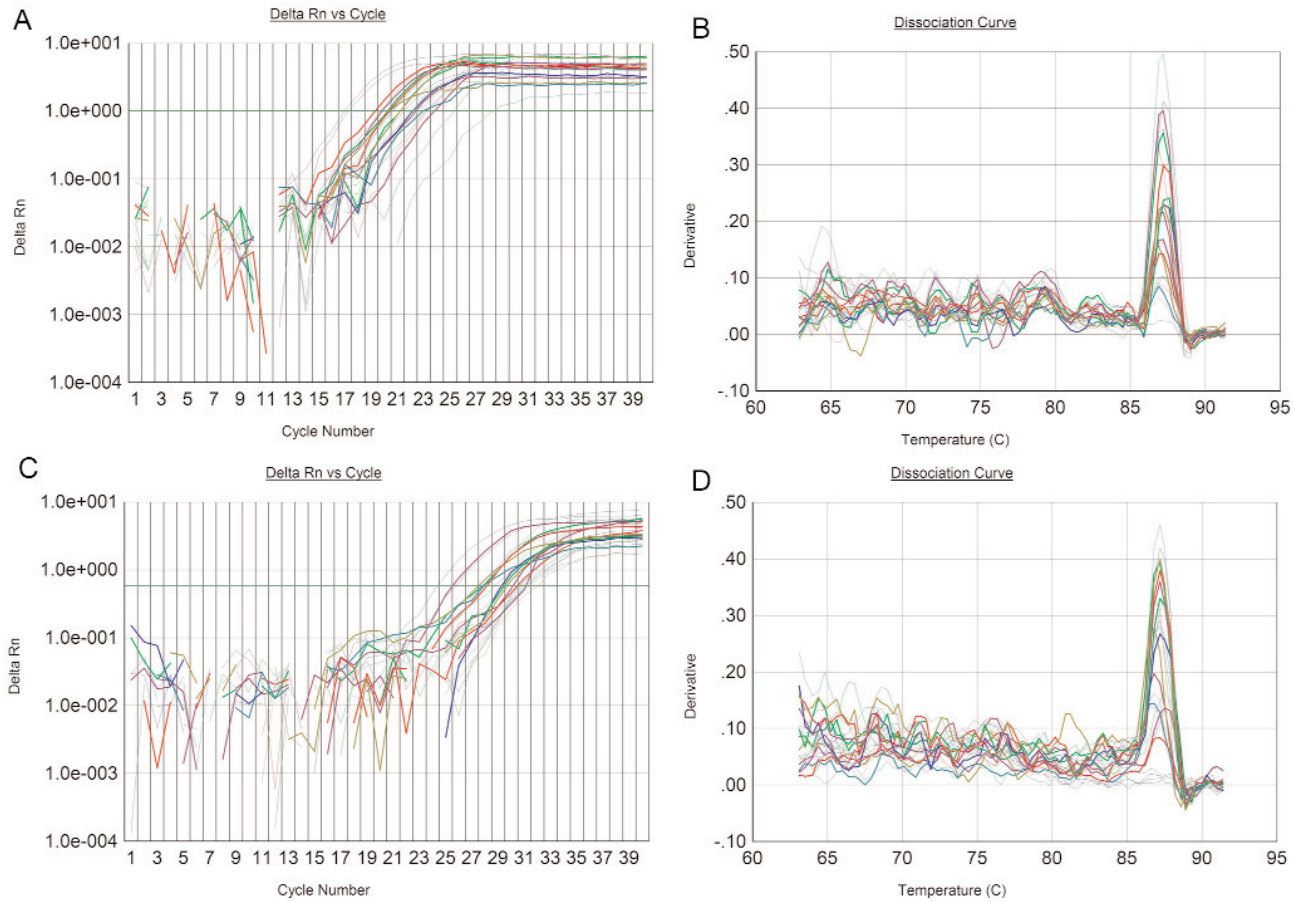

Fig. 2. Real-time RT-PCR with SYBR Green I. (**A**): Amplication curve of *GAPDH* gene; (**B**): Melting curve of *GAPDH* gene; (**C**): Amplication curve of *TGF-β_1* gene; (**D**): Melting curve of of *TGF-β_1* gene.

After 12 weeks of treatment, PCG animals exhibited significant HHcy (Table 2, Fig. 1). The UCG group developed moderate Hcy concentrations.

TGF-β_1 mRNA and TGF-β_1 protein expression decreased in UCG

Expression levels of *TGF-β_1* mRNA in renal cortex of NCG,PCG and UCG measured by real-time RT-PCR (Table 2, Fig. 2,3). Expression of *TGF-β_1* mRNA at renal cortex in PCG was elevated compared with NCG ($p<0.05$). However, the expression level in UCG was lower than that in PCG ($p<0.05$)

Expression of *TGF-β_1* protein at renal tubules and glomerulus in PCG and UCG was elevated compared with NCG ($p<0.05$). However, the index in UCG was lower than that in PCG ($p<0.05$) (Table 2, Fig. 4).

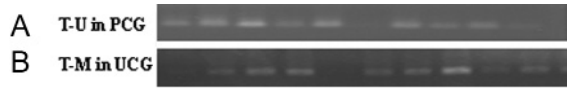

Fig. 3. Agarose gel electrophoresis of RT-PCR productions. (**A**): Productions of *GAPDH* gene; (*B*): Productions of TGF-β_1 gene; Band 1~4: NCG; Band 5~8: UCG; Band 5~8: PCG.

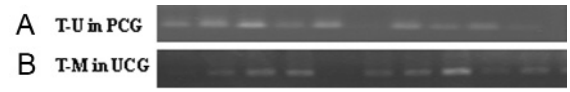

Fig. 5. TGF-β_1 promoter MSP result. (**A**): Seven demethylation fragments in PCG; (**B**): Seven methylation fragments in UCG.

Methylation status of TGF-β1 promoter

Several methods exist to detect changes in the DNA methylation pattern. Most of these techniques use bisulphate treatment to uncover the methylation status. Sodium bisulphate induces methylation-dependent single nucleotide polymorphisms by converting unmethylated cytosine to uracil and, upon PCR amplification, to thymine. 5-methylcytosine is highly resistant to sodium bisulphate treatment and thus becomes amplified as cytosine. Two primers: set one specific for the C-containing sequence, set two binding to T-containing DNA, are used to discriminate between the methylated and unmethylated status of gDNA.

The results were shown as Fig. 5 and 6. To the same DNA sequence, after PCR amplification with methylated primers M and non-methylated primers U, there are 8 specimens showing amplified methylation fragment in NCG, 3 specimens in PCG, and 7 specimens in UCG. On the other hand, there are 2 specimens showing amplified demethylation fragments in NCG, 7 specimens in PCG, and 3 specimens in UCG.

Three PCR products of M primer and 3 PCR products of U primer were sequenced. There were 3 C/T polymorphism in the *TGF-β_1* gene promoter, loci (-471), (-467) and (-335). The 3 loci were ^{m}C in NCG and UCG rats' *TGF-β_1* promoter while which were C in PCG rats' *TGF-β_1* promoter. In the sequence results, the 3 loci were T in PCG as a result of sodium bisulfate treatment and PCR amplification. The 3 loci were demethylated in PCG and recovered to methylation in UCG.

C (-487) G C T A C C G C C T G C A C A C/T (-471) G G C C/T (-467) G C G G G T G G C A C A G T G C A C C T T G G T A T C G G T C T G C T G C T G C G C C A C T T T G A T C C T C C A G A C A G C T A G G C C C C C G G C C G G G G G C A G G G G G G A C G C C C C T T C G G G G G C A C C C C C G G C T C T G A G C C G C C C G C G G A A C C/T (-335) G G C C T C C G C T G G G A G C C (-318)

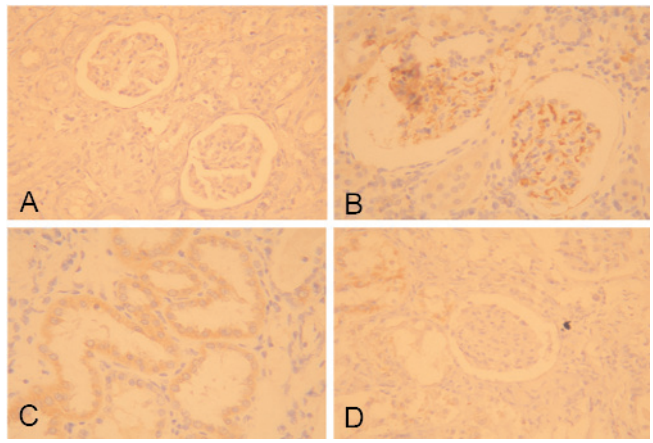

Fig. 4. Immunohistochemical staining of TGF-β_1, image at ×100. (**A**): No brown stains (TGF-β_1 protein expression) in NCG; (**B**): Heavy brown stains (TGF-β_1 protein expression) distributed in cytoplasm of glomerulus in PCG; (**C**): Heavy brown stains distributed in cytoplasm of renal tubules in PCG; (**C**): Less heavy brown stains distributed in cytoplasm in NCG, especially in glomerulus.

Discussion

Adenine causes renal disorders in rats. 2,8-dihydroxyadenine (DHOA), an adenine metabolites, was only slightly soluble in water and crystallizes and /or forms stones in the kidney. Although adenine does not have direct effect on rat kidney, DHOA shows cytotoxicity [8]. Experimented on the model of CRF in Wistar rats, Uremic Clearance Granule, was proved to be able to reduce serum Cr, BUN and Hcy. This result indicated that Uremic Clearance Granule has a protecting effects on kidney function during progression of CRF.

TGF-β_1 was named "transforming growth factor" due to its ability to induce normal rat kidney fibroblasts to grow and form colonies in soft agar. *TGF-β_1* was a powerful multifunctional cytokine that plays roles in cell proliferation, differentiation, migration, immunomodulation and regulation of ECM turnover in the kidney. The dysregulation of *TGF-β_1* has been linked with the development of nephropathy. It was highly expressed in the kidney and most commonly associated with pathological changes of the kidney. *TGF-β_1* became the treatment target recently [9-14]. In this study, we have examined the protein and mRNA expression of the *TGF-β_1* gene in 3 groups by IHC and real-time RT-PCR. A complete loss or low level of *TGF-β_1* protein and mRNA expression were in NCG. In contrast, a rich level of *TGF-β_1* protein and mRNA expression were identified in PCG. After the treatment of Uremic Clearance Granule, the level of *TGF-β_1* protein and mRNA expression decreased in UCG.

DNA methylation in eukaryotes involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction was catalyzed by DNA methyltransferase in the context of the sequence 5'-CG-3', which is

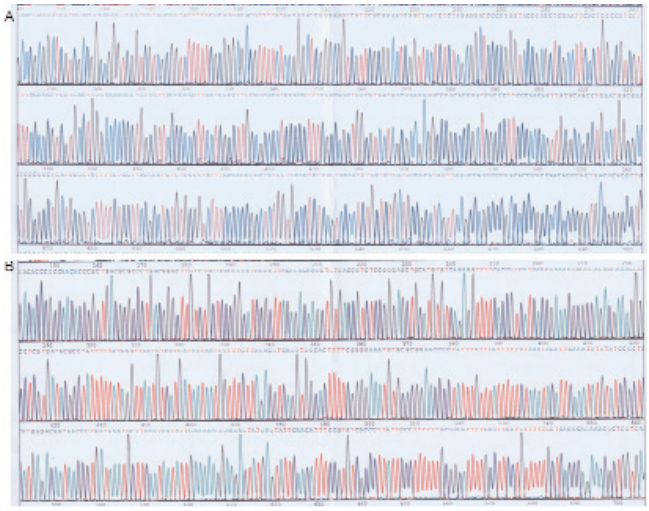

Fig. 6. DNA sequencing results. (**A**): DNA sequencing result of demethylation fragment in PCG; (**B**): DNA sequencing result of methylation fragment in UCG.

also referred to as a CpG dinucleotide. It is the most common eukaryotic DNA modification and is one of the many epigenetic (alteration in gene expression without a change in nucleotide sequence) phenomena. However, methylation is a reversible modification of DNA participating in epigenetic regulation of gene expression. If we were going to start looking for 'epigenetic signatures' associated with various diseases on the basis of genome-wide chromatin profiling or merely concentrate on specific loci or groups of genes, knowledge of where CpG methylation occurs within the genome (or epigenome) will be invaluable [14]. In addition to advancing basic research, the methylation research has immediate applications for diagnostics, and as epigenetic alterations are potentially reversible, it would has potential applications for therapeutics as well. Genomic DNA methylation profile for human and model organisms would not only provide a reference for subsequent profiling in complex diseases but also promise to enable the characterization of distinct methylation signatures for complex diseases with diagnostic, therapeutic and pharmacodynamics implications. The research on DNA methylation would point to epigenetic marks as potential novel tools for prevention and therapy of complex diseases, such as cardiovascular diseases and renal failure.

DNA methylation helps stabilize chromatin and hypomethylation can lead to genomic instability by predisposing to DNA strand breakage and recombination within dispersed repetitive sequences. Furthermore, promoter CpG hypomethylation can result in gene activation, also contributing to aberrant gene expression [15]. For example, increased heparanase expression during the development of bladder cancer is due to promoter CpG hypomethylation of early growth response gene-1 (EGR1) [16]. An earlier study from our laboratory demonstrated a global hypomethylation pattern of genomic DNA in CRF rat's renal cortex [17]. Pervious study has shown that demethylation of the *TGF-β_1* promoter was in human lung and prostate cancer [18]. 509C→T polymorphism in the *TGF-β_1* promoter impact on the hepatocellular carcinoma risk in Chinese patients with chronic hepatitis B virus infection [19]. In this study, we have examined the promoter methylation status of the *TGF-β_1* gene in 3 groups using an optimized MSP method. A complete loss or low level of *TGF-β_1* mRNA expression was in NCG. In contrast, only sparsely methylated or unmethylated CpG sites were identified in PCG with a rich level of *TGF-β_1* mRNA. Our study revealed that change of *TGF-β_1* promoter methylation was correlated with the development of CRF, and with the treatment of Uremic Clearance Granule. Increased *TGF-β_1* expression during the development of CRF was a critical step in rat and human glomerulosclerosis. *TGF-β_1* promoter CpG hypomethylation may be one of the mechanisms causing *TGF-β_1* overexpression in glomerulosclerosis.

In CRF, high plasma homocysteine levels are a common finding and in uremia almost the rule. Ingrosso D's study suggested that hyperhomocysteinaemia affects epigenetic control of gene expression and the toxic action of homocysteine can be mediated by macromolecule hypomethylation. Hypomethylation is a major mechanism of adverse Hcy effects [20,21]. Hyperhomocysteinaemia and unbalanced methylation were also found in uremia patients [1]. Our results showed that an increased Hcy concentration and downgrading of the DNA methylation level [17]. Aberrant DNA methylation status of individual genes was a hallmark of cancer and had been shown to play an important role in neurological disorders such as Rett syndrome. We extrapolated that the renal genomic DNA demethylation promoted some cytokines, such as *TGF-β_1* gene expression, caused proliferation of mesangial cell and ECM, and then participated progression of glomerulosclerosis. Uremic Clearance Granule treatment could restored DNA methylation to normal levels and corrected the patterns of gene expression. Clearance of Hcy has leaded to recovery of some genes mathylation statue may be one of molecular mechanisms of Uremic Clearance Granules as an effective treatment for CRF.

Compositions of Uremic Clearance Granule included Radix et Rhizoma Rhei, Radix Astragali, Raeix Glycyrrhizae, Poria, Rhizoma Atractylodis Macrocephalae, Radix Polygoni Multiflori, Rhizoma Chuanxiong, Flos Chrgsanthemi, Radix Salviae Miltiorrhizae, and Rhizoma Pinelliae.

Radix Astragali was the principal component of Uremic Clearance Granule. Previous clinical and experimental studies have confirmed that Radix Astragali and the formula with Radix Astragali as the principal com-

ponent had a wide range of effectiveness on CRF treatment, such as reduce proteinuria, inhibit mesangial cell proliferation, reduce IL-6 secretion and regulate immune function [22-26]. This study showed that Uremic Clearance Granule which takes Radix Astragali as the principal component could reduce not only *TGF-β_1* mRNA expression but *TGF-β_1* protein expression. The inhibition effect on glomerulosclerosis of Radix Astragali may be one of the mechanisms of Uremic Clearance Granule as an effective treatment of CRF and delaying chronic progressive renal function deterioration. The study, in cellular and molecular level, provides some theoretical basis for Uremic Clearance Granule to prevent of glomerulosclerosis in clinical application.

Emodin (EMD) was the most widespread type of single-anthraquinone nuclear l, 8-dihydroxyanthraquinone derivatives in anthracene derivatives. EMD was the main active ingredient of Radix et Rhizoma Rhei and Rhizoma Atractylodis Macrocephalae [27]. Studies showed that in recent years, EMD had a variety of pharmacological effects, including tumor suppression, anti-virus, anti-oxidation and immune suppression, etc. Studies also found that, in patients with CRF, EMD could not only inhibit serum tumor necrosis factor secretion and mesangial cell proliferation, but also reduce IL-6 secretion of mesangial cells and the ECM accumulation [28,29]. In the human embryonic kidney fibroblasts (KFB), EMD could reduce PAI-1 secretion and increase ECM degradation [30]. In accordance with the results of this study, we would speculate that *TGF-β_1* mRNA expression decrease was likely associated with the role of EMD.

Studies have shown that Radix Astragali could inhibit the proliferation of vascular smooth muscle cells [31]. In our experiment, it was also found that the heart size of rats in UCG was smaller than which in PCG. We would speculate the result may be associated with the inhibition of Radix Astragali on vascular smooth muscle cells proliferation. Min Liang [32] suggests that homocysteine can stimulate the proliferation of myocardial cells involved in CRF patients with the occurrence of cardiac hypertrophy. In this study, rats in PCG suffered from serious cardiac hypertrophy, while rats in UCG did slightly.

In conclusion, the present results indicate that the CRF Wistar rat's kidney function was recovered after treatment with Uremic Clearance Granules. The recovery may be result of the remethylation of *TGF-β_1* promoter and then lead to *TGF-β_1* be transcripted normally. Clearance of Hcy may be the important mechanism of Uremic Clearance Granules. Lan Wang [33] suggested that dissecting the mode of action of clinically effective formulae at the molecular, cellular, and organism levels may be a good strategy in exploring the value of Chinese Traditional Medicine. Study on epigenetic changes in process of CRF and TCM treatment could provide a new theoretical foundation for the prevention and treatment to CRF. With the development of biotechnology, improvement of diagnosis and treatment of CRF at the molecular level may be the direction of TCM research and development.

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References

[1] Ingrosso D, Cimmino A, Perna AF, *et al.* Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uremia. *Lancet.* 2003;361:1693-1699.
[2] Yue-Ming Yu, Fan-Fan Hou, Xun Zhang, *et al.* Hyperhomocysteinemia in patients with chronic renal failure. *Chin J Nephrol.* 2002;18:34-37. (In Chinese)
[3] Yue-Ming Yu, Fan-Fan Hou, Hua Zhou, *et al.* The role of hyperhomocysteinemia on atherosclerosis in patients with chronic renal failure. *Chin J Int Med.* 2002;41:517-520.
[4] Murrell A, Rakyan VK, Beck S. From genome to epigenome. Hum Mol Genet. 2005;14:R3-R10.
[5] Jiang Y, Sun T, Xiong J, *et al.* Hyperhomocysteinemia-mediated DNA hypomethylation and its potential epigenetic role in rats. *Acta Biochim Biophys Sin (Shanghai)*. 2007;39:657-667.
[6] Herman JG, Graff JR, Myöhänen S, *et al.* Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *PNAS*. 1996;93:9821-9826.
[7] Lindstedt KA, Wang Y, Shiota N, *et al.* Activation of paracrine TGF-beta1 signaling upon stimulation and degranulation of rat serosal mast cells: a novel function for chymase. *FASEB J.* 2001;15:1377-1388.
[8] Yokozawa T, Muto Y, Wakaki K, *et al.* Site of methylguanidine production and factors that influence production levels. *Nephron.* 2002;92(2):356-362.
[9] Maeda A, Horikoshi S, Gohda T, *et al.* Pioglitazone attenuates TGF-beta(1)-induction of fibronectin synthesis and its splicing variant in human mesangial cells via activation of peroxisome proliferator-activated receptor (PPAR) gamma. *Cell Biol Int.* 2005;29:422-428.
[10] Blush J, Lei J, Ju W, *et al.* Estradiol reverses renal injury in Alb/TGF-β1 transgenic mice. *Kidney Int.* 2004;66:2148-2154.
[11] Krag S, Danielsen CC, Carmeliet P, *et al.* Plasminogen activator inhibitor-1 gene deficiency attenuates TGF-beta1-induced kidney disease. *Kidney Int.* 2005;68:2651-2666.
[12] Benigni A, Zoja C, Corna D, *et al.* Add-on anti-TGF-? antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol.* 2003;14:1816-1824.
[13] Ma LJ, Jha S, Ling H, *et al.* Divergent effects of low versus high dose anti-TGF-beta antibody in puromycin aminonucleoside nephropathy in rats. *Kidney Int.* 2004;65:106-115.
[14] Kutz SM, Providence KM, Higgins PJ. Antisense targeting of c-fos transcripts inhibits serum and TGF-beta1-stimulated PAI-1 gene expression and directed mobility in renal epithelial cells. *Cell Motil Cytoskeleton.* 2001;48:163-174.
[15] Attwood JT, Yung RL, Richardson BC. DNA methylation and the regulation of gene transcription. *Cell Mol Life Sci.* 2002;59:241-257.

[16] Ogishima T, Shiina H, Breault JE, *et al.* Promoter CpG hypomethylation and transcription factor EGR1 hyperactivate herparanase expression in bladder cancer. *Oncogene.* 2005; 24:6765-6772.
[17] Xu-Hong Miao, Guan-Nan Su, Bao-Quan Hu, *et al.* Detection of DNA methylation changes in Chronic Renal Failure rats using Methylation Sensitive Amplification Polymorphism (MSAP). *Acta Sci Natural Univers Nankaiensis*. In Press (In Chinese).
[18] Shah JN, Shao G, Hei TK, *et al.* Methylation screening of the TGF-β1 promoter in human lung and prostate cancer by methylation-specific PCR. *BMC Cancer.* 2008;284:1471-1482.
[19] Qi P, Chen YM, Wang H, *et al.* -509C>T polymorphism in the TGF-beta1 gene promoter, impact on the hepatocellular carcinoma risk in Chinese patients with chronic hepatitis B virus infection. *Cancer Immunol Immunother.* 2009;58:1433-1440.
[20] Huang T, Yuan G, Zhang Z, *et al.* Cardiovascular pathogenesis in hyperhomocysteinemia. *Asia Pac J Clin Nutr.* 2008;17: 8-16.
[21] Ingrosso D, Perna AF. Epigenetics in hyperhomocysteinemic states. A special focus on uremia. *Biochim Biophys Acta.* 2009;1790(9):892-9.
[22] Qian-Chun Yang,Ni-Zhi Yang, Bo-Jun Chen. Clinical observation of Radix Astragali injection for chronic glomerulonephritis with Qi-Deficiency and Blood-Stasis. *Chin J Inform Trad Chin Med.* 2004;11:253-254. (In Chinese)
[23] Ni-Zhi Yang, Li-Xin Wang, Qi-Zhan Lin, *et al.* Treatment of chronic nephritis of qi-deficiency and blood-stasis type by tongmai oral liquid: a clinical observation of 32 cases. *J New Trad Chin Med.* 2003;35:19. (In Chinese)
[24] Kun Bao, Wei Mao, Yi Pang. Effect of polyglucosides from Astragalus Membranaceus and Tripterygium Wilfordii on mesangium cellar IL-6 in rats. *Trad Chin Med Res.* 2005;18: 9-11. (In Chinese)
[25] Kun Bao, Wei Mao, Yi Pang, *et al.* Comparison of effects of Radix Astragali and Triperygium Glucosides on Glomerular Mesangial Cells Proliferation and Interleukin-6 Secretion. *J Guangzhou Univer Trad Chin Med.* 2005;22:292-295. (In Chinese)
[26] Ni-Zhi Yang, Dan Zhong, Dai-Xin Zhao. Effects of Tongmai Oral Solution on cell proliferation and Interleckin-β1, Interleukin-10 production in Mesangial Cells. *Chin J Integrated Trad West Nephrol.* 2007;8:332-336. (In Chinese)
[27] Zhao-Sheng Huang, Zong-Wei Wang. Study on the original plant and pharmacological effects of Emodin. *For Med Sci Section Chin Med.* 1997;19:9-12. (In Chinese)
[28] Yang Qiu, Yu-Xiu Yang. Inhibition effects of emodin on tumor necrosis factor secreted by patients with chronic renal failure. *Chin J Nephrol.* 1998;14:188-189. (In Chinese)
[29] Xi-Li Wu, Wan-Sen Sun, Cheng-Lin Qiao. Effects of emodin on cell proliferation and IL-6 production in mesangial cells. J Chin Medi Materials. 2006;29:53-55. (In Chinese)
[30] Gao-Xiang Chen, Sui-Lin Qu, Qin Fang. Effects of emodin on human embryonic kidney fibroblast cells to produce plasminogen activator inhibitor. *Traffic Medicine.* 2000;14:576-580. (in Chinese)
[31] Jin-Zi Huang, Jiang Shan, Gen Xu, *et al.* Astragalus inhibits proliferation on vascular smooth muscle cells and its mechanism. *Chin J Modern App Pharmacy.* 2003;20:277-282. (In Chinese)
[32] Min Liang, Yue-Ming Yu. Homocysteine induces cardiomyocyte hypertrophy and increases intracellular free calcium concentration. *Chin J Nephrol.* 2003;19:71-74. (In Chinese)
[33] Lan Wang, Guang-Biao Zhou, Ping Liu, *et al.* Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. *PNAS.* 2008;105:4826-4831.

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